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Review

Nucleic Acid-Based Theranostics for Tackling Alzheimer's Disease

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Abstract

Nucleic acid-based technologies have received significant interest in recent years as novel theranostic strategies for various diseases. The approval by the United States Food and Drug Administration (FDA) of Nusinersen, an antisense oligonucleotide drug, for the treatment of spinal muscular dystrophy highlights the potential of nucleic acids to treat neurological diseases, including Alzheimer's disease (AD). AD is a devastating neurodegenerative disease characterized by progressive impairment of cognitive function and behavior. It is the most common form of dementia; it affects more than 20% of people over 65 years of age and leads to death 7–15 years after diagnosis. Intervention with novel agents addressing the underlying molecular causes is critical. Here we provide a comprehensive review on recent developments in nucleic acid-based theranostic strategies to diagnose and treat AD.

Key words: nucleic acids; Alzheimer's disease; amyloid beta peptides; tau peptide; chemically modified oligonucleotides; nucleic acid therapeutics.

Introduction

Nucleic acid-based technologies typically use synthetic oligonucleotides 8–50 nucleotides in length, most of which bind to RNA through Watson-Crick base pairing to alter the expression of the targeted RNA and protein. Novel chemical modifications and conjugation strategies have been developed to improve pharmacokinetics and tissue-specific delivery. Vitravene, Kynamro, Nusinersen and Eteplirsen are antisense oligonucleotides (AOs) approved by the FDA to treat cytomegalovirus retinitis, familial hypercholesterolemia, spinal muscular atrophy, and Duchenne muscular dystrophy respectively [1-3]. The nucleic acid aptamer drug Macugen was approved for age-related macular degeneration [4]. These successful clinical translations demonstrate the potential of nucleic acid-based technologies and provide scope for developing novel therapeutics for AD. AD is the most common form of dementia; it accounts for 70% of cases with that diagnosis. Globally there are ~47 million current cases; 7.7 million new cases are added each year [5]. AD is characterized by a progressive loss of memory and cognitive function [6]. Patients eventually need 24-hour care that places emotional and economic burdens on the community. There is no cure for AD, nor any treatment that addresses its underlying molecular cause [5]. Current treatments use cholinesterase inhibitors [7] and *N*-methyl-D-aspartate receptor (NMDA) antagonists [8] that improve cognitive function and reduce symptoms temporarily but do not stop the progression of the disease. The current approach to diagnosis relies on a combination of cognitive and clinical assessment, genetic profiling, and magnetic resonance imaging to measure anatomical changes in the brain [9], but confirmation relies on post-mortem neuropathological assessment

and misdiagnosis is common [6]. Two hallmarks of the disease are extracellular amyloid-β (Aβ) plaques (mainly an agglomeration of Aβ peptides) and intracellular neurofibrillary tangles (hyperphosphorylated tau peptides). In this review we focus on the potential of nucleic acid therapeutic, diagnostic, and research strategies that target both Aβ and tau pathologies to help diagnose and treat AD.

Amyloid β (Aβ) hypothesis

The Aβ hypothesis states that there is an imbalance of toxic Aβ peptide production and clearance [10-12]. The main Aβ species, $Aβ_{1-40}$ and $Aβ_{1-42}$, can aggregate to form fibrils and plaques [10-12]. $Aβ_{1-40}$ and $Aβ_{1-42}$ are produced by the aberrant splicing of amyloid precursor protein (APP) by β-site APP cleaving enzyme 1 (BACE1) and γ-secretase (Figure 1) [11-15]. Mutations in the APP and Presenilin genes (*PSEN1* codes for the catalytic subunits of γ-secretase) increase $Aβ_{1-42}$ levels [10-12, 14, 16-18] and lead to early-onset familial AD. Down syndrome cases have an extra copy of chromosome 23, and hence of the *APP* gene, and develop Aβ plaques early in adulthood [19]. Oligomers of Aβ promote synaptic loss, neuronal dysfunction, and cell death [20, 21]. $Aβ_{1-42}$ inhibits the maintenance of hippocampal long-term potentiation, resulting in altered memory function [10, 22] and reduced synaptic neurotransmission through NMDA receptor-mediated signaling [10, 22, 23]. Aβ toxicity has also been implicated in inflammation [11], oxidative stress [11, 24], cholinergic transmission [23], glucose metabolism [25, 26], and cholesterol metabolism [27].

Tau hypothesis

Microtubule-associated protein tau (tau), predominantly expressed in neuronal axons, is involved in microtubule assembly and stability. Tau is regulated by phosphorylation [28, 29]. Hyperphosphorylation decreases the ability of tau to bind to microtubules, leading to reduced trafficking, destabilization of microtubules, and synaptic loss [29, 30] (Figure 2). Abnormal tau can aggregate into paired helical filaments to form neurofibrillary tangles [31] in the cytosol and sequester normal tau to inhibit microtubule assembly [29]. Alternatively, tau aggregation may be a protective mechanism to stop hyperphosphorylated tau sequestering normal tau and inhibit microtubule assembly [29]. Tau hyperphosphorylation is detrimental in various neurodegenerative diseases termed "tauopathies" [28, 32]. Hyperphosphorylation of tau correlates with neurodegeneration and cognitive decline [29, 32]. Other post-translational modifications of tau, including abnormal glycosylation and reduced β-linked acylation of N-acetylglucosamine, increase hyperphosphorylation [29, 33]. Inhibition of the ubiquitin-proteasome system may also increase the aggregation of hyperphosphorylated tau [31].

Other hypothesis of AD

Drugs currently approved by the FDA for the treatment of AD are Donepezil, Rivastigmine, Galantamine and Memantine (Table 1) [34-37]. These agents enhance cholinergic and glutamatergic neurotransmission and improve cognitive function temporarily. However, they do not slow the progression of the disease. Oxidative stress [38], inflammation [39], insulin impairment [40, 41] and abnormal cholesterol metabolism [27] may also play roles (Table 1), but will not be considered in depth here.

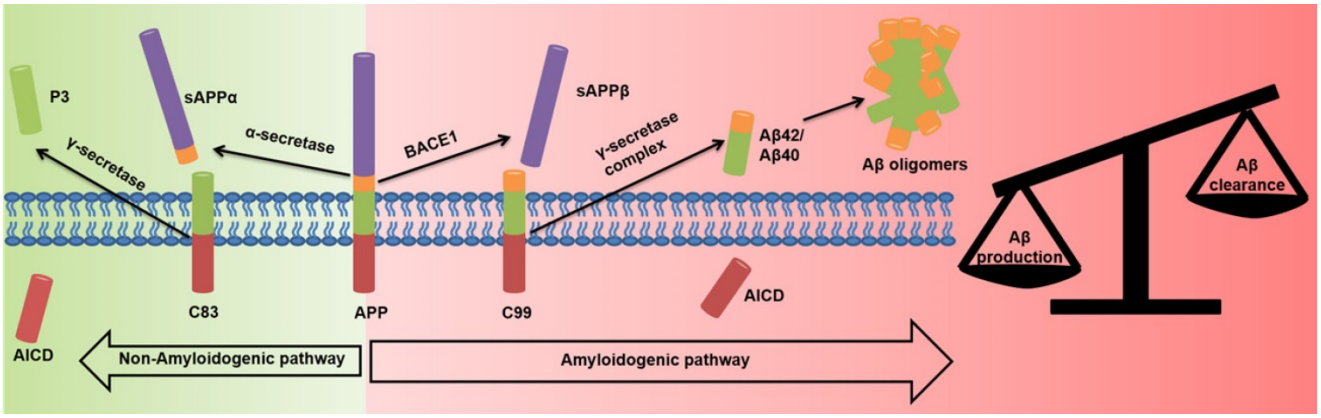

Figure 1. Non-amyloidogenic and amyloidogenic pathways in AD neurons. In the amyloidogenic pathway the APP is aberrantly spliced by BACE1 and γ-secretase to overproduce toxic Aβ species.

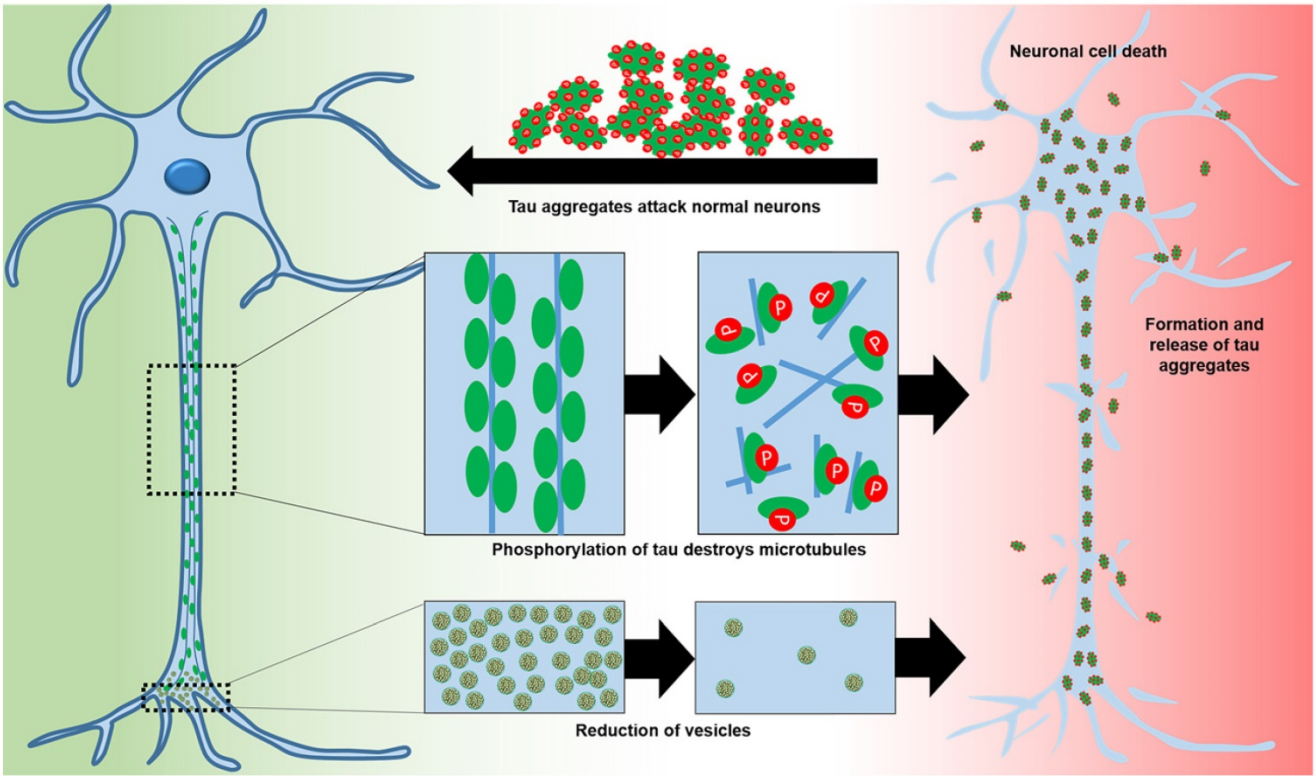

Figure 2. The roles of tau in normal neurons and of hyperphosphorylation in AD neurons that lead to neuronal toxicity.

Table 1. Therapeutic molecules in clinical trials, their targets, and trial outcomes.

Drug molecule	Role/ Target	Trial stage	Results	References
Donepezil (Pfizer)	Cholinesterase inhibitor	FDA approved- Although they improve the symptoms temporarily these drugs do not stop the progression of the disease.		[34-37]
Rivastigmine (Novartis)	Cholinesterase inhibitor			
Galantamine (Jansen-Cilag)	Cholinesterase inhibitor			
Memantine (Lundbeck)	NMDA receptor antagonist			
Tramiprosate	Aβ aggregation inhibitor	Phase III	No significant benefit. May promote abnormal tau aggregation	[47-49]
Colostrinin	Aβ aggregation inhibitor	Phase III	Modest improvements not sustained	[50-52]
Scyllo-inositol	Stabilizes Aβ aggregates and inhibits toxicity	Phase II	No statistically significant effect. Reduced Aβ in cerebrospinal fluid	[53]
Aβ vaccination	Aβ aggregation inhibitor	Phase II	Halted because patients developed meningo-encephalitis	[54]
Bapineuzumab	Aβ aggregation inhibitor	Phase III	End points not significantly different	[55]
Solanezumab	Aβ aggregation inhibitor	Phase III	End points not significantly improved	[56]
Anti-amyloid Ab	Aβ aggregation inhibitor	Phase III	No positive primary outcome	[57]
Other mAbs	Aβ aggregation inhibitor	Various	No positive outcome	[42, 58, 59]
Tarenflurbil	γ-secretase inhibitor	Phase III	No significant improvement	[60-62]
LY450139 (Eli Lilly)	γ-secretase inhibitor	Phase III	Discontinued: no $A\beta_{40/42}$ reduction	[63]
BMS-708163 (B-M Squibb)	γ-secretase inhibitor	Phase II	Terminated due to lack of favorable pharmacodynamics	[42, 64]
Verubecestat	BACE1 inhibitor	Phase III	Currently running	[65]
Rogiglitazone	BACE1 inhibitor and Type 2 diabetes drug	Phase III	No positive outcome	[66]
Pioglitaozone	BACE1 inhibitor and Type 2 diabetes drug	Phase III	No positive outcome	[66]
Methyl thionium chloride	Tau aggregation inhibitor	Phase II	Significantly improved cognitive function	[67, 68]
Tideglusib	GSK3β	Phase IIb	No positive outcome	[68, 69]
Davunetide	Microtubule stabilizer	Phase III	No significant improvement	[30]
Antioxidants	ROS	Phase III	No positive outcome	[42, 70]
Anti-inflammatories	Inflammation	Phase III	No significant improvement	[25, 39, 59, 71-73]
Intranasal insulin	Insulin impairment	Pilot	Improvement in patients without APOE-ε4 allele	[40, 74]
Other anti-diabetics	Insulin impairment	Phase III	Currently running	
Statins	Cholesterol metabolism	Phase III	Preliminary results positive; mechanism unknown.	[27, 75]

Current therapeutic molecules and clinical trials for the treatment of AD

Many disease-modifying therapeutics show positive outcomes in animal models but disappointing results in clinical trials (for drug candidates and ongoing trials see Table 1). Current strategies have been comprehensively reviewed [42]. Poor outcomes might have arisen because each agent is targeting a single pathway, whereas AD is a complex disease and it may be important to aim at multiple targets [43, 44]. Success in developing a suitable therapeutic approach is challenging because the pathogenesis of AD is unknown [45]. Trials might be affected by factors such as genetics, metabolism, and diet [46], but there is clearly a need to develop novel therapeutics for this disease.

Nucleic-acid based molecules for tackling AD

Unlike conventional small-molecule drugs, nucleic acid-based therapeutic agents such as AOs, small-interfering RNAs (siRNAs), microRNA moieties that target oligonucleotides (antimiRs and miRNA mimics), and DNAzymes/ribozymes can regulate the expression of key proteins by selectively targeting their mRNAs. The outcome is mRNA cleavage, repair, or steric blockade (Figure 3). The class of modified nucleic acids called aptamers can target proteins and inhibit their function (Figure 3). Nucleic acid-based strategies could be an effective alternative to drug development for AD because they can target a range of pathological features.

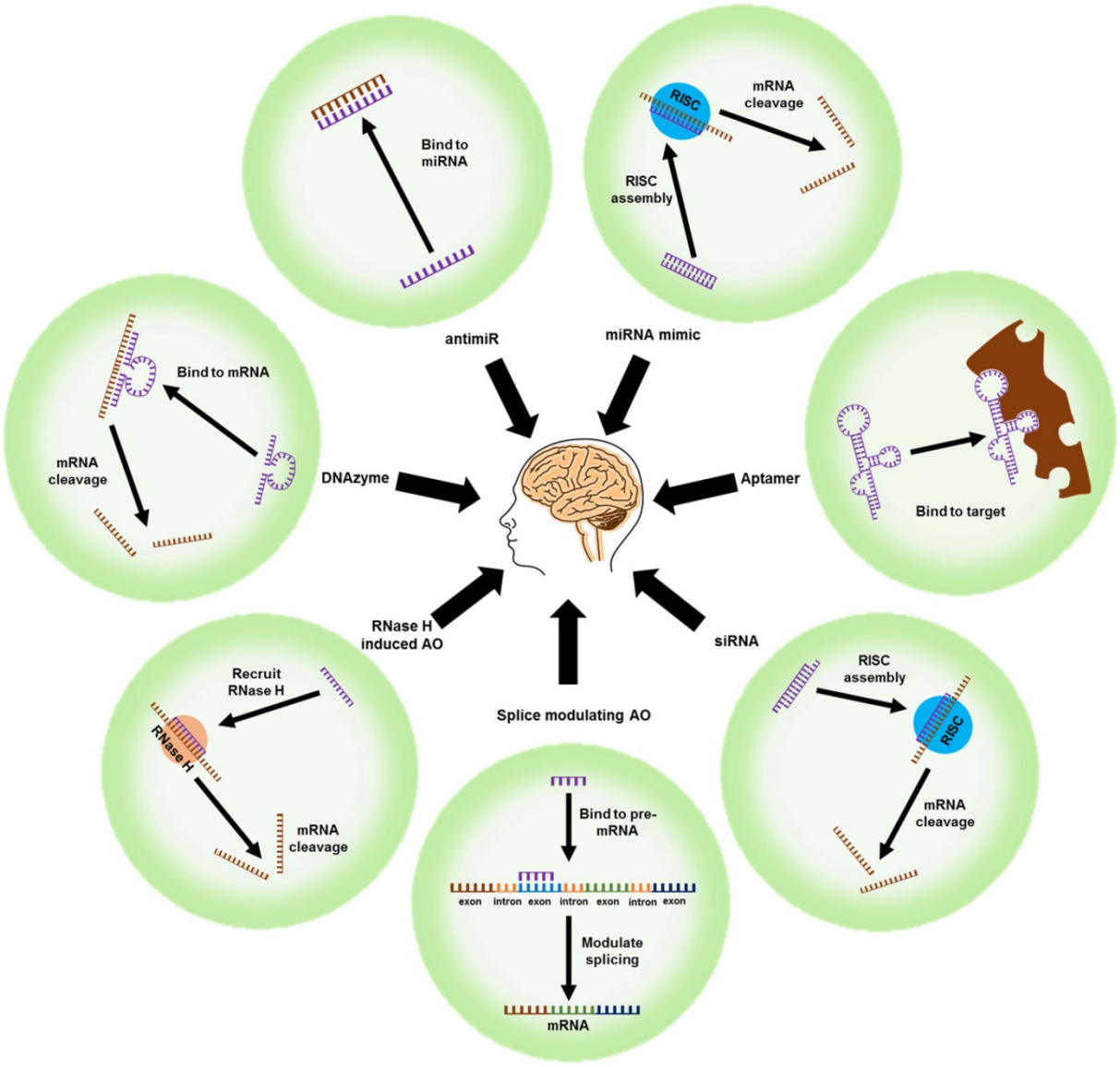

Figure 3. Nucleic acid-based therapeutic strategies. mRNA: messenger RNA; RNase H: ribonuclease H; siRNA: small interfering RNA; RISC: RNA inducing silencing complex; AO: antisense oligonucleotide; antimiR: anti-microRNA; miRNA mimic: microRNA mimic

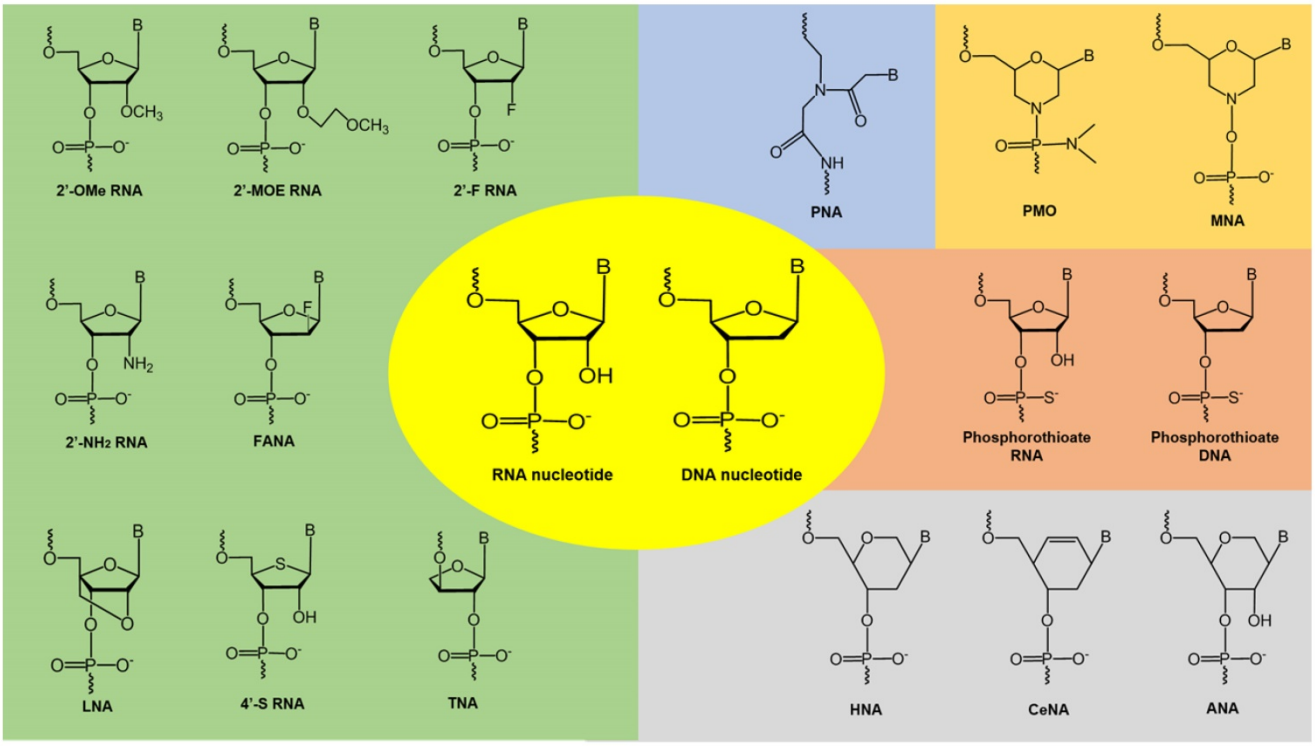

Figure 4. Examples of chemically-modified nucleotide analogues. 2'-OMe: 2'-*O*-methyl; 2'-MOE:2'-*O*-methoxyethyl; 2'-F: 2'-fluoro; 2'-NH_2: 2'-amino; FANA: fluoroarabinonucleotide; LNA: locked nucleic acid; TNA: threose nucleic acid; PNA: peptide nucleic acid; PMO: phosphorodiamidate morpholino oligomer; MNA: morpholino nucleic acid; HNA: hexitol nucleic acid; CeNA: cyclohexenyl nucleic acid; ANA: anhydrohexitol nucleic acid

Improving the stability and efficacy of nucleic acid-based therapeutics

Therapeutic oligonucleotides composed of naturally occurring nucleotides are rapidly degraded *in vivo*, which makes them unsuitable for drug development. To improve their pharmacokinetic properties, chemically modified nucleotide analogues with high resistance to nucleases are normally used. A number of analogues have been developed by modifying the base or sugar moieties, or the inter-nucleotide linkages (see Figure 4) [76-78]. Phosphorothioate DNA [79], 2'-*O*-methyl (2'-OMe) RNA [80], 2'-fluoro (2'-F) RNA [81], 2'-*O*-methoxyethyl (2'-MOE) RNA [82], and phosphorodiamidate morpholino (PMO) [83] analogues have been successfully utilized in FDA-approved oligonucleotide drugs. Analogues such as locked nucleic acids (LNA) [84, 85], peptide nucleic acids (PNA) [86], tricyclo-DNA (tcDNA) [87], and cyclohexenyl nucleic acids (CeNA) [88] also show excellent biophysical properties and offer further scope for novel oligonucleotide development. These chemistries can be used to construct fully modified or mixmer oligonucleotides. Aptamers can be modified during the selection or post-selection stages to improve their affinity and bioavailability [77]. Another challenge in the clinical utilization of unmodified oligonucleotides is rapid renal clearance from the blood due to their small size that falls under the renal filtration threshold. To increase their bioavailability, oligonucleotides can be conjugated with polyethylene glycol (PEG) to increase their size, which could also improve their resistance to nucleases [89]. Several PEGylated drugs have been approved by the FDA for clinical use [89, 90]. Other strategies include conjugating the oligonucleotides to albumin, which has a size of around 7 nm and shows reduced renal clearance and can therefore increase the circulation half-life of the oligonucleotides. Phosphorothioate modified oligonucleotides also showed reduced renal clearance by binding to plasma proteins like albumin to avoid glomerular filtration [91]. Another strategy is the synthesis of neutral siRNA (masking the negative charge on the phosphate backbone). Neutral siRNA showed reduced renal clearance [92].

Recent progress in modified nucleic acids for AD

Antisense oligonucleotides

A classical nucleic acid approach to controlling the expression of proteins is to use AOs, short single-stranded synthetic oligonucleotides, which can precisely target an mRNA transcript to regulate the

expression of the protein it codes for. Antisense mechanisms include RNase H recruitment and cleavage of mRNA, modulation of splicing in pre-mRNA, and steric blockade of either mature or pre-mRNAs (Figure 3). RNase H-mediated cleavage involves designing a short DNA oligonucleotide that binds to the target mRNA to form a RNA-DNA duplex [93]. The duplex is recognized and cleaved by endogenous RNase H. AOs that modulate pre-mRNA splicing can be used to repair defective RNA and eliminate disease-associated splice variants [94]. Many pre-mRNA transcripts are alternatively spliced to produce different mRNA, and hence protein, variants [94].

APP

Many groups have designed AOs that target APP to reduce APP expression. An early study by Allinquant *et al*. [95] developed AOs that successfully blocked rat APP synthesis. Administration of the AOs showed that APP played a role in axonal and dendritic growth, and thus in neuronal differentiation [95]. ISIS Pharmaceuticals (now Ionis Pharma) have patented (US 2003/0232435 A1) 78 gapmer AOs with 2'-MOE wings and central DNA region. The AOs target various regions of APP mRNA and inhibit 39–82% of APP protein expression [96].

Kumar and colleagues [97] developed phosphorothioated DNA AOs against sequences that correspond to the Aβ region of APP (17-42 amino acids). Administration of the AOs led to improved cognitive function in senescence-accelerated mouse-prone 8 (SAMP8) mice. SAMP8 mice have a natural mutation that leads to APP over-expression, impaired Aβ removal, and loss of memory with increasing age. The AOs that target the mid-Aβ region reduced APP levels by 43–68% in the amygdala, septum and hippocampus [97]. The mice showed improvement in acquisition and retention in the footshock avoidance paradigm, which reversed their deficits in learning and memory [97]. AOs that target the sequences that correspond to the region of APP coding for the first 17–30 amino acids of Aβ were the subject of intellectual property protection [98]. Banks and colleagues [99] showed that a radioactively tagged phosphorothioate DNA AO targeting the Aβ region of APP could transit the blood-brain barrier (BBB) of mice to enter the cerebrospinal fluid. When a 100-fold higher dose of the AO was injected into the brain by intracerebroventricular injection it reversed the learning and memory deficits in SAMP8 mice, possibly through reduced oxidative stress. Poon *et al*. [100] used proteomics to show that lower Aβ levels result in reduced oxidative stress in brain.

Opazo *et al*. [101] transfected the AOs described by Kumar and colleagues [97] into the CTb cell line, a neuronal line from mice that overexpresses APP, and the CNh cell line from normal mice. The AOs resulted in APP knockdown in CTb cells by 36%, 40% and 50% compared with normal CNh cells after 24 h, 48 h and 72 h respectively [101]. By 72 h after AO transfection, choline uptake was similar to that in CNh cells and there was increased choline release in response to glutamate, nicotine and KCl depolarization, which reached similar levels to those observed in CNh cells. The CTb cells come from a Down syndrome mouse model, which show some learning deficits and cholinergic dysfunction that are similar to those found in AD [102]. Similarly, Rojas *et al*. [103] showed that APP overexpression reduced the expression and retrograde transport of nerve growth factor. This reduced nicotine-induced stimulation of $\alpha_3\beta_2$ nicotinic acetylcholine receptor and in consequence lowered intracellular Ca^{2+} responses in CTb cells. The effects of APP overexpression were restored close to normal by treatment with AOs targeting APP expression.

Chauhan and colleagues [104] designed gapmer AOs with 2'-OMe and DNA nucleotides on a phosphorothioate backbone that target the β-secretase cleavage site of APP and found that they reduced brain $A\beta_{40}$ and $A\beta_{42}$ levels in a mouse model of AD. The AOs were delivered intracerebroventricularly and showed rapid uptake and retention for 30 minutes. They efficiently crossed cell membranes into the nuclear and cytoplasmic compartments of neuronal and non-neuronal cells. Chauhan and Siegel [105] designed two additional AOs targeting the β- and γ-secretase site of APP in the Tg2576 mouse model that expresses APP. The AO targeting the mutated β-secretase site increased soluble APPα by 43% and decreased soluble $A\beta_{40}$ and $A\beta_{42}$ levels by 39%, whereas the AO targeting the γ-secretase site had no effect. The AO targeting β-secretase also inhibited acetylcholinesterase activity, increasing acetylcholine by five-fold in cortex compared with controls.

Erickson *et al*. [106] peripherally administered an APP AO to SAMP8 mice. This resulted in a 30% increase in APP levels but no change in soluble Aβ levels. The treated mice showed improved memory. They also showed [107] that AO-mediated APP knockdown in Tg2576 mouse brains reduced cytokine expression and improved learning and memory. Attenuating APP overexpression may improve learning and memory by reducing inflammation (also implicated in AD pathology).

BACE1

Yan *et al*. [108] developed two AOs that target β-secretase aspartyl protease and found that they

reduced the release of $A\beta_{40}$ and $A\beta_{42}$ by 50–80%. Vassar and colleagues [109] also used AOs that target β-secretase to reduce $A\beta_{40}$ and $A\beta_{42}$ production by around 30%. These studies showed that β-secretase is important for the production of $A\beta_{40}$ and $A\beta_{42}$ and highlighted BACE1 as an important target for AD. Wolfe *et al*. [110] designed splice-modulating AOs to target BACE1, since alternatively spliced transcript variants at exons 2 and 3 do not show β-secretase activity. The AOs reduced Aβ production significantly in cells without altering total BACE1 mRNA.

Presenilin 1 (PSEN1)

Refolo *et al.* [111] found that AOs targeting PSEN1 in a human cell line reduced PSEN1 holoprotein by 80% 12 days after treatment and by 90% after 14 days. This was correlated with a two-fold increase in $A\beta_{42}$ levels. Grilli *et al.* [112] found that hippocampal primary neurons overexpressing mutant PSEN1 were vulnerable to excitotoxic and hypoxia-hypoglycemic damage and increased cell death. They designed two phosphorothioate AOs targeting PSEN1 in wild type mice. In contrast to Refolo *et al.* [111] they found that lower PSEN1 expression reduced cell death and provided neuroprotection [112]. Fiorini *et al*. [113] administered AOs targeting PSEN1 to aged SAMP8 mice and found they reduced brain oxidative stress biomarkers. In the T-maze foot shock avoidance and novel object recognition tests the mice showed a reversal of learning and memory deficits.

Tau

Caceres *et al*. [114] showed that an AO targeting the 5' end of the tau gene, in the region before the start codon, showed strong inhibition of neurite elongation in primary rat neurons. Immunoblotting revealed that the tau protein level was reduced in AO-treated mice but not in control mice. The effect of AO treatment on cognition needs to be assessed. DeVos *et al.* [115] screened 80 AOs targeting tau and selected the three that showed the best knockdown of tau to test *in vivo*. The latter reduced tau mRNA levels by more than 75%. The best AO was selected for further testing in mice; it lowered brain tau mRNA and protein significantly in a dose-dependent manner. Behavioral impacts and neurotoxicity were not measured. Kalbfuss *et al.* [116] developed splice-modulating AOs modified with 2'-OMe nucleotides to target the tau exon 10 splice junctions to reduce exon 10 inclusion. Exclusion of exon 10 increases the ratio of tau proteins lacking the microtubule-binding domain. In consequence, the microtubule cytoskeleton becomes destabilized as observed in frontotemporal dementia and parkinsonism.

Peacey *et al.* [117] designed bipartite AOs that bound to the hairpin structure at the boundary between exon 10 and intron 10 of tau to inhibit exon 10 splicing, reversing the effect of disease-causing mutations in cells. Liu *et al.* [118] developed a small-molecule (mitoxantrone) conjugated to a bipartite AO that binds to the tau RNA hairpin structure. The conjugate also inhibited exon 10 splicing in cell-free conditions more effectively than mitoxantrone or the bipartite AO alone, but induced cytotoxicity. The same group used a PNA-modified bipartite AO conjugated to mitoxantrone that inhibited tau splicing but was also cytotoxic [110].

Sud *et al.* [119] developed PMO AOs to modulate the splicing of tau and tau expression. The AOs were designed to target sequences at the donor and acceptor splice sites, the splicing branch points, and splicing enhancers and inhibitors to induce exon skipping. Exons 0, 1, 4, 5, 7, 9, and 10 were targeted. Exons 1, 4, 5, 7, and 9 are found in all 6 isoforms of tau while exon 10 is present in only three of the six isoforms. Of the 31 AOs tested, AO E1.4 targeting the splice donor site at the exon 1 – intron 1 junction reduced tau mRNA expression by 50%. The other AOs effective in this region were a combination of AOs that targeted the splice donor and acceptor sites and the start codon. AO E5.3 targeted the splice donor site at the exon 5 –intron 5 junction and reduced total tau mRNA expression by 29–46%. It also reduced tau protein level by 58–62%. The resulting transcript was missing exons 4 to exon 7 using the normal splice sites. AO E7.7 targeted the splice donor site on exon 7; it reduced tau mRNA expression by 30% and tau protein levels by 67%. E5.3 injected into mice *in vivo* produced lower tau mRNA levels than in non-injected regions.

GSK-β

Farr *et al*. [120] showed that a phosphorothioated AO that targets GSK-3β decreased GSK-3β protein levels in the cortex of SAMP8 mice. There were improvements in learning and memory, reduced oxidative stress, increased levels of the antioxidant transcription factor nuclear factor erythroid-2 related factor 2, and decreased tau phosphorylation.

Acetylcholinesterase (AChE)

Fu *et al.* [121] found that AOs against human AChE mRNA reduced AChE activity in an AD mouse model after 8 h; the effect lasted till 42 h. Lower enzymic activity was accompanied by improvement on behavioral tasks, which showed increased memory retention and improved water maze performance (shorter swimming time).

Apolipoprotein E receptor 2 (ApoER2)

ApoER2 may be a primary risk factor for late-onset AD [122, 123]. Dysregulation of ApoER2 splicing may result in impaired synaptic homeostasis. Cerebral injection of mice with AOs targeting the adjacent introns enhanced exon 19 inclusion, an effect that persisted for up to 6 months [123]. The mice showed improvement in Aβ-induced cognitive defects. It was postulated that the AOs bind to the splicing factor SRF1 to reduce its expression and increase the inclusion of exon 19, thereby increasing the level of the active form of ApoER2 to enhance NMDA receptor phosphorylation.

siRNA

These are short synthetic double stranded RNA oligonucleotides that target complementary mRNA and silence gene expression through the assembly of the RNA-induced silencing complex (RISC) (Figure 3) [93, 124]. Chemical modifications can be introduced into the siRNA to increase its stability against nucleases and increase its selectivity for the target (Figure 4).

APP

Miller and colleagues [125] found that siRNAs against the Swedish mutant in APP that causes a familial form of AD silenced the expression of mutant alleles. The siRNAs were designed to ensure that they bound specifically to the mutant alleles and not the wild-type. The mutation was placed in the central region of the siRNA duplex to achieve high silencing efficiency.

BACE1

McSwiggen and colleagues [126] patented 325 siRNAs that target BACE (NCBI ID: NM_012104). The patent covers sequences with various chemically modified siRNAs that include 2'-deoxy, 2'-F and 2'-OMe pyrimidine and purine nucleotides, phosphorothioate internucleotide linkages and inverted deoxyabasic caps. Four of the siRNAs reduced BACE expression by 40–90% at 25 nM concentration, but there was no data on whether this altered $A\beta_{40}$ and $A\beta_{42}$ expression. Basi *et al*. [127] made a siRNA that reduced the BACE1 mRNA level by 50% and BACE protein level by more than 90%. It decreased the secretion of Aβ peptide without affecting BACE2 expression, indicating specificity for BACE1. Kao *et al.* [128] also designed siRNAs, where two of the siRNAs reduced BACE1 mRNA by more than 90% and Aβ production by 36–41%. Pretreatment of neurons with the siRNA increased neuroprotection against hydrogen peroxide-induced oxidative stress. Modarresi *et al.* [129] injected LNA-modified siRNAs targeting BACE1 antisense transcripts into the third ventricle of Tg-19959 mice to downregulate BACE1 and BACE1 antisense transcripts, which led to lower BACE1 protein levels and less Aβ production and aggregation in the brain. Notably, Cai *et al*. [130] showed that siRNAs targeting BACE1 inhibited it in mice and increased choroidal neovascularization: BACE1 is also expressed in the neural retina and in *in vitro* and *in vivo* angiogenesis. Although BACE1 inhibition may be therapeutically beneficial in AD, it may contribute to retinal pathologies and exacerbate conditions such as age-related macular degeneration.

Heterogeneous nuclear ribonucleoprotein H

A G-rich region in exon 3 of BACE1 may form a G-quadruplex structure and recruit a splicing regulator, heterogeneous nuclear ribonucleoprotein H, that regulates splicing to increase generation of the BACE1 501 isoform. Fisette *et al*. [131] reported that siRNA and short hairpin RNA candidates that target heterogeneous nuclear ribonucleoprotein H reduced its expression and thereby decreased BACE1 501 isoform levels and Aβ production.

AntimiRs and miRNA mimics

miRNAs are short non-coding RNAs that regulate protein expression post-transcriptionally. miRNA mimics can modulate RNA and protein expression by acting like their endogenous miRNA counterparts. AntimiRs can modulate RNA and protein expression by inhibiting endogenous miRNA similar to AOs (Figure 3). miRNAs silence gene expression by translational repression and/or mRNA degradation [132, 133]. miRNAs are first transcribed by RNA polymerases II or III to form long primary miRNA with a 5′ CAP and a poly(A) tail [133-135]. These are then processed in the nucleus into short 70-nucleotide hairpin structures called precursor miRNAs (pre-miRNA) by the microprocessor complex [133-135]. The pre-miRNAs are exported to the cytoplasm by Exportin 5 and processed by Dicer into double-stranded miRNA duplexes, which are approximately 22 nucleotides long [133-135].

BACE1

An endogenous non-coding BACE1 antisense transcript stabilizes the BACE1 transcript and may upregulate BACE1 in AD cases. BACE1 antisense binds to BACE1 at the miR-485-5p binding site and suppresses BACE1 expression. Faghihi *et al.* [136] found that LNA-antimiRs that target miR-485-5p decreased miRNA-induced suppression of BACE1 and increased BACE1 antisense expression. Hebért *et al.* [137] showed that miR-29a/b-1 cluster was significantly reduced in sporadic AD patients and correlated with increased BACE1 expression and Aβ

generation, and therefore may be potential targets for miRNA mimics as a therapeutic strategy for AD.

Tau

Mi34a reduces endogenous tau expression at both the mRNA and protein level in M17D cells by binding to the 3' UTR region of tau [110], whereas miR-34c levels are elevated in the hippocampus of AD patients and mouse AD models [138]. Wolfe *et al.* [110] used LNA antimiRs to inhibit miR-34a, -34b and -34c and found increased tau expression. Zovolis *et al.* [138] found that an antimiR that targets miR-34c rescued learning in mouse models.

Acetyl-CoA acyl transferase

Acetyl-CoA acyl transferase has a role in lipid metabolism that has been implicated in the pathogenesis of AD. Murphy *et al.* [139] inhibited Acetyl-CoA acyl transferase using an artificial miRNA to reduce Aβ plaque burden and improve cognition in a mouse model of AD. The miRNA also reduced full-length human APP levels.

Brain-derived neurotropic factor

Brain-derived neurotropic factor regulates synaptic plasticity and memory and is decreased in AD brains [140-142], while miR-206 suppresses brain-derived neurotropic factor levels and memory function in AD mice [143]. Lee *et al.* [143] injected an anti-miR candidate AM-206 that targets miR-206 into the third ventricle of Tg2576 mice. It increased brain levels of brain-derived neurotropic factor, enhanced hippocampal synaptic density, neurogenesis, and memory. Intranasally administered AM-206 also reached the brain and had similar effects to the injected AM-206.

DNAzymes/Ribozymes as therapeutic candidates for AD

A target RNA can be cleaved to reduce its expression using catalytic oligonucleotides such as DNAzymes and ribozymes [144]. The arms of these enzymes hybridise with the target RNA and cleave their targets through the catalytic loop in the middle. DNAzymes and ribozymes cleave the phosphodiester bond at the purine-pyrimidine or purine-purine junction (Figure 3).

BACE1

Nawrot *et al.* [145] designed RNA-cleaving hammerhead ribozymes that downregulated BACE1 mRNA expression by more than 90% in HEK293 and SH-SY5Y cells and reduced $A\beta_{40}$ and $A\beta_{42}$ production by more than 80%. They also showed that a DNAzyme with the 10–23 catalytic loop reduced BACE mRNA expression by 70%. However, whether the reduced BACE mRNA expression leads to reduced Aβ production is unknown and requires validation.

Nucleic acid aptamers

Aptamers are short single stranded RNA or DNA oligonucleotides with unique three-dimensional structure that bind to targets with high affinity and specificity. Aptamers can be developed against a variety of targets ranging from small molecules to complex proteins over whole cells. Aptamers can be used for therapeutic, diagnostic (biosensors and molecular imaging), and targeted drug delivery applications. They are typically selected from large DNA and RNA oligonucleotide libraries through a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX) [146, 147].

Aβ

Ylera *et al.* [148] were the first to report novel RNA aptamers that bound to $A\beta_{1\text{-}40}$ fibrils with high affinity (29–48 nM). Bunka *et al.* [149] made aptamers against amyloid-like fibrils from β_2-microglobulin. They bound to the target with high affinity, but also bound to other amyloid fibrils including, but not confined to, those found in dialysis-related amyloidosis patients. Rahimi *et al.* [150] also developed RNA aptamers against Aβ fibrils, but these also interacted with other amyloidogenic proteins by binding to a common β-sheet motif. They bound to fibrils with ≥15-fold higher sensitivity than thioflavin-T, suggesting that aptamers might be diagnostic tools for AD. Takahashi *et al.* [151] isolated two RNA aptamers, N2 and E2, that bound to monomeric $A\beta_{40}$ with dissociation constants of 21.6 and 10.9 μM respectively. Though the affinities were quite low for clinical use, enzyme-linked immunosorbent assay (ELISA) showed that they could inhibit Aβ aggregation efficiently. When conjugated to AuNP gold nanoparticles, N2 and E2 bound to both Aβ monomers and oligomers. Mathew *et al.* [152] showed that the N2 aptamer conjugated to curcumin-polymer nanoparticles enhanced binding to, and disaggregated, amyloid plaques, which were then cleared by phagocytosis. The study targeted peripheral amyloid as peripheral organs may also generate amyloid proteins, which have also been implicated in AD. Targeting peripheral amyloid is easier due to the challenges in the brain delivery of aptamers.

Farrar *et al.* [153] developed a fluorescently tagged aptamer that bound to Aβ oligomers in both AD and transgenic mouse brain tissue. The aptamer may be useful for Aβ imaging, which has diagnostic implications. Similarly, Babu and colleagues [154]

developed an aptamer complexed with ruthenium that binds to, and inhibits the formation of, Aβ oligomers. The aptamer-ruthenium interaction increases luminescence intensity, which is reduced when the aptamer binds to Aβ monomer or oligomers.

BACE1

Rentmeister *et al.* [155] made an RNA aptamer that binds to the short cytoplasmic tail of BACE1. It is a good research tool to investigate the biological function of the cytoplasmic tail without interfering with BACE1 transport and localization. Liang *et al.* [156] developed two DNA aptamers, A1 and A4, that bind to the extracellular domain of BACE1 with high affinity (K_d 15–69 nM) and specificity. They have similar affinities to the anti-BACE1 antibody. *In vitro*, APP Swedish mutant cells treated with A1 showed lower $A\beta_{40}$ and $A\beta_{42}$ levels than control cells. sAPPβ expression decreased with A1 treatment compared with untreated controls.

Tau

Kim *et al.* [157] used recombinant his-tagged tau40 to select aptamers from an RNA library through SELEX. 12 rounds of selection produced a tau-1 aptamer, which represented ~76% of identified aptamers, that reduced the levels of oligomeric tau (by ~94%) *in vitro* in a dose-dependent manner. However, it could not de-oligomerize pre-existing tau oligomers and had no effect on tau degradation. The aptamer bound to tau protein and inhibited its oligomerization, unlike control aptamers. Primary neurons treated with tau-1 aptamer showed less cytotoxicity than controls but no difference in membrane integrity or viability; there was little effect on normal tau function. Primary rat cortical neurons administered tau oligomers and treated with tau-1 aptamers showed significantly less oligomeric tau phosphorylation at Ser199/202 but there was no effect on monomeric tau. Extracellular tau oligomers also stress neighboring neurons. Administration of tau oligomers leads to severe neurotoxicity, which was reduced by tau-1 aptamer treatment. Tau-1 aptamers can prevent or reverse cytotoxicity mediated by tau oligomerization both in a non-neuronal cell line and in primary rat cortical neurons. Unfortunately, the tau-1 aptamers isolated by Kim *et al.* bound only to one of the six isoforms of tau. Therefore, the effects of tau-1 aptamers observed in mice may not translate clinically, because six isoforms are prone to aggregation and implicated in neurodegeneration. To be successful clinically, the aptamers must be able to cross the BBB and the neuronal cell membrane, and disaggregate the neurofibrillary tangles after binding [158]. Kim *et al.* [159] reported a DNA aptamer-antibody sandwiched to the tau-381 isoform that detected tau in human plasma at femtomolar concentrations by surface plasmon resonance.

The ubiquitin-proteasome system

Lee *et al.* [160] developed an aptamer against USP14, an enzyme that delays protein degradation by the ubiquitin-proteasome system. Recombinant USP14 was incubated with a random RNA library for SELEX. Three aptamers, USP14-1, USP14-2 and USP-14-3, were identified, all of which bound to USP14 with high affinity. USP14-3 showed the strongest inhibition of deubiquitination, which may be due to its ability to bind both USP14 and UCH37. UCH37 is a protein that also slows protein degradation in the proteasome. The aptamers have yet to be tested in mice for their effect on tau oligomerization and degeneration.

Prion protein

Mashima *et al.* [161] isolated aptamers against bovine prion protein by SELEX that may have therapeutic potential in prion diseases and AD. Aβ oligomers bind to the prion protein to block long-term potentiation. Thus, prion protein may mediate Aβ oligomer-induced synaptic dysfunction.

Brain delivery of nucleic acid molecules

Receptor-mediated endocytosis or nanoparticle conjugation strategies

The barrier for any successful drug to treat neurological diseases is its failure to cross the BBB. Various approaches have been made to overcome this issue, and in many instances the drug can be conjugated with other molecules to improve brain delivery. Lipophilic molecules under 500 Da can cross the BBB by simple diffusion. Therapeutic nucleic acids are typically too large to cross the BBB, although nanotechnology is now starting to overcome this problem. This was the subject of a comprehensive review by Kanwar *et al.* [162]. Nucleic acids can be transported through the BBB by receptor-mediate endocytosis when conjugated to molecules such as transferrin, insulin, leptin, and insulin-like growth factor 1: these bind to their receptors on the BBB, which allows them to cross the BBB. Many studies have used nucleic acids conjugated to molecules that target the transferrin receptor. Transferrin-conjugated nanoparticles, or nanoparticles conjugated to transferrin receptor antibodies, can transport drugs across the BBB [163, 164]. An aptamer that targeted the mouse transferrin receptor allowed a lysosomal enzyme to enter cells via endocytosis; this can be applied to drug transport [165]. Two aptamers that

bound to epithelial cell adhesion molecule and transferrin receptor were fused together; the product could bind to cancer cells expressing the epithelial cell adhesion molecule after crossing the BBB by transferrin-receptor targeting [166].

Cell-penetrating peptide-based delivery systems

The use of cell-penetrating peptide-based delivery systems is another approach for transporting nucleic acids across the BBB. These systems generally contain between 8 and 30 amino acids. Fluorescein isothiocyanate-labeled cell-penetrating peptides were conjugated to a morpholino AO targeting the mutant *ataxia telangiectasia* gene and were able to cross the BBB [167]. Heitz *et al*. [168] reviewed the development of cell-penetrating peptides.

Intracerebroventricular infusion

Nucleic acids can also be introduced directly into the cerebrospinal fluid by intracerebroventricular infusion. The advantage of this over the use of targeting molecules is that the drugs are delivered at therapeutic concentrations quickly. However, intracerebroventricular infusions are highly invasive and rely on diffusion of the drugs throughout the ventricular system. The drugs can then enter the blood stream, because the cerebrospinal fluid turns over every 4–5 hours [169]. Pardridge *et al.* [169] described the advantages and disadvantages of different drug delivery methods to the brain. Intranasal methods are non-invasive and can deliver nucleic acids directly to the brain. They have been used successfully to deliver insulin to AD patients [170]. Various molecules delivered intranasally have improved cognitive function in a mouse model of AD and in clinical trials, as summarized in the review by Hanson *et al.* [171]. Many reviews describe how nose-to-brain delivery occurs, the different drugs that have been successfully delivered this way, and its potential for treating neurodegenerative diseases such as AD [172, 173].

Conclusions and Future Perspectives

Nucleic acid-based approaches offer great promise for developing novel therapeutics for AD, a complex neurodegenerative disease with several pathological features. Confounds include genetic factors, metabolic disorders including high cholesterol levels, insulin resistance due to impaired glucose metabolism, and dysfunction in various molecular pathways. Existing therapies only treat AD symptoms, not the underlying molecular causes. Although many drug molecules have shown success in cell and animal models, this effect often cannot be replicated in human trials. There is an unmet need for better theranostic strategies. The drug Nusinersen, recently approved by the FDA for spinal muscular atrophy, shows that nucleic acids have potential for the treatment of neurological diseases, including AD. Their efficacy in targeting several pathways that underlie AD highlights their potential to be developed as novel therapeutics for AD.

Abbreviations

FDA: Food and Drug Administration; AD: Alzheimer's disease; AO: antisense oligonucleotides; NMDA: N-methyl-D- aspartate receptor; Aβ: amyloid β; APP: amyloid precursor protein; BACE1: β-site amyloid precursor protein cleaving enzyme 1; PSEN: Presenilin; tau: microtubule associated protein; siRNA: small interfering RNA; antimiR: anti-microRNA; 2′-OMe: 2′-O-methyl; 2′-MOE: 2′-O-methoxyethyl; 2′-F: 2′-fluoro; LNA: locked nucleic acids; PNA: peptide nucleic acids; PMO: phosphorodiamidate morpholino; tcDNA: tricyclo-DNA; CeNA: cyclohexenyl nucleic acid; PEG: polyethylene glycol; mRNA: messenger RNA; SAMP8: senescence- accelerated mouse-prone 8; BBB: blood brain barrier; AChE: acetylcholinesterase; ApoER2: Apolipoprotein E receptor 2; RISC: RNA induced silencing complex; SELEX: systemic evolution of ligands by exponential enrichment.

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Competing Interests

The authors have declared that no competing interest exists.

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A/Prof. Peter R. Dodd obtained his PhD from Imperial College, University of London, and is currently the Director of Queensland Brain Bank at the School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia. His lab uses human autopsy tissues to study amino acid neurotransmission, and has developed protocols to prepare good-quality mRNA, miRNA, and proteins for mapping and quantification. His lab studied phenotype-genotype interactions in alcoholics and dementia cases, partitioned by sex and comorbid disease, with key findings including altered expression of GABA receptors in alcoholics without comorbid disease and of glutamate receptors in cirrhotic alcoholics, and altered expression of glutamate transporters and receptors in Alzheimer disease. Prof. Dodd's group was the first to use microarrays to study the human brain transcriptome. Overall, his lab researches the pathogenesis of human neurological diseases.

Rakesh N. Veedu is currently leading the precision nucleic acid theranostics group at Murdoch University and Perron Institute for Neuro-logical and Translational Science. He obtained his PhD in synthetic organic chemistry in 2006 from The University of Queensland, Australia under the supervision of Prof. Curt Wentrup after completing his MSc from Griffith University, Australia. He then continued his postdoctoral career under the supervision of Prof. Jesper Wengel at the Nucleic Acid Center, University of Southern Denmark in the field of nucleic acid chemical biology. Later in 2009, he was appointed as a Research Associate Professor within the Nucleic Acid Center. He then returned to The University of Queensland in mid-2010 and established his independent research career in Functional Nucleic Acid Theranostics Development. His current research is focused on developing novel precision nucleic acid theranostics for tackling solid cancers, neurological diseases including Alzheimer's disease and infectious diseases using nucleic acid aptamers, antisense oligonucleotides, siRNA, antimiRs, molecular beacons and DNAzymes.