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Self-assembling Modified β-Cyclodextrin Nanoparticles as Neuronal siRNA Delivery Vectors: Focus on Huntington's Disease

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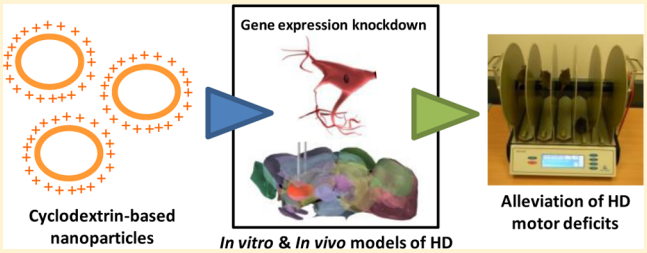
Supporting Information

ABSTRACT: Huntington's disease (HD) is a rare autosomal dominant neurodegenerative disease caused by the expression of a toxic Huntingtin (HTT) protein. The use of short interfering RNAs (siRNAs) to silence the mutant protein is one of the most promising therapeutic strategies under investigation. The biggest caveat to siRNA-based approaches is the lack of efficient and nontoxic delivery vectors for siRNA delivery to the central nervous system. In this study, we investigated the potential of modified amphiphilic β-cyclodextrins (CDs), oligosaccharide-based molecules, as novel siRNA neuronal carriers. We show that CDs formed nanosize particles which were stable in artificial cerebrospinal fluid. Moreover, these complexes were able to reduce the expression of the HTT gene in rat striatal cells (ST14A-HTT120Q) and in human HD primary fibroblasts. Only limited toxicity was observed with CD·siRNA nanoparticles in any of the *in vitro* models used. Sustained knockdown effects were observed in the striatum of the R6/2 mouse model of HD after single direct injections of CD·siRNA nanoparticles. Repeated brain injections of CD·siRNA complexes resulted in selective alleviation of motor deficits in this mouse model. Together these data support the utility of modified β-CDs as efficient and safe siRNA delivery vectors for RNAi-based therapies for neuropsychiatric and neurodegenerative disorders.



KEYWORDS: *nonviral vectors, gene therapy, rotarod, neurodegenerative disease, RNAi CNS delivery*

INTRODUCTION

Huntington's disease (HD) is a rare but devastating autosomal dominant neurodegenerative disease caused by a mutation within the Huntingtin (HTT) gene.[1] The mutation consists of an abnormal CAG repeat expansion that leads to the expression of a toxic HTT protein. Accumulation of the mutant HTT protein compromises survival and normal neuronal functioning in the striatum and progressively in other brain structures, such as the cortex.[1]

Reducing expression of the mutant HTT gene by means of RNA interference (RNAi) has been recently suggested as one of the most promising therapeutic strategies for HD.[1] Briefly, the RNAi pathway is an endogenous post-transcriptional mechanism whereby short double stranded RNA molecules inhibit the translation of specific mRNAs by ribosomal arrest or degradation.[2] Exogenously introduced synthetic short interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and pre-micro RNAs (miRNAs) are also able to evoke specific gene silencing effects via this pathway. However, such nucleic acids have poor cell penetrating proprieties, and therefore, an appropriate delivery method is required.[2] Moreover, for reasons not clearly understood, neurons are particularly resistant to RNAi, and therefore, delivering such molecules to the central nervous system (CNS) is very challenging.[3] Viral and nonviral approaches for RNAi delivery have been evaluated to facilitate the transport of genetic material into neurons.[4] Lentiviruses and adeno-associated viruses (AAV) have been by far the most widely used viral vectors for CNS applications due to their ability to transduce nondividing cells and to their relatively low immunogenicity. Several *in vitro* and *in vivo* studies have demonstrated the potential utility of such viral particles for treating neurological disorders, such as HD, Alzheimer's, and amyotrophic lateral sclerosis (ALS).[5,6] Although viral vectors have great tropism over a wide range of cell types, their potential for RNAi-based therapies in the CNS is limited by their toxicity, immunogenicity, risk of insertional mutagenesis, and high cost of large-scale production.[7,8] Alternatively, nonviral approaches for siRNA delivery have also been considered to enable RNAi in the CNS. *In vivo* studies have demonstrated that long-term treatment with large amounts of unmodified naked

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siRNAs was able to induce widespread gene silencing effects in the brain when delivered intracerebroventricularly using osmotic minipumps or to specific structures in the brain by convection-enhanced delivery.[9−11] In order to reduce the large amounts of siRNA required for *in vivo* applications, a number of strategies have been adopted to improve their stability, nuclease resistance, and cell penetrating properties. Chemical modifications of siRNA duplexes using functional groups such as peptides,[12] lipids, and steroid derivatives,[13,14] and other modifications[15,16] have been shown to be advantageous for *in vivo* delivery and have improved delivery of siRNAs to the brain and spinal cord.[17] On the other hand, several lipid-based,[18,19] polymer-based,[20,21] and peptide-based carriers[22] and other nanoparticles[23,24] have been engineered and used to complex, condense, and transport siRNAs into CNS *in vitro* and *in vivo* models. These vary in their efficacy, toxicity, and applicability to neuronal systems.

Despite the advances in the design and development of such nonviral RNAi vectors, there remains a great need to develop more effective and less toxic carriers for siRNA delivery into the CNS.[2] Modified cyclodextrins (CD), based on naturally occurring oligosaccharide molecules, are promising nucleic acid carriers that have been shown to bind and complex siRNA, protecting it from enzymatic degradation.[25] Our group has previously demonstrated efficacy of these nano carriers to deliver both plasmid DNA and siRNA into hepatocyte, enterocyte, prostate cancer, and neuronal *in vitro* models.[26−30] Moreover, the potential of CD polymer-based carriers for human therapy has been recently demonstrated by the first phase I clinical trial in patients with metastatic melanoma.[31,32]

The aim of the present study was to investigate the use of modified β-CDs to deliver HTT targeted siRNAs to multiple *in vitro* models and to the most widely used *in vivo* model of HD (R6/2 mouse model). To this end, a rat striatal cell line (ST14A-HTT120Q) stably cloned with a fragment of the human HTT gene and human primary fibroblasts naturally harboring the human mutant HTT gene were used to validate this technology *in vitro*. In order to evaluate if any effects translated to the *in vivo* setting, preclinical testing of CD·siRNA nanoparticles was carried out in the R6/2 mouse model of HD.

EXPERIMENTAL SECTION

Synthetic siRNAs. Synthetic duplexed siRNAs were obtained from Sigma-Aldrich (France) or QIAGEN (United Kingdom). HTT target siRNAs (HTTsiRNA) as per Wang et al.[33] sense strand, 5′-GCCUUCGAGUCCCUCAAGUCC-3′; antisense strand, 5′-ACUUGAGGGACUCGAAGGCCU-3′. Nonsilencing siRNAs (NSsiRNA): sense strand, 5′-UUCUCCGAACGUGUCACGUdTdT-3′; antisense strand, 5′-ACGUGACACGUUCGGAGAAdTdT-3′. FAM-labeled siRNA (FAMsiRNA): sense strand, 5′-[6FAM] UUCUCCGAACGUGUCACGUdTdT-3′; antisense strand, 5′-ACGUGACACGUUCGGAGAAdTdT-3′.

Preparation, Physicochemical Characterization, and Stability of CD·siRNA Nanoparticles. CD solutions were prepared as previously described in sterile water or 5% glucose solution.[28] Details on the preparation of CD solutions are described in the Supporting Information; CD·siRNA complexes were diluted in deionized water (DIW), and particle size and charge were assessed using a Malvern's Zetasizer Nano ZS. Stability studies were carried out in artificial cerebrospinal fluid (aCSF) (NaCl 148 mM, $MgCl_2$ 0.8 mM, KCl 3 mM, $CaCl_2$ 1.4 mM, Na_2HPO_4 1.5 mM, NaH_2PO_4 0.23 mM (all from Sigma-Aldrich, Germany). Complexes were incubated in aCSF at 37 °C for different periods of time, and siRNA binding was assessed by the gel retardation assay described in ref 28. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, Germany) was used to disrupt nanoparticles and enable release of siRNA. For *in vivo* studies, CD·siRNA nanoparticles were prepared as above and concentrated by ultrafiltration using Vivaspin 500 centrifugal units (Sartorius, Germany) to a final concentration of 1 μg/μL of siRNA.

Cell Culture and RNAi Transfection. Rat striatal cells expressing exon 1 and 120 polyQ of the human HTT gene (ST14A-HTT120Q cells) and primary human HD fibroblasts (GM04691) were obtained from Coriell Institute for Medical Research (Camden, NJ). Detailed information on subculture is described in the Supporting Information. CD·siRNA nanoparticles were prepared in sterile water and left to incubate for 20 min and thereafter diluted in optiMEM (GIBCO, United Kingdom). Cells were transfected with CD·siRNA nanoparticles 24 h after being seeded.

Toxicity Assays. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) (Sigma-Aldrich, Germany) was added to each well and left to incubate for 4 h either at 33 or 37 °C and 5% CO_2. The formazan product was then dissolved with 200 μL of dimethyl sulfoxide (Sigma-Aldrich, Germany) and left to incubate at room temperature at least for 30 min. Absorbance was measured at 590 nm using a SpectraMax Plus384 plate reader.

Confocal Microscopy. Confocal microscopy was carried out in living cells. Nonsilencing [6FAM] 5′-labeled siRNAs were obtained from Sigma-Aldrich (France) or QIAGEN (United Kingdom) and used at a final concentration of 200 nM. Cells were seeded in glass bottom plates and transfected for 24 h. Cells were incubated for 30 minutes with LysoTracker Red DND-99 endosomal marker (Invitrogen, Molecular Probes, Eugene, OR) following manufacturer instructions. Images were acquired on a FluoView FV1000 confocal microscope and analyzed using Olympus Fluoview ver 2.1b software.

Quantitative Real-Time PCR. RNA was isolated from tissue using a Trizol-based method (Invitrogen, United Kingdom). 300 ng of total RNA was reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, MO). Gene expression was assessed by fluorescent real time quantitative PCR using a 7300 Real Time PCR System. Cycling conditions were as follows: 10 min (min) at 95 °C, 40 cycles of [15 s (sec) at 95 °C; 1 min at 60 °C]. TaqMan rat or mouse b-actin VIC labelled probes were acquired from Applied Biosystems (United Kingdom) (part number 4352340E and 4352341E). Previously validated set of primer sequences (forward: CGACCCTGGAAAAGCTGATGAA, reverse: CTGCTGCTGCTGGAAGGA)[34] for detection of human HTT mRNA (ref. Seq. NM_002111) were used to design a TaqMan HTT FAM-labeled probe. Each sample was analyzed in triplicate wells, and average CT values were used for gene expression calculations. β-actin was used as endogenous control, and all CT values were normalized to the expression of β-actin.

Western Blot Analysis. ST14A HTT120Q cells were harvested 72 h after addition of transfection complexes and lysed in lysis buffer (10 mM HEPES, 100 mM KCl, 1.5 mM $MgCl_2$, 0.1% Igepal, 0.1% SDS, 2.5 mM CHAPS, 0.5% sodium deoxicholate) containing a protease and proteinase inhibitor cocktail (P8340, Sigma-Aldrich). Total protein content was quantified using a bicinchoninic acid (BCA) assay according to manufacturer's instructions (Pierce, Thermo Scientific, Rockford, IL). 5−10 μg of total protein were loaded onto each well of a precast NuPAGE Novex 4−12% Bis-Tris gel (Invitrogen, Carlsbad, CA).

Protein electrophoresis was carried out at 100 V for ~2.5 h. Protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for 1.5 h at 200 mA, and transfer was confirmed by Ponceau S staining. Membrane was incubated in a blocking solution containing 0.1% Tween and 5% milk and incubated overnight with antihuman HTT antibody (dilution 1:2,500) (MAB2166, Millipore) or anti-β-actin (dilution 1:3000) (A5441, Sigma). Membrane was washed and then incubated with a 1:10000 dilution of a goat antimouse antibody (IRDye 800CW, LI-COR) for 1 h. Scans were carried out using a LICOR Odyssey near-infrared scanner. Densitometry analysis of bands was performed using ImageJ software, and all results were normalized to β-actin controls.

R6/2 Colony Maintenance. R6/2 colony was maintained by breeding B6CBAF1 ovarian transplanted females (HD exon 1, 62Gpb/3J) and B6CBAF1 males (Stock # 006494, The Jackson Laboratories, Bar Harbor, ME). Pups were weaned at 3 weeks, and DNA samples for genotyping were collected from tail clips. Additional details on the genotyping protocol are described in the Supporting Information. Animals were group-housed in groups of 4−5 mice in cages containing regular sawdust bedding. After surgical procedures animals were recovered and kept single-housed until the end of the experiments. Animals were closely monitored and euthanized under ethical grounds if in pain and/or severe distress.

All animal experimental procedures were approved by the ethical committee at the University College Cork and performed in accordance with the European Union directive 2010/63/EU for animals used for scientific purposes.

Stereotaxic Surgery and Behavioral Assessment. Simulation of brain injections was carried out in the Brain Navigator (http://www.brainnav.com). Stereotaxic surgery was performed in animals with 4−5 weeks of age in order to implant cannulas (PlasticOne, Roanoke, VA) for chronic administration of CD·siRNA nanoparticles or to perform acute direct injections into the striatum (AP = +0.7, ML = 2.0, V = −3.0). During all surgical procedures animals were anaesthetized under a continuous flow of Isofluorane (IsoFlo, Abbott, United Kingdom), and Carprofen (Rimadyl, Pfizer Animal Health, Netherlands) was injected subcutaneously to provide analgesia. 2.5 μL of each treatment was injected bilaterally at a rate of 0.5 μL/min. In *in vivo* HTT mRNA knockdown studies, a 2 mm slice from the site of injection was isolated using a mouse brain slicer matrix. Tissue was kept in RNA Later (Sigma) and 4 °C overnight and, thereafter, stored at −80 °C until analysis. For behavioral studies a total of seven injections of naked HTTsiRNAs or CD·HTTsiRNA nanoparticles were given over a period of 5 weeks and behavior deficits were assessed up to 10 weeks after first injection (Figure 5a). All behavior tests were conducted after a 10−15 min habituation period to the testing room. Behavior tasks were carried out as previously described in refs 33−35; for further details on behavioral tasks, see the Supporting Information.

Statistical Analysis. All results are expressed as mean ± standard error of mean (SEM) unless otherwise stated. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by Bonferroni Post Hoc test for all comparisons. Two-way repeated measures ANOVA was carried out to investigate the overall effect of treatment over time on the rotarod, grip strength, and spontaneous locomotor activity data. Thereafter, one-way ANOVA followed by Bonferroni's Post Hoc test was used to analyze each specific time point of behavioral assessment. Finally, paw clasping behavior data was analyzed by χ^2 tests at each age separately. All inferential statistics were carried out using PAWS 18 Statistical package.

RESULTS

Physicochemical Characterization of CD·siRNA Nanoparticles and Stability in Artificial Cerebrospinal Fluid (aCSF). Chemical modifications have been previously introduced onto a β-CD to form polycationic amphiphilic molecules (SC12 CD (Click) propylamine) (Figure 1a).[28] Electrostatic interaction between these modified β-CDs and polyanionic siRNAs results in nucleic acid condensation and formation of nanoparticles (Figure 1a). HTT targeted siRNAs (HTTsiRNA) were mixed and complexed at different mass ratios (MR) (MR are expressed by μg of CD: μg of siRNA) with this modified β-CD. Gel retardation assays showed that modified β-CDs are able to bind and fully complex HTT targeted siRNAs from MR 5 (Supporting Information Figure S1a). Moreover, these complexes were found to have a hydrodynamic diameter between 100 and 350 nm and a net positive surface charge (Figure 1b). A reduction in particle size and an increase in net charge were noted as increasing MRs of modified β-CD were used. CD·siRNA nanoparticles were found to remain stable and undisrupted in aCSF up to 6 h (Figure 1c). aCSF and physiological temperatures (37 °C) seemed not to affect siRNA binding and complexation as shown by gel retardation assays. Furthermore, the size and surface charge of the CD·siRNA nanoparticles did not reveal remarkable changes up to 6 h (Figure S1b).

Delivery of CD·siRNA Nanoparticles to a Rat Neuronal *in Vitro* Model of HD. Cytotoxicity studies revealed that, even at high MRs, CD·siRNA nanoparticles maintained a good mitochondrial dehydrogenase activity profile when compared to untreated cells (Figure 2a). Reduced mitochondrial dehydrogenase activity has been widely used as an indication of cytotoxicity. Even after 48 h transfection with CD·siRNA complexes, cell viability was maintained above 80% and no statistically significant differences were found when compared to untreated or naked siRNA-treated cells.

Cellular uptake of a fluorescently labeled siRNA (FAMsiRNA) was observed by confocal microscopy (Figure 2b) and quantified by fluorescent activated cell sorting (FACS) flow cytometry (Supporting Information Figure S2a,b). Fluorescent CD·FAMsiRNA nanoparticles (green) were taken up by this neuronal cell line in a time-dependent fashion. After 48 h post-transfection, up to ~38% (38.3 ± 7.1%) cells were found to be positive for fluorescent CD·FAMsiRNA complexes. Furthermore, our data shows that only a few CD·FAMsiRNA complexes were colocalized (yellow) with acidic endosomes (red) after 24 h. In contrast, no significant uptake was observed in cells treated with naked FAMsiRNA (Figure 2b).

Mutant HTT gene expression was assessed by real time quantitative PCR (RT-qPCR) (Figure 2c). Results showed that CD·HTTsiRNA nanoparticles at different MR were able to effectively knockdown expression of the HTT gene by ~51% (50.9 ± 4.8%) after 24 h transfection. The HTTsiRNAs sequences used in the present study which allow for specific knockdown have been previously screened and validated by others.[33,36] No significant differences were observed in knockdown efficiency among the different MRs of CD·HTTsiRNAs used. In contrast, naked HTT siRNA and CD·siRNA complexes bearing a nonsilencing siRNA (NSsiRNA) sequence were not able to significantly reduce expression of HTT when compared with untreated cells. Additionally, HTT protein levels were found to

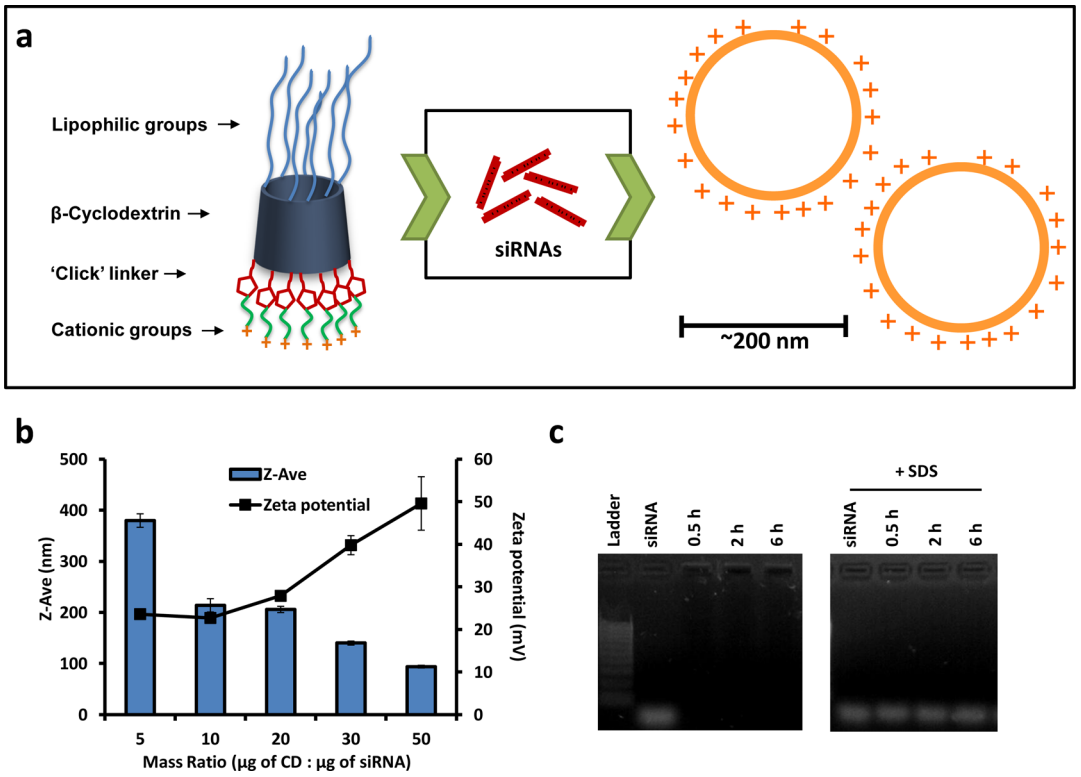

Figure 1. Physicochemical characterization of CD·siRNA nanoparticles and stability in artificial aCSF. (a) Schematic showing the formation of nanoparticles. Complex formation is enabled by electrostatic interactions between positively charged modified β-CD units and the negatively charged siRNA phosphate backbone. (b) Hydrodynamic radius and ζ potential of CD·siRNA nanoparticles measured through dynamic light scattering and electrophoretic light scattering, respectively. Results are expressed in mean ± SD. (c) siRNA binding and nanoparticle stability in aCSF was assessed through a gel retardation assay after different time points. 0.3 μg of siRNA loaded onto each well. Free siRNA migrates through the gel; complexed siRNA remains in the wells. SDS was used to release siRNA from nanoparticles and prove its integrity.

be reduced by ~35% (35.1 ± 7.1%) after 72 h transfection with CD·HTTsiRNAs (Figure 2d). Commercially available cationic lipids (Lipofectamine 2000) were also able to successfully transfect ST14A-HTT120Q cells, evoke silencing of HTT mRNA, and reduce expression of the HTT protein (Table 1). However, it is worth noting that Lipofectamine 2000 exerted much greater cytotoxic effects, as indicated by the reduced mitochondrial dehydrogenase activity when compared to CD·siRNA MR 10 (Table 1).

Delivery of CD·siRNA Nanoparticles to a Human *in Vitro* Model of HD. MTT assays revealed that even at high MRs CD·siRNA nanoparticles maintained a good cell viability profile when compared to untreated cells in these primary human cells (Figure 3a). After 48 h transfection with CD·siRNA complexes, cell viability was maintained above 78% and no statistically significant differences were found when compared to untreated or naked siRNA-treated cells.

CD·FAMsiRNA nanoparticles (green) were also actively taken up by this primary human cell line as shown by confocal images (Figure 3b). FACS flow cytometry revealed that after 48 h transfection up to ~40% (40.2 ± 1.6%) of cells were found to be positive for fluorescent CD·FAMsiRNA complexes (Supporting Information Figure S2c,d). Moreover, our data shows that only a few CD·FAMsiRNA complexes were colocalized (yellow) with acidic endosomes (red) after 24 h. In contrast, no significant uptake was observed in cells treated with naked fluorescent siRNA.

CD·HTTsiRNA nanoparticles at different MR were able to silence the expression of the HTT gene by ~78% (78.2 ± 8.5%) after 24 h transfection (Figure 3c). Alternatively, naked HTTsiRNA and CD·NSsiRNA complexes were not able to significantly reduce expression of the HTT gene when compared with untreated cells.

CD·siRNA Nanoparticles Mediate HTT mRNA Knockdown in the R6/2 Mouse Brain. Based on the physicochemical properties of the nanoparticles, cellular uptake, and mRNA knockdown efficiency in both *in vitro* models of HD, MR10 was chosen as the optimal ratio between CD and siRNA to carry out *in vivo* studies. Prior to *in vivo* experiments, formulation of CD·siRNA complexes in several physiological buffer solutions was also investigated. Preparation of CDs in 5% glucose, saline (150 mM NaCl), and aCSF resulted in clear solutions and did not affect complex formation with siRNAs (Supporting Information Figure S3a,b). Additionally, the size and surface charge of complexes prepared in these buffers were not affected to a great extent when compared to complexes prepared in water (Supporting Information Figure S3c,d). In contrast, CDs prepared in phosphate buffered saline (PBS) resulted in cloudy solutions, failed to fully complex siRNAs, and caused abrupt changes in the size and surface charge of particles (Supporting Information Figure S3). Since 5% glucose has been widely used for direct brain injections in previous studies and the stability of complexes was not affected, we have selected this buffer as a vehicle for brain delivery of CD·siRNA nanoparticles.[20]

In order to investigate knockdown efficiency *in vivo*, R6/2 mice were treated with vehicle (5% glucose),[20] HTT naked siRNA, CD·HTTsiRNA, or CD·NSsiRNA. A total of 2.5 μg of siRNA was injected bilaterally into the striatum of R6/2 males and females (Figure 4a).

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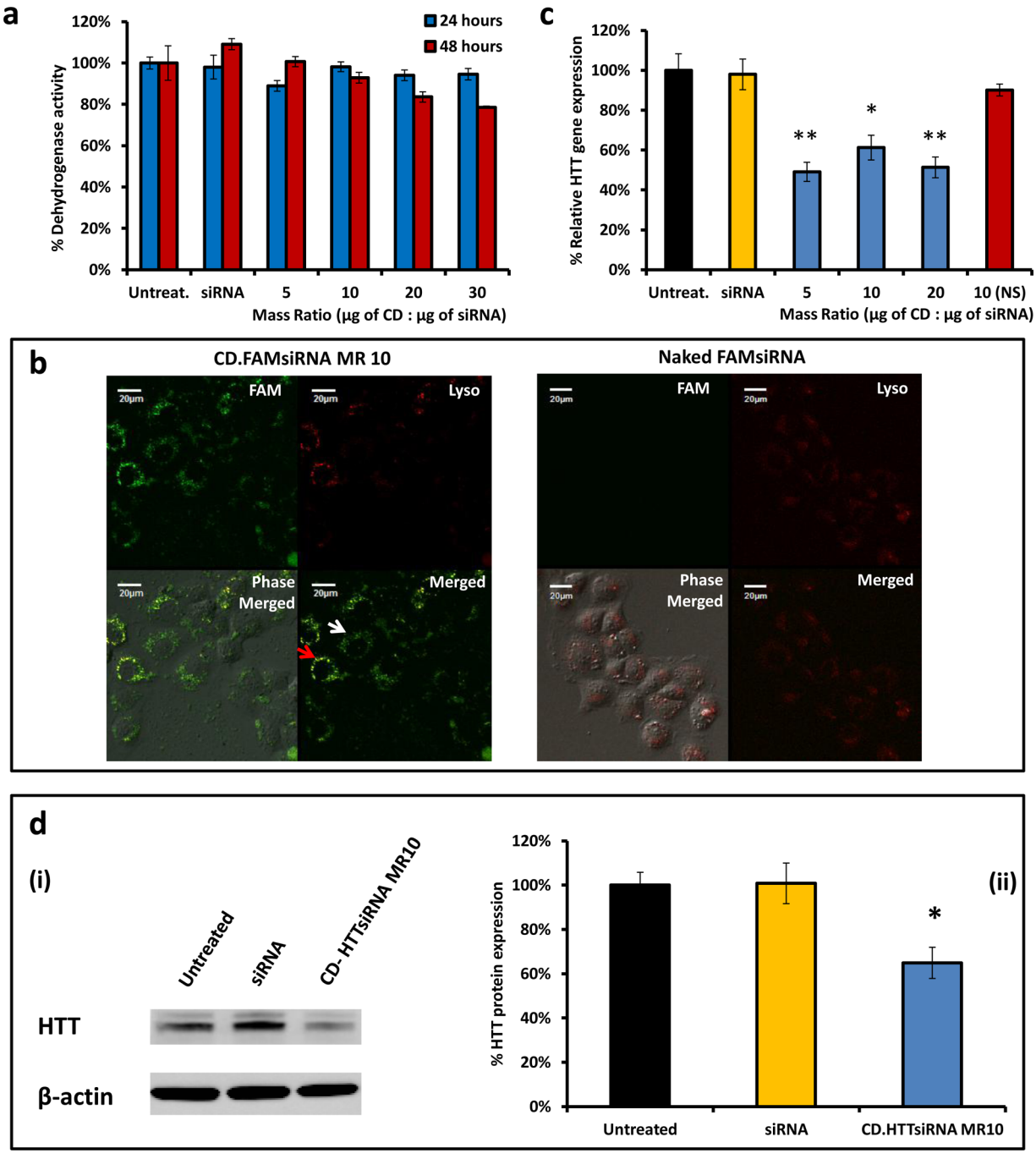

Figure 2. Delivery of CD·siRNA nanoparticles to a rat neuronal *in vitro* model of HD (ST14A-HTT120Q cells). (a) Cytotoxicity profiles of CD·siRNA nanoparticles in ST14A-HTT120Q cells were assessed by MTT assay after 24 and 48 h transfection. (b) Cellular uptake of fluorescently labeled CD·siRNA nanoparticles by confocal microscopy. *FAM*, Green CD·FAMsiRNAs nanoparticles; *Lyso*, acidic endosomes stained red with Lysotracker endosomal marker; *merged*, FAM and Lyso; *phase merged*, phase contrast and merged. Red arrow indicates CD·siRNA complexes colocalized with acidic endosomes. White arrow indicates CD·siRNAs nanoparticles free from endosomes. Scale bar = 20 μm (c) Knockdown of HTT gene expression in a rat striatal cell line. ST14A-HTT120Q cells were transfected with naked HTT siRNA (siRNA), CD·HTTsiRNA nanoparticles at different MR (blue), and CD.NSsiRNA at MR10 (10(NS)). Total RNA was extracted and reverse transcribed to cDNA. Relative expression of HTT mRNA was assessed by quantitative PCR. HTT gene expression was normalized against the expression of β-actin. (d) Western blot analysis of HTT protein expression in ST14A-HTT120Q cells. (i) Cell protein extracts were subjected to Western blotting with anti-HTT antibody (MAB2166). Five micrograms of total protein was loaded onto each lane. (ii) Densitometry analysis. All results were normalized to β-actin protein expression prior to densitometry analysis. The final concentration of siRNA for all experiments was 100 nM (except for microscopy experiments, 200 nM), and cells were transfected for 24, 48 or 72 h (HTT protein expression). All results are expressed in mean ± SEM, $n = 3$ per group, $^{*}p < 0.05$, and $^{**}p < 0.01$ compared to untreated cells.

CD·HTTsiRNA nanoparticles were able to significantly reduce the expression of the HTT gene *in vivo* (Figure 4b). The time course study revealed that, 4 h postinjection, HTT gene expression was reduced by ~85% (84.7 ± 3.8%). Moreover, gene silencing effects were found to be maintained up to seven days with HTT gene expression still reduced by ~66% (65.5 ± 8.3%). However,

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knockdown was no longer apparent at three weeks postinjection. In comparison, no significant gene expression knockdown was achieved either with naked HTTsiRNAs or with CD·NSsiRNA.

The spread of HTT gene expression knockdown in the brain after a single injection of CD·HTTsiRNA nanoparticles into the striatum was also assessed. Results showed that while there was a trend toward a significant knockdown in areas close to the site of injection (hippocampus, p = 0.061 by ANOVA followed by Bonferroni's post hoc test for multiple comparisons), no significant reduction in HTT gene expression was observed in a region distal from the site of injection such as the cerebellum (Supporting Information Figure S4a,b).

Table 1. Efficiency and Cytotoxicity of CD·siRNA Nanoparticles vs Lipofectamine 2000 in ST14A-HTT120Q Cells

vector characteristic	CD·siRNA MR10 (%)	Lipofectamine 2000 (%)
cellular uptake	30−50	50−60
knockdown of HTT gene expression	40−60	60−63
reduction in HTT protein levels (72 h)	35	36
dehydrogenase activity	82−98	58−60

Multiple Dosing with CD·siRNA Nanoparticles into the R6/2 Mouse Brain and Behavioral Assessment. On assessing behavioral differences between the R6/2 mice and their wildtype controls, significant differences in their latency to fall from a rotating rod were already evident from 5 weeks in both treated and untreated R6/2 mice (Figure 5b). Results revealed that CD·HTTsiRNA nanoparticles significantly alleviated rotarod deficits in R6/2 mice when compared to untreated or naked siRNA treated animals ($F(2,26)$ = 3.906, P = 0.033). By 6 weeks of age, CD·HTTsiRNA treated animals performed better than untreated R6/2 mice and naked siRNA treated animals, however significant differences were only observed from 7 weeks of age. When brain injections were ceased by week 9, deterioration in rotarod performance was then observed in animals treated with CD·HTTsiRNA nanoparticles.

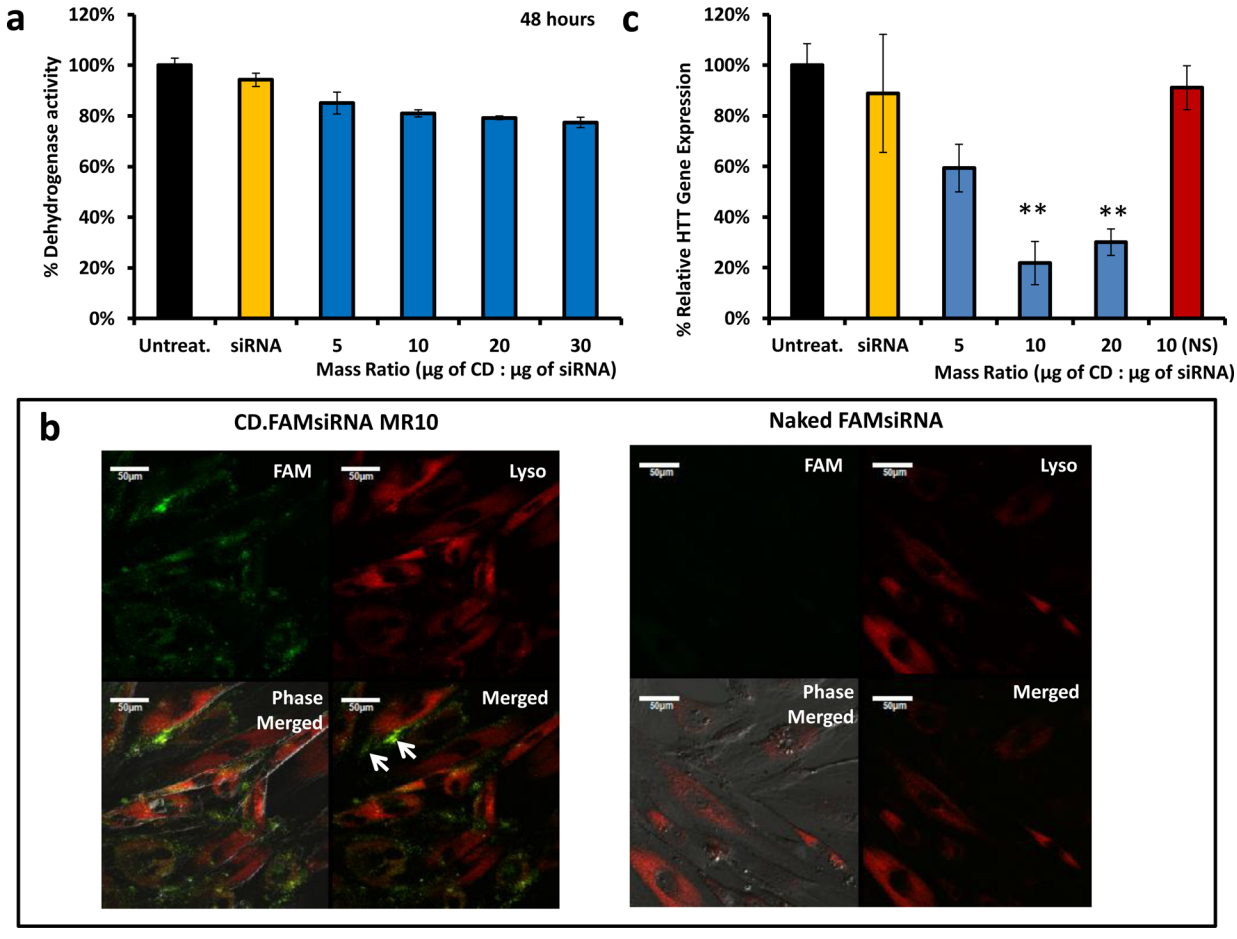

Figure 3. Delivery of CD·siRNA nanoparticles to a human *in vitro* model of HD (HD human primary fibroblasts). (a) Cytotoxicity profiles of CD·siRNA nanoparticles in human HD fibroblast primary cells were assessed by MTT assays after 48 h transfection. (b) Cellular uptake of fluorescently labeled CD·siRNA nanoparticles by confocal microscopy. *FAM*, Green CD·FAMsiRNAs nanoparticles; *Lyso*, acidic endosomes stained red with Lysotracker endosomal marker; *merged*, FAM and Lyso; *phase merged*, phase contrast and merged. White arrow indicates CD·siRNAs nanoparticles free from acidic endosomes. Scale bar = 50 μm. (c) Knockdown of HTT gene expression in a human *in vitro* model of HD. Human HD fibroblast primary cells were transfected with naked HTT siRNA (siRNA), CD·HTTsiRNA nanoparticles at different MR (blue) and CD·NSsiRNA at MR10 (10(NS)). Total RNA was extracted and reverse transcribed to cDNA. Relative expression of HTT mRNA was assessed by quantitative PCR. HTT gene expression was normalized against the expression of β-actin. Final concentration of siRNA for all experiments was 100 nM (except for microscopy experiments 200 nM), and cells were transfected for 24 h or 48 h. All results are expressed in mean ± SEM, n = 3 per group, $^{**}P < 0.01$ compared to untreated cells.

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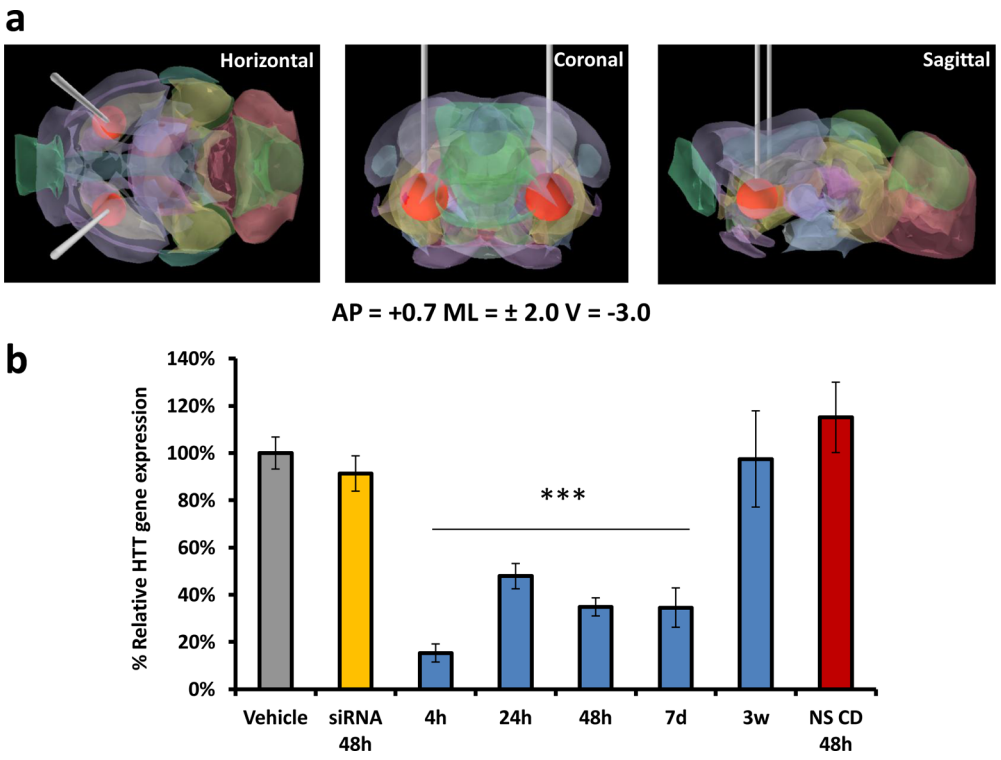

Figure 4. CD·HTTsiRNAs mediate *in vivo* HTT mRNA knockdown in the R6/2 mouse model. (a) CD·HTTsiRNA nanoparticles were injected into the striatum of R6/2 mice. 2.5 μL brain injections were simulated on BrainNavigator and shown in horizontal, coronal, and sagittal planes. Stereotaxic coordinates for brain injections are as follows: Anterior-Posterior (AP) = +0.7, Medio-lateral (ML) = ±2.0, and Ventral (V) = −3.0. (b) Knockdown of HTT gene expression in the R6/2 mouse brain. Mice were injected directly into the striatum with vehicle, naked siRNA, CD·HTTsiRNA, and CD·NSsiRNA nanoparticles. Tissue was harvested at different time points. RNA was extracted and reverse transcribed to cDNA. Relative expression of HTT mRNA was assessed by quantitative PCR. HTT gene expression was normalized against the expression of β-actin. All results are expressed in mean ± SEM, n = 3−8 per group, ***P < 0.001 compared to vehicle treated animals.

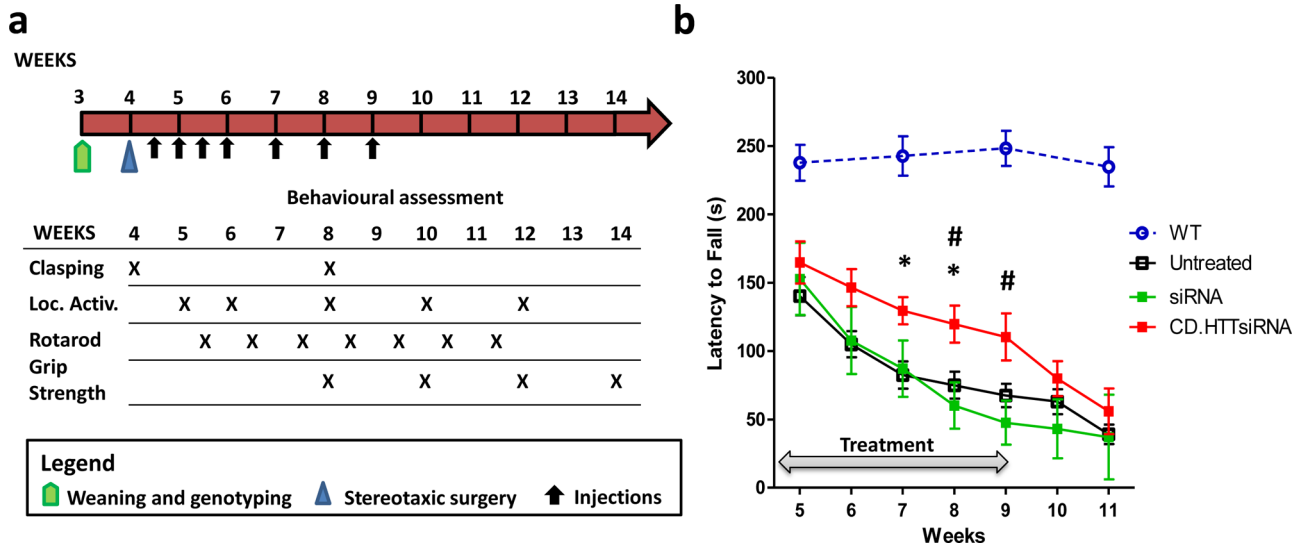

Figure 5. Multiple dosing with CD·siRNA nanoparticles into the R6/2 mouse brain and behavioral assessment. (a) Study design. Briefly, stereotaxic surgery to implant cannulas in the striatum (bilaterally) was carried out at 4 weeks of age. Seven injections of naked HTT siRNA and CD·HTTsiRNA nanoparticles were given over 5 weeks. Motor behavior was assessed as per figure. (b) Motor coordination and balance was assessed through rotarod task in 3 consecutive days. Mice were placed on top of a rotating rod and their latency to fall was recorded. All results are expressed in mean ± SEM. Statistical analysis by ANOVA with repeated measures F (2, 26) = 3.906, P = 0.033. *p < 0.05 compared with untreated mice, #p < 0.05 compared with naked HTT siRNA treated mice. R6/2 mice: n (Untreated) = 12, n (HTT siRNA) = 6, n (CD·HTTsiRNA) = 11. Wild-type mice n = 24.

No significant improvements in grip strength, locomotor activity, and clasping behavior were observed in the CD·HTTsiRNA treated group (Supporting Information Figure S5a,b,c). Moreover, no significant changes were

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observed in bodyweight profiles (Supporting Information Figure S5d).

DISCUSSION

Progress in the development of RNAi-based therapies for neurodegenerative diseases has been hindered by the lack of an effective and nontoxic delivery vector. Here we report, what is to our knowledge, the first use of a modified amphiphilic cationic β-CD vector for siRNA delivery to the brain and to multiple *in vitro* and *in vivo* models of HD. The synthesis and physicochemical characterization of this modified β-CD has been previously described by our group.[28] These positively charged modified β-CDs are thought to interact with negatively charged siRNAs by electrostatic interactions, as found for other cationic delivery systems.[2] In the current study, this self-assembling nanoparticle system was able to successfully bind and complex HTT targeted siRNAs forming particles in the nanosize range and with a positive net charge. Small particle size and positive surface charge are important physicochemical characteristics to aid cellular uptake by facilitating interactions with the negatively charged cellular membrane.[37,38] Furthermore, ensuring stability in physiological fluids such as CSF and at body temperature is crucial to enable delivery to the target site.[39−41] Our data shows that CD·siRNA complexes were found to be stable in aCSF and 37 °C up to 6 h, therefore assuring adequate protection of siRNA from degradation.

CD-based vectors have recently been considered as an attractive gene delivery vector due to their improved toxicity profiles when compared to other cationic lipid- or polymer-based vectors.[25] In comparison, cationic lipid-based gene transfer reagents induce rapid activation of innate immune response after local and systemic administrations and are shown to have an elevated risk of cellular toxicity.[42−44] Our *in vitro* data support the concept that CDs are an appropriate choice as a delivery vector, with limited toxicity shown here in both neuronal and human HD *in vitro* models after treatment with CD·siRNA complexes.

Gene and RNAi transfer into neurons is an extremely challenging task, most likely due to their post-mitotic nature or specific characteristics of their cellular membranes.[3] In the present study, CD·siRNA nanoparticles were able to transfect a rat striatal cell line (ST14A-HTT120Q) that stably expresses a fragment of the human mutant HTT gene and evokes specific silencing effects on the expression of the HTT gene and consequently reduces the expression of this protein. It is important to note that the toxic effects of the mutant HTT mainly affect the neurons of the striatum in the brain, and therefore, these findings are of great relevance. In comparison, a commercially available transfection reagent (Lipofectamine 2000) was also able to transfect and evoke silencing effects in ST14A-HTT120Q cells to a similar extent of CD·siRNA nanoparticles. However, the use of this cationic lipid reagent resulted in greater cytotoxic effects in this neuronal cell line. Previous work in our group has shown similar toxic effects of Lipofectamine 2000 in an immortalized hypothalamic cell line and also in primary hippocampal cultures.[30] Additionally, CD·siRNA nanoparticles were also able to transfect and evoke HTT gene expression knockdown in human fibroblasts naturally harboring the mutant HTT gene. Although, both mutant and wild type human HTT alleles were silenced in this HD fibroblast *in vitro* model, previous studies have demonstrated that allele-specific HTT gene expression knockdown is feasible.[45,46] Intriguingly, our *in vitro* studies in the rat neuronal cell line have shown that HTT gene silencing effects occur independently of particle size, whereas smaller CD·siRNA complexes seemed to be more efficacious in human HD fibroblasts. The underlying cause for these differential effects between both *in vitro* models still remains unknown. Although sedimentation of larger particles on top of the cells may facilitate interaction with cellular membranes and uptake *in vitro*, *in vivo* sedimentation is not relevant and smaller particles have been generally associated with greater cellular uptake and knockdown efficiencies (as reviewed by Guo et al., 2010).[47]

Importantly, our data also showed that CD·siRNAs complexes were able to reduce HTT gene expression in the R6/2 mouse model of HD by ∼85% after only 4 h and that these effects sustained silencing up to at least 7 days postinjection. Although HTT gene silencing effects have been previously observed using other delivery methods, to our knowledge, no other delivery vector has been able to achieve such an immediate and strong knockdown of HTT gene expression *in vivo* after local injection into the brain.[13,33,48] In addition, little to no overt gross toxicity has been observed after direct brain injections using CD·siRNA nanoparticles (unpublished results). On the other hand, the unfavorable toxicity profiles of viral and lipid-based delivery vectors may have precluded repeated dosing regimens which were not used in previous studies.[13,33,48] Thus, in contrast with other gene delivery vectors, the low toxicity profiles of CDs enabled multiple dosing in the present study and further advocate the promise of this technology. Nevertheless, further investigation of the effects of multiple dosing of CD·siRNA nanoparticles in the brain is required to ensure its safety for human therapy.

To investigate if such changes could translate into any behavioral effects and to determine the impact of sustained treatment, we repeatedly injected CD·siRNA nanoparticles into the striatum of the R6/2 mouse model. Results showed that sustained CD-based HTT gene expression knockdown in such a localized structure in the brain was able to alleviate balance and motor coordination deficits in this mouse model. Interestingly, when injections of CD·HTTsiRNA complexes were ceased, a relatively rapid deterioration of rotarod deficits was observed. Moreover, despite the significant level of knockdown of the HTT mRNA levels achieved in the brain and the partial improvements in the rotarod task, CD·siRNA nanoparticles failed to improve spontaneous locomotor activity, grip strength, and clasping behavior in this animal model. Differential effects on improvements of specific motor behaviors have also been observed in previous studies using both nonviral and viral-based RNAi delivery approaches to study HD *in vivo*.[33,36] In fact, it is worth noting that the most widely used preclinical model of HD, the R6/2 mouse model, is an early onset and more severe model of HD, and therefore, the observed benefits of the delivery vector might be underestimated. Thus, future studies should also be carried out in other rodent and primate models of HD which have a more delayed progression of the disease. In addition, caution is needed when silencing HTT gene expression exclusively in the striatum, as the behavioral phenotype in HD is likely to be due to dysfunctions in other extra-striatal brain structures.[1,49] Indeed, we speculate that accumulation of toxic N-terminal HTT fragments in other structures of the R6/2 mouse brain, such as the cortex and cerebellum, might account to the observation of such modest behavioral improvements. Moreover, the initial delay to observe therapeutic improvements in the rotarod task and the relatively fast decline when treatment was ceased might also be related to accumulation of the mutant HTT in untargeted regions of the brain. Therefore, it is crucial that future studies assess the effects of a widespread suppression of the mutant HTT gene throughout the brain.

The progression of RNAi-based therapies to the clinic is highly dependent on the efficacy and safety of the delivery vector.[50]

Here we have shown that modified CDs significantly increase the intracellular delivery of siRNAs, leading to dramatic reduction of HTT mRNA levels in neuronal and human *in vitro* models but also in an *in vivo* model of HD. Furthermore, modified β-CDs have exhibited favorable toxicity profiles in our *in vitro* models. Other *in vivo* studies in nonhuman primates have shown that multiple dosing with CD-polymer based nanoparticles was well tolerated and did not elicit major immune responses.[51] On this basis, the U.S. Food and Drug Administration has recently approved a clinical trial using CD-polymer based nanoparticles for RNAi delivery for cancer treatment.[32] In conclusion, although there has been a renaissance in the applicability of neurosurgical approaches to treat complex brain disorders, systemic RNAi delivery approaches might be more attractive when translating this potential therapeutic strategy to the clinic.[50,52] Here, we show that CDs have great potential in facilitating specific gene silencing effects once they are targeted to the site of greatest importance to disease pathology. However, it is important to note that these modified β-CDs are very versatile molecules and further pharmaceutical functionalization is feasible which may enable targeting across the blood brain barrier in the future. Finally, the potential application of these modified β-CDs as siRNA carriers for CNS delivery is not restricted to HD but applicable to other neurodegenerative diseases, such as Alzheimer's, Parkinson, and ALS.

■ ASSOCIATED CONTENT

Supporting Information

Data regarding the stability of CD·siRNA nanoparticles in aCSF (Figure S1); quantification of cellular uptake of fluorescent CD·siRNA nanoparticles by FACS (Figure S2); formulation of CD·siRNA complexes in physiological buffers suitable for *in vivo* brain delivery (Figure S3); spread of HTT gene expression knockdown in the brain after single injection of CD·siRNA nanoparticles into the striatum (Figure S4); effects of localized HTT gene expression knockdown on other motor behavior deficits of R6/2 mice (Figure S5); and further details regarding the materials and methods used in the Experimental Section. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Zuccato, C.; Valenza, M.; Cattaneo, E. Molecular Mechanisms and Potential Therapeutical Targets in Huntington's Disease. *Physiol. Rev.* **2010**, *90* (3), 905−981.

(2) Guo, J.; Fisher, K. A.; Darcy, R.; Cryan, J. F.; O'Driscoll, C. Therapeutic targeting in the silent era: advances in non-viral siRNA delivery. *Mol. BioSyst.* **2010**, *6* (7), 1143−1161.

(3) Krichevsky, A. M.; Kosik, K. S. RNAi functions in cultured mammalian neurons. *Proc. Natl. Acad. Sci.* **2002**, *99* (18), 11926−11929.

(4) Boudreau, R. L.; Davidson, B. L. RNAi therapeutics for CNS disorders. *Brain Res.* **2010**, *1338*, 112−121.

(5) Grondin, R.; Kaytor, M. D.; Ai, Y.; Nelson, P. T.; Thakker, D. R.; Heisel, J.; Weatherspoon, M. R.; Blum, J. L.; Burright, E. N.; Zhang, Z.; Kaemmerer, W. F. Six-month partial suppression of Huntingtin is well tolerated in the adult rhesus striatum. *Brain* **2012**, *135* (4), 1197−209.

(6) Sah, D. W. Y. Therapeutic potential of RNA interference for neurological disorders. *Life Sci.* **2006**, *79* (19), 1773−1780.

(7) Snove, O.; Rossi, J. J. Toxicity in mice expressing short hairpin RNAs gives new insight into RNAi. *Genome Biol.* **2006**, *7* (8), 231.1−231.5.

(8) Nayak, S.; Herzog, R. W. Progress and prospects: immune responses to viral vectors. *Gene Ther.* **2010**, *17* (2), 294−294.

(9) Stiles, D. K.; Zhang, Z.; Ge, P.; Nelson, B.; Grondin, R.; Ai, Y.; Hardy, P.; Nelson, P. T.; Guzaev, A. P.; Butt, M. T.; Charisse, K.; Kosovrasti, V.; Tchangov, L.; Meys, M.; Maier, M.; Nechev, L.; Manoharan, M.; Kaemmerer, W. F.; Gwost, D.; Stewart, G. R.; Gash, D. M.; Sah, D. W. Y. Widespread suppression of huntingtin with convection-enhanced delivery of siRNA. *Exp. Neurol.* **2012**, *233* (1), 463−471.

(10) Thakker, D. R.; Natt, F.; Husken, D.; Maier, R.; Muller, M.; Van Der Putten, H.; Hoyer, D.; Cryan, J. F. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101* (49), 17270−17275.

(11) Thakker, D. R.; Natt, F.; Husken, D.; Van Der Putten, H.; Maier, R.; Hoyer, D.; Cryan, J. F. siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol. Psychiatry* **2005**, *10* (8), 782−789.

(12) Davidson, T. J.; Harel, S.; Arboleda, V. A.; Prunell, G. F.; Shelanski, M. L.; Greene, L. A.; Troy, C. M. Highly efficient small interfering RNA delivery to primary mammalian neurons induces MicroRNA-like effects before mRNA degradation. *J. Neurosci.* **2004**, *24* (45), 10040−10046.

(13) DiFiglia, M.; Sena-Esteves, M.; Chase, K.; Sapp, E.; Pfister, E.; Sass, M.; Yoder, J.; Reeves, P.; Pandey, R. K.; Rajeev, K. G. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proc. Natl. Acad. Sci.* **2007**, *104* (43), 17204−17209.

(14) Chen, Q.; Butler, D.; Querbes, W.; Pandey, R. K.; Ge, P.; Maier, M. A.; Zhang, L.; Rajeev, K. G.; Nechev, L.; Kotelianski, V. Lipophilic siRNAs mediate efficient gene silencing in oligodendrocytes with direct CNS delivery. *J. Controlled Release* **2010**, *144* (2), 227−232.

(15) Nakajima, H.; Kubo, T.; Semi, Y.; Itakura, M.; Kuwamura, M.; Izawa, T.; Azuma, Y.-T.; Takeuchi, T. A rapid, targeted, neuron-selective, in vivo knockdown following a single intracerebroventricular injection of a novel chemically modified siRNA in the adult rat brain. *J. Biotechnol.* **2012**, *157* (2), 326−333.

(16) Wang, H.; Ghosh, A.; Baigude, H.; Yang, C.; Qiu, L.; Xia, X.; Zhou, H.; Rana, T. M.; Xu, Z. Therapeutic gene silencing delivered by a chemically modified small interfering RNA against mutant SOD1 slows amyotrophic lateral sclerosis progression. *J. Biol. Chem.* **2008**, *283* (23), 15845−15852.

(17) Watts, J. K.; Deleavey, G. F.; Damha, M. J. Chemically modified siRNA: tools and applications. *Drug Discovery Today* **2008**, *13* (19), 842−855.

(18) Salahpour, A.; Medvedev, I. O.; Beaulieu, J. M.; Gainetdinov, R. R.; Caron, M. G. Local knockdown of genes in the brain using small interfering RNA: a phenotypic comparison with knockout animals. *Biol. Psychiatry* **2007**, *61* (1), 65−69.

(19) Cardoso, A. L. C.; Simoes, S.; de Almeida, L. P.; Plesnila, N.; Pedroso de Lima, M. C.; Wagner, E.; Culmsee, C. Tf-lipoplexes for neuronal siRNA delivery: a promising system to mediate gene silencing in the CNS. *J. Controlled Release* **2008**, *132* (2), 113−123.

(20) Tan, P. H.; Yang, L. C.; Shih, H. C.; Lan, K. C.; Cheng, J. T. Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Therapy* **2004**, *12* (1), 59−66.
(21) Kim, I.-D.; Lim, C.-M.; Kim, J.-B.; Nam, H. Y.; Nam, K.; Kim, S.-W.; Park, J.-S.; Lee, J.-K. Neuroprotection by biodegradable PAMAM ester (e-PAM-R)-mediated HMGB1 siRNA delivery in primary cortical cultures and in the postischemic brain. *J. Controlled Release* **2009**, *142* (3), 422−430.
(22) Ifediba, M. A.; Medarova, Z.; Ng, S.; Yang, J.; Moore, A. siRNA delivery to CNS cells using a membrane translocation peptide. *Bioconjugate Chem.* **2010**, *21* (5), 803−806.
(23) Bonoiu, A. C.; Bergey, E. J.; Ding, H.; Hu, R.; Kumar, R.; Yong, K. T.; Prasad, P. N.; Mahajan, S.; Picchione, K. E.; Bhattacharjee, A.; Ignatowski, T. Gold nanorod-siRNA induces efficient in vivo gene silencing in the rat hippocampus. *Nanomedicine* **2011**, *6* (4), 617−630.
(24) Wong, Y.; Markham, K.; Xu, Z. P.; Chen, M.; Lu, G. Q.; Bartlett, P. F.; Cooper, H. M. Efficient delivery of siRNA to cortical neurons using layered double hydroxide nanoparticles. *Biomaterials* **2010**, *31* (33), 8770−8779.
(25) Chaturvedi, K.; Ganguly, K.; Kulkarni, A. R.; Kulkarni, V. H.; Nadagouda, M. N.; Rudzinski, W. E.; Aminabhavi, T. M. Cyclodextrin-based siRNA delivery nanocarriers: a state-of-the-art review. *Expert Opin. Drug Delivery* **2011**, *8* (11), 1455−1468.
(26) McMahon, A.; O'Neill, M. J.; Gomez, E.; Donohue, R.; Forde, D.; Darcy, R.; O'Driscoll, C. M. Targeted gene delivery to hepatocytes with galactosylated amphiphilic cyclodextrins. *J. Pharm. Pharmacol.* **2012**, *54*, 1063−1073.
(27) O'Neill, M. J.; Guo, J.; Byrne, C.; Darcy, R.; O'Driscoll, C. M. Mechanistic studies on the uptake and intracellular trafficking of novel cyclodextrin transfection complexes by intestinal epithelial cells. *Int. J. Pharm.* **2011**, *413* (1−2), 174−183.
(28) O'Mahony, A. M.; Ogier, J.; Desgranges, S.; Cryan, J. F.; Darcy, R.; O'Driscoll, C. M. A click chemistry route to 2-functionalised PEGylated and cationic β-cyclodextrins: co-formulation opportunities for siRNA delivery. *Org. Biomol. Chem.* **2012**, *10* (25), 4954−4960.
(29) Guo, J.; Ogier, J. R.; Desgranges, S.; Darcy, R.; O'Driscoll, C. Anisamide-targeted cyclodextrin nanoparticles for siRNA delivery to prostate tumours in mice. *Biomaterials* **2012**, *33* (31), 7775−7784.
(30) O'Mahony, A. M.; Godinho, B. M. D. C.; Ogier, J.; Devocelle, M.; Darcy, R.; Cryan, J. F.; O'Driscoll, C. M. Click-Modified Cyclodextrins as Nonviral Vectors for Neuronal siRNA Delivery. *ACS Chem. Neurosci.* **2012**, DOI: 10.1021/cn3000372.
(31) Davis, M. E. The First Targeted Delivery of siRNA in Humans via a Self-Assembling, Cyclodextrin Polymer-Based Nanoparticle: From Concept to Clinic. *Mol. Pharmaceutics* **2009**, *6* (3), 659−668.
(32) Davis, M. E.; Zuckerman, J. E.; Choi, C. H. J.; Seligson, D.; Tolcher, A.; Alabi, C. A.; Yen, Y.; Heidel, J. D.; Ribas, A. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. *Nature* **2010**, *464* (7291), 1067−1070.
(33) Wang, Y. L.; Liu, W.; Wada, E.; Murata, M.; Wada, K.; Kanazawa, I. Clinico-pathological rescue of a model mouse of Huntington's disease by siRNA. *Neurosci. Res.* **2005**, *53* (3), 241−249.
(34) Dragatsis, I.; Goldowitz, D.; Del Mar, N.; Deng, Y. P.; Meade, C. A.; Liu, L.; Sun, Z.; Dietrich, P.; Yue, J.; Reiner, A. CAG repeat lengths $\geq$ 335 attenuate the phenotype in the R6/2 Huntington's disease transgenic mouse. *Neurobiol. Disease* **2009**, *33* (3), 315−330.
(35) Menalled, L. B.; Patry, M.; Ragland, N.; Lowden, P. A. S.; Goodman, J.; Minnich, J.; Zahasky, B.; Park, L.; Leeds, J.; Howland, D. Comprehensive behavioral testing in the R6/2 mouse model of Huntington's disease shows no benefit from CoQ10 or minocycline. *PLoS ONE* **2010**, *5* (3), e9793.
(36) Rodriguez-Lebron, E.; Denovan-Wright, E. M.; Nash, K.; Lewin, A. S.; Mandel, R. J. Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol. Ther.* **2005**, *12* (4), 618−633.
(37) Gratton, S. E. A.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. The effect of particle design on cellular internalization pathways. *Proc. Natl. Acad. Sci.* **2008**, *105* (33), 11613−11618.
(38) Hillaireau, H.; Couvreur, P. Nanocarriers' entry into the cell: relevance to drug delivery. *Cell. Mol. Life Sci.* **2009**, *66* (17), 2873−2896.
(39) Agrawal, A.; Min, D.-H.; Singh, N.; Zhu, H.; Birjiniuk, A.; von Maltzahn, G.; Harris, T. J.; Xing, D.; Woolfenden, S. D.; Sharp, P. A.; Charest, A.; Bhatia, S. Functional Delivery of siRNA in Mice Using Dendriworms. *ACS Nano* **2009**, *3* (9), 2495−2504.
(40) Lu, J.; Owen, S. C.; Shoichet, M. S. Stability of self-assembled polymeric micelles in serum. *Macromolecules* **2011**, *44* (15), 6002−6008.
(41) Lu, J. J.; Langer, R.; Chen, J. A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery. *Mol. Pharmaceutics* **2009**, *6* (3), 763−771.
(42) Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Yan, J. Toxicity of cationic lipids and cationic polymers in gene delivery. *J. Controlled Release* **2006**, *114* (1), 100−109.
(43) Souto, E. B.; Martins-Lopes, P.; Lopes, C. M.; Gaivao, I.; Silva, A. M.; Guedes-Pinto, H. A note on regulatory concerns and toxicity assessment in lipid-based delivery systems (LDS). *J. Biomed. Nanotechnol.* **2009**, *5* (4), 317−322.
(44) Tonges, L.; Lingor, P.; Egle, R.; Dietz, G. P. H.; Fahr, A.; Bahr, M. Stearylated octaarginine and artificial virus-like particles for transfection of siRNA into primary rat neurons. *RNA* **2006**, *12* (7), 1431−1438.
(45) Lombardi, M. S.; Jaspers, L.; Spronkmans, C.; Gellera, C.; Taroni, F.; Di Maria, E.; Donato, S. D.; Kaemmerer, W. F. A majority of Huntington's disease patients may be treatable by individualized allele-specific RNA interference. *Exp. Neurol.* **2009**, *217* (2), 312−319.
(46) Fiszer, A.; Mykowska, A.; Krzyzosiak, W. J. Inhibition of mutant huntingtin expression by RNA duplex targeting expanded CAG repeats. *Nucleic Acids Res.* **2011**, *39* (13), 5578−5585.
(47) Guo, P.; Coban, O.; Snead, N. M.; Trebley, J.; Hoeprich, S.; Guo, S.; Shu, Y. Engineering RNA for targeted siRNA delivery and medical application. *Adv. Drug Delivery Rev.* **2010**, *62* (6), 650−666.
(48) Harper, S. Q.; Staber, P. D.; He, X.; Eliason, S. L.; Martins, I. H.; Mao, Q.; Yang, L.; Kotin, R. M.; Paulson, H. L.; Davidson, B. L. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (16), 5820−5825.
(49) Li, J. Y.; Popovic, N.; Brundin, P. The Use of the R6 Transgenic Mouse Models of Huntington's Disease in Attempts to Develop Novel Therapeutic Strategies. *NeuroRx* **2005**, *2* (3), 447−464.
(50) Gao, K.; Huang, L. Nonviral methods for siRNA delivery. *Mol. Pharmaceutics* **2009**, *6* (3), 651−658.
(51) Heidel, J. D.; Yu, Z.; Liu, J. Y. C.; Rele, S. M.; Liang, Y.; Zeidan, R. K.; Kornbrust, D. J.; Davis, M. E. Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc. Natl. Acad. Sci.* **2007**, *104* (14), 5715−5721.
(52) Chiocca, E. A. Gene therapy: a primer for neurosurgeons. *Neurosurgery* **2003**, *53* (2), 364.