

Title: Novel siRNA delivery strategy: A new "strand" in CNS translational medicine?

Authors: Lisa Gherardini¹, Giuseppe Bardi², Mariangela Gennaro³, Tommaso Pizzorusso^{3,4}

- 1. Institute of Clinical Physiology, CNR, Siena, Italy**
- 2. Center for MicroBioRobotics @SSSA, Istituto Italiano di Tecnologia, Viale Rinaldo Piaggio 34, 56025 Pontedera, Italy**
- 3. Institute of Neuroscience, CNR, Pisa, Italy.**
- 4. Department of Neuroscience, Psychology, Drug Research and Child Health NEUROFARBA, University of Florence, Italy**

Corresponding Author: Tommaso Pizzorusso

Inst Neuroscience, CNR

Via Moruzzi,1 56124 Pisa ITALY

e.mail: pizzorusso@in.cnr.it

tel: +390503153167

fax: +390503153220

Abstract:

RNA interference has been envisaged as powerful tool for molecular and clinical investigation with a great potential for clinical applications. In recent years increased understanding of cancer biology and stem cell biology has dramatically accelerated the development of technology for cell and gene therapy in these areas. This paper is a review of the most recent report of innovative use of siRNA to benefit several central nervous system diseases. Furthermore, a description is made of innovative strategies of delivery into the brain by means of viral and non-viral vectors with high potential for translation into clinical use. Problems are also highlighted that might hamper the transition from bench to bed, analysing the lack of reliable preclinical models with predictive validity and the lack of effective delivery systems which are able to overcome biological barriers and specifically reach the brain site of action.

Keywords: neurodegeneration, gene therapy, RNA interference, nanoparticles,

Introduction

“Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene”. The real meaning of this sentence was probably far from being appreciated at the time Fire and Mello wrote their manuscript [1,2], but since then RNA interference has been investigated extensively in the laboratory setting and there is great interest in translating siRNA into clinical application. In recent years, increased understanding of cancer biology and stem cell biology has dramatically accelerated technological advances in cell and gene therapy in these areas. This led to important medical progress, enabling therapists to design rationale-based personalised interventions. In this context, RNAi interference (RNAi) has become a powerful gene

silencing technology widely exploited as research tool. Most relevantly, RNAi quickly progressed to being probed as a potential treatment for the vast array of human conditions that could benefit from regulation of disease-associated genes. Nonetheless, a significant gap still remains between basic science and medical applications, in part due to the shortage of preclinical models with predictive validity. Translational research is the mission of scientists who take on significant challenges to develop innovative clinical trial designs; to accelerate the development of protocols for evaluating safety and efficacy by minimizing the number of patients required; to interpret biologic effects of cell and/or gene based therapies in patients; and to dissect the impact of therapeutic combinations. The journey from bench to bedside has never seemed so short however finding new ways to overcome this gap is still a major challenge [3]. Numerous reviews have preceded our overview on siRNA, a clear sign of ongoing interest in such an innovative and yet only partially explored biological mechanism. Here, we focus on the most recent reports of innovative use of siRNA in several central nervous system (CNS) diseases, and the successful attempts to deliver siRNA into the CNS with attendant high potential for translation into clinical use. Intriguingly, recent work raises the possibility that endogenous short RNAs (microRNA) may also have therapeutic potential in the CNS. This subject has already been reviewed elsewhere [4].

The RNA interference machinery

The ability of the cells to interfere with RNA translation is a conserved mechanism in eukaryotic organisms that use it to regulate, through genes expression, cellular metabolism, growth and differentiation, to maintain genome integrity, to fight viral infections and null mobile genetic elements. The components of the RNAi machinery were identified soon after the RNAi epiphany. By the time the 2006 Nobel Prize was awarded, the whole chains of intracellular events and the molecular players

involved were recognised. However, it has only recently become evident that specific intracellular compartmentalisation of components seems to play an important role in the silencing cascade[5,6]. In the cytoplasm RNAi pathway involves two specialized ribonucleases that control the production and function of small regulatory RNAs [7,8]. Pre-siRNAs are first processed by the endonuclease Dicer into 21–23 nucleotide fragments, leaving 2 single-stranded nucleotide overhanging at the 3'end. These small RNAs are transferred to Argonaute proteins (Ago), and subsequently to the RNA-inducing silencing complex (RISC). The duplex helix is unwound and the “guide strand” is used to direct sequence-specific cleavage of complementary RNAs to guide the sequence-specific silencing of expression of targeted gene expression for research or therapeutic applications (Fig. 1). This mechanism is also, in part, shared with the endogenous silencing mechanism agent miRNA. This is produced in the nucleus throughout several maturation steps that form pre-miRNA/pri-miRNA and is exported into the cytoplasm by the active carrier, exportin, where it encounters Dicer, which in turn cleaves endogenous pre-miRNA sequences that regulate gene expression. miRNA biogenesis results predominantly in translational repression of target genes and, in some cases, degradation of target mRNAs.

Once inside the cytoplasm the fate of siRNA is quite certain, although unexpected events might dictate the degree of complementarity held by the “guide strand”, causing reduced silencing of selected mRNAs and protein downregulation, as well as possible misregulation caused by off-target side effects. The main challenge in using siRNA as a therapeutic agent is that of selectively targeting the host cell inside the living organism. Biological barriers, such as the blood brain barrier (BBB) in the central nervous system (CNS), and enzymatic degradation tend to hamper the systemic use of siRNA-based therapeutics.

To stay on target

RNA silencing is now one of the most widely used techniques for gene expression regulation used in both research and clinical applications. However, recent insights on the possible undesired side effects of siRNA represent a major obstacle before it can be used as a drug [9]. Nonspecific effects of siRNA both in an animal model or patients have been thoroughly reviewed elsewhere[10]. They are generally described as sequence dependent and sequence independent events. Interference with the endogenous miRNA machinery and stability of the circulating RNA molecules are thought to be occurrences related to specific sequence (homology of the siRNA towards a miRNA target) and concentration. On the other hand, sequence-non-specific responses depending on siRNA length and structure may induce inherent toxicity given by off-target effects and triggering of immune responses toward dsRNA through cellular sensors of foreign RNA, such as RIG-I or Toll-like receptors, involved in innate immune antiviral responses[11]. Traditionally, chemical modifications of siRNA structure, such as 2' O- methylation of the second base of the guide strand of the siRNA and the introduction of modifications with locked nucleic acids (LNA) help to reduce most of the unwanted side effects without affecting the degree of silencing of the intended target. Alternatively, modifications at the 5' end or 3' end have been described to make the designed antisense strand more available for the RISC complex than the sense strand[12]. Similarly, the introduction of a controlled degree of asymmetry in the guide and passenger strands reduced disadvantages due to siRNA off target effect. To reduce the incidence of possible off target interactions, basic and translational research may benefit from the several websites, recently made available for the design of the effective siRNAs.

While structurally inherent drawbacks of siRNA substantially compromise its *in vivo* gene silencing activity, cell-targeted delivery may represent another obstacle for siRNA-based drug clinical

development. One of the trickiest **aspect** of the use of siRNA as a therapeutic agent is in fact the possibility to selectively reach the host cell inside the living organism. Degradation by serum nucleases and rapid elimination *via* the kidneys rapidly reduce siRNA concentration. Biological barriers, such as the BBB in the central nervous system CNS and enzymatic degradation hampered systemic use of siRNA-based therapeutics, however the development of biovectors and nanocarriers has recently **incentivized** the development of siRNA based new therapeutic strategies[13].

Delivery of siRNA to the CNS

Delivery of therapeutics based on RNA interference to the CNS is one of the major challenges currently hindering **its** use in clinical applications [14]. In the laboratory setting, several vectors have been used to transport and release nucleic acids into cells [15], but these are not yet suitable for clinical applications. Despite being used successfully in laboratory models, for many of these vehicles it is unclear whether they can safely transport and release the siRNA cargo into the neural parenchyma [16,17]. The CNS is protected by the BBB, a specialized capillary wall impermeable to most of the blood molecules and surrounded by perivascular astrocytes, macrophages, oligodendrocytes, as well as microglia and neural terminations. In addition to safety, technologies used to deliver siRNA therapeutics must possess other critical features such as vector stability, protection of the nucleic acid during administration and efficient release of that nucleic acid in the targeted tissue. Despite recent proof-of-concept reports and a growing body of siRNA research, off-target effects and inappropriate immune responses may jeopardise potential siRNA drug candidates even if they reach the advanced phase of clinical trials [18].

Due to their dimensions, siRNA-vector systems are often identified as nano-systems, with respect to either biological molecules (i.e. peptides, viruses, natural polymers) or artificial materials customized at the nanometric scale (i.e. lipid nanoparticles, polymeric nanoparticles, lipoplexes) (Fig. 1). From a translational medicine perspective, siRNA carriers can be divided into viral and non-viral systems. Both types have been demonstrated to be effective *in vitro* and some are also effective *in vivo*.

Viral vectors

Viral-mediated interference of gene expression by siRNA in the brain was demonstrated **ten years ago** in a seminal paper, by Xia and colleagues [19]. Potent gene suppression of GFP in eGFP-transgenic mice striatum was achieved by direct intraventricular administration of recombinant adenovirus. The same group also demonstrated therapeutic potential of siRNA in pre-clinical studies with a transgenic mouse model of the monogenetic disorder spinocerebellar ataxia (SCA) [20]. Specifically, the authors introduced viral vectors expressing DNA encoding short hairpin RNAs (shRNAs) directed against the transgenic mutated human ataxin-1 gene, thereby reducing the pathology in the mouse model.

This viral expression-based system engages the RNAi pathway at the pri-miRNA/pre-miRNA stage in the nucleus (Fig. 1). Efficient viral gene delivery strategies employed preferentially lentiviruses, adenoviruses (AV) and adeno-associated viruses (AAVs), each of which has distinct advantages and disadvantages [21].

Lentiviruses, which belong to the retrovirus family, can integrate into the genome of the host cell, thereby maintaining gene expression through cell division. Long-term transcription of shRNAs can be necessary for diseases affecting proliferating cell types but is generally not essential for non-dividing cells as neurons. Moreover, the possibility of introducing harmful insertion mutations means that clinical translation with lentiviral vectors is deemed high risk. AVs have the advantage of efficient penetration in different cell types including differentiated cells, since their infection is independent of the cell cycle. Gene delivery by AVs typically results in high levels of RNAi expression and, in

contrast to lentiviruses, integration of adenoviral DNA into the host genome is rare, with little chance of insertional mutagenesis. Despite these properties, AVs have been shown to induce immune system responses resulting in transient transgene expression in various immunocompetent mice models relevant to gene therapy research [22]. Nevertheless, improvements in vector design have significantly enhanced AV vector performance. Due to their lower immunogenicity, stability in the episomal form and penetration of non-dividing cells, AAVs have recently emerged as the preferred viral vectors for targeting neurodegenerative diseases (e.g. Huntington disease) that require downregulation of a single mutant gene, at least in pre-clinical models [23].

In addition to possible unexpected inflammatory immune reactions and insertional mutagenesis induced within the host genome, siRNA expressed in an uncontrolled manner by viral vectors may provoke cellular toxicity due to oversaturation of RNAi pathways [24]. Several obstacles are still present before viral vectors can be deemed safe carriers for siRNA therapeutics. However, some improvements may be obtained with the manipulation of viral capsids and envelopes, allowing a change of tropism and immunogenicity, a process called “pseudotyping” [21]. Another means of overcoming peripheral immune surveillance is the direct infusion of viral vectors into the brain parenchyma or by intraventricular injection, which have already been applied in clinical trials for gene therapy[16]. Obviously, the use of such invasive techniques should be confined to “undrugable” diseases. However, recent results on the safety of these viral vectors from both pre-clinical gene therapy studies on primates and clinical trials using AAVs vectors seem promising with both demonstrating good safety tolerance, including in patients with neurological diseases such as Parkinson’s Disease [25-28] or late infantile neuronal ceroid lipofuscinosis [29,30].

Non-viral vectors

Peptides

Molecular engineering of viral proteins allowed their use of viral infection mechanisms to selectively target the CNS after systemic injection [31]. Kumar et al. showed the potential of a modified rabies virus peptide, which binds the alpha-7 subunit of the acetylcholine receptor (AChR), to selectively transport and release functional siRNA into neurons. This method of delivery requires an appropriate siRNA-binding site on the vector, which has been achieved by linking nine arginine residues to the targeting peptide. Once inside the cell, siRNA was efficacious, although the mechanism of detachment remains unclear. The demonstration that such carrier was suitable for non-invasive systemic injection, able to cross the BBB and specifically release siRNA against viral encephalitis, thereby improving the survival of infected mice, was a seminal one. It has somewhat paved the way towards therapeutic application of siRNA in the CNS using peptide vectors.

Different peptide vectors have also been designed to target receptor-mediated transcytosis across the BBB facilitating delivery to the brain parenchyma. Among those peptides, lipoprotein receptor-related protein (LPR) binding peptides, called Angiopeps, seem to be efficiently transcytosed *in vitro* and *in vivo* [32].

Nanoparticles

Many aspects of delivery systems can be refined to improve their efficacy. Selective targeting, by peptides or specific antibodies is one possibility for improving delivery [31]. Serum degradation of nucleic acids occurring in the blood stream can be avoided by cargo encapsulation into nanoparticles. Although several and diverse types of non-viral nanoparticles have been proved functional for invasive siRNA release into the CNS [33-36], the most promising carriers for non-invasive delivery are based

on polymers [37,2] or lipids [38]. Among polymeric materials, dendrimers seem to be promising nucleic acid-carriers for translational medicine [39] due to their versatile properties and solubility. These hyperbranched star-shaped nanocarriers can be efficiently **functionalised** (e.g. with neuro-specific peptides) to purposely cross the BBB and target neurons [40].

However clinical applications, that often require long term and repeated administrations of therapeutics would require biodegradable nanomaterials approved by regulatory authorities. Furthermore, biodegradability is a major prerequisite for any **nanomaterial** targeting the CNS, since accumulation of non-degradable particles can result in unexpected dangerous side effects. Polylactide-co-glycolide (PLGA) and polylactide (PLA) are FDA-approved polymers for clinical use [41]. PLGA and PLA autocatalysis produces lactic and glycolic acids which are substrates for the Krebs cycle, resulting in complete and safe degradation of the carrier [42]. Notably, however, PLA and PLGA nanoparticles, and many other nanoparticles, are quickly removed from the body by the reticulo-endothelial system (RES) when injected in the blood circulation [43]. A common mechanism of their elimination is by serum protein adsorption onto engineered nanoparticles that regulates their interaction with blood cells, endothelial cells and surrounding tissues. To overcome degradation and removal from the peripheral circulation before reaching and crossing the BBB, nanoparticles can be further modified with specific moieties that are strategically designed on purpose for therapeutic applications [44]. For example, **modification of nanoparticles or therapeutics** with hydrophilic polymeric coating, such as polyethylene glycol (PEG), is widely used to avoid opsonisation, prolong the life span in the blood and help to targeted delivery [45]. Lipid nanostructure engineered particles, (i.e. liposomes, core-shell nanoparticles, lipoplexes) have also been widely used as non-viral vectors for siRNA delivery [46-48]. PEGylated liposomes modified with specific monoclonal antibodies, called Trojan Horses Liposomes (THL) are good candidate vectors for siRNA delivery into the CNS after systemic administration [49]. THL, modified with specific brain-targeting antibodies, have been already proven as both efficient and

specific gene delivery carriers *in vivo* after intravenous administration that can mediate functional motor improvement in an experimental model of Parkinson's disease [2]. However, nanoparticles require further pre-clinical investigation with regard to their ability to mediate neurotoxicity and immunogenicity *in vivo* prior to their therapeutic application for delivering siRNA.

A recent report combining biotechnology with naturally-occurring nanoparticles [50] showed that the translational potential of siRNA delivery in the CNS is promising. Exosomes obtained from primary dendritic cells harvested from murine bone marrow, were purified and engineered. To confer targeting specificity, the authors fused central nervous system-specific rabies viral glycoprotein (RVG) to the extra-exosomal N-terminus of murine LAMP2b, a protein abundantly found in exosomal membranes, limiting off-target side effects and toxicity[51]. After siRNA encapsulation, exosomes have been systemically injected and, subsequently, gene knockdown was observed in different regions of the brain. Using an autologous source of exosomes, the immunoreactivity was negligible, *in vitro* and *in vivo*, suggesting that siRNA-loaded exosomes are potentially suitable for long-term silencing, via repeated multiple administration without loss in delivery efficacy. It is maybe possible therefore to exploit a piggy-back mechanism using exosomes loaded with exogenous siRNA [51]in the future clinical treatment of chronic neurodegenerative disorders. Finally, Nakajima and co-workers [52] reported that intracerebroventricular injection of chemically modified naked Accell siRNA (Dharmacon), without any transfection reagent, achieved gene downregulation in several areas of the brain, including cortical layers I and II and other sub-cortical regions such striatum. As ICV injections are considered reasonably non-invasive, Accell siRNA delivery may have the potential for neurotherapeutic exploitation when it is necessary to reach vast areas of the brain [53,54]. Moreover, as gene knockdown was selectively achieved in differentiated mature neurons, this strategy might be exploited in neuron-specific diseases, such as neurodegenerative disorders.

Intranasal: an old route for new delivery

In an attempt to explore new delivery strategies to reach the CNS, the intranasal route is a simple and compliance-friendly approach which is worth serious consideration as it represents the ‘door’ to the olfactory bulb and brain [55]. The majority of studies on intranasal delivery describe brain distribution of neurotrophic proteins such as interferon [56], NGF [57,58] and BDNF [59,60] in Alzheimer models [61]. A new pilot clinical trial involves the use of old molecules such as insulin to revoke mild cognitive impairment [62]. The translational potential of intranasal delivery is confirmed by the large number of commercial patents for pulmonary disorders describing the use of siRNA in either naked form or along with a single/multiple delivery vectors. Moreover, accounts of siRNA intranasal delivery to the CNS have been very recently published. Rennen and co-workers [63] using fluorescent-labelled siRNA (siSTABLE, Dharmacon) were able to trace the nerve pathways that led siRNA molecules to their neuronal target. Labelled siRNA was concentrated in vesicles near the surface of the olfactory mucosa. In the *lamina propria*, siRNA was found within Bowman’s Glands and associated with both blood vessels and olfactory nerve bundles. Delivery progressed along the length of olfactory nerves, exiting the olfactory mucosa, crossing the cribriform plate and involving the anterior regions of the olfactory bulbs. Perez et al., [64] demonstrated that intranasal administration of a mucoadhesive gel containing siRNA dendriplexes increases their direct brain delivery. Most relevantly, Kim and co-workers achieved neuroprotection by intranasal delivery of high mobility group box 1 (HMGB1) siRNA in a rat model of focal cerebral ischemia [65]. HMGB1 plays a major role as endogenous danger signal, which is released by necrotic cells and activated macrophages and monocytes. HMGB1 mediates inflammation and acute damage leading to apoptotic neuronal death in the post-ischemic brain. HMGB1 siRNA was delivered using a biodegradable PAMAM dendrimer to rat brain after ischemia, and resulted in a significant reduction of infarct volume as well as improvements in motor function and other neurological deficits.

siRNA delivery strategies for neurodegenerative diseases

Many neurological conditions that have been to date considered 'undruggable', arise from alteration of gene and protein synthesis and could theoretically be treated by using siRNA, primarily directed to rebalance altered neuronal functions [66]. Since the earliest reports, the use of siRNA as an investigative tool has provided valuable information about CNS function and helped to determine the relevance of gene-based therapies for neuronal diseases[67,68]. Many reviews in the recent years have reported about the pre-clinical progress in RNA interference-based therapeutics [69] on delivery strategies [70] and on the use of RNA interference to identify novel therapeutic targets [71] in neurodegenerative CNS disorders such as Alzheimer's disease , Parkinson's, Huntington's diseases and amyotrophic lateral sclerosis (ALS) (Table 1).

Alzheimer's disease (AD) is a neurodegenerative disease leading to progressive cognitive decline and memory loss. Abnormal behaviour, agitation and mood swings arise along with dementia. Amyloid plaques and neurofibrillary tangles, which are formed via aggregation of extracellular amyloid β -peptide ($A\beta$) and intracellular hyper-phosphorylated tau, respectively, are the most characteristic feature of the Alzheimer brain, together with pronounced neuronal cell and synaptic loss. The search for target molecules associated with AD neuronal degeneration and inflammation reaction continues both *in vitro* [72-74] and *in vivo*, using siRNA sequences directed against potential molecular key players, however not many of these approaches have yield potential therapies, even at the preclinical stage. γ -secretase (γ -site APP-cleaving enzyme 1, BACE1) is a key component of the chain of enzymes that generate $A\beta$ from a type I membrane protein, amyloid precursor protein (APP), through sequential proteolytic cleavage events. Since $A\beta$ production strongly depends on BACE1, downregulation of BACE1 has long been considered as a good potential target for genetic therapy and an alternative to

pharmacological treatment in AD [75,76]. Similarly, targeted reduction of expression of APP expression may be of therapeutic benefit[67]. Lentivirus-mediated gene transfer of BACE1 siRNA in APP transgenic mice caused a significant drop in BACE1 and consequent improvement of impaired neuronal integrity [77]. More recently, Alvarez-Erviti et al., reported the systemic delivery of BACE-1 siRNA using an RVG-ligand-targeted-exosome-mediated technique [50]. Specifically, tail vein injection of BACE-1 siRNA-loaded exosomes achieved protein knockdown in several brain regions expressing the RVG-ligand (the nicotine receptor AchR), with a significant decrease in total β -amyloid 1-42 levels. Another strategy for reducing inflammation and amyloid burden in AD might be silencing of DNA damage inducible gene 153 (GADD153; also called CHOP) that plays a role in AD as well as Parkinson's disease, amyotrophic lateral sclerosis (ALS) and Huntington's disease. In AD, CHOP is activated by cholesterol-oxidized metabolite (oxysterol) 27-hydroxycholesterol (27-OHC), causing an increase in both A β 40-42 expression and the pro-apoptotic proteins Bax and caspase-3. In organotypic rabbit hippocampus slices, GADD153 siRNA reduced the effect of 27-OHC, protected neurons against oxidative damage by reactive oxygen species (ROS) and regulated basal expression of the antiapoptotic protein Bcl-2 [78]. This study provides supporting evidence indicating that [75], siRNA-mediated inhibition of β -amyloid expression and BACE-1 activity, among all possible targets, may be the most promising target for clinical translation in the short-term.

Silencing mutated genes in Huntington disease

The potential of gene silencing in Huntington's disease (HD) has been extensively studied [79,23]. HD is caused by dominant heterozygous expansion of a CAG repeat in the *Huntingtin (Htt)* gene, which generates an extended polyglutamine in exon1 of the multifunctional protein HTT. The central nervous system is especially sensitive to expression of mutant HTT, with striatal neurons suffering the most severe degeneration [80]. Abnormal protein folding and protein-protein interactions cause HTT protein

toxicity. HD post-mortem brains contain inclusion bodies expressing both mutant and wild type huntingtin. Silencing mutant HTT mRNA has been found to provide therapeutic benefit [81]. However, other proteins involved in HTT metabolism, such as huntingtin-associated protein 1 (HAP1) that plays a role in HTT transportation, may also be targets for silencing therapy [82]. As cleaved mutant HTT induces apoptosis, silencing of those proteins responsible for HTT cleavage such as metalloproteases [81] is one strategy for reducing HTT-induced toxicity. Moreover, siRNAs have also been employed for the study of downstream HTT-triggered caspase cascades leading to neurodegeneration by apoptosis [83]. HD research effort is now focussed on the important issue of optimising RNAi for therapeutic use, minimizing side-effects [17,84], and regulating the scalability of preclinical models (for a dedicated review see [23]). Numerous studies have stressed that siRNA used against HTT does not discriminate between mutated and wild type alleles both during development [85] and in adults. Preservation of physiological levels of HTT is a crucial spatial and temporal requirement for neural development and cell migration. Moreover, wild-type HTT has been documented to provide a positive effect on cell survival and can mitigate the effects of the mutant HTT. Studies carried out on patients showed that the mutant *htt* allele often contains single nucleotide polymorphisms (SNPs) [86,87]. Thus targeting SNPs might achieve a higher degree of selective inhibition for the mutated alleles. However, this approach would require somewhat laborious development of different compounds, selective for each of the five SNPs detected in humans. Another strategy to achieve mutated allele silencing is to hijack the interference pathways naturally used by miRNA. miRNA-induced silencing occurs as sequence mismatch is carried on the guide strand of the duplex. This strategy was used in a patient-derived fibroblast cell line GM04281 [69 CAG repeats/mutant; 17 repeats/wild-type allele [88] (locked oligonucleotides)]. In that study, several sequences with different mismatches placed sequentially throughout the duplexes on the guide strand complementary to the CAG repeat were used, thereby achieving more selective mismatch-related mutated allele silencing.

Scalability is another crucial bottleneck in the translation of basic research to the clinical setting. Translational medicine relies on preclinical experiments preferably carried out on reliable close-to-human situations, such as non-human primates [89]. Recombinant adeno-associated viral vectors were used to deliver RNAi silencing constructs for *htt* gene to Rhesus monkey striatum (rAAV-miRNA). Reduction in total expression of HTT protein was well tolerated in this model with no evident immune reaction development of gliosis or motor impairment in treated animals [90]. More recently, Stiles et al., [91] reported the silencing of mutated HTT using siRNA, by convection-enhanced delivery (CED). Several challenges were overcome in this work, including the use of an implanted catheter fixed in the brain for as long as 28 days. Furthermore, positive pressure was required to overcome the resistance of the brain tissue against siRNA movement through the interstitial space, with relevant distribution of radiolabelled siRNA being achieved in the putamen. The most important take home message in this study was that the technique is able to provide silencing, for long time, in wide areas of the brain using a well-tolerated dose of siRNA. Studies in non-human primates are laborious and expensive however they do represent a crucial physiological step toward clinical translation.

Developing siRNA strategies for Parkinson disease.

Parkinson's disease (PD) is one of the most common neurological disorders mainly characterized by the death of dopamine (DA) producing neurons in the *substantia nigra*. PD is predominantly idiopathic in its late-onset form, however familial, early onset forms of disease have been described. Since early reports [92] it was clear that applied siRNA technology in PD could be potentially beneficial. The use of **lentivirus_siRNA** technology in PD was recently reviewed [93], as was research on PD gene therapy [94] in preclinical mouse models. The majority of reports however have focused on the use of siRNA as a research strategy, mostly investigating relevant neurodegenerative and inflammatory patterns in

established or primary cell lines. These early preclinical investigations can only partially shed light on the translational potential of siRNA in PD.

One common feature among the various forms of PD is protein accumulation and aberrant protein clearance. PD is characterised by intracytoplasmic inclusions called Lewy bodies (LB). Synphilin-1, α -synuclein, and Parkin represent the major components of LB and are likely to be involved in the pathogenesis of Parkinson's disease. Several authors have targeted genetic loci involved in familial forms [92], such as PARK1, most of which are related to the formation of α -synuclein. A single point mutation (A53T) in α -synuclein gene is thought to give rise to the presence of misfolded protein in LB causing the autosomal dominant form of familial PD. Silencing of this gene with siRNA could hold therapeutic potential reducing the tendency for α -synuclein to aggregate and induce neuronal toxicity. Mutations in the parkin gene (PARK2 locus, chromosome 6q) are accountable for the formation as well as the maintenance of LB, representing potential risk factors in sporadic PD. LB formation has been also ascribed to alterations of the ubiquitin-proteasome system [95]. Recently, LB formation was reverted regulating monoubiquitination of α -synuclein by enzymes such as SIAH that have been reported to promote ubiquitin transfer [96]. However, the role of SIAH in ubiquitination of α -synuclein has been questioned by findings suggesting that Siah-1 might play a role in Parkinson's LB formation through the regulation of α -synphilin-1 function [97]. Thus, the possibility of using SIAH as a target for a siRNA based approach to revert LB formation remains controversial. Another enzyme involved in **deubiquitinating** α -synuclein is USP9X. The use of siRNA to silence USP9X in SH-SY5Y cells reduced synuclein aggregation [98]. The synuclein structurally related protein 14-3-3 was also targeted with neuroprotective effects using *in vivo* delivery to PD models of short hairpin RNA carried by viral vectors [99].

A role for PKC δ in dopaminergic neuron degeneration was described [100]. 6-OHDA, a neurotoxicant used for modelling *in vitro* neurodegeneration, induces apoptotic cell death through PKC δ activation. Suppression of PKC δ expression by siRNA protected N27 cells from 6-OHDA-induced apoptotic cell death [101]. siRNAs were also employed to study mediators involved in chaperone-mediated autophagy such as lysosomal LAMP2A [102] and heatshock (HSC) 70 protein expression, and genes involved in selective clearance of damaged mitochondria such as PARKIN and PINK1[103].

The progressive loss of dopaminergic regulation occurring in Parkinson's disease (PD) provokes a cascade of functional changes in the basal ganglia circuitry, which may sustain the development of the symptoms. One of the major metabolic changes in the basal ganglia circuitry after nigrostriatal denervation and loss of DA, is the cellular up-regulation of the messenger RNA coding for the 67 kDa isoform of glutamic acid decarboxylase (GAD67 mRNA), the synthetic enzyme of GABA, an indirect marker of GABAergic activity. Counteracting GAD67 increase, by means of siRNA delivered into the striatum using lentiviral vectors, was able to restore normal neuronal activity [104].

Enzymes involved in the degradation of dopamine are obvious targets for **siRNA therapies** in PD, therefore the list of attempts will probably become longer in the future. The current data did not allow an immediate translation of results into pre/clinical application.

Employing siRNA to tackle schizophrenia symptoms

Schizophrenia is a multifactorial syndrome believed to arise from a 'nurture and nature' interplay in which genetic and environmental causes coexist [105]. In contrast to PD, the so-called dopaminergic hypothesis states that overproduction of dopamine in the brain is common in people with schizophrenia [106]. The etiological DA hypothesis of schizophrenia is a classic, but perhaps simplistic, interpretation that excess dopaminergic activation may cause some symptoms of schizophrenia. Clinical treatment of this disease generally involves medications that block dopamine receptors in the brain [107]. Target-

specific siRNA sequences have been used to define the relevance for schizophrenia of selected metabolic [108,109] as well as signalling pathways. For example, silencing of Disrupted-in-schizophrenia 1 (DISC1) or dysbindin-1 [110,111] - both implicated in neurodevelopmental regulation of axonal growth - might shed some light on the cause of neuronal disarray, which is a characteristic morphological feature of the hippocampus and prefrontal cortex in schizophrenia. Similarly, synapsin II silencing in the prefrontal cortex may provide a new model for studying the role of prefrontal excitatory circuitry alteration in schizophrenia [112]. The only recent *in vivo* study, aimed at supporting the therapeutic use of siRNA in schizophrenia [113], demonstrated that intraventricular injection of a plasmid expressing D2DR siRNA achieved downregulation of dopamine receptor expression that, importantly, correlated with a reduction in schizophrenia-like hyperactivity induced by the dopamine receptor agonist apomorphine.

siRNA use in amyotrophic lateral sclerosis.

Among the neurodegenerative diseases, amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of “lower motor neurons” in the spinal cord and brainstem, and degeneration of the descending motor pathway in the corticospinal tracts, leading to paralysis and death. ALS **occurs in** the majority of cases in a sporadic form. However familial forms causally linked to nonsense single point mutations in the Cu, Zn superoxide dismutase (SOD1) gene has been discovered. At least 150 mutations in the **coding** sequence have been identified that are able to induce misfolding and aggregation of the protein in the motor neuron cytoplasm[114].

RNAi has previously been demonstrated to be a suitable strategy for silencing SOD1 and inducing the slowdown of the disease in genetically relevant animal models [115-117]. Until recently, silencing strategies were designed using siRNA against SOD1 allele harbouring missense point mutations while preserving wild-type allelic functions, however, recent reports [118,119] suggest that

the product of the normal SOD1 allele modulates the toxicity of mutant SOD1 in familial ALS and concur at the formation of aggregates and inclusion bodies generated by mutant SOD1. Generally, viral transfection of shRNA [120] is used to achieve selective mutant allele silencing. Several reports have described lentiviral vector shRNA delivery leading to long-lasting transgene expression *in vivo*, as would be required for chronic diseases such as ALS. Specifically, shRNA silencing of mutant SOD1 expression *in vivo* delays the onset of ALS and extends the survival of SOD1G93A mice, which is correlated with improved motor performance and motor neuron survival [121,122]. However, Towne et al., could not find any therapeutic benefit after multiple injections of AAV-shRNA for SOD1 into the neonatal muscles of a familial ALS mouse model [123], although transduction of motoneurons did occur at all spinal cord levels up to the brain stem. Protection from muscle atrophy, neuromuscular junction denervation, and motoneuron loss did not improve quality of life or *lifespan* in these animals. These authors emphasised the importance of precise endpoint reading, which accurately parallels the human pathology in the strive towards translational success. Also in an ALS mouse model, siRNA SOD1G93A was, for the first time, reported to be retrogradely transported, from the surgically severed end to the nuclear region of the sciatic nerves [124].

Other mediators of ALS pathogenesis have been investigated as potential therapeutic candidates. For example, CHOP is involved in the ER-dependent stress pathways that lead to neurodegeneration and cyclin-dependent kinase 5 (CDK5), regulated by p25, causes neuroinflammation [125]. Targeting the natural gene silencing mechanism of DNA methyltransferase Dnmt1 and 3, which are overexpressed in motoneurons during apoptosis, might also provide neuroprotection [126]. In all these cases, however, siRNA is used primarily as a research tool. Despite the significant effectiveness of potential therapeutics observed in preclinical trials [127], SOD1 RNAi has not yet reached clinical trials for patients with ALS. Moreover, the development of effective treatments of ALS depends strictly on

selective delivery, and the development of non-viral carriers to selectively reach motoneuron populations.

siRNA therapeutic attempt in stroke

Cerebral stroke can leave surviving patients with long-lasting physical mental and psychological symptoms that affect many aspects of their lives. Ischemic stroke represents the majority of cases and is caused by oxygen flow reduction or interruption to neurons, which consequentially suffer hypoxia leading to cell death by apoptosis and necrosis. Stroke may also be mediated by intracerebral hemorrhagia. The goal of therapeutic intervention is aimed at restoring lost neurological functions in affected patients. In recent years, with advances in silencing technology, many attempts have been made to induce neuroprotection and reduce inflammation [128,129,65], delay scar tissue formation and activate adult neuronal plasticity [84,3], enhance neurogenesis from the SVZ [130,131], and replace lost cells through stem cell insertions [132,133] in models of stroke. All of these strategies rely on the regulation of protein pathways to restoring impaired function after insult. As such, an RNA interference strategy either with shRNA [132] or siRNA is used [134-137] to silence protein pathways activated after stroke and to identify new molecular targets both in cell culture and animal models. However, to date, very few reports suggest a realistic possibility of using siRNA formulations as clinical therapeutics for preserving neuronal function in stroke [33]. This gap between bench and bedside is, in part, due to the widespread nature of damage induced by the unpredictable occurrence of a sudden ischemic insult. It also partially reflects the inaccessibility of damaged sites within the brain parenchyma, as well as the lack of selective targeting of therapeutics. Although frequently damaged during and after ischemia or haemorrhagic events, the BBB still represents an obstacle to efficient delivery of agents. Therefore, optimisation of delivery [128,138,139] and of administration regimes will be necessary for more efficient evaluation of siRNA-based therapeutics in stroke.

Inducing neuroprotection after stroke seems to be the preferred intervention route to restore neuronal activity and function. Blocking apoptotic pathways [140] using siRNA against apoptosis signal-regulating kinase 1 (Ask1) down-regulates the expression of Ask1 and prevents apoptotic neuronal cell death after intracerebroventricular infusion with osmotic minipumps. This treatment rescued brain damage after ischemia/reperfusion (I/R) in mice that underwent occlusion of the middle cerebral artery for 1h, followed by reperfusion. Although Ask1-siRNA attenuates upregulation of Ask1, and reduced infarction in ischemic brain after I/R, there were no reports of behavioural outcome in treated animals. A classic neuroprotective intervention is to target apoptotic gene translation that regulates intracellular caspase-dependent apoptosis. Delayed apoptosis and other genetically-based cell death signalling triggered under conditions of both transient focal and global ischemia represent suitable drug target. The transcription factor C/EBP homologous protein (CHOP, DDIT3/GADD153) functions mainly as a pro-apoptotic mediator after ER stress in several pathologies[141] and participates in delayed adaptation in cortical neurons after hypoxia [142]. CHOP acts at a post-transcriptional level through p38 MAPK in response to severe ER stress, activating the expression of Bim, leading to Caspase-3 dependent apoptosis. He and collaborators [143] recently proved that ICV pre-treatment with CHOP siRNA in a subarachnoid hemorrhage (SAH) model resulted in the significant upregulation of antiapoptotic Bcl2, and downregulation of the executioner Caspase-3. Interestingly, neurological deficits[144] were reduced in siRNA-treated animals, suggesting some translational potential for siRNA-based therapeutics targeting apoptotic mechanisms after SAH. Recently caspase-3 was targeted directly through delivering of siRNA locally by intraparenchymal injection. In an endothelin-induced ischemia rat model, acute local delivery of caspase-3-siRNA-loaded carbonanotubes in the primary motor cortex 24 hours prior to stroke induction reduced neuronal apoptosis and prevented microglia activation after stroke [33]. Most importantly, forelimb motor function was completely restored in animals treated with caspase-3 siRNA. Internalization of carbonanotubes into neurons, verified by

TEM, suggested that intracellular release of siRNA from the vector was achieved. Post-ischemic delivery of siRNA loaded carbo-nanovectors was not so effective in restoring motor functions. A constant, although not significant, improvement in a motor skilled reaching test was described, suggesting a potential for clinical application. Even if carbonanotubes do not seem to produce major adverse effects after intraparenchymal injection [145], limitations do remain as their delivery requires an invasive procedure that is not translatable to clinical practice.

In many other situations, siRNA has been used to elucidate the role of potential neurodegenerative mediators [135]. For example, CysC, an endogenous inhibitor of cysteine protease activity, regulates autophagy, a protection mechanism activated after cell damage. Blocking autophagy in oxygen deprivation cell culture model of N2A culture model and in primary neurons using beclin1 siRNA eliminated the protective effect of CysC from serum deprivation-induced death [136]. Using a proprietary dendriplex complex (TRANSGEDEN) for siRNA delivery, knockdown of Beclin-1 in rat cortical primary neuron was demonstrated [146] to prevents autophagy and NMDA mediated Ca^{2+} cellular influx, leading to cell death. Beclin-1 **prevention of** autophagy can therefore be considered a protective mechanism against **excitotoxicity**, and thus a potential strategy for neuroprotection after stroke damage. siRNA silencing of sigma-1 receptor induced neuroprotection, demonstrating the important role of this protein, which is expressed by reactive astrocytes and neurons, and its neuroplastic regulation of axonal elongation in primary neurons[147].

As a consequence of stroke, inflammation and immune system activation **occur** and inflammatory mechanisms **substantially** contribute to secondary brain damage [148]. The maturation and propagation of the damage is largely sustained by activation of the local immune system and a major role is played by activation of adhesion molecules. A possible anti-inflammatory restorative strategy consists in targeting adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells that mediate lymphocyte trafficking into the damaged brain via interaction with leukocyte very late

antigen-4 (VLA-4). Inhibiting this interaction by hydrodynamic in vivo administration of VCAM-1 siRNA significantly reduced VCAM-1 protein expression and, in turn, cerebral granulocyte and T cell trafficking and activation of cytotoxic IFN- γ . Together with circulating immune system elements, activation of local microglia occurs after disruption of the BBB or blood extravasations. Vascular adhesion protein-1 (VAP-1) a cell-surface expressed glycoprotein, has recently emerged as a potential target for inflammatory regulation in the brain as it supports leukocyte adhesion to the cells. Ma and co-workers [139] recently found that blocking of VAP-1 via ICV injection of siRNA, inhibits leukocyte migration, reduces the infiltration of systemic immune cells and downregulates the expression of adhesion molecule ICAM-1. siRNA-based VAP-1 inhibition of the inflammatory cascade also reduced treated animal neuro-behavioural impairment, suggesting that VAP-1 siRNA has potential therapeutic efficacy.

Overall in stroke, the general limitations of siRNA for clinical use are its delivery across the BBB and the narrow therapeutic time window for intervention. siRNA delivery to the brain could exploit the transient increased permeability of the BBB following brain insults. However, given its characteristic variability, to rely on increased BBB permeability to achieve effective therapeutic delivery is considered risky. New siRNA modifications or **delivery systems are needed** to overcome the vascular endothelial cell barrier of the CNS. Moreover, in most of the cases considered, experimental protocols included pretreatment of animals with selected siRNA molecules. This does not **reflect** a clinical setting and appropriate modification must take place with the view of validating the protocols. This uncertainty might result in lack of pharmaceutical investments in siRNA-based clinical trials for stroke therapy

siRNA anticancer therapy in CNS

Glioblastoma (GBM) is the most common primary brain tumour in adults and has a devastating prognosis, with median survival of less than two years. Notwithstanding poor molecular stability, targeting and delivery efficacy, siRNA technology has been employed to determine differences in type, stage and prevalence of candidate biomarkers in GBM [149]. Moreover, dedicated delivery technology has been developed to overcome CNS barriers [150,151]. Here we focus on those studies of siRNA delivery to the brain [152] that seem to have potential for clinical drug development for central nervous system tumours. GBM arises due to the summation of multiple activating and inactivating genetic lesions. Moreover, neovasculature processes [153] and cell migration [154] can determine growth progression and metastasis. As for many other research areas, RNA interference technology in GBM represents a first choice tool for investigation of cancer-related intracellular pathways, to identify mechanisms that sustain GBM cell survival, [155-157] metastatic evolution [158,159] and stem cell recruitment, as well as extracellular matrix protein activation [160,161]. siRNA silencing strategy is used to highlight gene products that, when suppressed, sensitize GBM cells to radiotherapy [162] and chemotherapy, allowing for the potential development of siRNA-drug combined therapy [149]. We report several examples of such studies that employed, for example, siRNA-mediated NF- κ B silencing to reduce growth [163] involving EGFR activation, to sustain cell infiltration via Akt signalling pathways [164] or through interleukin 8 [165]. Silencing of the urokinase proteolytic pathway, in glioma and meningioma cells, inhibits extracellular matrix proteolysis and cell signalling, thus reducing cell migration, proliferation and survival [166]. Cathepsin-B activates pro-urokinase-type plasminogen activator, a serine protease involved, via urokinase-type plasminogen activator receptor (uPAR), in ECM degradation, matrix metalloproteinase (MMP) activation [167] and tumour cell invasion. In the U251 glioma cell line, uPAR and cathepsin B siRNA-mediated downregulation suppressed Bcl-2 expression, possibly through inhibition of the PI3/Akt pathway. For translational purposes, *in vivo* studies are more relevant, and many reports indicated the inhibitor of apoptosis protein, survivin, as a

likely candidate for RNAi therapy. Survivin is a member of the family of inhibitor of apoptosis proteins (IAPs) involved in cell division and inhibition of apoptosis [168] through interaction with TRAIL /NF- κ B pathways [169]. It is highly expressed in cancer tissues and cancer cell lines and barely detected in normal differentiated tissues. Survivin-shRNA inhibited growth and reduced angiogenesis in an U251 transfected cell xenograft nude mouse model [170]. More recently, in an intracranial nude mouse model of human glioma U87, survivin-siRNA was conjugated to a single-chain variable fragment (scFv) of TfR to elevate the neuronal targeting efficiency of its BBB receptor-mediated endocytic transport systems [171]. Tail vein injection of scFv-TfR-survivin-siRNA suppressed survivin levels and prolonged the survival times of these mice. Similarly, i.p administration of polyethylenimine (PEI)/siRNA complexes produced efficient knock down of survivin and arrested subcutaneous U373-MG tumour growth, enhancing the survival rate of NMRInu/nu mice orthotopically transplanted with U87-MG [150]. Silencing with siRNA to survivin in syngenic immunocompetent mice also demonstrated that survivin down regulation mediates its anticancer effect also through the TRAIL pathway, which has been shown to increase the cytotoxic responses of human NK cells [172]. Although syngenic xenograft models are widely used, a humanized mouse model might more closely resemble the cascade of events seen in patients and confer a higher confidence for translational purposes [173,174].

The feasibility of a new delivery strategy using siRNA-PEG/solid lipid nanoparticle complexes for the systemic clinical treatment of GBM was demonstrated in orthotopic the U87-MG xenograft model [151]. The potential of c-Met silencing in growth and metastasis was previously investigated in glioma U251 cells using shRNA interference [175]. c-Met is overexpressed in brain tumours and its level frequently correlates with tumour grade and poor prognosis. Silencing of receptor tyrosine kinase (RTK) c-Met by intravenous administration of siRNA-PEG/SLN complexes suppressed tumour growth without showing any systemic toxicity in mice. Together with c-Met, the receptor tyrosine kinase

family also includes the EGF receptor (EGFR), the PDGF receptor (PDGFR) and the VEGF receptor (VEGFR,) and their expression is frequently deregulated in gliomas [176,177]. Michiue et al. [178] successfully inhibited tumour growth *in vitro* (human T98MG cells) and *in vivo* (xenograft) using a combined approach to silence both the overexpressed upstream receptor (EGFR), or its truncated form EGFRvIII, as well as members of the Akt kinase family involved in downstream cell growth and survival. To allow for efficient delivery, siRNA was bound to a peptide transduction delivery domain fused to a dsRNA-binding domain (PTD-DRBD) to mask the siRNA anionic negative charge. *In vivo* PTD-DRBD delivery of EGFR and Akt2 siRNAs induced tumour-specific apoptosis and significantly increased survival in intracerebral GBM mouse models. Selected mRNA targeting optimized delivery and the synergistic strategy contributed to the observed success; however the intraparenchymal route still represents a drawback for translational application in clinics. Recently, in an *in vitro* study, cyclodextrin-modified multivalent dendritic polyamines (carrying different siRNA at the same time) significantly inhibited cell proliferation and induced apoptosis more efficiently than individual treatments. To date, however, of the wide range of possible therapeutic agents for the treatment of GBM, none has been selected as a suitable candidate for siRNA-based clinical trials. Instead one clinical trial, which includes the use of siRNA as a research tool, has been initiated for neuroblastoma (NB), the most common and deadly extra-cranial solid tumour in children. In that study, siRNA is used to silence the expression of developmentally regulated 4-N-acetylgalactosaminyltransferase III on differentiating neuronal cells. This protein is fundamental for the development and differentiation of the nervous system, through regulation of cell contact and signalling [179]. β 1,4-N-acetylgalactosaminyltransferase III (B4GALNT3) exhibits GalNAc transferase activity to express the GalNAc β 1,4GlcNAc structure on neuroblasts. Its altered expression is associated with the development of NB whereas its increased expression is positively correlated with favourable prognosis. This clinical trial protocol aims to investigate the administration of B4GALNT3-siRNA to nude mice

bearing xenografts, to establish the role of glycosyltransferases regulating NB cell behaviour, as a possible oncogenic therapeutic target.

Retinoblastoma

Another common paediatric tumour is retinoblastoma, which occurs due to a mutation of the retinoblastoma tumour suppressor gene, and may occur in both eyes. Although several therapeutic strategies have been recently developed, in severe cases, enucleation is still a therapeutic option. Downregulation of gene expression with siRNA is a credible strategy to prevent or suppress tumour growth over extended periods, with the aim of sparing remaining sight. Recent in vitro studies used interference to different molecular targets in the human Y79 retinoblastoma [180] cell line to induce apoptosis and increase chemosensitivity in cultured cells [181]. Connexin 46 (Cx46) gap junction protein is involved in the development of neoplastic and malignant progression. Cx46 is found in solid tumours with a hypoxic component, including human Y79 retinoblastoma cells, where it is believed to act as a regulator of tumour progression and aggressiveness. In an in vivo xenograft model of human retinoblastoma Y79 cells [182] it was demonstrated that **intratumour** injections of Anti-Cx46 siRNA **significantly** reduced the mass tumour, probably by reducing resistance to hypoxia. Optimization of siRNA formulation to confer a longer half-life might improve the translational application of siRNA in retinoblastoma therapy, as well as in other solid tumours where Cx46 is highly expressed.

Retinal disease

Several clinical trials based on siRNA drugs have been conducted in the area of retinal degeneration. Wet age-related macular degeneration (AMD) is an eye disease characterized by the growth of abnormal retinal blood vessels that leak blood or fluid [183]. This disease was the first target for siRNA therapy. The macula consists of a thin layer of photoreceptors and its degeneration causes rapid and

severe central vision loss leading to visual impairment, with patients partially losing their central field of vision. Although it does not lead to total blindness, AMD severely affects a vast proportion of the over-50 population, severely impinging on quality of life and social health costs. To date, the aetiology of AMD is not clear and cures are not available, however pathological angiogenesis mediated by endothelial growth factor receptors (VEGFR) is considered the major cause [184]. Stimulation of VEGF and placental growth factor (PIGF) results in the growth of new blood vessels. The first siRNA-based clinical trial sponsored by Allergan (ClinicalTrials.gov Identifier: NCT00395057) is aimed at silencing the expression of VEGF Receptor-1 (VEGFR-1) on ocular vascular endothelial cells, to downregulate associated signalling pathways. Intravitreal injection of a modified siRNA drug, AGN 211745, has demonstrated an improved pharmacokinetic profile in pre-clinical studies compared to unmodified siRNAs. Studies progressed to Phase II to complete dose scaling (2008) and biological and anatomical assessment in the retina. A further update reported on a 24-month study to evaluate multiple doses in the treatment of subfoveal choroidal neovascularization associated AMD.

Quark Pharmaceuticals is recruiting patient cohorts for an escalation study to evaluate the effect of PF-04523655 (PF), a small interfering RNA (siRNA) with 2'-O-methyl nucleosides in every pair of the oligonucleotide sequence, to inhibit the expression of the hypoxia-inducible gene RTP801. This gene has long been implicated in the induction of retinopathy as a complication of *Diabetes Mellitus* where it is associated with retinal neovascularisation and increased vascular permeability causing increased retinal thickness and eventual loss of visual acuity [185]. The MATISSE clinical trial aims to evaluate the toxicity and efficacy of PF in combination, or not, with Ranibizumab (DME). This trial is supported by preliminary clinical evidence that a smaller group of patients participating in a underpowered pre-trial study benefitted from the combined therapy [184]

A further incentive to develop siRNA-based drugs for retinopathy derives from the relatively easy and immune-privileged access to the eye compartment. Until recently, intravitreal delivery of siRNA was considered an optimal route, as many of the drawbacks regarding, siRNA degradation, off-target delivery and immunogenicity encountered using other administration routes seemed absent. However, a warning against the use of naked unmodified double strand-siRNA in human clinical trials arose from studies [186] demonstrating that a 21er naked double stranded siRNA, injected into the eye stopped angiogenesis in mouse models of age-related macular degeneration regardless of their sequence. Kleinman et al. [18] also demonstrated that treatment efficacy was not exerted through RNAi, but instead through an already described immune reaction caused by extracellular interaction of dsRNAs with Toll-like receptor-3 (TLR3). This interaction caused upregulation of gamma interferon and interleukin 12, setting off a cascade of events that downregulated the neovascular processes and induced caspase 3-dependent apoptotic death of the retinal pigment epithelium. Misinterpretation of this clinical trial data forced Opko Health Inc. to withdraw from a stage III clinical trial for the study of a combinatory protocol of bevasiranib and lucentis, two approved drugs, for AMD (Opko Health, Inc. NCT00259753).

Treatment of Pain

Pain is an evolutionary component of the sensory system, which is critical for survival when facing environmental stresses. Translational pain research aims at understanding pain phenomena in humans, limiting direct and corollary suffering [187]. Chronic pain develops as a syndrome and has major socio-economic impact. Neuropathic pain is a component of chronic pain caused by an initial primary lesion to, or dysfunction of, the peripheral nervous system (PNS) which, in turn, causes modification at the cortical level [2]. Although the causes remain poorly understood, chronic pain correlates with altered expression and distribution of several proteins in sensory peripheral neurons, mostly excitatory channel

components such as sodium- or calcium-dependent channel subunits. Molecular strategies for therapeutic targeting of primary sensory neurons in chronic pain syndrome involving RNA interference (RNAi) is a novel approach to human treatment of neuropathic pain [188]. In rats, the intrathecal delivery route has been used to target the ganglion protein pain modulator activin β C [189], or excitation channel components such as P2X and NaV1.8 [190] with reduced neuropathic pain symptoms. Chitosan-siRNA nanoparticles were prepared with siRNA sequences directed against M2, M3, and M4 mAChR and administered intrathecally [191].

Another relevant target for pain treatment in the DRG sensory neurons is the transient receptor potential vanilloid subtype 1 (TRPV1) which plays a key role in visceral pain [192]. TRPV channels respond to several stresses to induce pain, inflammation and tissue fibrosis [190]. Most notably, this receptor family is functionally expressed in human conjunctivae epithelial [193] cells, and TRPV channel activation in ocular tissues is associated with symptoms occurring in patients suffering from dry eye syndrome [194]. Major advances in TRPV1-siRNA delivery in the eye compartment led to Phase 1 clinical trials for dry eye syndrome. Sylentis, has received authorisation from the Spanish Medicines and Health Products Agency to commence clinical trials with SYL1001 for treating or preventing eye discomfort. A phase I Study has been set up to evaluate the ocular tolerance of SYL1001 in healthy volunteers.

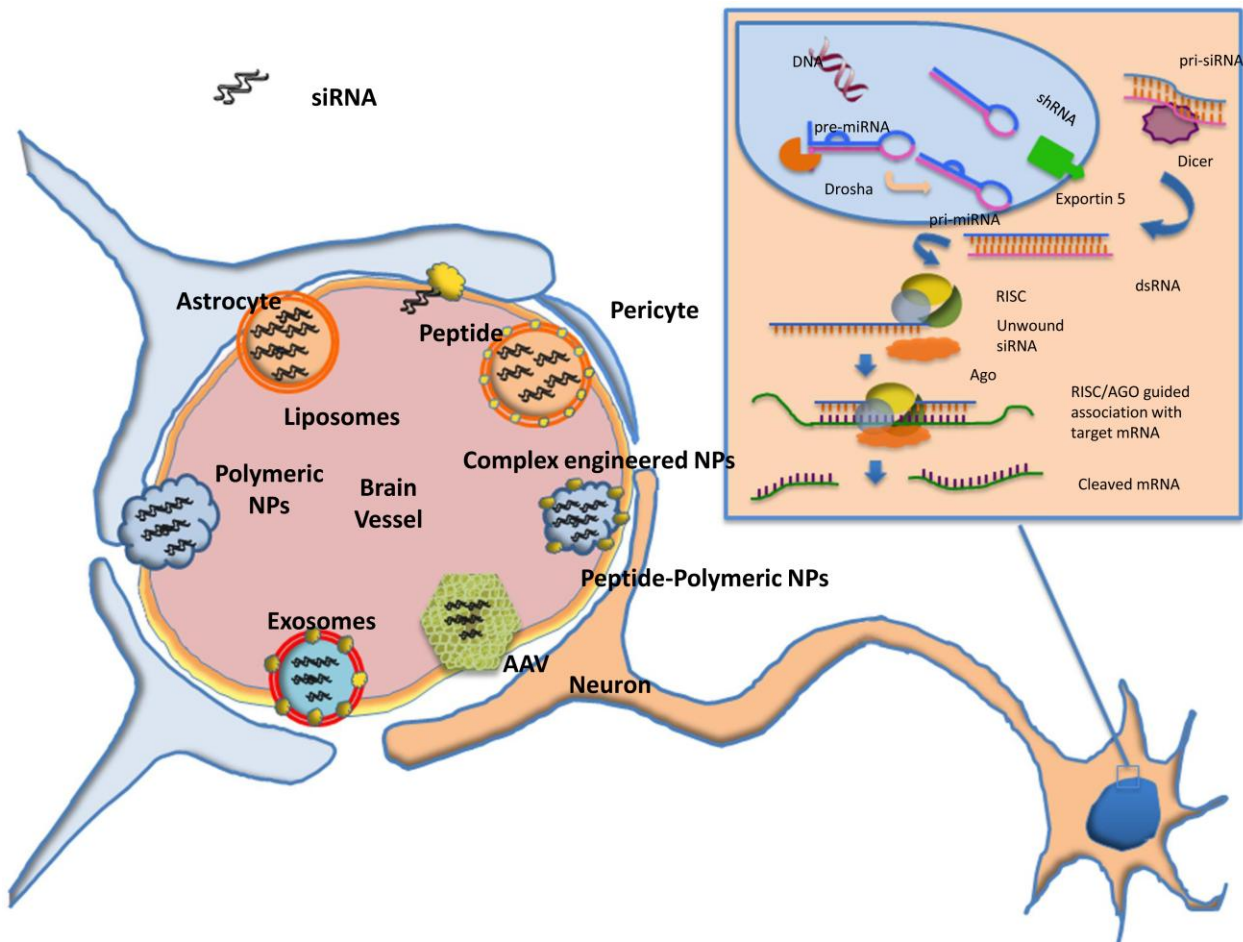
Final comments

Great expectations abound whenever scientists announce exciting advances in neuroscience and genomics, however not so many of these discoveries have been translated into clinical medicine. siRNA has become the fastest chart topper in drug delivery. A simplistic way to approach the challenge of siRNA could be “if you can bring it there in one piece it will work”. Over the years, many

phrases have been used to describe the potential of siRNA technology, but ‘magic bullet’ is one phrase that perseveres. However, this line of thinking overshadows its biological and clinical shortcomings. Another definition for this misplaced excitement is “clinical naïveté” as the key question remains of how to translate the laboratory experience to a clinical setting, which somehow involves personalised medicine. This transition is hampered by the lack of reliable preclinical models with predictive validity, which is jeopardising the effort put into genomic probing for relevant biomarkers. The lack of efficacious delivery systems able to overcome biological barriers and specifically reach the brain as site of action is another major hurdle. Finally, approval and regulatory problems abound with siRNA, as evidenced by the granting of FDA approval to only few siRNA-based therapeutics. To date at least twenty-five registered clinical trials are based on, or involve, siRNA technology. Among these, the large majority are using siRNA to target the CNS (neuropathic molecular oedema, and ocular pain) while others are developed for cancer treatment (i.e. neuroblastoma). For some trials, research did not successfully translate for either patients or investors due to lack of expected therapeutic effects or misleading interpretation of preliminary data. Such setbacks have shed a dim light on the entire siRNA technology platform, which has already suffered from lack of pharmaceutical investment. In this scenario, the bench-to-bedside gap can only be reconciled with a leap of faith, but new and groundbreaking reports [195,64,196,113,52] are still coming through that raise hope for the future application of siRNA.

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