ANTISENSE OLIGONUCLEOTIDES TARGETING ALPHA-SYNUCLEIN AND USES THEREOF

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

[0001] The content of th2019e electronically submitted sequence listing (Name: 3338_107PC01_SequenceListing_ST25.txt, Size: 10,458 bytes; and Date of Creation: January 10, 2019) submitted in this application is incorporated herein by reference in its entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to an antisense oligomeric compound (ASO) that targets the junction of intron 1 and exon 2 of alpha-synuclein (*SNCA*) transcript in a cell, leading to reduced expression of alpha-synuclein (SNCA) protein. Reduction of SNCA protein expression can be beneficial for a range of medical disorders, such as multiple system atrophy, Parkinson's disease, Parkinson's Disease Dementia (PDD), and dementia with Lewy bodies.

BACKGROUND OF THE DISCLOSURE

[0003] Alpha-synuclein (SNCA), a member of the synuclein protein family, is a small soluble protein that is expressed primarily within the neural tissues. *See* Marques O *et al.*, *Cell Death Dis.* 19: e350 (2012). It is expressed in many cell types but is predominantly localized within the presynaptic terminals of neurons. While the precise function has yet to be fully elucidated, SNCA has been suggested to play an important role in the regulation of synaptic transmission. For instance, SNCA functions as a molecular chaperone in the formation of SNARE complexes, which mediate the docking of synaptic vesicles with the presynaptic membranes of neurons. SNCA can also interact with other proteins like the microtubule-associated protein tau, which helps stabilize microtubules and regulate vesicle trafficking.

[0004] Due to SNCA's role in the regulation of synaptic transmission, alterations of SNCA expression and/or function can disrupt critical biological processes. Such disruptions have been thought to contribute to α-synucleinopathies, which are

neurodegenerative diseases characterized by abnormal accumulation of SNCA protein aggregates within the brain. Accordingly, insoluble inclusions of misfolded, aggregated, and phosphorylated SNCA protein are a pathological hallmark for diseases such as Parkinson's disease (PD), Parkinson's Disease Dementia (PDD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). *See* Galvin JE *et al.*, *Archives of Neurology* 58: 186-190 (2001); and Valera E *et al.*, *J Neurochem* 139 Suppl 1: 346-352 (Oct. 2016).

[0005] α-Synucleinopathies, such as Parkinson's disease, are highly prevalent progressive neurodegenerative brain disorders, especially among the elderly. *See* Recchia A *et al.*, *FASEB J.* 18: 617-26 (2004). It is estimated that approximately seven to ten million people worldwide are living with such disorders, with about 60,000 new cases each year in the United States alone. Medication costs for an individual person can easily exceed $2,500 a year and therapeutic surgery can cost up to $100,000 per patient. Therefore, a more robust and cost-effective treatment options are greatly needed.

BRIEF SUMMARY OF THE DISCLOSURE

[0006] The present disclosure is directed to an antisense oligonucleotide (ASO) comprising, consisting essentially of, or consisting of the contiguous nucleotide sequence of AtTcctttacaccACAC (SEQ ID NO: 4), wherein the upper letter is beta-D-oxy-LNA and the lower letter is DNA. In other embodiments, the ASO comprises an internucleotide linkage selected from the group consisting of a phosphodiester linkage, a phosphotriester linkage, a methylphosonate linkage, a phosphoramidate linkage, a phosphorothioate linkage, and combinations thereof. In certain embodiments, the internucleotide linkage is a phosphorothioate linkage..

[0007] In some embodiments, the ASO comprises, consists essentially of, or consists of OxyAs DNAts OxyTs DNAcs DNAcs DNAts DNAts DNAts DNAas DNAcs DNAas DNAcs DNAcs OxyAs OxyMCs OxyAs OxyMC, wherein OxyA, OxyT, and Oxy MC are adenine beta D-oxy-LNA, thymine beta D-oxy-LNA, and methyl cytosine beta D-oxy-LNA, respectively, and wherein DNAt, DNAc, and DNAa are thymine DNA, cytosine DNA, and adenine DNA, respectively. In some embodiments, the ASO of the present disclosure comprises a molecular formula of $C_{171}H_{214}N_{56}O_{90}P_{16}S_{16}$ and a structure as shown in FIG. 1B, wherein M^+ is a counterion. In some embodiments, the counterion is

selected from the group consisting of H^+, Na^+, $NH4^+$, and any combination thereof. In certain embodiments, the counterion is Na^+.

[0008] The present disclosure also provides a conjugate comprising the ASO as disclosed herein, wherein the ASO is covalently attached to at least one non-nucleotide or non-polynucleotide moiety. In some embodiments, the non-nucleotide or non-polynucleotide moiety comprises a protein, a fatty acid chain, a sugar residue, a glycoprotein, a polymer, or any combinations thereof.

[0009] Also provided herein is a pharmaceutical composition comprising the ASO or the conjugate as disclosed herein and a pharmaceutically acceptable carrier. In some embodiments, the composition further comprises a therapeutic agent. In certain embodiments, the therapeutic agent is an alpha-synuclein antagonist. In some embodiments, the alpha-synuclein antagonist is an anti-alpha-synuclein antibody or fragment thereof.

[0010] The present disclosure further provides a kit comprising the ASO, the conjugate, or the composition as disclosed herein. Also disclosed is a diagnostic kit comprising the ASO, the conjugate, or the composition of the present disclosure.

[0011] The present disclosure is also directed to method of inhibiting or reducing SNCA protein expression in a cell, the method comprising administering the ASO, the conjugate, or the composition as disclosed herein to the cell expressing SNCA protein, wherein the SNCA protein expression in the cell is inhibited or reduced after the administration. In some embodiments, the ASO inhibits or reduces expression of *SNCA* mRNA in the cell after the administration. In certain embodiments, the expression of *SNCA* mRNA is reduced by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, or about 100% after the administration compared to a cell not exposed to the ASO. In other embodiments, the ASO reduces expression of SNCA protein in the cell after the administration by at least about 60%, at least about 70%, at least about 80%, or at least about 90% compared to a cell not exposed to the ASO. In some embodiments, the cell is a neuron.

[0012] Provided herein is a method for treating a synucleinopathy in a subject in need thereof, comprising administering an effective amount of the ASO, the conjugate, or the composition of the present disclosure. In some embodiments, the synucleinopathy is selected from the group consisting of Parkinson's disease, Parkinson's Disease Dementia

(PDD), multiple system atrophy, dementia with Lewy bodies, and any combinations thereof.

[0013] Also provided herein is a use of the ASO, the conjugate, or the composition of the present disclosure for the manufacture of a medicament. The present disclosure also provides the use of the ASO, the conjugate, or the composition for the manufacture of a medicament for the treatment of a synucleinopathy in a subject in need thereof. In some embodiments, the ASO, the conjugates, or the composition of the present disclosure is for use in therapy of a synucleinopathy in a subject in need thereof. In other embodiments, the ASO, the conjugates, or the composition of the present disclosure is for use in therapy.

[0014] In some embodiments, the subject is a human. In some embodiments, the ASO, the conjugates, or the compositions is administered orally, parenterally, intrathecally, intra-cerebroventricularly, pulmonarily, topically, or intraventricularly.

BRIEF DESCRIPTION OF THE FIGURES

[0015] FIG. 1A shows the contiguous nucleotide sequence of ASO-005459. OxyA, OxyT, and Oxy MC are adenine beta D-oxy-LNA, thymine beta D-oxy-LNA, and methyl cytosine beta D-oxy-LNA, respectively; DNAt, DNAc, and DNAa are thymine DNA, cytosine DNA, and adenine DNA, respectively; and s is a phosphorothioate linkage.

[0016] FIG. 1B shows the molecular structure of ASO-005459 as disclosed herein. The provided structure has a molecular formula of $C_{171}H_{214}N_{56}O_{90}P_{16}S_{16}$ and each of the M+ is a pharmaceutically acceptable counterion such as H^+, Na^+, or NH_4^+.

[0017] FIGs. 2A and 2B show the effect of ASO-005459 on SNCA and tubulin (Tub) protein expression in primary neurons isolated from A53T-PAC transgenic mice. Neurons were treated with a 10-point titration of ASO-005459 and amounts of SNCA and tubulin protein measured. Percent inhibition of α-Syn/Tub ratio (FIG. 2A) and Tub levels (FIG. 2B) are shown. Each data point represents an individual replicate.

[0018] FIG. 3 shows the effect of ASO-005459 on the expression level of *SNCA* (circle), Protein S (alpha) (*PROS1* (square)), and Tubulin (*TUBB3* (triangle)) mRNAs in human neurons. The neurons were treated with various concentrations of ASO-005459 for 6 days, and then, the mRNA levels were measured by QUANTIGENE® assay. The expression level of the mRNAs is shown as a percent of the control. Data shown represents the mean ± SD from duplicate determinations.

[0019] FIG. 4 shows the *SNCA* mRNA expression levels in the hippocampus of A53T-PAC mice at three days post ICV administration of 100 μg of ASO-005459 (open square) or control vehicle (closed circle). *SNCA* mRNA expression levels were measured by qRT-PCR, normalized to GAPDH mRNA, and then expressed relative to the mean expression level of the vehicle group. The horizontal line marks the reference value of 1 (*i.e.*, value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group). Data shown represents the mean ± SD from duplicate determination. Statistics shown is based on 1-way ANOVA with Dunnett's post-test. *** $p<0.001$.

[0020] FIGs. 5A and 5B show a comparison of the average body weight of mice treated with ASO-005459. In FIG. 5A, the A53T-PAC mice were dosed with 3.13 μg, 12.5 μg, 25 μg, or 50 μg of ASO-005459 and their bodyweights measured at weeks 0, 1, and 2 post-treatment. In FIG. 5B, C57BL/6 mice were dosed with 100 μg of ASO-005459 and the animals' body weight was measured once a week during a course of 28 days. In both FIGs. 5A and 5B, animals receiving the vehicle control were used as controls. Data shown represents the mean ± SD from multiple animals (n = 5). Statistics shown is based on 2-way ANOVA.

[0021] FIGs. 6A, 6B, and 6C show the *SNCA* mRNA expression levels in the hippocampus (FIG. 6A), brainstem (FIG. 6B), and striatum (FIG. 6C) of A53T-PAC mice at 14 days post ICV administration of ASO-005459 (3.13 μg, 12.5 μg, 25 μg, or 50 μg) or the vehicle control. *SNCA* mRNA levels were measured by qRT-PCR, normalized to GAPDH mRNA, and then expressed relative to the mean of the vehicle group. Data shown represents the mean ± SD from multiple animals (n = 5). Each circle represents an individual animal. The horizontal line marks the reference value of 1 (*i.e.*, value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group). Statistics shown is based on 1-way ANOVA with Dunnett's post-test. *** $p<0.001$.

[0022] FIG. 7 shows the level of ASO-005459 detected in the hippocampus (black), brainstem (light gray), and the striatum (dark gray) of A53T-PAC mice at 14 days post ASO-005459 treatment. The mice received 3.13 μg, 12.5 μg, 25 μg, or 50 μg of ASO-005459 via ICV administration. Data shown represents the mean ± SD from multiple animals (n = 5).

[0023] FIGs. 8A, 8B, 8C, and 8D show the relationship between ASO-005459 exposure levels and *SNCA* mRNA expression in the hippocampus (FIG. 8A), brainstem (FIG. 8B), and striatum (FIG. 8C) of A53T-PAC mice at fourteen days post ASO-005459 treatment. FIG. 8D shows the data from the hippocampus (circle), brainstem (square), and striatum (triangle) in combination. Each data point represents an individual animal. A four-parameter, nonlinear fit is shown for the hippocampus (FIG. 8A) and the brainstem (FIG. 8B).

[0024] FIGs. 9A and 9B show the dose response curve of the effect of ASO-005459 on *SNCA* mRNA expression level in A53T-PAC mice. The animals received (via ICV injection) 12.5 μg (circle), 25 μg (box), or 50 μg (triangle) of ASO-005459 and were sacrificed at 24 hours, 3 days, 4, 8, 12, 16, and 20 weeks post-dosing. *SNCA* mRNA expression levels both in the brain stem (FIG. 9A) and in the striatum (FIG. 9B) were assessed via qRT-PCR and then normalized to the vehicle control. The mean data is shown. The horizontal line marks the reference value of 100% (*i.e.*, value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group).

[0025] FIGs. 10A and 10B show the effect of ASO-005459 on *SNCA* mRNA expression level in A53T-PAC mice after 4 weeks post ASO-005459 administration. The animals received either the vehicle control or varying concentration s of ASO-005459 (12.5, 25, or 50 μg). The relative *SNCA* mRNA expression level (normalized to the vehicle control) are shown for both the brain stem (FIG. 10A) and the striatum (FIG. 10B). Each data point represents an individual animal. The mean ± SD from multiple animals is also shown. Statistics shown is based on comparison to the vehicle group using 1-way ANOVA with Dunnett's correction for multiple comparisons. The horizontal line marks the reference value of 1 (*i.e.*, value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

[0026] FIGs. 11A and 11B show the dose response curve of the effect of ASO-005459 on SNCA protein expression level in the brain tissues of A53T-PAC mice. The animals received (via ICV injection) 12.5 μg (circle), 25 μg (box), or 50 μg (triangle) of ASO-005459 and were sacrificed at 24 hours, 3 days, 4, 8, 12, 16, and 20 weeks post-dosing. SNCA proteins levels were measured both in the brain stem (FIG. 11A) and in the striatum (FIG. 11B) by ELISA and then normalized to the vehicle control. The mean data

is shown. The horizontal line marks the reference value of 100% (*i.e.,* value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group).

[0027] FIGs. 12A and 12B show the effect of ASO-005459 on SNCA protein expression level in A53T-PAC mice at 8 weeks post ASO-005459 administration. The animals received either the vehicle control or varying concentration s of ASO-005459 (12.5, 25, or 50 μg). The relative SNCA protein expression levels (normalized to the vehicle control) are shown for both the brain stem (FIG. 12A) and the striatum (FIG. 12B). Each data point represents an individual animal. The horizontal line marks the reference value of 100% (*i.e.,* value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group). The mean ± SD from multiple animals is also shown. Statistics shown is based on comparison to the vehicle group using 1-way ANOVA with Dunnett's correction for multiple comparisons.

[0028] FIGs. 13A and 13B show the kinetics of *SNCA* mRNA and SNCA protein expression levels in cyno monkeys after ASO-005459 administration. Each of the animals received either the vehicle control or ASO-005459 (8 mg) and then were sacrificed at 24 hours, 3 days, 2, 4, 8, 13, or 20 weeks post-dosing. At each time point, the expression levels of *SNCA* mRNA (FIG. 13A) and SNCA protein (FIG. 13B) were assessed in the following tissues: medulla (top left panel), caudate putamen (top middle panel), pons (top right panel), cerebellum (bottom left panel), lumbar spinal cord (bottom middle panel), and frontal cortex (bottom right panel). The expression levels are shown as a percentage of the vehicle control. Both the data for the individual animals and the mean are shown. The horizontal line marks the reference value of 100% (*i.e.,* value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group).

[0029] FIGs. 14A and 14B show the relative expression level (as percentage of the vehicle control) for both the *SNCA* mRNA (FIG. 14A) and SNCA protein (FIG. 14B) in cyno monkeys at 2 weeks post ASO-005459 administration. The animals received either the vehicle control or varying concentrations of the ASO-005459 (2, 4, or 8 mg). The expression levels were assessed in the following tissues: medulla (top left panel), caudate putamen (top middle panel), pons (top right panel), cerebellum (bottom left panel), lumbar spinal cord (bottom middle panel), and frontal cortex (bottom right panel). The expression levels are shown as a percentage of the vehicle control. Both the data for the

individual animals and the mean are shown. The horizontal line marks the reference value of 100% (*i.e.,* value at which the *SNCA* mRNA expression would be equivalent to expression level observed in the vehicle control group).

DETAILED DESCRIPTION OF THE DISCLOSURE

I. Definitions

[0030] It is to be noted that the term "a" or "an" entity refers to one or more of that entity; for example, "a nucleotide sequence," is understood to represent one or more nucleotide sequences. As such, the terms "a" (or "an"), "one or more," and "at least one" can be used interchangeably herein.

[0031] Furthermore, "and/or" where used herein is to be taken as specific disclosure of each of the two specified features or components with or without the other. Thus, the term "and/or" as used in a phrase such as "A and/or B" herein is intended to include "A and B," "A or B," "A" (alone), and "B" (alone). Likewise, the term "and/or" as used in a phrase such as "A, B, and/or C" is intended to encompass each of the following aspects: A, B, and C; A, B, or C; A or C; A or B; B or C; A and C; A and B; B and C; A (alone); B (alone); and C (alone).

[0032] It is understood that wherever aspects are described herein with the language "comprising," otherwise analogous aspects described in terms of "consisting of" and/or "consisting essentially of" are also provided.

[0033] Unless defined otherwise, 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 disclosure is related. For example, the Concise Dictionary of Biomedicine and Molecular Biology, Juo, Pei-Show, 2nd ed., 2002, CRC Press; The Dictionary of Cell and Molecular Biology, 3rd ed., 1999, Academic Press; and the Oxford Dictionary Of Biochemistry And Molecular Biology, Revised, 2000, Oxford University Press, provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0034] Units, prefixes, and symbols are denoted in their Système International de Unites (SI) accepted form. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleotide sequences are written left to right in 5' to 3' orientation. Amino acid sequences are written left to right in amino to carboxy orientation. The headings provided herein are not limitations of the various aspects of the

disclosure, which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification in its entirety.

[0035] The term "about" is used herein to mean approximately, roughly, around, or in the regions of. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" can modify a numerical value above and below the stated value by a variance of, *e.g.*, 10 percent, up or down (higher or lower). For example, if it is stated that "the ASO reduces expression of SNCA protein in a cell following administration of the ASO by at least about 60%," it is implied that the SNCA levels are reduced by a range of 50% to 70%.

[0036] The term "antisense oligonucleotide" (ASO) refers to an oligomer or polymer of nucleosides, such as naturally-occurring nucleosides or modified forms thereof, that are covalently linked to each other through internucleotide linkages. The ASO useful for the disclosure includes at least one non-naturally occurring nucleoside. An ASO is complementary to a target nucleic acid, such that the ASO hybridizes to the target nucleic acid sequence. The terms "antisense ASO," "ASO," and "oligomer" as used herein are interchangeable with the term "ASO."

[0037] The term "nucleic acids" or "nucleotides" is intended to encompass plural nucleic acids. In some embodiments, the term "nucleic acids" or "nucleotides" refers to a target sequence, *e.g.,* pre-mRNAs, mRNAs, or DNAs *in vivo* or *in vitro*. When the term refers to the nucleic acids or nucleotides in a target sequence, the nucleic acids or nucleotides can be naturally occurring sequences within a cell. In other embodiments, "nucleic acids" or "nucleotides" refer to a sequence in the ASOs of the disclosure. When the term refers to a sequence in the ASOs, the nucleic acids or nucleotides are not naturally occurring, *i.e.,* chemically synthesized, enzymatically produced, recombinantly produced, or any combination thereof. In one embodiment, the nucleic acids or nucleotides in the ASOs are produced synthetically or recombinantly, but are not a naturally occurring sequence or a fragment thereof. In another embodiment, the nucleic acids or nucleotides in the ASOs are not naturally occurring because they contain at least one nucleotide analog that is not naturally occurring in nature. The term "nucleic acid" or "nucleoside" refers to a single nucleic acid segment, *e.g.*, a DNA, an RNA, or an analog thereof, present in a polynucleotide. "Nucleic acid" or "nucleoside" includes naturally occurring nucleic acids

or non-naturally occurring nucleic acids. In some embodiments, the terms "nucleotide", "unit" and "monomer" are used interchangeably. It will be recognized that when referring to a sequence of nucleotides or monomers, what is referred to is the sequence of bases, such as A, T, G, C or U, and analogs thereof.

[0038] The term "nucleotide" as used herein, refers to a glycoside comprising a sugar moiety, a base moiety and a covalently linked group (linkage group), such as a phosphate or phosphorothioate internucleotide linkage group, and covers both naturally occurring nucleotides, such as DNA or RNA, and non-naturally occurring nucleotides comprising modified sugar and/or base. Herein, a single nucleotide (unit) can also be referred to as a monomer or nucleic acid unit.

[0039] The term "nucleoside" as used herein is used to refer to a glycoside comprising a sugar moiety and a base moiety, which can be covalently linked by the internucleotide linkages between the nucleosides of the ASO. In the field of biotechnology, the term "nucleoside" is often used to refer to a nucleic acid monomer or unit. In the context of an ASO, the term "nucleoside" can refer to the base alone, *i.e.*, a nucleobase sequence comprising cytosine (DNA and RNA), guanine (DNA and RNA), adenine (DNA and RNA), thymine (DNA) and uracil (RNA), in which the presence of the sugar backbone and internucleotide linkages are implicit. Likewise, particularly in the case of oligonucleotides where one or more of the internucleotide linkage groups are modified, the term "nucleotide" can refer to a "nucleoside." For example the term "nucleotide" can be used, even when specifying the presence or nature of the linkages between the nucleosides.

[0040] As one of ordinary skill in the art would recognize, the 5' terminal nucleotide of an oligonucleotide does not comprise a 5' internucleotide linkage group, although it can comprise a 5' terminal group.

[0041] The term "downstream," when referring to a nucleotide sequence, means that a nucleic acid or a nucleotide sequence is located 3' to a reference nucleotide sequence. In certain embodiments, downstream nucleotide sequences relate to sequences that follow the starting point of transcription. For example, the translation initiation codon of a gene is located downstream of the start site of transcription.

[0042] Unless otherwise indicated, the sequences provided herein are listed from 5' end (left) to 3' end (right).

[0043] The term "upstream" refers to a nucleotide sequence that is located 5' to a reference nucleotide sequence.

[0044] The term "transcript" as used herein can refer to a primary transcript that is synthesized by transcription of DNA and becomes a messenger RNA (mRNA) after processing, *i.e.,* a precursor messenger RNA (pre-mRNA), and the processed mRNA itself. The term "transcript" can be interchangeably used with "pre-mRNA" and "mRNA." After DNA strands are transcribed to primary transcripts, the newly synthesized primary transcripts are modified in several ways to be converted to their mature, functional forms such as mRNA, tRNA, rRNA, lncRNA, miRNA and others. Thus, the term "transcript" can include exons, introns, 5' UTRs, and 3' UTRs.

[0045] The term "expression" as used herein refers to a process by which a polynucleotide produces a gene product, for example, a RNA or a polypeptide. It includes, without limitation, transcription of the polynucleotide into messenger RNA (mRNA) and the translation of an mRNA into a polypeptide. Expression produces a "gene product." As used herein, a gene product can be either a nucleic acid, *e.g.*, a messenger RNA produced by transcription of a gene, or a polypeptide which is translated from a transcript. Gene products described herein further include nucleic acids with post transcriptional modifications, *e.g.*, polyadenylation or splicing, or polypeptides with post translational modifications, *e.g.*, methylation, glycosylation, the addition of lipids, association with other protein subunits, or proteolytic cleavage.

[0046] The term "naturally occurring variant" of the SNCA polypeptide refers to variants of the SNCA polypeptide sequence or *SNCA* nucleic acid sequence (*e.g.,* transcript) which exist naturally within the defined taxonomic group, such as mammalian, such as mouse, monkey, and human. Typically, when referring to "naturally occurring variants" of a SNCA polynucleotide the term can also encompass any allelic variant of the *SNCA*-encoding genomic DNA which is found at Chromosomal position 17q21 by chromosomal translocation or duplication, and the RNA, such as mRNA derived therefrom. "Naturally occurring variants" can also include variants derived from alternative splicing of the *SNCA* mRNA. When referenced to a specific polypeptide sequence, *e.g.*, the term also includes naturally occurring forms of the protein, which can therefore be processed, *e.g.,* by co- or post-translational modifications, such as signal peptide cleavage, proteolytic cleavage, glycosylation, etc.

[0047] The term "complement" as used herein indicates a sequence that is complementary to a reference sequence. It is well known that complementarity is the base principle of DNA replication and transcription as it is a property shared between two DNA or RNA sequences, such that when they are aligned antiparallel to each other, the nucleotide bases at each position in the sequences will be complementary, much like looking in the mirror and seeing the reverse of things. Therefore, for example, the complement of a sequence of 5' "ATGC" 3' can be written as 3' "TACG" 5' or 5' "GCAT" 3'. The terms "reverse complement", "reverse complementary" and "reverse complementarity" as used herein are interchangeable with the terms "complement", "complementary" and "complementarity." Therefore, the sequence of 5' attcctttacaccacac 3'(SEQ ID NO: 4) can be complementary to 5' gtgtggtgtaaaggaat 3'.

[0048] As used herein, a reference to a SEQ ID number (*i.e.*, SEQ ID NO: 4) includes a particular nucleobase sequence, but does not include any design or full chemical structure. When this specification refers to a specific ASO number (*i.e.*, ASO-005459), the reference includes the sequence, the specific ASO design, and the chemical structure.

[0049] "Potency" is normally expressed as an IC_{50} or EC_{50} value, in μM, nM or pM unless otherwise stated. Potency can also be expressed in terms of percent inhibition. IC_{50} is the median inhibitory concentration of a therapeutic molecule. EC_{50} is the median effective concentration of a therapeutic molecule relative to a vehicle or control (*e.g.,* saline). In functional assays, IC_{50} is the concentration of a therapeutic molecule that reduces a biological response, e.g., transcription of mRNA or protein expression, by 50% of the biological response that is achieved by the therapeutic molecule. In functional assays, EC_{50} is the concentration of a therapeutic molecule that produces 50% of the biological response, *e.g.,* transcription of mRNA or protein expression. IC_{50} or EC_{50} can be calculated by any number of means known in the art.

[0050] By "subject" or "individual" or "animal" or "patient" or "mammal," is meant any subject, particularly a mammalian subject, for whom diagnosis, prognosis, or therapy is desired. Mammalian subjects include humans, domestic animals, farm animals, sports animals, and zoo animals including, *e.g.*, humans, non-human primates, dogs, cats, guinea pigs, rabbits, rats, mice, horses, cattle, bears, and so on.

[0051] The term "pharmaceutical composition" refers to a preparation which is in such form as to permit the biological activity of the active ingredient to be effective, and which

contains no additional components which are unacceptably toxic to a subject to which the composition would be administered. Such composition can be sterile.

[0052] An "effective amount" of an ASO as disclosed herein is an amount sufficient to carry out a specifically stated purpose. An "effective amount" can be determined empirically and in a routine manner, in relation to the stated purpose.

[0053] Terms such as "treating" or "treatment" or "to treat" or "alleviating" or "to alleviate" refer to both (1) therapeutic measures that cure, slow down, lessen symptoms of, and/or halt progression of a diagnosed pathologic condition or disorder and (2) prophylactic or preventative measures that prevent and/or slow the development of a targeted pathologic condition or disorder. Thus, those in need of treatment include those already with the disorder; those prone to have the disorder; and those in whom the disorder is to be prevented. In certain embodiments, a subject is successfully "treated" for a disease or condition disclosed elsewhere herein according to the methods provided herein if the patient shows, *e.g.*, total, partial, or transient alleviation or elimination of symptoms associated with the disease or disorder.

II. ASO-005459

[0054] The ASO of the disclosure (*i.e.*, ASO-005459) comprises a contiguous nucleotide sequence of 17 nucleotides in length, which corresponds to the complement of a region (*i.e.*, junction between intron 1 and exon 2) of *SNCA* transcript, *i.e.*, nucleotides 7,604-7,620 of SEQ ID NO: 1. The ASO of the disclosure has the nucleotide sequence as set forth in SEQ ID NO: 4 (*i.e.*, attcctttacaccacac) with an ASO design of LDLDDDDDDDDDDLLLL (*i.e.*, AtTcctttacaccACAC), wherein the L indicates a locked nucleic acid nucleoside (*i.e.*, LNA, e.g., beta-D-oxy-LNA) and the D indicates a deoxyribonucleic acid (DNA). Accordingly, the 1^{st}, 3^{rd}, and the 14^{th}-17^{th} nucleotides from the 5' end of ASO-005459 is beta-D-oxy-LNA and each of the other nucleotides is, DNA. The ASO disclosed herein also has the following chemical structure: OxyAs DNAts OxyTs DNAcs DNAcs DNAts DNAts DNAts DNAas DNAcs DNAas DNAcs DNAcs OxyAs OxyMCs OxyAs OxyMC, wherein "s" indicates a phosphorothioate linkage. The structural formula for ASO-005459 is provided in FIG. 1B, wherein M+ is a pharmaceutically acceptable counterion. The term "pharmaceutically acceptable counterion," as used herein," refers to an ion that accompanies an ionic species in order to maintain electric neutrality that is not biologically or otherwise undesirable and thereby,

allowing for the production of a pharmaceutically acceptable salt form. Accordingly, in some embodiments, pharmaceutically acceptable counterion can be H^+, Na^+, K^+, NH_4^+, Li^+, or any other cation with a charge of 1^+. In some embodiments, the pharmaceutically acceptable counterion is H^+, Na^+, NH_4^+, and combinations thereof.

[0055] The term "pharmaceutically acceptable salts" as used herein refers to derivatives of ASO-005459 wherein the ASO-005459 is modified (*e.g.*, addition of a cation disclosed herein) by making salts thereof. Such salts retain the desired biological activity of the ASO without imparting undesired toxicological effects. The ASO of the disclosure can be in any salt form. In some embodiments, the ASO of the disclosure is in the form of a sodium salt. In other embodiments, the ASO is in the form of a potassium salt.

[0056] ASO-005459 can bind to intron1/exon2 junction of *SNCA* mRNA and prevent translation of *SNCA* mRNA. In some embodiments, because of the sugar modification, the ASO of the disclosure has a binding affinity to a target RNA sequence that is enhanced by at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 100% compared to a control (*e.g.,* an ASO without such sugar modification).

[0057] The monomers of the ASO described herein are coupled together via linkage groups. Suitably, each monomer is linked to the 3' adjacent monomer via a linkage group.

[0058] The person having ordinary skill in the art would understand that, in the context of the present disclosure, the 5' monomer at the end of an ASO does not comprise a 5' linkage group, although it may or may not comprise a 5' terminal group.

[0059] The terms "linkage group" and "internucleotide linkage" are intended to mean a group capable of covalently coupling together two nucleotides. Examples include phosphate groups and phosphorothioate groups.

[0060] Examples of internucleotide linkages include phosphodiester linkage, a phosphotriester linkage, a methylphosphonate linkage, a phosphoramidate linkage, a phosphorothioate linkage, and combinations thereof. *See also* WO2007/031091, which is hereby incorporated by reference in its entirety.

[0061] In one aspect of the ASO of the disclosure, the nucleotides are linked to each other by means of phosphorothioate groups.

[0062] It is recognized that the inclusion of phosphodiester linkages, such as one or two linkages, into an otherwise phosphorothioate ASO, particularly between or adjacent to

nucleotides can modify the bioavailability and/or bio-distribution of an ASO – *see* WO2008/113832, hereby incorporated by reference.

[0063] In some embodiments, such as the embodiments referred to above, where suitable and not specifically indicated, all remaining linkage groups are either phosphodiester or phosphorothioate, or a mixture thereof.

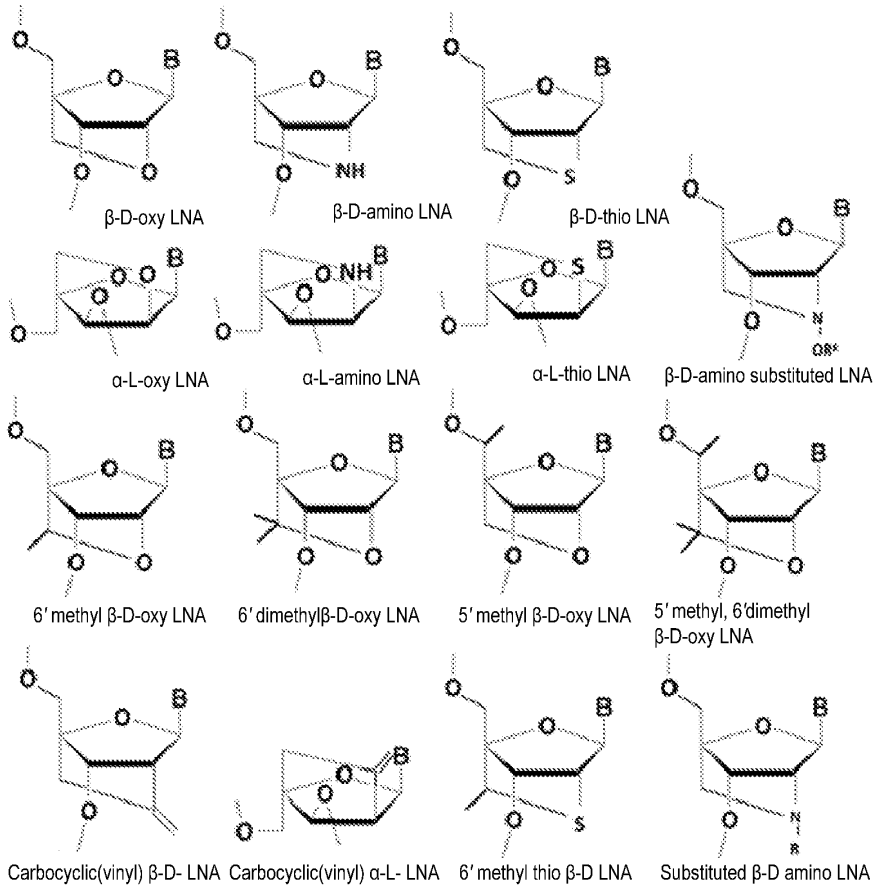
[0064] In some embodiments, all the internucleotide linkage groups are phosphorothioate.

[0065] A "LNA nucleoside" is a 2'- modified nucleoside which comprises a biradical linking the C2' and C4' of the ribose sugar ring of said nucleoside (also referred to as a "2'- 4' bridge"), which restricts or locks the conformation of the ribose ring. These nucleosides are also termed bridged nucleic acid or bicyclic nucleic acid (BNA) in the literature. The locking of the conformation of the ribose is associated with an enhanced affinity of hybridization (duplex stabilization) when the LNA is incorporated into an oligonucleotide for a complementary RNA or DNA molecule. This can be routinely determined by measuring the melting temperature of the oligonucleotide/complement duplex.

[0066] Non limiting, exemplary LNA nucleosides are disclosed in WO 99/014226, WO 00/66604, WO 98/039352 , WO 2004/046160, WO 00/047599, WO 2007/134181, WO 2010/077578, WO 2010/036698, WO 2007/090071, WO 2009/006478, WO 2011/156202, WO 2008/154401, WO 2009/067647, WO 2008/150729, Morita et al., Bioorganic & Med.Chem. Lett. 12, 73-76, Seth et al. J. Org. Chem. 2010, Vol 75(5) pp. 1569-81, and Mitsuoka et al., Nucleic Acids Research 2009, 37(4), 1225-1238, and Wan and Seth, J. Medical Chemistry 2016, 59, 9645−9667.

[0067] Further non limiting, exemplary LNA nucleosides are disclosed in Scheme 1.

Scheme 1



[0068] In a particular embodiment, the LNA useful for the disclosure is beta-D-oxy-LNA.

II.A. Conjugates

[0069] The term conjugate as used herein refers to ASO-005459 covalently linked to a non-nucleotide moiety.

[0070] Conjugation of ASO-005459 to one or more non-nucleotide moieties may improve the pharmacology of the ASO, *e.g.*, by affecting the activity, cellular distribution, cellular uptake or stability of the ASO. In some embodiments the conjugate moiety modify or enhance the pharmacokinetic properties of the ASO by improving cellular distribution, bioavailability, metabolism, excretion, permeability, and/or cellular uptake of the ASO. In particular the conjugate may target the ASO to a specific organ, tissue or cell type and thereby enhance the effectiveness of the ASO in that organ, tissue or cell type. At the same time the conjugate can serve to reduce activity of the ASO in non-target cell types, tissues or organs, *e.g.*, off target activity or activity in non-target

cell types, tissues or organs. WO 93/07883 and WO2013/033230 provide suitable conjugate moieties. Further suitable conjugate moieties are those capable of binding to the asialoglycoprotein receptor (ASGPr). In particular tri-valent N-acetylgalactosamine conjugate moieties are suitable for binding to the ASGPr, *see* for example WO 2014/076196, WO 2014/207232, and WO 2014/179620.

[0071] ASO conjugates and their synthesis have also been reported in comprehensive reviews by Manoharan in Antisense Drug Technology, Principles, Strategies, and Applications, S.T. Crooke, ed., Ch. 16, Marcel Dekker, Inc., 2001 and Manoharan, *Antisense and Nucleic Acid Drug Development*, 2002, 12, 103.

[0072] In some embodiments, the non-nucleotide moiety (conjugate moiety) is selected from the group consisting of carbohydrates, cell surface receptor ligands, drug substances, hormones, lipophilic substances, polymers, proteins, peptides, toxins (*e.g.*, bacterial toxins), vitamins, viral proteins (*e.g.*, capsids), and combinations thereof.

II.B. Activated ASOs

[0073] The term "activated ASO," as used herein, refers to an ASO of the disclosure that is covalently linked (*i.e.,* functionalized) to at least one functional moiety that permits covalent linkage of the ASO to one or more conjugated moieties, *i.e.,* moieties that are not themselves nucleic acids or monomers, to form the conjugates herein described. Typically, a functional moiety will comprise a chemical group that is capable of covalently bonding to the ASO via, *e.g.,* a 3'-hydroxyl group or the exocyclic NH_2 group of the adenine base, a spacer that can be hydrophilic and a terminal group that is capable of binding to a conjugated moiety (*e.g.,* an amino, sulfhydryl or hydroxyl group). In some embodiments, this terminal group is not protected, *e.g.,* is an NH_2 group. In other embodiments, the terminal group is protected, for example, by any suitable protecting group such as those described in "Protective Groups in Organic Synthesis" by Theodora W Greene and Peter G M Wuts, 3rd edition (John Wiley & Sons, 1999).

[0074] In some embodiments, ASO-005459 is functionalized at the 5' end in order to allow covalent attachment of the conjugated moiety to the 5' end of the ASO. In other embodiments, the ASO of the disclosure can be functionalized at the 3' end. In still other embodiments, the ASO of the disclosure can be functionalized along the backbone or on the heterocyclic base moiety. In yet other embodiments, the ASO of the disclosure can be

functionalized at more than one position independently selected from the 5' end, the 3' end, the backbone, and the base.

[0075] In some embodiments, activated ASO of the disclosure is synthesized by incorporating during the synthesis one or more monomers that is covalently attached to a functional moiety. In other embodiments, activated ASO of the disclosure is synthesized with monomers that have not been functionalized, and the ASO is functionalized upon completion of synthesis.

III. Pharmaceutical Compositions and Administration Routes

[0076] ASO-005459 can be used in pharmaceutical formulations and compositions. Suitably, such compositions comprise a pharmaceutically acceptable diluent, carrier, salt, or adjuvant.

[0077] ASO-005459 can be included in a unit formulation such as in a pharmaceutically acceptable carrier or diluent in an amount sufficient to deliver to a patient a therapeutically effective amount without causing serious side effects in the treated patient. However, in some forms of therapy, serious side effects may be acceptable in terms of ensuring a positive outcome to the therapeutic treatment.

[0078] The formulated drug can comprise pharmaceutically acceptable binding agents and adjuvants. Capsules, tablets, or pills can contain for example the following compounds: microcrystalline cellulose, gum or gelatin as binders; starch or lactose as excipients; stearates as lubricants; various sweetening or flavoring agents. For capsules, the dosage unit may contain a liquid carrier like fatty oils. Likewise, coatings of sugar or enteric agents may be part of the dosage unit. The oligonucleotide formulations can also be emulsions of the active pharmaceutical ingredients and a lipid forming a micellular emulsion.

[0079] 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. Administration can be (a) oral (b) pulmonary, *e.g.,* by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, (c) topical including epidermal, transdermal, ophthalmic and to mucous membranes including vaginal and rectal delivery; or (d) parenteral including intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.,* intrathecal, intra-cerebroventricular, or intraventricular, administration.

In one embodiment ASO-005459 is administered IV, IP, orally, topically or as a bolus injection or administered directly in to the target organ. In some embodiments, ASO-005459 is administered intrathecal or intra-cerebroventricular as a bolus injection.

[0080] Pharmaceutical compositions and formulations for topical administration can include transdermal patches, ointments, lotions, creams, gels, drops, sprays, suppositories, liquids, and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Examples of topical formulations include those in which ASO-005459 is in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents, and surfactants. Compositions and formulations for oral administration include but are not limited to powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets, or minitablets. Compositions and formulations for parenteral, intrathecal, intra-cerebroventricular, or intraventricular 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.

[0081] Pharmaceutical compositions of the present disclosure include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Delivery of drug to the target tissue can be enhanced by carrier-mediated delivery including, but not limited to, cationic liposomes, cyclodextrins, porphyrin derivatives, branched chain dendrimers, polyethylenimine polymers, nanoparticles and microspheres (Dass CR. *J Pharm Pharmacol* 2002; 54(l):3-27).

[0082] 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.

[0083] For parenteral, subcutaneous, intradermal or topical administration the formulation can include a sterile diluent, buffers, regulators of tonicity and antibacterials. The active ASOs can be prepared with carriers that protect against degradation or immediate elimination from the body, including implants or microcapsules with controlled release properties. For intravenous administration the carriers can be physiological saline or phosphate buffered saline. International Publication No. WO2007/031091 (A2), published March 22, 2007, further provides suitable pharmaceutically acceptable diluent, carrier and adjuvants - which are hereby incorporated by reference.

IV. Diagnostics

[0084] This disclosure further provides a diagnostic method useful during diagnosis of SNCA related diseases, *e.g.,* a synucleinopathy. Non-limiting examples of synucleinopathy include, but are not limited to, Parkinson's disease, Parkinson's Disease Dementia (PDD), dementia with Lewy bodies, and multiple system atrophy.

[0085] ASO-005459 can be used to measure expression of *SNCA* transcript in a tissue or body fluid from an individual and comparing the measured expression level with a standard *SNCA* transcript expression level in normal tissue or body fluid, whereby an increase in the expression level compared to the standard is indicative of a disorder treatable by ASO-005459.

[0086] ASO-005459 can be used to assay *SNCA* transcript levels in a biological sample using any methods known to those of skill in the art. (Touboul *et. al.*, *Anticancer Res.* (2002) 22 (6A): 3349-56; Verjout *et. al.*, *Mutat. Res*. (2000) 640: 127-38); Stowe *et. al.*, *J. Virol. Methods* (1998) 75 (1): 93-91).

[0087] By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source of cells potentially expressing *SNCA* transcript. Methods for obtaining tissue biopsies and body fluids from mammals are well known in the art.

V. Kits comprising ASO

[0088] This disclosure further provides kits that comprise ASO-005459 described herein and that can be used to perform the methods described herein. In certain embodiments, a kit comprises at least ASO-005459 in one or more containers. In some embodiments, the

kits contain all of the components necessary and/or sufficient to perform a detection assay, including all controls, directions for performing assays, and any necessary software for analysis and presentation of results. One skilled in the art will readily recognize that ASO-005459 can be readily incorporated into one of the established kit formats which are well known in the art.

VI. Methods of Using

[0089] ASO-005459 can be utilized for therapeutics and prophylaxis. SNCA is a 140 amino acid protein preferentially expressed in neurons at pre-synaptic terminals where it is thought to play a role in regulating synaptic transmission. It has been proposed to exist natively as both an unfolded monomer and a stable tetramer of α-helices and has been shown to undergo several posttranslational modifications. One modification that has been extensively studied is phosphorylation of SNCA at amino acid serine 129 (S129). Normally, only a small percentage of SNCA is constitutively phosphorylated at S129 (pS129), whereas the vast majority of SNCA found in pathological intracellular inclusions is pS129 SNCA. These pathological inclusions consist of aggregated, insoluble accumulations of misfolded SNCA proteins and are a characteristic feature of a group of neurodegenerative diseases collectively known as synucleinopathies (or α-synucleinopathies).

[0090] In synucleinopathies, SNCA can form pathological aggregates in neurons known as Lewy bodies, which are characteristic of both Parkinson's Disease (PD), Parkinson's Disease Dementia (PDD), and dementia with Lewy bodies (DLB). ASO-005459 therefore can reduce the number of the SNCA pathological aggregates or prevent formation of the SNCA pathological aggregates. Additionally, abnormal SNCA-rich lesions called glial cytoplasmic inclusions (GCIs) are found in oligodendrocytes, and represent the hallmark of a rapidly progressing, fatal synucleinopathy known as multiple systems atrophy (MSA). In some embodiments, ASO-005459 reduces the number of GCIs or prevents formation of GCIs. Reports of either undetectable or low levels of *SNCA* mRNA expression in oligodendrocytes suggest that some pathological form of SNCA is propagated from neurons, where it is highly expressed, to oligodendrocytes. In certain embodiments, ASO-005459 reduces or prevents propagation of SNCA, *e.g.,* pathological form of SNCA, from neurons.

[0091] ASO-005459 can be used in research, *e.g.,* to specifically inhibit the synthesis of SNCA protein (typically by degrading or inhibiting the mRNA and thereby prevent protein formation) in cells and experimental animals thereby facilitating functional analysis of the target or an appraisal of its usefulness as a target for therapeutic intervention. Further provided are methods of down-regulating the expression of *SNCA* mRNA and/or SNCA protein in cells or tissues comprising contacting the cells or tissues, *in vitro* or *in vivo*, with an effective amount of ASO-005459, conjugates, or compositions of the disclosure.

[0092] For therapeutics, an animal or a human, suspected of having a disease or disorder, which can be treated by modulating the expression of *SNCA* transcript and/or SNCA protein is treated by administering ASO-005459 in accordance with this disclosure. Further provided are methods of treating a mammal, such as treating a human, suspected of having or being prone to a disease or condition, associated with expression of *SNCA* transcript and/or SNCA protein by administering a therapeutically or prophylactically effective amount of ASO-005459 or compositions of the disclosure. The ASO, a conjugate, or a pharmaceutical composition according to the disclosure is typically administered in an effective amount. In some embodiments, the ASO or conjugate of the disclosure is used in therapy.

[0093] The disclosure further provides for ASO-005459 for use in treating one or more of the diseases referred to herein, such as a disease selected from the group consisting of Parkinson's disease, Parkinson's Disease Dementia (PDD), dementia with Lewy bodies, multiple system atrophy, and any combinations thereof.

[0094] The disclosure further provides for a method for treating α-synucleinopathies, the method comprising administering an effective amount of the ASO, conjugates, or pharmaceutical compositions thereof to an animal in need thereof (such as a patient in need thereof).

[0095] In certain embodiments, the disease, disorder, or condition is associated with overexpression of *SNCA* gene transcript and/or SNCA protein.

[0096] The disclosure also provides for methods of inhibiting (*e.g.,* by reducing) the expression of *SNCA* gene transcript and/or SNCA protein in a cell or a tissue, the method comprising contacting the cell or tissue, *in vitro* or *in vivo*, with an effective amount of the ASO, conjugates, or pharmaceutical compositions thereof, of the disclosure to affect degradation of expression of *SNCA* gene transcript thereby reducing SNCA protein.

[0097] In certain embodiments, ASO-005459 is used to reduce the expression of *SNCA* mRNA in one or more sections of brain, *e.g.,* hippocampus, brainstem, striatum, or any combinations thereof. In other embodiments, ASO-005459 reduces the expression of *SNCA* mRNA, *e.g.,* in brain stem and/or striatum, less than 70%, less than 60%, less than 50%, less than 40%, less than 30%, less than 20%, less than 10%, or less than 5% compared to the *SNCA* mRNA expression after administration of or exposure to a vehicle (no ASO), at day 3, day 5, day 7, day 10, day 14, day 15, day 20, day 21, or day 25. In some embodiments, the expression of *SNCA* mRNA is maintained below 70%, below 60%, below 50%, below 40%, below 30%, below 20%, below 10%, or below 5% compared to the *SNCA* mRNA expression after administration of or exposure to a vehicle (no ASO) until day 28, day 30, day 32, day 35, day 40, day 42, day 45, day 49, day 50, day 56, day 60, day 63, day 70, or day 75.

[0098] In other embodiments, ASO-005459 reduces *SNCA* mRNA and/or SNCA protein expression in medulla, caudate putamen, pons cerebellum, lumbar spinal cord, frontal cortex, and/or any combinations thereof.

[0099] The disclosure also provides for the use of ASO-005459 or a conjugate of the disclosure as described for the manufacture of a medicament. The disclosure also provides for a composition comprising ASO-005459 or a conjugate thereof for use in treating a disorder as referred to herein, or for a method of the treatment of as a disorder as referred to herein. The present disclosure also provides ASO-005459 or the conjugates for use in therapy. The present disclosure additionally provides ASO-005459 or the conjugates for use in the treatment of synucleinopathy.

[0100] The disclosure further provides for a method for inhibiting SNCA protein in a cell which is expressing SNCA comprising administering ASO-005459 or a conjugate according to the disclosure to the cell so as to affect the inhibition of SNCA protein in the cell.

[0101] The disclosure includes a method of reducing, ameliorating, preventing, or treating neuronal hyperexcitability in a subject in need thereof comprising administering ASO-005459 or a conjugate according to the disclosure.

[0102] The disclosure also provides for a method for treating a disorder as referred to herein the method comprising administering ASO-005459 or a conjugate according to the disclosure as herein described and/or a pharmaceutical composition according to the disclosure to a patient in need thereof.

[0103] ASO-005459 and other compositions according to the disclosure can be used for the treatment of conditions associated with over expression or expression of mutated version of SNCA protein.

[0104] The disclosure provides for ASO-005459 or the conjugate according to disclosure, for use as a medicament, such as for the treatment of α-Synucleinopathies. In some embodiments the α-Synucleinopathy is a disease selected from the group consisting of Parkinson's disease, Parkinson's Disease Dementia (PDD), dementia with Lewy bodies, multiple system atrophy, and any combinations thereof.

[0105] The disclosure further provides use of ASO-005459 in the manufacture of a medicament for the treatment of a disease, disorder or condition as referred to herein. In some embodiments, ASO-005459 or a conjugate thereof is used for the manufacture of a medicament for the treatment of a α-Synucleinopathy, a seizure disorder, or a combination thereof.

[0106] Generally stated, one aspect of the disclosure is directed to a method of treating a mammal suffering from or susceptible to conditions associated with abnormal levels of SNCA (*i.e.*, a α-synucleinopathy), comprising administering to the mammal a therapeutically effective amount of the ASO as described herein.

[0107] The disease or disorder, as referred to herein, can, in some embodiments, be associated with a mutation in the *SNCA* gene or a gene whose protein product is associated with or interacts with SNCA protein. Therefore, in some embodiments, the target mRNA is a mutated form of the *SNCA* sequence.

[0108] An interesting aspect of the disclosure is directed to the use of ASO-005459 as defined herein or a conjugate as defined herein for the preparation of a medicament for the treatment of a disease, disorder or condition as referred to herein.

[0109] The methods of the disclosure can be employed for treatment or prophylaxis against diseases caused by abnormal levels of SNCA protein. In some embodiments, diseases caused by abnormal levels of SNCA protein are α-synucleinopathies. In certain embodiments, α-synucleinopathies include Parkinson's disease, Parkinson's Disease Dementia (PDD), dementia with Lewy bodies, and multiple system atrophy.

[0110] Alternatively stated, in some embodiments, the disclosure is furthermore directed to a method for treating abnormal levels of SNCA protein, the method comprising administering ASO-005459, or a conjugate of the disclosure, or a pharmaceutical composition of the disclosure to a patient in need thereof.

[0111] The disclosure also relates to ASO-005459, a composition or a conjugate as defined herein for use as a medicament.

[0112] The disclosure further relates to use of a compound, composition, or a conjugate as defined herein for the manufacture of a medicament for the treatment of abnormal levels of SNCA protein or expression of mutant forms of SNCA protein (such as allelic variants, such as those associated with one of the diseases referred to herein).

[0113] A patient who is in need of treatment is a patient suffering from or likely to suffer from the disease or disorder.

[0114] The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, Sambrook *et al.*, ed. (1989) Molecular Cloning A Laboratory Manual (2nd ed.; Cold Spring Harbor Laboratory Press); Sambrook *et al.*, ed. (1992) Molecular Cloning: A Laboratory Manual, (Cold Springs Harbor Laboratory, NY); D. N. Glover ed., (1985) DNA Cloning, Volumes I and II; Gait, ed. (1984) Oligonucleotide Synthesis; Mullis *et al.* U.S. Pat. No. 4,683,195; Hames and Higgins, eds. (1984) Nucleic Acid Hybridization; Hames and Higgins, eds. (1984) Transcription And Translation; Freshney (1987) Culture Of Animal Cells (Alan R. Liss, Inc.); Immobilized Cells And Enzymes (IRL Press) (1986); Perbal (1984) A Practical Guide To Molecular Cloning; the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Miller and Calos eds. (1987) Gene Transfer Vectors For Mammalian Cells, (Cold Spring Harbor Laboratory); Wu *et al.*, eds., Methods In Enzymology, Vols. 154 and 155; Mayer and Walker, eds. (1987) Immunochemical Methods In Cell And Molecular Biology (Academic Press, London); Weir and Blackwell, eds., (1986) Handbook Of Experimental Immunology, Volumes I-IV; Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986);); Crooke, Antisense drug Technology: Principles, Strategies and Applications, 2nd Ed. CRC Press (2007) and in Ausubel *et al.* (1989) Current Protocols in Molecular Biology (John Wiley and Sons, Baltimore, Md.).

[0115] All of the references cited above, as well as all references cited herein, are incorporated herein by reference in their entireties.

[0116] The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Construction of ASO-005459

[0117] The ASO described herein (*i.e.*, ASO-005459) was designed to target the junction between intron 1 and exon 2 of *SNCA* pre-mRNA (*i.e.*, nucleotides 7,604 to 7,620 of SEQ ID NO: 1). ASO-005459 was designed to be a gapmer (*e.g.,* alternating gapmer) and contains locked nucleic acids – LNAs (upper case letters), beta-deoxy LNA at the 5' end and the 3' end, and a phosphorothioate backbone. But the backbone can be other types of backbones (*e.g.,* a phosphodiester linkage, a phosphotriester linkage, a methylphosphonate linkage, a phosphoramidate linkage, or combinations thereof).

[0118] The ASO of the present disclosure was synthesized using methods well known in the art. Exemplary methods of preparing such ASOs are described in Barciszewski *et al.*, Chapter 10 – " Locked Nucleic Acid Aptamers" in *Nucleic Acid and Peptide Aptamers: Methods and Protocols*, vol. 535, Gunter Mayer (ed.) (2009), the entire contents of which is hereby expressly incorporated by reference herein.

Example 2. High Content Assay to Measure Reduction of SNCA Protein in Primary Neurons

[0119] ASO-005459 was tested for its ability to reduce SNCA protein expression in primary mouse neurons. The primary neuronal cultures were established from the forebrain of PAC-Tg($SNCA^{A53T}$)$^{+/+}$;$SNCA^{-/-}$ ("PAC-A53T") mice carrying the entire human *SNCA* gene with a A53T mutation on a mouse *SNCA* knockout background. *See* Kuo Y *et al.*, *Hum Mol Genet.*, 19: 1633-50 (2010). All procedures involving mice were conducted according to Animal Test Methods (ATM) approved by the Bristol-Myers Squibb Animal Care and Use Committee (ACUC). Primary neurons were generated by papain digestion according to manufacturer's protocol (Worthington Biochemical Corporation, LK0031050). Isolated neurons were washed and resuspended in Neurobasal medium (NBM, Invitrogen) supplemented with B27 (Gibco), 1.25 μM Glutamax (Gibco), 100 unit/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml Amphotericin B.

[0120] Cells were plated on multi-well poly D-Lysine coated plates at 5,400 cells/cm^2 (for example in 384 well plates 6,000 cells/well in 25 μl NBM). The ASO was diluted in water and added to the cells at DIV01 (*i.e.,* 1 day post plating). The ASO was added to 2x final concentration in medium then delivered to cells manually. Alternatively, the ASO in water was dispensed using a Labcyte ECHO acoustic dispenser. For ECHO dispense, 250

nl of ASO in water was added to cells in medium followed by the addition of an equal volume aliquot of fresh aliquot of NBM. For primary screening, the ASO was added to final concentrations of 5 μM, 3.3 μM, 1 μM, 200 nM, or 40 nM. For potency determination, 8-10 point titrations of the ASO was prepared from 0.75 mM stock then delivered to cultured cells for a final concentration range of 2.7-4000 nM or 4.5-10,000 nM. ASO-000010 (TCTgtcttggctTTG, SEQ ID NO: 5) and ASO-000838 (AGAaataagtggtAGT, SEQ ID NO: 6) (5 μM) were included in each plate as reference control inhibitors for tubulin and SNCA, respectively. The cells were incubated with the ASOs for 14 days to achieve steady state reduction of mRNA.

[0121] After the 14-day incubation, the cells were fixed by the addition of fixative to final concentrations of 4% formaldehyde (J.T. Baker) and 4% sucrose (Sigma) in the wells. The cells were fixed for 15 minutes, and then, the fixative aspirated from the wells. Then, the cells were permeabilized for 20 minutes with a phosphate buffered saline (PBS) solution containing 0.3% Triton-X 100 and 3% bovine serum albumin (BSA) or 3% Normal goat serum. Afterwards, the permeabilization buffer was aspirated from the wells, and the cells were washed once with PBS. The primary antibodies were then diluted in PBS containing 0.1% Triton X-100 and 3% BSA. Dilutions of 1:1000 of rabbit anti-SNCA (Abcam) and 1:500 of chicken anti-tubulin (Abcam) were used. Cells were incubated with the primary antibodies between 2 hours to overnight. Following the incubation, the primary antibody staining solution was aspirated, and the cells were washed 2-times with PBS. A secondary staining solution containing 1:500 dilution of goat-anti-chicken Alexa 567 antibody, goat anti-rabbit-Alexa 488 antibody, and Hoechst (10 μg/ml) in PBS containing 0.1% Triton X-100 with 3% BSA was added to the wells, and the plates were incubated for 1 hour. Afterwards, the secondary staining solution was aspirated from the wells, and the cells were washed 3-times with PBS. After washing the cells, 60 μl of PBS was added to each well. Plates were then stored in the PBS until imaging.

[0122] For imaging, the plates were scanned on a Thermo-Fisher (Cellomics) CX5 imager using the Spot Detector bio-application (Cellomics) to quantify nuclei (Hoechst stain, Channel 1), tubulin extensions (Alexa 567, channel 2) and SNCA (Alexa 488, channel 3). Object count (nuclei) was monitored but not published to the database. The total area covered by tubulin was quantified as the feature SpotTotalAreaCh2 and total intensity of staining for SNCA quantified as SpotTotalIntenCh3. The tubulin measure was

included to monitor toxicity. To determine the reduction of SNCA protein, the ratio of SNCA intensity to the tubulin staining area was calculated and results normalized as % inhibition median using the median of vehicle treated wells as total and ASO-000010 or ASO-000838 wells as maximally inhibited wells for tubulin or SNCA, respectively.

Example 3: QUANTIGENE® Analysis (96-well assay) to Measure mRNA Reduction in Human Neurons

[0123] The ability of ASO-005459 to reduce human *SNCA* mRNA and/or possible human off target mRNA species was measured *in vitro* by QUANTIGENE® analysis. Human neurons (Cellular Dynamics Inc., "iNeurons"), were thawed, plated, and cultured per manufacturer's specifications. These iNeurons are highly pure population of human neurons derived from induced pluripotent stem (iPS) cells using Cellular Dynamic's proprietary differentiation and purification protocols.

[0124] Lysis: Cells were plated on poly-L-ornithine/laminin coated 96-well plates at 50,000 to 100,000 cells per well (dependent on the expression of the off target being investigated) and maintained in Neurobasal media supplemented with B27, glutamax, and Penicillin-Streptomycin. The ASO was diluted in water and added to cells at DIV01 (*i.e.*, 1 day post plating). For single point measurements, a final ASO concentration of 0.5 μM was typically used. For IC_{50} determinations, the neurons were treated with a seven-point concentration response dilution of 1:4, with the highest concentration as 5 μM to define the IC_{50}. The cells were then incubated at 37°C and 5% CO_2 for 6 days to achieve steady state reduction of mRNA.

[0125] After the incubation, the media was removed and cells were washed 1X in DPBS and lysed as follows. Measurement of lysate messenger RNA was performed using the QUANTIGENE® 2.0 Reagent System (AFFYMETRIX®), which quantitates RNA using a branched DNA-signal amplification method reliant on the specifically designed RNA capture probe set. The working cell lysis buffer solution was made by adding 50 μl proteinase K to 5ml of pre-warmed (37°C) Lysis mix and diluted in dH_2O to a 1:4 final dilution. The working lysis buffer was added to the plates (100 to 150 μl/ well, depending on the expression of the off target being investigated), triturated 10 times, sealed and incubated for 30 min at 55°C. Following the lysis, the wells were triturated 10 more times, and the plates were stored at -80°C or assayed immediately.

[0126] Assay: Depending on the specific capture probe used (*i.e., SNCA*, *PROS1*, or tubulin), the lysates were diluted (or not diluted) in the lysis mix. Then, the lysates were added to the capture plates (96-well polystyrene plate coated with capture probes) at a total volume of 80 µl/well. Working probe sets reagents were generated by combining nuclease-free water (12.1 µl), lysis mixture (6.6 µl), blocking reagent (1 µl), and specific 2.0 probe set (0.3 µl) (human *SNCA* catalogue #SA-50528, human PROS1 catalogue #SA-10542, or human beta 3 tubulin catalogue #SA-15628) per manufacturer's instructions (QUANTIGENE® 2.0 AFFYMETRIX®). Next, 20 µl working probe set reagents were added to 80 µl lysate dilution (or 80 µl lysis mix for background samples) on the capture plate. Plates were centrifuged at 240g for 20 seconds and then incubated for 16-20 hours at 55°C to hybridize (target RNA capture).

[0127] Signal amplification and detection of target RNA began by washing plates with buffer 3 times (300 µl/well) to remove any unbound material. Next, the 2.0 Pre-Amplifier hybridization reagent (100 µl/well) was added, incubated at 55°C for 1 hour, then aspirated, and wash buffer was added and aspirated 3 times. The 2.0 Amplifier hybridization reagent was then added as described (100 µl/well), incubated for 1 hour at 55°C and the wash step repeated as described previously. The 2.0 Label Probe hybridization reagent was added next (100 µl/well), incubated for 1 hour at 50°C and the wash step was repeated as described previously. The plates were again centrifuged at 240g for 20 seconds to remove any excess wash buffer and then, the 2.0 Substrate was added (100 µl/well) to the plates. Plates were incubated for 5 minutes at room temperature and then, the plates were imaged on a PerkinElmer Envision multilabel reader in luminometer mode within 15 minutes.

[0128] Data determination: For the gene of interest, the average assay background signal was subtracted from the average signal of each technical replicate. The background-subtracted, average signals for the gene of interest were then normalized to the background-subtracted average signal for the housekeeping tubulin RNA. The percent inhibition for the treated sample was calculated relative to the control treated sample lysate. Results of QUANTIGENE® assays for cells treated with the ASOs are shown in *e.g.,* FIG. 3.

Example 4: QUANTIGENE® Analysis (96-well assay) to Measure mRNA Reduction in Ramos Cells

[0129] To measure possible human off target IKZF3 (IKAROS family zinc finger 3) mRNA reduction, Ramos cells (a human lymphocytic cell line) were used. Since Ramos cells do not express *SNCA*, *RB1* (RB transcriptional corepressor 1), which is expressed in Ramos cells, was used as a positive control for assessing ASO-mediated knockdown IKZF3 mRNA expression. Two ASOs were synthesized to bind to and knockdown human RB1 mRNA expression. Beta-2 microglobulin (β2M) was used as a housekeeping gene control. The Ramos cells were grown in suspension in RPMI media supplemented with FBS, glutamine, and Pen/Strep.

[0130] Lysis: Cells were plated on poly-L-ornithine/laminin coated 96 well plates at 20,000 cells per well and maintained in Neurobasal media containing B27, glutamax and Penicillin-Streptomycin. The ASO was diluted in water and added to cells at 1 day post plating (DIV01) to a final concentration of 1 μM. Following ASO treatment, the cells were incubated at 37°C for 4 days to achieve steady state reduction of mRNA. After the incubation, the media was removed and cells lysed as follows. Measurement of lysate messenger RNA was performed using the QUANTIGENE® 2.0 Reagent System (AFFYMETRIX®), which quantitated RNA using a branched DNA-signal amplification method reliant on the specifically designed RNA capture probe set. Lysis mix (QUANTIGENE® 2.0 AFFYMETRIX®) was pre-warmed in an incubator at 37°C for 30 minutes. For lysing cells in suspension, 100 μl of 3X Lysis Buffer (with 10 μl/ml proteinase K) was added to 200 μl of cells in suspension. The cells were then triturated 10 times to lyse, and the plate sealed and incubated for 30 min at 55°C. Afterwards, the lysates were stored at -80°C or assayed immediately.

[0131] Assay: Depending on the specific capture probe used (*i.e.,* IKZF3, RB1, and β2M), the lysates were diluted (or not diluted) in the lysis mix. Then, the lysates were added to the capture plate (96 well polystyrene plate coated with capture probes) at a total volume of 80 μl/well. Working probe sets reagents were generated by combining nuclease-free water 12.1 μl, lysis mixture 6.6 μl, blocking reagent 1 μl, specific 2.0 probe set 0.3 μl (human IKZF3 catalogue #SA-17027, human RB1 catalogue #SA-10550, or human beta-2 microglobulin catalogue #SA-10012) per manufacturer instructions (QUANTIGENE® 2.0 AFFYMETRIX®). Then 20 μl working probe set reagents were added to 80 μl lysate dilution (or 80 μl lysis mix for background samples) on the capture

plate. Plates were then incubated for 16-20 hours at 55°C to hybridize (target RNA capture). Signal amplification and detection of target RNA was begun by washing plates with buffer 3 times (300 µl/well) to remove any unbound material. Next, the 2.0 Pre-Amplifier hybridization reagent (100 µl/well) was added, incubated at 55°C for 1 hour then aspirated and wash buffer was added and aspirated 3 times. The 2.0 Amplifier hybridization reagent was then added as described (100 µl/well), incubated for 1 hour at 55°C and the wash step was repeated as described previously. The 2.0 Label Probe hybridization reagent was added next (100 µl/well), incubated for 1 hour at 50°C and the wash step again was repeated as described previously. The plates were again centrifuged at 240g for 20 seconds to remove any excess wash buffer and then, the 2.0 Substrate was added (100 µl/well) to the plates. Plates were incubated for 5 minutes at room temperature, and then, the plates were imaged on a PerkinElmer Envision multilabel reader in luminometer mode within 15 minutes.

[0132] Data determination: For the gene of interest, the average assay background signal (*i.e.,* no lysate, just 1X lysis buffer) was subtracted from the average signal of each technical replicate. The background-subtracted, average signals for the gene of interest were then normalized to the background-subtracted average signal for the housekeeping mRNA (for Ramos cells, it was beta-2-microglobulin). The percent inhibition for the treated sample was calculated relative to the average of the untreated sample lysate. Results of QUANTIGENE® assays for cells treated with ASO-005459 is shown in Table 4.

Example 5: qPCR assay to measure reduction of *SNCA* mRNA in SK-N-BE(2) cells

[0133] ASO-005459 targeting *SNCA* was tested for its ability to reduce *SNCA* mRNA expression in human SK-N-BE(2) neuroblastoma cell acquired from ATCC (CRL-2271).

[0134] SK-N-BE(2) cells were grown in cell culturing media (MEM [Sigma, cat.no M2279] supplemented with 10% Fetal Bovine Serum [Sigma, cat.no F7524], 1x GlutamaxTM [Sigma, cat.no 3050-038] 1x MEM Non-essential amino acid solution [Sigma, cat.no M7145] and 0.025 mg/ml Gentamycin [Sigma, cat.no G1397]). Cells were trypsinized every 5 days, by washing with Phosphate Buffered Saline (PBS), [Sigma cat.no 14190-094] followed by addition of 0.25% Trypsin-EDTA solution (Sigma, T3924), 2-3 minutes incubation at 37°C, and trituration before cell seeding. Cells were maintained in culture for up to 15 passages.

[0135] For experimental use, 12,500 cells per well were seeded in 96 well plates (Nunc cat.no 167008) in 100 µL growth media. Oligonucleotides were prepared from a 750 µM stock. ASO dissolved in PBS was added approximately 24 hours after the cells were seeded to a final concentration of 25 µM for single point studies. Cells were incubated for 4 days without any media change. For potency determination, 8 concentrations of ASO were prepared for a final concentration range of 16-50,000 nM. ASO-004316 (CcAAAtcttataataACtAC, SEQ ID NO: 7) and ASO-002816 (TTCctttacaccACAC, SEQ ID NO: 8) were included as controls.

[0136] After incubation, cells were harvested by removal of media followed by addition of 125 µL PURELINK® Pro 96 Lysis buffer (Invitrogen 12173.001A) and 125 µL 70% ethanol. RNA was purified according to the manufacture's instruction and eluted in a final volume of 50 µL water resulting in an RNA concentration of 10-20 ng/µl. RNA was diluted 10 fold in water prior to the one-step qPCR reaction. For one-step qPCR reaction qPCR-mix (qScript TMXLE 1-step RT-qPCR TOUGHMIX® Low ROX from QauntaBio, cat.no 95134-500) was mixed with two Taqman probes in a ratio 10:1:1 (qPCR mix: probe1:probe2) to generate the mastermix. Taqman probes were acquired from LifeTechnologies: SNCA: Hs01103383_m1; PROS1: Hs00165590_m1: TBP: 4325803; GAPDH 4325792. Mastermix (6 µL) and RNA (4 µL, 1-2 ng/µL) were then mixed in a qPCR plate (MICROAMP® optical 384 well, 4309849). After sealing, the plate was given a quick spin, 1000g for 1 minute at RT, and transferred to a ViiaTM 7 system (Applied Biosystems, Thermo), and the following PCR conditions used: 50°C for 15 minutes; 95°C for 3 minutes; 40 cycles of: 95°C for 5 sec followed by a temperature decrease of 1.6°C/sec followed by 60°C for 45 sec. The data was analyzed using the QuantStudioTM Real_time PCR Software.

Example 6: *In vitro* Analysis of ASO-005459 on the Reduction of Human *SNCA* mRNA

Potency of ASO-005459 in Mouse Neurons

[0137] Using the methods described above in Example 2, ASO-005459 was tested for its ability to reduce SNCA protein expression as a downstream result of reduction in *SNCA* mRNA. Briefly, primary neurons derived from PAC-A53T mice were treated with ASO-005459 or control ASOs for 14 days. Cells were then fixed and the levels of SNCA

protein and tubulin protein were measured by high content imaging. Tubulin levels were measured to monitor toxicity and to normalize SNCA protein reduction.

[0138] As shown in Table 1 below, incubation of cells with 4 nM of ASO-005459 resulted in a 21% reduction in the SNCA protein expression. With 40 nM and 5 μM of ASO-005459, the SNCA protein expression was reduced by 84% and 86%, respectively. In contrast, ASO-005459 had minimal to no effect on the level of tubulin protein expression.

Table 1: ASO-005459 activity in A53T-PAC neurons

ASO-005459 concentration	aSyn/tub %inh	SD	N	Tub %inh	SD	N
4 nM	21.11	33.71	3	-8.60	12.07	4
40 nM	84.28	13.21	8	4.32	28.65	7
5 μM	86.45	7.91	2	29.01	18.06	2

SD = standard deviation
N = number of tests

[0139] To further evaluate potency, A53T-PAC neurons were treated with a 10-point titration of ASO-005459, as described above in Example 2A, and the IC_{50}s for the effect on SNCA and tubulin proteins determined. As shown in FIGs. 2A and 2B, and Table 2 (below), a concentration-dependent reduction in α-Syn/tubulin ratio was observed, with an average IC_{50} of 7.4 nM. This observation was consistent with the single point activity data shown in Table 1 (above). Moreover, ASO-005459 produced negligible effects on Tub levels. Taken together these results indicate that ASO-005459 potently reduces SNCA protein levels with minimal effect on overall cell viability.

Table 2: Potency and selectivity estimate for ASO-005459 for SNCA and tubulin protein in A53T-PAC neurons

aSyn/tub IC_{50} (nM)	SD	N	tub IC_{50} (nM)	SD	N
7.42	2.73	6	>4000	NA	5

SD = standard deviation
N = number of tests

Efficacy of ASO-005459 in SK-N-BE(2) cells

[0140] Using the method described in Example 5, ASO-005459 was tested for its ability to reduce SNCA mRNA in SK-N-BE(2) after 4 days treatment.

[0141] Incubation of cells with 25 μM ASO-005459 resulted in 92% reduction in SNCA mRNA in SK-N-BE(2) cells.

Potency of ASO-005459 in Human Neurons

[0142] ASO-005459 potency for SNCA was confirmed using primary human neurons as described above in Example 3. Briefly, human neurons were derived from induced pluripotent stem (iPS) cells. Cells were treated with ASO-005459 or control ASOs for 6 days and then, mRNA levels were measured by QUANTIGENE® Assay. Because human neurons also express PROS1, a potential off-target for ASO-005459, the *PROS1* mRNA level was also measured to assess the effect of ASO-005459 on off-target genes. In addition, tubulin (*TUBB*) mRNA was measured to monitor toxicity.

[0143] As shown in FIG. 3 and Table 3 (below), ASO-005459 induced a concentration-dependent reduction in *SNCA* expressed in human neurons, with an average IC_{50} of 100 nM. This IC_{50} is ~13-fold weaker than the IC_{50} for reducing SNCA protein in PAC-A53T neurons (Table 2, above). The reason for this potency shift is not clear but could be due to differences in ASO uptake, metabolism, or kinetics of SNCA knock-down. While ASO-005459 also exhibited concentration-dependent reductions of *PROS1* mRNA in the human neurons; the IC_{50} was 30-50 fold weaker than the IC_{50} for *SNCA*. These results confirm ASO-005459 activity for *SNCA* in human neurons and indicate that ASO-005459 is 30-50-fold more selective for *SNCA* compared to *PROS1*.

Table 3: Potency and Selectivity of ASO-005459 on *SNCA* and *PROS1* mRNA in human neurons

Assay Date	Batch	*SNCA* IC_{50} (nM)	*PROS1* IC_{50} (nM)	*TUBB3* IC_{50} (nM)	PROS1/*SNCA* IC_{50} ratio
8/26/2016	01-001	42	2127	>5000	51
12/8/2016	01-004	157	>5000	>5000	>32

Batch = particular lot-number of ASO-005459 used

Potency of ASO-005459 in Ramos Cells

[0144] IKZF3 is another potential off-target for ASO-005459, but, unlike PROS1, it is not expressed in human neurons. Instead, IKZF3 is robustly expressed in Ramos cells, a human lymphocytic cell line. Ramos cells were treated with 1 μM ASO-005459 and the effect on IKZF3 mRNA expression was measured. To monitor toxicity, beta-2 microglobulin mRNA was also measured. Since Ramos cells do not express *SNCA*, another gene, RB1 (RB transcriptional corepressor 1), was used as a positive control for

ASO-mediated knockdown. ASO-006754 (GGTgaggtttggtaGA, SEQ ID NO: 9) and ASO-006755 (GGTgaggtttggtagaAG, SEQ ID NO: 10) target RB1 and were included the study. As shown in Table 4 (below), at 1 μM concentration, ASO-005459 did not affect IKZF3 mRNA expression, demonstrating ASO-005459's specificity for *SNCA*. ASO-005459 also did not affect the expression of RB1 and β2M mRNAs, indicating that ASO-005459 is not toxic to Ramos cells. In contrast, ASO-006754 and ASO-006755 (the control ASOs) reduced RB1 levels by 87% and 83%, respectively, confirming the ASO activity in Ramos cells.

Table 4: Effect of 1 μM ASO-005459 on IKZF3 in Ramos cells

ASO	Target	Batch	*IKZF3* (%con)	*RB1* (%con)	*β2M* (%con)
ASO-005459	*SNCA*	01-004	102	99	94
ASO-006754	*RB1*	01-001	104	13	87
ASO-006755	*RB1*	01-001	116	17	83

Batch = particular lot-number of the ASO used

[0145] Collectively, the results presented here demonstrate that ASO-005459 is potent and highly selective for reducing *SNCA* mRNA, which in turn mediates the reduction of SNCA protein levels. The results also show ASO-005459 is well tolerated both in mouse and in human neurons. These findings support the continued development of ASO-005459 as a disease-modifying therapeutic for the treatment of synucleinopathies.

Example 7: *In Vivo* Tolerability and *In Vivo SNCA* mRNA Reduction

[0146] The *in vivo* tolerability of the ASO of the present disclosure was tested to see how the ASO was tolerated when injected into different animal models (*i.e.*, mice and cynomolgous monkeys).

Mice

[0147] *Subjects*: Male and female (2-3 months old) PAC-Tg($SNCA^{A53T}$)$^{+/+}$;$SNCA^{-/-}$ ("PAC-A53T") mice carrying the entire human *SNCA* gene with a A53T mutation on a mouse *SNCA* knockout background were used for acute, long term, and PK/PD *in vivo* efficacy studies. In some cases wild-type (WT) C57B/6 mice were used for long term (*i.e.*, 4 weeks) health assessment. Mice were housed in groups of 4 or 5 in a temperature controlled housing room with food and water available *ad libitum*. All procedures

involving mice were conducted according to Animal Test Methods (ATM) approved by the Bristol-Myers Squibb Animal Care and Use Committee (ACUC).

[0148] *ASO Dosing Solution Preparation*: Sterile saline (1 mL) syringes fitted with 0.2 μm Whatman filters and nuclease free centrifuge tubes were used to prepare dosing solutions. Indicated volume of water or saline was added to an ASO powder and was vortexed (~1 min) to dissolve the ASO powder. The solution was then allowed to sit for 10 min and was vortexed again for ~1 min. The tubes were briefly centrifuged to return all of the liquid to the bottom of the tube, and then, the solution was filtered through a 0.2 μm sterile filter into a 2nd RNase free tube. A small aliquot of the primary stock was diluted to 1 mg/ml for analysis of the concentration using Nanodrop. The analytical sample was vortexed three times with manual inversion to mix thoroughly. Then, the UV absorbance of the sample was measured twice at 260 nm with Nanodrop (the pedestal was rinsed and wiped three times before applying the sample). The test sample was discarded once the analysis was complete. The sample was considered ready for dosing if UV absorbance was between 90 and 110% of the sample. If UV absorbance exceeded 110% of the sample, a secondary dilution was prepared; if the absorbance was < 90%, the sample was prepared at a higher initial concentration and similar steps were followed as described above. Samples were stored at 4°C until use.

[0149] *Freehand Intracerebroventricular (ICV) Injection*: ICV injections were performed using a Hamilton micro syringe fitted with a 27 or 30-gauge needle, according to the method of Haley and McCormick. The needle was equipped with a polyethylene guard at 2.5-3 mm from the tip in order to limit its penetration into the brain. Mice were anesthetized using isoflurane anesthetic (1-4%). Once sufficiently anesthetized, the mice were held by the loose skin at the back of the neck with the thumb and first fingers of one hand. Applying gentle but firm pressure, the head of the animal was then immobilized by pressing against a firm flat level surface. Dosing was conducted using 10 μL Hamilton syringes fitted with a 27 ½ g needle. The needle tip was then inserted through the scalp and the skull, about 1 mm lateral and 1 mm caudal to bregma (*i.e.*, right of the midline, about 3mm back as measured from the eye line). Once the needle was positioned, the ASO was given in a volume of 5 μl in saline vehicle and injected over ~30 seconds. The needle was left in place for 5-10 seconds before removal. The mice were returned to their home cage and allowed to recover for ~2-4 min. Mice were observed continuously for 30 minutes immediately after dosing for adverse behavioral effects of drug and/or dosing.

During this time, any mouse that convulsed more than 3 separate times was immediately euthanized and given an automatic score of 20. Drug tolerability was scored 1 hr ± 15min post dosing. Animals dosed with non-tolerated compounds (tolerability score > 4) were euthanized immediately following the 1 hr evaluation.

[0150] *ASO Tolerability Assessment*: Animals dosed with the ASOs were evaluated right after the dosing and monitored for 2 hours for any adverse effects. For acute tolerability (AT) studies, mice were evaluated at the time of dosing and again at the takedown, *i.e.,* 3 days post ASO injection. For long term health assessment, the mice were weighed weekly and monitored for any health and behavioral issues until the completion of the experiment. Mice that had weight losses of greater than 15% of their initial body weight or exhibited tolerability issues were removed from the studies and euthanized. Health and tolerability assessments were conducted according to the following chart:

Table 5: Tolerability scoring system[18]

Category	Score 1	Score 2	Score 3	Score 4
Hyperactivity, stereotypies, home cage behavior	•Very slightly increased home cage exploration or rearing compared to controls	•Increased home cage exploration (e.g. digging, burying, etc.) •Increased grooming	•Moderately increased home cage activity •Detectable stereotypies (e.g. circling, repetitive behaviors, etc.)	•Marked hyperactivity •Marked stereotypies
Decreased vigilance, exploration and responsiveness	•Some reduction in exploratory activity •Responds normally to stimulation	•Drowsiness •Slightly reduced response to touch or handling	•Stupor (reduced responsiveness, decreased corneal reflex)	•Coma (does not respond to stimulation, e.g. pinch), no corneal reflex
Motor coordination and strength	•Mild change to gait or grip strength (falls between 5-10sec) •No falls, normal righting response	•Reduced grip strength (falls in less than 5sec) •Mild ataxia (e.g. slow righting response, swaying)	•Highly reduced grip strength (falls in less than 2sec) •Ataxia (e.g. staggering, falling impaired walking)	•Severe ataxia (e.g. crawling, fails to grip bar) •No ability to right
Posture, appearance, breathing	•Very slight abnormal posture (subtle)	•Slight abnormal posture (e.g. hunched, extended, low posture, tail position, straub tail) •Piloerection or ptosis •unkempt coat	•Moderately abnormal posture (e.g. ventral recumbency) •Shallow breathing	•Markedly abnormal posture (e.g. lateral recumbency) •Facial paralysis (e.g. drooling, protruding tongue) •Labored breathing

Table 5: Tolerability scoring system[a]

Category	Score 1	Score 2	Score 3	Score 4
Tremor, hyper-activity, convulsion	•Detectable tremor	•Hyper-responsive to stimuli (e.g. noise) •Marked tremors	•Few or partial seizures, rearing and falling as part of convulsing	•Repeated or continuous seizure (running, bouncing, clonic and/or tonic)

[a] Normal is scored as "0". Animals are scored on an individual basis at successive time points post dosing. Observations are rated at 1 h ± 15 min, then 24 h ± 2 h, then 7 days (if appropriate). Convulsions count for the 1 hr timepoint, even if they occur prior to the observation window. A total tolerability score is calculated based on the sum of the individual category scores, with a maximum possible score of 20.

[0151] *Tissue Collection*: Following final behavioral and health assessments, mice were decapitated on a guillotine and the brains were quickly removed. Each brain was split into two hemispheres and a) hippocampus was dissected for mRNA measurements in the 3-day acute tolerability studies; b) hippocampus, brain stem, and striatum from one hemisphere were dissected for mRNA measurements, whereas the same regions were dissected from the second hemisphere for protein/PK measurements in the dose-response time course PK/PD studies.

[0152] In some of the studies, the blood and the cerebrospinal fluid (CSF) were also collected for PK (blood) and PK/protein (CSF) measurements. To collect the blood and the CSF, the mice were deeply anesthetized with Isoflurane (4%). Blood was collected via cardiac puncture using 23G needle. Once removed, the blood was transferred into 2 ml BD Microtainer (K2EDTA BD #365974) tubes and placed on ice until processing. To process the blood, the tubes were centrifuged at 4500xg for 10 min at 4°C. Then, the plasma was removed and placed into 0.5 ml Eppendorf tubes and stored at -80°C until use. To collect the CSF, the thoracic cavity was opened exposing the heart, and as much of the blood was drained to avoid contamination of the CSF. The CSF samples were collected via Cisterna magna using micropipettes and placed into lo-bind protein Eppendorf tubes. Then, the tubes were centrifuged at 4500xg for 15min at 4°C. The CSF was carefully transferred to clean lo-bind 0.5ml Eppendorf tubes and stored at -80°C until further use.

Cyno Data

[0153] *Subject*: Male cynomolgous monkeys weighing 3.5-10.0 kg at the start of the study were used. Each was implanted with an intrathecal cerebrospinal fluid (CSF) catheter entering at the L3 or L4 vertebrae. The distal tip of the polyurethane catheter

extended within the intrathecal space to approximately the L1 vertebrae. The proximal end was connected to a subcutaneous access port located on the animal's lower back. Animals were allowed to heal for at least two weeks prior to the start of the study. Laboratory animal care was according to Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Guide for the Care and use of Laboratory Animals NRC (2011) (National Research Council: Guide for the Care and Use of Laboratory Animals (The National Academies Collection: Reports funded by National Institutes of Health). National Academies Press (US), Washington (DC)). The protocol was approved by the Wallingford Animal Care and Use Committee of the Bristol-Myers Squibb Company.

[0154] *CSF & Blood Sampling*: The CSF port was accessed subcutaneously using aseptic techniques, and CSF was sampled from awake animals sitting upright in a primate restraint chair. Approximately 0.1 ml of CSF was discarded at the start of collection to clear dead space in the catheter and port. CSF was collected by gravity flow to a maximum of 0.5 ml CSF per sample. CSF was spun at 2,000 g at 4° C for 10 min. The supernatant was frozen on dry ice or in liquid nitrogen and kept at -90° C until analyzed.

[0155] Blood was sampled from an available vein, typically the saphenous vein. Blood samples were prepared in a number of procedures depending upon the particular measure in question. For plasma, blood was collected into EDTA-treated tubes. For serum, blood was collected into serum-separator tubes and allowed to clot for at least 30 min prior to centrifugation. For measures of clotting and clotting factors, blood was collected into citrated tubes, and for analysis of RNA, blood was collected into tubes containing RNA later. After processing, samples were frozen on dry ice or in liquid nitrogen and kept frozen until analyzed.

[0156] *Intrathecal Dosing*: Animals were trained to be dosed while awake and using modified commercially-available restraint chairs, animals were maintained in a prone position. *SNCA*-targeted anti-sense oligonucleotides (ASOs) were dissolved in saline, sterilized by filtration, and administered at 0.33 ml/min in a 1.0 ml volume followed by a 0.5 ml sterile water flush. Total infusion time was 4.5 min. Animals remained in the prone position for 30 min post infusion.

[0157] *Necropsy*: Cynomolgus monkeys were administered the appropriate volume of a commercially available euthanasia solution while anesthetized with ketamine and/or isoflurane. Necropsy tissues were obtained immediately thereafter and the brain was

transferred to wet ice for dissection. Areas of interest were dissected using 4-6mm slices in an ASI Cyno Brain Matrix as well as free handed techniques. Samples were placed fresh in RNAlater®, or frozen on dry ice for later analysis. CNS tissue was rapidly dissected form cynomolgus monkeys and pieces no longer than 4 mm on any axis were collected and placed in 5mLs of RNA later. Samples were stored at 4°C overnight then transferred to -20°C for storage until analyzed.

[0158] Brain regions analyzed included medulla, pons, midbrain, cerebellum, caudate-putamen (left and right), hippocampus (left and right), frontal cortex (left and right), temporal cortex (left and right), parietal cortex (left and right), occipital cortex (left and right) and cortical white matter. Additionally, spinal cord was sampled at the cervical, thoracic and lumbar regions. Samples were also collected from liver, kidney and heart. On some occasions, samples of trigeminal nuclei, tibial nerve and the aorta were collected to examine off-target pharmacology in those areas.

ELISA quantitation of ASO concentration in mouse or monkey tissue, plasma, and CSF:

[0159] Tissue was homogenized with plasma and water in a 1:1 ratio. Standard curve was generated by 2-fold serial dilution from 5000 to 4.9 nM in plasma (for plasma and CSF) and in plasma:water (for tissues samples) and then further diluted to 5000-fold total with 5X SSCT (750 mM NaCl, and 75 mM sodium citrate, pH 7.0, containing 0.05 % (v/v) Tween-20) alone and in 5X SSCT containing 35 nM capture and 35 nM detection reagents to obtain a standard range of 1–1000 pM. The dilution factor used varied depending on the expected sample concentration range. The capture probe was AAAGGAA with a 3' Biotin (Exiqon) and the detection probe was 5' DigN-isopropyl 18 linker--GTGTGGT (Exiqon).

[0160] Experimental samples and standards were added to Clarity lysis buffer (Phenomenex, cat#AL0-8579) in a 1:1 ratio prior to dilution with capture and detection buffer and before transferring to the ELISA plate. CSF samples were diluted with plasma (2-fold) prior to addition of lysis buffer. A streptavidin-coated plate (Thermo 15119) was washed 3 times with 5X SSCT buffer. 100 µl samples were added and incubated for 60 min at room temperature. The detection probe, 100 µl anti-Dig-AP Fab fragment diluted 1:4000 in PBS containing 0.05% Tween-20 (Roche Applied Science, Cat. No. 11 093 274 910), was added and incubated for 60 min at room temperature. After washing the plate with 2X SSCT buffer, 100 µl Tropix CDP-star Sapphire II substrate (Applied Biosystems)

was added for 30 min at room temperature. ASO concentrations were measured by luminescence (Enspire-PerkinElmer).

Alpha-synuclein protein measurements:

[0161] Brain tissue samples were homogenized at 10 ml/g tissue in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) using bead homogenizer Qiagen Tissuelyser II for 25 cycles/sec, with a 5 mm stainless steel bead for 2 min total. Homogenized samples were incubated 30 min on ice. 50 µL aliquot of each sample was retained for PK analysis. The remaining samples were centrifuged 20,800g, for 60 min, 4°C. The supernatant was retained and used for analysis. Total protein was measured using Pierce BCA protein assay kit (23227).

[0162] *Brain tissue extracts*: SNCA protein was measured using the MJFR1+4B12 ELISA. Briefly, ELISA plates (Costar) were coated with 100 µl of the anti-SNCA antibody MJFR1 (Abcam) at a concentration of 0.1 µg/ml diluted in BupH carbonate-bicarbonate buffer, pH 9.4 (Thermo Scientific) overnight (O/N) at 4°C. The next day plates were washed 4-times with Dulbecco's PBS (Life Technologies) and blocked with 3% BSA (bovine serum albumin, protease free, Fraction V, Roche Diagnostic) in PBS for 2~3h at room temperature (RT) or overnight at 4°C. Both the standards and the brain samples were diluted with 1% BSA/0.05% Tween/PBS containing Roche protease inhibitor (Roche 11836145001, 1 pellet/25 ml) and Phosphatase Inhibitor 2&3 (Sigma, 1:100). SNCA wild-type (rPeptide) was used as a standard. Samples were loaded in duplicate (50 µl/well) and incubated for O/N at 4°C. After plates were equilibrated to RT, 50 µl of the detection antibody 4B12 (Biolegend) (diluted 1:4000 in 1% BSA/0.1% Tween/DPBS) was added to each well and co-incubated with the samples at RT for ~2 hours. Detection antibody was pre-conjugated with alkaline phosphatase (AP kit from Novus Biologicals). Plates were then washed 4-times with 0.05% Tween/PBS and developed with 100 µl of alkaline phosphatase substrate (Tropix CDP Star Ready-to-Use with Sapphire II, T-2214, Life Technologies) for 30 minutes. Luminescence counts were measured with Perkin Elmer EnVision (2102 Multilabel Reader). The plates were kept constant shaking (Titer plate shaker, speed 3) during the assay. Data was analyzed using GraphPad Prism. Total protein in brain tissue was measured using a Micro protein assay kit (Thermofisher #23235) according to manufacturer's instructions.

[0163] *Cerebral spinal fluid (CSF)*: SNCA protein was measured using the U-PLEX Human SNCA Kit: (cat# K151WKK-2, Meso Scale Discovery) according to manufacturer's instructions. CSF samples were diluted 10-fold. Hemoglobin was measured in CSF samples using the Abcam mouse Hemoglobin ELISA kit (ab157715). CSF samples were diluted 40-fold for the hemoglobin measurements.

mRNA measurements by qRT-PCR

[0164] Brain regions were harvested and placed in 1.5 ml RNA-later Tissue Protect tubes (Qiagen cat#76514) that were prefilled with RNA-later, a RNA stabilization solution. Tissue in RNA-later solution can be stored at 4°C for 1 month, or at -20°C or -80°C indefinitely.

[0165] RNA Isolation*: RNeasy Plus Mini Kit*: RNA from mouse hippocampus and cortex and was isolated using the RNeasy Plus Mini Kit (Qiagen cat#74134). Tissue samples were homogenized in a volume of 600 µL or 1200 µL RLT Plus buffer containing 10 µl/ml of 2-mercaptoethanol and 0.5% Reagent Dx. 600 µL lysis buffer was used if the tissue sample was <20 mg, 1200 µl lysis buffer was used for tissue samples >20 mg. For homogenization, tissue sample was transferred to a 2.0 mL round-bottom Eppendorf Safe-Lock tube (Eppendorf cat#022600044) containing 600 µL RLT Plus Buffer (plus 10 ul/ml of 2-mercaptoethanol and 0.5% Reagent Dx), and a 5 mm stainless steel Bead (Qiagen cat#69989) Samples were homogenized, using a Qiagen's TissueLyser II instrument. Samples were processed for 2.0 min at 20Hz, samples rotated 180° and processed for another 2.0 min at 20Hz. Samples were then processed 2.0 min at 30Hz, samples rotated 180° and processed for another 2.0 min at 30Hz. Longer and/or at higher frequency homogenization used if processing not complete. A 600 µL of the tissue lysate was then transferred into a gDNA Eliminator spin column in a 2.0 mL collection tube and samples centrifuged for 30 secs at 10,000g. All centrifugation steps were performed at RT. The flow-through was collected and an equal volume of 70% ethanol added and mixed. 600 µL was transferred to RNeasy spin column placed in a 2.0 mL collection tube and samples centrifuged for 15 secs at 10,000g. The flow-through was discarded and the remaining 600 µL sample added to the spin column. The spin columns were centrifuged and the flow-through discarded. Columns were washed with 700 µL of Wash Buffer RW1, centrifuged for 15 secs at 10,000g, and the flow-through discarded The columns were then washed 2-times with 500 µL of Buffer RPE containing 4 volumes of ethanol as

described in kit protocol. Columns were first centrifuged for 15 secs at 10,000g for first wash and then for 2.0 min at 10,000g for the second wash.. After second wash, columns were centrifuged once for 1.0 min at 10,000g to dry the membranes. Columns were then transferred to a new 1.5 mL collection tube and 30 µL of RNase-free water was added directly to the center of the membrane. The membranes were allowed to incubate for 10 min at RT. Then, the columns were centrifuged for 1.0 min at 10,000g to elute the RNA. The elution, containing the RNA, was collected and stored on ice until the RNA concentrations could be determined by UV absorbance using a NanoDrop Spectrophotometer (Thermo). RNA samples were stored at -80°C.

[0166] RNA Isolation: RNEASY® Plus Universal Mini Kit: RNA from all other Cyno, Mouse, and Rat tissue samples was isolated using RNEASY® Plus Universal Mini Kit (Qiagen cat#73404). For homogenization, 50 µg or less of tissue sample was transferred to a 2.0mL round-bottom Eppendorf Safe-Lock tube (Eppendorf cat#022600044) containing 900 µL QIAZOL® Lysis Reagent, and a 5mm stainless steel Bead (Qiagen cat#69989) Samples were homogenized, using a Qiagen's TissueLyser II instrument. Samples were processed for 2.0min at 20 Hz, samples rotated 180° and processed for another 2.0 min at 20 Hz. Samples were then processed 2.0 min at 30 Hz, samples rotated 180° and processed for another 2.0 min at 30 Hz. Longer and/or at higher frequency homogenization used if processing not complete.. Homogenized tissue lysate was then transferred into a new 2.0 mL round-bottom Eppendorf Safe-Lock tube and left at RT for 5.0 min. 100 µL of gDNA Eliminator Solution was added to each tube and tubes were vigorously shaken for 30 secs. 180 µL of Chloroform (Sigma cat#496189) was added to each tube and tubes were vigorously shaken for 30 secs. Tubes were left at RT for 3 min. Centrifuge tubes at 12,000g for 15 min at 4°C. After centrifugation the upper aqueous phase was transferred to a new 2.0 mL round-bottom Eppendorf Safe-Lock tube ~500 µL. An equal volume of 70% ethanol added and mixed. All future centrifugation steps were performed at RT. 500 µL was transferred to RNeasy spin column placed in a 2.0 mL collection tube and samples centrifuged for 15 secs at 10,000g. The flow-through was discarded and the remaining 500 µL sample added to the spin column. The spin columns were centrifuged and the flow-through discarded and the columns washed with 700 µL of Wash Buffer RWT containing 2 volumes of ethanol. Columns were centrifuged for 15 secs at 10,000g, the flow-through discarded. The columns were then washed twice with 500 µL of Buffer RPE containing 4-volumes of ethanol as described in kit protocol.

Columns were first centrifuged for 15 secs at 10,000 g for first wash and then for 2.0 min at 10,000g for the second wash.. After second wash, columns were centrifuged once for 1.0 min at 10,000g to dry the membranes. Columns were then transferred to a new 1.5 mL collection tube and 30 µL of RNase-free water added directly to the center of the membrane. Membranes were allowed to incubate for 10 min at RT. Columns were centrifuged for 1.0 min at 10,000 g to elute the RNA. The elutions, containing the RNA, were collected and stored on ice until RNA concentration determined by UV absorbance using a NanoDrop Spectrophotometer (Thermo). RNA samples were stored at -80°C.

[0167] cDNA Synthesis by Reverse Transcription*:* 300 ng of RNA was diluted to a final volume of 10.8 µL using nuclease-free water (Invitrogen cat#10977-015) in a PCR-96-AB-C microplate (Axygen cat#321-65-051). Added 6.0 µL to each well of reaction mix 1 containing the following: 2.0 µL of 50 µM random decamers (Ambion cat#AM5722G) and 4.0 µL of a 1X dNTP mix (Invitrogen cat#10297-018). The plate was sealed with optical sealing tape (Applied Biosystems cat# 4360954) and centrifuged for 1.0 min at 1,000 x g at RT. Next, the plate was heated for 3.0 min at 70°C using a 96-well Thermal Cycler GeneAmp PCR System 9700 (Applied Biosystems). The plate was then cooled completely on ice. Next, 3.25 µL of the reaction mix 2 (containing 2 µL of 10X strand buffer, 1.0 µL of MMLV-RT 200U/µL reverse transcriptase enzyme (Ambion cat#2044), and 0.25 µL of RNase inhibitor 40U/µL (Ambion cat#AM2682)) were added to each of the wells. Plate was sealed with optical sealing tape and centrifuged for 1.0 min at 1,000 x g at RT. Using a 96-well Thermal Cycler, the plate was heated at 42°C for 60 min proceeded by 95°C for 10min. Then, the plates were cooled on ice. The cDNA plates were stored at -20°C until ready to use for PCR analysis.

[0168] qPCR for amplification and quantification of *SNCA* and GAPDH mRNA expression: cDNA was diluted 5-fold in nuclease free water in a PCR-96-AB-C microplate. 16 µL of Master Mix solution consisting of the following: 10 µL of 2X Taqman Gene Expression Master Mix (Applied Biosystems cat#4369016), 1.0 µL of 20X Taqman primer-probe set (Applied Biosystems), and 5.0 µL of nuclease-free water, was added to each well of a 384-well optical PCR plate (Applied Biosystems cat#4483315). 4.0 µL of diluted cDNA was added to each well of the 384-well optical PCR plate. Plate was sealed with optical sealing tape and centrifuged for 1.0 min at 1,000 x g at RT. PCR was performed on the Applied Biosystems 700 HT Fast Real-Time PCR System using the

following parameters in standard mode: 50°C for 2.0 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 secs and 60°C for 1.0 min.

[0169] qRT-PCR primer-probe sets: Primer-probes sets from Applied Biosystems (Thermo Fisher) included the following:

1) Human alpha synuclein (cat#Hs01103383_m1) FAM labelled
2) Human PROS1 (cat#HS00165590_m1) FAM labelled
3) Cyno alpha synuclein (cat#Mf02793033_m1) FAM labelled
4) Cyno GAPDH (cat#Mf04392546_g1) FAM labelled
5) Cyno GAPDH (cat#Mf04392546_g1) VIC labelled Primer Limited
6) Rat alpha synuclein (cat#Rn01425141_m1) FAM labelled
7) Rat GAPDH (cat#Rn01775763-g1) FAM labelled
8) Rat GAPDH (cat#4352338E) VIC labelled Primer Limited
9) Mouse GAPDH (cat#Mm99999915-g1) FAM labelled
10) Mouse GAPDH (cat#4352339E) VIC labelled Primer Limited.

Example 8: Analysis of *in vivo* Activity and Tolerability of ASO-005459 in Mice

[0170] ASO-005459 is a LNA-modified ASO specific for human *SNCA*. The *in vitro* results (described above) demonstrate that ASO-005459 is potent and selective for reducing *SNCA* mRNA in primary neurons. The *in vitro* results also suggest that that ASO-005459 is well tolerated.

A53T-PAC mice

[0171] To evaluate whether these results are also true *in vivo*, 100 µg of ASO-005459 was administered to A53T-PAC mice via ICV injection, and both the tolerability and the *SNCA* mRNA knockdown (KD) in the hippocampus were assessed at 3 days post injection, as described in Example 4 (above).

[0172] As shown in Tables 6 and 7 (below), ASO-005459 was well tolerated with an overall mean tolerability score of 1. In addition, ASO-005459 significantly reduced *SNCA* mRNA levels by >90% in the hippocampus at 3 days post administration. (FIG. 4).

Table 6.

	Tox Score @1D	3D mRNA %KD	Tox Score @28D	Tox Score WT @28D
ASO-005459	0.50	90.83		0.00

Table 7: ASO-005459 tolerability in A53T-PAC mice, 3-day study

Animal#	Hyperactivity	Vigilance	Motor S&C	Posture/ Breathing	Tremor/ Convulsions	Total Score
31	0	2	1	2	0	5
32	0	0	0	0	0	0
33	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	0	0	0	0
mean						1.00
SEM						1.00

[0173] To evaluate activity *in vivo*, A53T-PAC mice were dosed with ASO-005459 at 3.13 µg, 12.5 µg, 25 µg, or 50 µg concentration via ICV injection. Tolerability was assessed by measuring body weights at days 7 and 14 post dosing. The expression of *SNCA* mRNA was assessed at 14 days post dosing, when the animals were sacrificed and their tissues harvested. *SNCA* mRNA knockdown was measured in three brain regions: Hippocampus, brainstem, and striatum. Brainstem and striatum are two of the regions that are most affected in the brains of MSA and PD patients. In a separate study, C57BL/6 mice were dosed with 100 µg of ASO-005459 and tolerability was assessed by measuring the body weight of the animals at days 7, 14, 21, and 28 post dosing. 100 µg ASO-005459 was dosed ICV in wild-type (WT) C57BL/6 mice, and the body weight and behavior were monitored over a 4-week period. Because ASO-005459 does not target mouse *SNCA*, reduction in *SNCA* mRNA expression was not measured in these animals.

[0174] As shown in FIGs. 5A and 5B, there were no significant differences in the body weight of the mice (both A53T-PAC mice and C57BL/6 mice) treated with ASO-005459 (for all concentrations) and those treated with the control vehicle. The animals (C57BL/6) did not exhibit any abnormal behaviors during the course of the experiment. *See* Table 8 (below). Such results demonstrate that ASO-005459 was well tolerated. Also, in mice treated with ASO-005459, there was a significant and dose-dependent reduction in *SNCA* mRNA expression in all three brain regions tested. *See* FIGs. 6A-6C. In the hippocampus, the *SNCA* mRNA expression was reduced by 53%, 73%, 80%, and 96% for 3.13, 12.5, 25, and 50 µg ASO-005459, respectively. Similar dose-dependent knockdowns were also observed in the brainstem. In the striatum, *SNCA* mRNA knockdowns were more variable and less robust compared to the other regions (FIGs. 6A-6C): a 75% and 46% knockdown was observed with 50 µg and 25 µg of ASO-005459, respectively. However, with 12.5 µg

and 3.13 µg of ASO-005459, there was no significant reduction in *SNCA* mRNA expression. Possible explanations for the lower activity observed in the striatum could be due to differences in the ASO levels or the kinetics of *SNCA* mRNA knockdown.

Table 8: ASO-005459 tolerability in 28-day study

Animal#	Timepoint (day)	Hyperactivity	Vigilance	Motor S&C	Posture/ Breathing	Tremor/ Convulsions	**Total Score**
26	1	0	0	0	0	0	**0**
26	28	0	0	0	0	0	**0**
27	1	0	0	0	0	0	**0**
27	28	0	0	0	0	0	**0**
28	1	0	0	0	0	0	**0**
28	28	0	0	0	0	0	**0**
29	1	0	0	0	0	0	**0**
29	28	0	0	0	0	0	**0**
30	1	0	0	0	0	0	**0**
30	28	0	0	0	0	0	**0**

[0175] The ASO-005459 levels were measured in all three brain regions of the A53T-PAC mice. As shown in FIG. 7 and Table 9 (below), brain exposures were approximately dose proportional and similar across all three regions, including the striatum. The ASO-005459 exposure-response relationships for the different brain regions are shown in FIGs. 8A-8D. Similar exposure-response relationships were observed in the hippocampus and brainstem with estimated IC_{50}s of 179 and 206 nM, respectively. In comparison, the exposure response relationship in the striatum was relatively steep suggesting slower kinetics of *SNCA* mRNA reduction in that region. *See* FIG. 8C.

Table 9: Summary of ASO-005459 brain exposure in 14-day A53T-PAC study

	Dose (µg)	**Mean (nM)**	**SD**	**Relative to hippo**
Hippocampus	3.13	214	43	
	12.5	411	140	
	25	600	223	
	50	1916	807	
Brainstem	3.13	160	47	0.7
	12.5	266	47	0.6

Table 9: Summary of ASO-005459 brain exposure in 14-day A53T-PAC study

	Dose (µg)	Mean (nM)	SD	Relative to hippo
	25	440	102	0.7
	50	707	202	0.4
Striatum	3.13	212	38	1.0
	12.5	438	103	1.1
	25	586	109	1.0
	50	1332	302	0.7

[0176] To provide a more extensive dose-response/time course data, ASO-005459 was again administered (0, 12.5, 25, or 50 µg) directly into the cerebral ventricles of A53T-PAC mice by ICV freehand injection. The animals were sacrificed at 24 hours, 3 days, 4, 8, 12, 16, and 20 weeks post dosing and the *SNCA* mRNA expression in the brain stem and the striatum was assessed.

[0177] As shown in FIGs. 9A and 9B (and in agreement with the data provided above), administration of ASO-005459 to the A53T-PAC mice resulted in significant reduction of *SNCA* mRNA expression level (relative to the vehicle control) in both the brain stem and the striatum. The reduction appeared to be both time- and dose-dependent, with peak reduction (~90%) observed in the brain stem with 50 µg ASO-005459 at about 4 weeks post dosing (FIG. 9A). In the striatum, peak reduction (~55%) was observed with 50 µg ASO-005459 at about 3 days post dosing (FIG. 9B). *SNCA* mRNA expression levels remained significantly reduced compared to the vehicle control at 4 weeks post dosing (FIGs. 10A and 10B) and returned to baseline control by about 16 weeks post-dosing (FIGs. 9A and 9B).

[0178] As shown in FIGs. 11A and 11B, administration of ASO-005459 to the animals also resulted in a time- and dose-dependent reduction in the SNCA protein level in both the brain stem and the striatum brain tissue. Peak reduction (~75%) was observed in the brain stem with a dose of 50 µg at 8 weeks post dosing (FIG. 11A). For the striatum brain tissue, peak reduction (~75%) was also observed with the 50 µg dose but at 4 weeks post dosing (FIG. 11B). Expression levels for the individual mice at 8 weeks post dosing are provided in FIGs. 12A (brain stem) and 12B (striatum). While SNCA protein level returned close to baseline by about 12 weeks post dosing in the striatum, expression level

was still significantly reduced (~25%) in the brain stem as far out as 16 weeks post-dosing.

Example 9: Analysis of *in vivo* Activity and Tolerability of *SNCA*-Targeted Antisense Oligonucleotides (ASOs) in Cynomolgous Monkeys

[0179] To further evaluate the ASO activity and tolerability *in vivo*, an intrathecal ported Cynomolgus monkey model (Cyno IT) was developed. This model enables the evaluation of ASO-005459-mediated knockdown of *SNCA* as well as knockdown of the potential 1-basepair mismatch off-targets PROS1 and IKZF3. The ASO-005459 target sites in *SNCA*, PROS1, and IKZF3 are completely conserved between human and cyno.

[0180] As described above in Example 6, each animal was implanted with an intrathecal cerebrospinal fluid (CSF) catheter entering at the L3 or L4 vertebrae. ASO-005459 was dissolved in saline and administered to the animals (8 mg / animal), infused over 4.5 min using the IT port (2 animals per dose group). Animals were then euthanized at 24 hours and 3 days post dosing, when the tissues were harvested for analysis of the ASO exposure and activity. Brain regions analyzed included medulla (Med), pons (V-Pons), midbrain (V-MB), cerebellum (CBL), caudate-putamen (left and right) (CauP), hippocampus (left and right) (Hip), frontal cortex (left and right) (FrC), temporal cortex (left and right) (TeC), parietal cortex (left and right) (PaC), occipital cortex (left and right) (Occ), and cortical white matter (WM). Additionally, spinal cord was sampled at the cervical (CSC), thoracic (TSC), and lumbar (LSC) regions. Samples were also collected from liver, kidney, heart, trigeminal nuclei, tibial nerve, and aorta to examine off-target pharmacology in those areas.

[0181] As was observed in mice, ASO-005459 was well tolerated in cyno with no adverse effects being observed (data not shown). And as shown in Table 10 below, the administration of ASO-005459 exhibited robust knockdown of *SNCA* mRNA in various regions of the cyno brain.

Table 10: Effect of ASO-005459 on brain *SNCA* mRNA levels in cyno brain

ASO No.	Dose (mg)	Timepoint (weeks)	Med	CBL	FrC	PaC	CauP	TeC	Occ	Hip	V-MB	V-Pons	CSC	TSC	LSC	WM
ASO-00545	8	24 hours	174	140	146		149					137			73	

9	8	24 hours	129	108	138		133					145			90	
	8	3 days	162	69	69		112					62			19	
	8	3 days	127	67	72		114					87			31	
	2	2	133	140	109		161					104			21	
	2	2	117	127	100		126					180			111	
	4	2	62	86	22		116					49			4	
	4	2	81	72	50		145					97			13	
	8	2	31	97	7		124					66			14	
	8	2	30	38	15	26	75	12	29	29	86	29	13	6	2	79
	8	4	26	50	4	12	53	3	13	9	24	21	4	6	4	87
	8	4	38	61	9		120					30			10	
	8	8	121	85	40		98					78			5	
	8	8	93	52	14		61					36			11	
	8	13	30	63	8		81					27			1	
	8	13	25	28	8		49					22			1	
	8	20	40	38	35		76					63			9	
	8	20	22	52	35		76					26			11	

[0182] To further characterize the reduction of *SNCA* mRNA described-above, cyno monkeys were dosed with ASO-005459 (8 mg total per animal) and sacrificed at 24 hour,

3 days, 2, 4, 8, 13, or 20 weeks post-dosing to assess the *SNCA* mRNA expression level in the different tissues. As shown in FIG. 13A, peak reduction was observed between 2 weeks and 13 weeks post-dosing. Peak reductions of 70%, 65%, 75%, 35%, 94% and 99% were observed in the medulla, cerebellum, pons, caudate-putamen, frontal cortex and lumbar spinal cord, respectively. This reduction in *SNCA* mRNA expression level also correlated with a time-dependent reduction in the SNCA protein expression level (FIG. 13B).

[0183] Next, to assess whether the reduction in expression level in the cyno monkeys was also dependent on the dose, the animals received 2, 4, or 8 mg of ASO-005459 and then, were sacrificed at 2 weeks post-dosing. As shown in FIGs. 14A and 14B, the reduction in both the *SNCA* mRNA and the SNCA protein expression levels was also dependent on the dose, with the greatest reduction observed with 8 mg of ASO-005459.

[0184] The results presented here demonstrate that ASO-005459 is potent and selective for reducing *SNCA* mRNA and that ASO-005459 is well tolerated in neurons and in preclinical species *in vivo*. Moreover, results from the A53T-PAC neurons confirm that ASO-005459-mediated reductions of mRNA result in reductions of SNCA protein levels *in vitro* and *in vivo*. In addition, results in A53T-PAC mice and cyno monkeys demonstrate that ASO-005459 reduces *SNCA* mRNA and SNCA protein in brain at doses that are well tolerated. Taken together, these findings support the continued development of ASO-005459 as a disease-modifying therapeutic for the treatment of synucleinopathies.

[0185] This PCT application claims priority benefit of U.S. Provisional Application No. 62/616,937, filed January 12, 2018, which is incorporated herein by reference in its entirety.

What is Claimed

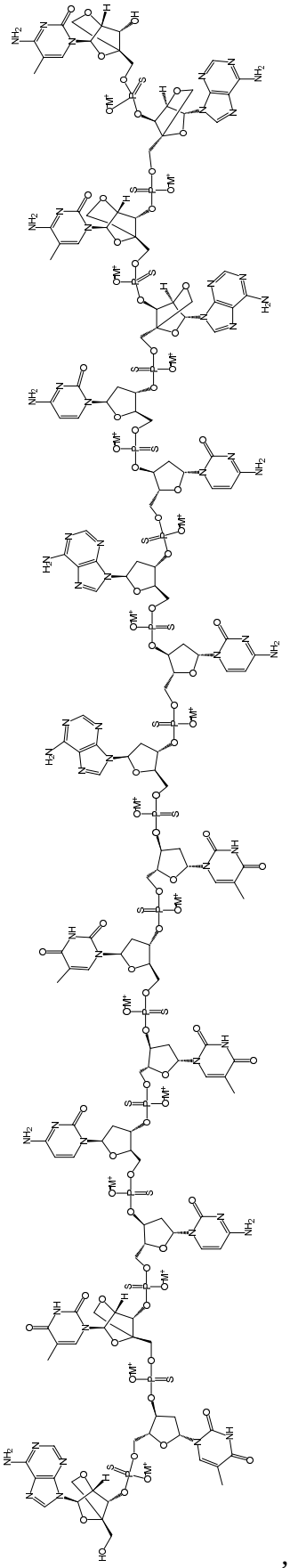
1. An antisense oligonucleotide (ASO) comprising the contiguous nucleotide sequence of AtTcctttacaccACAC (SEQ ID NO: 4), wherein the upper letter is beta-D-oxy-LNA and the lower letter is DNA.

2. The ASO of claim 1, which comprises an internucleotide linkage selected from the group consisting of a phosphodiester linkage, a phosphotriester linkage, a methylphosonate linkage, a phosphoramidate linkage, a phosphorothioate linkage, and combinations thereof.

3. The ASO of claim 2, wherein the internucleotide linkage is a phosphorothioate linkage.

4. The ASO of claim 1, wherein the contiguous nucleotide sequence is OxyAs DNAts OxyTs DNAcs DNAcs DNAts DNAts DNAts DNAas DNAcs DNAas DNAcs DNAcs OxyAs OxyMCs OxyAs OxyMC, wherein OxyA, OxyT, and Oxy MC are adenine beta D-oxy-LNA, thymine beta D-oxy-LNA, and methyl cytosine beta D-oxy-LNA, respectively, wherein DNAt, DNAc, and DNAa are thymine DNA, cytosine DNA, and adenine DNA, respectively, and wherein s is a phosphorothioate linkage between two nucleotides.

5. The ASO of claim 1, comprising the structure:



,

wherein M^+ is a counterion.

6. The ASO of claim 5, wherein the counterion is selected from the group consisting of H^+, Na^+, $NH4^+$, and any combination thereof.

7. The ASO of claim 6, wherein the counterion is Na^+.

8. A conjugate comprising the ASO of claim 1, wherein the ASO is covalently attached to at least one non-nucleotide or non-polynucleotide moiety.

9. The conjugate of claim 8, wherein the non-nucleotide or non-polynucleotide moiety comprises a protein, a fatty acid chain, a sugar residue, a glycoprotein, a polymer, or any combinations thereof.

10. A pharmaceutical composition comprising the ASO of claim 1 and a pharmaceutically acceptable carrier.

11. The composition of claim 10, which further comprises a therapeutic agent.

12. The composition of claim 11, wherein the therapeutic agent is an alpha-synuclein antagonist.

13. A kit comprising the ASO of claim 1 and instructions for use.

14. Use of the ASO of claim 1 for the manufacture of a medicament for inhibiting or reducing expression of SNCA mRNA and/or SNCA protein in a cell, wherein the medicament is to be administered to the cell expressing SNCA mRNA and/or SNCA protein to inhibit or reduce the expression of SNCA mRNA and/or SCNA protein in the cell.

15. Use of the ASO of claim 1 for the manufacture of a medicament for the treatment of a synucleinopathy in a subject in need thereof, wherein an effective amount of the medicament is to be administered to the subject.

17. The ASO of claim 1 for use in therapy.

18. The ASO of claim 1 for use in therapy of inhibiting or reducing expression of SNCA mRNA and/or SNCA protein in a cell, wherein the ASO is to be administered to the cell expressing SNCA mRNA and/or SNCA protein to inhibit or reduce the expression of SNCA mRNA and/or SCNA protein in the cell.

19. The ASO of claim 1 for use in therapy of a synucleinopathy in a subject in need thereof.

20. The use of claim 15 or the ASO for use of claim 19, wherein the synucleinopathy is selected from the group consisting of Parkinson's disease, Parkinson's Disease Dementia (PDD), multiple system atrophy, dementia with Lewy bodies, and any combinations thereof.

21. A pharmaceutical composition comprising the ASO of any one claims 3 to 5 or 7 and a pharmaceutically acceptable carrier.