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Modified Cyclodextrins as Novel Non-Viral Vectors for Neuronal siRNA Delivery: Focus on Huntington's Disease

Thesis presented by

Bruno M.D.C. Godinho

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Doctor of Philosophy

under the supervision of

Prof. John F. Cryan
Prof. Caitriona O'Driscoll

Head of School: Prof. Stephen Byrne

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“Não sou nada (...)
À parte isso,
tenho em mim todos os sonhos do mundo."
(Fernando Pessoa)

"I am nothing (...)
Apart from that,
I have in me all the dreams in the world.”
(Fernando Pessoa)

À minha família.

To my family.

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Declaration

This thesis has not been previously submitted, in part or in whole, to this or any other university for any degree and is, unless otherwise stated, the original work of the author.

Author Contribution

All the work described herein was performed independently by the author, with the following exceptions:

Chapter II

Dr. Susan Grenham assisted with the optimisation of genotyping protocols.

Chapter III

Dr. Susan Grenham assisted with the design of qPCR probes. Ms. Sheila O'Loughlin and Mr. Dinesh Rasiah assisted with the *in vitro* work in HD fibroblasts and qPCR analyses.

Chapter IV

Dr. Caroll J Beltran assisted with western blots. Dr. Cristina Torres-Fuentes assisted with HCA experiments. Mr. David J McCarthy assisted with *in vitro* work in U87 cells and qPCR analyses. Ms. Aoife Quinlan assisted with stereotaxic brain surgeries.

Chapter V

Ms. Aoife Quinlan assisted with pharmacokinetic study.

Signed, 

Bruno MDC Godinho

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List of publications

Peer-reviewed articles

1. **Godinho BMDC**, McCarthy DJ, Torres-Fuentes C, Beltrán CJ, McCarthy J, Quinlan A, Ogier JR, Darcy R., O'Driscoll CM., & Cryan JF. (2014). Differential nanotoxicological and neuroinflammatory liabilities of non-viral vectors for RNA interference in the central nervous system. Biomaterials, 35(1), 489-499.
2. **Godinho BMDC**, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. (2013). Self-assembling modified β-Cyclodextrin nanoparticles as neuronal siRNA delivery vectors: Focus on Huntington's Disease. Molecular Pharmaceutics, 10 (2), pp 640–649.
3. O'Mahony AM, **Godinho BMDC**, Cryan JF, & O'Driscoll CM. (2013). Non-viral nanosystems for gene and siRNA delivery to the central nervous system: formulating the solution. J. Pharm. Sci., 102(10), 3469-3484.
4. O'Mahony AM, O'Neill MJ, **Godinho BMDC**, Darcy R, Cryan JF, O'Driscoll CM. (2013). Cyclodextrins for non-viral gene and siRNA delivery. Pharmaceutical Nanotechnology, 1(1) pp. 6-14.
5. O'Mahony AM, **Godinho BMDC**, Ogier J, Devocelle M, Darcy R, Cryan JF, O'Driscoll CM. (2012). Click-modified cyclodextrins as non-viral vectors for neuronal siRNA delivery. ACS Chemical Neuroscience, 3 (10), pp 744–752.

Oral communications

6. **Godinho BMDC**, McCarthy DJ, Quinlan A, Beltrán C, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. A nanotoxicological appraisal of non-viral vectros for RNAi in the CNS. XIII Meeting of the Portuguese Neuroscience Society, Luso, Portugal, June, 2013.
7. **Godinho BMDC**, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Nanoparticles as disease-modifying mediators for brain therapy: focus on Huntigton's Disease. European College of Neuropsycopharmacology workshop 2013, Nice, France, March 2013.

Poster communications

8. **Godinho BMDC**, McCarthy DJ, Beltrán C, Quinlan A, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Modified cyclodextrins as biocompatible siRNA vectors for Huntington's Disease: a nanotoxicological and neuroinflammatory comparative study. Neuroscience Ireland 2013, Cork, Ireland, September 2013.
9. McCarthy DJ, **Godinho BMDC**, Gooding M, Ogier JR, Darcy R, Cryan JF, O'Driscoll CM. Gene silencing in the blood brain barrier using modified beta cyclodextrins as non-viral vectors for siRNA. Neuroscience Ireland 2013, Cork, Ireland, September 2013.
10. **Godinho BMDC**, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Nanoparticles for treating Huntington's Disease: Cyclodextrin-based siRNA delivery to the striatum improves motor deficits on the rotarod of the R6/2 mouse. 8th Forum of the Federation of Neuroscience Societies 2012, Barcelona, Spain, July 2012.
11. **Godinho BMDC**, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Silencing toxic gene expression in *in vitro* and *in vivo* models of Huntington's Disease using a cyclodextrin-based siRNA delivery system. Society for Neuroscience Annual Meeting 2011, Washington DC, November 2011.
12. **Godinho BMDC**, Tramullas M, Ogier JR, Grenham S, Darcy R, O'Driscoll CM, Cryan JF. Cyclodextrins as an effective siRNA delivery system in multiple models of Huntington's Disease. Annual Meeting of the American Association of Pharmaceutical Scientists 2011, Washington DC, United States of America, October 2011.
13. **Godinho BMDC**, O'Loughlin S, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Modified cyclodextrins as stable and effective siRNA delivery systems in cell-based and in animal models of Huntington's Disease. Neuroscience Ireland 2011, National University of Ireland, Maynooth, Republic of Ireland, September 2011.
14. **Godinho BMDC**, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Cyclodextrin-based siRNA delivery in an *in vitro* model of Huntington's Disease. XII Meeting of the Portuguese Neuroscience society, Universidade de Lisboa – Faculdade de Medicina, Lisboa, Portugal, May 2011.
15. **Godinho BMDC**, O'Mahony AM, Ogier JR, Darcy R, O'Driscoll CM, Cryan JF. Cylcodextrins as nonviral-vectors for siRNA delivery to the Central Nervous System. Neuroscience Ireland 2010, University College Dublin, Dublin, Republic of Ireland. September 2010.

Abstract

Huntington's Disease (HD) is a rare autosomal dominant neurodegenerative disease caused by the expression of a mutant Huntingtin (muHTT) protein, leading to neuronal dysfunction and death in specific regions of the central nervous system (CNS). Thus, preventing the expression of muHTT is a key research avenue for developing novel therapies for HD. In this regard, harnessing the specificity of the RNAi pathway using synthetic short interfering RNA (siRNA) to silence the expression of the muHTT gene holds great promise. However, the biggest caveat in the RNA interference (RNAi) approach is delivering siRNAs to neurons, which are notoriously difficult to transfect. Indeed, despite the advances in the design and development of non-viral vectors, efficient and safe siRNA delivery systems for CNS applications are still lacking. Thus, the aim of this thesis was to investigate the utility of modified amphiphilic β-cyclodextrins (CDs), olygosacharride-based molecules, as non-viral vectors for siRNA delivery for HD.

Our studies demonstrated that modified CDs were able to bind and complex siRNAs forming nanoparticles which were stable in artificial cerebrospinal fluid. Modified CDs efficiently delivered siRNAs to two different *in vitro* models of HD, a rat striatal cell line (ST14A-HTT120Q) and human HD fibroblasts, significantly reducing the expression of the HTT gene and only causing minimal cytotoxicity. Moreover, direct single injections of CD.siRNA nanoparticles into the R6/2 mouse brain resulted in significant HTT gene expression knockdown, which was found to be sustained for 7 days. In addition, repeated brain injections of CD.siRNA complexes resulted in selective alleviation of rotarod motor deficits in this mouse model of HD.

To further expand on previous preliminary toxicity data, a comparative study was carried out in multiple brain-derived cell lines (ST14-HTT120Q striatal cells, BV2 microglial cells and U87 astroglioma cells) using commercially available vectors and CD.siRNA nanoparticles. Differential cytotoxic effects were observed among commercially available cationic vectors,

whereas CD.siRNA nanoparticles only induced limited disruptions of cellular membrane integrity and of mitochondrial metabolic activity. Additionally, and in contrast with commercially available vectors, CD.siRNA nanoparticles only induced limited neuroinflammatory responses *in vitro*, in BV2 cells, and after single injections into the mouse brain. Although these data support CDs as an efficient and safe siRNA delivery vector suitable for intracerebral injections, further studies to investigate the effects of chronic dosing and escalating doses are warranted.

Progression towards developing CD-based formulations for systemic administration demands greater focus on increasing the stability of nanoparticles. Thus, to this end we have described a PEGylation method for post-modification of pre-formed CD.siRNA nanoparticles using amphiphilic PEGylated CDs. Resulting PEGylated CD.siRNA nanoparticles showed increased stability in physiological salt-conditions and, to some extent, reduced protein-induced aggregation. We have also identified polyethylene glycol length (PEG) as a major determinant for stability of CD.siRNA nanoparticles and now further studies are required with longer PEGs to improve the pharmacokinetic profile of siRNA.

Taken together, the work outlined in this thesis identifies modified CDs as efficient non-viral vectors for siRNA delivery to the brain, with minimal toxicity. Furthermore, we have established a strategy for modification of pre-formed CD.siRNA nanoparticles to improve the prospects of systemic delivery. These preliminary results indicate the potential of these nanosystems to treat a variety of CNS disorders, including other neuropsychiatric disorders and brain cancers, and indicate that further development towards the clinic is warranted.

List of abbreviations

3-NP	3-nitropropionic acid
A	Adenosine
AAT	α-1 antitrypsin
AAV	Adeno-associated viral
Ab	Antibodies
Ach	Acetylcholine
AchR	Acetylcholine receptor
aCSF	Artificial cerebrospinal fluid
AD	Alzheimer's disease
Ad	Adamantane
ALAT	Alanine aminotrasnferase
ALP	Alkaline phosphatase
ALS	Amyotrophic lateral sclerosis
APP	Amyloid precursor protein
ANG	Angiopep-2
ANOVA	One-way Analysis of Variance
ASAT	Aspartate transaminase
ASO	Antisense short oligonucleotides
AST	Aspartate transaminase
ATP	Adenosine triphosphate
AUC	Area under the curve
BAC	Bacterial artificial chromosome
BACE-1	β secretase 1
BBB	Blood Brain Barrier
BCA	Bicinchoninic acid
BCEC	Brain capillary endothelial cells
BCSFB	Blood-cerebrospinal fluid barrier
BDNF	Brain derived neurotrophic factor
bEND3	Brain endothelial cell-model
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
cAMP	Cyclic adenosine monophosphate
cc-siRNA-HTT	Cholesterol-conjugated HTT siRNA
CD	Cyclodextrin
cDNA	Complementary DNA
CDP	Cylcodextrin polymer
CED	Convection enhanced delivery
CHS	Chalcone synthase
CL	Clearance
CNS	Central Nervous System
COX-2	Cyclooxygenase 2
CPP	Cell penetrating peptide
CPu	Caudate Putamen
CRISPR	Clustered regulatory interspaced short palindromic repeat
DAMPS	Damage-associated molecular pattern molecules
DAPI	Diamidinophenylindole
DARPP-32	Dopamine and cAMP-responsive phophoprotein 32 kDa
DC-Chol	Dimethylaminoethanecarbamoyl cholesterol
DIW	Deionised water
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

dNTPs	Deoxy nucleotides
DOPE	Dioleoylphosphatidylethanolamine
DOSPA	Dioleoylsperminecarboxamidoethyldimethylpropanetrifluoroacetate
DOTAP	Dioleoyltrimethylammoniumpropane
DOTMA	Dioleyloxypropyltrimethylammonium
DOX	Doxycycline
Doxorub	Doxorubicin
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immune sorbent assay
ELS	Electrophoretic Light Scattering
EPA	Eicosapentaenoic acid
FACS	Fluorescent Activated Cell Sorting
FALS	Familial amyotrophic sclerosis
FAMsiRNA	Fluorescently labelled siRNA
FBS	Foetal bovine serum
FDA	Food and Drug Administration
G	Guanine
GABA	g-aminobutyric acid
GAPDH	Glyceraldehyde phosphate dehydrogenase
GDNF	Glial-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green fluorescent protein
GLT-1	Glial glutamate transporter 1
GT1b	Triasialoganglioside receptor
HA2	N-terminal hemagglutinin
HAP1	Huntingtin-associated protein 1
HCA	High Content Analysis
HD	Huntington's Disease
HDAC	Histone deacetylases
Hdh	Mouse homologue HTT gene
HEK	Human embryonic kidney
hESC	Human embryonic stem cells
hGDNF	Human glial cell line-derived neurotrophic factor
HIP1	Huntingtin-interacting protein 1
HIPPI	HIP1 interacts with HIP1 protein interactor
HIR	Human insulin receptor
HIV	Human Immunodeficiency Virus
HSV-1	Herpes simplex virus – 1
HTT	Huntingtin
HTTsiRNA	HTT target siRNA
i.c.v.	Intracerebroventricular
IFN	Interferon
IL	Interleukin
LDH	Lactate Dehydrogenase
iPSC	Induced-Pluripotent Stem cells
IP-9	Interferon gamma-induced protein 9
IP-10	Interferon gamma-induced protein 10
IT	Interesting transcript
i.v.	Intravenous injection
JEV	Japanese encephalitis virus
LDH	Lactate Dehydrogenase
Lf2000	Lipofectamine2000®
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein

LTF	Lactoferrin
LTFR	Lactoferrin receptor
LV	Lentiviral
mAb	Monoclonal antibody
MCP-1	Monocyte chemotactic protein-1
mGlu	Metabotropic glutamate receptors
MIG	Multicentric Intracerebral Grafting
miRISC	Micro RNA RISC
miRNA	Micro RNA
MMP	Mitochondrial membrane potential
MPS	Mononuclear phagocyte system
mRNA	Messenger RNA
MR	Mass ratio
MRI	Magnetic resonance imaging
MRIgFUS	Magnetic resonance imaging guided focused ultrasound
MSD®	Meso Scale Discovery®
MTT	Methyl thiazolyl tetrazolium
mu*Hdh*	Mutant homologous Huntingtin gene
muHTT	Mutant Huntingtin
Mw	Molecular weight
N	Amino
NID	Non-ionic detergent
NMDA	N-methyl-D-aspartic acid
NMDAR	N-methyl-D-aspartate receptor
NPC	Neuron progenitor cells
NRSF	Neuron-restrictive silencer factor
NSsiRNA	Non-silencing siRNA
NT	Neuortensin
Nt.	Nucleotide
NTS1	Neurotensin receptor
O-MWNTs	Oxidized multiwalled carbon nanotubes
ODNs	Oligodeoxynucleotides
OT	Ovarian transplanted
p75NTR	p75 Neurotrophin receptor
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
pDNA	Plasmid DNA
PDI	Polydispersity index
PEG	Poly(ethylene glycol)
PEI	Polyethylenimine
PEO	Poly(ethylene oxide)
PEO-PPO	Polyethylene oxide-polypropylene oxide.
PI	Propidium Iodide
PK	Pharmacokinetic
PKR	Protein kinase R
PLGA	Poly(lactic-*co*-glycolic) acid or Poly(lactide-*co*-glycolide)
PLL	Poly-L-lysine
PolyQ	Polyglutamine
ppENK	Preproenkephalin
PPO	poly(propylene oxide)
PrP^C	Prion protein cellular
PS	Phosphatidylserine
PTGS	Post transcriptional gene silencing
PVDF	Polyvinylidene difluoride
QA	Quinolinic acid

R9	Nona-arginine
RES	Reticuloendothelial system
REST	Repressor element-1 silencing transcription factor
RISC	RNA induced silencing complex
RITS	RNA-induced transcriptional silencing
RLC	RISC loading complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase III	Ribonuclease III
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RRM2	Ribonucleotide reductase subunit b2
RT	Room temperature
RT-qPCR	Real time quantitative PCR
RVG	Rabies virus glycoprotein
SCA	Spinocerebellar ataxia
SD	Standard deviation
SDS	Sodium Docecyl Sulfate
SEM	Standard Error of Mean
SF	Superfect®
shRNA	Short harpin RNA
SI	Supplementary Information
siRISC	siRNA activated RISC
siRNA	Short interfering RNA
SOD1	Superoxide dismutase 1
SNP	Single nucleotide polymorphism
Stat1	Signal transducers and activators of transcription 1
SV40	Virus 40
$t_{1/2}$	Half-life
$t_{1/2\beta}$	Elimination half-life
$t_{1/2\alpha}$	Distribution half-life
TAE	Tris-acetate-Ethylenediaminetetraacetic acid
TALEN	Transcription activator-like effector nuclease
Tat	Transcription-transactivating protein
TEM	Transmission electron microscope
Tf	Transferrin
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRBP	Transactivation response –RNA-binding protein
USA	United States of America
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
VMAT2	Vesicular monoamine transporter 2
WT	Wild-type
wtHTT	Wild-type Huntingtin
X-SCID	X-linked severe combined immune deficiency
XTT	Methoxynitrosulfophenyltetrazoliumcarboxanilide
YAC	Yeast artificial chromosome
ZFN	Zinc finger nucleases
ZFP	Zinc finger protein

Chapter I

Introduction

1.1 Huntington's Disease

Huntington's Disease (HD) is a rare neurodegenerative disease which affects ~5-10 in 100,000 people in European, Australasian and American populations (Morrison, 2010; Pringsheim et al., 2012). According to the HD association of Ireland, there are ~500 HD patients and ~2500 people are at risk of developing the disease in Ireland. In addition, HD mainly affects middle-aged individuals and leads to death 15-18 years after onset of symptoms (Bates et al., 2002; Wexler, 2004). The hallmarks of HD are involuntary choreiform movements, progressive motor and cognitive impairment, depressive-like behaviour and mood disorders (Bates et al., 2002; Myers, 2004; Novak et al., 2010).

The first accurate description of Huntington's chorea was made by George Huntington in 1872 in his classical paper "On Chorea" (Huntington, 2004). However, it is believed that HD was first mentioned by Paracelsus (1493-1541), who suggested that this movement disorder had origin in the central nervous system (CNS) (Zuccato et al., 2010). Despite all efforts, it was only in 1993 that The Collaborative Huntington's Research Group identified the mutation in the interesting transcript (IT)15 gene, now named Huntingtin (HTT) gene, as the cause for the disease (MacDonald et al., 1993).

Despite the great advances in the understanding of HD neuropathology, current pharmacotherapy is only able to provide relief for some of the symptoms and does not target the underlying cause of the disease (Ross et al., 2011). Therefore, the development of new therapeutic strategies to stop disease progression is crucial to improve the standard of care for HD patients.

1.2 Genetic mechanism of Huntington's Disease

We now know that HD is an autosomal dominant disease caused by an unstable and elongated repetitive deoxyribonucleic acid (DNA) element containing cytosine (C), adenine (A) and guanine (G) in exon 1 of the HTT gene (Chromosome 4, 4p16.3) (MacDonald et

al., 1993). Thus, HD is part of a family with 8 other neurodegenerative disorders that are caused by CAG triplet expansions which include a number of spinocerebellar ataxias (SCA), spinal bulbar muscular atrophy and dentatorubral-pallidoluysian atrophy (Shao et al., 2007). As the CAG codon codes for glutamine, these diseases are collectively called "polyglutamine" (polyQ) disorders (Shao & Diamond, 2007). In the HTT gene, the number of CAG repeats in normal individuals varies between 6-39, however 17-20 are the most commonly found (Imarisio et al., 2008; Novak & Tabrizi, 2010). Symptomatic HD patients usually present over 40 CAG repeats and complete dominance of the disease is now widely accepted to occur above this number of CAG repeats (Imarisio et al., 2008; Sathasivam et al., 1997). Patients with very high numbers of CAG repeats, 180 CAG repeats (Sathasivam et al., 1997) and 250 CAG reapeats (Nance et al., 1999), are rare but have been reported. Also, CAG expansions greater than 60 have been associated with the juvenile form of the disease and with severe disease progression (Andrew et al., 1993; Langbehn et al., 2004). Indeed, clinical studies have shown that the number of CAG repeats is inversely correlated with the age of onset of clinical symptoms (Andrew et al., 1993; Brinkman et al., 1997). However, it is important to remark that this correlation only accounts for approximately 50-70% of the variance in the age of onset of symptoms (Andrew et al., 1993; Brinkman et al., 1997; MacDonald et al., 1993; Zuccato et al., 2010). Thus, although it is clear that HD has an age-dependent penetrance, it is common to find variability in the age of onset and severity of symptoms among patients with the same CAG repeat length (Myers, 2004). Other environmental and genetic factors have also been suggested to influence the age of onset and disease progression (Andresen et al., 2007; Wexler, 2004).

HD is inherited in an autosomal dominant manner, meaning that a single copy of the mutant HTT (muHTT) gene is enough for disease manifestation (Myers, 2004). Thus, offspring of affected individuals have a 50% chance of expressing the disease regardless of their gender (Wexler, 2004). Furthermore, most HD patients are heterozygous individuals carrying one single copy of the mutant allele and very few patients have been found homozygous

(Squitieri et al., 2003). Although homozygosity has no apparent effect on the age of onset, it was recently found to clearly impact on severity of HD phenotype and disease progression (Squitieri et al., 2003). Nowadays, a precise and cost-effective predictive genetic test is available to identify individuals at risk before the onset of symptoms or as tool to confirm diagnosis based on pathological and symptom assessment (Levin et al., 2006). However, pre-symptomatic genetic testing for HD has raised many ethical issues mostly founded/based on the patient's right "not to know" and on the fact that no cure is yet available (Terrenoire, 1992). Despite this, in the future, if an effective disease-modifying therapy arises this test may be of particular interest to enable therapeutic interventions at early stages of the disease to block, or even reverse, disease progression.

1.3 Wild-type and mutant Huntingtin: What are their functions in the brain?

1.3.1 Wild-type Huntingtin

Wild-type HTT (wtHTT) is a 348-kDa protein ubiquitously expressed in a wide variety of tissues/organs, including liver, colon, pancreas and brain, with the highest levels being reported in neurons in the brain (Sharp et al., 1995; Strong et al., 1993). Although substantial efforts have been made to understand the cellular roles of wtHTT, its specific functions are still not completely understood. wtHTT is mostly found in the cytoplasm but also in small amounts in the nucleus and is suggested to be implicated in a wide variety of cellular processes (Kegel et al., 2002). Moreover, wtHTT is known to interact with over 200 other patterns and several roles in transcriptional regulation, endocytosis, endosomal motility, axonal transport and synaptic transmission have been described (Imarisio et al., 2008; Zuccato et al., 2010). Here we will briefly overview the most studied functions attributed to wtHTT.

HTT has a crucial role in ***embryonic development*** and ***CNS formation*** (Reiner et al., 2003). Complete inactivation of the mouse homologue HTT gene (*Hdh*) in knockout mice leads to embryonic death before formation of the nervous system (before gastrulation) (Duyao et al., 1995b; Zeitlin et al., 1995). This has been suggested to occur due to increased apoptosis in the embryonic ectoderm. In addition, other studies have demonstrated that mice with reduced levels of wtHTT expression (~50%) survive after birth but show profound changes in cortical and striatal structures as well as enlarged brain ventricles due to abnormal brain development (Auerbach et al., 2001). On the other hand, studies in Rhesus macaque have shown that 45% reduction of the HTT gene in the adult brain does not compromise motor movement nor induced neuronal degeneration (Grondin et al., 2012; McBride et al., 2011). Thus, this suggests that despite that HTT is required at specific stages of development and plays an important role in neurogenesis, partial supression in adulthood might be well tolerated up to several months (Grondin et al., 2012; McBride et al., 2011).

Other studies have suggested that wtHTT also has an ***anti-apoptotic*** and ***pro-survival function*** in the adult brain (Leavitt et al., 2001). Cattaneo and collaborators have shown that overexpression of wtHTT *in vitro* has a protective effect against toxic stimuli caused by starvation, mitochondrial toxins or toxic genes (Rigamonti et al., 2000; Rigamonti et al., 2001). Additionally, Hayden and colleagues have conducted *in vivo* studies and reported that overexpression of wtHTT in transgenic yeast-derived artificial chromosome (YAC)18 mice confers protection against excitotoxicity-triggered apoptosis (Leavitt et al., 2006). The anti-apoptotic effects mediated by wtHTT are believed to occur through several mechanisms including: direct interaction with caspase 3; inhibition of the processing of pro-caspase 9; and the sequestration of pro-apoptotic Huntingtin-interacting protein 1 (HIP1) (Gervais et al., 2002; Rigamonti et al., 2000; Rigamonti et al., 2001; Zhang et al., 2006). On the other hand, wtHTT may also act as a substrate for the Serine/threonine kinase Akt which in turn activates and enhance pro-survival pathways and gene expression (Rangone et al., 2004).

wtHTT is believed to be involved in ***transcriptional regulation*** interacting with multiple transcription factors and other intracellular partners. As an example, wtHTT increases transcription of pro-survival brain derived neurotrophic factor (BDNF) by sequestering Repressor element-1 silencing transcription factor (REST)/Neuron-restrictive silencer factor (NRSF) in the cytoplasm and inhibiting the formation of co-repressor complexes in the BDNF promoter exon II (Imarisio et al., 2008; Zuccato et al., 2003). Increased production of BDNF has been reported not only *in vitro* in brain-derived cells overexpressing wtHTT, but also *in vivo* in YAC18 mice where high striatal levels of BDNF were detected (Zuccato et al., 2001; Zuccato et al., 2003). Through interaction with REST/NRSF wtHTT promotes transcription of many other genes involved in neuronal development and normal neuronal functions, including genes coding for ion channels and neurotransmitter receptors (Cattaneo et al., 2005). Thus, wtHTT may have a role of facilitating gene transcription in the nervous system.

wtHTT is also thought to play an important role in ***axonal transport*** of vesicles and mitochondria along the microtubules. This may occur by direct interaction or via Huntingtin-associated protein 1 (HAP1) with dynein/dynactin molecular motor complex enabling anterograde and retrograde transport along microtubles. In fact, wtHTT has been reported to facilitate vesicular transport of BDNF from the cortex to the striatum perhaps working as a scaffolding protein (Gauthier et al., 2004). Finally, wtHTT interacts with various cytoskeletal and other proteins present at synaptic terminals involved in endo- and exocytosis, and has been suggested to play an active role in modulating ***synaptic transmission*** (Smith et al., 2005).

Thus, due to the potential involvement of wtHTT in many cellular processes, loss-of-function of wtHTT has been suggested to be implicated to some extent in the development of HD pathology (Dragatsis et al., 2000).

1.3.2 Mutant Huntingtin

An increasing body of evidence suggests that HD is mainly caused by a toxic gain-of-function of muHTT rather than only a loss-of-function of wtHTT (Shao & Diamond, 2007). In fact, muHTT has been found to disrupt multiple intracellular and intercellular processes directly causing toxicity or rendering specific neurons more susceptible to toxic stimulus (Ross & Tabrizi, 2011). Selected intracellular mechanisms of HD pathogenesis caused by muHTT are depicted on Figure 1.1.

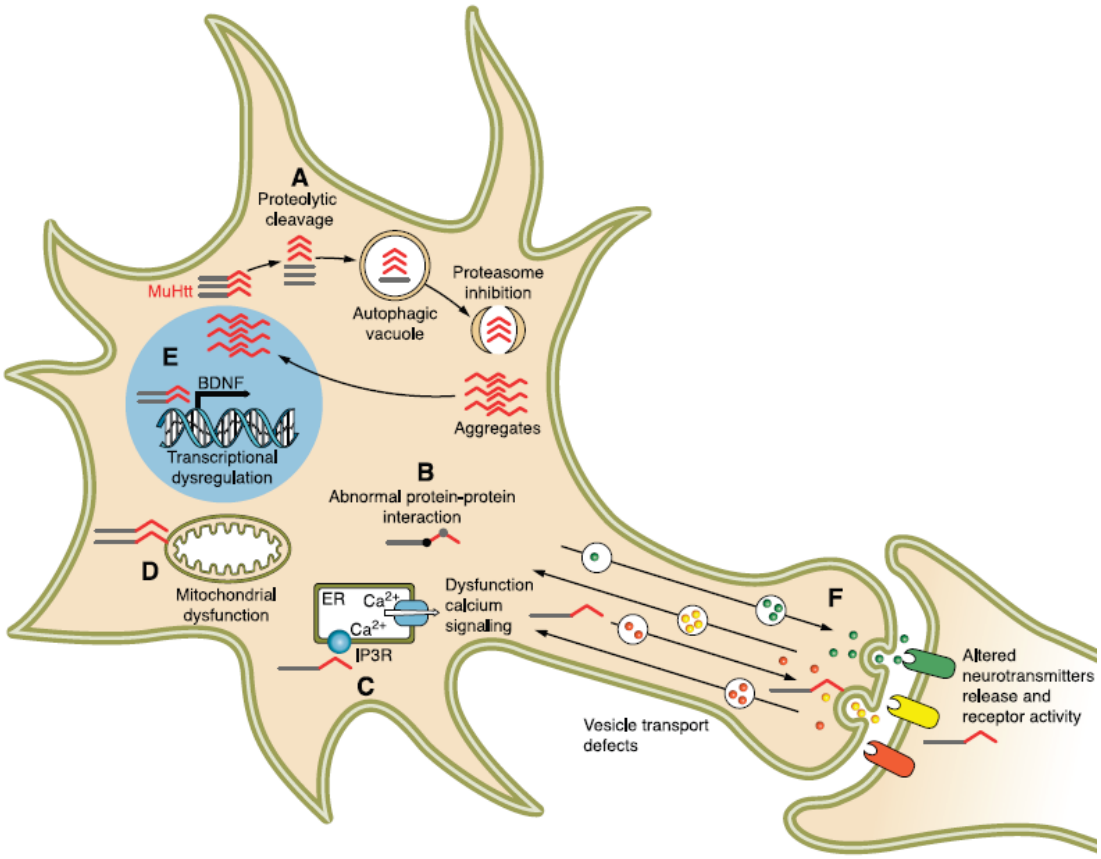


Figure 1.1. Selected intracellular mechanisms of pathogenesis induced by muHTT *(Zuccato et al., 2010). (A) Proteolytic cleavage of full length muHTT in the cytoplasm by proteases and subsequent targeting for proteasome degradation. Dysfunctional proteasome is not able to clear mutant protein and cytoplasmic aggregates are formed. (B-F) N-terminal muHTT interacts with multiple partners deregulating their normal function. (C) muHTT binds to inositol triphosphate receptors leading to calcium store release (D) muHTT induces mitochondrial dysfunction with a decrease in ATP and an increase of ROS production (E) muHTT shuttles into the nucleus forming inclusions and also impairing gene transcription (F) muHTT disrupts synaptic function in both pre- and post-synaptic terminals, by affecting vesicle transport, exocytosis and post-synaptic receptor activity.* ***Abbreviations****: ATP, adenosine triphosphate; BDNF, Brain derived neurotrophic factor; muHTT, Mutant HTT; ROS, reactive oxygen species.*

The anomalous CAG triplet expansion in muHTT gene leads to the formation of an abnormal protein which has an elongated polyQ tract close to its amino (N)-terminus (MacDonald et al., 1993). Caspases and/or cysteine aspartic proteases cleave the missfolded full length muHTT down to toxic N-terminal fragments, containing the expanded polyQ, and have been found to be one of the key steps in HD pathogenesis. Indeed, the expression of muHTT N-terminal fragments in rodent and nonhuman primates seem to suffice to induce HD-like symptoms (Mangiarini et al., 1996; Palfi et al., 2007; Schilling et al., 1999).

muHTT is believed to interact with many intracellular targets disrupting their normal function (Figure 1.1), consequently leading to: mitochondrial dysfunction by impairing mitochondrial metabolic function and motility; proteolysis and apoptosis induced by increased caspase activities; synaptic dysfunction and excitotoxicity. Furthermore, muHTT degradation and clearance seems to be impaired due dysfunctional ubiquitin-proteasome system (Tydlacka et al., 2008; Zuccato et al., 2010). As a result ubiquitinated-muHTT proteins accumulate in the cytoplasm and contribute to aggregate formation (Waelter et al., 2001). Indeed, cytoplasmic N-terminal fragments and/or full length missfolded muHTT are believed to cluster and form β-sheet-rich fibrils with amyloid-like features. In turn, these amyloid-like structures will align resulting in the formation of aggregates or inclusions that can remain in the cytoplasm or can be shuttled to the nucleus (Bates, 2003; Cooper et al., 1998). Nuclear inclusions comprise mostly of fragments containing the N-terminus of the muHTT, whereas cytoplasmic aggregates are composed of both full-length and truncated forms of the protein (Hackam et al., 1998; Zuccato et al., 2010). Additionally, it is believed that the length of the protein fragment and the length of the PolyQ repeats are crucial factors in aggregation and toxicity processes (Cooper et al., 1998; Hackam et al., 1998; Martindale et al., 1998a). Morever, several *in vitro* studies have shown that inclusion formation increases susceptibility to cell death in non-neuronal and neuronal mammalian cell lines (Hackam et al., 1998; Ho et al., 2001). It has also been suggested that this is achieved by sequestration of specific proteins (e.g. soluble motor proteins responsible for axonal

transport) (Gunawardena et al., 2003; Li et al., 2001) and transcription factors (Cha, 2007) that may lose their physiological function (Zuccato et al., 2010). In addition, an *in vivo* study in a conditional mouse model of HD have also demonstrated that aggregate formation correlates with disease progression and that reversal of the HD-like phenotype is observed when the muHTT gene is "turned off" and aggregates cleared (Yamamoto et al., 2000). Importantly, this study also highlights that symptoms arise prior neuronal death, indicating that neuronal dysfunction prevails at early stages of disease, a time window that could be explored for specific therapeutic interventions (Yamamoto et al., 2000). In contrast, other lines of evidence support aggregate formation as a cellular neuroprotective mechanism to avoid toxic effects from soluble forms of muHTT (Imarisio et al., 2008; Zuccato et al., 2010). In fact, inclusion formation was found to improve survival of cells in culture perhaps by sequestering toxic soluble fragments (Arrasate et al., 2004). Additionally, in a study by Hayden and co-workers, HD mice bearing an expanded and truncated form of muHTT presented abundant inclusions, but neither behavioural impairment nor neuronal loss were reported (Slow et al., 2005). Furthermore, pharmacological promotion of inclusion formation has been shown to reduce the toxic effects of soluble muHTT in cell culture models (Bodner et al., 2006). Thus, although the role of aggregates is yet to be clarified and may depend on factors such as cell type and disease stage, aggregate formation is a widely known hallmark of HD. The presence of such aggregates has been reported in cortical and striatal structures in the brain of HD patients, but not in non-diseased control individuals (DiFiglia et al., 1997).

In addition to detrimental effects within cells, muHTT also perturbs intercellular interactions between neurons themselves and neuron-glia interactions (Gu et al., 2007; Ross & Tabrizi, 2011). muHTT is believed to increase release of glutamate excitatory neurotransmitter from cortical afferents and to enhance N-methyl-D-aspartate receptor (NMDAR) activity causing excitotoxicity in striatal neurons. This is further aggravated by reduced glutamate re-uptake from the synaptic cleft by glial glutamate transporter 1 (GLT-1) in glial cells. GLT-1

expression has been found to be reduced in post-mortem brain tissues from HD patients (Arzberger et al., 1997). On the other hand, deficient functioning of other neurotransmitter systems, such as adenosine A2 receptors, cannabinoidd receptors, and dopamine receptors, have been reported in different models of HD and may also play an important role mediating excitotoxicity in the HD brain (Cha et al., 1998; Zuccato et al., 2010).

Finally, a systematic assessment of human cortices have revealed that HD patients present a reduction in BDNF levels and its cognate receptor (Zuccato et al., 2008). The loss of BDNF production in cortical neurons due to muHTT transcriptional interference, and the disruption of its vesicular transport and delivery to the striatum may increase vulnerability of striatal neurons to excitotoxicity and to intracellular muHTT toxicity (Zuccato et al., 2007; Zuccato et al., 2001).

Although the relative contributions of cell-autonomous and non-cell-autonomous dysfunctions to the pathogenesis of HD are still unclear, ultimately they lead to neuronal dysfunction and death in the striatum, but also other brain regions such as cortex (Ross & Tabrizi, 2011; Zuccato et al., 2010). Furthermore, the extent to which symptoms of disease arise from neuronal death and/or from neuronal dysfunction still warrants further investigations, and may be key for the timing and application of different therapeutic strategies.

1.4 Neuropathology and symptomatology

As mentioned earlier HD pathology is largely brain specific and leads to major cell loss and atrophy of basal ganglia structures, specifically in the striatum (caudate nucleus and putamen) (Harper, 1996; Kowall et al., 1998; Reiner et al., 1988; Vonsattel et al., 1998; Vonsattel et al., 1985). In early and middle stages of HD progression, degeneration occurs preferentially in projecting g-aminobutyric acid (GABA)-ergic medium-sized spiny neurons, while medium-sized aspiny interneurons are not affected to a great extent (Albin et al., 1990; Vonsattel, 2008). Enkephalin-containing GABAergic neurons are believed to be affected

first and substance P-containing GABAergic neurons seem to be affected at later stages (Reiner et al., 1988). Furthermore, among substance P-containing neurons, neurons projecting to susbtantia nigra reticulata seem to be much more affected than neurons projecting to substantia nigra compacta (Reiner et al., 1988; Vonsattel, 2008). Despite the different degrees of degeneration among projecting neurons observed in early and middle stages of HD, at advanced stages both populations are largely affected (Vonsattel & DiFiglia, 1998; Zuccato et al., 2010). However, it is worth noting that the first symptoms (cognitive and motor) arise before the onset of overt neuronal loss in the striatum, thus suggesting that cellular dysfunctions may play an important role in early stage HD symptoms (Vonsattel et al., 1985). This is consistent with an increasing body of evidence that shows that aggregate formation and other biochemical alterations in the brain of HD patients occurs many years prior to the onset of symptoms (Gómez-Tortosa et al., 2001; Gutekunst et al., 1999).

Other structures, such as cerebral cortex, hippocampus, cerebellum, substantia nigra, white matter, thalamus and hypothalamus, are also clearly affected in HD (Kassubek et al., 2004; Politis et al., 2008). In fact, morphometric analyses using magnetic resonance imaging (MRI) revealed that these brain structures have significant volume reductions in HD patients (Monte et al., 1988; Rosas et al., 2003; Vonsattel, 2008). In more severe HD patients this is more evident with patients sometimes presenting with up to a 30% of reduction in brain weight and 20-30% reduction in total area of the cortex, hippocampus, amygdala and thalamus (Monte et al., 1988). The degeneration observed in other CNS structures, especially in cortical structures, might explain to a great extent the heterogeneity of symptoms among HD patients (Rosas et al., 2008). As an example, degeneration and damage in cortical structures have been implicated in personality changes and dementia (Monte et al., 1988). Furthermore, although evidence suggests that most clinical features of HD are mainly attributable to degeneration within the CNS, other aspects of HD are believed by effects of muHTT in other peripheral tissues. As an example, patients may suffer

endocrine disturbances, metabolic dysfunctions, muscle atrophy and weight loss which have been associated, at least in part, with adverse effects of muHTT in skeletal muscle, liver, heart, pancreas and testes (Sanberg et al., 1981; van der Burg et al., 2009).

The clinical features of HD include general motor dysfunctions, cognitive decline and psychological disturbances (Vonsattel & DiFiglia, 1998). Symptoms in early-stage human HD include alterations in mood, reward and sexual behaviour, disturbance of the circadian rhythm and progressive weight loss (Bates et al., 2002; Politis et al., 2008). However, early cognitive impairment has also been described prior to the onset of classical motor symptoms (Foroud et al., 1995; Lawrence et al., 1998). At peak-age of adult-onset, HD is classically associated with motor dysfunction, such as involuntary rapid movements (chorea), rigidity and dystonia; cognitive impairment, starting with loss of memory and progressing to dementia; and more pronounced neuropsychiatric manifestations, such as severe depression and anxiety (Bates et al., 2002; Rosenblatt, 2007). As HD progresses patients become unable to care for themselves and life threatening complications usually arise from poor nutrition, infection and inflammation, serious falls, among others (Zuccato et al., 2010). Swallowing difficulties that lead to aspiration pneumonia is the most common cause of death among HD sufferers (Bates et al., 2002).

1.5 Modelling Huntington's Disease

Mainly due to methodological limitations when using post mortem brain samples from patients, research in HD has been largely dependent on model systems (Ross & Tabrizi, 2011; Zuccato et al., 2010). Indeed, different *in vitro* and *in vivo* models have been widely used to gain insight into HD neuropathology and as tools to screen for potential new therapeutic interventions. The following is a short overview of the most commonly used *in vitro* systems and also the different animal models used to model HD.

1.5.1 *In vitro* models

Cell model systems expressing muHTT have been invaluable to understand the mechanisms of intracellular toxicity caused by muHTT. The most widely used *in vitro* models of HD present a fast pathological course and consist of transiently transfected cell lines that overexpress the muHTT (Li et al., 2003a). Non-neuronal cell lines, such as 2-2 Monkey kidney cells and human embryonic kidney (HEK) 293T cells, and neuronal-derived cell lines, such as N2a neuroblastoma cells, have been used to evaluate the toxic consequences of different muHTT fragments, to study muHTT half-life and intracellular localization (Cooper et al., 1998; Hackam et al., 1998; Kaytor et al., 2004; Martindale et al., 1998b). Several stably transfected cell lines consistently expressing the muHTT (or wtHTT) under the control of an exogenous promoter have also been engineered. As an example, a rat striatal ST14A-HTT120Q cell line, derived from embryonic day 14 rat striatal primordia, has been stably cloned with a fragment of the human muHTT gene including the promoter region, exon 1 and 120 CAG repeats (Cattaneo et al., 1998; Rigamonti et al., 2000). muHTT expression is consistent up to the 25^{th} passage and these cell cultures present an increased susceptibility to toxicity (Rigamonti et al., 2000). Parental ST14A cells express neuronal specific markers such as βIII-tubulin and neuron-specific enolase (Ehrlich et al., 2001). These cells also retain characteristics from the immature primary neurons from which they are derived, expressing markers including nestin. However, sub-type specific markers for medium sized spiny neurons, such as dopamine and cAMP-responsive phophoprotein 32 kDa (DARPP-32) and GABA are also expressed in undifferentiated ST14A cells. Expression of DARPP-32 increases upon cell differentiation and cells also express functional D_1 and D_2 dopamine receptors (Ehrlich et al., 2001). Other cell lines have been developed such that they contain an inducible system that permits regulation of the expression of the transgene. Examples of such are ST14 cells (Bari et al., 2013), rat adrenal phaeochromocytoma PC12 cells (Kazantsev et al., 1999; Wyttenbach et al., 2001) and neuroblastoma-glioma hybrid NG108-15 cells (Lunkes et al., 1999) stably cloned with

muHTT (e.g HD43 or HD-Q74) (or wtHTT (e.g. HD-Q23) and under the control of non-steroid- or tetracycline transcriptional activator systems. All these cell-model systems have been used not only to study aggregate formation and toxicity, but also as models to screen potential therapies for HD (Wyttenbach et al., 2001). Despite their usefulness, *in vitro* models based on cell lines have several limitations, such as (i) they might not recapitulate cell biology of post-mitotic neurons, and (ii) they carry genetic artifacts due to their malignant nature or due to modifications introduced to drive immortal growth (Park et al., 2008; Ross & Tabrizi, 2011).

In contrast, primary neurons recapitulate many features observed *in vivo*. Primary striatal neurons obtained from transgenic mouse or rat HD models have been used to evaluate the protective effects of novel treatments for HD (Tang et al., 2003; Wu et al., 2009). On the other hand, primary human fibroblasts harvested from human HD patients, naturally harbouring the full length muHTT protein, have been used to investigate muHTT toxicity (Cray et al., 1980). Additionally, HD fibroblasts have recently been used for the identification of single nucleotide polymorphisms (SNP) associated with the mutant allele and as a model to test allele-specific silencing of the muHTT (Carroll et al., 2011; Lombardi et al., 2009; Zhang et al., 2009). However, a limitation of primary cells is their short life span *in vitro* upon their isolation from native tissues (Park et al., 2008). Finally, it has been recently demonstrated that through application of induced-Pluripotent Stem (iPS) technology on HD fibroblasts it is possible to generate disease-specific pluripotent cells (Park et al., 2008). These cells can virtually differentiate to any cell-type, including neurons, and may be a promising model to study disease pathogenesis in a human system as well as to screen novel therapies (Park et al., 2008). Despite their versatility, recent studies have demonstrated that "epigenetic memory" of the tissues of origin may restrict cell differentiation. Therefore, in order to generate suitable *in vitro* models of disease and/or eventual therapies, it is crucial to select approriate methods to control for "epigenetic memory" (Kim et al., 2010b).

1.5.2 *In vivo* models

A large variety of species ranging from invertebrates to widely used rodents, and more recently nonhuman primates, have been used to gain better understanding of HD pathology but also as invaluable resources for preclinical testing of potential therapies. Recently, several reviews have focused in the exhaustive description of the features and limitations of existing *in vivo* models of HD (Crook et al., 2011; Marsh et al., 2003; Morton et al., 2013; Pouladi et al., 2013; Ramaswamy et al., 2007; Xi et al., 2011; Yang et al., 2011a). Thus, we will only give a brief overview of the most widely used models.

1.5.2.1 Non-mammalian *in vivo* models

Invertebrate models, such as *Drosophila melanogaster* (a fruitfly) and *Caenorhabditis elegans* (nematode worm), are convenient model systems due to their ease of maintenance, allowing for the use of large number of animals in a cost-effective manner (Ramaswamy et al., 2007; Zuccato et al., 2010). Furthermore these systems are relatively easy to manipulate genetically to generate transgenic models. *D. Melanogaster* and *C. Elegans* transgenic models bearing an N-terminal truncated form of human muHTT have been shown to present polyQ length-dependent neurodegeneration and progressive loss of motor coordination/motility (Faber et al., 1999; Gunawardena et al., 2003; Jackson et al., 1998; Parker et al., 2001). In addition, these systems have also constituted a rapid and inexpensive option for high-throughput screening of numerous candidate therapies (e.g. histone deacetylase (HDAC) inhibitors in Drosophila) (Nichols, 2006; Steffan et al., 2001; Voisine et al., 2007). Non-mammalian vertebrate models, such as transgenic *Danio rerio* (Zebrafish) have also been successfully engineered and used to replicate aggregation and polyQ length-dependent toxicity (Xi et al., 2011). In addition to their rapid development, zebrafish embryos develop externally and they are transparent enabling direct analysis of organs and tissues, which makes it an attractive model system for screening new therapeutics (Miller et al., 2005; Xi et al., 2011).

Despite the above mentioned advantages and the positive correlation with aspects of HD pathology, when using non-mammalian models one should consider that the molecular pathways associated may be different across species. The neuroanatomy and immune systems of these animals are very simple when compared to the mammalian counterparts and, therefore discoveries in these models might require subsequent validation in mammalian models (Ross & Tabrizi, 2011).

1.5.2.2 Mammalian *in vivo* models

Broadly, mammalian *in vivo* models can be categorized in genetic and non-genetic models, and span from rodents to nonhuman primates (Morton & Howland, 2013; Ramaswamy et al., 2007). In addition to the insights regarding HD pathology, mammalian *in vivo* models have been crucial in pre-clinical testing of novel therapies, been widely applied in proof of concept studies.

- ***Non-genetic models***

Non-genetic rodent and nonhuman primate models dominated HD research prior to the discovery of the genetic mutation in the IT15 gene (Ramaswamy et al., 2007). In these early models, HD-like pathology was induced by direct injection of toxins into the striatum and therefore they are usually referred as chemical-based *in vivo* models.

Glutamate, quinolinic acid (QA) or kainic acid have been used as "excitotoxic" agents to induce cell death in rodents and nonhuman primates through NMDAR or kainate receptors (Beal et al., 1991; Beal et al., 1986; Coyle et al., 1976; Emerich et al., 2006; McGeer et al., 1976). The pattern of cell death in excitotoxic-models replicates in part the neuropathology observed HD patients, with enkephalin- and substance-P-containing medium spiny neurons being mainly affected (Beal et al., 1991; Beal et al., 1986). In addition, these models display HD-like motor symptoms, such as hyperkinesia and dyskinesia, but also cognitive symptoms (Ramaswamy et al., 2007). On the other hand, mitochondrial toxins, such as 3-nitropropionic acid (3-NP) and malonic acid, have also been used and caused degeneration of GABAergic

medium size spiny neurons in the striatum in a similar pattern that occurs in human HD brains (Beal et al., 1993). These toxins have been shown to irreversibly inhibit succinate dehydrogenase interfering with mitochondrial metabolic activity, causing cell death by reducing ATP production and increasing ROS formation. Although strain variability has been reported (Ouary et al., 2000), metabolic models also mimic hyperkinetic and hypokinetic symptoms of HD, and aspects of cognitive impairment (Borlongan et al., 1997; Palfi et al., 1996).

Both excitotoxic and metabolic animal models of HD have not only played important roles unravelling the implications of excitotoxicity and mitochondrial impairment in HD pathology, but also in the evaluation of potential neurorestorative and neuroprotective therapies (e.g. coenzyme Q10, glial-derived neurotrophic factor (GDNF) gene delivery and GDNF/BDNF-producing cell grafts) (Kasparová et al., 2006; McBride et al., 2003; Pérez Navarro et al., 1999; Pérez Navarro et al., 2000). Despite their many advantages as simpler and convenient models, chemical-models of HD present several limitations: no muHTT protein is produced and consequently no aggregate formation is observed; no association between the mechanism of action and the actual genetic cause of the disease; the rapid cell death mechanism as opposed to the progressive neuronal loss in human HD; and finally, the interlaboratory variability, in regards to both neuropathology and behaviour, which may rise from differences in the genetic background of the rodents used but also in the technique and precision of surgical procedures (Ramaswamy et al., 2007).

- ***Genetic models***

The discovery of the mutant HTT gene as cause of HD and the development of genetic engineering techniques has enabled the generation of classic transgenic models and transgenic models via gene targeting (knock-in models), that account for the hereditary and molecular processes underlying HD. Table 1.1 summarises the most widely used mouse models of HD and their main features (Yang & Chan, 2011a).

Transgenic models via gene targeting (Knock-in models)

Mouse knock-in models have been generated by introducing an elongated CAG track into the murine *Hdh* or/and by replacing the corresponding mouse *Hdh* exon 1 for a portion of the human muHTT gene containing exon 1 and elongated CAG repeats (chimeric knock-in) (Menalled, 2005). Since these models only contain 2 copies of the HTT gene and their expression is regulated by the endogenous mouse *Hdh* promoter, protein synthesis is spatially and temporally accurate. Thus, knock-in models are considered the most faithful reproduction of HD from the genetic standpoint given that the mutation is expressed in the homologous HD gene (mu*Hdh)* (Menalled, 2005; Ramaswamy et al., 2007; Yang et al., 2011b). Unfortunately, the first models generated did not represent HD neuropathology and models displayed unusual aggressive behaviour and no motor or cognitive deficits (Shelbourne et al., 1999). However, subsequent studies of knock-in mice with longer polyQ (Hdh111Q line) revealed increased gliosis and moderate neuropathology in the brain after 24 months, but only subtle HD-like behavioural deficits (Wheeler et al., 2000). Other models containing, longer CAG repeats (~140) have also been generated which form muHTT inclusions in the striatum, cerebellum, cortex and hippocampus after 2 months, with motor symptoms only apparent from 12 months (Menalled et al., 2002; Menalled et al., 2003). Recently, in addition to aggregate formation, CAG150 knock-in mice have been shown to develop locomotor deficits, including limb clasping and gait abnormalities, as early as 4 months (Lin et al., 2001; Yu et al., 2003). In general, knock-in models present a slow progression of molecular, cellular, pathological and behavioural features of the disease. Thus, they have been mainly applied to study the pathological pathways associated with muHTT, but also in some cases used to identification of novel molecular targets for the development of therapeutic approaches (Menalled, 2005). The lack of significant neuronal loss, no reduction in life span and relatively subtle behavioural deficits are the main limitations of knock-in models (Ramaswamy et al., 2007).

Traditional transgenic models

Transgenic models bear a truncated form or the full length human muHTT gene randomly inserted into the host's genome (Menalled, 2005; Morton & Howland, 2013). In these models, the expression of the transgene is achieved in addition to the endogenous HTT homologue and is usually driven by an exogenous promoter. Several species have been used when generating transgenic models including mice, rats (von Horsten et al., 2003), minipigs (Baxa et al., 2013), sheep (Jacobsen et al., 2010) and nonhuman primates (Wang et al., 2008a; Yang et al., 2008). However, for the scope of the present thesis here we focused on the most widely-used transgenic mouse models.

It is now almost 20 years since Gillian Bates and co-workers generated the first transgenic mouse model of HD – the R6/2 mouse model, which was also the first genetic model of HD. This model expresses a truncated muHTT containing the human exon 1 and ~140-150 CAG repeats under the control of the human promoter (Mangiarini et al., 1996). Furthermore, the R6/2 model showed the presence of HTT aggregates and, severe anatomical and behavioural deficits, demonstrating that N-terminal muHTT is sufficient to induce HD-like pathology. The characteristics of this model will be discussed in detail in Section 1.6 of this thesis.

A similar model containing fewer CAG repeats (~115 CAG) was also generated in the same mouse strain and presented a less dramatic disease progression, with later onset of symptoms and longer survival than R6/2 mice (Mangiarini et al., 1996). This effect has been attributed to the shorter CAG expansion but also to the lower level of expression of the transgene (~31% of the endogenous mouse HTT gene) and possible different sites of integration into the mouse genome (Naver et al., 2003). Both R6 lines presented nuclear and neurophil aggregates, overall brain atrophy, motor and congnitive impairment, weight loss and decreased survival when compared to wild-type littermates (Mangiarini et al., 1996). Borchelt and colleagues generated the N171-82Q transgenic mouse through insertion of a truncated fragment of the human muHTT gene containing the first 171 aminoacids and 82

CAG repeats, into the mouse genome (Schilling et al., 1999). The transgene is under control of the mouse prion promoter and is specifically expressed in neurons but not in glia. The N171-82Q mouse model present HTT inclusions in various brain regions and a late onset of motor symptoms (~10-15 weeks) (Schilling et al., 1999). Thus, it has been suggested as a good model for assessing pre-symptomatic therapies. More recently, transgenic mouse models expressing the full length human muHTT (containing different lengths of CAG repeats) under control of the human promoter have been engineered using yeast and bacterial artificial chromosome (YAC128 and BACHD97, respectively) vector systems (Gray et al., 2008; Slow et al., 2005). Thus, these models are particularly useful in studies that aim to study cleavage of the muHTT protein. Alternatively to N-terminal-based models, full-length transgenic models have been more successful in mimicking the neuronal loss and the age-dependent degeneration in the striatum and cortex, also exhibiting progressive motor and cognitive deficits (Gray et al., 2008; Slow et al., 2005). However, relatively to other models YAC128 and BACHD97 mice display unusually long survival and no overall weight loss (Menalled et al., 2009), and therefore they have been suggested as a good models for the investigation of the long-term effects of therapeutic strategies (Ramaswamy et al., 2007).

Even though transgenic models recapitulate most of the molecular, neuropathological and clinical features of HD, they also present several pitfalls. Containing multiple copies of the HTT gene these models are not considered, by many, representative of HD genetics. In addition, due to random insertion of the human muHTT transgene in the mouse genome the normal functioning of other genes might be affected (Ramaswamy et al., 2007). Furthermore, the expression of the muHTT transgene driven by the exogenous promoter may not reflect an endogenous physiological production of the protein and HD-unrelated pathologic mechanisms in mouse (Ramaswamy et al., 2007). Finally, another limitation that has affected genetic mice models of HD in general is the variability of neuropathological and behavioural phenotypes across laboratories. Thus, efforts have been put together for the

standardisation of behavioural tasks and general husbandry, in order to increase consistency across future studies (Hockly et al., 2003b; Menalled et al., 2009; Yang & Gray, 2011b).

Although none of the current existing mice models of HD can claim to be "the best" due to their specific limitations, genetic models are seen by many as "gold-standard" models for pre-clinical testing (Yang & Gray, 2011b; Zuccato et al., 2010). Thus, reversal of the phenotype in these models are generally required if the efficacy of a new experimental therapy is being evaluated (Yang & Gray, 2011b).

Table 1.1 Summary of most widely used mouse models of HD and their features. *Adapted from (Yang & Chan, 2011a).*

	Transgenic				Knockin		
Mouse model	**R6/2**	**N171-82Q**	**YAC128**	**BACHD**	***Hdh*^{111}**	**CAG140**	***Hdh*$^{(CAG)150}$**
Promoter	Human HTT	Murine prion	Human HTT locus	Human HTT locus	Murine *Hdh*	Murine *Hdh*	Murine *Hdh*
PolyQ repeat	150 CAG	82 CAG	128 CAG	97 CAA/CAG	111 CAG	140 CAG	150 CAG
Protein expression level (Relative to endogenous *Hdh*)	75%	20%	75%	150%	50% or 100%	50% or 100%	100%
Repeat stability	Unstable	Unstable	N.D.	Stable	Unstable	N.D.	N.D.
Motor phenotypes							
Open field	+++ (8 wk)		++ (8 wk)	++ (8 wk)		++ (4 wk)	++ (8 wk)
Rotarod	+++ (10–12 wk)		++ (24 wk)	+++ (24 wk)			100 wk
Grip strength	+++ (10–12 wk)						
Gait					104 wk	52 wk	100 wk
Wheel running	+++ (4.5–5.5 wk)						
Climbing	+++ (4.5–5.5 wk)						
Cognitive phenotypes							
Reversal learning	++		++ (8 wk)				
Morris water maze	++						
Instrumental learning				++ (24 wk)			
Anxiety	++			++			
Neuropathology							
Selective neuropathology	Nonselective	Nonselective	Selective	Selective	Selective	Selective	Selective
Brain weight	20% (12 wk)	N.D.	10% (52 wk)	14% (52 wk)	N.D.	N.D.	N.D.
Striatal volume			15% (52 wk)	28% (52 wk)			40% (100 wk)
Cortical volume			7% (52 wk)	32% (52 wk)			
Striatal cell loss (stereology)			18% (52 wk)				40% (100 wk)
Striatal dark neurons	+	+	N.D.	10–15% (52 wk)	3.5% (104 wk)	N.D.	N.D.
Mutant HTT aggregates	+++	+++	++	+	++	++	++
Gliosis		++					
Other phenotypes							
Early lethality	12-13 wk.	—	—	—	—	—	—
Body weight	Loss (7 wk)	Loss (12 wk)	Gain (8 wk)	Gain (8 wk)			Loss (70 wk)

1.6 R6/2 Mouse model of Huntington's Disease

As stated earlier (Section 1.5.2.2), the R6/2 mouse was the first and most widely used mouse model of HD. The R6/2 transgenic line was engineered through microinjections into single cell CBA x C57BL/6J F1 embryos. Insertions consisted of a 1.9-kb truncated fragment of the human muHTT gene containing the human HTT promoter, exon 1, an elongated CAG repeat tract (~140-150 CAG) and 262 base pairs (bp) of intron 1 (Mangiarini et al., 1996).

This is currently the most widely used transgenic animal model of HD for assessment of novel therapeutic strategies and presents a robust behavioural phenotype and HD neuropathology, in part attributed to the long polyQ repeat (Li et al., 2005).

1.6.1 Neuropathology

In the R6/2 line, the muHTT transgene was found to be ubiquitously expressed and at about 75% of the level of expression of the murine wild-type gene (Mangiarini et al., 1996), thus suggesting that the transgene is to some extent overexpressed when compared to the human disease. Although the first studies only reported the appearance of N-terminal HTT aggregates in the striatum by weeks 3-4 (Davies et al., 1997; Meade et al., 2002; Morton et al., 2000), subsequent studies demonstrated that they can be readily detected from birth in the neostriatum, S1 somatosensory cortex and hippocampal neurons (Stack et al., 2005). Furthermore, age-dependent increases in the size and number of inclusions (constituted by the muHTT protein and ubiquitin, which binds and tags proteins for degradation at the proteasome system) have been described, and at the terminal stage the vast majority of the neurons in the striatum express inclusions (Meade et al., 2002; Stack et al., 2005). At ~15 weeks of age 98% of the striatal projecting neurons contain numerous inclusions, whereas only few are found in interneurons (Kosinski et al., 1999; Meade et al., 2002; Sathasivam et al., 1999). Despite that the first studies failed to demonstrate significant neuronal loss in the striatum (Sun et al., 2002), Stack *et al.* reported moderate dark neuron degeneration and astrogliosys by ~12-14 weeks of age (Stack et al., 2005). In addition, this study

demonstrated a significant reduction in the total striatal cell counts in the brain of R6/2 mice (-25%) when compared to wild-type littermates (Stack et al., 2005). In a different study, these reductions seemed to be selective for enkephalin-containing neurons in the striatum, whereas substance P-containing neurons exhibit no major change by ~12 weeks of age (Sun et al., 2002). The fact that selective neuronal death in the R6/2 striatum is only observed at later stages of the disease suggests that the observed symptoms are mainly due to neuronal dysfunction rather than due to neurodegeneration (Cha et al., 1998; Sathasivam et al., 1999). Indeed, R6/2 mice show downregulation of several neurotransmitter receptors such as, metabotropic glutamate receptors type I and III (mGlu1 and mGlu3, respectively), dopamine receptors D1 and D2, muscarinic cholinergic receptors, and cannabinoid receptor CB1 (Cha et al., 1998). Alterations in neurotransmitter production, release and reuptake have also been reported and associated with multiple changes in neuron and glial function (Li et al., 2005).

As previously mentioned, R6/2 mice also display muHTT inclusions in many other brain regions, such as cortex, hippocampus, hypothalamus and cerebellum, yet in different proportions (Davies et al., 1997; Li et al., 2005). In the hippocampus, muHTT aggregates seem to affect CA1 at first instance (and later in other areas, CA3) resulting in reduced long term potentiation in this area of the hippocampus (Lione et al., 1999; Murphy et al., 2000); on the other hand, in the cortex of R6/2 mice muHTT impairs BDNF production and BDNF delivery to the striatum, but also leads to malfunctioning of the corticostriatal neuronal circuitry (Cepeda et al., 2003; Zuccato et al., 2005); HTT aggregates in the lateral hypothalamus are believed to cause death of orexin-positive neurons and affect gonado-tropin-release hormone, consequently causing gonadal atrophy and infertility in adult R6/2 mice (Papalexi et al., 2005; Petersén et al., 2005); muHTT mediates transcriptional dysregulation in the cerebellum affecting the expression of cyclic adenosine monophosphate (cAMP) and retinoid-responsive genes (Luthi-Carter et al., 2002). In addition to the cerebellum, gene expression dysregulation is found in multiple areas of the R6/2 mice brain,

including hippocampus and cortex, and has been documented from ~6 weeks of age, aggravating with disease progression (Luthi-Carter et al., 2002).

R6/2 mice demonstrate robust brain atrophy, with brains weighing ~20% less than the brains of wild-type littermates at 12 weeks of age (Davies et al., 1997). Subsequent studies of brain morphology reported age-dependent reduction of striatal and total brain volumes, significant from 60 days and achieving reductions up to ~41% and ~44%, respectively at ~12-13 weeks of age. Conversely, an increase of up to 6-fold in the ventricular volume was also reported (Mangiarini et al., 1996; Stack et al., 2005). Due to lack of massive neuronal loss in the R6/2 model, the reduction in striatal and brain volume has been associated with atrophy of individual neurons (cell bodies of medium size spiny neurons reduce in size by ~20% but also their dendrites) (Klapstein et al., 2001).

Whilst major focus has been given to the pathological effects of muHTT in the R6/2 brain, other body systems also seem to be affected. In fact, muHTT inclusions have been found in several other organs and tissues, such as: adrenal glands, hepatocytes, stomach wall, testes, kidney, cardiac and skeletal muscles and in pancreatic Langerhans islets (Sathasivam et al., 1999). As examples, (i) R6/2 mice develop insulin-dependent diabetes by ~12 weeks of age, which is believed to be caused by the formation of intranuclear HTT inclusions in β-cells pancreatic islets (Hurlbert et al., 1999); (ii) R6/2 mice also develop progressive muscle atrophy which is thought to be caused, at least in part, by specific gene expression dysregulation (Luthi-Carter et al., 2002; Sathasivam et al., 1999).

1.6.2 Behavioural phenotype, weight loss and survival

The behavioural phenotype of the R6/2 mouse model recapitulates most of the symptoms observed in human HD patients (see below). However, the progression of HD symptoms in this *in vivo* model is rapid and aggressive, and therefore it has been referred to as a good model of the rare juvenile variant of the disease (Ramaswamy et al., 2007). Table 1.2

summarises the most widely studied phenotypical deficits in R6/2 mice and the different behavioural tests commonly used for their assessment.

Table 1.2 Summary of phenotypical deficits in R6/2 mice and the different behavioural tests commonly used for their assessment.

Deficits	Behavioural assessment		R6/2 mice characterisation
	Task	**Description**	
Motor	Open field	Evaluates spontaneous locomotor activity. The animal is usually placed in the centre of an arena and left to explore.	From ~8 weeks, R6/2 mice present a reduced total distance travelled in the open field when compared to wild-type littermates.
	Rotarod (Jones et al., 1968)	Tests motor coordination and balance. Mice are place on top of a rod which rotates at a constant speed or accelerates according to a specific protocol. The latency to fall (in seconds) is the most widely used outcome.	R6/2 mice tend to have shorter latencies to fall when compared to wild-type littermates from ~5 weeks of age.
	Clasping behaviour (after tail suspension)	The clasping behaviour consists in the adoption of a dystonic posture, after tail suspension, where fore- or/and hind limbs are tightly pressed against the thorax and/or abdomen.	When suspended wild-type mice spread their four limbs, whereas R6/2 mice crunch towards their thorax/abdomen. Clearly present by 8-9 weeks of age.
	Grip Strength	Assesses muscle strength and grasping reflex of the forelimbs. Animals are allowed to grasp a trapeze/bar and are then pulled back gently until release.	R6/2 mice tend to present lower peak strengths than wild-type littermates from ~10 weeks of age.
Cognitive	Morris water maze (Morris, 1981)	Assesses spatial memory. Typically consists of a circular tank filled with water where the animal is placed and tries to escape by climbing to a platform just below the water level. Platform may be removed and time spent at the location is recorded.	R6/2 mice have in average longer escape latencies, taking longer to find the platform based on visual landmarks than wild-type littermates. Early onset by ~3.5 weeks if age.
	Alternating T-maze (Olton, 1979)	Tests alternation, spatial and non-spatial learning. In this test, mice have to alternate between the arms so that they receive a reward.	R6/2 mice made fewer alternations between the arms since they have a higher tendency to perseverate to the first stimulus. Poor performance for ~5-6 weeks of age.
Neuropsychiatric-like	Elevated plus-maze (Hogg, 1996)	Assesses anxiety-like behaviours. Consists of plus maze with two open and two closed arms, elevated from the floor 40-70 cm and roof open. Low anxious behaviour is measured by increased time spent in the open arms	R6/2 mice have been reported to present reduced anxiety-like behaviours in this test from ~8 weeks of age. Note that other behavioural tests have shown conflicting results.

The onset of overt ***motor symptoms*** occurs on average at ~8 weeks of age and mice are severely affected by week 12 (Li et al., 2005). In spite of being initially hyperactive in the open field test by 3 weeks of age (Lüesse et al., 2001), by 4.5 weeks mice have been reported to present decreased activity in the running wheel (Hickey et al., 2005). Indeed, hypoactivity in the open field task has been reported as early as 4 weeks (Hickey et al., 2005). However, subsequent studies have only reported reduction of the spontaneous locomotor activity by ~8 weeks of age (Carter et al., 1999; Lüesse et al., 2001; Menalled et al., 2009). The normal rearing-climbing behaviour in mice is also found to be significantly impaired in R6/2 mice from ~4-6 weeks of age, and it has been noted that at later stages mice are not able to reach food (which will thereafter need to be provided in the form of soft food at the bottom of the cages) (Li et al., 2005; Menalled et al., 2009). In addition, R6/2 mice exhibit a progressive motor coordination decline which is detected by poor rotarod performance from ~5 weeks of age and this is found to be further aggravated by 12 weeks, with animals presenting clear difficulties in performing the task (Menalled et al., 2009; Stack et al., 2005). Furthermore, when suspended by their tail R6/2 mice show paw clasping behaviour which instigates at around 8-9 weeks, however the pathophysiology of this abnormal behaviour is not well understood (Mangiarini et al., 1996). Also, grip strength deficits have been described to begin at about ~10 weeks of age (Menalled et al., 2009; Stack et al., 2005), and are likely to be correlated to the muscle atrophy observed from ~8 weeks of age (Sathasivam et al., 1999). Other gradual changes in motor behaviour including resting tremor, involuntary jerky movements, stereotypical grooming and changes in gait and circadian rhythm have also been described (Carter et al., 1999; Mangiarini et al., 1996; Morton et al., 2005; Stack et al., 2005). Furthermore, R6/2 mice suffer, and even in some instances die, from epileptic seizures which may be triggered by handling or unexpected noises (Cepeda-Prado et al., 2012; Mangiarini et al., 1996).

Cognitive deficits arise at earlier stages than motor deficits (at about ~3.5 weeks of age) with impairment in performing spatial learning tasks such as Morris water maze task, and at

weeks ~7-8 R6/2 mice are already unable to learn and carry out this task (Lione et al., 1999; Lüesse et al., 2001; Murphy et al., 2000). These deficits have been associated with HTT-induced hippocampal pathology and the inability to learn at later stages of the disease could be in part explained by the worsening of the visual acuity and motor performance (deterioration of swimming abilities) (Lione et al., 1999; Murphy et al., 2000). Additionally, mice also show difficulties in reversing pre-learned tasks and perform poorly in the alternating T-maze task from ~5-6 weeks of age. Consequently, it has been suggested that these mice may have disturbances in the innate motivational program or tendency to perseverate, which is also common to HD patients (Lione et al., 1999). On the other hand, R6/2 mice have been found to mimic some of the ***neuropsychiatric manifestations*** of human HD. Despite that File *et al.* reported that by 8 weeks of age R6/2 mice are less anxious than wild-type littermates in the elevated plus-maze (increased time spent in the open arms of the maze) (File et al., 1998), recent studies have shown progressive anxiety-like behaviour in R6/2 mice in the light-dark choice test (Menalled et al., 2009). The fast progression of symptoms in the R6/2 mice makes this model less adequate to study psychopathologies associated to HD (Du et al., 2013).

Body weight in R6/2 mice reaches a plateau at about ~ 9 weeks of age, decreasing to as little as 30-40% of their wild-type littermates at later stages (Mangiarini et al., 1996). Additionally, the dramatic progression of the phenotype also results in premature death, with R6/2 mice dying at about 13-16 weeks of age (Stack et al., 2005). However, factors such as, housing conditions and handling, introduce great variability in these outcomes and therefore standardized living conditions are recommended (Hockly et al., 2002; Hockly et al., 2003b). Despite variability, the R6/2 model is the only mouse model that has consistently demonstrated reduced survival in systematic studies (Menalled et al., 2009). Furthermore, as the etiology for early death and body weight loss are not fully understood, these markers are only normally used to support other neuropathological or/and behavioural findings (Hockly et al., 2003b; Yang & Chan, 2011a).

In summary, the R6/2 model reproduces, at least in part, the molecular, cellular and behavioural deficits of HD in a robust and reproducible phenotype. Furthermore, this model is available from commercial breeding facilities and can be readily accessed by researchers. Thus, this model has been regarded as an excellent tool for high throughput screening of new therapies for HD in a relatively easy, rapid and inexpensive way (Li et al., 2005; Menalled et al., 2009).

1.7 Therapeutic strategies for Huntington's Disease

Current drug therapies are unable to prevent, cure or stop disease progression, but are only able to provide relief for some of the symptoms of HD. Medical management of the symptoms is carried out using several classes of medications including: neuroleptics, antidepressants, anticonvulsants, benzodiazepines, acetylcholinesterase inhibitors, skeletal muscle relaxants, among others, which have been reviewed elsewhere (Adam et al., 2008). In addition, recently the United States of America (USA) Food and Drug Administration (FDA) approved tetrabenazine specifically for the treatment of HD chorea and other hyperkinetic disorders (Hayden et al., 2009). Tetrabenazine acts mainly as an inhibitor of the vesicular monoamine transporter (VMAT), depleting the release of monoamine neurotransmitters, such as dopamine, into the synaptic cleft. This was the first drug to be specifically approved by the FDA for the treatment of a symptom of HD (Hayden et al., 2009; Phillips et al., 2008).

Other measures, in addition to pharmacotherapy, can be taken to improve patient's quality of life. For instance, since at later stages patients develop swallowing difficulties, thickening agents can be used to increase thickness of fluids making them safer and easier to swallow. Moreover, physiotherapy, and occupational and speech therapy are aimed to improve functional activity, and may be recommended on an individual basis according to clinical decision (Bilney et al., 2003). Despite that little evidence exists regarding the usefulness of these approaches to address motor deficits of HD (Bilney et al., 2003), environment

enrichment and exercise has been shown to partially reverse HD-like behaviour deficits in the R/2 mouse model of HD (Hockly et al., 2002; Wood et al., 2010). Thus, this aspect warrants further investigations.

As mentioned earlier, no disease-modifying treatments are yet available and therefore efforts are being made to develop such therapies. Indeed, the identification of the causative gene and the better understanding of HD pathophysiology has brought to light new molecular targets (Johnson et al., 2010). Broadly, novel emerging therapeutic approaches can be classified into three main categories (Figure 1.2): (i) cell replacement therapies; (ii) therapies that aim to target the underlying pathologic mechanisms HD; and (ii) therapies that aim to silence the toxic muHTT. As an exhaustive description of all novel therapies is out of the scope of the present thesis, a brief overview of the most widely investigated will be given.

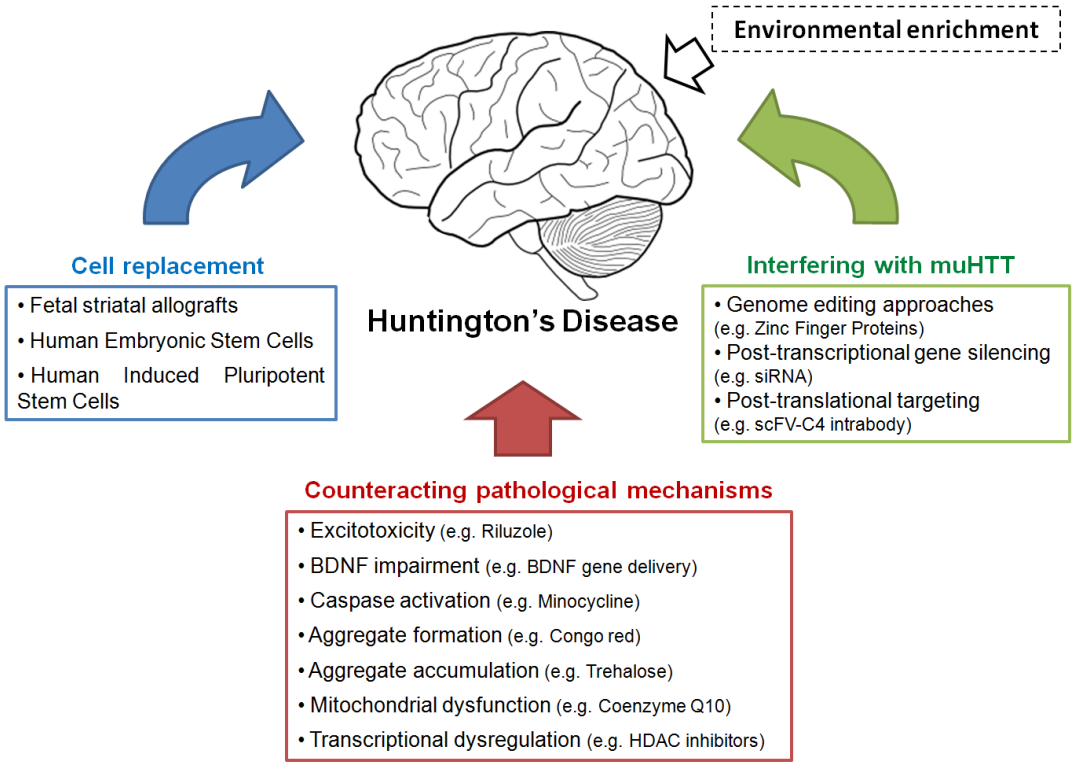


***Figure 1.2. Novel emerging therapeutic approaches for HD. Abbreviations**: BDNF, Brain Neurotrophic Factor; HDAC, Histone deacetylase; muHTT, Mutant Huntingtin; siRNA, short interfering RNA.*

1.7.1 Cell replacement therapies

Cell transplantation into the striatum has been postulated as a potential therapy to stabilize HD-related symptoms by replacing the affected neurons (Björklund et al., 2000). Indeed, initial preclinical studies in excitoxin-lesioned animals revealed that transplantation of fetal striatal progenitors restores neuronal activity in the globus pallidus, functionally repairing the damaged pathway (Nakao et al., 1999). Subsequent clinical studies revealed that fetal striatal allografts were well tolerated and improvements in motor behaviour and cognitive deficits were also reported (Bachoud-Lévi et al., 2006; Bachoud-Lévi et al., 2000). However, about 2 years after surgery a plateau on amelioration was observed and the improvements faded off around 4-6 years (Bachoud-Lévi et al., 2006). Thus, it seems that this strategy is able to stabilize HD symptoms but is not able to provide a cure. Further investigations are now being carried out in phase II clinical trials (Multicentric Intracerebral Grafting in HD (MIG-HD). ClinicalTrials.gov Identifier: NCT00190450).

Additionally, human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSC) also hold great therapeutic potential. Indeed, exciting results with *in vivo* xenografts were recently obtained (Aubry et al., 2008). However, challenges such as cell differentiation to specific neuronal phenotypes, controlled formation of functional circuits, and absence of tumour growth, still warrants further investigations before the clinic stage (Ross & Tabrizi, 2011).

1.7.2 Targeting the underlying pathogenic mechanisms of HD

Several therapies targeting the underlying pathogenic mechanisms of HD have been extensively evaluated *in vitro* and *in vivo*. These include strategies targeting excitotoxicity, BDNF impairment, muHTT cleavage, aggregate/inclusion formation, energy impairment, and transcriptional dysregulation, all of which are deregulated in HD (Figure 1.1).

Drugs that counteract ***excitotoxicity*** by blocking pre-synaptic glutamate release or reduce N-methyl-D-aspartic acid (NMDA) receptor activity have been extensively evaluated for their efficacy in HD. Riluzole was shown to inhibit glutamate neurotransmition and improve motor symptoms in genetic mouse models of HD. However, pilot studies in humans have showed only transient beneficial effects (Seppi et al., 2001). On the other hand, an open labelled trial has revealed that memantine, a NMDA receptor blocker, slows progression of the disease and improves cognitive deficits (Cankurtaran et al., 2006). Finally, dopamine pathway inhibitors, such as tetrabenazine, have also been tested and showed encouraging results regarding the control of chorea (Savani et al., 2007). In fact, this drug has been approved by the USA FDA and is now available for symptoms management (Phillips et al., 2008).

Different approaches have been considered when targeting ***BDNF impairment*** as a therapeutical strategy for HD. Since recombinant BDNF has poor pharmacokinetic proprieties, low ability to penetrate through the blood brain barrier (BBB) and showed limited effects in other neurodegenerative diseases (Kasarskis et al., 1999; Zuccato & Cattaneo, 2007), researchers have considered the use of viral gene delivery to achieve a local and sustained production of BDNF. Lentiviral (LV) and adeno-associate viral delivery of BDNF have been successfully achieved in animal models of the disease with significant improvements in neuronal survival (Bemelmans et al., 1999; Zuccato & Cattaneo, 2007). Recently, synthetic molecules that mimic BDNF have also been designed and showed improved BBB penetrability, which allows for a systemic administration rather than a local invasive administration (Fletcher et al., 2009). Alternatively, indirect strategies of increasing BDNF expression have also been evaluated and have mainly focused on the reduction of REST activity, which is increased in HD and represses BDNF expression (Zuccato et al., 2003). Abrogating REST activity has been achieved by sequestering this transcription factor through overexpression of wtHTT (Zuccato et al., 2003); dominant-negative REST

constructs (Conforti et al., 2012); using synthetic oligonucleotides decoys (Soldati et al., 2011), but also using low molecular weight drugs (Conforti et al., 2013).

In order to prevent muHTT cleavage to its toxic N-terminal fragments, pharmacological inhibition of ***caspase activation*** has also been considered as a therapeutic approach for HD. As an example, researchers have used minocycline, a tetracycline antibiotic, as an inhibitor of caspase 1 and 3. Pre-clinical trials using minocycline in the R6/2 mouse model have revealed ameilioration of the symptoms, however results have been hard to reproduce (Ona et al., 1999; Wang et al., 2003). An open label study with minocylcine has been carried out in HD patients and showed an improvement after 6 months and a stabilization of motor and neuropsychological symptoms after 24 months (Bonelli et al., 2003). On the other hand, results from a double-blinded, randomised, multicentre clinical trial suggested futility of carrying out larger Phase III clinical studies (Cudkowicz, 2010). However, this study only assessed a specific dose of minocycline and based statistical analysis on a 25% improvement of the Total Functional Capacity (Cudkowicz, 2010). Thus, further investigations using higher doses may be warranted.

Inclusion/aggregation formation is a widely accepted hallmark of HD, however its precise role in the toxic events is still unclear (Zuccato et al., 2010). Although both strategies promoting or blocking aggregate formation have been considered, a greater focus has been given to the latter. Congo red disrupts oligomerisation of polyQ and also increases clearance of polyQ expansions. Despite a reduction in the number of aggregates *in vitro* and improvement of the behavioural phenotype of a HD mouse model having been demonstrated already, results have been hard to replicate by others (Sánchez et al., 2003). C2-8, another polyQ inhibitor, has been shown to be able to penetrate the BBB and reduce both the size of HTT inclusions and neuronal atrophy in R6/2 HD model (Chopra et al., 2007). Furthermore, drugs that promote clearance of toxic muHTT fragments by induction autophagy, a process by which the cells degrade large portions of the cytosol and other cellular components by the internal lysosomal machinery, have also been evaluated. As an example, rapamycin and

trehalose significantly reduced the number of HTT aggregates and improved pathology in *in vivo* models of HD (Sarkar et al., 2007; Sarkar et al., 2008). However, the immunosupression induced by some of these compounds raise concerns about their use in humans (Sarkar et al., 2007; Sarkar et al., 2008).

In HD ***mitochondrial dysfunction*** may lead to oxidative stress, increased vulnerability to excitotoxicity and apoptosis. Drugs, such as creatine and coenzyme Q10, have known antioxidant effects and therefore reduce ROS production (Zuccato et al., 2010). These drugs have been shown to have protective effects in toxin-based animal models and R6/2 mice (Ferrante et al., 2002; Ferrante et al., 2000). On the other hand, early stage clinical trials revealed amelioration of neurological scores for creatine. A larger clinical trial (2CARE study) is now being conducted for coenzyme Q10. Moreover, mitochondrial proliferators such as eicosapentaenoic acid (EPA) improve behavioural deficits in various HD animal models (Van Raamsdonk et al., 2005). Furthermore, this drug has significantly delayed cerebral atrophy and improved the orofacial component of HD in a stage III human trial (Puri et al., 2005). Finally, cysteamine blocks oxidative damage and has a pro-survival effect *in vitro* (Mao et al., 2006b). Preclinical studies have revealed that cysteamine is able to improve motor deficits in the R6/2 HD mouse model (Dedeoglu et al., 2002).

Early-stage ***transcriptional dysregulation*** of a large number of genes significantly affects the molecular pathogenesis of HD (Cha, 2007). HDAC inhibitors improve histone acetylation leading to more relaxed chromatin structure prone to be transcribed. Moreover, it has been suggested that HDAC inhibitors exert effects beyond enhacing transcription and have also anti-inflammatory effects and anti-apoptotic effects (Giorgini et al., 2008). Examples of this type of drugs are: Suberoylanilide hydroxamic acid, sodium butyrate, phenylbutyrate and pimelic diphenylamide. All the above mentioned drugs improved motor deficits in animal models of HD (Hockly et al., 2003a; Thomas et al., 2008). Human trials are now ongoing for sodium phenylbutyrate to ensure its tolerability and safety in humans (Hogarth et al., 2007). Additionally, valproic acid, a well-tolerated antiepileptic drug, has

shown great potential as HDAC inhibitor in other neurodegenerative diseases, such as Retinitis Pigmentosa (Shanmugam et al., 2012). Indeed, initial clinical testing has shown very promising results (Shanmugam et al., 2012) and a Phase II clinical trial is currently ongoing (ClinicalTrial.gov Identifier: NCT01233609). However, it is also important to highlight that growth arrest and chromosomal instability can occur upon treatment with these drugs and therefore caution should be taken. Furthermore, compounds that may interact directly with DNA are also being evaluated for therapeutic efficacy in HD. Anthracycline antibiotics, such as chromomycin and mithramycin, modulate epigenetic histone modifications and influence transcription (Ferrante et al., 2004). Preclinical studies with these drugs showed a significant increase in survival and a significant improvement in the motor behaviour deficits in R6/2 mice (Ferrante et al., 2004). Clinical trials are currently being designed for these compounds.

1.7.3 Silencing the mutant Huntingtin

Targeting the muHTT specifically is now being considered as a potential therapeutic approach for HD. This can be achieved at the transcriptional level by specifically repressing expression of the muHTT gene or through genome editing techniques; but also post-transcriptionally by targeting the respective messenger RNA (mRNA) or post-translationally, by targeting the muHTT protein itself.

Blocking muHTT toxicity at a ***post-translational level*** has been achieved using artificial polypeptides and/or intrabodies. First studies with artificial polypeptides were undertaken in *Drosophila Melanogaster* and revealed that small molecules, such as peptides, are able to bind HTT and avoid polyQ aggregation (Kazantsev et al., 2002). On the other hand, intrabodies are recombinant fragments of antibodies (Ab) that exert their activity against a specific intracellular antigen. Intrabodies have high affinity and specificity to the target antigen site and have been previously used for cancer and human immunodeficiency virus (HIV) therapies. Intrabodies targeting HTT exon 1 have effectively reduced HTT protein

levels, aggregates and cell death *in vitro* (Lecerf et al., 2001). Wang *et al.* showed that adeno-associated viral (AAV)2/1 delivery of scFV-C4 intrabody is able to reduce size and number of HTT inclusions in the striatum of R6/1 mice. Additionally, AAV delivery of scFV-EM48 to N171-82Q mice reduced motor deficits and cytoplasmic aggregate formation (Wang et al., 2008b). Recently, AAV2/1 delivery of Happ1 have been shown to improve HD neuropathology and motor and cognitive deficits in several other HD mouse models (Southwell et al., 2009). Although, the use of intrabodies as a potential therapeutic strategy for neurodegenerative diseases seems promising, this technology is still in a very early stage of development and improvements are needed regarding their stability and solubility.

Post-transcriptional gene silencing (PTGS) approaches for HD have undergone considerable research and, include ribozymes, antisense oligonucleotides (ASOs) and RNA interference (RNAi) (Scanlon, 2004). Ribozymes are RNA molecules with self-cleaving capabilities consisting of an effector catalytic core and two flanking sequences that allow for specific binding to the mRNA (Scanlon, 2004; Tanner, 1999). Hammerhead ribozymes are believed to cleave mRNAs at a preferred site with rapid degradation of mRNA fragments, and have been successfully used *in vitro* and in transgenic mouse models of HD to silence the expression of muHTT (Scanlon, 2004; Tanner, 1999). Artificial ribozymes have also recently been considered as potential therapeutics for Alzheimer's Disease (AD) (Aissa et al., 2012). On the other hand, ASO technology involves the use of single stranded DNA molecules, typically ~20 bp long, which have complementary sequence to the target mRNA (Chan et al., 2006; Smith et al., 2006). Hybridization can occur at the pre-mRNA level in the nucleus with inhibition of 5' cap formation, inhibition of splicing and/or activation of RNase (Ribonuclease) H degradation (Chan et al., 2006). Moreover, when hybridisation takes place in the cytoplasm translation is inhibited by steric hindrance or by RNase H degradation of the mRNA transcript. ASOs are limited to the inhibition of one mRNA copy (Bertrand et al., 2002). In early *in vivo* studies, although ASOs successfully penetrated neurons with no remarkable toxicity, no significant reduction in muHTT was observed (Haque et al., 1997).

The lack of efficacy in these studies were speculated to be due to high susceptibility to nuclease degradation (Bertrand et al., 2002). In contrast, modified ASOs have recently been shown to successfully reduce expression of HTT in human fibroblasts (Hu et al., 2009). Furthermore, it was recently demonstrated in several rodent models of HD that modified ASOs are able to successfully reduce muHTT expression, improve HD-like neuropathology and ameliorate symptoms of disease (Carroll et al., 2011; Kordasiewicz et al., 2012). In addition, ASOs have very recently undergone clinical trials as a potential therapy for amyolateral sclerosis, further advocating its promise for other neurodegenerative diseases (Miller et al., 2013). Despite their therapeutic potential, ribozymes and ASOs, have been largely superseded by the gene silencing potency and lasting effects of RNAi approaches (Bertrand et al., 2002; Miyagishi et al., 2003). This approach will be further discussed in the following sections.

Alternatively, specific gene targeting/silencing can also be achieved at the ***transcriptional level*** through engineered nucleases, such as meganucleases, zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas systems (Gaj et al., 2013). These chimeric nucleases are able to bind to specific DNA sequences, repressing gene transcription and/or inducing DNA double strand breaks and enabling correction of mutated genes (Gaj et al., 2013). Some of these approaches have been successfully applied in X-linked severe combined immune deficiency (X-SCID) (Urnov et al., 2005), hemophilia B (Li et al., 2011b), sickle-cell disease (Zou et al., 2011), Parkinson's Disease (Soldner et al., 2011), Retinitis Pigmentosa (Mussolino et al., 2011) and HIV (Holt et al., 2010). In fact, ZFNs that interefere with the C-C chemokine receptor type 5, which in turn confers HIV resistance, are now undergoing clinical testing (ClinicalTrial.gov Identifiers: NCT01252641, NCT00842634 and NCT01044654). In the specific case of HD, it has recently been demonstrated that zinc finger proteins (ZFP) are able to effectively silence the muHTT, without affecting the expression of wtHTT, *in vitro* and in the R6/2 mouse brain (~40%

reduction in muHTT) (Garriga-Canut et al., 2012). In this study, ZFP repressors were delivered intraparenchymally using an AAV delivery system, resulting in significant improvements in HD-related neuropathology and motor deficits (Garriga-Canut et al., 2012). Although these technologies are still at their infancy, they hold great promise not only for HD but for other monogenic disorders.

1.8 RNA interference

RNAi has emerged as one of the most exciting areas for gene therapy development in the past two decades. RNAi is an endogenous cellular pathway that allows post-transcriptional regulation of gene expression. This intracellular pathway enables cells to auto-regulate gene expression and has been shown to have a crucial role during development (He et al., 2004; Krützfeldt et al., 2006; Stefani et al., 2008). Moreover, the same pathway may also be used to restrain the expression of parasitic invaders, such as viruses (Ding et al., 2007; Stefani & Slack, 2008). The key elements of this gene silencing machinery are small double stranded RNA (dsRNA) molecules, consisting of ~20-30 oligonucleotides (Agrawal et al., 2003; Bartel, 2004). These dsRNA molecules target complementary mRNA sequences to degradation or induce ribosomal arrest, blocking the translation of those transcripts into protein (Agrawal et al., 2003; Bartel, 2004). Napoli *et al*. (1990) were the first to unexpectedly observe the activation of the RNAi pathway in petunia plants while trying to overexpress a chalcone synthase (CHS) gene. Plants revealed a loss of pigmentation and CHS mRNA levels were reduced by 50-fold (Napoli et al., 1990). The realisation that this RNAi technology also generalised to animal systems was provided in 1998 by Fire and Mello, who were subsequently awarded the Nobel Prize in Physiology & Medicine for their groundbreaking research (Fire et al., 1998). The authors reported gene knockdown of a myofilament protein *unc22* in a nematode worm, *Caenorhabditis elegans* using long dsRNAs (Fire et al., 1998). Moreover, Fire and Mello reported that unrelated dsRNAs sequences did not yield gene expression knockdown of the target gene, further supporting that RNAi is highly specific (Fire et al., 1998). Tuschel and colleagues demonstrated that

RNAi technology can also be used in mammalian cells to evoke gene silencing (Elbashir et al., 2001). In this study, gene expression of luciferase reporter plasmids were suppressed 3- to 25-fold in several mammalian cell lines, such as HEK293, HeLA, NIH/3T3 and COS-7 (Elbashir et al., 2001), using short interfering RNAs. The same study shows significant gene silencing effects in *Drosophila* S2 cells indicating that the RNAi pathway is also conserved in insects.

Although significant differences between animals and plants in terms of their RNAi mechanisms have been reported, homologous key proteins of the pathway have been shown to be highly conserved. Thus, this might suggest that the pathway is being conserved from the last eukaryotic common ancestor (Agrawal et al., 2003; Shabalina et al., 2008). Moreover, it has been described that prokaryotes possess an analogous RNAi-like defensive system that has evolved independently from eukaryotes (Shabalina & Koonin, 2008).

1.8.1 RNA interference gene silencing mechanism

PTGS through the RNAi pathway is mainly accomplished through two categories of small dsRNAs, endogenous microRNAs (miRNA) and short interfering RNAs (siRNA) (Agrawal et al., 2003; Meister et al., 2004; Tang, 2005). Figure 1.3 represents a schematic illustration of the RNAi pathway and its major components.

Endogenous miRNAs are small non-coding RNAs derived from primary precursors encoded in the genome (He & Hannon, 2004; Siomi et al., 2009). Transcription of primary transcripts (pri)-miRNAs is carried out by RNA polymerase II in the nucleus originating single stranded RNAs which back fold and contain secondary structures, such as hairpin stem-loops and mismatch sequences. Before being exported to the cytoplasm, pri-miRNAs are processed by a ribonuclease (RNase III nuclease), Drosha, originating pre-miRNA molecules of ~60-70 oligonucleotides. Pre-miRNA molecules are thereafter exported to the cytoplasm through the GTP-powered exportin-5 transporter (He & Hannon, 2004). In the cytoplasm these dsRNA pre-miRNAs are further processed by another RNase III, Dicer, generating small dsRNA

molecules with ~21-25 oligonucleotides (Agrawal et al., 2003; Esau et al., 2007). miRNAs bind to a RNA induced Silencing Complex (RISC) loading complex (RLC), a multimeric protein complex containing a transactivation response-RNA-binding protein (TRBP) (Kim et al., 2012b). Activation of the RISC complex occurs after unwinding of miRNA and thermodynamic selection of the guide/antisense strand (Haley et al., 2004; Siomi & Siomi, 2009; Tang, 2005). An Argonaute protein with endonuclease capabilities subsequently attaches to the proteic complex (Agrawal et al., 2003; Rana, 2007). Due to oligonucleotide mismatch within miRNAs, miRNA activated RISCs (miRISCS) (also called micro Ribonucleprotein complex, miRNP) continuously search the transcriptome for partially complementary mRNA sequences to bind (Meister & Tuschl, 2004). Moreover, it has been suggested that miRNAs perform gene silencing effects by binding to target- 3'UTR sequences in specific mRNA transcripts (He & Hannon, 2004). Thus, miRNAs function mainly as translational repressors by promoting ribosomal translational arrest rather than mRNA cleavage (He & Hannon, 2004; Lai, 2002; Meister & Tuschl, 2004; Tang, 2005). However, the exact mechanism through which miRNA perform their gene silencing effects is still to be fully elucidated (Siomi & Siomi, 2009). On the other hand, siRNAs derive from long dsRNAs introduced during viral infections or as a result of transcription of genetic elements. In the cytoplasm, these dsRNAs are processed by Dicer to form perfectly matched dsRNAs with ~21-25 nt, or siRNAs (Agrawal et al., 2003). Synthetic siRNAs can also be artificially introduced in the cell and bypass the nuclear processing steps by Drosha, following essentially the same cytoplasmic pathway of miRNAs (Siomi & Siomi, 2009). siRNA activated RISCs (siRISCs) thereafter search the transcriptome for specific complementary mRNAs targeting them to degradation (Martinez et al., 2002; Siomi & Siomi, 2009; Zamore et al., 2000). siRNA and miRNA base pairing with target mRNA sequences is crucial for recognition, binding and cleavage. Indeed, it has been suggested that the seed region near the 5' end of the antisense strand (2-7th oligontucelotide) plays a key role in this regard (Bartel, 2004; Haley & Zamore, 2004; Lai, 2002). Ultimately, both siRNA and miRNA inhibit translation of mRNA transcripts to their protein product.

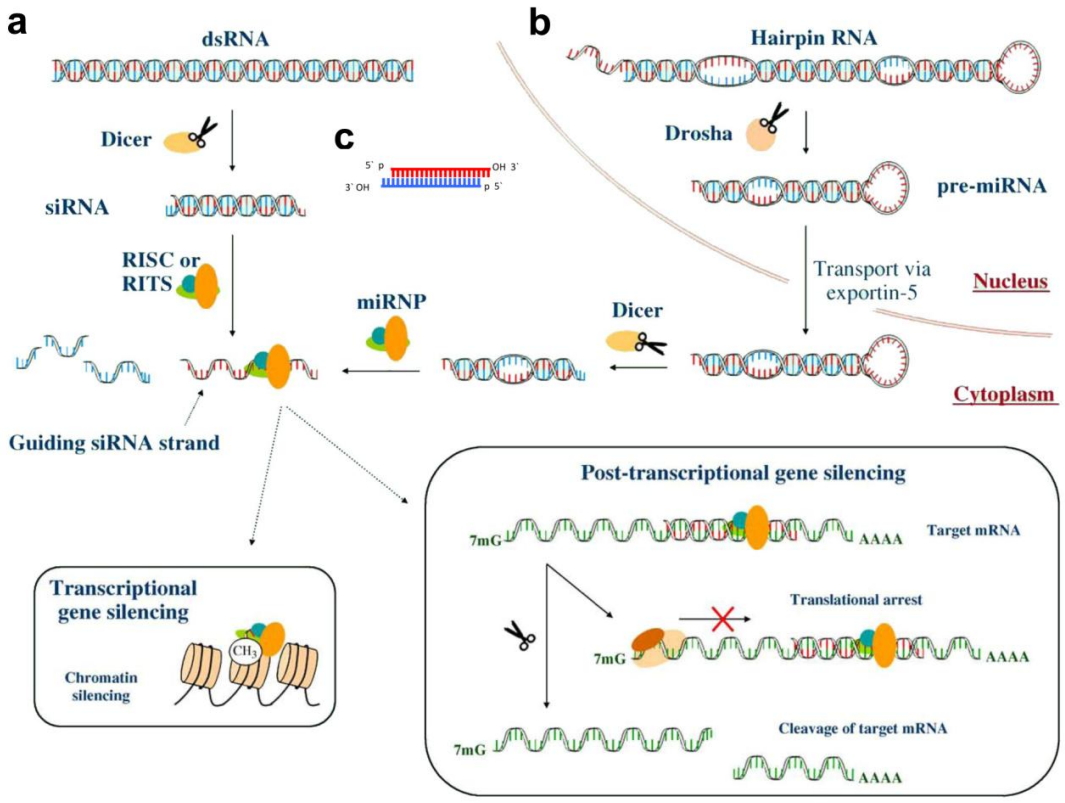


Figure 1.3. RNA interference post transcriptional gene silencing mechanism. *Adapted from (Guo et al., 2010a; Thakker et al., 2006). (a) Long dsRNA molecules are processed by Dicer in the cytoplasm originating siRNAs (~21-25 oligonucleotides). (b) Nuclear dsRNAs are processed by Drosha and exported to the cytoplasm for further processing by Dicer. (c) Synthetic and artificially introduced siRNAs by pass Drosha and follow the RNAi pathway. (a-c) Assembly of siRNA/miRNA in RISC or RITS complexes allow for transcriptional and post-transcriptional gene silencing.* ***Abbreviations****: dsRNAs, Double-stranded RNA; miRNA, Micro RNA; RISC, RNA induced silencing complex; RITS, RNA-induced transcriptional silencing; siRNAs, Short interfering RNA.*

Despite the differences in their biogenesis, miRNAs and siRNAs are both generated by Dicer and are thought to share components of the cytoplasmic RNAi machinery (Bartel, 2004; Esau & Monia, 2007; Siomi & Siomi, 2009). It has also been argued that siRISCS and miRISCS are very similar and their functions sometimes interchangeable (Doench et al., 2003; Saxena et al., 2003; Tang, 2005). Although less well described in the literature, siRNAs and miRNAs may also induce gene silencing at the transcriptional level by forming a RNA-induced transcriptional silencing (RITS) complex (Kawasaki et al., 2004; Morris et al., 2004). RITS bind to complementary regions in the chromatin recruiting enzymes that enable heterochromatin remodelling (Kawasaki & Taira, 2004; Meister & Tuschl, 2004;

Verdel et al., 2004). Thus, induction of transcriptional and/or PTGS through siRNAs and miRNAs is dependent on the different proteins and factors recruited in these proteic complexes (Meister & Tuschl, 2004).

1.8.2 Approaches for artificial induction of RNAi

The RNAi machinery can be artificially hijacked to induce specific gene expression knockdown. This is commonly performed using synthetic siRNAs, but also through short hairpin RNAs (shRNA) (Pardridge, 2007b; Rao et al., 2009; Singh et al., 2011).

siRNAs (~21-25 nucleotides) are chemically synthesised double stranded RNA macromolecules with ~14 kDa and a net negative charge. As previously mentioned these synthetic siRNAs are able to bypass nuclear processing by Drosha being active upon efficient delivery and release to the cytoplasm. siRNAs allow for potent and specific gene expression knockdown in mammalian cells, however their effects have been found to be transient only lasting up to 5-7 days (Bartlett et al., 2006, 2007; Raab et al., 2004; Shin et al., 2011). Dilution effect due to cell division has been suggested as one of the main reasons for short gene silencing effects in proliferating cells (Bartlett & Davis, 2007; Raab & Stephanopoulos, 2004). In addition, siRNA is not amplified intracellularly and probably more susceptible to cellular metabolism. Thus, if continuous gene expression knockdown is required, multiple administrations should be performed. Alternatively, synthetic siRNAs have presented long-lasting gene expression knockdown in cells with low proliferating rates, such as neurons (Bartlett & Davis, 2006; Omi et al., 2004).

Alternatively, shRNAs can be used for stable RNAi gene silencing and are usually encoded within an expression vector (plasmid DNA or viral vector) (Paddison et al., 2002; Raab & Stephanopoulos, 2004; Rubinson et al., 2003; Sui et al., 2002). Transcription of shRNAs and miRNA constructs is carried out by RNA polymerase II or III in the nucleus and requires a suitable promoter (Singh et al., 2011). Pri-shRNAs are thereafter processed by Drosha, exported to the cytoplasm and further processed by Dicer. The amplification of the

expression of shRNAs by the intracellular transcription machinery makes this approach more resistant to cellular metabolism than the synthetic siRNA approach. Despite the convenience of long-term gene silencing effects, the need for translocation of constructs to the nucleus may restrict the use of such approaches in quiescent cells or cells with low proliferating activity (Wang et al., 2010a; Zou et al., 2010). Moreover, depending on the final application, inclusion of a "turn-off" system might be crucial for controlling intracellular levels of shRNA (Singh et al., 2011). Finally, plasmid DNA (pDNA) encoding shRNA or miRNA have a molecular weight (Mw) in average 100x times higher than synthetic siRNAs, which might further complicate packaging to adequate vectors and delivery to target cells (Wang et al., 2010a).

1.8.3 Current applications of RNAi technology

Experimentally RNAi technology has mostly been applied as a research tool to provide greater understanding of gene and protein functions (Agrawal et al., 2003). The recognised applicability of RNAi in a wide variety of organisms has lead to the identification of genes with crucial roles in embryonic development, biochemical signalling cascades and other cellular processes (Cheng et al., 2003). Furthermore, RNAi has allowed for high-throughput screening of gene function that is not achievable through conventional methods and therefore has an increasingly important role in drug development. Indeed, pharmaceutical companies use RNAi as a mean to validate novel drug targets by protein loss-of-function analyses in model systems, such as *C. Elegans* (Jain, 2004). Homologue relevant genes for disease detected in these systems are thereafter tracked back to the human genome and re-validated by other means (Jain, 2004).

In addition, RNAi has been successfully used to induce specific gene expression knockdown with the aim of generating *in vivo* models of disease (Gao et al., 2007; Hitz et al., 2009; Zhang, 2008). Examples of animal models engineered using this technology are an inducible RNAi rat model of Diabetes Mellitus (Kotnik et al., 2009) and a mouse model of human

Barth syndrome (Acehan et al., 2011). Although it is unlikely that RNAi will supplant knockout technology, it has been particularly useful when knockout models are not viable. Inducible RNAi *in vivo* models are able to circumvent the issues of embryonic impaired development, allowing the study of gene dysfunction in later stages of development (Agrawal et al., 2003; Gao & Zhang, 2007; Hitz et al., 2009; Zhang, 2008).

Finally, harnessing the RNAi pathway to induce specific gene silencing effects has also shown great potential as a therapeutic strategy for incurable diseases (Aagaard et al., 2007; Cheng et al., 2003; Fougerolles et al., 2007; Kim et al., 2007). Diseases caused by specific dysfunctional or mutated genes are the main candidates for RNAi therapeutic approaches (Fougerolles et al., 2007). For instance, RNAi strategies for cancer therapy mainly target oncogenes and other pro-survival genes that are overexpressed in tumour tissue (Aagaard & Rossi, 2007). By suppressing multidrug resistance genes it has been suggested that RNAi might also be used to increase efficacy of other treatments such as chemo- and radiotherapy (Aagaard & Rossi, 2007). RNAi strategies may also facilitate treatment of autosomal dominant neurodegenerative diseases by targeting their respective cognate causative genes (Martínez et al., 2013). Suppression of such specific causative genetic targets is expected to halt progression of disease and ameliorate symptoms. Moreover, RNAi technologies might constitute a new alternative to conventional therapies which largely target proteins, by enabling post-transcriptional silencing of the so-called "undrugable" therapeutic targets. Therefore, efforts have been put together to support translation of this strategy to the clinic (Cheng et al., 2003). Indeed, numerous Phase I and Phase II clinical trials are currently underway mainly for solid cancers and age-related macular degeneration, but also for several infectious diseases (for further information on ongoing trials see (Davidson et al., 2011; Guo et al., 2010a; Kubowicz et al., 2013)). Finally, recent updates have shown very promising results regarding RNAi delivery to humans (Davis, 2009; Kaiser et al., 2010; Tabernero et al., 2013).

1.9 RNAi therapeutics for CNS disorders: focusing on neurodegeneration

In the specific context of the CNS, RNAi holds great promise for the treatment of neurological incurable diseases, including neurodegenerative diseases and brain cancers (Raoul et al., 2005b; Sah, 2006). Although there have been significant advances in RNAi-based approaches for brain tumors (Erdmann et al., 2009), for the purpose of this thesis a special emphasis will be given to neurodegenerative diseases.

Neurodegenerative diseases are often associated with age-related dysfunction and are characterised by a loss of selective subpopulations of neurons within the CNS, leading to subsequent decline in neurological functions (Bossy-Wetzel et al., 2004; Forman et al., 2004). These diseases are a relatively heterogeneous group of disorders with distinctive pathological and clinical features. Table 1.3 contains candidate neurodegenerative diseases that have been considered for RNAi therapeutics, their potential genetic targets, molecular pathological aspects, affected structures within the CNS and main symptoms. Dominantly inherited neurodegenerative diseases, such as HD, SCA-1 -2, -3 and -7, and familial amyotrophic sclerosis (FALS), are associated with causative mutant genes that lead to toxic gain of function of the protein (Bossy-Wetzel et al., 2004; Forman et al., 2004). Thus, although the precise molecular mechanisms of these diseases are not fully understood yet, reduction of the cellular load of abnormal disease-causing protein through RNAi has been a major area of focus.

Table 1.3 Main candidate neurodegenerative diseases for RNAi. *Adapted from (Ralph et al., 2005a).*

Disease	Potential gene targets for RNAi	Molecular pathological aspects	Main CNS structures affected	Main Symptoms	First *in vivo* RNAi study
HD	HTT	Cytoplasmic and nuclear misfolded muHTT protein aggregates	Striatum and cortex	Chorea, rigidity, cognitive impairment	(Harper et al., 2005)
SCA	SCA-1, -2, -3, -7	Cytoplasmic and nuclear misfolded mutant ataxin protein aggregates	Cerebellum	Loss of coordination of the gait, hands, speech and eye movement	(Xia et al., 2004)
FALS	SOD-1	Hyaline inclusions containing SOD-1 deposits	Spinal cord and brain stem neurons	Muscle atrophy, stiffness	(Ralph et al., 2005b; Raoul et al., 2005a)
PD	α-synuclein, LRRK2	Lewy bodies	Substantia nigra	Tremor, bradykinesia, rigidity, neuropsychiatric manifestations	(Sapru et al., 2006)
AD	BACE-1, Tau, APP	Extracellular amyloid plaques, intracellular neurofibrillary tangles	Cortex and hippocampus	Memory loss, confusion, trouble with language	(Singer et al., 2005)

Abbreviations: *AD, Alzheimer's Disease; APP, amyloid precursor protein; BACE, β-secretase; CNS, Central Nervous System; FALS, familial amyotrophic lateral sclerosis; HD, Huntington's Disease; LRRK2, Leucine-rich repeat kinase 2; muHTT, Mutant Huntingtin; PD, Parkinson's Disease; RNAi, RNA interference; SCA, Spinocerebellar ataxia; SOD, superoxide dismutase.*

The therapeutic effects of RNAi for polyQ disorders was first reported *in vivo* in a model of SCA by Davidson and colleagues (Xia et al., 2004). In this study, transduction of ~10% of Purkinje cells resulted in significant SCA-1 gene expression knockdown, reduction of the cerebellar pathology and improvements in the motor coordination deficits (Xia et al., 2004).The first RNAi preclinical studies for ALS showed significant suppression of the mutant superoxide dismutase (SOD-1) after intramuscular injections (and retrograde transport to motor neurons) (Ralph et al., 2005b) and/or after lumbar injections (Raoul et al., 2005a) in a transgenic mouse model of ALS. Both studies described improvements in pathological and behavioural abnormalities. Additionally, pioneering studies on the application of RNAi for AD targeted BACE-1 in the hippocampus of a transgenic mouse model of AD (Singer et al., 2005). Reduction of BACE-1 in mice overexpressing mutant amyloid precursor protein (APP) resulted in decreased amyloid plaque formation, improved neuronal survival and improved spatial learning and memory (Singer et al., 2005).

Furthermore, initial preclinical studies have also been conducted for PD and showed significant suppression of human α-synuclein overexpressed in the rat striatum (Sapru et al., 2006). However, further *in vivo* studies are yet to be carried out in other animal models of PD to provide insights into potential pathological and behavioural improvements of RNAi. Finally, several RNAi preclinical studies in rodents and primates have also been carried out for HD. These will be discussed and reviewed in the following section (Section 1.9.1).

Many other *in vivo* studies have since been conducted and the progress on the application of RNAi for neurodegenerative diseases has recently been reviewed elsewhere (AD (Chen et al., 2013b), ALS (Rizvanov et al., 2011), HD (Mantha et al., 2012), SCA (Keiser et al., 2013) (Gonzalez-Alegre, 2007; Maxwell, 2009; Thakker et al., 2006).

1.9.1 Progress in RNAi therapeutics for Huntington's Disease

Davidson and colleagues were also pioneers in the use of RNAi as a therapeutic strategy for HD *in vivo* (Harper et al., 2005). Since then many studies in various *in vivo* models of HD, from rodents to nonhuman primates, have been carried out. Table 1.4 summarises a large selection of RNAi *in vivo* studies for HD, including details regarding the main neuropathological and behavioural outcomes.

In their initial study, Davidson and colleagues performed bilateral single injections of AAV delivery system, coding anti-HTT shRNA, into the striatum of N171-82Q mice (Harper et al., 2005). Significant reductions in muHTT mRNA levels (~55%) and in the number of HTT inclusions were observed. Moreover, behavioural improvements in stride length and in rotarod deficits were also reported (Harper et al., 2005). However, in this study there were no improvements in weight profiles and this was attributed to the systemic nature of the disease or to muHTT-mediated hypothalamic dysfunction. Subsequently, Rodriguez-Lebron *et al.* also reported reduction in HTT mRNA levels (~75%), decreased number of HTT inclusions (25-38%) and improvement of hindlimb clasping behaviour in the R6/1 mouse model, upon delivery of specific shRNAs (shHUNT1 and shHUNT2) targeting human

muHTT using a AAV-delivery system (Rodriguez-Lebron et al., 2005a). In addition, muHTT supression in the striatum increased expression of DARPP-32 and preproenkephalin (ppENK) when compared to untreated R6/1 (Rodriguez-Lebron et al., 2005a). Nevertheless, in this study RNAi treatment failed to improve weight gain and performance in the rotarod task of R6/1 mice (Rodriguez-Lebron et al., 2005a).

In 2005 the first pre-clinical study for HD using lipid-formulated siRNAs emerged (Wang et al., 2005). Therein Wang and co-workers demonstrated that siRNA-HDExon1, targeting a sequence upstream of the CAG repeats of the human muHTT transcript, was successfully delivered into the intracerebroventricular (i.c.v.) of postnatal day 2 R6/2 mice yielding a significant reduction in HTT mRNA levels (~70%), coupled with sustained effects up to 7 days (Wang et al., 2005). This suppression of HTT resulted in a reduced number of nuclear aggregates and general brain atrophy. Additionally, RNAi treatment delayed the onset of the clasping behaviour, improved spontaneous locomotor activity in the open field and improved rotarod motor deficits of the R6/2 mouse. Furthermore, less severe weight loss and increased survival when compared to untreated R6/2 mice were also reported (Wang et al., 2005). In another approach, the use of cholesterol-conjugated siRNAs (cc-siRNA-HTT) for RNAi in HD was first demonstrated by DiFiglia *et al.* in a AAV-based mouse model of HD. Co-administration of AAV-HTT100Q and cc-siRNA-HTT into adult mouse striatum resulted ~66% knockdown of the HTT transcript and reduction of HTT aggregates in the striatum (DiFiglia et al., 2007). Results also showed increased neuronal survival and significant behavioural improvements in beam walking and in the clasping behaviour (DiFiglia et al., 2007).

In all of the above mentioned *in vivo* studies, RNAi treatment was initiated when animals were still pre-symptomatic, and therefore limited conclusions in regards to reversal of neuropathology could be drawn. However, following studies were carried out in symptomatic rodents and have shown that RNAi treatment is able to reverse the number of HTT inclusions and improve striatal dysfunction (Drouet et al., 2009; Machida et al., 2006).

Further investigations are now warranted to assess if such improvements in HD neuropathology also result in reversal or only block progression of behavioural deficits.

Additionally, it is also worth noting that siRNAs/shRNAs molecules used in most preclinical trials described so far are unable to distinguish between human mutant and wild-type HTT alleles, and may cause disruption of both alleles if directly applied in human therapy. Although it has been recently shown in rodents and nonhuman primates that partial suppression of wtHTT can be well-tolerated in the adult brain up to 6 months (Grondin et al., 2012; McBride et al., 2011; Stiles et al., 2012), the effects of long term suppression of wtHTT have yet to be investigated. Thus, in order to circumvent the issue of long-term unwanted suppression of wtHTT, allele-specific siRNAs targeting SNP associated with the mutant allele have been developed (Lombardi et al., 2009; Zhang et al., 2009). Despite that initial *in vitro* studies in human HD fibroblasts have shown effective knockdown of muHTT alone leaving wtHTT undisrupted, *in vivo* studies are now warranted (Lombardi et al., 2009; Zhang et al., 2009). Alternatively, if generic RNAi approaches targeting both wtHTT and muHTT alleles are to be used in the long run, one may consider gene replacement as a strategy to maintain adequate levels of expression of the wild-type protein. Such combined approaches have been previously used with success in dominant Retinitis Pigmentosa where a RNAi-resistant gene construct was used to supplement the functional wild-type gene (Millington-Ward et al., 2011).

Taken together, these great advances have demonstrated the utility of RNAi as a therapeutic approach for HD. However, the progression to the clinic has mainly been hindered by the lack of effective and safe siRNA delivery systems able to overcome the different CNS barriers.

Table 1.4. RNAi in vivo studies for Huntington's Disease in mammalian models

Category	Animal model	Disease stage/Age of intervention	Delivery system	Route of administration	HTT gene expression knockdown*	Protein knockdown*	Improvement in HD pathology*	Behavioural outcomes	Ref.
Rodents	HD-N171-82Q mice	Pre-symptomatic 4-week old	AAV1 shRNA (shHD2.1)	Intrastriatal injection (bilateral). Intracerebellar injection.	↓ 51-55% in the striatum	↓ HTT inclusions (striatum and cerebellum)	↓ HTT inclusions (striatum and cerebellum)	✓Rotarod deficits ✓Gait deficits (Front and rear stride length) ×Weight loss	(Harper et al., 2005)
	R6/1 mice	Pre-symptomatic 6-week old	AAV5 shRNA (siHUNT-1 and -2)	Intrastriatal injection (bilateral)	↓~75% in the striatum	↓ 25-38% in the striatum	↓HTT nuclear inclusions ↑ 24% ppENK, ↑ 16% DARPP-32 mRNA	✓Clasping behaviour ×Weight loss ×Rotarod deficits	(Rodriguez-Lebron et al., 2005a)
	R6/2 mice	Pre-symptomatic Post natal day 2	Lipofectamine2000 siRNA-HDExon1	i.c.v. injection	↓70% in the striatum	↓ HTT nuclear aggregates in the striatum	↓General brain atrophy ↓ HTT nuclear aggregates in the striatum	✓Survival ✓Weight loss ✓Rotarod deficits ✓Clasping behaviour ✓Spontaneous locomotor activity	(Wang et al., 2005)
	HD190Q EGFP mice	Symptomatic 12-week old	AAV2/AAV5 shRNA (shEGFP)	Intrastriatal injection (unilateral)	Not reported	↓ ~82% human HTT-positive aggregates, ↓ ~65.9% ubiquitin aggregates	↑ ppENK and ↑DARPP-32 mRNA	No improvement in behaviour and survival (due to unilateral injection)	(Machida et al., 2006)
	AAV1/8-based mouse model overexpressing HTT100Q	Pre-symptomatic	cc-siRNA-HTT (co-injection with the AAV1/8 HTT100Q)	Intrastriatal injection	Not reported	↓~66% human HTT	↓Size of nuclear inclusions ↓Neurophil aggregates ↑Survival of striatal neurons (Nissl-stain)	✓ Clasping behaviour ✓ Beam walking	(DiFiglia et al., 2007)

Table 1.4. (Cont.)

Category	Animal model	Disease stage/Age of intervention	Delivery system	Route of administration	HTT gene expression knockdown*	Protein knockdown*	Improvement in HD pathology*	Behavioural outcomes	Ref.
Rodents (Cont.)	Adenoviral-based mouse model overexpressing HTTN171Q128 R6/2 mice	R6/2 symptomatic. 5 week-old	Ad shRNA (shHTT) (co-injection with Ad HTTN171Q12)	Intrastriatal injection (bilateral)	Not reported	↓ HTT aggregates in transduced areas	↓ HTT aggregates in transduced areas	Not reported	(Huang et al., 2007a)
	CAG140 heterozygous knock in mice	5 week-old	AAV2/1 shRNA and miRNA	Intrastriatal injection (bilateral)	~50-60% in transduced areas	Not reported	Not reported	Not reported	(McBride et al., 2008)
	AAV1/2-based rat model	Pre-symptomatic	AAV2/1 shRNA (shHD2)	Intrastriatal injection (bilateral)	↓~80-90% in the striatum	↓~50% HTT in the striatum	↑ Neuronal survival ↓Number of degenerating neurons	✓ Spontaneous exploratory forepaw use	(Franich et al., 2008)
	Lentiviral-based rat model overexpressing HTT171-82Q	Symptomatic 2 months after expression started	DOX regulated lentiviral shRNA sihtt1.1system	Intrastriatal injection	Not reported for muHTT	↓ HTT inclusions	↑DARPP-32 mRNA, ↓ubiquitin inclusions	Not reported	(Drouet et al., 2009)
	HD-N171-82Q mice	Pre-symptomatic 7 week-old	AAV2/1 shRNA (sh2.4) and miRNA (mi2.4) (also targeted endogenous HTT homologue)	Intrastriatal injection (bilateral)	↓ ~60-75% in the striatum	Not reported	Not reported	✓ Rotarod deficits ✓ Trend to improved survival ✗ Weight loss	(Boudreau et al., 2009b)
	BACHD mice	Not reported	AAV2/1 miRNA (miHDS1) (also targeted endogenous HTT homologue)	Intrastriatal injections	↓ ~60% in the striatum	Not reported	Not reported	Not reported	(McBride et al., 2011)
	Wistar rats	N/A	cc-siRNA-HTT (targeting endogenous HTT homologue)	MRIgFUS combined with i.v. injection	↓ ~35% in the striatum	Not reported	N/A	N/A	(Burgess et al., 2012)

Table 1.4. (Cont.)

Category	Animal model	Disease stage/Age of intervention	Delivery system	Route of administration	HTT gene expression knockdown*	Protein knockdown*	Improvement in HD pathology*	Behavioural outcomes	Ref.
Nonhuman primates	Adult rhesus monkeys (males)	N/A	AAV2/1 miRNA (miHDS1) (targeting endogenous HTT homologue)	Intrastriatal injections (3 injections per hemisphere)	↓ ~45% in mid and caudal putamen	Not reported	N/A	N/A	(McBride et al., 2011)
	Adult rhesus monkeys (females)	N/A	AAV2 shRNA (shHD5) (targeting endogenous HTT homologue)	Intrastriatal injections (5 injections per hemisphere)	↓~30%	↓~45% ↓ HTT immunostain-ing	N/A	N/A	(Grondin et al., 2012)
	Adult rhesus monkeys (females)	N/A	^{14}C-siRNA (siHTT) (targeting endogenous HTT homologue)	CED in the striatum for 28 days	↓ ~44% in the putamen	↓ ~32% in the putamen ↓HTT immunostain-ing with decreasing distance from the catheter	N/A	N/A	(Stiles et al., 2012)

* (vs diseased control or sham treated)

Abbreviations*: (↑) Increase; (↓) reduction; AAV, adeno-associated virus; cc-siRNA, cholesterol-conjugated siRNA; CED, convection enhanced delivery; DARPP-32, dopamine and cAMP-responsive phophoprotein 32 kDa; DOX, doxycycline; EGFP, enhanced green fluorescent protein; HTT, huntingtin; i.v., intravenous injection; ppENK, preproenkephalin; MRIgFUS, magnetic resonance imaging guided focused ultrasound; mRNA, messenger RNA; N/A, not applicable; shRNA, short hairpin RNA; siRNA, short interfering RNA.*

1.10 Barriers and challenges for therapeutic RNAi delivery to the CNS

In order to achieve gene silencing, synthetic siRNAs and/or pDNA coding shRNAs (from now on called shRNA) need to be successfully introduced into cells and reach their respective subcellular processing sites (O'Mahony et al., 2013b; Singh et al., 2011; Wang et al., 2010a; Wiethoff et al., 2003). Depending on the route of administration and target organ/tissue, these macromolecules must overcome several other extracellular barriers (O'Mahony et al., 2013b; Wang et al., 2010a; Wiethoff & Middaugh, 2003). The following sections give a brief overview of the various barriers that need to be circumvented, with a particular focus on siRNA delivery and CNS barriers. Figure 1.4 represents a schematic illustration of specific challenges to be overcome when delivering RNAi therapeutics to the CNS.

1.10.1 Extracellular barriers

Although administration of RNAi therapies by the systemic route may be more attractive when translating such therapy to the clinic, it poses significant delivery challenges (Dominska et al., 2010; O'Mahony et al., 2013b). ***Stability and nuclease degradation*** are among the major hurdles for naked nucleic acids when administered systemically (Whitehead et al., 2009). Exposure to serum and tissue endonucleases leads to degradation and enhances elimination, limiting efficacy. In addition, due to their unfavourable physicochemical characteristics (size, net negative charge and hydrophilic nature), nucleic acids have poor pharmacokinetic profiles and are subject to rapid ***systemic elimination***. Elimination of degraded fragments through renal glomerular filtration seems to be the prevailing mechanism of clearance of nucleic acids when administered intravenously (Liu et al., 2007; Soutschek et al., 2004; Van de Water et al., 2006). However, an alternative intestinal elimination route for siRNAs after liver accumulation has recently been suggested (Huang et al., 2011). Introducing chemical modifications or bioconjugation of synthetic siRNAs (Bumcrot et al., 2006; Choung et al., 2006; Gao et al., 2009b; Soutschek et al.,

2004) and/or complexing with appropriate non-viral vectors (O'Mahony et al., 2013a; Pulford et al., 2010) have been shown to significantly improve stability and circulation half-lives (t1/2). However, unmodified, modified and/or vector-formulated siRNAs can be rapidly phagocytosed by the ***mononuclear phagocyte system*** (MPS) distributing to organs of the ***reticuloendothelial system*** (RES)**,** such as the liver and the spleen (Guo et al., 2011). This may happen as a result of the interaction with components of the blood, including immunoglobulins, components of the complement cascade and other proteins, subsequently leading to an extensive accumulation in RES organs (Wang et al., 2010a).

Upon reaching a specific organ, siRNAs need to overcome the ***vascular endothelial barrier*** and reach tissue interstitium. Extravasation to specific tissues is strongly dependent on the pore size limit of the capillary and specific characteristics of the endothelium of that organ. For instance, fenestrated/discontinuous endothelium of the liver present large pore sizes of ~150 nm whereas the continuous endothelium in the retina and in the brain form tight junctions with pore sizes <2 nm (Singh et al., 2011; Wang et al., 2010a). The latter constitute the so-called ***blood brain barrier*** (BBB), which limits free diffusion of nutrients, drugs and nanoparticles to the brain. Although less restrictive, the spinal cord possesses the ***blood-cerebrospinal fluid barrier*** (BCSFB) formed by the choroid plexus epithelial cells which also modulate transport to the CNS (Johanson et al., 2011). Effectively overcoming the BBB and/or the BCSFB constitutes one of the major hurdles in delivery to the CNS and is strongly dependent on the characteristics of the RNAi delivery system (O'Mahony et al., 2013b). Several strategies that are being investigated to enhance circulating times and targeting across CNS barriers are discussed in Section 1.11.3.

After crossing the epithelial barrier, RNAi vectors have to traverse the ***extracellular matrix*** (ECM), containing structural proteins and polysaccharides, in order to interact with target cells. Increased deposition of ECM macromolecules in certain disease states (such as cancer) may significantly hinder transport, reducing uptake of drug carriers and increasing the

likelihood of phagocytosis by tissue macrophages (Singh et al., 2011; Zámecník et al., 2004).

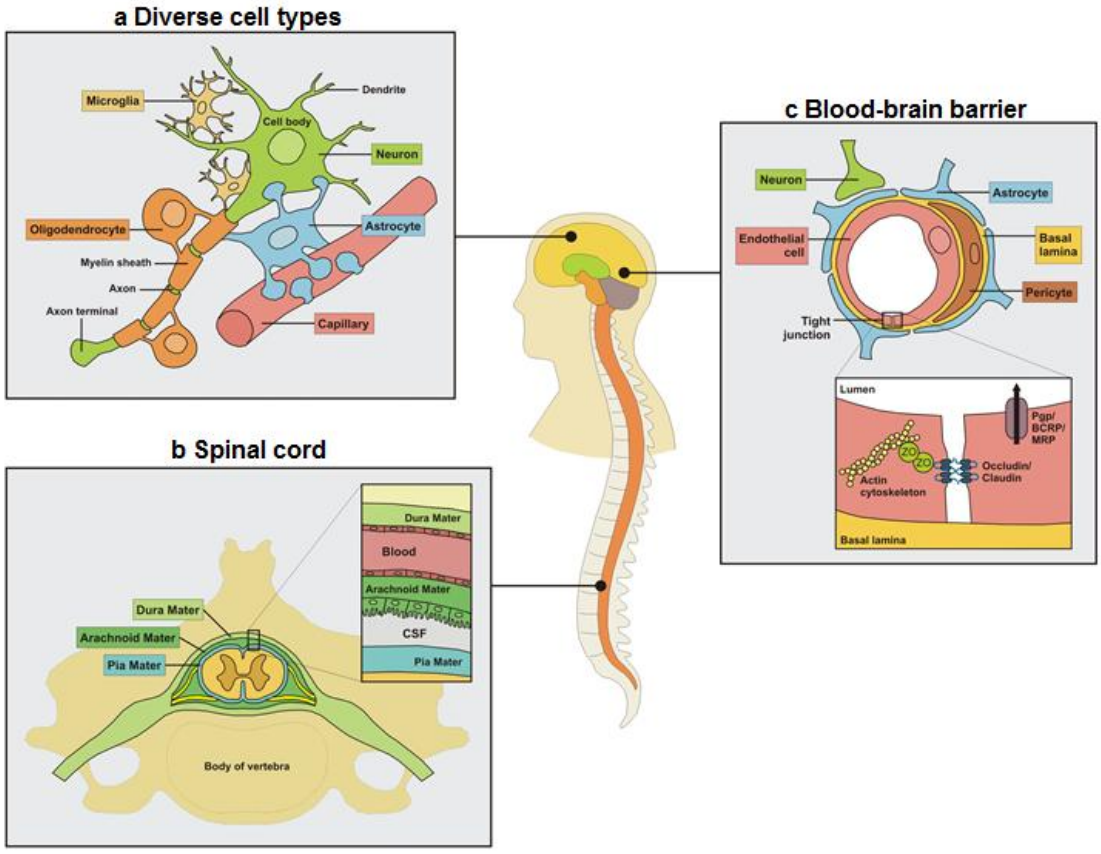


Figure 1.4. Challenges to delivery RNAi therapeutics to the CNS by non-viral vectors. *(O'Mahony, Godinho et al. 2013b). The CNS includes the brain and spinal cord and consists of various different cell types, all of which contribute to the difficulty in achieving effective delivery of nucleic acids. Highlighted here are. (a) diverse cell types, (b) the spinal cord and (c) the blood–brain barrier, including neurons, astrocytes and other glial cells.*

1.10.2 Cellular uptake and endosomal release

Nucleic acids, such as siRNAs and shRNA, have poor ***plasma membrane*** permeability due to their size, high Mw and net negative charge (phosphate backbone) which restrict passive diffusion (Whitehead et al., 2009). Formulation of nucleic acids using cationic non-viral vectors yields particles with net positive charges that significantly increase cellular uptake. The majority of positively charged siRNA.nanoparticles interact with anionic heparin sulphate proteoglycans (syndecans) on cellular membranes through electrostatic interactions, thereby facilitating endocytosis (Benfer et al., 2012; Evans et al., 2011; Lu et al., 2009;

Madani et al., 2011; O'Neill et al., 2011). However, specific characteristics of plasma membranes, such as protein and lipid constitution, may vary significantly among different cell types and in some instances significantly affect cellular uptake (Alberts et al., 2002). In the context of the CNS, ***multiple and different cell types*** exist, including neurons, microglia and astrocytes, which may vary in their ability to endocytose naked or formulated nucleic acids. Indeed, primary cultured astrocytes and microglial cells have previously presented differential cellular uptake profiles of lipid-formulated siRNA (Ki et al., 2010). In addition, neurons have been reported to be notoriously difficult to transfect for reasons not yet fully understood (Krichevsky et al., 2002; Ohki et al., 2001). Although neuronal uptake of siRNA nanoparticles has been reported to primarily occur at the cell soma, internalization has also been shown to occur at the neurites (Bergen et al., 2008b). In most cases, following cellular uptake, cholesterol modified siRNAs or cationic nanoparticles are localised inside endocytic vesicles. ***Endosomal escape*** of siRNAs or shRNAs is crucial to avoid lysosomal degradation and to achieve higher levels of gene expression knockdown. In this regard, the use of non-viral vectors containing pH-sensitive polymers has been shown to aid nucleic acid release by causing endosomal swelling and rupture (Agrawal et al., 2009; Creusat et al., 2010). This is believed to occur by means of a "proton sponge effect", whereby multiple amine groups become protonated upon acidification of endosomes. This buffers the pH inside the late endosome driving inward currents of protons, along with chloride and water leading to osmotic rupture (Agrawal et al., 2009; Creusat et al., 2010). Other examples of strategies to improve endosomal release are: (i) the inclusion of fusogenic lipids, such as dioleoylphosphatidylethanolamine (DOPE), which will fuse with the endosomal membrane enabling release of the nucleic acids (Farhood et al., 1995; Litzinger et al., 1992); and (ii) the use of fusogenic peptides, such as GALA and influenza-derived fusion domain based on N-terminal hemagglutinin (HA2), which mimic the interaction of viral envelops with the endosomal membrane causing its destabilization (Hatakeyama et al., 2009; Navarro-Quiroga et al., 2002; Oliveira et al., 2007; Wadia et al., 2004).

The ***nuclear membrane*** is an additional barrier faced by shRNAs in order to elicit gene silencing effects. Translocation of pDNA and shRNAs to the nucleus has been achieved using nuclear localization signal peptides, such as VP1 capsid protein from the simian virus 40 (SV40) and bis- and trisacridine conjugates of nuclear localization signal peptides (Navarro-Quiroga et al., 2002; Shiraishi et al., 2005). On the other hand, using appropriate viral vector, such as adeno-associated viruses (AAV) and/or lentiviruses, shRNAs can be delivered directly in the nucleus (Harper et al., 2005; Rubinson et al., 2003).

1.11 Improving RNAi delivery to the CNS

The major setback for the application of RNAi technologies in the treatment of CNS disorders is the lack of effective and non-toxic strategies for delivery. Neurons are hard to transfect and this is most likely due to their post-mitotic nature (Bergen et al., 2008a; Ohki et al., 2001). Indeed, relatively high doses have been required to induce gene silencing effects in primary neuronal cultures but also when administering to the brain *in vivo*.

In order to improve delivery to the CNS, various strategies have been extensively evaluated. Among these, the introduction of chemical modifications in siRNAs and the use of appropriate delivery vectors, such as viral and non-viral vectors, have been given considerable attention. In this section we will overview the most widely used (i) delivery methods for RNAi, (ii) routes of administration and (iii) strategies to improve systemic brain delivery.

1.11.1 Delivery methods for RNAi in the CNS

1.11.1.1 Chemically modified siRNAs

siRNAs are amenable to chemical modifications at several sites, including their ribose moieties and phosphate backbone (Bumcrot et al., 2006; Choung et al., 2006; Guo et al., 2010b). Indeed, different chemical modifications have been introduced to siRNAs to enhance stability against enzymatic degradation and to reduce immunogenicity (Bumcrot et

al., 2006; Choung et al., 2006). These modifications have been extensively reviewed elsewhere (Behlke, 2008; Watts et al., 2008). As an example of such, the 2'-*O*-methylation of the ribose moiety is usually used to confer resistance to nucleases but also to prevent recognition of the siRNA by the immune system (Judge et al., 2006). On the other hand, modifications to enhance delivery have also been carried out. In fact, cholesterol conjugation to the antisense strand aided siRNA delivery in the striatum and corpus callosum, after brain administration (Chen et al., 2010; DiFiglia et al., 2007). Additionally, conjugation of sertraline to the sense strand enabled specific targeting upon intranasal administration (Bortolozzi et al., 2012).

However, since modifications may impact on gene silencing potency there are limits to the extent to which modifications can be carried out (Behlke, 2008; Choung et al., 2006). In addition and for obvious reasons, sense strands can be more extensively modified than antisense strands without significant reductions in gene silencing efficiency (Choung et al., 2006).

1.11.1.2 Viral delivery

Recombinant viral vectors have been widely applied in gene therapy approaches as they present great tropism across a broad range of cell types, including neurons (Davidson et al., 2003; Thomas et al., 2003). Recently, viruses have also been considered for mediating RNAi delivery to the CNS (Raoul et al., 2005b). Briefly, the concept is based on the use of a shRNA encoded in the viral genome that, once inside the nucleus, is transcribed by the host's transcriptional machinery and exported to the cytoplasm for gene silencing. Recombinant AAV and LV vectors have been by far the most widely used viral vectors for RNAi in the CNS (Davidson & Breakefield, 2003; Lentz et al., 2012).

AAV are small (~20 nm) ssDNA viruses derived from human parvovirus and devoid of viral genes. Several serotypes have been generated, but AAV1, AAV2 and AAV5 are the most widely used for CNS gene and RNAi delivery. Great tropism across large areas of the brain

and significant knockdown of target genes has been reported. Although AAV does not commonly integrate to the host's genome, sustained transgene expression in the brain has been reported (Klein et al., 1999). Probably due to its low immunogenic profile, AAV has been widely used in many pre-clinical RNAi studies for neurodegenerative diseases, such as HD (Grondin et al., 2012; Harper et al., 2005), Parkinson's Disease (PD) (Khodr et al., 2011) and SCA 1 (Xia et al., 2004). Additionally, it is also worth noting that AAV delivery systems have also been successfully used in the clinical setting for gene replacement therapies in the human retina (Bainbridge et al., 2008; Maguire et al., 2008) and for haemophilia B (Nathwani et al., 2011), presenting good expression levels of the transgenes and no major adverse effects. Although it might constitute a major drawback for some gene replacement approaches, the limited packaging capacity of AAV (~4.2 kb) is not a problem for RNAi since shRNA constructs are relatively small (Gonzalez-Alegre, 2007). Thus, AAVs are very attractive delivery systems to conduct RNAi in the CNS.

LV, commonly derived from the human immunodeficiency virus, are ssRNA viruses and are of particular interest for CNS delivery since they are capable of transducing post-mitotic cells, such as neurons (Davidson & Breakefield, 2003; Lentz et al., 2012). In addition, these retroviruses have the advantage of integrating into the host cell genome. This has allowed for long-term/stable expression of the shRNA in the striatum (Van den Haute et al., 2003). LV have also been shown to undergo retrograde transport through motor neurons to the spinal cord and brain stem after focal injections to the muscle (Ralph et al., 2005b). Successful application of LV in various preclinical RNAi studies for neurodegenerative diseases, such as HD (Drouet et al., 2009), PD (Sapru et al., 2006), ALS (Ralph et al., 2005b) and AD (Singer et al., 2005), has been reported. Despite the many advantages, the main drawback of this vector system is that it can cause insertional mutagenesis by activation of cellular proto-oncogenes.

Other viruses, such as neurotrophic herpes simplex virus – 1 (HSV-1) and recombinant adenoviruses also mediate shRNA delivery to the CNS. Indeed, significant gene silencing

effects were shown in a murine model of AD using HSV-1 viral particles encoding shRNA against β-amyloid peptide (Hong et al., 2006a). On the other hand, recombinant adenoviruses have been stereotaxically injected into the R6/2 mouse model of HD models with successful reduction in HTT aggregate load (Huang et al., 2007a). However, the development of adenoviral vectors for brain delivery of shRNA has been delayed due to their strong immunogenicity (Wood et al., 1996).

Viral vectors are the most widely used delivery vectors in gene and RNAi clinical trials (Ginn et al., 2013; Thomas et al., 2003). Nevertheless, and broadly speaking, immunogenicity, neutralizing Ab and the high cost of large-scale production are still the main obstacles and need further consideration.

1.11.1.3 Non-viral delivery

As an alternative to viral vectors, several types of non-viral vectors have been developed for RNAi delivery to the CNS (O'Mahony et al., 2013b). In the vast majority of cases, these consist of cationic nanosystems which rely on electrostatic interactions with negatively charged nucleic acid for self-assembly. Furthermore, the net positive charge of the siRNA nanoparticle enables interaction with cellular membranes and internalization. Subsequently, siRNA is released to the cytoplasm where it is available for gene expression knockdown (O'Mahony et al., 2013b).

Figure 1.5 contains a schematic representation of selected non-viral vectors for RNAi delivery in the CNS. Non-viral vectors for neuronal nucleic acid delivery have recently been reviewed elsewhere (Bergen et al., 2008a; O'Mahony et al., 2013b; Posadas et al., 2010). Thus, an exhaustive description of all existing non-viral vectors is out of the scope of this thesis; instead a focused overview of the most widely used non-viral vectors for RNAi in the CNS will be given.

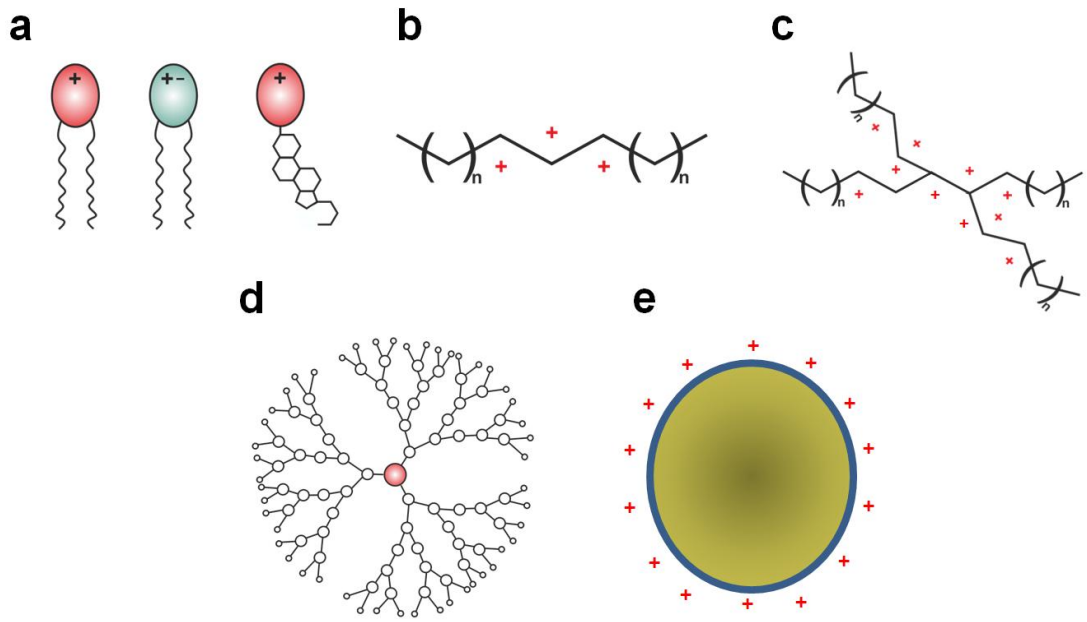


Figure 1.5. Schematic diagrams of selected delivery systems. *(a) Lipid-based delivery systems including cationic and neutral lipids with long aliphatic chains, and cationic non-viral vectors containing cholesterol domains. (b) Cationic linear and (c) branched natural and/or synthetic polymers. (d) Hyperbranched symmetrical synthetic dendrimers. (e) Gold nanoparticles.*

- ***Lipids***

Cationic synthetic lipids have been by far the most widely used non-viral vectors for CNS delivery of nucleic acids (Bergen et al., 2008a). These are amphiphilic molecules typically consisting of a cationic hydrophilic head group (mono- or multivalent), a hydrophobic lipid moiety, and a linker group. Long aliphatic chains or cholesterol domains have been adopted as hydrophobic moieties (Figure 1.5a). Dioleyloxypropyltrimethylammonium (DOTMA), dioleoylsperminecarboxamidoethyldimethylpropanetrifluoroacetate (DOSPA) (Lipofectamine®) and dioleoyltrimethylammoniumpropane (DOTAP) are examples of cationic lipids containing long aliphatic chains, whereas the newer dimethylaminoethanecarbamoyl cholesterol (DC-Chol) is an example of cationic lipid containing cholesterol.

The first transfection *in vitro* using cationic lipids was reported for pDNA using DOTMA (Felgner et al., 1987). Although in early studies low transfection efficiencies where achieved

in primary neuronal cultures with DOTAP and DOSPA (~3%) (Kaech et al., 1996), subsequent formulations of DOSPA with a neutral "helper" lipid DOPE improved delivery (~27%) (Ohki et al., 2001). Indeed, incorporation of fusogenic lipid DOPE is believed to aid endosomal escape (Farhood et al., 1995). However, recently DOTAP has achieved successful gene expression knockdown when delivering siRNA to the adult mouse brain (Salahpour et al., 2007), suggesting that unsuccessful translocation of pDNA to the nucleus might have been the cause of early low trasnfection. Lipofectamine®2000 (Lf2000) (DOSPA:DOPE formulation) has also proved useful for enabling RNAi with siRNA and shRNA (encoded in plasmid DNA) in primary neuronal cultures (Dalby et al., 2004; Kao et al., 2004; Krichevsky & Kosik, 2002; Lingor et al., 2004; Omi et al., 2004; Tönges et al., 2006; Yu et al., 2002). Due to its relatively good efficiency, Lf2000 is commonly used as standard transfection reagent for comparison when developing novel non-viral vectors. Furthermore, Lf2000 has been used in several RNAi studies *in vivo* and enabled significant gene expression knockdown after brain administration (Fu et al., 2007; Lei et al., 2008; Wang et al., 2005). Other proprietary amphiphilic cationic molecules, such as INTERFERin®, have also enabled gene silencing in primary neuronal cultures and in the mouse brain (Badaut et al., 2011; Rohn et al., 2012).

Different formulation methods have been employed to enhance the steric stability of cationic nanoparticles and/or to further functionalise the vector for targeted delivery (Cardoso et al., 2007; Pulford et al., 2010). Despite their relatively good efficiency, cationic lipid-based delivery systems have been usually associated with cytotoxic effects (Lv et al., 2006).

- ***Polymers***

Both synthetic and natural polymers have also been used to facilitate gene and RNAi delivery in the CNS (Bergen et al., 2008a) (Figure 1.5b). Polyethylenimine (PEI) and poly-L-lysine (PLL) are examples of synthetic polymers whereas chitosan and polylactic-co-glycolic acid (PLGA) are examples of natural polymers. PLL, PEI and chitosan are cationic

polymers with nucleic acid condensing capabilities, while PLGA protects nucleic acid by entrapment.

PEI's repeating unit is composed of an amine group and two carbon aliphatic CH_2CH_2 spacer (Park et al., 2006; Smedt et al., 2000). Linear and branched forms, as well as low and high Mw PEI, have been used for gene and RNAi delivery applications in the CNS (Bergen et al., 2008a). Early studies with high Mw PEI showed successful gene transfer to chick embryonic neurons and in the neonatal mouse brain (Boussif et al., 1995). In general, high Mw PEI enables greater transfection probably due to higher number of protonable amino groups available in the structure which aids endosomal release of the nucleic acid to the cytoplasm. siRNA (Campbell et al., 2012; Tan et al., 2004; Wang et al., 2005) and shRNA (encoded in pDNA) (Hassani et al., 2007) have been successfully delivered to the brain in preclinical studies using high Mw PEI. However, the high level of cytotoxicity is one of the main disadvantages of PEI. As an example, high Mw PEI (ExGen500) delivered to the R6/2 mouse model of HD proved to be more efficient than Lf2000, however it was also more toxic (Wang et al., 2005). Chemical modifications have been introduced and shown to reduce, in part, its toxicity and aid targeting to neurons (Park et al., 2007; Son et al., 2011; Zeng et al., 2007).

PLL is a linear polymer with L-lysine repeating units (Park et al., 2006; Smedt et al., 2000). At physiological pH, the primary amines within the polymer are fully protonated and therefore PLL has poor endosomal escape abilities when compared to PEI and dendrimers (Sonawane et al., 2003). However, functionalization with targeting ligands such as neurotensin, have shown potential to improve delivery to the rat brain (Gonzalez-Barrios et al., 2006; Martinez-Fong et al., 1999).

Chitosans are linear polysaccharides consisting of D-glucosamine and N-acetyl-D-glucosamine units connected through glycosidic bonds. This polymer is produced by deacetylation of chitin, it exists in a wide range of Mw (3.8-2000 kDa) and has been largely

applied for siRNA delivery (Rudzinski et al., 2010). The interest in this polymer might have arisen from its nucleic acid binding abilities and high biocompatibility. Indeed, chitosans in general present good toxicity profiles probably due to their biodegradability by lysozymes (Nordtveit et al., 1996; Varum et al., 1997). However, a significant limitation of unmodified chitosans is their low transfection efficiency and therefore several strategies have been adopted to improve delivery (Ishii et al., 2001; Mansouri et al., 2004). As an example, imidazole-modified and targeted chitosans have improved gene delivery to neuronal-like cell lines and also dorsal root ganglia primary neurons (Oliveira et al., 2010). Furthermore, modified-chitosan nanoparticles incorporated onto a biofunctionalized microfiber implant have shown significant knockdown and good biocompatibility when compared to Lf2000 in PC12 neuronal-like cells (Mittnacht et al., 2010). In addition, studies with targeted-chitosan nanoparticles have also improved delivery to Neuro2a cells (Malhotra et al., 2013).

- ***Dendrimers***

Dendrimers are synthetic polymers with a hyperbranched symmetrical structure derived from a central core (single atom of group of atoms) (Figure 1.5c) (Dufès et al., 2005; Perez-Martinez et al., 2012). Dendrimers display a globular shape and tend to have high solubility and surface reactivity that allows for nucleic acid complexation (Posadas et al., 2010). Dendrimers are also believed to avail of the "proton sponge effect", initially described for PEI, to enhance endosomal release (Haensler et al., 1993). Moreover, the geometric progression of the concentric layers allows for the classification of dendrimers in generations (G) (Dufès et al., 2005; Perez-Martinez et al., 2012). Although polyamidoamine (PAMAM) dendrimers have been by far the most widely used in gene and RNAi delivery to primary neurons and to the brain (Agrawal et al., 2009; Huang et al., 2008; Ke et al., 2009; Kim et al., 2010a; Kim et al., 2006; Liu et al., 2009; Rodrigo et al., 2011), carbosilane dendrimers have also been successfully applied (Posadas et al., 2009). In general, high generation dendrimers have been found to be more effective than low generation, but they have also been associated with higher toxicities (Haensler & Szoka Jr, 1993; Omidi et al.,

2005). Functionalization approaches have been employed to reduce their cytotoxicity and to enhance targeted delivery to neuronal cells (Posadas et al., 2010).

- ***Other novel nanoparticles***

In addition to the well-established non-viral vectors, other novel nanoparticles for siRNA are emerging for RNAi delivery to the CNS. *Carbon nanotubes* consist of graphene sheets arranged in concentric layers and they can be single- or multi-walled (Lacerda et al., 2008). Although further investigations are needed on the mechanisms of siRNA binding and internalization in neurons, this vector has been successfully used in primary neuron cultures and *in vivo* after brain administration (Al-Jamal et al., 2011; Ladeira et al., 2010; Ren et al., 2012). Furthermore, brain-targeted nanotubes have also been successfully administered systemically to deliver other drug loads, such as doxiciclin, to the mouse brain (Ren et al., 2012).

Other nanoparticles based on inorganic elements, such as gold nanoparticles (Figure 1.5d), have also been considered for siRNA delivery in the CNS. In addition to efficient gene expression knockdown in primary neuronal cultures and in the brain after direct injections, these nanoparticles have been shown to effectively traverse an *in vitro* model of the BBB (Bonoiu et al., 2011; Bonoiu et al., 2009; Wang et al., 2010b).

Finally, functionalized cyclodextrins (CDs) have been recently applied to deliver siRNA to primary hippocampal neurons (O'Mahony et al., 2012b). A more detailed description about CDs and their use as non-viral vectors for gene and siRNA delivery is given in Section 1.14.

1.11.2 Routes of administration for RNAi delivery to the brain

The selection of an appropriate route of administration is critical for the successful delivery of RNAi to the CNS (O'Mahony et al., 2013b). In the first instance, the route of administration determines the interaction of nanoparticles with specific organs and tissues (Karra et al., 2012). In addition, the delivery route impacts on the dose and length of

treatment and subsequently on eventual side effects of the therapy. Choosing a route of administration is largely dependent on the target structure within the CNS to be treated; the capabilities of the delivery vector; and the need for localised or widespread gene expression knockdown.

Direct injections into the brain parenchyma have been largely employed when localised gene silencing effects are required. In rodents, intraparenchymal injections of naked or formulated siRNAs have been carried out to induce gene silencing effects in structures such as the striatum (Cardoso et al., 2010; Cardoso et al., 2008; Salahpour et al., 2007), hypothalamus (Makimura et al., 2002), hippocampus (Bonoiu et al., 2011), nucleus accumbens (Jean et al., 2007), cortex (Badaut et al., 2011; Kim et al., 2010a) and basolateral amygdala (Fu et al., 2007). Accurate injections to such specific structures enabled the use of significantly lower siRNA doses (for example, 0.2 µg (Manrique et al., 2009), 0.4 µg (Badaut et al., 2011), 3 µg (Cardoso et al., 2008)) when compared to other administration methods. It is worth noting that gene silencing effects in these studies were mostly short-lived and restricted to the site of injection (Jean et al., 2007). ***Convection enhanced delivery*** (CED) consists of intraparenchymal infusions that employ the local positive pressure gradient from the effluent (vehicle) to enhance distribution of particles in the brain interstitium (Bobo et al., 1994). Delivery of siRNA by CED using osmotic minipumps has resulted in widespread suppression of gene expression in the mouse and primate brains with the highest levels of knockdown achieved at the site of infusion (Agrawal et al., 2009; Kato et al., 2010; Stiles et al., 2012). Biodistribution and gene expression knockdown is likely to be dependent on the type of catheter used, the dose and length of treatment. Distribution of nanoparticles in the brain by CED has been found to be highly reproducible and predictable (Krauze et al., 2008). This delivery strategy was initially developed to treat brain tumors (Allhenn et al., 2012; Barnett et al., 2007), but is now being considered as a potential administration method to treat other brain disorders, such as HD (Stiles et al., 2012). ***Injections or infusions into intracerebroventricular*** *(i.c.v.)* ***system*** have also been

extensively used to deliver siRNAs to the brain in the pre-clinical setting. In rodents, i.c.v. injections into the lateral ventricle (Hayakawa et al., 2012; Wang et al., 2005) and infusions in the dorsal third ventricle (Bortolozzi et al., 2012; Senechal et al., 2007; Thakker et al., 2004; Thakker et al., 2005) using osmotic minipumps enabled wide spread gene silencing in the brain. In general, administration through the i.c.v. system allows for the use of higher volumes and/or higher doses. Indeed, relatively high doses of siRNA have been used in most studies when using i.c.v. infusions (for example, Thakker et al 0.4 mg/day (Thakker et al., 2004)). The additional ventricular epithelial barrier and dependence on diffusion of siRNAs to the brain parenchyma might have been the underlying causes for the use of higher doses. Intraparenchymal injections, CED and i.c.v. infusions are possible through well-established stereotactic brain surgical techniques which allow delivery of therapeutics to specific regions within the brain (Athos et al., 2001; Cetin et al., 2006).

Although less explored ***intranasal administration*** has also been considered as potential delivery route for nanoparticle-based therapeutics (Ali et al., 2010; Lochhead et al., 2012; Pardridge, 2007a). Transport to the brain has been suggested to occur mainly by intracellular uptake by the olfactory or trigeminal ganglion nerves and subsequent transport to the olfactory bulb, or extracellularly across the arachnoid membrane into the olfactory cerebrospinal fluid (Allhenn et al., 2012; Lochhead & Thorne, 2012; Pardridge, 2007a). Trans-nasal delivery of siRNA in rodents has resulted in efficient siRNA uptake in the olfactory bulb but also in deeper regions of the mouse brain, such as amygdala, raphe nuclei and hypothalamus (Bortolozzi et al., 2012; Kim et al., 2012a; Perez et al., 2012; Renner et al., 2012). Gene silencing effects have also been reported in several studies (Bortolozzi et al., 2012; Kim et al., 2009b; Kim et al., 2012a). Intranasal and stereotactic-based delivery allow the BBB, one of the major limiting factors in the progress of RNAi and gene therapy for brain disorders, to be circumvented (Cetin et al., 2006).

Intravenous delivery of RNAi is among the most convenient and less invasive methods of administration, however the systemic route poses additional challenges to siRNA delivery

(see Section 1.10.1). In addition, due to difficulties targeting the brain when using the systemic route, higher doses of siRNA or formulated siRNA nanoparticles might be required to achieve the desired gene expression knockdown effect (O'Mahony et al., 2013b). This may increase the likelihood of toxicity in organs such as blood, liver, lungs and spleen. Despite the recent developments on strategies to effectively overcome the BBB (discussed in Section 1.11.3.2), transvascular delivery of RNAi to the brain is still limited and very challenging.

1.11.3 Strategies to improve systemic brain delivery

Systemically administered siRNA nanoparticles should have suitable physicochemical properties that allow for enhanced circulating times and/or facilitate translocation to the brain. However, in most cases, when administered intravenously cationic siRNA nanoparticles are rapidly opsonised and removed by the MPS (Gref et al., 2000; Owens III et al., 2006). Additionally, these nanoparticles also have poor stability in physiological conditions leading to aggregation, premature unpacking of the siRNA cargo and rapid subsequent removal from the blood circulation (O'Mahony et al., 2013b). Thus, multiple strategies are being investigated to improve blood residency times and/or improve brain delivery by translocation across the BBB.

1.11.3.1 Strategies to improve stability and circulating times

PEGylation is one of the most common approaches used to increase stability and circulating times of nanoparticles and involves the use of polyethylene glycol (PEG), a polymer of ethylene oxide, to modify the surface of the nanoparticles. PEG is a hydrophilic polymer that shields the positive charge at the surface of cationic nanoparticles and reduces aggregation (Owens III & Peppas, 2006). PEGylation confers steric hindrance by creating a "hydrophilic cloud" which reduces opsonisation and capture by the MPS (Gref et al., 2000; Owens III & Peppas, 2006). A variety of delivery systems including, lipid- (Li et al., 2007), PEI- (Kunath et al., 2002), PLL- (Guo et al., 2012a), and CD-based (O'Mahony et al., 2013a; Pun et al.,

2002) nanoparticles have been successfully PEGylated, and have shown improved stability and circulating $t_{1/2}$ *in vivo*. Indeed, in order to improve stability of these nanoparticles, different PEG lengths and polymer densities at the surface have been evaluated, however, no general consensus has yet been reached on the ideal PEG length or density (Gref et al., 2000; Kunath et al., 2002; Mao et al., 2006a). Furthermore, this is likely to be dependent on the vector type, and also on the PEGylation strategy adopted for modification of the nanoparticles. Finally, in this regard, a wide variety of methods have been developed, spanning from chemical to physical methods, to covalently attach PEG to the delivery system or to incorporate the polymer in a pre-formed assembled siRNA nanoparticle through post-modification methods (e.g. post-insertion) (O'Mahony et al., 2013b; Wang et al., 2012).

Despite its usefulness in enhancing the stability and circulating times of siRNA nanoparticles (Li et al., 2007), PEGylation reduces interaction with cell membranes and may consequently impact on uptake by relevant tissues (Davis, 2009; O'Mahony et al., 2013d; O'Mahony et al., 2012d). Moreover, PEGylation as a strategy is not designed to enhance brain delivery *per se* thus it must be coupled with alternative methods to enable penetration to the brain such as by transient disruption of the BBB; receptor-mediated transport across the BBB using targeting ligands or monoclonal Ab coupled to the nanosystem; or through cell penetrating peptides (O'Mahony et al., 2013b). Figure 1.6 displays selected mechanisms to overcome the BBB which are currently under investigation for non-viral gene and RNAi delivery to the brain (O'Mahony et al., 2013b).

1.11.3.2 Strategies for translocation across the blood brain barrier

Transient disruption of the BBB has been previously applied to enhance delivery of conventional pharmaceuticals. To this end, mannitol, a sugar alcohol derived from mannose, has been used to cause osmotic shrinkage of endothelial cells and facilitate extravasion of drugs and nanoparticles across the BBB (Hwang et al., 2011; Iwadate et al., 1993). Indeed, in the context of RNAi, this method has been used to enhance delivery of modified-PEI

nanoparticles carrying miRNA to the brain (Hwang et al., 2011). Alternatively, magnetic resonance image (MRI)-guided focused ultrasound, another method to widen BBB tight junctions, has also improved delivery of cholesterol conjugated siRNAs to the rat brain (Burgess et al., 2012). However, the safety of these approaches to enable chronic administrations of siRNA still remains to be elucidated.

Targeting ligands have also been used to enhance neuronal uptake and targeting across the BBB. Attachment of certain ligands to the delivery system, such as rabies virus glycoprotein peptide (RVG) (Alvarez-Erviti et al., 2011; Kumar et al., 2007; Pulford et al., 2010), transferrin (Tf) (Cardoso et al., 2007; Cardoso et al., 2008), lactoferrin (LTF) (Huang et al., 2010) and angiopep (Ke et al., 2009), have been shown to enhance cellular uptake in neuronal cells or in the brain. Targeted-nanoparticles associate with cell-surface receptors and internalized through a receptor-mediated endocytotic process, whereas untargeted nanoparticles rely on unspecific electrostatic interactions (Sahay et al., 2010; Wang et al., 2011). After transvascular delivery, targeted-nanoparticles have also been successfully transported across the BBB and delivered their nucleic acid into the brain, successfully achieving gene expression or gene expression knockdown (e.g. (Kumar et al., 2007). Although further investigations are needed, transcytosis is believed to be the main mechanism involved. Similarly, the "molecular Trojan horse" strategy employed ***peptidomimetic monoclonal antibodies*** (mAb) which target specific receptors expressed in the BBB and in neuronal cells. Indeed, TFR- or human insulin receptor (HIR)- Ab grafted on the tip of PEG2000 chains of anionic liposomes – PEGylated immunoliposomes – have facilitated shRNA delivery to intracranial tumors (Zhang et al., 2004). TFR Ab enabled targeting across the BBB, while HIR Ab enabled delivery to human glioma cancer xenografts (Zhang et al., 2004). Additionally, Ab against the p75 neurotrophin receptor (p75NTR) have been conjugated to a modified-PLL vector and successfully delivered shRNAs against TrkA in the rat brain (Berhanu et al., 2008).

Cell penetrating peptides such as penetratin and transcription-transactivating (TAT) protein of the HIV virus, have also been described for delivery of drugs and proteins into the brain (Heitz et al., 2009; O'Mahony et al., 2013b). Despite recent encouraging *in vitro* results using Tat-modified chitosans to silence the SCA1 gene, implicated in a spinocerebellar neurodegenerative disease (Malhotra et al., 2013), *in vivo* application of cell penetrating peptides for RNAi needs further investigation. Finally, other novel approaches such as using magnetic force to traverse the BBB are being considered for brain therapeutics and diagnostics, and may be applicable to brain-targeted RNAi (Kong et al., 2012).

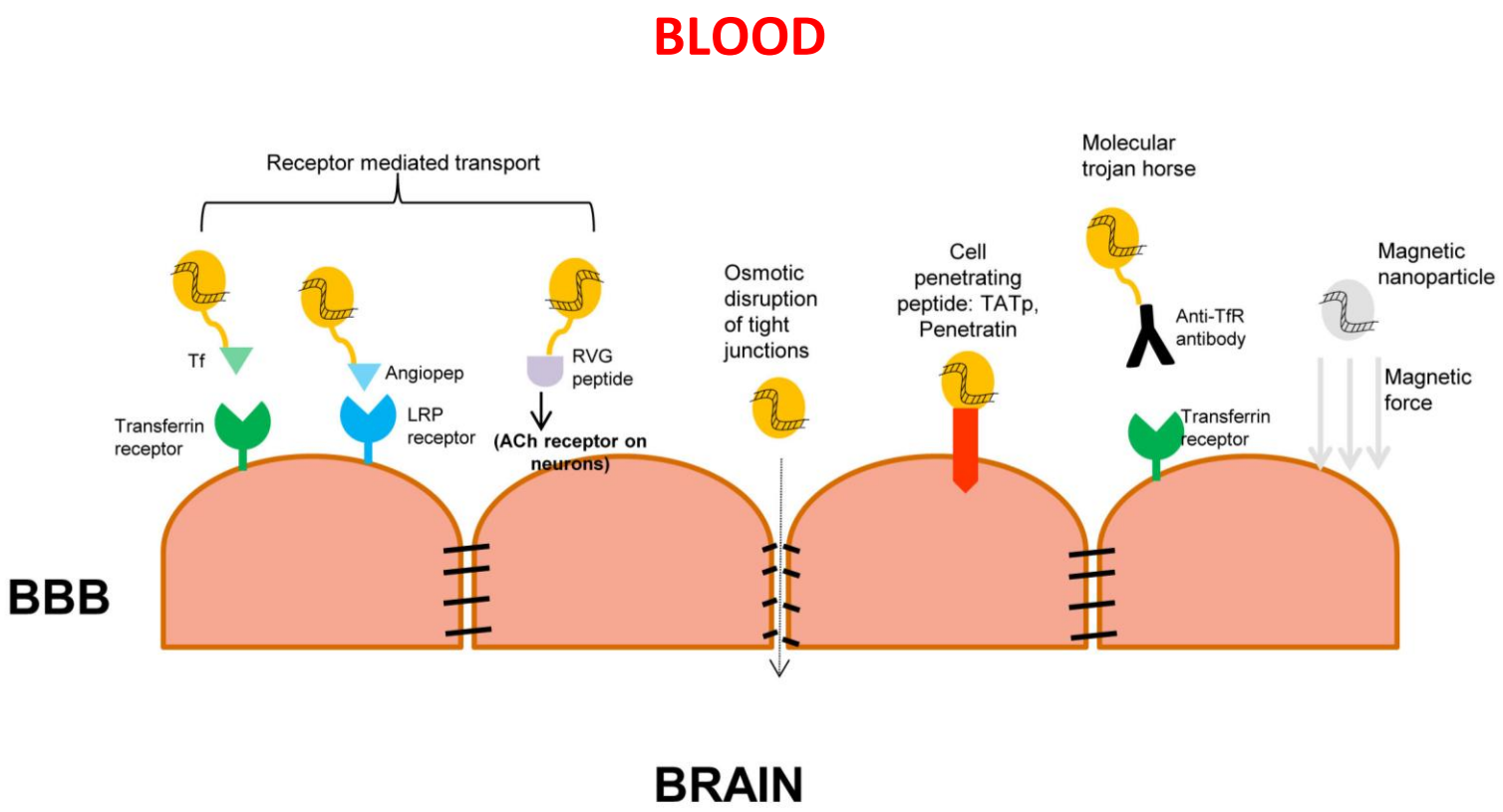


Figure 1.6. Selected examples of the mechanisms for transport of non-viral gene delivery vectors from the systemic circulation across the blood–brain barrier into the brain. *(O'Mahony, Godinho et al. 2013b). These include receptor-mediated transport, molecular Trojan horse approach, osmotic disruption of tight junctions, cell-penetrating peptides and magnetic force.* ***Abbreviations****: Ach, Acetylcholine; BBB, Blood brain barrier; LRP, lipoprotein receptor-related protein; RVG, Rabies Virus Glycoprotein; TATp, transcripition-transactivating protein; Tf, transferrin; TfR, transferrin receptor.*

1.12 Limitations of RNAi

RNAi technologies are, without question, a useful tool to study gene function, however translation of such technology as a therapeutic to the clinic still presents some challenges. "Off-target effects", saturation of the RNAi machinery and the lack of efficient and non-toxic delivery vectors are the main obstacles in the progress of this technology (O'Mahony et al., 2013b).

1.12.1 "Off-target" effects

"Off-target effects" can arise from hybridization of the antisense strand (or in some cases the sense strand) to other non-target mRNA transcripts or from undesired activation of components of the immune system. The so-called "miRNA-like off-target effects" whereby siRNA acts as an endogenous miRNA regulating gene expression by partial complementarity, can induce unwanted gene silencing effects. In fact, it has been found that one or two perfect matches between 2^{nd} -7^{th} nucleotide (or 2^{nd} -8^{th} nucleotide) of the antisense strand and the 3'-UTR of an unrelated mRNA are all that is required for off-target gene silencing (Jackson et al., 2006). These undesired effects can induce false-positive phenotypes, induce toxicity and considerably complicate the interpretation of therapeutic outcomes (Jackson et al., 2010). Furthermore, microarray data have shown that "off-target" effects occur in a siRNA concentration-independent fashion, meaning that reducing dose alone will not be enough for reducing "off-target effects" (Jackson et al., 2003). On the other hand, conflicting microarray studies have shown that unspecific gene silencing is more likely to occur in a dose-dependent manner and therefore the use of a minimal dose is critical (Persengiev et al., 2004). Thus, rational algorithmic design tools should be used to guarantee correct loading of the antisense strand to the RISC (G/C content influences thermodynamic selection of strands), to generate highly complementary siRNA/shRNA to their target mRNAs, but also to ensure low potential for cross-hybridization with untargeted mRNA transcripts (Gong et al., 2004; Naito et al., 2004; Reynolds et al., 2004). On the other

hand, long dsRNAs (>30 bp) are known to induce host immune response by activation of the Interferon (IFN) pathway (Elbashir et al., 2001). Although siRNAs were initially believed to bypass undesired activation of the immune system, recent reports have demonstrated that this might not always be the case (Marques et al., 2005). Indeed, siRNAs and shRNA were shown to activate RNA helicases, such as protein kinase R (PKR) and RIG-1, causing protein synthesis arrest through phosphorylation of a general translation factor eIF2α and upregulation of a subset of genes from the IFN pathway (Bridge et al., 2003; Judge et al., 2008; Moss et al., 2003; Pebernard et al., 2004; Sledz et al., 2003). siRNAs have also been shown to activate endosomal pattern-recognition toll-like receptors (TLR) 3, 7 and 8, thereby increasing the expression of pro-inflammatory cytokines (Hornung et al., 2005; Karikó et al., 2004; Sioud, 2005). Recognition of "danger motifs", such as CpG motifs in oligodeoxynucleotides (ODNs) and UG rich regions in siRNAs, are believed to be responsible for such induction of immunostimulatory effects (Hornung et al., 2005; Judge et al., 2005). Several chemical modifications have been introduced to the backbone of siRNAs to overcome these issues and have been shown to be advantageous in reducing immunological activation (Judge et al., 2006; Sioud et al., 2007). Furthermore, induction of immune responses by siRNAs has been found to be cell type-, duplex length-dependent (Hornung et al., 2005; Judge et al., 2005; Reynolds et al., 2006; Sioud, 2005). Immune stimulatory effects were also found to be dose-dependent, further advocating the use of minimal doses (Persengiev et al., 2004). Although the reported activation of IFN response was only moderate in the above mentioned studies, this is an issue that should not be overlooked and further investigations are needed (Moss & Taylor, 2003).

1.12.2 Saturation of RNAi endogenous pathway

Saturation of the RNAi machinery can occur with both shRNAs (Borel et al., 2011; Grimm et al., 2006; Martin et al., 2011; Snove et al., 2006) and siRNAs (Bitko et al., 2005; Khan et al., 2009) leading to dysregulation of endogenous miRNA function (Barik, 2006). Dose-dependent saturation of nuclear exportin-5 by shRNAs has led to liver toxicity and increased

morbidity in mice after intravenous injection of a shRNA-AAV expressing vector (Borel et al., 2011; Grimm et al., 2006). In these studies, shRNAs prevented endogenous miRNA maturation and led to a global shutdown of the miRNA pathway (Barik, 2006; Martin et al., 2011). Selection of adequate promoters for modest expression of shRNAs or co-expression of recombinant exportin-5 are the main approaches being evaluated to reduce shRNA-mediated toxicity (Grimm et al., 2006; Yi et al., 2005). In addition, others have suggested using an artificial miRNA-based expression system which is well tolerated *in vitro* and *in vivo* (Boudreau et al., 2009a; McBride et al., 2008). On the other hand, synthetic siRNAs bypass nuclear processing and do not overload nuclear transport, thereby circumventing this issue. However, siRNAs compete with endogenous miRNAs for RISC binding and therefore the use of the lowest possible dose is key to avoid dysregulation of the endogenous miRNA pathway (Barik, 2006).

1.12.3 Vector-mediated toxicity

Viruses and non-viral vectors for RNAi delivery can also induce toxicity and activate immune responses in the host organism (Sakurai et al., 2008). Despite their great tropism over a wide number of cell types, viruses have recently been associated with extreme adverse reactions (Thomas et al., 2003). The first human fatality in gene therapy occurred in 1999 upon direct administration of an adenovirus vector (expression vector for ornithine-transcarbamylase) into the hepatic artery (Marshall, 1999). The vector caused a systemic inflammatory response with respiratory distress, multi-organ failure and subsequent death of the 18-year-old patient (Marshall, 1999). Furthermore, in a gene therapy trial for X-SCID disease, using a γc Moloney retrovirus-derived vector, two patients were reported to develop a leukaemia-type disorder due to insertional mutagenesis (Check, 2002; Kaiser, 2003). In spite of the excellent clinical outcomes in most of the patients treated, the trial was halted (Cavazzana-Calvo et al., 2000). In contrast, several other gene therapy trials for head- and neck- cancer have used viral vectors with negligible or no remarkable induction of host's immune response (Raty et al., 2008).

The nanotoxicological and immunological aspects of non-viral vectors for gene and RNAi delivery will be addressed in the following section (Section 1.13).

1.13 Nanotoxicology of engineered biomaterials

Biomaterials used for gene and RNAi delivery have, for some time, been considered as relatively inert materials with negligible or no toxic effects (O'Mahony et al., 2013b). In spite of their considerable advantages over their viral counter parts regarding toxicology, non-viral vectors are now known to cause several biological, genomic and inflammatory disturbances (Akhtar et al., 2007; Dobrovolskaia et al., 2007; Merkel et al., 2011). Thus, in addition to uptake and gene silencing requirements, biocompatibility of non-viral formulations is one of the emerging hurdles to their translation to the clinic (Ballarín-González et al., 2012; Reischl et al., 2009). Nanoparticle-induced toxicity is dependent on the physicochemical characteristics of the nanoparticle, the subsequent tissue susceptibility and on other treatment-dependent factors, such as route of administration, dose and length of treatment required (Albanese et al., 2012).

1.13.1 Mechanisms of nanoparticle-mediated toxicity: cytotoxicity and inflammatory susceptibilities in the CNS

Assembled nanoparticles for RNAi can instigate detrimental effects at the cellular and intracellular level and can induce inflammatory responses with local or systemic consequences (Akhtar, 2010; Akhtar & Benter, 2007; Dobrovolskaia & McNeil, 2007; Merkel et al., 2011).

1.13.1.1 Nanoparticle-mediated cytotoxic effects

Nanosystems for siRNA delivery have been reported to cause ***cellular membrane destabilization and lysis***. Disturbance of lipid raft assemblies and pore hole formation have been suggested as the main mechanisms for disruption of biological membranes by polymeric nanoparticles (Hong et al., 2004; Hong et al., 2006b; Moghimi et al., 2005).

Detrimental interactions with cellular membranes may also compromise cellular and intracellular signalling, influencing cellular and tissue homeostasis (Karra & Borlak, 2012). In addition, nanoparticles have also been shown to interfere with the ***metabolic activity of the mitochondria*** (Hunter et al., 2010). Indeed, leakage of mitochondrial enzymes (such as cytochrome-c) to the cytoplasm and reduction of mitochondrial membrane potential (MMP) have been observed in several cell-types with delivery vectors such as PEI, PLL and PAMAM dendrimers (Hibbitts et al., 2011; Lee et al., 2009; Moghimi et al., 2005; Symonds et al., 2005). Release of cytochrome-c consequently triggers caspases-3 and -9, ***activating the apoptotic pathway*** eventually culminating in loss of membrane integrity (Hunter & Moghimi, 2010). In addition, disruption of the mitochondrial respiratory chain by polymeric nanoparticles results in increased ***production of reactive oxygen species*** (ROS) (Hunter & Moghimi, 2010). Similarly, the commercially available lipid-based vector Lipofectamine® has been shown to increase multiple ROS and trigger the apoptotic pathway (Dokka et al., 2000; Kongkaneramit et al., 2008). Nanoparticles may also interact with the cell nucleus causing ***permeabilization of the nuclear membrane*** (Karra & Borlak, 2012). Furthermore, polymer-, lipid- and dendrimer-based nanoparticles have been shown to induce ***global changes in gene expression*** profiles (Akhtar, 2010; Akhtar & Benter, 2007; Choi et al., 2010; Omidi et al., 2011). Indeed, microarray studies of the transcriptome have shown that biomaterials *per se* and assembled siRNA nanosystems induce different and specific "gene fingerprints" (Choi et al., 2010; Hollins et al., 2007). In this regard, cationic polymer- and lipid-based nanoparticles disrupt the expression of different genes related to cell proliferation and differentiation, apoptosis and mechanisms of DNA repair (Omidi et al., 2005; Omidi et al., 2011). Therefore, adequate selection of the delivery system is crucial in order to reduce additional undesired "off-target" effects in RNAi therapies (Akhtar & Benter, 2007; Merkel et al., 2011).

The extent and magnitude of the above mentioned biological and genotoxic adverse effects have been found to be ***cell-type dependent*** (Omidi et al., 2005). Thus, neurons, microglia

and astroglia – in the particular case of the CNS – may present ***different cellular susceptibilities*** due to differences in their intracellular regulation and nanoparticle uptake kinetics. Moreover, certain CNS disorders, such as HD, might render specific neuronal populations more susceptible to toxic stimuli (Rigamonti et al., 2000; Rigamonti et al., 2001). In addition to the adverse effects directly exerted on neurons, toxic interactions of nanoparticles in astroglia and microglia populations may prompt oxidative stress, subsequently causing neuronal injury. This reduction in brain homeostasis and exacerbated inflammatory response may mask benefits of RNAi treatment and even aggravate the progress of neurodegenerative diseases.

1.13.1.2 Nanoparticle-mediated immuno-inflammatory effects

Cationic lipid- (Kawakami et al., 2006; Li et al., 1999; Loisel et al., 2001; Sakurai et al., 2002; Tousignant et al., 2000; Whitmore et al., 1999; Yew et al., 1999; Zhao et al., 2004) and polymer-based (Gautam et al., 2001) gene delivery systems have been previously reported to induce ***production of pro-inflammatory cytokines*** upon systemic administration. However, these immunostimulatory effects were deemed to be due to unmethylated CpG sequences contained within the plasmid DNA to be delivered (Tousignant et al., 2000; Tousignant et al., 2003; Zhao et al., 2004). Lack or modification of such immunogenic sequences reduced cytokine release but did not completely eliminate it (Whitmore et al., 1999). Furthermore, comparative studies have shown that linear PEI.pDNA complexes cause only limited increases in serum cytokine levels (TNF-α, interferon gamma (IFN-γ), interleukin 6 (IL-6), interleukin (IL-12)) when compared to lipid-based formulations (DOTMA:Chol and DOTAP:Chol) (Bonnet et al., 2008; Kawakami et al., 2006). Taken together this suggests that other underlying mechanisms might be implicated (Sakurai et al., 2008). On the other hand, only recently siRNA nanoparticles have been reported to cause activation of the innate immune response upon intravenous administration (Bonnet et al., 2008; Kedmi et al., 2010; Ma et al., 2005; Tao et al., 2011). Systemical delivery of linear PEI.siRNA nanoparticles induced mild increases in serum cytokine levels (TNF-α, IFN-γ,

IFN-β, IL-6 and IL-12) (Bonnet et al., 2008). In contrast, systemically administered DOTAP-based siRNA nanoparticles induced the expression of IFN responsive genes as well as the expression of pro-inflammatory cytokines, such as TNF-α (Kedmi et al., 2010). This pro-inflammatory response was also detected at the cellular level in monocytes (CD11b+), T cells (CD3+) and B cells (B220+) and was found to be dependent on the ***activation of pattern recognition toll-like receptor*** 4 (TLR4) (Kedmi et al., 2010). However, investigations in various dendritic cell models have demonstrated that this is likely to be a structure activity dependent-effect and therefore specific to certain lipids (Lonez et al., 2009; Tanaka et al., 2008; Vangasseri et al., 2006). Other *in vivo* studies have also reported differential activation of the immune system by polymer- and lipid-based nanoparticles containing the same siRNA cargo (Bonnet et al., 2008), further indicating that specific non-viral vectors may be more likely to enhance siRNA-mediated immune response than others. In support, charge-dependent activation of the components of the ***complement system*** have also been reported for cationic lipids and polymers (Chanan-Khan et al., 2003; Plank et al., 1996).

Interestingly, strong drug-based suppression of IFN-γ, IL-6 and monocyte chemotactic protein-1 (MCP-1) (IFN-γ inducible cytokine) and partial suppression of TNF-α improved toxic effects in the liver and spleen of animals treated intravenous injection (i.v.) with cholesterol-based nanoparticles (Tao et al., 2011). These results suggest that cytokines are key mediators of nanoparticle-induced toxicities and premedication might be a viable strategy of reducing side effects.

In the case of the brain, immune responses are mainly mediated by microglia, resident immune effector cells involved in phagocytosis and antigen-presentation, and to some extent by astroglia, cells that provide biochemical support to neurons (Block et al., 2005; Dong et al., 2001). Despite the importance of cytokine expression in vector-mediated toxicity, to our knowledge there are only few reports exploring these effects in CNS models (Gorina et al., 2009; Kateb et al., 2007). Gorina *et al.* demonstrated in primary astroglial cultures that lipid

(oligofectamine)-based siRNA nanoparticles enhanced expression of Signal Transducers and Activators of Transcription 1 (Stat1), involved in the upregulation of a subset of IFN genes, but also enhanced the expression of cyclooxygenase 2 (COX-2). In the same study authors reported release of IL-6 and IFN-γ induced protein 9 and 10 (IP-9 and IP-10). Curiously, in these studies astroglial cultures were more sensitive to lipid.siRNA nanoparticles than microglial cultures (Gorina et al., 2009). The latter only presented a modest increase in cytokine release upon stimulation with a lipid-formulated non-silencing siRNA (Gorina et al., 2009). In addition, siRNAs formulated in multi-walled carbon nanotubes have shown mild induction of TNF-α and IL-10 in an immortalised murine microglial cell model (BV2 cells) (Kateb et al., 2007). Although not in the context of gene or RNAi delivery, other cationic nanoparticles have also been able to induce expression of pro-inflammatory cytokines and membrane TLR2 in cultured BV2 microglial cells (Hutter et al., 2010). Stimulation of the innate immune response might be advantageous when delivering RNAi in the particular case of brain cancers, however nanoparticle-induced exacerbated immune responses can be detrimental when treating non-malignant diseases, such as HD (Block & Hong, 2005).

1.13.2 Correlating physicochemical properties with cellular toxicity

Physicochemical characteristics of the assembled nanosystem, such as size, charge, shape and architecture, surface functionalisation, chemical composition and biodegradability are key features for efficient cellular uptake and gene expression knockdown (Albanese et al., 2012; Karra & Borlak, 2012). The influence of such characteristics on the interaction of nanoparticles with cellular membranes, specific receptors, organelles and other intracellular targets is critical and, therefore, it is not surprising that they may also contribute to their toxicity profile (Albanese et al., 2012; Karra & Borlak, 2012).

Size is a major determinant for tissue penetration, biodistribution and elimination. Large particles are cleared by the MPS whereas small particles (<10 nm) are removed by

glomerular filtration (Karra & Borlak, 2012; Moghimi et al., 2001). On the other hand, at the cellular level this feature is thought not only to determine the specific pathway of internalisation as well as the extent of exocytosis and retention (Harush-Frenkel et al., 2008; Nel et al., 2009; Rejman et al., 2004; Resina et al., 2009). Additionally, others have found that the ***shape/morphology*** of the nanoparticle plays an important role in neuronal and microglial uptake (Albanese et al., 2010; Albanese et al., 2012). Although not specifically in the context of RNAi, Hutter *et al*. demonstrated that microglial cells tend to phagocytose irregularly-shaped nanoparticles while neurons favoured uptake of rod-like nanoparticles (Hutter et al., 2010).

In spite of providing a clear advantage from the delivery stand point facilitating membrane interaction and subsequent internalization, positive ***surface charge*** has been associated with detrimental effects (Ballarín-González & Howard, 2012; He et al., 2010; Karra & Borlak, 2012). Indeed, polycation-based nanoparticles are well-known to disrupt cellular and mitochondrial biological membranes and to induce global changes in gene expression eventually leading to cell death (He et al., 2010; Lv et al., 2006). Additionally, cationic nanoparticles are also more likely to induce inflammatory responses (Kedmi et al., 2010; Vangasseri et al., 2006). In contrast, neutral or anionic systems seem to be less correlated with cytotoxic adverse effects (Dokka et al., 2000; Karra & Borlak, 2012; Lee et al., 2003; Mozafari et al., 2007). Nevertheless, significant disruptions in gene expression profiles have also been recently detected with anionic polymers such as PEG and PEG-poly glutamic acid (Kabanov et al., 2005). Therefore, when assessing biological and genomic effects other physicochemical factors must also be taken in account and toxicological categorization of delivery systems based solely on surface charge should be avoided (Akhtar, 2010).

In most cases, ***surface functionalisation*** of nanoparticles has proven to be a useful strategy to reduce nanoparticle-mediated toxicities (Ballarín-González & Howard, 2012). The use of hydrophilic polymer, PEG, has helped prolong circulating halve-lives by reducing opsonisation and clearance through phagocytosis (Gref et al., 1994). PEGylation has also

been shown to improve *in vitro* and *in vivo* cytotoxicity profiles of PEI and PAMAM dendrimers (Beyerle et al., 2010; Hibbitts et al., 2011; Wang et al., 2010b). Moreover, toxicity profiles of G4 PAMAM dendrimers in brain endothelial cell-model (bEND3) and mixed glial cultures were improved upon introduction of surface lipid-modifications (Bertero et al., 2013). Further functionalization via grafting of targeting ligands (see Section 1.11.3.2) enhances specific cellular uptake, thereby preventing toxicity in non-targeted tissues (Ballarín-González & Howard, 2012; Karra & Borlak, 2012). However, it must be considered that use of such targeting components at high density may also increase the immunogenic potential of the nanoparticles (Karra & Borlak, 2012).

Chemical composition and biodegradability of nanomaterials are important determinants in their bioaccumulation and clearance from the body. Upon release of their cargo, biocompatible nanomaterials should be amenable to enzymatic degradation, generating non-toxic by-products (Karra & Borlak, 2012). Persistence of non-degradable nanoparticles may increase the risk of biological and inflammatory responses (Akhtar & Benter, 2007; Karra & Borlak, 2012). Engineered biodegradable nanosystems based on synthetic or natural polymers, such as PLGA and modified chitosans, have shown improved toxicity profiles when compared to cationic lipids and PAMAM dendrimers (Chan et al., 2007; Chen et al., 2013a; Li et al., 2003b; Mansouri et al., 2006). Moreover, introduction of cleavable ester linkers to a PAMAM dendrimer has shown to improve its degradability and consequently its toxicity profile (Kim et al., 2010a). In summary, although the adverse effects of high-positive surface charge have been well-documented, other factors such as biodegradability and processing of the biomaterial in the intracellular compartment might play important roles and must also be considered.

1.13.3 *In vitro* and *in vivo* assessment of nanoparticle safety for CNS delivery

Evaluation of cytotoxic effects of siRNA nanoparticles in relevant *in vitro* and *in vivo* models is vital to understanding the potential risks of these delivery nanosystems in the

CNS. A large number of RNAi studies for CNS delivery have based their biosafety assumptions on experiments conducted in cancer-derived and other immortalised cell lines (van Gaal et al., 2011). Despite their undisputable usefulness for transfection and high-throughput screening, these cell-models have expression and proliferative patterns that in turn make them more resistant to toxic stimuli. Furthermore, in these studies researchers have mainly focused on cancer-derived neuron-like cell lines with little or no consideration has been given to microglia and astroglia. In contrast, some studies have been conducted in more representative primary mixed neuronal and glial cultures derived from healthy rodents (Gorina et al., 2009; O'Mahony et al., 2012b). Although considered a more representative and sensitive *in vitro* model, primary neuronal and glial cultures are models of healthy brain cells rather than *in vitro* models of CNS disease. Thus, the selection of *in vitro* models should be relevant to the purpose of the study. For instance, in the specific case of HD, ST14A-HTT120Q cells which harbour a muHTT protein that renders these cells more susceptible to toxic stimulus (Rigamonti et al., 2000; Rigamonti et al., 2001), might constitute an appropriate model for testing of RNAi nanosystems.

A wide range of well-established methodologies for *in vitro* testing of biocompatibility of siRNA nanoparticles are available to researchers (Kepp et al., 2011). Table 1.5 summarises the most widely used methodologies for *in vitro* assessment of cytoxicity, their main advantages and limitations. Depending on the cytotoxic assay, the *in vitro* cell model system selected and the specific experimental conditions, different results can be obtained (Kim et al., 2009a; van Gaal et al., 2011; Weyermann et al., 2005). Thus, in order to avoid underestimation of adverse effects and have a more complete insight of the underlying mechanisms multiple assays must be used (van Gaal et al., 2011). Alternatively, High Content Analysis assays, based on automated cell imaging analysis, can be used for high throughput screening of multiple cytotoxic parameters. Indeed, due to its high sensitivity and specificity this technique is now gaining popularity for the assessment of efficacy and cytotoxicity of gene and RNAi delivery vectors (Hibbitts et al., 2011; Solmesky et al., 2011).

Evaluation of nanoparticle-mediated toxicities *in vivo* depends largely on the route of administration chosen for delivery. Most studies carrying out i.v. injections assess the serum levels of hepatic enzymes (such as, Lactate Dehydrogenase (LDH), aspartate transaminase (ASAT), alanine aminotrasnferase (ALAT) and alkaline phosphatase (ALP)) and serum levels of pro-inflammatory cytokines (such as TNFα, IFN-γ, IL-1β and IL-6), but also histopathological examination of representative sections of organs such as liver and spleen (e.g. of selected studies (Kawakami et al., 2006; Kedmi et al., 2010; Tao et al., 2011). On the other hand, evaluation of biological adverse effects of RNAi delivery systems when directly injected into the brain have been usually limited to histopathological studies of the injection sites and surrounding areas. Protocols for cresyl violet staining and immunostaining for glial fibrillary acidic protein (GFAP) and CD11b marker have been used (e.g. of selected studies (Cardoso et al., 2010)). Additionally, the vast majority of preclinical studies for RNAi in the brain conducted to date are proof-of-concept studies of short duration and, therefore, only limited conclusions about safety can be drawn. Finally, the use of relevant disease models is crucial for gaining an integrated insight to the toxic responses to RNAi nanosystems during CNS disease states (Karra & Borlak, 2012; Silva, 2009).

Table 1.5. List of parameters and assays commonly used for in vitro assessment of biocompatibility of non-viral gene and RNAi delivery vectors

Parameter	Assay (Ref.)	Principle	Advantage	Limitation	Biocompat. studies Select. Ref.
Cell morphology/cell density	Light microscopy	Visual comparison of treated and untreated cells. Characteristics to observe: Cell detachment, changes in morphology and pronounced cell debris due to cellular membrane lysis	Inexpensive, rapid and routinely employed	Unable to differentiate cell death mode	(Fischer et al., 2003; Merkel et al., 2011)
Plasma Membrane integrity	Trypan Blue exclusion assay (Strober, 2001)	Trypan blue dye is not permeable through intact functional cellular membranes. Viable cells exclude the dye whereas nonviable cells incorporate the dye	Dead cells can be visualised. Inexpensive, routinely employed	Unable to differentiate cell death mode	(O'Mahony et al., 2012b)
Plasma Membrane integrity	LDH assay (Decker et al., 1988)	Leakage of cytoplasmic lactate dehydrogenase enzyme to the cell culture supernatant. LDH assay can detect minimal damages to the membrane	Rapid, routinely employed	Unable to identify cause of membrane disruption	(Moghimi et al., 2005)
Mitochondrial Metabolic activity	MTT and XTT assay (Mosmann, 1983)	Conversion of soluble tetrazolium salt in insoluble formazan product by metabolically active cells. A measure of mitochondrial dehydrogenase activity. Colorimetric read-out	Inexpensive, rapid and routinely employed	Unable to discriminate cytotoxicity and proliferation arrest	(Hamid et al., 2004)
Mitochondrial Metabolic activity	Alamar Blue (O'Brien et al., 2000)	Based on oxidation-reduction by mitochondrial respiratory chain (similar to MTT and XTT). Colorimetric or fluorimetric read-out	Inexpensive, rapid and routinely employed	Unable to discriminate cytotoxicity and proliferation arrest	(Cardoso et al., 2007; Hamid et al., 2004)
Mitochondrial Membrane Potential	MMP JC-1 staining (Johnson et al., 1981)	Probe is sequestered in healthy mitochondrion. Loss of mitochondrial potential leads to dissipation of JC-1 aggregates in the cytoplasm with loss of fluorescence. Cells can be visualised by fluorescent microscopy or sorted by flow cytometry	Rapid, inexpensive dyes available	MMP dissipation can occur independently from cell death events	(Hibbitts et al., 2011; Moghimi et al., 2005)
Lysosome detection	LysoTracker or Neutral red uptake (Borenfreund et al., 1985)	Viable cells take up neutral red which accumulates in lysosomes	Rapid, inexpensive dyes available	Unable to differentiate cell death mode.	(Fotakis et al., 2006)
Apoptosis/ Necrosis	Annexin V / PI assay (Jones et al., 1985; Vermes et al., 1995)	Annexin V labels PS residues in cells undergoing apoptosis. PI is membrane impermeant and DNA intercalating dye. Cells can be visualised by fluorescent microscopy or sorted by flow cytometry	Rapid, identification of early/late apoptosis. Dead cells can be visualised	PS exposure may occur independently of apoptosis	(Moghimi et al., 2005)

Table 1.5. (Cont.)

Parameter	Assay	Principle	Advantage	Limitation	Biocompat. studies Select. Ref.
Apoptosis	Caspase 3 activity assay	Assay carried out in cell lysates. Hydrolysis of Caspase-3 substrate and release of p-nitroanilide or fluorescent 7-amino-4-methylcoumarin. Colorimetric or fluorimetric read-out	Rapid	Caspase activation may occur independently from cell death events	(Moghimi et al., 2005)
Nuclear morphology/DNA damage	Hoescht and DAPI staining (Durand et al., 1982; Otto, 1990)	Membrane permeant dyes intercalate DNA allowing the observation of nuclear morphology. DNA fragmentation	Well established inexpensive dyes, useful in co-staining protocols	DAPI not so effective in living cells, fixing might be needed	(Hibbitts et al., 2011)
DNA damage	Comet assay (Tice et al., 2000)	Single Cell Gel electrophoresis where upon cell lysis breakage of DNA strands is assessed based on the intensity of the comet trail	Rapid	Technical variability	(Choi et al., 2010)
Protein quantification	BCA assay (Bradford, 1976)	Quantification of total protein content in viable cells upon removal of detached cells. Provides an indirect measure of viability	Rapid, inexpensive and routinely employed	Unable to differentiate cell death mode. Indirect measure	(Fotakis & Timbrell, 2006)

Abbreviations: *BCA, Bicinchoninic acid; DAPI, Diamidinophenylindole; LDH, Lactate dehydrogenase; MMP, Mitochondrial membrane potential; MTT, Methylthiazolyltetrazolium; PI, Propidium Iodide; PS, Phosphatidylserine; XTT, methoxynitrosulfophenyltetrazoliumcarboxanilide.*

1.14 Cyclodextrins as non-viral vectors for RNAi delivery to the CNS

CDs are enzymatically derived from starch and consist of cyclic oligosaccharides with a torus-like ring shape (Stella et al., 2008). CDs are formed by a number of glucopyranose units connected through α-(1,4)-glycosidic bonds (Stella & He, 2008). Each basic unit contains one hydroxyl group in the primary face (position C-6) and two in the secondary face (positions C-2 abd C-3). Furthermore, CDs are classified according to the number of glucopyranose units that they contain in α-CD (6 units), β-CD (7 units) and γ-CD (8 units) (Challa et al., 2005). CDs contain a hydrophobic cavity where a guest molecule can be accommodated (Brewster et al., 2007). The diameter of the cavity is dependent on the type of CD and determines the size of the guest molecule that can be accommodated (Brewster & Loftsson, 2007).

CDs have been widely used to enhance physicochemical properties of drug molecules that have poor solubility or/and bioavailability, for instance by forming non-covalent inclusion complexes with lipophilic drugs (Challa et al., 2005; Loftsson et al., 2007). Among all CDs, β-CD is the most widely used in the pharmaceutical sector, probably due to the favourable characteristics of its cavity, low cost and ready availability (Loftsson & Duchêne, 2007). In fact, β-CDs are well tolerated, have USA FDA approval for human consumption and are used in various drug formulations currently on the market (Loftsson et al., 2010). Despite of being virtually non-toxic on oral administration (Brewster & Loftsson, 2007), high i.v. doses of unmodified β-CDs have been associated with haemolysis and renal toxicity (Loftsson et al., 2005; Ohtani et al., 1989). These limitations have led to the introduction of modifications to the simple β-CD molecule, to form hydroxypropyl-β-CD, thereby improving its toxicity and solubility profiles (Davis et al., 2004; Loftsson et al., 2005). Indeed, functionalization of CDs is possible at each hydroxyl group in the primary and secondary face of the glycopyranose unit and this has been exploited to generate a wide variety of modified CDs (Davis & Brewster, 2004; Sallas et al., 2008; Szejtli, 1998).

CDs have recently attracted significant interest as component biomaterials for gene and RNAi delivery vectors (Chaturvedi et al., 2011; Mellet et al., 2011; O'Mahony et al., 2013c). Broadly speaking, two different approaches for the use of CDs in nucleic acid delivery have been used: (i) inclusion of CDs as a component of an existing delivery system, for its surface functionalization, toxicity profile improvement or enhancement of assembly properties; and (ii) as main starting materials, or scaffolds, onto which other chemical groups are attached achieving monodisperse self-assembling nanosystems (Chaturvedi et al., 2011; O'Mahony et al., 2013c).

1.14.1 Cyclodextrin-containing delivery systems

A wide range of CD-containing delivery systems have recently been developed and are represented in Figure 1.7. Mark Davis's research group has pioneered studies in this area with the development of a linear CD-containing polymer (CDP). CDP synthesis is based on the condensation of a difunctionalised CD co-monomer with a co-monomer containing charge centres (Figure 1.7a) (Gonzalez et al., 1999). Electrostatic interactions of the charge centres of the polymer back bone facilitated interaction with negatively nucleic acids (including pDNA and siRNA) forming cationic nanoparticles in the range of ~60-150 nm (Bartlett et al., 2007; Gonzalez et al., 1999). Further functionalization was achieved with adamantyl-PEG 5000 derivatives by inclusion complex formation within the CD cavity (Pun & Davis, 2002). This minimised aggregation in high salt physiological conditions and interaction with components of the bloodstream (Bartlett & Davis, 2007; Pun & Davis, 2002). Furthermore, the use of specific targeting ligands, such as galactose and Tf, coupled to the adamantyl-PEG 5000 chains enhanced delivery to hepatocytes and cancer cells, respectively (Bellocq et al., 2003; Hu-Lieskovan et al., 2005; Pun & Davis, 2002).

CDPs have been successfully used in various gene silencing experiments *in vitro* and *in vivo* mainly aimed at cancer (Bartlett et al., 2008; Bartlett et al., 2007a; Hu-Lieskovan et al., 2005). In addition, a recent safety trial in non-human primates for CALAA-01, a transferrin-

targeted CDP-based delivery system containing siRNA for ribonucleotide reductase subunit M2 (RRM2), has shown that CDPs are well tolerated after i.v. administration with no significant immune responses after multiple dosing (Davis, 2009; Heidel et al., 2007). Although no Ab for PEGylated CDPs were detected after a washout period, low levels were detected for the Tf component of the delivery system (Davis, 2009; Heidel et al., 2007). Moreover, slight changes in creatinine and blood urea nitrogen were detected at higher doses (100-200 times higher than in mice) (Heidel et al., 2007). The success in pre-clinical studies has advanced this technology to Phase I safety clinical trials for solid tumor melanoma cancers (Davis et al., 2010). First results were recently published demonstrating dose-dependent accumulation of the nanoparticles at the tumor site, efficient reductions in the levels of RRM2 mRNA and protein (Davis et al., 2010). Safety data should soon follow. Furthermore, application of the CDP-based technology has been reported as a new anticancer formulation CALAA-02, against hypoxia inducible factor-2α (Davis, 2009).

In a similar approach, Srinivasachari *et al.* synthesised a CDP-based delivery system by coupling diazido-β-CD monomers to dialkyne-oligoethylenemine monomers with different numbers of ethylenemine units (Figure 1.7b) (Srinivasachari et al., 2009). Higher levels of pDNA transfection and lower levels of cytotoxicity than jet-PEI were achieved. This was the first report on the use of "click chemistry" for CDP synthesis.

CDs have been conjugated to high Mw (25kDa) linear and branched PEI polymers improving the well-documented high toxicity profiles of these polymers *in vitro* and *in vivo* (the latter only for CD-branched PEI) (Pun et al., 2004). These formulations also explored the CD cavity for inclusion of adamantyl-PEG components to improve nanoparticle stability (Pun et al., 2004). Other studies have coupled CDs to low Mw (600 Da) PEIs and shown comparable gene delivery to high Mw PEI in the CNS, without causing significant toxicity (Tang et al., 2006).

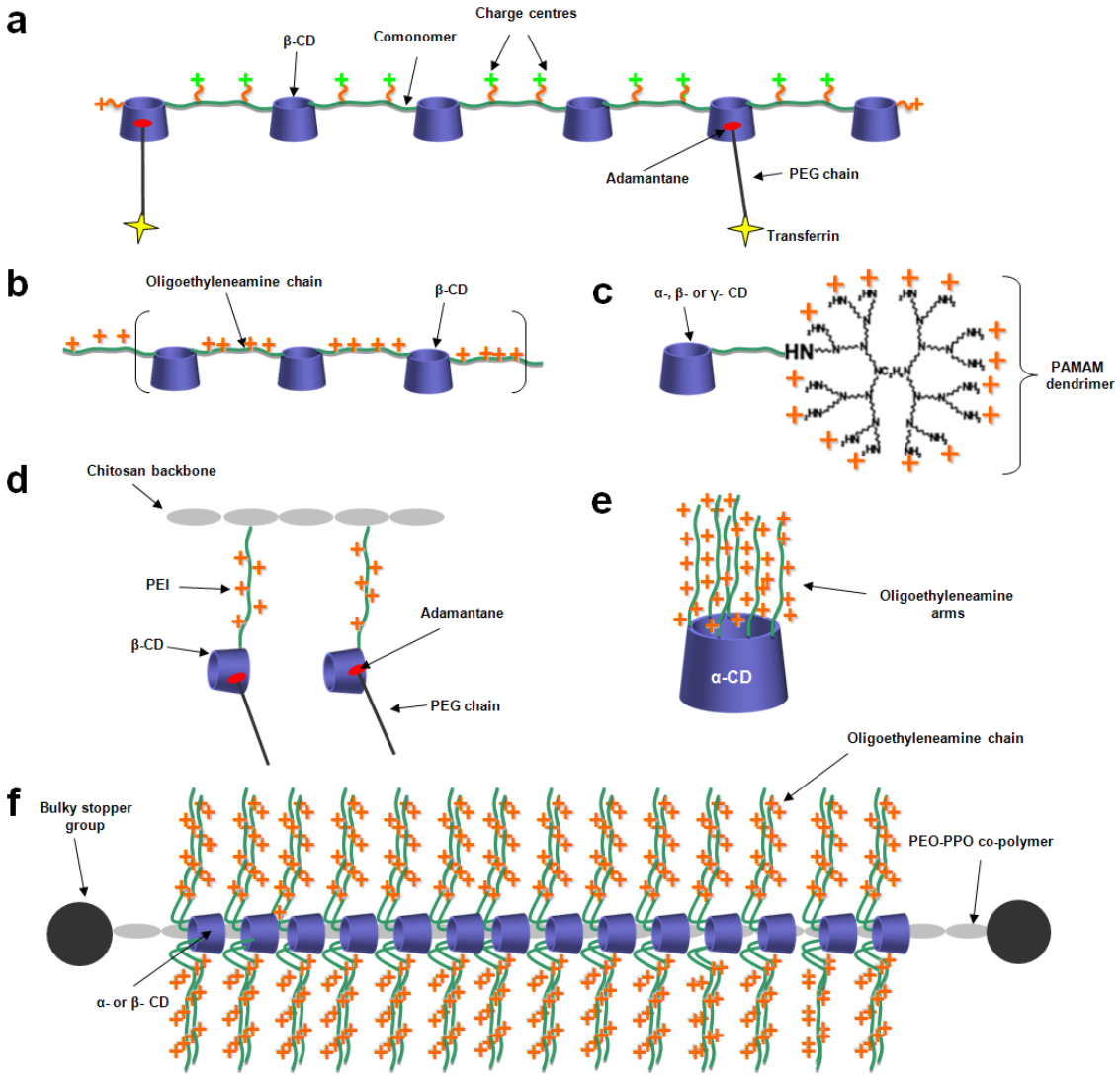


Figure 1.7. Cyclodextrin-containing delivery systems. *Adapted from (O'Mahony et al. 2013c). (a) CD-containing polymer + Ad-PEG-Tf (Davis, 2009). (b) CD-containing linear olygoethyleneamine polymer (Srinivasachari & Reineke, 2009). (c) CDs conjugated to PAMAM dendrimers (Arima et al., 2009). (d) Chitosan-graft-PEI-β-CD polymer + Ad-PEG (Ping et al., 2011). (e) Olygoethyleneamine-CD star polymer (Yang et al., 2007). (f) Olygoethyleneamine-modified CDs on a PEO-PPO co-polymer (Li et al., 2006).* ***Abbreviations:*** *Ad, adamantane; CD, cyclodextrins; PAMAM, Polyamidoamine; PEG, Polyethylenegycol; PEI, Polyethyleneimine; PEO-PPO, polyethylene oxide-polypropylene oxide.*

In addition, CDs can also be directly conjugated to PAMAM dendrimers (Figure 1.7c). Modifications with α- β- or γ-CD improved gene delivery efficiencies of lower generation G2 and G3 PAMAM dendrimers (Arima et al., 2001; Arima & Motoyama, 2009). Attachment of targeting ligands enhanced gene delivery to cultured hepatocytes *in vitro* and

in vivo after tail injection (Arima et al., 2010). Further modification of the delivery system with PEG chains and folate allowed gene delivery to cancer cells (Arima et al., 2012). In addition, successful delivery of shRNA and siRNA have also been achieved in hepatocytes *in vitro* and *in vivo*, with superior gene silencing effects and toxicological profiles compared to PEI and Lf2000 (Arima et al., 2011; Tsutsumi et al., 2007, 2008).

Other CDP strategies used in gene and RNAi delivery include: (i) the attachment of CDs to chitosan polymers through PEI linkers and inclusion formation with adamantyl-PEG derivatives (Figure 1.7d) (Ping et al., 2011); (ii) cationic "star" polymers based on oligoethyleneamine chains grafted onto a α-CD core (Figure 1.7e) (Srinivasachari et al., 2008; Yang et al., 2007); (iii) oligoethylenimine-conjugated CDs threaded onto a polyrotaxane polymer chain (co-polymers of poly(propylene oxide) (PPO) and poly(ethylene oxide) (PEO)) (Li et al., 2006; Yang et al., 2009) (Figure 1.7f). In general, these approaches have also yielded vectors which achieved successful binding to nucleic acids, high levels of transgene expression and lower levels of cytotoxicity than high Mw PEI.

1.14.2 Functionalised cyclodextrin delivery systems

CDs can be directly functionalised by modification of the hydroxyl groups in the primary and secondary face (Sallas & Darcy, 2008). Selected examples of functionalised CD delivery systems are represented on Figure 1.8. O'Driscoll's and Cryan's groups have been pioneers in this area with the development of various modified β-CDs for gene and RNAi delivery. In early stage studies, alkylimidazole, pyridylamino, methoxyethylamino groups were conjugated to the C-6 hydroxyl group of each glucopyranose unit yielding a series of cationic CDs (Figure 1.8a) (Cryan et al., 2004b). Electrostatic interactions between the cationic moieties of the modified β-CD and the negatively charged backbone of the nucleic acid allowed for self-assembly of the nanosystem. Gene delivery to COS-7 cells, a monkey fibroblast-like cell line derived kidney tissue, revealed that CDs modified with pyridylamino groups achieved similar transfection efficiencies to DOTAP, a lipid-based transfection

reagent (Cryan et al., 2004a). In subsequent studies, CDs were functionalized by attachment of short C6 and long C16 lipophilic chains to the primary face and oligoethyleneglycol chains on the secondary face to form neutral derivatives (Cryan et al., 2004a; Donohue et al., 2002). Further coupling of primary amino groups resulted in cationic amphiphilic CDs (Figure 1.8b). In these studies, while delivering luciferase pDNA to COS-7 cells, cationic amphiphilic CDs performed significantly better than neutral compounds (Cryan et al., 2004a). Moreover, studies in HepG2 cells, human liver carcinoma cell line, revealed that CDs modified with long (C16) hydrophobic lipid chains achieve better transfection efficiencies than those containing shorter (C6) lipid chains (McMahon et al., 2008). Lipid chains are believed to aid interaction with cellular membranes, thereby enhancing uptake of CD-siRNA complexes. In addition, reversing the arrangement of the cationic and lipid moieties in this CD delivery system did not affect transfection efficiency in undifferentiated and differentiated Caco-2 cells (human epithelial colorectal adenocarcinoma cells) with macropinocytosis identified as the major route of internalization (Figure 1.8c) (O'Neill et al., 2011). On the other hand, differences between these systems where observed while delivering siRNA to mHypo N41 cells, mouse hypothamalic cell line, probably due to changes in assembly properties (O'Mahony et al., 2012a).

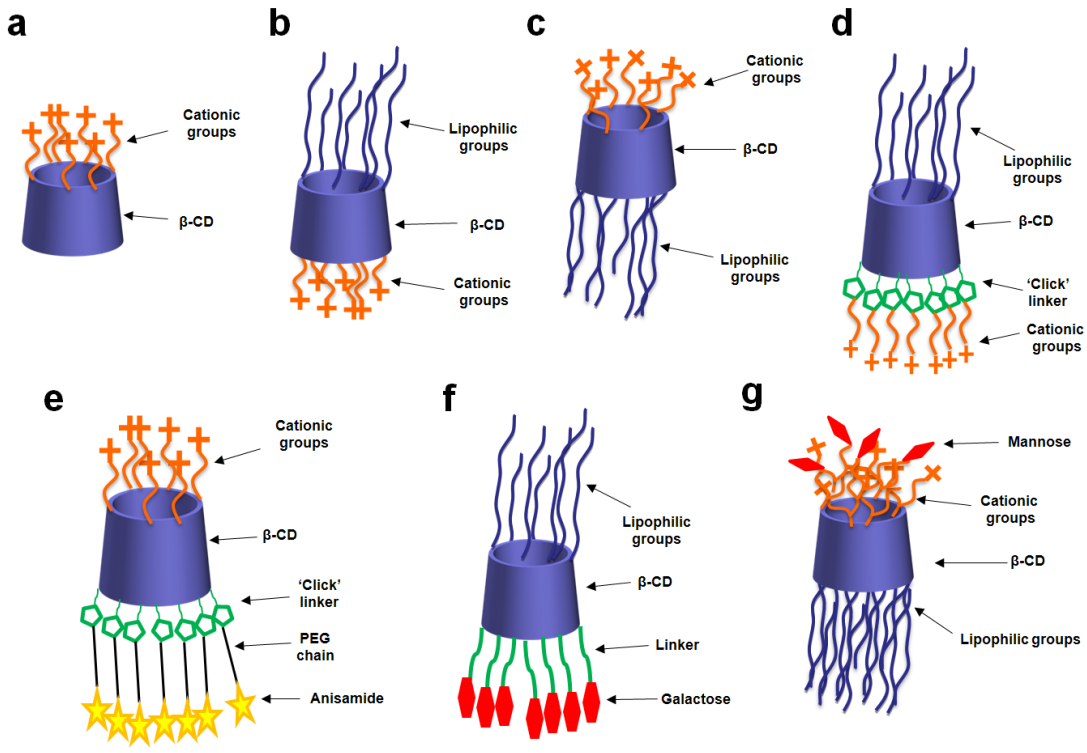


Figure 1.8. Functionalised cyclodextrin delivery systems. *Adapted from (O'Mahony et al. 2013c). (a) Polycationic CDs. (b) Polycationic amphiphilic CDs (Cryan et al., 2004b). (c) Reverse polycationic amphiphilic CDs (O'Neill et al., 2011). (d) Polycationic amphiphilic CDs containing "click" linkers (O'Mahony et al., 2012d). (e) Polycationic anisamide-targeted PEGylated CD containing "click" linkers (Guo et al., 2012b). (f) Galactosyl-targeted amphiphilic CD (McMahon et al., 2012). (g) Mannosyl-targeted polycationic amphiphilic CD (Díaz-Moscoso et al., 2011).* ***Abbreviations****: CD, Cyclodextrin; PEG, Polyethyleneglycol.*

The application of "click" chemical reactions (copper catalyzed reaction between alkyne and azide groups (Binder et al., 2007)) in the synthesis of modified CD scaffolds has recently enabled the rapid synthesis of amphiphilic CD derivatives with high yields. Cationic amphiphilic click-modified CDs facilitated siRNA delivery to mHypo N41 cells and primary hipocamppal neuronal cultures with comparable efficiencies to Lf2000 (Figure 1.8d) (O'Mahony et al., 2012b). In these studies exogenous luciferase and endogenous glyceraldehyde phosphate dehydrogenase (GAPDH) gene expression were successfully silenced (~40-70%) (O'Mahony et al., 2012b). The same delivery system was also used for eliciting TNF-α gene expression knockdown in RAW264.7 cells, a murine macrophage-like cell line, and in a murine model of acute colitis (McCarthy et al., 2013). Significant

cytokine gene expression knockdown and partial amelioration of the clinical signs of colitis were achieved (McCarthy et al., 2013). A PEGylated click-modified CD delivery system containing an anisamide targeting ligand enabled specific delivery of vascular endothelial growth factor (VEGF) to prostate cancer cells *in vitro* and *in vivo*, via the sigma receptor (Figure 1.8e) (Guo et al., 2012b). Functionalization of cationic amphiphilic CD.siRNA complexes with cell penetrating peptides has also been achieved by "post-insertion" of a DSPE-PEG-octaarginine (O'Mahony et al., 2013a). Octaarginine-containing formulations improved luciferase and GAPDH gene expression knockdown in mHypo N41 cells. In addition, PEGylated-octaarginine formulations presented improved stability in high salt buffers and enhanced pharmacokinetic profiles when compared to non-PEGylated nanoparticles and naked siRNAs, respectively (O'Mahony et al., 2013a).

Studies with "co-formulated" CDs, where amphiphilic CD-derivatives are blended together, have also been carried out. Galactosylated amphiphilic CDs (Figure 1.8f) were co-formulated with cationic amphiphilic CDs and enhanced gene delivery to HepG2 cells, especially when formulated in the presence of DOPE fusogenic lipid (McMahon et al., 2012). In different studies, cationic click-modified amphiphilic CD and a PEGylated click-modified CD were formulated together (O'Mahony et al., 2013d; O'Mahony et al., 2012c). In these studies, the cationic CD enabled complexation of siRNA whereas PEGylated CD improved stabilisation of the nanoparticle in high salt buffers. Although the gene silencing effects, in both Caco-2 cells and mHypo N41 cells, were blocked after PEGylation, the group is now focusing on the addition of targeting ligands to allow specific-targeted delivery (O'Mahony et al., 2013d; O'Mahony et al., 2012c).

The research group of García-Fernandez and collaborators have also reported extensive work in direct functionalization of CDs, mainly for gene delivery. Their initial studies were based on CDs, modified with primary amino groups at the primary face and short (C6) lipids at the secondary face (Díaz-Moscoso et al., 2008). High expression of a luciferase plasmid in BNL-CL2 murine embryonic cells with very low toxicity profile was reported (Díaz-

Moscoso et al., 2008). Reversing such modifications abolished transfection unless long (C12) lipid chains were used, further advocating the importance of hydrophobic-hydrophilic balance (Ortega-Caballero et al., 2008). However, when long hydrocarbon lipid chain was grafted to the secondary face it was shown to reduce binding capability to pDNA (Díaz-Moscoso et al., 2009). Recently, mannosyl and galactosyl amphiphilic CDs enabled targeted delivery of pDNA to a RAW264.7 cells and HepG2 cells, respectively (Figure 1.8g) (Díaz-Moscoso et al., 2011; Symens et al., 2012). In most of the above mentioned studies functionalised CDs presented comparable (or higher) levels of transfection to PEI with improved toxicity profiles.

1.15 Cyclodextrins as siRNA non-viral vectors for Huntington's Disease

CDs are highly versatile pharmaceutical molecules which have recently demonstrated great potential as non-viral vectors for gene and RNAi delivery (O'Mahony et al., 2013c). Indeed, from the formulation point of view CDs present multiple sites for modification and further functionalization can be accomplished through inclusion formation in the CD hydrophobic cavity (Sallas & Darcy, 2008). From the biological point of view, modified CDs have achieved high levels of transfection in multiple cell lines and also presented encouraging results in animal models (Chaturvedi et al., 2011; O'Mahony et al., 2013c). Additionally, no major cytotoxic or immunological effects have been reported in gene or RNAi delivery studies so far (Chaturvedi et al., 2011; O'Mahony et al., 2013c).

Our group has recently reported advances on the application of modified CDs for siRNA delivery to neurons. Indeed, CDs have enabled gene silencing effects in neuronal cell lines and primary hippocampal cultures (O'Mahony et al., 2012b). However, there is limited information on the applicability of such delivery systems for the treatment of neurodegenerative diseases, such as HD. Thus, this thesis focuses on the investigation of modified CDs as non-viral vectors for siRNA delivery for the treatment of HD.

1.16 Aims of Thesis

The main overarching goal of this thesis is to investigate the application of modified amphiphilic CDs as potential non-viral vectors for siRNA delivery in HD.

1.17 Specific Aims

To achieve this goal, three specific aims were defined:

Aim 1. Can modified amphiphilic CDs deliver siRNAs to relevant in vitro and in vivo models of HD?

To this end we first describe and validate a murine pre-clinical model of HD for testing of CD.siRNA nanoparticles ***(Chapter 2)***. We subsequently assessed the efficiency and toxicity of CD.siRNA nanoparticles in a rat striatal cell line and in human HD fibroblasts ***(Chapter 3)***. Further assessments of efficiency were carried by direct injections into the R6/2 mice brain and subsequent evaluations of motor improvements ***(Chapter 3)***.

Aim 2. Can modified amphiphilic CDs deliver siRNAs to the CNS without causing marked toxicity and/or local neuroinflammatory responses?

To address this question we conducted a comprehensive comparison of the nanotoxicological implications of CD-based siRNA delivery in a range of brain-derived cell lines using multiple methods for cytotoxicity assessment ***(Chapter 4)***. Furthermore, neuroinflammatory responses were assessed *in vitro*, in a microglia cell line, and *in vivo* after direct injections into the mouse brain. In all experiments a comparison with other commonly used commercial vectors was established ***(Chapter 4)***.

Aim 3. Can CD formulations be further optimised to enhance key proprieties such as stability?

To this end we investigated post-PEGylation as a formulation alternative to improve stability of pre-formed CD.siRNA nanoparticles, elucidating the influence of PEG length and density on nanoparticles stability ***(Chapter 5)***.

Chapter II

Establishment of a Huntington's Disease Animal Model Platform for Assessing the Efficacy of Non-Viral siRNA Nanoparticles

Bruno M. D. C. Godinho[1,2], *Susan Grenham*[2,3], *Caitriona M. O'Driscoll*[1], *John F. Cryan*[2,3]

[1]Pharmacodelivery group, School of Pharmacy, University College Cork, Cork, Ireland

[2]Dept. Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3]Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, Cork, Ireland

2.1 Abstract

Huntington's Disease (HD) is a rare autosomal dominant neurodegenerative disease caused by the expression of a toxic Huntingtin (HTT) protein. The disease is characterised by dysfunction and death of neurons in the brain, mainly in the striatum. Symptoms include chorea, dyskinesia, rigidity, cognitive deficits and neuropsychiatric manifestations. Several animal models have been developed to further understand the pathophysiology of the disease and also as platforms for preclinical testing of novel therapies. Indeed, several genetic rodent models have been engineered to express a form of the human muHTT gene and these have proven to be very important tools for the field. The R6/2 mouse model is perhaps the most utilised transgenic model of HD and has been shown to display many of the neuropathological aspects of HD including aggregate formation. Additionally, the R6/2 model presents a robust phenotype with progressive motor deficits, cognitive impairment and fast disease progression. However, and despite the robust phenotype, variability in behavioural outcomes has been previously reported and attributed to different laboratory and testing conditions. The goal of the present study was to establish a colony of transgenic mice hemizygous for the human HTT transgene (R6/2 mice) and characterise them behaviourally to allow for intervention studies. To this end, we show that R6/2 mice display motor coordination deficits in the rotarod task, grip strength impairment, reduced locomotor activity and enhanced clasping behaviour in a time dependent process. These results validate the model for use in the preclinical assessment of novel cyclodextrin short interfering ribonucleic acid nanoparticle-based therapeutic approach for HD.

2.2 Introduction

Huntington's Disease (HD) is a rare autosomal dominant neurodegenerative disease caused by the expression of an aberrant toxic Huntingtin (HTT) protein, which contains an expanded polyglutamine tract (polyQ) (Ross & Tabrizi, 2011; Zuccato et al., 2010). Significant neuronal cell death occurs mainly in the striatum but also in the cortex, leading to the manifestation of a wide range of symptoms including: chorea, stereotypic movements, cognitive impairment and depression (Zuccato et al., 2010). Although no cure or preventative treatments are available as yet, great advancements have been made in the understanding of HD neuropathology with the aid of animal models (Ross & Tabrizi, 2011).

Research in HD has been largely based on model systems of HD, in particular animal models (Ross & Tabrizi, 2011). Although initial studies were conducted in toxin-based models, since the identification of the mutant HTT (muHTT) as cause of HD, genetic models have grown in popularity (Ramaswamy et al., 2007). These models have been engineered in several species spanning from non-mammalian systems to non-human primates, however, transgenic and knock-in rodent models are by far the most widely used in the pre-clinical setting (Crook & Housman, 2011; Morton & Howland, 2013).

Bates and co-workers generated the first transgenic mouse models of HD, the R6/1 and R6/2 lines (Mangiarini et al., 1996). The R6/2 line, in particular, express a truncated form of the human muHTT gene containing exon 1 and ~144 CAG repeats, under the control of the human promoter (Mangiarini et al., 1996). This mouse model has been shown to display characteristic signs of HD neuropathology, such as HTT inclusion formation, gene transcriptional dysregulation and general brain atrophy (Luthi-Carter et al., 2002; Schilling et al., 1999; Stack et al., 2005). In addition, transgenic mice hemizygous for the human HTT transgene (R6/2 mice) mimic most of the symptoms of HD, including choreiform-like movements, motor deficits, clasping behaviour and cognitive impairment (Carter et al., 1999; Lione et al., 1999). Furthermore, progression of HD-like pathology and respective

symptoms occurs rapidly and mice present decreased survival rates compared to wild-type (WT) littermates. In fact, the R6/2 mouse model is the most widely used mouse model for pre-clinical screening of novel therapies, at least in part due to its early onset of behavioural deficits and the its ease of maintenance (Gil et al., 2009; Li et al., 2005).

Despite their advantages over toxin-based models, the use of rodent genetic models has been plagued by the variability in results across laboratories, in terms of both their neuropathological and behavioural phenotypes. Despite its robust behavioural phenotype, inconsistent results have also been reported for the R6/2 mouse model (Hockly et al., 2003b). Indeed, factors such as genetic instability of the CAG tract and environmental factors have been shown to contribute to the variability in neuropathology and behavioural deficits in this model (Carter et al., 2000; Dragatsis et al., 2009; Hockly et al., 2003b). Additionally, it is worth noting that inter-laboratory variability regarding behavioural outcomes has been also documented for other mouse strains and found to be dependent on the specific behavioural test being carried out, housing conditions, experimenter and mouse genetic background (Butcher et al., 1979; Crabbe et al., 1999; Lewejohann et al., 2006). Thus it is important to undertake behavioural validation of any mouse model prior to therapeutic intervention studies.

Thus, the aim of the present chapter is to establish *de novo* the R6/2 model in a new laboratory setting, the Biological Science Unit Annex at University College Cork. To this end, we conducted phenotypic and behavioural assessment of this mouse model, cross validating and comparing results with previous studies.

2.3 Materials and methods

2.3.1 R6/2 mouse breeding, housing and animal care

R6/2 colony was maintained by cross breeding B6CBAF1 ovarian transplanted (OT) females (HD exon 1, 62Gpb/3J) and B6CBAF1 males (Stock # 006494, The Jackson Laboratories, Bar Harbor, ME). Figure 2.1 depicts the breeding scheme and the pattern of inheritance of the human muHTT transgene. Two cohorts of breeders were purchased (Cohort A, with 6 breeding pairs; Cohort B, with 16 breeding pairs) and kept as individualized pairs. B6CBAF1 mice were noted to be poor breeders (~6 pups per litter), and a total number of 6-8 litters per female were allowed before retiring breeders. See Supplementary Information (SI) Supplementary Table S2.1 for further detail on husbandry, breeding and colony development.

Pups were weaned at 3 weeks of age and group-housed in groups of 4-5 mice of mixed genotype. Cages contained minimally enriched living conditions with regular sawdust bedding, paper shred, and a cardboard play tunnel. Dry food pellets in a food hoper and water were available *ad libitum* and cages regularly changed on a weekly basis. Soft diet (mashed chow mixed with water) was provided on a small petri dish at the bottom of the cage at late stages of disease (~8 weeks of age onwards). Mice were maintained on a 12/12 hour light-dark cycle with temperature (22 ± 1 ºC) and humidity (~55 %) controlled conditions. 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.

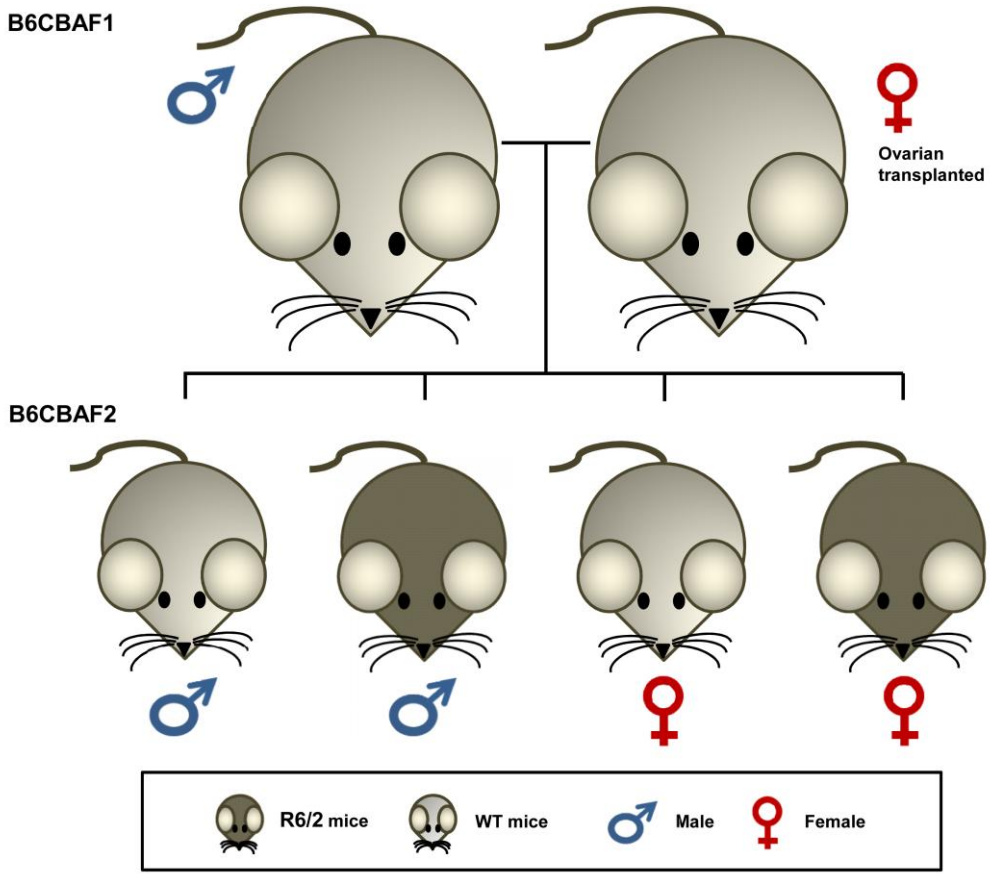


Figure 2.1. Breeding scheme and pattern of inheritance. *R6/2 mouse colony was maintained by crossing OT B6CBAF1 females with B6CBAF1males. Breeders were kept together until retired. Pups were weaned at 3 weeks of age and group-housed in mixed genotype cages. Male and female pups were housed separately.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, wild-type mice.*

2.3.2 Genotyping

2.3.2.1 DNA collection and extraction

At the time of weaning (3 weeks of age), tissue samples were collected from tails (2-3 mm) and placed in nuclease-free tubes. Deoxyribonucleic acid (DNA) extraction was carried out by adding 200 µL of non-ionic detergent (NID)-buffer [(50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2 mM $MgCl_2$; 0.1 mg/ml gelatin; 0.45 % NP40; 0.45 % Tween-20)] and 3 µL of Proteinase K, and incubating samples overnight at 56 °C. Samples were further incubated for 10 minutes at 95 °C and centrifuged at 14000 rotations per minute (rpm) for 5 minutes to pellet debris. Supernatant was transferred into a new nuclease-free tube and stored at -20 °C until polymerase chain reaction (PCR) was performed.

2.3.2.2 PCR

PCR assays for genes containing long trinucleotide repeat regions, such as the N-terminal HTT transgene in the R6/2 mouse, are particularly challenging. Indeed, the extremely high guanine and cytosine content and the presence of secondary structures are believed to hamper amplification within the CAG region (Wallace, 1996). The initial genotyping protocol described by Mangiarini *et al.* and other similar protocols using primer sequences spanning the CAG repeat region (Mangiarini et al., 1996), were found to be very sensitive to assay conditions when performed in our lab. Thus, an alternative region prior to the CAG expansion was selected as target region for amplification.

Genotyping PCR was carried out on genomic DNA samples (collected at 3 weeks of age) using previously published forward (CGCAGGCTAGGGCTGTCAATCATGCT) and reverse (TCATCAGCTTTTCCAGGGTCGCCAT) primers specific for the human HTT gene (Hockly et al., 2003b). Each 20 µL PCR reaction contained: 0.4 µL of 10 mM Deoxy nucleotides (dNTPs) (Sigma-Aldrich, United Kingdom); 0.4 µL of 20 µM forward primer; 0.4 µL of 20 µM reverse primer; 0.6 µL of $MgCl_2$; 1 µL dimethyl sulfoxide (DMSO); 1 µL of β-mercaptoethanol 1 mM (Fluka, Germany); 2 µL of Immolase buffer (Bioline, United Kingdom); 0.25 µL of Immolase Taq Polymerase (Bioline, United Kingdom); 1 µL of the specific tail DNA sample; and completed to the volume with nuclease-free water. Cycling conditions used were: 95°C for 10 minutes; 35 cycles [95 °C for 30 seconds; 60 °C for 30 seconds; 72 °C for 1 m 30 seconds]; 72 °C for 10 minutes; and 4 °C indefinitely. The presence of the 272 base pairs (bp) amplicon was confirmed by running all amplified DNA samples in a 2% agarose gel. A representative gel is depicted on SI, Supplementary Figure S2.1.

2.3.3 Phenotypical characterisation of the R6/2 model

The study design for phenotypical and behavioural validation of the R6/2 model is represented in table 2.1.

Table 2.1. Study design for phenotypical and behavioural validation of the R6/2 mouse model.

Activity		Weeks															
		3	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
Weaning		**X**															
Genotyping		**X**															
Weight		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
Behavioural testing	Clasping		**X**		**X**		**X**		**X**		**X**						
	Rotarod		**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**				
	Grip Strength							**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**	**X**
	Loc. Activ.																**X**

2.3.3.1 Body weight and survival

Body weight was assessed on a weekly basis to the nearest 0.1 g. Mice were monitored and examined daily to determine their health state. Animals that were found moribund meeting criteria for euthanasia under ethical guidelines were removed from the study.

2.3.3.2 Rotarod

Balance and motor coordination were assessed using a rotarod apparatus (Harvard Apparatus) in a similar protocol to that described by Menalled *et al.* and Hockly *et al.* (Hockly et al., 2003b; Menalled et al., 2009). In order to familiarize animals with the procedure, rotarod task was introduced to animals by a 5-minute trial at a constant speed of 4 rpm. Thereafter animals were tested using an accelerating protocol with a linear increase of velocity from 4 to 40 rpm in 300 seconds. Tests were conducted weekly in 3 consecutive days and animals were given 3 trials per day. An interval of at least 10 minutes was given between trials. Locomotor performance was measured as "latency to fall" in seconds, with a cut off of 300 seconds.

2.3.3.3 Grip Strength

Muscle strength was assessed using a grip strength meter (Ugo Basile, Italy) in a similar protocol to that described by Menalled *et al.* (Menalled et al., 2009). Mice were held by the tail and brought close to the grip strength apparatus. Mice were allowed to grasp the grid with the front paws and were gently pulled back until they released their grip. The apparatus registered the peak strength for that trial. Each animal had five trials with at least 15-30 seconds rest between trials. The 5 trial test did not exceed 5 minutes.

2.3.3.4 Spontaneous locomotor activity

Spontaneous locomotor activity in the open field task was assessed at 18 weeks of age using a similar protocol to that previously described by Menalled *et al.* (Menalled et al., 2009). After 15-20 minutes habituation to the testing room, animals were placed individually in the middle of a 40 x 32 cm arena containing usual bedding. Locomotor activity was then recorded for 15 minutes using a JVC Everio GZ-MG21EK camera. Total distance travelled by each animal was tracked using Ethovision 3.0 software.

2.3.3.5 Paw clasping behaviour

Clasping phenotype was assessed by suspending mice by their tails for 60 seconds. Animals exhibiting feet-clasping (front or hind paws) were scored positive. Similar protocols have been used by Hickey *et al.* and Rodriguez-Lebron *et al.* (Hickey et al., 2005; Rodriguez-Lebron et al., 2005b).

2.3.4 Statistical analysis

All results are expressed as mean ± standard error of mean (SEM) unless otherwise stated. Three-way repeated measures Analysis of Variance (ANOVA) was carried out to investigate the overall effect of genotype, gender and age on rotarod, grip strength and body weight profile data. Significant genotype x age interactions were further investigated using One-way ANOVA followed by Bonferroni's Post Hoc test to determine from which age

transgenic and WT differ. One-way ANOVA followed by Bonferroni's Post Hoc test was also used to investigate significant statistical differences in spontaneous locomotor activity. Kaplan-Meier analysis and Mantel-Cox log-rank statistic were used to analyze survival data. All inferential statistics were carried out using PAWS 18 Statistical package.

2.4 Results

The validation of the phenotype and behavioural deficits of the R6/2 mouse model was carried out according to the study design represented in Table 2.1. Animals were observed closely on a daily basis for development and progression of the phenotype, and a battery of behavioural tests was carried out to quantify and allow comparisons with previous studies.

2.4.1 General appearance, body weight and survival

At birth, R6/2 mice were indistinguishable from their WT littermates in terms of size and homecage behaviour. However, with progression of disease, R6/2 mice displayed piloerection, jerky and choreiform-like movements, tremor, and epileptic seizures. Also, in several instances premature death occurred after seizures, which were commonly triggered by handling and unexpected noises. Furthermore, R6/2 mice also exhibited unusual vocalizations and clicking sounds when at rest. In addition, considerable differences in animals' size when compared to WT littermates were observed for both R6/2 males and females at late stages of disease. As an example, Figure 2.2 shows a R6/2 female and respective WT littermate at ~16 weeks of age.

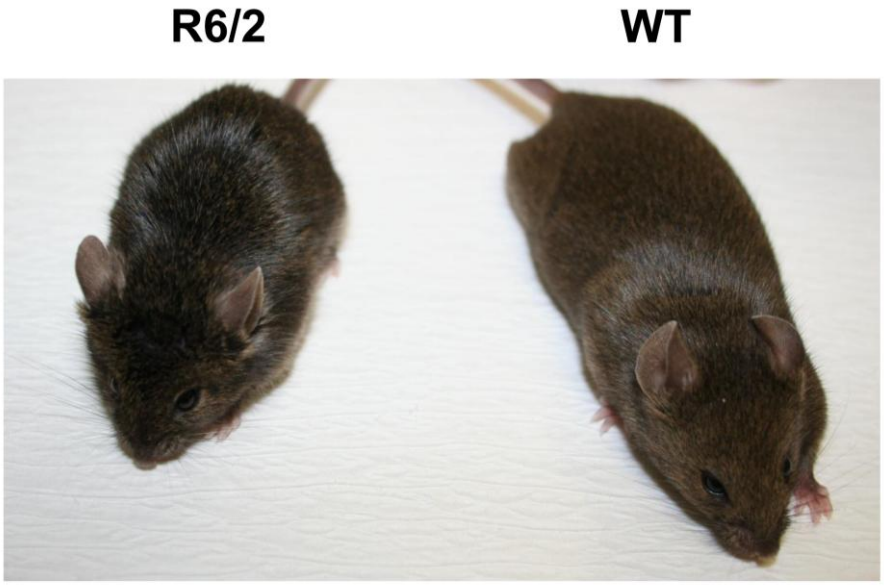


Figure 2.2. General appearance of R6/2 and WT mice. *Picture denotes a R6/2 and a WT female with ~16 weeks of age. At this late stage of disease progression there are considerable size differences between R6/2 mice and WT mice. It is also clear from the picture that R6/2 mice display piloerection whereas WT do not.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

In concordance with the above mentioned observations regarding the size of animals, body weight profile was significantly influenced by the genotype ($F(1, 31) = 38{,}78$, $P<0.0001$), but also affected by gender ($F(1,31) = 106.36$, $P<0.0001$) (Figure 2.3). Indeed, body weight profile of R6/2 mice plateau and animals fail to gain weight from ~7-8 weeks in the case of females and from ~10 weeks in the case of males, whereas WT littermates gain weight throughout the study. Furthermore, when compared to WT counterparts, differences in body weight profile became significant from 10 weeks of age for R6/2 males ($P<0.01$) and from 13 weeks of age for R6/2 females ($P<0.001$). The effect of gender was apparent in both R6/2 and WT mice, with males consistently weighing more than females.

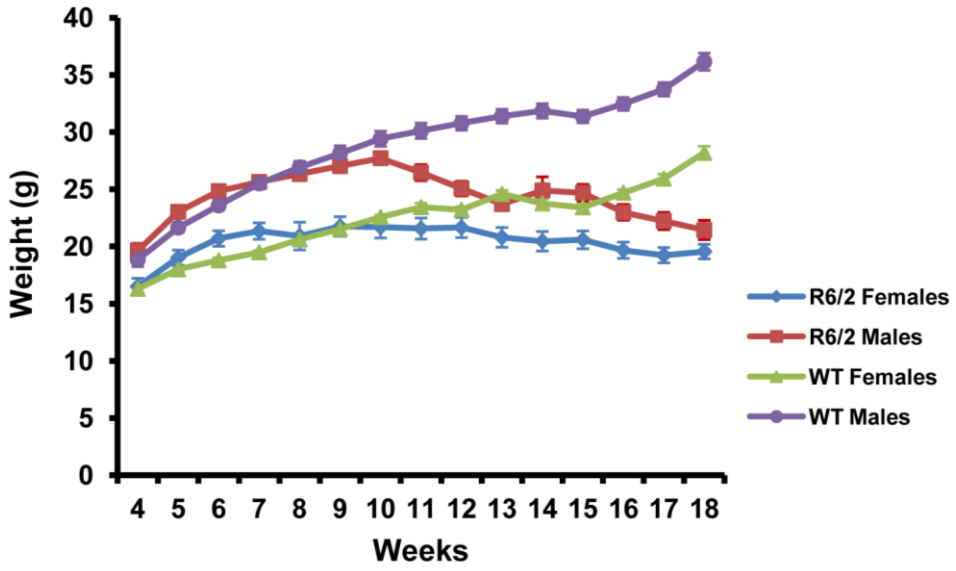


Figure 2.3. Weight profiles as function of genotype, gender and age. *Results are expressed mean ± SEM. As disease progresses R6/2 mice fail to gain weight. n = 7-10/group.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

In addition, R6/2 mice presented significantly shorter life span than WT counter parts, with a median survival of 154 days (Mantel-Cox: $P<0.0001$) (Figure 2.4). No significant differences were found between median survivals of R6/2 males (156 days) and R6/2 females (152 days) (Mantel-Cox: $P=0.7962$), and the last animal survived for 168 days.

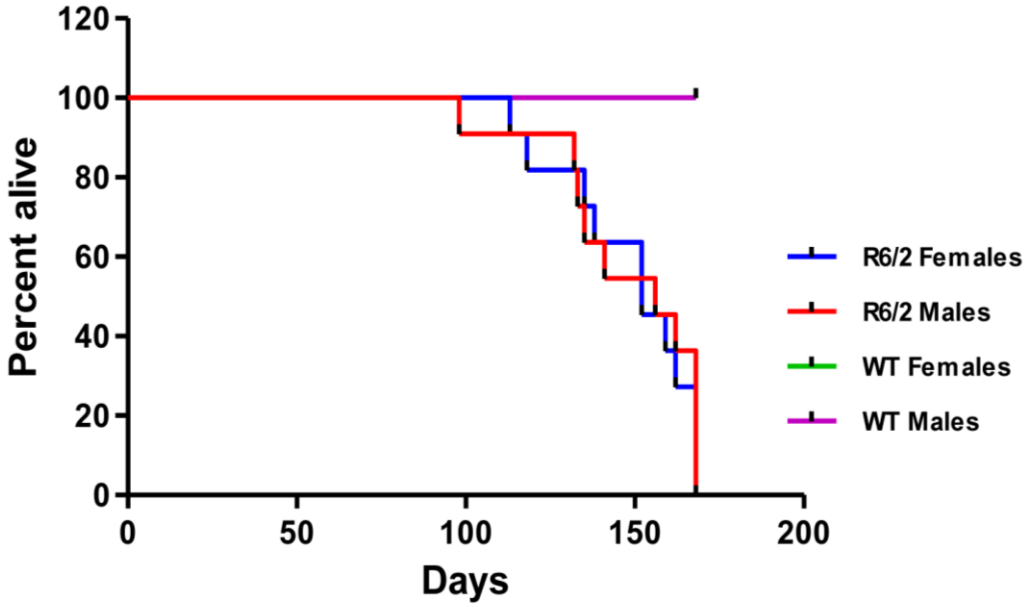


Figure 2.4. Kaplan-Meier survival curves as a function of genotype, gender and age. *Both R6/2 males and females presented shorter life spans than their WT counter parts. By the end of the study the percentage alive for both WT males and females was of 100%.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

2.4.2 Behavioural characterisation

Significant motor impairments were observed for R6/2 mice in the rotarod task as they aged, with shorter latencies to fall compared to their WT littermates ($F(1,33) = 343.64$, $P<0.0001$) (Figure 2.5). Although no significant overall differences were observed among females and males ($F(1,33) = 3.22$, $P = 0.082$), significant differences were seen in R6/2 mice compared to WT counterparts from 4 weeks of age ($P<0.01$) and from 5 weeks of age in the females ($P<0.001$). At late stages of disease, R6/2 mice were no longer able to hold on to the rod.

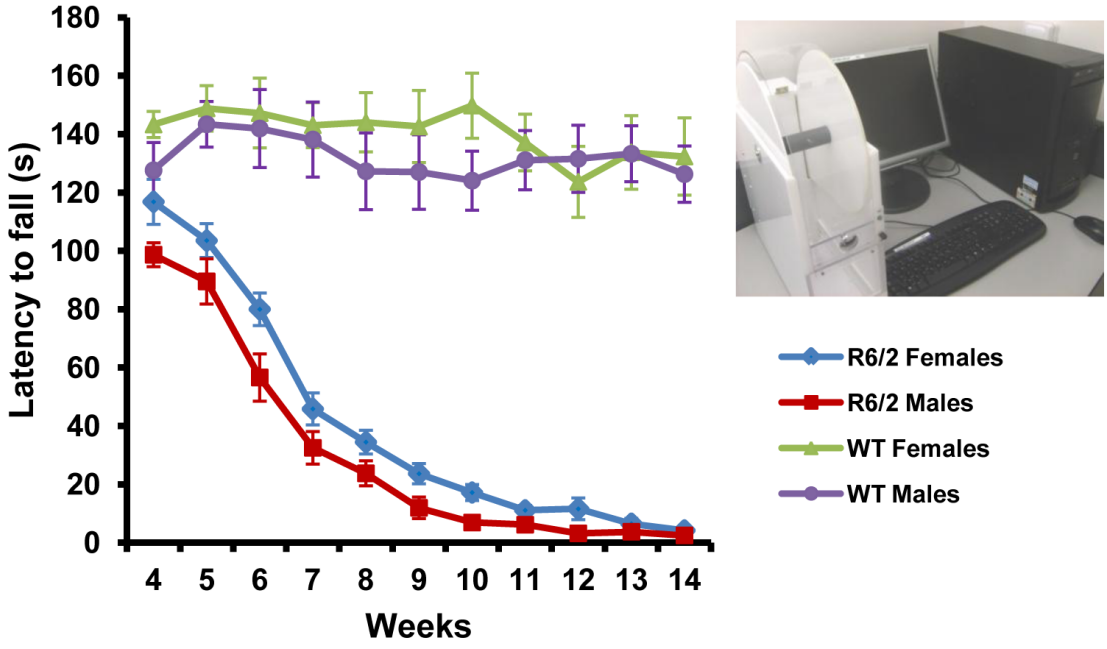


Figure 2.5. Motor coordination and balance in the rotarod task as a function of genotype, gender and age. *Mice were placed on top of a rotating rod and their latency to fall was recorded. Average of the best performances achieved in 3 consecutive days was recorded. All results are expressed in mean ± SEM, n =8-10/group.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

Furthermore, R6/2 mice displayed progressive deterioration of muscle strength ($F(1, 26) = 328.66$, $P<0.0001$) (Figure 2.6). Although no overall significant differences in grip strength were found between genders ($F(1,26) = 0.085$), differences compared to WT counter parts became evident for R6/2 males at 10 weeks of age ($P<0.01$) and by 11 weeks of age for R6/2 females ($P<0.001$). At late stages of disease, R6/2 mice were not motivated to perform the grip strength task and did not hold the grid with their front paws, thus testing was stopped after 18 weeks of age.

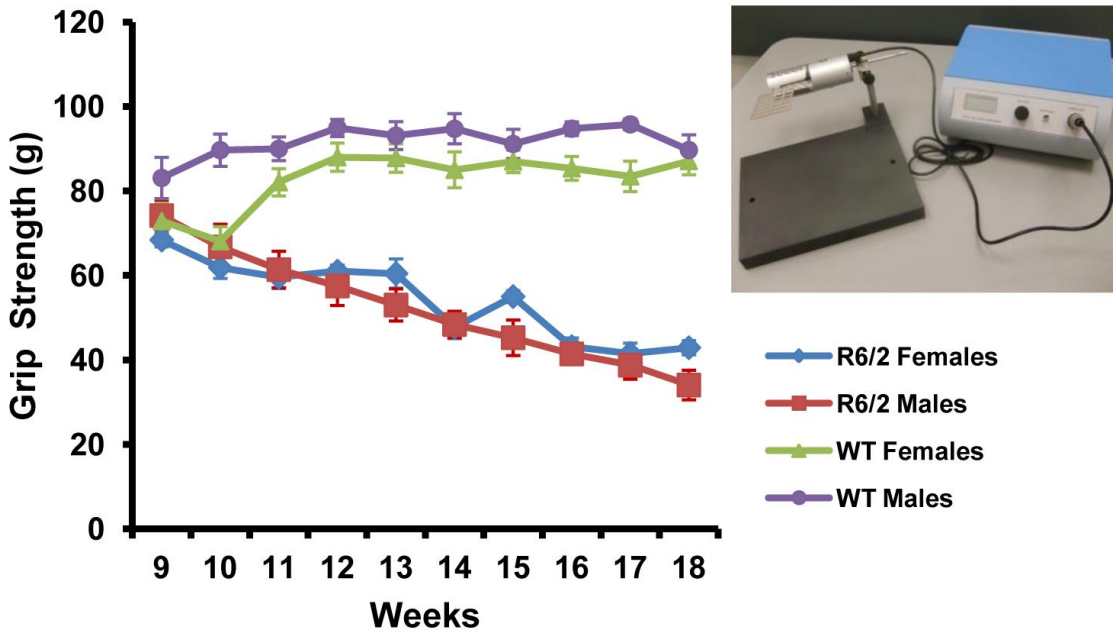


Figure 2.6. Muscle strength in the grip strength task as a function of genotype, gender and age. *Mice were allowed to grasp a grid with their fore limbs and gently pulled back until release of their grip. Average of 5 trials was recorded for each animal. All results are expressed in mean ± SEM, n =7-10/group.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

As seen in Figure 2.7a, at 18 weeks of age both R6/2 males and females were found to be hypoactive when compared to their WT counterparts, presenting significantly reduced spontaneous locomotor activity in the open field task ($P<0.001$) (Figure 2.7a,b). Indeed, at this late stage of disease, hypoactivity in R6/2 mice was also clearly evident through mere observation of their homecage behaviour. In addition, for both R6/2 and WT mice, no significant differences in distance travelled were found between males and females.

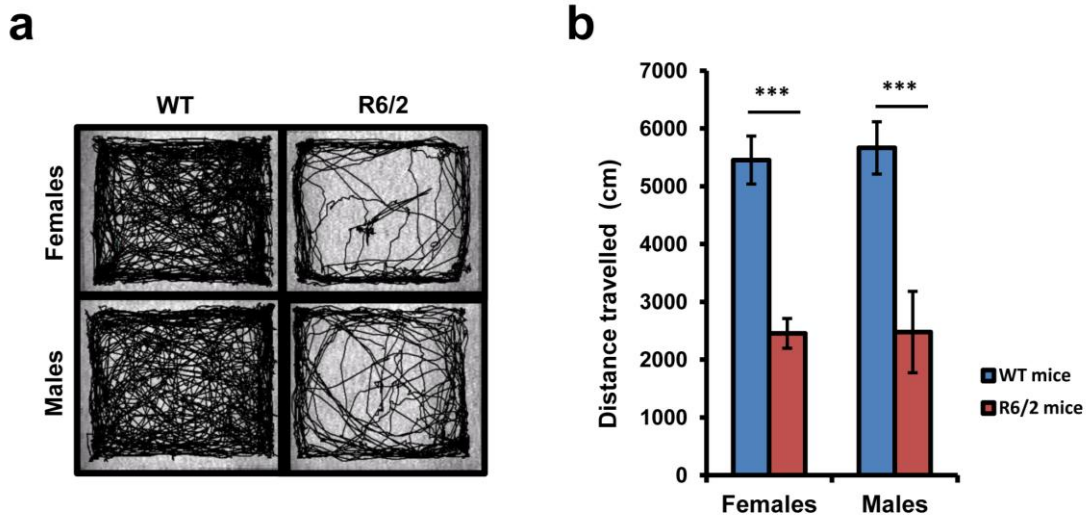


Figure 2.7. Spontaneous locomotor activity in the open field task as a function of genotype and gender. *Mice were placed in the centre of an arena and allowed to move freely for 15 minutes. (a) Movement of R6/2 and WT females and males in the open field was tracked with Ethovision software. (b) Total distance moved as function of genotype and gender represented as mean ± SEM.***P<0.001, n=8-10/group.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

For the time course of the study, characteristic feet-clasping behaviour was observed from 4 weeks of age in R6/2 mice but not in WT mice. Figure 2.8a depicts the classical feet-clasping posture adopted by R6/2 mice and the "normal" position adopted by WT mice. Although, initially R6/2 females seemed to be affected to a greater extent (~36%) than R6/2 males (~9%), by 12 weeks of age week nearly ~100% of R6/2 mice presented feet-clasping behaviour (Figure 2.8b).

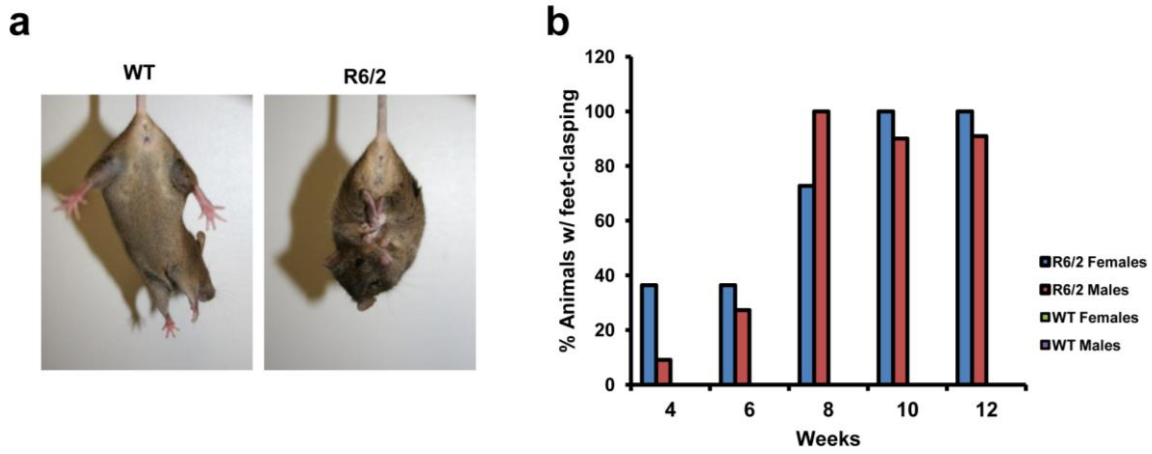


Figure 2.8. Clasping behaviour as a function of genotype, gender and age. *Mice were held by their tails and scored positive if presenting clasping behaviour. (a) Characteristic clasping posture adopted by R6/2 mouse and "normal" posture by WT mouse when suspended by their tails. (b) Percentage of animals with clasping behaviour as a function of genotype, gender and age. n=9-11/group.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

2.5 Discussion

In this study we have successfully validated the R6/2 mouse model in our laboratory, establishing the basis for testing the therapeutic potential of modified cyclodextrins as non-viral vectors for ribonucleic acid (RNA) interference treatment strategies for HD. R6/2 mice displayed early and progressive HD-like symptoms which did not arise in WT animals. These data are in agreement with previous findings by Mangiarini *et al.* As disease progressed, our R6/2 colony displayed piloerection, irregular gait, resting tremors, seizures and involuntary shuddering noises (Mangiarini et al., 1996). At the late stages of disease, R6/2 mice were considerably less active in their home-cage and consistently smaller than WT littermates. Additionally, as the disease progressed R6/2 mice fail to gain weight with significant differences in weight compared to their respective WT littermates from about ~ 10 weeks for R6/2 males and ~ 13 weeks for females. Similar results have been reported by others (Carter et al., 1999; Hickey et al., 2005; Hockly et al., 2003b), however, earlier drops in the body weight of R6/2 mice have also been observed (Menalled et al., 2009; Stack et al., 2005). In the latter studies, abrupt weight loss was most likely caused by the inability of R6/2 mice to reach for food and water in standard laboratory cages at later stages of disease. In the present study animals were provided with soft diet at the bottom of the cage, consisting of mashed chow mixed with water (enhanced diet), which may have delayed weight loss at late stages of disease. Other studies focusing on the effect of feeding regimes have also found that enhanced diets improved weight profiles and also survival of R6/2 mice when compared to animals fed with standard diets (Carter et al., 2000). The present R6/2 colony displayed longer survivals (median survival of 158 days) than has been commonly reported before (~92-113 days) (Menalled et al., 2009; Stack et al., 2005). Enhanced diet, but also other factors such as minimal environmental enrichment, animal handling and behavioural testing have been demonstrated to improve weight loss, longevity and motor deficits in the R6 transgenic lines (Carter et al., 2000; Hockly et al., 2002; Spires et al., 2004; Wood et al., 2010), and might have played an important role in the current study.

Thus, in order to enable a fair comparison among studies carried out in R6/2 mice, there is an urgent call for standardisation of housing conditions, feeding regimens, handling and behavioural testing routines (Hockly et al., 2003b). Indeed such standardisation has also been called for in all aspects of behavioural neuroscience (Van der Staay et al., 2002). However, it is also worth noting that excessive standardisation has been shown to reduce reproducibility of studies between laboratories by increasing test sensitivity within a specific laboratory setting (Richter et al., 2010; Richter et al., 2009). All of this points to the need for behavioural validation of relevant mouse models prior to preclinical testing of novel therapeutics.

Behavioural data in this study confirmed an early onset of motor symptoms in R6/2 mice. Indeed, motor coordination and balance in the rotarod task was found to be impaired as early as 4 weeks of age in the rotarod task. Although some studies have also reported similar early rotarod deficits (Menalled et al., 2009), others only reported significant differences from WT littermates from 5 weeks of age (Carter et al., 1999; Stack et al., 2005). We reason that differences in testing protocols (constant vs. accelerating protocols) and rotarod equipments are, most likely, the cause of such dissimilarities between studies. On the other hand, grip strength data showed that disease progression affects R6/2 mice grip by ~10-11 weeks of age, which is in agreement with previous behavioural studies (Menalled et al., 2009). It is also worth noting that the deterioration of grip strength follows on from progressive muscle atrophy, which has been reported to start by 6 weeks of age (Sathasivam et al., 1999). Furthermore, at 4 weeks of age R6/2 mice already present dyskinetic clasping behaviour which progresses rapidly, and by 10 weeks of age almost all animals clasp after being suspended by their tails. Similar results have been reported in other studies (Hansson et al., 2003; Stack et al., 2005). Finally, spontaneous locomotor activity at 18 weeks of age was found to be reduced in R6/2 mice compared to their wild type littermates. Although R6/2 mice have been found by others to be hyperactive at early stages of their life (Lüesse et al., 2001), from ~ 8 weeks of age these mice tend to be hypoactive in the open field task

(Menalled et al., 2009). Importantly, no overall significant differences were found between R6/2 males and females in the behavioural tests undertaken here. It is worth noting that WT and R6/2 males consistently weighed more than females of their respective genotype as reported by others (Hockly et al., 2003b; Stack et al., 2005).

Although R6/2 mice also display other symptoms characteristic of human HD, such as increased cognitive impairment (Lione et al., 1999; Murphy et al., 2000), co-founding factors including reduction of visual acuity and the onset of motor symptoms confound/hinder the clear assessment of cognitive function in the R6/2 mouse model (Murphy et al., 2000; Murphy et al., 1998). Similarly, assessment of anxiety and depressive-like behaviours in R6/2 mice are believed to be in part affected by their generalised hypoactivity at later stages of disease (Hickey et al., 2005). Thus, most preclinical studies in the R6/2 model which aim to evaluate the therapeutic potential of novel approaches for HD have majorly concentrated on the assessment of motor improvements (Gil & Rego, 2009; Li et al., 2005). Furthermore, behavioural tests such as rotarod, grip strength and spontaneous activity in the open field provide reliable quantitative measures of disease progression and are, therefore, less likely to be biased by the experimenter. To this end, the R6/2 colony established here has been successfully validated in the above mentioned behavioural tests, presenting a similar progressive dysfunctional motor behaviour to that previously described.

2.6 Conclusion

Animal models of HD have revolutionized the field, giving new insights into the pathophysiology of HD and also providing a platform for testing novel therapies. Although none of the current animal models of HD fully reproduce the complexity of human HD neuropathology, the R6/2 mouse model offers a robust representation of the poor motor coordination and muscle wasting seen in human disease. The early onset of motor symptoms in this model enables rapid testing, with the possibility of obtaining results within 3 months, reducing costs and accelerating preclinical studies of new therapies. Thus, the R6/2 model, which we have validated here, seems to be a suitable model for initial studies of potential new disease-modifying therapies, such as cyclodextrin-based short interfering RNA (siRNA) nanoparticles for muHTT gene silencing. However, in spite of its unequivocal economical convenience, the aggressive progression of the disease phenotype in this model may pose other challenges, for instance in terms of the timeframe & logistics of experiments including surgical intervention, if required. Furthermore, it is worth nothing that, prior to human trials, positive results obtained in R6/2 mice will likely need further confirmation in other animal HD models which show different temporal and functional correlates of the disease (see Chapter I, Table 1.1). In conclusion we have shown that the R6/2 model produces behavioural deficits relevant to HD and is thus a suitable platform for initial therapeutic intervention studies.

2.7 Supplementary Information

The SI section in this chapter includes further details concerning R6/2 mouse husbandry, breeding and development (Supplementary Table S2.1), and also additional data regarding the PCR-based method for genotyping R6/2 mice.

2.7.1 R6/2 mouse husbandry, breeding and development

Two different cohorts of B6CBAF1 breeders (OT B6CBAF1females x B6CBAF1 males) were used to expand and maintain the R6/2 colony. Breeders were obtained from Jackson Laboratories and paired together when they were ~6 weeks of age. Although none of OT B6CBAF1 females expressed the disease, they were previously transplanted with the ovaries of a histocompatible R6/2 female and therefore were able to produce eggs containing the transgenic allele. Most OT B6CBAF1females delivered their first litter ~60 days after pairing with males. In some cases, for instance in breeding pairs 2 (Cohort A), 9 and 12 (Cohort B), OT B6CBAF1females failed to deliver their first litter after >120 days. To investigate if this was due to infertility of the male breeder, a male from a different breeding pair was introduced, but no litter was still delivered and therefore females were deemed unproductive. Furthermore, OT B6CBAF1 females sometimes displayed excessive grooming and in those cases the breeding pair was separated. However, this was only carried out in extreme cases since females were less likely to breed when put back together with males after a period of separation (e.g. female from breeding pair 4 (cohort B)).

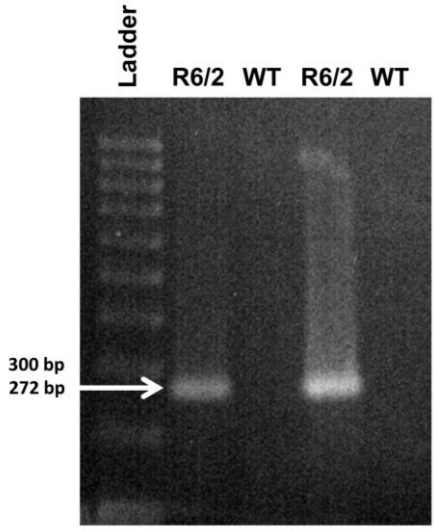
From both cohorts of breeders a total number of 529 mice was obtained and reached adulthood (Supplementary table S2.1). Rare cases where litters were lost are not included. From all B6CBAF2 mice generated, 275 were males (52.0% of total), 254 females (48.0% of total), 262 carried the muHTT transgene (49.5% of total) and 267 were WT (50.5% of total). From total, the percentage of R6/2 males was of 26.1% and of R6/2 females was of 24.6%. In conclusion, from this analysis the percentage of R6/2 and WT mice obtained very closely matched the expected dominant inheritance pattern (~50%) of mendelian traits when one of the progenitors is affected.

Supplementary Table S2.1. R6/2 Mouse Husbandry, Breeding and Development

Cohort	Breeding pair	Pups	Males	Females	R6/2	WT	R6/2 F	R6/2 M	WT F	WT M
A	1	45	18	27	27	18	17	10	10	8
	2	-	-	-	-	-	-	-	-	-
	3	23	13	10	10	13	5	5	5	8
	4	14	8	6	7	7	3	4	3	4
	5	18	9	9	8	10	4	4	5	5
	6	20	13	7	8	12	2	6	5	7
	Subtotal	120	61	59	60	60	31	29	28	32
	% Subtotal	N/A	50.8	49.2	50.0	50.0	25.8	24.2	23.3	26.7
B	1	11	8	3	6	5	2	4	1	4
	2	41	25	16	24	17	10	14	6	11
	3	20	9	11	11	9	7	4	4	5
	4	6	2	4	4	2	2	2	2	0
	5	25	16	9	14	11	5	9	4	7
	6	38	23	15	17	21	4	13	11	10
	7	20	10	10	9	11	4	5	6	5
	8	29	19	10	17	12	5	12	5	7
	9	-	-	-	-	-	-	-	-	-
	10	56	25	31	32	24	17	15	14	10
	11	32	14	18	14	18	10	4	8	10
	12	-	-	-	-	-	-	-	-	-
	13	16	10	6	5	11	0	5	6	5
	14	44	20	24	16	28	10	6	14	14
	15	44	21	23	23	21	10	13	13	8
	16	27	12	15	10	17	7	3	8	9
	Subtotal	409	214	195	202	207	93	109	102	105
	% Subtotal	N/A	52.3	47.7	49.4	50.6	22.7	26.7	24.9	25.7
TOTAL		529	275	254	262	267	124	138	130	137
% TOTAL		N/A	52.0	48.0	49.5	50.5	23.4	26.1	24.6	25.9

Abbreviations*: N/A, Not applicable; R6/2 F, R6/2 females; R6/2 M, R6/2 males; WT, Wild-type.*

2.7.2 PCR-based method for genotyping R6/2 mice



Supplementary Figure S2.1. PCR-based method for genotyping R6/2 mice. *Agarose gel electrophoresis of the PCR product amplified from DNA samples collected from tail tips. Arrow indicates ~272 bp amplicon product from the human muHTT transgene only expressed in R6/2 mice. Absence of specific band indicates that the transgene is not present and therefore the animal belongs to the WT genotype group. Ladder 1000 Kb.* ***Abbreviations****: R6/2 mice, transgenic mice hemizygous for the human HTT transgene; WT, Wild-type.*

.

Chapter III

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

Bruno M. D. C. Godinho[1,2], Julien R. Ogier[3], Raphael Darcy[3], Caitriona M. O'Driscoll[1], John F. Cryan[2,4]

[1]Pharmacodelivery group, School of Pharmacy, University College Cork, Cork, Ireland

[2]Dept. Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3]Centre for synthesis and Chemical Biology, University College Dublin, Dublin, Ireland

[4]Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, Cork, Ireland

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3.1 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 ribonucleic acids (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 non-toxic 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 rivonucleic acid interference based (RNAi-based) therapies for neuropsychiatric and neurodegenerative disorders.

3.2 Introduction

Huntington's Disease (HD) is a rare but devastating autosomal dominant neurodegenerative disease caused by a mutation within the Huntingtin (HTT) gene (Zuccato et al., 2010). The mutation consists of an abnormal CAG repeat expansion that leads to the expression of a toxic HTT protein. Accumulation of the mutant HTT (muHTT) protein compromises survival and normal neuronal functioning in the striatum and progressively in other brain structures, such as the cortex (Zuccato et al., 2010).

Reducing expression of the muHTT gene by means of ribonucleic acid interference (RNAi) has been recently suggested as one of the most promising therapeutic strategies for HD (Zuccato et al., 2010). Briefly, the RNAi pathway is an endogenous post-transcriptional mechanism whereby short double stranded RNA molecules inhibit the translation of specific messenger RNA (mRNA) by ribosomal arrest or degradation (Guo et al., 2010a). Exogenously introduced synthetic short interfering RNAs (siRNAs), short harpin 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 (Guo et al., 2010a). 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 (Krichevsky & Kosik, 2002). Viral and non-viral approaches for RNAi delivery have been evaluated to facilitate the transport of genetic material into neurons (Boudreau et al., 2010). 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 disease (AD) and amyotrophic lateral sclerosis (ALS) (Grondin et al., 2012; Sah, 2006). 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 (Nayak et al., 2010; Snove & Rossi, 2006). Alternatively, non-viral 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 siRNAs were 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 (Stiles et al., 2012; Thakker et al., 2004; Thakker et al., 2005). 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 (Davidson et al., 2004), lipids and steroid derivatives (Chen et al., 2010; DiFiglia et al., 2007), and other modifications (Nakajima et al., 2012; Wang et al., 2008c) have been shown to be advantageous for *in vivo* delivery and have improved delivery of siRNAs to the brain and spinal cord (Watts et al., 2008). On the other hand, several lipid-based (Cardoso et al., 2008; Salahpour et al., 2007), polymer-based (Kim et al., 2010a; Tan et al., 2004), peptide-based carriers (Ifediba et al., 2010) and other nanoparticles (Bonoiu et al., 2011; Wong et al., 2010) 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 non-viral RNAi vectors, there remains a great need to develop more effective and less toxic carriers for siRNA delivery into the CNS (Guo et al., 2010a). Modified cyclodextrins (CD), based on naturally occuring oligosaccharide molecules, are promising nucleic acid carriers that have been shown to bind and complex siRNA protecting it from enzymatic degradation (Chaturvedi et al., 2011). Our group has previously demonstrated efficacy of these nano carriers to deliver both plasmid deoxyribonucleic acid (DNA) and siRNA into hepatocyte, enterocyte, prostate cancer and neuronal *in vitro* models (Guo et al., 2012b; McMahon et al., 2012; O'Mahony et al., 2012b;

O'Mahony et al., 2012d; O'Neill et al., 2011). 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 (Davis, 2009; Davis et al., 2010).

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 harbouring the human muHTT gene were used to validate this technology *in vitro*. In order to evaluate if any effects translated to the *in vivo* setting, pre-clinical testing of CD.siRNA nanoparticles was carried out in the R6/2 mouse model of HD.

3.3 Materials and methods

3.3.1 Synthetic siRNAs

Synthetic duplexed siRNAs were obtained from Sigma-Aldrich (France) or QIAGEN (United Kingdom). HTT target siRNAs (HTTsiRNA) as per Wang *et al.* (Wang et al., 2005) sense strand, 5'-GCCUUCGAGUCCCUCAAGUCC-3'; antisense strand, 5'-ACUUGAGGGACUCGAAGGCCU-3'. Non-silencing siRNAs (NSsiRNA): sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. FAM-labelled siRNA (FAMsiRNA): sense strand, 5'-[6FAM] UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

3.3.2 Preparation, physicochemical characterisation and stability of CD.siRNA nanoparticles

CD solutions were prepared as previously described in sterile deionised water (DIW) or 5% glucose solution (O'Mahony et al., 2012d). Details on preparation of CD solutions are described in Supplementary Information (SI, Supplementary Materials and Methods), CD.siRNA complexes were diluted 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_2PO4 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 gel retardation assay described in O'Mahony *et al.* (O'Mahony et al., 2012d). Sodium dodecyl sulphate (SDS) (Sigma-Aldrich, Germany) was used to disrupt nanoparticles and enable release of siRNA. For *in vivo* studies CD.siRNAs 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.

3.3.3 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 described in SI (SI Materials and Methods). CD.siRNA nanoparticles were prepared in sterile DIW and left to incubate for 20 minutes and thereafter diluted in optiMEM® (GIBCO, United Kingdom). Cells were transfected with CD.siRNA nanoparticles 24 hours after being seeded.

3.3.4 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 hours either at 33 °C or 37 °C and 5% CO_2. The formazan product was then dissolved with 100 µL of dimethyl sulfoxide (Sigma-Aldrich, Germany) and left to incubate at room temperature at least for 30 minutes. Absorbance was measured at 590 nm using a SpectraMax Plus384 plate reader.

3.3.5 Confocal Microscopy

Confocal microscopy was carried out in living cells. Non-silencing [6FAM] 5'-labelled 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 hours. 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 analysed using Olympus Fluoview ver 2.1b software.

3.3.6 Quantitative real-time PCR

Ribonucleic acid (RNA) was isolated from tissue using a Trizol®-based method (Invitrogen, United Kingdom). 300 ng of total RNA was reverse transcribed to complementary DNA (cDNA) using a High Capacity cDNA Reverse Transcription kit from Applied Biosystems (Foster City, MO). Gene expression was assessed by fluorescent real time quantitative polymerase chain reaction (RT-qPCR) using a 7300 Real Time polymerase chain reaction (PCR) System. Cycling conditions were: 10 minutes at 95 °C, 40 cycles of [15 seconds at 95 °C; 1 minute at 60 °C]. TaqMan® rat or mouse b-actin VIC® labelled probes were acquired from Applied Biosystems (United Kingdom) (part number 4352340E and 4352341E). Primer sequences (forward: CGACCCTGGAAAAGCTGATGAA, reverse: CTGCTGCTGCTGGAAGGA) were validated for detection of human HTT mRNA (Ref. Seq. NM_002111) and used to design a TaqMan® HTT FAM-labelled probe. Each sample was analysed 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.

3.3.7 Western blot analysis

ST14A HTT120Q cells were harvested 72 hours after addition of transfection complexes and lysed in lysis buffer (10 mM HEPES, 100 mM KCl, 1.5 mM MgCl2, 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 ug 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 volts for ~2.5 hours. Protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) for 1.5 hours at 200 mA and transfer confirmed by Ponceau S staining. Membrane was incubated in a blocking solution

containing 0.1% Tween and 5% milk and incubated overnight with anti-human HTT antibody (dilution 1:2,500) (MAB2166, Millipore) or anti-β-actin (dilution 1:3,000) (A5441, Sigma). Membrane was washed and then incubated with a 1:10,000 dilution of a goat anti-mouse antibody (IRDye 800CW, LI-COR) for 1 hour. Scans were carried out using a LICOR Odyssey near-infrared scanner. Densitometry analysis of bands was performed using ImageJ software and all results were normalised to β-actin controls.

3.3.8 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. Detailed description of the genotyping protocol has been given in Chapter II Materials and Methods. 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.

3.3.9 Stereotaxic surgery and behavioural 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 (Anterior-Posterior = + 0.7, Medio-lateral = ± 2.0, Ventral = -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 behavioural studies a total of 7 injections of naked HTTsiRNAs or CD.HTTsiRNA nanoparticles were given over a period of 5 weeks and behaviour deficits were assessed up to 10 weeks after first injection (Figure 3.5a). All behaviour tests were conducted after 10-15 minutes habituation period to the testing room. Behaviour tasks were carried out as previously described in (Dragatsis et al., 2009; Menalled et al., 2010; Wang et al., 2005) for further details on behavioural tasks see SI, Supplementary Materials and Methods.

3.3.10 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 analyse each specific time point of behavioural assessment. Finally, paw clasping behaviour data was analysed by Chi Square tests at each age separately. All inferential statistics were carried out using PAWS 18 Statistical package.

3.4 Results

3.4.1 Physicochemical characterisation of CD.siRNA nanoparticles and stability in artificial cerebrospinal fluid

Chemical modifications have been previously introduced onto a β-CD to form polycationic amphiphilic molecules (SC12 CD (Click) Propylamine) (Figure 3.1a) (O'Mahony et al., 2012d) Electrostatic interaction between these modified β-CDs and polyanionic siRNAs results in nucleic acid condensation and formation of nanoparticles (Figure 3.1a). 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 (SI, Supplementary Figure S3.1a). Moreover, these complexes were found to have a hydrodynamic diameter between 100 nm and 350 nm and a net positive surface charge (Figure 3.1b). A reduction in particle size and an increase in net charge were noted as increasing MRs of modified β-CD were used. CD.siRNAs nanoparticles were found to remain stable and undisrupted in aCSF up to 6 hours (Figure 3.1c). aCSF and physiological temperatures (37 °C) seemed not to affect siRNA binding and complexation as shown by gel retardation assays. Furthermore, size and surface charge of the CD.siRNA nanoparticles did not reveal remarkable changes up to 6 hours (SI, Supplementary Figure S3.1b).

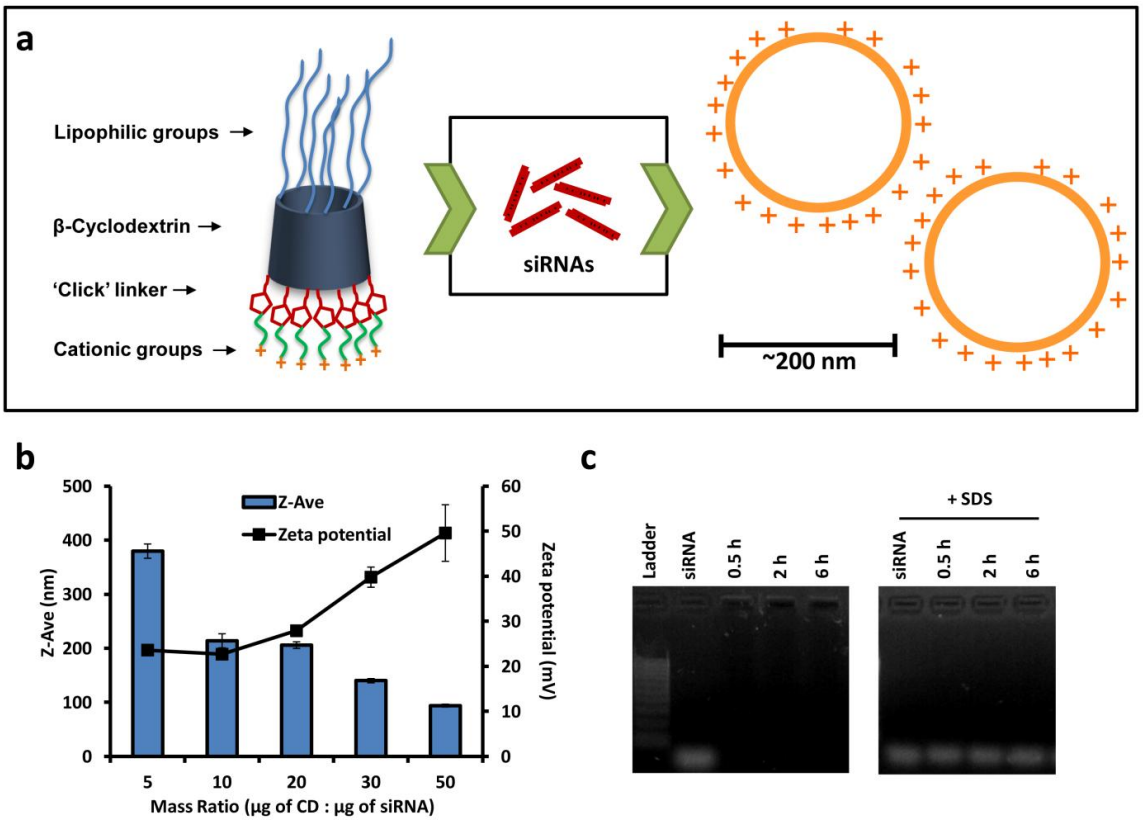


Figure 3.1. Physicochemical characterisation of CD.siRNA nanoparticles and stability in artificial aCSF. *(a) Schematic showing the formation of nanoparticles. Complex formation is enabled by electrostatic interactions between positively charged modified β-CD units and negatively charged siRNA phosphate back bone. (b) Hydrodynamic radius and zeta potential of CD.siRNA nanoparticles measured through Dynamic Light Scattering and Electrophoretic Light Scattering, respectively. Results are expressed in mean ± standard deviation (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 remain in the wells. SDS was used to release siRNAs from nanoparticles and prove its integrity.* ***Abbreviations****: aCSF, Artificial cerebrospinal fluid; CD, Cyclodextrin; SD, Standard deviation; SDS, Sodium Docecyl Sulfate; siRNA, Short interfering RNA.*

3.4.2 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 3.2a). Reduced mitochondrial dehydrogenase activity has been widely used as an indication of cytotoxicity. Even after 48 hours 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 FAMsiRNA was observed by confocal microscopy (Figure 3.2b) and quantified by Fluorescent Activated Cell Sorting (FACS) Flow cytometry (SI, Supplementary Figure S3.2a,b). Fluorescent CD.FAMsiRNA nanoparticles (green) were taken up by this neuronal cell line in a time-dependent fashion. After 48 hours post transfection, up to ~38% (38.3 ± 7.1%) of cells were found to be positive for fluorescent CD.FAMsiRNA complexes. Furthermore, our data shows that only a few CD.FAMsiRNA complexes were co-localised (yellow) with acidic endosomes (red) after 24 hours. In contrast, no significant uptake was observed in cells treated with naked FAMsiRNA (Figure 3.2b).

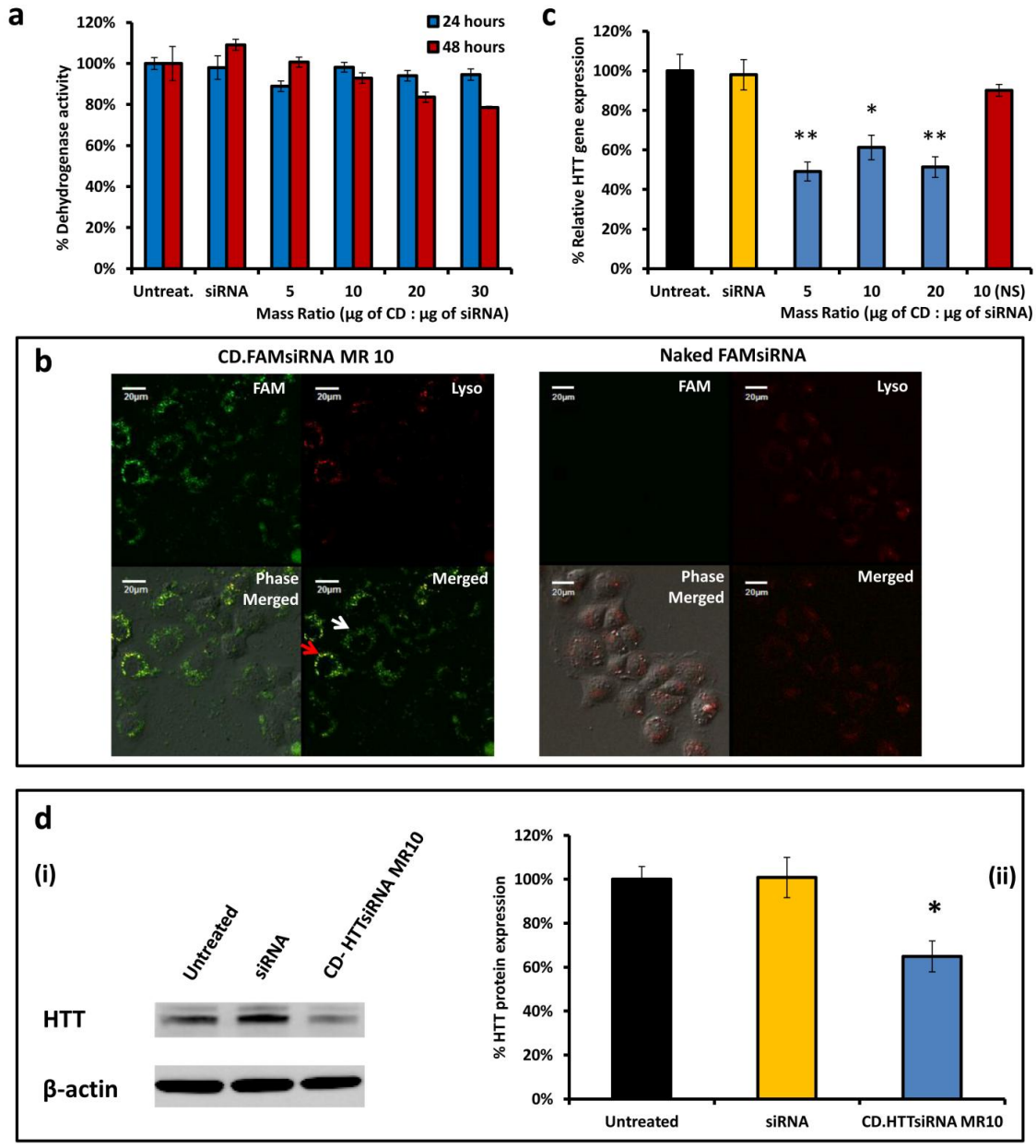


Figure 3.2. Delivery of CD.siRNA nanoparticles to a rat neuronal in vitro model of HD (ST14A-HTT120Q cells). *(a) Cytotoxicity profiles of CD.siRNA nanoparticles in ST14A-HTT120Q cells were assessed by MTT assay after 24 and 48 hours transfection. (b) Cellular uptake of fluorescently labelled CD.siRNA 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 co-localised 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). 5 µg of total protein was loaded onto each lane. (ii) Densitometry analysis. All results were normalised to β-actin protein expression prior densitometry analysis. 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.*

MuHTT gene expression was assessed by RT-qPCR (Figure 3.2c). 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 hours transfection. The HTTsiRNAs sequences used in the present study which allow for specific knockdown have been previously screened and validated by others (Rodriguez-Lebron et al., 2005a; Wang et al., 2005). 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 NSsiRNA sequence were not able to significantly reduce expression of HTT when compared with untreated cells. Additionally, HTT protein levels were found to be reduced by ~35% (35.1 ± 7.1%) after 72 hours transfection with CD.HTTsiRNAs (Figure 3.2d). 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 3.1). 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 3.1).

Table 3.1. Efficiency and cytotoxicity of CD.siRNA nanoparticles vs. Lipofectamine® 2000 in ST14A-HTT120Q cells

Vector characteristic	Lipofectamine®2000	CD.siRNA MR10
Cellular uptake	50-60%	30-50%
Knockdown in HTT gene expression	60-63%	40-60%
Reduction in HTT protein expression (72 hours)	36%	35%
Dehydrogenase activity	58-60%	82-98%

3.4.3 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 3.3a). After 48 hours 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 3.3b). FACS flowcytometry revealed that after 48 hours transfection up to ~40% (40.2 ± 1.6%) of cells were found to be positive for fluorescent CD.FAMsiRNA complexes (SI, Supplementary Figure S3.2c,d). Moreover, our data shows that only few CD.FAMsiRNA complexes were co-localised (yellow) with acidic endosomes (red) after 24 hours. In contrast, no significant uptake was observed in cells treated with naked fluorescent siRNA. Additionally, CD.HTTsiRNA nanoparticles at different MR were able to silence the expression of the HTT gene by ~78% (78.2 ± 8.5%) after 24 hours transfection (Figure 3.3c). Alternatively, naked HTTsiRNA and CD.NSsiRNA complexes were not able to significantly reduce expression of the HTT gene when compared with untreated cells.

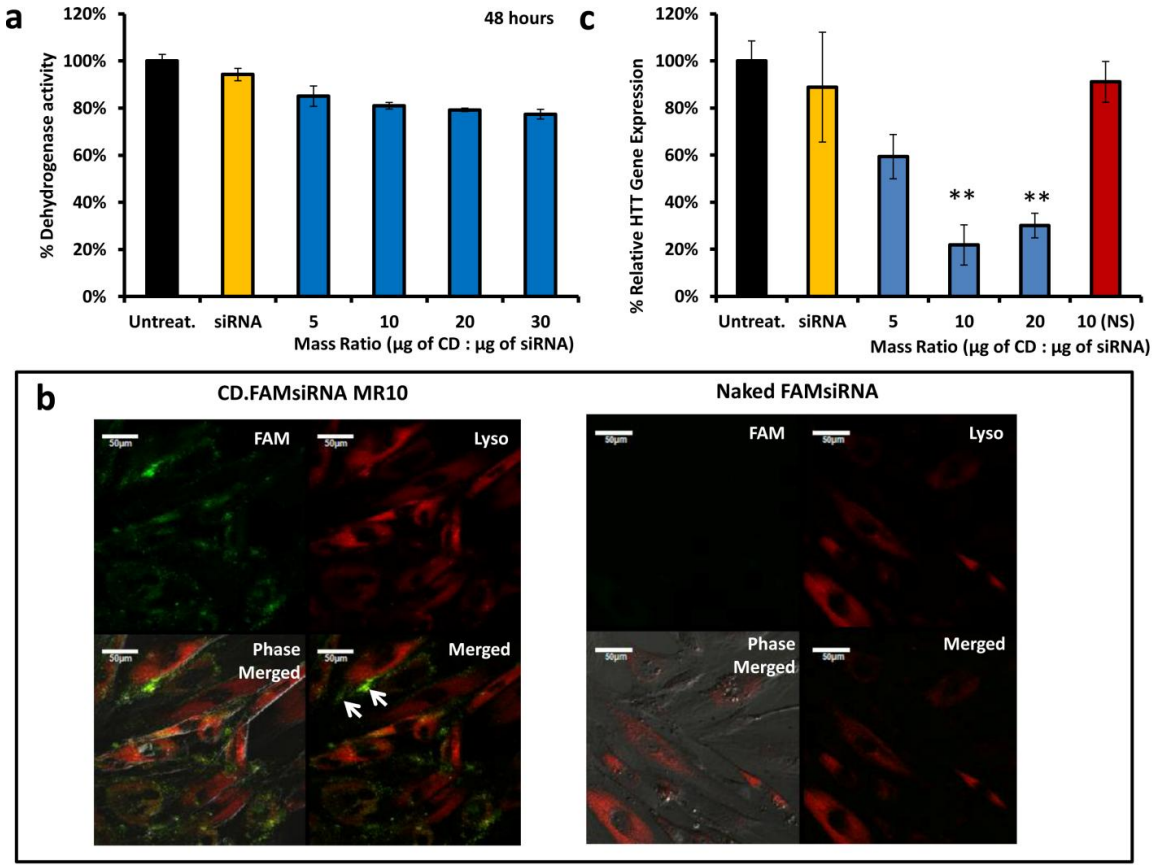


Figure 3.3. Delivery of CD.siRNA nanoparticles to a human in vitro model of HD (HD human primary fibroblasts). *(a) Cytotoxicity profiles of CD.siRNA nanoparticles in human HD fibroblast primary cells were assessed by MTT assays after 48 hours transfection. (b) Cellular uptake of fluorescently labelled 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 or 48 h. All results are expressed in mean ± SEM, n = 3 per group, **P < 0.01 compared to untreated cells.*

3.4.4 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 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 (SI, Supplementary Figure S3.3a,b). Additionally, size and surface charge of complexes prepared in these buffers were not affected to a great extent when compared to complexes prepared in DIW (SI, Supplementary Figure S3.3c,d). In contrast, CDs prepared in phosphate buffered saline (PBS) resulted in cloudy solutions, failed to fully complex siRNAs and caused abrupt changes in size and surface charge of particles (SI, Supplementary Figure S3.3). Since 5 % glucose has been widely used for direct brain injections in previous studies and stability of complexes was not affected, we have selected this buffer as vehicle for brain delivery of CD.siRNA nanoparticles (Tan et al., 2004).

In order to investigate knockdown efficiency *in vivo*, R6/2 mice were treated with vehicle (5% glucose) (Tan et al., 2004), 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 3.4a).

CD.HTTsiRNA nanoparticles were able to significantly reduce the expression of the HTT gene *in vivo* (Figure 3.4b). The time course study revealed that, 4 hours post-injection, 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, knockdown was no longer apparent at three weeks post-injection.

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 whilst there was a trend towards 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 (SI, Supplementary Figure S3.4a,b).

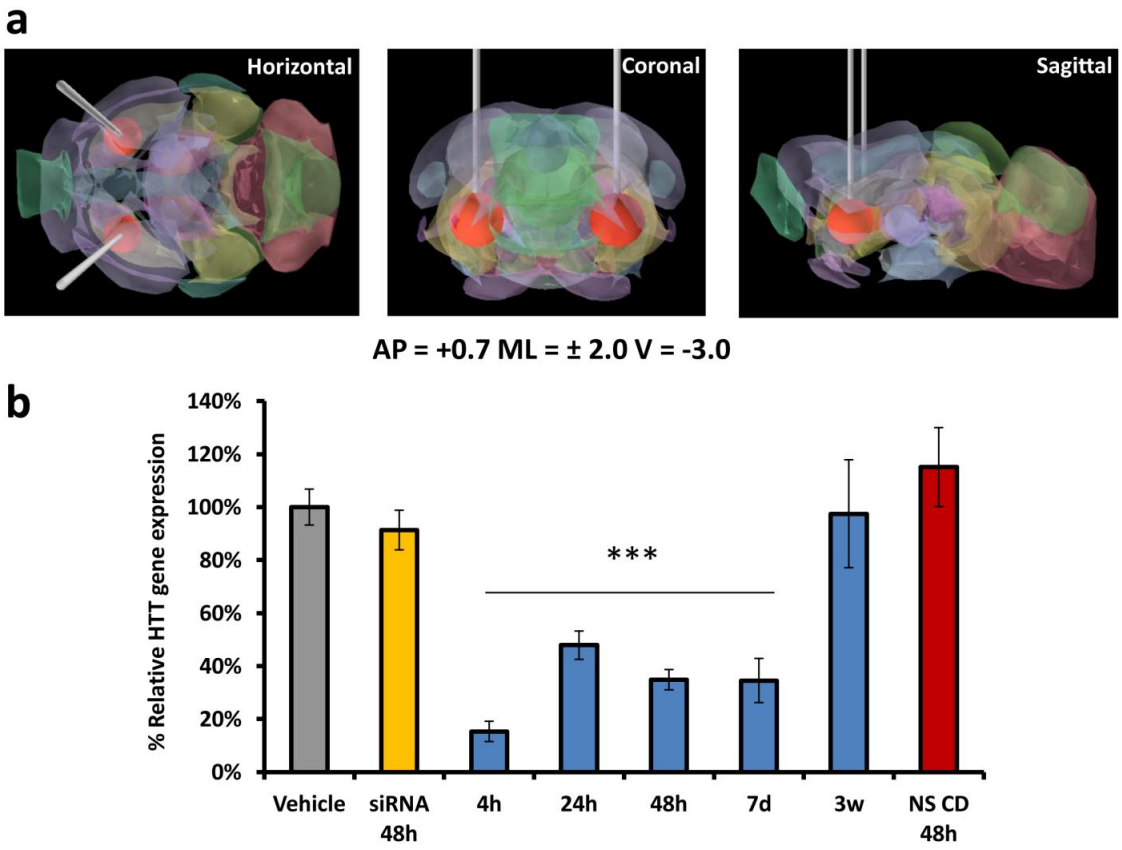


Figure 3.4. CD.HTTsiRNAs mediate in vivo HTT mRNA knockdown in the R6/2 mouse model . *(a) CD.HTTsiRNA nanoparticles were injected into the Striatum of R6/2 mice. 2.5 uL brain injections were simulated on BrainNavigator™ and shown in horizontal, coronal and sagittal planes. Stereotaxic coordinates for brain injections are as follow 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 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.*

3.4.5 Multiple dosing with CD.siRNA nanoparticles into the R6/2 mouse brain and behavioural assessment

On assessing behavioural differences between the R6/2 mice and their wild-type 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 3.5b). 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.

No significant improvements in grip strength, locomotor activity and clasping behaviour were observed in the CD.HTTsiRNA treated group (SI, Supplementary Figure S3.5a,b,c). Moreover, no significant changes were observed in bodyweight profiles (SI, Supplementary Figure S3.5d).

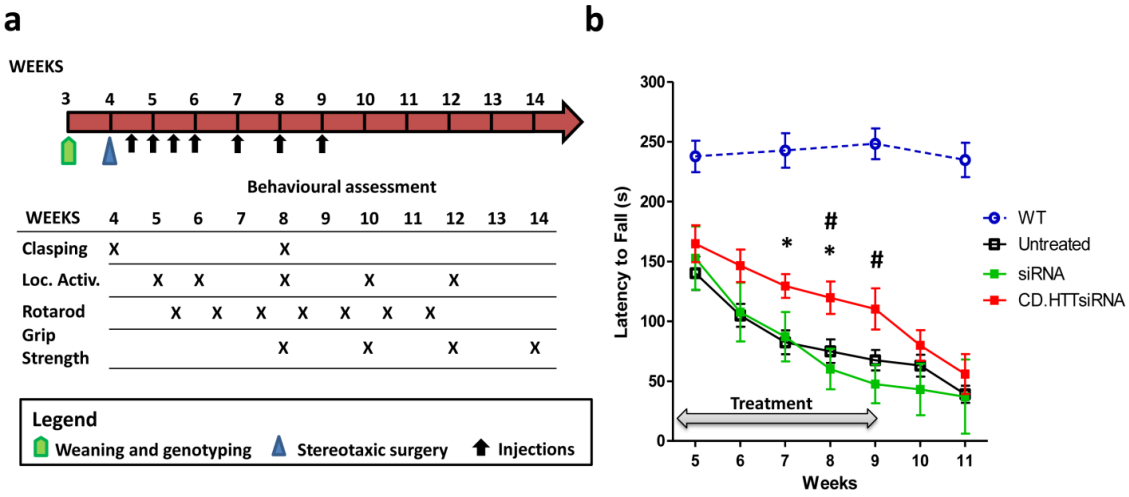


Figure 3.5. Multiple dosing with CD.siRNA nanoparticles into the R6/2 mouse brain and behavioural assessment*. (a) Study design. Briefly, stereotaxic surgery to implant cannulas in the striatum (bilaterally) was carried out at 4 weeks of age. 7 injections of naked HTT siRNA and CD.HTTsiRNA nanoparticles were given over 5 weeks. Motor behaviour 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.*

3.5 Discussion

Progress in the development of RNAi-based therapies for neurodegenerative diseases has been hindered by the lack of an effective and non-toxic 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 characterisation of this modified β-CD has been previously described by our group (O'Mahony et al., 2012d). These positively charged modified β-CDs are thought to interact with negatively charged siRNAs by electrostatic interactions, as found for other cationic delivery systems (Guo et al., 2010a). In the current study, this self-assembling nanoparticle system was able to successfully bind and complex HTT targeted siRNAs forming particles in the nano-size 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 (Gratton et al., 2008; Hillaireau et al., 2009). Furthermore, ensuring stability in physiological fluids such as CSF and at body temperature is crucial to enable delivery to the target site (Agrawal et al., 2009; Lu et al., 2011; Lu et al., 2009). Our data shows that CD.siRNA complexes were found to be stable in aCSF and 37 °C up to 6 hours, 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 (Chaturvedi et al., 2011). 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 (Lv et al., 2006; Souto et al., 2009; Tönges et al., 2006). 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 (Krichevsky & Kosik, 2002). 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 muHTT gene and evoke specific silencing effects on the expression of the HTT gene and consequently reduce the expression of this protein. It is important to note that the toxic effects of the muHTT mainly affects 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 immortalised hypothalamic cell line and also in primary hippocampal cultures (O'Mahony et al., 2012b). Additionally, CD.siRNA nanoparticles were also able to transfect and evoke HTT gene knockdown in human fibroblasts naturally harbouring the muHTT 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 (Fiszer et al., 2011; Lombardi et al., 2009). 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*.) (Guo et al., 2010b).

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 hours and that these effects sustained silencing up to at least 7 days post-injection. 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 (DiFiglia et al., 2007; Harper et al., 2005; Wang et al., 2005). 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 unfavourable toxicity profiles of viral and lipid-based delivery vectors may have precluded repeated dosing regimens which were not used in previous studies (DiFiglia et al., 2007; Harper et al., 2005; Wang et al., 2005). Thus, in contrast with other gene delivery vectors, the low toxicity profiles of CDs enabled multiple dosing in the present study and further advocates 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 behavioural 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 localised 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 behaviour in this animal model. Differential effects on improvements of specific motor behaviours have also been observed in previous studies using both non-viral and viral-based RNAi delivery approaches to study HD *in vivo* (Rodriguez-Lebron et al., 2005a; Wang et al.,

2005). In fact, it is worth noting that although the most widely used pre-clinical 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 behavioural phenotype in HD is likely to be due to dysfunctions in other extra-striatal brain structures (Li et al., 2005; Zuccato et al., 2010). Indeed, we speculate that accumulation of toxic N-terminal HTT fragments in other structures of the R6/2 mouse brain, such as cortex and cerebellum, might account for the observation of such modest behavioural 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 muHTT in untargeted regions of the brain. Therefore, it is crucial that future studies assess the effects of a widespread suppression of the muHTT 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 (Gao et al., 2009a). 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 favourable toxicity profiles in our *in vitro* models. Other *in vivo* studies in non-human primates have shown that multiple dosing with CD-polymer based nanoparticles was well tolerated and did not elicit major immune responses (Heidel et al., 2007). On this basis, the United States of America Food and Drug Administration has recently approved a clinical trial using CD-polymer based nanoparticles for RNAi delivery for cancer treatment (Davis et al., 2010).

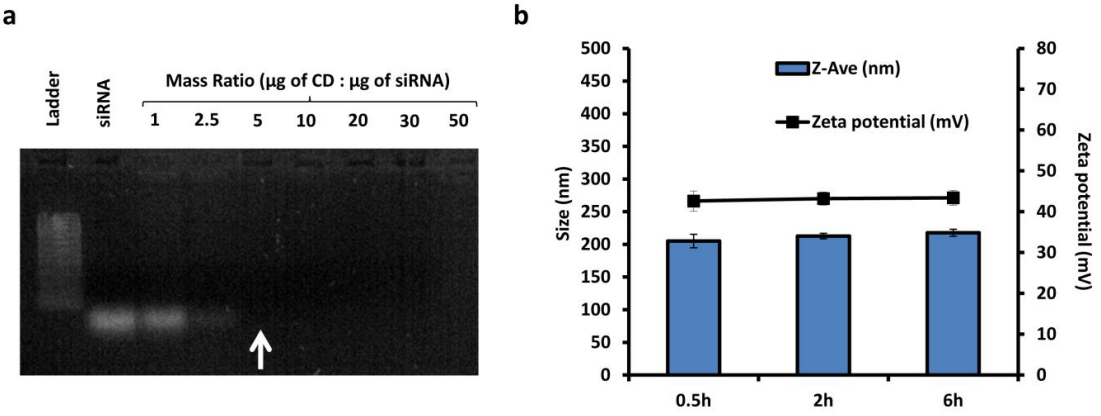
3.6 Conclusions

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 (Chiocca, 2003; Gao & Huang, 2009a). 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 disease such as AD, Parkinson's Disease and ALS.

3.7 Supplementary Information

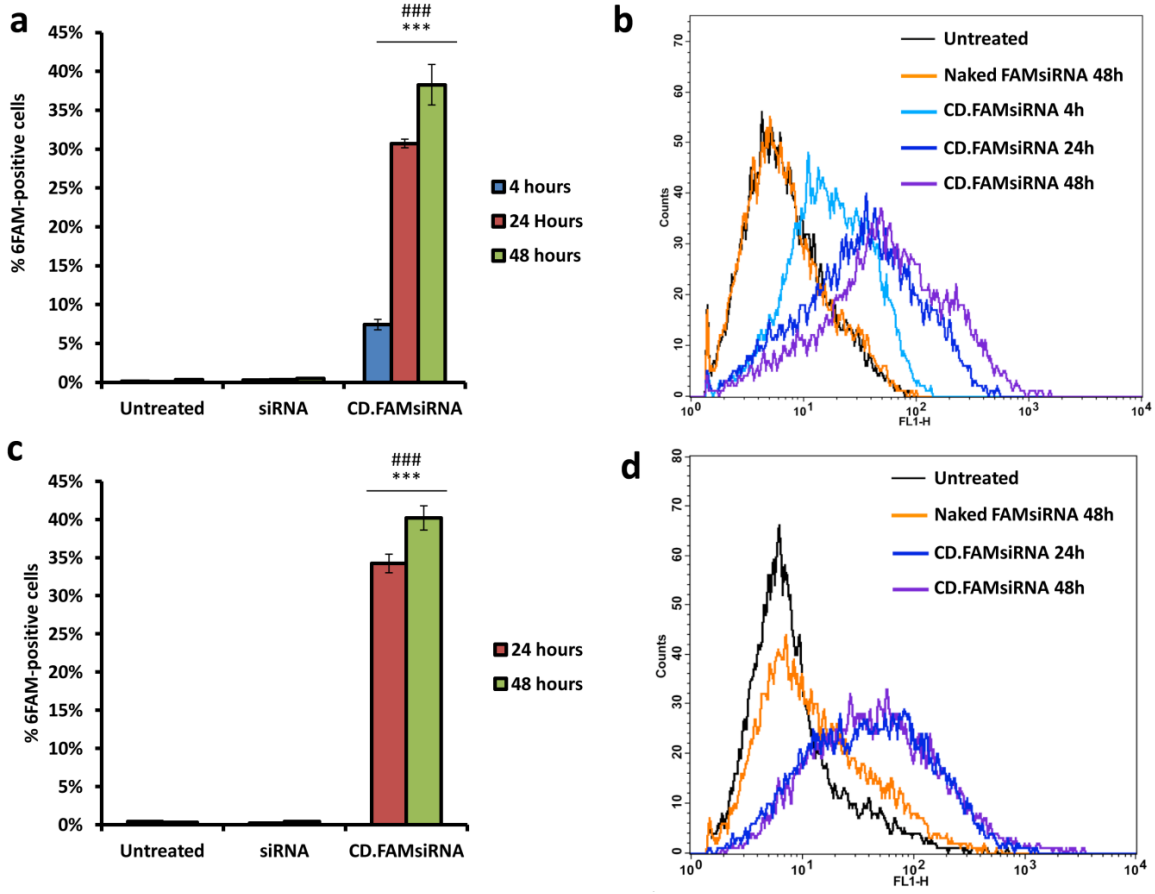
The SI section in this chapter includes data regarding stability of CD.siRNA nanoparticles in aCSF (Supplementary Figure S3.1); quantification of cellular uptake of fluorescent CD.siRNA nanoparticles by FACS (Supplementary Figure S3.2); formulation of CD.siRNA complexes in physiological buffers suitable for *in vivo* brain delivery (Supplementary Figure S3.3); spread of HTT gene expression knockdown in the brain after single injection of CD.siRNA nanoparticles into the striatum (Supplementary Figure S3.4); effects of localised HTT gene expression knockdown on other motor behaviour deficits of R6/2 mice (Supplementary Figure S3.5); and further details are given regarding materials and methods used in the experimental section (Supplementary material and methods).

3.7.1 Modified amphiphilic β-CDs bind HTT targeted siRNAs and are stable in aCSF



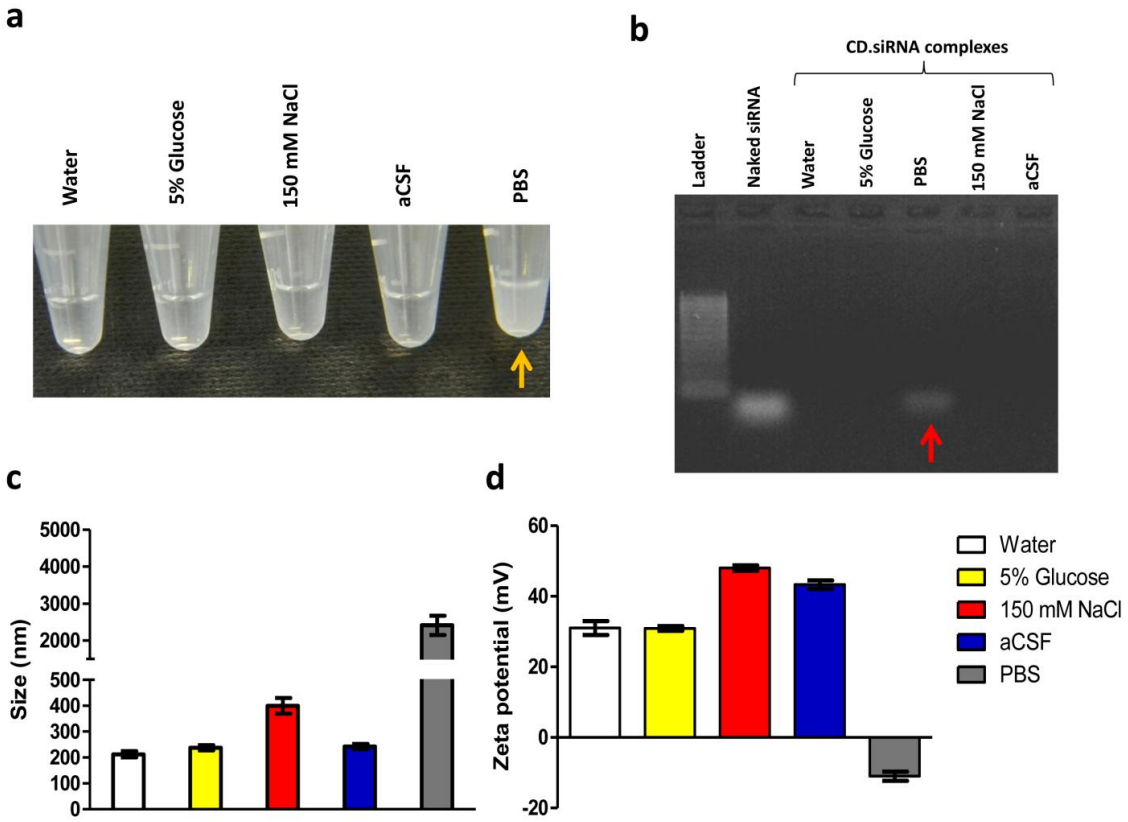
Supplementary Figure S3.1. Modified amphiphilic β-CDs bind HTT targeted siRNAs and are stable in aCSF.*(a) Gel retardation assay. Modified amphiphilic β-CDs complexed with siRNAs at different MR. Free siRNA migrates through the gel whereas complexed siRNAs fail to migrate from wells. White arrow indicates full complexation of siRNA from MR 5. 0.3 µg of siRNA loaded into each well. (b) CD.siRNA nanoparticles complexed at MR10 were incubated in aCSF for different amounts of time. Particle size and zeta potential were assessed using Dynamic Light Scattering and Electrophoretic light scattering respectively. All values represent mean ± SD.* ***Abbreviations****: aCSF, Artificial cerebrospinal fluid; CD, Cyclodextrin; HTT, Huntingtin; MR, Mass ratio; SD, Standard deviation; siRNA, Short interfering RNA.*

3.7.2 Quantification of cellular uptake of fluorescent CD.siRNA nanoparticles by FACS



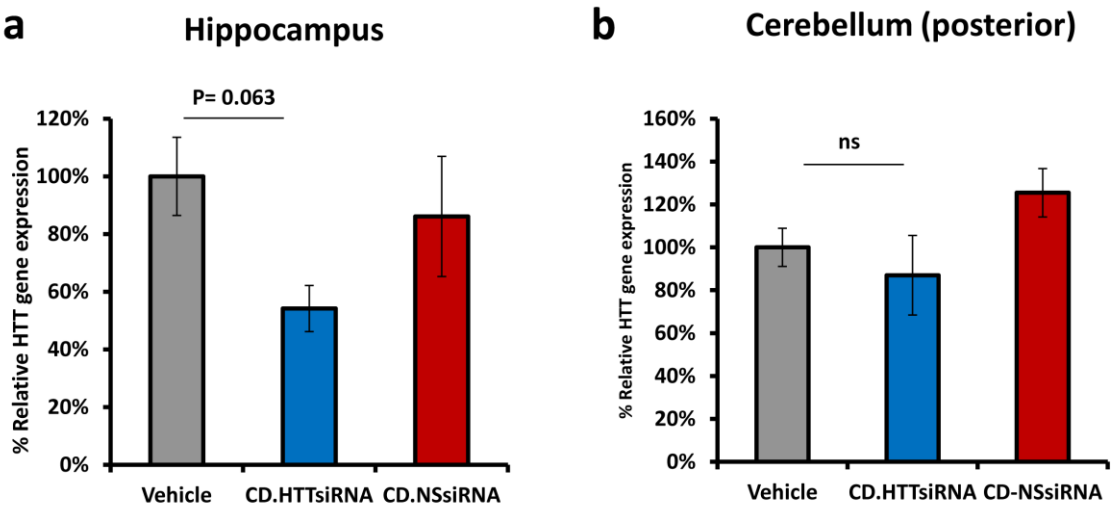
Supplementary Figure S3.2. Quantification of cellular uptake of fluorescent CD.siRNA nanoparticles by FACS. *[6FAM] 5'- labelled siRNAs were complexed with modified amphiphilic β-CDs at MR 10. ST14A-HTT120Q neuronal cells and HD human primary fibroblasts were transfected for 4, 24 or 48 hours. Prior to FACS cells were treated with Cell Scrub™ buffer in order to remove complexes bound to the cellular membrane. Representative flowcytometry histograms for ST14A-HTT120Q cells (a) and HD primary fibroblasts (c). Percentage of FAM-positive cells per time point for ST14A-HTT120Q cells (b) and HD primary fibroblasts (d). n = 3 per group and a total of 10,000 cells were counted per sample. Final concentration of FAMsiRNA was of 100 nM. All values represent mean ± SEM. ***p<0.001 compared to untreated cells, ###p<0.001 compared to naked 6FAM-siRNA. All statistics performed by ANOVA followed by Bonferroni's Post hoc test.*

3.7.3 Formulation of CD.siRNA complexes in physiological buffer solutions suitable for *in vivo* brain delivery



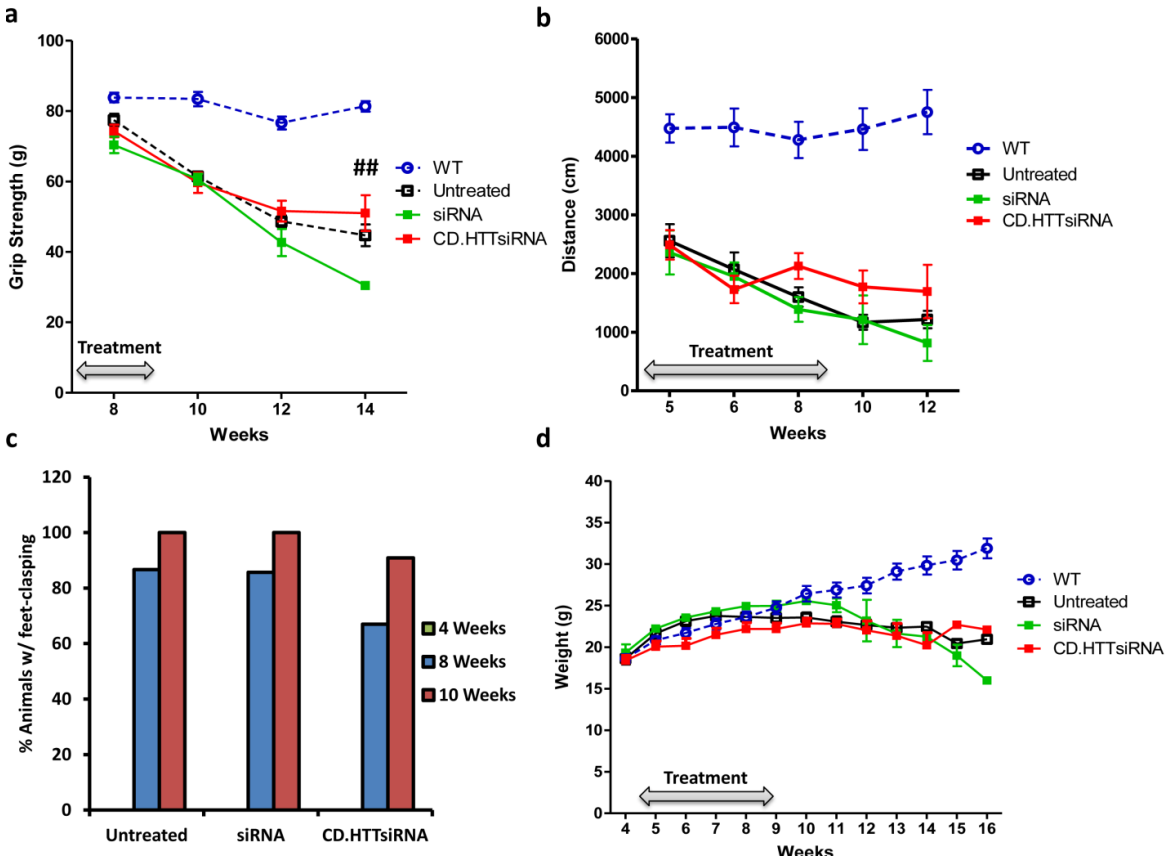
Supplementary Figure S3.3. Formulation of CD.siRNA complexes in physiological buffer solutions suitable for in vivo brain delivery. *(a) Photograph of modified amphiphilic β-cyclodextrins reconstituted in different physiological buffer solutions. Yellow arrow indicates cloudy solution. (b) Gel retardation assay. Free siRNA migrates through the gel whereas complexed siRNA fails to migrate from wells. Red arrow indicates uncomplexed siRNA. (c, d) Particle size and zeta potential assessed using Dynamic Light Scattering and Electrophoretic light scattering respectively. All values represent mean ± SD.*

3.7.4 Spread of HTT gene expression knockdown in the brain after single injection of CD.siRNA nanoparticles into the striatum



Supplementary Figure S3.4. Spread of HTT gene expression knockdown in the brain after single injection of CD.siRNA nanoparticles into the striatum. *HTT gene expression in the hippocampus (a) and cerebellum (b) 24 hours after direct injection of CD.HTTsiRNA or CD.NSsiRNA complexes. Total RNA was extracted and reverse transcribed to cDNA. HTT gene expression was assessed by quantitative PCR. n = 4-5 per group. All values represent mean ± SEM. All statistics performed by ANOVA followed by Bonferroni's Post hoc test.*

3.7.5 Effects of localised HTT gene expression knockdown in other motor behaviour deficits of R6/2 mice



Supplementary Figure S3.5. Effects of localised HTT gene expression knockdown in other motor behaviour deficits of R6/2 mice. *(a) Despite an overall positive effect on grip strength (F(2, 14) = 9.08, P= 0.003), and a beneficial effect of CD.HTTsiRNAs when compared to naked HTT siRNAs (##p<0.01 by ANOVA with Bonferroni's Post hoc test), the treatment failed to improve grip strength when compared to untreated R6/2 mice (n (Untreated) = 10 n (siRNA) = 2 n (HTT CD) = 5). CD-based HTT siRNA delivery did not significantly improve (b) spontaneous locomotor activity (F(2, 16) = 1.535, P= 0.246) (n (Untreated) = 10 n (siRNA) = 3 n (HTT CD) = 6) or (c) feet clasping behaviour (chi-square = 0.603, df= 1, p= 0.213). No significant improvements in weight profile were observed in animals treated repeatedly with CD.HTTsiRNA nanoparticles when compared to untreated or naked HTT siRNA-treated animals.*

3.7.6 Supplementary Materials and Methods

Preparation of CD.siRNA nanoparticles

As previously described (O'Mahony et al., 2012b), modified CDs were dissolved in chloroform, and chloroform evaporated under a stream of nitrogen. CDs were reconstituted in different physiological buffers (5% glucose, 150 mM NaCl, aCSF or DIW) and sonicated for 60 minutes. siRNAs were also diluted in different buffers, mixed with CDs and incubated for ~20 minutes at room temperature.

Cell culture

ST14A-HTT120Q cells were grown at permissive temperature 33 °C and 5% CO2 and sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, United Kingdom) + 10% Foetal bovine serum (FBS) (Sigma-Aldrich, Germany) up to passage 25. HD fibroblasts were grown at 37 °C and 5% CO2 and subcultured in DMEM + 20% FBS + 5% vitamins (GIBCO, United Kingdom) + 5% aminoacids (GIBCO, United Kingdom) up to passage 15.

Behavioural assessment

Rotarod

Balance and motor coordination were assessed using a rotarod (Model 7650, Ugo Basile, Italy) in a similar protocol to that described by Menalled *et al.* (Menalled et al., 2009). Rotarod task was introduced to animals by a 5-minute trial at a constant speed of 4 rpm (data not included in the analysis). Thereafter, animals were tested using an accelerating protocol with a linear increase of velocity from 4 to 40 rpm in 300 seconds. Tests were conducted weekly in 3 consecutive days and animals were given 3 trials per day. An interval of at least 10 minutes was given between trials. Locomotor performance was measured as "latency to fall" in seconds, with a cut off of 300 seconds.

Grip Strength

Muscle strength was assessed using a grip strength meter (Ugo Basile, Italy) in a similar protocol to that described by Menalled *et al* (Menalled et al., 2009). Mice were held by the tail and brought to the grip strength apparatus. Mice were allowed to grasp the grid with the front paws and were gently pulled back until they released their grip. The apparatus registers the peak strength for that trial. Each animal had five trials with at least 15-30 seconds rest between trials. The 5 trial test did not exceed 5 minutes.

Spontaneous locomotor activity

Spontaneous locomotor activity was assessed using a similar protocol to that previously described by Menalled *et al.* (Menalled et al., 2009). Animals were placed individually in the middle of a 25x19 cm arena in a transparent acrylic cage containing usual bedding. Locomotor activity was then recorded for 60 minutes using Video Monitor Control Package from Med Associates Inc. Total distance travelled by each animal was tracked using SOF-840-VHCM software (version 1.70).

Paw clasping behaviour

Typical clasping phenotype of HD was assessed by suspending mice for 60 seconds. Animals exhibiting feet-clasping (front or hind paws) clasping were scored positive. Similar protocols have been used by Rodriguez-Lebron *et al.* and Hickey *et al.* (Hickey et al., 2005; Rodriguez-Lebron et al., 2005a).

3.8 Addendum

Delivery of CD.siRNA nanoparticles to a human in vitro model of HD (HD human primary fibroblasts).

Figure 3.3. shows that CD.FAMsiRNA nanoparticles are able to effectively transfect human HD Fibroblasts. It is also possible to observe that CD.FAMsiRNAs nanoparticles have successfully escaped the endossomal pathway, with very few nanoparticles being co-localised with late acidic endossomes/lysosomes. Interestingly, HD fibroblasts transfected with CD.FAMsiRNA nanoparticles presented stronger late acidic endossomal/lysosomal staining than cells transfected with naked FAMsiRNAs. We reason that high cellular uptake of CD.FAMsiRNAs resulting in an increased number of endocytic vesicles within the cell, may have in turn led to an enhanced production of lysosome vesicles. Conversely, naked FAMsiRNAs which are not easily taken up by HD fibroblasts, generate very few endocytic vesicles and consequently present reduced late endossoamal/lysosomal staining.

CD-based siRNA delivery as therapeutical approach for HD

In this chapter we have demonstrated, using HD as a model disease, the utility of modified CDs as effective and safe siRNA delivery systems for CNS applications. Although CD.HTTsiRNAs were very effective silencing the muHTT gene, it is worth noting that HTTsiRNAs here used do not distinguish between the wild-type and mutant alleles and were designed and developed in 2005 (Wang et al., 2005). Thus, despite their utility for proof-of-concept studies as this one, when developing a formulation for clinical application for HD, it would be wise designing new siRNAs based on up-to-date algorithms. Furthermore, this new siRNAs would ideally be able to target the mutant HTT without affecting the wild-type allele, eventually by targeting disease-associated single nucleotide polymorphisms (SNP).

Chapter IV

Differential Nanotoxicological and Neuroinflammatory Liabilities of Non-Viral Vectors for RNA Interference in the Central Nervous System

Bruno M. D. C. Godinho[1,2], David J. McCarthy[1,2], Cristina Torres-Fuentes[3], Caroll J. Beltrán[4,5], Joanna McCarthy[1], Aoife Quinlan[1], Julien R. Ogier[6], Raphael Darcy[6], Caitriona M. O'Driscoll[1], John F. Cryan[2,4]

[1]Pharmacodelivery group, School of Pharmacy, University College Cork, Cork, Ireland

[2]Dept. Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3]Food for Health Ireland, University College Cork, Cork, Ireland

[4]Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, Cork, Ireland

[5]Department of Medicine, Faculty of Medicine, Universidad de Chile, Hospital Clínico Universidad de Chile, Chile

[6]Centre for synthesis and Chemical Biology, University College Dublin, Dublin, Ireland

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4.1 Abstract

Progression of ribonucleic acid (RNA) interference-based gene silencing technologies for the treatment of disorders of the central nervous system (CNS) depends on the availability of efficient non-toxic nanocarriers. Despite advances in the field of nanotechnology undesired and non-specific interactions with different brain-cell types occur and are poorly investigated. To this end, we studied the cytotoxic and neuroinflammatory effects of widely-used transfection reagents and modified amphiphilic β-cyclodextrins (CDs). All non-viral vectors formed positively charged nanoparticles with distinctive physicochemical properties. Differential and significant cytotoxic effects were observed among commercially available cationic vectors, whereas CDs induced limited disruptions of cellular membrane integrity and mitochondrial dehydrogenase activity. Interestingly, murine derived BV2 microglia cells and a rat striatal *in vitro* model of Huntington's Disease (ST14A-HTT120Q) were more susceptible to toxicity than human U87 astroglioma cells. BV2 microglia presented significant increases in cytokine, toll-like receptor 2 and cyclooxygenase-2 gene expression after transfection with selected commercial vectors but not with CD.siRNA nanoparticles. Non-viral short interfering RNA (siRNA) nanoparticles formulated with G6 polyamidoamine (PAMAM) dendrimers also significantly increased cytokine gene expression in the brain following injections into the mouse striatum. Together our data identify modified CDs as nanosystems that enable siRNA delivery to the brain with low levels of cytotoxicity and immunological activation.

4.2 Introduction

Therapeutic gene silencing by harnessing the specificity of the endogenous ribonucleic acid interference (RNAi) pathway offers great promise for the treatment of neurological disorders, such as Huntington's Disease (HD) (Sah, 2006). However, the lack of efficient and safe delivery vectors has tempered the progression of this technology for the treatment of disorders of the central nervous system (CNS) (O'Mahony et al., 2013b). To date, both viral and non-viral approaches have been investigated. Despite their ability to transduce a wide range of cell types, several concerns have been raised against viral vectors regarding their immunogenicity and safety (Thomas et al., 2003). On the other hand, efforts in the field of nanotechnology have been put together to develop more effective and safe non-viral alternatives for short interfering RNA (siRNA) delivery to the CNS (O'Mahony et al., 2013b).

Non-viral vectors are chemically synthesised or derived from naturally occurring polymers and often contain cationic moieties that facilitate electrostatic interaction with anionic siRNAs, enabling complexation and protection from serum degradation (O'Mahony et al., 2013b). These nanosystems have been able to successfully deliver siRNA and elicit gene silencing effects in a variety of cell models, including cultured neurons, but also *in vivo* in the brain of relevant models of CNS disorders (e.g. (Badaut et al., 2011; Godinho et al., 2013; O'Mahony et al., 2012b; Wang et al., 2005)). However, and in addition to cellular uptake and gene silencing requirements, biocompatibility of non-viral formulations is one of the emerging hurdles (Ballarín-González & Howard, 2012). Although until recently biomaterials were considered to be relatively inert, advancements have shown that they are capable of causing toxic biological responses and inducing specific genomic signatures (Akhtar & Benter, 2007; Ballarín-González & Howard, 2012). Indeed, several delivery vectors (e.g. formulations containing cationic/neutral lipids, cationic linear and branched polymers, polyamidoamine (PAMAM) dendrimers) have been reported to cause cellular membrane destabilization and lysis, and to interfere with mitochondrial metabolic activity

leading to increased cellular oxidative stress (Hong et al., 2006b; Hunter & Moghimi, 2010; Lee et al., 2009; Moghimi et al., 2005). Furthermore, global changes in gene expression profiles, activation of the apoptotic pathway and induction of immune responses have also been reported to occur in a vector-dependent fashion both *in vitro* and *in vivo* upon systemic delivery (Bonnet et al., 2008; Gorina et al., 2009; Hollins et al., 2007; Hunter & Moghimi, 2010; Kedmi et al., 2010; Omidi et al., 2005).

Key contributors to the toxicological and immunological profiles of nanoparticles are the physicochemical properties of the assembled nanosystem as well as tissue and cell susceptibility (Albanese et al., 2012; O'Mahony et al., 2013b). In fact, surface functionalization, shape, size, charge, and architecture are fundamental aspects for cellular uptake and gene silencing efficiency, and have now been found to be also crucial in nanoparticle-mediated toxicity (Albanese et al., 2012; Gary et al., 2011; Rejman et al., 2004). On the other hand, as ultimate targets in the CNS, neurons are notoriously difficult to transfect and are also very sensitive to cytotoxicity mediated by non-viral vectors (Krichevsky & Kosik, 2002; Tönges et al., 2006). In addition, neurodegenerative diseases, such as HD, may render specific neuronal populations more susceptible to toxic stimuli and therefore adequate non-toxic carriers must be used (Rigamonti et al., 2000). Furthermore, inducing gene silencing effects in the brain requires, in various circumstances, interaction of nanoparticles with different cell types, including microglia and astroglia. Thus, non-specific toxic interactions with these cell types may reduce brain homeostasis, induce inflammatory processes and eventually accelerate progression of neurological diseases (Amor et al., 2010). However, despite its importance, the nanotoxicological and neuroinflammatory impact of nanoparticles for gene and RNAi in the intricate context of the CNS is still relatively poorly investigated. In fact, most studies have focused on single CNS cell types, essentially providing efficacy data and only presenting limited data on the cytoxicity and inflammatory profiles of delivery systems. Thus, a systematic and integrated assessment of the cytotoxic

and neuroinflammatory effects of commonly used transfection reagents in multiple brain-derived cells is warranted.

To this end the present study aims to assess the toxicological and immunological profiles of three commercially available and widely used cationic vectors and a modified cationic amphiphilic cyclodextrin (CD) delivery system. These biomaterials were chosen on the basis of their particular molecular architecture and/or in order to cover the most widely used polycation-based delivery systems. Potential biological adverse effects and neuroinflammatory responses were assessed in three different brain-derived cell lines: ST14A-HTT120Q cells derived from rat striatal primordia and previously cloned with the mutant Huntingtin (HTT) gene were chosen as we are interested in developing non viral therapeutic approaches for HD (Godinho et al., 2013); mouse BV2 microglial cells were chosen as model of CNS resident immune cells; and U87 human astroglioma cells were chosen as brain cancer *in vitro* model. Moreover, we investigated local immune responses to these distinctive biomaterials *in vivo* after single bilateral injections into the striatum of mice.

4.3 Materials and methods

4.3.1 Synthetic siRNAs

Synthetic duplexed siRNAs were obtained from QIAGEN (United Kingdom) or Sigma-Aldrich (France). Non-silencing siRNAs (NSsiRNA): sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. Non-silencing FAM-labelled siRNA (FAMsiRNA): sense strand, 5'-[6FAM] UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'. HTT siRNAs as per Wang *et al*. 2005 (Wang et al., 2005): 5'-GCCUUCGAGUCCCUCAAGUCC-3'; antisense strand, 5'-ACUUGAGGGACUCGAAGGCCU-3.

4.3.2 Nanoparticle preparation and characterisation

Modified cationic amphiphilic CDs were prepared as previously described in O'Mahony *et al.* (O'Mahony et al., 2012b) and Godinho *et al.* (Godinho et al., 2013). Briefly, CDs were dissolved in chloroform and evaporated under a stream of gaseous nitrogen. CDs were then rehydrated in sterilised deionised water (DIW) and sonicated for 1 hour before complexation with siRNAs. For nanoparticle formation, CDs were mixed with equal volumes of siRNA solutions and left to incubate at room temperature (RT) for 20 minutes. Commercially available cationic vectors, Lipofectamine^{TM}2000 (Lf2000) (Invitrogen, Carlsbad, CA), INTERFERin® (Interferin) (PolyPlus®, France) and Superfect® (SF) (QIAGEN, United Kingdom) were complexed with siRNAs as per manufacturer's instructions. CD.siRNA nanoparticles were used at a mass ratio 10:1 (10 µg CD : 1 µg siRNA). The final vector/siRNA ratios for commercially available transfection reagents were selected or adapted from manufacturer's recommendations to facilitate comparisons across vectors *in vitro* and also to facilitate comparison with *in vivo* studies (Lf2000 (1 µL Lf2000 : 20 pmol siRNA), SF (5 µL SF : 1 µg siRNA) and Interferin (1-1.2 µL Interferin : 0.1-0.2 µg siRNA).

For physicochemical characterisation all nanoparticles were prepared in sterilised DIW and further diluted in DIW up to 1 mL. Size and charge measurements were assessed at RT by dynamic light scattering (DLS) and electrophoretic light scattering, respectively, using a Malvern's Zetasizer Nano ZS as previously described in O'Mahony *et al.* (O'Mahony et al., 2012b) and Godinho *et al.* (Godinho et al., 2013). Results are expressed in mean ± standart deviation (DS) of 3 independent experiments. For *in vivo* studies nanoparticles were prepared in 5% glucose solution (Sigma-Aldrich, Germany) and CD.siRNA nanoparticles concentrated by ultrafiltration using Vivaspin 500 spin columns (Sartorius, Germany) to a final concentration of siRNA of 0.08 µg/µL.

4.3.3 Cell culture and RNAi transfection

ST14A-HTT120Q cells derived from rat striatal primordia and cloned with the human HTT gene were obtained from Coriell Institute for Medical Research (Camden, NJ). BV2 cells derived from primary mouse microglia cells were obtained from Banca Biologica e Cell Factory – IST (Italy, Genova). U87 astroglioma cells were a kind gift from Dr. Paul Young (University College Cork). ST14A-HTT120Q cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, Germany). BV2 cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) (GIBCO, United Kingdom) medium supplemented with 10% FBS and 2 mM L-glutamine (GIBCO, United Kingdom). U87 cells were grown in DMEM supplemented with 10% FBS and 2 mM L-glutamine (GIBCO, United Kingdom). For passaging ST14A-HTT120Q and U87 cells 0.05% Trypsin-EDTA (GIBCO, United Kingdom) was used, for passaging BV2 cells 0.25% Trypsin-EDTA (Sigma, United Kingdom) was used. All cultures were kept in a humidified incubator with 5% CO2 and at 33 °C (ST14A-HTT120Q) or 37 °C (BV2 and U87). ST14A-HTT120Q, BV2 and U87 cells were seeded in 96-well plates at a density of 7.5×10^3, 1×10^4 and 1×10^4 cells / well, respectively. For experiments carried out on 12-well plates cells were seeded at a density of 1.7×10^5, 0.3×10^6, and 2×10^5 cells / well, respectively.

RNAi transfection or stimulation with lipopolysaccharide (LPS) (Sigma, Germany) was carried out for 4, 24 or 48 hours according to the experiment. Nanoparticles were prepared as described above and diluted in optiMEM®. The volume of transfection sample accounted for 20% of the total volume of the well, the remaining 80% consisted of complete growth media. The final concentration of siRNA in all RNAi-treated groups was of 100 nM.

4.3.4 Trypan blue exclusion assay

The trypan blue assay is a well established method for the evaluation of cell viability in cell suspensions (Kepp et al., 2011). This is a dye exclusion assay technique whereby viable cells, with intact cellular membranes, exclude the dye and nonviable cells incorporate the dye (Kepp et al., 2011). The method was conducted essentially as previously described in O'Mahony *et al.* (O'Mahony et al., 2012b). Briefly, cells were seeded in 12-well plates and transfected as described above. After 24 hours cell supernatants were collected, spun down, decanted into new tubes and stored at -80 ºC. Cells were washed with phosphate buffered saline (PBS) (Sigma, United Kingdom) and detached using 0.25% trypsin-EDTA (Sigma, United Kingdom). Cell suspensions were spun down at 1,000 revolutions per minute (rpm) for 5 minutes and the supernatant decanted. Cell pellet was resuspended in 1 mL of respective growth media. A 1:1 dilution of the cell suspension in a trypan blue solution 0.4% (Sigma, United Kingdom) was carried out, and cell counts (total and living cells) were obtained from BioRad TC10™ Automated Cell Counter.

4.3.5 Lactate Dehydrogenase release assay

Lactate Dehydrogenase (LDH) release assay measures early and even transient damages to the cellular membrane (Kepp et al., 2011). An increased leakage of cytosolic LDH to the cell supernatant has been associated with an increase in cytototoxicity (Kepp et al., 2011). LDH assay was carried out on cell supernatants using CytoTox® 96 Non-radioactive Cytotoxicity Assay from Promega (Madison, WI) as per manufacturer's instructions. Briefly, cell supernatants were defrosted on ice and 50 µL of each sample was placed in triplicate on 96-

well plates and respective complete media used as control. 50 µL of substrate solution was added into each well and incubated at RT for 30 minutes protected from light. 50 µL of stop solution was added to each well and absorbance measured at 490 nm using a SpectraMax Plus384 plate reader.

4.3.6 Methyl thiazolyl tetrazolium assay

Methyl thiazolyl tetrazolium (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) (Sigma, St. Louis, MO) assay assesses mitochondrial reductase activity and therefore is a good measure of cellular metabolism (Kepp et al., 2011). Reduction in mitochondrial dehydrogenase activity has been associated with reduced cell viability. MTT assays were carried out in 96-well plates as previously described in Godinho *et al*. (Godinho et al., 2013) and O'Mahony *et al*. (O'Mahony et al., 2012b).

4.3.7 Cell Integrity Assay by High Content Analysis

High Content Analysis (HCA) is a high throughput technique that allows for screening of multiple cellular features based on automated cell imaging analysis. In this study, Cytiva™ Cell Integrity HCA Assay was used to investigate different cell viability parameters such as plasma membrane integrity, mitochondrial viability and apoptosis (Cat. #. 29-0244-69, GE Healthcare, UK). Briefly, dye cocktails containing membrane permeable / impermeable DNA, mitochondrial and phosphatidylserine dyes were prepared following manufacturer's instructions. Cells incubated with Ionomycin 20 µM for 2 hours were validated and included as positive control for cytotoxicity and apoptosis. Three images per well were acquired using the IN Cell Analyser 1000 (GE Healthcare, United Kingdom) with a 20x objective. Further information on excitation and emission wavelengths used for detection of each dye is described in Supplementary Information (SI), Supplementary Materials and Methods. After acquisition, data were analysed using In Cell® 1,000 Workstation software (GE Healthcare, United Kingdom) using multitarget analysis. Specific details on the settings used for analysis are given in SI, Supplementary Materials and Methods.

4.3.8 Gene expression

RNA was isolated using GenELUTE™ Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO). 300 ng of total RNA was reverse transcribed to complementary DNA (cDNA) using the High-capacity cDNA reverse transcription kit from Life technologies, Applied Biosystems (Foster City, MO). Real-time quantitative Polymerase chain reaction (RT-qPCR) was performed using a 7300 Real Time Polymerase chain reaction (PCR) system under the cycling conditions previously described in Godinho *et al*. (Godinho et al., 2013). Mouse Tumour Necrosis Factor (TNF)-α (Mm00443258_m1), Interleukin (IL)-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), Toll-like receptor (TLR) 2 (Mm00442346_m1), cyclooxygenase 2 (COX-2) (Mm00478374_m1) and β-actin (4352341E) Taqman® gene expression assays were acquired from Life technologies, Applied Biosystems (United Kingdom). Custom TaqMan® HTT FAM-labelled probe was designed on previously validated primers as per Godinho *et al.* (Godinho et al., 2013). Samples were run in triplicate and average CT values were used for gene expression calculations. β-actin gene expression was used as endogenous control and relative cytokine gene expression was calculated on normalized CT values.

4.3.9 Brain stereotaxic surgery

Bilateral injections into the striatum (CPu) of 6-week old C57/BL6 male mice (Harlan, United Kingdom) were carried out through brain stereotaxic surgery. Previously optimised coordinates from bregma were used (Anterior-posterior = + 0.7, Medio-lateral = ± 2.0 and Ventral = - 3.0) and a total volume of 2.5 µL was delivered bilaterally at a rate of 0.5 µL/min. In RNAi treated animals 0.2 µg of siRNA was delivered in each side and in positive control animals LPS (3 µg) was injected. Following the injection 5 minutes extra were given before the syringe was retracted to avoid flush back. Bone wax (ETHICON, Johnson&Johnson, Belgium) was used to cover the burr hole and sterile sutures (ETHICON Mersilk, Belgium) were used to sew the skin. All procedures were conducted under gaseous

anaesthetic Isofluorane (IsoFlo®, Abbott, United Kingdom). After 24 hours animals were euthanized and brain tissue collected using a brain slicer matrix. Tissue for western blotting was snap frozen in dry ice and tissue for gene expression analysis was kept in RNA later (Sigma, United Kingdom) at 4 ºC overnight. All tissues were thereafter kept in -80 ºC until further analysis. 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.

4.3.10 Western blotting

Brain tissue from the site of injection was disrupted by homogenization in lysis buffer and total protein quantified using a bicinchoninic acid assay as described in Godinho *et al.* (Godinho et al., 2013). 30 µg of total protein was loaded on NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA). Protein electrophoresis, protein transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) and membrane blocking was carried out as described in Godinho *et al*. (Godinho et al., 2013). Membranes were incubated overnight with anti-Glial Fibrillary Acidic Protein (GFAP) antibody (dilution 1:1,000) (MAB3402, Millipore, Temecula, CA) or anti-β-actin (dilution 1:3,000) (A5441, Sigma, St Louis, MO). Membrane was washed with Tris-buffered saline solution containing 0.1% Tween 20 (Fisher Scientific, Fair Lawn, NJ) and incubated for 1 hour with anti-mouse antibody (dilution 1:10,000) (IRDye 800CW, LI-COR). LICOR Odyssey near-infrared scanner was used to scan membranes and ImageJ software to carry out densitometry analysis. All results were normalised to the house keeping gene β-actin.

4.3.11 Statistical analysis

Unless otherwise stated results are expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by Bonferroni's Post Hoc test was carried to determine statistical significant differences in particle size and surface charge among all non-viral vectors. ANOVA followed by Dunnett's Post Hoc test was used to determine

significant statistical differences between naked siRNA, CD, Lf2000, Interferin and SF against untreated controls. Student's t-tests were carried out to investigate significant differences between LPS-positive controls and untreated controls. In *in vivo* studies statistical significant differences were investigated against vehicle, whereas untreated animals were only kept as a reference. All statistics were carried out using PAWS 18 Statistical package.

4.4 Results

4.4.1 Physicochemical characterisation of non-viral siRNA nanoparticles

The non-viral delivery systems investigated in this study have been represented in Figure 4.1 a. Cationic amphiphilic CDs are siRNA nanocarriers consisting of click-modified β-CDs (Godinho et al., 2013; O'Mahony et al., 2012b). On the other hand, Lf2000 consists of a cationic liposome formulation (3:1 DOSPA:DOPE (Fiszer-Kierzkowska et al., 2011; Vangasseri et al., 2006)), Interferin is a proprietary cationic non-liposomal amphiphile and SF is a 6^{th} generation fractured PAMAM dendrimer (Hollins et al., 2007). Lf2000, Interferin and SF are commercially available and have been widely used for nucleic acid transfection. Although all cationic vectors were able to successfully bind and complex siRNAs as shown in gel retardation assays (Figure 4.1 b), the hydrodynamic radius, polydispersity and surface charge of these non-viral siRNA nanoparticles varied significantly (Figure 4.1 c,d). CD (192.34 ± 9.89 nm) and Lf2000 siRNA nanoparticles (222.37 ± 4.96 nm) were significantly larger than Interferin (122.83 ± 7.86) and SF (148.81 ± 16.33). Furthermore, polydispersity index (PDI) of these nanoparticles decreased in the following order CD (0.329 ± 0.033) > Interferin (0.173 ± 0.038) > SF (0.129 ± 0.048) > Lf2000 (0.071 ± 0.007), suggesting different degrees of homogeneity within samples. Finally, zeta potential measurements demonstrated that all non-viral siRNA nanoparticles were positively charged and that CD.siRNA nanoparticles presented the lowest surface charge. However, no statistically significant differences were found among the different systems (Figure 4.1 d).

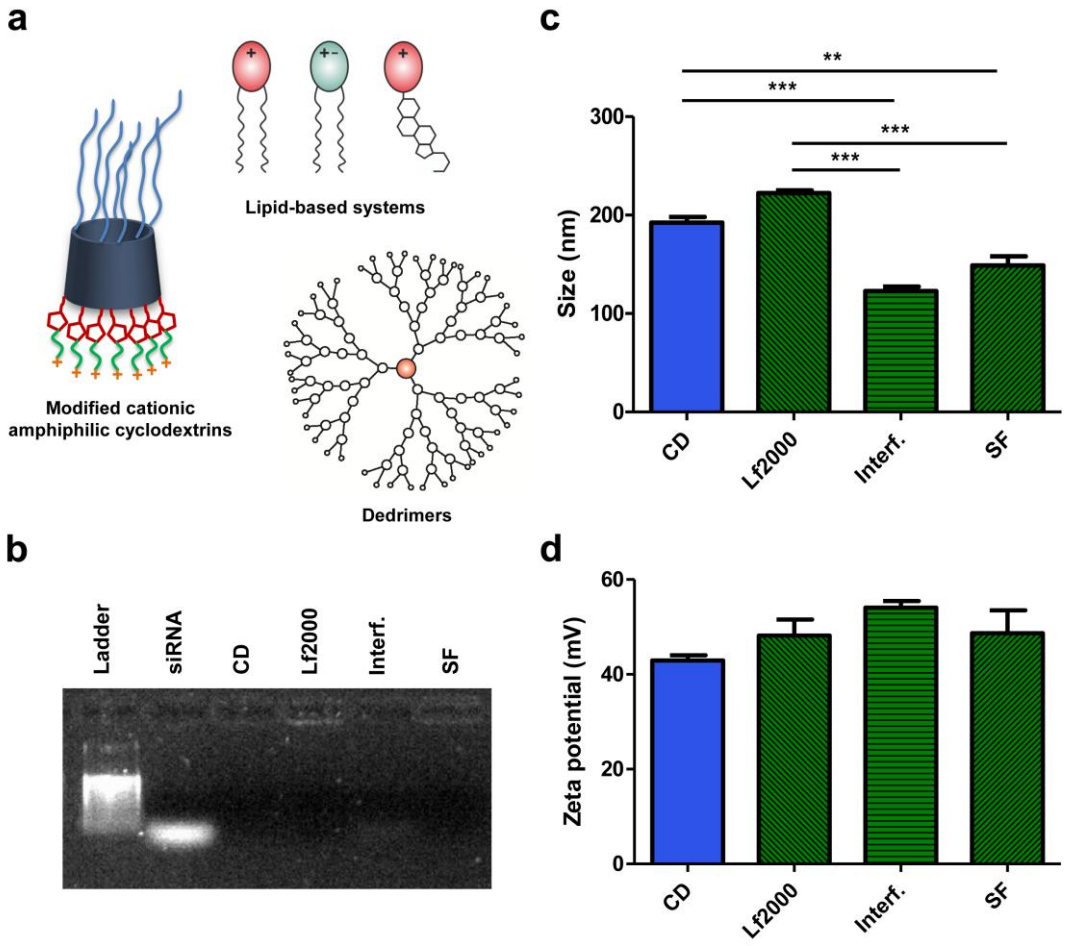


Figure 4.1. Physicochemical characterisation of non-viral siRNA nanoparticles. *(a) Schematic representation of non-viral vectors. (b) Gel retardation assay for siRNA binding and complexation. Free siRNA migrates through the gel. 0.3µg siRNA per well. (c) Hydrodynamic radius of non-viral siRNA nanoparticles measured by DLS. (d) ζ potential measured through electrophoretic light scattering. Results are expressed as Mean ± SD. **P<0.01 and *** P<0.001. n = 3 per group.* ***Abbreviations:*** *CD, Cylcodextrin; DLM, Dynamic light scattering; Interf., Interferin; Lf2000, Lipofectamine2,000; SF, Superfect®.*

4.4.2 Gene silencing efficiency in ST14A-HTT120Q cells

For completion and to enable further comparison among the different vectors their gene silencing efficiency was investigated in ST14A-HTT120Q cells, an *in vitro* model of HD. Transfection with Lf2000 and Interferin induced the highest levels of HTT gene expression knockdown in this cell line (Table 4.1). Furthermore, CD.siRNA nanoparticles also induced a very high level of HTT gene expression knockdown, whereas SF was the nanosystem that achieved the lowest level of gene expression knockdown in this cell line (Table 4.1).

Table 4.1. HTT gene expression knockdown efficiency of non-viral vectors in ST14-HTT120Q cells

Non-viral delivery system	HTT gene expression knockdown (% of untreated controls)
Cyclodextrin	45.06 ± 16.49
Lipofectamine®2000	69.90 ± 6.42
INTERFERin®	63.74 ± 12.13
Superfect®	29.25 ± 6.80

4.4.3 Direct biological adverse effects of non-viral siRNA nanoparticles in brain-derived cell lines

Assessment of direct biological adverse effects using conventional end-point methods revealed differential toxicity profiles of non-viral siRNA nanoparticles within the same brain-derived cell line. Moreover, trypan blue exclusion assays (Figure 4.2 a – c), LDH assays (Figure 4.2 d – f) and MTT assays (Figure 4.2 g – i) provided insights into the various aspects of cellular toxicity. Trypan blue dye exclusion assays provided robust live/dead cell evaluation based on permanent cellular membrane damage, LDH assays detected transient and early injury to the cellular membrane and MTT assays were used as a measure of cellular metabolic activity (Jurisic et al., 2008; Kepp et al., 2011; Lappalainen et al., 1994).

In the rat striatal cell line (ST14A-HTT120Q) siRNA transfections using Lf2000 and Interferin resulted in significant reduction in cell viability (53.2 ± 3.1% and 37.6 ± 7.5% viable cells after 24 hours, respectively), increased LDH release (4.08 ± 0.08 and 5.06 ± 0.39 fold-increase after 24 hours, respectively) and reduction in mitochondrial dehydrogenase activity (46.29 ± 0.53%, 76.36 ± 2.39% metabolically active cells after 48 hours, respectively) (Figure 4.2 a,d,g). Although after 24 hours transfection SF.siRNA nanoparticles did not affect cell viability or LDH release, they had significant effects in mitochondrial metabolic activity after 48 hours (71.72 ± 0.54%). No significant adverse

effects were detected for CD.siRNA nanoparticles in all toxicity tests carried out in ST14A-HTT120Q cells (Figure 4.2 a,d,g).

Interferin and SF siRNA nanoparticles significantly reduced cell viability (56.47 ± 5.29% and 43.88 ± 1.44% viable cells after 24 hours, respectively), increased LDH release (2.59 ± 0.06, 2.93 ± 0.08 fold-increase after 24 hours, respectively) and reduced dehydrogenase activity (12.60 ± 1.85%, 53.13 ± 4.00% metabolically active cells after 48 h, respectively) in BV2 microglia cells (Figure 4.2 b,e,h). Although Lf2000 did not reduce cell viability, it significantly increased LDH release after 24 hours (2.10 ± 0.084 fold-increase) and reduced dehydrogenase activity (36.78 ± 2.97% metabolically active cells) after 48 hours. On the other hand, CD.siRNA nanoparticles only modestly affected cellular metabolic activity (79.59 ± 6.13% metabolically active cells) in BV2 cells after 48 hours (Figure 4.2 b,e,h).

Although not dramatically, Lf2000 and interferin siRNA nanoparticles significantly reduced cell viability (92.09 ± 1.50% and 92.43 ± 1.91% viable cells after 24 hours transfection, respectively) and increased LDH release (3.13 ± 0.51% and 3.85 ± 0.30 fold-increase after 24 hours, respectively) in U87 astroglioma cells (Figure 4.2 c,f,i). However, after 48 hours Interferin.siRNA nanoparticles did not induce significant changes in mitochondrial metabolic activity in this cell line whereas Lf2000 did (81.50 ± 0.63% metabolically active cells). Although no changes were observed in trypan blue and LDH assays after 24 hours, SF.siRNA nanoparticles induced mitochondrial adverse effects detected by MTT assay after 48 hours (47.39 ± 0.70% metabolically active cells). No toxic effects in U87 cells were observed with CD.siRNA nanoparticles in the tests performed (Figure 4.2 c,f,i). Trypan blue dye exclusion assays, LDH and MTT biochemical assays did not detect any detrimental effects of naked siRNAs in the brain-derived cell lines tested in this study.

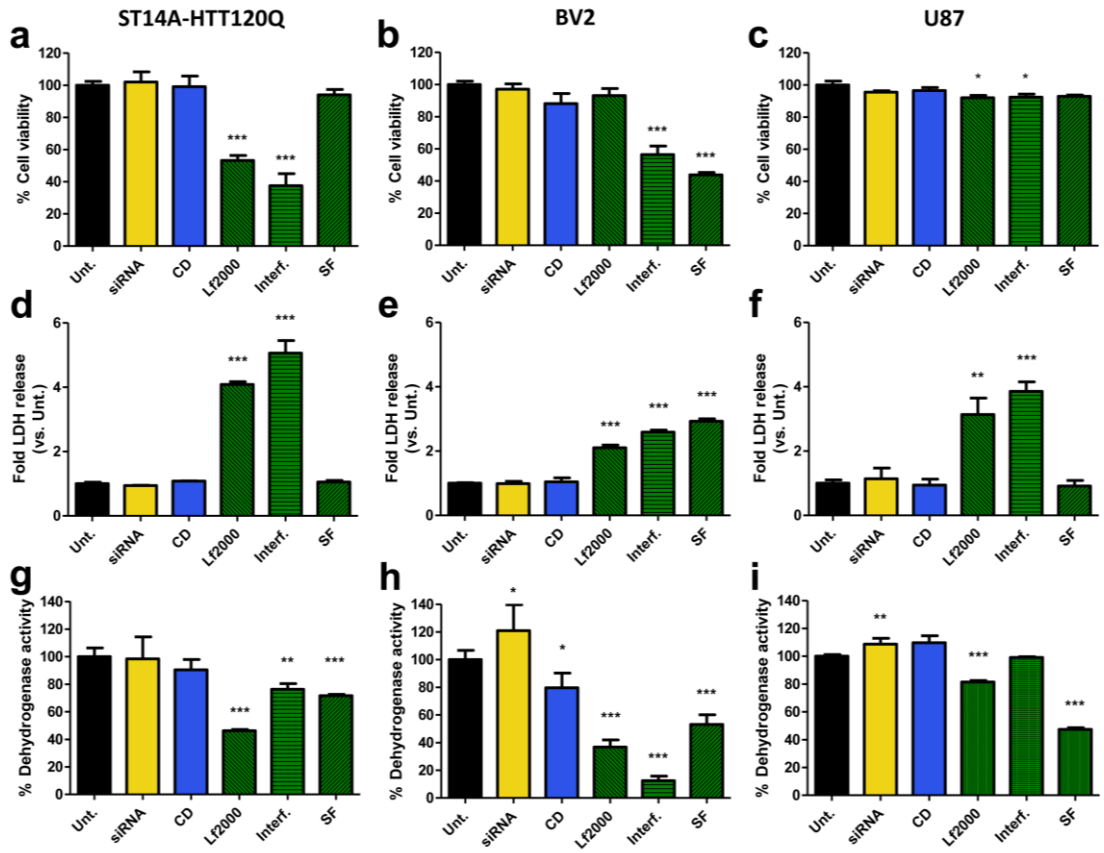


Figure 4.2. Evaluation of nanoparticle-induced cytotoxicity in multiple brain-derived cell lines using conventional methods. *ST14A-HTT120Q cells (a, d, g), BV2 microglial cells (b, e, h) and U87 cells (c, f, i) were transfected using different non-viral siRNA nanoparticles. Final concentration of siRNA in RNAi-treated groups was of 100 nM for all experiments. Trypan blue exclusion assays (a-c) and LDH release assays (d-f) were carried out after 24 hours of transfection. MTT assays were performed after 48 hours transfection (g-i). Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 against untreated control. n = 3-5 per group.* ***Abbreviations****: CD, Cylcodextrin; Interf., Interferin; Lf2000, Lipofectamine2000; MTT, Methyl thiazolyl tetrazolium ; siRNA, Naked siRNA; SF, Superfect®; Unt., Untreated.*

HCA was used to further investigate nanoparticle-induced cytotoxicity in the ST14A-HTT120Q *in vitro* model of HD (Figure 4.3). Figure 4.3 a shows fused images of the HCA cell integrity assay where membrane permeant nuclear blue stain identifies viable living cells, membrane impermeant red nuclear dye identifies dead cells (co-staining with blue yields magenta), phosphatidylserine green marker identifies apoptotic cells and red mitochondrial stain identifies healthy mitochondria. After 24 hours, Lf2000 and Interferin significantly reduced the number of viable cells (51.61 ± 3.62% and 18.93 ± 5.17% of total number of cells, respectively) and increased the number of late apoptotic (43.72 ± 2.98% and 71.98 ± 5.48% of total number of cells, respectively) and dead cells (4.68 ± 1.90% and

9.09 ± 2.67% of total number of cells, respectively) (Figure 4.3 b). In addition, Lf2000 and Interferin also significantly decreased cell density by 45.12 ± 3.32% and 49.86 ± 1.69%, respectively, when compared to untreated controls (Figure 4.3 c). Furthermore, HCA revealed that Lf2000 and Interferin increased membrane permeability by 147.80 ± 5.17% and 241.60 ± 8.69%, respectively, when compared to untreated controls following cellular insult (Figure 4.3 d). Despite obvious differences in sensitivity, these results are in accordance with trypan blue and LDH assays, respectively. Nuclear morphology analysis showed significant shrinkage of the nuclear area in Lf2000 (-23.49 ± 2.33%) and Interferin (-43.35 ± 0.84%) transfected cells (Figure 4.3 e). Additionally, Lf2000 and Interferin significantly reduced mitochondrial membrane potential (MMP) by 38.4 ± 0.63% and 45.04 ± 2.79%, respectively (Figure 4.3 f). On the other hand, naked siRNAs, CDs and SF did not alter significantly any of the above mentioned cell integrity parameters when compared to untreated controls. However, and in contrast with CDs which have efficiently transfected ST14A-HTT120Q cells in the present study, it is worth noting that the good viability profile observed for SF might have been associated with the lower levels of transfection achieved in this particular cell line. Finally, cells stimulated for 2 hours with a calcium ionophore (Ionomycin 20 µM), known to increase release of intracellular calcium and to induce an apoptotic like process, presented reduction in cell densities, number of viable cells, nuclear area and the MMP. Ionomycin also significantly increased the number of late apoptotic cells and plasma membrane permeability when compared to untreated controls (Figure 4.3 a-f).

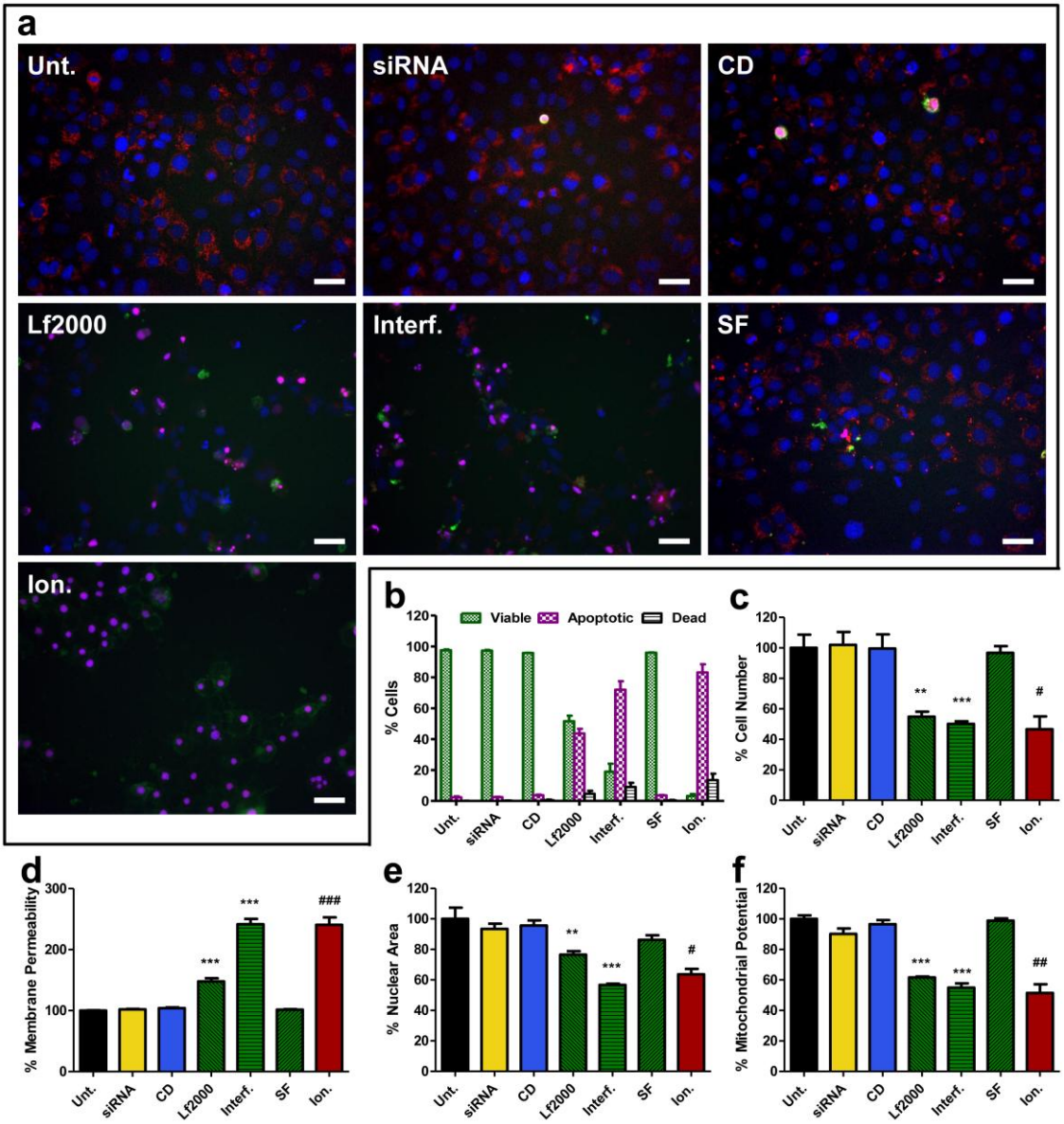


Figure 4.3. Nanoparticle-induced cytotoxicity in ST14A-HTT120Q striatal cells assessed by High Content Analysis. *ST14A-HTT120Q cells were transfected for 24 h with different non-viral siRNA nanoparticles. Final concentration of siRNA in RNAi-treated groups was of 100 nM. Ionomycin 20 µM incubated for 2 h was used as positive control for apoptosis. (a) Representative fused images obtained from HCA consisting of (blue) nuclear permeant dye indicating viable cells, (red) mitochondrial stain identifying healthy mitochondrion, (green) marker for presence of phosphatidylserine in outer plasma membrane and (magenta) indicating co-localization of blue nuclear stain with membrane impermeant dye, identifying late apoptotic cells. (b) Percentage of viable, apoptotic and dead cells from total cell count. (c) Cell number (d) Membrane permeability (e) Nuclear area and (f) Mitochondrial membrane potential presented as a percentage of untreated controls. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01 and ***P<0.001 against untreated control. n = 3 per group.* ***Abbreviations:*** *CD, Cylcodextrin; Interf, Interferin; Ion, Ionomycin; Lf2000, Lipofectamine2,000; SF, Superfect®; Unt, Untreate; siRNA, Naked siRNA.*

4.4.4 Nanoparticle-induced neuroinflammatory responses in brain-derived cell lines

Immune responses in the CNS are mainly mediated by microglia and astroglia. Therefore here we tested BV2 microglia cells and U87 astroglioma cells for the expression of pro-inflammatory markers, such as cytokines, after transfection with different non-viral vectors.

Results showed that after only 4 hours Lf2000, Interferin and SF siRNA nanoparticles had significantly increased TNF-α gene expression (2.65 ± 0.28, 1.98 ± 0.24, 2.26 ± 0.4 fold-increase, respectively), which was further increased for Interferin and SF (29.59 ± 2.18 and 46.35 ± 2.75, respectively) after 24 hours (Figure 4.4 a). IL-1β gene expression was only found to be significantly increased in cells transfected with SF.siRNA nanoparticles after 4 hours (26.58 ± 11.22 fold-increase), however after 24 hours both Interferin and SF induced significant increases in IL-1β gene expression (225.88 ± 63.65 and 386.51 ± 115.07, respectively) (Figure 4.4 b). In contrast, no significant changes from untreated controls were observed for cells treated with naked siRNA or CD.siRNA nanoparticles for any of the cytokines assessed at any of the time points (Figure 4.4 a,b). A positive control for cytokine release, LPS induced a significant increase in TNF-α and IL-1β gene expression immediately after 4 hours stimulation. The expression of IL-6 was also assessed in this study, yet no expression of this cytokine was detected with either LPS positive control or with the different non-viral vectors (data not shown). For completion of these results we investigated cytokine release to the culture medium in BV2 and U87 cells through multi-spot enzyme-linked immune sorbent assay (ELISA) after 24 hours transfection. In BV2 cells, Lf2000, Interferin and SF significantly increased TNF-α release when compared to untreated controls (2.55 ± 0.11, 1.80 ± 0.48 and 1.54 ± 0.23 pg/mL, respectively), however this was only a modest increase at this particular time point (SI, Supplementary Figure S4.1). Release of IL-1β in BV2 cells was only found to be modestly increased with LPS and none of the vectors induced significant release of this cytokine (SI, Supplementary Figure S4.1). In the U87 astroglioma cell line, Lf2000 and Interferin were found to significantly increase release

of IL-6 (51.89 ± 6.44, 49.08 ± 7.38 pg/mL, respectively) (SI, Supplementary Figure S4.1). Moreover, stimulation of U87 cells with LPS resulted in low levels of expression of TNF-α and IL-1β release after 24 hours (data not shown).

The expression of the pattern recognition TLR2 was also assessed and found to be significantly increased in BV2 cells after 4 hours transfection with Lf2000 (3.16 ± 0.14 fold-increase) and Interferin (3.47 ± 0.54 fold-increase) (Figure 4.4 c). Further increases were observed at 24 hours for Interferin (11.73 ± 0.64 fold-increase) and SF siRNA nanoparticles (11.51 ± 1.13 fold-increase). On the other hand, neither naked siRNAs nor CD.siRNA nanoparticles induced significant increases in the expression of this pattern recognition receptor (Figure 4.4 c). Finally, the expression of the pro-inflammatory prostaglandin synthase COX-2 was found to be significantly increased in BV2 cells treated with SF.siRNA nanoparticles (84.25 ± 7.95 fold-increase) (Figure 4.4 d). Despite a modest increase observed with siRNA nanoparticles formulated with CD (2.56 ± 0.90 fold-increase), Lf2000 (2.74 ± 0.56 fold-increase) and Interferin (2.85 ± 0.75 fold-increase) results for these nanoparticles did not reach significance when compared to untreated controls.

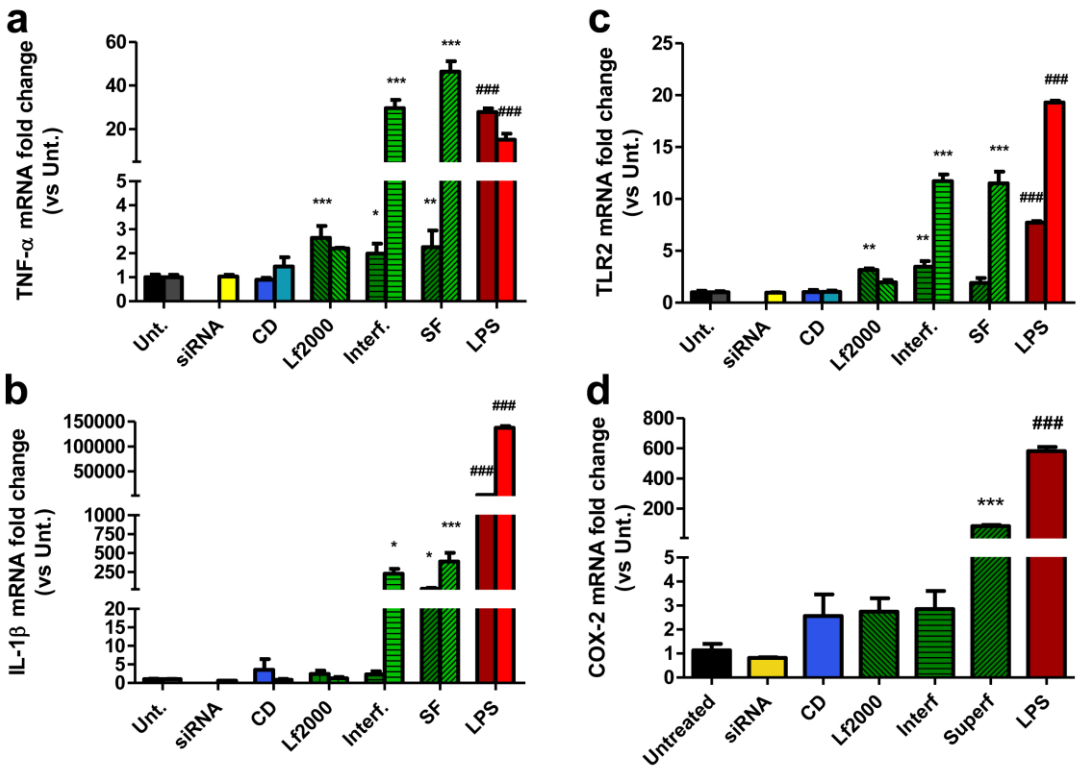


Figure 4.4. Nanoparticle-induced pro-inflammatory gene expression in BV2 microglia cells. *BV2 microglia cells were transfected for 4 or 24 hours using different nanoparticles. Final siRNA concentration was of 100 nM for all experiments. Total RNA was extracted, reverse transcribed to cDNA and gene expression assessed by RT-qPCR. (a-c) First bar series correspond to gene expression at 4 hours and second bar series to 24 hours. All results were normalized to the expression of β-actin endogenous control. LPS was used as positive control. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ###P<0.001 against untreated control. n = 3-5 per group.* ***Abbreviations****: CD, Cylcodextrin; cDNA, complementary DNA; Interf, Interferi; Lf2000, Lipofectamine2000; siRNA, Naked siRNA; SF, Superfect®; LPS, Lipopolysaccharide; RT-qPCR, Real time quantitative PCR; Unt, Untreated.*

4.4.5 Acute *in vivo* neuroinflammatory responses to non-viral siRNA nanoparticles in the brain

In order to investigate local activation of immune response in the brain caused by non-viral siRNA nanoparticles, direct injections into the striatum of C57/BL6 mice were performed. Subsequently, gene expression of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 gene expression were assessed through RT-qPCR (Figure 4.5). After 24 hours, all animals subjected to stereotaxic brain surgery revealed an expected increase in the expression of TNF-α due to mechanical lesion and trauma. However, only SF.siRNA nanoparticles

significantly increased the expression of this cytokine when compared to vehicle-treated animals (527.40 ± 137.10 fold-increase) (Figure 4.5 a). Furthermore, the expression of IL-1β was found to be undetectable in untreated control animals and only SF.siRNA nanoparticles significantly increased its expression when compared to vehicle-treated animals (Figure 4.5 b). Finally, expression of IL-6 was found to be significantly enhanced in animals treated with SF.siRNA nanoparticles (259.50 ± 94.54 fold-increase) and a trend towards significance was found for animals treated with Interferin.siRNA nanoparticles (Figure 4.5 c). Naked siRNA did not stimulate the expression of any of the cytokines screened in this study. In contrast, LPS caused a significant and dramatic increase in TNF-α, IL-1β and IL-6 after 24 hours.

Furthermore, astroglia activation was evaluated by assessing GFAP levels across the different treatment groups (Figure 4.5 d box). All animals subjected to brain surgery presented increased levels of GFAP when compared to untreated animals. Although a positive trend towards significance is clear for animals treated with Lf2000 and SF siRNA nanoparticles, no statistical significance was achieved (Figure 4.5 d). Moreover, only modest weight loss was noted in all RNAi-treated animals, except for the SF-treated group where significant differences were observed when compared to vehicle-treated animals (SI, Supplementary Figure S4.2).

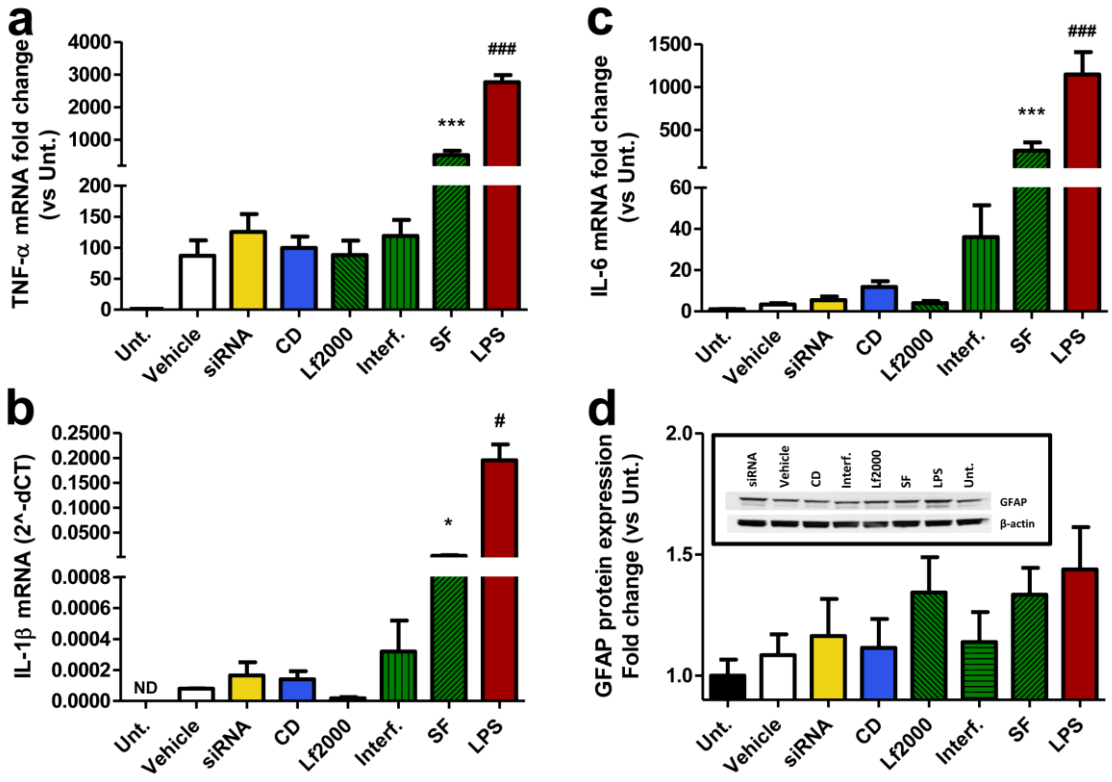


Figure 4.5. Acute in vivo neuroinflammatory responses to non-viral siRNA nanoparticles in the brain. *Different non-viral siRNA nanoparticles were injected bilaterally (2 x 0.2 µg siRNA / 2.5µL) into the striatum of 6-week old C57/BL6 male mice. After 24 hours, total RNA was extracted, reverse transcribed to cDNA and cytokine gene expression assessed by RT-qPCR (a-c). (d) Western blot (box) and densitometry analysis for GFAP protein expression. LPS was used as positive control. All results were normalized to the expression of β-actin endogenous control. Results are expressed as Mean ± SEM. ***P<0.001 and ###P<0.001 against vehicle control. n = 3-12 per group.* ***Abbreviations:*** *CD, Cylcodextrin; cDNA, Complementary DNA; Interf, Interferin; GFAP, Glial Fibrillary Acidic Protein; Lf2000, Lipofectamine2000; LPS, Lipopolysaccharide; ND, Not detected; SF, Superfect®; siRNA, Naked siRNA; RT-qPCR, Real time quantitative PCR; Unt, Untreated; Vehicle, 5% glucose.*

4.5 Discussion

Developing nanosystems for RNAi delivery is a difficult balancing act between inducing an appropriate level of efficacy versus the biocompatibility and safety liabilities of the assembled nanosystem. This is particularly cogent for disorders of the CNS where neuronal and glial cells are highly sensitive to cytotoxic insults. Moreover, the rapid developments in nanotechnology have resulted in the establishment of a wide range of non-viral vectors whose biological and immunological effects in the CNS are still to be comprehensively elucidated and compared. Thus, this report aims to solely evaluate the differential nanotoxicological and neuroinflammatory effects of widely used non-viral vectors for siRNA delivery to the CNS.

The physicochemical characteristics of the assembled nanosystem have been shown to dictate cellular uptake and gene knockdown efficiency, but also their cytotoxic effect. In this study, the different vectors (CD, Lf2000, Interferin and SF) yielded nanoparticles with comparable surface charges but with varying hydrodynamic sizes. Similar particle sizes have been previously reported by our group for CD.siRNA nanoparticles in DLS studies (Godinho et al., 2013; O'Mahony et al., 2012b), and further confirmed by morphological studies using transmission electron microscopy (O'Mahony et al., 2013d). Furthermore, vectors achieved different degrees of gene silencing of the mutant HTT gene in an *in vitro* model of HD (ST14A-HTT120Q), with CD.siRNA nanoparticles having a similar potency to that described previously (Godinho et al., 2013). However, and despite the fact that all nanoparticles presented comparable surface charges, only CD.siRNA nanoparticles have consistently presented safer cytotoxic profiles across most cell lines and assays here performed. Thus, in spite of the well documented detrimental effects of high positive surface charges (Bertero et al., 2013; Hong et al., 2006b; Hunter, 2006; Kedmi et al., 2010), we suggest that the differences in cytotoxicity and also in the degree of HTT suppression observed, are probably in part associated with other characteristics of the nanoparticles, such as size and/or morphology. Indeed, others have found that nanoparticle size is a key factor in

determining the specific cellular uptake and intracellular trafficking pathways whereas nanoparticle morphology may determine selective uptake by neurons and/or microglia (Albanese et al., 2012; Rejman et al., 2004). We also reason that, biodegradability and clearance of the nanosystem from the intracellular compartment could have played an important role in cellular toxicity, however, further investigations are needed to clarify the mechanisms implicated. On the other hand, it is of interest to note that recent microarray data show that different biomaterials induce cell-specific "gene fingerprints", deregulating various genes related to apoptosis, cell proliferation and differentiation and mechanisms of DNA repair (Choi et al., 2010; Hollins et al., 2007; Merkel et al., 2011; Omidi et al., 2005). In turn, these genomic disruptions significantly differ between empty non-viral vectors and assembled nanosystems (containing their nucleic acid cargo) (Choi et al., 2010; Hollins et al., 2007; Omidi et al., 2005). Therefore, this may suggest that cells recognize assembled nanosystems as singular entities distinct from the individual components, and that pathways implicated in subsequent cytotoxicity may also be different (Akhtar & Benter, 2007).

Inducing gene silencing effects in the brain requires in various circumstances interaction of nanoparticles with different cell types, including neurons and glia (O'Mahony et al., 2013b). Thus, here we emphasise important differences in cellular susceptibility to the toxic stimulus mediated by non-viral vectors in brain-derived cell lines. Our results showed that ST14A-HTT120Q striatal cells and U87 astroglioma cells seemed to be more susceptible to toxic adverse effects from Lf2000 and Interferin siRNA nanoparticles, whereas BV2 microglia cells seemed to be more susceptible to toxicity from Interferin and SF. In agreement with our results, others have found that cellular uptake and cytotoxic profiles of widely used commercially available vectors are largely cell type-dependent (Gebhart et al., 2001; Kiefer et al., 2004; Uchida et al., 2002; Yamano et al., 2010). Furthermore, in the specific context of the CNS, primary cultured astrocytes and microglial cells have also presented differential cellular uptake profiles when transfected with lipid-formulated siRNA (Ki et al., 2010). Together, the differential toxicological cellular responses found in these studies may be

related to the specific composition of cellular membranes of each cell-type, differences in the interaction of the biomaterials with intracellular components, but also in how cells are able to process and degrade these biomaterials. Overall, ST14A-HTT120Q cells, an *in vitro* model of HD, and the BV2 microglia cells used in this study seemed to be the most sensitive to adverse effects of non-viral siRNA nanoparticles. Indeed, it has been previously shown that the expression of the mutant and toxic HTT protein in this striatal cell line renders these cells more prone to toxic insults (Rigamonti et al., 2000). At the other end of the transfection spectrum, U87 astroglia cells, derived from a human astroglioma cancer, seemed to be more resistant to cell death. Thus, the selection of appropriate CNS *in vitro* models and appropriate toxicity assays is crucial for the assessment of biological adverse effects of non-viral vectors.

The majority of studies assessing *in vitro* cytotoxicity of widely used delivery systems have based their biosafety assumptions on a single end-point toxicity assay (e.g. (Gebhart & Kabanov, 2001; Kiefer et al., 2004; Uchida et al., 2002; Yamano et al., 2010)). In fact, despite the vast number of well established toxicity assays available to researchers for monitoring cell death, such as trypan blue exclusion, LDH and MTT assays, there is limited comparative information on the relative utility of these tests (Kepp et al., 2011). In summary, each of the conventional cytotoxicity methods employed in this study assesses a specific parameter involved in cell death and should be used together for a more complete assessment of cytotoxicity. As an example, no dramatic reduction in cell viability was detected in U87 cells upon transfection, nevertheless remarkable increases in LDH release revealed that early disruptions of membrane permeability might be occurring. Furthermore, MTT assays complement these results demonstrating that nanoparticles have also altered mitochondrial metabolic activity significantly. Thus, assumptions regarding biocompatibility of nanomaterials using a single conventional end-point toxicity assay are limited and should be avoided. Alternatively, HCA is a high-throughput technique that allows the evaluation of multiple cellular morphological and biochemical parameters with high sensitivity and

specificity (Giuliano et al., 2003). Although this technique is lately becoming popular to assess cytotoxicity of active pharmaceutical compounds (Giuliano et al., 2003), was only recently that HCA has been applied to evaluate efficiency and cytotoxic effects of non-viral vectors for gene delivery and other nanoparticles *in vitro* (Hibbitts et al., 2011; Rawlinson et al., 2010). HCA cell integrity assay revealed that Lf2000 and Interferin siRNA nanoparticles reduced cell densities and the number of viable cells, and increased the number of late apoptotic and dead cells. Presence of phosphatildylserine in the outer face of the plasma membrane and co-staining of the nucleus with nuclear impermeable dye due to increased plasmatic membrane permeability, enabled identification of these cells as late apoptotic (Van Cruchten et al., 2002). Additionally, RNAi transfection with these vectors induced nuclear contraction and chromatin condensation, both of which are typical features of cells undergoing apoptosis (Rawlinson et al., 2010; Van Cruchten & Van Den Broeck, 2002). Lf2000 and Interferin also triggered loss of MMP indicating that these vectors compromise healthy mitochondrial function, eventually leading to cytochrome C release and induction of several other signalling cascades. Thus, the HCA results bolster our data obtained with conventional methods, however at a much higher degree of sensitivity, while also allowing for specific identification of the cell death mechanism activated by these biomaterials.

Safety of non-viral vectors for RNAi in the CNS is also dependent on a reduced activation of the local immune system. Interestingly, our data showed that the non-viral vectors that induced greater cytotoxic effects in microglia and astroglia cells are more likely to trigger neuroinflammatory responses. Indeed, in BV2 microglia cells, Interferin and SF induced the highest expression of major pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) among all vectors used. Consistent with our results, others have also reported increased cytokine release in primary glial cultures and/or *in vivo*, after systemic administrations, when using lipid- and/or polymer-based siRNA/pDNA nanoparticles (Gautam et al., 2001; Gorina et al., 2009; Sakurai et al., 2002). In addition, although these immunostimulatory effects could have been triggered by the nucleic acid cargo itself rather than the biomaterial, other studies

have highlighted that this might be a vector-dependent effect. Indeed, these studies demonstrate that delivery of the same nucleic acid cargo (including unmodified siRNAs) by different vectors leads to differential immune responses after intravenous injection (i.v.) (Bonnet et al., 2008; Kawakami et al., 2006; Kedmi et al., 2010). Thus, certain vectors seem to be more likely to enhance the immunostimulatory effects of siRNA than others, and these effects have been suggested to be closely related to sequestration of siRNA within a TLR7 rich environment in the endosomes (Ballarín-González & Howard, 2012). This further supports the need to develop non-viral vectors with endosomolytic properties and with low cytotoxic effects. Furthermore, it has also been recently suggested that the induction of cytokine expression by nanoparticles and biomaterials may occur through the activation of TLRs (Hutter et al., 2010; Kedmi et al., 2010). Investigations in various denditric cell models have demonstrated that this is likely to be a structural activity dependent-effect and therefore specific to certain lipids (Lonez et al., 2009; Tanaka et al., 2008; Vangasseri et al., 2006). Although not in the particular context of RNAi or gene delivery, several biomaterials and delivery systems (e.g. PAMAM dendrimers) have been shown to activate microglia, resident immune cells of the CNS, and to increase the expression of specific inflammatory receptors such as TLRs and CC-chemokine receptor 2 (Bertero et al., 2013; Hutter et al., 2010). Expression of TLR2 was found to be enhanced following the administration of Interferin and SF, however further studies are needed to reach a better understanding of the mechanism underlying these effects. Additionally, despite marked increased in cytokine gene expression *in vitro* with SF, Lf2000 and Inteferin, only SF nanoparticles lead to a significant increase gene expression of the pro-inflammatory enzyme COX-2, a key enzyme responsible for the synthesis of prostaglandins. Thus, although expression of COX-2 in the brain is closely regulated by growth factors and cytokines (Ramsay et al., 2003), this differential response of the nanosystems indicates that additional underlying mechanisms are probably responsible for its activation by this G6 PAMAM dendrimer.

Route of administration, length of treatment and dosing regimens have also been identified as important determinants for toxic and inflammatory responses to delivery systems (O'Mahony et al., 2013b). Indeed, here we demonstrate that the mechanical damage during brain intraparenchymal injections *per se* is able to enhance cytokine gene expression and also GFAP levels, effect which is clearly observed in all surgical animals including vehicle-treated animals. In agreement with our *in vitro* data, SF nanoparticles caused significant increases in cytokine gene expression *in vivo* and induced weight loss when compared to vehicle-treated animals. However, in a previous study only moderate glial activation was reported upon intracortical injections with G4 PAMAM dendrimers (Albertazzi et al., 2012). Thus, we speculate that the increased activation of the immune response in our study might be related to the increased cytotoxic effects of the G6 PAMAM dendrimer in the brain. Indeed, *in vitro* mechanistic studies in mammalian cells have demonstrated that dendrimers induce cytotoxic effects in a generation-dependent manner (Mukherjee et al., 2010). On the other hand, although no significant immune activation was found for Interferin in the present *in vivo* study, increased immunological responses upon brain delivery have been reported elsewhere (Badaut et al., 2011). In contrast, a previous study in our group showed that multiple injections with CD.HTTsiRNA nanoparticles into the striatum of the R6/2 mouse model of HD selectively improved rotarod motor deficits without causing detrimental effects on body weight profiles (Godinho et al., 2013). In addition, other CD-containing polymer delivery systems for siRNA (CALAA-01) have been shown to be well tolerated in non-human primates after multiple i.v. administrations revealing no significant activation of the immune system (Heidel et al., 2007). Therefore, further studies should be carried out for Lf2000, Interferin and SF to assess the effects of multiple injections into this susceptible structure.

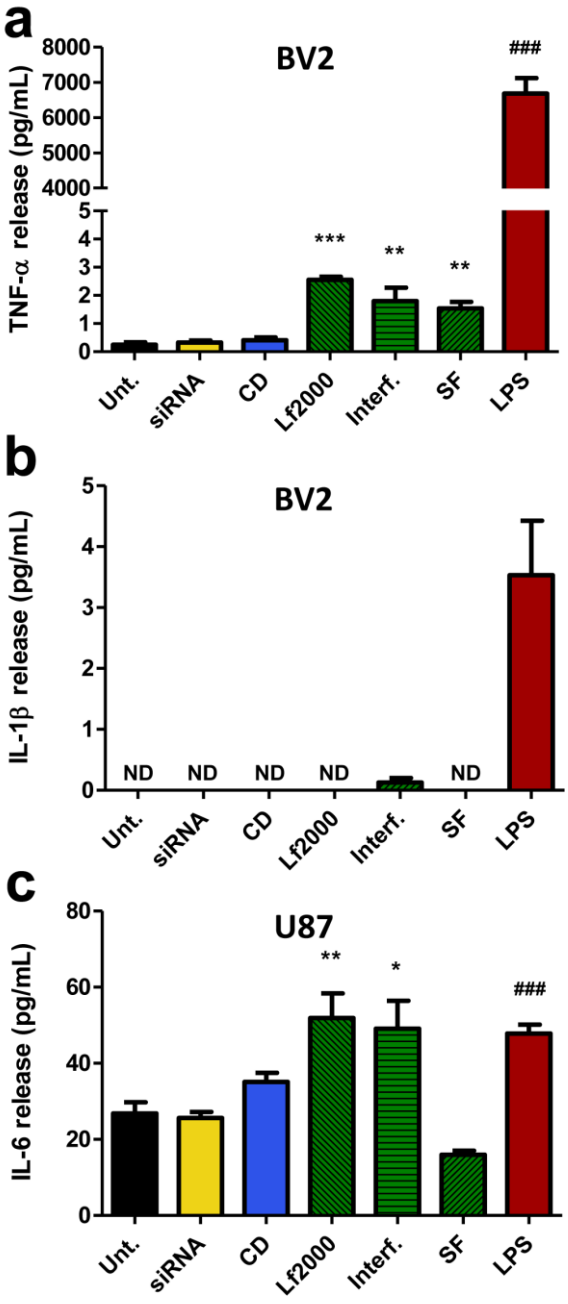
4.6 Conclusion

The functional importance of examining toxicity profiles of nanosystems is obvious when one is extrapolating to *in vivo* analysis. Although brain stereotaxic surgery and direct administration of non-viral siRNA nanoparticles into the CNS is a common practice in research and pre-clinical testing (e.g. (Badaut et al., 2011; Godinho et al., 2013; Wang et al., 2005)), the translation of this approach to the clinic requires a better understanding of the interaction of non-viral siRNA nanoparticles and the CNS cellular milieu. Intrinsic toxicity of nanoparticles might be advantageous when treating brain cancers, but the application of such technologies to neurodegenerative disorders demands low cytotoxic and immunological adverse effects. Thus, taken together our data enable us to identify modified CDs as promising nanocarriers that enable siRNA delivery to the brain with low levels of cytotoxicity and immunological activation.

4.7 Supplementary information

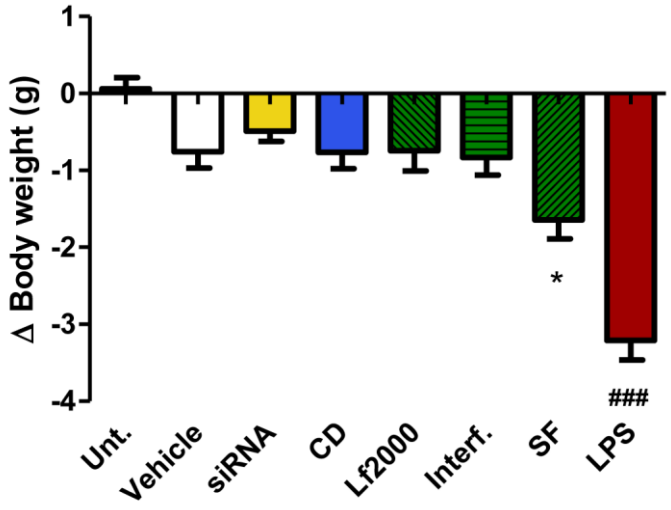
The SI section in this chapter includes data regarding nanoparticle-induced cytokine release in brain-derived cell lines (Supplementary Figure S4.1); body weight changes after stereotaxic injections of different non-viral siRNA nanoparticles into the mouse brain (Supplementary Figure S4.2); and further details are given regarding materials and methods used in the experimental section (Supplementary Materials and Methods).

4.7.1 Nanoparticle-induced cytokine release in brain-derived cell lines



Supplementary Figure S4.1. Nanoparticle-induced cytokine release in brain-derived cell lines. *BV2 microglia cells and U87 astroglioma cells were transfected for 24 hours using different non-viral siRNA nanoparticles. Final concentration of siRNA in all RNAi-treated groups was of 100 nM. (a-c) TNF-α, IL-1β and IL-6 release to cell supernatants was assessed by using Multi Spot MSD ELISA. LPS was used as positive control. Results are expressed as Mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ###P<0.001 against untreated control. n = 3 per group.* ***Abbreviations****: CD, Cylcodextrin; Lf2000, Lipofectamine2000; Interf., Interferin; SF, Superfect®; LPS, Lipopolysaccharide ND, Not detected; Unt., Untreated; siRNA, Naked siRNA.*

4.7.2 Body weight changes after stereotaxic injections of different non-viral siRNA nanoparticles into the mouse brain



Supplementary Figure S4.2. Body weight changes after stereotaxic injections of different non-viral siRNA nanoparticles into the mouse brain. *C57/BL6 mice were bilaterally injected into the striatum (2 × 0.2 μg siRNA/2.5 μL) with different non-viral siRNA nanoparticles. Differences in body weights observed 24 hours after stereotaxic injections were recorded. Results are expressed as Mean ± SEM. *P<0.05 and ###P<0.001 against vehicle control. n = 9-11 per group.* ***Abbreviations****: CD, Cylcodextrin; Interf, Interferin; Lf2000, Lipofectamine2000; SF, LPS, Lipopolysaccharide; Superfect®; siRNA, Naked siRNA; Unt., Untreated.*

4.7.3 Supplementary Materials and Methods

Cell Integrity Assay by High Content Analysis

Supplementary Table S4.1. Cytiva™ Cell Integrity Assay kit dye cocktail for HCA assay.

Dye	Excitation/Emission λ (nm)	Dye function	Objects identified
A	360/535	Membrane permeant nuclear stain	All Nuclei
B	535/620	Membrane impermeant nuclear stain	Dead cells nuclei
C	535/620	Membrane permeant indicator for mitochondrial membrane potential	Mitochondria
E	475/535	Phosphatidylserine detection at the plasma membrane	Apoptotic cells

Supplementary Table S4.2. Multitarget analysis settings cytotoxicity assay analysis with In Cell® 1000 Workstation software.

Object	Source	Segmentation	Others
Nuclei	Wave 1 (360/535)	Top-hat	Min. area: 32 μm^2 / Sensitivity: 90
Cells	Wave 2 (475/535)	Collar	Radius: 7 μm
Reference 1 (dye B)	Wave 3 535/620	Pseudo-nuclei	-
Mitochondria	Wave 3 535/620	Organelles	Multiscale top-hat Range: 1-32 μm Sensitivity: 80 # scales: 3
Reference 2 (dye B)	Wave 3 535/620	Pseudo-cells	-

Cytokine release

BV2 microglia cells and U87 astroglioma cells were transfected for 24 hours with different non-viral siRNA nanoparticles. Cytokine release was assessed from cell supernatants by ELISA. Meso Scale Discovery® (MSD®, Gaithersburg, MD) 96-well plate multi-spot mouse and human pro-inflammatory cytokine assays were purchased for detection of TNF-α, IL-1β, IL-6 and IFN-γ. Mouse assay also included the detection of IL-12p70, IL-10 and KC. Specific manufacturer's instructions were followed for plate preparation. SECTOR® Imager 2400 reader was used to quantify cytokine release.

Chapter V

PEGylated Cyclodextrins as Novel siRNA Nanosystems: Correlations between Polyethylene Glycol Length and Nanoparticle Stability

Bruno M. D. C. Godinho[1,2], Julien R. Ogier[3], Aoife Quinlan[1], Raphael Darcy[3], John F. Cryan[2,4], Caitriona M. O'Driscoll[1]

[1]Pharmacodelivery group, School of Pharmacy, University College Cork, Cork, Ireland

[2]Dept. Anatomy and Neuroscience, University College Cork, Cork, Ireland

[3]Centre for synthesis and Chemical Biology, University College Dublin, Dublin, Ireland

[4]Laboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, Cork, Ireland

5.1 Abstract

Silencing disease-related genes in the central nervous system (CNS) using short interfering RNA (siRNA) holds great promise for treating neurological disorders. Yet, delivering RNAi therapeutics to the brain poses major challenges to non-viral systems especially given that the systemic route is preferred. Cationic nanoparticles have been widely investigated for siRNA delivery, but these are hindered with their aggregation potential in physiological environments which limits their intravenous application. Thus, strategies to increase the stability of nanoparticles, such as incorporation of polyethylene glycol (PEG), have being considered. In the current study we investigated the utility of modified cationic amphiphilic or PEGylated amphiphilic cyclodextrins (CD), oligosaccharide-based molecules, to formulate stable CD.siRNA nanoparticles. To this end, we describe a simple method of post-modifying pre-formed CD.siRNA nanoparticles at their surface using PEGylated CDs of different PEG lengths. Resulting PEGylated CD.siRNA nanoparticles presented reduced surface charges and increased stability in physiological salt conditions indicating that PEG has been incorporated. Stability of PEGylated CD.siRNA nanoparticles *in vitro* increased with both PEG length and PEG density at the surface. Furthermore, in a comparative pharmacokinetic (PK) study, improved blood residency times were achieved with CD-formulations when compared to naked siRNAs. However, no significant differences were observed among non-PEGylated and PEGylated CD.siRNAs suggesting that longer PEG lengths might be required for improving stability *in vivo*. Once a stable CD-based formulation is optimised further modification with specific targeting ligands will enable tailoring of this technology for CNS delivery across the blood brain barrier (BBB).

5.2 Introduction

Therapeutic gene silencing by harnessing the endogenous RNA interference (RNAi) pathway using synthetic short interfering RNAs (siRNA) holds great promise for the treatment of neurological disorders, such as Huntington's Disease (Sah, 2006; Thakker et al., 2006). However, silencing disease-related genes in the central nervous system (CNS) constitutes a significant challenge for current non-viral delivery systems. In addition to the difficulty of transfecting neuronal cells, siRNA nanoparticles have to overcome multiple biological barriers, including the blood brain barrier (BBB), which limits the diffusion of these nanoparticles to the brain (O'Mahony et al., 2013b). Indeed, most successful preclinical studies so far consisted of stereotaxic injections into specific structures within the brain (Bonoiu et al., 2011; Cardoso et al., 2010; Cardoso et al., 2008; Godinho et al., 2013) and/or infusion into the intracerebroventricular (i.c.v.) (Thakker et al., 2004; Thakker et al., 2005) in order to overcome the hurdles of systemic delivery. Although gene expression knockdown has been efficiently achieved using the strategies mentioned above, direct and continuous administration into the brain may be less practical when transferring to the clinical setting. Thus, the development of efficient and non-toxic non-viral formulations for systemic administration and subsequent transport across the BBB has received great attention (O'Mahony et al., 2013b).

Cyclodextrins (CD) are starch-derived molecules which have been recently modified to form cationic amphiphilic siRNA delivery systems (O'Mahony et al., 2012d). We have previously used this non-viral vector to deliver specific siRNAs and silence the expression of the mutant Huntingtin (muHTT) gene in the R6/2 mouse brain through stereotaxic injections into the striatum (Godinho et al., 2013). However, the development of such cationic CD formulations for systemic administration will require, at first, further improvements to their stability in physiological salt and serum conditions. Polyethylene glycol (PEG), a polymer of ethylene oxide commonly used in medical applications, has been widely used to confer "steric" stability to nanoparticles, reducing interactions with plasma and blood components

(Gref et al., 2000; Monfardini et al., 1998). In addition, PEGylation has also been shown to minimise recognition of nanoparticles by the mononuclear phagocyte system (Gref et al., 2000; Owens III & Peppas, 2006). Thus, by these means, PEGylation has improved the circulating times of several non-viral vectors including CD-containing polymers (Davis, 2009; Pun & Davis, 2002), lipid-based nanoparticles (Li et al., 2007; Sonoke et al., 2008), and cationic polymer (e.g polyethylenimine (PEI)) (Malek et al., 2009). In order to enhance the stability of nanoparticles, different PEG lengths and polymer densities have been evaluated, however, no general consensus has been reached yet on the ideal PEG length or polymer density (Gref et al., 2000; Kunath et al., 2002; Mao et al., 2006a). Indeed, this is likely to be dependent on the vector type, and also on the PEGylation strategy adopted for modification of the nanoparticles.

Different strategies for PEGylating nanoparticles have been employed including: chemical methods to covalently couple PEG chains to the non-viral vector (Guo et al., 2012b; Mao et al., 2006a); and physical methods, such as post-insertion (Mendonça et al., 2009; Morille et al., 2011; O'Mahony et al., 2013a) and co-formulation (O'Mahony et al., 2013d), used to incorporate PEG into the final formulation. Regarding CD-based formulation approaches, our group has previously described a co-formulation strategy whereby a cationic amphiphilic CD and a PEGylated amphiphilic CD were blended together prior to siRNA complexation. This PEGylation method yielded nanoparticles with increased stability in physiological salt conditions (O'Mahony et al., 2013d). Alternatively, pre-formed CD.siRNA nanoparticles have also been surface-modified by post-insertion of PEGylated lipids yielding a formulation with enhanced pharmacokinetic (PK) profiles when compared to naked siRNAs (O'Mahony et al., 2013a). However, although different methods for PEGylating CD-based nanoparticles are available, the influence of PEG length and polymer density in these delivery systems still warants further investigation.

The present study aims to determine the influence of PEG density and length on the properties of CD nanosystems. To this end, a post-PEGylation approach was used to modify

pre-formed CD.siRNA nanoparticles at the surface using different amphiphilic CDs containing a range of molecular weight (Mw) PEGs. Physicochemical characterisatics and *in vitro* stability in physiological salt conditions were investigated. Subsequently, an optimal polymer density was selected and the PK behaviour of PEGylated CD.siRNA nanoparticles was assessed *in vivo* and compared to non-PEGylated CD.siRNA nanoparticles and naked siRNA.

5.3 Materials and Methods

5.3.1 Synthetic siRNAs

Synthetic duplexed siRNAs were obtained from Sigma-Aldrich (France) or QIAGEN (United Kingdom). HTT target siRNAs (HTTsiRNA) as per Wang *et al.* (Wang et al., 2005) sense strand, 5'-GCCUUCGAGUCCCUCAAGUCC-3'; antisense strand, 5'-ACUUGAGGGACUCGAAGGCCU-3'. FAM-labelled siRNA (FAMsiRNA): sense strand, 5'-[6FAM] UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

5.3.2 Preparation of PEGylated CD.siRNA nanoparticles

The synthesis of the modified cationic amphiphilic β-cyclodextrin (SC12 CD Click Propylamine) and PEGylated amphiphilic β-cyclodextrin (SC12 CD Click PEG500) used in the present study has been previously described (O'Mahony et al., 2012d). Similarly, PEGylated amphiphilic β-cyclodextrins with larger Mw PEG chains (SC12 CD Click PEG1000, SC12 CD Click PEG2000) were synthesised using the same method. SC12 CD Click Propylamine and SC12 CD Click PEGx will from here after be referred as cationic CD and PEG CDs, respectively. Modified amphiphilic CDs were dissolved in chloroform, and chloroform evaporated under a stream of nitrogen. Prior to formulation with siRNAs, modified amphiphilic CDs were reconstituted in sterile dionised water (DIW) and sonicated for 60 minutes. Complexes with anionic siRNA were formed using the cationic CD at mass ratio 10 (µg of CD : µg of siRNA). Equal volumes of cationic CD and siRNA solution were mixed together and incubated at room temperature (RT) for 20 minutes. Thereafter, PEGylated CDs were added to pre-formed cationic CD.siRNA nanoparticles in defined molar ratios between cationic CD and PEGylated CD. A specific volume of each of the PEGylated CDs was gently mixed with corresponding pre-formed CD.siRNA nanoparticles and further incubated at RT for 20 minutes.

For *in vivo* studies CD.siRNA nanoparticles were prepared as outlined above in 5% glucose and concentrated by ultrafiltration using Vivaspin 500 centrifugal units (Sartorius, Germany) to a final concentration of 0.267 µg/µL of siRNA.

5.3.3 Physicochemical characterisation

Binding and complexation of siRNA was confirmed by gel retardation assay. CD.siRNA nanoparticles containing ~0.3 µg of siRNA were mixed with 2 µL of Blue Juice Loading Buffer (Invitrogen, Carlsbad, CA) and sufficient DIW to a final volume of ~30 µL. Samples were loaded in a 2% agarose gel and electrophoresis performed at 90 mV for 20 minutes in Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer (Fisher Scientific, Fair Lawn, NJ). The gel was post-stained using GelRed™ nucleic acid stain (Biotium, Hayward, CA) and visualised using the DNR Bioimaging Systems and Gel Capture version 7.0.9 software.

Size and surface charge measurements were carried out using Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively. CD.siRNA nanoparticles, containing ~ 3 µg of siRNA, were diluted up to 1 mL with filtered sterilised DIW and assessed by DLS and ELS using a Malvern Zetasizer Nano ZS. A total of five readings for size and charge were taken per sample and the refractive index (1.33) and viscosity (0.8872 mPa·s) of water were taken into to account in data analysis.

5.3.4 *In vitro* stability studies in physiological buffer conditions

The stability of PEGylated CD.siRNA nanoparticles was investigated in salt-containing medium (optiMEM®, Invitrogen, UK) and in foetal bovine serum (FBS, Sigma-Aldrich, Germany). Complexes were prepared as above, diluted up to 1 mL in optiMEM® or FBS and incubated at 37 ºC. Size measurements were carried out at different time points (24, 48 and 72 hours) using a Malvern Zetasizer Nano ZS.

5.3.5 Comparative pharmacokinetic study

Male 8-week old Balb/c mice (~24 grams) were obtained from Harlan Laboratories (United Kingdom). Mice were given approximately one week to habituate to the animal facility and maintained on a 12/12 hour light-dark cycle with temperature (22 ± 1 ºC) and humidity (~55 %) controlled conditions.

For PK study, mice were injected with naked FAMsiRNA or with CD.FAMsiRNA nanoparticles. A single i.v. bolus dose of 40 µg of formulated or non-formulated FAMsiRNA in a total of 150 µL 5% glucose solution was administered through the tail vein. Thereafter, blood samples (~30 µL) were collected from the saphenous vein at various time points. Plasma was isolated, snap frozen and kept at -80 °C until analysis. Plasma concentrations of FAMsiRNA were assessed by post-extraction as previously described (Li et al., 2007; O'Mahony et al., 2013a). Briefly, 10 µL of plasma was incubated at 65 °C for 10 minutes with 90 µL of lysis buffer (0.1 % sodium dodecyl sulphate in phosphate buffer saline (PBS)). Methanol (200 µL) was added and samples incubated for 10 minutes at 90 °C. Samples were spun down at 14,000 for 5 minutes and 100 µL of supernatant was transferred in duplicates to a black 96-well plate. Fluorescence was measured using a fluorescent plate reader (λ_{ex} 465 nm and λ_{em} 520 nm) and the concentration of FAMsiRNA determined from a standard curve. The final concentration of FAMsiRNA was corrected for extraction efficiency. Data were fitted to a two-compartmental model with biexponential function $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ for estimation of plasma PK parameters. Estimates were calculated for each individual animal and averaged by group.

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.

5.3.6 Statistical analysis

Data are expressed as mean ± standard deviation (SD) and mean ± standard error of mean (SEM) according to the experiment. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc was used to investigate statistical significant differences against naked siRNA regarding PK parameters. ANOVA followed by Bonferroni's post hoc test was used to investigate statistical significant differences among CD formulations in regards to PK parameters. All inferential statistics were carried out using PAWS 18 Statistical package.

5.4 Results

Cationic amphiphilic β-cyclodextrins and PEGylated amphiphilic β-cyclodextrins with different Mw PEG chains are represented in Figure 5.1. These CDs were previously synthesised using copper catalysed "click" chemistry which enabled coupling of cationic propylamine groups or different Mw PEG chains to the 2-position of the β-CD. This route for functionalisation is a versatile method for coupling functional groups to a variety of vectors, as well as achieving high yields (O'Mahony et al., 2012d).

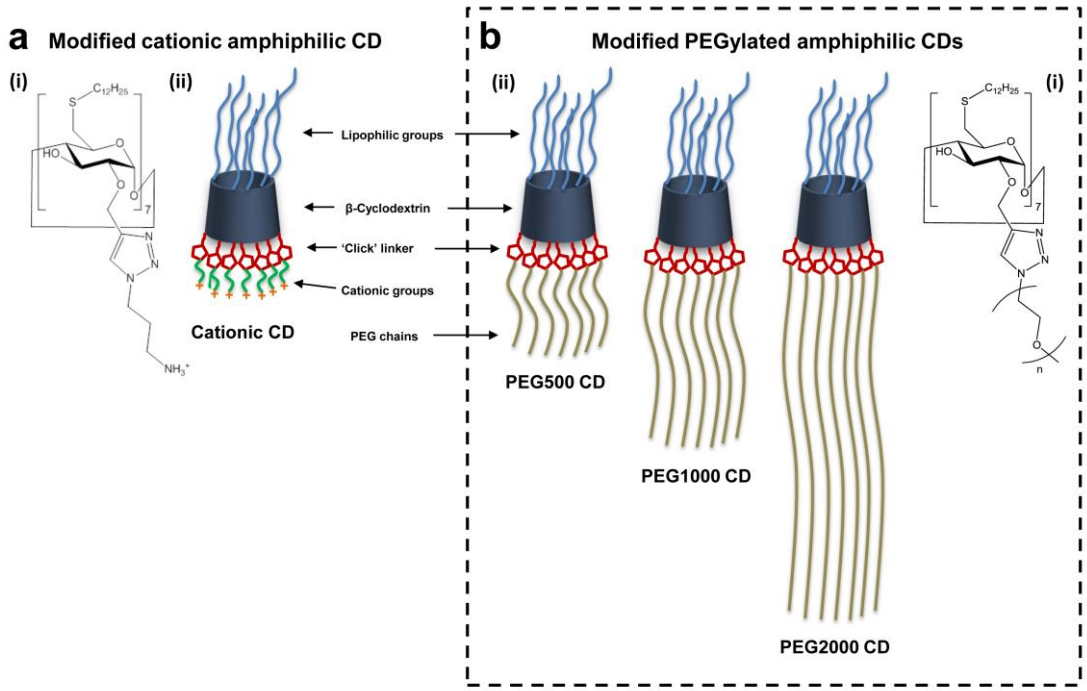


Figure 5.1. Chemical structures and schematic diagrams of modified amphiphilic CDs. *(a) Modified cationic amphiphilic CD and (b) modified PEGylated amphiphilic CDs with different molecular weight PEG chains. (i) Chemical structures of modified CDs and (ii) schematic diagrams.* ***Abbreviations:*** *CD, Cyclodextrin; PEG, Polyethyleneglycol.*

5.4.1 Physicochemical characterisation of non-PEGylated and PEGylated CD.siRNA nanoparticles

Pre-formed CD.siRNA nanoparticles were modified at the surface using different amounts of PEGylated CDs and this was expressed as molar ratio of cationic amphiphilic CD to PEGylated amphiphilic CDs (Cationic CD : PEG CD). Cationic CD successfully complexed

siRNAs (ratio 1:0, Figure 5.2a,c,e) and modification of CD.siRNA nanoparticles with PEGylated CDs did not affect binding and complexation of siRNA as shown in gel retardation assay studies (Figure 5.2a,c,e). In contrast, gel retardation assays showed that PEGylated amphiphilic CDs *per se* were not able to complex anionic siRNA in the absence of cationic CD (Supplementary Information (SI), Supplementary Figure S5.1).

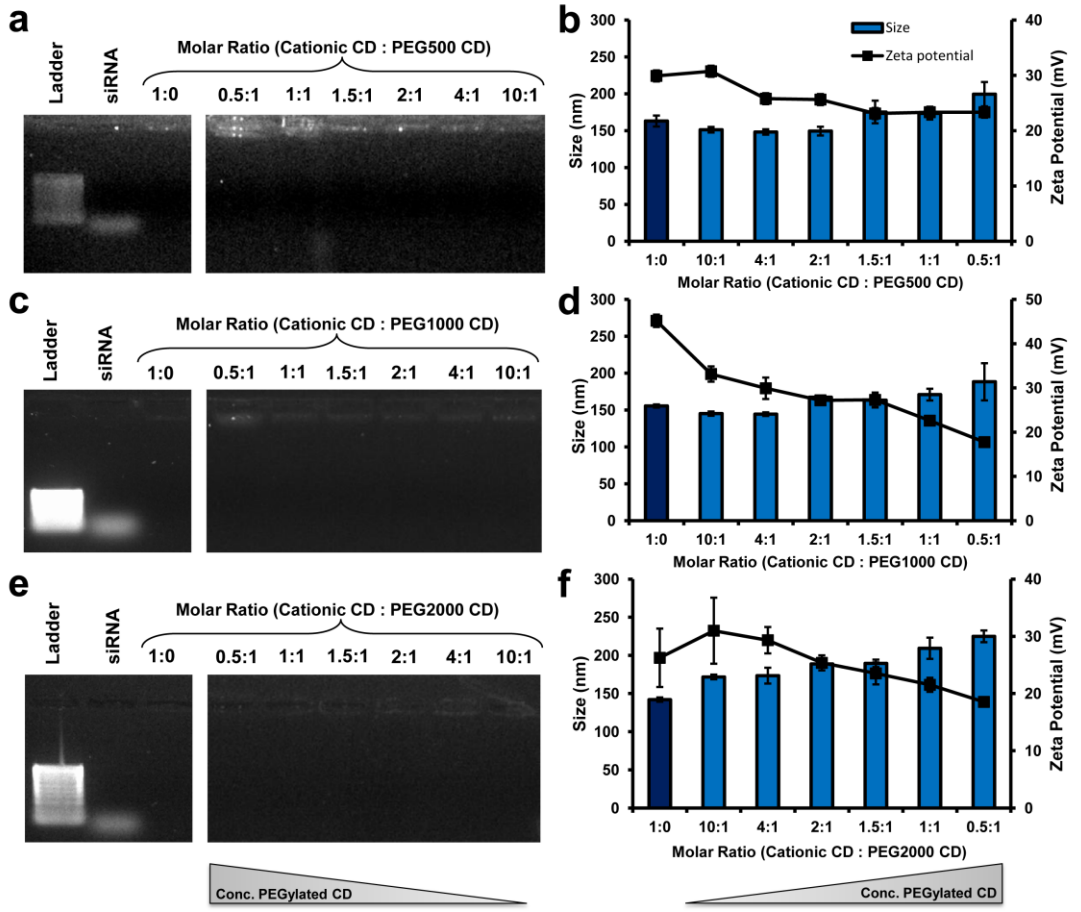


Figure 5.2. Physicochemical characterisation of non-PEGylated and PEGylated CD.siRNA nanoparticles. *CD.siRNA nanoparticles surface-modified with PEG500 CD (a,b), PEG1000 CD (c,d) and PEG2000 CD (e,f). (a,c,e) Gel retardation assay to investigate binding and complexation of siRNA. Free siRNA migrates through the gel, whereas successfully complexed siRNAs fail to migrate from wells. (b,d,f) Particle size and zeta potential of PEGylated CD.siRNA nanoparticles measured by DLS and ELS, respectively. Data are represented as mean ± SD (n = 5).* ***Abbreviations****: CD; Cyclodextrin; DLS, Dynamic light scattering; ELS, Electrophoretic Light Scattering; PEG, Polyethyleneglycol; siRNA, Short interfering RNA.*

Surface modification of CD.siRNA nanoparticles with increasing amounts of PEGylated CDs resulted in modest increases in particle size in all cases (Figure 5.2b,d,f). PEGylation with PEG500 CD increased size of nanoparticles from 151.28 ± 3.80 nm at the lowest molar ratio between cationic CD : PEG500 CD to 199.46 ± 16.48 nm at the highest molar ratio. Similarly, PEG1000 CD led to an increase from 145.14 ± 2.92 nm to 188.30 ± 25.22 nm and PEG2000 CD increased particle size from 171.76 ± 3.04 to 224.98 ± 7.71 nm. The polydispersity index (PDI) of samples tended to increase with increasing amounts of PEGylated CDs, i.e. lowest cationic CD : PEG CD ratios (Table 5.1), indicating a higher degree of heterogeneity within the sample.

Table 5.1. PDI of non-PEGylated and PEGylated CD.siRNA nanoparticles in deionised water

Molar ratio Cationic CD : PEG CD	PEG500 CD		PEG1000 CD		PEG2000 CD	
	PDI		PDI		PDI	
	Mean	SD	Mean	SD	Mean	SD
1:0	0.346	0.048	0.275	0.011	0.316	0.035
10:1	0.378	0.038	0.292	0.030	0.392	0.048
4:1	0.265	0.008	0.347	0.035	0.413	0.021
2:1	0.369	0.035	0.400	0.028	0.361	0.036
1.5:1	0.320	0.042	0.429	0.024	0.336	0.038
1:1	0.337	0.045	0.518	0.049	0.366	0.068
0.5:1	0.415	0.023	0.621	0.078	0.383	0.007

Abbreviations: *PDI, Polydispersity index; PEG CD, PEGylated cyclodextrins; SD, Standard deviation.*

Furthermore, PEGylation reduced surface charge of pre-formed CD.siRNA nanoparticles. Subtle reductions were observed with PEG500 CD with surface charge of nanoparticles been reduced from +30.74 ± 2.19 mV to +23.34 ± 0.6 mV. On the other hand, PEG1000 CD and PEG2000 CD caused greater reductions in surface charge from +33.14 ± 1.72 to +17.74 ± 0.71 mV and +30.98 ± 5.77 to +18.54 ± 0.39 mV, respectively (Figure 5.2b,d,f).

5.4.2 Stability of non-PEGylated and PEGylated CD.siRNA nanoparticles in physiological salt conditions

CD.siRNA nanoparticles were exposed to optiMEM® and incubated at physiological temperatures (37 ºC) for different periods of time (Figure 5.3a-c). As expected, non-PEGylated cationic nanoparticles (molar ratio 1:0) showed the greatest tendency to aggregate even after only 4 hours (Figure 5.3a). Similarly, CD.siRNA nanoparticles with the lowest degree of PEGylation, namely the ratio 10:1 between cationic CD : PEGylated CD), tended to aggregate after 4 hours incubation. However, this effect was only evident in nanoparticles modified with lower Mw PEG CDs (500 and 1000). Lower molar ratios of cationic CD to PEGylated CD (2:1 – 0.5:1) tended to be more resistant to salt-induced aggregation at early time points (4 and 24 hours) (Figure 5.3a,b). Although in general, lower molar ratios of cationic CD : PEGylated CD showed improved stability in salt-containing medium, subtle increases in particle size were observed over time (SI, Supplementary Table S1-3). In addition, over a longer time period (48 hours), lower ratios of cationic CD to PEG500 CD also aggregated, an effect that was not observable with PEG1000 CD and PEG2000 CD (Figure 5.3c). Surface modification of CD.siRNA nanoparticles with PEG2000 CD provided the greatest resistance to salt-induced aggregation for all molar ratios tested and at all time points tested (Figure 5.3a-c). Overall, the ratio 1.5:1 of cationic CD : PEGylated CD presented favourable hydrodynamic diameters across all PEGylated CDs at early time points (Figure 5.3 and SI, Supplementary Table S1-3). Furthermore, PEGylated CD.siRNA nanoparticles in this particular molar ratio display low PDI values when prepared in DIW (Table 5.1) and in aggregation studies in optiMEM® (Supplementary Table S5.1 – S5.2). Thus, this particular ratio was selected for subsequent studies with PEGylated CDs containing PEG2000 in serum.

Stability of non-PEGylated CD.siRNA nanoparticles and PEGylated (PEG2000 CD) CD.siRNA nanoparticles was investigated in FBS (Supplementary Figure S5.2). After 4 hours a broadening of the main peak in particle size distribution for both non-PEGylated and

PEGylated formulations was observed, suggesting an increase in PDI. However, a clear shift of the main peak of particle size distribution was only observable for non-PEGylated nanoparticles at 24 hours, suggesting that protein-induced nanoparticles aggregation has occurred. No abrupt shifts in size distribution were observed for PEGylated nanoparticles, possibly suggesting that PEG2000 CD confer some degree of stability to CD.siRNA nanoparticles in serum-containing environment.

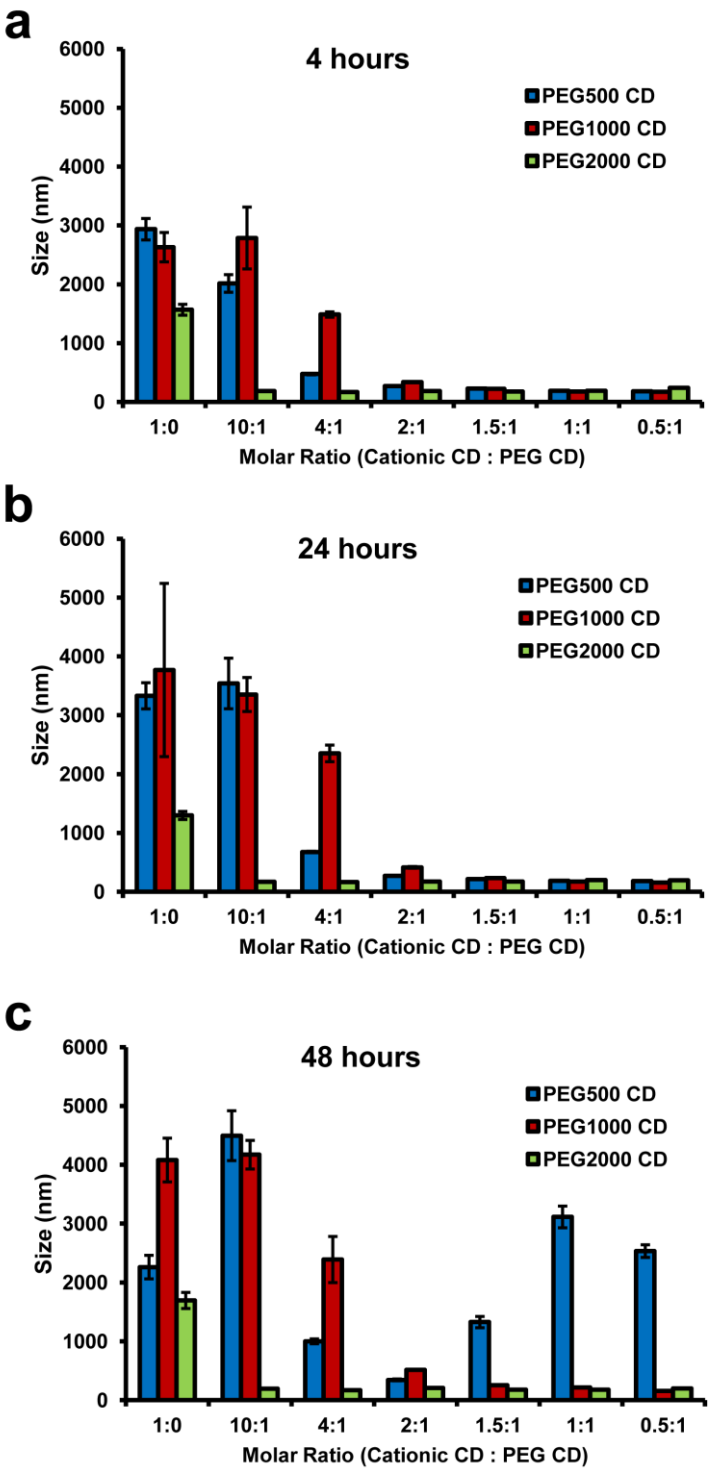


Figure 5.3. Stability of non-PEGylated and PEGylated CD.siRNA nanoparticles in physiological salt conditions. *Changes in particle size after incubation of nanoparticles in optiMEM® at 37 ºC for different periods of time. Assessed by DLS. Results are expressed as mean ± SD (n=5).* ***Abbreviations:*** *Cat. CD : PEG CD, Ratio of cationic cyclodextrin to PEGylated cyclodextrin; PEG, Polyethyleneglycol; SD, Standard deviation.*

5.4.3 Comparative pharmacokinetic study

The *in vivo* PK behaviour of naked FAMsiRNAs, non-PEGylated and PEGylated CD.siRNAs were compared after administration of a single intravenous injection (i.v.) bolus dose, corresponding to a total of 40 µg of nucleic acid. The optimal molar ratio for PEGylation of CD.siRNA nanoparticles was determined taking into account the physicochemical properties and the *in vitro* aggregation studies up to 24 hours. The ratio Cationic CD : PEGylated CD (ratio of 1.5 : 1) was kept constant for all PEGylated CDs to enable investigation of optimal PEG length for *in vivo* applications.

The plasma concentration profiles of naked and CD-formulated FAMsiRNAs over the time course of the study are represented in Figure 5.4. These plasma concentration profiles were best described by a biexponential 2-compartmental model with a first fast deposition phase and a slower elimination phase (Table 5.2 and Table 5.3). No significant differences were found between areas under the curve (AUC) estimated by the 2-compartmental model and AUCs derived by a model independent method (Trapezoidal rule). Additionally, ratios of model-dependent : model independent AUCs were between 0.92 – 1.11 further reinforcing the predictability of the model (Table 5.3). Altogether, these data validate the suitability of the model for calculation of PK parameters in this study.

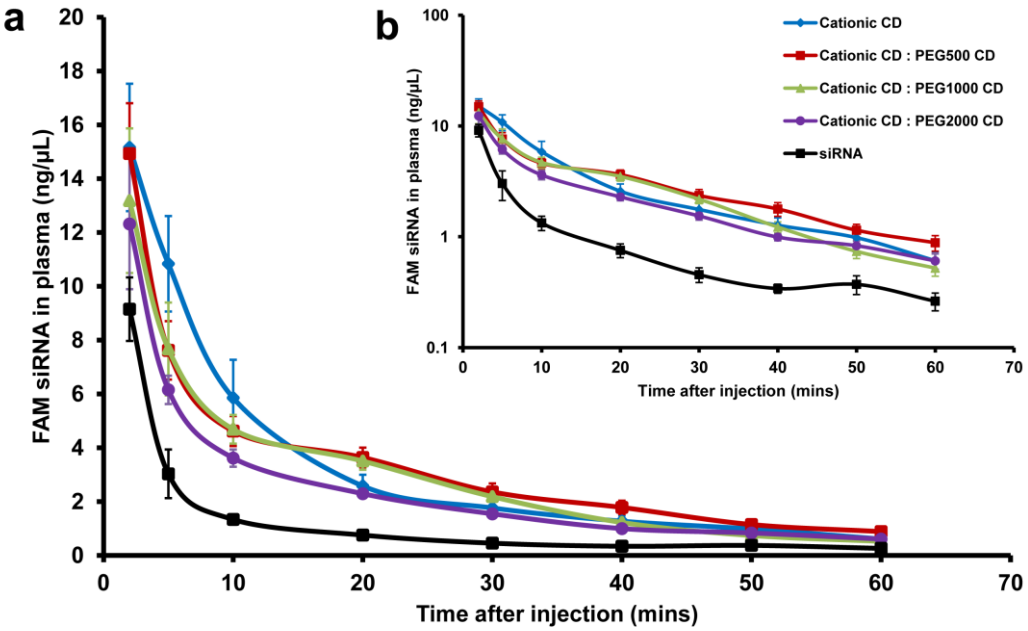


Figure 5.4. Plasma concentration profiles of naked and CD-formulated FAMsiRNA after single i.v. bolus dose. *Male Balb-c mice were injected through the tail vein with 40 µg of naked or CD-formulated FAMsiRNA. PEGylated formulations consisted of a molar ratio 1.5:1 of cationic CD to a specific PEGylated CD (PEG500 CD, PEG1000 CD and PEG2000 CD). (a) Plasma concentration of FAMsiRNA expressed in a continuous scale and (b) in a semi-logarithmic scale. Data are presented as mean ± SEM (n=3-8).* ***Abbreviations****: CD, Cyclodextrin; FAMsiRNA, Fluorescently labelled siRNA; PEG, Polyethyleneglycol; siRNA, Naked siRNA.*

Immediately after injection, plasma concentrations of naked FAMsiRNAs dramatically decreased whereas CD-formulated FAMsiRNAs displayed a more delayed profile. Indeed, the distribution half-life ($t_{1/2\alpha}$) of naked FAMsiRNA was short and fast (2.09 ± 0.17 min.) compared to CD-formulated FAMsiRNAs (2.50 – 4.24 min.), however, these differences did not reach statistical significance. Conversely, all treatments presented slower β elimination phases, but with the exception of CD.siRNA nanoparticles containing PEG1000 CD, no significant differences were found in the elimination half-lives ($t_{1/2\beta}$). Typically, $t_{1/2\beta}$ were 20 – 30 minutes.

Naked FAMsiRNAs and all CD formulations tested were rapidly cleared from the blood compartment with only residual levels detected by 60 minutes. However, CD formulations,

with the exception of CD.siRNA nanoparticles containing PEG2000 CD, showed significantly improved AUCs (211-253 ng/µL x min.) when compared to naked siRNAs (82 ± 14.98 ng/µL x min.). It is worth noting that although no statistical significance was reached for PEGylated CD.siRNA nanoparticles formulated with PEG2000 CDs, a positive trend was observed (168.55 ± 12.17 ng/µL x min.). Importantly, CD formulations also exerted profound effects on clearance (CL) rates (162 – 246 µL/min), showing reduced CL when compared to naked FAMsiRNAs (528.18 ± 114.93 µL/min). In summary, these data suggest that CD-formulations in general exhibit significant increases in circulating times when compared to naked FAMsiRNAs, however, no statistically significant differences were observed among non-PEGylated and PEGylated CD.siRNA nanoparticles.

Table 5.2 Comparison of PK parameters of non-formulated and CD-formulated FAMsiRNA upon single i.v. bolus dose

PK parameter	A (ng/µL)		α (min^{-1})		B (ng/µL)		β (min^{-1})		$t_{1/2\ \alpha}$ (min)		$t_{1/2\ \beta}$ (min)		AUC (ng/µL x min)		Vd (µL)		CL (µL/min)	
Formulation	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Cationic CD	20.82	2.80	0.2265#	0.0371	3.51#	0.99	0.0361	0.0070	3.51	0.50	22.15	2.73	225.06*	38.90	1840.52	265.03	199.35***	21.79
Cat. CD : PEG500 CD	24.43	6.99	0.3476	0.1063	5.39*	0.97	0.0322	0.0084	4.24	2.61	24.89	4.29	252.92*	22.69	1634.00	484.32	162.15***	14.91
Cat. CD : PEG1000 CD	20.65	5.17	3.1156	1.3208	6.80**	0.66	0.0464	0.0044	2.50	1.73	15.61**	1.47	210.58*	30.73	1816.81	377.18	210.48***	29.15
Cat. CD : PEG2000 CD	21.23	6.16	0.3775#	0.0567	3.68	0.58	0.0347	0.0017	2.95	1.22	20.30	1.05	168.55	12.17	2450.36	568.82	246.23***	18.06
siRNA	11.82	4.19	0.3373	0.0300	1.06	0.16	0.0241	0.0036	2.09	0.17	30.05	4.30	82.22	14.98	4310.44	1931.01	528.18	114.93

P<0.05 and **P<0.01 vs. siRNA and #P<0.05 vs. Cat. CD : PEG1000 CD by One-way ANOVA followed by Dunnett's posthoc test or Bonferroni's post hoc test, respectively.* ***Abbreviations:* *$Ae^{-\alpha t} + Be^{-\beta t}$, A, B, α, and β refer to the biexponential function C(t); AUC, Area under the curve; Cat. CD, Cationic Cyclodextrin; CL, Clearance; PEG CD, PEGylated cyclodextrin; SEM, Standard error of mean; siRNA, Naked siRNA. $t_{1/2}\alpha$, half-life in alpha phase; $t_{1/2}\beta$, half-life in elimination phase; Vd, Volume of distribution.*

Table 5.3 Comparison of model-dependent and model-independent area under the curve of non-formulated and CD-formulated FAMsiRNA upon single i.v. bolus dose

Formulation	Cationic CD		Cat. CD : PEG500 CD		Cat. CD : PEG1000 CD		Cat. CD : PEG2000 CD		siRNA	
AUC (ng/µL x min)	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
AUC 2-comp. Model	225.06	38.90	252.92	22.69	210.58	30.73	168.55	12.17	82.22	14.98
AUC (0-∞) Model Indep.	202.16	37.91	238.88	28.59	201.15	20.28	155.98	6.30	89.36	18.15
Ratio (2-comp/Mod.Indep.)	1.11	N/A	1.06	N/A	1.05	N/A	1.08	N/A	0.92	N/A

Abbreviations: *AUC 2-comp. Model, AUC derived from parameter estimates from 2-compartmental model; AUC (0-∞) Model Indep., Area under the curve derived through the trapezoidal method; Cat. CD, Cationic Cyclodextrin; CL, Clearance; PEG CD, PEGylated cyclodextrins; N/A, Not applicable; SEM, Standard error of mean; siRNA, Naked siRNA.*

5.5 Discussion

Optimising formulation strategies to obtain stable non-viral vectors for systemic administration of RNAi-based therapies with subsequent transfer to the brain is critical to the progression of this technology to the clinical setting. In this study, we investigated a simple and efficient post-PEGylation approach to surface-modify pre-formed CD.siRNA nanoparticles and increase their steric stability in physiological conditions.

Physicochemical characterisation of PEGylated CD.siRNA nanoparticles showed that post-modifications with higher molar content of PEGylated CDs tended to yield nanoparticles with slightly bigger sizes. This is in accordance with other methods of post-modification of liposome with PEGylated lipids (Mendonça et al., 2009), and possibly suggests that in our case PEGylated amphiphilic CDs are located at the surface of pre-formed CD.siRNA nanoparticles. On the other hand, increasing the molar content of PEGylated CDs within the formulation decreased the surface charge of the PEGylated CD.siRNA nanoparticles. This surface charge reduction is also in agreement with previous findings in our group where cationic and PEGylated CDs were blended together to form stable CD nanoparticles with reduced charge (O'Mahony et al., 2013d; O'Mahony et al., 2012d). This has been hypothesised to occur due to the charge shielding capacity of highly hydrated PEG chains which form a stealth corona at the surface of the nanoparticles and has also been observed in other polymeric vectors (Kunath et al., 2002). In support, it is also interesting to note that in the present study PEGylation using higher Mw PEG CDs (PEG1000 and PEG2000) yielded greater reductions in surface charge, probably due to better charge masking abilities of large PEG molecules.

Despite that PEGylated amphiphilic CDs do not have cationic groups in their structure and therefore *per se* have poor siRNA binding capabilities, they have been used in the present study to post-modify pre-formed CD.siRNA nanoparticles. Post-modification of pre-formed CD.siRNA nanoparticles with PEGylated CDs did not affect binding and complexation of

anionic siRNAs. In fact, this represents an advantage of the current method relative to other PEGylation methods, such as covalent grafting of PEG chains to the delivery system, which can interfere with complex formation by reducing the affinity to bind siRNA (Mao et al., 2006a). Covalent and non-covalent post-PEGylation methods have also been successfully applied to other DNA and siRNA delivery vectors, such as PEI (Ogris et al., 1999) and poly(2-(dimethylamino) ethyl methacrylate (Kong et al., 2009), to enhance stability.

In this study, highly PEGylated CD.siRNA nanoparticles were resistant to salt-induced aggregation *in vitro* for up to 24 hours, whereas non-PEGylated CD.siRNA nanoparticles readily aggregated after 4 hours (Figure 5.3). Similar results have been previously reported by our group using a different PEGylation method which consisted of co-formulation of cationic and low Mw CDs (PEG500 CD) (O'Mahony et al., 2013d; O'Mahony et al., 2012d). Here we expand on this previous work providing further comparative information with higher Mw PEG CDs to clarify the importance of PEG length and polymer density on the stability of the final assembly. Interestingly, CD.siRNA nanoparticles PEGylated with PEG2000 CDs required lower polymer densities at the nanoparticle's surface, than PEG500 and PEG1000 CDS, to produce nanoparticles resistant to salt-induced aggregation. In addition, these PEGylated CD.siRNA nanoparticles were stable up to 48 hours of incubation in physiological salt conditions, whereas, such long-lasting anti-aggregation effects were not apparent for PEG500 CDs. Therefore, at early time points PEG CD density at the nanoparticle's surface seems to be of greater importance to improve stability when using PEGylated CDs of lower Mw. On the other hand, high Mw PEGylated CDs consistently present longer lasting stabilities *in vitro* even when present at low densities, and were overall more efficient in conferring stability against salt-induced aggregation. Furthermore, surface-modification with PEG2000 CDs also conferred some degree of stability in the presence of serum. In contrast, a previous serum-stability study with PEG500 CDs have shown the lack of effectiveness of this low Mw PEGylated CD to protect against interactions with serum proteins (O'Mahony et al., 2013d). Thus, suggesting that high Mw PEG CDs will be more

likely to be able to stabilise and enhance the circulating times of CD.siRNA nanoparticles in the *in vivo* setting.

Finally, our PK study showed that FAMsiRNAs formulated in CD-based delivery systems display longer plasma residency times, as indicated by higher AUCs and lower CLs, than naked FAMsiRNAs. In fact, others have also reported very short circulating times for naked siRNAs upon systemic administration, which has been associated with its rapid renal CL (Li et al., 2007; Malek et al., 2009; O'Mahony et al., 2013a). Although, CD-formulations have improved circulating times up to what might be comparable to other delivery systems (Malek et al., 2009; Merdan et al., 2005), no significant differences were observed among the different non-PEGylated and PEGylated CD.siRNA nanoparticles. After i.v. injection the opsonisation of nanoparticles with plasma proteins might have enhanced their removal by the mononuclear phagocyte system. Indeed, instability of the nanoparticles leading to premature complex disassembly have been suggested as possible reasons for rapid CL of siRNA nanoparticles (Merdan et al., 2005). This is not an effect solely observed in CD nanosystems but has also been observed in other polymer-based delivery systems (Malek et al., 2009; Merkel et al., 2009). Although it seemed likely from our aggregation studies that CD.siRNA nanoparticles PEGylated with PEG2000 CDs would perform better *in vivo*, the reason underlying its poor performance might be again related to the PEG length used. Previous studies have revealed that nanoparticles PEGylated with PEG blocks of Mw below Mw 5000 are more likely to be opsonised and phagocytised by the mononuclear phagocyte system (Gref et al., 2000). On the other hand, it seems that PEG lengths above Mw 5000 are enough to avoid opsonisation in human plasma (Gref et al., 2000). Indeed, CD-containing polymers surface-modified by inclusion formation with an adamantane-PEG5000 derivative have shown low aggregation and improved circulating times (Davis et al., 2010; Pun & Davis, 2002). This further advocates that higher Mw PEG lengths may be required for modification of CD-based delivery systems to achieve appropriate stealth effects *in vivo*. In addition, although higher Mw PEGs have also been successfully used to improve circulating

times of liposomes and other active proteins, it has been reported that high Mw PEG may cause immunogenicity and therefore reduce effectiveness of a second dose (Cheng et al., 1999; Garay et al., 2011; Wang et al., 2007). Thus, PEGylating siRNA nanoparticles is a challenging balancing act between achieving suitable stability of the delivery system upon systemic administration and no immunogenic activation.

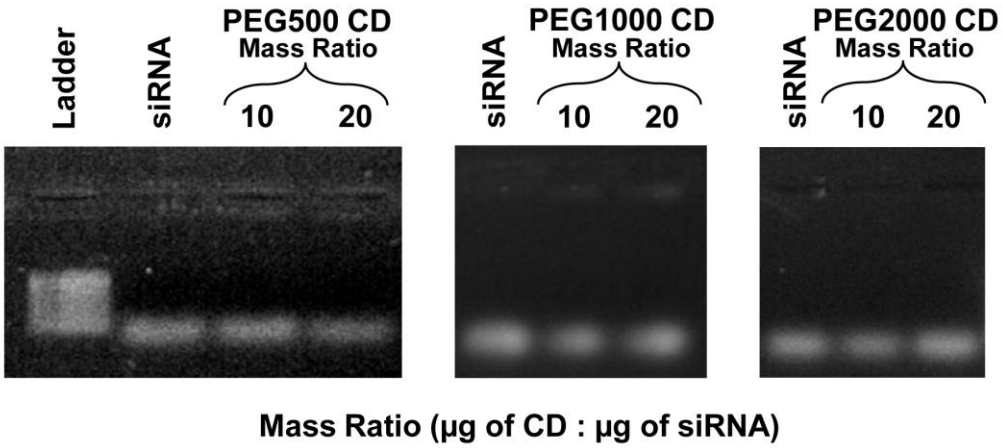
5.6 Conclusions

Although there has been a revitalised interest in neurosurgical approaches for treating complex brain disorders, systemic delivery of RNAi therapeutics is by far the preferred route of administration. Thus, developing suitable non-viral vectors for systemic administration with subsequent transfer to the brain is one of the current hurdles in the field of nanotechnology. Here we have shown that post-PEGylation is a successful approach to surface-modify pre-formed CD.siRNA nanoparticles. We have also explored the importance of surface PEG density and PEG length modifications on the physicochemical properties and stability of CD.siRNA nanoparticles. Although both PEG densities and PEG length appeared to have a profound impact on salt-induced aggregation, it seems that PEG length is the major determinant factor in the *in vivo* setting. Thus, when modifying CD-delivery systems with higher Mw PEGs should be preferred. Once a stable CD delivery system has been developed, coupling of specific targeting ligands, such as transferrin or the fragment of rabies virus glycoprotein, will facilitate tailoring of these formulations for potential CNS delivery across the BBB.

5.7 Supplementary Information

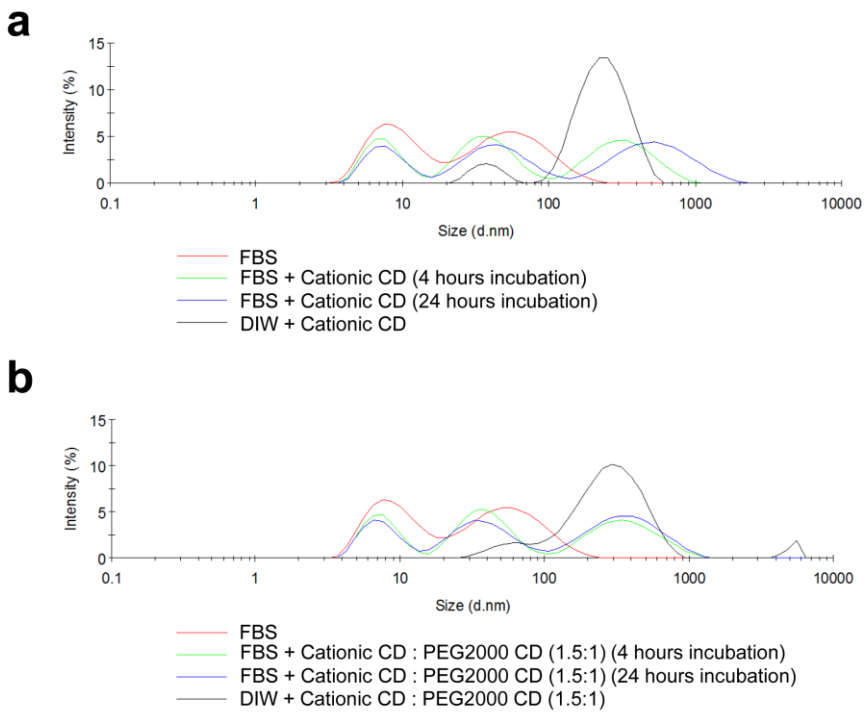
The SI in this chapter includes data regarding the limited ability of PEGylated amphiphilic CDs to bind and complex siRNA (Supplementary Figure S5.1); the stability of non-PEGylated and PEGylated CD.siRNA nanoparticles in FBS (Supplementary Figure S5.2); and further details on sizes and polydispersity indexes of non-PEGylated and PEGylated CD.siRNA nanoparticles after incubation in physiological salt conditions at 37 °C (Supplementary Table S5.1 – S5.3).

5.7.1 Binding and complexation of siRNA by PEGylated amphiphilic CDs



Supplementary Figure S5.1. Gel retardation assay for assessing binding and complexation of siRNA by PEGylated amphiphilic CDs . *PEGylated CDs were mixed with 0.3 µg of siRNA to achieve specific mass ratios (µg of CD : µg of siRNA) and allowed 20 minutes for complexation. Samples were mixed with loading buffer and run in a 2% agarose gel. Gel electrophoresis carried out for 20 minutes at 90 volts. Free siRNA migrates through the gel whereas complexed siRNAs fail to migrate from wells. PEGylated cyclodextrins fail to effectively complex siRNA at the mass ratios studied, and therefore a band is seen and stained by the nucleic acid stain.* ***Abbreviations****: CD, Cyclodextrin; PEG, Polyethyleneglycol; siRNA, Short interfering RNA.*

5.7.2 Stability of non-PEGylated and PEGylated CD.siRNA nanoparticles in serum



Supplementary Figure S5.2. Stability of non-PEGylated and PEGylated CD.siRNA nanoparticles in serum. (a) *Non-PEGylated and (b) PEGylated CD.siRNA nanoparticles were incubated in FBS at 37 ºC for 4 or 24 hours. Size distribution was assessed by DLS. FBS (red line) and nanoparticles prepared in DIW (black line) were included as controls (n=5).* ***Abbreviations****: CD, Cyclodextrin; DIW, Deionised water; DLS, Dynamic light scattering; FBS, Foetal bovine serum, PEG, Polyethyleneglycol; siRNA, Short interfering RNA.*

Supplementary Table S5.1. Sizes and polydispersity indexes of non-PEGylated and PEGylated CD.siRNA nanoparticles after 4 hours incubation in physiological salt conditions at 37 °C

4 hours	PEG500				PEG1000				PEG2000			
	Size		PDI		Size		PDI		Size		PDI	
Cat.CD : PEG CD	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1:0	2936.8	182.5	0.251	0.052	2631.0	249.6	0.282	0.179	1567.6	92.5	0.401	0.133
10:1	2013.8	149.7	0.274	0.038	2786.2	525.8	0.312	0.186	184.3	4.6	0.277	0.016
4:1	475.6	14.2	0.312	0.039	1486.6	46.0	0.273	0.034	168.9	1.8	0.258	0.012
2:1	271.2	2.0	0.245	0.019	338.7	5.9	0.258	0.013	186.5	3.3	0.347	0.035
1.5:1	227.6	4.8	0.266	0.012	222.3	2.6	0.198	0.011	179.3	7.4	0.282	0.014
1:1	192.7	2.7	0.255	0.030	176.2	1.0	0.221	0.015	192.6	5.6	0.357	0.013
0.5:1	182.9	2.9	0.332	0.022	172.2	6.4	0.419	0.020	242.8	6.5	0.464	0.091

Abbreviations: *Cat. CD : PEG CD, Ratio of cationic cyclodextrin to PEGylated cyclodextrin; PDI, Polydispersity index; SD, Standard deviation.*

Supplementary Table S5.2. Sizes and polydispersity indexes of non-PEGylated and PEGylated CD.siRNA nanoparticles after 24 hours incubation in physiological salt conditions at 37 °C

24 hours	PEG500				PEG1000				PEG2000			
	Size		PDI		Size		PDI		Size		PDI	
Cat.CD : PEG CD	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1:0	3329.6	222.2	0.327	0.156	3769.0	1471.6	0.612	0.328	1297.8	68.4	0.782	0.053
10:1	3541.0	429.9	0.410	0.154	3352.4	288.7	0.418	0.358	170.3	1.4	0.214	0.008
4:1	676.8	11.4	0.324	0.067	2351.6	140.7	0.373	0.144	163.7	3.7	0.257	0.013
2:1	271.6	4.4	0.239	0.020	415.2	14.6	0.277	0.010	173.1	2.1	0.283	0.026
1.5:1	214.5	3.7	0.220	0.013	233.4	1.1	0.202	0.013	173.3	3.9	0.266	0.015
1:1	188.5	2.6	0.247	0.010	175.5	1.1	0.216	0.015	199.5	7.1	0.372	0.009
0.5:1	183.2	3.1	0.356	0.029	158.8	3.7	0.401	0.021	194.9	4.1	0.346	0.029

Abbreviations: *Cat. CD : PEG CD, Ratio of cationic cyclodextrin to PEGylated cyclodextrin; PDI, Polydispersity index; SD, Standard deviation.*

Supplementary Table S5.3. Sizes and polydispersity indexes of non-PEGylated and PEGylated CD.siRNA nanoparticles after 48 hours incubation in physiological salt conditions at 37 °C

48 hours	PEG500				PEG1000				PEG2000			
	Size		PDI		Size		PDI		Size		PDI	
Cat.CD : PEG CD	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
1:0	2261.2	199.9	0.446	0.218	4081.2	372.0	0.499	0.361	1693.6	137.2	0.658	0.066
10:1	4495.0	423.3	0.491	0.355	4171.6	243.9	0.421	0.389	195.0	4.3	0.278	0.021
4:1	1000.4	40.8	0.300	0.062	2389.4	392.5	0.498	0.084	171.2	3.3	0.298	0.023
2:1	343.8	15.7	0.368	0.018	513.5	7.6	0.286	0.037	209.4	7.0	0.435	0.060
1.5:1	1327.2	95.7	0.850	0.056	255.4	4.0	0.223	0.021	176.5	3.7	0.281	0.016
1:1	3113.4	185.1	1.000	0.000	214.4	8.0	0.315	0.018	179.8	2.1	0.289	0.028
0.5:1	2533.0	108.3	1.000	0.000	157.1	6.0	0.380	0.025	198.8	3.1	0.374	0.015

Abbreviations: *Cat. CD : PEG CD, Ratio of cationic cyclodextrin to PEGylated cyclodextrin; PDI, Polydispersity index; SD, Standard deviation.*

Chapter VI

General discussion

6.1 Overview & Summary

Silencing disease-related genes using RNAi therapeutics holds great promise as a strategy for the treatment of disorders of the central nervous system (CNS), such as neurodegenerative diseases. Indeed, autosomal dominant diseases with well-identified genetic targets, such as Huntington's Disease (HD), are probably the most well-suited candidate-diseases for this potential therapeutic approach. However, achieving efficient and safe delivery of short interfering RNAs (siRNAs) to the brain, and specifically to neurons, poses great challenges to the progression of such a strategy to the clinic. Thus, efforts have emerged in the field of nanotechnology to develop more efficient and safe delivery systems for siRNAs into the CNS.

In this thesis we investigated the suitability of modified cyclodextrins (CD) as non-viral vectors for siRNA delivery to the CNS, focusing on HD as a disease-model. To this end, an animal model for pre-clinical testing of CD.siRNA nanoparticles was established (Chapter II). The R6/2 mouse model was successfully validated and enabled us to optimise behavioural tasks for the purpose of our pre-clinical studies. Subsequently, we demonstrated that modified cationic amphiphilic CDs were able to bind and complex siRNAs forming nanoparticles which were stable in artificial cerebrospinal fluid (Chapter III). Moreover, *in vitro* evaluations showed that CD.siRNA nanoparticles could transfect a rat striatal cell line (ST14A-HTT120Q) and human HD fibroblasts with minimal toxicity. Additionally, CD.siRNAs nanoparticles efficiently reduced the expression of the Huntingtin (HTT) gene in both *in vitro* models and also after stereotaxic injection into the R6/2 mouse brain. Repeated brain injections of CD.siRNA nanoparticles into the R6/2 mouse brain resulted in a selective alleviation of rotarod deficits (Chapter III). Thus, these data indentify CDs as effective non-viral vectors for siRNA delivery to the CNS *in vivo* and highlights their potential as a therapeutic approach for the treatment of HD.

To expand on previously obtained preliminary cytotoxicity data (Chapter III), we further investigated the nanotoxicological and neuroinflammatory liabilities of CD.siRNA nanoparticles, and siRNA nanoparticles formulated with other commercially available non-viral vectors, in multiple brain-derived cell lines. Overall these investigations demonstrated that CD.siRNA nanoparticles did not cause marked disruptions of the cellular or nuclear membrane, and did not disrupt normal mitochondrial metabolic activity to a great extent. Furthermore, we observed that different brain-derived cells presented different susceptibilities to toxic stimuli caused by commercially available non-viral vectors, with rat striatal cell line (ST14A-HTT120Q cells) and the mouse microglia cell line (BV2 cells) being generally more sensitive than the human astroglioma cell line (U87 cells). In addition to the low cytotoxicity, CD.siRNA nanoparticles did not elicit marked inflammatory responses *in vitro*, when transfecting BV2 microglia cells and U87 cells, and *in vivo* upon direct injections into the brain of a naive mouse. In contrast, significant increases in neuroinflammatory markers were observed with siRNA nanoparticles formulated with commercial non-viral vectors, in particular with dendrimer-based delivery systems (Chapter IV). Overall, these data identify CDs as safe and non-toxic non-viral vectors for delivering siRNAs through intraparenchymal injections to the brain, further supporting its suitability for neurodegenerative diseases, such as HD.

On the other hand, in order to circumvent the need for brain surgery, cationic CD.siRNA nanoparticles need to be further modified to achieve greater stability in physiological conditions and allow systemic administration. In the final studies of this thesis we focused on stabilisation of CD.siRNA nanoparticles by PEGylation. PEGylated formulations showed improved resistance to salt-induced aggregation and, at least to some extent, to protein-induced aggregation. These effects were found to be dependent on both PEG density and length. Furthermore, all CD-formulations displayed enhanced circulating times *in vivo* when compared to naked siRNAs, however, we were unable to differentiate between PEGylated and non-PEGylated CD.siRNA nanoparticles. Thus, here we have described a successful

post-modification approach to PEGylate pre-formed CD.siRNA nanoparticles and identified the need for longer PEG lengths to increase nanoparticle's stability *in vivo* in subsequent studies.

In summary, in this thesis we have outlined two possible CD-based formulation strategies for HD, one for intracerebral administration and another for systemic delivery. Figure 6.1 depicts the main stages of drug discovery and indicates where the studies in this thesis contribute to the effort of developing CD-based siRNA formulations for HD. However, it is important to highlight that prior reaching the clinical trial stage, selected formulations should undergo extensive preclinical toxicological and biodistribution testing in animals, such as human primates. Furthermore, when developing products for human application their production should follow the regulatory and good manufacturing practices guidelines to ensure that standards are met. Upon approval, these products should be tested in well-designed Phase I, II and III clinical trials.

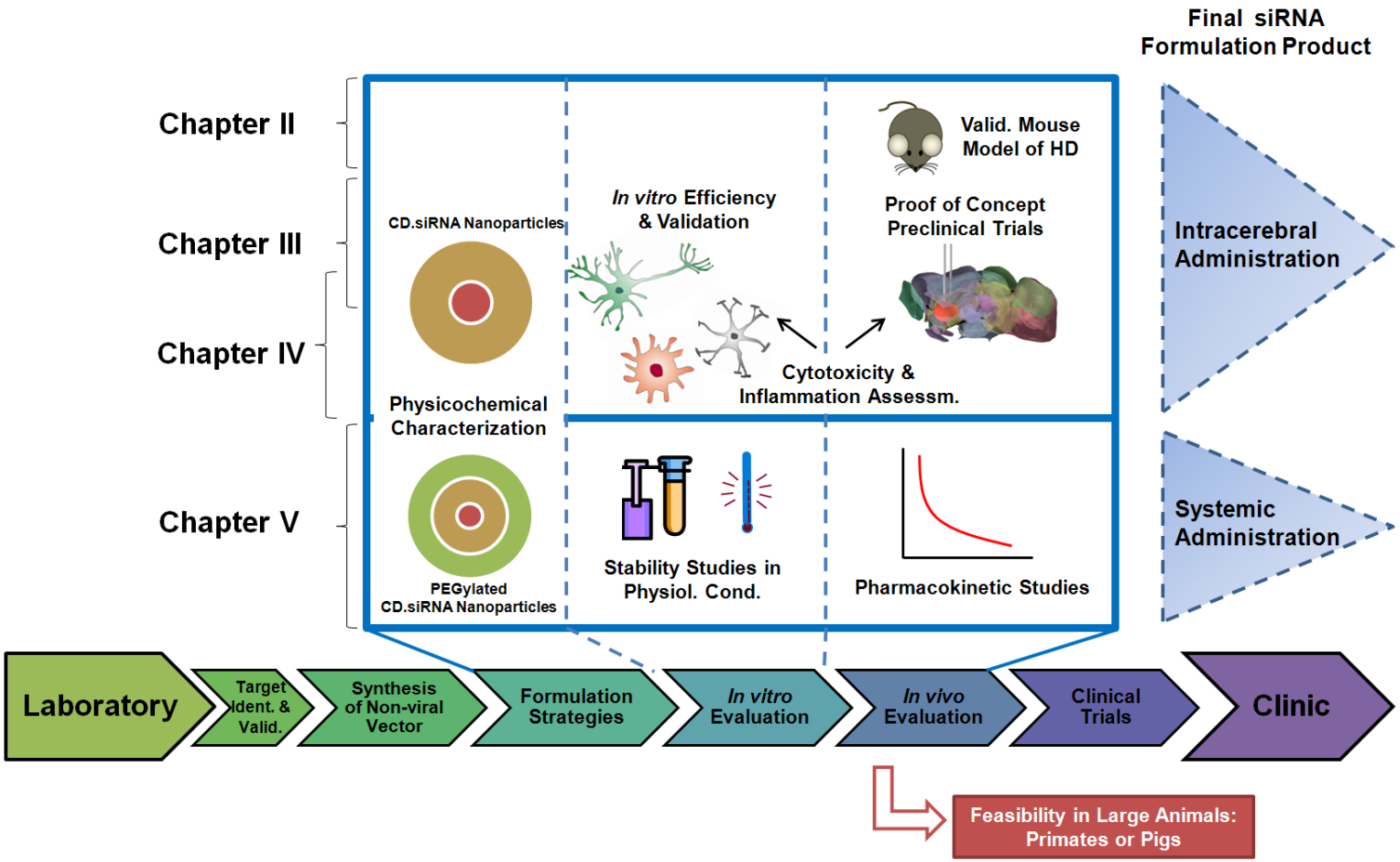


Figure 6.1. Drug discovery timeline for novel gene therapy approaches for HD*. This diagram depicts the main stages of drug discovery and indicates where the studies in this thesis contribute to the effort of developing CD-based siRNA formulations for HD. Upon identification of a specific genetic target, siRNAs must be designed and validated using appropriate algorithms. Synthesis and formulation of modified CDs is carried out according to the specific requirements of the treatment and selected route of administration. Gene silencing efficiency, cytotoxicity and eventual inflammatory responses are investigated in relevant in vitro models. Proof of concept preclinical trials are carried out in a validated animal model of the disease to investigate therapeutic efficacy, along with the first in vivo toxicological studies. Further assessments of efficacy and extensive animal toxicological testing are also conducted in large animals prior clinical trials. Phase I, II, and III clinical trials conducted when permission is granted.* ***Abbreviations****: CD, Cyclodextrin; HD, Huntington's Disease; siRNA, Short interfering RNA.*

6.2 Towards an "ideal" siRNA delivery system for CNS applications: How do CD-formulations stand-up?

Delivery to the CNS, and in particular to the brain, is a major challenge faced by delivery systems for RNAi therapeutics. Indeed, producing an "ideal" delivery system for CNS applications is a fine balancing act between efficiency and toxicity. Thus, these two main aspects need to be considered when developing non-viral vectors for siRNA delivery to the brain and are further discussed below.

6.2.1 Improving delivery efficiency

When designing non-viral vectors for brain delivery, one should be aware that several extracellular and intracellular biological barriers exist and must be overcome in order to achieve the required silencing effect at the specific target site. Based on the knowledge about these different barriers (see Section 1.10) and according to the selected route of administration, it is possible to foresee the "ideal" characteristics required by the non-viral vector to effectively deliver to the brain. In this regard, Kostarelos and Miller (Kostarelos et al., 2005) have suggested a practical and meaningful paradigm for optimisation of non-viral vectors based on the self-assembly "ABCD" nanoparticles concept depicted in Figure 6.2.

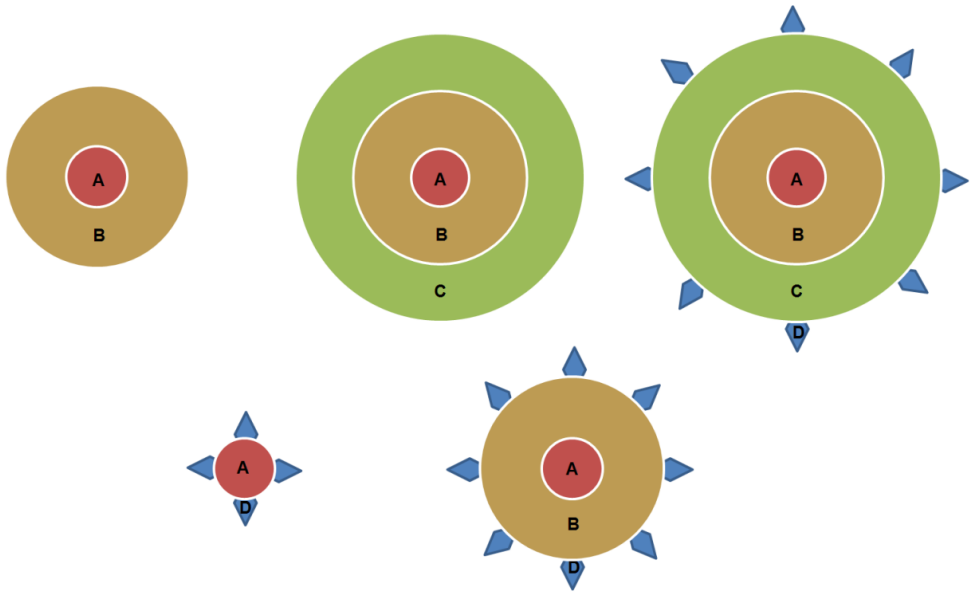


Figure 6.2. "ABCD" structural paradigm for non-viral vectors. *(A) Nucleic acid cargo (e.g. siRNA, pDNA). (B) Condensing or encapsulating layer. (C) Stealth layer to confer stability. (D) Biological recognition layer.* ***Abbreviations****: pDNA, Plasmid DNA; siRNA, Short interfering RNA.*

Based on this structural paradigm, if non-viral vectors are to be administered through localised intraparenchymal administrations to a specific target site within the brain, as in Chapter III, simpler "AB" formulations may suffice. However, in order to achieve successful gene silencing effects, this "AB" delivery systems should still: protect siRNA from enzymatic degradation; be able to transfect relevant target cell-types within the CNS; and, escape endosomal degradation releasing the siRNAs to the cytoplasm (Guo et al., 2010a; O'Mahony et al., 2013b). In addition, it is worth noting that neurons are notoriously difficult to transfect, most likely due to their post-mitotic nature, and therefore pose great challenges to non-viral delivery systems (Krichevsky & Kosik, 2002). Here we have shown that modified cationic amphiphilic CDs (SC12 CD Click Propylamine) (Chapter III) fulfil these criteria by condensing siRNAs into positively charged nanoparticles, which in turn are capable of interacting with cellular membranes and facilitating cellular uptake. In addition, CD.siRNA nanoparicles have effectively reduced the expression of the mutant HTT (muHTT), both *in vitro* and in the R6/2 mouse brain, also suggesting successful escape from

endosomal degradation. Therefore, this cationic CD-formulation holds great promise for administrations to be carried out directly in the brain. Moreover, it is worth noting that the innovative "click" chemistry methodology used to synthesise these non-viral vectors is a very promising approach. This is especially true in the context of the high yields garnared which is poised to be very advantageous over other cationic CDs when industrial scale-up is required.

Alternatively, and given that the systemic route is preferred, cationic non-viral vectors may need to be further stabilised by forming "ABC"-type nanoparticles. This is commonly achieved by addition of a stealth layer usually consisting of PEG to improve stability under physiological conditions (reducing salt- and serum-induced aggregation). In this thesis, we have described a modification strategy towards improving the *in vivo* stability of CD.siRNA nanoparticles. Surface post-modification through incorporation of other complementary amphiphilic PEGylated CDs conferred enhanced stability against salt-induced aggregation, and in part against serum protein-induced aggregation. Although, in our case these PEGylated CD.siRNA nanoparticles failed to demonstrate any improved blood residency, others have achieved this by using larger PEG molecules (Mw 5000) (Davis, 2009; Davis et al., 2010). Also, stable "ABC"-type CD.siRNA nanoparticles have been previously formed by: direct grafting of PEG chains to the CD scaffold; by co-formulation with PEGylated CDs; or even by using the hydrophobic CD cavity to form inclusion complexes with PEGylated "guest molecules" such as adamantane-PEG derivatives. Thus, CDs as non-viral vectors have the advantage of being versatile pharmaceutical molecules which allow several functional modifications to be carried out in order to generate stable and efficient delivery systems (Sallas & Darcy, 2008).

Finally, once stable siRNA nanoparticles have been formulated, targeting across the BBB is likely to be required to access the brain. To this end, several targeting ligands have been investigated to enhance transport across the BBB or to increase delivery to neurons (Table 6.1). Among such peptides, the 29 amino acid fragment derived from the rabies virus

glycoprotein (RVG) has lately received much attention (Hwang et al., 2011; Kumar et al., 2007; Liu et al., 2009). This targeting ligand interacts with acetylcholine (Ach) receptors expressed in the BBB and neuronal cells, leading to translocation across the BBB by receptor-mediated endocytosis. Transferrin (Cardoso et al., 2010; Cardoso et al., 2007), lactoferrin (Huang et al., 2010) and angiopep (Ren et al., 2012) have also been widely investigated for CNS delivery, however, the receptors for this proteins/peptides are widely expressed in other tissues and may not confer specificity for the CNS. In this thesis the "AB" and "ABC" delivery systems investigated lack neuronal targeting. However, it is worth noting specific targeting has been successfully achieved in our lab, for example, using anisamide-targeted PEGylated CD delivery systems for prostate cancer (Guo et al., 2012b). Thus, this demonstrates that further improvements of current CD-formulations for specific targeting to neurons, and eventually to specific sub-populations of neurons, may be feasible in the future.

Table 6.1. In vivo studies using targeting ligands to improve neuronal and brain delivery of drugs and nucleic acids

Target ligand	Receptor	Basic nanosystem	Other modifications	Target/disease	Cargo	System model (*in vivo*)	Outcome Across BBB Enhanced neuronal	Route of Admin.	Ref.
TF	TFR	Liposomes	N/A	Luciferase	siRNA	NF-kB-luciferase reporter mice	40% reduction in luciferase expression in the striatum	Single intrastriatal injection	(Cardoso et al., 2008)
TF	TFR	PAMAM dendrimer	PEG3400	pGL2/pEGFP gene delivery	pDNA	Balb/c mice	Several regions of the brain compared to untargeted formulations	i.v.	(Huang et al., 2007b)
LTF	LTFR	PAMAM dendrimer	PEG3400	pGL2/pEGFP gene delivery	pDNA	Nude mice and Balb/c	Enhanced gene expression in the mid brain	i.v.	(Huang et al., 2010)
RVG	AchR	R9	N/A	GFP, SOD1 and FvE^{J}	siRNA	GFP transgenic, Balb-c, JEV-infected mice	~50% reduction in GFP and SOD1. Improved survival in JEV-infected mice	i.v	(Kumar et al., 2007)
RVG	AchR	Liposomes	Imidazole containing lipid	PrP^{C}	siRNA	FVB mice	25% decrease in PrP^{C} in the brain	i.v.	(Pulford et al., 2010)
RVG	AchR	PEI	PEG5000	Delivery of neurogenic miR-124a	miRNA	Balb/c mice	Accumulation of Cy5.5-miR124.a in the brain	i.v.	(Hwang et al., 2011)
RVG	AchR	PAMAM	PEG3400	pGL2/pEGFP gene delivery	pDNA	Balb/c mice	Increased uptake in the brain. High gene expression	i.v.	(Liu et al., 2009)
ANG	LRP	O-MWNTS	PEG2000	Brain glioma	Doxorub.	Intracranial glioma bearing Balb/c	Enhanced delivery of DOX to the brain and anti-glioma effects.	i.v.	(Ren et al., 2012)
ANG	LRP	PAMAM dendrimer	PEG3400	GFP gene delivery	pDNA	Nude mouse	Increased uptake in the brain, GFP expression in several regions	i.v.	(Ke et al., 2009)
NT	NTS1	PLL	HA2 fusigenic peptide and Vp1 SV40 karyophilic peptide	hGDNF gene expression	pDNA	6-OHDA hemiparkinsonian rats	Increased hGDNG expression in the striatum and substantia nigra. Biochemical and functional recovery.	Injection into the substantia nigra	(Gonzalez-Barrios et al., 2006)
Tet1	GT1b	PEI	PEG5000	Luciferase/β galactosidase plasmid	pDNA	C57/BL6 mice	Expression of Luciferase sustained for 10 days. Targets mainly NPC	Left lateral cerebral ventriculum	(Kwon et al., 2010)

Abbreviations: *AchR, Acetylcholine Receptor; ANG, Angiopep-2; BCEC, Brain Capillary Endothelial Cells; Doxorub., Doxorubicin; GFP, Green fluorescent protein; GT1b, triasialoganglioside receptor; hGDNF, human glial cell line-derived neurotrophic factor; JEV, japanese encephalitis virus; LTF, lactoferrin; LTFR, lactoferrin receptor; LRP, low-density lipoprotein receptor-related protein; miRNA, micro RNA; NT, Neuortensin; NPC, neuron progenitor cells; NTS1, neurotensin receptor; O-MWNTs, oxidized multiwalled carbon nanotubes; PAMAM, polyamidoamine; PEG, Poly(ethylene glycol); PrP^{C}, prion protein cellular; R9, nona-arginine; RVG, rabies virus glycoprotein; SOD1, superoxide dismutase 1.*

6.2.2 Reducing nanoparticle-mediated toxicity

Cell death and/or exacerbated inflammatory responses to siRNA nanoparticles in the CNS may mask the benefits of RNAi-based therapies. This is of particular importance in the context of HD, where an underlying inflammatory condition exists (Möller, 2010), and diseased neurons have been found to be particularly sensitive to toxic stimuli (Rigamonti et al., 2000). Furthermore, unspecific interactions of siRNA nanoparticles with other cell types within the brain, such as microglia and astroglia, may lead to direct toxic effects in these cells with subsequent reduction in brain homeostasis (Figure 6.3 bottom box). As a result, neurons can be indirectly affected due to a reduction in the biochemical support provided and/or due to the inflammatory response triggered. Thus, investigating the cytotoxicity caused by the final assembled nanosystem in multiple brain cell types is crucial to provide an integrated and more complete evaluation of these effects.

In addition to the therapeutic efficacy, gene silencing in the CNS requires the use of safe and non-toxic delivery vectors. In this thesis, commercially available delivery systems, such as Lipofectamine2000® (lipid-based vector) and INTERFERin® (proprietary cationic non-liposomal amphiphile), were very effective transfecting a rat striatal cell line (ST14A-HTT120Q) and silencing the expression of muHTT gene. However, these vectors also displayed high cytotoxicity at concentrations and time points commonly used to silence genes in *in vitro* neuronal cell cultures (O'Mahony et al., 2012b). Indeed, polycation-based nanoparticles are well-known to cause biological adverse effects, such as membrane lysis and disruption of mitochondrial activity (Figure 6.3 top box) (Ballarín-González & Howard, 2012; He et al., 2010). However, although CD.siRNA nanoparticles are also cationic in nature, in this study they exhibited low cytotoxicity and comparable gene silencing effects to that seen with commercial vectors. Thus, we hypothesise that other characteristics associated with the physicochemical properties of the nanoparticle, such as the size and morphology, but also the biodegradability of the nanocarrier may play a pivotal role in toxicity. However, mechanistic studies are still warranted to further elucidate the different pathways involved

and the importance of each of the above factors in cytotoxicity. Interestingly, our *in vitro* studies also showed that vectors that commonly induced high cytotoxicity in BV2 microglial cells, also induced high gene expression of pro-inflammatory cytokines (Chapter IV). Thus, the immune activation observed in our studies might be closely related to the cytotoxicity of the vector and may involve the activation of Toll-like receptors (TLR) (Figure 6.3 top box). It is thus possible that a vector-induced activation of TLRs may occur after the release of danger associated molecular patterns (DAMPS), or even by direct activation of these receptors. In fact, it has been recently suggested that cationic siRNA nanoparticles may directly activate TLRs at the cellular membrane when injected intravenously (Kedmi et al., 2010). On the other hand, it has also been argued that siRNA itself, rather than the biomaterial may be the culprit of the immune response. However, in general these effects have been suggested to be vector-dependent (Bonnet et al., 2008; Kawakami et al., 2006; Kedmi et al., 2010), and closely related to the sequestration of siRNA within a TLR rich environment in endosomes (Ballarín-González & Howard, 2012). In the particular case of modified CDs, low activation of the immune response was observed, thus suggesting that these vectors are able to avoid TLR activation at the cellular membrane and also escape the TLR-rich environment in the endosomes.

Several approaches have been investigated to reduce nanoparticle-mediated toxicity. As an example, introduction of cleavable ester linkers in synthetic polyamidoamine (PAMAM) dendrimers have been shown to improve its biodegradability and consequently its toxicity profile (Kim et al., 2010a). Alternative strategies to reduce cytotoxicity have consisted of surface modifications with PEG in order to reduce charge-related toxic effects (Beyerle et al., 2010; Hibbitts et al., 2011; Wang et al., 2010b). Further functionalisation of the nanoparticle with targeting ligands (Table 6.1) may enhance cellular uptake by specific cell types, thereby preventing toxicity in non-target cells and tissues when nanoparticles are applied *in vivo*. This strategy might be of particular utility to avoid uptake by microglial and astroglial cells and circumvent, at least in part, the activation of the inflammatory response.

Furthermore, the addition of fusogenic lipids and/or peptides to the formulation has been shown to improve endosomal escape (Farhood et al., 1995; Hatakeyama et al., 2009; Litzinger & Huang, 1992; Oliveira et al., 2007), and therefore might constitute valid and feasible strategies to minimise immune activation and enhance gene silencing effects.

Although our studies revealed favourable toxicity and neuroinflammatory profiles for modified CDs *in vitro* and after single injections into the striatum, further extensive toxicological evaluations are still required in different brain regions and using escalating doses. Also, although no apparent toxic effects were seen after multiple dosing into the R6/2 mouse brain, an evaluation of the nanotoxicological effects of CD-formulations after chronic administration is warranted. In addition, PEGylated CD.siRNA nanoparticles used in this thesis were not assessed for toxicological and inflammatory responses after i.v. administration. However, previous work in our lab has demonstrated no significant increases in liver enzymes, alanine transaminases (ALAT) or aspartate transaminases (AST), following i.v. injections with anisamide-targeted cationic PEGylated CDs (Guo et al., 2012b). In addition, encouraging results have also been obtained in recent clinical trials where CD-containing formulations have been used with no apparent or significant immune responses (Davis, 2009; Davis et al., 2010). Thus, taken together, this further advocates that CD formulations may also be used through this route of administration with success.

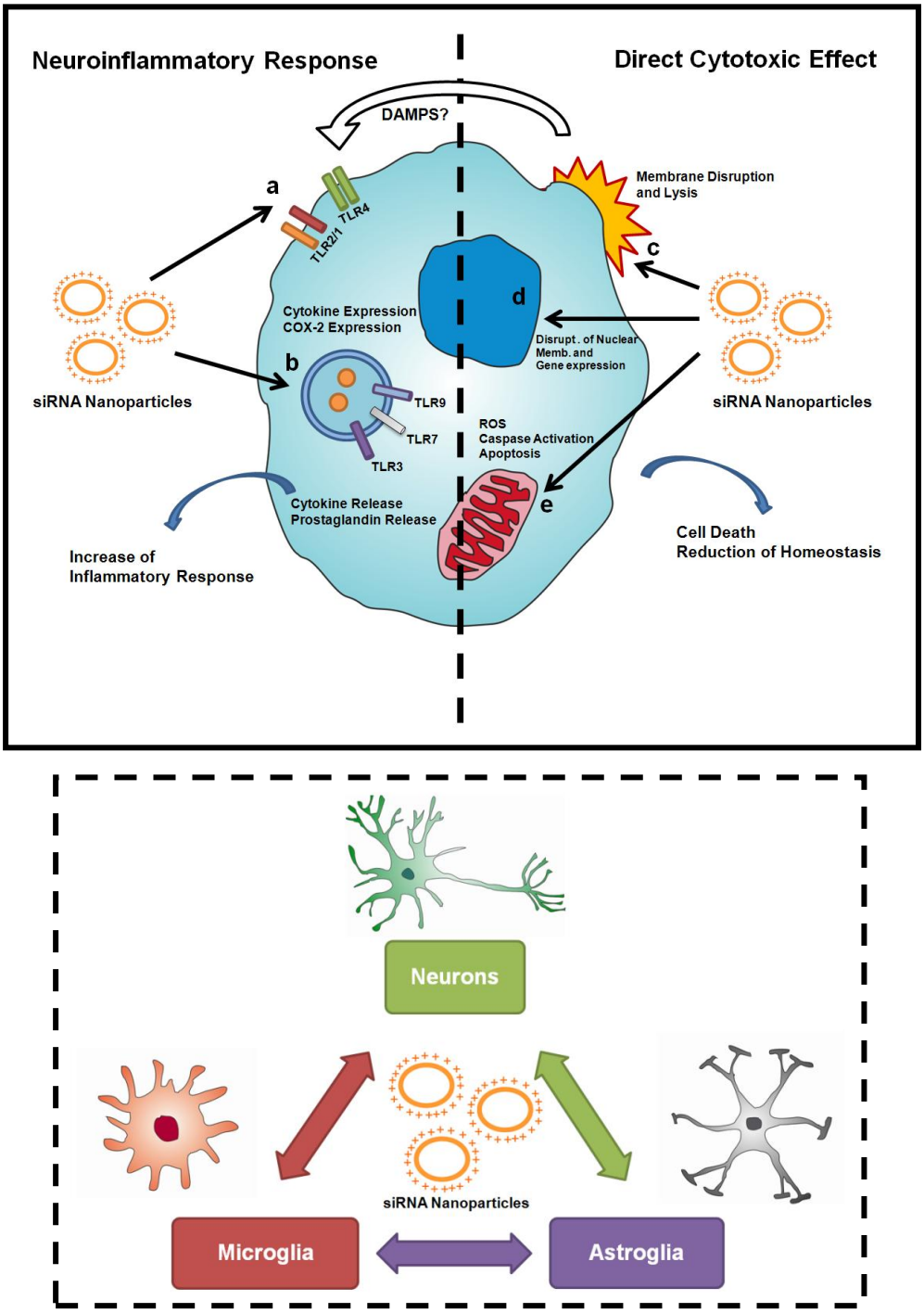


Figure 6.3. Nanotoxicological and neuroinflammatory liabilities of cationic siRNA nanoparticles. *Cationic siRNA nanoparticles may unspecifically interact with different cell types in the CNS milieu eventually causing cytotoxicity or triggering inflammatory response. Top box: (a) Cationic siRNA nanoparticles may directly activate TLRs at the surface of the cell membrane. (b) Poor endosomal release capabilities may also result on the activation of TLRs by siRNAs. Cationic siRNA nanoparticles may (c) disrupt the cellular membrane causing lysis and release of Damage-associated molecular pattern molecules (DAMPS). DAMPS can subsequently activate TLRs. (d) Permeation of the nuclear membrane and disruption of gene expression. (e) Interference with mitochondria may cause disruptions in the metabolic activity, pore hole formation and release of mitochondrial enzymes. Bottom box: Cytotoxicity over one cell-type might reduce homeostasis in the brain.* ***Abbreviations:*** *CNS, Central Nervous System; DAMPS, Damage-associated molecular pattern molecules; TLR, Toll-like receptor; siRNA, Short interfering RNA.*

6.3 Silencing the mutant Huntingtin: is allele-specificity merely an alternative or a compulsory prerequisite?

In dominantly inherited neurodegenerative diseases, such as HD, most of the affected individuals are heterozygous, carrying one copy of the normal allele and another mutated form of the allele (Squitieri et al., 2003). Although mutant proteins are causative of disease, wild-type proteins may have essential or unknown functions. Therefore, non-specific silencing of the wild-type alleles could have detrimental effects, especially if chronic administration is need, and allele-specific targeting of mutant genes might be an alternative to circumvent this issue (Rodriguez-Lebron & Paulson, 2005b). Indeed, this has been successfully achieved by exploiting the nucleotide differences between mutated and wild-type genes and the specificity of the RNAi mechanism. Rational design of siRNA (or shorth hairpin RNAs (shRNAs)) targeting the site of the mutations has enabled allele-specific silencing of the mutant forms of superoxide dismutase (SOD)-1 *in vitro* and *in vivo* (Xia et al., 2006), and of tau and amyloid precursor protein (APP) *in vitro* (Miller et al., 2003). However, targeting of the mutant CAG expansion using siRNAs in polyglutamine (polyQ) disorders, such as HD and spinocerebellar ataxia (SCA), leads to an unintended suppression of the normal allele and also of other genes normally containing CAG repeats (Hu et al., 2009; Miller et al., 2003). In this case, allele-specificity might only be achievable by targeting disease-linked polymorphisms.

Approximately 60 single nucleotide polymorphisms (SNP) have been identified in the coding and in the 3' untranslated regions (UTR) of the human HTT gene (Bilsen et al., 2008). From those, a selection of 25 SNPs and a GAG deletion (in exon 58) were recently found to have enough heterozygosity among a cohort of HD Caucasian Europeans, with 86% of patients being heterozygous for at least one of these polymorphisms (Lombardi et al., 2009). Initial studies showed specific silencing of the muHTT allele in artificial HeLa cell systems containing plasmids with the nucleotide sequence of the SNP incorporated (Schwarz et al., 2006). Further studies revealed that allele-specific silencing is also possible

by targeting HTT polymorphisms in human HD fibroblasts, naturally harbouring the muHTT (Lombardi et al., 2009; Zhang et al., 2009). Additionally, rationally designed siRNAs against a subset of 3-7 SNPs have achieved allele-specific knockdown of the muHTT *in vitro* and may be suitable to treat at least three quarters of the US and European HD populations (Pfister et al., 2009; Zhang et al., 2009). It is also important to note that allele-specific silencing has also been achieved using ASO technology in several *in vitro* and *in vivo* studies, further supporting the feasibility of this approach (Carroll et al., 2011). However, this approach would require genotyping of all SNP sites of interest, selection of the SNP to be targeted and of the allele-specific siRNAs, which may lead to increased costs.

Alternatively, non-allele specific targeting has been recently suggested as a valid approach that would circumvent the economic cost of individualized therapy. Complete (or almost complete) ablation of the mouse homologue *Hdh* gene has led to complications in embryogenesis and progressive degeneration in the adult brain (Dragatsis et al., 2000; Duyao et al., 1995a). However, recent *in vivo* preclinical studies have shown that partial reduction of the wild-type protein might be tolerable (Boudreau et al., 2009b; Drouet et al., 2009; McBride et al., 2011). In these studies, partial silencing of the endogenous HTT homologue did not exacerbate HD pathology or cause detrimental effects in neuronal survival, and has been found to be well tolerated for several months (Boudreau *et al.*; Drouet *et al.*). Furthermore, studies carried out in nonhuman primates have also shown that reduction of endogenous HTT homologue by ~45% does not induce neuronal degeneration, astrogliosis or even motor deficits (Grondin et al., 2012; McBride et al., 2011; Stiles et al., 2012). Thus, the residual levels of wildtype protein may be sufficient to maintain cellular needs. However, silencing of endogenous HTT lead to transcriptomic changes in other genes related to the functions of HTT and therefore a clear assessment of the impact of these is needed before progressing to the clinic (Boudreau et al., 2009b; Drouet et al., 2009). Finally, despite the favourable outcomes, most of these *in vivo* studies were conducted up to 6-9

months and therefore the effect of long term gene expression knockdown of wild-type Huntingtin (wtHTT) still needs further investigations.

A novel combinatory therapy has been reported as a useful strategy to target diseases where mutations have a high level of heterogeneity, such as dominant retinitis pigmentosa (Millington-Ward et al., 2011). The method consists of utilising RNAi for non-allele specific gene suppression of the mutated gene and gene therapy for supplementing a RNAi-resistant wild-type. This approach has been successfully used *in vivo* for α-1 antitrypsin (AAT) deficiency, preventing liver pathology and increasing blood levels of AAT, and for dominant retinitis pigmentosa , improving retinal structures and function (Li et al., 2011a; Millington-Ward et al., 2011). The applicability to neurodegenerative diseases has also been explored with initial studies for SOD1 and SCA showing effective knockdown and replacement; however no functional effects were reported (Kubodera et al., 2011; Kubodera et al., 2005). The suppression and replacement approach could also be possibly used for HD, however this has not yet been investigated.

6.4 Conclusions & Future Perspectives

The work carried out in this thesis identified a CD-based delivery system capable of efficiently delivering siRNAs to the brain, inducing significant gene silencing effects with low levels of toxicity and immunological activation. Here we also demonstrated that the application of such nanosystem for delivering RNAi therapeutics in the context of HD is feasible through direct injections into the brain. Additionally, and taking into account that the systemic route is preferred, we have described a method for post-modification of CD.siRNA nanoparticles in order to increase stability in physiological conditions, and identified PEG length as a major determinant for stability. However, certain limitations still remain including lack of improved half-lives *in vivo* and lack of specific neuronal targeting.

In order to overcome the limitations above mentioned, at first, future studies should concentrate on further stabilisation of CD.siRNA nanoparticles with higher molecular

weight (Mw) PEG. To this end, PEGs of Mw > 2,000 should be selected and incorporated to CD-formulations using the post-modification strategy described in this thesis, or other strategies previously described by our group. Alternatively, other PEGylation approaches that have been found successful elsewhere might also be employed (Davis, 2009), for example, by using the hydrophobic cavity of the CD to form inclusion complexes with adamantane-PEG derivatives. Once the desired stability *in vivo* has been achieved, transport of CD.siRNA nanoparticles across the BBB must be guaranteed. To achieve this, transient disruptions of the BBB and/or the use of specific targeting ligands are two possible strategies. The later most likely will be preferred since it may also facilitate specific neuronal uptake.

Moreover, in this thesis, HD has been used as a disease-model for investigating the applicability of CD-based formulations for siRNA delivery to the CNS. Thus, the applicability of these nanosystems for the treatment of other CNS disorders, including other neuropsychiatric disorders and brain cancers, still warrants further investigations and all of which requires clinical validation.

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Appendices

Appendix A:

Optimisation of cell culture conditions for Methyl thiazolyl tetrazolium colorimetric assays

A.1. Background information

Methyl thiazolyl tetrazolium (MTT) colorimetric assays assess mitochondrial dehydrogenase activity and therefore are a good measure of cellular metabolism (Kepp et al., 2011). Indeed, MTT assays are commonly used to measure cell proliferation, but also as a mean to evaluate *in vitro* cytotoxicity of specific treatments. However, MTT assays have been found to be sensitive to several assay conditions, including cell densities, culture medium, and exposure times (Sylvester, 2011). Thus, in order to obtain reliable results in cytotoxicity experiments based on MTT assays, here we have investigated the optimal cell density to be used for each specific cell line.

(Data in this appendix were obtained prior carrying out experiments outlined in Chapter III and IV)

A.2. Materials and Methods

A.2.1. Cell culture

ST14A-HTT120Q cells derived from rat striatal primordia and cloned with the human Huntingtin (HTT) gene were obtained from Coriell Institute for Medical Research (Camden, NJ). Primary human fibroblasts harvested from a patient with Huntington's Disease (HD) (HD fibroblasts, GM04691) were obtained from Coriell Institute for Medical Research (Camden, NJ). BV2 cells derived from primary mouse microglia cells were obtained from Banca Biologica e Cell Factory – IST (Italy, Genova). U87 astroglioma cells were a kind gift from Dr. Paul Young (University College Cork). ST14A-HTT120Q cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, Germany) up to passage 25. HD fibroblasts were grown in DMEM + 20% FBS + 5% vitamins (GIBCO, United Kingdom) + 5% aminoacids (GIBCO, United Kingdom) up to passage 15. BV2 cells were maintained in Roswell Park Memorial Institute medium 1640 (RPMI) (GIBCO, United Kingdom) medium supplemented

with 10% FBS and 2 mM L-glutamine (GIBCO, United Kingdom) up to passage 25. U87 cells were grown in DMEM supplemented with 10 % FBS and 2 mM L-glutamine (GIBCO, United Kingdom). All cultures were kept in a humidified incubator with 5 % CO2 and at 33 °C (ST14A-HTT120Q) or 37 °C (HD fibroblasts, BV2 and U87).

A.2.2. MTT assay

Cells were seeded at different cell densities in 96-well plates and after 48 hours MTT assays were carried out. Briefly, medium was removed from wells and replaced with 100 µL of fresh medium. 20 µL of a 5 mg/mL solution of MTT reagent was added to each well and incubated at 37 °C for 4 hours. The formazan crystal product was dissolved in 100 µL of dimethyl sulfoxide. Absorbance was measured at 590 nm using a using a SpectraMax Plus384 UV plate reader.

A.3. Results and Discussion

Our results demonstrate that different cell types reduce the MTT reagent to the respective formazan product differently (Figure A.1). Indeed, we observed that BV2 and U87 cells reduced the MTT reagent to a greater extent (resulting in higher absorbances), than ST14-HTT120Q and HD fibroblast cells. This may be due to differences in plasma membrane permeability to the MTT dye from cell to cell, or possibly due to higher mitochondrial metabolic activity rates in BV2 and U87 cells.

For the purpose of our cytotoxicity studies (Chapter III and IV), a cell density within the growth phase (before absorbance reaches plateau) was selected for each of the cell lines independently. Table A.1 summarises the cell densities used for each of the cell lines in subsequent cytotoxicity experiments using MTT assays.

Table A.1. Cell densities for MTT assays in 96-well plates.

Cell line	Cell density (Cells/Well)
ST14A-HTT120Q striatal cells	7.5×10^3
HD Fibroblast	7.5×10^3
BV2 microglial cells	1.0×10^4
U87 astroglioma cells	1.0×10^4

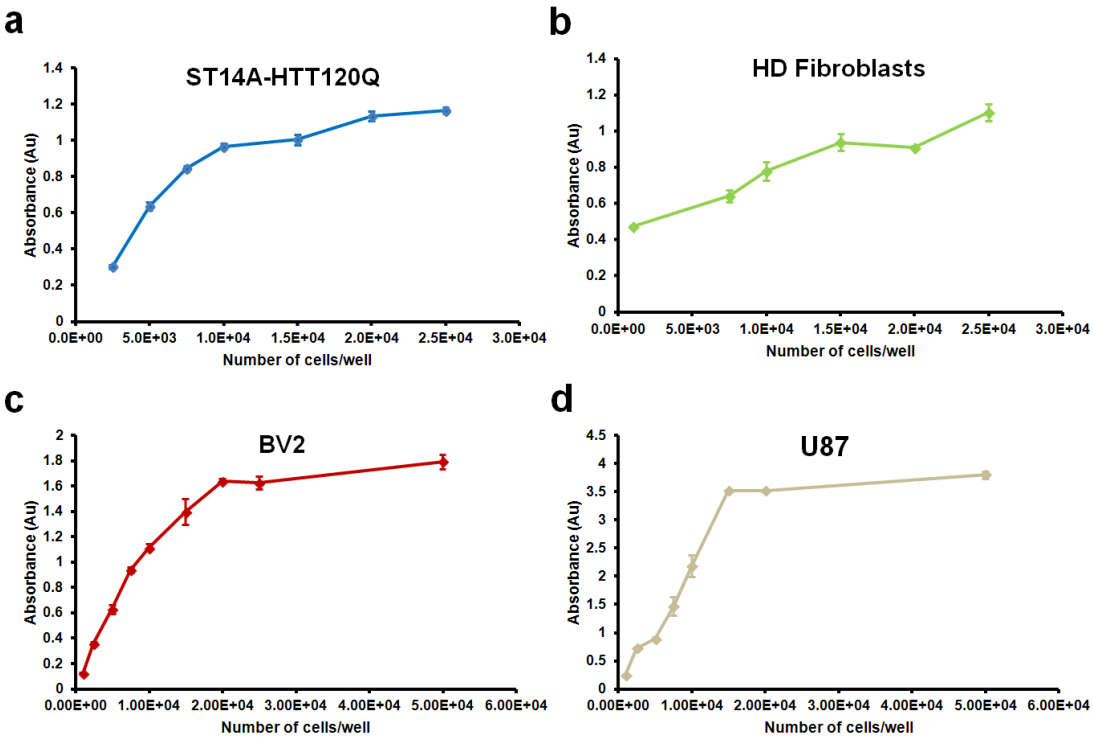

Figure A.1. Optimisation of cell culture conditions for MTT assays. *(a) ST14A-HTT120Q striatal cells, (b) human HD fibroblast, (c) BV2 microglia cells, and (d) U87 human astroglioma cells were seeded at different cell densities in 96-well plates and MTT assay carried out after 48 hours. (n = 3).*

Appendix B:

Investigation of ultrafiltration as a method for concentration of CD.siRNA nanoparticles: Effects on the physicochemical proprieties and gene silencing efficiency

B.1. Background information

Delivering short interfering RNAs (siRNAs) to the central nervous system (CNS) and in particular to the brain poses significant challenges to delivery systems. Neurons are notoriously difficult to transfect (Krichevsky & Kosik, 2002), and the existence of specialised biological barriers, such as the blood brain barrier (BBB), limits diffusion of siRNA nanoparticles to the brain (O'Mahony et al., 2013b). Indeed, in order to circumvent the hurdles of systemic delivery, most successful preclinical studies for brain delivery of siRNA have consisted of stereotaxic injections into specific structures (Bonoiu et al., 2011; Cardoso et al., 2010; Cardoso et al., 2008) and/or infusion into the intracerebroventriculum (Thakker et al., 2004). However, when carrying out local administrations to the brain brain one is constricted to small volumes, and therefore additional processing steps in the formulation of nanoparticles are necessary to meet this requirement. To this end several methods have been employed, including ultrafiltration.

Ultrafiltration is a gentle method that uses pressure for separation and concentration of high molecular weight solutes by retention, whereas, low molecular weight solutes and solvent pass through a semi-permeable membrane (Pingoud et al., 2002). Indeed, this method has been successfully applied to concentrate cationic lipoplexes and polyplexes carrying DNA (Howard et al., 2004; Nchinda et al., 2003), and recently applied to siRNA nanoparticles formulated with modified cyclodextrins (CDs) (O'Mahony et al., 2013a).

Thus, here we investigated the effects of ultrafiltration on the physicochemical proprieties and gene silencing efficiency of CD.siRNA nanoparticles. To this end, complexes were prepared according to standard protocols previously described by our group, and concentrated up to 20x from the initial concentrations. Physicochemical properties, including binding and complexation of siRNA, and size and charge were assessed. Subsequently, gene silencing efficiency of concentrated and non-concentrated CD.siRNA

nanoparticles was assessed in ST14A-HTT120Q cells. targeting the mutant Huntingtin (muHTT) mRNA.

(Data in this appendix were obtained prior carrying out experiments outlined in Chapter III and IV)

B.2. Materials and Methods

B.2.1. Synthetic siRNAs

Synthetic duplexed siRNAs were obtained from Sigma-Aldrich (France) or QIAGEN (United Kingdom). HTT target siRNAs (HTTsiRNA) as per Wang et al. (Wang et al., 2005) sense strand, 5'-GCCUUCGAGUCCCUCAAGUCC-3'; antisense strand, 5'-ACUUGAGGGACUCGAAGGCCU-3'. Non-silencing siRNAs (NSsiRNA): sense strand, 5'-UUCUCCGAACGUGUCACGUdTdT-3'; antisense strand, 5'-ACGUGACACGUUCGGAGAAdTdT-3'.

B.2.2. Preparation and concentration of CD.siRNA nanoparticles

Modified cationic CDs were dissolved in chloroform to give a 1 mg/mL solution. Chloroform was evaporated, under a stream of gaseous nitrogen, leaving a CD film which was rehydrated using a solution of 5% glucose to achieve a solution containing 1 mg/mL of CD. CDs were vortexed for 5 minutes and sonicated for 60 minutes. CDs were mixed with siRNAs at a mass ratio 10 (µg CD : µg siRNA), using equal volumes of both solutions, and incubated at room temperature (RT) for 20 minutes. CD.siRNAs nanoparticles were concentrated by ultrafiltration using Vivaspin 500 centrifugal units (Sartorius, Germany) with a molecular weight cut off of 3,000 Da. Concentrator tubes were centrifuged at 1,500 g at RT to a final concentration of 1 µg/µl of siRNA.

B.2.3. Physicochemical characterisation

Binding and complexation of siRNA was confirmed by gel retardation assay. Concentrated and non-concentrated CD.siRNA nanoparticles containing ~1 µg of siRNA were mixed with 2 µL of Blue Juice Loading Buffer (Invitrogen, Carlsbad, CA) and sufficient DIW to a final volume of ~30 µL. Samples were loaded in a 2% agarose gel and electrophoresis performed at 90 mV for 20 minutes in Tris-acetate-Ethylenediaminetetraacetic acid (TAE) buffer (Fisher Scientific, Fair Lawn, NJ). The gel was post-stained using GelRedTM nucleic acid stain (Biotium, Hayward, CA) and visualised using the DNR Bioimaging Systems and Gel Capture version 7.0.9 software.

Size and surface charge measurements were carried out using Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS), respectively. CD.siRNA nanoparticles, containing ~ 3 µg of siRNA, were diluted up to 1 mL with filtered sterilised DIW and assessed by DLS and ELS using a Malvern Zetasizer Nano ZS. A total of five readings for size and charge were taken per sample and the refractive index (1.33) and viscosity (0.8872 mPa·s) of water were taken into to account in data analysis.

B.2.4. Cell culture and RNAi transfection

ST14A-HTT120Q cells were obtained from Coriell Institute for Medical Research (Camden, NJ) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, Germany) up to passage 25.

For RNAi transfection experiments cells were seeded in 12-well plates at a density of 1.7-2.0 x 105 cells/well. Nanoparticles were prepared as described above and diluted in optiMEM. The volume of transfection sample accounted for 20% of the total volume of the well, the remaining 80% consisted of complete growth media. Transfection was carried out for 24 hours and the final concentration of siRNA in all RNAi-treated groups was of 100 nM.

B.2.5. Gene expression

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: 10 minutes at 95 °C, 40 cycles of [15 seconds 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). Primer sequences (forward: CGACCCTGGAAAAGCTGATGAA, reverse: CTGCTGCTGCTGGAAGGA) were validated for detection of human HTT mRNA (Ref. Seq. NM_002111) and used to design a TaqMan® HTT FAM-labelled probe. Each sample was analysed 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.

B.2.6. Statistical analysis

Statistical analysis was performed by One-way Analysis of Variance (ANOVA) followed by Dunnett's Post Hoc test. Unpaired t-student test was carried out for comparisons between concentrated and non-concentrated CD.siRNA nanoparticles.

B.3. Results and Discussion

Direct local administrations of siRNAs into the mouse brain require small volumes. To this end, we investigated the suitability of ultrafiltration as a method for concentration of CD.siRNA nanoparticles. CD.siRNA nanoparticles were prepared in optimal volume as previously described (O'Mahony et al., 2012b),and thereafter concentrated by ultrafiltration. Our results showed that concentration of CD.siRNA nanoparticles by ultrafiltration up to 20-fold from the initial solution did not affect binding and complexation of siRNA (Figure

B.1a). Furthermore, after incubation of concentrated CD.siRNA nanoparticles with SDS, confirming that siRNA was still present within the CD-formulation after the concentration process (Figure B.1b). On the other hand, CD.siRNA nanoparticles concentrated up to 20-fold from the initial solution and with a final concentration of siRNA of 1 mg/mL did not present marked increases in particle size nor major changes in zeta potential.

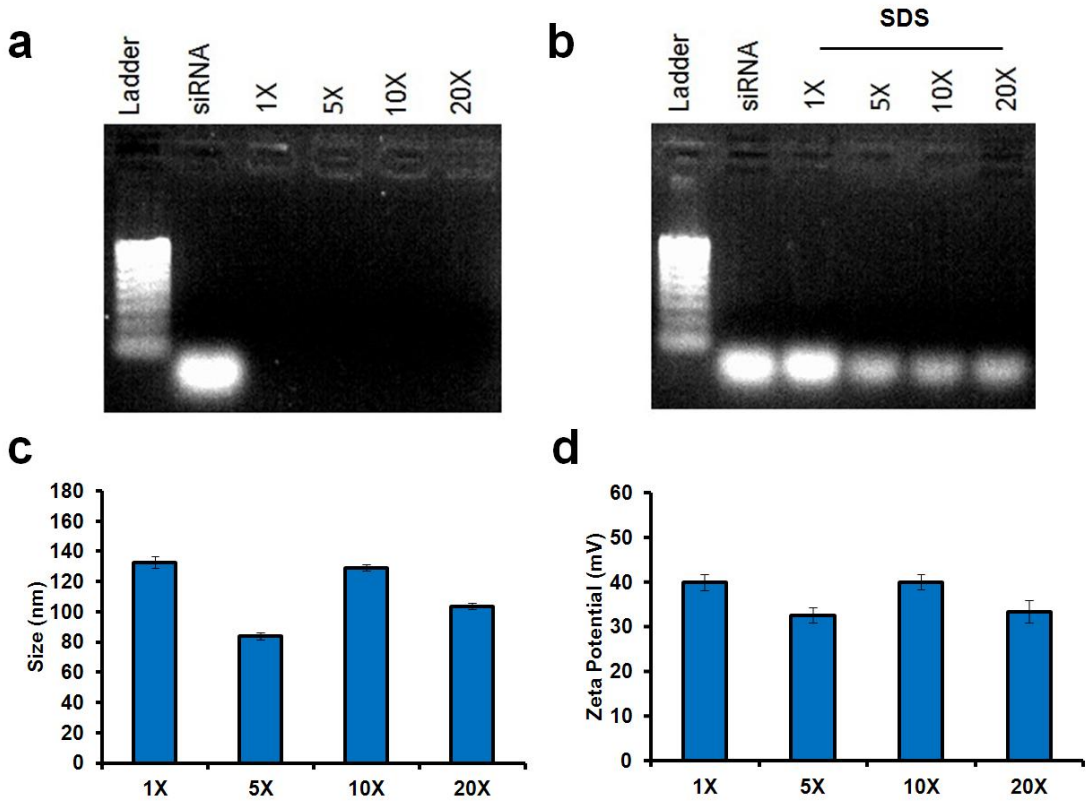

Figure B.1. Physicochemical characterisation of concentrated and non-concentrated CD.siRNA nanoparticles. *CD.siRNA nanoparticles were concentrated from 1x up to 20x by ultrafiltration. Samples for characterisation were taken at different stages of the concentration process. (a,b) Gel retardation assay where free siRNA migrates from the well. (a) Concentrated CD.siRNA nanoparticles were not disrupted by the concentration process. (b) Release of siRNA from the complexes after incubation of samples with sodium dodecyl sulfate confirms that siRNA is still present within the nanoparticle after the concentration process. (c) Hydrodynamic diameter measured by Dynamic Light Scattering. (d) Zeta potential measured by Electrophoretic Light Scattering. Data represented as Mean ± SD (n = 5).*

Subsequently, we investigated if CD.siRNA nanoparticles concentrated 20-fold from the starting solution would maintain comparable gene silencing efficiencies to the non-concentrated nanoparticles. Results showed that concentrated CD.siRNA nanoparticles were capable of delivering siRNAs and efficiently silence the expression of the muHTT gene in ST14-HTT120Q cells. No statistical significant differences were found when compared to non-concentrated CD.siRNA nanoparticles or Lipofectamine2000® (commercially available lipid-based formulation).

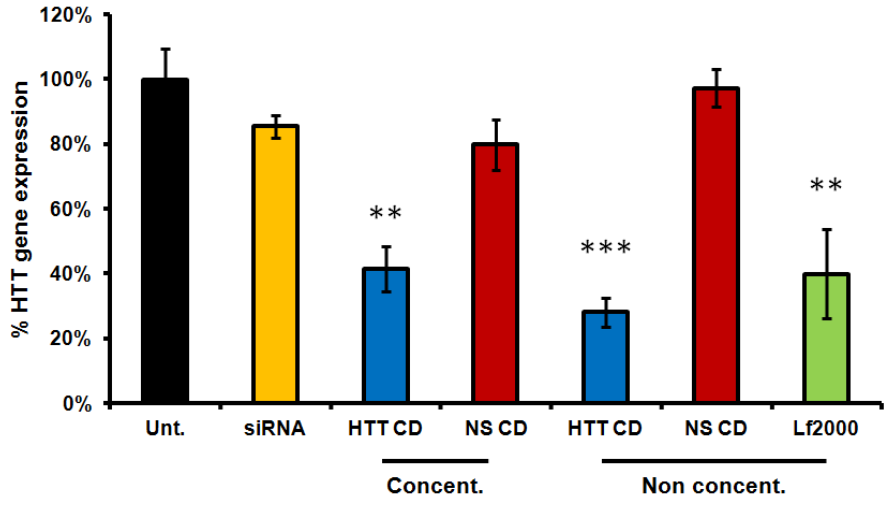

Figure B.2. Gene silencing efficiencies of concentrated and non-concentrated CD.siRNA nanoparticles in ST14A-HTT120Q cells. *ST14A-HTT120Q cells were transfected for 24 hours. RNA was extracted and reverse transcribed to cDNA. HTT gene expression assessed by RT-qPCR. Final concentration of siRNA = 100 nM. Data represented as Mean ± SEM (n = 2-4). **P<0.01 and ***P<0.001.* ***Abbreviations:*** *HTT CD, CD.HTTsiRNA nanoparticles; NS CD, CD.NSsiRNA nanoparticles; Lf2000, Lipofectamine2000®; Unt., Untreated; siRNA, naked siRNA.*

Ultrafiltration has been previously used to concentrate polyplexes and lipoplexes containing pDNA for *in vivo* applications (Howard et al., 2004; Nchinda et al., 2003). In these studies, concentration by ultrafiltration did not disturb to a great extent the physical properties of the nanoparticles and in some cases enhanced delivery of the nuclei acid *in vivo* (Howard et al., 2004; Nchinda et al., 2003). Taken together with our *in vitro* results, these data suggests that ultrafiltration may be a suitable method for concentration of CD.siRNA nanoparticles for *in vitro* and *in vivo* applications.