Expert Opinion

Systemic delivery of therapeutics to neuronal tissues: a barrier modulation approach

Matthew Campbell[†], Ema Ozaki & Peter Humphries
Smurfit Institute of Genetics, Trinity College Dublin, Ocular Genetics Unit, Dublin 2, Ireland

Importance of the field: Efficient systemic delivery of low-molecular-mass therapeutics to neuronal tissue remains a central issue not only to drug development but also to the chronic treatment of a range of neurodegenerative disorders.

Areas covered in this review: This review discusses the potential of using RNA interference to modulate tight junction proteins at the blood–brain barrier and inner blood–retina barrier. Both systemic delivery of short-interfering RNA and viral-mediated delivery of short hairpin RNA are discussed, highlighting the therapeutic area relevant to each.

What the reader will gain: Readers will gain an insight into the potential of size-selective and reversible modulation of neuronal barriers and the types of low-molecular-mass molecule that could be used in the treatment of various neurodegenerative or neuromalignant disorders.

Take home message: The purpose of this review is to describe a new therapeutic strategy for systemic delivery of low-molecular-mass therapeutics to neuronal tissues.

Keywords: blood–brain barrier, blood–retina barrier, claudin-5, RNA interference, tight junctions

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1. Introduction

1.1 The blood–brain barrier and the blood–retinal barrier

The CNS accounts for ~ 20% of oxygen consumption in humans and the retina has the highest oxygen consumption per weight of any tissue. This, combined with the delicacy of neuronal tissue and its requirement of a highly regulated extracellular environment (e.g., osmotic balance and ionic concentration), underlines the necessity of a separation of the CNS and the retina from the circulating blood. These separations are mediated by the blood–brain barrier (BBB) and the blood–retina barrier (BRB), respectively, which are capable of supplying an abundant and oxygenated blood supply, while simultaneously preventing passive diffusion of harmful blood-borne agents such as antibodies, pathogens, immune cells and anaphylatoxins (reviewed in [1,2]).

In 1885, Paul Ehrlich first described the phenomenon of the BBB in a series of experiments designed to compare the oxygen consumptions of different organs [2]. He observed a lack of coloration in the brain and spinal cord compared with the peripheral organs, after intravenous injection of aniline dyes. Ehrlich attributed the phenomenon to the neuronal tissues having lower affinity to the dye. However, nearly 30 years later, Ehrlich's student, Edwin Goldman, observed the opposite phenomenon, coloration confined to the CNS, after injecting Trypan blue into the cerebrospinal fluid. After it was illustrated that these phenomena were not due to

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Article highlights.

- The blood–brain barrier (BBB) and the blood–retina barrier (BRB) remain key challenges in drug delivery strategies to the brain and retina.
- RNA interference (RNAi) can be used to modulate the BBB and BRB and improve drug delivery to neuronal tissues.
- There are numerous delivery strategies associated with RNAi, ranging from polymer-mediated carriers to virally delivered material.
- RNAi-mediated modulation of TJ proteins is an efficient and new method for enhancing systemic drug delivery to the brain and retina.
- The potential 'off-target' effects of RNAi include activation of the innate immune system and suppression of transcripts other than that targeted. These can be controlled, however, by efficient design of short-interfering/short hairpin RNA.
- The clinical potential of using barrier modulation for the treatment of acute and chronic diseases is immense and the number of applications is potentially very far reaching.

This box summarizes key points contained in the article.

the different compositions of blood and CSF influencing the speed of dye diffusion [3], the presence of a barrier between neuronal tissues and the circulation was widely accepted.

The BBB comprises a series of barriers in parallel, including the vascular BBB and the blood–cerebrospinal fluid (blood–CSF) barrier. The former, located at the endothelial cells of the brain capillaries, is the focus of most drug delivery systems, whereas the latter is located at the choroid plexus and meninges. The BBB is collectively composed of brain microvascular endothelial cells, astrocytes, basement membrane, pericytes and neurons. Brain microvascular endothelial cells are distinguished from peripheral vascular endothelial cells by the increased number of mitochondria, the absence of fenestrations, reduced pinocytosis and the presence of complex tight junctions (TJs). It is primarily these TJs that confer the low paracellular permeability, high electrical resistance and extremely low rates of fluid phase transcytosis that characterise the BBB [4]. Pathological dysfunction of the BBB has been described in several neurodegenerative diseases (Alzheimer's disease) and inflammation-related diseases in the brain (stroke, multiple sclerosis and vascular dementia) [5-8].

Highly similar in structure and function, the inner BRB (iBRB) and outer BRB (oBRB) allow for the maintenance of the delicate neuronal environment of the retina. Indeed, the retina has been described as an 'accessible part of the brain' and is ultimately an exterior portion of CNS that can be stimulated by light. At the molecular level, the iBRB is extremely similar to the vascular BBB and is made up of TJs of retinal endothelial cells sheathed in pericytes and glial cells, which together constitute the iBRB. Breakdown and pathological dysfunction of the iBRB result in visual loss in many ocular diseases, including diabetic retinopathy, sickle-cell disease and cystoid macular oedema [9-12]. Unlike the BBB, however, the retina also contains what is termed the oBRB. The oBRB comprises a single layer of retinal pigment epithelium (RPE) cells located directly adjacent to the neural retina at the back of the eye. It is involved in the regulation of transport between the retina and fenestrated capillaries of the choroid, in the regulation of the ionic environment of the subretinal space, phagocytosis of shed photoreceptor cell outer segments, and also plays a fundamental role in visual phototransduction. Diseases of the oBRB result in retinal degeneration and degeneration of the choriocapillaris [13].

1.2 Tight junctions

Tight junctions are contact points between the plasma membranes of adjacent epithelial or endothelial cells. The junctions make complete contact and were first identified by electron microscopy, where a fibrillary appearance was observed between two epithelial cells. Each TJ strand is paired to and associates laterally with another TJ strand on the membrane of an adjacent cell and the intercellular space is completely occluded. Tight junctions are involved in vesicle targeting, cytoskeletal dynamics, signalling during proliferation and transcription, defining cellular polarity and, most importantly in the context of the BBB and iBRB, functioning as paracellular barriers to the diffusion of solutes. Tight junctions are major components of the BBB and BRB that form a rate-limiting barrier to passive paracellular diffusion of water-soluble substances from the peripheral circulation to the CNS and retina, respectively. Tight junctions are composed of integral transmembrane proteins, claudins (of which up to 24 have been identified), occludin and junctional adhesion molecules (JAMs). There are also several accessory/anchoring proteins called the Zonula occludens (ZO-1, ZO-2 and ZO-3), which are members of the membrane-associated guanylate kinase (MAGUK) protein family [14,15].

JAM-1 (40 kDa) is a member of the IgG superfamily and mediates the early attachment of adjacent cell membranes by means of homophilic interactions. The claudins (20 – 27 kDa) and occludin (65 kDa) have similar membrane topology and locations, yet little sequence homology. However, there is much evidence suggesting that claudins function as the main sealing proteins of TJs. For example, occludin knockout mice show morphologically and functionally normal TJs [16]. Nevertheless, the presence of occludin at the BBB is correlated with increased tightness (electrical resistance) across the barrier and may also be important for cell signalling.

Up to 24 claudins have been identified so far in mammals that share high sequence homology in the first and fourth transmembrane domains. Claudins are predicted to have four transmembrane domains, two extracellular loops that project into the paracellular space, and a short internal amino terminus and an internal carboxyl terminus, both of which are cytoplasmic. These proteins are believed to form dimmers,

and through their extracellular loops interact with homologous regions on adjacent endothelial or epithelial cells to form the primary seal of TJs. The first extracellular loop has a set of highly conserved amino acids (W-GLW-C-C), and it is highly likely that the two cysteines form a disulphide bond. The carboxyl terminus possesses a PDZ domain that binds to the PDZ domains of other scaffolding proteins such as ZO-1, ZO-3 and ZO-3 [17,18].

The expression patterns of claudins vary immensely among different cell types and tissues. Some claudins are widely expressed, for example claudin-1, whereas others are expressed only in certain cell types, for example claudin-5 is more or less specific to endothelial cells, or during certain periods of development, for example claudin-6 is developmentally restricted and is not expressed in the adult [19]. This, together with the large number of different claudins expressed in mammals, suggests that different subtypes of claudins function in different tissue types and consequently the TJs in these tissues vary in electrical conductance, charge selectivity, solute permeability and size discrimination [20,21].

Claudins-1, -3, -5 and -12 have been detected in brain capillary endothelial cells [22], and so are likely to be key to the 'seal' characteristic of the BBB and iBRB. One recent study, measuring the mRNA expression levels of claudin subtypes in mouse brain capillary endothelial cells, reported that claudin-5 was most highly expressed, at a level that was at least 593-fold greater than that of claudin-1, -3 or -12 [23]. Indeed, knockout studies in mice also highlight the importance of claudin-5 in BBB integrity [24]. Claudin-5-deficient mice were born alive, but died within 1 day of birth without any morphological abnormalities reported. However, tracer experiments and MRI analyses revealed a size-selective (approximately < 800 Da) loosening of the BBB in these mice. This finding of a size-selective BBB in the absence of claudin-5 gave rise to the hypothesis that modulation of claudin expression at the BBB and iBRB may represent a new therapeutic strategy to deliver potential low-molecular-mass drugs into the CNS. In this regard, it is of note that recently Campbell and co-workers successfully suppressed claudin-5 expression in adult mice using RNA interference (RNAi) and showed that reversible modulation of the BBB and iBRB in mice allowed for a phenotype similar to that observed in the claudin-5 knockout mice without the formation of cerebral or retinal oedema and with very few differentially regulated genes in neuronal tissues [25,26].

It is well accepted that an estimated 98% of drugs with established anti-neovascular, neuroprotective, anti-inflammatory or anti-apoptotic potential do not easily passively diffuse across the BBB or iBRB, rendering systemic delivery of such compounds either impractical or highly inefficient. Poor penetration of these compounds is a result not only of the low rates of fluid phase transcytosis, but also the presence at the BBB and iBRB of P-glycoprotein efflux pump activity and a range of specific efflux receptors present in the microvascular endothelium [27,28].

Permanent and uncontrolled opening of these barriers to large molecules such as anaphylatoxins, antibodies, other soluble proteins or pathogens would be disastrous for neuronal viability. However, controlled, size-selective and transient modulation of these barriers for short periods of time to allow passive diffusion of very low-molecular-mass compounds could have substantial therapeutic potential, avoiding the necessity for regular invasive delivery to ocular tissues.

2. RNA interference

RNA interference is a term coined by Fire and co-workers in 1998, subsequent to their observation that long double-stranded RNA (dsRNA) molecules can elicit potent suppression of expression of a target gene in *Caenorhabditis elegans* [29]. During that time, it was thought that sense or antisense single-stranded RNAs (ssRNAs) had the ability to induce gene silencing. Surprisingly, however, Fire *et al.* found that dsRNA was over 100-fold more effective in silencing the *unc-22* gene than either ssRNA strand. They later realised that the sense and antisense ssRNA preparations in previous experiments had been contaminated with small amounts of dsRNA. Ultimately, the same phenomenon had been observed previously in the plant petunia by Jorgensen in 1990 and was termed 'co-suppression'. RNAi is believed to have evolved as an antiviral defence mechanism in mammalian systems.

In contrast to *C. elegans,* when long dsRNA was introduced into mammalian cells, an interferon response resulting in a nonspecific halt in translation, activation of interferon-stimulated genes and often cell death was observed, limiting their use in mammals. To overcome or limit the interferon response, small dsRNA molecules ~ 20 nt in length termed short-interfering RNAs (siRNAs) can be used [30].

siRNAs typically consist of two 21-nucleotide ssRNAs that form a 19-base pair duplex with 2-nucleotide 3′ overhangs. These siRNA duplexes are transfected into cells with a transfection reagent that facilitates entry of the duplex into cells. The duplex then becomes incorporated into a complex of cytoplasmic proteins already present in the cell. This protein complex, known collectively as the RNA-induced silencing complex (RISC), directs the duplex to the appropriate target transcript and initiates its degradation. Only one strand of the duplex – the guide strand, which is complementary to the targeted transcript – becomes incorporated into RISC, where it binds the complementary nucleotide sequence in the targeted mRNA. RISC then cleaves the target mRNA at this point, leading to its destruction and prevention of translation to the target protein. A Slicer protein called Argonaut 2 is one of the components of the RISC complex and is involved in the cleavage of the target RNA. Crystal structure analysis identified a domain in Argonaut 2 that resembles RNase H, a well-known protein that cleaves RNA in DNA/RNA duplexes.

siRNA-mediated suppression using chemically synthesised siRNA in mammalian cells has been found to be transient.

Potential solutions to increasing the stability and longevity of suppression include synthesising modified siRNAs to increase longevity or to provide a stable source of siRNA from a plasmid or viral vector. dsRNA delivered by means of plasmid or viral vector is termed short hairpin RNA (shRNA). In this approach, plasmids or viral vectors engineered to express siRNA are introduced into cells. These systems express a shRNA, which consists of a sense sequence ~ 21 bases long followed by a 6 – 8-base non-complementary loop and another 21-base sequence complementary to the sense sequence. Once the shRNA expression plasmid is delivered into the cytoplasm, it is transported into the nucleus for transcription. The shRNA is transcribed from specific promoters, typically a polymerase III promoter such as the H1 or U6 promoter, but this can be created to be cell/tissue type specific. The primary transcripts (pre-shRNA) are processed by a complex containing the Rnase III enzyme Drosha and the dsRNA-binding protein DGCR8, and are then transported to the cytoplasm via exportin-5. Here, they enter another complex containing the RNase III enzyme Dicer and TRBP/PACT to form a double-stranded siRNA with two nucleotide 3′ overhangs. These double-stranded siRNAs are then incorporated into the RISC pathway for directed suppression of the target mRNA [31].

3. Therapeutic potential of RNAi-mediated modulation of the BBB and iBRB

RNAi-based treatment of ocular diseases is an area where there has been great progress in recent years both at the basic research level and, indeed, clinically. Using RNAi, Campbell *et al.* have recently developed a method to enhance reversibly the permeability of the iBRB. Previously, the same authors showed that RNAi-mediated knockdown of claudin-5 in the brain microvasculature of adult mice resulted in a transient and size-selective increase in diffusion of low-molecular-mass compounds across the BBB, without any detrimental effects to such mice [25]. This process is outlined in the schematics in **Figures 1** and **2**. Owing to the similarities between the BBB and iBRB, the same approach was performed on the iBRB. Mouse tail-vein injection of claudin-5 siRNA resulted in lower levels of claudin-5 protein 24 and 48 h after injection, but not 72 h after injection, which correlated with the modulation of the iBRB observed by a small molecule (742 Da) MRI contrast agent. To explore the therapeutic potential of transient and reversible modulation of the iBRB, they assessed the delivery of therapeutic molecules to the retinas of two mouse models of retinopathy. Following systemic drug delivery, visual function was shown to be improved in IMPDH1$^{-/-}$ mice with systemic delivery of guanosine-5′-triphosphate (GTP) in a model of autosomal recessive retinitis pigmentosa, and photoreceptor cell death was shown to be reduced in a model of light-induced retinal degeneration with enhanced delivery of the calpain inhibitor ALLM.

The systemic siRNA-based approach described by Campbell *et al.* could potentially be modified using adeno-associated virus (AAV) vectors that can stably persist in the retinal endothelial cells following a single local injection in the retina or indeed in distinct regions of the brain, while allowing for the inducible expression of shRNA directed against claudin-5 transcripts. This approach could be used to enhance delivery of low-molecular-mass drugs to treat several degenerative retinopathies such as age-related macular degeneration (AMD), diabetic retinopathy and retinitis pigmentosa (RP).

Worldwide, the pharmaceutical industry invests heavily in the development of drugs for the treatment of neurological disorders. However, the low permeability of the iBRB and BBB to such agents reduces their efficacies substantially. Indeed, many of these drugs are of low molecular mass (< 1 kDa). If a safe means were available for reversible opening of the barrier to molecules of this size, the therapeutic potential could be enormous, particularly given the fact that neurodegenerative, malignant and neuropsychiatric diseases are steadily increasing in prevalence in ageing populations.

4. RNAi delivery strategies

The key challenge in the development of RNAi-based therapies is the delivery of these macromolecules to the desired cell type, tissue or organ. siRNAs can be delivered locally or systemically *in vivo*. Lower doses are needed for local administration of siRNAs because nonspecific delivery to other organs is reduced, as is renal or hepatic elimination. Also, as intracellular immune responses to siRNAs have been shown to be concentration-dependent, local delivery may reduce some of these off-target effects. However, local delivery is invasive and limited to tissues that are sufficiently reachable and tolerable [32].

4.1 Non-viral delivery

'Naked' or uncomplexed siRNA refers to delivery of siRNA (unmodified or modified) in simple media such as saline or 5% dextrose. However, owing to their relatively large molecular mass and polyanionic nature, these naked siRNAs do not diffuse freely across cell membranes, and delivery systems are required to mediate intracellular delivery. Non-viral delivery methods rely on the use of synthetic compounds that can imitate the viral infection process. Such features include the ability to condense DNA, to protect it against degradation, and to mediate its cellular uptake and nuclear delivery.

Liposomes are physiologically stable vesicles that consist of an aqueous compartment where the carried DNA is enclosed and is surrounded by a phospholipid bilayer. This bilayer is made up of a cationic or fusogenic lipid component, cholesterol and a polyethylene glycol (PEG) lipid. Lipoplexes are spontaneously formed on interaction of cationic lipids and negatively charged nucleic acids, and are structurally more heterogeneous and unstable than liposomes and aggregate over time in solution. They are typical of most commercial transfection agents such as Lipofectamine 2000 [33].

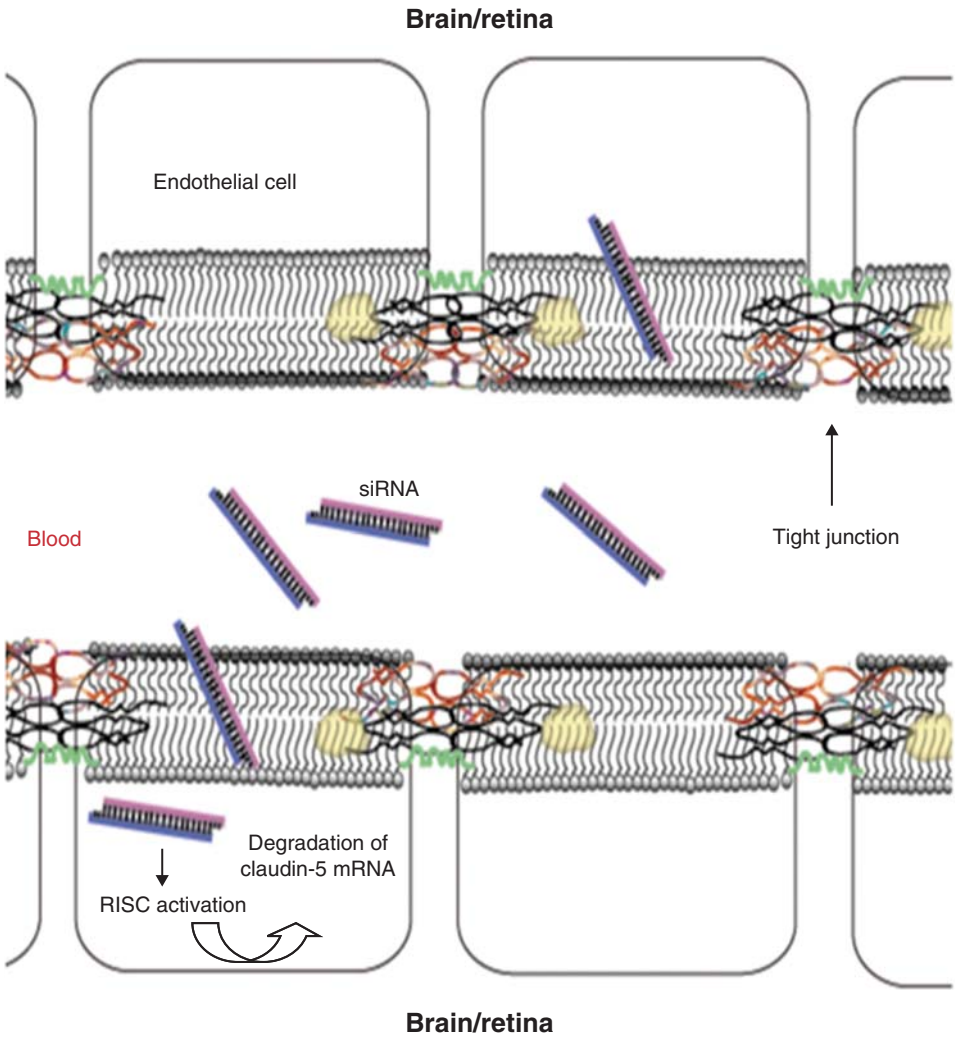

Figure 1. siRNA injected into the tail veins of mice either hydrodynamically or with *in vivo* transfection reagents will diffuse across the plasma membranes of endothelial cells lining the retinal or brain microvasculature. The siRNA will activate the RISC, unwind, and the antisense strand will bind to transcripts encoding the tight junction protein claudin-5.
RISC: RNA-induced silencing complex; siRNA: Short-interfering RNA.

Stable nucleic acids lipid particles (SNALP) technology is a recently developed approach for delivering liposome-mediated RNAi [34]. This system relies on the enhanced permeability and retention effects that persist as these nucleic acid-containing particles have a long blood circulation time leading to an increased accumulation in areas of vascular leakage that are often found at sites of tumour cell growth, infection or inflammation. Once at the target sites, cells endocytose these particles and the DNA is delivered intracellularly.

Polyethylenimines (PEIs) are a family of branched or linear synthetic DNA carriers. They are different from other polymers as they have a high cationic charge and only a third of the amino groups are protonated at pH 4. The high gene transfer efficiency results from this buffering capacity of PEIs. When endosomes become acidic, PEIs are able to seize protons, which results in osmotic swelling and endosome disruption, releasing the endocytosed DNA in the cytosol. Furthermore, PEIs are capable of condensing and compacting the carried DNA into small colloidal particle complexes. These two characteristics also protect the carried DNA from degradation (reviewed in [32]). Claudin-5 siRNA complexed with PEIs could allow for the systemic delivery of siRNA to the brain microvasculature in conditions where acute treatment would be necessary, as in neuronal malignancies, and where systemic delivery of siRNA could be ethically justified. Some other delivery strategies are summarised in **Table 1**.

4.2 Viral-mediated delivery of RNAi material

For the past 20 years, viral vectors have been used to introduce therapeutically beneficial genes into patients. Advantages of using virus-mediated siRNA/shRNA delivery systems are that cells that are often difficult to transduce by other

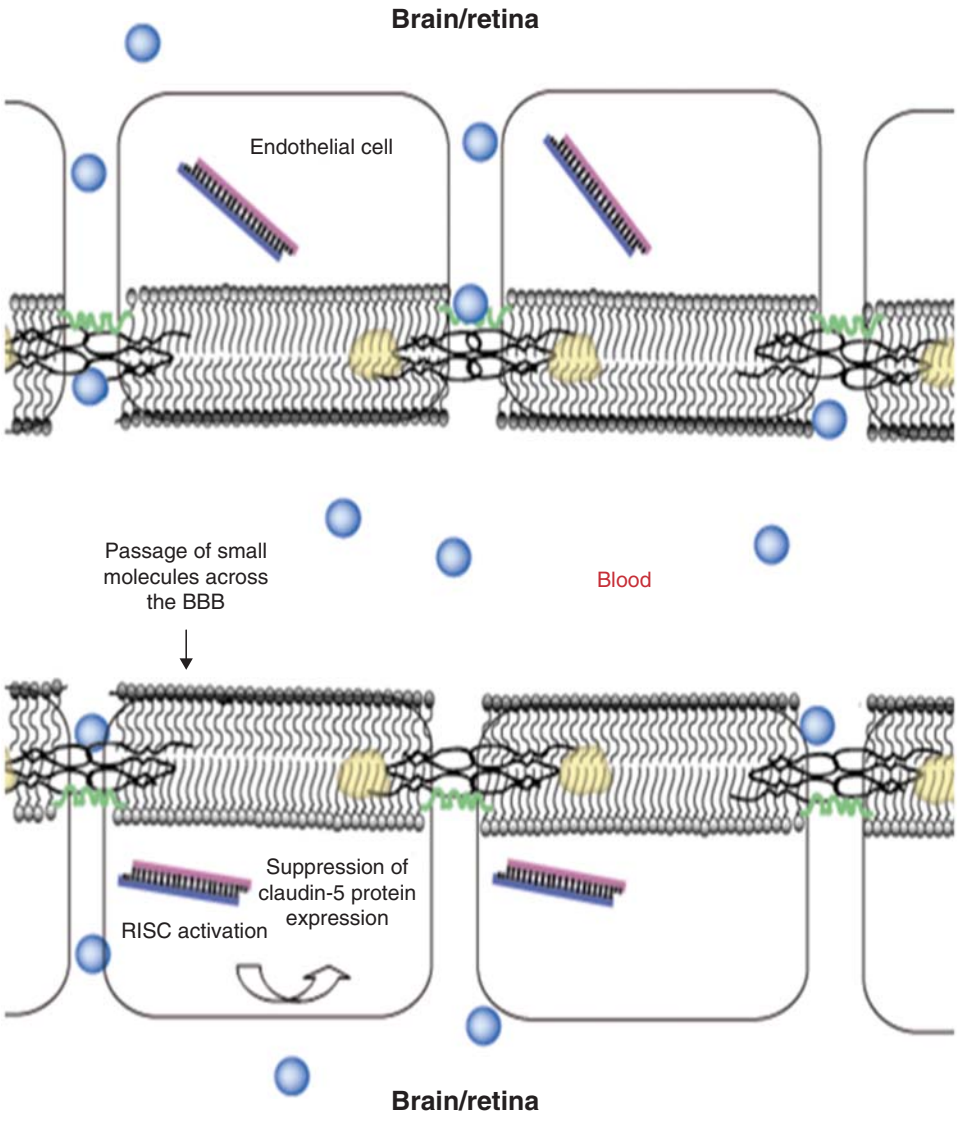

Figure 2. Once claudin-5 transcripts have been prevented from translating to protein, the claudin-5 present at the endothelial cell tight junctions will be suppressed and will allow for the formation of pores in the paracellualar pathway of the cells. This size-selective barrier modulation will subsequently allow for the passive diffusion from blood to brain of molecules below ~ 1 kDa while excluding larger potentially harmful blood-borne molecules.
BBB: Blood–brain barrier; RISC: RNA-induced silencing complex.

methods, such as non-dividing cells, can be transduced with these vectors, and transduction efficiency is higher than with other non-viral methods. The viral vectors primarily used are AAV vectors, adenoviral (AdV) vectors, lentiviral vectors and retroviral vectors.

Specifically, and directly relevant to ocular therapeutics, AAV is a small (4681 nt), single-stranded, icosahedral and non-enveloped virus that belongs to the class Parvoviridae. AAV relies on other viruses, such as adenovirus or herpes simplex virus, to facilitate productive infection and replication. AAV encodes the proteins rep and cap, which can be provided in *trans* for viral replication between the 5′ and 3′ inverted terminal repeats (ITRs) of the viral genome. In the laboratory setting, recombinant AAV (rAAV) vectors are produced by deleting rep and cap, leaving the ITRs, which are essential for viral replication and integration. DNA of ~ 4.5 kb in size, which can express shRNAs, can then be cloned/packaged into this vector, commonly under the control of pol-II or pol-III promoters, but this can be altered as required.

Since the establishment of the first infectious clone of AAV serotype 2 (AAV2) in 1982, rAAV vectors have been used exponentially in gene therapy clinical trials throughout the world. Its popularity as a vector system in gene therapy applications is because of some of its unique characteristics.

Table 1. Some RNAi delivery approaches with their advantages and disadvantages.

Delivery strategy	Advantages	Disadvantages
Aptamer	Highly target-specific, large-scale manufacturing possible, can be backbone modified for stability *in vivo*	Large relative to siRNA, repeated treatment might be necessary, need to be modified for enhanced circulation and pharmacodynamics, costly to manufacture
Antibodies	Highly target-specific, can use monoclonals or antibody fragments	Costly to produce, repeated treatments might be necessary, possibly immunogenic
Cholesterol	Non-immunogenic, proven *in vivo* delivery in non-human primates, potentially low cost	Possible liver toxicity if used repeatedly, not useful for delivery to most organs other than liver, very large doses
Synthetic nanoparticles	Specific targeting possible, can be synthesised in large scale, able to accommodate large amounts of siRNAs and can be engineered to escape endosome	Need to be conjugated to specific ligands for tissue-specific delivery, costly manufacturing, repeated administration necessary

First, AAV vectors can transduce both dividing and non-dividing cells *in vivo* and *in vitro* with very high efficiency and have a broad host and cell type tropism. Second, AAVs are one of the safest of the available viral delivery methods at present because they are more or less non-pathogenic and do not induce immune responses like AdVs. Third, they have very low rates of random integration into the host genome. AAV vectors hold onto only ~ 300 nucleotides of their viral genome, which greatly reduces the risk of recombination. Finally, AAV vectors maintain persistent expression of shRNAs *in vivo* and the vector genomes can be modified to aid in tissue-specific delivery or to allow inducible expression of shRNAs [35,36].

There are few drawbacks in the use of AAV vectors for gene therapy, however some do exist. First, their small size limits the size of the transgene that can be inserted into the vector. Second, the ssAAV DNA must be converted into dsDNA as an initial step, therefore gene expression is usually of slow onset. Importantly though, there are at present several clinical trials that are using AAV vectors to treat diseases such as cancer, cystic fibrosis, heart failure, Parkinson's and Alzheimer's disease, haemophilia, muscular dystrophies and some ocular diseases [37]. It is now possible to create AAV viruses containing shRNA targeting claudin-5 that in principle will allow for the localised expression of claudin-5 shRNA and subsequently localised iBRB or BBB modulation. It is also of note that there are now numerous inducible vector systems whereby shRNA expression can be controlled by an inducer such as tetracycline, mifepristine, hypoxia and light, to name but a few.

5. RNAi-mediated modulation of TJ proteins

The iBRB remains a key element in slow rate of development of new therapeutics for the treatment of many retinopathies, including AMD, RP and diabetic retinopathy. The process of modulating the iBRB using siRNA directed against claudin-5 has been shown, in principle, to allow for the experimental delivery of low-molecular-mass therapeutics to improve vision radically in a murine model of autosomal dominant RP and to inhibit light-induced damage to the retina, in each case by systemic drug delivery following barrier modulation. Based on these findings, the process could potentially be used as an effective means of periodic systemic delivery to the retina of neuroprotective, anti-inflammatory and anti-neovascular drugs in human subjects showing early signs of retinal degeneration, including hereditary retinopathies, AMD and diabetic retinopathy. However, rather than using siRNA directed against claudin-5, a system whereby claudin-5 shRNA could be expressed would be necessary. By incorporating shRNA directed against transcripts of claudin-5 into the genome of an AAV, it should be possible to allow for localised and controlled modulation of the iBRB for the chronic treatment of retinopathies using known drugs with proven efficacy as anti-apoptotic, anti-neovascular or indeed anti-neurodegenerative effects. The process may also facilitate cytotoxic drug delivery to the retina in cases of ocular malignancy.

Although therapeutic application of RNAi-mediated modulation of the iBRB and BBB by siRNA and shRNA holds huge promise for future treatments of a range of neurodegenerative disorders, this potential therapy is not without its side effects. One such side effect is the potential of RNAi material to activate innate immune system and cause 'off-target' effects.

6. Potential 'off-target' effects of RNAi

6.1 Off-target effects

The first indication of off-target gene regulation in siRNAs came from studies by Jackson and co-workers, where gene array profiling demonstrated that ectopically applied siRNAs could alter the expression levels of dozens of non-targeted transcripts. siRNAs could silence transcripts that had as few as 11 contiguous nucleotides of identity to the siRNA [38,39]. After the discovery and understanding of the miRNA pathway Jackson *et al.* realised that this off-target effect was likely to

work by means of miRNA-like functions and the problem was controlled by a single 2′-OMe substitution on the ribose of the second nucleotide, which reduced off-target effects [39].

6.2 Crossreactions with the miRNA pathway

As the siRNA and miRNA pathways are similar, the endogenous miRNA pathway may become saturated by overexpressing shRNAs, resulting in cellular depletion of one or more miRNAs, which could lead to eventual cellular toxicity. This was demonstrated by Grimm *et al.* when they investigated the long-term effects of sustained high-level shRNA expression in livers of adult mice [40]. Pol III expressed shRNAs delivered in an AAV by tail-vein injection into mice resulted in acute liver failure in many of these animals. Morbidity was associated with the downregulation of liver-derived miRNAs, indicating possible competition of the latter with shRNAs for cellular factors such as exportin-5 required for the processing of small RNAs. This study highlights that therapeutic shRNA dosage must be carefully controlled at the preclinical stage to avert potential side effects during the clinical trial stage. The risk of saturation can also be reduced by selective shRNA sequence consideration.

6.3 Innate immune response

Another potential drawback of RNAi is that certain motifs, in particular GU-rich regions, in siRNAs can trigger an endosomal TLR7/8-mediated immune response that leads to secretion of inflammatory cytokines in a cell-type and sequence-specific manner [41]. This has raised concerns recently about the safety of RNAi use in therapeutics. However, the inclusion of at least one 2′-OMe in either the sense or the antisense strand of the siRNA has been shown to revoke strongly cytokine induction while maintaining silencing activity in *in vivo* models [42].

Furthermore, Kleinman *et al.* have demonstrated that naked siRNA activates TLR3 on the surface of vascular endothelial cells, resulting in IFN-γ and IL-12 release, which subsequently facilitates nonspecific anti-angiogenic effects [43]. Angiogenesis is often seen in cancer metastases and also occurs in the choroidal neovascularisation (CNV) associated with AMD. Clinical trials for AMD are premised on gene silencing by means of using siRNAs targeting vascular endothelial growth factor-A (VEGFA) or its receptors (VEGFR-1 and VEGFR-2). However, Kleinman *et al.* found that CNV inhibition can be induced in a nonspecific manner. 21-nucleotide or longer siRNAs targeting non-mammalian genes, non-expressed genes, non-genomic sequences, pro- and anti-angiogenic genes, and RNAi-incompetent siRNAs all suppressed CNV in mice by binding to cell-surface TLR3. TLR3 activation leads to a signalling pathway that culminates in the induction of IFN-γ and IL-12, which are involved in angiogenesis inhibition. This effect was not observed in knockout mice lacking TLR3. A minimum of 21 nucleotides was required for CNV inhibition and a docking model confirmed an interaction between 21-nucleotide siRNA and TLR3. siRNAs shorter than 21 nucleotides could interact with TLR3, but with a free energy of binding lower than the threshold required for dimer stabilisation and receptor activation. This finding could have huge implications in RNAi-based therapeutics. Besides inducing effects on vasculatures, siRNAs could have other undesirable side effects through activating TLR3 as TLR3 signalling is also involved in pregnancy, immune privilege and neuronal growth.

The potential 'off-target' effects of RNAi are well documented and should form a fundamental section of any RNAi-based study with clinically relevant translation. Indeed, using RNAi to modulate the iBRB or BBB will need to occur in combination with a known low-molecular-mass drug, and the permutations of these combinations will also need to be considered.

7. Clinical potential and safety of iBRB and BBB modulation

From broad-scale transcriptional analysis of neuronal tissues post barrier modulation, it is clear that there are few if any differentially regulated genes when using claudin-5 siRNA. In a clinical sense, this is a promising finding as any toxic effects of barrier modulation will need to be addressed when translating this technology to humans. Indeed, in all preclinical experiments carried out so far there have been no adverse effects to retinal electrophysiology or no neuronal cell death. Coupled with this, no signs of retinal or cerebral edema were observed in mice when the iBRB and/or the BBB were modulated [26]. Although the experimental platform used a hydrodynamic approach to deliver siRNA to the neuronal microvasculature, for acute systemic use in humans as the case may be in Glioblastoma multiforme (GBM) treatment, siRNA could potentially be conjugated to polymer agents such as *in vivo-jetPEI*® (Polyplus Transfection, Strasbourg, France) that could allow for siRNA delivery in low volumes by means of intravenous administration and without anaesthesia. Hypothetically, this would then be followed by systemic administration of chemotherapeutic agents when claudin-5 levels are suppressed, which would in turn be able to diffuse passively into the brain.

With regard to chronic and localised barrier modulation, it will be necessary to optimise an inducible and virally mediated system whereby claudin-5 shRNA, cloned into the genome of a clinically safe virus, can be inducibly expressed in distinct regions of the brain or indeed in the retina. Such a therapeutic strategy would allow for an inducing agent such as doxycycline or mifipristine to be administered to an individual before a low-molecular-mass drug with proven efficacy against a particular condition.

8. Expert opinion

Although RNAi-based therapeutics hold massive promise for the treatment of otherwise incurable conditions, like all new therapies, a full work-up of the safety profile of this therapy

will be necessary. The RNAi strategy described here, whereby suppression of claudin-5 in the microvasculature of the retina or the brain will allow for enhanced diffusion of low-molecular-mass drugs into neuronal tissue, is unlike other 'gene therapy' approaches described so far. If the required safety toxicology and pharmacological data can be obtained for this technology, the applications of iBRB and BBB modulation could be potentially far reaching. The ultimate goal in this field is to generate a safe, localised and inducible system whereby the iBRB or BBB can be modulated in a chronic sense so that drug therapies can be taken by an individual without the need for regular and costly intra-ocular injections. In the case of neurodegenerative conditions such as Alzheimer's disease, a new therapeutic strategy for the management of disease progression could be put in place for the first time.

It is the authors' opinion that with the evaluation of a safety profile of barrier modulation in non-human primates, a case could be made for the selection of one or more neurodegenerative or neuronal malignant conditions to pursue a Phase I/II clinical trial of localised and inducible iBRB or BBB modulation for the delivery of low-molecular-mass therapeutics to neuronal tissues. In the coming years and with the characterisation of function of other TJ-associated proteins at the iBRB and BBB, it may be possible to develop a greater size-selectivity of barrier modulation by using a combination of RNAi materials targeting numerous TJ proteins at the same time. Although exposure of the brain or retina to blood-borne molecules could be detrimental, the barriers could potentially be modulated to allow for the diffusion of molecules up to possibly 15 kDa for short periods of time. In this case, small therapeutic proteins could be used for enhanced systemic delivery to the brain or retina.

With regard to Alzheimer's disease, there is now a clear consensus that the amyloid-β-peptide is causally involved in the progression of neurodegeneration observed in this disease. Therefore, therapeutic strategies targeting this peptide may hold significant promise in slowing disease progression. Recently, it has emerged that platinum-based compounds may act as inhibitors of the amyloid-β neurotoxicity associated with Alzheimer's. It has been shown that Pt(II)-1,10-phenanthroline compounds act as potent inhibitors of amyloid-β by binding to specific histidine residues on the amyloid-β-peptide. These Pt-based compounds potently inhibited both the neurotoxic and the synaptotoxic actions of amyloid-β. $Pt(II)Cl_2$(1,10-phenanthroline) compounds form stable adducts by coordinating selectively to the histidine residues of the amyloid-β-peptide. It has been demonstrated that the formation of these adducts reduces the neurotoxic and synaptotoxic activities of amyloid-β in mouse hippocampal slices [44]. Importantly, however, the compounds do not freely passively diffuse across the BBB and could represent new therapeutics to be used in conjunction with barrier-modulating technology.

Finally, with respect to neuronal malignancies, BBB modulation could be used for the treatment of primary GBMs, which are notoriously resistant to both chemotherapy and radiation. Indeed, several clinically relevant chemotherapeutics could have significant efficacy if their delivery to the tumours could be enhanced [45].

In all, modulation of the iBRB or the BBB could hold immense potential for the treatment of conditions with little or no therapeutics available at present. Although significant work still needs to be completed in the validation of the safety of this method, this work is continuing and it is hoped that the technology will have major clinical applications within 3 years.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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Affiliation

Matthew Campbell[†], Ema Ozaki & Peter Humphries
[†]Author for correspondence
Smurfit Institute of Genetics,
Trinity College Dublin,
Ocular Genetics Unit,
Dublin 2, Ireland
Tel: +353 1 8962164; Fax: +353 1 8963848;
E-mail: Matthew.Campbell@tcd.ie