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Potential application of RNAi for understanding and therapy of neurodegenerative diseases

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1. ABSTRACT

RNA interference (RNAi) technique has become a valuable tool in biology and biomedicine. In the future, it has the potential for application in many different fields including clinical medicine and agriculture. RNAi is a revolution in biology, representing the natural biological process in which genes are turned off in cells, and a completely new approach to drug discovery and development. This review focuses on the therapeutic potential of RNAi for central nervous system diseases. It gives an overview of the progress which has been made in this field to date, including the application of RNAi *in vitro* (to neurons) and *in vivo* (animal disease models) and addresses challenges in developing RNAi-based therapies. This review attempts to describe the future prospective of the clinical application of RNAi for neurodegenerative disorders.

2. INTRODUCTION

Neuroprotection is described as the continuous adaptation of the neuron to new functional horizons and responsibilities (1). It represents the sum of all mechanisms and strategies used to protect against neuronal injury or degeneration in the Central Nervous System (CNS) following acute disorders such as stroke or nervous system injury or as a result of chronic neurodegenerative diseases such as Parkinson's (PD), Alzheimer's (AD), Multiple Sclerosis and others. Despite years of research, the treatment and prevention of neuronal injury or degeneration in the CNS remains a major medical challenge. One of the general reasons is the long process of the identification of highly specific and potent drug candidates due to established pharmaceutical approaches. Furthermore, many targets can not be affected by traditional small-molecule and protein approaches.

RNAi, a fundamental cellular mechanism for silencing gene expression that is initiated or triggered by double-stranded RNA (dsRNA), can be used for the development of new drugs (2, 3, 4, 5). Two American scientists, Andrew Z. Fire and Craig C. Mello, received the Nobel Prize in Physiology or Medicine in 2006 for their discovery of RNAi. Less than a decade has passed since they first published their findings about the primary characterization of RNAi in the nematode *Caenorhabditis elegans (*6). A few years later another very important paper reported that RNAi occurs in mammalian cells in response to double-stranded small interfering RNAs (siRNAs) working as the effector molecules of sequence-specific gene silencing (7). Now, the RNAi mechanism is widely known and has become promising new tools for treating different diseases.

One of the major advantages of the RNAi approach is the application of all classes of molecular targets, including those that are difficult or even impossible to regulate selectively with traditional pharmaceutical approaches. Also, RNAi technology could significantly reduce the time needed for target validation and overall drug development, accelerating the drug discovery process. Thus, RNAi-based therapeutics can potentially have a transformational effect on modern medicine in general (8). Specifically, in the area of neurodegenerative diseases, RNAi can be used in treating some disorders, such as Prion disease or any disease from the polyglutamine (polyQ)-repeat disease family for which reduction of mutant or toxic gene expression may provide a therapeutic benefit (9, 10, 11). These neurodegenerative diseases are caused by dominant, gain-of-function types of gene mutations where one allele is mutated and produces a toxic protein, whereas the other allele is normal and performs essential functions. In addition, RNAi technology can be applied to study neurodegeneration pathways (through the specific knockdown of each protein in these pathways) in order to better understand the mechanism of the most common neurodegenerative diseases such as AD and PD and in the future to develop therapies to prevent and/or control them (12, 13).

Animal models (mostly the mouse) of human neurodegenerative diseases provide an excellent platform to test and validate new RNAi therapeutics. The main challenges for RNAi therapy in the field of neurodegenerative diseases are delivery across the blood brain barrier into the brain and expression of siRNA in neurons. Both viral (with viral vectors expressing short hairpin RNA) and non-viral (with siRNAs formulated with transfection reagent or in phosphate-buffered saline) delivery strategies are being undertaken for *in vivo* silencing of molecular targets by RNAi. Several groups have demonstrated efficacy by using this technique in animal models of neurodegenerative diseases, including AD, Amyotrophic Lateral Sclerosis (ALS) Huntington's disease, spinocerebellar ataxia Prion disease and many others (11, 14, 15, 16, 17, 18). In 2007, several reviews have been published about the different aspects of the application of RNAi technology for neurodegenerative diseases (19, 20, 21, 22, 23).

This review briefly introduces the main steps of the RNAi mechanism in mammalian cells in order to better understand how we can manipulate this pathway for therapeutic purposes. It then discusses the progress and perspectives of strategies for effective delivery of siRNA to neurons (an *in vitro* approach) and to the CNS (an *in vivo* approach). Moreover, this review summarizes up to date preclinical trials of therapeutic RNAi in animal disease models and addresses prospectives of the preclinical development of the RNAi therapeutic candidates for the treatment of neurodegenerative diseases in humans by using as examples, Prion, Huntington's diseases, Spinocerebellar ataxia, AD, PD and ALS. Finally, it discusses the commercial potential of RNAi in the neuroprotection strategy and the challenges which may arise on the way of development of successful treatments for neurodegenerative diseases.

3. THE PRINCIPAL STEPS OF THE MECHANISMS OF RNA INTERFERENCE IN MAMMALIAN CELLS

RNAi technology takes advantage of the cell's natural machinery, facilitated by siRNAs molecules, to effectively knock down expression of a gene of interest. RNAi can be triggered by various sources of RNA molecules (also known as RNAi effector molecules), including RNA viruses, transposons, exogenously introduced dsRNAs (known as siRNAs) and endogenous small non-coding RNAs, or microRNA (miRNAs) (24). In addition to miRNAs and exogenous siRNAs, endogenous siRNAs have been discovered in various organisms (25, 26, 27). Since the first ground-breaking paper of Fire et al. on *C. elegans,* the understanding of the mechanisms of RNAi-mediated gene silencing has greatly increased (6, 24, 28, 29, 30). Now, scientists have a greater comprehension of the components of the RNAi pathway in general, the functional role of these components, and many of the key requirements for designing and generating extremely effective RNAi reagents for *in vivo* analysis and, most importantly, the potential to use RNAi in the development of therapeutics.

RNAi effector molecules induce gene silencing in two general ways: first, the direct sequence-specific cleavage of perfectly complementary mRNAs (the short interfering RNAs pathway) (Figure 1); second, the translational repression and transcript degradation for imperfectly complementary targets (the miRNA pathway) (Figure 2) (20). Based on these two pathways, all RNAi effector molecules can be divided into two groups: the siRNAs and the miRNAs effector molecules (Chapters 3.1.1 and 3.2). Each group has two subgroups: endogenous and exogenous RNAi effector molecules. For both groups: the endogenous RNAi effector molecules exist naturally in cells, the exogenous molecules come from infection by a virus with RNA genome, or laboratory manipulations. In mammalian cells, naturally occurring post-trancriptional gene silencing is mainly mediated by miRNA. However, researchers use the siRNAs molecules more frequently to manipulate RNAi.

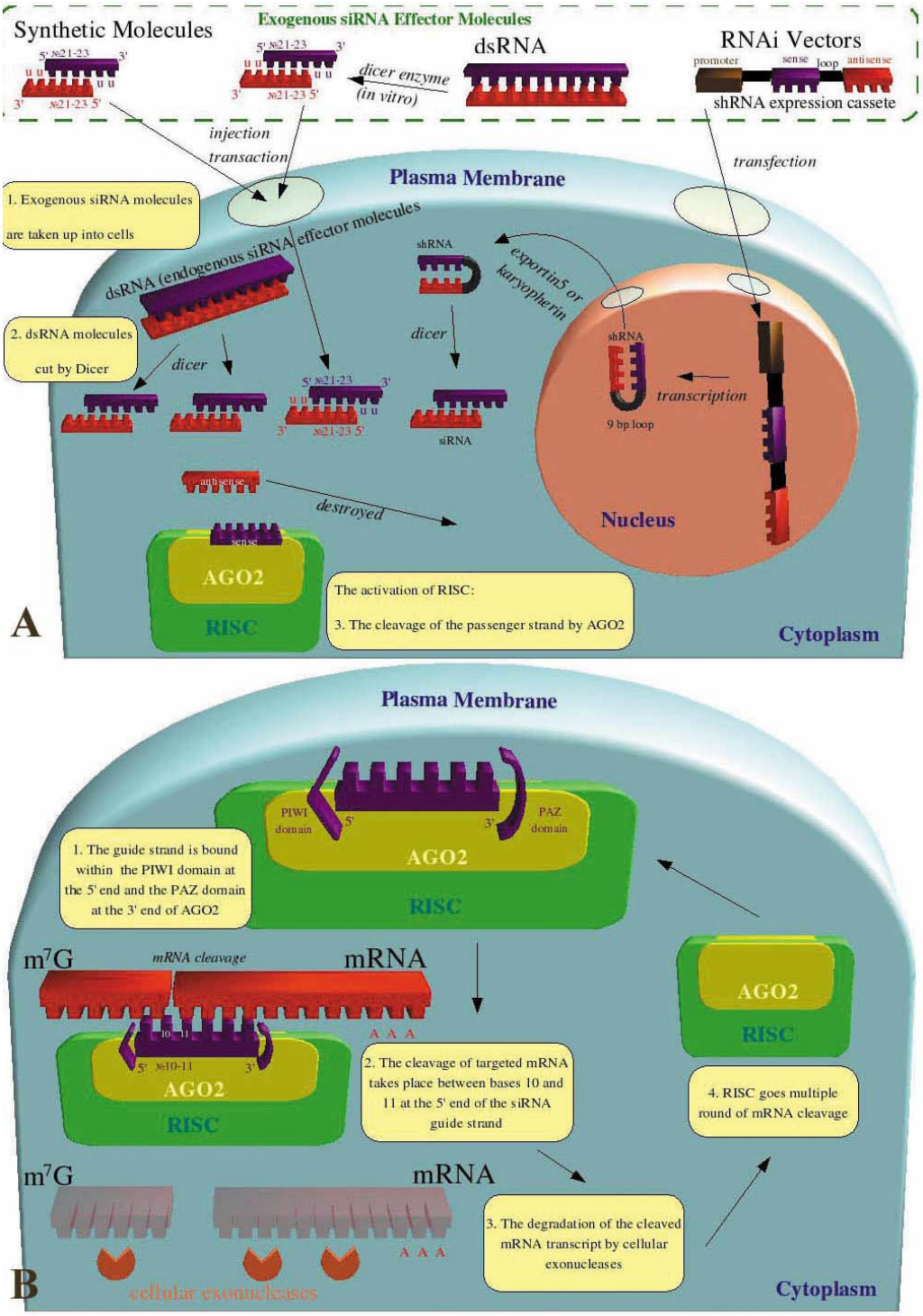

Figure 1. The short interfering RNAs pathway. A. The assembly phase. 1. The dsRNA molecules (or hairpin-RNA substrate) are taken up into cells; 2. The dsRNA molecules are cut by Dicer; 3. The activation of RISC (a cleavage mechanism): the cleavage of the passenger strand by the AGO2. B. The effector phase. 1. The guide strand is bound within the PIWI domain at the 5' end and the PAZ domain at the 3' end of AGO2; 2. The cleavage of targeted mRNA takes place between bases 10 and 11 at the 5'end of the siRNA guide strand; 3. The degradation of the cleaved mRNA transcript by cellular exonucleases occurs;4. RISC goes multiple rounds.

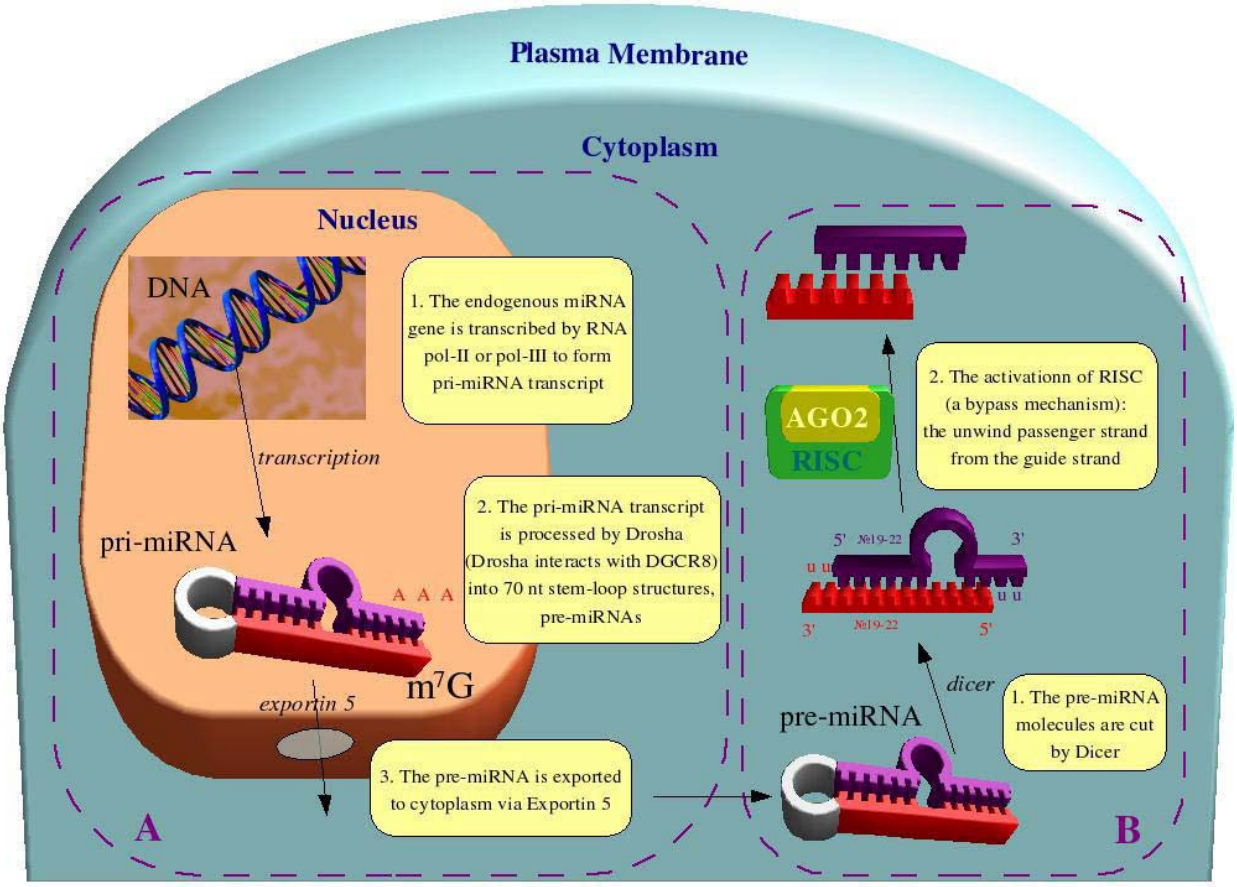

Figure 2. The microRNA (miRNA) pathway. A. The preassembly phase. 1. The endogenous miRNA gene is transcribed by RNA pol-II or pol-III to form pri-miRNA transcript; 2. The pri-miRNA transcript is processed by Drosha (Drosha interacts with DGCR8) into 70 nt stem-loop structures, pre-miRNAs; 3. The pre-miRNA is exported to cytoplasm via Exportin-5. B. The assembly phase. 1. The pre-miRNA matures are cut by Dicer; 2. The activation of RISC (a bypass mechanism): the unwind passenger strand from the guide strand. C. The effector phase – the same as for the siRNAs pathway: Figure 1B.

3.1. The siRNA pathway

Figure 1 summarizes the main steps of the RNAi mechanism by siRNAs in mammalian cells. Endogenous siRNAs are dsRNA molecules of about 20–25 nucleotides. They have been described in the entire phylogenetic eukaryotic tree. There are several ways to induce RNAi by exogenous siRNAs effector molecules: synthetic molecules, RNAi vectors (DNA vectors that express hairpin RNAs used to induce RNAi), and *in vitro* dicing (these mechanisms will be described below). In general, the RNAi response can be divided into two distinct steps (31).

The first step, also known as the assembly phase (Figure 1A), begins when the dsRNA molecules (or hairpin-RNA) are taken up into cells where these molecules, recognized by the host cell as being "foreign," are cut by the RNase III enzyme called Dicer into fragments about 21-23 nt in length with about 2 nt 3' overhangs (32). These fragments are commonly known as siRNAs (7). Then siRNAs are shuttled into the RNA-induced silencing complex (RISC) to form an RNA-protein complex (33, (34, 35). The activation of RISC by an Argonaute 2 protein (AGO2) begins through the cleavage of the sense or passenger strand of the double-stranded siRNAs (33, 35). For each siRNA duplex, only one strand, the guide, is assembled into the active RISC; the other strand, the passenger, is destroyed.

In the second step, called the effector phase (Figure 1B), RISC uses this single-stranded RNA molecule as a guide to endonucleolytically cleave the complementary RNAs (36, 37). This strand (the single-stranded guide) is bound within the catalytic, RNase H-like PIWI (named for the protein piwi) domain of AGO2 at the 5'end and a PIWI–Argonaute–Zwille (PAZ) domain at the 3'end (Figure 1B) (38, 39) . The cleavage of the targeted mRNA takes place between bases 10 and 11 relative to the 5' end of the siRNA guide strand, leading to subsequent degradation of the cleaved mRNA transcript by cellular exonucleases (40, 41). It results in the reduction of target mRNA levels and decreasing target protein levels. RISC can undergo multiple rounds of mRNA cleavage to mediate a robust post-transcriptional gene silencing response against the target gene (42).

RNAi pathways can also direct transcriptional gene silencing in the nucleus however this pathway is not yet well established in mammalian systems (43, 44, 45).

3.1.1. The siRNAs effector molecules

3.1.1.1. Chemically synthesized siRNAs

Chemically synthesized siRNAs have been successfully used in different cell types (including neurons), plants and animals (e.g. *C elagans* and *Drosophila*). Most of the proposed clinical applications of RNAi include chemically synthesized 21 nt siRNA duplexes that have 2 nt, 3' overhangs; sometimes the siRNA duplexes have different chemical modifications that increase their stability (20). The main advantage of chemically synthesized siRNAs is the easy method of transfection. Usually, they are directly transfected into target cells in a straightforward and robust experimental design without viral production. The main disadvantage of using the synthetic siRNAs is a transient effect of knockdown of gene expression due to rapid degradation.

3.1.1.2. RNAi vectors (RNAi inducing DNA vectors)

The siRNA can also be taken into cells by DNA vectors that express short hairpin RNA (shRNA) which can mediate constant gene silencing after stable integration of the most often virally encoded expression cassette into the host genome (46, (47, 48, 49). Commonly, shRNA encode two perfectly complementary short RNA strands of 19–29 bp; there is one strand identical in sequence to the target mRNA. These two strands are linked by a 9 bp loop sequence and usually transcribed from DNA polymerase III (pol-III) promoters (50). The eukaryotic H1 and U6 small nuclear RNA pol-III promoters are more often used in RNAi vectors to transcribe shRNAs (47, 51). These promoters work well for this purpose due to their ability to generate high levels of small, non-coding RNA transcripts lacking the polyA-tail and initiate from a position +1 of the transcripts, unaffected by an inhibitory 5'-nucleotide. Moreover, pol-III promoters have the advantage of high activity in all cell types, which means the capacity for stronger knockdowns and a smaller size of the entire expression cassette (52). After transcription from a DNA pol-III promoter, shRNA are then processed into a perfectly complementary short RNA duplex, mimicking the miRNA pathway. The main difference between these pathways is the absent Drosha processing **(**see the description of this in the miRNA pathways chapter and as well more specific reviews) in the shRNA pathway (20, 24, 28). Exportin 5 or karyopherin then export the perfectly complementary short RNA duplex to the cytoplasm (53, 54, 55, (56). There can be additional shRNA nuclear export pathways since the export process is not well understood. Once exported into the cytoplasm, shRNA is processed by Dicer and is incorporated into RISC. Since shRNA usually exhibits perfect pairing, they lead to target cleavage of mRNA transcript rather than translational repression (21). shRNA has been used often in preclinical trials for animal models of neurodegenerative disease (Chapter 6.1).

3.1.1.3. The post-Dicer cleavage products, siRNAs (*in vitro* dicing)

The post-Dicer cleavage products, siRNAs, can be exogenously introduced into cells to induce RNAi. Recent studies have shown that pre Dicer cleavage dsRNA (Diced siRNA pools) are highly potent for gene knockdown and provide a cost-effective approach to RNAi (57, 30, 58). First, a long, dsRNA duplex is created *in vitro*; second, the Dicer enzyme generates the shorter 21 to 23-nt double-stranded siRNA duplexes. Then, the siRNA can be transfected into cells. Several kits for generating a Dicer reaction substrate (for example, the BLOCK-iT™ RNAi TOPO® Transcription kit from Invitrogen) are commercially available. While siRNA has been used by researchers more often, naturally occurring post-trancriptional gene silencing in mammalian cells is mainly mediated by miRNA. The biogenesis, processing and function of miRNAs are described below.

3.2. The microRNA pathway

As mentioned above, the RNAi machinery can also be programmed for gene silencing by endogenous miRNAs. The endogenous miRNAs pathway has important roles in the post-transcriptional regulation of genes during development and differentiation in many organisms including mammals (59, 60, 61, 62, 63). Interestingly, the brain expresses different miRNAs at specific developmental stages (55, 64, 65, 66). In the review of Gonzalez-Alegre there are two very important conclusions based on this fact. First, neurons contain the necessary machinery for the manipulation of RNAi. Second, this pathway plays a relevant role in the CNS and its disruption through exogenous intervention could alter normal neuronal homeostasis (21). The recent experimental paper where miRNAs abundance in the hippocampal region of fetal, adult and AD brain have been examined supports these conclusions very well. In this paper, it was shown that altered miRNA-mediated processing of messenger RNA populations may contribute to atypical mRNA abundance and neural dysfunction in AD brain (67). Thus, all features of the nuclear processing of miRNAs have to be considered very carefully when planning therapeutic RNAi experiments.

The main steps of the RNAi mechanism by miRNAs in mammalian cells are summarized in Figure 2. Similar to siRNAs, miRNAs are dsRNA molecules of about 20–25 nucleotides but exhibit several differences (68). In general, the miRNA pathway can be divided into three steps: the initiation step (or preassemble phase) which takes place in the nucleus (this step is missing in the siRNA pathway) and two additional steps (the assembly and the effector phases) which take place in the cytoplasm. In the initiation step in mammalian cells, the endogenous miRNA gene is transcribed by RNA polymerase II (pol-II) or by RNA pol-III to form primary miRNA (pri-miRNA) transcript that contains a 5'-end cap structure and a polyA-tail sequence (69, 70). Then this pri-miRNA transcript is processed by Drosha into 70 nt stem-loop structures known as precursor miRNAs (pre-miRNAs) (71). Drosha is a nuclear RNase III that interacts with its cofactor, DiGeorge syndrome critical region gene 8 (DGCR8), and produces

RNA products that contain 5' phosphate groups and two nucleotide overhangs at their 3' ends (30). Pre-miRNA is then exported to the cytoplasm via Exportin 5 in a Ran-GTP-dependent manner where pre-miRNA matures into miRNA, a 19-22 base pair dsRNA with two 3' nucleotide overhangs at both ends, by the cytoplasmic process mediated by Dicer. This step, similar to siRNA pathway, is known as the assembly phase. Mature miRNA is then loaded into RISC for gene silencing (72, 73, 74). In contrast to the siRNA pathway, the activation of RISC in the miRNA pathway does not involve cleavage of the miRNA passenger strand, instead using a bypass mechanism that requires helicase activity to unwind and discard the passenger strand. Most likely, the imperfect sequence homology between the mature miRNA strand and its complementary passenger strand prevents AGO2 from cleaving the passenger strand, but more investigation of this mechanism is needed (74).

As in the siRNA pathway, during the second step (the effector phase) which takes place in the cytoplasm, RISC uses this single-stranded RNA molecule as a guide to endonucleolytically cleave complementary RNAs. Similarly to the siRNA pathway, two different domains in AGO2, Piwi and PAZ, bind the 5′ and 3′ ends of the guide strand, respectively. In some cases, unlike siRNAs, miRNAs do not cleave the mRNA of a target gene, but instead suppress mRNA translation (75, 76). In another instance similar to the siRNA pathway, AGO2 cleaves the mRNA between bases 10 and 11 of the guide strand, independent of the total length of this short RNA. Base-pairing is usually required in those nucleotides for effective cleavage (77). The 5′ and 3′ mRNA fragments generated are then degraded by different cellular exonucleases (68). This results in mRNA degradation and sequential silencing of gene expression. This process occurs in cytoplasmic compartments known as processing bodies (P-bodies) (78, 79, 80). Once in P-bodies, translationally repressed mRNA can stay in oligomeric structures for storage or can form a complex with decapping enzymes and cap-binding proteins that trigger mRNA decay. Thus, the miRNA in RISC can provide sequence specificity (24).

Using all the detailed information about the miRNA pathway will help researchers design therapeutic constructs which allow modification of target specificity (81). For example, nucleotides 2–11 of potential therapeutic constructs could be critical for target recognition and cleavage and also could be used for the design of allele-specific silencing strategies (77, 82).

4. NEURODEGENERATIVE DISEASE: PUBLIC HEALTH CHALLENGES

Together, AD and PD with Dementia represent nearly 80% of the neurodegenerative diseases that affect patients in the United States. According to the report issued in 2007 by the World Health Organization entitled "Neurological disorders: Public Health Challenges," nearly one billion people worldwide are affected by neurological disorders. An estimated 6.8 million people die every year as a result of neurological disorders. Thus, as a result of increased life expectancy and changing population demographics (i.e., the aging of baby boomers), neurodegenerative diseases and movement disorders are becoming more common (83). These disorders remain among the most devastating and critical health issues for millions of people around the world. The impact on individuals, families and healthcare costs has forced these disorders to the forefront of the healthcare industry. The market value for the neurodegenerative drug industry exceeded $16 billion in 2006, with revenues growth anticipated to surpass $18 billion in 2007 (84). Despite the huge neurodegenerative drug market and the years of research in the area, the treatment and prevention of neuronal injury or degeneration in the CNS remains a major medical challenge. Currently, there are no effective therapies for most neurodegenerative diseases (85, 86). For disorders where pharmacological treatments are available, such as AD or PD, these treatments provide only relief of symptoms (87, 86). Unfortunately, current medical therapies have so far failed to change the natural course of neurodegeneration in humans (21).

Currently, investigations are focused on the identification of biomarkers to facilitate the diagnosis in the earlier stages of the disease, the finding of molecular mediators of neurodegenerative diseases and the development of therapies aimed at preventing, stopping or slowing down the process of neuronal failure (86, 88, 89). Recently, many new molecular mediators of neurodegenerative diseases and neurological disorders have been identified and yet few novel therapies have emerged (90, 91). In some cases, considerable pre-clinical validation exists, but the molecular target has not been readily amenable with small molecules, proteins or antibodies, which traditional pharmaceutical approach provides.

RNAi represents a therapeutic approach applicable to such inaccessible targets. Moreover, the relatively short time for efficacy testing of potential therapeutic RNAi molecules raises the potential of RNAi for treating a wide range of diseases. The key therapeutic advantage of using RNAi for neurodegenerative diseases is in its ability to specifically and potently knock down the expression of disease-causing genes of known sequence. In these neurodegenerative diseases, such as Prion, Huntington's and many others, one allele is mutated and produces a toxic protein, while the other allele is normal and performs essential functions. Another strategy for using RNAi for neurodegenerative diseases is suppresing the expression of genes implicated in disease pathology; for example, down-regulation of amyloid precursor protein (APP), or other proteins involved in the formation of plaques in AD (22).

5. RNAi APPLICATION IN THE NEUROSCIENCE FIELD

In the field of neuroscience, RNAi has been applied to a variety of research objectives, including the study of the function of proteins involved in human disease, dissection of neurodegeneration pathways, generation of animal models or therapeutic goals (19, 20, 21, 22, 23). In

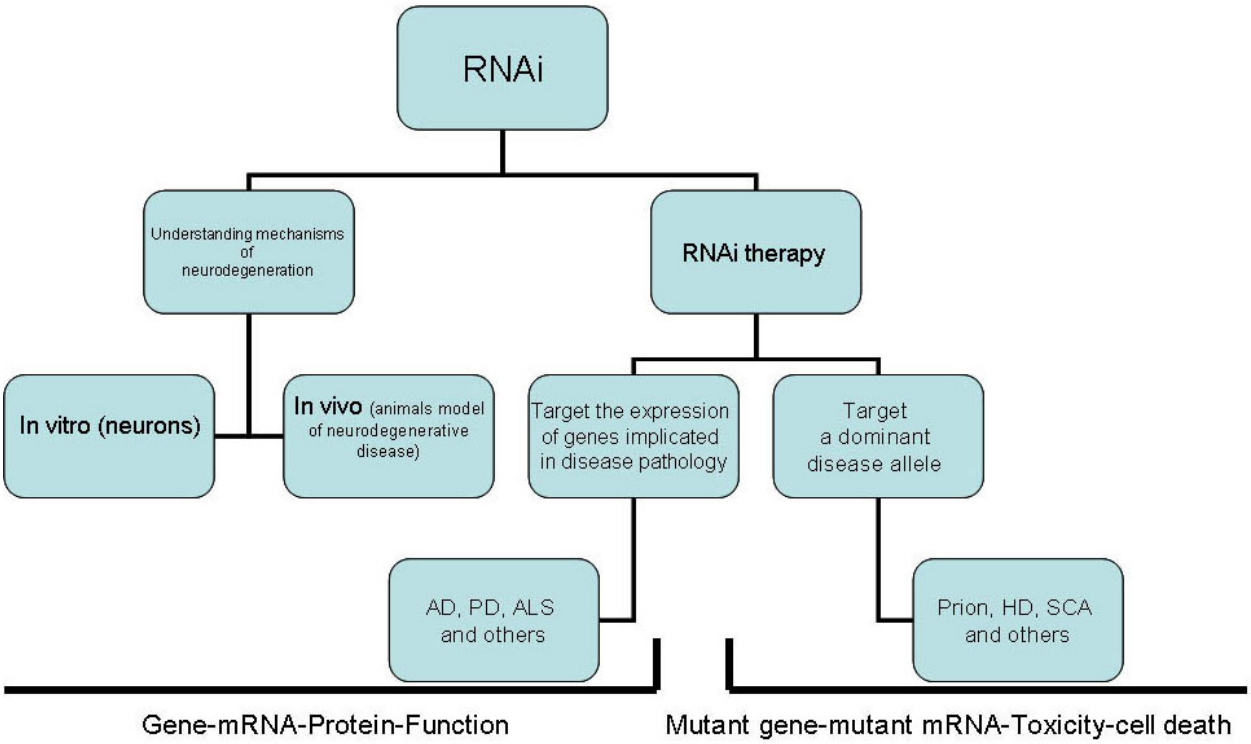

Figure 3. The different strategies of RNAi application in the neuroscience field. Understanding mechanisms of neurodegeneration: The RNAi approach is used to block the expression of specific genes, and then determine the cellular changes as a result of the missing gene, and based on this, the function of this gene may be described (the methodology scheme: gene-mRNA-protein-function). RNAi therapy: RNAi approach is applied to two therapeutic strategies. First, to target the expression of genes implicated in disease pathology. The methodology scheme is the same as above. Second, to target a dominant disease allele: The RNAi approach is used to block the expression of specific mutant proteins in order to decrease or eliminate their toxicity (the methodology scheme: mutant gene-mutant mRNA-toxicity-cell death).

all these research objectives, RNAi application was based on the different aspects of the natural mechanism of RNAi by using both the siRNA and the miRNA pathways. Figure 3 summarizes the strategies of RNAi application in the neuroscience field (Figure 3). The experimental approach selected for each RNAi application will depend on the aim of silencing the expression of a given gene. A step-by-step approach for the application of RNAi for each research direction in the field of neuroscience will be described, emphasizing the advantages and disadvantages of the different methods.

5.1. Application of RNAi in understanding the mechanism of neurodegeneration in vitro

RNAi induced by introduction of siRNA has become an important research tool for knocking down targeted genes in neuronal cells. Effective gene silencing by siRNA depends on its sequences and the delivery methods. The homology to the target, the internal stability at the 5' end of the antisense-strand and the position-specific sequences - these factors together contribute to the overall activity (92). Many companies offer free software that designs siRNA sequences by optimizing the above factors (Qiagene, Ambion, Invitrogen and many others). siRNA can be delivered to cells in two forms: chemically synthesized siRNA and RNAi expression cassettes (or RNAi gene, usually expressing shRNA) via vectors. In neurodegenerative diseases, neurons are initially functionally damaged and subsequently die in disease-specific regions of the nervous system at later stages. Rat neurons are frequently used as an experimental model to study neural cell function and to develop new therapies for neurodegenerative diseases. Unfortunately, neurons are not easy to transfect with general transfection reagents and with standard expression vectors, apparently because they are postmitotic cells and therefore do not undergo M-phase in normal circumstances. Interested readers can find descriptions of general approaches using RNAi *in vitro* in several reviews (93, 94, 95). This part of the review will focus on new approaches for delivering siRNA and/or shRNA to primary mammalian (mostly rat) neurons *in vitro* and on RNAi libraries (mammalian RNAi Libraries) which are powerful tools for discovering novel functional genes, pathways and new drug targets.

5.1.1. New delivery methods for siRNA into primary neurons

5.1.1.1. Peptide-mediated and polymer/lipid-based cellular delivery methods

Many commercial transfection reagents are available for the introduction of chemically synthesized siRNA or RNAi expression cassettes into cell lines. The

most frequently used method is based on cationic lipid based reagents (96). Unfortunately, the cationic lipid-based transfection reagents are toxic for many primary mammalian cells including primary neurons (97, 98).

Recently, various peptide-based strategies have been developed to improve the delivery of oligonucleotides both *in vitro* and *in vivo* using either covalent or complex approaches (99, 100, 101). One of them is a lipophilic polypeptide - stearylated octaarginine (Stearyl-R8), which is as effective as other usual lipid-based reagents (102). The stearylation on the N terminus of the peptides increases the efficiency of transfection by facilitating the absorption of the complex to the membranes through its hydrophobic moiety (96).

Polymeric vectors combined with liposomal artificial virus-like envelopes, also known as artificial virus-like particles (AVPs) provide another transfection method for neurons. In a paper from Bähr's laboratory, this method has been modified, by using siRNA condensed with transfection reagent (polyethyleneimine or protamine sulfate) then complexed with a liposomal envelope, artificial viral envelope (AVE), that mimics the lipid composition of naturally enveloped viruses (96). This research used both novel peptide-based (Stearyl-R8) and lipid-based AVP delivery methods for the transfection of siRNAs into rat primary neurons and was compared to a widely used liposomal transfection reagent (Lipofectamine). Stearyl-R8 and AVPs both promoted siRNA transfection into primary hippocampal neurons via the endosomal pathway. In comparison to the commonly used cationic liposome transfection agent, both novel reagents were less detrimental to cell metabolic activity. The authors concluded that these novel transfection methods yield performances comparable to cationic liposome-mediated transfection for siRNA, while being less cytotoxic to the primary neurons (96). Thus, Stearyl-R8 and AVPs may represent novel and more cost-efficient alternatives to conventional siRNA-transfection reagents (96). Another peptide-based strategy for siRNA delivery uses short amphipathic peptide (MPG), which that is able to form stable nanoparticles with siRNA (103). MPG-based particles enter the cell independently of the endosomal pathway and can efficiently deliver siRNA in a fully biologically active form to a variety of cell lines and *in vivo*. Transfection reagents (DeliverX Transfection Reagents) based on MPG delivery technology are commercially available from Panomics (www.panomics.com). The MPG strategy has been used successfully for the delivery of siRNA to a large panel of cell lines including adherent and suspension cell lines, as well as primary and embryonic stem cells which cannot be transfected using other non-viral approaches (104). Thus, this strategy has great potential for delivering siRNA to primary mammalian neurons *in vitro*. Even though MPG technology is still new and needs to be optimized for systematic *in vivo* applications, it is already a powerful tool for basic research and will have a major impact on the use of siRNA for future therapies.

5.1.1.2. The nucleofector technology

In additional to transfection agents, electroporation is a general approach for delivering genetic material inside cells. The nucleofector technology marketed by Amaxa Biosystems (www.amaxa.com) is intended for the cell lines that are difficult to transfect. It is a non-viral method which is based on a unique combination of electrical parameters and the cell-type specific solutions. Nucleofection is the first non-viral transfection technology that delivers DNA not only into the cytoplasm, but also straight into the nucleus of a cell. Thus, it provides the ability to transfect even non-dividing cells, such as neurons and resting blood cells. The nucleofector technology has been used intensively in the neuroscience field, showing transfection efficiency with plasmid DNA (RNAi expression cassettes) of up to 59 % and up to 70% with siRNA duplexes for primary neurons (105, 106, 107, 108). In a recent review paper, novel possibilities for using the nucleofector technology not only in basic research and development, also in *ex vivo* gene therapeutic applications are discussed (109).

5.1.2 Mammalian RNAi libraries

Application of high-throughput RNAi libraries for mammalian cells provides a new approach for discovering novel functional genes, pathways and potential new drug targets, including combinatorial gene inactivation. In general, the library is introduced into cells that are screened for a particular phenotype (s) associated with the particular disease state of interest. Based on the library screen, it is possible to identify the particular siRNA responsible for the phenotype of interest, and then use this as a target for drug discovery. This part of the review will describe the recent examples of using the different types of mammalian RNAi libraries in the neuroscience field. Interested readers can find descriptions of all types of RNAi libraries and their role in the drug target discovery in more specific reviews (110, 111, 112). Several groups reported the creation of comprehensive RNAi libraries and their application for the discovery of functional genes implicated in the neurodegenerative diseases. Dr. Li's group has recently created a novel RNAi library based on partially randomized consensus sequences of nuclear receptors (113). This siRNA library was created based on the pHUMU vector by using partially randomized sequences targeting the consensus region in the C4 zinc-finger motif of nuclear hormone receptors and thus against the entire receptor superfamily. This library has been screened for receptors that might be involved in reducing amyloid β (Aβ) peptide accumulation which is a marker for AD. The negative effect has been discovered with retinoid-related orphan receptor-γ, a potential receptor target, on the degradation of the toxic Aβ peptides. This receptor target could be useful as a therapeutic target in the treatment of AD.

Other researchers used high-throughput siRNA screening to define the role of 15,200 genes in $A\beta_{42}$ secretion, which is believed to cause a rare form of AD. They identified the gene leucine-rich repeat transmembrane (LRRTM)3 as a promoter of APP processing by β-secretase and, when overexpressed, promoted Aβ secretion (114). Thus, siRNAs targeting LRRTM3 inhibit the secretion of $A\beta_{40}$, $A\beta_{42}$, and sAPPβ, the N-terminal APP fragment produced by β-secretase cleavage in cultured cells and primary neurons by up to 60%. Interestingly, LRRTM3 is

expressed almost exclusively in the CNS, including regions affected during AD, such as the dentate gyrus of the hippocampus. Moreover, LRRTM3 is related to a family of neuronal receptors such as NOGO, an inhibitor of neuronal regeneration and APP processing. Thus, LRRTM3 is a functional and positional candidate gene for AD, and, given its receptor-like structure and restricted expression, a potential therapeutic target. Thus, the use of RNAi approach *in vitro* in understanding the mechanisms of neurodegeneration has grown intensively in recent years. In mammalian cells, RNAi has been applied to identify α-synuclein, a protein that is involved in PD pathogenesis and other proteins that involve different pathways of neurodegeneration including PAG608, spastin, neurosin and many others (115, 116, 117, 118).

5.2. Development of *in vivo* RNAi technology

Based on the rapid progress of RNAi technology for drug target discovery and validation in an *in vitro* culture system, interest is quickly growing in the extension of its application to *in vivo* systems, such as the development of gene-based RNAi technology, animal disease models and human therapeutics (Chapter 6.1).

5.2.1 Recent progress in the development of gene-based RNAi technology

The main problem of gene-based RNAi technology is developing a delivery method that accomplishes effective systemic distribution to the cell cytoplasm *in vivo*. Recently, more specific reviews have described approaches for *in vivo* transfection of siRNA or shRNA (94, 119, 120, 121). This part of the review will focus on approaches which can be applied to create neurodegeneration models and recent progress in the development of the delivery methods of siRNA (or shRNA) across the blood–brain barrier.

Viral-mediated RNAi vectors described by Hommel et al. can become a tool for the rapid production and testing of new genetic disease models (122). The adeno-associated virus carrying the antisense tyrosine hydroxylase shRNA vector was administered to the substantia nigra by the local stereotaxic surgery. A decrease of dopamine and phenotypic changes consistent with dopamine depletion were observed. This localized gene knockdown also resulted in behavioral changes, including a motor performance deficit and reduced response to a psychostimulant. Despite showing promising results this approach has a few disadvantages. Every animal needs to be injected with the virus through stereotaxic surgery, and the transduction efficiency may vary from animal to animal (123).

An alternative to viral transduction is to create transgenic animals with gene cassettes that express shRNA. Xia et al. described the technology of generating transgenic mice using the conventional zygote injection technique (123). Gene cassettes have been used that express shRNAs against several different genes, mutations of which are associated with neurodegeneration. They used, for example, gene SOD1 (Chapter 6.1) which causes ALS; SOD2 which causes widespread neurodegeneration; and DJ-1 mutations which cause Parkinson's syndrome. Either U6 or an enhanced U6 promoter has been used to direct the synthesis of these shRNAs (124). Unfortunately, the overall positive rate of transgenicity was low; approximately 10% and few transgenic lines expressed detectable levels of siRNA. None of these transgenic lines have shown definitive protein knockdown. The major difficulties in generating transgenic RNAi are low frequency of transgenic positives that express high levels of siRNA, failure in spreading the lines that express high levels of siRNA and a lack of consistent knockdowns.

One approach to avoid these difficulties is to generate knockdown of embryonic stem (ES) cell lines with transgenic shRNA. This approach has been described by Kunath et al. (125). It has been reported that transgenic RNAi in ES cell-derived embryos recapitulates a genetic null phenotype, though unfortunately not in all tissues of adult transgenic mice.

Another approach which was developed recently is to use conditional transgenic RNAi expression of shRNA by using a Cre-loxP inducible U6 promoter. Zhou et al. used this technique against the Pink1 gene, whose loss-of-functional mutations causes one form of familiar PD (126). The expression of shRNA was tightly regulated and induced the silence of the Pink1 gene product by more than 95 % in the mouse brain. However, these mice did not develop dopaminergic neurodegeneration. The authors suggested that the silencing of the Pink1 gene expression from mouse embryos is not enough to cause similar biochemical or morphological changes to those observed in PD. Thus, the results demonstrated that silencing of the Pink1 gene does not induce a reliable mouse model for PD, but most importantly that technically the inducible U6 promoter is useful for conditional RNAi *in vivo* and can be successfully used in gene-based RNAi technology.

The general approach in gene therapy experiments for injection of siRNA (or shRNA) and viral or DNA vectors directly into the brain is stereotactic surgery. However, the use of this technique results only in the localized delivery around the injection site, with no widespread effects throughout the brain. This method is also too invasive for human therapy (121).

The main challenge for targeting CNS with siRNA (or shRNA) therapeutics is the blood–brain barrier (BBB), which is only permeable to lipophilic molecules of less than 400 Da (127). In a recent paper, Kumar et al showed that a short peptide derived from rabies virus glycoprotein (RVG) enables the transvascular delivery of siRNA to the brain (121). This 29-amino-acid peptide specifically binds to the acetylcholine receptor expressed by neuronal cells. After intravenous injection into mice, RVG-9R delivered siRNA to the neuronal cells, resulting in specific gene silencing within the brain. Moreover, intravenous treatment with RVG-9R-bound antiviral siRNA afforded robust protection against fatal viral encephalitis in mice. The authors also repeated the administration of RVG-9R-bound siRNA. This experiment

did not induce inflammatory cytokines or anti-peptide antibodies. Thus, RVG-9R provides a safe and noninvasive approach for the delivery of siRNA and potentially other therapeutic molecules across the BBB (Kumar, Wu et al. 2007). Another promising alternative approach for delivery of siRNA (or shRNA) to the CNS is the "Trojan Horse Liposome" (THL) technology (127, 128, 129, 130). The THL is engineered with lipids containing approximately 1% of the polyethyleneglycol residues joined with peptidomimetic monoclonal antibodies. These antibodies bind to specific endogenous receptors (i.e. insulin and transferrin receptors) located on both the BBB and the brain cellular membranes, respectively. A few recent reviews provided an overview of the THL technology and its current application to gene-based RNAi therapy (131, 132, 133). The efficiency of THL technology for the delivery of therapeutic genes to the brain has been tested in the rat and the mouse PD model (130, 134). Both studies show promising results, providing evidence that the treatment of PD is possible with THL technology (132). The advantage of the THL approach is that the molecular Trojan horses that cross the BBB via receptor-mediated transport can be formulated with different technology platforms, depending on the pharmaceutical molecule which needs to across the BBB (131). For example, siRNA may be delivered with avidin-biotin technology; genes encoding shRNA can be targeted to the brain with Trojan horse liposomes, which may be engineered with shRNA expression vectors driven by the specific promoters.

Utilizing avidin-biotin, the therapeutic is monobiotinylated in parallel with a genetically engineered fusion protein of avidin or streptavidin and the molecular Trojan horse (the MTH). The bond between avidin or streptavidin and biotin is extremely tight, and is not disrupted by serum proteins. Owing to the extremely high affinity of avidin or streptavidin for biotin, there is immediate formation of conjugate between the siRNA and the targeting monoclonal antibodies (133). Several successful examples of in vivo CNS pharmacologic effects where the pharmaceutical was attached to the MTH with the fused avidin–biotin have been published. Below is a description of a few papers related to neurodegenerative diseases, but interested readers can see all these examples in the recent review on drug targeting to the brain (135). A 65–80% reduction in stroke volume was shown in response to the intravenous delivery of brain-derived neurotrophic factor or fibroblast growth factor attached to the MTH conjugated with avidin-biotin to rats raised with the middle cerebral artery occlusion (MCAo) model of ischemia (136), (137), (138). The same brain delivery approach has been used in an AD transgenic mouse model. It has been demonstrated that $A\beta^{1-40}$, a potential peptide radiopharmaceutical, can be used to image the brain Abeta amyloid of AD *in vivo* (139). Also, *in vivo* expression of the glial fibrillary acidic protein gene in brain ischemia was possible following the intravenous administration of sequence specific antisense radiopharmaceuticals using peptide nucleic acids. This antisense RNA fragment was conjugated to a BBB molecular Trojan horse (140).

Non-viral plasmid DNA and genes encoding shRNA can be targeted to the brain with Trojan horse liposomes, which may be engineered with shRNA expression vectors driven by the U6 promoter and encoding a T5 terminator sequence for RNA polymerase III at the 3′-end of the shRNA (141). **Z**hang et al. demonstrated the production of THL packaged with anti-luciferase shRNA expression plasmids and an intra-cranial brain cancer model of rat glioma cells permanently transfected with the luciferase gene (129). It has been shown that the combination of the THL gene delivery system and shRNA expression plasmids allows for a 90 % knockdown of brain cancer specific gene expression. This effect persists for 5 days after a single intravenous injection of a low dose (10 μg/rat) of plasmid DNA (129). Other successful examples of using the combination of shRNA expression vectors and THL technology for the treatment of brain disease has been described (142). It used the shRNA construct and targeted the kinase domain of the epidermal growth factor receptor tyrosine kinase gene participating in the oncogenic growth of brain tumor. It has been shown that this RNAi gene therapy of brain tumors resulted in an 88% increase in survival time with weekly intravenous gene therapy using this construct in combination with the THL gene delivery system. Importantly, this increase in survival time was not due to a non-specific effect of THL administration because prior work had shown no change in survival with the weekly administration of Trojan horse liposomes carrying a luciferase expression plasmid gene (129). Thus, siRNA can be delivered to the brain *in vivo* with the MTH and avidin–biotin technology and shRNA expressing plasmid DNA delivered with Trojan horse liposomes. The genetic engineering of molecular Trojan horses has been completed, and this will enable future clinical applications in humans (132, 133).

6. RNAi THERAPY FOR NEURODEGENERATIVE DISEASES

The modern drug discovery process is a multidisciplinary approach that begins by identifying a specific medical need, such as treatment for neurodegenerative diseases RNAi methodology can facilitate each step of this process. Moreover, RNAi-based therapeutics can provide a promising new therapeutic strategy for many untreatable disorders. Excellent reviews have been published in 2007 describing the whole process of developing RNAi-based therapeutics starting from *in vitro* lead design and identification, to *in vivo* pre-clinical drug delivery and testing (20, 143, 144, 145 and others). For many important reasons, including the huge increase in the number of people dying every day as a result of neurological disorders, the lack of effective therapies for most of these disorders, and other reasons previously described in this review, interest has grown sharply in the use of a potential RNAi therapeutic for neurodegenerative diseases. By way of comparison, only a few reviews have been published over the last five years, but already this year (2007) seven excellent reviews have been published about the application of RNAi technology to different neurodegenerative diseases (19, 21, 22, 23, 81, 95). This chapter of the review will focus on the most important

aspects of RNAi therapy development for neurodegenerative diseases which the pharmaceutical industry will need to address in order to develop clinical applications of RNAi for future neuroprotective cures.

6.1. Overview of preclinical application of RNAi for neurodegenerative diseases

In general, the components of preclinical drug development (for example, RNAi-based therapy) can be divided into four general areas that include: 1) *in vitro* studies defining a new agent's pharmacologic properties, 2) drug supply and manufacturing, 3) drug formulation, and, finally, 4) *in vivo* studies in animal models demonstrating a potential for clinical efficacy and proof of therapeutic principle (146). *In vivo* animal models of neurodegenerative diseases are now generally employed for later testing after promising agents have been identified in preliminary drug screening and discovery programs. Nonetheless, studies in animal models are extremely important for demonstrating potential clinical efficacy and establishing proof of therapeutic principle. Also, preclinical animal studies are the first opportunity to define the toxicity profile of a new RNAi-based therapy and to perform detailed single-dose and multiple-dose toxicology studies. Practical issues that can also be addressed in animal models include the initial assessment of drug pharmacokinetics and pharmacodynamics as well as the determination of a safe starting dose and schedule of treatment for the initial Phase I clinical trials in humans (146). A number of animal model systems have been developed that mimic the clinical situations of the different neurodegenerative diseases. Based on RNAi therapeutic strategies (Figure 3, Chapter 5), neurodegenerative diseases can be divided into two groups: 1) diseases where the reduction of mutant or toxic gene expression may provide a therapeutic benefit. These neurodegenerative diseases are caused by dominant, gain-of-function types of gene mutations, for example, Prion diseases and any disease from the (polyQ)-repeat disease family, such as Huntington's disease (HD) and Spinocerebellar ataxia (SCA) (9, 10, 11, 147). 2) diseases where decreasing (or the complete knockout of) the expression of genes involved in disease pathology can have therapeutic effect, for example, down-regulation of APP, or other proteins involved in the formation of plaques in AD, or SOD1 which causes ALS, or DJ-1 mutations which cause Parkinson's syndrome and many others (123)**.** This part of Chapter 6 will briefly discuss pathological and clinical aspects of the most important examples of neurodegenerative diseases from each group, will summarize the different preclinical trials of therapeutic RNAi in animal disease models, and will address perspectives of the preclinical development of the RNAi therapeutic candidate for the treatment of these neurodegenerative diseases in humans.

6.1.1. The first RNAi therapeutic strategy – targets a dominant disease allele.

6.1.1.1. Prion diseases

Prion diseases or transmissible spongiform encephalopathies, are lethal for both humans and animals, characterized by neuronal loss, spongiform lesions, and astrogliosis (23). Despite many years of attempts to develop effective therapeutics for these disorders, until now no efficient drug has been commercially available for treatment or cure (148). Creutzfeldt–Jakob disease is the human form of prion diseases. A variety of evidence suggests that Prion diseases are characterized by the accumulation of an abnormal proteinase K–resistant isoform (PrPsc) of the host-encoded prion protein (PrPc) in the CNS (149). Thus, down-regulation or silencing of the prion protein gene (Prnp) may provide a therapeutic approach. Pfeifer et al mimicked the clinical aspects of prion diseases by generating chimeric mice derived from lentivector-transduced embryonic stem cells (150). Depending on the degree of chimerism, these animals carried the lentiviral shRNAs in a certain percentage of brain cells and expressed reduced levels of PrPc. Also, it has been shown that after intracranial injection, lentiviral shRNA reduced PrPc expression in transgenic mice carrying multiple copies of Prnp. It is important to note that survival of mice infected with scrapie, a model representing Creutzfeldt-Jakob disease in humans, for which no treatment is currently available, significantly increased after exposure to the appropriate RNAi molecules. This suggests that lentivector-mediated RNAi could be a promising approach for the treatment of prion diseases (148, 150). The problem is that a high proportion of cells (about 65% of prion genes need to be turned off) must be treated with RNAi before a curing effect can be observed. Unfortunately, if these observations are extrapolated to Creutzfeldt-Jakob patients, it is obvious that the approach is still a long way from a cure for prion diseases in humans (151). The 37-kDa/67-kDa laminin receptor (LRP/LR) acts as a receptor for the PrPc and the PrPsc, the abnormal form of the prion protein (152). Leucht et al have generated transgenic mice ectopically expressing antisense LRP RNA in the brain under control of the neuron-specific enolase promoter (153). A significant reduction of LRP/LR protein levels in hippocampal and cerebellar brain regions has been shown. Thus, these findings lead to the conclusion that gene silencing by RNAi targeting PrPc and LRP is a promising approach to slow the progression of prion diseases (148).

6.1.1.2. The (polyQ)-repeat disease

The (polyQ)-repeat disease family are autosomal-dominant neurodegenerative diseases that result from multiplication of cytosine-adenine-guanine triplets expanding within the genes. The consequent toxic gain of function of mutant expanded proteins, polyQ-repeats, leads to neuronal intranuclear inclusions and neurodegeneration (154). Despite the fact that many therapeutic approaches have been created for this group of diseases, the ideal therapy has to involve reduction of the respective mutant proteins. Thus, the RNAi approach is a promising tool for developing a powerful therapeutic strategy for this group of diseases (155). For two diseases from this family, HD and SCA, preclinical trials in animal models have already been completed (21).

6.1.1.3. Huntington's disease

Huntington's disease is a fatal progressive neurodegenerative disorder in which affected individuals

suffer from involuntary movement disorder (chorea) and behavioral and cognitive changes (156). HD is associated with the expansion of a CAG repeat codon in the first exon of the gene coding the protein huntingtin (Htt). This polyQ-repeat expansion (CAG is a codon for Gln) in exon 1 of Htt results in a toxic gain of function of Htt, which causes neuronal cell death (95). Currently, HD is one of the most intensively studied neurodegenerative diseases in the field of experimental RNAi therapeutics (21, 81, 157). Several mouse models of HD have been generated and used to test attractive therapies (19). RNAi has been demonstrated to improve motor deficits and neuropathological abnormalities in various murine models of HD (17, 158). Harper et al. have shown that shRNA against mutant human Htt reduced Htt mRNA and protein expression in transgenic HD mouse brain (17). Importantly, Htt gene silencing improved behavioral and neuropathological abnormalities associated with HD, providing support for the further development of RNAi for HD therapy. Another example of using shRNA HD in young transgenic mouse brains (6-8 weeks old) has been described in a paper by Rodriguez-Lebron et al. (158). Treated HD mice had reductions in both mRNA (by 78%) and mutant protein (by 28%). Moreover, shRNA-HD had decreased neuronal inclusions in HD mice. These results demonstrate that even a limited down-regulation of protein level results in significant functional improvement (95, 158). Other research groups have shown similar data (159). Also, Wang et al. performed a single intraventricular injection of naked siRNA in the early postnatal period by using a nonviral delivery approach (160). Transient suppression of Htt expression and remarkably long-lasting beneficial effect on pathological and behavioral effects has been shown. Thus, all these studies demonstrate, using different delivery methods, animal models and experimental conditions, that the RNAi approach effectively suppresses expression of human mutant Htt and reduces the presence of neuronal inclusions in the mammalian brain. Unfortunately, the requirement for widespread delivery and the need for allele specificity, not addressed by any of these reports, represent the major problems that need to be solved before planning HD therapeutic trials in humans (21). Currently, Targeted Genetics Corporation (www.targen.com) is working with Sirna/Merck on the preclinical development of an AAV-based RNAi therapeutic candidate for the treatment of HD. Their goal is to enter human clinical trials in the first half of 2008.

6.1.1.4. Spinocerebellar ataxia Type 1

Spinocerebellar ataxia Type 1 (SCA1) is a neurodegenerative disease characterized by ataxia (lack of coordinated movement), dysarthria (difficulty in articulating words) and progressive bulbar dysfunction (difficulty in speech and swallowing) (156). Similar to HD, SCA1 is a fatal polyQ disease. In SCA1, an abnormal CAG increase in the gene encoding ataxin1 relates to selective degeneration of Purkinje cells in the cerebellar cortex (161). The transgenic mouse model expressing mutant SCA1 gene exhibits ataxia, neuronal inclusions, and Purkinje cell loss with other severe neurological deficits (19, 162, 163). It has been shown that intracerebellar injection of shRNA against SCA1 delivered through adeno-associated virus improved motor coordination, cellular morphology, and reduced pathological inclusions in Purkinje cells in a mouse model of SCA1 (11). Remarkably, even though only about 10% of Purkinje cells in the lobules were affected by the therapeutic shRNA vector, they achieved very significant improvements in pathological and motor phenotypes. This data demonstrated that a limited down regulation of protein level of SCA1 still results in significant functional improvement. A similar effect was seen with shRNA-HD vector applied to young transgenic mice brains (158). This example was described above. Thus, these findings prove the significant therapeutic effect of the RNAi approach for neurodegenerative diseases. However, in order to apply a potential RNAi therapeutic candidate, for example shRNA against SCA1 in humans, there has to be a significant improvement shown by the therapeutic agent. For example, the cerebellum in humans is proportionally larger than in mice, by a significant extent. Thus, the demonstration of effective Purkinje cell transduction by this vector (shRNA-SCA1) in other animals before applying the method to humans would help determine the number and location of injections required to achieve a similar level of transduction (21). Next, the transgenic mouse model used in this study carried the mutant human ataxin-1 transgene and two copies of normal mouse ataxin-1 (11). In these mice, it was possible to specifically silence the human mutant allele. However, in SCA1 disease in humans, this RNAi therapeutic candidate (shRNA-SCA1) would silence both the wild-type and mutant alleles. This could cause unpleasant site effects. One strategy to avoid this problem is to target a single nucleotide polymorphism that segregates with the mutant allele of polyQ-repeat disease gene rather than the CAG repeat mutation itself (120). This strategy was successfully applied with the gene for Machado-Joseph disease (SCA3) (155). This study established that siRNA could be engineered to silence disease genes differing by a single nucleotide and suggested a possible role for SNPs in the RNAi approach in dominantly inherited disorders. Another problem with applying the RNAi approach in humans (specifically for SCA1 disease) is fairly widespread brain degeneration involving both the cerebellum and brainstem in this disorder. The brainstem is a very difficult target for RNAi delivery. Moreover, a successful preclinical RNAi study was performed in transgenic mice in which the mutant gene was only expressed in the cerebellum (Purkinje cells). Thus, additional studies in SCA1 knock-in mice, which more reliably recreate the widespread pattern of neurodegeneration in humans and express the disease allele at physiological levels, might be helpful before a clinical trial is initiated in humans (81).

6.1.2. The second RNAi therapeutic strategy – target the expression of genes involved in disease pathology

6.1.2.1. Alzheimer's disease

AD is the most common cause of dementia in humans. This disorder is an age related and fatal neurodegenerative disease seen with increasing frequency in recent decades due to increased life expectancy and changing population demographics (the aging of Baby Boomers). Extracellular amyloid plaques formed by accumulated Aβ, a metabolic

product of APP, are a pathological marker in the brain of AD patients. More generally, AD is associated with increased β-secretase and/or γ-secretase activities, neurodegeneration and accumulation of APP products (95). In neurons, β-secretase activity is mediated by an aspartyl protease cleaving enzyme named β-site APP Cleaving Enzyme1 (BACE1) (164). Transgenic models that develop Aβ deposits and neuritic plaques have provided valuable experimental tools for understanding the mechanisms of AD and provide an opportunity to test potential therapies (165, 166). The recent review of Federici and Boulis summarized the potential therapeutic targets for RNAi-based treatment of AD which include BACE1 (the main target for siRNA intervention) and APP, neprilysin, and tau genes, which are also related to the formation of amyloid plaques (23). Singer et al. used a mouse model of amyloidosis (mutant APP mice) (167). It has been shown that lowering BACE1 levels using lentiviral vectors expressing β-secretase-directed siRNAs reduced Aβ production and the neurodegenerative and behavioral deficits in an Alzheimer's model in APP transgenic mice. This finding indicated that inhibiting BACE1 can reduce existing pathology and behavioral deficits in different amyloidosis mouse models, and emphasized the potential for RNAi against BACE1 as a therapeutic approach for AD. Tau is a microtubule-associated protein. Tauopathies are a group of neurodegenerative diseases, including AD, characterized by mutations in the tau gene. siRNA-based strategies to target missense Tau mutations can be useful in the treatment of these types of diseases (23). siRNAs and shRNAs have been generated that successfully silence the Tau mutation V337M *in vitro*, indicating that this approach can be useful in experimental and therapeutic studies of AD (155, 168). Another potential therapeutic target for the treatment of AD is APP. Miller et al. constructed replication-defective herpes simplex virus vectors carrying siRNA expression cassettes to target APP that successfully silenced the mutant APP allele in AD mouse models (168). The same approach has been used to down-regulate the neprilysin gene, which is a potent β-amyloid–degrading protease (169). All these findings demonstrated that BACE1, APP, neprilysin, and tau genes could be potential therapeutic targets for RNAi-based treatment of AD.

6.1.2.2. Parkinson's disease

PD is one of the most common neurodegenerative diseases, characterized clinically by the cardinal features of resting tremor, rigidity, bradykinesia, and gait difficulty with postural instability. One of the reasons for motor dysfunction, in the early stages, is the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta (170). Despite advances in understanding the pathogenesis of PD, the progress in development of a therapy to delay or reverse disease progression is slow. Currently, no treatment that shows neuroprotective effect for PD is available (171). Thus, clinical and basic research focused on identifying neuroprotective strategies for the development of specific therapies for PD is a high priority. The main characteristics of PD are the loss of pigmented dopaminergic neurons with the appearance of characteristic cytoplasmic protein aggregates known as Lewy bodies and dystrophic neurites in the substantia nigra pars compacta (SNc) (172). Thus, the SNc, a relatively small midbrain structure, would be the major anatomical target in PD. Hommel et al. created a viral-mediated shRNA vector targeting tyrosine hydroxylase within midbrain neurons of adult mice (122). Serious behavioral changes, including a motor performance deficit and reduced response to a psychostimulant were observed in mice injected with this vector (122). Another potential molecular target for therapeutics' in PD could be α-synuclein, which is one of the core proteins identified in Lewy bodies (173). Viral vector-based RNAi has demonstrated effective silencing of human α-synuclein expression *in vivo* in rat brain (174). Despite the fact that this study showed successful suppression of overexpressed α-synuclein in neurons, it did not address its efficacy in reversing the pathological and behavioral expression of PD (21). Thus, future studies should be performed in different animal models of PD, covering the different pathological and behavioral abnormalities. An additional target in PD could be the leucine-rich repeat kinase 2 (LRRK2) gene encoding dardarin. It has been discovered that the mutation in this gene can frequently cause PD (175). LRRK2 is extraordinarily large and complex, with multiple enzymatic and protein-interaction domains, each of which is targeted by pathogenic mutations in familial PD (176). Thus, additional targets could be identified to develop RNAi based therapies that could be applied to a large number of patients.

The major complication for the development of therapeutic RNAi for AD and PD is the effective delivery of a potential RNAi therapeutic across the BBB. A promising alternative approach for delivery of siRNA (or shRNA) to the CNS is THL technology (Chapter 5.2.1). As was described above, the efficiency of THL technology for the delivery of therapeutic genes to the brain has been successfully tested in the rat and the mouse PD model (134, 142). The genetic engineering of THL technology has been completed, and this will allow future clinical applications in humans (132, 133). Also, it is important to emphasize that when an optimal therapeutic agent for AD and PD is identified in mouse models, it should be tested in nonhuman primates to ensure appropriate distribution and safety before moving onto human trials (81).

6.1.2.3. Amyotrophic Lateral Sclerosis

ALS is the most common motor neuron disease in humans, also known as Lou Gehrig's disease (177). It is a progressive neurodegenerative disease which is characterized by progressive weakness, muscle atrophy, and spasticity caused by selective loss of motor neurons in specific regions of the brain and spinal cord (178). There are still no other effective therapies 10 years after the approval of riluzole for the treatment of ALS, but advances in drug development and screening are substantially increasing the number of potential therapeutic agents (179). The majority (90-95%) of the disease cases are potentially sporadic in nature. Only 5–10% of patients with ALS have a genetic form of the disease, usually inherited in a dominant manner; of those, 20% carry a dominant inherited mutation in Cu/Zn superoxide dismutase 1 (SOD1) in the gene encoding SOD1 (180). There are many lines of mutant

SOD1 mice that have been generated and characterized (181, 182, 183). Three different papers have described the effectiveness of shRNA against SOD1 in mutant SOD1 mice (184, 185, 186). These findings concluded that shRNA against SOD1 delayed ALS pathology and motor deficits in SOD1 mice, providing evidence that the RNAi approach may be effective therapy for ALS caused by the mutant SOD1. In support of this conclusion, multiple abnormalities in different organs, including the nervous system, were found in mice lacking SOD1 (187). The challenge of creating a potential RNAi therapeutic for ALS is due to the large number (more than 100) of different SOD1 mutations which have been identified in ALS. Thus, the targeting construct should be described for specific families because a single therapeutic vector would not be helpful. An overview of recent *in vivo* therapeutic trials for ALS was described (81). All of these therapeutic trials have been performed in transgenic mouse models that overexpress human SOD1 carrying the disease-linked mutation G93A. Generally, these studies demonstrated that suppressing expression of mutant SOD1 in motor neurons in early stages of disease (before behavioral and pathological abnormalities) significantly delays the beginning of ALS and extends life, even in the absence of allele specificity (81). As for any neurodegenerative diseases, a potential therapeutic agent for ALS, which was identified in mouse models, should be tested in nonhuman primates. Smith et al. used antisense technology to target SOD1 in rodents and primates through continuous intraventricular infusion near to disease onset (188). This work demonstrates the potential for developing a similar delivery method for modified siRNA and applying this approach to rodents and primates which is a necessary step before moving onto human trials.

6.2 Commercial potential of RNAi

The commercial potential of RNAi can apply in three areas: (A) drug discovery and research (currently the biggest input); (B) potential therapeutic applications; and (C) the role of miRNA in molecular diagnostics (189). The role of RNAi technology in areas (A) and (B) has been described in this review in detail. The role of microRNA in molecular diagnostics is related mainly to cancer which is beyond the main direction of this review. The RNAi market for drug discovery and research is relatively young with global sales of approximately $447 million in 2006. It is projected to reach $900 million in sales by 2010, a compound annual growth rate of 19% between 2006-2010 (190). The evaluation of RNAi-based therapies in animal models and preclinical studies has accelerated dramatically in the last few years, and many companies have been established with the aim of developing the technology to create therapeutic agents. Currently, there are more than 140 companies involved in developing RNAi technologies in the USA. They are a mix of companies that supply reagents and technologies (nearly half of the total) and companies that use the technologies for drug discovery. Of these, 24 are developing RNAi-based therapeutics (191). An example of these companies is Sirna Therapeutics (www.sirna.com). Originally founded in 1992 as Ribozyme Pharmaceuticals, the company reorganized to focus on RNAi in 2003. One of the leading compounds of this company is Sirna-027, which is a RNAi-therapeutic for the treatment of age-related macular degeneration (AMD). Sirna has presented results of a phase I clinical trial of Sirna-027 that forms the basis for the initiation of a phase II clinical trial (192). The Acuity Pharmaceuticals (http://www.acuitypharma.com), another company involved in developing RNAi-based therapeutics, is also targeting AMD with siRNA, and has reported phase II results for their drug, bevasiranib, which targets the same gene as Sirna-027 - vascular endothelial growth factor (VEGF). The study confirmed the safety of the drug shown in earlier studies (192). The results demonstrated an effect at all doses with encouraging durability of response in patients with early aggressive AMD, and the company notes that it is moving toward a phase III study of bevasiranib in combination with VEGF antagonists. Thus, even though there is no RNAi-based drug on the market, it is projected that some may reach the market in the next few years and by the year 2010 will be worth $3.5 billion in a total pharmaceutical market of $560 billion (189). One of the encouraging signs for the commercial potential of RNAi technology is the involvement in this field of Big Pharma, for example, Merck. Merck has been involved in the field since its 2001 acquisition of Rosetta Inpharmatics (192). This year, Merck has announced the acquisition of Sirna. Merck is also collaborating with Alnylam (www.alnylam.com), another major player in the RNAi field, to develop RNAi treatments for spinal cord injury. Based on Jain PharmaBiotech Publications, the RNAi-based therapeutic market will grow to $10.5 billion by the year 2015 when the total pharmaceutical market will be worth $892 billion (189). Currently, the main use of RNAi technologies in the neurodegenerative field is in reagents for research and in finding potential targets for drug discovery. Initial animal studies give us optimism about the future of clinical application of this therapeutic approach to humans suffering from neurodegenerative diseases. These applications form the basis of estimation of commercial potential of RNAi-based therapeutics and estimation of markets. The RNAi approach can interact with some of the other technologies such as drug delivery and molecular diagnostics. This approach has a lot of advantages over traditional pharmaceuticals which were described in detail in this review. Thus, all of these futures of the RNAi technology show that the commercial prospects of RNAi-based therapies are presumed to be good.

6.3 Challenges in developing RNAi therapy for neurodegenerative diseases

Translation of any promising therapy from the laboratory into an approved clinical application involves technical and regulatory issues, as well as intellectual properties. Technical issues cover all challenges in developing RNAi therapy for clinical applications which include safety and efficacy. The initial studies on therapeutic RNAi for neurodegenerative diseases have investigated the efficacy of this approach to prevent or reverse pathological phenotypes such as off-target effects (81). Two separate studies in cellular systems demonstrated widespread silencing of off target effects triggered by the introduction of siRNA constructs into cells (193, 194). Unfortunately, these off target effects could cascade

downstream, causing divergent changes in the levels of unrelated proteins (120). Off target effects can be minimized by selecting siRNAs with minimal matches to off-target genes. Moreover, chemical modification of the base at position 2 in the guide strand of the siRNA has been shown to significantly reduce off-target effects (195). Also, several software packages have been created to analyze siRNAs for off-target effects and can be used for more effective siRNA design (196).

The main reason for unexpected changes in non-targeted gene expression levels could be the activation of immune responses or the saturation of endogenous RNAi machinery which are safety concerns of RNAi technology. An interferon response (IR), which can be activated by dsRNA, results in a signaling cascade activation of interferon responsive genes (IRG) which cause global translational repression (197). There is controversial data published in the literature about the role of different types of RNAi-mediating constructs in activation of IRG. Some researchers showed that RNAi-mediating constructs trigger the IR through the activation of IRG whereas others failed to find this effect in vivo (198, 199, 200). Thus, a check of the state of the IR in the targeted tissue should be performed when using the RNAi therapeutic molecule for neurodegenerative diseases. Gonzalez-Alegre et al. showed that a therapeutic lentiviral vector for early-onset primary dystonia did not trigger an IR in mammalian neuronal cultures, although it did achieve effective target silencing (201). Another work showed also no the IR activation in mouse models of ALS expressing shRNA targeting mutant SOD1 (187). Therefore, there is no solid evidence that neuronal IR activation is caused by RNAi-mediating constructs. Thus, the IR does not seem to cause a significant challenge for the development of therapeutic RNAi. Another challenge of safety issue for the development the RNAi therapy is preventing saturation of endogenous silencing pathways (RNAi machinery). Two studies have shown that abundant shRNA expression can be toxic. Grimm et al showed that high-level expression of shRNA delivery vector based on adeno-associated virus caused saturation of exportin 5 and the resulting inhibition of endogenous pre-miRNA nuclear export (56). It caused death in 23 of 49 mice. Importantly, administering lower doses of shRNA-expressing AAV vectors was non-lethal and effective in prolonged therapeutic intervention against hepatitis B virus (20). Another study in human primary lymphocytes demonstrated that U6 promoter-driven shRNAs induced cytotoxicity, whereas using a weaker H1 promoter for expression of the same shRNAs seemed to eliminate the toxic effect (202). These findings showed the importance of the optimization of shRNA expression levels in RNAi therapeutics.

Another challenging part of developing RNAi therapy for neurodegenerative diseases is related to regulatory issues. Currently, regulatory authorities (FDA) have no special guidelines for RNAi-based biological products. RNAi is related to and competes with antisense oligonucleotides and gene therapy approaches. Both of these related approaches have their own guidelines as special forms of biotechnology. RNAi is more closely related to gene therapy than to antisense oligonucleotides technology. Currently, the gene therapy approach is strictly regulated by the FDA due to a few misfortunes in the past years. Therefore, in order to get FDA approval for any new RNAi therapeutic for neurodegenerative diseases it is better to follow guidelines which were specifically created for this type of therapy. Hopefully, these guidelines will established by FDA in the close future.

Intellectual property of RNAi technology is a very complicated issue and should be described independently in a specific review. Currently, only one review has been published regarding the issue of RNAi patents (203). According to this paper more than 2,000 RNAi patent applications for new inventions have been filed with the US Patent and Trademark Office and only a few have been issued. Due to the Supreme Court's 2005 decision in Merck verses Integra, biomedical scientists can use patented inventions for research as long as they're working towards an approved drug (203). Thus, although currently RNAi researchers don't have to worry much about patent infringement, this situation could change in the future when some RNAi-based drug reaches the market.

7. CONCLUSIONS AND PERSPECTIVES

RNAi is a biological process of gene silencing that takes place in different organisms from plants to mammals. Due to the natural procedure of RNAi occurring in our cells, a new class of medicines, known as RNAi therapeutics, has been developed. RNAi therapeutics (RNAi technology) targets the cause of diseases by suppressing the expression of disease-causing proteins through silencing specific mRNAs. Thus, RNAi technology has the potential to treat disease and help patients in a fundamentally new way. It is remarkable that less than a decade after its discovery, RNAi has generated such a wealth of data, activity and promise of clinical application. The transition of RNAi therapy from research to clinical trials (currently, the drug bevasiranib from Acuity Pharmaceuticals which targets AMD with siRNA has successfully passed its phase II clinical trial) within the last few years was much faster than that of other new technologies. Last year developments in RNAi technology resulted in a lot of progress in the neuroscience field which included new strategies for effective delivery of siRNA to neurons (an *in vitro* approach) and to the CNS (an *in vivo* approach). Currently, there are no effective cures for neurodegenerative diseases. The limitations in clinical options for many neurological disorders necessitate the design of new experimental approaches, such as gene therapy and RNAi application. Recent progress in preclinical trials of therapeutic RNAi in animal models of neurodegenerative diseases allow us to be optimistic about the future application of this therapeutic approach to humans suffering from these types of diseases.

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9. REFERENCES

1. Muresanu D.F.: Neuroprotection and neuroplasticity - a holistic approach and future perspectives. *J Neurol Sci* 257, 38-43 (2007)

2. Dykxhoorn D.M. and J. Lieberman: Running Interference: Prospects and Obstacles to Using Small Interfering RNAs as Small Molecule Drugs. *Annu Rev Biomed Eng* (2006)

3. Sah D.W.: Therapeutic potential of RNA interference for neurological disorders. *Life Sci* 79, 1773-80 (2006)

4. Sen C.K. and S. Roy: miRNA: licensed to kill the messenger. *DNA Cell Biol* 26, 193-4 (2007)

5. Kourtidis A., C. Eifert and D.S. Conklin: RNAi applications in target validation. *Ernst Schering Res Found Workshop*1-21 (2007)

6. Fire A., S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver and C.C. Mello: Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391, 806-11 (1998)

7. Elbashir S.M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494-8 (2001)

8. Bumcrot D., M. Manoharan, V. Koteliansky and D.W. Sah: RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol* 2, 711-9 (2006)

9. Kong Q.: RNAi: a novel strategy for the treatment of prion diseases. *J Clin Invest* 116, 3101-3 (2006)

10. Boudreau R.L. and B.L. Davidson: RNAi therapy for neurodegenerative diseases. *Curr Top Dev Biol* 75, 73-92 (2006)

11. Xia H., Q. Mao, S.L. Eliason, S.Q. Harper, I.H. Martins, H.T. Orr, H.L. Paulson, L. Yang, R.M. Kotin and B.L. Davidson: RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med* 10, 816-20 (2004)

12. Hardy J. and D.J. Selkoe: The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 297, 353-6 (2002)

13. Smith W.W., H. Jiang, Z. Pei, Y. Tanaka, H. Morita, A. Sawa, V.L. Dawson, T.M. Dawson and C.A. Ross: Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. *Hum Mol Genet* 14, 3801-11 (2005)

14. Laird F.M., H. Cai, A.V. Savonenko, M.H. Farah, K. He, T. Melnikova, H. Wen, H.C. Chiang, G. Xu, V.E. Koliatsos, D.R. Borchelt, D.L. Price, H.K. Lee and P.C. Wong: BACE1, a major determinant of selective vulnerability of the brain to amyloid-beta amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J Neurosci* 25, 11693-709 (2005)

15. Huttunen H.J., C. Greco and D.M. Kovacs: Knockdown of ACAT-1 reduces amyloidogenic processing of APP. *FEBS Lett* 581, 1688-92 (2007)

16. Azzouz M.: Gene Therapy for ALS: progress and prospects. *Biochim Biophys Acta* 1762, 1122-7 (2006)

17. Harper S.Q., P.D. Staber, X. He, S.L. Eliason, I.H. Martins, Q. Mao, L. Yang, R.M. Kotin, H.L. Paulson and B.L. Davidson: RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc Natl Acad Sci U S A* 102, 5820-5 (2005)

18. Korth C. and P.J. Peters: Emerging pharmacotherapies for Creutzfeldt-Jakob disease. *Arch Neurol* 63, 497-501 (2006)

19. Farah M.H.: RNAi silencing in mouse models of neurodegenerative diseases. *Curr Drug Deliv* 4, 161-7 (2007)

20. Kim D.H. and J.J. Rossi: Strategies for silencing human disease using RNA interference. *Nat Rev Genet* 8, 173-84 (2007)

21. Gonzalez-Alegre P.: Therapeutic RNA interference for neurodegenerative diseases: From promise to progress. *Pharmacol Ther* 114, 34-55 (2007)

22. Jagannath A. and M. Wood: RNA interference based gene therapy for neurological disease. *Brief Funct Genomic Proteomic* 6, 40-9 (2007)

23. Federici T. and N.M. Boulis: Ribonucleic acid interference for neurological disorders: candidate diseases, potential targets, and current approaches. *Neurosurgery* 60, 3-15; discussion 15-6 (2007)

24. Rana T.M.: Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol* 8, 23-36 (2007)

25. Lee R.C., C.M. Hammell and V. Ambros: Interacting endogenous and exogenous RNAi pathways in Caenorhabditis elegans. *RNA* 12, 589-97 (2006)

26. Bouche N., D. Lauressergues, V. Gasciolli and H. Vaucheret: An antagonistic function for Arabidopsis DCL2 in development and a new function for DCL4 in generating viral siRNAs. *EMBO J* 25, 3347-56 (2006)

27. Watanabe T., A. Takeda, T. Tsukiyama, K. Mise, T. Okuno, H. Sasaki, N. Minami and H. Imai: Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes

and germline small RNAs in testes. *Genes Dev* 20, 1732-43 (2006)

28. Scherr M. and M. Eder: Gene silencing by small regulatory RNAs in mammalian cells. *Cell Cycle* 6, 444-9 (2007)

29. van Rij R.P. and R. Andino: The silent treatment: RNAi as a defense against virus infection in mammals. *Trends Biotechnol* 24, 186-93 (2006)

30. Kim V.N.: MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol* 6, 376-85 (2005)

31. Filipowicz W.: RNAi: the nuts and bolts of the RISC machine. *Cell* 122, 17-20 (2005)

32. Bernstein E., A.A. Caudy, S.M. Hammond and G.J. Hannon: Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363-6 (2001)

33. Matranga C., Y. Tomari, C. Shin, D.P. Bartel and P.D. Zamore: Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* 123, 607-20 (2005)

34. Liu J., M.A. Carmell, F.V. Rivas, C.G. Marsden, J.M. Thomson, J.J. Song, S.M. Hammond, L. Joshua-Tor and G.J. Hannon: Argonaute2 is the catalytic engine of mammalian RNAi. *Science* 305, 1437-41 (2004)

35. Rand T.A., S. Petersen, F. Du and X. Wang: Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* 123, 621-9 (2005)

36. Hammond S.M., S. Boettcher, A.A. Caudy, R. Kobayashi and G.J. Hannon: Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 293, 1146-50 (2001)

37. Martinez J., A. Patkaniowska, H. Urlaub, R. Luhrmann and T. Tuschl: Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 110, 563-74 (2002)

38. Parker J.S., S.M. Roe and D. Barford: Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature* 434, 663-6 (2005)

39. Ma J.B., K. Ye and D.J. Patel: Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* 429, 318-22 (2004)

40. Elbashir S.M., W. Lendeckel and T. Tuschl: RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 15, 188-200 (2001)

41. Orban T.I. and E. Izaurralde: Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *RNA* 11, 459-69 (2005)

42. Hutvagner G. and P.D. Zamore: A microRNA in a multiple-turnover RNAi enzyme complex. *Science* 297, 2056-60 (2002)

43. Matzke M.A. and J.A. Birchler: RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 6, 24-35 (2005)

44. Wassenegger M.: The role of the RNAi machinery in heterochromatin formation. *Cell* 122, 13-6 (2005)

45. Huettel B., T. Kanno, L. Daxinger, E. Bucher, J. van der Winden, A.J. Matzke and M. Matzke: RNA-directed DNA methylation mediated by DRD1 and Pol IVb: a versatile pathway for transcriptional gene silencing in plants. *Biochim Biophys Acta* 1769, 358-74 (2007)

46. Zamore P.D., T. Tuschl, P.A. Sharp and D.P. Bartel: RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 101, 25-33 (2000)

47. Hemann M.T., J.S. Fridman, J.T. Zilfou, E. Hernando, P.J. Paddison, C. Cordon-Cardo, G.J. Hannon and S.W. Lowe: An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. *Nat Genet* 33, 396-400 (2003)

48. Rubinson D.A., C.P. Dillon, A.V. Kwiatkowski, C. Sievers, L. Yang, J. Kopinja, D.L. Rooney, M. Zhang, M.M. Ihrig, M.T. McManus, F.B. Gertler, M.L. Scott and L. Van Parijs: A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 33, 401-6 (2003)

49. Cullen B.R.: Induction of stable RNA interference in mammalian cells. *Gene Ther* 13, 503-8 (2006)

50. Amarzguioui M., J.J. Rossi and D. Kim: Approaches for chemically synthesized siRNA and vector-mediated RNAi. *FEBS Lett* 579, 5974-81 (2005)

51. Paul C.P., P.D. Good, I. Winer and D.R. Engelke: Effective expression of small interfering RNA in human cells. *Nat Biotechnol* 20, 505-8 (2002)

52. Paddison P.J. and G.J. Hannon: RNA interference: the new somatic cell genetics? *Cancer Cell* 2, 17-23 (2002)

53. Yi R., B.P. Doehle, Y. Qin, I.G. Macara and B.R. Cullen: Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. *RNA* 11, 220-6 (2005)

54. Bohnsack M.T., K. Czaplinski and D. Gorlich: Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA* 10, 185-91 (2004)

55. Kim J., A. Krichevsky, Y. Grad, G.D. Hayes, K.S. Kosik, G.M. Church and G. Ruvkun: Identification of many

microRNAs that copurify with polyribosomes in mammalian neurons. *Proc Natl Acad Sci U S A* 101, 360-5 (2004)

56. Grimm D., K.L. Streetz, C.L. Jopling, T.A. Storm, K. Pandey, C.R. Davis, P. Marion, F. Salazar and M.A. Kay: Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441, 537-41 (2006)

57. Gregory R.I., T.P. Chendrimada, N. Cooch and R. Shiekhattar: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* 123, 631-40 (2005)

58. Siolas D., C. Lerner, J. Burchard, W. Ge, P.S. Linsley, P.J. Paddison, G.J. Hannon and M.A. Cleary: Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* 23, 227-31 (2005)

59. Bartel D.P.: MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-97 (2004)

60. Wienholds E. and R.H. Plasterk: MicroRNA function in animal development. *FEBS Lett* 579, 5911-22 (2005)

61. Ambros V.: The functions of animal microRNAs. *Nature* 431, 350-5 (2004)

62. Neilson J.R., G.X. Zheng, C.B. Burge and P.A. Sharp: Dynamic regulation of miRNA expression in ordered stages of cellular development. *Genes Dev* 21, 578-89 (2007)

63. Visvanathan J., S. Lee, B. Lee, J.W. Lee and S.K. Lee: The microRNA miR-124 antagonizes the anti-neural REST/SCP1 pathway during embryonic CNS development. *Genes Dev* 21, 744-9 (2007)

64. Miska E.A., E. Alvarez-Saavedra, M. Townsend, A. Yoshii, N. Sestan, P. Rakic, M. Constantine-Paton and H.R. Horvitz: Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol* 5, R68 (2004)

65. Wheeler G., S. Ntounia-Fousara, B. Granda, T. Rathjen and T. Dalmay: Identification of new central nervous system specific mouse microRNAs. *FEBS Lett* 580, 2195-200 (2006)

66. Cheng H.Y., J.W. Papp, O. Varlamova, H. Dziema, B. Russell, J.P. Curfman, T. Nakazawa, K. Shimizu, H. Okamura, S. Impey and K. Obrietan: microRNA modulation of circadian-clock period and entrainment. *Neuron* 54, 813-29 (2007)

67. Lukiw W.J.: Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *Neuroreport* 18, 297-300 (2007)

68. Valencia-Sanchez M.A., J. Liu, G.J. Hannon and R. Parker: Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes Dev* 20, 515-24 (2006)

69. Lee Y., M. Kim, J. Han, K.H. Yeom, S. Lee, S.H. Baek and V.N. Kim: MicroRNA genes are transcribed by RNA polymerase II. *EMBO J* 23, 4051-60 (2004)

70. Borchert G.M., W. Lanier and B.L. Davidson: RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* 13, 1097-101 (2006)

71. Lee Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V.N. Kim: The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415-9 (2003)

72. Yi R., Y. Qin, I.G. Macara and B.R. Cullen: Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* 17, 3011-6 (2003)

73. Lund E., S. Guttinger, A. Calado, J.E. Dahlberg and U. Kutay: Nuclear export of microRNA precursors. *Science* 303, 95-8 (2004)

74. Preall J.B. and E.J. Sontheimer: RNAi: RISC gets loaded. *Cell* 123, 543-5 (2005)

75. Doench J.G., C.P. Petersen and P.A. Sharp: siRNAs can function as miRNAs. *Genes Dev* 17, 438-42 (2003)

76. Zeng Y., R. Yi and B.R. Cullen: MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc Natl Acad Sci U S A* 100, 9779-84 (2003)

77. Rodriguez-Lebron E. and H.L. Paulson: Allele-specific RNA interference for neurological disease. *Gene Ther* 13, 576-81 (2006)

78. Liu J., M.A. Valencia-Sanchez, G.J. Hannon and R. Parker: MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* 7, 719-23 (2005)

79. Sen G.L. and H.M. Blau: Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol* 7, 633-6 (2005)

80. Pillai R.S., S.N. Bhattacharyya, C.G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand and W. Filipowicz: Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science* 309, 1573-6 (2005)

81. Gonzalez-Alegre P. and H.L. Paulson: Technology insight: therapeutic RNA interference--how far from the neurology clinic? *Nat Clin Pract Neurol* 3, 394-404 (2007)

82. Aronin N.: Target selectivity in mRNA silencing. *Gene Ther* 13, 509-16 (2006)

83. WorldHealthOrganization: Neurological disorders: public health challenges

http://www.who.int/mental_health/neurology/neurodiso/en/index.html (2007)

84. Visiongain F.: Neurodegenerative Disease Drug Market Analysis and Forecasts 2007. *http://www.marketresearch.com* (2007)

85. DeKosky S.T. and K. Marek: Looking backward to move forward: early detection of neurodegenerative disorders. *Science* 302, 830-4 (2003)

86. Shaw L.M., M. Korecka, C.M. Clark, V.M. Lee and J.Q. Trojanowski: Biomarkers of neurodegeneration for diagnosis and monitoring therapeutics. *Nat Rev Drug Discov* 6, 295-303 (2007)

87. Youdim M.B. and J.J. Buccafusco: Multi-functional drugs for various CNS targets in the treatment of neurodegenerative disorders. *Trends Pharmacol Sci* 26, 27-35 (2005)

88. Cardone M.: Prospects for gene therapy in inherited neurodegenerative diseases. *Curr Opin Neurol* 20, 151-8 (2007)

89. Farlow M.R. and J.L. Cummings: Effective pharmacologic management of Alzheimer's disease. *Am J Med* 120, 388-97 (2007)

90. Muramatsu S.: Gene therapy for Parkinson's disease. *Brain Nerve* 59, 425-30 (2007)

91. Cole G.M., B. Teter and S.A. Frautschy: Neuroprotective effects of curcumin. *Adv Exp Med Biol* 595, 197-212 (2007)

92. Ui-Tei K., Y. Naito, F. Takahashi, T. Haraguchi, H. Ohki-Hamazaki, A. Juni, R. Ueda and K. Saigo: Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res* 32, 936-48 (2004)

93. Banan M. and N. Puri: The ins and outs of RNAi in mammalian cells. *Curr Pharm Biotechnol* 5, 441-50 (2004)

94. Martineau H.M. and I.T. Pyrah: Review of the application of RNA interference technology in the pharmaceutical industry. *Toxicol Pathol* 35, 327-36 (2007)

95. Hoyer D.: RNA interference for studying the molecular basis of neuropsychiatric disorders. *Curr Opin Drug Discov Devel* 10, 122-9 (2007)

96. Tonges L., P. Lingor, R. Egle, G.P. Dietz, A. Fahr and M. Bahr: Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA* 12, 1431-8 (2006)

97. Davidson T.J., S. Harel, V.A. Arboleda, G.F. Prunell, M.L. Shelanski, L.A. Greene and C.M. Troy: Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation. *J Neurosci* 24, 10040-6 (2004)

98. Kiefer K., J. Clement, P. Garidel and R. Peschka-Suss: Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells. *Pharm Res* 21, 1009-17 (2004)

99. Morris M.C., L. Chaloin, F. Heitz and G. Divita: Translocating peptides and proteins and their use for gene delivery. *Curr Opin Biotechnol* 11, 461-6 (2000)

100. Audouy S. and D. Hoekstra: Cationic lipid-mediated transfection in vitro and in vivo (review). *Mol Membr Biol* 18, 129-43 (2001)

101. Gait M.J.: Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues. *Cell Mol Life Sci* 60, 844-53 (2003)

102. Futaki S., W. Ohashi, T. Suzuki, M. Niwa, S. Tanaka, K. Ueda, H. Harashima and Y. Sugiura: Stearylated arginine-rich peptides: a new class of transfection systems. *Bioconjug Chem* 12, 1005-11 (2001)

103. Crombez L., A. Charnet, M.C. Morris, G. Aldrian-Herrada, F. Heitz and G. Divita: A non-covalent peptide-based strategy for siRNA delivery. *Biochem Soc Trans* 35, 44-6 (2007)

104. Simeoni F., M.C. Morris, F. Heitz and G. Divita: Peptide-based strategy for siRNA delivery into mammalian cells. *Methods Mol Biol* 309, 251-60 (2005)

105. Seng S., H.K. Avraham, S. Jiang, S. Venkatesh and S. Avraham: KLHL1/MRP2 mediates neurite outgrowth in a glycogen synthase kinase 3beta-dependent manner. *Mol Cell Biol* 26, 8371-84 (2006)

106. Yang Y.C., C.H. Lin and E.H. Lee: Serum- and glucocorticoid-inducible kinase 1 (SGK1) increases neurite formation through microtubule depolymerization by SGK1 and by SGK1 phosphorylation of tau. *Mol Cell Biol* 26, 8357-70 (2006)

107. Dail M., M. Richter, P. Godement and E.B. Pasquale: Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion. *J Cell Sci* 119, 1244-54 (2006)

108. Hood C., A.L. Cunningham, B. Slobedman, A.M. Arvin, M.H. Sommer, P.R. Kinchington and A. Abendroth: Varicella-zoster virus ORF63 inhibits apoptosis of primary human neurons. *J Virol* 80, 1025-31 (2006)

109. Hagemann C., C. Meyer, J. Stojic, S. Eicker, S. Gerngras, S. Kuhnel, K. Roosen and G.H. Vince: High efficiency transfection of glioma cell lines and primary cells for overexpression and RNAi experiments. *J Neurosci Methods* 156, 194-202 (2006)

110. van Es H.H. and G.J. Arts: Biology calls the targets: combining RNAi and disease biology. *Drug Discov Today* 10, 1385-91 (2005)

111. Root D.E., N. Hacohen, W.C. Hahn, E.S. Lander and D.M. Sabatini: Genome-scale loss-of-function screening with a lentiviral RNAi library. *Nat Methods* 3, 715-9 (2006)

112. Chang K., S.J. Elledge and G.J. Hannon: Lessons from Nature: microRNA-based shRNA libraries. *Nat Methods* 3, 707-14 (2006)

113. Yang J.P., W. Fan, C. Rogers, J.E. Chatterton, J. Bliesath, G. Liu, N. Ke, C.Y. Wang, K. Rhoades, F. Wong-Staal and Q.X. Li: A novel RNAi library based on partially randomized consensus sequences of nuclear receptors: identifying the receptors involved in amyloid beta degradation. *Genomics* 88, 282-92 (2006)

114. Majercak J., W.J. Ray, A. Espeseth, A. Simon, X.P. Shi, C. Wolffe, K. Getty, S. Marine, E. Stec, M. Ferrer, B. Strulovici, S. Bartz, A. Gates, M. Xu, Q. Huang, L. Ma, P. Shughrue, J. Burchard, D. Colussi, B. Pietrak, J. Kahana, D. Beher, T. Rosahl, M. Shearman, D. Hazuda, A.B. Sachs, K.S. Koblan, G.R. Seabrook and D.J. Stone: LRRTM3 promotes processing of amyloid-precursor protein by BACE1 and is a positional candidate gene for late-onset Alzheimer's disease. *Proc Natl Acad Sci U S A* 103, 17967-72 (2006)

115. Fountaine T.M. and R. Wade-Martins: RNA interference-mediated knockdown of alpha-synuclein protects human dopaminergic neuroblastoma cells from MPP (+) toxicity and reduces dopamine transport. *J Neurosci Res* 85, 351-63 (2007)

116. Asanuma M., I. Miyazaki, Y. Higashi, F.J. Diaz-Corrales, M. Shimizu, K. Miyoshi and N. Ogawa: Suppression of p53-activated gene, PAG608, attenuates methamphetamine-induced neurotoxicity. *Neurosci Lett* 414, 263-7 (2007)

117. Trotta N., G. Orso, M.G. Rossetto, A. Daga and K. Broadie: The hereditary spastic paraplegia gene, spastin, regulates microtubule stability to modulate synaptic structure and function. *Curr Biol* 14, 1135-47 (2004)

118. Iwata A., M. Maruyama, T. Akagi, T. Hashikawa, I. Kanazawa, S. Tsuji and N. Nukina: Alpha-synuclein degradation by serine protease neurosin: implication for pathogenesis of synucleinopathies. *Hum Mol Genet* 12, 2625-35 (2003)

119. Pluta K., W. Diehl, X.Y. Zhang, R. Kutner, A. Bialkowska and J. Reiser: Lentiviral vectors encoding tetracycline-dependent repressors and transactivators for reversible knockdown of gene expression: a comparative study. *BMC Biotechnol* 7, 41-3 (2007)

120. Wood M., H. Yin and G. McClorey: Modulating the expression of disease genes with RNA-based therapy. *PLoS Genet* 3, 109-15 (2007)

121. Kumar P., H. Wu, J.L. McBride, K.E. Jung, M.H. Kim, B.L. Davidson, S.K. Lee, P. Shankar and N. Manjunath: Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 448, 39-43 (2007)

122. Hommel J.D., R.M. Sears, D. Georgescu, D.L. Simmons and R.J. DiLeone: Local gene knockdown in the brain using viral-mediated RNA interference. *Nat Med* 9, 1539-44 (2003)

123. Xia X.G., H. Zhou and Z. Xu: Promises and challenges in developing RNAi as a research tool and therapy for neurodegenerative diseases. *Neurodegener Dis* 2, 220-31 (2005)

124. Xia X.G., H. Zhou, H. Ding, B. Affar el, Y. Shi and Z. Xu: An enhanced U6 promoter for synthesis of short hairpin RNA. *Nucleic Acids Res* 31, 100-102 (2003)

125. Kunath T., G. Gish, H. Lickert, N. Jones, T. Pawson and J. Rossant: Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotechnol* 21, 559-61 (2003)

126. Zhou H., B.H. Falkenburger, J.B. Schulz, K. Tieu, Z. Xu and X.G. Xia: Silencing of the Pink1 gene expression by conditional RNAi does not induce dopaminergic neuron death in mice. *Int J Biol Sci* 3, 242-50 (2007)

127. Pardridge W.M.: Drug and gene delivery to the brain: the vascular route. *Neuron* 36, 555-8 (2002)

128. Shi N., R.J. Boado and W.M. Pardridge: Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm Res* 18, 1091-5 (2001)

129. Zhang Y., R.J. Boado and W.M. Pardridge: In vivo knockdown of gene expression in brain cancer with intravenous RNAi in adult rats. *J Gene Med* 5, 1039-45 (2003)

130. Zhang Y., F. Schlachetzki, Y.F. Zhang, R.J. Boado and W.M. Pardridge: Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism with intravenous nonviral gene therapy and a brain-specific promoter. *Hum Gene Ther* 15, 339-50 (2004)

131. Pardridge W.M.: Blood-brain barrier delivery. *Drug Discov Today* 12, 54-61 (2007)

132. Boado R.J.: Blood-brain barrier transport of non-viral gene and RNAi therapeutics. *Pharm Res* 24, 1772-87 (2007)

133. Pardridge W.M.: shRNA and siRNA delivery to the brain. *Adv Drug Deliv Rev* 59, 141-52 (2007)

134. Kaneda N., T. Sasaoka, K. Kobayashi, K. Kiuchi, I. Nagatsu, Y. Kurosawa, K. Fujita, M. Yokoyama, T.

Nomura, M. Katsuki and et al.: Tissue-specific and high-level expression of the human tyrosine hydroxylase gene in transgenic mice. *Neuron* 6, 583-94 (1991)

135. Pardridge W.M.: Drug targeting to the brain. *Pharm Res* 24, 1733-44 (2007)

136. Zhang Y. and W.M. Pardridge: Neuroprotection in transient focal brain ischemia after delayed intravenous administration of brain-derived neurotrophic factor conjugated to a blood-brain barrier drug targeting system. *Stroke* 32, 1378-84 (2001)

137. Zhang Y. and W.M. Pardridge: Conjugation of brain-derived neurotrophic factor to a blood-brain barrier drug targeting system enables neuroprotection in regional brain ischemia following intravenous injection of the neurotrophin. *Brain Res* 889, 49-56 (2001)

138. Song B.W., H.V. Vinters, D. Wu and W.M. Pardridge: Enhanced neuroprotective effects of basic fibroblast growth factor in regional brain ischemia after conjugation to a blood-brain barrier delivery vector. *J Pharmacol Exp Ther* 301, 605-10 (2002)

139. Lee H.J., Y. Zhang, C. Zhu, K. Duff and W.M. Pardridge: Imaging brain amyloid of Alzheimer disease in vivo in transgenic mice with an Abeta peptide radiopharmaceutical. *J Cereb Blood Flow Metab* 22, 223-31 (2002)

140. Suzuki T., Y. Zhang, Y.F. Zhang, F. Schlachetzki and W.M. Pardridge: Imaging gene expression in regional brain ischemia in vivo with a targeted (111in)-antisense radiopharmaceutical. *Mol Imaging* 3, 356-63 (2004)

141. Boado R.J.: RNA interference and nonviral targeted gene therapy of experimental brain cancer. *NeuroRx* 2, 139-50 (2005)

142. Zhang Y., Y.F. Zhang, J. Bryant, A. Charles, R.J. Boado and W.M. Pardridge: Intravenous RNA interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin Cancer Res* 10, 3667-77 (2004)

143. Natt F.: siRNAs in drug discovery: target validation and beyond. *Curr Opin Mol Ther* 9, 242-7 (2007)

144. de Fougerolles A., H.P. Vornlocher, J. Maraganore and J. Lieberman: Interfering with disease: a progress report on siRNA-based therapeutics. *Nat Rev Drug Discov* 6, 443-53 (2007)

145. Liu G., F. Wong-Staal and Q.X. Li: Development of new RNAi therapeutics. *Histol Histopathol* 22, 211-7 (2007)

146. Takimoto C.H. and S.H. Khleif: Preclinical Drug Development. In: *Principles of Clinical Pharmacology*. Ed A. J. Atkinson, C. H. Daniels, R. L. Dedrick, C. V. Grudzinskas&S. P. Markey. Academic Press, New York (2001)

147. Gatchel J.R. and H.Y. Zoghbi: Diseases of unstable repeat expansion: mechanisms and common principles. *Nat Rev Genet* 6, 743-55 (2005)

148. Ludewigs H., C. Zuber, K. Vana, D. Nikles, I. Zerr and S. Weiss: Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* 5, 613-30 (2007)

149. Glatzel M., K. Stoeck, H. Seeger, T. Luhrs and A. Aguzzi: Human prion diseases: molecular and clinical aspects. *Arch Neurol* 62, 545-52 (2005)

150. Pfeifer A., S. Eigenbrod, S. Al-Khadra, A. Hofmann, G. Mitteregger, M. Moser, U. Bertsch and H. Kretzschmar: Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest* 116, 3204-10 (2006)

151. D'Haeze W.: RNA Interferences. http://www.scienceboard.net/community/perspectives.183.html (2006)

152. Gauczynski S., D. Nikles, S. El-Gogo, D. Papy-Garcia, C. Rey, S. Alban, D. Barritault, C.I. Lasmezas and S. Weiss: The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* 194, 702-9 (2006)

153. Leucht C., K. Vana, I. Renner-Muller, D. Dormont, C.I. Lasmezas, E. Wolf and S. Weiss: Knock-down of the 37-kDa/67-kDa laminin receptor in mouse brain by transgenic expression of specific antisense LRP RNA. *Transgenic Res* 13, 81-5 (2004)

154. Everett C.M. and N.W. Wood: Trinucleotide repeats and neurodegenerative disease. *Brain* 127, 2385-405 (2004)

155. Miller V.M., H. Xia, G.L. Marrs, C.M. Gouvion, G. Lee, B.L. Davidson and H.L. Paulson: Allele-specific silencing of dominant disease genes. *Proc Natl Acad Sci U S A* 100, 7195-200 (2003)

156. Gusella J.F. and M.E. MacDonald: Molecular genetics: unmasking polyglutamine triggers in neurodegenerative disease. *Nat Rev Neurosci* 1, 109-15 (2000)

157. Denovan-Wright E.M. and B.L. Davidson: RNAi: a potential therapy for the dominantly inherited nucleotide repeat diseases. *Gene Ther* 13, 525-31 (2006)

158. Rodriguez-Lebron E., E.M. Denovan-Wright, K. Nash, A.S. Lewin and R.J. Mandel: Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol Ther* 12, 618-33 (2005)

159. Machida Y., T. Okada, M. Kurosawa, F. Oyama, K. Ozawa and N. Nukina: rAAV-mediated shRNA ameliorated neuropathology in Huntington disease

model mouse. *Biochem Biophys Res Commun* 343, 190-7 (2006)

160. Wang Y.L., W. Liu, E. Wada, M. Murata, K. Wada and I. Kanazawa: Clinico-pathological rescue of a model mouse of Huntington's disease by siRNA. *Neurosci Res* 53, 241-9 (2005)

161. Humbert S. and F. Saudou: Toward cell specificity in SCA1. *Neuron* 34, 669-70 (2002)

162. Clark H.B., E.N. Burright, W.S. Yunis, S. Larson, C. Wilcox, B. Hartman, A. Matilla, H.Y. Zoghbi and H.T. Orr: Purkinje cell expression of a mutant allele of SCA1 in transgenic mice leads to disparate effects on motor behaviors, followed by a progressive cerebellar dysfunction and histological alterations. *J Neurosci* 17, 7385-95 (1997)

163. Skinner P.J., C.A. Vierra-Green, H.B. Clark, H.Y. Zoghbi and H.T. Orr: Altered trafficking of membrane proteins in purkinje cells of SCA1 transgenic mice. *Am J Pathol* 159, 905-13 (2001)

164. Vassar R.: Beta-secretase (BACE) as a drug target for Alzheimer's disease. *Adv Drug Deliv Rev* 54, 1589-602 (2002)

165. Borchelt D.R., T. Ratovitski, J. van Lare, M.K. Lee, V. Gonzales, N.A. Jenkins, N.G. Copeland, D.L. Price and S.S. Sisodia: Accelerated amyloid deposition in the brains of transgenic mice coexpressing mutant presenilin 1 and amyloid precursor proteins. *Neuron* 19, 939-45 (1997)

166. Wong P.C., H. Cai, D.R. Borchelt and D.L. Price: Genetically engineered mouse models of neurodegenerative diseases. *Nat Neurosci* 5, 633-9 (2002)

167. Singer O., R.A. Marr, E. Rockenstein, L. Crews, N.G. Coufal, F.H. Gage, I.M. Verma and E. Masliah: Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat Neurosci* 8, 1343-9 (2005)

168. Miller V.M., C.M. Gouvion, B.L. Davidson and H.L. Paulson: Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. *Nucleic Acids Res* 32, 661-8 (2004)

169. Hong C.S., W.F. Goins, J.R. Goss, E.A. Burton and J.C. Glorioso: Herpes simplex virus RNAi and neprilysin gene transfer vectors reduce accumulation of Alzheimer's disease-related amyloid-beta peptide in vivo. *Gene Ther* 13, 1068-79 (2006)

170. Hung A.Y. and M.A. Schwarzschild: Clinical trials for neuroprotection in Parkinson's disease: overcoming angst and futility? *Curr Opin Neurol* 20, 477-83 (2007)

171. Suchowersky O., G. Gronseth, J. Perlmutter, S. Reich, T. Zesiewicz and W.J. Weiner: Practice Parameter: neuroprotective strategies and alternative therapies for Parkinson disease (an evidence-based review): report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 66, 976-82 (2006)

172. Braak H., K. Del Tredici, U. Rub, R.A. de Vos, E.N. Jansen Steur and E. Braak: Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24, 197-211 (2003)

173. Eriksen J.L., T.M. Dawson, D.W. Dickson and L. Petrucelli: Caught in the act: alpha-synuclein is the culprit in Parkinson's disease. *Neuron* 40, 453-6 (2003)

174. Sapru M.K., J.W. Yates, S. Hogan, L. Jiang, J. Halter and M.C. Bohn: Silencing of human alpha-synuclein in vitro and in rat brain using lentiviral-mediated RNAi. *Exp Neurol* 198, 382-90 (2006)

175. Paisan-Ruiz C., S. Jain, E.W. Evans, W.P. Gilks, J. Simon, M. van der Brug, A. Lopez de Munain, S. Aparicio, A.M. Gil, N. Khan, J. Johnson, J.R. Martinez, D. Nicholl, I.M. Carrera, A.S. Pena, R. de Silva, A. Lees, J.F. Marti-Masso, J. Perez-Tur, N.W. Wood and A.B. Singleton: Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44, 595-600 (2004)

176. Mata I.F., W.J. Wedemeyer, M.J. Farrer, J.P. Taylor and K.A. Gallo: LRRK2 in Parkinson's disease: protein domains and functional insights. *Trends Neurosci* 29, 286-93 (2006)

177. Irwin D., C.F. Lippa and J.M. Swearer: Cognition and Amyotrophic Lateral Sclerosis (ALS). *Am J Alzheimers Dis Other Demen* 22, 300-12 (2007)

178. Martin L.J., Z. Liu, K. Chen, A.C. Price, Y. Pan, J.A. Swaby and W.C. Golden: Motor neuron degeneration in amyotrophic lateral sclerosis mutant superoxide dismutase-1 transgenic mice: mechanisms of mitochondriopathy and cell death. *J Comp Neurol* 500, 20-46 (2007)

179. Bhatt J.M. and P.H. Gordon: Current clinical trials in amyotrophic lateral sclerosis. *Expert Opin Investig Drugs* 16, 1197-207 (2007)

180. Rosen D.R., P. Sapp, J. O'Regan, D. McKenna-Yasek, K.S. Schlumpf, J.L. Haines, J.F. Gusella, H.R. Horvitz and R.H. Brown, Jr.: Genetic linkage analysis of familial amyotrophic lateral sclerosis using human chromosome 21 microsatellite DNA markers. *Am J Med Genet* 51, 61-9 (1994)

181. Wong P.C., C.A. Pardo, D.R. Borchelt, M.K. Lee, N.G. Copeland, N.A. Jenkins, S.S. Sisodia, D.W. Cleveland and D.L. Price: An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria. *Neuron* 14, 1105-16 (1995)

182. Gurney M.E., H. Pu, A.Y. Chiu, M.C. Dal Canto, C.Y. Polchow, D.D. Alexander, J. Caliendo, A. Hentati, Y.W. Kwon, H.X. Deng and et al.: Motor neuron degeneration in mice that express a human Cu,Zn superoxide dismutase mutation. *Science* 264, 1772-5 (1994)

183. Bruijn L.I., M.W. Becher, M.K. Lee, K.L. Anderson, N.A. Jenkins, N.G. Copeland, S.S. Sisodia, J.D. Rothstein, D.R. Borchelt, D.L. Price and D.W. Cleveland: ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions. *Neuron* 18, 327-38 (1997)

184. Ralph G.S., P.A. Radcliffe, D.M. Day, J.M. Carthy, M.A. Leroux, D.C. Lee, L.F. Wong, L.G. Bilsland, L. Greensmith, S.M. Kingsman, K.A. Mitrophanous, N.D. Mazarakis and M. Azzouz: Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med* 11, 429-33 (2005)

185. Raoul C., T. Abbas-Terki, J.C. Bensadoun, S. Guillot, G. Haase, J. Szulc, C.E. Henderson and P. Aebischer: Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med* 11, 423-8 (2005)

186. Miller T.M., B.K. Kaspar, G.J. Kops, K. Yamanaka, L.J. Christian, F.H. Gage and D.W. Cleveland: Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. *Ann Neurol* 57, 773-6 (2005)

187. Xia X., H. Zhou, Y. Huang and Z. Xu: Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. *Neurobiol Dis* 23, 578-86 (2006)

188. Smith R.A., T.M. Miller, K. Yamanaka, B.P. Monia, T.P. Condon, G. Hung, C.S. Lobsiger, C.M. Ward, M. McAlonis-Downes, H. Wei, E.V. Wancewicz, C.F. Bennett and D.W. Cleveland: Antisense oligonucleotide therapy for neurodegenerative disease. J Clin Invest 116, 2290-6 (2006)

189. Jain K.K.: Commercial potential of RNAi. Mol Biosyst 2, 523-6 (2006)

190. Sirna: Outlook For RNAi, 2007: Sirna And Mirna In Biology, Diagnostics And Therapeutics. http://www.mindbranch.com (2007)

191. JainPharmaBiotech: RNAi - technologies, markets and companies. http://pharmabiotech.ch/reports/rnai/ (2007)

192. Frederickson R.M.: RNAi gets vote of confidence from big pharma. Mol Ther 15, 221-2 (2007)

193. Jackson A.L., S.R. Bartz, J. Schelter, S.V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P.S. Linsley: Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* 21, 635-7 (2003)

194. Persengiev S.P., X. Zhu and M.R. Green: Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA* 10, 12-8 (2004)

195. Jackson A.L., J. Burchard, D. Leake, A. Reynolds, J. Schelter, J. Guo, J.M. Johnson, L. Lim, J. Karpilow, K. Nichols, W. Marshall, A. Khvorova and P.S. Linsley: Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. *RNA* 12, 1197-205 (2006)

196. Yamada T. and S. Morishita: Accelerated off-target search algorithm for siRNA. *Bioinformatics* 21, 1316-24 (2005)

197. Stark G.R., I.M. Kerr, B.R. Williams, R.H. Silverman and R.D. Schreiber: How cells respond to interferons. *Annu Rev Biochem* 67, 227-64 (1998)

198. Karpala A.J., T.J. Doran and A.G. Bean: Immune responses to dsRNA: implications for gene silencing technologies. *Immunol Cell Biol* 83, 211-6 (2005)

199. Judge A.D., V. Sood, J.R. Shaw, D. Fang, K. McClintock and I. MacLachlan: Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* 23, 457-62 (2005)

200. Heidel J.D., S. Hu, X.F. Liu, T.J. Triche and M.E. Davis: Lack of interferon response in animals to naked siRNAs. *Nat Biotechnol* 22, 1579-82 (2004)

201. Gonzalez-Alegre P., N. Bode, B.L. Davidson and H.L. Paulson: Silencing primary dystonia: lentiviral-mediated RNA interference therapy for DYT1 dystonia. *J Neurosci* 25, 10502-9 (2005)

202. An D.S., F.X. Qin, V.C. Auyeung, S.H. Mao, S.K. Kung, D. Baltimore and I.S. Chen: Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther* 14, 494-504 (2006)

203. Schmidt C.: Negotiating the RNAi patent thicket. *Nat Biotechnol* 25, 273-5 (2007)

Abbreviations: AD: Alzheimer's disease, ALS: Amyotrophic Lateral Sclerosis, AMD: age-related macular degeneration, Aβ peptide: amyloid β peptide, APP: amyloid precursor protein, AVE: artificial viral envelope, AVPs: artificial virus-like particles, BACE1: β-site APP Cleaving Enzyme1, BBB: the blood–brain barrier, CNS: Central Nervous System, DGCR8: DiGeorge syndrome critical region gene 8, dsRNA: double-stranded RNA, ES: embryonic stem, HD: Huntington's disease, Htt: the protein huntingtin, IRG: interferon responsive genes, IR: interferon response, LRP/LR: The 37-kDa/67-kDa laminin receptor, LRRK2: the leucine-rich repeat kinase 2, LRRTM: leucine-rich repeat transmembrane, MCAo: the middle cerebral artery occlusion, MPG: amphipathic peptide, MTH: the molecular Trojan horse, PAZ: PIWI–Argonaute–Zwille, PD: Parkinson's disease, P-bodies: processing bodies, polyQ-repeat: the polyglutamine-repeat, pre-miRNAs:

precursor miRNAs, pri-miRNA: primary miRNA, Prnp: the prion protein gene, PrPc: the host-encoded prion protein, PrPsc: proteinase K–resistant isoform, RNA pol-II: RNA polymerase II, RNA pol-III: RNA polymerase III, RVG: rabies virus glycoprotein, SCA: Spinocerebellar ataxia, SCA1: Spinocerebellar ataxia type 1, SCA3: Spinocerebellar ataxia type 3, siRNA: small interfering RNA, SNc: the substantia nigra pars compacta, SOD1: Cu/Zn superoxide dismutase 1, Stearyl-R8: stearylated octaarginine, THL: Trojan Horse Liposome, VEGF: vascular endothelial growth factor.

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