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Synthesis and Evaluation of Parenchymal Retention and Efficacy of a Metabolically Stable, O-phosphocholine-*N*-docosaheptaenoyl-L-serine siRNA Conjugate in Mouse Brain

Mehran Nikan^{1,2,†,§}, Maire F. Osborn^{1,2,†,*}, Andrew H. Coles^{1,2,†}, Annabelle Biscans^{1,2}, Bruno M.D.C. Godinho^{1,2}, Reka A. Haraszi^{1,2}, Ellen Sapp³, Dimas Echeverria^{1,2}, Marian DiFiglia³, Neil Aronin^{1,4}, and Anastasia Khvorova^{1,2,*}

¹RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, USA

²Department of Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, USA

³Department of Neurology, Mass General Institute for Neurodegenerative Disease, Charlestown, MA, USA

⁴Department of Medicine, University of Massachusetts Medical School, Worcester, MA, USA

Abstract

Ligand-conjugated siRNAs have potential to achieve targeted delivery and efficient silencing in neurons following local administration in the central nervous system (CNS). We recently described the activity and safety profile of a docosaheptaenoic acid (DHA)-conjugated, hydrophobic siRNA (DHA-hsiRNA) targeting Huntingtin (*Htt*) mRNA in mouse brain. Here, we report the synthesis of an amide-modified, phosphocholine-containing DHA-hsiRNA conjugate (PC-DHA-hsiRNA), which closely resembles the endogenously esterified biological structure of DHA. We hypothesized that this modification may enhance neuronal delivery *in vivo*. We demonstrate that PC-DHA-hsiRNA silences *Htt* in mouse primary cortical neurons and astrocytes. After intrastriatal delivery, *Htt*-targeting PC-DHA-hsiRNA induces ~80% mRNA silencing and 71% protein silencing after one week. However, PC-DHA-hsiRNA did not substantially outperform DHA-hsiRNA under the conditions tested. Moreover, at the highest locally

*Co-corresponding authors: Maire F. Osborn, maire.osborn@umassmed.edu. Phone: (508) 654-6215 and Anastasia Khvorova, anastasia.khvorova@umassmed.edu. Phone: (774) 455-3638.

†These authors contributed equally to this work

§Present address: Ionis Pharmaceuticals, Carlsbad, CA

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Oligonucleotide synthesis experimental details and spectra, supporting figures and methods.

Author contributions

M.N. conceived of the PC-modified DHA concept, designed and executed the multistep synthesis, and assisted in manuscript preparation, A.B. optimized the protection group synthetic strategy, characterized all intermediates and final products, and prepared the Supporting Information, A.H.C. and B.G. performed all *in vivo* efficacy and distribution experiments, R.A.H. quantified the tissue accumulation of each compound, E.S. evaluated protein silencing, M.N., D.E. and A.B. synthesized oligonucleotide variants, M.D., N.A., and A.K. supervised experimental design and assisted with manuscript editing, and M.F.O. performed all *in vitro* experiments, assisted with the *in vivo* distribution experiments, critically analyzed the data, prepared figures, and wrote the final manuscript.

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administered dose (4 nmol, 50 μ g), we observe evidence of PC-DHA-hsiRNA-mediated reactive astrogliosis. Lipophilic ligand conjugation enables siRNA delivery to neural tissues, but rational design of functional, non-toxic siRNA conjugates for CNS delivery remains challenging.

TOC Graphic

Introduction

The clinical success of therapeutic siRNA depends on efficient delivery to relevant tissues and uptake by the target cell types. Therapeutic siRNAs suffer from poor serum stability, rapid renal clearance, inadequate tissue retention due to inefficient cellular uptake, and nonproductive intracellular localization.^{2–3} Many diverse strategies have been investigated to improve tissue delivery following systemic (e.g. intravenous or subcutaneous) administration of therapeutic siRNA, including nanoparticle encapsulation, formulation with cationic carrier molecules, and direct chemical conjugation.^{4–11} The most clinically advanced technology employs a trivalent N-acetylgalactosamine (GalNAc)-siRNA conjugate^{12–14}, in which a single injection was shown to induce several months of gene silencing in the liver¹⁵. Productive delivery to organs beyond the liver and kidney, particularly the brain, remains a challenge^{16–19}. Typical lipid-mediated siRNA transfection strategies show acute toxicity when administered locally in the brain^{18, 20–21}. Moreover, unmodified (retaining 2'-OH) or partially modified siRNAs require continuous, high dose local infusions to maintain gene silencing in neighboring tissues^{19, 22}.

There are many genetically defined neurological disorders that would benefit greatly from a technology permitting non-toxic and targeted delivery to the brain. One synthetic strategy that holds promise for non-formulated siRNA delivery to the CNS is direct conjugation of biologically occurring ligands, including hydrophobic modifications such as cholesterol or free fatty acids^{23–25}. Efficient delivery to neuronal cells has been described for polyunsaturated fatty acid-siRNA conjugates both *in vitro* and *in vivo*^{1, 26–27}. We recently demonstrated that a docosahexaenoic acid (DHA) conjugate permits enhanced intracranial distribution and Huntingtin mRNA (*Htt*) silencing compared to an unconjugated siRNA following direct intrastriatal administration in wild-type FVB/NJ mice (Scheme 1a)¹. DHA was conjugated to a chemically stabilized, hydrophobic siRNA scaffold (hsiRNA), which is necessary for stability *in vivo* (Hassler *et al.*, manuscript in review). Notably, this compound did not elicit an innate immune response or induce neuronal toxicity over a broad range of concentrations, as cholesterol-conjugated hsiRNAs of the same chemical composition are strongly retained around the site of injection and induce local neuronal toxicity in FVB/NJ mice²⁸. These results strongly suggest that chemical tuning of hydrophobic conjugates is a promising strategy for enhancing CNS distribution and improving their safety profile.

DHA is highly enriched in the membrane phospholipids of brain and eye tissues, and is actively transported across the blood-brain barrier (BBB)^{29–30}. The major endothelial receptor implicated in BBB trafficking is major facilitator superfamily domain containing 2a (MFSD2A)³¹. This receptor preferentially recognizes and transports DHA as a lysophosphatidylcholine (LPC) ester, which is synthesized from unesterified DHA in the liver (Scheme 1b)³². In human plasma, ~55% of DHA is present as the LPC ester³². Based on these considerations, we were interested in investigating whether the use of an esterified DHA bioconjugate (phosphocholine-DHA; PC-DHA) would improve siRNA retention and neuronal uptake throughout the brain following local or systemic administration³³. Although lipid-based conjugates and nanoparticles are routinely used in siRNA formulation, direct phospholipid conjugation to oligonucleotides is rare and generally involves major alteration of the phospholipid head group^{34–36}. In our original scheme for DHA-hsiRNA synthesis, we conjugated DHA through its terminal carboxyl group, rendering it inert to endogenous esterification. Here, we report the design, synthesis, *in vitro*, and *in vivo* activity of an *O*-phosphocholine-*N*-docosahexaenoyl-L-serine siRNA conjugate (PC-DHA-hsiRNA) that is structurally analogous to naturally occurring phosphocholines and sphingomyelins (Scheme 1a,b) and is orthogonally compatible with solid-phase RNA synthesis.

Results

Synthesis of PC-DHA-hsiRNA Bioconjugates

A synthetic route for the preparation of PC-DHA-hsiRNAs was developed utilizing mild conditions and simple precursors, including docosahexaenoic acid, a protected L-serine, a phosphocholine head group, and a commercially available bifunctional linker immobilized on CPG (see Supplementary Information, Fig. S1-S10, Scheme 2). This route involved reacting Na-Fmoc-L-serine tert-butyl ester (**4**) with 2-cyanoethyl N,N-diisopropylchlorophosphoramidite to afford **5** as two diastereomers (S, Sp and S, Rp) in 95% yield. **5** was then reacted with choline tosylate³⁷ in the presence of 5-ethylthio-1*H*-tetrazole (ETT) as activator and oxidized with meta-chloroperoxybenzoic acid (mCPBA) to afford **6** as a mixture of tetrazolium (~95%) and tosylate salt (~5%) in 69% yield. Following this, the ester group of **6** was removed by treatment with trifluoroacetic acid (TFA) in dichloromethane (DCM) and the cyanoethyl group was then eliminated by using 10% diisopropylamine in acetonitrile (ACN) to obtain **7** in 74% yield (over two steps). This last deprotection is advantageous from a synthetic perspective as it affords a phosphodiester (**7**), which is stable to base catalyzed β-elimination of phosphoserines^{38–39}. In a parallel line, the Fmoc group on the 1-*O*-DMT-6-*N*-Fmoc-2-hydroxymethylhexane⁴⁰ support (**2**) was removed using a solution of 20% piperidine in N,N-dimethylformamide (DMF) to give **3**. Subsequently, **7** and **3** were coupled in the presence of (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), hydroxybenzotriazole (HOBt) and 2,4,6-collidine to yield **8**. The Fmoc group on **8** was then removed using 20% piperidine in DMF. Lastly, the free amine on **9** was coupled to DHA in the presence of 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxidhexafluorophosphate (HATU) and diisopropylethylamine (DIPEA) to afford the final functionalized support **10**. All sense strands were synthesized on this support following standard solid-phase synthesis protocols. Following synthesis, the oligonucleotides were

cleaved and deprotected with 40% methylamine in water (45°C, 1 hour). Deprotected oligonucleotides were purified by high-performance liquid chromatography (HPLC) and characterized by liquid chromatography–mass spectrometry (LC-MS) (Figure S10).

PC-DHA-hsiRNA Exhibits Intracellular Uptake and Enhanced Huntingtin mRNA Silencing In Vitro

The hsiRNA duplexes used in this study (hsiRNA^{Htt}) were designed to target both human and mouse huntingtin mRNA (*Htt*). Their sequences and chemical modification patterns are presented in Table S1. These oligonucleotides contain alternating 2'-fluoro and 2'-O-methyl ribose modifications to enhance stability⁴¹ and a partially phosphorothioated backbone to increase serum half-life and improve cellular uptake (Hassler *et al.*, manuscript in revision).

We first evaluated the ability of PC-DHA-hsiRNA to enter mouse primary cortical neurons (Figure 1a). Here, the duplex was labeled for imaging by addition of a Cy3 fluorophore to the 5'-end of the sense strand. Modifications on the 5'-end of the sense strand, unlike the antisense strand, do not interfere with RNA Induced Silencing Complex (RISC) loading⁴². Cy3-labeled PC-DHA-hsiRNA^{Htt} (Figure 1a, red) was readily detectable in the cytoplasm of neuronal biomarker NeuN-positive neurons (Figure 1a, green) after 48 hours, demonstrating both cytoplasmic and membrane localization and few intracellular foci. Intracellular staining was most concentrated in the perinuclear space, where productive RISC loading and mRNA silencing is thought to occur^{43–44}. We also observed a small fraction of nuclear-localized PC-DHA-hsiRNAs. Active nuclear RISC complexes have been reported previously^{45–46}. The slow kinetics of delivery were analogous to the first generation of DHA-hsiRNA conjugates⁴⁷. We directly compared the ability of PC-DHA-hsiRNA^{Htt} and DHA-hsiRNA^{Htt} to silence Huntingtin mRNA in purified primary mouse cortical neurons or astrocytes. Following passive delivery in neuronal culture (*i.e.* no transfection reagent), PC-DHA-hsiRNA^{Htt} achieves a 68% reduction in *Htt* mRNA, while DHA-hsiRNA^{Htt} silences by 55% (Figure 1b, 1.5 µM dose). The calculated IC₅₀ values for both compounds are similar (135 nM and 133 nM, respectively). Furthermore, at the 1.5 µM dose, the PC-DHA-hsiRNA non-targeting control shows no effect on *Htt* expression (Figure 1b, green point). A non-conjugated hsiRNA control is capable of silencing *Htt* mRNA by ~40% *in vitro* (Figure 1b, black line); this residual activity has been reported from non-specific cellular internalization mediated by the single-stranded phosphorothioate tail, in a manner similar to that of conventional therapeutic antisense oligonucleotides²⁸. Treatment of mouse primary cortical neurons with non-conjugated hsiRNA, DHA-hsiRNA, or PC-DHA-hsiRNA with doses from 0.05 – 3 µM has no significant effect on cell viability as measured by the Alamar Blue cytotoxicity assay (Figure S11).

PC-DHA-siRNA Shows Broad Intracranial Distribution and Parenchymal Retention Within the Injected Hemisphere

We first qualitatively examined the local intracranial distribution of Cy3-labeled PC-DHA-hsiRNA in wild-type (FVB/NJ) mouse brain by fluorescence microscopy. Animals (n = 2 per group) were administered 2 nmol (25 µg, 2 µL) of active Cy3-labeled DHA-hsiRNA^{Htt} or PC-DHA-hsiRNA^{Htt}, which were compared to an equimolar injection of a control construct lacking the phosphocholine and DHA moieties (C₇linker-hsiRNA^{Htt}). After 48

hours, animals were perfused with phosphate-buffered saline and fixed with formalin. Brains were processed into 4 μ m coronal sections, stained with DAPI to visualize nuclei, and imaged on a Leica DMI8 inverted microscope (Figure 2a). At this dose, both DHA-hsiRNA^{Htt} and PC-DHA-hsiRNA^{Htt} exhibited diffuse staining throughout the ipsilateral (injected) striatum and cortex, while the C₇linker-hsiRNA control was barely detectable. Next, we directly measured the striatal and cortical accumulation of each compound using a quantitative peptide-nucleic acid (PNA) hybridization assay (n = 5 per group) (Figure 2b,c). In these studies, hsiRNAs were not labeled with Cy3, to remove any contribution of the fluorophore to distribution and cellular uptake. Animals were injected with 2 nmol (25 μ g, 2 μ L) of non-Cy3 labeled unconjugated hsiRNA, C₇linker-hsiRNA, DHA-hsiRNA, or PC-DHA-hsiRNA. After 48 hours, animals were perfused with phosphate-buffered saline and punch biopsies (2 mm) were lysed, hybridized to a fully complementary PNA probe, and analyzed by HPLC as described previously¹. At the 2 nmol (25 μ g) dose, we observe that PC-DHA-hsiRNA (40.4 $\pm$ 16.0 ng/mg tissue) and DHA-hsiRNA (26.5 $\pm$ 5.4 ng/mg tissue) are both highly retained in the striatum, compared to the unconjugated and C₇linker-conjugated hsiRNA controls (2.2 $\pm$ 0.9 and 5.6 $\pm$ 0.9 ng/mg tissue, respectively) (Figure 2b). At this dose and time point, we also observe significant cortical retention of both PC-DHA-hsiRNA (6.7 $\pm$ 3.7 ng/mg tissue) and DHA-hsiRNA (3.6 $\pm$ 2.1 ng/mg tissue), compared to the unconjugated and C₇linker-conjugated hsiRNA controls (0.8 $\pm$ 0.5 and 1.6 $\pm$ 0.5 ng/mg tissue, respectively) (Figure 2c). Statistical significance was calculated using a Kruskal-Wallis one-way ANOVA with Dunn's post hoc analysis.

Striatal Huntingtin mRNA and Protein Silencing by PC-DHA-hsiRNA^{Htt}

We investigated whether addition of the phosphocholine head group to the DHA-hsiRNA scaffold was capable of sustaining or increasing gene-silencing activity *in vivo* following local administration in mouse brain. Wild-type mice (FVB/NJ, n = 8 per group) were injected into the right striatum with aCSF, a non-targeting control hsiRNA (PC-DHA-hsiRNA^{NTC}, 4 nmol, 50 μ g), DHA-hsiRNA^{Htt} (2 nmol, 25 μ g), and PC-DHA-hsiRNA^{Htt} (2 nmol, 25 μ g). After one week, the levels of *Htt* mRNA expression in the ipsilateral striatum and cortex were measured by the QuantiGene® assay, normalized to a housekeeping gene (*Hprt1*), and presented as percentage of aCSF-treated control⁴⁸. Huntingtin mRNA levels are reduced by 72% and 77% in the striatum after administration of 2 nmol of DHA-hsiRNA or PC-DHA-hsiRNA^{Htt}, respectively (Figure 3a). We assessed protein levels at the 4 nmol dose by Western blot and detected 71% Huntingtin protein silencing in the striatum (Figure 3c,d). In the cortex, DHA-hsiRNA^{Htt} and PC-DHA-hsiRNA^{Htt} silenced *Htt* mRNA by 49% and 52% after treatment with 2 nmol, respectively (Figure 3b). A statistical comparison of DHA-hsiRNA and PC-DHA-hsiRNA at this dose was performed using a Kruskal-Wallis one-way ANOVA with Dunn's post-hoc analysis. Although significant levels of mRNA silencing were achieved in both striatum and cortex, there was no discernable difference between DHA-hsiRNA conjugates with or without the phosphocholine head group (Figure 3a,b).

We observed a 21% reduction of *Htt* mRNA in animals receiving a non-targeting control (PC-DHA-hsiRNA^{NTC}) around the site of injection (Figure 3a). This resulted in 42% nonspecific silencing of Huntingtin protein expression after normalization to tubulin (compared to an untreated control, Figure 3d, left). To ascertain the cause of this aberrant

activity, we examined the expression of dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) levels in treated tissues. DARPP-32 expression is routinely used to monitor dopamine receptor integrity and cell viability in medium spiny neurons⁴⁹. We noted that DARPP-32 staining was not significantly reduced in animals treated with the highest dose (4 nmol) of PC-DHA-hsiRNA^{Htt} (Figure 3d, middle panel). This result suggested that PC-DHA-hsiRNA is not inducing acute neuronal toxicity after local administration. We also monitored changes in glial fibrillary acidic protein (GFAP) expression. GFAP upregulation is a hallmark of reactive astrogliosis, an inflammatory response to mechanical or chemical brain trauma⁵⁰. We observed a ~two-fold increase in GFAP expression in animals receiving PC-DHA-hsiRNA^{NTC} and PC-DHA-hsiRNA^{Htt} compared to an aCSF-injected control (Figure 3d, right). These data suggested that astrocyte activation was sequence-independent and therefore mediated by the PC-DHA-hsiRNA bioconjugate or oligonucleotide chemical modifications.

Transvascular brain delivery of DHA-hsiRNA^{Htt} and PC-DHA-hsiRNA^{Htt}

The original motivation behind the design of the PC-DHA bioconjugate was to enable efficient BBB transfer. To determine if DHA-hsiRNA or PC-DHA-hsiRNA could penetrate the blood-brain or blood-cerebrospinal fluid (CSF) barriers after intravenous delivery, we injected wild-type animals (FVB/NJ, n = 2) with a two-day regimen of PBS, DHA-hsiRNA^{Htt} or PC-DHA-hsiRNA^{Htt} (20 mg/kg per injection). Animals were perfused and brains were sectioned and DAPI-stained as described previously. After systemic injection, we observed low, but detectable levels of Cy3 fluorescence in the brains of both DHA-hsiRNA- and PC-DHA-hsiRNA-treated animals. This fluorescence was limited to the epithelial cells of the choroid plexus, the principal source of CSF production (Figure 4). Oligonucleotide accumulation in the choroid plexus is unsurprising, as its fenestrated endothelium is highly permeable to ions, small molecules, and immune cells⁵¹. We did not observe significant accumulation in other brain regions, minimizing the applicability of this delivery method to the treatment of Huntington's disease, which primarily affects the striatum and cortex²⁸. We quantified oligonucleotide accumulation in wild-type animals at the same dose (FVB/NJ, n = 3) by PNA hybridization, and noted that both DHA-hsiRNA and PC-DHA-hsiRNA are retained in the choroid plexus (DHA-hsiRNA: 0.1 ng/mg; PC-DHA-hsiRNA: 0.3 ng/mg). Under these conditions, the levels of DHA-hsiRNA and PC-DHA-hsiRNA accumulation are not high enough to support gene silencing (data not shown). However, a higher bolus intravenous dosing regimen may improve BBB penetration.

Conclusion

Gene silencing by RNA interference (RNAi) is a highly promising approach for the pharmaceutical treatment of genetically defined neurological disorders, including Huntington's disease. Many non-viral strategies have been attempted to efficiently deliver siRNAs into the CNS, including liposome formulation and conjugation of cell-penetrating peptides, with limited success^{16, 52}. Previously, we validated the efficacy and safety of *Htt*-targeting DHA-hsiRNA for CNS delivery. We reported efficient *Htt* silencing in the ipsilateral striatum and cortex with no neural cell death or measureable innate immune activation at concentrations over 20-fold above the efficacious dose¹. Here, we have

investigated the utility of further modifying DHA-hsiRNA to mimic the structure of lysophosphatidylcholine DHA ester, a molecule which is actively transported into neurons. We report that PC-DHA-hsiRNA shows similar intracranial distribution and tissue retention compared to the previous generation of DHA-hsiRNA conjugates⁴⁷. Both PC-DHA-hsiRNA and DHA-hsiRNA are significantly more hydrophobic than an unconjugated hsiRNA, as evidenced from their higher retention time on a reverse-phase column (Figure S12). This property may enable the dramatic increase in retention of these compounds compared to the unconjugated control¹.

PC-DHA-hsiRNA induced *Htt* mRNA and protein silencing in the striatum (~80% mRNA and ~71% protein silencing following a single, 50 µg intrastriatal injection). This degree of *Htt* reduction is on par with current antisense oligonucleotides under clinical investigation⁵³ ([ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02519036), NCT02519036). However, this silencing activity occurs with a disturbing increase in neuronal toxicity and astrogliosis. The precise mechanism behind these adverse effects is unknown. There is a precedent for phosphorothioate and 2'-fluoro-mediated toxicity in heavily modified oligonucleotides⁵⁴⁻⁵⁶. We do not exclude the possibility that the phosphocholine itself is stimulating an immunogenic response⁵⁷.

We observed that PC-DHA-hsiRNA is capable of low, but measurable transfer across the blood-CSF barrier into the choroid plexus. Although oligonucleotide accumulation in this tissue is not optimal for the treatment of Huntington's disease, there are several potential disorders which may benefit from an RNAi-based therapeutic which enters the choroid epithelium, including leptomenigeal amyloidosis. An antisense oligonucleotide that suppresses choroid transthyretin (TTR) production in a leptomenigeal amyloidosis mouse model has been previously described⁵⁸. For these applications, delivery of PC-DHA-hsiRNA to the choroid plexus following intravenous injection could be further improved by the use of a large, bolus dose, and supported by a multi-day dosing regimen.

After a direct intrastriatal injection, we observed no diffusion or *Htt* silencing within the contralateral (non-injected) striatum or cortex (Figures 2a, S13). Different routes of administration can be explored to promote more diffuse distribution throughout the CSF, including intrathecal (lumbar spinal cord) and intracerebroventricular (lateral ventricle) injections. These different routes of injection may help to reduce the local toxicity observed following a direct parenchymal injection.

The synthetic methodology described herein can be used for the preparation of a broad range of biologically active siRNA conjugates containing at least one saturated or unsaturated fatty acid and a polar or neutral head group. Current efforts are focused on expanding this chemistry to other classes of fatty acids and on studying their distribution and efficacy following both CNS and systemic administration.

Materials and Methods

Oligonucleotide synthesis and purification

All oligonucleotide synthetic schemes and corresponding spectra are presented in the Supporting Information.

Preparation and treatment of primary cortical neurons and astrocytes

Primary cortical neurons were isolated and cultured from FVN/NJ embryos (E15.5) as described previously¹. Briefly, the cortices from both embryonic brain hemispheres were dissected, and a solution of papain and DNase I (Worthington, Lakewood, NJ) in Hibernate E medium (Brainbits, Springfield, IL) was used to disrupt the extracellular matrix prior to trituration to a single-cell suspension using a fire-polished glass pipette. For mRNA silencing experiments, cells were plated at 1×10^5 cells/well in a poly-L-lysine coated 96-well plate and treated with oligonucleotides on day 3 after plating. For cellular uptake experiments, neurons were plated at 2×10^5 cells per poly-L-lysine coated, 35 mm glass-bottom dish. For imaging, neurons were maintained in phenol red-free NbActiv4 medium (BrainBits). Prior to imaging, cells were stained according to the manufacturer's protocol with NucBlue (Life Technologies), washed three times with PBS, and fixed with 4% paraformaldehyde.

Fluorescence imaging

All fluorescent images were acquired with a Leica DMI8 inverted tiling microscope (Leica Microsystems). Images were processed using the Leica software suite (LAS X and LAS X Lite).

mRNA quantification in cells and tissue punches

mRNA was quantified from both cells and tissue punches using the QuantiGene® 2.0 DNA Assay (Affymetrix) exactly as described previously^{1, 48}. Probe sets (Mouse *Htt* or *Hprt*) were diluted and used according to the manufacturer's recommended protocol. Briefly, tissue punches (5 mg) were homogenized in 300 μ L of Homogenizing Buffer (Affymetrix) containing 2 μ g/ μ L proteinase K in 96-well plate format on a QIAGEN TissueLyser II, and 40 μ L of each lysate was added to a bDNA capture plate. *Htt* or *Hprt* probe sets (60 μ L) were added to each well of the capture plate for a final volume of 100 μ L. Signal was amplified according to the Affymetrix protocol. Luminescence was detected on a Tecan M1000 luminometer.

PNA hybridization assay

Levels of hsiRNA guide (antisense) strand accumulation in mouse brain were quantified using a PNA hybridization assay, as described previously¹. Briefly, tissue punches were homogenized in MasterPure Tissue Lysis Solution (EpiCentre) with added proteinase K (2 mg/mL, Invitrogen) and homogenized using a TissueLyser II (Qiagen), using 100 μ L of lysis solution per 10 mg tissue. Following lysis, sodium dodecyl sulfate was precipitated with KCl (3 mol/L) and cleared supernatant was hybridized to a Cy3-labeled PNA oligonucleotide fully complementary to the guide strand (PNABio). This mixture was analyzed by high-performance liquid chromatography. Cy3-labeled peaks were integrated and plotted on an internal calibration curve. Mobile phase for HPLC was 50% water, 50% acetonitrile, 25 mmol/L Tris-HCl (pH 8.5) and 1 mmol/L ethylenediamine-tetraacetate. The salt gradient was 0–800 mmol/L NaClO₄.

Stereotaxic injections and tissue collection

Wild-type (FVB/NJ) mice (6–8 weeks old) were deeply anesthetized with 1.2% Avertin (Sigma) and microinjected by stereotactic placement into the right striatum (coordinates relative to bregma: 1.0 mm anterior, 2.0 mm lateral, and 3.0 mm ventral). For efficacy studies, animals were euthanized after seven days, 300 μ m coronal sections were collected using a cryotome, and a 2 mm punch biopsy was taken from both injected and non-injected hemispheres. Each punch was placed in RNA later (Ambion) for 24 hours at 4°C. Punches were processed for the QuantiGene® 2.0 DNA Assay as described previously¹. For biodistribution studies, animals were injected as described, euthanized after 48 hours, perfused with PBS and 10% formalin. Brains were postfixed for 48 hours, paraffin-embedded, and sliced into 4 μ m sections. Each brain section was mounted on a glass slide and stained with DAPI to visualize nuclei prior to image collection on a Leica DMI8 inverted microscope. All animal procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC, protocol number A-2411).

Western blot

Protein levels were quantified from tissue punches collected and snap-frozen seven days after intrastratial injection, as described previously²⁸. Briefly, cell lysates (25 μ g) were separated by SDS-PAGE using 3–8% Tris-acetate gels (Life Technologies) and transferred to nitrocellulose using a TransBlot Turbo apparatus (BioRad). Blots were blocked in 5% nonfat dry milk (BioRad) diluted in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 hour at room temperature and then incubated with primary anti-huntingtin Ab1, anti-DARPP-32, or anti-GFAP antibodies overnight at 4°C with agitation. Blots were washed with TBST and incubated with peroxidase-labeled antirabbit IgG (Jackson ImmunoResearch) for 1 hour at room temperature. Protein signal was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and Hyperfilm ECL (GE Healthcare). Blots were reprobed with anti- β tubulin antibody (Sigma) as a loading control. After films were scanned (Epson Perfection V750), bands were measured and densitometry was performed using ImageJ software (v. 1.47). Huntingtin, DARPP-32, and GFAP signals were normalized to the tubulin signal for each set of samples ($n = 5$).

Statistical analysis

Data were analyzed using GraphPad Prism 6 software. Concentration-dependent IC50 curves for primary neuron experiments were fitted using a log(inhibitor) versus response—variable slope (four parameter). *In vivo* data were analyzed using a Kruskal-Wallis one-way ANOVA with Dunn's post-hoc analysis

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Neuronal uptake and efficacy of PC-DHA-hsiRNA

(a) Cellular internalization of Cy3-labeled PC-DHA-hsiRNA in mouse primary cortical neurons. Cells incubated for 0, 24, or 48 h with PC-DHA-hsiRNA^{Htt}. Images acquired with Leica DMi8 microscope (63X). Nuclei (Hoechst), blue; PC-DHA-hsiRNA (Cy3), red; NeuN (AlexaFluor 488), green (b) Primary mouse cortical neurons were incubated with 0–1.5 μ M *Htt*-targeting compounds at concentrations shown for one week. *Htt* mRNA levels were measured using QuantiGene® (Affymetrix), normalized to a housekeeping gene, *Hprt*

(Hypoxanthine-guanine phosphoribosyl transferase), and presented as percent of untreated control (n=3, mean $\pm$ SD). UNT—untreated; NTC—non-targeting control.

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Figure 2. PC-DHA-hsiRNA shows enhanced distribution and retention in mouse striatum and cortex

(a) aCSF, Cy3-C₇linker-hsiRNA, Cy3-DHA-hsiRNA, and Cy3-PC-DHA-hsiRNA were administered by unilateral intrastratial injection (2 nmol, 25 μ g). After 48 hours, fluorescent images were acquired with a Leica DMI8 tiling microscope using equivalent exposure and acquisition settings (10X). aCSF and C₇linker-hsiRNA^{Htt} images were published previously.¹ (b) Tissue concentrations of animals injected with 2 nmol of non-Cy3-labeled unconjugated hsiRNA, C₇linker-hsiRNA, DHA-hsiRNA, and PC-DHA-hsiRNA (n=8) measured by RNA hybridization from the ipsilateral (injected) striatum (b) or cortex (c). (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Kruskal-Wallis one-way ANOVA with Dunn's post hoc analysis). aCSF – artificial cerebrospinal fluid (vehicle); n.s. - non-significant.

Figure 3. Huntingtin mRNA and protein are efficiently silenced by PC-DHA-hsiRNA^{Htt}
aCSF, PC-DHA-hsiRNA^{NTC} (4 nmol, 50 μ g), DHA-hsiRNA^{Htt}, and PC-DHA-hsiRNA^{Htt} (2 nmol, 25 μ g) were unilaterally injected into the striatum of FVB/NJ mice (n = 8 per group). Punch biopsies (5 mg) of the striatum (a) and cortex (b) were collected after one week. Level of *Htt* mRNA was measured using QuantiGene® (Affymetrix), normalized to a housekeeping gene (*Hprt*), and presented as percentage of untreated control (mean $\pm$ SD). DHA-hsiRNA^{Htt} efficacy was previously reported.¹ (c,d) Protein expression levels of Huntingtin, Dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32), and

Glial fibrillary acidic protein (GFAP) levels in the ipsilateral striatum of wild-type (FVB/NJ) mice after a one-week treatment with PC-DHA-hsiRNA^{NTC} or PC-DHA-hsiRNA^{Htt} (4 nmol, 25 μ g). (n=5, mean $\pm$ SD). NTC—non-targeting control; aCSF—artificial cerebrospinal fluid; n.s. – nonsignificant (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, Kruskal-Wallis one-way ANOVA with Dunn's post hoc analysis).

Figure 4. DHA-hsiRNA and PC-DHA-hsiRNA_{Htt} accumulation in mouse brain choroid plexus after systemic administration (left panel) PBS (vehicle), Cy3-DHA-hsiRNA^{Htt}, and Cy3-PC-DHA-hsiRNA^{Htt} (two daily 20 mg/kg) were administered by intravenous injection in FVB/NJ mice (n=3 per group). After 48 hours, fluorescent images were acquired with a Leica DMi8 tiling microscope (63X). (right panel) Whole brain lysate measurement of *Htt* antisense strand accumulation following intravenous administration PBS, DHA-hsiRNA^{Htt}, and PC-DHA-hsiRNA^{Htt} (two daily 20 mg/kg) via PNA hybridization. PBS—phosphate-buffered saline.

Scheme 1. Chemical structures of DHA-hsiRNA conjugates and derivatives

(a) Fully chemically stabilized (alternating 2'-fluoro, 2'-O-methyl substituted), hydrophobically modified siRNA (hsiRNA) containing a 5'-phosphate on the antisense strand and a 3'-DHA or phosphocholine-DHA conjugate attached via a carbon linker on the sense strand. Molecular models of hsiRNA represented to scale using PyMOL. The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC. (b) Chemical structures of 1-oleoyl-2-docosahexaenoyl phosphatidylcholine (a naturally esterified form of DHA) and sphingomyelin (an important lipid component of nerve cell membranes).

Scheme 2. Synthetic route to PC-DHA-controlled pore glass (CPG)

Reagents and conditions: (a) 20% piperidine in DMF (2×15 min); (b) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite, DIEA, DCM, 2 h, rt, 95%; (c) choline tosylate, ETT, MeCN, 2 h, rt, followed by mCPBA, 10 min, rt, 69%; (d,e) TFA in dry DCM (1:1), triisopropylsilane, 2 h, rt, then 10% diisopropylamine in MeCN, 1.5h, rt 74%(f) **3**, BOP, HOBT, DMF, 2,4,6-collidine, rt, 12 h; (g) 20% piperidine in DMF (2×15 min), rt; (h) DHA, HATU, DMF, rt, 12 h