SNCA-TARGETING SIRNA COMPOSITIONS FOR TREATING SNCA-ASSOCIATED DISEASE

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is related to and claims priority under 35 U.S.C. § 119(e) to U.S. provisional patent application No. 63/326,813, entitled "SNCA-Targeting siRNA Compositions for Treating SNCA-Associated Disease," filed April 2, 2022; and to U.S. provisional patent application No. 63/326,770, entitled "SNCA-Targeting siRNA Compositions for Treating SNCA-Associated Disease," filed April 1, 2022. The entire contents of the aforementioned patent applications are incorporated herein by this reference.

FIELD OF THE INVENTION

The instant disclosure relates generally to SNCA-targeting RNAi agents and methods.

SEQUENCE LISTING

The instant application contains a Sequence Listing which has been filed electronically in eXtensible Markup Language (XML) format and is hereby incorporated by reference in its entirety. Said XML copy, created on March 29, 2023, is named ALN-461 WO.xml and is 3242 KB in size.

BACKGROUND OF THE INVENTION

The SNCA gene encodes a presynaptic neuronal protein, α-synuclein (also referred to as alpha-synuclein or synuclein-alpha herein), which has been linked genetically and neuropathologically to Parkinson's disease (PD) (Stefanis, L. *Cold Spring Harb Perspect Med.* 2: a009399). α-Synuclein is viewed to contribute to PD pathogenesis in a number of ways, but it is generally believed that aberrant soluble oligomeric conformations of α-synuclein, termed protofibrils, are the toxic species that mediate disruption of cellular homeostasis and neuronal death, through effects on various intracellular targets, including synaptic function. Furthermore, secreted α-synuclein is believed to exert deleterious effects on neighboring cells, thus possibly contributing to disease propagation. Although the extent to which α-synuclein is involved in all

cases of PD is not clear, targeting the toxic functions conferred by this protein when it is dysregulated presents a potentially valuable therapeutic strategy, not only for PD, but also for other neurodegenerative conditions, termed synucleinopathies, which all exhibit common neuropathological hallmarks as a result of alpha-synuclein accumulation, referred to as Lewy bodies (LBs) and Lewy neurites (LNs). In addition to PD, such documented or suspected SNCA-related synucleinopathies include, without limitation, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

PD, LBD, and MSA are the three most prevalent examples of neurodegenerative disorders with SNCA brain pathology. PD is the most common movement disorder and is characterized by rigidity, hypokinesia, tremor and postural instability. PD is believed to affect approximately four to six million people worldwide. LBD represents 5-15 % of all dementia. In addition to forgetfulness and other dementing symptoms that often fluctuate, LBD patients typically suffer from recurrent falls and visual hallucinations. MSA is a rapidly progressing orphan disorder leading to severe motor disability in an affected subject within a few years. The prevalence of MSA is reported to be between 3.4-4.9 cases per 100,000 population.

Apart from the neuropathological changes observed in α-synucleinopathies, levels of α-synuclein protein are generally increased in affected brain regions (Klucken *et al.*, 2006).

α-Synuclein monomers, tetramers and fibrillar aggregates are a major component of Lewy body (LB)-like intraneuronal inclusions, glial inclusions and axonal spheroids in neurodegeneration with brain iron accumulation. Lewy-related pathology (LRP), primarily comprised of α-synuclein, is present in a majority of Alzheimer's autopsies, and higher levels of α-synuclein in patients have been linked to cognitive decline (Twohig et al. (2019) *Molecular Neurodegeneration*). Autosomal dominant mutations in the SNCA gene including, among others, A53T, A30P, E46K, and H50Q (Zarranz et al. (2004) *Ann. Neurol.* 55,164-173, Choi et al. (2004) *FEBS Lett.* 576, 363-368, and Tsigelny et al. (2015) *ACS Chem. Neurosci.* 6, 403-416), A53T

(Polymeropoulos et al. (1997) *Science*), as well as triplications and duplications, have been identified to run in families afflicted with associated neurodegenerative diseases. The preceding indicates that not only pathogenic mutations in SNCA, but also increases in alpha-synuclein protein, impact disease outcome.

The role of SNCA mutations in disease onset is not well understood; however, evidence points to a toxic gain-of-function inherent in the normal α-synuclein protein when it exceeds a certain level (Stefanis et al. (2012) *Cold Spring Harb Perspect Med.*) and/or interacts aberrantly with cellular lipids and vesicles (reviewed in Kiechler *et al.* (2020) *Front. Cell Dev. Biol*). In apparent agreement with this, SNCA null mice, in contrast to transgenic over-expressors, displayed no overt neuropathological or behavioral phenotype (Abeliovich et al. (2000) *Neuron*). Posttranscriptional regulation of SNCA was also shown to occur through endogenous microRNAs, binding to the 3′ end of the gene (Junn et al. (2009) *PNAS* 106: 13052–13057; Doxakis (2010), *JBC*). Further, studies on the familial point mutations in SNCA demonstrated suppressed expression, especially in cases with prolonged disease onset (Markopoulou et al. (1999) *Ann Neurol.* 46(3):374-81 and Kobayashi et al. (2003) *Brain* 126(Pt 1):32-42). Similarly, Voutsinas et al. (2010) *Hum Mutat.* 31(6):685-91) found that over-expression of even wild-type SNCA messenger RNA (mRNA) was responsible for disease onset. These data indicate that suppression of total SNCA levels would lower α-synuclein-induced toxicity.

There are no disease modifying treatments for synucleinopathies, including PD, multiple system atrophy, and Lewy body dementia, and treatment options are limited, e.g., merely palliative. For example, at present, only symptomatic treatments are available for PD patients (by substituting the loss of active dopamine in the brain) and AD patients (*i.e.*, cholinesterase inhibitors). None of the existing treatment strategies for α-synucleinopathies are directed against the underlying disease processes.

Thus, noting the described involvement of SNCA in several neurodegenerative disorders (synucleinopathies), there remains a need for an agent – particularly a central nervous system (CNS)-directed agent, given the described functionality of α-synuclein in CNS tissue – that can selectively and efficiently silence the SNCA gene (e.g., eliminating or reducing the effect of toxic α-synuclein species) using the cell's own RNAi machinery that has both high biological activity and *in vivo* stability, and that can effectively inhibit expression of a target SNCA gene.

BRIEF SUMMARY OF THE INVENTION

The present disclosure provides RNAi agent compositions which affect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a Synuclein alpha (SNCA) gene. The SNCA gene may be within a cell, *e.g.*, a cell within a subject, such as a human. In certain embodiments, the RNAi agent is designed and directed for knockdown of SNCA in cells and/or tissues of the CNS. The present disclosure also provides methods of using the RNAi agent compositions of the disclosure for inhibiting the expression of a SNCA gene or for treating a subject who would benefit from inhibiting or reducing the expression of a SNCA gene, *e.g.*, a subject suffering or prone to suffering from a SNCA-associated neurodegenerative disease or disorder, *e.g.*, PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease and Huntington's disease.

Accordingly, in one aspect, the instant disclosure provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA, where the dsRNA agent includes a sense strand having a 5'-terminus and a 3'-terminus and an antisense strand having a 5'-terminus and a 3'-terminus, where the sense strand and the antisense strand form a double stranded region, where: the sense strand includes a nucleotide sequence of SEQ ID NO: 150, SEQ ID NO: 157, SEQ ID NO: 164, or SEQ ID NOs: 142-149, 151-156, 158-163, or 165-184 of **Table 3**, with 0 or 1 mismatches; the sense strand of the dsRNA agent includes a lipophilic moiety attached at position 6 or 16, counting from the 5'-terminus of the sense strand; the antisense strand includes a nucleotide sequence of SEQ ID NO: 193, SEQ ID NO: 200, SEQ ID NO: 207, or SEQ ID NOs: 185-192, 194-199, 201-206, or 208-227 of **Table 3**, with 0 or 1 mismatches; the dsRNA agent does not include a GalNAc modification; and the dsRNA agent includes eight phosphorothioate internucleotide linkages positioned at the penultimate and ultimate internucleotide linkages from the respective 3'- and 5'-termini of each of the sense and antisense strands of the dsRNA agent.

Accordingly, in one aspect, the instant disclosure provides a double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA, where the dsRNA agent includes a sense

strand having a 5′-terminus and a 3′-terminus and an antisense strand having a 5′-terminus and a 3′-terminus, where the sense strand and the antisense strand form a double stranded region, where: the sense strand includes a nucleotide sequence of **Table 3** (SEQ ID NOs: 142-184), with 0 or 1 mismatches; the sense strand of the dsRNA agent includes a lipophilic moiety attached at position 6 or 16, counting from the 5′-terminus of the sense strand; the antisense strand includes a nucleotide sequence of **Table 3** (SEQ ID NOs: 185-227), with 0 or 1 mismatches; the dsRNA agent does not include a GalNAc modification; and the dsRNA agent includes six phosphorothioate internucleotide linkages, wherein the six phosphorothioate internucleotide linkages are positions at the penultimate and ultimate internucleotide linkages at the 5′ and 3′ ends of the antisense strand and at the penultimate and ultimate internucleotide linkages at the 5′ end of the sense strand.

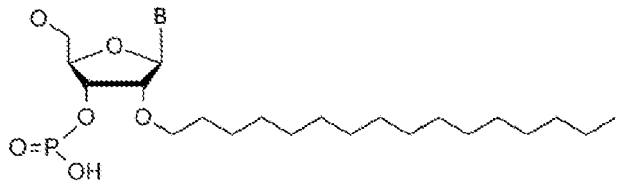
In certain embodiments, the lipophilic moiety is an aliphatic, alicyclic, or polyalicyclic compound.

In some embodiments, the lipophilic moiety is lipid, cholesterol, retinoic acid, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O(hexadecyl)glycerol, geranyloxyhexyanol, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine.

In one embodiment, the lipophilic moiety contains a saturated or unsaturated C_4-C_{30} hydrocarbon chain, and an optional functional group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, or alkyne.

In some embodiments, the lipophilic moiety contains a saturated or unsaturated C_6-C_{18} hydrocarbon chain. Optionally, the lipophilic moiety contains a saturated or unsaturated C_{16} hydrocarbon chain.

In an embodiment, the lipophilic moiety is



, where B is a nucleotide base or a nucleotide base analog. Optionally, B is adenine, guanine, cytosine, thymine or uracil.

In certain embodiments, the lipophilic moiety is attached via a linker or carrier. Optionally, the lipophilic moiety is conjugated via a carrier that replaces the nucleotide at position 6 or 16 of the sense strand (counting from the 5'-terminal nucleotide of the sense strand as position 1). In a related embodiment, the carrier is a cyclic group (*e.g.*, a pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, or decalinyl) or is an acyclic moiety based on a serinol backbone or a diethanolamine backbone.

In some embodiments, the lipophilic moiety is conjugated to the dsRNA agent via a linker containing an ether, thioether, urea, carbonate, amine, amide, maleimide-thioether, disulfide, phosphodiester, sulfonamide linkage, a product of a click reaction, or carbamate.

In one embodiment, the lipophilic moiety is conjugated to the dsRNA agent via a bio-cleavable linker that is DNA, RNA, disulfide, amide, functionalized monosaccharides or oligosaccharides of galactosamine, glucosamine, glucose, galactose, and/or mannose, or a combination thereof.

In certain embodiments, substantially all of the nucleotides of the sense strand, or of the antisense strand, or both, are modified nucleotides.

In one embodiment, all of the nucleotides of the sense strand are modified nucleotides.

In another embodiment, all of the nucleotides of the antisense strand are modified nucleotides.

In an additional embodiment, all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides.

In certain embodiments, the dsRNA agent has one or more modified nucleotides, where at least one of the one or more modified nucleotides is a deoxy-nucleotide, a 3'-terminal deoxythimidine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxy-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a

cyclohexenyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, a nucleotide comprising a 5'-methylphosphonate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic, a nucleotide comprising vinyl phosphate, a nucleotide comprising adenosine-glycol nucleic acid (GNA), a nucleotide comprising thymidine-glycol nucleic acid (GNA) S-Isomer, a nucleotide comprising 2-hydroxymethyl-tetrahydrofuran-5-phosphate, a nucleotide comprising 2'-deoxythymidine-3'phosphate, a nucleotide comprising 2'-deoxyguanosine-3'-phosphate, or a terminal nucleotide linked to a cholesteryl derivative and a dodecanoic acid bisdecylamide group.

In another embodiment, the dsRNA agent includes at least one modified nucleotide that is a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-C16 (2'-O-hexadecyl) modified nucleotide, or a nucleotide including vinyl phosphate. Optionally, the dsRNA agent includes at least one of each of the following modifications: 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-C16 (2'-O-hexadecyl) modified nucleotide, and a nucleotide including vinyl phosphate.

In certain embodiments, the dsRNA agent further includes a phosphate or phosphate mimic at the 5'-end of the antisense strand. Optionally, the phosphate mimic is a 5'-vinyl phosphonate (VP).

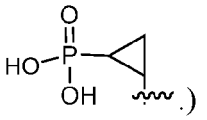
In some embodiments, the dsRNA agent includes a pattern of modified nucleotides as shown in **Table 2** (where locations of 2'-C16, 2'-O-methyl, 2'-deoxy, GNA, phosphorothioate, vinyl phosphonate, and 2'-fluoro modifications are as displayed in **Table 2**, irrespective of the individual nucleotide base sequences of the displayed dsRNA agents).

In one embodiment, the dsRNA agent has a sense strand nucleotide sequence of AD-1804698, AD-1804699, AD-1747575, AD-1747576, AD-1804700, AD-1747577, AD-1747578, AD-1747579, AD-1747580, AD-1747581, AD-1804701, AD-1747582, AD-1804702, AD-1804703, AD-1804704, AD-1747583, AD-1804705, AD-1804706, AD-1804707, AD-1804708, AD-1804709, AD-1747591, AD-1747585, AD-1804710, AD-1804711, AD-1747586, AD-1804712, AD-1747587, AD-1804713, AD-1804714, AD-1747588, AD-1804715, AD-1804716, AD-1804717, AD-1804718, AD-1804719, AD-1804720, AD-1804721, AD-1804722, AD-1804723, AD-1804724, AD-1804725, or AD-1804726. Optionally, the dsRNA agent has a sense strand nucleotide sequence of AD-1747580, AD-1747583, or AD-1747585.

In certain embodiments, the sense strand of the dsRNA agent has one of the following modification patterns: 5'-nsnsnnn(Nhd)NfnNfNfNfnnnnnnnnsnsn-3', 5'-nsnsnnnnnnNfNfNfnnnn(Nhd)nnnsnsn-3', or 5′-nsnsnnn(Nhd)nnNfNfNfnnnnnnnnsnsn-3′, where n is a 2'-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2'-fluoro-nucleotide, and (Nhd) is a 2'-O-hexadecyl-nucleotide.

In embodiments, the sense strand of the dsRNA agent has the following modification pattern: 5'-(L1)(inv)snnnnnnnnNfNfNfnnnnnnnnns(inv)(L2)-3', wherein each (inv) is an inverted nucleotide (e.g., an inverted abasic nucleotide, such as an inverted abasic ribonucleotide) and at least one of (L1) and (L2) is a ligand comprising a lipophilic group (e.g., comprising an C16 alkyl or C16 alkenyl group) and the other of (L1) and (L2) is absent or hydrogen.

In some embodiments, the antisense strand of the dsRNA agent has one of the following modification patterns: 5'-VPnsdNsnndNndNnnnndNnNfnnnnnnnsnsn-3', 5'-VPnsNfsnndNn(Ngn)nnnnnnNfnNfnnnnnsnsn-3', or 5′-VPnsNfsnndNn(N2p)nnnnnnNfnNfnnnnnsnsn-3′, where VP is Vinyl-phosphonate, n is a 2'-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, dN is a 2'-deoxy-nucleotide, Nf is a 2'-fluoro-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer and N2p is a 2′-phosphate nucleotide.

In embodiments, the antisense strand of the dsRNA agent has one of the following modification patterns: 5'-ZnsdNsnndNndNnnnndNnNfnnnnnsnsn-3' , 5'-ZnsNfsnndNn(Ngn)nnnnnnNfnNfnnnsnsn-3', 5'-ZnsNfsnNfnNfnNfnNfnNfnNfnNfnNfnsNfsn-3', 5'-ZnsNfsnNfnNfnNfnNfnNfnNfnNfnNfnsnsn-3', 5'-ZnsNfsnNfnNfnnnnnNfnNfnNfnNfnsNfsn-3', 5'-ZnsNfsnsNfnNfnNfnNfnNfnNfnNfnNfnNfsn-3', 5'-ZnsNfsnsNfnNfnnnnnNfnNfnNfnNfnNfsn-3', or 5'-ZnsNfsnsNfnNfnNfnNfnNfnNfnNfnNfnnsn-3', where Z is a 5'-phosphate mimic, such as a 5'-cyclopropylphosphonate (5'-CP). (The structure of 5'-CP, as used herein, is .)

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)NfnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsdNsnndNndNnnnndNnNfnnnnnnnsnsn-3′, wherein VP is Vinyl-

phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, and (Nhd) is a 2′-O-hexadecyl-nucleotide.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)NfnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(Ngn)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)NfnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(N2p)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, (Ngn) is a glycol nucleic acid, S-isomer, and N2p is a 2′-phosphate nucleotide.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnnnnnNfNfNfnnnn(Nhd)nnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsdNsnndNndNnnnndNnNfnnnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, and (Nhd) is a 2′-O-hexadecyl-nucleotide.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnnnnnNfNfNfnnnn(Nhd)nnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(Ngn)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnnnnnNfNfNfnnnn(Nhd)nnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(N2p)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, (Ngn) is a glycol nucleic acid, S-isomer, and N2p is a 2′-phosphate nucleotide.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)nnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsdNsnndNndNnnnndNnNfnnnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)nnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(Ngn)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer.

In certain embodiments, the sense strand of the dsRNA agent comprises the modification pattern: 5′-nsnsnnn(Nhd)nnNfNfNfnnnnnnnnsnsn-3′ and the antisense strand comprises the modification pattern: 5′-VPnsNfsnndNn(N2p)nnnnnnNfnNfnnnnnsnsn-3′, wherein VP is Vinyl-phosphonate, n is a 2′-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2′-fluoro-nucleotide, dN is a 2′-deoxy-nucleotide, (Nhd) is a 2′-O-hexadecyl-nucleotide, (Ngn) is a glycol nucleic acid, S-isomer, and N2p is a 2′-phosphate nucleotide.

Another aspect of the instant disclosure provides a dsRNA agent for inhibiting expression of SNCA, where the dsRNA agent includes a sense strand having a 5'-terminus and a 3'-terminus and an antisense strand having a 5'-terminus and a 3'-terminus, where the sense strand and the antisense strand form a double stranded region, where: the sense strand includes a nucleotide sequence and modifications of **Table 2** (SEQ ID NOs: 13-55), with 0 or 1 mismatches; and the antisense strand includes a nucleotide sequence and modifications of **Table 2** (SEQ ID NOs: 56-98), with 0 or 1 mismatches.

In one embodiment, the dsRNA agent has one of the following sense strand nucleotide sequences: 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35), 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28), 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21), 5'-gsusaca(Ahd)GfuGfCfUfcaguuccasasa-3' (SEQ ID NO: 34), 5'-cscsauc(Ahd)gcAfGfUfgauugaagsusa-3' (SEQ ID NO: 43), 5'-

uscsccag(Uhd)uUfCfUfugagaucusgsa-3' (SEQ ID NO: 261), or 5'-uscsaug(Ahd)aaGfGfAfcuuucaaasgsa-3' (SEQ ID NO: 19), where a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate, (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

In another embodiment, the dsRNA agent has one of the following antisense strand nucleotide sequences: 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78), 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71), 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64), 5'-VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg-3' (SEQ ID NO: 77), 5'-VPusdAscudTcdAaucadCuGfcugauggsasa-3' (SEQ ID NO: 86), 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' (SEQ ID NO: 262), or 5'-VPusdCsuudTgdAaagudCcUfuucaugasasu-3' (SEQ ID NO: 62), where VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG is a 2`-deoxyguanosine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Agn) is an Adenosine-glycol nucleic acid (GNA), S-isomer.

In a further embodiment, the dsRNA agent has one of the following duplex pairs of nucleotide sequences: (i) sense strand: 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35) and antisense strand: 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78); (ii) sense strand: 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28) and antisense strand: 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71); (iii) sense strand: 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21) and antisense strand: 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64); (iv) sense strand: 5'-gsusaca(Ahd)GfuGfCfUfcaguuccasasa-3' (SEQ ID NO: 34) and antisense strand: 5'-

VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg-3' (SEQ ID NO: 77); (v) sense strand: 5'-cscsauc(Ahd)gcAfGfUfgauugaagsusa-3' (SEQ ID NO: 43) and antisense strand: 5'-VPusdAscudTcdAaucadCuGfcugauggsasa-3' (SEQ ID NO: 86); (vi) sense strand: 5'-uscsccag(Uhd)uUfCfUfugagaucusgsa-3' (SEQ ID NO: 261) and antisense strand: 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' (SEQ ID NO: 262); or (vii) sense strand: 5'-uscsaug(Ahd)aaGfGfAfcuuucaaasgsa-3' (SEQ ID NO: 19) and antisense strand: 5'-VPusdCsuudTgdAaagudCcUfuucaugasasu-3' (SEQ ID NO: 62), where VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG is a 2`-deoxyguanosine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, (Agn) is an Adenosine-glycol nucleic acid (GNA), S-isomer, (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate, (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

An additional aspect of the instant disclosure provides a dsRNA agent for inhibiting expression of SNCA, the dsRNA agent having sense strand sequence 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35) and antisense strand sequence 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78), where VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

Another aspect of the instant disclosure provides a dsRNA agent for inhibiting expression of SNCA, the dsRNA agent having sense strand sequence 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28) and antisense strand sequence 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71), where VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate

internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG is a 2`-deoxyguanosine-3`-phosphate, and (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate.

A further aspect of the instant disclosure provides a dsRNA agent for inhibiting expression of SNCA, the dsRNA agent having sense strand sequence 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21) and antisense strand sequence 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64), where VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate.

In another embodiment, the RNAi agent is a pharmaceutically acceptable salt thereof. "Pharmaceutically acceptable salts" of each of the RNAi agents herein include, but are not limited to, a sodium salt, a calcium salt, a lithium salt, a potassium salt, an ammonium salt, a magnesium salt, and mixtures thereof. One skilled in the art will appreciate that the RNAi agent can be provided as a polycationic salt having one cation per free acid group of the optionally modified phosophodiester backbone and/or any other acidic modifications (*e.g.*, 5'-terminal phosphonate groups). For example, an oligonucleotide of "n" nucleotides in length contains n-1 optionally modified phosophodiesters, so that an oligonucleotide of 21 nt in length may be provided as a salt having up to 20 cations (*e.g.*, 20 sodium cations). Similarly, an RNAi agent having a sense strand of 21 nt in length and an antisense strand of 23 nt in length may be provided as a salt having up to 42 cations (*e.g.*, 42 sodium cations). In the preceding example, where the RNAi agent also includes a 5'-terminal phosphate or a 5'-terminal vinylphosphonate group, the RNAi agent may be provided as a salt having up to 44 cations (*e.g.*, 44 sodium cations).

Another aspect of the instant disclosure provides a cell containing a dsRNA agent disclosed herein.

An additional aspect of the instant disclosure provides a pharmaceutical composition for use in inhibiting expression of α-synuclein that includes a dsRNA agent disclosed herein.

In certain embodiments, the dsRNA agent and/or the pharmaceutical composition is administered in an unbuffered solution. Optionally, the unbuffered solution is saline or water.

In some embodiments, the dsRNA agent is administered with a buffer solution. Optionally, the buffer solution includes acetate, citrate, prolamine, carbonate, or phosphate, or a combination thereof. In certain embodiments, the buffer solution is phosphate buffered saline (PBS).

A further aspect of the instant disclosure provides a pharmaceutical composition that includes a dsRNA agent disclosed herein and a lipid formulation.

In one embodiment, the lipid formulation includes or is a lipid nanoparticle (LNP).

Another aspect of the instant disclosure provides a method of inhibiting expression of an α-synuclein (SNCA) gene in a cell and/or preventing the formation of alpha-synuclein aggregates in a cell or subject, the method involving: (a) contacting the cell or subject with a dRNA agent or pharmaceutical composition disclosed herein; and (b) maintaining the cell or subject produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of an SNCA gene, thereby inhibiting expression of the SNCA gene in the cell and/or preventing the formation of alpha-synuclein aggregates in the cell or subject.

In one embodiment, the cell is within a subject. Optionally, the subject is a human. Alternatively, the subject is a rhesus monkey, a cynomolgous monkey, a mouse, or a rat.

In certain embodiments, the human subject suffers from a SNCA-associated disease. Optionally, the SNCA-associated disease is a synucleinopathy. Optionally the synucleinopathy is PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down’s syndrome, psychosis, schizophrenia and/or Creutzfeldt-Jakob disease.

In some embodiments, SNCA expression in the cell or the subject is inhibited by at least about 50%, at least about 40%, at least about 30%, at least about 20%, or at least about 10% by the dsRNA agent, as compared to a control cell or control subject.

An additional aspect of the instant disclosure provides a method of treating a subject diagnosed with a SNCA-associated neurodegenerative disease, the method involving administering to the subject a therapeutically effective amount of a dsRNA agent or pharmaceutical composition disclosed herein, optionally wherein the subject is re-dosed with a therapeutically effective amount of the dsRNA agent or pharmaceutical composition disclosed herein, thereby treating the subject.

In certain embodiments, treating includes amelioration of at least one sign or symptom of the SNCA-associated neurodegenerative disease.

In some embodiments, treating includes prevention of progression of the disease.

In one embodiment, the SNCA-associated neurodegenerative disease is characterized by one or more of the following symptoms: tremors, slowed movement (bradykinesia), rigid muscles, impaired posture and balance, loss of automatic movements, speech changes, writing changes, visual, auditory, olfactory, or tactile hallucinations, poor regulation of body functions (autonomic nervous systems) such as dizziness, falls and bowel issues, cognitive problems such as confusion, poor attention, visual-spatial problems and memory loss, sleep difficulties such as rapid eye movement (REM) sleep behavior disorder (in which dreams are physically acted out while asleep), fluctuating attention including episodes of drowsiness, long periods of staring into space, long naps during the day or disorganized speech, depression, and apathy, orthostatic hypotension (a sudden drop in blood pressure that occurs when a person stands up, causing a person to feel dizzy and lightheaded, and the need to sit, squat, or lie down in order to prevent fainting), clumsiness or incoordination, bladder control problems, contractures (chronic shortening of muscles or tendons around joints, which prevents the joints from moving freely) in the hands or limbs, Pisa syndrome (an abnormal posture in which the body appears to be leaning to one side), antecollis (in which the neck bends forward and the head drops down), and/or involuntary and uncontrollable sighing or gasping.

In certain embodiments, the dsRNA agent is administered to the subject at a dose of about 0.01 mg/kg to about 50 mg/kg.

In some embodiments, the dsRNA agent or pharmaceutical composition is administered to the subject intrathecally.

In one embodiment, the method further involves administering to the subject an additional agent or a therapy suitable for treatment or prevention of a SNCA-associated neurodegenerative disease or disorder.

In certain embodiments, the SNCA-associated neurodegenerative disease is Parkinson's Disease (PD).

In one embodiment, the SNCA-associated neurodegenerative disease is Lewy body dementia (LBD) or multiple system atrophy (MSA).

In some embodiments, SNCA expression is inhibited by at least about 30%.

In another embodiment, the method further involves administering an additional therapeutic agent to the subject.

In certain embodiments, the method reduces the expression of SNCA in a brain or spinal cord tissue. Optionally, the brain or spinal cord tissue is cerebral cortex, cerebellum, basal ganglia, hippocampus, amygdala, thalamus, brainstem, cervical spinal cord, lumbar spinal cord, and/or thoracic spinal cord.

Another aspect of the instant disclosure provides a method of inhibiting the expression of SNCA in a subject, the method involving administering to the subject a therapeutically effective amount of a dsRNA agent or the pharmaceutical composition disclosed herein, thereby inhibiting the expression of SNCA in the subject.

An additional aspect of the instant disclosure provides a method for treating or preventing an SNCA-associated disease in a subject, the method involving administering to the subject a therapeutically effective amount of a dsRNA agent or the pharmaceutical composition disclosed herein, thereby treating or preventing an SNCA-associated disease in the subject.

In certain embodiments, the step of administering produces at least 60% knockdown of SNCA mRNA or α-synuclein protein in one or more tissues of the subject. Optionally, the one or more tissues of the subject include CSF, prefrontal cortex, midbrain, thoracic spine, hippocampus, medulla pons, striatum caudate, and/or cerebellum.

A further aspect of the instant disclosure provides a kit for performing a method disclosed herein, the kit including a dsRNA agent disclosed herein, and instructions for its use. Optionally, the kit also includes a means for administering the dsRNA agent to a subject.

Definitions

That the present disclosure may be more readily understood, certain terms are first defined. In addition, it should be noted that whenever a value or range of values of a parameter are recited, it is intended that values and ranges intermediate to the recited values are also intended to be part of this disclosure.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element, *e.g.*, a plurality of elements.

The term "including" is used herein to mean, and is used interchangeably with, the phrase "including but not limited to".

The term "or" is used herein to mean, and is used interchangeably with, the term "and/or," unless context clearly indicates otherwise.

The term "about" is used herein to mean within the typical ranges of tolerances in the art. For example, "about" can be understood as about 2 standard deviations from the mean. In certain embodiments, about means ±10%. In certain embodiments, about means ±5%. When about is present before a series of numbers or a range, it is understood that "about" can modify each of the numbers in the series or range.

The term "at least" prior to a number or series of numbers is understood to include the number adjacent to the term "at least", and all subsequent numbers or integers that could logically be included, as clear from context. For example, the number of nucleotides in a nucleic acid molecule must be an integer. For example, "at least 18 nucleotides of a 21 nucleotide nucleic acid molecule" means that 18, 19, 20, or 21 nucleotides have the indicated property. When at least is present before a series of numbers or a range, it is understood that "at least" can modify each of the numbers in the series or range.

As used herein, "no more than" or "less than" is understood as the value adjacent to the phrase and logical lower values or integers, as logical from context, to zero. For example, a duplex with an overhang of "no more than 2 nucleotides" has a 2, 1, or 0 nucleotide overhang. When "no more than" is present before a series of numbers or a range, it is understood that "no more than" can modify each of the numbers in the series or range. As used herein, ranges include both the upper and lower limit.

As used herein, methods of detection can include determination that the amount of analyte present is below the level of detection of the method.

In the event of a conflict between an indicated target site and the nucleotide sequence for a sense or antisense strand, the indicated sequence takes precedence.

In the event of a conflict between a chemical structure and a chemical name, the chemical structure takes precedence.

The term "SNCA," "α-synuclein," "synuclein alpha," or "alpha-synuclein," refers to a gene associated with neurodegenerative diseases, termed "synucleinopathies," as well as the proteins encoded by that gene. The human SNCA gene region covers approximately 114 kb. The SNCA transcript contains 13 exons, and 15 mRNA isoforms have been identified or otherwise predicted as produced. Nucleotide and amino acid sequences of SNCA may be found, for example, at GenBank Accession No. NM_007308.3 (*Homo sapiens* SNCA, SEQ ID NO: 1, reverse complement, SEQ ID NO: 2); GenBank Accession No. XM_005555421 (*Macaca fascicularis* SNCA, SEQ ID NO: 3, reverse complement, SEQ ID NO: 4); GenBank Accession No.: NM_009221 (*Mus musculus* SNCA, SEQ ID NO: 5, reverse complement, SEQ ID NO: 6); GenBank Accession No. NM_019169.2 (*Rattus norvegicus* SNCA, SEQ ID NO: 7, reverse complement, SEQ ID NO: 8); and GenBank Accession No. XM_535656.7 (*Canis lupus familiaris* SNCA, SEQ ID NO: 228, reverse complement, SEQ ID NO: 229).

The term "SNCA" as used herein also refers to variations of the SNCA gene including naturally occurring sequence variants provided, for example, isoform 1 transcript NM_000345.4 (SEQ ID NO: 232), which encodes polypeptide NP_000336.1; isoform 2 transcript NM_001146054.2 (SEQ ID NO: 230), which encodes polypeptide NP_001139526.1; isoform 3 transcript NM_001146055.2 (SEQ ID NO: 231), which encodes polypeptide NP_001139527.1; isoform 4 transcript NM_007308.3 (SEQ ID NO: 1) as mentioned above, which encodes polypeptide NP_009292.1; isoform 5 transcript NM_001375285.1 (SEQ ID NO: 233), which encodes polypeptide NP_001362214.1; isoform 6 transcript NM_001375286.1 (SEQ ID NO: 234), which encodes polypeptide NP_001362215.1; isoform 7 transcript NM_001375287.1 (SEQ ID NO: 235), which encodes polypeptide NP_001362216.1; isoform 8 transcript NM_001375288.1 (SEQ ID NO: 236), which encodes polypeptide NP_001362217.1; isoform 9 transcript NM_001375290.1 (SEQ ID NO: 237), which encodes polypeptide NP_001362219.1; as well as predicted isoform X1 transcript XM_011532203.1 (SEQ ID NO: 238), which encodes polypeptide XP_011530505.1; predicted isoform X2 transcript XM_011532204.3 (SEQ ID NO: 239), which encodes polypeptide XP_011530506.1; predicted isoform X3 transcript XM_011532205.2 (SEQ

ID NO: 240), which encodes polypeptide XP_011530507.1; predicted isoform X4 transcript XM_011532206.1 (SEQ ID NO: 241), which encodes polypeptide XP_011530508.1; predicted isoform X5 transcript XM_011532207.1 (SEQ ID NO: 242), which encodes polypeptide XP_011530509.1; and predicted isoform X8 transcript XM_017008563.1 (SEQ ID NO: 243), which encodes polypeptide XP_016864052.1 (the unique sequence associated with each of the preceding Accession Numbers is incorporated herein by reference in the form available on the filing date of the instant application). Additional examples of SNCA sequences can be found in publicly available databases, for example, GenBank, OMIM, UniProt, NCBI dbSNP (see, *e.g.*, www.ncbi.nlm.nih.gov/gene/6622), and the Macaca genome project web site (macaque.genomics.org.cn/page/species/index.jsp). Additional information on SNCA can be found, for example, at www.ncbi.nlm.nih.gov/gene/6622. The entire contents of each of the foregoing GenBank Accession numbers and the Gene database numbers are incorporated herein by reference as of the date of filing this application.

Three protein isoforms of α-synuclein have been described in UniProt. The longest α-synuclein isoform is an approximately 14 kDa protein (Isoform 1 UniProt, P37840 of 140 amino acids). Other α-synuclein isoforms in UniProt include: Isoform 2-4, P37840-2 of 112 amino acids; and Isoform 2-5, P37840-3 of 126 amino acids. The 140 amino acid α-synuclein protein is encoded by 5 exon pairs mapping to chromosome loci 4q21.3-q22. The α-synuclein protein has an N-terminal region composed of incomplete KXKEGV (SEQ ID NO: 244) motifs, an extremely hydrophobic NAC domain and a highly acidic C-terminal domain. At physiological conditions, SNCA is believed to be an intrinsically disordered monomer or helically folded tetramer. α-Synuclein composes 1% of all proteins in the cytosol of brain cells, and is predominantly expressed in the neocortex, hippocampus, substantia nigra, thalamus, and cerebellum. α-Synuclein is also expressed in lower amounts in the heart, skeletal muscle, pancreas, lymph and blood cells. Although the function of SNCA is not well understood, evidence suggests it plays an important role in maintaining an adequate supply of synaptic vesicles in presynaptic terminals. α-Synuclein is implicated in the regulation of dopamine release and transport, fibrillization of microtubule associated protein tau, and the regulation of a neuroprotective phenotype in non-dopaminergic neurons by regulating the inhibition of both p53 expression and transactivation of proapoptotic genes, leading to decreased caspase-3 activation. The primary mechanism by which α-synuclein induces neurodegenerative diseases such as Parkinson's, Lewy body dementia, and multiple

system atrophy, appears to be elevated levels of the α-synuclein protein resulting in α-synuclein fibrillary aggregates.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a SNCA gene, including mRNA that is a product of RNA processing of a primary transcription product. In one embodiment, the target portion of the sequence will be at least long enough to serve as a substrate for RNAi-directed cleavage at or near that portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a SNCA gene. In one embodiment, the target sequence is within the protein coding region of the SNCA gene. In another embodiment, the target sequence is within the 3' UTR of the SNCA gene.

The target sequence may be from about 9-36 nucleotides in length, *e.g.*, about 15-30 nucleotides in length. For example, the target sequence can be from about 15-30 nucleotides, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length. In some embodiments, the target sequence is about 19 to about 30 nucleotides in length. In other embodiments, the target sequence is about 19 to about 25 nucleotides in length. In still other embodiments, the target sequence is about 19 to about 23 nucleotides in length. In some embodiments, the target sequence is about 21 to about 23 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the disclosure.

As used herein, the term "strand comprising a sequence" refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

"G," "C," "A," "T", and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine, and uracil as a base, respectively in the context of a modified or unmodified nucleotide. However, it will be understood that the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety (see, *e.g.*, **Table 1**). The skilled person is well aware that guanine, cytosine, adenine, thymidine, and uracil can be replaced by other moieties without substantially altering the

base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base can base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured in the disclosure by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods featured in the disclosure.

The terms "iRNA", "RNAi agent," "iRNA agent," "RNA interference agent" as used interchangeably herein, refer to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript *via* an RNA-induced silencing complex (RISC) pathway. RNA interference (RNAi) is a process that directs the sequence-specific degradation of mRNA. RNAi modulates, *e.g.,* inhibits, the expression of SNCA in a cell, *e.g.,* a cell within a subject, such as a mammalian subject.

In one embodiment, an RNAi agent of the disclosure includes a single stranded RNAi that interacts with a target RNA sequence, *e.g.,* a SNCA target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory it is believed that long double stranded RNA introduced into cells is broken down into double-stranded short interfering RNAs (siRNAs) comprising a sense strand and an antisense strand by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev.* 15: 485). Dicer, a ribonuclease-III-like enzyme, processes these dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) *Nature* 409: 363). These siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) *Cell* 107: 309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, *et al.*, (2001) *Genes Dev.* 15: 188). Thus, in one aspect the disclosure relates to a single stranded RNA (ssRNA) (the antisense strand of a siRNA duplex) generated within a cell and which promotes the formation of a RISC complex to effect silencing of the target gene, *i.e.,* a SNCA gene. Accordingly, the term "siRNA" is also used herein to refer to an RNAi as described above.

In another embodiment, the RNAi agent may be a single-stranded RNA that is introduced into a cell or organism to inhibit a target mRNA. Single-stranded RNAi agents bind to the RISC endonuclease, Argonaute 2, which then cleaves the target mRNA. The single-stranded siRNAs are generally 15-30 nucleotides and are chemically modified. The design and testing of single-stranded RNAs are described in U.S. Patent No. 8,101,348 and in Lima *et al.*, (2012) *Cell* 150: 883-894, the entire contents of each of which are hereby incorporated herein by reference. Any of the antisense nucleotide sequences described herein may be used as a single-stranded siRNA as described herein or as chemically modified by the methods described in Lima *et al.*, (2012) *Cell* 150: 883-894.

In another embodiment, a "RNAi agent" for use in the compositions and methods of the disclosure is a double stranded RNA and is referred to herein as a "double stranded RNAi agent," "double stranded RNA (dsRNA) molecule," "dsRNA agent," or "dsRNA". The term "dsRNA" refers to a complex of ribonucleic acid molecules, having a duplex structure comprising two anti-parallel and substantially complementary nucleic acid strands, referred to as having "sense" and "antisense" orientations with respect to a target RNA, *i.e.,* a SNCA gene. In some embodiments of the disclosure, a double stranded RNA (dsRNA) triggers the degradation of a target RNA, *e.g.*, an mRNA, through a post-transcriptional gene-silencing mechanism referred to herein as RNA interference or RNAi.

In general, a dsRNA molecule can include ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide, a modified nucleotide. In addition, as used in this specification, an "RNAi agent" may include ribonucleotides with chemical modifications; an RNAi agent may include substantial modifications at multiple nucleotides.

As used herein, the term "modified nucleotide" refers to a nucleotide having, independently, a modified sugar moiety, a modified internucleotide linkage, or a modified nucleobase. Thus, the term modified nucleotide encompasses substitutions, additions or removal of, *e.g.,* a functional group or atom, to internucleoside linkages, sugar moieties, or nucleobases. The modifications suitable for use in the agents of the disclosure include all types of modifications disclosed herein or known in the art. Any such modifications, as used in a siRNA type molecule, are encompassed by "RNAi agent" for the purposes of this specification and claims.

In certain embodiments of the instant disclosure, inclusion of a deoxy-nucleotide – which is acknowledged as a naturally occurring form of nucleotide – if present within an RNAi agent can be considered to constitute a modified nucleotide.

The duplex region may be of any length that permits specific degradation of a desired target RNA through a RISC pathway, and may range from about 9 to 36 base pairs in length, *e.g.*, about 15-30 base pairs in length, for example, about 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, or 36 base pairs in length, such as about 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the invention.

The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting RNA chain is referred to as a "hairpin loop." A hairpin loop can comprise at least one unpaired nucleotide. In some embodiments, the hairpin loop can comprise at at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides or nucleotides not directed to the target site of the dsRNA. In some embodiments, the hairpin loop can be 10 or fewer nucleotides. In some embodiments, the hairpin loop can be 8 or fewer unpaired nucleotides. In some embodiments, the hairpin loop can be 4-10 unpaired nucleotides. In some embodiments, the hairpin loop can be 4-8 nucleotides.

In certain embodiments, the two strands of double-stranded oligomeric compound can be linked together. The two strands can be linked to each other at both ends, or at one end only. By linking at one end is meant that 5'-end of first strand is linked to the 3'-end of the second strand or 3'-end of first strand is linked to 5'-end of the second strand. When the two strands are linked to each other at both ends, 5'-end of first strand is linked to 3'-end of second strand and 3'-end of first strand is linked to 5'-end of second strand. The two strands can be linked together by an oligonucleotide linker including, but not limited to, (N)n; wherein N is independently a modified

or unmodified nucleotide and n is 3-23. In some embodiments, n is 3-10, *e.g.*, 3, 4, 5, 6, 7, 8, 9, or 10. In some embodiments, the oligonucleotide linker is selected from the group consisting of GNRA, (G)4, (U)4, and (dT)4, wherein N is a modified or unmodified nucleotide and R is a modified or unmodified purine nucleotide. Some of the nucleotides in the linker can be involved in base-pair interactions with other nucleotides in the linker. The two strands can also be linked together by a non-nucleosidic linker, *e.g.*, a linker described herein. It will be appreciated by one of skill in the art that any oligonucleotide chemical modifications or variations describe herein can be used in the oligonucleotide linker.

Hairpin and dumbbell type oligomeric compounds will have a duplex region equal to or at least 14, 15, 15, 16, 17, 18, 19, 29, 21, 22, 23, 24, or 25 nucleotide pairs. The duplex region can be equal to or less than 200, 100, or 50, in length. In some embodiments, ranges for the duplex region are 15-30, 17 to 23, 19 to 23, and 19 to 21 nucleotides pairs in length.

The hairpin oligomeric compounds can have a single strand overhang or terminal unpaired region, in some embodiments at the 3', and in some embodiments on the antisense side of the hairpin. In some embodiments, the overhangs are 1-4, more generally 2-3 nucleotides in length. The hairpin oligomeric compounds that can induce RNA interference are also referred to as "shRNA" herein.

Where the two substantially complementary strands of a dsRNA are comprised by separate RNA molecules, those molecules need not, but can be covalently connected. Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, an RNAi may comprise one or more nucleotide overhangs.

In one embodiment, an RNAi agent of the disclosure is a dsRNA, each strand of which is 24-30 nucleotides in length, that interacts with a target RNA sequence, *e.g.*, a SNCA target mRNA sequence, to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev.* 15: 485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3'

overhangs (Bernstein, *et al.*, (2001) *Nature* 409: 363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) *Cell* 107: 309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, *et al.*, (2001) *Genes Dev.* 15: 188). In one embodiment, an RNAi agent of the disclosure is a dsRNA of 24-30 nucleotides that interacts with a SNCA RNA sequence to direct the cleavage of the target RNA.

In one embodiment, an RNAi agent of the disclosure is a dsRNA agent, each strand of which comprises 19-23 nucleotides that interacts with a SNCA RNA sequence to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.* (2001) *Genes Dev.* 15: 485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, *et al.*, (2001) *Nature* 409: 363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) *Cell* 107: 309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleave the target to induce silencing (Elbashir, *et al.*, (2001) *Genes Dev.* 15: 188). In one embodiment, an RNAi agent of the disclosure is a dsRNA of 19-23 nucleotides that interacts with a SNCA RNA sequence to direct the cleavage of the target RNA.

As used herein, the term "nucleotide overhang" refers to at least one unpaired nucleotide that protrudes from the duplex structure of an RNAi agent, *e.g.*, a dsRNA. For example, when a 3'-end of one strand of a dsRNA extends beyond the 5'-end of the other strand, or vice versa, there is a nucleotide overhang. A dsRNA can comprise an overhang of at least one nucleotide; alternatively, the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end or both ends of either an antisense or sense strand of a dsRNA.

In one embodiment of the dsRNA, at least one strand comprises a 3' overhang of at least 1 nucleotide. In another embodiment, at least one strand comprises a 3' overhang of at least 2 nucleotides, *e.g.*, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, or 15 nucleotides. In other embodiments, at least one strand of the RNAi agent comprises a 5' overhang of at least 1 nucleotide. In certain embodiments, at least one strand comprises a 5' overhang of at least 2 nucleotides, *e.g.*, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, or 15 nucleotides. In still other embodiments, both the 3' and the 5' end of one strand of the RNAi agent comprise an overhang of at least 1 nucleotide.

In one embodiment, the antisense strand of a dsRNA has a 1-10 nucleotide, *e.g.*, 0-3, 1-3, 2-4, 2-5, 4-10, 5-10, *e.g.*, a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide, overhang at the 3'-end, the 5'-end, at both ends, or at neither end. In one embodiment, the sense strand of a dsRNA has a 1-10 nucleotide, *e.g.*, a 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide, overhang at the 3'-end, the 5'-end, at both ends, or at neither end. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

In certain embodiments, the overhang on the sense strand or the antisense strand, or both, can include extended lengths longer than 10 nucleotides, *e.g.*, 1-30 nucleotides, 2-30 nucleotides, 10-30 nucleotides, or 10-15 nucleotides in length. In certain embodiments, an extended overhang is on the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'end of the sense strand of the duplex. In certain embodiments, an extended overhang is present on the 5'end of the sense strand of the duplex. In certain embodiments, an extended overhang is on the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 3'end of the antisense strand of the duplex. In certain embodiments, an extended overhang is present on the 5'end of the antisense strand of the duplex. In certain embodiments, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate. In certain embodiments, the overhang includes a self-complementary portion such that the overhang is capable of forming a hairpin structure that is stable under physiological conditions.

The terms "blunt" or "blunt ended" as used herein in reference to a dsRNA mean that there are no unpaired nucleotides or nucleotide analogs at a given terminal end of a dsRNA, *i.e.*, no nucleotide overhang. One or both ends of a dsRNA can be blunt. Where both ends of a dsRNA are blunt, the dsRNA is said to be blunt ended. To be clear, a "blunt ended" dsRNA is a dsRNA that is blunt at both ends, *i.e.*, no nucleotide overhang at either end of the molecule. Most often such a molecule will be double stranded over its entire length.

In some embodiments, an dsRNA comprises a 3′ overhang. In some embodiments, a dsRNA comprises a 1 nucleotide 3′ overhang. In some embodiments, a dsRNA comprises a 2 nucleotide 3′ overhang. In some embodiments, an dsRNA comprises a 5′ overhang. In some embodiments, a dsRNA comprises a 1 nucleotide 5′ overhang. In some embodiments, a dsRNA comprises a 2 nucleotide 5′ overhang.

In some embodiments, a dsRNA has one end having an overhang and one end having a blunt end. The overhang can be a sense strand 3′ overhang, a sense strand 5′ overhang, an antisense strand 3′ overhang, or an antisense strand 5′ overhang. In some embodiments, the overhang is a 1 nucleotide overhang. In some embodiments, the overhang is a 2 nucleotide overhang. In some embodiments, an dsRNA has two blunt ends. In some embodiments, a dsRNA has overhangs at both ends. The overhangs at each end are independently a sense strand 3′ overhang, a sense strand 5′ overhang, an antisense strand 3′ overhang, or an antisense strand 5′ overhang. In some embodiments, the overhang is a 1 nucleotide overhang. In some embodiments, the overhang is a 2 nucleotide overhang. One or more overhang nucleotides can be a modified nucleotide, an inverted nucleotide, an abasic nucleotide, or an inverted abasic nucleotide. An inverted nucleotide may be linked via a 3′-3′ phosphodiester linkage.

In some embodiments, the sense strand is 23 nucleotides in length and the antisense strand is 21 nucleotides in length. In some embodiments, the sense strand is 23 nucleotides in length and the antisense strand is 21 nucleotides in length, wherein the 3′ and 5′ terminal nucleotide positions of the sense strand are inverted abasic residues. The sense strand 3′ and 5′ terminal inverted abasic residues may be overhangs.

In some embodiments, the sense strand is 21 nucleotides in length and the antisense strand is 23 nucleotides in length, wherein the antisense strand contains a 2 nucleobase 3′ overhang.

The term “antisense strand” or "guide strand" refers to the strand of an RNAi agent, *e.g.*, a dsRNA, which includes a region that is substantially complementary to a target sequence, *e.g.*, a SNCA mRNA.

As used herein, the term “region of complementarity” refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, *e.g.*, a SNCA nucleotide sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches can be in the internal or terminal regions

of the molecule. Generally, the most tolerated mismatches are in the terminal regions, *e.g.*, within 5, 4, 3, or 2 nucleotides of the 5'- or 3'-terminus of the RNAi agent.

In some embodiments, a double stranded RNA agent of the disclosure includes a nucleotide mismatch in the antisense strand.

In some embodiments, the antisense strand of the double stranded RNA agent of the disclosure includes no more than 4 mismatches with the target mRNA, *e.g.*, the antisense strand includes 4, 3, 2, 1, or 0 mismatches with the target mRNA. In some embodiments, the antisense strand double stranded RNA agent of the disclosure includes no more than 4 mismatches with the sense strand, *e.g.*, the antisense strand includes 4, 3, 2, 1, or 0 mismatches with the sense strand. In some embodiments, a double stranded RNA agent of the disclosure includes a nucleotide mismatch in the sense strand. In some embodiments, the sense strand of the double stranded RNA agent of the invention includes no more than 4 mismatches with the antisense strand, *e.g.*, the sense strand includes 4, 3, 2, 1, or 0 mismatches with the antisense strand. In some embodiments, the nucleotide mismatch is, for example, within 5, 4, 3 nucleotides from the 3'-end of the iRNA. In another embodiment, the nucleotide mismatch is, for example, in the 3'-terminal nucleotide of the iRNA agent. In some embodiments, the mismatch(s) is not in the seed region.

Thus, an RNAi agent as described herein can contain one or more mismatches to the target sequence. In one embodiment, an RNAi agent as described herein contains no more than 3 mismatches (*i.e.*, 3, 2, 1, or 0 mismatches). In one embodiment, an RNAi agent as described herein contains no more than 2 mismatches. In one embodiment, an RNAi agent as described herein contains no more than 1 mismatch. In one embodiment, an RNAi agent as described herein contains 0 mismatches. In certain embodiments, if the antisense strand of the RNAi agent contains mismatches to the target sequence, the mismatch can optionally be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, in such embodiments, for a 23 nucleotide RNAi agent, the strand which is complementary to a region of a SNCA gene, generally does not contain any mismatch within the central 13 nucleotides. The methods described herein, or methods known in the art can be used to determine whether an RNAi agent containing a mismatch to a target sequence is effective in inhibiting the expression of a SNCA gene. Consideration of the efficacy of RNAi agents with mismatches in inhibiting expression of a SNCA gene is important, especially if the particular region of complementarity in a SNCA gene is known to have polymorphic sequence variation within the population.

The term “sense strand” or "passenger strand" as used herein, refers to the strand of an RNAi agent that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

As used herein, “substantially all of the nucleotides are modified” are largely but not wholly modified and can include not more than 5, 4, 3, 2, or 1 unmodified nucleotides.

As used herein, the term “cleavage region” refers to a region that is located immediately adjacent to the cleavage site. The cleavage site is the site on the target at which cleavage occurs. In some embodiments, the cleavage region comprises three bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage region comprises two bases on either end of, and immediately adjacent to, the cleavage site. In some embodiments, the cleavage site specifically occurs at the site bound by nucleotides 10 and 11 of the antisense strand, and the cleavage region comprises nucleotides 11, 12 and 13.

As used herein, and unless otherwise indicated, the term “complementary,” when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions can include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50oC or 70oC for 12-16 hours followed by washing (see, *e.g.*, “Molecular Cloning: A Laboratory Manual, Sambrook, *et al.* (1989) Cold Spring Harbor Laboratory Press). Other conditions, such as physiologically relevant conditions as can be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

Complementary sequences within an RNAi agent, *e.g.*, within a dsRNA as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence over the entire length of one or both nucleotide sequences. Such sequences can be referred to as “fully complementary” with respect to each other herein. However, where a first sequence is referred to as “substantially complementary” with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3 or 2

mismatched base pairs upon hybridization for a duplex up to 30 base pairs, while retaining the ability to hybridize under the conditions most relevant to their ultimate application, *e.g.*, inhibition of gene expression via a RISC pathway. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, can yet be referred to as "fully complementary" for the purposes described herein.

"Complementary" sequences, as used herein, can also include, or be formed entirely from, non-Watson-Crick base pairs or base pairs formed from non-natural and modified nucleotides, in so far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs include, but are not limited to, G:U Wobble or Hoogstein base pairing.

The terms "complementary," "fully complementary" and "substantially complementary" herein can be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of an RNAi agent and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide that is "substantially complementary to at least part of" a messenger RNA (mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding SNCA). For example, a polynucleotide is complementary to at least a part of a SNCA mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding SNCA.

Accordingly, in some embodiments, the antisense strand polynucleotides disclosed herein are fully complementary to the target SNCA sequence.

In certain embodiments, the antisense strand polynucleotides disclosed herein are substantially complementary to the target SNCA sequence and comprise a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of SEQ ID NOs: 1, 3, 5, 7, or 228 for SNCA, or a fragment of SEQ ID NOs: 1, 3, 5, 7, or 228, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In other embodiments, the antisense polynucleotides disclosed herein are substantially complementary to the target SNCA sequence and comprise a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to any one of the sense strand nucleotide sequences in **Tables 2 or 3**, or a fragment of any one of the sense strand nucleotide sequences in **Tables 2 or 3**, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In one embodiment, an RNAi agent of the disclosure includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is the same as a target SNCA sequence, and wherein the sense strand polynucleotide comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to the equivalent region of the nucleotide sequence of SEQ ID NOs: 2, 4, 6, 8, or 229, or a fragment of any one of SEQ ID NOs: 2, 4, 6, 8, or 229, such as about 85%, about 86%, about 87%, about 88%, about 89%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% complementary.

In some embodiments, an iRNA of the disclosure includes a sense strand that is substantially complementary to an antisense polynucleotide which, in turn, is complementary to a target SNCA sequence, and wherein the sense strand polynucleotide comprises a contiguous nucleotide sequence which is at least about 80% complementary over its entire length to any one of the antisense strand nucleotide sequences in **Tables 2 or 3**, or a fragment of any one of the antisense strand nucleotide sequences in **Tables 2 or 3**, such as about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% complementary

In some embodiments, the double-stranded region of a double-stranded iRNA agent is equal to or at least, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotide pairs in length.

In some embodiments, the antisense strand of a double-stranded iRNA agent is equal to or at least 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

In some embodiments, the sense strand of a double-stranded iRNA agent is equal to or at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each 15 to 30 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each 19 to 25 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are each 21 to 23 nucleotides in length.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are 21 to 23 nucleotides in length, respectively.

In one embodiment, the sense and antisense strands of the double-stranded iRNA agent are 23 to 21 nucleotides in length, respectively.

In one embodiment, the sense strand of the iRNA agent is 21-nucleotides in length, and the antisense strand is 23-nucleotides in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 2-nucleotide long single stranded overhangs at the 3'-end.

In one embodiment, the sense strand of the iRNA agent is 23 nucleotides in length, and the antisense strand is 21 nucleotides in length, wherein the strands form a double-stranded region of 21 consecutive base pairs having a 1-nucleotide long single stranded overhang at the 3′- and 5′ ends.

In some embodiments, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include one or more non-ribonucleotides, *e.g.*, a deoxyribonucleotide or a modified nucleotide. In addition, an "iRNA" may include ribonucleotides with chemical modifications. Such modifications may include all types of modifications disclosed herein or known in the art. Any such modifications, as used in an iRNA molecule, are encompassed by "iRNA" for the purposes of this specification and claims.

In one aspect of the disclosure, an agent for use in the methods and compositions of the disclosure is a single-stranded antisense nucleic acid molecule that inhibits a target mRNA *via* an antisense inhibition mechanism. The single-stranded antisense RNA molecule is complementary to a sequence within the target mRNA. The single-stranded antisense oligonucleotides can inhibit translation in a stoichiometric manner by base pairing to the mRNA and physically obstructing the translation machinery, see Dias, N. *et al.*, (2002) *Mol Cancer Ther* 1: 347-355. The single-stranded antisense RNA molecule may be about 15 to about 30 nucleotides in length and have a sequence that is complementary to a target sequence. For example, the single-stranded antisense RNA

molecule may comprise a sequence that is at least about 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from any one of the antisense sequences described herein.

In one embodiment, at least partial suppression of the expression of a SNCA gene, is assessed by a reduction of the amount of SNCA mRNA which can be isolated from or detected in a first cell or group of cells in which a SNCA gene is transcribed and which has or have been treated such that the expression of a SNCA gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition may be expressed in terms of:

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} * 100\%$$

The phrase "contacting a cell with an RNAi agent," such as a dsRNA, as used herein, includes contacting a cell by any possible means. Contacting a cell with an RNAi agent includes contacting a cell *in vitro* with the RNAi agent or contacting a cell *in vivo* with the RNAi agent. The contacting may be done directly or indirectly. Thus, for example, the RNAi agent may be put into physical contact with the cell by the individual performing the method, or alternatively, the RNAi agent may be put into a situation that will permit or cause it to subsequently come into contact with the cell.

Contacting a cell *in vitro* may be done, for example, by incubating the cell with the RNAi agent. Contacting a cell *in vivo* may be done, for example, by injecting the RNAi agent into or near the tissue where the cell is located, or by injecting the RNAi agent into another area, *e.g.*, the central nervous system (CNS), optionally *via* intrathecal, intravitreal or other injection, or to the bloodstream or the subcutaneous space, such that the agent will subsequently reach the tissue where the cell to be contacted is located. For example, the RNAi agent may contain or be coupled to a ligand, *e.g.*, a lipophilic moiety or moieties as described below and further detailed, *e.g.*, in PCT/US2019/031170, which is incorporated herein by reference, that directs or otherwise stabilizes the RNAi agent at a site of interest, *e.g.*, the CNS. In some embodiments, the RNAi agent may contain or be coupled to a lipophilic moiety or moieties, optionally in the absence of GalNAc derivatives. Combinations of *in vitro* and *in vivo* methods of contacting are also possible. For example, a cell may also be contacted *in vitro* with an RNAi agent and subsequently transplanted into a subject.

In one embodiment, contacting a cell with an RNAi agent includes "introducing" or "delivering the RNAi agent into the cell" by facilitating or effecting uptake or absorption into the cell. Absorption or uptake of an RNAi agent can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. Introducing an RNAi agent into a cell may be *in vitro* or *in vivo*. For example, for *in vivo* introduction, an RNAi agent can be injected into a tissue site or administered systemically. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below or are known in the art.

The term "lipophile" or "lipophilic moiety" broadly refers to any compound or chemical moiety having an affinity for lipids. One way to characterize the lipophilicity of the lipophilic moiety is by the octanol-water partition coefficient, $\log K_{ow}$, where K_{ow} is the ratio of a chemical's concentration in the octanol-phase to its concentration in the aqueous phase of a two-phase system at equilibrium. The octanol-water partition coefficient is a laboratory-measured property of a substance. However, it may also be predicted by using coefficients attributed to the structural components of a chemical which are calculated using first-principle or empirical methods (see, for example, Tetko *et al.*, *J. Chem. Inf. Comput. Sci.* 41: 1407-21 (2001), which is incorporated herein by reference in its entirety). It provides a thermodynamic measure of the tendency of the substance to prefer a non-aqueous or oily milieu rather than water (*i.e.* its hydrophilic/lipophilic balance). In principle, a chemical substance is lipophilic in character when its $\log K_{ow}$ exceeds 0. Typically, the lipophilic moiety possesses a $\log K_{ow}$ exceeding 1, exceeding 1.5, exceeding 2, exceeding 3, exceeding 4, exceeding 5, or exceeding 10. For instance, the $\log K_{ow}$ of 6-amino hexanol, for instance, is predicted to be approximately 0.7. Using the same method, the $\log K_{ow}$ of cholesteryl N-(hexan-6-ol) carbamate is predicted to be 10.7.

The lipophilicity of a molecule can change with respect to the functional group it carries. For instance, adding a hydroxyl group or amine group to the end of a lipophilic moiety can increase or decrease the partition coefficient (*e.g.*, $\log K_{ow}$) value of the lipophilic moiety.

Alternatively, the hydrophobicity of the double-stranded RNAi agent, conjugated to one or more lipophilic moieties, can be measured by its protein binding characteristics. For instance, in certain embodiments, the unbound fraction in the plasma protein binding assay of the double-stranded RNAi agent could be determined to positively correlate to the relative hydrophobicity of

the double-stranded RNAi agent, which could then positively correlate to the silencing activity of the double-stranded RNAi agent.

In one embodiment, the plasma protein binding assay determined is an electrophoretic mobility shift assay (EMSA) using human serum albumin protein. An exemplary protocol of this binding assay is illustrated in detail in, *e.g.*, PCT/US2019/031170. The hydrophobicity of the double-stranded RNAi agent, measured by fraction of unbound siRNA in the binding assay, exceeds 0.15, exceeds 0.2, exceeds 0.25, exceeds 0.3, exceeds 0.35, exceeds 0.4, exceeds 0.45, or exceeds 0.5 for an enhanced *in vivo* delivery of siRNA.

Accordingly, conjugating the lipophilic moieties to the internal position(s) of the double-stranded RNAi agent provides optimal hydrophobicity for the enhanced *in vivo* delivery of siRNA.

The term "lipid nanoparticle" or "LNP" refers to a vesicle comprising a lipid layer encapsulating a pharmaceutically active molecule, such as a nucleic acid molecule, *e.g.*, an RNAi agent or a plasmid from which an RNAi agent is transcribed. LNPs are described in, for example, U.S. Patent Nos. 6,858,225, 6,815,432, 8,158,601, and 8,058,069, the entire contents of which are hereby incorporated herein by reference.

As used herein, a "subject" is an animal, such as a mammal, including a primate (such as a human, a non-human primate, *e.g.*, a monkey, and a chimpanzee), or a non-primate (such as a a rat, or a mouse). In a preferred embodiment, the subject is a human, such as a human being treated or assessed for a disease, disorder, or condition that would benefit from reduction in SNCA expression; a human at risk for a disease, disorder, or condition that would benefit from reduction in SNCA expression; a human having a disease, disorder, or condition that would benefit from reduction in SNCA expression; or human being treated for a disease, disorder, or condition that would benefit from reduction in SNCA expression as described herein.

As used herein, the terms "treating" or "treatment" refer to a beneficial or desired result including, but not limited to, alleviation or amelioration of one or more signs or symptoms associated with SNCA gene expression or SNCA protein production, *e.g.*, SNCA-associated neurodegenerative disease, *e.g.*, synucleinopathies, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous

sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease, decreased expression or activity of SNCA in regions of increased neuronal dysfunction or death, in subjects having such neurodegenerative diseases. "Treatment" can also mean prolonging survival as compared to expected survival in the absence of treatment.

The term "lower" in the context of the level of SNCA in a subject or a disease marker or symptom refers to a statistically significant decrease in such level. The decrease can be, for example, at least 10%, 15%, 20%, 25%, 30%, %, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more. In certain embodiments, a decrease is at least 20%. In certain embodiments, the decrease is at least 50% in a disease marker, e.g., protein or gene expression level. "Lower" in the context of the level of SNCA in a subject is optionally down to a level accepted as within the range of normal for an individual without such disorder. In certain embodiments, "lower" is the decrease in the difference between the level of a marker or symptom for a subject suffering from a disease and a level accepted within the range of normal for an individual, e.g., the level of decrease in speed of movement (bradykinesia) and ability to regulate posture and balance in an individual having Parkinson's and an individual not having Parkinson's or having symptoms that are within the range of normal.

As used herein, "prevention" or "preventing," when used in reference to a disease or disorder, that would benefit from a reduction in expression of a SNCA gene or production of SNCA protein, e.g., in a subject susceptible to a SNCA-associated disorder due to, e.g., genetic factors or age, wherein the subject does not yet meet the diagnostic criteria for the SNCA-associated disorder. As used herein, prevention can be understood as administration of an agent to a subject who does not yet meet the diagnostic criteria for the SNCA-associated disorder to delay or reduce the likelihood that the subject will develop the SNCA-associated disorder. As the agent is a pharmaceutical agent, it is understood that administration typically would be under the direction of a health care professional capable of identifying a subject who does not yet meet the diagnostic criteria for a SNCA-associated disorder as being susceptible to developing a SNCA-associated disorder.

The term "synucleinopathies" refers to a group of neurodegenerative disorders characterized by fibrillary aggregates of α-synuclein protein that tend to accumulate in the

cytoplasm of selective populations of neurons and glia. Synucleinopathies are therefore a class of SNCA-associated neurodegenerative diseases and disorders, which include Parkinson's disease (PD), Lewy body dementia (LBD), pure autonomic failure (PAF), and multiple system atrophy (MSA), among other neurodegenerative diseases. Clinically, synucleinopathies are characterized by a chronic and progressive decline in motor, cognitive, behavioral, and autonomic functions, depending on the distribution of the lesions in the brain. Because of clinical overlap, differential diagnosis is sometimes very difficult. Parkinsonism is the predominant symptom of PD, but it can be indistinguishable from the parkinsonism of LBD and MSA. Autonomic dysfunction, which is an isolated finding in PAF, may be present in PD and LBD, but is usually more prominent and appears earlier in MSA. LBD could be the same disease as PD but with widespread cortical pathological states, leading to dementia, fluctuating cognition, and the characteristic visual hallucinations.

The likelihood of developing a synucleinopathy, e.g., PD, LBD, etc., is reduced, for example, when an individual having one or more risk factors for PD or for LBD (or other synucleinopathy) either fails to develop PD or LBD (or other synucleinopathy) or develops PD or LBD (or other synucleinopathy) with less severity relative to a population having the same risk factors and not receiving treatment as described herein. The failure to develop a SNCA-associated disorder, e.g., PD or LBD (or other synucleinopathy), or a delay in the time to develop PD or LBD (or other synucleinopathy) by months or years is considered effective prevention. Prevention may require administration of more than one dose of the iRNA agent. Provided with appropriate methods to identify subjects at risk to develop any of the SNCA-associated diseases above, the iRNA agents provided herein can be used as pharmaceutical agents for or in methods of prevention of SNCA-associated diseases. Risk factors for various SNCA-associated diseases are discussed herein.

As used herein, the term “Parkinson’s disease” or “PD” refers to a progressive nervous system disorder that affects movement. The main pathological characteristics of PD are cell death in the brain's basal ganglia (affecting up to 70% of the dopamine secreting neurons in the substantia nigra pars compacta by the end of life) and the presence of Lewy bodies (accumulations of the SNCA-encoded α-synuclein protein) in many of the remaining neurons. Symptoms start gradually, sometimes with a barely noticeable tremor in just one hand, or stiffness or slowing of movement. Other early symptoms include lack of facial expression, lack of arm movement while walking, and

slurring during speech. Parkinson's disease symptoms worsen over time. The average onset of PD is age 60, and later onset is associated with greater symptom severity. Clinical features include, but are not limited to, more severe tremors, slowed movement (bradykinesia), rigid muscles, impaired posture and balance, loss of automatic movements, speech changes, and eventually, dementia, hallucinations, and wheelchair confinement.

As used herein, the term "Lewy body dementia (LBD)" refers to a type of progressive dementia that leads to a decline in thinking, reasoning and independent function caused by the aggregation of α-synuclein protein within diseased brain neurons, known as Lewy bodies and Lewy neurites. Aggregates of α-synuclein protein lead to sub-optimal functioning and eventual death of the affected neurons. Symptoms include visual, auditory, olfactory, or tactile hallucinations, signs of Parkinson's disease (parkinsonian signs), poor regulation of body functions (autonomic nervous system) such as dizziness, falls and bowel issues, cognitive problems such as confusion, poor attention, visual-spatial problems and memory loss, sleep difficulties such as rapid eye movement (REM) sleep behavior disorder (in which dreams are physically acted out while asleep), fluctuating attention including episodes of drowsiness, long periods of staring into space, long naps during the day or disorganized speech, depression, and apathy.

As used herein, the term "Multiple System Atrophy (MSA)" refers to a rare, degenerative neurological disorder affecting a subject's involuntary (autonomic) functions, including blood pressure, breathing, bladder function and motor control. Formerly called Shy-Drager syndrome, olivopontocerebellar atrophy or striatonigral degeneration, MSA shares many Parkinson's disease-like symptoms, such as slow movement, rigid muscles and poor balance. MSA treatment includes medications and lifestyle changes to help manage symptoms, but there is no cure. MSA causes deterioration and shrinkage (atrophy) of portions of the brain (cerebellum, basal ganglia and brainstem) that regulate internal body functions, digestion and motor control. The damaged brain tissue of MSA subjects shows nerve cells (neurons) that contain an abnormal amount of alpha-synuclein. MSA subjects typically live about seven to 10 years after MSA symptoms first appear. However, the survival rate with MSA varies widely. Occasionally, MSA subjects can live for 15 years or longer with the disease. Death is often due to respiratory problems. MSA progresses gradually and eventually leads to death. Affecting many parts of the body. MSA symptoms typically develop in adulthood, usually in the 50s or 60s. MSA is classified by two types: parkinsonian and cerebellar. The type depends on the symptoms observed at diagnosis.

Parkinsonian type MSA is the most common type of MSA, with signs and symptoms similar to those of Parkinson's disease, such as rigid muscles, difficulty bending arms and legs, slow movement (bradykinesia), tremors (rare in MSA compared with classic Parkinson's disease), and problems with posture and balance. Cerebellar type MSA signs and symptoms include problems with muscle coordination (ataxia), also possibly including impaired movement and coordination, such as unsteady gait and loss of balance, slurred, slow or low-volume speech (dysarthria), visual disturbances, such as blurred or double vision and difficulty focusing the eyes, difficulty swallowing (dysphagia) or chewing, as well as general signs and symptoms. In addition, the primary sign of multiple system atrophy is postural (orthostatic) hypotension, a form of low blood pressure that makes a subject feel dizzy or lightheaded, or even faint, when a subject stands up from sitting or lying down. MSA subjects may also develop dangerously high blood pressure levels while lying down (supine hypertension). Other difficulties associated with MSA include involuntary (autonomic) body functions, such as urinary and bowel dysfunction, constipation, loss of bladder or bowel control (incontinence), sweating abnormalities, reduced production of sweat, tears and saliva, heat intolerance due to reduced sweating, impaired body temperature control, often causing cold hands or feet, sleep disorders, agitated sleep due to "acting out" dreams, abnormal breathing at night, sexual dysfunction, inability to achieve or maintain an erection (impotence), loss of libido, cardiovascular problems, color changes in hands and feet caused by pooling of blood, cold hands and feet, psychiatric problems, and difficulty controlling emotions, such as laughing or crying inappropriately. Possible complications of MSA include breathing abnormalities during sleep, injuries from falls caused by poor balance or fainting, progressive immobility that can lead to secondary problems such as a breakdown of your skin, loss of ability to care for oneself in day-to-day activities, vocal cord paralysis, which makes speech and breathing difficult, and increased difficulty swallowing.

In one embodiment, a SNCA-associated disease or disorder (synucleinopathy) is one of Parkinson's disease, Lewy body dementia, multiple system atrophy (MSA), and pure autonomic failure (PAF).

"Therapeutically effective amount," as used herein, is intended to include the amount of an RNAi agent that, when administered to a subject having a SNCA-associated disease, is sufficient to effect treatment of the disease (*e.g.*, by diminishing, ameliorating, or maintaining the existing

disease or one or more symptoms of disease). The "therapeutically effective amount" may vary depending on the RNAi agent, how the agent is administered, the disease and its severity and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the subject to be treated.

"Prophylactically effective amount," as used herein, is intended to include the amount of an RNAi agent that, when administered to a subject having a SNCA-associated disorder, is sufficient to prevent or ameliorate the disease or one or more symptoms of the disease. Ameliorating the disease includes slowing the course of the disease or reducing the severity of later-developing disease. The "prophylactically effective amount" may vary depending on the RNAi agent, how the agent is administered, the degree of risk of disease, and the history, age, weight, family history, genetic makeup, the types of preceding or concomitant treatments, if any, and other individual characteristics of the patient to be treated.

A "therapeutically-effective amount" or "prophylactically effective amount" also includes an amount of an RNAi agent that produces some desired local or systemic effect at a reasonable benefit/risk ratio applicable to any treatment. An RNAi agent employed in the methods of the present disclosure may be administered in a sufficient amount to produce a reasonable benefit/risk ratio applicable to such treatment.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human subjects and animal subjects without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, manufacturing aid (*e.g.*, lubricant, talc magnesium, calcium or zinc stearate, or steric acid), or solvent encapsulating material, involved in carrying or transporting the subject compound from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject being treated. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such

as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) lubricating agents, such as magnesium state, sodium lauryl sulfate and talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates or polyanhydrides; (22) bulking agents, such as polypeptides and amino acids (23) serum component, such as serum albumin, HDL and LDL; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

The term "sample," as used herein, includes a collection of similar fluids, cells, or tissues isolated from a subject, as well as fluids, cells, or tissues present within a subject. Examples of biological fluids include blood, serum and serosal fluids, plasma, cerebrospinal fluid, ocular fluids, lymph, urine, saliva, and the like. Tissue samples may include samples from tissues, organs or localized regions. For example, samples may be derived from particular organs, parts of organs, or fluids or cells within those organs. In certain embodiments, samples may be derived from the brain (*e.g.*, whole brain or certain segments of brain, *e.g.*, striatum, or certain types of cells in the brain, such as, *e.g.*, neurons and glial cells (astrocytes, oligodendrocytes, microglial cells)). In other embodiments, a "sample derived from a subject" refers to blood drawn from the subject or plasma or serum derived therefrom. In further embodiments, a "sample derived from a subject" refers to brain tissue (or subcomponents thereof) or retinal tissue (or subcomponents thereof) derived from the subject.

It will be understood that, although the sequences in **Table 2** are described as modified or conjugated sequences, in certain embodiments, the RNA of the RNAi agent of the disclosure *e.g.*, a dsRNA of the disclosure, may comprise any one of the sequences set forth in **Tables 2 or 3** that is modified or conjugated differently than described therein. A lipophilic ligand can be included in any of the positions provided in the instant application, except where a specific position/location is specified.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGs. 1A and 1B show the effects of selected SNCA-targeting RNAi agents on SNCA levels in human BE(2)-C neuroblastoma cells, a dual luciferase reporter system in COS-7 African green monkey fibroblast-like cells, and in human SNCA-AAV over-expressing mice. ***FIG. 1A*** shows SNCA mRNA knockdown results obtained for SNCA-targeting RNAi agents AD-464778, AD-464782, AD-464694, AD-464634, AD-464779, AD-464314, AD-464313, AD-464590, AD-464585, AD-464229, AD-464586, and AD-464592 ("parental" siRNAs harboring triantennary GalNAc modifications, relative to the CNS-directed siRNAs of the instant disclosure) in BE(2)-C cells, and using a dual luciferase reporter system in COS-7 cells, respectively for each siRNA. ***FIG. 1B*** shows *in vivo* liver SNCA knockdown results for indicated duplexes in human SNCA-AAV over-expressing mice. To identify RNA *in vivo* efficacy of the RNAi compounds in mice, a full-length human SNCA was first transduced by AAV. At 7 days post AAV-administration, the following selected duplexes were delivered: duplexes targeting the 3'UTR of human SNCA AD-464778, AD-464782, AD-464694, AD-464634, AD-464779; and duplexes targeting the coding sequence of SNCA AD-464314, AD-464313, AD-464590, AD-464585, AD-464229, AD-464586, and AD-464592. Data were normalized to PBS-treated samples.

FIG. 2 shows SNCA knockdown results for the forty most potent SNCA-targeting siRNAs (liver targeting duplexes) in a combined hot spot and structure activity relationship (SAR) assessment performed upon 360 total duplexes. The forty indicated siRNAs showed the highest SNCA knockdown results at 0.1 nM duplex concentration and exhibited a clear linear dose-response.

FIG. 3 shows a schematic diagram for the exemplary AD-1549290 duplex, which possessed sense strand sequence 5'-uscscca(Ghd)uuUfCfUfugagaucugaL96-3' (SEQ ID NO: 245) and antisense strand sequence 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' (SEQ ID NO: 246).

FIG. 4 shows results of an *in vivo* AAV titration study, in which AAV was employed to administer human SNCA to mouse liver and the extent of delivery and expression was then assessed. At left, the cycle threshold for PCR-meditated human SNCA detection used to evaluate human SNCA expression in mouse liver was shown to drop in a dose-responsive manner when AAV was employed to administer human SNCA. At right, previously characterized SNCA-targeting siRNAs AD-464634 (targeting SNCA 3' UTR sequences) and AD-464314 (targeting

SNCA coding sequences) were demonstrated to inhibit SNCA expression robustly *in vivo* in mouse liver, when administered at either 3 mg/kg (mpk) or 10 mpk levels to huSNCA-expressing mice.

FIGs. 5A and 5B show *in vivo* SNCA knockdown results in mouse liver for all 17 SNCA-targeting siRNAs evaluated in *in vivo* AAV studies, as well as schematic diagrams of duplex sequence modification patterns. ***FIG. 5A*** shows *in vivo* human SNCA knockdown results observed in mice administered human SNCA via AAV. The 17 indicated newly evaluated SCNA-targeting siRNAs were compared to previously identified SNCA-targeting duplex AD-464634.2 and to a PBS control. ***FIG. 5B*** shows schematic diagrams of sequences and modification patterns for potent liver-targeting duplexes AD-1549333 (sense strand: 5'-asasgug(Chd)ucAfGfUfuccaaugugaL96-3' SEQ ID NO: 247; antisense strand: 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' SEQ ID NO: 248), AD-1746465 (sense strand: 5'-uscsuuugcuCfCfCfaguu(Uhd)cuugaL96-3' SEQ ID NO: 249; antisense strand: 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' SEQ ID NO: 250), AD-1571188 (sense strand: 5'-gsusaca(Ahd)GfuGfCfUfcaguuccaaaL96-3' SEQ ID NO: 251; antisense strand: 5'-VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg-3' SEQ ID NO: 252), AD-1549401 (sense strand: 5'-cscsauc(Ahd)gcAfGfUfgauugaaguaL96-3' SEQ ID NO: 253; antisense strand: 5'-VPusdAscudTcdAaucadCuGfcugauggsasa-3' SEQ ID NO: 254), AD-1549054 (sense strand: 5'-gsasgca(Ahd)guGfAfCfaaauguuggaL96-3' SEQ ID NO: 255; antisense strand: 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' SEQ ID NO: 256), AD-1746466 (sense strand: 5'-uscsccag(Uhd)uUfCfUfugagaucugaL96-3' SEQ ID NO: 257; antisense strand: 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' SEQ ID NO: 258), and AD-1548886 (sense strand: 5'-uscsaug(Ahd)aaGfGfAfcuuucaaagaL96-3' SEQ ID NO: 259; antisense strand: 5'-VPusdCsuudTgdAaagudCcUfuucaugasasu-3' SEQ ID NO: 260), which were selected for potential use as parental siRNAs for non-human primate (NHP) studies.

FIGs. 6A to 6C show comparisons of murine *in vivo* knockdown, *in vitro* knockdown and location information, across the above-identified seven SNCA-targeting siRNAs, which yielded three lead candidate parental siRNAs for NHP studies, with structures also shown. ***FIG. 6A*** shows the comparison of murine *in vivo* knockdown and *in vitro* knockdown across the above-identified seven SNCA-targeting siRNAs AD-1549333, AD-1746465, AD-1571188, AD-1549401, AD-1549054, AD-1746466, and AD-1548886. ***FIG. 6B*** shows target SNCA mRNA locations for each of candidate lead parental siRNAs AD-1549333, AD-1746465, AD-1571188, AD-1549401, AD-1549054, AD-1746466, and AD-1548886. ***FIG. 6C*** shows sequences and modification patterns

for selected lead candidate parent duplexes AD-1549333 (sense strand: SEQ ID NO: 247; antisense strand: SEQ ID NO: 248), AD-1746465 (sense strand: SEQ ID NO: 249; antisense strand: SEQ ID NO: 250), and AD-1549054 (sense strand: SEQ ID NO: 255; antisense strand: SEQ ID NO: 256), from top to bottom. These parental duplexes were respectively adapted to create lead SNCA-targeting duplexes AD-1747585, AD-1747583, and AD-1747580, having sequences and modification patterns as shown in **Table 2**, for CNS administration in NHP studies.

FIG. 7 presents a schematic showing three duplexes selected for NHP studies, AD-1747585 (having sense strand SEQ ID NO: 35 and antisense strand SEQ ID NO: 78), AD-1747583 (having sense strand SEQ ID NO: 28 and antisense strand SEQ ID NO: 71), and AD-1747580 (having sense strand SEQ ID NO: 21 and antisense strand SEQ ID NO: 64).

FIGs. 8A and 8B show initial selection and pharmacokinetic distribution of three candidate lead duplexes that were moved forward into testing in non-human primate (NHP) animals. ***FIG. 8A*** shows initial in vivo efficacy results and ED_{50} values for three candidate lead duplexes selected to move into NHP animal studies. ***FIG. 8B*** shows that each of three candidate lead SNCA-targeting duplexes dosed to non-human primate (NHP) animals *via* intrathecal injection with a single 60 mg dose of siRNA showed similar pharmacokinetics across dosed animals for the immediate 24 hour period after injection.

FIGs. 9A and 9B show pharmacokinetic (PK) and pharmacodynamic (PD) data obtained in NHP animal studies of three candidate lead SNCA-targeting duplexes administered via intrathecal injection. ***FIG. 9A*** shows that at day 29 ("D29") post-injection, each of the three tested SNCA-targeting duplexes exhibited high potency reduction of SNCA mRNA in NHP tissues of both the prefrontal cortex and the midbrain (left panel, PD results). Strong correlation between tissue PK (tissue concentration of tested duplex) and tissue SNCA mRNA PD was observed throughout tested NHP prefrontal cortex and midbrain tissues (right panel, PK/PD results), with each of the three tested duplexes showing similar potencies (no statistically significant differences were observed between the three leads at 29 days, based on mRNA knockdown results). Nominally, the following IC_{50} ranking was observed in NHPs at day 29: AD-1747583 (0.532 μg/g) < AD-1747580 (1.050 μg/g) < AD-1747585 (1.300 μg/g). ***FIG. 9B*** shows that at day 29 ("D29") post-injection, α-Synuclein protein levels were reduced in the brain and spinal cord of NHPs that received an intrathecal dose of a SNCA-targeting duplex, and little differentiation was observed between the three tested candidate lead agents.

FIG. 10 shows a summary of Day 29 NHP data.

FIG. 11 shows that at day 84 ("D84") after intrathecal administration of SNCA-targeting duplex or control molecules, significant levels of SNCA mRNA knockdown were observed in both prefrontal cortex and midbrain tissues, across all 3 candidate lead duplexes tested (left panel). Consistent with results observed above at day 29 post-administration, day 84 samples exhibited good correlation between tissue PK and tissue mRNA PD, with similar potencies observed for each of the three candidate lead duplexes examined (right panel). Notably, the AD-1747585-administered cohort only had one animal that met dosing criteria, which resulted in the remainder of the AD-1747585-administered cohort being re-dosed.

FIGs. 12A to 12E show that both SNCA mRNA and α-Synuclein protein levels were reduced at day 84 post-administration in different brain regions, with little differentiation observed between the three candidate leads tested (AD-1747580, AD-1747583, and AD-1747585 duplexes), and also shows SNCA mRNA/α-Synuclein protein correlation and redosing data. ***FIG. 12A*** shows that SNCA mRNA was reduced in NHPs at day 84 after administration of 60 mg of duplex agent via the intrathecal route. ***FIG. 12B*** shows that α-Synuclein protein levels were also reduced at day 84 in the brain and spinal cord (across all examined regions), again with little differentiation observed between the three candidate lead duplexes. ***FIG. 12C*** shows that α-Synuclein protein levels exhibited high correlation to SNCA mRNA levels across the different brain regions examined at day 84 post-administration. ***FIG. 12D*** shows that in certain NHPs that were identified as mis-dosed at initial administration, redosing and examining brain and spinal cord tissues at beyond day 84 demonstrated that SNCA NHP tissue protein pharmacodynamics (PD) exhibited robust knockdown, specifically in AD-1747580-re-dosed NHPs, across various brain regions. ***FIG. 12E*** shows α-Synuclein protein levels in cerebrospinal fluid (CSF) across an extended time course that included redose and monitoring of certain originally mis-dosed NHPs at time points beyond day 84. In such re-dosed NHP animals, CSF α-Synuclein protein levels exhibited up to a 75% reduction for animals (re)dosed with duplex AD-1747585, while CSF α-Synuclein protein levels exhibited up to a 50% reduction for animals (re)dosed with duplexes AD-1747580 and AD-1747583.

FIG. 13 shows that at day 84 after duplex administration, observed levels of α-Synuclein protein in NHP CSF were up to 90% reduced (PD effect) for animals dosed with duplexes AD-1747580 and AD-1747583. Meanwhile, duplex AD-1747585 showed ~75% CSF α-Synuclein

protein knockdown, while duplex AD-1747585 notably also exhibited variable CSF α-Synuclein protein knockdown, potentially due to blood contamination, as SNCA is highly expressed in blood cells.

FIGs. 14A to 14D show a divergence observed between tissue SNCA mRNA levels of knockdown and α-Synuclein protein levels of knockdown in the same tissues, at day 29 (as an "early" timepoint), and that such discrepancies resolved at the day 84 timepoint, indicative of such effects being attributable to a long half-life of α-Synuclein protein in the examined tissues. ***FIG. 14A*** shows that at day 29 post-administration, α-Synuclein protein (PD effect of duplexes) in the cortex and midbrain of treated NHPs exhibited only modest (<60%) knockdown for all three lead duplexes tested (left panel). Such modest reductions in α-Synuclein protein levels in cortex and midbrain were indicative of an extended half-life for α-Synuclein protein in such tissues. Tissue protein PK/PD was observed to be scattered for all three tested duplexes (middle panel); however, tissue α-Synuclein protein level was highly correlated with tissue SNCA mRNA level (right panel). ***FIG. 14B*** shows that α-Synuclein protein knockdown in CSF correlated with cortex (left panel) and striatal (middle panel) α-Synuclein protein knockdown at day 29 post-administration. In contrast, midbrain tissue α-Synuclein protein knockdown did not correlate well with CSF α-Synuclein protein knockdown at day 29 post-administration (right panel). ***FIG. 14C*** shows that while SNCA mRNA levels were reduced at day 29 post-administration (here, of SNCA-targeting duplex AD-1747580) in prefrontal cortex and midbrain tissues, α-Synuclein protein levels were only modestly reduced, which indicated that a long protein half-life for α-Synuclein protein was likely to explain this discrepancy. ***FIG. 14D*** shows that at day 84 post-administration, α-Synuclein protein levels in the cortex and midbrain tissues of treated NHP animals showed robust knockdown for all 3 lead duplexes (PD effect, left panel). Robust α-Synuclein protein knockdown observed at day 84 in cortex and midbrain NHP tissues confirmed a long protein half-life for α-Synuclein. Tissue protein PK/PD curves showed similar profiles for all three duplexes (middle panel; AD-1747585 cohort is noted as only having had one animal that met dosing criteria, while the remainder of the cohort was re-dosed). α-Synuclein protein levels were highly correlated with mRNA levels in both cortex and midbrain tissues (right panel).

FIG. 15 shows that α-Synuclein protein knockdown in CSF in NHP animals was also observed to correlate with prefrontal cortex and midbrain α-Synuclein protein knockdown at day 84 post-duplex administration.

FIG. 16 shows siRNA tissue exposure across various tested brain regions of NHP animals, at day 84 post-duplex administration. With few exceptions, tissue penetration of siRNA agent was good, even if tissues of greatest exposure to siRNA duplexes (e.g., prefrontal cortex) did not necessarily show the greatest observed levels of SNCA mRNA and/or α-Synuclein protein knockdown.

FIG. 17 shows that none of the three candidate lead duplexes administered to NHP animals exhibited any significant effect on body weight, either at day 29 or day 84 post-administration, as compared to control NHP animals dosed in parallel with vehicle only.

FIG. 18 shows results obtained when detecting Neurofilament light chain (NfL) in CSF of candidate lead duplex-dosed cohorts of NHP animals, as compared to a control NHP animal cohort administered artificial CSF (aCSF) only. While a transient spike in NfL was observed after dosing for all three duplexes, such NfL spikes largely did not persist beyond day 29 post-administration, which indicated that the three SNCA-targeting candidate lead duplexes did not appear to have an NfL profile suggestive of duplex toxicity.

FIGs. 19A to 19D show RNA-Seq data for each of the three candidate lead duplexes examined. ***FIG. 19A*** shows a schematic diagram of the AD-1747580 duplex (sense strand: SEQ ID NO: 21; antisense strand: SEQ ID NO: 64), with RNA-Seq data indicating achievement of 93% knockdown of SNCA in cells administered the AD-1747580 duplex and subjected to RNA-Seq analysis, while no other loci across the genome showed greater than a 50% knockdown (or robust and significant elevation) in the presence of the AD-1747580 duplex, as compared to an appropriate control. ***FIG. 19B*** shows a potency-matched RNA-Seq profile for candidate lead duplex AD-1747580, where RNA-Seq results for parent duplex AD-1549054 are also shown. At left, a schematic diagram of the GalNAc-modified AD-1549054 parent duplex is displayed (sense strand: 5'-gsasgca(Ahd)guGfAfCfaaauguuggaL96-3' SEQ ID NO: 255; antisense strand: 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' SEQ ID NO: 256) above RNA-Seq data for this duplex, which demonstrated approx. 74% SNCA knockdown in treated cells (administered a 10 nM dose of AD-1549054 parent duplex). At center and right are a schematic diagram of the AD-1747580 duplex (sense strand: SEQ ID NO: 21; antisense strand: SEQ ID NO: 64) above RNA-Seq results for the duplex at respective 1 nM (center, showing approx. 78% SNCA knockdown) and 10 nM (right, showing approx. 93% SNCA knockdown) doses. ***FIG. 19C*** shows a potency-matched RNA-Seq profile for candidate lead duplex AD-1747583, where RNA-Seq results for

parent duplex AD-1549283 are also shown. At left, a schematic diagram of the GalNAc-modified AD-1549283 parent duplex is displayed (sense strand: 5'-uscsuuu(Ghd)cuCfCfCfaguuucuugaL96-3' SEQ ID NO: 268; antisense strand: 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' SEQ ID NO: 269) above RNA-Seq data for this duplex, which demonstrated approx. 79% SNCA knockdown in treated cells (administered a 10 nM dose of AD-1549283 parent duplex). At center and right are a schematic diagram of the AD-1747583 duplex (sense strand: SEQ ID NO: 28; antisense strand: SEQ ID NO: 71) above RNA-Seq results for the duplex at respective 1 nM (center, showing approx. 67% SNCA knockdown) and 10 nM (right, showing approx. 92% SNCA knockdown) doses. Notably, three off-target loci were identified as significantly impacted (> 50% reduction) by dosing with the AD-1747583 duplex: PYGB, NREP and LCLAT1. ***FIG. 19D*** shows a potency-matched RNA-Seq profile for candidate lead duplex AD-1747585, where RNA-Seq results for parent duplex AD-1549333 are also shown. At left, a schematic diagram of the GalNAc-modified AD-1549333 parent duplex is displayed (sense strand: 5'-asasgug(Chd)ucAfGfUfuccaaugugaL96-3' SEQ ID NO: 247; antisense strand: 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' SEQ ID NO: 248) above RNA-Seq data for this duplex, which demonstrated approx. 81% SNCA knockdown in treated cells (administered a 10 nM dose of AD-1549333 parent duplex). At center and right are a schematic diagram of the AD-1747585 duplex (sense strand: SEQ ID NO: 35; antisense strand: SEQ ID NO: 78) above RNA-Seq results for the duplex at respective 0.1 nM (left center, showing approx. 68% SNCA knockdown), 1 nM (right center, showing approx. 93% SNCA knockdown) and 10 nM (right, showing approx. 96% SNCA knockdown) doses. Notably, one off-target locus was identified as significantly impacted (> 50% reduction) by dosing with the AD-1747585 duplex: HMGB2.

FIG. 20 shows a table summarizing knockdown and RNA-Seq data for all three candidate lead SNCA-targeting duplexes administered to NHP animals.

Throughout the figures, the term "2-C16" refers to a 2'-O-hexadecyl modification. The present invention is further illustrated by the following detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure provides RNAi compositions, which effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of a SNCA gene. The SNCA gene may be within a cell, *e.g.*, a cell within a subject, such as a human. The present disclosure

also provides methods of using the RNAi compositions of the disclosure for inhibiting the expression of a SNCA gene or for treating a subject having a disorder that would benefit from inhibiting or reducing the expression of a SNCA gene, *e.g.*, a SNCA-associated disease, *e.g.*, a synucleinopathy, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

The RNAi agents of the disclosure include an RNA strand (the antisense strand) having a region which is about 30 nucleotides or less in length, *e.g.*, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a SNCA gene. In certain embodiments, the RNAi agents of the disclosure include an RNA strand (the antisense strand) having a region which is about 21-23 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a SNCA gene.

In certain embodiments, the RNAi agents of the disclosure include an RNA strand (the antisense strand) which can include longer lengths, for example up to 66 nucleotides, *e.g.*, 36-66, 26-36, 25-36, 31-60, 22-43, 27-53 nucleotides in length with a region of at least 19 contiguous nucleotides that is substantially complementary to at least a part of an mRNA transcript of a SNCA gene. These RNAi agents with the longer length antisense strands optionally include a second RNA strand (the sense strand) of 20-60 nucleotides in length wherein the sense and antisense strands form a duplex of 18-30 contiguous nucleotides.

The use of these RNAi agents enables the targeted degradation of mRNAs of a SNCA gene in mammals. Thus, methods and compositions including these RNAi agents are useful for treating a subject who would benefit by a reduction in the levels or activity of a SNCA protein, such as a

subject having a SNCA-associated neurodegenerative disease, *e.g.* a synucleinopathy, such as PD, multiple system atrophy, Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

Intraneuronal accumulation of α-synuclein has been described as either resulting in the formation of Lewy bodies, round eosinophilic hyaline 10-20 pm large inclusions, or Lewy neurites, elongated thread-like dystrophic axons and dendrites. Without wishing to be bound by theory, in the PD brain, deposition of Lewy bodies and Lewy neurites is widespread, though the most vulnerable region for such deposition is believed to be the substantia nigra. Pathology is widespread, as PD has both motor (substantia nigra-based) and non-motor (other parts of the brain, e.g., cortex, etc.) symptoms. Substantia nigra cells are crucial for the execution of movement and postural functions, explaining the nature of PD motor symptoms, while impact upon other cell types explains PD non-motor symptoms, such as dementia, mood changes and depression. In the LBD brain, widespread depositions of Lewy bodies and Lewy neurites are found both in midbrain and cortical areas.

α-Synuclein is a protein which is mainly found intraneuronally. Within the neuron, α-synuclein is predominantly located presynaptically and it has therefore been speculated that it plays a role in the regulation of synaptic activity. Three main isoforms of α-synuclein have been identified, of which the longest and most common form comprises 140 amino acids.

Oxidative stress has been implicated in a number of neurodegenerative disorders characterized by the pathological accumulation of misfolded α-synuclein. Various reactive oxygen species can induce peroxidation of lipids such as cellular membranes or lipoproteins and also result in the generation of highly reactive aldehydes from poly-unsaturated fatty acids (Yoritaka *et al.*, 1996)

Brain pathology indicative of Alzheimer's disease (AD), i.e., amyloid plaques and neurofibrillary tangles, are seen in approximately 50% of cases with LBD. It is unclear whether

the existence of parallel pathologies implies two different diseases or just represents a variant of each respective disorder. Sometimes the cases with co-pathology are described as having a Lewy body variant of AD (Hansen *et al.*, 1990).

Research has also implicated a role of SNCA in AD and Down's syndrome, as the α-synuclein protein has been demonstrated to accumulate in the limbic region in these disorders (Crews *et al.*, 2009).

Rare dominantly inherited forms of PD and LBD can be caused by point mutations or duplications of the SNCA gene. The pathogenic mutations A30P and A53T (Kruger *et al.*, 1998) (Polymeropoulos *et al.*, 1998) and duplication of the gene (Chartier-Harlin *et al.*, 2004) have been described to cause familial PD, whereas one other a-synuclein mutation, E46K (Zarranz *et al.*, 2004) as well as triplication of the a-synuclein gene (Singleton *et al.*, 2003) have been reported to cause either PD or LBD.

The pathogenic consequences of the α-synuclein mutations are only partly understood. However, *in vitro* data have shown that the A30P and A53T mutations increase the rate of aggregation (Conway *et al.*, 2000). A broad range of differently composed α-synuclein species (monomers, dimers, oligomers, including protofibrils) are involved in the aggregation process, all of which may have different toxic properties. It is not clear which molecular species exert toxic effects in the brain. However, research has indicated that oligomeric forms of α-synuclein are particularly neurotoxic. Additional evidence for the role of oligomers is given by the observation that certain α-synuclein mutations (A30P and A53T) causing hereditary Parkinson's disease, lead to an increased rate of oligomerization.

It is not completely known how the α-synuclein aggregation cascade begins. Possibly, an altered conformation of monomeric α-synuclein initiates formation of dimers and trimers, which continue to form higher soluble oligomers, including protofibrils, before these intermediately sized species are deposited as insoluble fibrils in Lewy bodies. It is also conceivable that the α-synuclein oligomers, once they are formed, can bind new monomers and/or smaller multimers of α-synuclein and hence accelerate the fibril formation process. Such seeding effects can possibly also occur in the extracellular space as some evidence suggests that α-synuclein pathology may propagate from neuron to neuron in the diseased brain.

The following detailed description discloses how to make and use compositions containing RNAi agents to inhibit the expression of a SNCA gene, as well as compositions and methods for

treating subjects having diseases and disorders that would benefit from inhibition or reduction of the expression of the genes.

I. RNAi Agents of the Disclosure

Described herein are RNAi agents which inhibit the expression of a SNCA gene. In one embodiment, the RNAi agent includes double stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a SNCA gene in a cell, such as a cell within a subject, *e.g.*, a mammal, such as a human having a SNCA-associated neurodegenerative disease, *e.g.*, a synucleinopathy, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease. The dsRNA includes an antisense strand having a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of a SNCA gene. In embodiments, the region of complementarity is about 15-30 nucleotides or less in length. Upon contact with a cell expressing the SNCA gene, the RNAi agent inhibits the expression of the SNCA gene (*e.g.*, a human gene, a primate gene, a non-primate gene) by at least 50% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by immunofluorescence analysis, using, for example, western blotting or flow cytometric techniques.

A dsRNA includes two RNA strands that are complementary and hybridize to form a duplex structure under conditions in which the dsRNA will be used. One strand of a dsRNA (the antisense strand) includes a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence. The target sequence can be derived from the sequence of an mRNA formed during the expression of a SNCA gene. The other strand (the sense strand) includes a region that is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. As described elsewhere herein and as known in the art, the complementary sequences of a dsRNA can also be

contained as self-complementary regions of a single nucleic acid molecule, as opposed to being on separate oligonucleotides.

Generally, the duplex structure is 15 to 30 base pairs in length, *e.g.,* 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs in length. In certain preferred embodiments, the duplex structure is 18 to 25 base pairs in length, *e.g.*, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-25, 20-24,20-23, 20-22, 20-21, 21-25, 21-24, 21-23, 21-22, 22-25, 22-24, 22-23, 23-25, 23-24 or 24-25 base pairs in length, for example, 19-21 base pairs in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the disclosure.

Similarly, the region of complementarity to the target sequence is 15 to 30 nucleotides in length, *e.g.,* 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20, 15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 nucleotides in length, for example 19-23 nucleotides in length or 21-23 nucleotides in length. Ranges and lengths intermediate to the above recited ranges and lengths are also contemplated to be part of the disclosure.

In some embodiments, the dsRNA is 15 to 23 nucleotides in length, or 24 to 30 nucleotides in length (optionally, 25 to 30 nucleotides in length). In general, the dsRNA can be long enough to serve as a substrate for the Dicer enzyme. For example, it is well known in the art that dsRNAs longer than about 21-23 nucleotides can serve as substrates for Dicer. As the ordinarily skilled person will also recognize, the region of an RNA targeted for cleavage will most often be part of a larger RNA molecule, often an mRNA molecule. Where relevant, a "part" of an mRNA target is a contiguous sequence of an mRNA target of sufficient length to allow it to be a substrate for RNAi-directed cleavage (*i.e.*, cleavage through a RISC pathway).

One of skill in the art will also recognize that the duplex region is a primary functional portion of a dsRNA, *e.g.*, a duplex region of about 15 to 36 base pairs, *e.g.*, 15-36, 15-35, 15-34, 15-33, 15-32, 15-31, 15-30, 15-29, 15-28, 15-27, 15-26, 15-25, 15-24, 15-23, 15-22, 15-21, 15-20,

15-19, 15-18, 15-17, 18-30, 18-29, 18-28, 18-27, 18-26, 18-25, 18-24, 18-23, 18-22, 18-21, 18-20, 19-30, 19-29, 19-28, 19-27, 19-26, 19-25, 19-24, 19-23, 19-22, 19-21, 19-20, 20-30, 20-29, 20-28, 20-27, 20-26, 20-25, 20-24,20-23, 20-22, 20-21, 21-30, 21-29, 21-28, 21-27, 21-26, 21-25, 21-24, 21-23, or 21-22 base pairs, for example, 19-21 base pairs. Thus, in one embodiment, to the extent that it becomes processed to a functional duplex, of *e.g.*, 15-30 base pairs, that targets a desired RNA for cleavage, an RNA molecule or complex of RNA molecules having a duplex region greater than 30 base pairs is a dsRNA. Thus, an ordinarily skilled artisan will recognize that in one embodiment, a miRNA is a dsRNA. In another embodiment, a dsRNA is not a naturally occurring miRNA. In another embodiment, an RNAi agent useful to target SNCA expression is not generated in the target cell by cleavage of a larger dsRNA.

A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs *e.g.*, 1, 2, 3, or 4 nucleotides. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) can be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5'-end, 3'-end or both ends of either an antisense or sense strand of a dsRNA. In certain embodiments, longer, extended overhangs are possible.

A dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc.

iRNA compounds of the disclosure may be prepared using a two-step procedure. First, the individual strands of the double stranded RNA molecule are prepared separately. Then, the component strands are annealed. The individual strands of the siRNA compound can be prepared using solution-phase or solid-phase organic synthesis or both. Organic synthesis offers the advantage that the oligonucleotide strands comprising unnatural or modified nucleotides can be easily prepared. Single-stranded oligonucleotides of the disclosure can be prepared using solution-phase or solid-phase organic synthesis or both.

An siRNA can be produced, *e.g.*, in bulk, by a variety of methods. Exemplary methods include: organic synthesis and RNA cleavage, *e.g.*, *in vitro* cleavage.

An siRNA can be made by separately synthesizing a single stranded RNA molecule, or each respective strand of a double-stranded RNA molecule, after which the component strands can then be annealed.

A large bioreactor, *e.g.*, the OligoPilot II from Pharmacia Biotec AB (Uppsala Sweden), can be used to produce a large amount of a particular RNA strand for a given siRNA. The OligoPilotII reactor can efficiently couple a nucleotide using only a 1.5 molar excess of a phosphoramidite nucleotide. To make an RNA strand, ribonucleotides amidites are used. Standard cycles of monomer addition can be used to synthesize the 21 to 23 nucleotide strand for the siRNA. Typically, the two complementary strands are produced separately and then annealed, *e.g.*, after release from the solid support and deprotection.

Organic synthesis can be used to produce a discrete siRNA species. The complementary of the species to a SNCA gene can be precisely specified. For example, the species may be complementary to a region that includes a polymorphism, *e.g.*, a single nucleotide polymorphism. Further the location of the polymorphism can be precisely defined. In some embodiments, the polymorphism is located in an internal region, *e.g.*, at least 4, 5, 7, or 9 nucleotides from one or both of the termini.

In one embodiment, RNA generated is carefully purified to remove ends. iRNA is cleaved *in vitro* into siRNAs, for example, using a Dicer or comparable RNAse III-based activity. For example, the dsiRNA can be incubated in an *in vitro* extract from *Drosophila* or using purified components, *e.g.*, a purified RNAse or RISC (RNA-induced silencing complex). See, *e.g.*, Ketting *et al. Genes Dev* 2001 Oct 15;15(20): 2654-9 and Hammond *Science* 2001 Aug 10;293(5532): 1146-50.

dsiRNA cleavage generally produces a plurality of siRNA species, each being a particular 21 to 23 nt fragment of a source dsiRNA molecule. For example, siRNAs that include sequences complementary to overlapping regions and adjacent regions of a source dsiRNA molecule may be present.

Regardless of the method of synthesis, the siRNA preparation can be prepared in a solution (*e.g.*, an aqueous or organic solution) that is appropriate for formulation. For example, the siRNA preparation can be precipitated and re-dissolved in pure double-distilled water, and lyophilized. The dried siRNA can then be resuspended in a solution appropriate for the intended formulation process.

In one aspect, a dsRNA of the disclosure includes at least two nucleotide sequences, a sense sequence and an antisense sequence. The sense strand sequence for SNCA may be selected from the group of sequences provided in **Tables 2 or 3**, and the corresponding nucleotide sequence of

the antisense strand of the sense strand may be selected from the group of sequences in **Tables 2 or 3**. In this aspect, one of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of a SNCA gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand (passenger strand) in **Tables 2 or 3**, and the second oligonucleotide is described as the corresponding antisense strand (guide strand) of the sense strand in **Tables 2 or 3** for SNCA.

In one embodiment, the substantially complementary sequences of the dsRNA are contained on separate oligonucleotides. In another embodiment, the substantially complementary sequences of the dsRNA are contained on a single oligonucleotide.

It will be understood that, although the sequences provided herein are described as modified or conjugated sequences, the RNA of the RNAi agent of the disclosure *e.g.*, a dsRNA of the disclosure, may comprise any one of the sequences set forth in in **Tables 2 or 3** that is modified or conjugated differently than described therein. One or more lipophilic ligands can be included in any of the positions of the RNAi agents provided in the instant application.

The skilled person is well aware that dsRNAs having a duplex structure of about 20 to 23 base pairs, *e.g.*, 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al.*, (2001) *EMBO J.*, 20: 6877-6888). However, others have found that shorter or longer RNA duplex structures can also be effective (Chu and Rana (2007) *RNA* 14: 1714-1719; Kim *et al.* (2005) *Nat Biotech* 23: 222-226). In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided herein, dsRNAs described herein can include at least one strand of a length of minimally 21 nucleotides. It can be reasonably expected that shorter duplexes minus only a few nucleotides on one or both ends can be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides derived from one of the sequences provided herein, and differing in their ability to inhibit the expression of a SNCA gene by not more than 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence using the *in vitro* assay with Be(2)-C cells and a 10 nM concentration of the RNA agent and the PCR assay as provided in the examples herein, are contemplated to be within the scope of the present disclosure.

One benchmark assay for inhibition of SNCA involves contacting human Be(2)-C cells with a dsRNA agent as disclosed herein, where sufficient or effective SNCA inhibition is identified

if at least 5% reduction, at least 10% reduction, at least 15% reduction, at least 20% reduction, at least 25% reduction, at least 30% reduction, at least 35% reduction, at least 40% reduction, at least 45% reduction, at least 50% reduction, at least 55% reduction, at least 60% reduction, at least 65% reduction, at least 70% reduction, at least 75% reduction, at least 80% reduction, at least 85% reduction, at least 90% reduction, at least 95% reduction, at least 97% reduction, at least 98% reduction, at least 99% reduction, or more of SNCA transcript or protein is observed in contacted cells, as compared to an appropriate control (e.g., cells not contacted with SNCA-targeting dsRNA). Optionally, a dsRNA agent of the disclosure is administered at 10 nM concentration, and the PCR assay is performed as provided in the examples herein (e.g., Example 2 below).

In addition, the RNAs described herein identify a site(s) in a SNCA transcript that is susceptible to RISC-mediated cleavage. As such, the present disclosure further features RNAi agents that target within this site(s). As used herein, an RNAi agent is said to target within a particular site of an RNA transcript if the RNAi agent promotes cleavage of the transcript anywhere within that particular site. Such an RNAi agent will generally include at least about 15 contiguous nucleotides, optionally at least 19 nucleotides, from one of the sequences provided herein coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a SNCA gene.

An RNAi agent as described herein can contain one or more mismatches to the target sequence. In one embodiment, an RNAi agent as described herein contains no more than 3 mismatches (*i.e.*, 3, 2, 1, or 0 mismatches). In one embodiment, an RNAi agent as described herein contains no more than 2 mismatches. In one embodiment, an RNAi agent as described herein contains no more than 1 mismatch. In one embodiment, an RNAi agent as described herein contains 0 mismatches. In certain embodiments, if the antisense strand of the RNAi agent contains mismatches to the target sequence, the mismatch can optionally be restricted to be within the last 5 nucleotides from either the 5'- or 3'-end of the region of complementarity. For example, in such embodiments, for a 23 nucleotide RNAi agent, the strand which is complementary to a region of a SNCA gene generally does not contain any mismatch within the central 13 nucleotides. The methods described herein or methods known in the art can be used to determine whether an RNAi agent containing a mismatch to a target sequence is effective in inhibiting the expression of a SNCA gene. Consideration of the efficacy of RNAi agents with mismatches in inhibiting

expression of a SNCA gene is important, especially if the particular region of complementarity in a SNCA gene is known to have polymorphic sequence variation within the population.

II. Modified RNAi Agents of the Disclosure

In preferred embodiments, the RNA of an RNAi agent of the disclosure, *e.g.*, a dsRNA, is chemically modified to enhance stability or other beneficial characteristics. In certain embodiments of the disclosure, substantially all of the nucleotides of an RNAi agent of the disclosure are modified. In other embodiments of the disclosure, all of the nucleotides of an RNAi agent of the disclosure are modified. RNAi agents of the disclosure in which "substantially all of the nucleotides are modified" are largely but not wholly modified and can include not more than 5, 4, 3, 2, or 1 unmodified nucleotides. In still other embodiments of the disclosure, RNAi agents of the disclosure can include not more than 5, 4, 3, 2 or 1 modified nucleotides.

The nucleic acids featured in the disclosure can be synthesized or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, end modifications, *e.g.*, 5'-end modifications (phosphorylation, conjugation, inverted linkages) or 3'-end modifications (conjugation, DNA nucleotides, inverted linkages, *etc.*); base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases; sugar modifications (*e.g.*, at the 2'-position or 4'-position) or replacement of the sugar; or backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of RNAi agents useful in the embodiments described herein include, but are not limited to, RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In some embodiments, a modified RNAi agent will have a phosphorus atom in its internucleoside backbone.

Modified RNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl

and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5'-linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, *e.g.,* sodium salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patent Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6, 239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and US Pat RE39464, the entire contents of each of which are hereby incorporated herein by reference.

Modified RNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, the entire contents of each of which are hereby incorporated herein by reference.

In other embodiments, suitable RNA mimetics are contemplated for use in RNAi agents, in which both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patent Nos. 5,539,082; 5,714,331; and 5,719,262, the entire contents of each of which are hereby incorporated herein by reference. Additional PNA compounds suitable for use in the RNAi agents of the disclosure are described in, for example, in Nielsen *et al.*, *Science*, 1991, 254, 1497-1500.

Some embodiments featured in the disclosure include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2-, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O--P--O--CH_2--] of the above-referenced U.S. Patent No. 5,489,677, and the amide backbones of the above-referenced U.S. Patent No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced U.S. Patent No. 5,034,506.

Modified RNAs can also contain one or more substituted sugar moieties. The RNAi agents, *e.g.*, dsRNAs, featured herein can include one of the following at the 2'-position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl can be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include O[$(CH_2)_nO$]$_mCH_3$, O$(CH_2)_nOCH_3$, O$(CH_2)_nNH_2$, O$(CH_2)_nCH_3$, O$(CH_2)_nONH_2$, and O$(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an RNAi agent, or a group

for improving the pharmacodynamic properties of an RNAi agent, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78: 486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.*, a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--$N(CH_2)_2$. Further exemplary modifications include: 5'-Me-2'-F nucleotides, 5'-Me-2'-OMe nucleotides, 5'-Me-2'-deoxynucleotides, (both R and S isomers in these three families); 2'-alkoxyalkyl; and 2'-NMA (N-methylacetamide).

Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$), 2'-*O*-hexadecyl, and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of an RNAi agent, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. RNAi agents can also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application. The entire contents of each of the foregoing are hereby incorporated herein by reference.

An RNAi agent of the disclosure can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl anal other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl

and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, these disclosed by Englisch *et al.*, (1991) *Angewandte Chemie, International Edition*, 30: 613, and those disclosed by Sanghvi, Y S., Chapter 15, dsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the disclosure. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., dsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent Nos. 3,687,808, 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 5,750,692; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, the entire contents of each of which are hereby incorporated herein by reference.

An RNAi agent of the disclosure can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1): 439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3): 833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12): 3185-3193).

An RNAi agent of the disclosure can also be modified to include one or more bicyclic sugar moities. A "bicyclic sugar" is a furanosyl ring modified by the bridging of two atoms. A "bicyclic nucleoside" ("BNA") is a nucleoside having a sugar moiety comprising a bridge connecting two carbon atoms of the sugar ring, thereby forming a bicyclic ring system. In certain embodiments, the bridge connects the 4′-carbon and the 2′-carbon of the sugar ring. Thus, in some embodiments an agent of the disclosure may include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. In other words, an LNA is a nucleotide comprising a bicyclic sugar moiety comprising a 4'-CH2-O-2' bridge. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1): 439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3): 833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12): 3185-3193). Examples of bicyclic nucleosides for use in the polynucleotides of the disclosure include without limitation nucleosides comprising a bridge between the 4′ and the 2′ ribosyl ring atoms. In certain embodiments, the antisense polynucleotide agents of the disclosure include one or more bicyclic nucleosides comprising a 4′ to 2′ bridge. Examples of such 4′ to 2′ bridged bicyclic nucleosides, include but are not limited to 4′-(CH2)—O-2′ (LNA); 4′-(CH2)—S-2′; 4′-(CH2)2—O-2′ (ENA); 4′-CH(CH3)—O-2′ (also referred to as "constrained ethyl" or "cEt") and 4′-CH(CH2OCH3)—O-2′ (and analogs thereof; see, *e.g.*, U.S. Pat. No. 7,399,845); 4′-C(CH3)(CH3)—O-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,283); 4′-CH2—N(OCH3)-2′ (and analogs thereof; see *e.g.*, US Patent No. 8,278,425); 4′-CH2—O—N(CH3)-2′ (see, *e.g.*,U.S. Patent Publication No. 2004/0171570); 4′-CH2—N(R)—O-2′, wherein R is H, C1-C12 alkyl, or a protecting group (see, *e.g.*, U.S. Pat. No. 7,427,672); 4′-CH2—C(H)(CH3)-2′ (see, *e.g.*, Chattopadhyaya *et al., J. Org. Chem.*, 2009, 74, 118-134); and 4′-CH2—C(=CH2)-2′ (and analogs thereof; see, *e.g.*, US Patent No. 8,278,426). The entire contents of each of the foregoing are hereby incorporated herein by reference.

Additional representative US Patents and US Patent Publications that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: US Patent Nos. 6,268,490; 6,525,191; 6,670,461; 6,770,748; 6,794,499; 6,998,484; 7,053,207; 7,034,133;7,084,125; 7,399,845; 7,427,672; 7,569,686; 7,741,457; 8,022,193; 8,030,467;

8,278,425; 8,278,426; 8,278,283; US 2008/0039618; and US 2009/0012281, the entire contents of each of which are hereby incorporated herein by reference.

Any of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α-L-ribofuranose and β-D-ribofuranose (see WO 99/14226).

An RNAi agent of the disclosure can also be modified to include one or more constrained ethyl nucleotides. As used herein, a "constrained ethyl nucleotide" or "cEt" is a locked nucleic acid comprising a bicyclic sugar moiety comprising a 4'-CH(CH3)-0-2' bridge. In one embodiment, a constrained ethyl nucleotide is in the S conformation referred to herein as "S-cEt."

An RNAi agent of the disclosure may also include one or more "conformationally restricted nucleotides" ("CRN"). CRN are nucleotide analogs with a linker connecting the C2'and C4' carbons of ribose or the C3' and C5' carbons of ribose. CRN lock the ribose ring into a stable conformation and increase the hybridization affinity to mRNA. The linker is of sufficient length to place the oxygen in an optimal position for stability and affinity resulting in less ribose ring puckering.

Representative publications that teach the preparation of certain of the above noted CRN include, but are not limited to, US 2013/0190383; and WO 2013/036868, the entire contents of each of which are hereby incorporated herein by reference.

In some embodiments, an RNAi agent of the disclosure comprises one or more monomers that are UNA (unlocked nucleic acid) nucleotides. UNA is unlocked acyclic nucleic acid, wherein any of the bonds of the sugar has been removed, forming an unlocked "sugar" residue. In one example, UNA also encompasses monomer with bonds between C1'-C4' have been removed (*i.e.* the covalent carbon-oxygen-carbon bond between the C1' and C4' carbons). In another example, the C2'-C3' bond (*i.e.* the covalent carbon-carbon bond between the C2' and C3' carbons) of the sugar has been removed (see *Nuc. Acids Symp. Series,* 52, 133-134 (2008) and Fluiter et al., *Mol. Biosyst.,* 2009, 10, 1039 hereby incorporated by reference).

Representative U.S. publications that teach the preparation of UNA include, but are not limited to, US8,314,227; and US Patent Publication Nos. 2013/0096289; 2013/0011922; and 2011/0313020, the entire contents of each of which are hereby incorporated herein by reference.

Potentially stabilizing modifications to the ends of RNA molecules can include N-(acetylaminocaproyl)-4-hydroxyprolinol (Hyp-C6-NHAc), N-(caproyl-4-hydroxyprolinol (Hyp-

C6), N-(acetyl-4-hydroxyprolinol (Hyp-NHAc), thymidine-2'-O-deoxythymidine (ether), N-(aminocaproyl)-4-hydroxyprolinol (Hyp-C6-amino), 2-docosanoyl-uridine-3'- phosphate, inverted 2'-deoxy-modified ribonucleotide, such as inverted dT(idT), inverted dA (idA), and inverted abasic 2'-deoxyribonucleotide (iAb) and others. Disclosure of this modification can be found in WO 2011/005861.

In one example, the 3' or 5' terminal end of an oligonucleotide is linked to an inverted 2'-deoxy-modified ribonucleotide, such as inverted dT(idT), inverted dA (idA), or an inverted abasic 2'-deoxyribonucleotide (iAb). In one particular example, the inverted 2'-deoxy-modified ribonucleotide is linked to the 3'end of an oligonucleotide, such as the 3'-end of a sense strand described herein, where the linking is via a 3'-3' phosphodiester linkage or a 3'-3'-phosphorothioate linkage.

In another example, the 3'-end of a sense strand is linked via a 3'-3'-phosphorothioate linkage to an inverted abasic ribonucleotide (iAb). In another example, the 3'-end of a sense strand is linked via a 3'-3'-phosphorothioate linkage to an inverted dA (idA).

In one particular example, the inverted 2'-deoxy-modified ribonucleotide is linked to the 3'end of an oligonucleotide, such as the 3'-end of a sense strand described herein, where the linking is via a 3'-3' phosphodiester linkage or a 3'-3'-phosphorothioate linkage.

In another example, the 3'-terminal nucleotides of a sense strand is an inverted dA (idA) and is linked to the preceding nucleotide via a 3'-3'- linkage (e.g., 3'-3'-phosphorothioate linkage).

Other modifications of an RNAi agent of the disclosure include a 5' phosphate or 5' phosphate mimic, *e.g.,* a 5'-terminal phosphate or phosphate mimic on the antisense strand of an RNAi agent. Suitable phosphate mimics are disclosed in, for example US 2012/0157511, the entire contents of which are incorporated herein by reference.

A. Modified RNAi agents Comprising Motifs of the Disclosure

In certain aspects of the disclosure, the double-stranded RNAi agents of the disclosure include agents with chemical modifications as disclosed, for example, in WO 2013/075035, the entire contents of which are incorporated herein by reference. As shown herein and in WO 2013/075035, a superior result may be obtained by introducing one or more motifs of three identical modifications on three consecutive nucleotides into a sense strand or antisense strand of an RNAi agent, particularly at or near the cleavage site. In some embodiments, the sense strand and antisense strand of the RNAi agent may otherwise be completely modified. The introduction

of these motifs interrupts the modification pattern, if present, of the sense or antisense strand. The RNAi agent may be optionally conjugated with a lipophilic ligand, *e.g.*, a C16 ligand, for instance on the sense strand. The RNAi agent may be optionally modified with a (*S*)-glycol nucleic acid (GNA) modification, for instance on one or more residues of the antisense strand. The resulting RNAi agents present superior gene silencing activity.

Accordingly, the disclosure provides double stranded RNAi agents capable of inhibiting the expression of a target gene (*i.e.*, a SNCA gene) *in vivo*. The RNAi agent comprises a sense strand and an antisense strand. Each strand of the RNAi agent may be 15-30 nucleotides in length. For example, each strand may be 16-30 nucleotides in length, 17-30 nucleotides in length, 25-30 nucleotides in length, 27-30 nucleotides in length, 17-23 nucleotides in length, 17-21 nucleotides in length, 17-19 nucleotides in length, 19-25 nucleotides in length, 19-23 nucleotides in length, 19-21 nucleotides in length, 21-25 nucleotides in length, or 21-23 nucleotides in length. In certain embodiments, each strand is 19-23 nucleotides in length.

The sense strand and antisense strand typically form a duplex double stranded RNA ("dsRNA"), also referred to herein as an "RNAi agent." The duplex region of an RNAi agent may be 15-30 nucleotide pairs in length. For example, the duplex region can be 16-30 nucleotide pairs in length, 17-30 nucleotide pairs in length, 27-30 nucleotide pairs in length, 17 - 23 nucleotide pairs in length, 17-21 nucleotide pairs in length, 17-19 nucleotide pairs in length, 19-25 nucleotide pairs in length, 19-23 nucleotide pairs in length, 19- 21 nucleotide pairs in length, 21-25 nucleotide pairs in length, or 21-23 nucleotide pairs in length. In another example, the duplex region is selected from 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, and 27 nucleotides in length. In preferred embodiments, the duplex region is 19-21 nucleotide pairs in length.

In one embodiment, the RNAi agent may contain one or more overhang regions or capping groups at the 3'-end, 5'-end, or both ends of one or both strands. The overhang can be 1-6 nucleotides in length, for instance 2-6 nucleotides in length, 1-5 nucleotides in length, 2-5 nucleotides in length, 1-4 nucleotides in length, 2-4 nucleotides in length, 1-3 nucleotides in length, 2-3 nucleotides in length, or 1-2 nucleotides in length. In preferred embodiments, the nucleotide overhang region is 2 nucleotides in length. The overhangs can be the result of one strand being longer than the other, or the result of two strands of the same length being staggered. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences

being targeted or can be another sequence. The first and second strands can also be joined, *e.g.*, by additional bases to form a hairpin, or by other non-base linkers.

In one embodiment, the nucleotides in the overhang region of the RNAi agent can each independently be a modified or unmodified nucleotide including, but no limited to 2'-sugar modified, such as, 2'-F, 2'-O-methyl, thymidine (T), and any combinations thereof.

For example, TT can be an overhang sequence for either end on either strand. The overhang can form a mismatch with the target mRNA or it can be complementary to the gene sequences being targeted or can be another sequence.

The 5'- or 3'- overhangs at the sense strand, antisense strand or both strands of the RNAi agent may be phosphorylated. In some embodiments, the overhang region(s) contains two nucleotides having a phosphorothioate between the two nucleotides, where the two nucleotides can be the same or different. In one embodiment, the overhang is present at the 3'-end of the sense strand, antisense strand, or both strands. In one embodiment, this 3'-overhang is present in the antisense strand. In one embodiment, this 3'-overhang is present in the sense strand.

The RNAi agent may contain only a single overhang, which can strengthen the interference activity of the RNAi, without affecting its overall stability. For example, the single-stranded overhang may be located at the 3'-terminal end of the sense strand or, alternatively, at the 3'-terminal end of the antisense strand. The RNAi may also have a blunt end, located at the 5'-end of the antisense strand (or the 3'-end of the sense strand) or *vice versa*. Generally, the antisense strand of the RNAi has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. While not wishing to be bound by theory, the asymmetric blunt end at the 5'-end of the antisense strand and 3'-end overhang of the antisense strand favor the guide strand loading into RISC process.

In one embodiment, the RNAi agent is a double ended bluntmer of 19 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 7, 8, 9 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In another embodiment, the RNAi agent is a double ended bluntmer of 20 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 8, 9, 10 from the 5'end. The antisense strand contains at least

one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In yet another embodiment, the RNAi agent is a double ended bluntmer of 21 nucleotides in length, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end.

In one embodiment, the RNAi agent comprises a 21 nucleotide sense strand and a 23 nucleotide antisense strand, wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides at positions 9, 10, 11 from the 5'end; the antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at positions 11, 12, 13 from the 5'end, wherein one end of the RNAi agent is blunt, while the other end comprises a 2 nucleotide overhang. Optionally, the 2 nucleotide overhang is at the 3'-end of the antisense strand. When the 2 nucleotide overhang is at the 3'-end of the antisense strand, there may be two phosphorothioate internucleotide linkages between the terminal three nucleotides, wherein two of the three nucleotides are the overhang nucleotides, and the third nucleotide is a paired nucleotide next to the overhang nucleotide. In one embodiment, the RNAi agent additionally has two phosphorothioate internucleotide linkages between the terminal three nucleotides at both the 5'-end of the sense strand and at the 5'-end of the antisense strand. In one embodiment, every nucleotide in the sense strand and the antisense strand of the RNAi agent, including the nucleotides that are part of the motifs are modified nucleotides. In one embodiment each residue is independently modified with a 2'-O-methyl or 3'-fluoro, *e.g.*, in an alternating motif. Optionally, the RNAi agent further comprises a ligand (*e.g.*, a lipophilic ligand, optionally a C16 ligand).

In one embodiment, the RNAi agent comprises a sense and an antisense strand, wherein the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1) positions 1 to 23 of the first strand comprise at least 8 ribonucleotides; the antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, comprises at least 8 ribonucleotides in the positions paired with positions 1- 23 of sense strand to form a duplex; wherein at least the 3 ' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand,

thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a substantially duplexed region between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene expression when the double stranded nucleic acid is introduced into a mammalian cell; and wherein the sense strand contains at least one motif of three 2'-F modifications on three consecutive nucleotides, where at least one of the motifs occurs at or near the cleavage site. The antisense strand contains at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at or near the cleavage site.

In one embodiment, the RNAi agent comprises sense and antisense strands, wherein the RNAi agent comprises a first strand having a length which is at least 25 and at most 29 nucleotides and a second strand having a length which is at most 30 nucleotides with at least one motif of three 2'-O-methyl modifications on three consecutive nucleotides at position 11, 12, 13 from the 5' end; wherein the 3' end of the first strand and the 5' end of the second strand form a blunt end and the second strand is 1-4 nucleotides longer at its 3' end than the first strand, wherein the duplex region which is at least 25 nucleotides in length, and the second strand is sufficiently complementary to a target mRNA along at least 19 nucleotide of the second strand length to reduce target gene expression when the RNAi agent is introduced into a mammalian cell, and wherein dicer cleavage of the RNAi agent preferentially results in an siRNA comprising the 3' end of the second strand, thereby reducing expression of the target gene in the mammal. Optionally, the RNAi agent further comprises a ligand.

In one embodiment, the sense strand of the RNAi agent contains at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at the cleavage site in the sense strand.

In one embodiment, the antisense strand of the RNAi agent can also contain at least one motif of three identical modifications on three consecutive nucleotides, where one of the motifs occurs at or near the cleavage site in the antisense strand.

For an RNAi agent having a duplex region of 17-23 nucleotide in length, the cleavage site of the antisense strand is typically around the 10, 11 and 12 positions from the 5'-end. Thus the motifs of three identical modifications may occur at the 9, 10, 11 positions; 10, 11, 12 positions; 11, 12, 13 positions; 12, 13, 14 positions; or 13, 14, 15 positions of the antisense strand, the count starting from the 1st nucleotide from the 5'-end of the antisense strand, or, the count starting from the 1st paired nucleotide within the duplex region from the 5'- end of the antisense strand. The cleavage site in the antisense strand may also change according to the length of the duplex region of the RNAi from the 5'-end.

The sense strand of the RNAi agent may contain at least one motif of three identical modifications on three consecutive nucleotides at the cleavage site of the strand; and the antisense strand may have at least one motif of three identical modifications on three consecutive nucleotides at or near the cleavage site of the strand. When the sense strand and the antisense strand form a dsRNA duplex, the sense strand and the antisense strand can be so aligned that one motif of the three nucleotides on the sense strand and one motif of the three nucleotides on the antisense strand have at least one nucleotide overlap, *i.e.*, at least one of the three nucleotides of the motif in the sense strand forms a base pair with at least one of the three nucleotides of the motif in the antisense strand. Alternatively, at least two nucleotides may overlap, or all three nucleotides may overlap.

In one embodiment, the sense strand of the RNAi agent may contain more than one motif of three identical modifications on three consecutive nucleotides. The first motif may occur at or near the cleavage site of the strand and the other motifs may be a wing modification. The term "wing modification" herein refers to a motif occurring at another portion of the strand that is separated from the motif at or near the cleavage site of the same strand. The wing modification is either adjacent to the first motif or is separated by at least one or more nucleotides. When the motifs are immediately adjacent to each other, the chemistry of the motifs are distinct from each other; and when the motifs are separated by one or more nucleotide, the chemistries can be the same or different. Two or more wing modifications may be present. For instance, when two wing modifications are present, each wing modification may occur at one end relative to the first motif which is at or near cleavage site or on either side of the lead motif.

Like the sense strand, the antisense strand of the RNAi agent may contain more than one motif of three identical modifications on three consecutive nucleotides, with at least one of the motifs occurring at or near the cleavage site of the strand. This antisense strand may also contain

one or more wing modifications in an alignment similar to the wing modifications that may be present on the sense strand.

In one embodiment, the wing modification on the sense strand or antisense strand of the RNAi agent typically does not include the first one or two terminal nucleotides at the 3'-end, 5'-end or both ends of the strand.

In another embodiment, the wing modification on the sense strand or antisense strand of the RNAi agent typically does not include the first one or two paired nucleotides within the duplex region at the 3'-end, 5'-end or both ends of the strand.

When the sense strand and the antisense strand of the RNAi agent each contain at least one wing modification, the wing modifications may fall on the same end of the duplex region, and have an overlap of one, two or three nucleotides.

When the sense strand and the antisense strand of the RNAi agent each contain at least two wing modifications, the sense strand and the antisense strand can be so aligned that two modifications each from one strand fall on one end of the duplex region, having an overlap of one, two or three nucleotides; two modifications each from one strand fall on the other end of the duplex region, having an overlap of one, two or three nucleotides; two modifications one strand fall on each side of the lead motif, having an overlap of one, two, or three nucleotides in the duplex region.

In one embodiment, the RNAi agent comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mismatch may occur in the overhang region or the duplex region. The base pair may be ranked on the basis of their propensity to promote dissociation or melting (*e.g.*, on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C (I=inosine) is preferred over G:C. Mismatches, *e.g.*, non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a universal base are preferred over canonical pairings.

In one embodiment, the RNAi agent comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'- end of the antisense strand independently selected from the group of: A:U, G:U, I:C, and mismatched pairs, *e.g.*, non-canonical or other than

canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

In one embodiment, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2 or 3 base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair.

In another embodiment, the nucleotide at the 3'-end of the sense strand is deoxy-thymine (dT). In another embodiment, the nucleotide at the 3'-end of the antisense strand is deoxy-thymine (dT). In one embodiment, there is a short sequence of deoxy-thymine nucleotides, for example, two dT nucleotides on the 3'-end of the sense or antisense strand.

In one embodiment, the sense strand sequence may be represented by formula (I):

$5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3'$ (I)

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein Nb and Y do not have the same modification; and

XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. Optionally YYY is all 2'-F modified nucleotides.

In one embodiment, the N_a or N_b comprise modifications of alternating pattern.

In one embodiment, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the RNAi agent has a duplex region of 17-23 nucleotides in length, the YYY motif can occur at or the vicinity of the cleavage site (*e.g.*: can occur at positions 6, 7, 8, 7, 8, 9, 8, 9, 10, 9, 10, 11, 10, 11,12 or 11, 12, 13) of - the sense strand, the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end.

In one embodiment, i is 1 and j is 0, or i is 0 and j is 1, or both i and j are 1. The sense strand can therefore be represented by the following formulas:

5' n_p-N_a-YYY-N_b-ZZZ-N_a-n_q 3' (Ib);

5' n_p-N_a-XXX-N_b-YYY-N_a-n_q 3' (Ic); or

5' n_p-N_a-XXX-N_b-YYY-N_b-ZZZ-N_a-n_q 3' (Id).

When the sense strand is represented by formula (Ib), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides.

Each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Ic), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Id), each N_b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Optionally, N_b is 0, 1, 2, 3, 4, 5 or 6. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X, Y and Z may be the same or different from each other.

In other embodiments, i is 0 and j is 0, and the sense strand may be represented by the formula:

5' n_p-N_a-YYY- N_a-n_q 3' (Ia).

When the sense strand is represented by formula (Ia), each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

In one embodiment, the antisense strand sequence of the RNAi may be represented by formula (II):

5' $n_{q'}$-N_a'-$(Z'Z'Z')_k$-N_b'-Y'Y'Y'-N_b'-$(X'X'X')_l$-N'_a-$n_{p'}$ 3' (II)

wherein:

k and l are each independently 0 or 1;

p' and q' are each independently 0-6;

each N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p' and n_q' independently represent an overhang nucleotide;

wherein N_b' and Y' do not have the same modification;

and

X'X'X', Y'Y'Y' and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, the N_a' or N_b' comprise modifications of alternating pattern.

The Y'Y'Y' motif occurs at or near the cleavage site of the antisense strand. For example, when the RNAi agent has a duplex region of 17-23nucleotidein length, the Y'Y'Y' motif can occur at positions 9, 10, 11;10, 11, 12; 11, 12, 13; 12, 13, 14 ; or 13, 14, 15 of the antisense strand, with the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end. Optionally, the Y'Y'Y' motif occurs at positions 11, 12, 13.

In one embodiment, Y'Y'Y' motif is all 2'-OMe modified nucleotides.

In one embodiment, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

The antisense strand can therefore be represented by the following formulas:

5' n_q'-N_a'-Z'Z'Z'-N_b'-Y'Y'Y'-N_a'-n_p' 3' (IIb);

5' n_q'-N_a'-Y'Y'Y'-N_b'-X'X'X'-n_p' 3' (IIc); or

5' n_q'-N_a'- Z'Z'Z'-N_b'-Y'Y'Y'-N_b'- X'X'X'-N_a'-n_p' 3' (IId).

When the antisense strand is represented by formula (IIb), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIc), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IId), each N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified

nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Optionally, N_b is 0, 1, 2, 3, 4, 5 or 6.

In other embodiments, k is 0 and l is 0 and the antisense strand may be represented by the formula:

5' $n_{p'}$-$N_{a'}$-Y'Y'Y'- $N_{a'}$-$n_{q'}$ 3' (Ia).

When the antisense strand is represented as formula (IIa), each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X', Y' and Z' may be the same or different from each other.

Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-hydroxyl, or 2'-fluoro. For example, each nucleotide of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. Each X, Y, Z, X', Y' and Z', in particular, may represent a 2'-O-methyl modification or a 2'-fluoro modification.

In one embodiment, the sense strand of the RNAi agent may contain YYY motif occurring at 9, 10 and 11 positions of the strand when the duplex region is 21 nt, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y represents 2'-F modification. The sense strand may additionally contain XXX motif or ZZZ motifs as wing modifications at the opposite end of the duplex region; and XXX and ZZZ each independently represents a 2'-OMe modification or 2'-F modification.

In one embodiment the antisense strand may contain Y'Y'Y' motif occurring at positions 11, 12, 13 of the strand, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y' represents 2'-O-methyl modification. The antisense strand may additionally contain X'X'X' motif or Z'Z'Z' motifs as wing modifications at the opposite end of the duplex region; and X'X'X' and Z'Z'Z' each independently represents a 2'-OMe modification or 2'-F modification.

The sense strand represented by any one of the above formulas (Ia), (Ib), (Ic), and (Id) forms a duplex with an antisense strand being represented by any one of formulas (IIa), (IIb), (IIc), and (IId), respectively.

Accordingly, the RNAi agents for use in the methods of the disclosure may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the RNAi duplex represented by formula (III):

sense: 5' n_p -N_a-(X X X)$_i$ -N_b- Y Y Y -N_b -(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'-n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p', q, and q' are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

each n_p', n_p, n_q', and n_q, each of which may or may not be present, independently represents an overhang nucleotide; and

XXX, YYY, ZZZ, X'X'X', Y'Y'Y', and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, i is 0 and j is 0; or i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 0; or both i and j are 1. In another embodiment, k is 0 and l is 0; or k is 1 and l is 0; k is 0 and l is 1; or both k and l are 0; or both k and l are 1.

Exemplary combinations of the sense strand and antisense strand forming an RNAi duplex include the formulas below:

5' n_p - N_a -Y Y Y -N_a-n_q 3'

3' n_p'-N_a'-Y'Y'Y' -N_a'n_q' 5'

(IIIa)

5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-Y'Y'Y'-N_b'-Z'Z'Z'-N_a'n_q' 5'

(IIIb)

5' n_p-N_a- X X X -N_b -Y Y Y - N_a-n_q 3'

3' n_p'-N_a'-X'X'X'-N_b'-Y'Y'Y'-N_a'-n_q' 5'

(IIIc)

5' n_p -N_a -X X X -N_b-Y Y Y -N_b- Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-X'X'X'-N_b'-Y'Y'Y'-N_b'-Z'Z'Z'-N_a-n_q' 5'

(IIId)

When the RNAi agent is represented by formula (IIIa), each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented by formula (IIIb), each N_b independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5 or 1-4 modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIIc), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIId), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a, N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N_a, N_a', N_b and N_b' independently comprises modifications of alternating pattern.

In one embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications. In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications and n_p′ >0 and at least one n_p′ is linked to a neighboring nucleotide a via phosphorothioate linkage. In yet another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications, n_p′ >0 and at least one n_p′ is linked to a neighboring nucleotide via phosphorothioate linkage, and the sense strand is conjugated to one or more C16 (or related) moieties attached through a bivalent or trivalent branched linker (described below). In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , n_p′ >0 and at least one n_p′ is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand

comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more lipophilic, *e.g.*, C16 (or related) moieties, optionally attached through a linker.

In one embodiment, when the RNAi agent is represented by formula (IIIa), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications, $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more lipophilic, *e.g.*, C16 (or related) moieties attached through a linker.

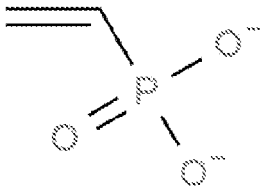
In one embodiment, the RNAi agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, the RNAi agent is a multimer containing three, four, five, six or more duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, two RNAi agents represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId) are linked to each other at the 5' end, and one or both of the 3' ends and are optionally conjugated to a ligand. Each of the agents can target the same gene or two different genes; or each of the agents can target same gene at two different target sites.

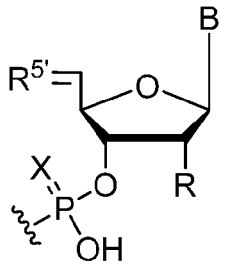
Various publications describe multimeric RNAi agents that can be used in the methods of the disclosure. Such publications include WO2007/091269, WO2010/141511, WO2007/117686, WO2009/014887, and WO2011/031520; and US 7858769, the entire contents of each of which are hereby incorporated herein by reference.

In certain embodiments, the compositions and methods of the disclosure include a 5'-phosphate mimic, such as a vinyl phosphonate (VP) modification of an RNAi agent as described herein. In exemplary embodiments, a vinyl phosphonate of the disclosure has the following structure:



A vinyl phosphonate of the instant disclosure may be attached to either the antisense or the sense strand of a dsRNA of the disclosure. In certain preferred embodiments, a vinyl phosphonate of the instant disclosure is attached to the antisense strand of a dsRNA, optionally at the 5' end of the antisense strand of the dsRNA.

In exemplary embodiments, a 5' vinyl phosphonate modified nucleotide of the disclosure has the structure:



wherein X is O or S;

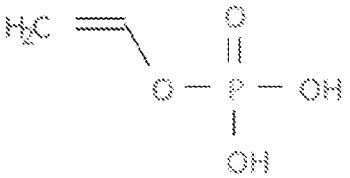
R is hydrogen, hydroxy, fluoro, or C_{1-20}alkoxy (*e.g.*, methoxy or n-hexadecyloxy);

$R^{5'}$ is =C(H)-P(O)$(OH)_2$ and the double bond between the C5' carbon and $R^{5'}$ is in the *E* or *Z* orientation (*e.g.*, *E* orientation); and

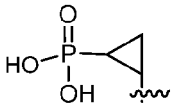
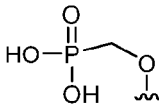
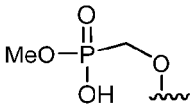
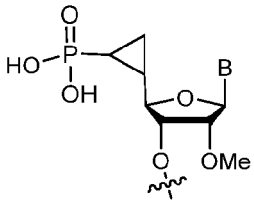
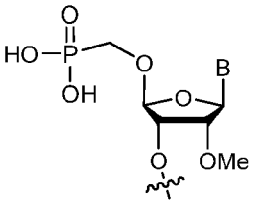
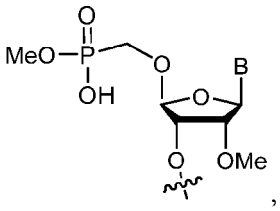
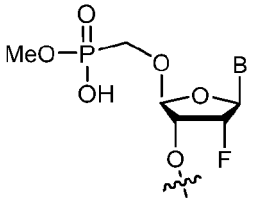
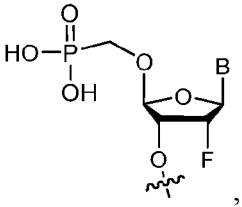
B is a nucleobase or a modified nucleobase, optionally where B is adenine, guanine, cytosine, thymine, or uracil.

In one embodiment, $R^{5'}$ is =C(H)-P(O)$(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation. In another embodiment, R is methoxy and $R^{5'}$ is =C(H)-P(O)$(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation. In another embodiment, X is S, R is methoxy, and $R^{5'}$ is =C(H)-P(O)$(OH)_2$ and the double bond between the C5' carbon and R5' is in the E orientation.

Vinyl phosphate modifications are also contemplated for the compositions and methods of the instant disclosure. An exemplary vinyl phosphate structure is:



Additional exemplary vinyl phosphate structures include the preceding structure, where R5' is =C(H)-OP(O)(OH)$_2$ and the double bond between the C5' carbon and R5' is in the E or Z orientation (*e.g.*, E orientation).

In some embodiments, the 5-phosphate mimic is selected from , , or , or a salt (e.g., sodium salt) thereof, where the broken bond is the covalent bond to the 4'-carbon of the 5'-terminal nucleotide. In some embodiments, the 5'-terminal nucleotide may be , , , , or , or a salt (e.g., sodium salt) thereof, wherein B is a modified or unmodified nucleobase (e.g., uracil or 5-methyluracil).

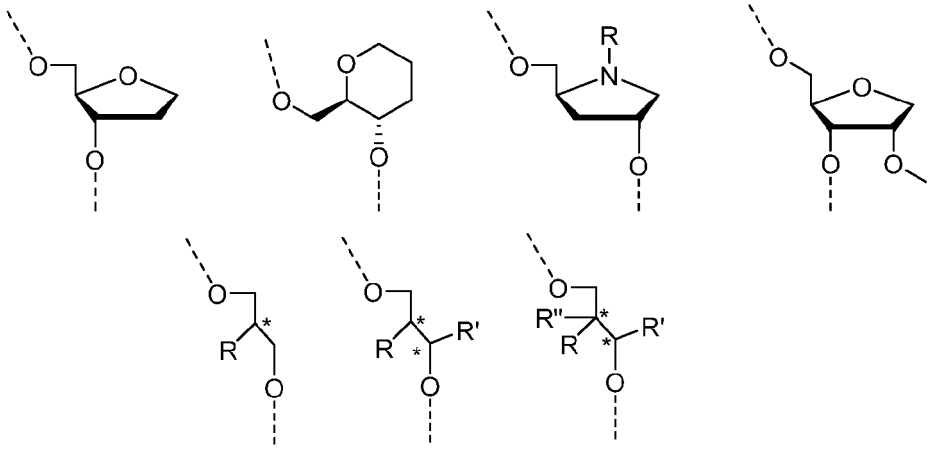
B. *Thermally Destabilizing Modifications*

In certain embodiments, a dsRNA molecule can be optimized for RNA interference by incorporating thermally destabilizing modifications in the seed region of the antisense strand (*i.e.*, at positions 2-9 of the 5'-end of the antisense strand) to reduce or inhibit off-target gene silencing. It has been discovered that dsRNAs with an antisense strand comprising at least one thermally destabilizing modification of the duplex within the first 9 nucleotide positions, counting from the 5' end, of the antisense strand have reduced off-target gene silencing activity. Accordingly, in some embodiments, the antisense strand comprises at least one (*e.g.*, one, two, three, four, five or

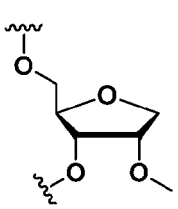
more) thermally destabilizing modification of the duplex within the first 9 nucleotide positions of the 5' region of the antisense strand. In some embodiments, one or more thermally destabilizing modification(s) of the duplex is/are located in positions 2-9, or optionally positions 4-8, from the 5'-end of the antisense strand. In some further embodiments, the thermally destabilizing modification(s) of the duplex is/are located at position 6, 7 or 8 from the 5'-end of the antisense strand. In still some further embodiments, the thermally destabilizing modification of the duplex is located at position 7 from the 5'-end of the antisense strand. The term "thermally destabilizing modification(s)" includes modification(s) that would result with a dsRNA with a lower overall melting temperature (Tm) (optionally a Tm with one, two, three or four degrees lower than the Tm of the dsRNA without having such modification(s). In some embodiments, the thermally destabilizing modification of the duplex is located at position 2, 3, 4, 5 or 9 from the 5'-end of the antisense strand.

The thermally destabilizing modifications can include, but are not limited to, abasic modification; mismatch with the opposing nucleotide in the opposing strand; and sugar modification such as 2'-deoxy modification, acyclic nucleotide, e.g., unlocked nucleic acids (UNA) or glycol nucleic acid (GNA); and 2'-5'-linked ribonucleotides ("3'-RNA").

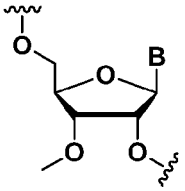
Exemplified abasic modifications include, but are not limited to the following:



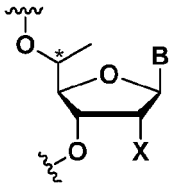
Wherein R = H, Me, Et or OMe; R' = H, Me, Et or OMe; R" = H, Me, Et or OMe



Mod2
(2'-OMe Abasic Spacer)

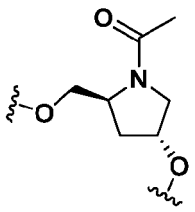


Mod3
(3'-OMe)



Mod4
(5'-Me)

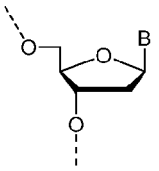
X = OMe, F



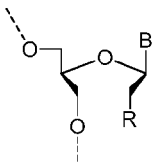
Mod5
(Hyp-spacer)

wherein B is a modified or unmodified nucleobase.

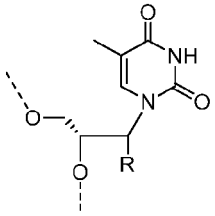
Exemplified sugar modifications include, but are not limited to the following:



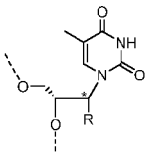
2'-deoxy



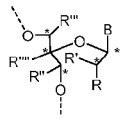
unlocked nucleic acid
R= H, OH, O-alkyl



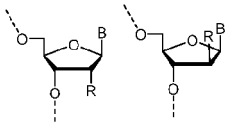
glycol nucleic acid
R= H, OH, O-alkyl



glycol nucleic acid
R= H, OH, O-alkyl



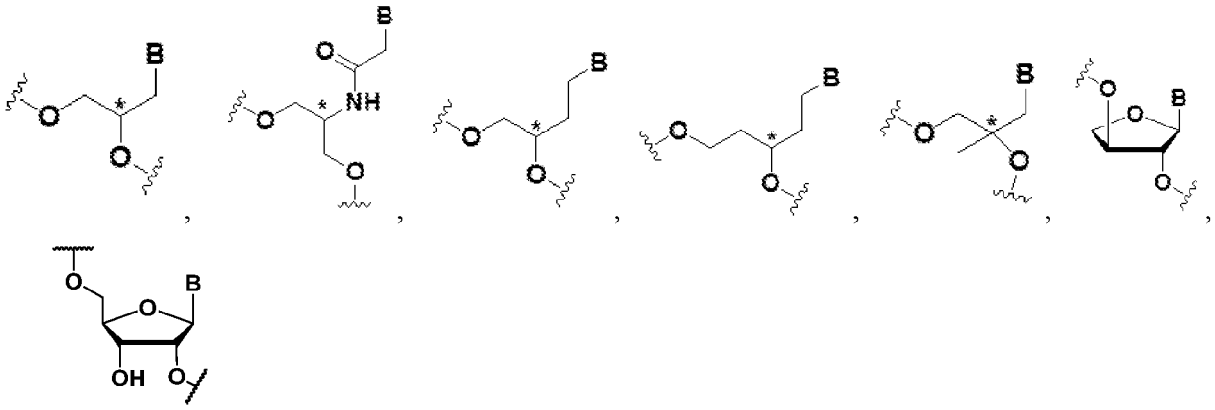
unlocked nucleic acid
R= H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R'' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R''' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2
R'''' = H, OH, CH_3, CH_2CH_3, O-alkyl, NH_2, NHMe, NMe_2



R = H, methyl, ethyl

wherein B is a modified or unmodified nucleobase.

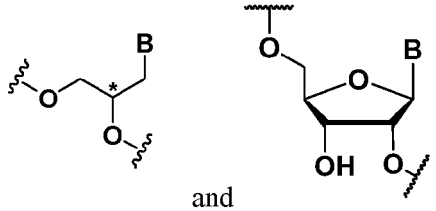
In some embodiments the thermally destabilizing modification of the duplex is selected from the group consisting of:



and (3'-RNA),

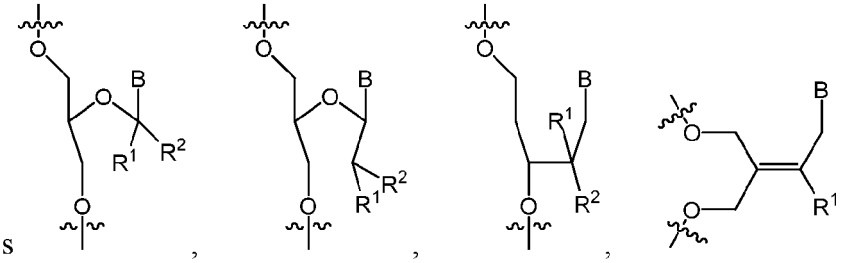
wherein B is a modified or unmodified nucleobase and the asterisk on each structure represents either *R*, *S* or *racemic*.

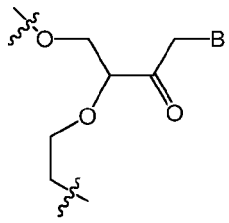
In some embodiments the thermally destabilizing modification of the duplex is selected from the group consisting of:



and

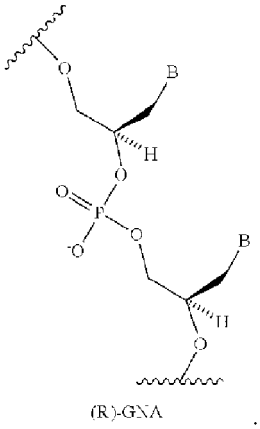
wherein B is a modified or unmodified nucleobase and the asterisk represents either *R*, *S* or *racemic* (e.g. S).

The term "acyclic nucleotide" refers to any nucleotide having an acyclic ribose sugar, for example, where any of bonds between the ribose carbons (*e.g.*, C1'-C2', C2'-C3', C3'-C4', C4'-O4', or C1'-O4') is absent or at least one of ribose carbons or oxygen (*e.g.*, C1', C2', C3', C4' or O4') are independently or in combination absent from the nucleotide. In some embodiments, acyclic nucleotide is  or



, wherein B is a modified or unmodified nucleobase, R^1 and R^2 independently are H, halogen, OR_3, or alkyl; and R_3 is H, alkyl, cycloalkyl, aryl, aralkyl, heteroaryl or sugar). The term "UNA" refers to unlocked acyclic nucleic acid, wherein any of the bonds of the sugar has been removed, forming an unlocked "sugar" residue. In one example, UNA also encompasses monomers with bonds between C1'-C4' being removed (*i.e.* the covalent carbon-oxygen-carbon bond between the C1' and C4' carbons). In another example, the C2'-C3' bond (*i.e.* the covalent carbon-carbon bond between the C2' and C3' carbons) of the sugar is removed (see Mikhailov et. al., Tetrahedron Letters, 26 (17): 2059 (1985); and Fluiter et al., Mol. Biosyst., 10: 1039 (2009), which are hereby incorporated by reference in their entirety). The acyclic derivative provides greater backbone flexibility without affecting the Watson-Crick pairings. The acyclic nucleotide can be linked via 2'-5' or 3'-5' linkage.

The term 'GNA' refers to glycol nucleic acid which is a polymer similar to DNA or RNA but differing in the composition of its "backbone" in that is composed of repeating glycerol units linked by phosphodiester bonds:

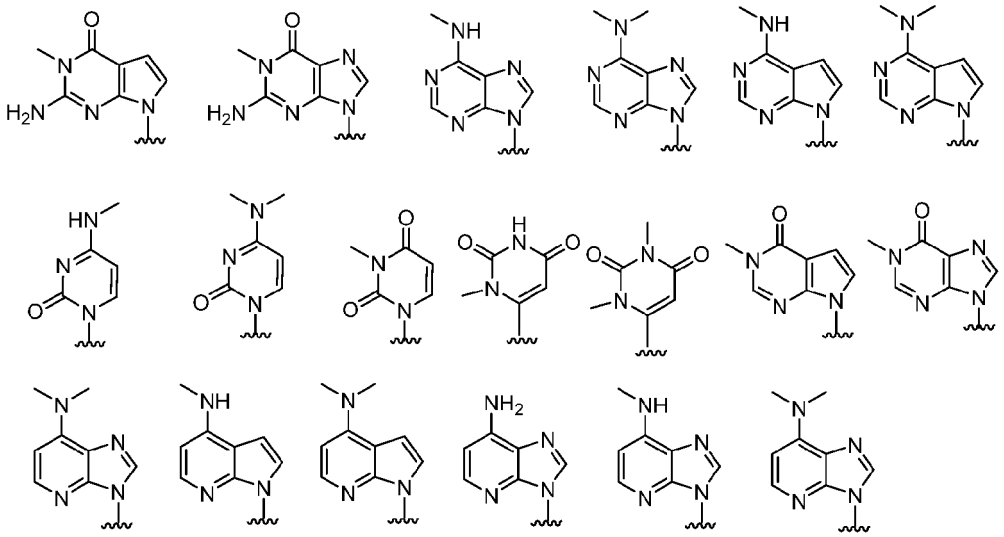


(R)-GNA

The thermally destabilizing modification of the duplex can be mismatches (*i.e.*, noncomplementary base pairs) between the thermally destabilizing nucleotide and the opposing nucleotide in the opposite strand within the dsRNA duplex. Exemplary mismatch base pairs include G:G, G:A, G:U, G:T, A:A, A:C, C:C, C:U, C:T, U:U, T:T, U:T, or a combination thereof. Other mismatch base pairings known in the art are also amenable to the present disclosure. A

mismatch can occur between nucleotides that are either naturally occurring nucleotides or modified nucleotides, *i.e.*, the mismatch base pairing can occur between the nucleobases from respective nucleotides independent of the modifications on the ribose sugars of the nucleotides. In certain embodiments, the dsRNA molecule contains at least one nucleobase in the mismatch pairing that is a 2'-deoxy nucleobase; *e.g.*, the 2'-deoxy nucleobase is in the sense strand.

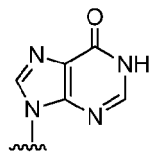
In some embodiments, the thermally destabilizing modification of the duplex in the seed region of the antisense strand includes nucleotides with impaired W-C H-bonding to complementary base on the target mRNA, such as:



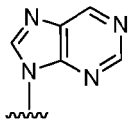
More examples of abasic nucleotide, acyclic nucleotide modifications (including UNA and GNA), and mismatch modifications have been described in detail in WO 2011/133876, which is herein incorporated by reference in its entirety.

The thermally destabilizing modifications may also include universal base with reduced or abolished capability to form hydrogen bonds with the opposing bases, and phosphate modifications.

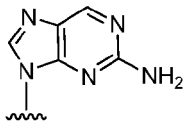
In some embodiments, the thermally destabilizing modification of the duplex includes nucleotides with non-canonical bases such as, but not limited to, nucleobase modifications with impaired or completely abolished capability to form hydrogen bonds with bases in the opposite strand. These nucleobase modifications have been evaluated for destabilization of the central region of the dsRNA duplex as described in WO 2010/0011895, which is herein incorporated by reference in its entirety. Exemplary nucleobase modifications are:



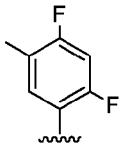
inosine



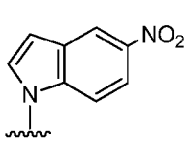
nebularine



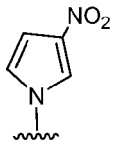
2-aminopurine



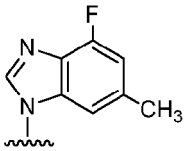
2,4-difluorotoluene



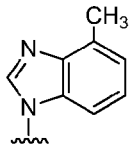
5-nitroindole



3-nitropyrrole

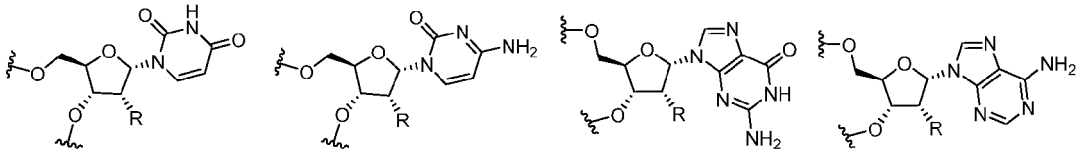


4-Fluoro-6-methylbenzimidazole



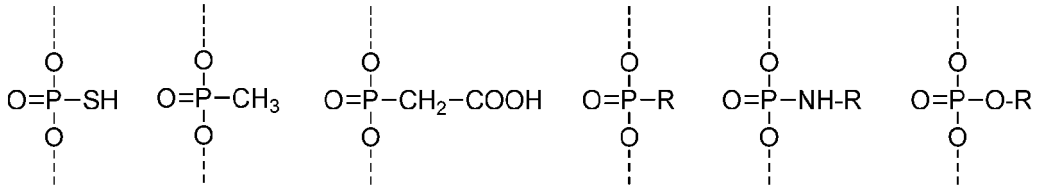
4-Methylbenzimidazole

In some embodiments, the thermally destabilizing modification of the duplex in the seed region of the antisense strand includes one or more α-nucleotide complementary to the base on the target mRNA, such as:



wherein R is H, OH, OCH_3, F, NH_2, NHMe, NMe_2 or O-alkyl.

Exemplary phosphate modifications known to decrease the thermal stability of dsRNA duplexes compared to natural phosphodiester linkages are:



R = alkyl

The alkyl for the R group can be a C_1-C_6alkyl. Specific alkyls for the R group include, but are not limited to methyl, ethyl, propyl, isopropyl, butyl, pentyl and hexyl.

As the skilled artisan will recognize, in view of the functional role of nucleobases is defining specificity of an RNAi agent of the disclosure, while nucleobase modifications can be performed in the various manners as described herein, *e.g.*, to introduce destabilizing modifications into an RNAi agent of the disclosure, *e.g.*, for purpose of enhancing on-target effect relative to off-target effect, the range of modifications available and, in general, present upon RNAi agents of the disclosure tends to be much greater for non-nucleobase modifications, *e.g.*,

modifications to sugar groups or phosphate backbones of polyribonucleotides. Such modifications are described in greater detail in other sections of the instant disclosure and are expressly contemplated for RNAi agents of the disclosure, either possessing native nucleobases or modified nucleobases as described above or elsewhere herein.

In addition to the antisense strand comprising a thermally destabilizing modification, the dsRNA can also comprise one or more stabilizing modifications. For example, the dsRNA can comprise at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, the stabilizing modifications all can be present in one strand. In some embodiments, both the sense and the antisense strands comprise at least two stabilizing modifications. The stabilizing modification can occur on any nucleotide of the sense strand or antisense strand. For instance, the stabilizing modification can occur on every nucleotide on the sense strand or antisense strand; each stabilizing modification can occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both stabilizing modification in an alternating pattern. The alternating pattern of the stabilizing modifications on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the stabilizing modifications on the sense strand can have a shift relative to the alternating pattern of the stabilizing modifications on the antisense strand.

In some embodiments, the antisense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, a stabilizing modification in the antisense strand can be present at any positions. In some embodiments, the antisense comprises stabilizing modifications at positions 2, 6, 8, 9, 14, and 16 from the 5'-end. In some other embodiments, the antisense comprises stabilizing modifications at positions 2, 6, 14, and 16 from the 5'-end. In still some other embodiments, the antisense comprises stabilizing modifications at positions 2, 14, and 16 from the 5'-end.

In some embodiments, the antisense strand comprises at least one stabilizing modification adjacent to the destabilizing modification. For example, the stabilizing modification can be the nucleotide at the 5'-end or the 3'-end of the destabilizing modification, *i.e.*, at position -1 or +1 from the position of the destabilizing modification. In some embodiments, the antisense strand comprises a stabilizing modification at each of the 5'-end and the 3'-end of the destabilizing modification, *i.e.*, positions -1 and +1 from the position of the destabilizing modification.

In some embodiments, the antisense strand comprises at least two stabilizing modifications at the 3'-end of the destabilizing modification, *i.e.*, at positions +1 and +2 from the position of the destabilizing modification.

In some embodiments, the sense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) stabilizing modifications. Without limitations, a stabilizing modification in the sense strand can be present at any positions. In some embodiments, the sense strand comprises stabilizing modifications at positions 7, 10, and 11 from the 5'-end. In some other embodiments, the sense strand comprises stabilizing modifications at positions 7, 9, 10, and 11 from the 5'-end. In some embodiments, the sense strand comprises stabilizing modifications at positions opposite or complimentary to positions 11, 12, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some other embodiments, the sense strand comprises stabilizing modifications at positions opposite or complimentary to positions 11, 12, 13, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some embodiments, the sense strand comprises a block of two, three, or four stabilizing modifications.

In some embodiments, the sense strand does not comprise a stabilizing modification in position opposite or complimentary to the thermally destabilizing modification of the duplex in the antisense strand.

Exemplary thermally stabilizing modifications include, but are not limited to, 2'-fluoro modifications. Other thermally stabilizing modifications include, but are not limited to, LNA.

In some embodiments, the dsRNA of the disclosure comprises at least four (*e.g.*, four, five, six, seven, eight, nine, ten, or more) 2'-fluoro nucleotides. Without limitations, the 2'-fluoro nucleotides all can be present in one strand. In some embodiments, both the sense and the antisense strands comprise at least two 2'-fluoro nucleotides. The 2'-fluoro modification can occur on any nucleotide of the sense strand or antisense strand. For instance, the 2'-fluoro modification can occur on every nucleotide on the sense strand or antisense strand; each 2'-fluoro modification can occur in an alternating pattern on the sense strand or antisense strand; or the sense strand or antisense strand comprises both 2'-fluoro modifications in an alternating pattern. The alternating pattern of the 2'-fluoro modifications on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the 2'-fluoro modifications on the sense strand can have a shift relative to the alternating pattern of the 2'-fluoro modifications on the antisense strand.

In some embodiments, the antisense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, or more) 2'-fluoro nucleotides. Without limitations, a 2'-fluoro modification in the antisense strand can be present at any positions. In some embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 6, 8, 9, 14, and 16 from the 5'-end. In some other embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 6, 14, and 16 from the 5'-end. In still some other embodiments, the antisense comprises 2'-fluoro nucleotides at positions 2, 14, and 16 from the 5'-end.

In some embodiments, the antisense strand comprises at least one 2'-fluoro nucleotide adjacent to the destabilizing modification. For example, the 2'-fluoro nucleotide can be the nucleotide at the 5'-end or the 3'-end of the destabilizing modification, *i.e.*, at position -1 or +1 from the position of the destabilizing modification. In some embodiments, the antisense strand comprises a 2'-fluoro nucleotide at each of the 5'-end and the 3'-end of the destabilizing modification, *i.e.*, positions -1 and +1 from the position of the destabilizing modification.

In some embodiments, the antisense strand comprises at least two 2'-fluoro nucleotides at the 3'-end of the destabilizing modification, *i.e.*, at positions +1 and +2 from the position of the destabilizing modification.

In some embodiments, the sense strand comprises at least two (*e.g.*, two, three, four, five, six, seven, eight, nine, ten or more) 2'-fluoro nucleotides. Without limitations, a 2'-fluoro modification in the sense strand can be present at any positions. In some embodiments, the antisense comprises 2'-fluoro nucleotides at positions 7, 10, and 11 from the 5'-end. In some other embodiments, the sense strand comprises 2'-fluoro nucleotides at positions 7, 9, 10, and 11 from the 5'-end. In some embodiments, the sense strand comprises 2'-fluoro nucleotides at positions opposite or complimentary to positions 11, 12, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some other embodiments, the sense strand comprises 2'-fluoro nucleotides at positions opposite or complimentary to positions 11, 12, 13, and 15 of the antisense strand, counting from the 5'-end of the antisense strand. In some embodiments, the sense strand comprises a block of two, three or four 2'-fluoro nucleotides.

In some embodiments, the sense strand does not comprise a 2'-fluoro nucleotide in position opposite or complimentary to the thermally destabilizing modification of the duplex in the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises a 21 nucleotides (nt) sense strand and a 23 nucleotides (nt) antisense, wherein the antisense strand contains at least one thermally destabilizing nucleotide, where the at least one thermally destabilizing nucleotide occurs in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), wherein one end of the dsRNA is blunt, while the other end is comprises a 2 nt overhang, and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4 or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4 or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA comprises a blunt end at 5'-end of the antisense strand. Optionally, the 2 nt overhang is at the 3'-end of the antisense.

In some embodiments, the dsRNA molecule of the disclosure comprises sense and antisense strands, wherein: the sense strand is 25-30 nucleotide residues in length, wherein starting from the 5' terminal nucleotide (position 1), positions 1 to 23 of said sense strand comprise at least 8 ribonucleotides; antisense strand is 36-66 nucleotide residues in length and, starting from the 3' terminal nucleotide, at least 8 ribonucleotides in the positions paired with positions 1- 23 of sense strand to form a duplex; wherein at least the 3 ' terminal nucleotide of antisense strand is unpaired with sense strand, and up to 6 consecutive 3' terminal nucleotides are unpaired with sense strand, thereby forming a 3' single stranded overhang of 1-6 nucleotides; wherein the 5' terminus of antisense strand comprises from 10-30 consecutive nucleotides which are unpaired with sense strand, thereby forming a 10-30 nucleotide single stranded 5' overhang; wherein at least the sense strand 5' terminal and 3' terminal nucleotides are base paired with nucleotides of antisense strand when sense and antisense strands are aligned for maximum complementarity, thereby forming a substantially duplexed region between sense and antisense strands; and antisense strand is sufficiently complementary to a target RNA along at least 19 ribonucleotides of antisense strand length to reduce target gene expression when said double stranded nucleic acid is introduced into a mammalian cell; and wherein the antisense strand contains at least one thermally destabilizing nucleotide, where at least one thermally destabilizing nucleotide is in the seed region of the antisense strand (*i.e.* at position 2-9 of the 5'-end of the antisense strand). For example, the

thermally destabilizing nucleotide occurs between positions opposite or complimentary to positions 14-17 of the 5'-end of the sense strand, and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4, or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; and (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA comprises a duplex region of 12-30 nucleotide pairs in length.

In some embodiments, the dsRNA molecule of the disclosure comprises sense and antisense strands, wherein said dsRNA molecule comprises a sense strand having a length which is at least 25 and at most 29 nucleotides and an antisense strand having a length which is at most 30 nucleotides with the sense strand comprises a modified nucleotide that is susceptible to enzymatic degradation at position 11 from the 5'end, wherein the 3' end of said sense strand and the 5' end of said antisense strand form a blunt end and said antisense strand is 1-4 nucleotides longer at its 3' end than the sense strand, wherein the duplex region which is at least 25 nucleotides in length, and said antisense strand is sufficiently complementary to a target mRNA along at least 19 nt of said antisense strand length to reduce target gene expression when said dsRNA molecule is introduced into a mammalian cell, and wherein dicer cleavage of said dsRNA preferentially results in an siRNA comprising said 3' end of said antisense strand, thereby reducing expression of the target gene in the mammal, wherein the antisense strand contains at least one thermally destabilizing nucleotide, where the at least one thermally destabilizing nucleotide is in the seed region of the antisense strand (*i.e.* at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5, or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4, or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages; and (vi) the dsRNA comprises at least four 2'-fluoro modifications; and (vii) the dsRNA has a duplex region of 12-29 nucleotide pairs in length.

In some embodiments, every nucleotide in the sense strand and antisense strand of the dsRNA molecule may be modified. Each nucleotide may be modified with the same or different modification which can include one or more alteration of one or both of the non-linking phosphate oxygens or of one or more of the linking phosphate oxygens; alteration of a constituent of the ribose sugar, *e.g.*, of the 2′ hydroxyl on the ribose sugar; wholesale replacement of the phosphate moiety with "dephospho" linkers; modification or replacement of a naturally occurring base; and replacement or modification of the ribose-phosphate backbone.

As nucleic acids are polymers of subunits, many of the modifications occur at a position which is repeated within a nucleic acid, *e.g.*, a modification of a base, or a phosphate moiety, or a non-linking O of a phosphate moiety. In some cases, the modification will occur at all of the subject positions in the nucleic acid but in many cases it will not. By way of example, a modification may only occur at a 3' or 5' terminal position, may only occur in a terminal region, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand. A modification may occur in a double strand region, a single strand region, or in both. A modification may occur only in the double strand region of an RNA or may only occur in a single strand region of an RNA. *E.g.*, a phosphorothioate modification at a non-linking O position may only occur at one or both termini, may only occur in a terminal region, *e.g.*, at a position on a terminal nucleotide or in the last 2, 3, 4, 5, or 10 nucleotides of a strand, or may occur in double strand and single strand regions, particularly at termini. The 5' end or ends can be phosphorylated.

It may be possible, *e.g.*, to enhance stability, to include particular bases in overhangs, or to include modified nucleotides or nucleotide surrogates, in single strand overhangs, *e.g.*, in a 5' or 3' overhang, or in both. *E.g.*, it can be desirable to include purine nucleotides in overhangs. In some embodiments all or some of the bases in a 3' or 5' overhang may be modified, *e.g.*, with a modification described herein. Modifications can include, *e.g.*, the use of modifications at the 2' position of the ribose sugar with modifications that are known in the art, *e.g.*, the use of deoxyribonucleotides, 2'-deoxy-2'-fluoro (2'-F) or 2'-O-methyl modified instead of the ribosugar of the nucleobase, and modifications in the phosphate group, *e.g.*, phosphorothioate modifications. Overhangs need not be homologous with the target sequence.

In some embodiments, each residue of the sense strand and antisense strand is independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'- O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-deoxy, or 2'-fluoro. The strands can contain more than one modification. In some

embodiments, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. It is to be understood that these modifications are in addition to the at least one thermally destabilizing modification of the duplex present in the antisense strand.

At least two different modifications are typically present on the sense strand and antisense strand. Those two modifications may be the 2'-deoxy, 2'- O-methyl or 2'-fluoro modifications, acyclic nucleotides or others. In some embodiments, the sense strand and antisense strand each comprises two differently modified nucleotides selected from 2'-O-methyl or 2'-deoxy. In some embodiments, each residue of the sense strand and antisense strand is independently modified with 2'-O-methyl nucleotide, 2'-deoxy nucleotide, 2′-deoxy-2'-fluoro nucleotide, 2'-O-N-methylacetamido (2'-O-NMA) nucleotide, a 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE) nucleotide, 2'-O-aminopropyl (2'-O-AP) nucleotide, or 2'-ara-F nucleotide. Again, it is to be understood that these modifications are in addition to the at least one thermally destabilizing modification of the duplex present in the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises modifications of an alternating pattern, particular in the B1, B2, B3, B1', B2', B3', B4' regions. The term "alternating motif" or "alternative pattern" as used herein refers to a motif having one or more modifications, each modification occurring on alternating nucleotides of one strand. The alternating nucleotide may refer to one per every other nucleotide or one per every three nucleotides, or a similar pattern. For example, if A, B and C each represent one type of modification to the nucleotide, the alternating motif can be "ABABABABABAB...," "AABBAABBAABB...," "AABAABAABAAB...," "AAABAAABAAAB...," "AAABBBAAABBB...," or "ABCABCABCABC...," etc.

The type of modifications contained in the alternating motif may be the same or different. For example, if A, B, C, D each represent one type of modification on the nucleotide, the alternating pattern, *i.e.*, modifications on every other nucleotide, may be the same, but each of the sense strand or antisense strand can be selected from several possibilities of modifications within the alternating motif such as "ABABAB...", "ACACAC..." "BDBDBD..." or "CDCDCD...," etc.

In some embodiments, the dsRNA molecule of the disclosure comprises the modification pattern for the alternating motif on the sense strand relative to the modification pattern for the alternating motif on the antisense strand is shifted. The shift may be such that the modified group of nucleotides of the sense strand corresponds to a differently modified group of nucleotides of the

antisense strand and vice versa. For example, the sense strand when paired with the antisense strand in the dsRNA duplex, the alternating motif in the sense strand may start with "ABABAB" from 5'-3' of the strand and the alternating motif in the antisense strand may start with "BABABA" from 3'-5'of the strand within the duplex region. As another example, the alternating motif in the sense strand may start with "AABBAABB" from 5'-3' of the strand and the alternating motif in the antisense strand may start with "BBAABBAA" from 3'-5'of the strand within the duplex region, so that there is a complete or partial shift of the modification patterns between the sense strand and the antisense strand.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand, where each A is an unmodified ribonucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand, where each A is a 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

In another particular example, the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is a 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand and the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is an unmodified ribonucleotide and each B is a 2'-Omethyl modified nucleotide.

In one particular example, the alternating motif in the sense strand is "ABABAB" sfrom 5'-3' of the strand and the alternating motif in the antisense strand is "BABABA" from 3'-5'of the strand, where each A is a 2'-deoxy-2'-fluoro modified nucleotide and each B is a 2'-Omethyl modified nucleotide.

The dsRNA molecule of the disclosure may further comprise at least one phosphorothioate or methylphosphonate internucleotide linkage. The phosphorothioate or methylphosphonate internucleotide linkage modification may occur on any nucleotide of the sense strand or antisense strand or both in any position of the strand. For instance, the internucleotide linkage modification may occur on every nucleotide on the sense strand or antisense strand; each internucleotide linkage modification may occur in an alternating pattern on the sense strand or antisense strand; or the

sense strand or antisense strand comprises two or more internucleotide linkage modifications in an alternating pattern. The alternating pattern of the internucleotide linkage modification on the sense strand may be the same or different from the antisense strand, and the alternating pattern of the internucleotide linkage modification on the sense strand may have a shift relative to the alternating pattern of the internucleotide linkage modification on the antisense strand.

In some embodiments, the dsRNA molecule comprises the phosphorothioate or methylphosphonate internucleotide linkage modification in the overhang region. For example, the overhang region comprises two nucleotides having a phosphorothioate or methylphosphonate internucleotide linkage between the two nucleotides. Internucleotide linkage modifications also may be made to link the overhang nucleotides with the terminal paired nucleotides within duplex region. For example, at least 2, 3, 4, or all the overhang nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage, and optionally, there may be additional phosphorothioate or methylphosphonate internucleotide linkages linking the overhang nucleotide with a paired nucleotide that is next to the overhang nucleotide. For instance, there may be at least two phosphorothioate internucleotide linkages between the terminal three nucleotides, in which two of the three nucleotides are overhang nucleotides, and the third is a paired nucleotide next to the overhang nucleotide. Optionally, these terminal three nucleotides may be at the 3'-end of the antisense strand.

In some embodiments, the sense strand of the dsRNA molecule comprises 1-10 blocks of two to ten phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said sense strand is paired with an antisense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of two phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising

any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of three phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of four phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of five phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of six phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination

of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of seven phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, 6, 7, or 8 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of eight phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, 4, 5, or 6 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the antisense strand of the dsRNA molecule comprises two blocks of nine phosphorothioate or methylphosphonate internucleotide linkages separated by 1, 2, 3, or 4 phosphate internucleotide linkages, wherein one of the phosphorothioate or methylphosphonate internucleotide linkages is placed at any position in the oligonucleotide sequence and the said antisense strand is paired with a sense strand comprising any combination of phosphorothioate, methylphosphonate and phosphate internucleotide linkages or an antisense strand comprising either phosphorothioate or methylphosphonate or phosphate linkage.

In some embodiments, the dsRNA molecule of the disclosure further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the termini position(s) of the sense or antisense strand. For example, at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides may be linked through phosphorothioate or methylphosphonate internucleotide linkage at one end or both ends of the sense or antisense strand.

In some embodiments, the dsRNA molecule of the disclosure further comprises one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the internal region of the duplex of each of the sense or antisense strand. For example, at least 2,

3, 4, 5, 6, 7, 8, 9, or 10 nucleotides may be linked through phosphorothioate methylphosphonate internucleotide linkage at position 8-16 of the duplex region counting from the 5'-end of the sense strand; the dsRNA molecule can optionally further comprise one or more phosphorothioate or methylphosphonate internucleotide linkage modification within 1-10 of the termini position(s).

In some embodiments, the dsRNA molecule of the disclosure further comprises one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 1-5 and one to five phosphorothioate or methylphosphonate internucleotide linkage modification(s) within position 18-23 of the sense strand (counting from the 5'-end), and one to five phosphorothioate or methylphosphonate internucleotide linkage modification at positions 1 and 2 and one to five within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate or methylphosphonate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate or methylphosphonate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides and between the 2nd and 3rd nucleotides at the 5′ end of the antisense strand. In some embodiments, the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides and between the 2nd and 3rd nucleotides at the 3′ end of the antisense strand. In some embodiments, the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides and between the 2nd and 3rd nucleotides at both the 5′ end and 3′ end of the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides and between the 2nd and 3rd nucleotides at the 5′ end of the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides and between the 2nd and

3rd nucleotides at the 5′ end of the sense strand and between the 1st and 2nd nucleotides and between the 2nd and 3rd nucleotides at both the 5′ and 3′ ends of the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure the dsRNA molecule of the disclosure comprises phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides, between the 2nd and 3rd nucleotides, and between the 3rd and 4th nucleotdies at the 5′ end of the antisense strand, a phosphorothioate internucleotide linkage modification between the 1st and 2nd nucleotides at the 3′ end of the antisense strand, and phosphorothioate internucleotide linkage modifications between the 1st and 2nd nucleotides at the 5′ and 3′ ends of the sense strand.

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and two phosphorothioate internucleotide linkage modifications within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at

positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modification at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification within position 1-5 (counting from the 5'-end) of the sense strand, and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 (counting from the 5'-end) of the sense strand, and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and one phosphorothioate internucleotide linkage modification within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications within position 1-5 and one

phosphorothioate internucleotide linkage modification within position 18-23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications within positions 18-23 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 20 and 21 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 20 and 21 the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 21 and 22 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 21 and 22 the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises two phosphorothioate internucleotide linkage modifications at position 1 and 2, and two phosphorothioate internucleotide linkage modifications at position 22 and 23 of the sense strand (counting from the 5'-end), and one phosphorothioate internucleotide linkage modification at

positions 1 and one phosphorothioate internucleotide linkage modification at position 21 of the antisense strand (counting from the 5'-end).

In some embodiments, the dsRNA molecule of the disclosure further comprises one phosphorothioate internucleotide linkage modification at position 1, and one phosphorothioate internucleotide linkage modification at position 21 of the sense strand (counting from the 5'-end), and two phosphorothioate internucleotide linkage modifications at positions 1 and 2 and two phosphorothioate internucleotide linkage modifications at positions 23 and 23 the antisense strand (counting from the 5'-end).

In some embodiments, compound of the disclosure comprises a pattern of backbone chiral centers. In some embodiments, a common pattern of backbone chiral centers comprises at least 5 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 6 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 7 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 8 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 9 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 10 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 11 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 12 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 13 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 14 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 15 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 16 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 17 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 18 internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises at least 19

internucleotidic linkages in the Sp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 8 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 7 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 6 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 5 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 4 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 3 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 2 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 1 internucleotidic linkages in the Rp configuration. In some embodiments, a common pattern of backbone chiral centers comprises no more than 8 internucleotidic linkages which are not chiral (as a non-limiting example, a phosphodiester). In some embodiments, a common pattern of backbone chiral centers comprises no more than 7 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 6 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 5 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 4 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 3 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 2 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises no more than 1 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 10 internucleotidic linkages in the Sp configuration, and no more than 8 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 11 internucleotidic linkages in the Sp configuration, and no more than 7 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 12 internucleotidic linkages in the Sp configuration, and

no more than 6 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 13 internucleotidic linkages in the Sp configuration, and no more than 6 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 14 internucleotidic linkages in the Sp configuration, and no more than 5 internucleotidic linkages which are not chiral. In some embodiments, a common pattern of backbone chiral centers comprises at least 15 internucleotidic linkages in the Sp configuration, and no more than 4 internucleotidic linkages which are not chiral. In some embodiments, the internucleotidic linkages in the Sp configuration are optionally contiguous or not contiguous. In some embodiments, the internucleotidic linkages in the Rp configuration are optionally contiguous or not contiguous. In some embodiments, the internucleotidic linkages which are not chiral are optionally contiguous or not contiguous.

In some embodiments, compound of the disclosure comprises a block is a stereochemistry block. In some embodiments, a block is an Rp block in that each internucleotidic linkage of the block is Rp. In some embodiments, a 5'-block is an Rp block. In some embodiments, a 3'-block is an Rp block. In some embodiments, a block is an Sp block in that each internucleotidic linkage of the block is Sp. In some embodiments, a 5'-block is an Sp block. In some embodiments, a 3'-block is an Sp block. In some embodiments, provided oligonucleotides comprise both Rp and Sp blocks. In some embodiments, provided oligonucleotides comprise one or more Rp but no Sp blocks. In some embodiments, provided oligonucleotides comprise one or more Sp but no Rp blocks. In some embodiments, provided oligonucleotides comprise one or more PO blocks wherein each internucleotidic linkage in a natural phosphate linkage.

In some embodiments, compound of the disclosure comprises a 5'-block is an Sp block wherein each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block is an Sp block wherein each of internucleotidic linkage is a modified internucleotidic linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block is an Sp block wherein each of internucleotidic linkage is a phosphorothioate linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 5'-block comprises 4 or more nucleoside units. In some embodiments, a 5'-block comprises 5 or more nucleoside units. In some embodiments, a 5'-block comprises 6 or more nucleoside units. In some embodiments, a 5'-block comprises 7 or more nucleoside units. In some embodiments, a 3'-block is an Sp block wherein each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block is an Sp block

wherein each of internucleotidic linkage is a modified internucleotidic linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block is an Sp block wherein each of internucleotidic linkage is a phosphorothioate linkage and each sugar moiety comprises a 2'-F modification. In some embodiments, a 3'-block comprises 4 or more nucleoside units. In some embodiments, a 3'-block comprises 5 or more nucleoside units. In some embodiments, a 3'-block comprises 6 or more nucleoside units. In some embodiments, a 3'-block comprises 7 or more nucleoside units.

In some embodiments, compound of the disclosure comprises a type of nucleoside in a region or an oligonucleotide is followed by a specific type of internucleotidic linkage, *e.g.*, natural phosphate linkage, modified internucleotidic linkage, Rp chiral internucleotidic linkage, Sp chiral internucleotidic linkage, etc. In some embodiments, A is followed by Sp. In some embodiments, A is followed by Rp. In some embodiments, A is followed by natural phosphate linkage (PO). In some embodiments, U is followed by Sp. In some embodiments, U is followed by Rp. In some embodiments, U is followed by natural phosphate linkage (PO). In some embodiments, C is followed by Sp. In some embodiments, C is followed by Rp. In some embodiments, C is followed by natural phosphate linkage (PO). In some embodiments, G is followed by Sp. In some embodiments, G is followed by Rp. In some embodiments, G is followed by natural phosphate linkage (PO). In some embodiments, C and U are followed by Sp. In some embodiments, C and U are followed by Rp. In some embodiments, C and U are followed by natural phosphate linkage (PO). In some embodiments, A and G are followed by Sp. In some embodiments, A and G are followed by Rp.

In some embodiments, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 21 and 22, and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the antisense comprises 3, 4 or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 1, 2, 3, 4 or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro

modifications; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, between nucleotide positions 2 and 3, between nucleotide positions 21 and 22, and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the sense strand is conjugated with a ligand and/or lipophilic moiety; (iii) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (iv) the sense strand comprises 1, 2, 3, 4 or 5 phosphorothioate internucleotide linkages; (v) the dsRNA comprises at least four 2'-fluoro modifications; (vi) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the sense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, and between nucleotide positions 2 and 3, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six, seven or all eight) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the antisense comprises 1, 2, 3, 4 or 5 phosphorothioate internucleotide linkages; (iii) the sense strand is conjugated with a ligand and/or lipophilic moiety; (iv) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (v) the sense strand comprises 3, 4 or 5 phosphorothioate internucleotide linkages; (vi) the dsRNA comprises at least four 2'-fluoro modifications; (vii) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (viii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the sense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, and between nucleotide positions 2 and 3, the antisense strand comprises phosphorothioate internucleotide linkages between nucleotide positions 1 and 2, between nucleotide positions 2 and 3, between nucleotide positions 21 and 22,

and between nucleotide positions 22 and 23, wherein the antisense strand contains at least one thermally destabilizing modification of the duplex located in the seed region of the antisense strand (*i.e.*, at position 2-9 of the 5'-end of the antisense strand), and wherein the dsRNA optionally further has at least one (*e.g.*, one, two, three, four, five, six or all seven) of the following characteristics: (i) the antisense comprises 2, 3, 4, 5 or 6 2'-fluoro modifications; (ii) the sense strand is conjugated with a ligand and/or lipophilic moiety; (iii) the sense strand comprises 2, 3, 4 or 5 2'-fluoro modifications; (iv) the sense strand comprises 3, 4 or 5 phosphorothioate internucleotide linkages; (v) the dsRNA comprises at least four 2'-fluoro modifications; (vi) the dsRNA comprises a duplex region of 12-40 nucleotide pairs in length; and (vii) the dsRNA has a blunt end at 5'-end of the antisense strand.

In some embodiments, the dsRNA molecule of the disclosure comprises mismatch(es) with the target, within the duplex, or combinations thereof. The mismatch can occur in the overhang region or the duplex region. The base pair can be ranked on the basis of their propensity to promote dissociation or melting (*e.g.*, on the free energy of association or dissociation of a particular pairing, the simplest approach is to examine the pairs on an individual pair basis, though next neighbor or similar analysis can also be used). In terms of promoting dissociation: A:U is preferred over G:C; G:U is preferred over G:C; and I:C is preferred over G:C (I=inosine). Mismatches, *e.g.*, non-canonical or other than canonical pairings (as described elsewhere herein) are preferred over canonical (A:T, A:U, G:C) pairings; and pairings which include a universal base are preferred over canonical pairings.

In some embodiments, the dsRNA molecule of the disclosure comprises at least one of the first 1, 2, 3, 4, or 5 base pairs within the duplex regions from the 5'- end of the antisense strand can be chosen independently from the group of: A:U, G:U, I:C, and mismatched pairs, *e.g.*, non-canonical or other than canonical pairings or pairings which include a universal base, to promote the dissociation of the antisense strand at the 5'-end of the duplex.

In some embodiments, the nucleotide at the 1 position within the duplex region from the 5'-end in the antisense strand is selected from the group consisting of A, dA, dU, U, and dT. Alternatively, at least one of the first 1, 2 or 3 base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair. For example, the first base pair within the duplex region from the 5'- end of the antisense strand is an AU base pair.

It was found that introducing 4'-modified or 5'-modified nucleotide to the 3'-end of a phosphodiester (PO), phosphorothioate (PS), or phosphorodithioate (PS2) linkage of a dinucleotide at any position of single stranded or double stranded oligonucleotide can exert steric effect to the internucleotide linkage and, hence, protecting or stabilizing it against nucleases.

In some embodiments, 5'-modified nucleoside is introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. For instance, a 5'-alkylated nucleoside may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The alkyl group at the 5' position of the ribose sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 5'-alkylated nucleoside is 5'-methyl nucleoside. The 5'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-modified nucleoside is introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. For instance, a 4'-alkylated nucleoside may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The alkyl group at the 4' position of the ribose sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 4'-alkylated nucleoside is 4'-methyl nucleoside. The 4'-methyl can be either racemic or chirally pure *R* or *S* isomer. Alternatively, a 4'-*O*-alkylated nucleoside may be introduced at the 3'-end of a dinucleotide at any position of single stranded or double stranded siRNA. The 4'-*O*-alkyl of the ribose sugar can be racemic or chirally pure *R* or *S* isomer. An exemplary 4'-*O*-alkylated nucleoside is 4'-*O*-methyl nucleoside. The 4'-*O*-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 5'-alkylated nucleoside is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 5'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 5'-alkylated nucleoside is 5'-methyl nucleoside. The 5'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-alkylated nucleoside is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 4'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 4'-alkylated nucleoside is 4'-methyl nucleoside. The 4'-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, 4'-*O*-alkylated nucleoside is introduced at any position on the sense strand or antisense strand of a dsRNA, and such modification maintains or improves potency of the dsRNA. The 5'-alkyl can be either racemic or chirally pure *R* or *S* isomer. An exemplary 4'-*O*-alkylated nucleoside is 4'-*O*-methyl nucleoside. The 4'-*O*-methyl can be either racemic or chirally pure *R* or *S* isomer.

In some embodiments, the dsRNA molecule of the disclosure can comprise 2'-5' linkages (with 2'-H, 2'-OH and 2'-OMe and with P=O or P=S). For example, the 2'-5' linkages modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

In another embodiment, the dsRNA molecule of the disclosure can comprise L sugars (*e.g.*, L ribose, L-arabinose with 2'-H, 2'-OH and 2'-OMe). For example, these L sugars modifications can be used to promote nuclease resistance or to inhibit binding of the sense to the antisense strand, or can be used at the 5' end of the sense strand to avoid sense strand activation by RISC.

Various publications describe multimeric siRNA which can all be used with the dsRNA of the disclosure. Such publications include WO2007/091269, US 7858769, WO2010/141511, WO2007/117686, WO2009/014887, and WO2011/031520 which are hereby incorporated by their entirely.

A ligand and/or lipophilic moiety may be attached to the polynucleotide via a carrier. Exemplary carriers include (i) at least one "backbone attachment point," optionally two "backbone attachment points" and (ii) at least one "tethering attachment point." A "backbone attachment point" as used herein refers to a functional group, *e.g.* a hydroxyl group, or generally, a bond available for, and that is suitable for incorporation of the carrier into the backbone, *e.g.*, the phosphate, or modified phosphate, *e.g.*, sulfur containing, backbone, of a ribonucleic acid. A "tethering attachment point" (TAP) in some embodiments refers to a constituent ring atom of the cyclic carrier, *e.g.*, a carbon atom or a heteroatom (distinct from an atom which provides a backbone attachment point), that connects a selected moiety. The moiety can be, *e.g.*, a liphophilic alkyl group, optionally a C16 lipophilic moiety. Optionally, the selected moiety is connected by an intervening tether to the cyclic carrier. Thus, the cyclic carrier can include a functional group, *e.g.*, an amino group, or generally, provide a bond, that is suitable for incorporation or tethering of another chemical entity, *e.g.*, a ligand and/or lipophilic moiety to the constituent ring.

The RNAi agents may be conjugated to a ligand and/or lipophilic moiety *via* a carrier, wherein the carrier can be a cyclic group or an acyclic group. Optionally, the cyclic group is selected from pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolane, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuryl and decalin. Optionally, the acyclic group is selected from serinol backbone and diethanolamine backbone.

In certain specific embodiments, the RNAi agent for use in the methods of the disclosure is an agent selected from the group of agents listed in **Tables 2 or 3**. These agents may further comprise a ligand, such as one or more lipophilic moieties.

III. iRNAs Conjugated to Ligands

Another modification of the RNA of an iRNA of the disclosure involves chemically linking to the iRNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution or cellular uptake of the iRNA, *e.g.*, into a cell. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acid. Sci. USA*, 1989, 86: 6553-6556), cholic acid (Manoharan *et al.*, *Biorg. Med. Chem. Let.*, 1994, 4: 1053-1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, 1992, 660: 306-309; Manoharan *et al.*, *Biorg. Med. Chem. Let.*, 1993, 3: 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.*, 1992, 20: 533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, *EMBO J*, 1991, 10: 1111-1118; Kabanov *et al.*, *FEBS Lett.*, 1990, 259: 327-330; Svinarchuk *et al.*, *Biochimie*, 1993, 75: 49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36: 3651-3654; Shea *et al.*, *Nucl. Acids Res.*, 1990, 18: 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, *Nucleosides & Nucleotides*, 1995, 14: 969-973), or adamantane acetic acid (Manoharan *et al.*, *Tetrahedron Lett.*, 1995, 36: 3651-3654), a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta*, 1995, 1264: 229-237), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, 1996, 277: 923-937).

In certain embodiments, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In some embodiments, a ligand provides an enhanced affinity

for a selected target, *e.g.*, molecule, cell or cell type, compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Typical ligands will not take part in duplex pairing in a duplexed nucleic acid.

Ligands can include a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an α-helical peptide.

Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic. In certain embodiments, the ligand is a multivalent galactose, *e.g.*, an N-acetyl-galactosamine.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-

(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by disrupting the cell's microtubules, microfilaments, or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In some embodiments, a ligand attached to an iRNA as described herein acts as a pharmacokinetic modulator (PK modulator). PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins *etc*. Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin *etc*. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, *e.g.*, oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbone are also amenable to the present disclosure as ligands (*e.g.* as PK modulating ligands). In addition, aptamers that bind serum components (*e.g.* serum proteins) are also suitable for use as PK modulating ligands in the embodiments described herein.

Ligand-conjugated iRNAs of the disclosure may be synthesized by the use of an oligonucleotide that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the oligonucleotide (described below). This reactive oligonucleotide may be reacted directly with commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto.

The oligonucleotides used in the conjugates of the present disclosure may be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems® (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates and alkylated derivatives.

In the ligand-conjugated oligonucleotides and ligand-molecule bearing sequence-specific linked nucleosides of the present disclosure, the oligonucleotides and oligonucleosides may be assembled on a suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

When using nucleotide-conjugate precursors that already bear a linking moiety, the synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. In some embodiments, the oligonucleotides or linked nucleosides of the present disclosure are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to the standard phosphoramidites and non-standard phosphoramidites that are commercially available and routinely used in oligonucleotide synthesis.

A. Lipid Conjugates

In certain embodiments, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule can typically bind a serum protein, such as human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, a non-kidney target tissue of the body. For example, the target tissue can be tissues of the CNS, e.g., brain tissue. Other molecules that can bind HSA can also be used as ligands. For example,

naproxen or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

A lipid-based ligand can be used to modulate, *e.g.*, control (*e.g.*, inhibit) the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In certain embodiments, the lipid-based ligand binds HSA. For example, the ligand can bind HSA with a sufficient affinity such that distribution of the conjugate to a non-kidney tissue is enhanced. However, the affinity is typically not so strong that the HSA-ligand binding cannot be reversed.

In certain embodiments, the lipid-based ligand binds HSA weakly or not at all, such that distribution of the conjugate to the kidney is enhanced. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid-based ligand.

In another aspect, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a target cell, *e.g.*, a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, *e.g.*, of the malignant or non-malignant type, *e.g.*, cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

B. Cell Permeation Agents

In another aspect, the ligand is a cell-permeation agent, such as a helical cell-permeation agent. In certain embodiments, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is typically an α-helical agent and can have a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular

recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp, or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO: 9). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO: 10)) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO: 11)) and the *Drosophila Antennapedia* protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 12)) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, *Nature*, 354: 82-84, 1991). Typically, the peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide for use in the compositions and methods of the disclosure may be linear or cyclic, and may be modified, *e.g.*, glycosylated or methylated, to facilitate targeting to a specific tissue(s). RGD-containing peptides and peptidiomimemtics may include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Preferred conjugates of this ligand target PECAM-1 or VEGF.

An RGD peptide moiety can be used to target a particular cell type, *e.g.*, a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, *Cancer Res.*, 62: 5139-43, 2002). An RGD peptide can facilitate targeting of an dsRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, *Cancer Gene Therapy* 8: 783-787, 2001). Typically, the RGD peptide will facilitate targeting of an iRNA agent to the

kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.*, glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver an iRNA agent to a tumor cell expressing $\alpha v\beta_3$ (Haubner *et al.*, *Jour. Nucl. Med.*, 42: 326-336, 2001).

A "cell permeation peptide" is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, *Nucl. Acids Res.* 31: 2717-2724, 2003).

C. Linkers

In some embodiments, the conjugate or ligand described herein can be attached to an iRNA oligonucleotide with various linkers that can be cleavable or non-cleavable.

The term "linker" or "linking group" means an organic moiety that connects two parts of a compound, *e.g.*, covalently attaches two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR8, C(O), C(O)NH, SO, SO_2, SO_2NH or a chain of atoms, such as, but not limited to, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl,

alkenylheteroaryl, alkynylhereroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, N(R8), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R8 is hydrogen, acyl, aliphatic or substituted aliphatic. In certain embodiments, the linker is of a length of about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18 atoms, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least about 10 times, 20, times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times or more, or at least about 100 times faster in a target cell or under a first reference condition (which can, *e.g.*, be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, *e.g.*, be selected to mimic or represent conditions found in the blood or serum).

Cleavable linking groups are susceptible to cleavage agents, *e.g.*, pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, *e.g.*, oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, *e.g.*, those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing a cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the cell to be targeted.

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood, cerebrospinal fluid (CSF), or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, *e.g.*, blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It can be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to CSF, blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

i. Redox cleavable linking groups

In certain embodiments, a cleavable linking group is a redox cleavable linking group that is cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, *e.g.*, a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In one, candidate compounds are cleaved by at most about 10% in the blood. In other embodiments, useful candidate compounds are degraded at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

ii. Phosphate-based cleavable linking groups

In certain embodiments, a cleavable linker comprises a phosphate-based cleavable linking group. A phosphate-based cleavable linking group is cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

iii. Acid cleavable linking groups

In certain embodiments, a cleavable linker comprises an acid cleavable linking group. An *a*cid cleavable linking group is a linking group that is cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (*e.g.*, about 6.0, 5.75, 5.5, 5.25, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

iv. Ester-based cleavable linking groups

In certain embodiments, a cleavable linker comprises an ester-based cleavable linking group. An ester-based cleavable linking group is cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

v. Peptide-based cleavable linking groups

In yet another embodiment, a cleavable linker comprises a peptide-based cleavable linking group. A peptide-based cleavable linking group is cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (*e.g.*, dipeptides, tripeptides *etc.*) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide-based cleavage group is generally limited to the peptide bond (*i.e.*, the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula –NHCHR$_A$C(O)NHCHR$_B$C(O)-, where R$_A$ and R$_B$ are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

"Chimeric" iRNA compounds or "chimeras," in the context of this disclosure, are iRNA compounds, optionally dsRNA agents, that contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of a dsRNA compound. These iRNAs typically contain at least one region wherein the RNA is modified so as to confer upon the iRNA increased resistance to nuclease degradation, increased cellular uptake, or increased binding affinity for the target nucleic acid. An additional region of the iRNA can serve as a substrate for enzymes capable of cleaving RNA: DNA or RNA: RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA: DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of iRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter iRNAs when chimeric dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the RNA of an iRNA can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to iRNAs in order to enhance the activity, cellular distribution or cellular uptake of the iRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid

moieties, such as cholesterol (Kubo, T. *et al., Biochem. Biophys. Res. Comm.*, 2007, 365(1): 54-61; Letsinger *et al., Proc. Natl. Acad. Sci. USA*, 1989, 86: 6553), cholic acid (Manoharan *et al., Bioorg. Med. Chem. Lett.*, 1994, 4: 1053), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al., Ann. N.Y. Acad. Sci.*, 1992, 660: 306; Manoharan *et al., Bioorg. Med. Chem. Let.*, 1993, 3: 2765), a thiocholesterol (Oberhauser *et al., Nucl. Acids Res.*, 1992, 20: 533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al., EMBO J.*, 1991, 10: 111; Kabanov *et al., FEBS Lett.*, 1990, 259: 327; Svinarchuk *et al., Biochimie*, 1993, 75: 49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al., Tetrahedron Lett.*, 1995, 36: 3651; Shea *et al., Nucl. Acids Res.*, 1990, 18: 3777), a polyamine or a polyethylene glycol chain (Manoharan *et al., Nucleosides & Nucleotides*, 1995, 14: 969), or adamantane acetic acid (Manoharan *et al., Tetrahedron Lett.*, 1995, 36: 3651), a palmityl moiety (Mishra *et al., Biochim. Biophys. Acta*, 1995, 1264: 229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al., J. Pharmacol. Exp. Ther.*, 1996, 277: 923). Representative United States patents that teach the preparation of such RNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of RNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction can be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

IV. *In Vivo* Testing of SNCA Knockdown

A wide variety of α-synuclein PD animal models are available (Gómez-Benito *et al. Front Pharmacol.* 11: 356). A number of rodent models of PD rely upon intracerebral or systemic administration of either α-synuclein pre-formed fibrils (PFFs) or brain extracts containing Lewy bodies and α-synuclein derived from PD patients or transgenic mice exhibiting α-synuclein pathology. More relevant to assessment of SNCA RNAi agents, genetic models of PD have also been made. Recombinant adeno-associated virus vectors (rAAV) overexpressing the SNCA gene have been used to model PD: overexpression of wild type α-synuclein or PD-associated mutants (A53T or A30P α-synuclein) utilizing rAAV has been described as leading to a progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), a loss of dopamine terminals

in the striatum (Koprich *et al. Mol Neurodegener*. 5: 43; Koprich *et al. PLoS One*. 6: e17698; Oliveras-Salvá *et al. Mol Neurodegener*. 8: 44; Bourdenx *et al. Acta Neuropathol Commun*. 3: 46; Caudal *et al. Exp Neurol*. 273: 243-52; Lu *et al. Biochem Biophys Res Commun*. 464: 988-993; Ip *et al. Biochem Biophys Res Commun*. 464: 988-993), and a reduction of striatal dopamine content (Koprich *et al. PLoS One*. 6: e17698; Ip *et al.*). However, the extent of neurodegeneration achieved with the rAAV model has been variable among the different studies. Several serotypes, promoters, α-synuclein species, doses, and time-course after injection have been tested, and all these factors influence the parkinsonian phenotype achieved.

Several transgenic mice lines expressing E46K α-synuclein have also been generated (Emmer *et al. J Biol Chem*. 286: 35104-18; Nuber *et al. Neuron*. 100: 75-90.e5), while E46K human α-synuclein has been overexpressed using viral vectors in mice. In the rAAV-α-synuclein model, the presence of pα-synuclein inclusions in the nigrostriatal system is concomitant with a significant loss of nigral dopaminergic neurons and the reduction in tyrosine hydroxylase immunoreactivity in the striatum. Overexpression of wild type or A53T human α-synuclein induces a progressive loss of dopaminergic neurons in the SN over time (Oliveras-Salvá *et al. Mol Neurodegener*. 8: 44).

Some studies have shown that rAAV-α-synuclein expression causes the development of motor alterations, such as an increased apomorphine or amphetamine-induced rotation, defects in the stepping test or increased forepaw asymmetry in the cylinder test (Kirik *et al. J Neurosci*. 22: 2780-91; Decressac *et al. Brain*. 134(Pt 8): 2302-11; Koprich *et al. PLoS One*. 6: e17698; Decressac *et al. Neurobiol Dis*. 45: 939-53; Gaugler *et al. Acta Neuropathol*. 123: 653-69; Gombash *et al. PLoS One*. 8: e81426; Oliveras-Salvá *et al. Mol Neurodegener*. 8: 44; Bourdenx *et al. Acta Neuropathol Commun*. 3: 46; Caudal *et al. Exp Neurol*. 273: 243-52; Ip *et al. Biochem Biophys Res Commun*. 464: 988-993). These motor deficits appear several weeks after injection in animals with a significant loss of dopaminergic neurons.

Such models have been used to develop and evaluate potential therapies aimed at reducing the aggregation of α-synuclein and preventing against neurodegeneration induced by α-synuclein (Decressac *et al. Proc Natl Acad Sci U S A*. 110: E1817-26; Xilouri *et al. Autophagy*. 9: 2166-8; Rocha *et al. Neurobiol Dis*. 82: 495-503), and can further be used to demonstrate the *in vivo* efficacy of the RNAi agents provided herein. Such models may contain constitutive or inducible expression, *e.g.*, overexpression, of, for example, human or rat SNCA, in some instances

comprising a pathogenic mutation. Examples of overexpression models used herein include AAV induced expression of the full-length *Homo sapiens* SNCA transcript Hs00240906_m1 and 3' UTR, and AAV induced expression of the full-length *Rattus norvegicus* SNCA transcript NM_019169.2 and 3' UTR.

V. Delivery of an RNAi Agent of the Disclosure

The delivery of an RNAi agent of the disclosure to a cell *e.g.*, a cell within a subject, such as a human subject (*e.g.*, a subject in need thereof, such as a subject having a SNCA-associated disorder, *e.g.*, PD, multiple system atrophy, Lewy body dementia (LBD), etc., can be achieved in a number of different ways. For example, delivery may be performed by contacting a cell with an RNAi agent of the disclosure either *in vitro* or *in vivo*. *In vivo* delivery may also be performed directly by administering a composition comprising an RNAi agent, *e.g.*, a dsRNA, to a subject. Alternatively, *in vivo* delivery may be performed indirectly by administering one or more vectors that encode and direct the expression of the RNAi agent. These alternatives are discussed further below.

In general, any method of delivering a nucleic acid molecule (*in vitro* or *in vivo*) can be adapted for use with an RNAi agent of the disclosure (see *e.g.*, Akhtar S. and Julian RL., (1992) *Trends Cell. Biol.* 2(5): 139-144 and WO94/02595, which are incorporated herein by reference in their entireties). For *in vivo* delivery, factors to consider for delivering an RNAi agent include, for example, biological stability of the delivered agent, prevention of non-specific effects, and accumulation of the delivered agent in the target tissue. The non-specific effects of an RNAi agent can be minimized by local administration, for example, by direct injection or implantation into a tissue or topically administering the preparation. Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that can otherwise be harmed by the agent or that can degrade the agent, and permits a lower total dose of the RNAi agent to be administered. Several studies have shown successful knockdown of gene products when an RNAi agent is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ. *et al.*, (2004) *Retina* 24: 132-138) and subretinal injections in mice (Reich, SJ. *et al.* (2003) *Mol. Vis.* 9: 210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a dsRNA in mice reduces tumor

volume (Pille, J. *et al.* (2005) *Mol. Ther.* 11: 267-274) and can prolong survival of tumor-bearing mice (Kim, WJ. *et al.*, (2006) *Mol. Ther.* 14: 343-350; Li, S. *et al.*, (2007) *Mol. Ther.* 15: 515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G. *et al.*, (2004) *Nucleic Acids* 32: e49; Tan, PH. *et al.* (2005) *Gene Ther.* 12: 59-66; Makimura, H. *et a.l* (2002) *BMC Neurosci.* 3: 18; Shishkina, GT., *et al.* (2004) *Neuroscience* 129: 521-528; Thakker, ER., *et al.* (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101: 17270-17275; Akaneya,Y., *et al.* (2005) *J. Neurophysiol.* 93: 594-602) and to the lungs by intranasal administration (Howard, KA. *et al.*, (2006) *Mol. Ther.* 14: 476-484; Zhang, X. *et al.*, (2004) *J. Biol. Chem.* 279: 10677-10684; Bitko, V. *et al.*, (2005) *Nat. Med.* 11: 50-55). For administering an RNAi agent systemically and/or intrathecally for the treatment of a disease, the RNA can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the dsRNA by endo- and exo-nucleases *in vivo*. Modification of the RNA or the pharmaceutical carrier can also permit targeting of the RNAi agent to the target tissue and avoid undesirable off-target effects (*e.g.*, without wishing to be bound by theory, use of GNAs as described herein has been identified to destabilize the seed region of a dsRNA, resulting in enhanced preference of such dsRNAs for on-target effectiveness, relative to off-target effects, as such off-target effects are significantly weakened by such seed region destabilization). RNAi agents can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, an RNAi agent directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and jejunum (Soutschek, J. *et al.*, (2004) *Nature* 432: 173-178). Conjugation of an RNAi agent to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO. *et al.*, (2006) *Nat. Biotechnol.* 24: 1005-1015). In an alternative embodiment, the RNAi agent can be delivered using drug delivery systems such as a nanoparticle, a dendrimer, a polymer, liposomes, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of molecule RNAi agent (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an RNAi agent by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an RNAi agent, or induced to form a vesicle or micelle (see *e.g.*, Kim SH. *et al.*, (2008) *Journal of Controlled Release* 129(2): 107-116) that encases an RNAi agent. The formation of vesicles or micelles further prevents degradation of the RNAi agent when

administered systemically. Methods for making and administering cationic- RNAi agent complexes are well within the abilities of one skilled in the art (see *e.g.*, Sorensen, DR., *et al.* (2003) *J. Mol. Biol* 327: 761-766; Verma, UN. *et al.*, (2003) *Clin. Cancer Res.* 9: 1291-1300; Arnold, AS *et al.* (2007) *J. Hypertens.* 25: 197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of RNAi agents include DOTAP (Sorensen, DR., et al (2003), supra; Verma, UN. *et al.*, (2003), supra), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS. *et al.*, (2006) *Nature* 441: 111-114), cardiolipin (Chien, PY. *et al.*, (2005) *Cancer Gene Ther.* 12: 321-328; Pal, A. *et al.*, (2005) *Int J. Oncol.* 26: 1087-1091), polyethyleneimine (Bonnet ME. *et al.*, (2008) *Pharm. Res.* Aug 16 Epub ahead of print; Aigner, A. (2006) *J. Biomed. Biotechnol.* 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) *Mol. Pharm.* 3: 472-487), and polyamidoamines (Tomalia, DA. *et al.*, (2007) *Biochem. Soc. Trans.* 35: 61-67; Yoo, H. *et al.*, (1999) *Pharm. Res.* 16: 1799-1804). In some embodiments, an RNAi agent forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of RNAi agents and cyclodextrins can be found in U.S. Patent No. 7,427,605, which is herein incorporated by reference in its entirety.

Certain aspects of the instant disclosure relate to a method of reducing the expression of a SNCA target gene in a cell, comprising contacting said cell with the double-stranded RNAi agent of the disclosure. In one embodiment, the cell is a CNS cell.

Another aspect of the disclosure relates to a method of reducing the expression of a SNCA target gene in a subject, comprising administering to the subject the double-stranded RNAi agent of the disclosure.

Another aspect of the disclosure relates to a method of treating a subject having a SNCA-associated disorder, comprising administering to the subject a therapeutically effective amount of the double-stranded RNAi agent of the disclosure, thereby treating the subject. Exemplary CNS disorders that can be treated by the method of the disclosure include synucleinopathies, such as PD, multiple system atrophy, Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal

degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

In one embodiment, the double-stranded RNAi agent is administered intrathecally. By intrathecal administration of the double-stranded RNAi agent, the method can reduce the expression of a SNCA target gene in a brain (*e.g.,* striatum) or spine tissue, for instance, cortex, cerebellum, cervical spine, lumbar spine, and thoracic spine.

For ease of exposition the formulations, compositions and methods in this section are discussed largely with regard to modified siRNA compounds. It may be understood, however, that these formulations, compositions and methods can be practiced with other siRNA compounds, *e.g.*, unmodified siRNA compounds, and such practice is within the disclosure. A composition that includes an RNAi agent can be delivered to a subject by a variety of routes. Exemplary routes include: intrathecal, intravenous, topical, rectal, anal, vaginal, nasal, pulmonary, and ocular.

The RNAi agents of the disclosure can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically include one or more species of RNAi agent and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The pharmaceutical compositions of the present disclosure may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, vaginal, rectal, intranasal, transdermal), oral, or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, or intrathecal or intraventricular administration.

The route and site of administration may be chosen to enhance targeting. For example, to target brain and other CNS cells, intrathecal injection would be a logical choice.

Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids, and powders. Conventional

pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions for oral administration include powders or granules, suspensions or solutions in water, syrups, elixirs or non-aqueous media, tablets, capsules, lozenges, or troches. In the case of tablets, carriers that can be used include lactose, sodium citrate and salts of phosphoric acid. Various disintegrants such as starch, and lubricating agents such as magnesium stearate, sodium lauryl sulfate and talc, are commonly used in tablets. For oral administration in capsule form, useful diluents are lactose and high molecular weight polyethylene glycols. When aqueous suspensions are required for oral use, the nucleic acid compositions can be combined with emulsifying and suspending agents. If desired, certain sweetening or flavoring agents can be added.

Compositions for intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents, and other suitable additives.

Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents, and other suitable additives. Intrathecal injection may be facilitated by an intrathecal catheter, for example, attached to a reservoir. For intrathecal use, the total concentration of solutes may be controlled to render the preparation isotonic.

In one embodiment, the administration of the siRNA compound, *e.g.*, a double-stranded siRNA compound, or ssiRNA compound, composition is parenteral, *e.g.*, intrathecal, intravenous (*e.g.*, as a bolus or as a diffusible infusion), intradermal, intraperitoneal, intramuscular, intraventricular, intracranial, subcutaneous, transmucosal, buccal, sublingual, endoscopic, rectal, oral, vaginal, topical, pulmonary, intranasal, urethral, or ocular. Administration can be provided by the subject or by another person, *e.g.*, a health care provider. The medication can be provided in measured doses or in a dispenser which delivers a metered dose. Selected modes of delivery are discussed in more detail below.

Intrathecal Administration

In certain embodiments, the double-stranded RNAi agent is delivered by intrathecal injection (*i.e.*, injection into the spinal fluid which bathes the brain and spinal cord tissue). Intrathecal injection of RNAi agents into the spinal fluid can be performed as a bolus injection or via minipumps which can be implanted beneath the skin, providing a regular and constant delivery of siRNA into the spinal fluid. The circulation of the spinal fluid from the choroid plexus, where

it is produced, down around the spinal cord and dorsal root ganglia and subsequently up past the cerebellum and over the cortex to the arachnoid granulations, where the fluid can exit the CNS, that, depending upon size, stability, and solubility of the compounds injected, molecules delivered intrathecally could hit targets throughout the entire CNS.

In some embodiments, the intrathecal administration is via a pump. The pump may be a surgically implanted osmotic pump. In one embodiment, the osmotic pump is implanted into the subarachnoid space of the spinal canal to facilitate intrathecal administration.

In some embodiments, the intrathecal administration is via an intrathecal delivery system for a pharmaceutical including a reservoir containing a volume of the pharmaceutical agent, and a pump configured to deliver a portion of the pharmaceutical agent contained in the reservoir. More details about this intrathecal delivery system may be found in WO 2015/116658, which is incorporated by reference in its entirety.

The amount of intrathecally injected RNAi agents may vary from one target gene to another target gene and the appropriate amount that has to be applied may have to be determined individually for each target gene. Typically, this amount ranges from 10 μg to 2 mg, optionally 50 μg to 1500 μg, more optionally 100 μg to 1000 μg.

Vector-encoded RNAi agents of the Disclosure

RNAi agents targeting the SNCA gene can be expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al.*, *TIG.* (1996), 12: 5-10; WO 00/22113, WO 00/22114, and US 6,054,299). Expression is optionally sustained (months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, (1995) *Proc. Natl. Acad. Sci. USA* 92: 1292).

The individual strand or strands of an RNAi agent can be transcribed from a promoter on an expression vector. Where two separate strands are to be expressed to generate, for example, a dsRNA, two separate expression vectors can be co-introduced (*e.g.*, by transfection or infection) into a target cell. Alternatively, each individual strand of a dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, a dsRNA is expressed as inverted repeat polynucleotides joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

RNAi agent expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, optionally those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of an RNAi agent as described herein. Delivery of RNAi agent expressing vectors can be to the CNS, such as by intrathecal administration, systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, *etc.*; (c) adeno-associated virus (AAV) vectors; (d) herpes simplex virus vectors; (e) SV 40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, *e.g.*, vaccinia virus vectors or avipox, *e.g.* canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. Replication-defective viruses can also be advantageous. Different vectors will or will not become incorporated into the cells' genome. The constructs can include viral sequences for transfection, if desired. Alternatively, the construct can be incorporated into vectors capable of episomal replication, *e.g.* EPV and EBV vectors. Constructs for the recombinant expression of an RNAi agent will generally require regulatory elements, *e.g.*, promoters, enhancers, *etc.*, to ensure the expression of the RNAi agent in target cells. Other aspects to consider for vectors and constructs are known in the art.

VI. Pharmaceutical Compositions of the Invention

The present disclosure also includes pharmaceutical compositions and formulations which include the RNAi agents of the disclosure. In one embodiment, provided herein are pharmaceutical compositions containing an RNAi agent, as described herein, and a pharmaceutically acceptable carrier. The pharmaceutical compositions containing the RNAi agent are useful for treating a disease or disorder associated with the expression or activity of SNCA, *e.g.*, a SNCA-associated neurodegenerative disease, such as a synucleinopathy, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma,

meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for direct delivery into the CNS, *e.g.*, by intrathecal or intravitreal routes of injection, optionally by infusion into the brain (*e.g.,* striatum), such as by continuous pump infusion.

In some embodiments, the pharmaceutical compositions of the disclosure are pyrogen free or non-pyrogenic.

The pharmaceutical compositions of the disclosure may be administered in dosages sufficient to inhibit expression of a SNCA gene. In general, a suitable dose of an RNAi agent of the disclosure will be in the range of about 0.001 to about 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of about 1 to 50 mg per kilogram body weight per day.

A repeat-dose regimen may include administration of a therapeutic amount of an RNAi agent on a regular basis, such as monthly to once every six months. In certain embodiments, the RNAi agent is administered about once per quarter (*i.e.*, about once every three months) to about twice per year.

After an initial treatment regimen (*e.g.*, loading dose), the treatments can be administered on a less frequent basis.

In other embodiments, a single dose of the pharmaceutical compositions can be long lasting, such that subsequent doses are administered at not more than 1, 2, 3, or 4 or more month intervals. In some embodiments of the disclosure, a single dose of the pharmaceutical compositions of the disclosure is administered once per month. In other embodiments of the disclosure, a single dose of the pharmaceutical compositions of the disclosure is administered once per quarter to twice per year.

The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health or age of the subject, and other diseases present.

Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments.

Advances in mouse genetics have generated mouse models for the study of SNCA-associated diseases that would benefit from reduction in the expression of SNCA. Such models can be used for *in vivo* testing of RNAi agents, as well as for determining a therapeutically effective dose. Suitable mouse models are known in the art and include, for example, the mouse models described elsewhere herein.

The pharmaceutical compositions of the present disclosure can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. In certain embodiments, administration is intrathecal, However, in certain embodiments, administration can be topical (*e.g.*, by a transdermal patch), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; subdermal, *e.g.*, via an implanted device; or intracranial, *e.g.*, by intraparenchymal, intrathecal or intraventricular, administration.

The RNAi agents can be delivered in a manner to target a particular tissue, such as the CNS (*e.g.*, neuronal, glial or vascular tissue of the brain).

Pharmaceutical compositions and formulations for topical administration can include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like can be necessary or desirable. Coated condoms, gloves and the like can also be useful. Suitable topical formulations include those in which the RNAi agents featured in the disclosure are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (*e.g.*, dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (*e.g.*, dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.*, dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). RNAi agents featured in the disclosure can be encapsulated within liposomes or can form complexes thereto, in particular to cationic liposomes. Alternatively, RNAi agents can be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are

not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-20} alkyl ester (*e.g.*, isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in US 6,747,014, which is incorporated herein by reference.

A. RNAi Agent Formulations Comprising Membranous Molecular Assemblies

An RNAi agent for use in the compositions and methods of the disclosure can be formulated for delivery in a membranous molecular assembly, *e.g.*, a liposome or a micelle. As used herein, the term "liposome" refers to a vesicle composed of amphiphilic lipids arranged in at least one bilayer, *e.g.*, one bilayer or a plurality of bilayers. Liposomes include unilamellar and multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the RNAi agent composition. The lipophilic material isolates the aqueous interior from an aqueous exterior, which typically does not include the RNAi agent composition, although in some examples, it may. Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomal bilayer fuses with bilayer of the cellular membranes. As the merging of the liposome and cell progresses, the internal aqueous contents that include the RNAi agent are delivered into the cell where the RNAi agent can specifically bind to a target RNA and can mediate RNAi. In some cases, the liposomes are also specifically targeted, *e.g.*, to direct the RNAi agent to particular cell types.

A liposome containing an RNAi agent can be prepared by a variety of methods. In one example, the lipid component of a liposome is dissolved in a detergent so that micelles are formed with the lipid component. For example, the lipid component can be an amphipathic cationic lipid or lipid conjugate. The detergent can have a high critical micelle concentration and may be nonionic. Exemplary detergents include cholate, CHAPS, octylglucoside, deoxycholate, and lauroyl sarcosine. The RNAi agent preparation is then added to the micelles that include the lipid component. The cationic groups on the lipid interact with the RNAi agent and condense around the RNAi agent to form a liposome. After condensation, the detergent is removed, *e.g.*, by dialysis, to yield a liposomal preparation of RNAi agent.

If necessary, a carrier compound that assists in condensation can be added during the condensation reaction, *e.g.*, by controlled addition. For example, the carrier compound can be a polymer other than a nucleic acid (*e.g.*, spermine or spermidine). pH can also be adjusted to favor condensation.

Methods for producing stable polynucleotide delivery vehicles, which incorporate a polynucleotide/cationic lipid complex as structural components of the delivery vehicle, are further described in, *e.g.*, WO 96/37194, the entire contents of which are incorporated herein by reference. Liposome formation can also include one or more aspects of exemplary methods described in Felgner, P. L. *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 8: 7413-7417; United States Patent No. 4,897,355; United States Patent No. 5,171,678; Bangham *et al.*, (1965) *M. Mol. Biol.* 23: 238; Olson *et al.*, (1979) *Biochim. Biophys. Acta* 557: 9; Szoka *et al.*, (1978) *Proc. Natl. Acad. Sci.* 75: 4194; Mayhew *et al.*, (1984) *Biochim. Biophys. Acta* 775: 169; Kim *et al.*, (1983) *Biochim. Biophys. Acta* 728: 339; and Fukunaga *et al.*, (1984) *Endocrinol.* 115: 757. Commonly used techniques for preparing lipid aggregates of appropriate size for use as delivery vehicles include sonication and freeze-thaw plus extrusion (see, *e.g.*, Mayer *et al.*, (1986) *Biochim. Biophys. Acta* 858: 161. Microfluidization can be used when consistently small (50 to 200 nm) and relatively uniform aggregates are desired (Mayhew *et al.*, (1984) *Biochim. Biophys. Acta* 775: 169. These methods are readily adapted to packaging RNAi agent preparations into liposomes.

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged nucleic acid molecules to form a stable complex. The positively charged nucleic acid/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.* (1987) *Biochem. Biophys. Res. Commun.*, 147: 980-985).

Liposomes, which are pH-sensitive or negatively charged, entrap nucleic acids rather than complex with them. Since both the nucleic acid and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some nucleic acid is entrapped within the aqueous interior of these liposomes. pH sensitive liposomes have been used to deliver nucleic acids encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.* (1992) *Journal of Controlled Release*, 19: 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid or phosphatidylcholine or cholesterol.

Examples of other methods to introduce liposomes into cells *in vitro* and *in vivo* include United States Patent No. 5,283,185; United States Patent No. 5,171,678; WO 94/00569; WO 93/24640; WO 91/16024; Felgner, (1994) *J. Biol. Chem.* 269: 2550; Nabel, (1993) *Proc. Natl. Acad. Sci.* 90: 11307; Nabel, (1992) *Human Gene Ther.* 3: 649; Gershon, (1993) *Biochem.* 32: 7143; and Strauss, (1992) *EMBO J.* 11: 417.

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu *et al.*, (1994) *S.T.P.Pharma. Sci.*, 4(6): 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives

from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, (1987) *FEBS Letters,* 223: 42; Wu *et al.*, (1993) *Cancer Research,* 53: 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (*Ann. N.Y. Acad. Sci.*, (1987), 507: 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (*Proc. Natl. Acad. Sci. U.S.A.*, (1988), 85: 6949). United States Patent No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. United States Patent No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al*).

In one embodiment, cationic liposomes are used. Cationic liposomes possess the advantage of being able to fuse to the cell membrane. Non-cationic liposomes, although not able to fuse as efficiently with the plasma membrane, are taken up by macrophages in vivo and can be used to deliver RNAi agents to macrophages.

Further advantages of liposomes include: liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated RNAi agents in their internal compartments from metabolism and degradation (Rosoff, in "Pharmaceutical Dosage Forms," Lieberman, Rieger and Banker (Eds.), 1988, volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

A positively charged synthetic cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) can be used to form small liposomes that interact spontaneously with nucleic acid to form lipid-nucleic acid complexes which are capable of fusing with the negatively charged lipids of the cell membranes of tissue culture cells, resulting in delivery of RNAi agent (see, *e.g.*, Felgner, P. L. *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 8: 7413-7417, and United States Patent No.4,897,355 for a description of DOTMA and its use with DNA).

A DOTMA analogue, 1,2-bis(oleoyloxy)-3-(trimethylammonia)propane (DOTAP) can be used in combination with a phospholipid to form DNA-complexing vesicles. Lipofectin™ Bethesda Research Laboratories, Gaithersburg, Md.) is an effective agent for the delivery of highly

anionic nucleic acids into living tissue culture cells that comprise positively charged DOTMA liposomes which interact spontaneously with negatively charged polynucleotides to form complexes. When enough positively charged liposomes are used, the net charge on the resulting complexes is also positive. Positively charged complexes prepared in this way spontaneously attach to negatively charged cell surfaces, fuse with the plasma membrane, and efficiently deliver functional nucleic acids into, for example, tissue culture cells. Another commercially available cationic lipid, 1,2-bis(oleoyloxy)-3,3-(trimethylammonia)propane ("DOTAP") (Boehringer Mannheim, Indianapolis, Indiana) differs from DOTMA in that the oleoyl moieties are linked by ester, rather than ether linkages.

Other reported cationic lipid compounds include those that have been conjugated to a variety of moieties including, for example, carboxyspermine which has been conjugated to one of two types of lipids and includes compounds such as 5-carboxyspermylglycine dioctaoleoylamide ("DOGS") (Transfectam™, Promega, Madison, Wisconsin) and dipalmitoylphosphatidylethanolamine 5-carboxyspermyl-amide ("DPPES") (see, *e.g.*, United States Patent No. 5,171,678).

Another cationic lipid conjugate includes derivatization of the lipid with cholesterol ("DC-Chol") which has been formulated into liposomes in combination with DOPE (See, Gao, X. and Huang, L., (1991) *Biochim. Biophys. Res. Commun.* 179: 280). Lipopolylysine, made by conjugating polylysine to DOPE, has been reported to be effective for transfection in the presence of serum (Zhou, X. *et al.*, (1991) *Biochim. Biophys. Acta* 1065: 8). For certain cell lines, these liposomes containing conjugated cationic lipids, are said to exhibit lower toxicity and provide more efficient transfection than the DOTMA-containing compositions. Other commercially available cationic lipid products include DMRIE and DMRIE-HP (Vical, La Jolla, California) and Lipofectamine (DOSPA) (Life Technology, Inc., Gaithersburg, Maryland). Other cationic lipids suitable for the delivery of oligonucleotides are described in WO 98/39359 and WO 96/37194.

Liposomal formulations are particularly suited for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer RNAi agent into the skin. In some implementations, liposomes are used for delivering RNAi agent to epidermal cells and also to enhance the penetration of RNAi agent into dermal tissues, *e.g.*, into skin. For example, the

liposomes can be applied topically. Topical delivery of drugs formulated as liposomes to the skin has been documented (see, *e.g.*, Weiner *et al.*, (1992) *Journal of Drug Targeting*, vol. 2,405-410 and du Plessis *et al.*, (1992) *Antiviral Research*, 18: 259-265; Mannino, R. J. and Fould-Fogerite, S., (1998) *Biotechniques* 6: 682-690; Itani, T. *et al.*, (1987) *Gene* 56: 267-276; Nicolau, C. *et al.* (1987) *Meth. Enzymol.* 149: 157-176; Straubinger, R. M. and Papahadjopoulos, D. (1983) *Meth. Enzymol.* 101: 512-527; Wang, C. Y. and Huang, L., (1987) *Proc. Natl. Acad. Sci. USA* 84: 7851-7855).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome II (glyceryl distearate/ cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver a drug into the dermis of mouse skin. Such formulations with RNAi agent are useful for treating a dermatological disorder.

Liposomes that include RNAi agents can be made highly deformable. Such deformability can enable the liposomes to penetrate through pore that are smaller than the average radius of the liposome. For example, transfersomes (highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles) are a type of deformable liposomes. Transfersomes can be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.*, they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. Transferosomes can be made by adding surface edge activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin. Transfersomes that include RNAi agent can be delivered, for example, subcutaneously by infection in order to deliver RNAi agent to keratinocytes in the skin. In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. In addition, due to the lipid properties, these transferosomes can be self-optimizing (adaptive to the shape of pores, *e.g.*, in the skin), self-repairing, and can frequently reach their targets without fragmenting, and often self-loading.

Other formulations amenable to the present disclosure are described in United States provisional application serial Nos. 61/018,616, filed January 2, 2008; 61/018,611, filed January 2, 2008; 61/039,748, filed March 26, 2008; 61/047,087, filed April 22, 2008 and 61/051,528, filed May 8, 2008. PCT application number PCT/US2007/080331, filed October 3, 2007, also describes formulations that are amenable to the present disclosure.

Surfactants find wide application in formulations such as those described herein, particularly in emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general, their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

The RNAi agent for use in the methods of the disclosure can also be provided as micellar formulations. "Micelles" are defined herein as a particular type of molecular assembly in which amphipathic molecules are arranged in a spherical structure such that all the hydrophobic portions of the molecules are directed inward, leaving the hydrophilic portions in contact with the surrounding aqueous phase. The converse arrangement exists if the environment is hydrophobic.

A mixed micellar formulation suitable for delivery through transdermal membranes may be prepared by mixing an aqueous solution of the siRNA composition, an alkali metal C_8 to C_{22} alkyl sulphate, and a micelle forming compounds. Exemplary micelle forming compounds include lecithin, hyaluronic acid, pharmaceutically acceptable salts of hyaluronic acid, glycolic acid, lactic acid, chamomile extract, cucumber extract, oleic acid, linoleic acid, linolenic acid, monoolein, monooleates, monolaurates, borage oil, evening of primrose oil, menthol, trihydroxy oxo cholanyl glycine and pharmaceutically acceptable salts thereof, glycerin, polyglycerin, lysine, polylysine, triolein, polyoxyethylene ethers and analogues thereof, polidocanol alkyl ethers and analogues thereof, chenodeoxycholate, deoxycholate, and mixtures thereof. The micelle forming compounds may be added at the same time or after addition of the alkali metal alkyl sulphate. Mixed micelles will form with substantially any kind of mixing of the ingredients but vigorous mixing in order to provide smaller size micelles.

In one method a first micellar composition is prepared which contains the siRNA composition and at least the alkali metal alkyl sulphate. The first micellar composition is then mixed with at least three micelle forming compounds to form a mixed micellar composition. In another method, the micellar composition is prepared by mixing the siRNA composition, the alkali metal alkyl sulphate and at least one of the micelle forming compounds, followed by addition of the remaining micelle forming compounds, with vigorous mixing.

Phenol or m-cresol may be added to the mixed micellar composition to stabilize the formulation and protect against bacterial growth. Alternatively, phenol or m-cresol may be added

with the micelle forming ingredients. An isotonic agent such as glycerin may also be added after formation of the mixed micellar composition.

For delivery of the micellar formulation as a spray, the formulation can be put into an aerosol dispenser and the dispenser is charged with a propellant. The propellant, which is under pressure, is in liquid form in the dispenser. The ratios of the ingredients are adjusted so that the aqueous and propellant phases become one, *i.e.*, there is one phase. If there are two phases, it is necessary to shake the dispenser prior to dispensing a portion of the contents, *e.g.*, through a metered valve. The dispensed dose of pharmaceutical agent is propelled from the metered valve in a fine spray.

Propellants may include hydrogen-containing chlorofluorocarbons, hydrogen-containing fluorocarbons, dimethyl ether and diethyl ether. In certain embodiments, HFA 134a (1,1,1,2 tetrafluoroethane) may be used.

The specific concentrations of the essential ingredients can be determined by relatively straightforward experimentation. For absorption through the oral cavities, it is often desirable to increase, *e.g.*, at least double or triple, the dosage for through injection or administration through the gastrointestinal tract.

Lipid particles

RNAi agents, *e.g.*, dsRNAs of in the disclosure may be fully encapsulated in a lipid formulation, *e.g.*, a LNP, or other nucleic acid-lipid particle.

As used herein, the term "LNP" refers to a stable nucleic acid-lipid particle. LNPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle (*e.g.*, a PEG-lipid conjugate). LNPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). LNPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in WO 00/03683. LNP-formulated particles typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, nucleic acids when present in the nucleic acid-lipid particles are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos.

5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; United States Patent publication No. 2010/0324120 and WO 96/40964.

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. Ranges intermediate to the above recited ranges are also contemplated to be part of the disclosure.

Certain specific LNP formulations for delivery of RNAi agents have been described in the art, including, *e.g.*, "LNP01" formulations as described in, *e.g.*, WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-dsRNA formulations are identified in the chart below.

	Ionizable/Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid: siRNA ratio**
SNALP-1	1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid: siRNA ~ 7: 1
2-XTC	2,2-Dilinolcyl-4-dimcthylaminocthyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid: siRNA ~ 7: 1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid: siRNA ~ 6: 1
LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid: siRNA ~ 11: 1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid: siRNA ~ 6: 1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolanc (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid: siRNA ~ 11: 1

LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA 10: 1
LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA 10: 1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA 10: 1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1)	Tech G1/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA 10: 1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA: 33: 1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid: siRNA: 11: 1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid: siRNA: 11: 1
LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA: 7: 1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid: siRNA: 10: 1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid: siRNA: 12: 1

LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid: siRNA: 8: 1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1.5 Lipid: siRNA: 10: 1
LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid: siRNA: 7: 1
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid: siRNA: 10: 1

DSPC: distearoylphosphatidylcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyristoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000)

PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of 2000)

PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000)

SNALP (l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in WO 2009/127060, which is hereby incorporated by reference.

XTC comprising formulations are described in WO 2010/088537, the entire contents of which are hereby incorporated herein by reference.

MC3 comprising formulations are described, *e.g.*, in United States Patent Publication No. 2010/0324120, the entire contents of which are hereby incorporated by reference.

ALNY-100 comprising formulations are described in WO 2010/054406, the entire contents of which are hereby incorporated herein by reference.

C12-200 comprising formulations are described in WO 2010/129709, the entire contents of which are hereby incorporated herein by reference.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders can be desirable. In some embodiments, oral formulations

are those in which dsRNAs featured in the disclosure are administered in conjunction with one or more penetration enhancer surfactants and chelators. Suitable surfactants include fatty acids or esters or salts thereof, bile acids or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.*, sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs featured in the disclosure can be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (*e.g.*, p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Patent 6,887,906, U.S. 2003/0027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration can include sterile aqueous solutions which can also contain buffers, diluents and other suitable additives such as, but not limited to,

penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present disclosure include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions can be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the brain when treating SNCA-associated diseases or disorders.

The pharmaceutical formulations of the present disclosure, which can conveniently be presented in unit dosage form, can be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present disclosure can be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present disclosure can also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions can further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol or dextran. The suspension can also contain stabilizers.

Additional Formulations

i. Emulsions

The compositions of the present disclosure can be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1μm in diameter (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301).

Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions can be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions can contain additional components in addition to the dispersed phases, and the active drug which can be present as a solution in either aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants can also be present in emulsions as needed. Pharmaceutical emulsions can also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise, a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion can be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that can be incorporated into either phase of the emulsion. Emulsifiers can broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in Pharmaceutical

Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants can be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize

emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that can readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used can be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

ii. Microemulsions

In one embodiment of the present disclosure, the compositions of RNAi agents and nucleic acids are formulated as microemulsions. A microemulsion can be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in

Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically, microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used, and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void

space generated among surfactant molecules. Microemulsions can, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase can typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase can include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385; Ho *et al.*, J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions can form spontaneously when their components are brought together at ambient temperature. This can be particularly advantageous when formulating thermolabile drugs, peptides or RNAi agents. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present disclosure will facilitate the increased systemic absorption of RNAi agents and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of RNAi agents and nucleic acids.

Microemulsions of the present disclosure can also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the RNAi agents and nucleic acids of the present disclosure. Penetration enhancers used in the microemulsions of the present disclosure can be classified as belonging to one of five broad categories--surfactants, fatty acids,

bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

iii. Microparticles

An RNAi agent of the disclosure may be incorporated into a particle, *e.g.*, a microparticle. Microparticles can be produced by spray-drying, but may also be produced by other methods including lyophilization, evaporation, fluid bed drying, vacuum drying, or a combination of these techniques.

iv. Penetration Enhancers

In one embodiment, the present disclosure employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly RNAi agents, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs can cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers can be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above-mentioned classes of penetration enhancers are described below in greater detail.

Surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of RNAi agents through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40, 252).

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-20} alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (see *e.g.*, Touitou, E., *et al.* Enhancement in Drug Delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44, 651-654).

The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79, 579-583).

Chelating agents, as used in connection with the present disclosure, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the

result that absorption of RNAi agents through the mucosa is enhanced. With regards to their use as penetration enhancers in the present disclosure, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(see *e.g.*, Katdare, A. *et al.*, Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al.*, J. Control Rel., 1990, 14, 43-51).

As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of RNAi agents through the alimentary mucosa (see *e.g.*, Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of RNAi agents at the cellular level can also be added to the pharmaceutical and other compositions of the present disclosure. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (WO 97/30731), are also known to enhance the cellular uptake of dsRNAs.

Other agents can be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

vi. Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient can be liquid or solid

and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc*).

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present disclosure. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids can include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions can also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

vii. Other Components

The compositions of the present disclosure can additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions can contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or can contain additional materials useful in physically formulating various dosage forms of the compositions of the present disclosure, such as dyes, flavoring agents,

preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present disclosure. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions can contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol or dextran. The suspension can also contain stabilizers.

In some embodiments, pharmaceutical compositions featured in the disclosure include (a) one or more RNAi agents and (b) one or more agents which function by a non-RNAi mechanism and which are useful in treating a SNCA-associated neurodegenerative disorder. Examples of such agents include, but are not limited to dopamine agonists and promoters, among others, including carbidopa-levodopa, levodopa, entacopone, tolcapone, opicapone, pramipexole, ropinirole, apomorphine, rotigotine, selegiline, rasagiline, safinamide, amantadine, istradefylline, trihexyphenidyl, benztropine, rivastigmine, donepezil, galantamine and memantine.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured herein in the disclosure lies generally within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the disclosure, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (*e.g.*, achieving a decreased concentration of the polypeptide) that includes the IC_{50} (*i.e.*, the concentration of the test

compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

In addition to their administration, as discussed above, the RNAi agents featured in the disclosure can be administered in combination with other known agents effective in treatment of pathological processes mediated by nucleotide repeat expression. In any event, the administering physician can adjust the amount and timing of RNAi agent administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

VII. Kits

In certain aspects, the instant disclosure provides kits that include a suitable container containing a pharmaceutical formulation of a siRNA compound, *e.g.*, a double-stranded siRNA compound, or siRNA compound, (*e.g.*, a precursor, *e.g.*, a larger siRNA compound which can be processed into a siRNA compound, or a DNA which encodes an siRNA compound, *e.g.*, a double-stranded siRNA compound, or siRNA compound, or precursor thereof). In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, *e.g.*, one container for a siRNA compound preparation, and at least another for a carrier compound. The kit may be packaged in a number of different configurations such as one or more containers in a single box. The different components can be combined, *e.g.*, according to instructions provided with the kit. The components can be combined according to a method described herein, *e.g.*, to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

VIII. Methods for Inhibiting SNCA Expression

The present disclosure also provides methods of inhibiting expression of a SNCA gene in a cell. The methods include contacting a cell with an RNAi agent, *e.g.*, double stranded RNAi agent, in an amount effective to inhibit expression of SNCA in the cell, thereby inhibiting expression of SNCA in the cell. In certain embodiments of the disclosure, SNCA is inhibited preferentially in CNS (*e.g.*, brain) cells.

Contacting of a cell with an RNAi agent, *e.g.*, a double stranded RNAi agent, may be done *in vitro* or *in vivo*. Contacting a cell *in vivo* with the RNAi agent includes contacting a cell or group of cells within a subject, *e.g.*, a human subject, with the RNAi agent. Combinations of *in vitro* and *in vivo* methods of contacting a cell are also possible.

Contacting a cell may be direct or indirect, as discussed above. Furthermore, contacting a cell may be accomplished via a targeting ligand, including any ligand described herein or known in the art.

The term "inhibiting," as used herein, is used interchangeably with "reducing," "silencing," "downregulating," "suppressing" and other similar terms, and includes any level of inhibition. In certain embodiments, a level of inhibition, *e.g.*, for an RNAi agent of the instant disclosure, can be assessed in cell culture conditions, *e.g.*, wherein cells in cell culture are transfected *via* Lipofectamine™-mediated transfection at a concentration in the vicinity of a cell of 10 nM or less, 1 nM or less, *etc*. Knockdown of a given RNAi agent can be determined *via* comparison of pre-treated levels in cell culture versus post-treated levels in cell culture, optionally also comparing against cells treated in parallel with a scrambled or other form of control RNAi agent. Knockdown in cell culture of, *e.g.*, optionally 50% or more, can thereby be identified as indicative of "inhibiting" or "reducing", "downregulating" or "suppressing", *etc*. having occurred. It is expressly contemplated that assessment of targeted mRNA or encoded protein levels (and therefore an extent of "inhibiting", *etc*. caused by an RNAi agent of the disclosure) can also be assessed in *in vivo* systems for the RNAi agents of the instant disclosure, under properly controlled conditions as described in the art.

The phrase "inhibiting expression of a SNCA gene" or "inhibiting expression of SNCA," as used herein, includes inhibition of expression of any SNCA gene (such as, *e.g.*, a mouse SNCA gene, a rat SNCA gene, a monkey SNCA gene, or a human SNCA gene) as well as variants or mutants of a SNCA gene that encode a SNCA protein. Thus, the SNCA gene may be a wild-type SNCA gene, a mutant SNCA gene, or a transgenic SNCA gene in the context of a genetically manipulated cell, group of cells, or organism.

"Inhibiting expression of a SNCA gene" includes any level of inhibition of a SNCA gene, *e.g.*, at least partial suppression of the expression of a SNCA gene, such as an inhibition by at least 20%. In certain embodiments, inhibition is by at least 30%, at least 40%, optionally at least 50%, at least about 60%, at least 70%, at least about 80%, at least 85%, at least 90%, at least 95%, at

least 96%, at least 97%, at least 98%, or at least 99%; or to below the level of detection of the assay method.

The expression of a SNCA gene may be assessed based on the level of any variable associated with SNCA gene expression, *e.g.*, SNCA mRNA level or SNCA protein level, or, for example, the level of neuroinflammation, *e.g.,* microglial and astrocyte activation, and SNCA deposition in areas of the brain associated with neuronal cell death and/or levels of SNCA mRNA/protein within exosomes (neuronal or otherwise).

Inhibition may be assessed by a decrease in an absolute or relative level of one or more of these variables compared with a control level. The control level may be any type of control level that is utilized in the art, *e.g.*, a pre-dose baseline level, or a level determined from a similar subject, cell, or sample that is untreated or treated with a control (such as, *e.g.*, buffer only control or inactive agent control).

In some embodiments of the methods of the disclosure, expression of a SNCA gene is inhibited by at least 20%, 30%, 40%, optionally at least 50%, 60%, 70%, 80%, 85%, 90%, or 95%, or to below the level of detection of the assay. In certain embodiments, the methods include a clinically relevant inhibition of expression of SNCA, *e.g.*, as demonstrated by a clinically relevant outcome after treatment of a subject with an agent to reduce the expression of SNCA.

Inhibition of the expression of a SNCA gene may be manifested by a reduction of the amount of mRNA expressed by a first cell or group of cells (such cells may be present, for example, in a sample derived from a subject) in which a SNCA gene is transcribed and which has or have been treated (*e.g.*, by contacting the cell or cells with an RNAi agent of the disclosure, or by administering an RNAi agent of the disclosure to a subject in which the cells are or were present) such that the expression of a SNCA gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has not or have not been so treated (control cell(s) not treated with an RNAi agent or not treated with an RNAi agent targeted to the gene of interest). The degree of inhibition may be expressed in terms of:

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \bullet 100\%$$

In other embodiments, inhibition of the expression of a SNCA gene may be assessed in terms of a reduction of a parameter that is functionally linked to a SNCA gene expression, *e.g.*, SNCA protein expression. SNCA gene silencing may be determined in any cell expressing SNCA,

either endogenous or heterologous from an expression construct, and by any assay known in the art.

Inhibition of the expression of a SNCA protein may be manifested by a reduction in the level of the SNCA protein that is expressed by a cell or group of cells (*e.g.*, the level of protein expressed in a sample derived from a subject). As explained above, for the assessment of mRNA suppression, the inhibiton of protein expression levels in a treated cell or group of cells may similarly be expressed as a percentage of the level of protein in a control cell or group of cells.

A control cell or group of cells that may be used to assess the inhibition of the expression of a SNCA gene includes a cell or group of cells that has not yet been contacted with an RNAi agent of the disclosure. For example, the control cell or group of cells may be derived from an individual subject (*e.g.*, a human or animal subject) prior to treatment of the subject with an RNAi agent.

The level of SNCA mRNA that is expressed by a cell or group of cells may be determined using any method known in the art for assessing mRNA expression. In one embodiment, the level of expression of SNCA in a sample is determined by detecting a transcribed polynucleotide, or portion thereof, *e.g.*, mRNA of the SNCA gene. RNA may be extracted from cells using RNA extraction techniques including, for example, using acid phenol/guanidine isothiocyanate extraction (RNAzol B; Biogenesis), RNeasy™ RNA preparation kits (Qiagen®) or PAXgene (PreAnalytix, Switzerland). Typical assay formats utilizing ribonucleic acid hybridization include nuclear run-on assays, RT-PCR, RNase protection assays, northern blotting, *in situ* hybridization, and microarray analysis. Circulating SNCA mRNA may be detected using methods the described in WO2012/177906, the entire contents of which are hereby incorporated herein by reference.

In some embodiments, the level of expression of SNCA is determined using a nucleic acid probe. The term "probe", as used herein, refers to any molecule that is capable of selectively binding to a specific SNCA nucleic acid or protein, or fragment thereof. Probes can be synthesized by one of skill in the art, or derived from appropriate biological preparations. Probes may be specifically designed to be labeled. Examples of molecules that can be utilized as probes include, but are not limited to, RNA, DNA, proteins, antibodies, and organic molecules.

Isolated mRNA can be used in hybridization or amplification assays that include, but are not limited to, Southern or northern analyses, polymerase chain reaction (PCR) analyses and probe arrays. One method for the determination of mRNA levels involves contacting the isolated mRNA

with a nucleic acid molecule (probe) that can hybridize to SNCA mRNA. In one embodiment, the mRNA is immobilized on a solid surface and contacted with a probe, for example by running the isolated mRNA on an agarose gel and transferring the mRNA from the gel to a membrane, such as nitrocellulose. In an alternative embodiment, the probe(s) are immobilized on a solid surface and the mRNA is contacted with the probe(s), for example, in an Affymetrix® gene chip array. A skilled artisan can readily adapt known mRNA detection methods for use in determining the level of SNCA mRNA.

An alternative method for determining the level of expression of SNCA in a sample involves the process of nucleic acid amplification or reverse transcriptase (to prepare cDNA) of for example mRNA in the sample, *e.g.*, by RT-PCR (the experimental embodiment set forth in Mullis, 1987, US Patent No. 4,683,202), ligase chain reaction (Barany (1991) *Proc. Natl. Acad. Sci. USA* 88: 189-193), self-sustained sequence replication (Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87: 1874-1878), transcriptional amplification system (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86: 1173-1177), Q-Beta Replicase (Lizardi *et al.* (1988) *Bio/Technology* 6: 1197), rolling circle replication (Lizardi *et al.*, US Patent No. 5,854,033) or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers. In particular aspects of the disclosure, the level of expression of SNCA is determined by quantitative fluorogenic RT-PCR (*i.e.*, the TaqMan™ System), by a Dual-Glo® Luciferase assay, or by other art-recognized method for measurement of SNCA expression or mRNA level.

The expression level of SNCA mRNA may be monitored using a membrane blot (such as used in hybridization analysis such as northern, Southern, dot, and the like), or microwells, sample tubes, gels, beads or fibers (or any solid support comprising bound nucleic acids). See US Patent Nos. 5,770,722, 5,874,219, 5,744,305, 5,677,195 and 5,445,934, which are incorporated herein by reference. The determination of SNCA expression level may also comprise using nucleic acid probes in solution.

In some embodiments, the level of mRNA expression is assessed using branched DNA (bDNA) assays or real time PCR (qPCR). The use of this PCR method is described and exemplified in the Examples presented herein. Such methods can also be used for the detection of SNCA nucleic acids.

The level of SNCA protein expression may be determined using any method known in the art for the measurement of protein levels. Such methods include, for example, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, fluid or gel precipitin reactions, absorption spectroscopy, a colorimetric assays, spectrophotometric assays, flow cytometry, immunodiffusion (single or double), immunoelectrophoresis, western blotting, radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, electrochemiluminescence assays, and the like. Such assays can also be used for the detection of proteins indicative of the presence or replication of SNCA proteins.

In some embodiments, the efficacy of the methods of the disclosure in the treatment of a SNCA-related disease is assessed by a decrease in SNCA mRNA level (*e.g*, by assessment of a CSF sample for SNCA level, by brain biopsy, or otherwise).

In some embodiments of the methods of the disclosure, the RNAi agent is administered to a subject such that the RNAi agent is delivered to a specific site within the subject. The inhibition of expression of SNCA may be assessed using measurements of the level or change in the level of SNCA mRNA or SNCA protein in a sample derived from a specific site within the subject, *e.g.*, CNS cells. In certain embodiments, the methods include a clinically relevant inhibition of expression of SNCA, *e.g.* as demonstrated by a clinically relevant outcome after treatment of a subject with an agent to reduce the expression of SNCA.

As used herein, the terms detecting or determining a level of an analyte are understood to mean performing the steps to determine if a material, *e.g.*, protein, RNA, is present. As used herein, methods of detecting or determining include detection or determination of an analyte level that is below the level of detection for the method used.

IX. Methods of Treating or Preventing SNCA-Associated Neurodegenerative Diseases

The present disclosure also provides methods of using an RNAi agent of the disclosure or a composition containing an RNAi agent of the disclosure to reduce or inhibit SNCA expression in a cell. The methods include contacting the cell with a dsRNA of the disclosure and maintaining the cell for a time sufficient to obtain degradation of the mRNA transcript of a SNCA gene, thereby inhibiting expression of the SNCA gene in the cell. Reduction in gene expression can be assessed

by any methods known in the art. For example, a reduction in the expression of SNCA may be determined by determining the mRNA expression level of SNCA using methods routine to one of ordinary skill in the art, *e.g.,* northern blotting, qRT-PCR; by determining the protein level of SNCA using methods routine to one of ordinary skill in the art, such as western blotting, immunological techniques, and mass-spectrometry.

In the methods of the disclosure the cell may be contacted *in vitro* or *in vivo, i.e.,* the cell may be within a subject.

A cell suitable for treatment using the methods of the disclosure may be any cell that expresses a SNCA gene. A cell suitable for use in the methods of the disclosure may be a mammalian cell, *e.g.,* a primate cell (such as a human cell or a non-human primate cell, *e.g.*, a monkey cell or a chimpanzee cell), a non-primate cell (such as a a rat cell, or a mouse cell. In one embodiment, the cell is a human cell, *e.g.,* a human CNS cell.

In certain embodiments, SNCA expression is inhibited in the cell by at least about 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99, or about 100%, *i.e.*, to below the level of detection. In preferred embodiments, SNCA expression is inhibited by at least 50 %.

The *in vivo* methods of the disclosure may include administering to a subject a composition containing an RNAi agent, where the RNAi agent includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the SNCA gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition can be administered by any means known in the art including, but not limited to CNS-directed and/or intracranial (*e.g.*, intraventricular, intraparenchymal, and intrathecal), oral, intraperitoneal, or other parenteral routes, including, intravenous, intramuscular, intravitreal, subcutaneous, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by intrathecal infusion or injection.

In some embodiments, the administration is *via* a depot injection. A depot injection may release the RNAi agent in a consistent way over a prolonged time period. Thus, a depot injection may reduce the frequency of dosing needed to obtain a desired effect, *e.g.*, a desired inhibition of SNCA, or a therapeutic or prophylactic effect. A depot injection may also provide more consistent serum concentrations. Depot injections may include intrathecal injections, subcutaneous injections or intramuscular injections.

In some embodiments, the administration is *via* a pump. The pump may be an external pump or a surgically implanted pump. In certain embodiments, the pump is a subcutaneously implanted osmotic pump. In other embodiments, the pump is an infusion pump. An infusion pump may be used for intracranial, intravenous, subcutaneous, arterial, or epidural infusions. In preferred embodiments, the infusion pump is a surgically implanted pump that delivers the RNAi agent to the CNS.

The mode of administration may be chosen based upon whether local or systemic treatment is desired and based upon the area to be treated. The route and site of administration may be chosen to enhance targeting.

In one aspect, the present disclosure also provides methods for inhibiting the expression of a SNCA gene in a mammal. The methods include administering to the mammal a composition comprising a dsRNA that targets a SNCA gene in a cell of the mammal and maintaining the mammal for a time sufficient to obtain degradation of the mRNA transcript of the SNCA gene, thereby inhibiting expression of the SNCA gene in the cell. Reduction in gene expression can be assessed by any methods known it the art and by methods, *e.g.* qRT-PCR, described herein. Reduction in protein production can be assessed by any methods known it the art and by methods, *e.g.* ELISA, described herein. In one embodiment, a CNS biopsy sample or a cerebrospinal fluid (CSF) sample serves as the tissue material for monitoring the reduction in SNCA gene or protein expression (or of a proxy therefore).

The present disclosure further provides methods of treatment of a subject in need thereof. The treatment methods of the disclosure include administering an RNAi agent of the disclosure to a subject, *e.g.*, a subject that would benefit from inhibition of SNCA expression, in a therapeutically effective amount of an RNAi agent targeting a SNCA gene or a pharmaceutical composition comprising an RNAi agent targeting a SNCA gene.

In addition, the present disclosure provides methods of preventing, treating or inhibiting the progression of a SNCA-associated neurodegenerative disease or disorder, such as a synucleinopathy, such as PD, multiple system atrophy, Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease,

lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

The methods include administering to the subject a therapeutically effective amount of any of the RNAi agent, *e.g.*, dsRNA agents, or the pharmaceutical composition provided herein, thereby preventing, treating or inhibiting the progression of the SNCA-associated neurodegenerative disease or disorder in the subject.

An RNAi agent of the disclosure may be administered as a "free RNAi agent." A free RNAi agent is administered in the absence of a pharmaceutical composition. The naked RNAi agent may be in a suitable buffer solution. The buffer solution may comprise acetate, citrate, prolamine, carbonate, or phosphate, or any combination thereof. In one embodiment, the buffer solution is phosphate buffered saline (PBS). The pH and osmolarity of the buffer solution containing the RNAi agent can be adjusted such that it is suitable for administering to a subject.

Alternatively, an RNAi agent of the disclosure may be administered as a pharmaceutical composition, such as a dsRNA liposomal formulation.

Subjects that would benefit from a reduction or inhibition of SNCA gene expression are those having a SNCA-associated neurodegenerative disease.

The disclosure further provides methods for the use of an RNAi agent or a pharmaceutical composition thereof, *e.g.*, for treating a subject that would benefit from reduction or inhibition of SNCA expression, *e.g.*, a subject having a SNCA-associated neurodegenerative disorder, in combination with other pharmaceuticals or other therapeutic methods, *e.g.*, with known pharmaceuticals or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. For example, in certain embodiments, an RNAi agent targeting SNCA is administered in combination with, *e.g.*, an agent useful in treating a SNCA-associated neurodegenerative disorder as described elsewhere herein or as otherwise known in the art. For example, additional agents and treatments suitable for treating a subject that would benefit from reduction in SNCA expression, *e.g.*, a subject having a SNCA-associated neurodegenerative disorder, may include agents currently used to treat symptoms of SNCA. The RNAi agent and additional therapeutic agents may be administered at the same time or in the same combination, *e.g.*, intrathecally, or the additional therapeutic agent can be administered as part of a separate composition or at separate times or by another method known in the art or described herein.

Exemplary additional therapeutics and treatments include dopamine-modulating agents, among others, for example, carbidopa-levodopa, levodopa, entacopone, tolcapone, opicapone, pramipexole, ropinirole, apomorphine, rotigotine, selegiline, rasagiline, safinamide, amantadine, istradefylline, trihexyphenidyl, benztropine, rivastigmine, donepezil, galantamine and memantine, as well as physical, occupational and speech therapy, an exercise program including cardiorespiratory, resistance, flexibility, and gait and balance exercises, and deep brain stimulation (DBS) involving the implantation of an electrode into a targeted area of the brain.

In one embodiment, the method includes administering a composition featured herein such that expression of the target SNCA gene is decreased, for at least one month. In certain embodiments, expression is decreased for at least 2 months, 3 months, or 6 months.

Optionally, the RNAi agents useful for the methods and compositions featured herein specifically target RNAs (primary or processed) of the target SNCA gene. Compositions and methods for inhibiting the expression of these genes using RNAi agents can be prepared and performed as described herein.

Administration of the dsRNA according to the methods of the disclosure may result in a reduction of the severity, signs, symptoms, or markers of such diseases or disorders in a patient with a SNCA-associated neurodegenerative disorder. By "reduction" in this context is meant a statistically significant or clinically significant decrease in such level. The reduction can be, for example, at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or about 100%.

Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. For example, efficacy of treatment of a SNCA-associated neurodegenerative disorder may be assessed, for example, by periodic monitoring of a subject's cognition, learning, or memory. Comparisons of the later readings with the initial readings provide a physician an indication of whether the treatment is effective. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In

connection with the administration of an RNAi agent targeting SNCA or pharmaceutical composition thereof, "effective against" a SNCA-associated neurodegenerative disorder indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as an improvement of symptoms, a cure, a reduction in disease, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating SNCA-associated neurodegenerative disorders and the related causes.

A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and optionally at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given RNAi agent drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

Alternatively, the efficacy can be measured by a reduction in the severity of disease as determined by one skilled in the art of diagnosis based on a clinically accepted disease severity grading scale. Any positive change resulting in *e.g.*, lessening of severity of disease measured using the appropriate scale, represents adequate treatment using an RNAi agent or RNAi agent formulation as described herein.

Subjects can be administered a therapeutic amount of dsRNA, such as about 0.01 mg/kg to about 200 mg/kg.

The RNAi agent can be administered intrathecally, via intravitreal injection, or by intravenous infusion over a period of time, on a regular basis. In certain embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. Administration of the RNAi agent can reduce SNCA levels, *e.g.*, in a cell, tissue, blood, CSF sample or other compartment of the patient by at least 20%, 30%, 40%, 50%, 55%, 60%, 65%, 70,% 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or at least about 99% or more. In a preferred embodiment, administration of the RNAi agent can reduce SNCA levels, *e.g.*, in a cell, tissue, blood, CSF sample or other compartment of the patient by at least 50%.

Before administration of a full dose of the RNAi agent, patients can be administered a smaller dose, such as a 5% infusion reaction, and monitored for adverse effects, such as an allergic reaction. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (*e.g.*, TNF-alpha or INF-alpha) levels.

One or more injections may be used to deliver the desired, *e.g.*, monthly dose of RNAi agent to a subject. The injections may be repeated over a period of time. The administration may be repeated on a regular basis. In certain embodiments, after an initial treatment regimen, the treatments can be administered on a less frequent basis. A repeat-dose regimine may include administration of a therapeutic amount of RNAi agent on a regular basis, such as monthly or extending to once a quarter, twice per year, once per year. In certain embodiments, the RNAi agent is administered about once per month to about once per quarter (*i.e.*, about once every three months).

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the RNAi agents and methods featured in the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

An informal Sequence Listing is also filed herewith and forms part of the specification as filed.

EXAMPLES

Example 1: Materials and Methods

Bioinformatics

A set of siRNAs targeting the human Synuclein alpha gene (SNCA; human NCBI refseq ID NM_007308.3; NCBI GeneID: 6622; SEQ ID NO: 1) as well the toxicology-species SNCA (XM_005555422.2; SEQ ID NO: 3) ortholog from cynomolgus monkey were designed using custom R and Python scripts. All the siRNAs were designed to have a perfect match to the human

SNCA transcripts and a subset either perfect or near-perfect matches to the cynomolgus monkey ortholog. The human SNCA NM_007308 REFSEQ mRNA, version 3 (SEQ ID NO: 1), has a length of 3312 bases. The rationale and method for the set of siRNA designs follows. The predicted efficacy for every potential 23mer siRNA from position 10 through the end was determined with a random forest model derived from the direct measure of mRNA knockdown from several thousand distinct siRNA designs targeting a diverse set of vertebrate genes. For each strand of the siRNA, a custom Python script was used in a brute force search to measure the number and positions of mismatches between the siRNA and all potential alignments in the human transcriptome. Extra weight was given to mismatches in the seed region, defined here as positions 2-9 of the antisense oligonucleotide, as well the cleavage site of the siRNA, defined here as positions 10-11 of the antisense oligonucleotide. The relative weight of the mismatches was 2.8, 1.2, 1 for seed mismatches, cleavage site, and other positions up through antisense position 19. Mismatches in the first position were ignored. A specificity score was calculated for each strand by summing the value of each weighted mismatch. Preference was given to siRNAs whose antisense score in human and cynomolgus monkey was >= 2 and predicted efficacy was >= 50% knockdown.

In Vitro Screening - Dual-Glo® Luciferase Assay

Cos-7 cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO_2 in DMEM (ATCC) supplemented with 10% FBS, before being released from the plate by trypsinization. Multi-dose experiments were performed at 10nM and 0.1nM. siRNA and psiCHECK2-SNCAs (human NM_007308 and mouse NM_009221) plasmid transfections were carried out with plasmids containing the 3' untranslated region (UTR). Transfection was carried out by adding 5 µL of siRNA duplexes and 5 µL (5 ng) of psiCHECK2 plasmid per well along with 4.9 µL of Opti-MEM plus 0.1 µL of Lipofectamine™ 2000 per well (Invitrogen, Carlsbad CA. cat # 13778-150) and then incubated at room temperature for 15 minutes. The mixture was then added to the cells which were re-suspended in 35 µL of fresh complete media. The transfected cells were incubated at 37°C in an atmosphere of 5% CO_2.

Forty-eight hours after the siRNAs and psiCHECK2 plasmid were transfected, Firefly (transfection control) and *Renilla* (fused to SNCA target sequence) luciferase were measured. First, media was removed from cells. Then Firefly luciferase activity was measured by adding a

mixture of 20 µL Dual-Glo® Luciferase Reagent and 20µL DMEM to each well. The mixture was incubated at room temperature for 30 minutes before luminescense (500nm) was measured on a Spectramax (Molecular Devices) to detect the Firefly luciferase signal. *Renilla* luciferase activity was measured by adding a mixture of 20 µL of room temperature of Dual-Glo® Stop & Glo® Buffer and 0.1µL Dual-Glo® Stop & Glo® Substrate to each well and the plates were incubated for 10-15 minutes before luminescence was again measured to determine the *Renilla* luciferase signal. The Dual-Glo® Stop & Glo® mixture quenches the firefly luciferase signal and sustained luminescence for the Renilla luciferase reaction. siRNA activity was determined by normalizing the *Renilla* (SNCA) signal to the Firefly (control) signal within each well. The magnitude of siRNA activity was then assessed relative to cells that were transfected with the same vector but were not treated with siRNA or were treated with a non-targeting siRNA. All transfections were done with n=4.

In Vitro Screening - Cell Culture and Transfections

Cells were transfected by adding 4.9 µL of Opti-MEM plus 0.1 µL of RNAiMAX per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5 µL of siRNA duplexes per well, with 4 replicates of each siRNA duplex, into a 384-well plate, and incubated at room temperature for 15 minutes. 40 µL of MEDIA containing ~5 $x10^3$ cells were then added to the siRNA mixture. Cells were incubated for 24 hours prior to RNA purification. Experiments were performed at 10 nM, 1 nM and 0.1 nM, alongside a PBS control. Transfection experiments were performed in human hepatoma Hep3B cells (ATCC HB-8064) with EMEM (ATCC catalog no. 30-2003), mouse neuroblastoma Neuro-2A cells (ATCC CCL-131) with EMEM media, and human neuroblastoma BE(2)-C, HeLa, and B16-F10 cells. BE(2)-C cells (ATCC CRL-2268) were grown in EMEM:F12 media (Gibco catalog no. 11765054). HeLa cells and B16-F10 cells were grown according to standard protocols.

In Vitro Screening - cDNA Synthesis Using ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, Cat #4368813)

12 µL of a master mix containing 1.2 µL 10X Buffer, 0.48 µL 25X dNTPs, 1.2 µL 10x Random primers, 0.6 µL Reverse Transcriptase, 0.6 µL RNase inhibitor and 7.92 µL of H2O per reaction was added to the bead bound RNA isolated above. Plates were sealed, mixed, and incubated on an electromagnetic shaker for 10 minutes at room temperature, followed by 2h

incubation at 37°C. Branched DNA assays were also performed using the aforementioned protocol.

In Vitro Screening - Real Time PCR

2 µL of cDNA were added to a master mix containing 0.5 µL of human or mouse GAPDH TaqMan Probe (ThermoFisher cat 4352934E or 4351309) and 0.5 µL of appropriate SNCA probe (Thermo Fisher Taqman human: Hs00268077, mouse: Mm00485946) and 5 µL Lightcycler 480 probe master mix (Roche Cat # 04887301001) per well in a 384 well plates (Roche cat # 04887301001). Real time PCR was done in a LightCycler480 Real Time PCR system (Roche). Each duplex was tested with N=4 and data were normalized to cells transfected with a non-targeting control siRNA. To calculate relative fold change, real time data were analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with a non-targeting control siRNA.

Example 2: Identification of Potent Candidate siRNAs to Adapt for CNS-Directed Use

In vitro and *in vivo* screening for active SNCA-targeting siRNAs (duplexes) was performed previously, and identified a number of knockdown "hot spots" in the SNCA gene (mRNA target). 356 duplexes were initially subjected to *in vitro* screening, across various cell types, including human BE(2)-C neuroblastoma cells, human HeLa epithelial cells, B16-F10 mouse epithelial cells, and in a dual luciferase reporter system in COS-7 African green monkey fibroblast-like cells. Exemplary 3'UTR SNCA-targeting RNAi agents AD-464778, AD-464782, AD-464694, AD-464634, AD-464779, and coding sequence (CDs) SNCA-targeting RNAi agents AD-464314, AD-464313, AD-464590, AD-464585, AD-464229, AD-464586, and AD-464592 were assessed in BE(2)-C cells and in the COS-7 cell dual luciferase reporter system, respectively, at 10 nM concentration (**FIG. 1A**), with all such duplexes showing significant knockdown in at least one *in vitro* system (e.g., > 50% SNCA mRNA reduction). A majority of potent duplexes for SNCA knockdown were observed to occur in the 3'UTR region. The 12 indicated duplexes (3'UTR-targeting siRNAs: AD-464778, AD-464782, AD-464694, AD-464634, AD-464779; coding sequence-targeting siRNAs: AD-464314, AD-464313, AD-464590, AD-464585, AD-464229, AD-464586, and AD-464592) were then assayed in human SNCA-AAV over-expressing mice for liver SNCA knockdown. Robust *in vivo* knockdown of liver SNCA levels was observed for various tested siRNAs, including all 3'UTR-targeting duplexes (AD-464778, AD-464782, AD-464694,

AD-464634 and AD-464779) and particularly for coding sequence-targeting AD-464314 and AD-464229 duplexes (**FIG. 1B**). *In vitro* cell line screening and *in vivo* liver AAV mouse liver data were observed to be well correlated.

The top 40 most potent SNCA-targeting siRNAs were identified from *in vitro* hot spot and structure activity relationship (SAR) assessments, which were performed for each tested liver-directed (GalNAc-modified) duplex across a concentration range of 0.1 nM, 1 nM and 10 nM, and RNAseq assessments were also performed upon SNCA-targeting duplexes, in identifying a select number of SNCA-targeting siRNAs to move forward for further *in vivo* assessment and adaptation to CNS-targeting siRNAs (including siRNAs of the instant disclosure). Specifically, SNCA-targeting siRNAs AD-1548851.1, AD-1548854.1, AD-1548869.1, AD-1548870.1, AD-1548884.1, AD-1548886.1, AD-1549052.1, AD-1549054.1, AD-1549245.1, AD-1549266.1, AD-1549267.1, AD-1549269.1, AD-1549272.1, AD-1549283.1, AD-1549284.1, AD-1549285.1, AD-1549290.1, AD-1549333.1, AD-1549334.1, AD-1549351.1, AD-1549354.1, AD-1549357.1, AD-1549359.1, AD-1549397.1, AD-1549401.1, AD-1549403.1, AD-1549406.1, AD-1549407.1, AD-1549439.1, AD-1549518.1, AD-1549525.1, AD-1549628.1, AD-1549641.1, AD-1571164.1, AD-1571187.1, AD-1571188.1, AD-1571191.1, AD-1571193.1, AD-1571194.1 and AD-1571262.1 were the forty duplexes identified as the most potent (**FIG. 2**) when selecting those duplexes that exhibited the highest knockdown level at 0.1 nM and that displayed a clear linear dose response.

Only two duplexes tested (AD-1549525.1 and AD-1549628.1) exhibited significant off-target effects, i.e., off-target gene changes that exceeded log two-fold changes.

Based upon the above results, 17 duplexes were selected for further evaluation for *in vivo* use (including administration via AAV to model mice). Five of the selected duplexes (AD-1549052.1, AD1549054.1, AD-1548886.1, AD-1548884.1, and AD-1549245.1) targeted coding sequences (CDs) in SNCA, while the remaining 12 selected duplexes (AD-1549359.1, AD-1549333.1, AD-1549407.1, AD-1548854.1, AD-1549283.1, AD-1549267.1, AD-1548869.1, AD-1571164.1, AD-1549354.1, AD-1571188.1, AD-1549401.1, and AD-1549290.1) targeted UTR sequences in SNCA. Among the selected duplexes was the UTR-targeting duplex AD-1549290.1 (**FIG. 3**). **Table 4** provides the targeted location within the SNCA mRNA for any individual duplex (it is further noted that CDs within the SNCA mRNA span nucleotide locations 226-648, with SNCA exon 1 spanning SNCA mRNA nucleotide residues 1-200, SNCA exon 2 spanning SNCA mRNA nucleotide residues 201-346, SNCA exon 3 spanning SNCA mRNA nucleotide

residues 347-388, SNCA exon 4 spanning SNCA mRNA nucleotide residues 389-531, SNCA exon 5 spanning SNCA mRNA nucleotide residues 532-615, and SNCA exon 6 spanning SNCA mRNA nucleotide residues 616-3177).

Example 3: *In Vivo* Evaluation of Candidate SNCA-Targeting RNAi Agents Identified Three Candidate Lead Compounds for Adaptation to CNS-Directed Use

The initial 17 selected duplexes were evaluated in an *in vivo* study in which human SNCA was administered *via* adeno-associated virus (AAV) and expressed in mice for seven days prior to administration of a SNCA-targeting siRNA. Knockdown was assessed at seven to 14 days post-siRNA administration (10 days was identified as most optimal for siRNA knockdown). Human SNCA expression by AAV in mouse liver was first verified (**FIG. 4**, at left, in which cycle threshold was used to detect AAV-mediated expression of SNCA in mouse liver at days 7, 14 and 21 in a dose-responsive manner). Previously described SNCA-targeting duplexes AD-464634 (3' UTR-targeting) and AD-464314 (coding sequence-targeting) were each identified to exhibit robust SNCA knockdown at both 7 day and 14 day timepoints, at both 3 mpk and 10 mpk amounts assessed (**FIG. 4**, at right). Testing of the 17 selected duplexes for *in vivo* SNCA knockdown in liver tissue of hSNCA AAV transfected mice was then performed and revealed robust hSNCA knockdown *in vivo* by a majority of the tested duplexes (**FIG. 5A**). In particular, duplexes AD-1549052.3 (CD-targeting), AD-1549359.3 (UTR-targeting), AD-1549054.3 (CD-targeting), AD-1549333.3 (UTR-targeting), AD-1746467.2 (UTR-targeting, having sense strand oligonucleotide A-3227795 5'-gscsagugauUfGfAfagua(Uhd)cuguaL96-3' (SEQ ID NO: 263), antisense strand oligonucleotide A-2861991 5'-VPusdAscadGadTacuudCaAfucacugcsusg-3' (SEQ ID NO: 264), corresponding unmodified sense strand oligonucleotide 5'-GCAGUGAUUGAAGUAUCUGUA-3' (SEQ ID NO: 265), and corresponding unmodified antisense strand oligonucleotide sequence 5'-UACAGATACUUCAAUCACUGCUG-3' (SEQ ID NO: 266), where a CNS-directed duplex has sense strand oligonucleotide 5'-gscsagugauUfGfAfagua(Uhd)cugsusa-3' (SEQ ID NO: 267) and antisense strand oligonucleotide SEQ ID NO: 264), AD-1746465.2 (UTR-targeting), AD-1571188.3 (UTR-targeting), AD-1549401.3 (UTR-targeting), AD-1548886.3 (CD-targeting), and AD-1746466.2 (UTR-targeting) each exhibited robust hSNCA knockdown in liver tissue of hSNCA AAV-transfected mice. Seven of these siRNAs, AD-1549054.3, AD-1549333.3, AD-1746465.2, AD-1571188.3, AD-1549401.3, AD-1548886.3, and AD-1746466.2, were selected for

further evaluation. Each of these duplexes harbored both GalNAc and C16 modifications (**FIG. 5B**) in the liver-tested forms; however, removal of the GalNAc moieties was contemplated for adaptation of these duplexes for CNS targeting.

Additional review of *in vivo* SNCA knockdown, *in vitro* SNCA knockdown, and RNAseq results resulted in selection of a preferred set of three SNCA-targeting siRNAs – AD-1549333 (3' UTR-targeting), AD-1746465 (3' UTR-targeting), and AD-1549054 (CD-targeting) – for further *in vivo* evaluation, including non-human primate (NHP) studies (**FIG. 6A**). This selection included one coding-sequence-targeting anti-SNCA siRNA and two UTR-targeting siRNAs (**FIG. 6B**), each of which exhibited high *in vivo* knockdown activity at 3 mg/kg administration and that included C16 modification at either position 6 or position 16 of the antisense strand (a chemistry that prior studies had de-risked; **FIG. 6C**). These three duplexes – AD-1549333, AD-1746465, and AD-1549054 – were respectively adapted for CNS-directed delivery, by removal of the 3'-terminal triantennary GalNAc from the sense strand and inclusion of two additional phosphorothioate modifications, located at the ultimate and penultimate inter-nucleotide linkages of the 3' end of the sense strand, as shown in **Table 2** for resulting lead duplexes AD-1747585 (having parent duplex AD-1549333), AD-1747583 (having parent duplex AD-1746465), and AD-1747580 (having parent duplex AD-1549054), respectively (**FIG. 7**).

Example 4: Evaluation of Candidate Lead Compound Efficacy and Safety in Non-Human Primates (NHPs)

Duplexes identified as the top three lead candidate CNS-directed SNCA-targeting siRNAs after rodent in vivo AAV screening – AD-1747580, AD-1747583, and AD-1747585 – were selected for evaluation of pharmacokinetic (PK) and pharmacodynamic (PD) properties in non-human primates (NHPs), as well as assessment of any off-target effects and toxicity evaluation (refer to **FIG. 8A** for initial rodent in vivo knockdown data for selected duplexes; selected duplexes AD-1747580, AD-1747583, and AD-1747585 are each noted as cross-reactive with the *Macaca fascicularis* synuclein alpha (SNCA), transcript variant X2, mRNA (XM_005555422.3), targeting the following positions: AD-1747580 targets nucleotides 257-279 in XM_005555422.3; AD-1747583 targets nucleotides 506-528 in XM_005555422.3; and AD-1747585 targets nucleotides 556-578 in XM_005555422.3). For PK/PD studies of the three SNCA-targeting siRNAs, study design was partitioned into 29 day and 84 day time courses, performed upon parallel animal

cohorts. For the 29 day study (designed to inform on potency and useful for further duplex selection and/or potential experimental modification at an earlier timepoint), three control animals were dosed with artificial cerebrospinal fluid (aCSF), while a second group of animals (n=5) was administered a single 60 mg dose of the first lead candidate siRNA (AD-1747580), a third group of animals (n=5) was administered a single 60 mg dose of the second lead candidate siRNA (AD-1747583), and a fourth group of animals (n=5) was administered a single 60 mg dose of the third lead candidate siRNA (AD-1747585). Similarly, for the parallel study terminating at day 84 (designed to evaluation duration), another group (group 5) of three control animals was dosed with artificial cerebrospinal fluid (aCSF), while a sixth group of animals (n=5) was administered a single 60 mg dose of the first lead candidate siRNA (AD-1747580), a seventh group of animals (n=5) was administered a single 60 mg dose of the second lead candidate siRNA (AD-1747583), and an eighth group of animals (n=5) was administered a single 60 mg dose of the third lead candidate siRNA (AD-1747585).

Serial cerebrospinal fluid (CSF) and plasma samples were collected from NHPs of each group for biomarker analysis (including exosome evaluation). Tissues were also collected for the PK/PD studies, to evaluate mRNA and protein levels in each of the following: CNS tissues = cortex, midbrain, striatum, hippocampus, cerebellum, pons, and spinal cord; kidney; liver; and heart.

In an initial observation at the time of injection of cohorts of day 29 experimental NHP animals, with each NHP animal dosed via intrathecal injection with a single 60 mg dose of siRNA, all three candidate lead SNCA-targeting duplexes examined showed similar pharmacokinetics across dosed animals for the immediate 24 hour period after dosing (**FIG. 8B**), consistent with reproducible dosing having occurred and reflective of platform stability.

When SNCA knockdown was examined at day 29 post-injection, pharmacodynamic (PD) effects of greater than 80% knockdown of SNCA mRNA levels in prefrontal cortex and greater than 60% knockdown of SNCA mRNA levels in midbrain were observed for each of the three candidate lead SNCA-targeting duplexes examined (**FIG. 9A**, left panel). For all three tested duplexes, the observed PD effects on SNCA mRNA levels in the NHPs correlated well with pharmacokinetic (PK) distribution of duplexes in examined tissues, as assessed by measurement of duplex tissue concentration. Duplex tissue concentration (PK) "dose"-tissue SNCA mRNA (PD) response curve IC_{50} values of 1.050 µg/g, 0.532 µg/g, and 1.300 µg/g were calculated for AD-

1747580, AD-1747583, and AD-1747585 duplexes, respectively (**FIG. 9A**, right panel). While the calculated differences in IC_{50} values between these three duplexes were not statistically significant, the IC_{50} value ranking of duplexes for target SNCA mRNA knockdown was noted as AD-1747583 < AD-1747580 < AD-1747585. α-synuclein protein levels were also significantly reduced in brain and spinal cord tissues of NHPs administered candidate lead duplexes AD-1747580, AD-1747583, and AD-1747585 (**FIG. 9B**, left panel). At day 29, the greatest α-synuclein protein knockdown was observed in the spinal cord (thoracic) and in CSF (shown in **FIG. 13** below), consistent with the intrathecal dosing route. In brain tissues, greater α-synuclein protein knockdown was observed in the striatum, while tissue protein PK/PD plots were much more scattered for all three candidate lead duplexes (**FIG. 9B**, right panel) than were corresponding plots for SNCA mRNA knockdown (**FIG. 9A**, right panel). The preceding data (including **FIG. 10** summary) indicated that all three duplexes tested (AD-1747580, AD-1747583, and AD-1747585) exhibited similar α-synuclein knockdown potencies, consistent with both mouse AAV in vivo screen data and mRNA PK/PD curves for NHP administrations.

Cohorts of NHPs, each administered one of the three candidate lead compounds, were subsequently assessed at day 84 post-injection. At day 84, high potency SNCA mRNA knockdown was observed in both prefrontal cortex and midbrain for all three duplexes tested (AD-1747580, AD-1747583, and AD-1747585). Specifically, 80-90% or more knockdown of SNCA mRNA was observed in prefrontal cortex across all three duplexes at day 84 (**FIG. 11**, left panel). Consistent with results previously observed at day 29 post-administration, day 84 samples exhibited good correlation between tissue PK and tissue mRNA PD, with similar potencies observed for each of the three candidate lead duplexes examined (**FIG. 11**, right panel). Calculated IC_{50} values of 0.985 μg/g, 0.239 μg/g, and 1.40 μg/g were measured for duplexes AD-1747580, AD-1747583, and AD-1747585, respectively. Notably, the AD-1747585-administered cohort only had one animal that met dosing criteria, which resulted in the remainder of the AD-1747585-administered cohort being re-dosed (six animals in total were re-dosed in this cohort, with only three re-dosed animals ultimately observed to have been well-dosed).

Closer examination of distinct brain regions of dosed NHPs at day 84 revealed significant reduction of both SNCA mRNA (**FIG. 12A**) and α-Synuclein protein (**FIG. 12B**) levels at day 84 post-administration in different brain regions, with little differentiation observed between the three candidate leads tested (AD-1747580, AD-1747583, and AD-1747585 duplexes). SNCA mRNA

was reduced in NHPs at day 84 after administration of 60 mg of duplex agent via the intrathecal route, with similar potencies observed between the three tested duplexes in various brain regions (prefrontal cortex, midbrain, hippocampus, medulla and pons, striatum caudate, and cerebellum) and in the spinal cord (thoracic spine) (**FIG. 12A**). The greatest levels of mRNA knockdown at day 84 were observed in the prefrontal cortex and hippocampus, consistent with the duplexes being distributed via the intrathecal route of administration upon the current platform. Specifically, >60% knockdown of SNCA mRNA was observed in the striatum and midbrain, with some well-dosed animals showing up to 90% knockdown in these deeper brain regions (**FIG. 12A**). α-Synuclein protein levels were also reduced at day 84 in the brain and spinal cord (with all of the following regions showing significant reductions in α-Synuclein protein levels: prefrontal cortex, midbrain, thoracic spine, hippocampus, medulla and pons, striatum caudate, and cerebellum; **FIG. 12B**), again with little differentiation observed between the three candidate lead duplexes. Notably, similar, robust α-Synuclein protein reduction was observed in the spinal cord (thoracic), prefrontal cortex, hippocampus and midbrain (**FIG. 12B**). Up to 75% reduction in α-Synuclein protein was also observed in the medulla pons and striatum caudate, for all three molecules. These data indicated that all 3 leads possessed similar potencies, as well as similar durability. α-Synuclein protein levels exhibited high correlation to SNCA mRNA levels across the different brain regions examined at day 84 (**FIG. 12C**), with R^2 values in excess of 0.9 for all brain/spinal cord regions other than medulla and pons, and correlations in each brain region achieving high levels of statistical significance. Certain NHPs were identified as mis-dosed at initial administration of duplex, particularly some animals that had received the AD-1747585 duplex. Such animals were re-dosed and monitored beyond day 84 (**FIG. 12D**, top and bottom panels, with bottom panel omitting data associated with the documented mis-dosing event(s)). Specifically, re-dosed NHPs examined at beyond day 84 showed robust α-Synuclein protein knockdown in cortex and midbrain tissues, while the greatest magnitude of α-Synuclein protein knockdown was observed in the striatum (caudate), where knockdown levels in excess of 90% reduction were detected in re-dosed animals (**FIG. 12D**). Indeed, even NHP animals that had not met successful dosing criteria at both dosing times (when measured at 24 hours post-administration for levels in cerebrospinal fluid (CSF)) at initial duplex administration exhibited knockdown levels that exceeded 90% in the caudate striatum. Notably, tissue protein pharmacodynamics exhibited similar profiles for all three duplexes in the various CNS regions examined. When re-dosed NHP animals were examined for

α-Synuclein protein levels in cerebrospinal fluid (CSF) across an extended time course that included redose and monitoring at time points beyond day 84, CSF α-Synuclein protein levels exhibited up to a 75% reduction for animals (re)dosed with duplex AD-1747585, while CSF α-Synuclein protein levels exhibited up to a 50% reduction for animals (re)dosed with duplexes AD-1747580 and AD-1747583 (**FIG. 12E**).

Properly dosed NHP animals were also assessed for α-Synuclein protein levels in CSF at day 84 post-duplex administration. Observed levels of α-Synuclein protein in NHP CSF were up to 90% reduced (PD effect) for animals dosed with duplexes AD-1747580 and AD-1747583 (**FIG. 13**). NHP animals dosed with duplex AD-1747585 showed ~75% α-Synuclein protein knockdown in CSF. Duplex AD-1747585-dosed animals notably also exhibited variable CSF α-Synuclein protein knockdown, potentially due to blood contamination, as SNCA is highly expressed in blood cells. Blood cell count data can be assessed, to verify whether blood contamination did indeed occur.

At day 29 post-duplex administration, α-Synuclein protein in the cortex and midbrain of treated NHPs exhibited only modest (<60%) knockdown for all three lead duplexes tested (**FIG. 14A**, left panel). Such modest reductions in α-Synuclein protein levels in cortex and midbrain were indicative of an extended half-life for α-Synuclein protein in such tissues. Tissue protein PK/PD was observed to be scattered for all three tested duplexes (**FIG. 14A**, middle panel); however, tissue α-Synuclein protein level was highly correlated with tissue SNCA mRNA level (**FIG. 14A**, right panel). When levels in CSF were also examined, α-Synuclein protein knockdown in CSF correlated with cortex (**FIG. 14B**, left panel) and striatal (**FIG. 14B**, middle panel) α-Synuclein protein knockdown at day 29 post-administration. Indeed, correlation in the cortex vs CSF α-Synuclein protein knockdown levels was observed to be similar to that seen previously with antisense oligonucleotides targeting SNCA in NHPs. In contrast, midbrain tissue α-Synuclein protein knockdown did not correlate well with CSF α-Synuclein protein knockdown at day 29 post-administration (**FIG. 14B**, right panel). While SNCA mRNA levels were reduced at day 29 post-administration (here, of SNCA-targeting duplex AD-1747580) in prefrontal cortex and midbrain tissues, α-Synuclein protein levels were only modestly reduced, which indicated that a long protein half-life for α-Synuclein protein was likely to explain this discrepancy (**FIG. 14C**). Indeed, the protein half-life of superoxide dismutase type 1 (SOD1) has been assessed to be ~28 days in tissue, and it was apparent that a similar half-life for α-Synuclein protein might apply in

cortex and midbrain tissues. Further evidence that an α-Synuclein protein half-life effect likely caused the disparity between SNCA mRNA and α-Synuclein protein knockdown results at day 29 post-administration was obtained when day 84 post-administration data were obtained. In such day 84 data, α-Synuclein protein levels in the cortex and midbrain tissues of treated NHP animals exhibited robust knockdown for all three lead duplexes (PD effect, **FIG. 14D**, left panel). Robust α-Synuclein protein knockdown observed at day 84 in cortex and midbrain NHP tissues confirmed a long protein half-life for α-Synuclein. Tissue α-Synuclein protein PK/PD curves showed similar profiles for all three duplexes (**FIG. 14D**, middle panel; AD-1747585 cohort is noted as only having had one animal that met dosing criteria, while the remainder of the cohort was re-dosed). α-Synuclein protein levels were highly correlated with mRNA levels in both cortex and midbrain tissues at day 84 (**FIG. 14D**, right panel), in contrast to those observed above at day 29 post-administration.

At day 84 post-duplex administration, the prefrontal cortex and midbrain tissue α-Synuclein protein knockdown levels also correlated well with α-Synuclein CSF protein knockdown (**FIG. 15**). Correlation in the cortex vs. CSF α-Synuclein protein knockdown levels (R squared = 0.7246, P value = 0.0004) was even better than previously described for antisense oligonucleotides targeting SNCA in NHP (R squared = 0.6, P value = 0.0089) (Cole *et al. JCI Insight* (2021), 6(5), e135633. doi: 10.1172/jci.insight.135633).

siRNA tissue exposure was also examined across brain regions at day 84 post-duplex administration. All three candidate duplexes showed good distribution into all examined target tissues, in most NHP animals that were dosed with such duplexes (**FIG. 16**). Interestingly, the tissues with high exposure to siRNA duplexes (e.g., cerebellum) did not necessarily show the highest levels of SNCA mRNA and/or α-Synuclein protein knockdown.

To assess and ultimately attempt to mitigate against any potential detrimental impacts of dosing of candidate lead duplexes to NHP animals, body weight of dosed animals and Neurofilament light chain (NfL) levels in CSF responsive to duplex dosing of animals were monitored as initial indicators of the health of such animals and potential toxicity of the tested duplexes to such animals, while genomic assessments for any significant off-target gene impacts in dosed NHP animals were also performed using RNA-seq. No impact upon body weight was observed for the three candidate lead duplexes administered to NHP animals, either at day 29 or day 84 post-administration, as compared to control NHP animals dosed in parallel with vehicle

only (**FIG. 17**). For NfL detection in CSF of duplex-dosed NHP animals, while transient spikes in NfL were observed after dosing for all three duplexes, such NfL spikes largely did not persist beyond day 29 post-administration, which indicated that the three SNCA-targeting candidate lead duplexes did not appear to have an NfL profile suggestive of duplex toxicity (**FIG. 18**). RNA-Seq data were also obtained and evaluated for cells dosed with each of the three candidate lead duplexes, to identify the extent of off-target effects caused by dosing with each such duplex. RNA-Seq data for each of the three candidate lead duplexes examined. For the AD-1747580 duplex, as well as for parent duplex AD-1549054, SNCA knockdown was uniformly robust and no significant off-target effects (dysregulated off-target genes at > 50%) were identified in potency-matched RNA-Seq profiles (**FIGs. 19A and 19B**). In contrast with the clean RNA-Seq results obtained for the AD-1747580 duplex, RNA-Seq results obtained for the AD-1747583 duplex revealed robust SNCA mRNA knockdown that was accompanied by three genes identified as dysregulated at > 50%, PYGB, NREP and LCLAT1 (**FIG. 19C**). For duplex AD-1747585, robust SNCA mRNA knockdown that was accompanied by one gene identified as dysregulated at > 50%, HMGB2 (**FIG. 19D**). Removal of GalNAc from parent duplexes was identified as associated with an increase in potency, both on- and off-target.

The AD-1747580 duplex was therefore identified via RNA-Seq to possess the most favorable selectivity profile among the three candidate lead duplexes tested (AD-1747580 exhibited 93% SNCA knockdown with no off-target gene dysregulation detected; AD-1747585 exhibited 96% SNCA knockdown with a single off-target gene dysregulated; and AD-1747583 exhibited 92% SNCA knockdown, but with three off-target genes dysregulated) (**FIG. 20**).

Example 5: SNCA-Targeting Duplexes for Parkinson's Disease Therapy

SNCA-targeting duplexes are administered to a subject to knock down SNCA as a Parkinson's disease (PD) therapy, particularly in subjects having a GBA_{PD} mutation (GBA encodes for the lysosomal enzyme glucocerebrosidase (GCase)), or having sporadic PD. Evaluations are performed to assess the extent to which administration of SNCA-targeting duplexes to a subject constitute a disease-modifying therapy for neurodegenerative diseases characterized by alpha synuclein aggregates in the brain (such as PD in subjects having a GBA_{PD} mutation or sporadic PD).

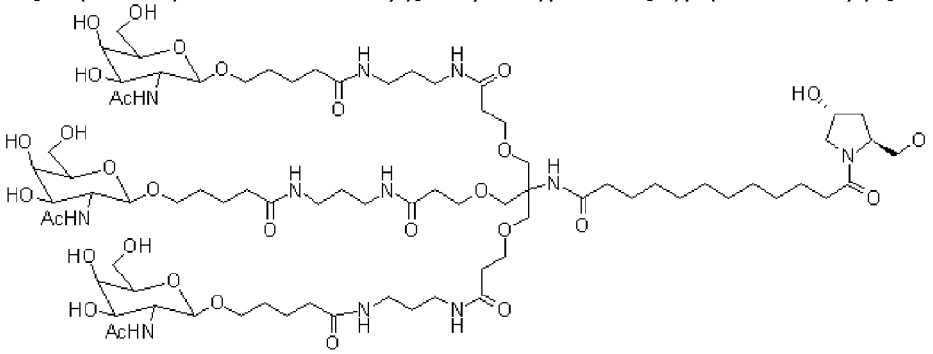
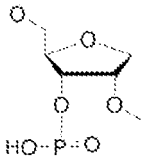
The "mRNA" sequences of the Informal Sequence Listing and certain of the "mRNA target" sequences listed herein may be noted as reciting thymine (T) residues rather than uracil (U) residues. As is apparent to one of ordinary skill in the art, such sequences reciting "T" residues rather than "U" residues can be derived from NCBI accession records that list, as "mRNA" sequences, the DNA sequences (not RNA sequences) that directly correspond to mRNA sequences. Such DNA sequences that directly correspond to mRNA sequences technically constitute the DNA sequence that is the complement of the cDNA (complementary DNA) sequence for an indicated mRNA. Thus, while the mRNA target sequence does, in fact, actually include uracil (U) rather than thymine (T), the NCBI record-derived "mRNA" sequence includes thymine (T) residues rather than uracil (U) residues.

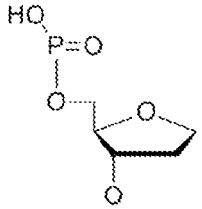
Table 1. Abbreviations of nucleotide monomers used in nucleic acid sequence representation

It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds; and it is understood that when the nucleotide contains a 2'-fluoro modification, then the fluoro replaces the hydroxy at that position in the parent nucleotide (*i.e.*, it is a 2'-deoxy-2'-fluoronucleotide). It will also be understood that the abbreviations correspond to nucleotides which omit the 3'-phosphate when found at the 3'-terminal position (i.e., they are 3'-OH).

Abbreviation	Nucleotide(s)
A	Adenosine-3'-phosphate
Aam	2`-O-(N-methylacetamide)adenosine-3`-phosphate
Aams	2`-O-(N-methylacetamide)adenosine-3`-phosphorothioate
Ab	beta-L-adenosine-3`-phosphate
Abs	beta-L-adenosine-3`-phosphorothioate
Af	2'-fluoroadenosine-3'-phosphate
Afs	2'-fluoroadenosine-3'-phosphorothioate
Agn	Adenosine-glycol nucleic acid (GNA), S-isomer
Ahd	2'-*O*-hexadecyl adenosine-3'-phosphate
Ahds	2'-O-hexadecyl-adenosine-3'-phosphorothioate
As	adenosine-3'-phosphorothioate
C	cytidine-3'-phosphate
Cb	beta-L-cytidine-3`-phosphate

Abbreviation	Nucleotide(s)
Cbs	beta-L-cytidine-3'-phosphorothioate
Cf	2'-fluorocytidine-3'-phosphate
Cfs	2'-fluorocytidine-3'-phosphorothioate
Cgn	Cytidine-glycol nucleic acid (GNA), S-isomer
Chd	2'-*O*-hexadecyl cytidine-3'-phosphate
Chds	2'-O-hexadecyl-cytidine-3'-phosphorothioate
Cs	cytidine-3'-phosphorothioate
G	guanosine-3'-phosphate
Gam	2`-O-(N-methylacetamide)guanosine-3`-phosphate
Gams	2`-O-(N-methylacetamide)guanosine-3`-phosphorothioate
Gb	beta-L-guanosine-3`-phosphate
Gbs	beta-L-guanosine-3`-phosphorothioate
Gf	2'-fluoroguanosine-3'-phosphate
Gfs	2'-fluoroguanosine-3'-phosphorothioate
Ggn	Guanosine-glycol nucleic acid (GNA), S-isomer
Ghd	2'-*O*-hexadecyl guanosine-3'-phosphate
Ghds	2'-O-hexadecyl-guanosine-3'-phosphorothioate
Gs	guanosine-3'-phosphorothioate
T	5'-methyluridine-3'-phosphate
Tam	2`-O-(N-methylacetamide)thymidine-3`-phosphate
Tams	2`-O-(N-methylacetamide)thymidine-3`-phosphorothioate
Tf	2'-fluoro-5-methyluridine-3'-phosphate
Tfs	2'-fluoro-5-methyluridine-3'-phosphorothioate
Tgn	Thymidine-glycol nucleic acid (GNA), S-Isomer
Ts	5-methyluridine-3'-phosphorothioate
U	Uridine-3'-phosphate
Uf	2'-fluorouridine-3'-phosphate
Ufs	2'-fluorouridine -3'-phosphorothioate
Uhd	2'-*O*-hexadecyl uridine-3'-phosphate
Uhds	2'-O-hexadecyl-uridine-3'-phosphorothioate
Us	uridine -3'-phosphorothioate
N	any nucleotide (G, A, C, T or U), modified or unmodified
a	2'-O-methyladenosine-3'-phosphate

Abbreviation	Nucleotide(s)
as	2'-O-methyladenosine-3'- phosphorothioate
c	2'-O-methylcytidine-3'-phosphate
cs	2'-O-methylcytidine-3'- phosphorothioate
g	2'-O-methylguanosine-3'-phosphate
gs	2'-O-methylguanosine-3'- phosphorothioate
t	2'-O-methyl-5-methyluridine-3'-phosphate
ts	2'-O-methyl-5-methyluridine-3'-phosphorothioate
u	2'-O-methyluridine-3'-phosphate
us	2'-O-methyluridine-3'-phosphorothioate
s	phosphorothioate linkage
(C2p)	cytidine-2`-phosphate
(G2p)	guanosine-2`-phosphate
(U2p)	uridine-2`-phosphate
(A2p)	adenosine-2`-phosphate
L96	N-[tris(GalNAc)-amido-dodecanoyl)]-4-hydroxyprolinol [Hyp-(GalNAc-alkyl)3]  i.e., (2S,4R)-1-[29-[[2-(acetylamino)-2-deoxy-β-D-galactopyranosyl]oxy]-14,14-bis[[3-[[3-[[5-[[2-(acetylamino)-2-deoxy-β-D-galactopyranosyl]oxy]-1-oxopentyl]amino]propyl]amino]-3-oxopropoxy]methyl]-1,12,19,25-tetraoxo-16-oxa-13,20,24-triazanonacos-1-yl]-4-hydroxy-2-hydroxymethylpyrrolidine
Y34	2-hydroxymethyl-tetrahydrofuran-4-methoxy-3-phosphate (abasic 2'-OMe furanose) 

Abbreviation	Nucleotide(s)
Y44	inverted abasic DNA (2-hydroxymethyl-tetrahydrofuran-5-phosphate) 
dA	2`-deoxyadenosine-3`-phosphate
dAs	2`-deoxyadenosine-3`-phosphorothioate
dC	2`-deoxycytidine-3`-phosphate
dCs	2`-deoxycytidine-3`-phosphorothioate
dG	2`-deoxyguanosine-3`-phosphate
dGs	2`-deoxyguanosine-3`-phosphorothioate
dT	2`-deoxythymidine-3`-phosphate
dTs	2`-deoxythymidine-3`-phosphorothioate
dU	2`-deoxyuridine
dUs	2`-deoxyuridine-3`-phosphorothioate
Aeo	2`-O-methoxyethyladenosine-3`-phosphate
Aeos	2`-O-methoxyethyladenosine-3`-phosphorothioate
Geo	2`-O-methoxyethylguanosine-3`-phosphate
Geos	2`-O-methoxyethylguanosine-3`-phosphorothioate
Teo	2`-O-methoxyethyl-5-methyluridine-3`-phosphate
Teos	2`-O-methoxyethyl-5-methyluridine-3`-phosphorothioate
m5Ceo	2`-O-methoxyethyl-5-methylcytidine-3`-phosphate
m5Ceos	2`-O-methoxyethyl-5-methylcytidine-3`-phosphorothioate
A3m	3`-O-methyladenosine-2`-phosphate
A3mx	3`-O-methyl-xylofuranosyladenosine-2`-phosphate
G3m	3`-O-methylguanosine-2`-phosphate
G3mx	3`-O-methyl-xylofuranosylguanosine-2`-phosphate
C3m	3`-O-methylcytidine-2`-phosphate
C3mx	3`-O-methyl-xylofuranosylcytidine-2`-phosphate
U3m	3`-O-methyluridine-2`-phosphate
U3mx	3`-O-methyl-xylofuranosyluridine-2`-phosphate
m5Cam	2`-O-(N-methylacetamide)-5-methylcytidine-3`-phosphate
m5Cams	2`-O-(N-methylacetamide)-5-methylcytidine-3`-phosphorothioate

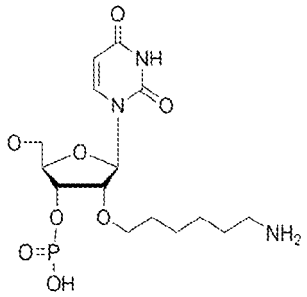
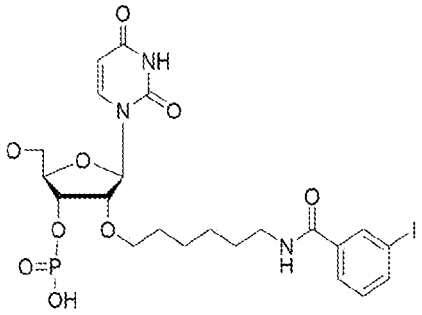
Abbreviation	Nucleotide(s)
P	Phosphate
pshe	Hydroxyethylphosphorothioate
VP	Vinyl-phosphonate
(Aah)	2'-O-(6-aminohexyl)-adenosine-3'-phosphate
(Gah)	2'-O-(6-aminohexyl)-guanosine-3'-phosphate
(Cah)	2'-O-(6-aminohexyl)-cytidine-3'-phosphate
(Uah)	2'-O-(6-aminohexyl)-uridine-3'-phosphate 
Y196	2'-O-(6-aminohexyl-metaiodobenzoyl)-uridine-3'-phosphate (Uah with iodo-benzoyl) 

Table 2. Modified Sense and Antisense Strand Sequences of CNS-Directed SNCA-Targeting siRNAs

Duplex Name	Sense Oligo Sequence	SEQ ID NO:	Antisense Oligo Sequence	SEQ ID NO:	mRNA Target Sequence	SEQ ID NO:	Parent Duplex Name
AD-1804698	gsusgug(Ghd)UfgUfAfAfaggaauucsasa	13	VPusUfsgadAu(Tgn)ccuuuaCfaCfcacacsusg	56	CAGUGUGGUGUAAAGGAAUUCAU	99	AD-1571164
AD-1804699	usgsugg(Uhd)guAfAfAfggaauucasusa	14	VPusdAsugdAadTuccudTuAfcaccacascsu	57	AGUGUGGUGUAAAGGAAUUCAUU	100	AD-1548851
AD-1747575	gsgsugu(Ahd)aaGfGfAfauucauuasgsa	15	VPusdCsuadAudGaauudCcUfuuacaccsasc	58	GUGGUGUAAAGGAAUUCAUUAGC	101	AD-1548854
AD-1747576	asusuag(Chd)caUfGfGfauguauucsasa	16	VPusdTsgadAudAcaucdCaUfggcuaausgsa	59	UCAUUAGCCAUGGAUGUAUUCAU	102	AD-1548869
AD-1804700	ususagc(Chd)auGfGfAfuguauucasusa	17	VPusdAsugdAadTacaudCcAfuggcuaasusg	60	CAUUAGCCAUGGAUGUAUUCAUG	103	AD-1548870
AD-1747577	asusuca(Uhd)gaAfAfGfgacuuucasasa	18	VPusdTsugdAadAguccdTuUfcaugaausasc	61	GUAUUCAUGAAAGGACUUUCAAA	104	AD-1548884
AD-1747578	uscsaug(Ahd)aaGfGfAfcuuucaaasgsa	19	VPusdCsuudTgdAaagudCcUfuucaugasasu	62	AUUCAUGAAAGGACUUUCAAAGG	105	AD-1548886
AD-1747579	asasgag(Chd)aaGfUfGfacaaaugususa	20	VPusdAsacdAudTugucdAcUfugcucuususg	63	CAAAGAGCAAGUGACAAAUGUUG	106	AD-1549052
AD-1747580	gsasgca(Ahd)guGfAfCfaaauguugsgsa	21	VPusdCscadAcdAuuugdTcAfcuugcucsusu	64	AAGAGCAAGUGACAAAUGUUGGA	107	AD-1549054
AD-1747581	asasggg(Uhd)auCfAfAfgacuacgasasa	22	VPusdTsucdGudAgucudTgAfuacccuuscsc	65	GGAAGGGUAUCAAGACUACGAAC	108	AD-1549245
AD-1804701	csusgaa(Ghd)ccUfAfAfgaaauaucsusa	23	VPusdAsgadTadTuucudTaGfgcuucagsgsu	66	ACCUGAAGCCUAAGAAAUAUCUU	109	AD-1549266
AD-1747582	usgsaag(Chd)cuAfAfGfaaauaucususa	24	VPusdAsagdAudAuuucdTuAfggcuucasgsg	67	CCUGAAGCCUAAGAAAUAUCUUU	110	AD-1549267
AD-1804702	asasgcc(Uhd)aaGfAfAfauaucuuusgsa	25	VPusdCsaadAgdAuauudTcUfuaggcuuscsa	68	UGAAGCCUAAGAAAUAUCUUUGC	111	AD-1549269
AD-1804703	cscsuaa(Ghd)aaAfUfAfucuuugcuscsa	26	VPusdGsagdCadAagaudAuUfucuuaggscsu	69	AGCCUAAGAAAUAUCUUUGCUCC	112	AD-1549272
AD-1804704	uscsuuu(Ghd)cuCfCfCfaguuucuusgsa	27	VPusdCsaadGadAacugdGgAfgcaaagasusa	70	UAUCUUUGCUCCCAGUUUCUUGA	113	AD-1549283
AD-1747583	uscsuuugcuCfCfCfaguu(Uhd)cuusgsa	28	VPusdCsaadGadAacugdGgAfgcaaagasusa	71	UAUCUUUGCUCCCAGUUUCUUGA	114	AD-1746465
AD-1804705	csusuug(Chd)ucCfCfCfAfguuucuugsasa	29	VPusdTscadAgdAaacudGgGfagcaaagsasu	72	AUCUUUGCUCCCAGUUUCUUGAG	115	AD-1549284

Duplex Name	Sense Oligo Sequence	SEQ ID NO:	Antisense Oligo Sequence	SEQ ID NO:	mRNA Target Sequence	SEQ ID NO:	Parent Duplex Name
AD-1804706	ususugc(Uhd)ccCfAfGfuuucuugasgsa	30	VPusdCsucdAadGaaacdTgGfgagcaaasgsa	73	UCUUUGCUCCCAG UUUCUUGAGA	116	AD-1549285
AD-1804707	uscscca(Ghd)uuUfCfUfugagaucusgsa	31	VPusdCsagdAudCucaadGaAfacugggasgsc	74	GCUCCCAGUUUCU UGAGAUCUGC	117	AD-1549290
AD-1804708	csusgua(Chd)AfaGfUfGfcucaguucscsa	32	VPusGfsgadAc(Tgn)gagcacUfuGfuacagsgsa	75	UCCUGUACAAGUG CUCAGUUCCA	118	AD-1571187
AD-1804709	usgsuac(Ahd)AfgUfGfCfucaguuccsasa	33	VPusUfsggdAa(C2p)ugagcaCfuUfguacasgsg	76	CCUGUACAAGUGC UCAGUUCCAA	119	AD-1571262
AD-1747591	gsusaca(Ahd)GfuGfCfUfcaguuccasasa	34	VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg	77	CUGUACAAGUGCU CAGUUCCAAU	120	AD-1571188
AD-1747585	asasgug(Chd)ucAfGfUfuccaaugusgsa	35	VPusdCsacdAudTggaadCuGfagcacuusgsu	78	ACAAGUGCUCAGU UCCAAUGUGC	121	AD-1549333
AD-1804710	asgsugc(Uhd)caGfUfUfccaaugugscsa	36	VPusdGscadCadTuggadAcUfgagcacususg	79	CAAGUGCUCAGUU CCAAUGUGCC	122	AD-1549334
AD-1804711	usgsccc(Ahd)guCfAfUfgacauuucsusa	37	VPusdAsgadAadTgucadTgAfcugggcascsa	80	UGUGCCCAGUCAU GACAUUUCUC	123	AD-1549351
AD-1747586	cscsagu(Chd)auGfAfCfauuucucasasa	38	VPusdTsugdAgdAaaugdTcAfugacuggsgsc	81	GCCCAGUCAUGAC AUUUCUCAAA	124	AD-1549354
AD-1804712	gsuscau(Ghd)acAfUfUfucucaaagsusa	39	VPusdAscudTudGagaadAuGfucaugacsusg	82	CAGUCAUGACAUU UCUCAAAGUU	125	AD-1549357
AD-1747587	csasuga(Chd)auUfUfCfucaaaguususa	40	VPusdAsaadCudTugagdAaAfugucaugsasc	83	GUCAUGACAUUUC UCAAAGUUUU	126	AD-1549359
AD-1804713	uscsuuc(Chd)auCfAfGfcagugauusgsa	41	VPusdCsaadTcdAcugcdTgAfuggaagascsu	84	AGUCUUCCAUCAG CAGUGAUUGA	127	AD-1549397
AD-1804714	ususcca(Uhd)CfaGfCfAfgugauugasasa	42	VPusUfsucdAa(Tgn)cacugcUfgAfuggaasgsa	85	UCUUCCAUCAGCA GUGAUUGAAG	128	AD-1571191
AD-1747588	cscsauc(Ahd)gcAfGfUfgauugaagsusa	43	VPusdAscudTcdAaucadCuGfcugauggsasa	86	UUCCAUCAGCAGU GAUUGAAGUA	129	AD-1549401
AD-1804715	cscsauc(Ahd)GfcAfGfUfgauugaagsusa	44	VPusAfscudTc(Agn)aucacuGfcUfgauggsasa	87	UUCCAUCAGCAGU GAUUGAAGUA	130	AD-1571192
AD-1804716	asuscag(Chd)agUfGfAfuugaaguasusa	45	VPusdAsuadCudTcaaudCaCfugcugausgsg	88	CCAUCAGCAGUGA UUGAAGUAUC	131	AD-1549403
AD-1804717	asuscag(Chd)AfgUfGfAfuugaaguasusa	46	VPusAfsuadCu(Tgn)caaucaCfuGfcugausgsg	89	CCAUCAGCAGUGA UUGAAGUAUC	132	AD-1571193
AD-1804718	asgscag(Uhd)gaUfUfGfaaguaucusgsa	47	VPusdCsagdAudAcuucdAaUfcacugcusgsa	90	UCAGCAGUGAUUG AAGUAUCUGU	133	AD-1549406

Duplex Name	Sense Oligo Sequence	SEQ ID NO:	Antisense Oligo Sequence	SEQ ID NO:	mRNA Target Sequence	SEQ ID NO:	Parent Duplex Name
AD-1804719	gscsagu(Ghd)auUfGfAfaguaucugsusa	48	VPusdAscadGadTacuudCaAfucacugcsusg	91	CAGCAGUGAUUGA AGUAUCUGUA	134	AD-1549407
AD-1804720	gscsagu(Ghd)AfuUfGfAfaguaucugsusa	49	VPusAfscadGa(Tgn)acuucaAfuCfacugcsusg	92	CAGCAGUGAUUGA AGUAUCUGUA	135	AD-1571194
AD-1804721	ususcac(Uhd)gaAfGfUfgaauacausgsa	50	VPusdCsaudGudAuucadCuUfcagugaasasg	93	CUUUCACUGAAGU GAAUACAUGG	136	AD-1549439
AD-1804722	usasagu(Ghd)acUfAfCfcacuuauususa	51	VPusdAsaadTadAguggdTaGfucacuuasgsg	94	CCUAAGUGACUAC CACUUAUUUC	137	AD-1549518
AD-1804723	csusacc(Ahd)cuUfAfUfuucuaaauscsa	52	VPusdGsaudTudAgaaadTaAfgugguagsusc	95	GACUACCACUUAU UUCUAAAUCC	138	AD-1549525
AD-1804724	csusacc(Ahd)CfuUfAfUfuucuaaauscsa	53	VPusGfsaudTu(Agn)gaaauaAfgUfgguagsusc	96	GACUACCACUUAU UUCUAAAUCC	139	AD-1571199
AD-1804725	usasugu(Ghd)agCfAfUfgaaacuausgsa	54	VPusdCsaudAgdTuucadTgCfucacauasusu	97	AAUAUGUGAGCAU GAAACUAUGC	140	AD-1549628
AD-1804726	asascua(Uhd)gcAfCfCfuauaaauascsa	55	VPusdGsuadTudTauagdGuGfcauaguususc	98	GAAACUAUGCACC UAUAAAUACU	141	AD-1549641

Table 3. Unmodified Sense and Antisense Strand Sequences of CNS-Directed SNCA-Targeting siRNAs

Duplex Name	Sense Oligo Sequence	SEQ ID NO:	Antisense Oligo Sequence	SEQ ID NO:
AD-1804698	GUGUGGUGUAAAGGAAUUCAA	142	UUGAAUTCCUUUACACCACACUG	185
AD-1804699	UGUGGUGUAAAGGAAUUCAUA	143	UAUGAATUCCUTUACACCACACU	186
AD-1747575	GGUGUAAAGGAAUUCAUUAGA	144	UCUAAUGAAUUCCUUUACACCAC	187
AD-1747576	AUUAGCCAUGGAUGUAUUCAA	145	UTGAAUACAUCCAUGGCUAAUGA	188
AD-1804700	UUAGCCAUGGAUGUAUUCAUA	146	UAUGAATACAUCCAUGGCUAAUG	189
AD-1747577	AUUCAUGAAAGGACUUUCAAA	147	UTUGAAAGUCCTUUCAUGAAUAC	190
AD-1747578	UCAUGAAAGGACUUUCAAAGA	148	UCUUTGAAAGUCCUUUCAUGAAU	191
AD-1747579	AAGAGCAAGUGACAAAUGUUA	149	UAACAUTUGUCACUUGCUCUUUG	192
AD-1747580	GAGCAAGUGACAAAUGUUGGA	150	UCCAACAUUUGTCACUUGCUCUU	193
AD-1747581	AAGGGUAUCAAGACUACGAAA	151	UTUCGUAGUCUTGAUACCCUUCC	194
AD-1804701	CUGAAGCCUAAGAAAUAUCUA	152	UAGATATUUCUTAGGCUUCAGGU	195
AD-1747582	UGAAGCCUAAGAAAUAUCUUA	153	UAAGAUAUUUCTUAGGCUUCAGG	196
AD-1804702	AAGCCUAAGAAAUAUCUUUGA	154	UCAAAGAUAUUTCUUAGGCUUCA	197
AD-1804703	CCUAAGAAAUAUCUUUGCUCA	155	UGAGCAAAGAUAUUUCUUAGGCU	198
AD-1804704	UCUUUGCUCCCAGUUUCUUGA	156	UCAAGAAACUGGGAGCAAAGAUA	199
AD-1747583	UCUUUGCUCCCAGUUUCUUGA	157	UCAAGAAACUGGGAGCAAAGAUA	200
AD-1804705	CUUUGCUCCCAGUUUCUUGAA	158	UTCAAGAAACUGGGAGCAAAGAU	201
AD-1804706	UUUGCUCCCAGUUUCUUGAGA	159	UCUCAAGAAACTGGGAGCAAAGA	202
AD-1804707	UCCCAGUUUCUUGAGAUCUGA	160	UCAGAUCUCAAGAAACUGGGAGC	203
AD-1804708	CUGUACAAGUGCUCAGUUCCA	161	UGGAACTGAGCACUUGUACAGGA	204
AD-1804709	UGUACAAGUGCUCAGUUCCAA	162	UUGGAACUGAGCACUUGUACAGG	205
AD-1747591	GUACAAGUGCUCAGUUCCAAA	163	UUUGGAACUGAGCACUUGUACAG	206
AD-1747585	AAGUGCUCAGUUCCAAUGUGA	164	UCACAUTGGAACUGAGCACUUGU	207
AD-1804710	AGUGCUCAGUUCCAAUGUGCA	165	UGCACATUGGAACUGAGCACUUG	208

Duplex Name	Sense Oligo Sequence	SEQ ID NO:	Antisense Oligo Sequence	SEQ ID NO:
AD-1804711	UGCCCAGUCAUGACAUUUCUA	166	UAGAAATGUCATGACUGGGCACA	209
AD-1747586	CCAGUCAUGACAUUUCUCAAA	167	UTUGAGAAAUGTCAUGACUGGGC	210
AD-1804712	GUCAUGACAUUUCUCAAAGUA	168	UACUTUGAGAAAUGUCAUGACUG	211
AD-1747587	CAUGACAUUUCUCAAAGUUUA	169	UAAACUTUGAGAAAUGUCAUGAC	212
AD-1804713	UCUUCCAUCAGCAGUGAUUGA	170	UCAATCACUGCTGAUGGAAGACU	213
AD-1804714	UUCCAUCAGCAGUGAUUGAAA	171	UUUCAATCACUGCUGAUGGAAGA	214
AD-1747588	CCAUCAGCAGUGAUUGAAGUA	172	UACUTCAAUCACUGCUGAUGGAA	215
AD-1804715	CCAUCAGCAGUGAUUGAAGUA	173	UACUTCAAUCACUGCUGAUGGAA	216
AD-1804716	AUCAGCAGUGAUUGAAGUAUA	174	UAUACUTCAAUCACUGCUGAUGG	217
AD-1804717	AUCAGCAGUGAUUGAAGUAUA	175	UAUACUTCAAUCACUGCUGAUGG	218
AD-1804718	AGCAGUGAUUGAAGUAUCUGA	176	UCAGAUACUUCAAUCACUGCUGA	219
AD-1804719	GCAGUGAUUGAAGUAUCUGUA	177	UACAGATACUUCAAUCACUGCUG	220
AD-1804720	GCAGUGAUUGAAGUAUCUGUA	178	UACAGATACUUCAAUCACUGCUG	221
AD-1804721	UUCACUGAAGUGAAUACAUGA	179	UCAUGUAUUCACUUCAGUGAAAG	222
AD-1804722	UAAGUGACUACCACUUAUUUA	180	UAAATAAGUGGTAGUCACUUAGG	223
AD-1804723	CUACCACUUAUUUCUAAAUCA	181	UGAUTUAGAAATAAGUGGUAGUC	224
AD-1804724	CUACCACUUAUUUCUAAAUCA	182	UGAUTUAGAAAUAAGUGGUAGUC	225
AD-1804725	UAUGUGAGCAUGAAACUAUGA	183	UCAUAGTUUCATGCUCACAUAUU	226
AD-1804726	AACUAUGCACCUAUAAAUACA	184	UGUATUTAUAGGUGCAUAGUUUC	227

Table 4. Duplex Target Locations Within SNCA mRNA

Sequence Name	Min	Max	Target region	Type
AD-1804698	198	220	Exon 1-2	5'UTR
AD-1804699	199	221	Exon 1-2	5'UTR
AD-1747575	202	224	Exon 2	5'UTR
AD-1747576	217	239	Exon 2	5'UTR

AD-1804700	218	240	Exon 2	5'UTR
AD-1747577	232	254	Exon 2	CDS
AD-1747578	234	256	Exon 2	CDS
AD-1747579	402	424	Exon 4	CDS
AD-1747580	404	426	Exon 4	CDS
AD-1747581	615	637	Exon 5-6	CDS
AD-1804701	636	658	Exon 6	CDS-3'UTR
AD-1747582	637	659	Exon 6	CDS-3'UTR
AD-1804702	639	661	Exon 6	CDS-3'UTR
AD-1804703	642	664	Exon 6	CDS-3'UTR
AD-1747583	653	675	Exon 6	3'UTR
AD-1804704	653	675	Exon 6	3'UTR
AD-1804705	654	676	Exon 6	3'UTR
AD-1804706	655	677	Exon 6	3'UTR
AD-1804707	660	682	Exon 6	3'UTR
AD-1804708	697	719	Exon 6	3'UTR
AD-1804709	698	720	Exon 6	3'UTR
AD-1747591	699	721	Exon 6	3'UTR
AD-1747585	703	725	Exon 6	3'UTR
AD-1804710	704	726	Exon 6	3'UTR
AD-1804711	721	743	Exon 6	3'UTR
AD-1747586	724	746	Exon 6	3'UTR
AD-1804712	727	749	Exon 6	3'UTR
AD-1747587	729	751	Exon 6	3'UTR
AD-1804713	767	789	Exon 6	3'UTR
AD-1804714	769	791	Exon 6	3'UTR
AD-1804715	771	793	Exon 6	3'UTR
AD-1747588	771	793	Exon 6	3'UTR
AD-1804716	773	795	Exon 6	3'UTR
AD-1804717	773	795	Exon 6	3'UTR
AD-1804718	776	798	Exon 6	3'UTR

AD-1804719	777	799	Exon 6	3'UTR
AD-1804720	777	799	Exon 6	3'UTR
AD-1804721	830	852	Exon 6	3'UTR
AD-1804722	920	942	Exon 6	3'UTR
AD-1804724	927	949	Exon 6	3'UTR
AD-1804723	927	949	Exon 6	3'UTR
AD-1804725	1090	1112	Exon 6	3'UTR
AD-1804726	1103	1125	Exon 6	3'UTR

Table 5. Additional SNCA-Targeting Duplexes

Duplex Name	Sense Sequence	SEQ ID NO:	Antisense Sequence	SEQ ID NO:
AD-1548851	usgsugg(Uhd)guAfAfAfggaauucauaL96	270	VPusdAsugdAadTuccudTuAfcaccacascsu	325
AD-1548854	gsgsugu(Ahd)aaGfGfAfauucauuagaL96	271	VPusdCsuadAudGaauudCcUfuuacaccsasc	326
AD-1548869	asusuag(Chd)caUfGfGfauguauucaaL96	272	VPusdTsgadAudAcaucdCaUfggcuaausgsa	327
AD-1548870	ususagc(Chd)auGfGfAfuguauucauaL96	273	VPusdAsugdAadTacaudCcAfuggcuaasusg	328
AD-1548884	asusuca(Uhd)gaAfAfGfgacuuucaaaL96	274	VPusdTsugdAadAguccdTuUfcaugaausasc	329
AD-1548886	uscsaug(Ahd)aaGfGfAfcuuucaaagaL96	275	VPusdCsuudTgdAaagudCcUfuucaugasasu	330
AD-1549052	asasgag(Chd)aaGfUfGfacaaauguuaL96	276	VPusdAsacdAudTugucdAcUfugcucuususg	331
AD-1549054	gsasgca(Ahd)guGfAfCfaaauguuggaL96	277	VPusdCscadAcdAuuugdTcAfcuugcucsusu	332
AD-1549245	asasggg(Uhd)auCfAfAfgacuacgaaaL96	278	VPusdTsucdGudAgucudTgAfuacccuuscsc	333
AD-1549266	csusgaa(Ghd)ccUfAfAfgaaauaucuaL96	279	VPusdAsgadTadTuucudTaGfgcuucagsgsu	334
AD-1549267	usgsaag(Chd)cuAfAfGfaaauaucuuaL96	280	VPusdAsagdAudAuuucdTuAfggcuucasgsg	335
AD-1549269	asasgcc(Uhd)aaGfAfAfauaucuuugaL96	281	VPusdCsaadAgdAuauudTcUfuaggcuuscsa	336
AD-1549272	cscsuaa(Ghd)aaAfUfAfucuuugcucaL96	282	VPusdGsagdCadAagaudAuUfucuuaggscsu	337
AD-1549283	uscsuuu(Ghd)cuCfCfCfaguuucuugaL96	283	VPusdCsaadGadAacugdGgAfgcaaagasusa	338
AD-1549284	csusuug(Chd)ucCfCfAfguuucuugaaL96	284	VPusdTscadAgdAaacudGgGfagcaaagsasu	339
AD-1549285	ususugc(Uhd)ccCfAfGfuuucuugagaL96	285	VPusdCsucdAadGaaacdTgGfgagcaaasgsa	340
AD-1549290	uscscca(Ghd)uuUfCfUfugagaucugaL96	286	VPusdCsagdAudCucaadGaAfacugggasgsc	341
AD-1549333	asasgug(Chd)ucAfGfUfuccaaugugaL96	287	VPusdCsacdAudTggaadCuGfagcacuusgsu	342
AD-1549334	asgsugc(Uhd)caGfUfUfccaaugugcaL96	288	VPusdGscadCadTuggadAcUfgagcacususg	343
AD-1549351	usgsccc(Ahd)guCfAfUfgacauuucuaL96	289	VPusdAsgadAadTgucadTgAfcugggcascsa	344
AD-1549354	cscsagu(Chd)auGfAfCfauuucucaaaL96	290	VPusdTsugdAgdAaaugdTcAfugacuggsgsc	345

AD-1549357	gsuscau(Ghd)acAfUfUfucucaaaguaL96	291	VPusdAscudTudGagaadAuGfucaugacsusg	346
AD-1549359	csasuga(Chd)auUfUfCfucaaaguuuaL96	292	VPusdAsaadCudTugagdAaAfugucaugsasc	347
AD-1549397	uscsuuc(Chd)auCfAfGfcagugauugaL96	293	VPusdCsaadTcdAcugcdTgAfuggaagascsu	348
AD-1549401	cscsauc(Ahd)gcAfGfUfgauugaaguaL96	294	VPusdAscudTcdAaucadCuGfcugauggsasa	349
AD-1549403	asuscag(Chd)agUfGfAfuugaaguauaL96	295	VPusdAsuadCudTcaaudCaCfugcugausgsg	350
AD-1549406	asgscag(Uhd)gaUfUfGfaaguaucugaL96	296	VPusdCsagdAudAcuucdAaUfcacugcusgsa	351
AD-1549407	gscsagu(Ghd)auUfGfAfaguaucuguaL96	297	VPusdAscadGadTacuudCaAfucacugcsusg	352
AD-1549439	ususcac(Uhd)gaAfGfUfgaauacaugaL96	298	VPusdCsaudGudAuucadCuUfcagugaasasg	353
AD-1549518	usasagu(Ghd)acUfAfCfcacuuauuuaL96	299	VPusdAsaadTadAguggdTaGfucacuuasgsg	354
AD-1549525	csusacc(Ahd)cuUfAfUfuucuaaaucaL96	300	VPusdGsaudTudAgaaadTaAfugguagsusc	355
AD-1549628	usasugu(Ghd)agCfAfUfgaaacuaugaL96	301	VPusdCsaudAgdTuucadTgCfucacauasusu	356
AD-1549641	asascua(Uhd)gcAfCfCfuauaaauacaL96	302	VPusdGsuadTudTauagdGuGfcauaguususc	357
AD-1571164	gsusgug(Ghd)UfgUfAfAfaggaauucaaL96	303	VPusUfsgadAu(Tgn)ccuuuaCfaCfcacacsusg	358
AD-1571187	csusgua(Chd)AfaGfUfGfcucaguuccaL96	304	VPusGfsgadAc(Tgn)gagcacUfuGfuacagsgsa	359
AD-1571188	gsusaca(Ahd)GfuGfCfUfcaguuccaaaL96	305	VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg	360
AD-1571191	ususcca(Uhd)CfaGfCfAfgugauugaaaL96	306	VPusUfsucdAa(Tgn)cacugcUfgAfuggaasgsa	361
AD-1571192	cscsauc(Ahd)GfcAfGfUfgauugaaguaL96	307	VPusAfscudTc(Agn)aucacuGfcUfgauggsasa	362
AD-1571193	asuscag(Chd)AfgUfGfAfuugaaguauaL96	308	VPusAfsuadCu(Tgn)caaucaCfuGfcugausgsg	363
AD-1571194	gscsagu(Ghd)AfuUfGfAfaguaucuguaL96	309	VPusAfscadGa(Tgn)acuucaAfuCfacugcsusg	364
AD-1571199	csusacc(Ahd)CfuUfAfUfuucuaaaucaL96	310	VPusGfsaudTu(Agn)gaaauaAfgUfgguagsusc	365
AD-1571262	usgsuac(Ahd)AfgUfGfCfucaguuccaaL96	311	VPusUfsggdAa(C2p)ugagcaCfuUfguacasgsg	366
AD-1746465	uscsuuugcuCfCfCfaguu(Uhd)cuugaL96	312	VPusdCsaadGadAacugdGgAfgcaaagasusa	367
AD-464229	asusgaaaGfgAfCfUfuucaaaggcaL96	313	VPusGfsccuUfuGfAfaaguCfcUfuucausgsa	368
AD-464313	asasagagGfgUfGfUfucucuauguaL96	314	VPusAfscauAfgAfGfaacaCfcCfucuuususg	369
AD-464314	asasgaggGfuGfUfUfcucuauguaaL96	315	VPusUfsacaUfaGfAfgaacAfcCfcucuususu	370
AD-464585	asasggguAfuCfAfAfgacuacgaaaL96	316	VPusUfsucgUfaGfUfcuugAfuAfcccuuscsc	371
AD-464586	asgsgguaUfcAfAfGfacuacgaacaL96	317	VPusGfsuucGfuAfGfucuuGfaUfacccususc	372
AD-464590	usasucaaGfaCfUfAfcgaaccugaaL96	318	VPusUfscagGfuUfCfguagUfcUfugauascsc	373
AD-464592	uscsaagaCfuAfCfGfaaccugaagaL96	319	VPusCfsuucAfgGfUfucguAfgUfcuugasusa	374
AD-464634	asgsuuucUfuGfAfGfaucugcugaaL96	320	VPusUfscagCfaGfAfucucAfaGfaaacusgsg	375
AD-464694	cscsagucAfuGfAfCfauuucucaaaL96	321	VPusUfsugaGfaAfAfugucAfuGfacuggsgsc	376
AD-464778	ususucacUfgAfAfGfugaauacauaL96	322	VPusAfsuguAfuUfCfacuuCfaGfugaaasgsg	377
AD-464779	ususcacuGfaAfGfUfgaauacaugaL96	323	VPusCfsaugUfaUfUfcacuUfcAfgugaasasg	378
AD-464782	ascsugaaGfuGfAfAfuacaugguaaL96	324	VPusUfsaccAfuGfUfauucAfcUfucagusgsa	379

INFORMAL SEQUENCE LISTING

SEQ ID NO: 1
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DEFINITION Homo sapiens synuclein alpha (SNCA), transcript variant 4, mRNA. VERSION NM_007308.3

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SEQ ID NO: 2
Reverse complement of SEQ ID NO: 1
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SEQ ID NO: 3
LOCUS XM_005555421 2955 bp mRNA linear PRI 25-JAN-2016
DEFINITION PREDICTED: Macaca fascicularis synuclein alpha (SNCA), transcript variant X7, mRNA.
VERSION XM_005555421.2

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SEQ ID NO: 4
Reverse complement of SEQ ID NO: 3
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SEQ ID NO: 5
LOCUS NM_009221 1208 bp mRNA linear ROD 06-SEP-2020
DEFINITION Mus musculus synuclein, alpha (Snca), transcript variant
2, mRNA. VERSION NM_009221.2
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1201 aaaaaaaa

SEQ ID NO: 6
Reverse complement of SEQ ID NO: 5
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SEQ ID NO: 7
LOCUS NM_019169 1176 bp mRNA linear ROD 23-AUG-2020
DEFINITION Rattus norvegicus synuclein alpha (Snca), mRNA.
VERSION NM_019169.2
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SEQ ID NO: 8
Reverse complement of SEQ ID NO: 7
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cttccgcagaactctgattcctcctgttttccccaatctaatttcaatgcaagaggggtgaaaggctctg
ggctgaggggcaagggcaggggagcacctgctggtctcctgccgtctgctgccgg

SEQ ID NO: 228
LOCUS XM_535656 1493 bp mRNA linear MAM
06-JAN-2021
DEFINITION PREDICTED: Canis lupus familiaris synuclein alpha (SNCA),
transcript variant X12, mRNA.
ACCESSION XM_535656
VERSION XM_535656.7
1 cggcagaggg gcggggagag gcgctgacaa atcagctgcg ggggcggtga gccgaggaga
61 aggaggagaa agaggaaggg gaggaagacc acgacgactt gcaggggacc cgagagaggg
121 ggtgagagac cgagcgcggc agcgtggggg tgagtgtggt gtgaacgaat tcattagcca
181 tggatgtatt catgaaagga ctttcaaagg ccaaggaggg agtcgtggct gctgctgaaa
241 aaaccaaaca gggtgtggca gaagcagcag gaaagacaaa agagggtgtc ctctatgtag
301 gctccaaaac caaggaagga gtggttcatg gtgtgacaac agtggctgag aagaccaaag
361 agcaagtgac aaatgttggt gaggccgtgg tgacaggggt gacagcagta gcacaaaaga
421 cagtggaggg agcagggagc atcgcagctg ctactggctt tggcaaaaag gatcagttgg
481 gcaagagtga agaaggaggc ccacaggaag gaattctgga agatatgcct gttgatcctg
541 acaatgaggc atatgaaatg ccttctgagg aagggtatca agactatgaa cccgaagcct
601 aagaaatact tttgctccca gtttcttgag acctactgac agatgttcca tcctgtacaa
661 gtactcagtt ccaaaatgcc cagtcataac attttctcaa aatttttaca gtgtatttta
721 aactcttcca tcagcagtga ttgaagttat ctgtaccagc ccctactcag catttcagtg
781 cttcctctc actgaagtga ttatatggta gcagggtcct cccttgtgtg ctgtgtggat
841 attgtggctt caaatctaaa atgttaaatt aaagcaccta agtgactacc acttatttct
901 aaatcttcac tatttttttg ttgctgttat tgagaagttg tgatttacta tcatatatta
961 taagatttct aggtgtcttt taatgattat ttctgtttaa aaaataatga tgtgttgtga
1021 aatttgttaa tatatacaat acttagaaac atgttagcat gaaactatgc acctataaat
1081 attaactatg aaatttttact gttttgtgat gtgttttatt aatttgtgtt tatatataaa
1141 tgctgaaaat taaaatgtta tctcattaca aaaatcttat ttttaatccc atctcacttt
1201 aataataaaa tcatgcttat aacaatatga actgagaact gacacaatta acttaaagct
1261 cttgacagcc atttgaagga gaaggaattt tagaagaatt aagcagacaa gatggaacat
1321 taatccttta ctctggaaat tcactgaagc aacactaccc aaagtatcct gacatgcagt
1381 ggtgtcttaa gaggttatat ggaaaaaaaa aaaaacgggt tccatggaat agtgagttta
1441 agaaattatt ttgactatgt ctgcttcaaa tattaataaa acatattagc aca

SEQ ID NO: 229
Reverse complement of SEQ ID NO: 228
tgtgctaatatgttttattaatatttgaagcagacatagtcaaaataatttcttaaactcactattccat
ggaacccgtttttttttttttccatataacctcttaagacaccactgcatgtcaggatactttgggtagt
gttgcttcagtgaatttccagagtaaaggattaatgttccatcttgtctgcttaattcttctaaaattcc
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tgattttattattaaagtgagatgggattaaaaataagatttttgtaatgagataacattttaattttca
gcatttatatataaacacaaattaataaaacacatcacaaaacagtaaaatttcatagttaatatttata
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gatgctccctgctccctccactgtcttttgtgctactgctgtcacccctgtcaccacggcctcaccaaca
tttgtcacttgctctttggtcttctcagccactgttgtcacaccatgaaccactccttccttggttttgg
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agcagccacgactccctccttggcctttgaaagtcctttcatgaatacatccatggctaatgaattcgtt
cacaccacactcacccccacgctgccgcgctcggtctctcacccccctctctcgggtcccctgcaagtcgt
cgtggtcttcctccccttcctctttctcctccttctcctcggctcaccgcccccgcagctgatttgtcag
cgcctctccccgcccctctgccg

EQUIVALENTS

Those skilled in the art will recognize or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments and methods described herein. Such equivalents are intended to be encompassed by the scope of the following claims.

We claim:

1. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA, wherein the dsRNA agent comprises a sense strand having a 5'-terminus and a 3'-terminus and an antisense strand having a 5'-terminus and a 3'-terminus, wherein the sense strand and the antisense strand form a double stranded region, wherein:

the sense strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 150, SEQ ID NO: 157, SEQ ID NO: 164, and SEQ ID NOs: 142-149, 151-156, 158-163, and 165-184 of **Table 3**, with 0 or 1 mismatches;

the sense strand of the dsRNA agent comprises a lipophilic moiety attached at position 6 or 16, counting from the 5'-terminus of the sense strand;

the antisense strand comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 193, SEQ ID NO: 200, SEQ ID NO: 207, and SEQ ID NOs: 185-192, 194-199, 201-206, and 208-227 of **Table 3**, with 0 or 1 mismatches;

the dsRNA agent does not comprise a GalNAc modification; and

the dsRNA agent comprises eight phosphorothioate internucleotide linkages positioned at the penultimate and ultimate internucleotide linkages from the respective 3'- and 5'-termini of each of the sense and antisense strands of the dsRNA agent.

2. The dsRNA agent of claim 1, wherein the lipophilic moiety is an aliphatic, alicyclic, or polyalicyclic compound.

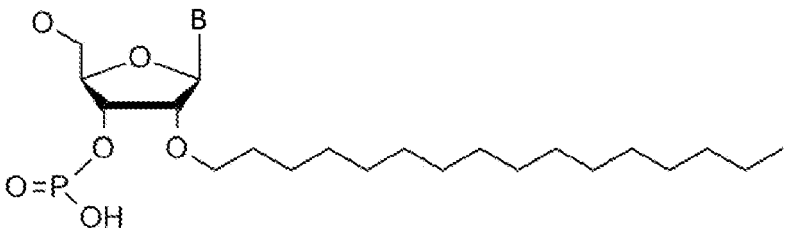
3. The dsRNA agent of claim 1 or claim 2, wherein the lipophilic moiety is lipid, cholesterol, retinoic acid, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-bis-O(hexadecyl)glycerol, geranyloxyhexyanol, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine.

4. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety contains a saturated or unsaturated C_4-C_{30} hydrocarbon chain, and an optional functional group selected from the group consisting of hydroxyl, amine, carboxylic acid, sulfonate, phosphate, thiol, azide, and alkyne.

5. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety contains a saturated or unsaturated C_6-C_{18} hydrocarbon chain.

6. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety contains a saturated or unsaturated C_{16} hydrocarbon chain.

7. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety is



,

wherein B is a nucleotide base or a nucleotide base analog, optionally wherein B is selected from the group consisting of adenine, guanine, cytosine, thymine and uracil.

8. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety is attached via a linker or carrier.

9. The dsRNA agent of any one of the preceding claims, wherein the lipophilic moiety is conjugated via a carrier that replaces the nucleotide at position 6 or 16 of the sense strand.

10. The dsRNA agent of claim 9, wherein the carrier is a cyclic group selected from the group consisting of pyrrolidinyl, pyrazolinyl, pyrazolidinyl, imidazolinyl, imidazolidinyl, piperidinyl, piperazinyl, [1,3]dioxolanyl, oxazolidinyl, isoxazolidinyl, morpholinyl, thiazolidinyl, isothiazolidinyl, quinoxalinyl, pyridazinonyl, tetrahydrofuranyl, and decalinyl; or is an acyclic moiety based on a serinol backbone or a diethanolamine backbone.

11. The dsRNA agent of any one of claims 1-8, wherein the lipophilic moiety is conjugated to the dsRNA agent via a linker containing an ether, thioether, urea, carbonate, amine, amide,

maleimide-thioether, disulfide, phosphodiester, sulfonamide linkage, a product of a click reaction, or carbamate.

12. The dsRNA agent of any one of claims 1-8 and 11, wherein the lipophilic moiety is conjugated to the dsRNA agent via a bio-cleavable linker selected from the group consisting of DNA, RNA, disulfide, amide, functionalized monosaccharides or oligosaccharides of galactosamine, glucosamine, glucose, galactose, mannose, and combinations thereof.

13. The dsRNA agent of any one of the preceding claims, wherein substantially all of the nucleotides of the sense strand are modified nucleotides.

14. The dsRNA agent of any one of the preceding claims, wherein substantially all of the nucleotides of the antisense strand are modified nucleotides.

15. The dsRNA agent of any one of the preceding claims, wherein all of the nucleotides of the sense strand are modified nucleotides.

16. The dsRNA agent of any one of the preceding claims, wherein all of the nucleotides of the antisense strand are modified nucleotides.

17. The dsRNA agent of any one of the preceding claims, wherein all of the nucleotides of the sense strand and all of the nucleotides of the antisense strand are modified nucleotides.

18. The dsRNA agent of any one of the preceding claims, having one or more modified nucleotides and wherein at least one of the one or more modified nucleotides is selected from the group consisting of a deoxy-nucleotide, a 3'-terminal deoxythimidine (dT) nucleotide, a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an unlocked nucleotide, a conformationally restricted nucleotide, a constrained ethyl nucleotide, an abasic nucleotide, a 2'-amino-modified nucleotide, a 2'-O-allyl-modified nucleotide, 2'-C-alkyl-modified nucleotide, 2'-hydroxy-modified nucleotide, a 2'-methoxyethyl modified nucleotide, a 2'-O-alkyl-modified nucleotide, a morpholino nucleotide, a

phosphoramidate, a non-natural base comprising nucleotide, a tetrahydropyran modified nucleotide, a 1,5-anhydrohexitol modified nucleotide, a cyclohexenyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, a nucleotide comprising a 5'-methylphosphonate group, a nucleotide comprising a 5' phosphate or 5' phosphate mimic, a nucleotide comprising vinyl phosphate, a nucleotide comprising adenosine-glycol nucleic acid (GNA), a nucleotide comprising thymidine-glycol nucleic acid (GNA) S-Isomer, a nucleotide comprising 2-hydroxymethyl-tetrahydrofuran-5-phosphate, a nucleotide comprising 2'-deoxythymidine-3'phosphate, a nucleotide comprising 2'-deoxyguanosine-3'-phosphate, and a terminal nucleotide linked to a cholesteryl derivative and a dodecanoic acid bisdecylamide group.

19. The dsRNA agent of any one of the preceding claims, wherein the dsRNA agent comprises at least one modified nucleotide selected from the group consisting of a 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-C16 (2'-O-hexadecyl) modified nucleotide, and a nucleotide comprising vinyl phosphate, optionally wherein the dsRNA agent comprises at least one of each of the following modifications: 2'-O-methyl modified nucleotide, a 2'-fluoro modified nucleotide, a 2'-C16 (2'-O-hexadecyl) modified nucleotide, and a nucleotide comprising vinyl phosphate.

20. The dsRNA agent of any one of the preceding claims, further comprising a phosphate or phosphate mimic at the 5'-end of the antisense strand.

21. The dsRNA agent of claim 20, wherein the phosphate mimic is a 5'-vinyl phosphonate (VP).

22. The dsRNA agent of any one of the preceding claims comprising a pattern of modified nucleotides as shown in Table 2 (wherein locations of 2'-C16, 2'-O-methyl, 2'-deoxy, GNA, phosphorothioate, vinyl phosphonate, and 2'-fluoro modifications are as displayed in Table 2, irrespective of the individual nucleotide base sequences of the displayed dsRNA agents).

23. The dsRNA agent of any one of the preceding claims having a sense strand nucleotide sequence and an antisense nucleotide sequence of a single duplex selected from the group

consisting of AD-1804698, AD-1804699, AD-1747575, AD-1747576, AD-1804700, AD-1747577, AD-1747578, AD-1747579, AD-1747580, AD-1747581, AD-1804701, AD-1747582, AD-1804702, AD-1804703, AD-1804704, AD-1747583, AD-1804705, AD-1804706, AD-1804707, AD-1804708, AD-1804709, AD-1747591, AD-1747585, AD-1804710, AD-1804711, AD-1747586, AD-1804712, AD-1747587, AD-1804713, AD-1804714, AD-1747588, AD-1804715, AD-1804716, AD-1804717, AD-1804718, AD-1804719, AD-1804720, AD-1804721, AD-1804722, AD-1804723, AD-1804724, AD-1804725, and AD-1804726, optionally having a sense strand nucleotide sequence and an antisense nucleotide sequence of a single duplex selected from the group consisting of AD-1747580, AD-1747583, and AD-1747585.

24. The dsRNA agent of any one of the preceding claims, wherein the sense strand of the dsRNA agent has a modification pattern selected from the group consisting of 5'-nsnsnnn(Nhd)NfnNfNfNfnnnnnnnnsnsn-3' and 5'-nsnsnnnnnnNfNfNfnnnn(Nhd)nnnsnsn-3', wherein n is a 2'-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, Nf is a 2'-fluoro-nucleotide, and (Nhd) is a 2'-O-hexadecyl-nucleotide.

25. The dsRNA agent of any one of the preceding claims, wherein the antisense strand of the dsRNA agent has a modification pattern selected from the group consisting of 5'-VPnsdNsnndNndNnnnndNnNfnnnnnnnsnsn-3' and 5'-VPnsNfsnndNn(Ngn)nnnnnnNfnNfnnnnnsnsn-3', wherein VP is Vinyl-phosphonate, n is a 2'-O-methyl-nucleotide, s is a phosphorothioate internucleotide linkage, dN is a 2'-deoxy-nucleotide, Nf is a 2'-fluoro-nucleotide, and (Ngn) is a glycol nucleic acid, S-isomer.

26. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA, wherein the dsRNA agent comprises a sense strand having a 5'-terminus and a 3'-terminus and an antisense strand having a 5'-terminus and a 3'-terminus, wherein the sense strand and the antisense strand form a double stranded region, wherein:

the sense strand comprises a nucleotide sequence and modifications of **Table 2** (SEQ ID NOs: 13-55), with 0 or 1 mismatches; and

the antisense strand comprises a nucleotide sequence and modifications of **Table 2** (SEQ ID NOs: 56-98), with 0 or 1 mismatches.

27. The dsRNA agent of any one of the preceding claims having a sense strand nucleotide sequence selected from the group consisting of 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35), 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28), 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21), 5'-gsusaca(Ahd)GfuGfCfUfcaguuccasasa-3' (SEQ ID NO: 34), 5'-cscsauc(Ahd)gcAfGfUfgauugaagsusa-3' (SEQ ID NO: 43), 5'-uscsccag(Uhd)uUfCfUfugagaucusgsa-3' (SEQ ID NO: 261), and 5'-uscsaug(Ahd)aaGfGfAfcuuucaaasgsa-3' (SEQ ID NO: 19), wherein a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate, (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

28. The dsRNA agent of any one of the preceding claims having an antisense strand nucleotide sequence selected from the group consisting of 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78), 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71), 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64), 5'-VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg-3' (SEQ ID NO: 77), 5'-VPusdAscudTcdAaucadCuGfcugauggsasa-3' (SEQ ID NO: 86), 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' (SEQ ID NO: 262), and 5'-VPusdCsuudTgdAaagudCcUfuucaugasasu-3' (SEQ ID NO: 62), wherein VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG

is a 2`-deoxyguanosine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Agn) is an Adenosine-glycol nucleic acid (GNA), S-isomer.

29. The dsRNA agent of any one of the preceding claims having a duplex nucleotide sequence selected from the group consisting of (i) sense strand: 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35) and antisense strand: 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78); (ii) sense strand: 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28) and antisense strand: 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71); (iii) sense strand: 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21) and antisense strand: 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64); (iv) sense strand: 5'-gsusaca(Ahd)GfuGfCfUfcaguuccasasa-3' (SEQ ID NO: 34) and antisense strand: 5'-VPusUfsugdGa(Agn)cugagcAfcUfuguacsasg-3' (SEQ ID NO: 77); (v) sense strand: 5'-cscsauc(Ahd)gcAfGfUfgauugaagsusa-3' (SEQ ID NO: 43) and antisense strand: 5'-VPusdAscudTcdAaucadCuGfcugauggsasa-3' (SEQ ID NO: 86); (vi) sense strand: 5'-uscscccag(Uhd)uUfCfUfugagaucusgsa-3' (SEQ ID NO: 261) and antisense strand: 5'-VPusdCsagdAudCucaadGaAfacugggasgsc-3' (SEQ ID NO: 262); and (vii) sense strand: 5'-uscsaug(Ahd)aaGfGfAfcuuucaaasgsa-3' (SEQ ID NO: 19) and antisense strand: 5'-VPusdCsuudTgdAaagudCcUfuucaugasasu-3' (SEQ ID NO: 62), wherein VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG is a 2`-deoxyguanosine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, (Agn) is an Adenosine-glycol nucleic acid (GNA), S-isomer, (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate, (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

30. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA having sense strand sequence 5'-asasgug(Chd)ucAfGfUfuccaaugusgsa-3' (SEQ ID NO: 35) and antisense strand sequence 5'-VPusdCsacdAudTggaadCuGfagcacuusgsu-3' (SEQ ID NO: 78),

wherein VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Uf is a 2'-fluorouridine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Chd) is a 2'-O-hexadecyl cytidine-3'-phosphate.

31. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA having sense strand sequence 5'-uscsuuugcuCfCfCfaguu(Uhd)cuusgsa-3' (SEQ ID NO: 28) and antisense strand sequence 5'-VPusdCsaadGadAacugdGgAfgcaaagasusa-3' (SEQ ID NO: 71), wherein VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dG is a 2`-deoxyguanosine-3`-phosphate, and (Uhd) is a 2'-O-hexadecyl uridine-3'-phosphate.

32. A double stranded ribonucleic acid (dsRNA) agent for inhibiting expression of SNCA having sense strand sequence 5'-gsasgca(Ahd)guGfAfCfaaauguugsgsa-3' (SEQ ID NO: 21) and antisense strand sequence 5'-VPusdCscadAcdAuuugdTcAfcuugcucsusu-3' (SEQ ID NO: 64), wherein VP is Vinyl-phosphonate, a is a 2'-O-methyladenosine-3'-phosphate, u is a 2'-O-methyluridine-3'-phosphate, g is a 2'-O-methylguanosine-3'-phosphate, c is a 2'-O-methylcytidine-3'-phosphate, s is a phosphorothioate internucleotide linkage, Af is a 2'-fluoroadenosine-3'-phosphate, Gf is a 2'-fluoroguanosine-3'-phosphate, Cf is a 2'-fluorocytidine-3'-phosphate, dA is a 2`-deoxyadenosine-3`-phosphate, dC is a 2`-deoxycytidine-3`-phosphate, dT is a 2`-deoxythymidine-3`-phosphate, and (Ahd) is a 2'-O-hexadecyl adenosine-3'-phosphate.

33. A cell containing the dsRNA agent of any one of claims 1-32.

34. A pharmaceutical composition for use in inhibiting expression of α-synuclein comprising the dsRNA agent of any one of claims 1-32.

35. The pharmaceutical composition of claim 34, wherein the dsRNA agent is administered in an unbuffered solution.

36. The pharmaceutical composition of claim 35, wherein said unbuffered solution is saline or water.

37. The pharmaceutical composition of claim 34, wherein said dsRNA agent is administered with a buffer solution.

38. The pharmaceutical composition of claim 37, wherein said buffer solution comprises acetate, citrate, prolamine, carbonate, or phosphate or any combination thereof.

39. The pharmaceutical composition of claim 37, wherein said buffer solution is phosphate buffered saline (PBS).

40. A pharmaceutical composition comprising the dsRNA agent of any one of claims 1-32, and a lipid formulation.

41. The pharmaceutical composition of claim 40, wherein the lipid formulation comprises a LNP.

42. A method of inhibiting expression of an α-synuclein (SNCA) gene in a cell and/or preventing the formation of alpha-synuclein aggregates in a cell or subject, the method comprising:

(a) contacting the cell or subject with the dsRNA agent of any one of claims 1-32 or the pharmaceutical composition of any one of claims 34-41; and

(b) maintaining the cell or subject produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of an SNCA gene, thereby inhibiting expression of the SNCA gene in the cell and/or preventing the formation of alpha-synuclein aggregates in the cell or subject.

43. The method of claim 42, wherein said cell is within a subject.

44. The method of claim 43, wherein the subject is a human.

45. The method of claim 43, wherein the subject is selected from the group consisting of a rhesus monkey, a cynomolgous monkey, a mouse, and a rat.

46. The method of claim 44, wherein the human subject suffers from a SNCA-associated disease.

47. The method of claim 46, wherein the SNCA-associated disease is a synucleinopathy, optionally a disease selected from the group consisting of PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, gangliоglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

48. The method of any one of claims 42-47, wherein SNCA expression in the cell or the subject is inhibited by at least about 50%, at least about 40%, at least about 30%, at least about 20%, or at least about 10% by the dsRNA agent as compared to a control cell or control subject.

49. A method of treating a subject diagnosed with a SNCA-associated neurodegenerative disease, the method comprising administering to the subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-32 or the pharmaceutical composition of any one of claims 34-42, optionally wherein the subject is re-dosed with a therapeutically effective amount of the dsRNA agent of any one of claims 1-32 or the pharmaceutical composition of any one of claims 34-42, thereby treating said subject.

50. The method of claim 49, wherein the subject is a human.

51. The method of claim 49 or claim 50, wherein treating comprises amelioration of at least one sign or symptom of the SNCA-associated neurodegenerative disease.

52. The method of any one of claims 49-51, where treating comprises prevention of progression of the disease.

53. The method of any one of claims 49-52, wherein the SNCA-associated neurodegenerative disease is characterized by one or more symptoms selected from the group consisting of tremors, slowed movement (bradykinesia), rigid muscles, impaired posture and balance, loss of automatic movements, speech changes, writing changes, visual, auditory, olfactory, or tactile hallucinations, poor regulation of body functions (autonomic nervous systems) such as dizziness, falls and bowel issues, cognitive problems such as confusion, poor attention, visual-spatial problems and memory loss, sleep difficulties such as rapid eye movement (REM) sleep behavior disorder (in which dreams are physically acted out while asleep), fluctuating attention including episodes of drowsiness, long periods of staring into space, long naps during the day or disorganized speech, depression, and apathy, orthostatic hypotension (a sudden drop in blood pressure that occurs when a person stands up, causing a person to feel dizzy and lightheaded, and the need to sit, squat, or lie down in order to prevent fainting), clumsiness or incoordination, bladder control problems, contractures (chronic shortening of muscles or tendons around joints, which prevents the joints from moving freely) in the hands or limbs, Pisa syndrome (an abnormal posture in which the body appears to be leaning to one side), antecollis (in which the neck bends forward and the head drops down), and involuntary and uncontrollable sighing or gasping.

54. The method of any one of claims 49-53, wherein the SNCA-associated neurodegenerative disease is selected from the group consisting of a synucleinopathy, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration,

frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

55. The method of any one of claims 49-54, wherein the dsRNA agent is administered to the subject at a dose of about 0.01 mg/kg to about 50 mg/kg.

56. The method of any one of claims 49-55, wherein the dsRNA agent is administered to the subject intrathecally.

57. The method of any one of claims 49-56, further comprising administering to the subject an additional agent or a therapy suitable for treatment or prevention of a SNCA-associated neurodegenerative disease or disorder.

58. The method of any one of claims 49-57, wherein the SNCA-associated neurodegenerative disease is Parkinson's Disease (PD).

59. The method of any one of claims 49-58, wherein the SNCA-associated neurodegenerative disease is selected from the group consisting of Lewy body dementia (LBD) and multiple system atrophy (MSA).

60. The method of any one of claims 49-59, wherein the SNCA expression is inhibited by at least about 30%.

61. The method of any one of claims 49-60, further comprising administering an additional therapeutic agent to the subject.

62. The method of any one of claims 49-61, wherein the dsRNA agent is administered to the subject intrathecally.

63. The method of claim 62, wherein the method reduces the expression of SNCA in a brain or spinal cord tissue.

64. The method of claim 63, wherein the brain or spinal cord tissue is selected from the group consisting of cerebral cortex, cerebellum, basal ganglia, hippocampus, amygdala, thalamus, brainstem, cervical spinal cord, lumbar spinal cord, and thoracic spinal cord.

65. A method of inhibiting the expression of SNCA in a subject, the method comprising:

administering to said subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-32 or the pharmaceutical composition of any one of claims 34-41, thereby inhibiting the expression of SNCA in said subject.

66. A method for treating or preventing an SNCA-associated disease in a subject, the method comprising

administering to said subject a therapeutically effective amount of the dsRNA agent of any one of claims 1-32 or the pharmaceutical composition of any one of claims 34-41, thereby treating or preventing an SNCA-associated disease in the subject.

67. The method of claim 66, wherein the SNCA-associated disease is selected from the group consisting of a synucleinopathy, such as PD, multiple system atrophy (MSA), Lewy body dementia (LBD), pure autonomic failure (PAF), Pick's disease, progressive supranuclear palsy, dementia pugilistica, parkinsonism linked to chromosome 17, Lytico-Bodig disease, tangle predominant dementia, Argyrophilic grain disease, ganglioglioma, gangliocytoma, meningioangiomatosis, subacute sclerosing panencephalitis, lead encephalopathy, tuberous sclerosis, Hallervorden-Spatz disease, lipofuscinosis, corticobasal degeneration, frontotemporal dementia, frontotemporal lobar degeneration, Alzheimer's disease, Huntington's disease, Down's syndrome, psychosis, schizophrenia and Creutzfeldt-Jakob disease.

68. The method of claim 66, wherein the step of administering produces at least 60% knockdown of SNCA mRNA or α-synuclein protein in one or more tissues of the subject, optionally wherein the one or more tissues of the subject are selected from the group consisting of CSF, prefrontal cortex, midbrain, thoracic spine, hippocampus, medulla pons, striatum caudate, and cerebellum.

69. A kit for performing the method of any one of claims 42-67, comprising

a) the dsRNA agent,

b) instructions for use, and

c) optionally, a means for administering the dsRNA agent to a subject.