Chaitanya R. Joshi, Astrocyte TIMP-1: Regulation and Gene Delivery in HAND Doctor of Philosophy (Biomedical Sciences), August 2018, 193 pp., 9 tables, 24 illustrations, bibliography, 427 titles.

Abstract

Despite antiretroviral therapy, HIV-associated neurocognitive disorders (HAND) persist in 30-70% of patients. During HAND, elevated matrix metalloproteinases (MMPs) in the brain exacerbate the disease by blood-brain barrier breakdown, neuroinflammation and direct neurotoxicity. Tissue inhibitors of metalloproteinases (TIMPs) counter MMP activity. In the brain, TIMP-1 is primarily produced by astrocytes in response to injury or inflammation. However, TIMP-1 is downregulated during chronic inflammation in astrocytes and in HIV encephalitis brain tissues. We propose that restoring astrocyte TIMP-1 levels could mitigate neurodegeneration due to its MMP-inhibitory and -independent neuroprotective functions.

HIV-1 non-productively infects astrocytes, which express viral proteins such as transactivator of transcription (Tat). As Tat mimics aspects of HAND by direct and indirect mechanisms, glial fibrillary acidic protein (GFAP) promoter-restricted Tat expressing (GT-Tg) mice were used to model HAND in our studies. Prolonged astrocyte Tat expression in GT-Tg mice resulted in HAND-relevant behavioral impairments characterized by higher anxiety, lower ambulation, impaired spatial learning, and memory. Importantly, behavioral deficits were accompanied by altered brain MMP/TIMP balance. Our data from GT-Tg mouse model confirmed neurocognitive decline and TIMP-1 dysregulation in the context of HAND.

As TIMP-1 was downregulated with prolonged Tat expression in mice, we focused on replenishing TIMP-1 *via* gene delivery to the brain using cationic polymers. Polyethylenimine (PEI) is a highly efficient polymer for transfecting mammalian cells, however, high cytotoxicity restricts

its use. Hence, PEI was modified using arginine (A) and stabilized with polyethylene glycol (P) to produce multiple A_nP_n analogues. A_nP_n analogues were biocompatible and successfully delivered reporter genes to primary neural cells. Select A_nP_n led to sustained reporter gene expression in human astrocytes and in mouse brains. In order to restrict gene expression to astrocytes, truncated GFAP promoters were used to drive gene expression. Subsequently, GFAP promoters were modified enhancing their activity and increasing gene expression. Lastly, successful polymer-mediated GFAP promoter-driven TIMP-1 gene delivery was demonstrated in human astrocytes.

Overall, these findings enhance our understanding HIV-1 Tat-mediated TIMP-1 regulation, provide a novel therapeutic TIMP-1 gene delivery system, and pave the way for future investigations geared towards preclinical translation of TIMP-1-based HAND therapy.

ASTROCYTE TIMP-1: REGULATION AND GENE DELIVERY IN HAND

DISSERTATION

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CONFLICT OF INTEREST:

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
Aβ	amyloid-β
AADC	amino acid decarboxylase
AAV	adeno-associated virus
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANI	asymptomatic neurocognitive impairment
ANOVA	analysis of variance
ApoE	apolipoprotein E
AQP	aquaporin
ART	antiretroviral therapy
AV	adenoviral vector
BBB	blood-brain barrier
Bcl-w	B-cell lymphoma-w
BDNF	brain-derived neurotrophic factor
BMVEC	brain microvascular endothelial cell
Cas9	CRISPR-associated system 9
C/EBP	CCAAT-enhancer-binding protein
CMV	cytomegalovirus

CNS	central nervous system
CNTF	ciliary neurotrophic factor
CPF	chondroitin polymerizing factor
CRISPR	Clustered regulatory interspaced short palindromic repeats
CS-1	chondroitin synthase-1
CSF	cerebrospinal fluid
Cx	connexin
DAPI	4′,6-diamidino-2-phenylindole
DOX	doxycycline
EAAT	excitatory amino acid transporter
Egr	early growth response protein
EPM	elevated plus maze
EPO	erythropoietin
GALC	galactocerebrosidase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
gfa2	GFAP essential promoter segment;
GFP	green fluorescent protein
GLAST	glutamate transporter
Gp	glycoprotein
GS	glutamine synthase
GT-Tg	GFAP promoter-driven Tat transgenic

H	human
HAD	HIV-associated dementia
HAND	HIV-associated neurocognitive disorders
HD	Huntington’s disease
HIV	human immunodeficiency virus
Htt	huntingtin
ICV	intracerebroventricular
IGF	insulin-like growth factor
IL	interleukin
i.p.	intraperitoneal
kb	kilobase
LCMV	lymphocytic choriomeningitis virus
LDH	lactate dehydrogenase
LMA	locomotor activity
LRP	low density lipoprotein receptor-related protein
LV	lentiviral vector
MAP	microtubule-associated protein
MBP	myelin basic protein
MCAO	middle cerebral artery occlusion
MES	2-(N-morpholino)ethanesulfonic acid
MHC	major histocompatibility complex
miRNA/miR	microRNA
MMP	matrix metalloproteinase

MND	mild neurocognitive disorder
MS	multiple sclerosis
muLV	murine leukemia virus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MWM	Morris water maze
Nab	neutralizing antibody
NDDs	neurological diseases and disorders
Nef	negative regulatory factor
NF	nuclear factor
NGF	nerve growth factor
NHP	non-human primate
NMDA	N-methyl-D-aspartate
NP	nanoparticle
NSE	neuron-specific enolase
NTRN	neurturin
ORF	open reading frame
PAMAM	polyamidoamine
PBS	phosphate buffer saline
PD	Parkinson’s disease
pDNA	plasmid DNA
PDGF	platelet-derived growth factor
PEG	polyethylene glycol
PEI	polyethylenimine

PGK	phosphoglycerate kinase
PLGA	poly(lactic-co-glycolic) acid
RBC	red blood cell
RNAi	RNA interference
scAAV	self-complementary AAV
ssAAV	single-stranded AAV
SCI	spinal cord injury
SD	Sprague-Dawley
shRNA	short hairpin RNA
siRNA	small interfering RNA
SN	substantia nigra
SVZ	subventricular zone
Syn	synapsin
Tat	transactivator of transcription
TTC	total trials to criteria
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TH	tyrosine hydroxylase
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus-G
WT	wild-type
w/w	weight/weight

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CHAPTER 1

INTRODUCTION

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CHAITANYA R JOSHI

1.1 HIV and HAND

Human immunodeficiency virus (HIV) infection is a global pandemic. As of 2017, over 36 million individuals were living with HIV and about 35 million people have died worldwide of HIV or HIV-related complications. Importantly, the rate of new infections has reduced by 47% since its peak in 1997 and about 59% HIV-infected individuals use antiretroviral therapy (ART).[1] HIV predominantly infects CD4+ T cells and weakens the immune system of infected patients, which makes them susceptible to opportunistic infections, malignancies, and accelerated aging. The central nervous system (CNS) is also affected during HIV infection resulting in morphological and functional changes, followed by neurocognitive deficits.[2]

The role of HIV infection in causing encephalitis was known since early the 1980s.[2] In 1991, the American Association of Neurology categorized the HIV-1 related CNS complications for the first time into two categories, HIV-associated dementia (HAD) and minor cognitive motor deficits.[2] The advent of combined ART in the mid to late 1990s[3] reduced the HIV mother to fetus transmissions, incidence of opportunistic infections in HIV patients, and HIV-related deaths by about 50%.[4] Similarly, cases with most severe neurocognitive complications *i.e.* HAD reduced from 10-15% to 2-8%.[5] However, milder forms of the disease remain prevalent with 30-70% HIV-infected patients experiencing neurological complications.[6,7] In 2007, the criteria for neurologic manifestations of HIV was revised into three categories, namely asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorders (MND), and HAD. Collectively, these are referred to as HIV-associated neurocognitive disorders (HAND). The criteria used to distinguish HAND are presented in **Table 1.1**

Table 1.1 HIV-associated neurocognitive disorders (HAND) classification

HAND Category*	Diagnostic criteria	Prevalence
Asymptomatic neurocognitive impairment (ANI)	• Impairment in ≥ 2 neurocognitive domains (at least 1 SD below age-education matched neuropsychological testing scores) • No interference with daily functioning	30%
Mild neurocognitive disorder (MND)	• Impairment in ≥ 2 neurocognitive domains (at least 1 SD below age-education matched neuropsychological testing scores) • Mild to moderate interference in daily functioning	20-30%
HIV-associated dementia (HAD)	• Marked impairment in ≥ 2 neurocognitive domains (at least 2 SD below age-education matched neuropsychological testing scores) • Marked interference in daily functioning	2-8%

***** Cannot be explained by other comorbidities

Neurocognitive domains: attention-information processing, language, abstraction-executive, complex perceptual motor skills, memory, including learning and recall, simple motor skills or sensory perceptual abilities

SD: Standard deviation (Adapted from [8])

Despite the lower prevalence of HAD in the ART era, HAND remains clinically relevant. Patients with milder forms of HAND have a high likelihood of developing HAD.[5,8] Further, CD4+ cell count and viral load may not correlate with cognitive impairment.[5] Additionally, age-related cognitive deficits, viral co-infections, use of illicit drugs, and psychiatric comorbidities are considered risk factors for HAND.[4] Above all, HIV neuropathogenesis remains a complex process with an interplay between multiple cell types and intracellular pathways, which presents a challenge in developing effective HAND therapeutics.

Brain structural changes have been seen as early as 100 days post HIV infection.[9] HIV enters the brain *via* infected macrophages or monocytes, a phenomenon commonly known as the 'trojan horse hypothesis'. Once in the brain, HIV actively infects the resident microglia and macrophages in a CD4-dependent manner spreading the infection. Though microglia are the predominant HIV targets in the brain, other cell types are directly or indirectly affected. For example, neurons are not infected by HIV, but virus- or viral protein have known neurotoxic effects. Some astrocytes are restrictively infected by HIV,[10,11] *i.e.* are unable to produce active virions. However, they can produce viral proteins such as transactivator of transcription (Tat) and glycoprotein (gp)120. The frequency of astrocyte HIV infection correlates with the extent of neurocognitive decline.[10] In this context, HAND-relevant structural and functional features of astrocytes are discussed in Section 1.2.

1.2 Astrocytes in HAND

Astrocytes, the principal support cells in the CNS, were first discovered in the late nineteenth century.[12] The term 'astrocytes' was coined due to their star-shaped appearance (*astro*, star and *cyte*, cell). Originally thought to only structurally support neurons, a more important role for astrocytes in CNS homeostasis has now been established that includes, but not limited to, neuronal activity, cerebral blood flow coupling, and glutamate synaptic transmission modulation.[13] Disruption of astrocyte function contributes to pathogenesis of neurological diseases and disorders (NDDs) (**Figure 1.1**). Astrocytes are an attractive cellular target for treating NDDs. In this regard, the structural and functional aspects of astrocytes are described in detail in Chapter 3 (Section 3.3.4). Here, contributions of astrocytes to HAND pathology relevant to this study are briefly discussed.

Multiple mechanisms have been proposed on how HIV infects astrocytes. Some studies inferred that infected CD4+T cells transfer the virus *via* cell to cell contact to astrocytes,[14,15] by a CD4-independent but CXCR4-dependent mechanism.[16] Another report proposed endocytosis of the free virus by astrocytes,[17,18] or uptake *via* gp120 and human mannose receptor-based mechanism.[16] In parallel, it was also argued that phagocytic properties of astrocytes could be responsible for uptake of viral proteins, nucleic acids, and integration products in astrocytes; and thus astrocytes are not 'infected' since cell-free HIV failed to fuse and infect human fetal astrocytes.[19] Nonetheless, these findings indicate that astrocytes can harbor virus and may serve as viral reservoirs in the brain causing direct or indirect neurotoxicity.

Infected cells, viral proteins, inflammatory cytokines activate astrocytes, a classic proinflammatory response of astrocytes,[20] affecting their normal functions like glutamate clearance, potentially causing excitotoxic neuronal damage.[21] In response to HAND-relevant stimuli, *i.e.* viral isolates, viral proteins, and inflammatory cytokine such as interleukin (IL)-1β, astrocytes have been shown to produce proinflammatory mediators such as CCL2, CXCL8, IL-6, IL-1β and tumor necrosis factor (TNF)-α.[22,23] Astrocytes infected with HIV can secrete HIV-1 proteins such as HIV-1 Tat, negative regulatory factor (Nef), and regulator of expression of viral proteins (Rev).[24] Astrocytes respond to multiple signals such as nitric oxide, TNF, and FasL impair neuroprotective functions, increase glutamate uptake, and apoptosis.[20] Astrocytes provide trophic support to neural progenitor cells, and thus altered astrocyte function may result in impaired neurogenesis in adults.[20]

Astrocytes are also implicated in HAND comorbidities such as drug abuse, including methamphetamine,[25,26] cocaine,[27] and alcohol;[28] and viral co-infections.[29] Treatment with ART resulted in oxidative and endoplasmic reticulum stress in astrocytes.[30] A number of rodent models investigate the role of astrocyte-produced HIV proteins; including HIV-1 Tat, gp120, and Nef, during HAND using a glial fibrillary acidic protein (GFAP) promoter-driven transgene approach.[31-33] One of these models, *i.e.* doxycycline-inducible, GFAP promoter-driven HIV-1 Tat transgenic (abbreviated as iTat or GT-Tg), was used for the work presented in this study (**Chapter 2**) and the literature reporting HIV-1 Tat effects on astrocyte function are discussed in detail in the next section.

1.3. HIV-1 Tat

Most, if not all, HIV-1 infected cells produce and release Tat protein *via* a 'golgi independent' and plasma membrane-dependent pathway.[24,34] The full-length protein consists of 101 amino acids (aa), coded by two exons in the HIV genome. Tat remodels chromatin at the HIV promoter, recruiting a positive transcription elongation factor that phosphorylates RNA polymerase II, allowing for efficient transcription.[35] Thus, absence of Tat impedes HIV replication caused by repressive chromatin architecture as well as a defect in transcription elongation.[35] An 86 aa Tat is produced by some strains of HIV, such as LAI (isolated from patients with initial LAI),[36] due to an premature stop codon in the second exon. In later stages of infection, a truncated Tat variant is produced due to cytoplasmic export of unspliced viral RNAs by HIV regulator of expression of viral proteins (Rev). This truncated Tat variant coded only by the first exon (72 aa) is sufficient for HIV promoter transactivation.[37] Tat is produced by infected cells even with ART drugs [38] and

induces direct or indirect toxic effects on other infected or non-infected cells, which contribute to HIV pathogenesis.[24]

1.3.1 Functional significance of Tat during HAND

Tat is one of the early HIV proteins, consisting of six functional regions. Among these, region II (spanning aa 22-37) is cysteine rich, region IV (spanning aa 48-59) is arginine rich, and region V (spanning aa 60-72) is glutamine rich.[39] The region II and basic regions (IV and V) are associated with Tat neurotoxicity, while region IV is responsible for transcriptional properties of Tat.[40]

Tat secretion from infected cells occurs in low serum conditions such as in the brain.[41] In this context, Tat mRNA was detected in postmortem HIV-1 encephalitic CNS tissues, while protein was not quantified due to short half-life and high instability of Tat.[42] Nonetheless, it is suggested that inflammatory responses in specific CNS regions or cell types could be associated with higher Tat levels during HAND.[39] For example, Tat induced TNF from monocytes and microglial cells *in vitro*.[43] Therefore it was suggested that higher TNF levels observed in the brain macrophages and microglia of HAD patients were associated with high brain Tat levels.[43]

Tat crosses the BBB bidirectionally,[41] and reduces the expression of tight junction proteins in brain endothelial cells,[44,45] potentially contributing to BBB disruption. Subsequently, Tat could increase monocyte invasion in the brain *via* increased CCL2 expression in astrocytes and microglia resulting in increased neuroinflammation.[22,46] Tat exerts its neurotoxic effects indirectly by multiple mechanisms including oxidative stress,[47,48] calcium signaling dysregulation,[49,50] autophagy,[51] and disruption of microRNAs.[52,53] Tat treatment led to Zn^{2+}-mediated inhibition of N-methyl-D-aspartate

receptor resulting in death of hippocampal neurons *in vitro*.[54] Neurotoxic effects of Tat might be associated with uptake and nuclear translocation of Tat in neurons *via* a low-density lipoprotein receptor-related protein (LRP) promoted mechanism, causing LRP ligands disruption and modulating gene expression in neurons.[55]

Collectively, Tat contributes to CNS pathology by several direct and indirect mechanisms. As Tat is produced from restrictively infected astrocytes, multiple studies investigated effects of Tat on astrocytes with respect to HAND pathogenesis.

1.3.2 HIV-1 Tat and astrocytes

Astrocyte Tat increased proinflammatory cytokines and chemokines, monocyte invasion, oxidative stress, calcium overload, and reactive astrogliosis, etc.[51,56-58] Critical intracellular pathways including p38, Jnk, and Akt signaling are associated with Tat-induced changes in astrocytes.[59] Tat mediated a translation-independent increase in nuclear factor (NF)-κB binding activity and cytoplasmic protein kinase C activity in human astrocytes.[60] Tat induced several cytokines and chemokines from astrocytes including CCL2,[22] CCL5, IL-6,[61] CXCL8,[62] and CXCL10.[59,62] In a separate study, Tat treatments increased IL-6, IL-1β and TNF-α protein levels in monocytes, while only increasing IL-6 in astrocytes.[63] Increased IL-1β mRNA was measured in astrocytes, which did not correspond with changes in protein levels.[63] These findings suggest that Tat-mediated regulation of astrocyte inflammatory responses is distinct from other cell types, highlighting the need for astrocyte focused investigations to evaluate Tat-mediated neuropathology.

Another Tat-mediated neurotoxic mechanism in astrocytes include inhibition wingless-integrated (Wnt)/β-catenin signaling,[35] increased connexin (Cx)43 expression,[64]

and lysosomal exocytosis.[58] The Wnt/β-catenin pathway, which potently repressed HIV progression in astrocytes, was inhibited by Tat.[35] Elevated Cx43 increased gap junction communication presenting a previously unknown cell-cell communication mechanism with potential neurotoxic effects.[64] Tat induced lysosomal exocytosis of cathepsin B from astrocytes coupled with neurotoxicity.[58]

Morphologically, Tat-expressing mouse astrocytes demonstrated a reactive phenotype with increased GFAP expression.[65] Subsequent reports established that increased GFAP expression was regulated by an early growth response (Egr)-1 element in Histone acetyltransferase p300 gene following a direct transactivation of Egr-1 by Tat.[58] These Tat-dependent changes in GFAP could be an early step in reactive gliosis and astrocyte dysfunction.[58]

1.3.3 Modeling HAND with HIV-1 Tat

Tat is used to mimic aspects of HAND in multiple *in vitro* and *in vivo* models since several neurotoxic effects observed with HIV infection are mediated by Tat.[32,33,66,67] Some studies use exogenous Tat for treating cells or rodents,[67,68] while some studies use Tat transgenic rodent models.[32,33] The GT-Tg mouse model was developed by Dr. Johnny He, in which the GFAP promoter restricts Tat expression to astrocytes allowing study of astrocyte-specific responses during HAND-relevant inflammation.[32,69] The GT-Tg mouse model has been used extensively over last 15 years to evaluate Tat-associated pathogenesis.[70] These changes include impaired neurogenesis, reactive astrogliosis, astrocyte endoplasmic reticulum stress, lysosomal exocytosis, and autophagy.[70] Concurrently, Tat induced cognitive and behavioral changes including anxiety,[71] depression,[72] impaired learning and memory.[73] A similar Tat model used in several

studies exhibited less severe CNS pathology.[74] Tat protein levels (1–5 ng/ml) in the brains of DOX-treated GT-Tg mice were similar to the Tat levels in the brains of HIV-infected patients.[75] Despite abundant research, the GT-Tg model has not been used to study the balance between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMP)s in the brain. Implications of altered MMP/TIMP from a HAND perspective are discussed in the next section and subsequent chapters.

1.4 MMP/TIMP balance and astrocyte TIMP-1

As a class of calcium-dependent, zinc-containing endopeptidases, MMPs are primarily responsible for extracellular matrix degradation and remodeling. There are over two dozen known MMPs. The activity of MMPs is regulated by four closely related TIMP proteins.[76] The MMP/TIMP balance is tightly regulated by transcription factors, cytokines, enzymes, and secondary messengers. This axis is crucial for homeostatic processes including cell migration, adhesion, proliferation, and apoptosis. On the other hand, MMP/TIMP imbalance is associated with inflammation in multiple NDDs. In this section, known literature on MMP and TIMPs, MMP/TIMP imbalance during HAND is discussed followed by a brief summary of our previous work on TIMP-1, and MMP-independent, therapeutically relevant TIMP-1 functions.

1.4.1 MMPs and TIMPs in the brain

In the CNS, MMP-2 and MMP-9 are the most investigated due to their involvement in most disease pathologies and ease of detection.[76] MMP-2 is predominantly astrocytic, while MMP-9 is predominantly neuronal.[76] TIMP-1 is an inducible protein produced by multiple cell types in the CNS and primarily produced by astrocytes during injury and inflammation.[76,77]. TIMP-2 expression in constitutive and it is the most abundantly

expressed TIMP in the brain.[76] Not much is known about functions of TIMP-3 and TIMP-4 during homeostasis and/or disease; however they are detected in the brain.[76]

The N-terminal domain of all TIMPs (~125 aa), including TIMP-1, folds and functions independently; and is principally responsible for inhibiting MMPs.[78] The C-terminal region (~65 aa) is involved in MMP-independent, direct signaling *via* cell surface receptors; and is responsible for growth factor like properties of TIMP-1 as well as TIMP-2. Among TIMPs, TIMP-1 is the most conserved member of the protein family and can inhibit non-membrane type MMPs.[79] In the CNS, TIMP-1 increased oligodendrocyte differentiation and neuronal myelination, regulated neural stem cell migration, and promoted neuroprotection following stroke.[80-82] Further, IL-1β, IL-6, and transforming growth factor (TGF)-β induced astrocyte TIMP-1.[77,83] The anti-apoptotic functions of TIMP-1 along with its cytokine/growth factor like properties led to TIMP-1 centric therapeutic research for multiple conditions such as stroke,[84] glioblastoma,[85] and HAND.[86,87]

1.4.2 Brain MMP/TIMP balance in HAND

The principal MMP-driven mechanisms contributing to HAND pathogenesis include blood-brain barrier (BBB) breakdown, inducing neuronal dysfunction, and myelin degradation.[41] Levels of MMP-2 and MMP-9 were elevated in the cerebrospinal fluid (CSF) of HAD patients,[88,89] and TIMP-1 but not TIMP-2 expression was increased but in the CSF of HAND patients.[90] These findings reflect the inducible nature of TIMP-1 as compared to TIMP-2. Correlating CSF MMP levels with cognitive changes indicated MMP-9 and MMP-2 had deleterious effects on verbal fluency and motor speed parameters, respectively.[89] The MMP/TIMP changes observed in HAND patients could

be due to direct and/or indirect effects of specific viral protein(s) including Tat and gp120. In a gp120-based rat model of HIV encephalopathy, CNS TIMP-1 and TIMP-2 levels were elevated. MMPs were elevated during gp120-mediated oxidative stress in a rat model of HIV encephalopathy, which in turn led to increased TIMPs to counteract MMPs.[91] Another study employing an HIV-1 gp120 mouse model demonstrated induction of MMP-2 activity in mouse brains.[92] Similar to these findings, HIV-1 Tat-induced changes in MMP/TIMP balance are discussed in more detail in Chapter 2.

TIMP-1 is produced in response to injury by multiple cell types including reactive astrocytes[93] and reactive astrogliosis is one of the hallmarks of chronic neuroinflammation characteristic of HAND.[94] Therefore, it would be interesting to understand astrocyte TIMP-1 regulation during HAND.

1.4.3 Differential regulation of astrocyte TIMP-1

Inflammatory stimuli, CNS myelin injury, ischemic injury, and acute HIV infection increased CNS TIMP-1 levels[80,90,95,96] often in a cell- and time-dependent manner.[80,90,95,96] Following an acute injury, brain MMP-2 and MMP-9 expression was elevated, first in neurons and then in astrocytes.[97] Subsequently, TIMP-1 mRNA was induced in neurons as an immediate early response gene, followed by elevated TIMP-1 in astrocytes with reactive astrogliosis.[97,98] Elevated TIMP-1 in astrocytes remained localized to the cell body or was secreted into the cell supernatant.[99] TIMP-2, which exhibits similar MMP-independent functions as TIMP-1, was expressed constitutively and did not respond to injury stimuli as TIMP-1.[76] Further, TIMP-1, and not TIMP-2, was shown to colocalize with both MMP-2 and MMP-9 in mouse astrocytes post-inflammatory stimuli indicating its pivotal role in modulating MMP/TIMP axis.[100]

Our laboratory extensively investigated multiple aspects of astrocytes TIMP-1 regulation. Our group demonstrated that astrocyte TIMP-1 levels increased following acute IL-1β treatment, while prolonged IL-1β activation led to decreased TIMP-1.[101] On the other hand, MMP-2 levels remained relatively high in astrocytes with prolonged inflammatory stimuli, tipping the MMP/TIMP axis towards MMPs.[101] Lowered TIMP-1 and higher MMP-2 levels with prolonged inflammation in human astrocytes were consistent with TIMP-1 and MMP-2 levels measured in the CSF and brain tissue samples from HAD patients.[101] Similar TIMP-1 downregulation and MMP-2 increase were also observed in astrocytes acutely co-stimulated with IL-1β and TGF-β, indicating TIMP-1 was regulated not only in a cell- and time- dependent manner, but also depending on type of stimuli. It must also be noted that, effects of increased cell death and potential interference of IL-1 signaling on reducing TIMP-1 were ruled out.[99]

Transcriptional control and loss of mRNA stabilization attributed downregulated TIMP-1 during prolonged inflammation.[99] In this regard, role of transcription factor CAAT enhancer-binding protein (C/EBP)β in TIMP-1 regulation was evaluated due to presence of five CCAAT boxes in the 1.7 kb sequence upstream of TIMP-1 exon 1 and intron 1.[102,103] Overexpressing C/EBPβ increased TIMP-1 promoter activity, mRNA, and protein levels in IL-1β-activated astrocytes, while C/EBPβ knockdown with siRNA decreased TIMP-1 mRNA and protein levels. Simultaneously, C/EBPβ mRNA levels were elevated in total brain tissue lysates of HIV-1-infected and HIV-1 encephalitis patients indicating C/EBPβ involvement in astrocyte TIMP-1 regulation during HIV-1 infection.[104] A follow-up study demonstrated that extracellular signal-regulated kinases (ERK)1/2 activation was critical for IL-1β-mediated astrocyte TIMP-1 expression. Concurrently, p38K

activation contributed to IL-1β-induced astrocyte TIMP-1 and C/EBPβ expression.[105] Our studies highlight differential TIMP-1 expression during acute versus prolonged inflammation (**Figure 1.2**) and the complex interplay of regulatory pathways involved in TIMP-1 regulation.

1.4.4 *Astrocyte TIMP-1 as a therapeutic option*

Due to its MMP-independent, cytokine-like functions, TIMP-1 has been proposed as therapeutic option for cancers,[106] ischemia,[84] and HAND.[86,87] TIMP-1 prevented excitotoxic death of rat hippocampal neurons [107] and protected rat brains during ischemia.[96] In another study, magnetic nanoparticles loaded with TIMP-1 were delivered across an *in vitro* BBB model, leading to reduced HIV infectivity, and reactive oxygen species, while also improving spine density in neuroblastoma cells.[87] Neuroprotective effects of TIMP-1 during HIV-induced apoptosis were shown in human neurons.[86] Overall, these findings suggest that TIMP-1 would be an excellent gene target for restoring CNS homeostasis during neural pathologies. Constitutive CNS TIMP-1 expression and suppression of MMP activity did not interfere with homeostatic brain functions[108] and thus TIMP-1 could be considered a suitable candidate for long-term therapeutic treatment of chronic inflammatory CNS diseases.[108] Taken together, these findings indicate a protective role for TIMP-1 in CNS function. As its levels diminish with chronic inflammation, TIMP-1 restoration has been proposed as a therapeutic option for neuroinflammatory conditions.[84,86,87]

1.5 Hypothesis and objectives of the present study

Brain HIV infection and viral proteins, such as HIV-1 Tat, cause direct and/or indirect neurotoxicity by multiple mechanisms including elevated MMP levels and

activity.[41,88,89] In this context, TIMP-1 could be an excellent therapeutic for treating HAND due to its MMP inhibitory and neuroprotective effects. However, the CNS is a difficult therapeutic target for TIMP-1 delivery. Nanomedicine[109] and gene therapy[110] are suggested approaches in designing brain-targeted therapeutics to overcome challenges such as structural complexity and low BBB permeability. Nanoparticle (NP)s of ~100-200 nm can cross the BBB[110] and gene therapy uses nucleic acids, such as plasmid DNA (pDNA), to alter gene expression and function in target cells.[109] Further, TIMP-1 expression could be restricted to astrocytes using cell-specific promoters to help eliminate off-target effects in the brain and/or periphery.

The availability of an appropriate HAND model would be needed to validate the therapeutic delivery and efficacy of TIMP-1. Since, HIV does not infect rodents, it is not feasible to mimic neuropathology of HAND in its entirety *in vivo* in rodent models. Yet, several rodent models mimicking aspects of HAND have been developed in the last two decades.[31,32,111,112] Some of these models consist of inducible or constitutively expressing transgenes for one or more HIV proteins, restricted to a cell type such as astrocytes. These mouse models are often used, compared to more relevant non-human primate models, due to ease of handling, lower cost, and established experimental protocols.(Reviewed by Jaegar and Nath).[11] In our studies, HIV-1 Tat-based GT-Tg model was used to mimic HAND-associated neuroinflammation. Our hypothesis was, ***astrocyte HIV-1 Tat induces HAND-relevant neuroinflammation, neurodegeneration and TIMP-1 downregulation; and an effective and biocompatible astrocyte gene delivery system will offer a novel HAND therapeutic for TIMP-1 restoration.*** The

studies carried out to test the hypothesis are described in the following chapters of this dissertation.

Chapter 2 *'Tipping the MMP/TIMP Balance and Neurocognitive Decline in a HIV-1 Tat Transgenic Mouse Model'*

In this chapter, we evaluated if and how HIV-1 Tat expression contributed to tissue and behavioral changes in the context of MMP/TIMP-1 axis? For this, behavioral impairments in GT-Tg mice were assessed following an acute or prolonged Tat expression. Subsequently, brains were harvested from these mice to measure the gene expression of principal brain MMPs, TIMPs and inflammatory biomarkers. The findings of these studies provided important insights about: 1. how duration of Tat expression altered rodent behavior as well as MMP/TIMP axis and 2. if GT-Tg could be used as a suitable model to test TIMP-1 therapeutic delivery in future studies.

Chapter 3 *'Destination Brain: The Past, Present, and Future of Therapeutic Gene Delivery'*

In this chapter, existing literature on CNS-targeted gene delivery, common challenges, cellular targets, gene delivery systems, and the current status of clinical translations was reviewed. We inferred that astrocytes are suitable gene therapy targets for treating NDDs compared to other CNS cell types and polymeric nanoparticles would be an attractive delivery system over viral vectors for astrocyte-targeted gene delivery.

Chapter 4 '*Reaching for the Stars in the Brain: Polymer-mediated TIMP-1 Gene Delivery to astrocytes*'

In this chapter, multiple arginine-modified, polyethylene glycol (PEG)-stabilized PEI analogues (A_nP_n) were tested for their gene delivery potential. A cytomegalovirus (CMV)

promoter-driven luciferase (luc) reporter plasmid was used to measure efficiency of transfection in primary human brain cells, *i.e.* astrocytes and neurons, as well as in nude mice. Biocompatibility, uptake-release profiles, cell-targeting potential of A_nP_n was determined first in neural cells, primarily astrocytes, followed by *in vivo* testing in mice. Next, GFAP promoter-driven luciferase (luc) and TIMP-1 plasmids were sub-cloned and tested successfully in human astrocytes using A_nP_n .

Chapter 5 '*Putting the TIMPuzzle Together*'

First, the findings from entire dissertation were summarized and analyzed. Ongoing and future studies were described with potential limitations of the project. Second, we delved into the overall impact of this dissertation in the context of HAND and other NDDs by discussing the rationale of using TIMP-1 gene therapy, optimal approaches for using TIMP-1, and implications for translating these findings into effective therapies for future.

1.6 Figures and Figure Legends

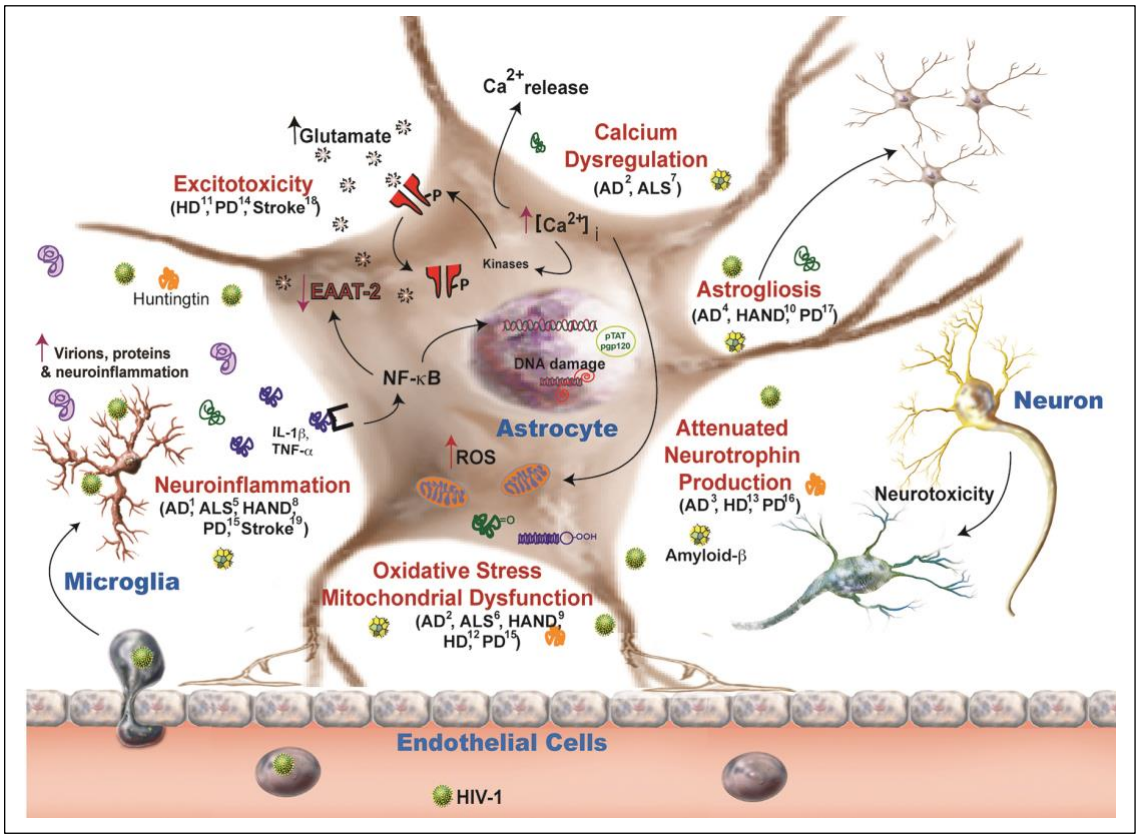

Figure 1.1 Neurotoxic mechanisms of astrocytes during neurodegenerative conditions CNS diseases are linked to one or more astrocyte dysfunctions that include excitotoxicity, neuroinflammation, oxidative stress and mitochondrial dysfunction, attenuated neurotrophin production, astrogliosis and calcium dysregulation. Astrocytes undergo diverse changes during CNS disease such as Alzheimer's disease (AD) [1[113], 2[114],3[115],4[116]]; Amyotrophic lateral sclerosis (ALS) [5[117], 6[118], 7[119]]; HIV-associated neurocognitive disorders (HAND) [8[120], 9[121], 10[122]]; Huntington's disease (HD) [11[123], 12[124], 13[125]], Parkinson's disease (PD) [14[126], 15[127], 16[128], 17[129]] and Stroke (18[130], 19[131]].

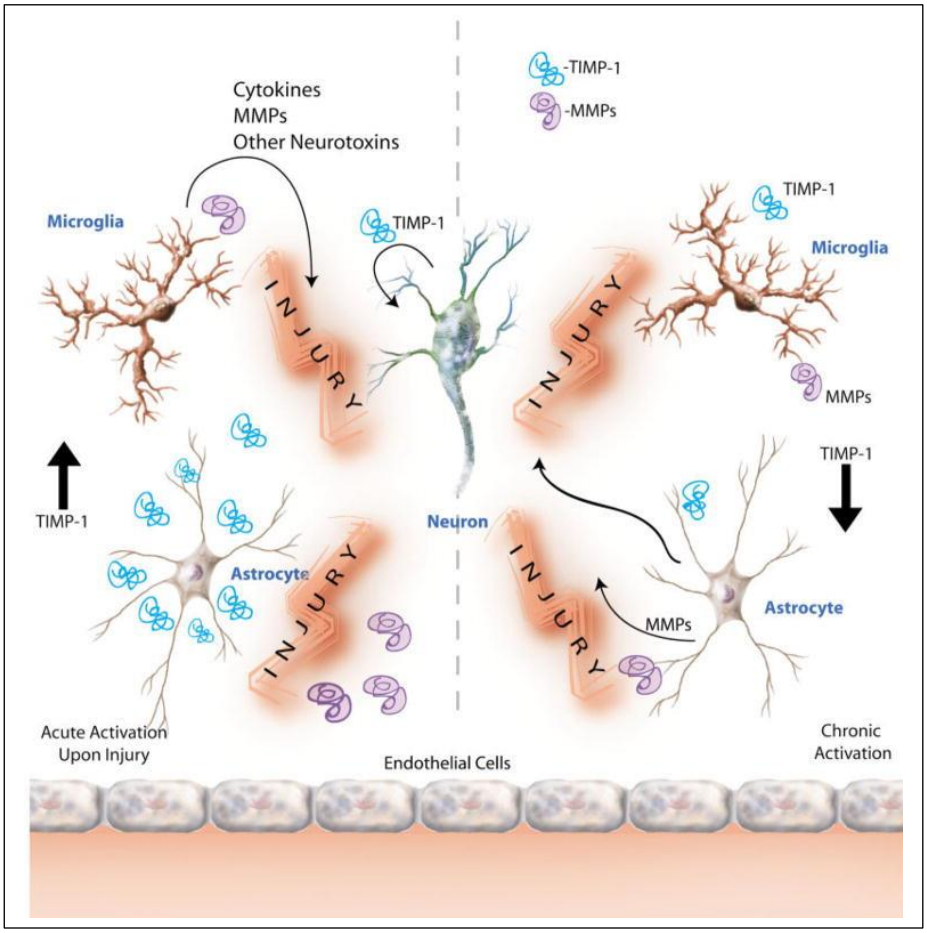

Figure 1.2 Differential regulation of astrocyte TIMP-1 during acute versus chronic inflammation Acute inflammation due to exposure to proinflammatory cytokines such as IL-1β lead to enhanced levels of TIMP-1 in brain cells including astrocytes, microglia, and neurons and elicit a typical repair response early in injury (left). However, under prolonged inflammation, such as those observed in NDDs, TIMP-1 levels in the CNS decline significantly below homeostatic levels (right). While the exact mechanisms driving this dichotomy remain unclear, TIMP-1 restoration would be an applicable therapeutic strategy owing to its neuroprotective functions (Illustration from Gardner and Ghorpade, 2003).[132]

CHAPTER 2

TIPPING THE MMP/TIMP BALANCE: LINKS TO NEUROCOGNITIVE DECLINE IN AN HIV-1 TAT TRANSGENIC MOUSE MODEL

AUTHORS

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2.1 Abstract

Though HIV-associated neurocognitive disorders (HAND) affect ~50% HIV-infected individuals, asymptomatic to milder forms of the disease prevail in the antiretroviral therapy era. Imbalance of brain matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) has been implicated in HAND neuropathogenesis. The MMP/TIMP balance is associated with cognition, learning, and memory; however, its role on cognitive and behavioral deficits in HAND is not known. In this study, we used doxycycline-**i**nduced, glial fibrillary acidic protein promoter-driven HIV-1 transactivator of transcription (Tat) transgenic (GT-Tg) mouse model to assess behavioral changes in HAND, simultaneously elucidating changes in MMP/TIMP balance. A series of behavior tests evaluating arousal, ambulation, anxiety, and cognition were employed followed by harvesting brain tissues for gene expression analysis. Behavior and gene expression in GT-Tg mice were measured in the context of acute versus prolonged Tat expression paradigms. We observed a mild neurocognitive impairment phenotype in prolonged Tat-expressing transgenic mice. Concurrently, MMP/TIMP mRNA axis was altered depending on the duration of Tat expression. These alterations were independent of *de novo* inflammatory biomarkers typically implicated in MMP/TIMP regulation. Based on these findings, we infer that tipping MMP/TIMP balance towards MMPs may be associated with Tat-mediated neurocognitive decline in HAND.

2.2 Introduction

Despite antiretroviral therapy (ART), HIV-associated neurocognitive disorders (HAND) remain a major concern affecting 40-50% of HIV-infected individuals.[5,133] While the incidence of severe HAND, *i.e.* HIV-associated dementia (HAD), has declined;

asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorders (MND) remain prevalent lowering the quality of life[133] and ART adherence.[134] Patients suffering from these milder forms of HAND exhibit difficulty with working memory, executive functioning, and speed of information processing.[135] Multiple underlying mechanisms are implicated in HAND pathogenesis and associated behavioral deficits. These include secretion of proinflammatory mediators from infected cells, blood-brain barrier (BBB) disruption, reactive astrogliosis, excitotoxicity, and imbalance of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs).[4,136,132]

During homeostatic conditions, MMPs are involved in the extracellular matrix remodeling and their levels are regulated by TIMPs. Additionally, TIMP-1 and TIMP-2 possess cytokine-like functions during cell differentiation, growth, and apoptosis.[78,137] Although more than 20 MMPs and 4 TIMPs are known, MMP-2, MMP-9, TIMP-1 and TIMP-2 are most investigated due to their critical role in modulating brain MMP/TIMP axis during multiple central nervous system (CNS) diseases and disorders.[76] Levels of MMPs were elevated in primary brain cell cultures treated with HIV or HIV-relevant stimuli[138] [2006,139] as well as in cerebrospinal fluid (CSF) specimens,[90] and postmortem brain tissues of infected patients.[101] Concurrently, TIMP-1 increased or decreased with acute or prolonged HIV-relevant inflammatory stimuli, respectively, in primary human astrocytes.[101] [2006] Reduced TIMP-1 levels were also observed in CSF and brain tissues of HIV-infected patients indicative of chronic inflammation.

Increased MMPs disrupt the BBB *via* breakdown of tight junction proteins, recruit immune cells in the CNS, and cause direct neuronal damage potentially contributing to HAND pathology.[41] [2011, Xu et al., 2012] Further, increased MMP levels are also linked to long-

term memory[140,141] as well as learning[142,143] impairments, and altered synaptic plasticity[144] suggesting their potential role in neurocognitive declines during HAND. However, links between the MMP/TIMP balance and neurological deficits in HAND are not well investigated. To address this 'knowledge gap', we employed a doxycycline (DOX)-inducible, glial fibrillary acidic protein (GFAP) promoter-driven HIV-1 transactivator of transcription (Tat)-expressing transgenic (abbreviated as iTat or GT-Tg) mouse model.[32] HIV-1 Tat is a key HIV protein involved in neuronal dysfunction,[50,145] BBB disruption,[139] oxidative stress,[49,146] elevating MMPs[147,148] and possesses chemokine-like abilities that promote immune infiltration into the brain.[22 2003] The GT-Tg mouse model, which expresses HIV-1 Tat only in astrocytes,[32] was previously used to evaluate Tat-associated changes in cognition and memory, anxiety, and depression.[71,72,149]

Most prior investigations elucidated effects of acute Tat expression on behavior i.e. a few days after Tat induction.[71,72,149] While these studies provided valuable insights, effects of prolonged Tat expression were not well-established. As TIMP-1 is differentially regulated with acute versus chronic neuroinflammation,[90,101] we hypothesized that duration of Tat expression would alter MMP/TIMP balance differently, which would in turn contribute to concurrent neurological impairments. To validate the hypothesis, two DOX induction paradigms were utilized to mimic acute versus prolonged Tat expression in the GT-Tg mouse model (**Figure 2.1A**). A battery of behavioral tests was employed to investigate changes in anxiety, arousal, ambulation, learning, and memory (**Table 2.1**). Behavioral testing was followed by assessing gene expression of MMPs, TIMPs and proinflammatory mediators.

Table 2.1 Battery of behavior tests used in the study

Test description	*Capacity or deficit measured*	*Primary anatomical target of test*
Elevated plus maze (EPM)	Anxiety/fear level	Limbic system
Locomotor activity (LMA) Horizontal, vertical	Ambulation, rearing, arousal	Limbic reward circuitry
Spatial water maze (WM)	Visual/spatial discrimination, explicit reference learning/memory; executive function/planning	Hippocampus, frontal cortex
Discrimination reversal (T-maze)	Associative learning (conditioned fear), cognitive flexibility	Frontal cortex, limbic system

2.3 Materials and Methods

Animals

All animal experiments were conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. An animal protocol was approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee in Fort Worth, TX. The iTat or GT-Tg mice, generated as previously described,[32] were provided by Dr. Johnny He for breeding purposes. The breeder mice were rederived by Jackson laboratories and then bred in house for experiments. The mice were genotyped to confirm the presence of two transgenes, i.e. *hiv tat* and reverse tetracycline-controlled transactivator (*rtTa)*. The mice were housed in groups of three to five in polycarbonate cages with corncob bedding, fed *ad libitum*, and maintained at an ambient temperature

(23 ± 1 °C), under a 12 h light/dark cycle. Breeder C57BL6/J (WT) mice (six weeks old) were purchased from Jackson laboratories. These mice were bred, housed, and fed in same conditions as GT-Tg mice. Males and females were used for all experiments.

Treatment

At the age of six to seven months old, GT-Tg mice were administered DOX by two different methods, *i.e.* i.p. injections or food, to delineate effects of acute versus prolonged Tat expression, respectively. Age- and sex-matched WT mice were used to control off-target DOX effects. The schematic representation of the experimental timeline is presented in **Figure 2.1**.

Acute Tat induction

Previous studies reported that Tat expression increased significantly after three DOX (100 mg/kg) i.p. injections and returned to baseline in two weeks after last injection.[71] Further, attrition rate increased with DOX concentration and/or injection frequency. Based on these findings, seven i.p. injections of 100 mg/kg DOX (Cat no. D9891, Sigma-Aldrich, St.Louis, MO) were used to acutely induce Tat expression. Mice were injected on four times in the week prior to, two times during the first week of, and once in the third week of behavior testing. The seven injections were administered over three weeks to maintain Tat expression above baseline during behavioral testing and at the time of brain harvesting.

Prolonged Tat induction

Mice were fed with chow containing DOX (1250 mg/kg, Cat no. TD.160353, +Maltodextrin, green, Harlan Laboratories, Indianapolis, IN) starting at four to five weeks of age for a total of 6 months prior to and four weeks during behavior studies to mimic

prolonged Tat expression responses. DOX food intake was monitored for two months prior to and during behavior studies (data not shown).

Behavioral Assessments

At 6-7 months, the mice were behaviorally characterized for anxiety, arousal, spatial learning and memory, and cognitive flexibility. All mice were euthanized within a week after the last behavioral test. The mice were weighed weekly during the behavior studies (**Supplementary Figure 2.1**). The tests were performed in the following order during the animals light cycle.

Elevated plus maze (EPM)

To measure anxiety,[71] a plus-sized maze elevated three feet was placed in a dimly lit test room (60 Watts). The maze consisted of two arms opened to the room and two arms enclosed such that the floor and rest of the room were not visible. An automated tracking system monitored the position of each mouse in the maze (Any-maze, Stoelting Co., Wood Dale, IL, USA). Mice were acclimated to the testing room for at least 10 min prior to testing. Each mouse was placed in the center of the maze facing an open arm and was given 5 min to explore the maze. The percent time spent in the open arms and distance covered on the maze were recorded.

Locomotor activity (LMA)

Spontaneous locomotor activity was measured as described previously.[150] In this test, each mouse was placed in a clear acrylic box (40.5 × 40.5 × 30.5 cm), surrounded by a photocells lined metal frame. Then, the test cage was placed in a dimly lit chamber equipped with a fan that provided background noise (80 dB). The test was conducted for 16 min, in which movements in the horizontal plane and vertical plane (7.6 cm above the

floor of the box) were detected by the photocells, and processed by a software program (Digiscan apparatus, Omnitech Electronics, model RXYZCM-16) to yield different measures including distance covered, vertical activity, and spatial components of spontaneous activity in the box.

Morris water maze (MWM)

Spatial learning and memory were measured using a MWM test. Testing was carried out as described previously.[151] Mice were acclimated to the testing room for at least 10 min prior to testing each day. During each trial, the mouse was put in a tank filled opaque water to swim and was able to escape the water by finding and climbing on a platform hidden 1.5 cm below the water surface. The water temperature was maintained at 24 ± 1 °C. An automated tracking system recorded various measures such as latency, path length and swimming speed for each trial (Any-maze; Stoelting Co., Wood Dale, IL, USA). The test consisted of two phases (1) Pre-training phase: the mice learned swimming and climbing onto a platform using a straight alley containing a platform at one end. During this phase, the tank was covered with a black curtain to hide surrounding visual cues. Each mouse was trained for a single session of five trials with a 5 min inter-trial interval. During each trial, mouse was allowed to swim until it climbed on the platform or for a maximum of 60 s. (2) Acquisition phase: the mice were tested for their ability to locate a hidden platform using spatial cues around the room over four sessions (one session/day). Each session consisted of five trials, at two min intervals. For each trial, a mouse was placed at one of four different starting points at the edge of tank and had to swim to the platform, which remained at the same location. The mouse was allowed to swim until it reached the platform or for a maximum of 90s. Path length (distance taken

to reach the platform) over all sessions was used as the primary measure of performance. The swim speed was calculated by dividing path length by the latency (to reach the platform) for each trial.

Discrimination reversal

The discrimination reversal testing assessed memory with a T-maze as described previously.[152] Briefly, the T-maze was constructed of acrylic with black sides to hide spatial cues for mouse and clear tops for tester to observe the mouse. The maze consisted of three compartments: a start box (10 × 6.3 × 6 cm), which extended into the stem (17.5 × 6.3 × 6 cm), and two arms (14.5 × 6.3 × 6 cm), each separated by acrylic flaps manually operated by the tester. The maze rested on a metal grid wired to deliver 0.69 mA scrambled shock to the feet. Mice were acclimated to the testing room for at least 10 min prior to testing. Each mouse was tested in three sessions separated by one hour. At the beginning of each trial, the mouse was placed in the start box, and the start flap was removed for mouse to enter the stem. During the first trial of the first session, the mouse received shock on entering an arm (preferred arm) and was allowed to avoid shock by running to the other arm, which then become the correct arm for the remainder of first session. For subsequent trials, shock was initiated five seconds after the opening of the start flap if the mouse had not entered the correct arm or immediately upon entry into the incorrect arm. The shock continued until the correct arm was entered or for a maximum of 60 s. Once the mouse entered the correct arm, the flap was closed to prevent escape, and the mouse was moved after 10 s, by detaching the arm, into a holding cage for one min before beginning next trial. This trial paradigm until the mouse fulfilled the correct avoidance criterion, *i.e.* running directly to the correct arm within 5 s, in four of the

five consecutive training trials including the last two. In the second and third sessions, there was a reversal in correct arm such that the mouse was required to run to the other arm compared to the one it was trained for in the previous session. The ability of the mice to learn is inversely proportional to number of trials required to fulfill the avoidance criteria.

Cardiac perfusion, euthanasia, and tissue harvesting

Mice were euthanized by i.p. injections of ketamine: xylazine (10:1 wt/wt, 1.5 μL/g body wt of mice), followed with cardiac perfusion using 1X phosphate buffer saline. Each harvested brain was cut in three parts. Rostral half of one hemisphere was used for RNA isolation and subsequent gene expression testing.

RNA isolation, cDNA synthesis, and real-time PCR

Tissue was homogenized with Trizol (Sigma Aldrich, MO). The homogenates were centrifuged to remove debris and viscous supernatants were used for RNA isolation. Total RNA was isolated using phenol-chloroform extraction method and then treated with DNAse (Thermo Fisher, Waltham, MA) as per manufacturer's instructions to remove residual genomic DNA contamination. Then, RNA was reverse transcribed into cDNA, which was then used for quantitative PCR measurements. Taqman gene expression assays for interleukin (IL)-1β (Cat no. Mm00434228), tumor necrosis factor (TNF)-α (Cat no. Mm00443258), CCL2 (Cat no. Mm00441242), TIMP-1 (Cat no. Mm01341361), TIMP-2 (Cat no. Mm00441825), MMP-2 (Cat no. Mm00439498), MMP-9 (Cat no. Mm00442991), GFAP (Cat no. Mm01253033), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cat no. 43-523-39E) and phosphoglycerate kinase (PGK)-1 (Cat no. Mm00435617) were purchased from Fisher (Waltham, MA). PGK-1 was used as a housekeeping control for all targets except Tat. For Tat mRNA measurements, mRNA

was isolated from total RNA using dynabeads mRNA direct purification kit (Cat no. 61012). Primers used for Tat detection were- 5' ggaagcatccaggaagtcag 3' and 5' ggaggtgggttgctttgata 3' with 5' cctcctcaaggcagtcagac 3' used as probe. Tat mRNA expression was evaluated using fast virus 1-step mastermix (Thermo Fisher, Waltham, MA). GAPDH was used as an internal housekeeping control. The data was analyzed to calculate fold changes in gene expression.

Statistical analysis

All data are presented as mean ± SEM. Differences between mean values were determined using analyses of variance (ANOVA). The data were subjected to two-way ANOVA, with Treatment Time (acute versus prolonged) and Strain (WT versus GT-Tg) as between group factors. Water maze and body weight data were subjected to three-way ANOVA, with Treatment Time and Strain as between-group factors, and Weeks or Session as within group factors. Planned individual comparisons between groups were made using the single degree-of-freedom F tests. The alpha level was set at 0.05 for all analyses. Behavioral statistics were done using Systat 13 and gene expression statistics were done using GraphPad Prism 7.0.

2.4 Results

Tat gene expression

Tat gene expression was quantified in mRNA isolated from GT-Tg mouse brain tissues using one-step PCR. Relative *tat* gene expression was higher in acutely Tat-expressing GT-Tg brains compared to prolonged Tat-expressing GT-Tg brains (**Figure 2.1B**) ($^{\#}p<0.05$). Tat expression was not detected in WT brain tissues (data not shown).

EPM

Anxiety was measured using the percent time spent in the open arms of the EPM (**Figure 2.2A**). Acutely Tat-expressing GT-Tg mice spent less time than their WT in the open arms, while there was no significant difference in the prolonged Tat expressing GT-Tg mice compared to their WT controls. Overall, the DOX chow-fed mice had lower times in the open arms than the DOX-injected mice. These observations were supported by an ANOVA which revealed significant main effects of Treatment Time ($p=0.002$) and Strain ($p=0.014$) on the percent time spent in the open arms, however, there was no significant interaction between the two factors ($p=0.17$).

Distance travelled by the mice in the maze was measured to determine if activity affected the time spent in the arms (**Figure 2.2B**). There was no difference between any of the groups which was supported by a lack of significant main effects of Treatment Time ($p=0.34$), strain ($p=0.34$), or their interaction ($p=0.59$).

LMA

The effect of Treatment Time and strain on the horizontal, vertical, and spatial components of spontaneous activity are presented in **Figure 2.3.** The horizontal distance travelled by the mice during the test was differentially affected depending on the Strain and Treatment (**Figure 2.3A**). The horizontal distance travelled by the mice during the test was differentially affected depending on the Treatment Time and Strain (**Figure 2.3A**). Acutely Tat-expressing GT-Tg mice travelled comparable distance to their WT, however prolonged Tat-expressing GT-Tg mice travelled lower distance compared to their Treatment Time and Strain counterparts. Two-way ANOVA revealed significant main

effect of Treatment Time (p=0.03), but no effect of Strain (p=0.2) or interaction of the two factors (p=0.07).

The number of rearing counts served as the measure for vertical activity and is presented in **Figure 2.3B**. Acutely Tat-expressing GT-Tg mice had higher rearing counts compared to their WT and prolonged Tat-expressing GT-Tg mice. There was no difference between the two WT groups. These findings reflected in the two-way ANOVA, which depicted significant main effect of Treatment Time (p=0.03), interaction of the two factors (p=0.04), however there was no effect of strain (p=0.18).

In **Figure 2.3C**, the amount of time spent in the center of the apparatus is presented. In both acute and prolonged treatment groups, the time spent in the center was similar between WT and GT-Tg. The WT from both treatment groups also did not differ, however the acutely treated GT-Tg mice spent more time in the center than the prolonged treated GT-Tg mice. A two-way ANOVA revealed main effect of Treatment Time (p=0.03), and no effect of strain (p=0.322) or interaction of the two factors (p=0.32).

MWM

Spatial memory was assessed measuring path length and swim speed of mice to locate a hidden platform under the water surface **(Figure 2.4)**. All mice learned to locate the platform across sessions, and a similar pattern of learning efficiency was observed across the groups. This was supported by a significant effect of Session ($p<0.01$) and a lack of interaction between Session and any other factors (all ps >0.4) following a repeated measure ANOVA (**Figure 2.4A**). The prolonged Tat-expressing GT-Tg mice seemed to take longer path length at almost every session than any of the other groups, which was supported by a main effect of Strain approaching significance (p=0.09). There

was no main effect of Treatment Time (p=0.5) or an interaction between the two factors (p=0.4).

Swim speed was analyzed and is presented in **Figure 2.4B.** The swimming speed of the mice varied across sessions (p<0.01), but the other factors did not have an effect across sessions (all ps >0.2). The GT-Tg mice swam faster than the WT (lines with squares versus lines with circles) and overall the DOX-injected group (solid lines) swam faster than the DOX chow-fed group (dashed lines). This observation was supported by a main effect of treatment time (p=0.049) and Strain (p<0.001) but not interaction between the two factors (p=0.676).

Discrimination reversal (T-Maze)

Data from the first session of the discriminated avoidance task (TTC1) represent a measure of learning/acquisition and the subsequent two sessions a measure of cognitive flexibility (TTC2, TTC3) (**Figure 2.5**). During the acquisition session, there was no effect of strain or treatment on the number of trials taken to reach criterion, even though the prolonged Tat-expressing GT-Tg mice seem to take more. A two-way ANOVA failed to show main effects of Treatment Time (p=0.29) and Strain (p=0.19), or their interaction (p=0.59). During the first reversal session (TTC2), the prolonged Tat-expressing GT-Tg mice took more trials compared to all other groups. This was supported by a main effect of Strain (p=0.02), along with no effect of Treatment Time or an interaction (all ps>0.2). In the last reversal session, the DOX-injected GT-Tg mice took less trials than the WT, while the DOX chow-fed GT-Tg mice took more trials than the WT to reach criterion. This observation was supported by a significant interaction between Strain and Treatment Time (p=0.04).

Gene expression of inflammatory markers

The gene expressions of IL-1β, TNF-α, CCL2, and GFAP were measured in the brains harvested from mice post-behavior tests to assess neuroinflammation (**Figure 2.6**). Proinflammatory cytokines IL-1β, TNF-α, and CCL2 mRNA levels were comparable among all treatment groups (**Figures 2.6A-C**). Subsequently, a two-way ANOVA failed to show main effects of Treatment Time and Strain or their interaction on gene expression of all tested inflammatory cytokines (all ps>0.3). GFAP, a marker of reactive astrogliosis, was marginally increased in acute and prolonged Tat expressing mice compared to their respective WT controls. Further, acutely Tat expressing mice showed significantly higher GFAP mRNA expression when compared to prolonged Tat-expressing mice (**Figure 2.6D**). This was reflected in main effect of Strain approaching significance ($p=0.06$), however, there was no effect of Treatment Time ($p=0.1$) and its interaction with Strain ($p=0.3$) on GFAP gene expression.

Gene expression of MMP/TIMP balance components

To elucidate MMP/TIMP balance in GT-Tg mice, gene expression profiles of TIMP-1, TIMP-2, MMP-9, and MMP-2 were analyzed (**Figure 2.7**). Among inducible proteins regulating the MMP/TIMP axis, TIMP-1 mRNA levels increased significantly in acute Tat-expressing mice compared to their WT controls, while prolonged Tat expressing GT-Tg mice showed comparable expression to their respective WT mice (**Figure 2.7A**). More importantly, TIMP-1 expression was significantly lower in prolonged Tat-expressing GT-Tg mice compared to their acute Tat-expressing counterparts. These trends were reflected in the two-way ANOVA, which showed main effects of Treatment Time ($p=0.02$) and Strain ($p=0.04$) as well as their interaction on TIMP-1 gene expression

(p=0.01). Acutely Tat-expressing GT-Tg mice had higher TIMP-2 mRNA expression compared to their WT control (**Figure 2.7B**). The levels of MMP-9 and MMP-2 were higher in GT-Tg mice compared to their WT (**Figures 2.7C-D**). A two-way ANOVA indicated a main effect of Stain (all ps<0.01) on TIMP-2, MMP-9, and MMP-2 expression, however, there were no effects of Treatment Time and its interaction with Strain (all ps> 0.13). The changes in mRNA expression were also analyzed as MMP/TIMP ratios (**Table 2.2**). Both MMP-2 and MMP-9 ratios with TIMP-1 indicated relative increase in MMPs in the prolonged Tat-induction paradigm compared to acutely Tat-expressing GT-Tg mice. On the other hand, MMP-2 ratio with TIMP-2 was high in acutely and prolonged Tat-expressing GT-Tg mice compared to respective WT controls, however it was not different across Treatment Time for the same Strain.

2.5 Discussion and Conclusion

This study investigated the behavioral deficits associated with Tat expression in the GT-Tg mouse model concurrent with changes in brain MMP/TIMP balance. Two DOX-administration methods *i.e.* i.p. injections and food were used to induce acute versus prolonged Tat expression, respectively. We showed that Tat induction in GT-Tg mice led to mild behavioral deficits when compared to WT controls including one or more of the following trends 1) higher anxiety levels depicted by reduced time spent in the open arms of EPM 2) altered ambulation during LMA and 3) longer path lengths and higher swim speeds in MWM and 4) higher number of trials to reach avoidance criteria in the T-maze. Simultaneously, significant changes in the gene expression of components maintaining the brain MMP/TIMP axis were observed in GT-Tg mice compared to their WT controls.

Further, the duration of Tat exposure changed MMP/TIMP ratio, which was primarily driven by changes in TIMP-1 expression.

Acute Tat expression was induced using DOX i.p. injection method, similar to previous studies investigating Tat-mediated behavioral changes in GT-Tg mice.[71,72,149] Additionally, prolonged Tat expression was induced *via* food, which has not been used in this model prior to behavior studies. We anticipated that higher Tat expression would correspond with increased behavioral impairments. In this context, the relative Tat expression was higher in DOX i.p. injected GT-Tg mice as compared to DOX chow administered GT-Tg mice. However, the behavioral deficits did not correspond directly with relative Tat levels. We infer that the Tat levels remained mildly elevated for a longer duration in the DOX food paradigm, resulting in consistent impairments as compared to i.p. injection method. Nevertheless, since none of the previous reports utilized two different methods in the same study to induce Tat expression, how induction method and resulting Tat expression may affect final outcomes must be interpreted carefully.

Consistent with previous studies, we report higher anxiety and lower ambulation in GT-Tg mice compared to WT.[71,153] However, the underlying intracellular mechanism driving these behavioral changes are not well established. In this context, increased MMPs expression, such as MMP-9, may be playing a role elevating anxiety and lowering ambulation. Past investigations support this claim as MMP-9 knockout exhibited lower anxiety in EPM and higher vertical activity in an open field test.[141] However, further investigations would be required to elucidate the role MMP-9 and the associated intracellular pathways.

The GT-Tg mice showed spatial memory and learning deficits following DOX i.p. injections.[149] We used MWM and T-maze to study spatial learning and memory, respectively. Though robust neurocognitive decline was not observed in our studies, prolonged Tat expressing mice exhibited mild spatial learning and memory impairments. Concomitantly, TIMP-1 expression was reduced with longer Tat expression compared to acute Tat expression, while MMP-9 remained comparable across Treatment Time in GT-Tg mice. Previous studies depicted that TIMP-1 impacted memory and cognition using an olfactory maze test[142,143] and MMP-9/TIMP-1 balance was associated to altered neuronal plasticity and memory.[140,144] Collectively, we propose that altered MMP/TIMP axis could be associated with behavioral changes observed in this study.

We and several others depicted inflammatory biomarkers TNF-α- or IL-1β-mediated MMP and/or TIMP expression regulation.[63,83,101,154] Further, upregulation of TNF-α was linked to Tat-mediated neurotoxicity.[155] A recent study in a similar Tat-transgenic model demonstrated increase in TNF-α, IL-1β, CCL2 gene expression in the cortex with prolonged Tat expression.[156] Contrary to these findings, the basal levels of TNF-α, IL-1β, and CCL2 were comparable to respective WT controls in our studies. It is possible that increased mRNA expression of these inflammatory mediators was not observed as RNA was isolated from hindbrain and not from frontal cortex as done in the previous study. Alternately, these results suggest that Tat expression may not regulate IL-1β and TNF$-\alpha$ transcriptionally in this model.

To our knowledge, this is a first study documenting changes in MMP/TIMP axis concurrent with behavioral deficits in the context of HAND. We observed that levels of MMP-9 and TIMP-2 remained elevated compared to WT controls during acute and

prolonged Tat induction. On the other hand, levels of MMP-2 and TIMP-1 reduced in prolonged Tat expression compared to acute Tat expression. These changes are reflected in the MMP/TIMP ratios calculated based on fold changes in each MMP and TIMP gene expression. It was evident that ratios of both MMPs with TIMP-2 did not change suggesting it does not play a critical role in maintaining the MMP/TIMP balance. On the other hand, TIMP-1 emerged as the key regulator in maintaining the MMP/TIMP balance as its ratio with both MMP-9 and MMP-2 increased in prolonged Tat expression.

Previous reports documented that Tat-induced behavioral impairments were sex dependent and males were more vulnerable than female mice.[74,153,157] Further, direct intervention with progesterone reduced anxiety-like effects in ovariectomized female GT-Tg mice indicating direct effect of sex hormones in modulating Tat-induced behavioral impairments.[71] In this context, we performed a preliminary analysis of data to evaluate sex-based differences in behavior (data not shown). It was observed that characteristic behavioral deficit phenotype was prominent in male mice compared to female mice. However, further testing with more mice and thorough analysis will be required to evaluate these trends further.

Though our study used distinct Tat induction methods with DOX i.p. injections and DOX food, potential caveats in our methods must be discussed. In previous reports, DOX inhibited MMPs and one of the proposed mechanisms include transcriptional inhibition [158]. Since we used appropriate WT controls that were either injected or fed with DOX, it potentially eliminated DOX-specific effects from interpretation. In this context, significant changes in MMPs observed in the data emphasize the potential role of MMP/TIMP axis during Tat-mediated neurotoxicity. We observed significant weight loss in DOX injected

WT and GT-Tg mice over the duration of the study. On the other hand, the DOX-fed mice weighed higher overall potentially due to presence of maltodextrin used as a binder in the manufacturing of DOX chow. One may argue that weight could be a confounding variable in the context of behavioral studies; however, we noticed possible differences only in swim speed in MWM. The DOX fed mice swam at a slower speed compared to their injection counterparts. However, the Overall, GT-Tg mice swam faster than their respective WT controls. In EPM, DOX-fed mice spent less time in the open arms of the maze compared to DOX-injected mice indicating that long-term DOX treatment may increase anxiety. Yet, the GT-Tg mice showed higher anxiety in DOX-fed mice indicating that the Tat expression increased anxiety. Overall, our WT controls for both acute and prolonged induction isolated DOX-specific effects. Lastly, shorter induction periods of 2 to 12 weeks have been used effectively in a similar Tat model,[33,74] we were unable to observe a distinct behavioral phenotype in our model with shorter durations of DOX feed (data not shown).

Conclusion

Overall, our data depicts that Tat expression tipped the MMP/TIMP axis towards MMPs, which may be associated with behavioral impairments observed in GT-Tg mice. There are multiple directions to pursue these investigations further, yet, we are particularly excited about the changes observed in TIMP-1. Consistent with our previous work, TIMP-1 expression increased with acute Tat expression and was reduced in comparison during prolonged Tat expression.[99,101] Considering the MMP-independent neuroprotective functions of TIMP-1,[82,86,87,159], these findings support our hypothesis of TIMP-1 restoration as a therapeutic strategy to prevent neurodegeneration in chronic neuroinflammatory

conditions such as HAND. In this context, we recently demonstrated a polymer-mediated gene delivery system for human astrocytes.[160]. Astrocytes are the primary producers of TIMP-1 in the brain following injury or inflammatory stimuli. Thus, a tested, efficient, astrocyte-targeted gene therapy approach to modulate TIMP-1 would be the most logical next step. These studies provide a viable model to test therapeutic potential of TIMP-1, which may have broader implications not just for HAND but other neurodegenerative conditions as well.

2.6 Figures and Figure Legends

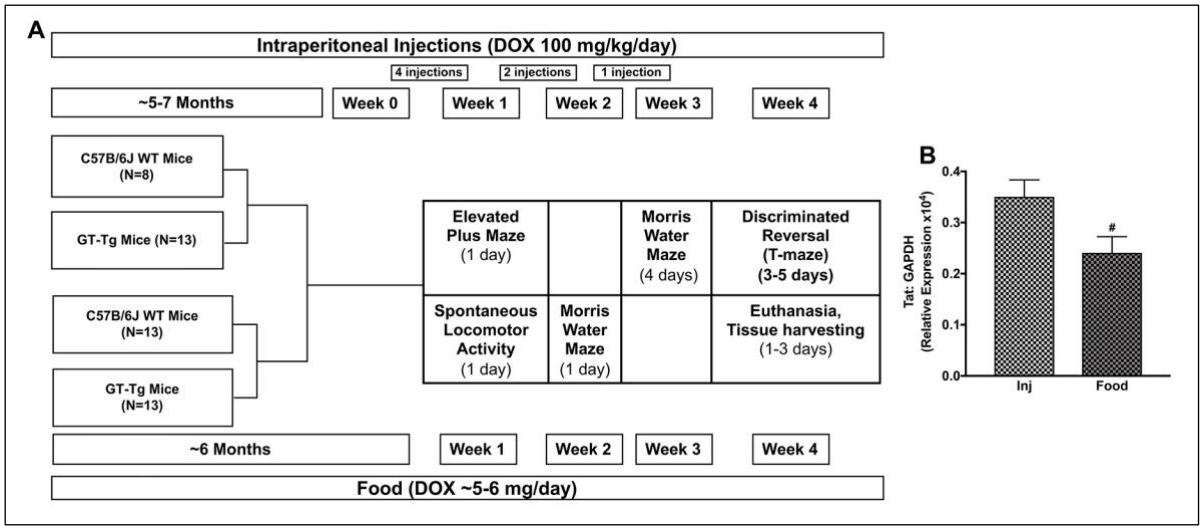


Figure 2.1 Experimental schematic and acute versus prolonged *Tat* expression.

(**A**) Five to seven months old WT and GT-Tg mice of both sexes were administered doxycycline (DOX) *via* intraperitoneal (i.p.) injections (100 mg/kg/dose) or DOX-containing food *ad libitum* (about 5-6 mg DOX/day). A total of seven i.p. injections were given over three weeks prior to and during the behavior testing to mice. DOX food was fed for about seven months to mice (six months prior to and during behavior testing) starting at four to five weeks of age. The behavior testing was carried out over four weeks. After the behavior testing, mice were euthanized, and their brains were harvested to isolate RNA and gene expression was measured for multiple targets. (**B**) Tat mRNA expression was measured in GT-Tg mice using one-step RT^2-PCR. GAPDH was used as an internal housekeeping control. The GT-Tg mice received acute Tat induction by DOX i.p. injections (n = 11, light gray patterned bar) and prolonged Tat induction *via* DOX food (n = 13 dark gray patterned bar). Each bar represents the mean ± SEM. $^{\#}p<0.05$ by unpaired T-test.

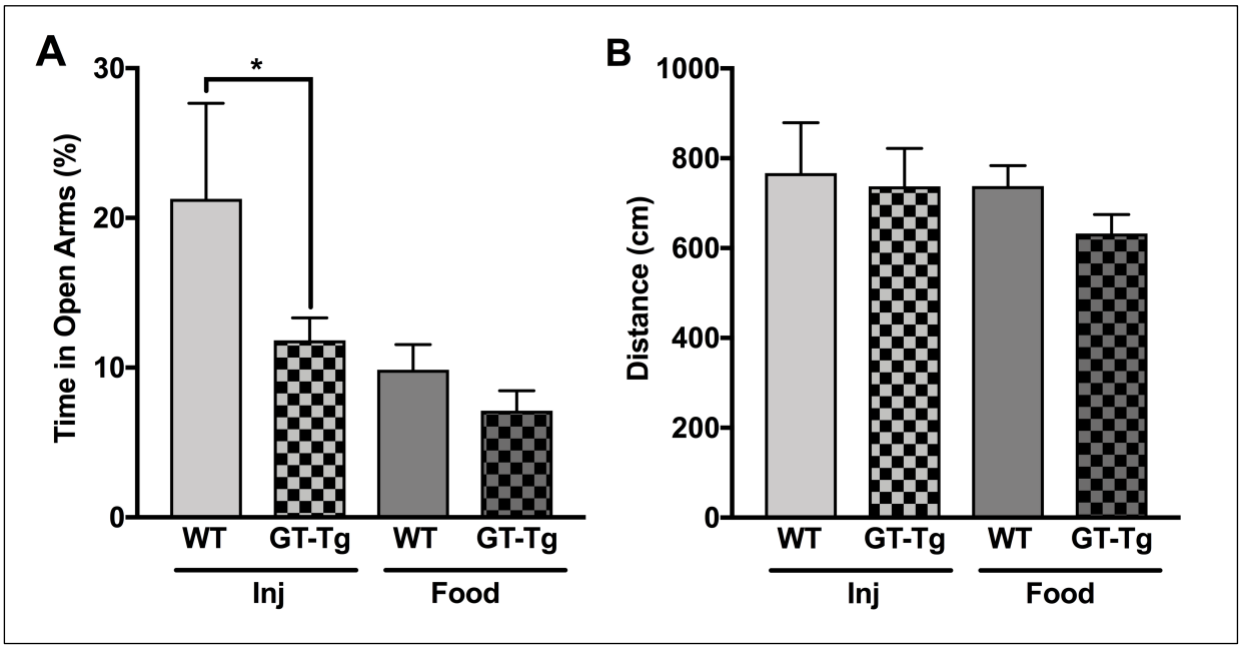


Figure 2.2 Higher anxiety levels were observed in GT-Tg mice compared to Treatment Time WT controls in elevated plus maze testing. Anxiety levels were measured by (**A**) the percent time spent in the open arms and (**B**) total distance covered on the maze in GT-Tg and C57BL/6 (WT) mice. The WT mice (*n* = 6, light gray bars) and the GT-Tg mice (*n* = 12, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 13, dark gray bars) and the GT-Tg mice (*n* = 13, dark gray patterned bars) received prolonged induction *via* DOX food. Each bar represents the mean ± SEM. *p<0.05 for same Treatment Time comparisons between strains by two-way ANOVA using Treatment Time and strain as variables.

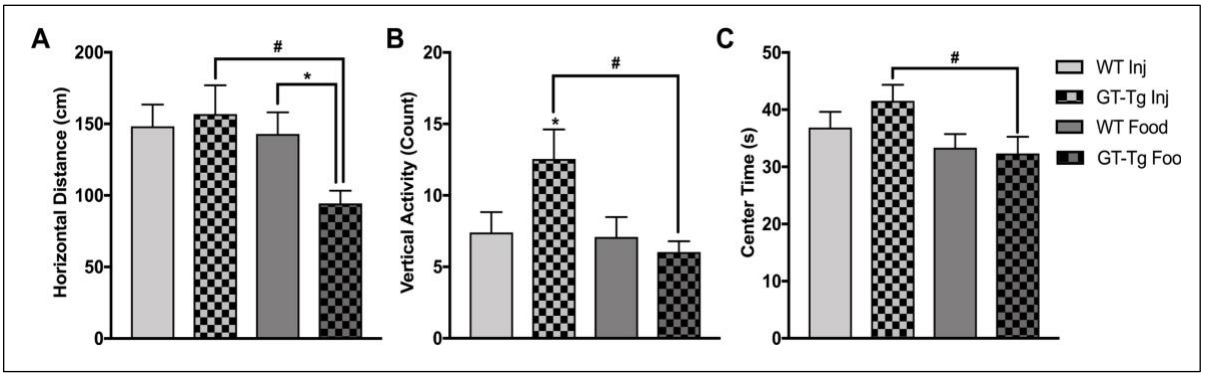


Figure 2.3 Prolonged HIV-1 Tat expression led to slower ambulation, reduced vertical activity, and reduced center time compared to acute Tat expression in GT-Tg mice. (**A**) Horizontal distance, (**B**) vertical activity, and (**C**) center time in GT-Tg and C57BL/6 (WT) mice were measured. Average per minute values for each parameter were plotted. The WT mice (*n* = 7-8, light gray bars) and the GT-Tg mice (*n* = 10-11, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 13, dark gray bars) and the GT-Tg mice (*n* = 13, dark gray patterned bars) received prolonged induction *via* DOX food. Each bar represents the mean ± SEM. TD: total distance, VAC: vertical activity count, CT: center time. *$p<0.05$ for same Treatment Time comparisons between strains, $^{\#}p<0.05$ for same strain comparisons across Treatment Time by two-way ANOVA using Treatment Time and strain as variables.

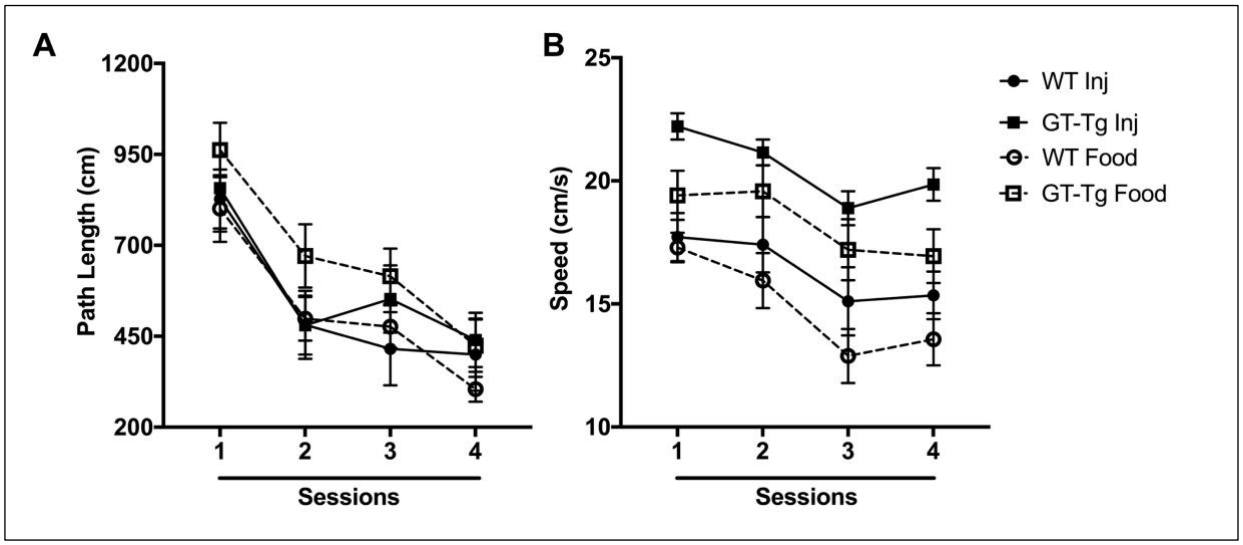


Figure 2.4 Morris water maze performance indicated higher swim speeds in GT-Tg mice during acute versus prolonged HIV-1 Tat expression. (**A**) Path length and (**B**) swimming speed were assessed in GT-Tg and C57BL/6 (WT) mice. The test was conducted over four sessions. Each data point represents the mean ± SEM from an average of four trials per session. One session is equivalent to one day. The WT mice (*n* = 7, solid line with circles) and the GT-Tg mice (*n* = 11, solid line with squares) received acute induction by DOX i.p. injections, while the WT mice (*n* = 13, dashed line with open circles) and the GT-Tg mice (*n* = 13, dashed line with open squares) received prolonged induction *via* DOX food.

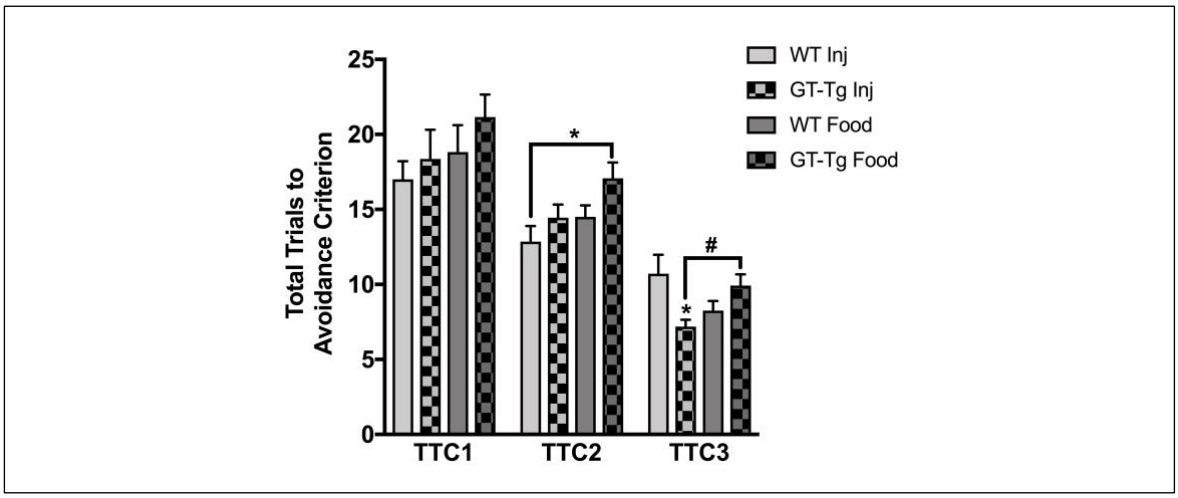


Figure 2.5 Prolonged HIV-1 Tat expression increased trials to reach avoidance criteria in GT-Tg mice in discrimination reversal test. The T-maze test measured the number of total trials taken by GT-Tg and C57BL/6 (WT) mice to reach discriminated avoidance criteria during three sessions. The WT mice (*n* = 7, light gray bars) and the GT-Tg mice (*n* = 11, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 12, dark gray bars) and the GT-Tg mice (*n* = 13, dark gray patterned bars) received prolonged induction *via* DOX food. Each bar represents the mean ± SEM, TTC: total trials to criteria, TTC1: Acquisition session, TTC2, TTC3: Reversal session, *p<0.05 for same treatment comparisons between strains, $^{\#}$p<0.05 for same strain comparisons across Treatment Time by two-way ANOVA using treatment and strain as variables.

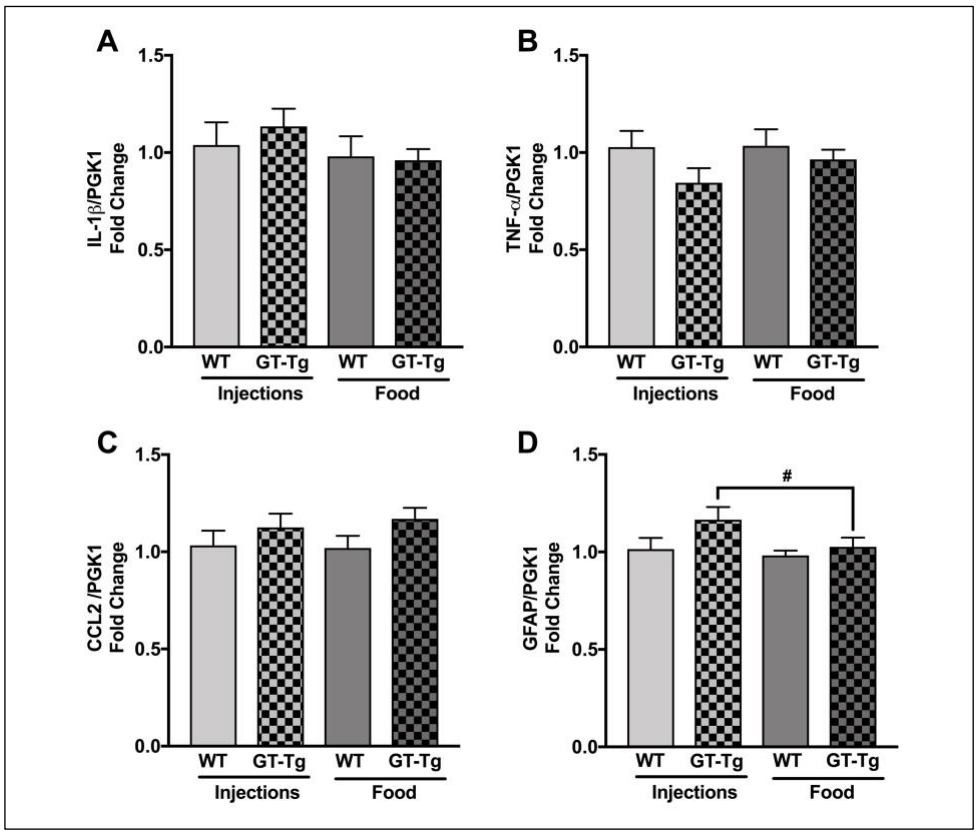


Figure 2.6 Inflammatory biomarkers gene expression remained unaltered with acute and prolonged HIV-1 Tat expression in GT-Tg mice. (**A**) IL-1β, (**B**) TNF-α, (**C**) CCL2, (**D**) GFAP mRNA expression was measured in GT-Tg and C57BL/6 (WT) mice. The WT mice (*n* = 7, light gray bars) and the GT-Tg mice (*n* = 11, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 12-13, dark gray bars) and the GT-Tg mice (*n* = 12-13, dark gray patterned bars) received prolonged induction *via* DOX food. Each bar represents the mean ± SEM. ***p<0.001 for same treatment comparisons between strains, $^{\#\#}$p<0.01 for same strain comparisons across treatments by one-way ANOVA.

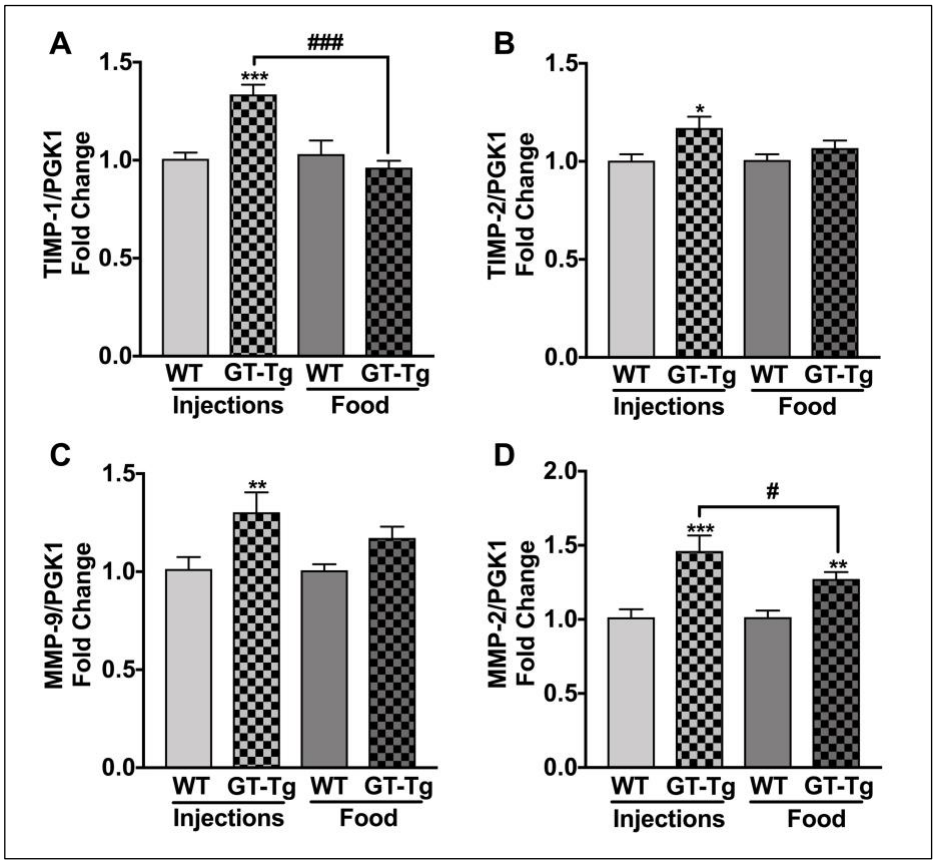
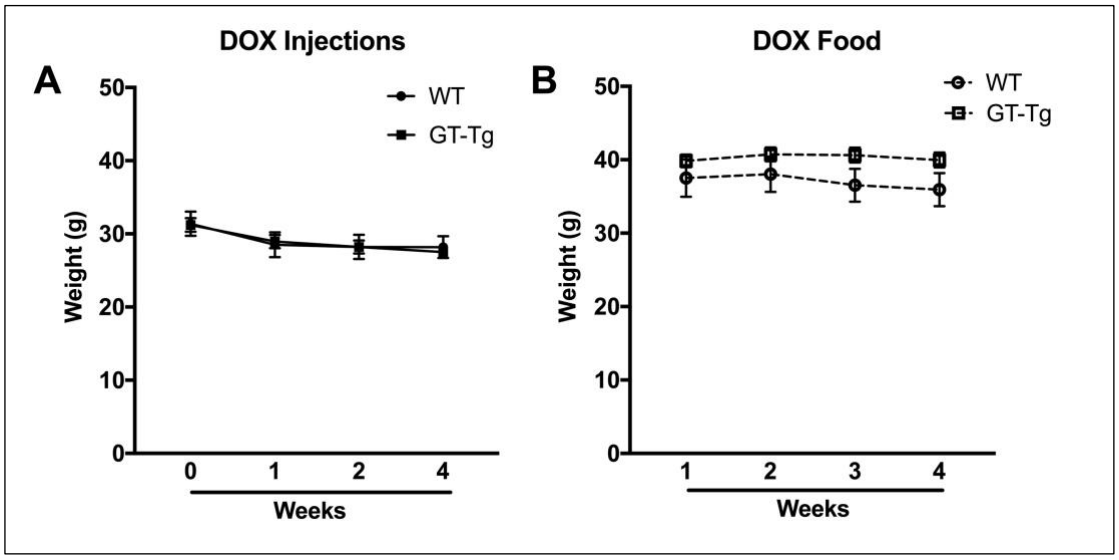


Figure 2.7 Acute versus prolonged HIV-1 Tat induction dysregulates of MMP/TIMP balance in GT-Tg mice. TIMP-1 (**A**)**,** TIMP-2 (**B**)**,** MMP9 (**C**)**,** and MMP2 (**D**) in GT-Tg and C57BL/6 (WT) mice. The WT mice (*n* = 7, light gray bars) and the GT-Tg mice (*n* = 11, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 12-13, dark gray bars) and the GT-Tg mice (*n* = 12-13, dark gray patterned bars) received prolonged induction *via* DOX food. Each bar represents the mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 for same treatment comparisons between strains, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 for same strain comparisons across treatments by one-way ANOVA.



Supplementary Figure 2.1 Animal weights measured every week during behavioral testing. Weights were measured every week in both WT and GT-Tg mice that were injected (**A**) or fed (**B**) DOX. Each data point represents the mean ± SEM. For acute DOX treatment by i.p. injections, The WT mice (*n* = 7, solid line with circles) and the GT-Tg mice (*n* = 11, solid line with squares) received acute induction by DOX i.p. injections, while the WT mice (*n* = 13, dashed line with open circles) and the GT-Tg mice (*n* = 13, dashed line with open squares) received prolonged induction *via* DOX food.

Table 2.2 MMP/TIMP ratios in acute and prolonged DOX-treated WT and GT-Tg mice

	WT Inj	GT-Tg Inj	WT Food	GT-Tg Food
MMP-9/TIMP-1	1.01	0.95	1.03	1.24[b]
MMP-2/TIMP-1	1.01	1.05	1.03	1.34[a,b]
MMP-9/TIMP-2	1.01	1.13	1.01	1.11
MMP-2/TIMP-2	1.01	1.26[a]	1.01	1.20[a]

TIMP-1, TIMP-2**,** MMP9**,** and MMP2 mRNA fold change values presented in Figure 7 were used to obtain the above ratios in GT-Tg and C57BL/6 (WT) mice. The WT mice (*n* = 7, light gray bars) and the GT-Tg mice (*n* = 11, light gray patterned bars) received acute induction by DOX i.p. injections, while the WT mice (*n* = 12-13, dark gray bars) and the GT-Tg mice (*n* = 12-13, dark gray patterned bars) received prolonged induction *via* DOX food. Each cell represents the mean ratio for the treatment group. [a]$p<0.05$ for same treatment comparison to WT controls, [b]$p<0.05$ for same strain comparisons across Treatment Time by one-way ANOVA.

CHAPTER 3

DESTINATION BRAIN: THE PAST, PRESENT, AND FUTURE OF THERAPEUTIC GENE DELIVERY

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3.1 Abstract

Neurological diseases and disorders (NDDs) present a significant societal burden and currently available drug- and biological-based therapeutic strategies have proven inadequate to alleviate it. Gene therapy is a suitable alternative to treat NDDs compared to conventional systems since it can be tailored to specifically alter select gene expression, reverse disease phenotype and restore normal function. The scope of gene therapy has broadened over the years with the advent of RNA interference and genome editing technologies. Consequently, encouraging results from central nervous system (CNS)-targeted gene delivery studies have led to their transition from preclinical to clinical trials. As we shift to an exciting gene therapy era, a retrospective of available literature on CNS-associated gene delivery is in order. This review is timely in this regard, since it analyzes key challenges and major findings from the last two decades and evaluates future prospects of brain gene delivery. We emphasize major areas consisting of physiological and pharmacological challenges in gene therapy, function-based selection of an ideal cellular target, available therapy modalities, and diversity of viral vectors and nanoparticles as vehicle systems. Further, we present plausible answers to key questions such as strategies to circumvent low blood-brain barrier permeability, most suitable CNS cell types for targeting. We compare and contrast pros and cons of the tested viral vectors in context of delivery systems used in past and current clinical trials. Gene vector design challenges are also evaluated in the context of cell-specific promoters. Key challenges and findings reported for recent gene therapy clinical trials, assessing viral vectors and nanoparticles, are discussed in the context of bench to bedside gene therapy translation. We conclude this review by tying together gene delivery challenges, available vehicle

systems and comprehensive analysis of neuropathogenesis to outline future prospects of CNS-targeted gene therapies.

3.2 Introduction

A comprehensive global burden of disease study indicated that years lived with disability increased by 59% for neurological disorders and by 83% for cerebrovascular disease from 1990 to 2013.[161] These neurological disorders include Alzheimer's disease (AD) and other dementias, Parkinson's disease (PD), and multiple sclerosis (MS), while ischemic and hemorrhagic stroke constitute cerebrovascular disease. Neurological and cerebrovascular conditions are discussed in this review as neurological diseases and disorders (NDDs) in the context of therapeutic gene delivery. Huntington's disease (HD), amyotrophic lateral sclerosis (ALS) and human immunodeficiency virus (HIV)-associated neurocognitive disorders (HAND) are also included in the NDDs spectrum. Available drug- and biological-based therapeutic strategies are inadequate to treat or cure NDDs. Several studies are testing safe, effective, non-invasive, therapeutic strategies for NDDs by attempting delivery of drugs,[162] proteins,[84] and genes[163] to cure disease or to slow its development. NDD progression could potentially be halted or reversed by targeting a single cell type, for instance neurons or astrocytes. Gene therapy is highly promising in this regard as it could be tailored for transient or long-term gene expression in specific cell types.

Researchers have actively investigated gene-based NDD therapies in the last two decades, and genes have been delivered successfully to central nervous system (CNS) cells.[164,165] Conventionally, challenges towards brain-targeted therapeutic delivery include low blood-brain barrier (BBB) permeability, brain heterogeneity, route of

administration and dosing. Though these challenges are common for drugs and genes, unique modifications to gene delivery vehicles could potentially circumvent the challenges (**Section 3.3**). In addition to tackling the physiological and pharmacological problems, the principal questions related to gene delivery have revolved around obtaining an ideal cellular target, suitable therapeutic gene(s), and an efficient gene delivery vehicle. Oligodendrocytes and microglia play key role(s) in the neuropathogenesis of NDDs, yet treatments have centered on neurons and astrocytes. We have analyzed the functional features and disease roles of these cell types to ascertain their suitability as cellular target(s) (**Section 3.4**). Gene therapy modalities increased after the advent of silencing by RNA interference (RNAi) technology and genome editing endonuclease systems in addition to augmentation. The pros and cons of each approach are discussed here from the perspective of NDDs (**Section 3.5**). Vectors derived from adenoviruses, adeno-associated viruses (AAV), and lentiviruses as well as non-viral transport systems, such as polymeric nanoparticles (NPs) and lipid complexes, are under investigation as vehicles for CNS gene delivery. Evaluating expression efficiency, duration, and targeting specificity is critical to validate a suitable delivery system (**Section 3.6**). Therapeutic genes driven by cell-specific promoters have been utilized to restrict expression. The selection of promoter during vector design process has evolved over time and unique trends for neural cell types have been examined (**Section 3.7**). Recent gene therapy clinical trials are evaluating safety and efficacy of novel viral vectors and nanoparticles. The findings reported thus far for these trials must be analyzed in the context of 'bench to bedside translational' challenges to postulate directions for future gene therapy research **(Section 3.8)**. To conclude, we present a synopsis of the overall trends

observed for each aspect of CNS-targeted gene delivery systems and propose future directions (**Section 3.9**).

3.3 Challenges towards CNS-targeted gene delivery

Effective gene delivery could restore normal cell function during NDDs by providing transient or long-term expression of the gene of interest, eliminating the need for frequent drug administration and continual penetration of the BBB. An ideal gene delivery system development would involve *in vitro* testing of delivery vehicle under consideration of biocompatibility, efficacy, dose-time kinetics, and sustained expression. Subsequently, *in vivo* studies would evaluate dosing, route of administration, and immune responses in addition to the same *in vitro* assessments followed by transition into clinical trials. However, each step of this process contains its own challenges. For example, our ongoing studies have revealed surprising difficulty in polymeric NP-mediated gene delivery to primary human neurons and astrocytes, let alone targeting them *in vivo*. Currently, we are looking into the NP-uptake mechanisms in these cells to determine feasible alternatives and to overcome gene expression challenges (unpublished data). Further, transgenic animal models [Reviewed by[166,167]] fail to mimic the complexities of the NDD-associated mechanisms making therapeutic assessments difficult. Diverse hosts (mouse, rat, rabbit, human), coupled with age differences have resulted in varying gene expression levels in preclinical studies.[168,169] Finally, the immunologically privileged status of the brain makes reaching brain cells difficult, given the low permeability of the intact BBB and complexity of brain structure. These barriers along with obstacles to routes of administration for gene therapies, and the difficulties in bench to bedside translation will be discussed in this section.

3.3.1 Low blood-brain barrier permeability

In the absence of trauma or disease, tight junctions between brain microvascular endothelial cells (BMVECs) covered by astrocyte foot processes seal the majority of the brain to peripheral immune surveillance and passive diffusion of water-soluble drugs.[170] The intricate vasculature of the brain promotes global perfusion since all brain cells are within 20 µm of a blood capillary.[171] Therefore, intravascular gene delivery systems could permeate the entire brain if the BBB can be traversed. Low permeability of the BBB remains the primary physical challenge and rate-limiting step for targeting brain cells. An ideal vascular gene delivery system would have very small size, high lipophilicity, and low plasma protein binding.[171]

Severe neuroinflammation compromises the BBB by activating BMVECs and astrocytes, increasing cellular adhesion molecule expression and decreasing tight junction and extracellular matrix protein levels. Thus, BBB permeability varies in health and disease.[172] Based on this, delivering gene(s) during NDDs, such as AD and PD, may be easier since chronic neuroinflammation enhances BBB permeability. However, compromised BBB during ischemia may not be helpful for gene therapies since the therapeutic window is small and a rapidly acting therapy is required.[173] According to a report, gene delivery was efficient in fetal mice compared to older mice, attributed underdeveloped BBB.[169] As classic NDDs (*e.g.* AD) are diagnosed and treated in older patients, improved gene delivery older mice will be more relevant. Disruption of BBB to enhance delivery has been explored *via* microbubbles, ultrasound[174] and chemicals (*e.g.* mannitol);[168,175] but the clinical implications of such approaches are not known. Arginine-modified polyplexes,[165] transactivator of transcription-conjugated NPs[176] and AAV vector

serotype 9[177] crossed the BBB when injected intravenously in rodent models, indicating that tailor-made NPs with surface modifications and genetically modified viral vectors with particular capsid sequences can overcome the BBB. Further, off-target gene expression in peripheral organs could be prevented by inclusion of cell-specific promoters (**discussed in Section 3.7**).

Gene vectors have been injected directly into the brain to circumvent the BBB.[178,179] The innate difficulty and risk during brain administration makes this method less applicable over long-term clinical trials. Convection-enhanced delivery, in which one or more catheters are carefully placed in the brain parenchyma for therapeutic delivery, could be a potential solution. While elaborate, this technology is currently in Phase III clinical trials for glioblastoma treatments[180] and could be a feasible delivery mechanism for future gene-based NDD therapies.

3.3.2 Brain structure complexity

The heterogeneity of the brain rules out a 'universal' delivery system for genes. In addition, unique pathogenesis of each NDD requires a tailored vehicle system designed to deliver a gene of interest to a certain cell type for a given disease. Though genes have been injected or infused directly in the brain,[178,179,181] obtaining global gene expression has been difficult. [182,183] This is attributed to low gene or vehicle diffusion within brain parenchyma,[181] less viral vector transduction efficiency,[182] and reduced gene expression driven by weak promoters.[184] Additional difficulties ensue in diseases where a precise region is to be targeted within the heterogeneous brain structure, *e.g.* the substantia nigra (SN) in PD. Age contributes to brain complexity in the context of the extent of BBB development[169] and the ratios of glia to neurons throughout the brain parenchyma.

Some viral vectors demonstrate select cell type and brain region tropism.[185,186] For instance, AAV2 preferentially transduced neurons while AAV5 transduced astrocytes *in vitro*. *In vivo*, AAV2-mediated expression was restricted to pyramidal and granular cells and AAV5 transduced only granular cells. [185,186] Further, neurons and astrocytes could respond dissimilarly to the same biomolecules, such as heme oxygenase,[187] requiring evaluation of off-target effects. Tagging specific cell surface receptor ligands on DNA-vehicle complex can target neurons or astrocytes (Discussed further in **Section 3.7**). Thus, thorough knowledge of neuropathology, cellular responses to particular stimuli, intrinsic cell tropisms of delivery systems, and gene vector or vehicle system modifications are critical to tackle the brain heterogeneity challenge in the treatment of each NDD.

3.3.3 Route of administration

Pharmacokinetic and pharmacodynamic profiles of drugs are studied to obtain optimal dose(s) and route of administration. Gene delivery is complicated because dose and frequency of administration depends on both the gene and the delivery system. Intranasal,[188] intracarotid,[168] intravitreal,[189] intrathecal,[190] and intramuscular[191] routes of administration have been explored for brain delivery. Currently, intravenous[192] and intracerebral[128] administrations are frequently used; yet, neither is an effective 'gold standard' for CNS gene delivery. Intracerebral delivery was shown to be efficient[192], but had brain diffusion issues.[128] Low diffusion is a noted disadvantage of intracerebroventricular (ICV) delivery as well.[171] Depending on the therapeutic strategy though, limited diffusion could be beneficial when a local effect is desired.[128] Intrathecal delivery revealed promising results in rodents with spinal cord injury (SCI);[190] however, it

led to severe neurotoxic effects in monkeys.[193] Overall, the route of administration must be tailored to the therapeutic usage, while reducing associated risks and optimizing ease of use and efficacy. Intravenous and intranasal routes offer ease of administration compared to intracerebral or intrathecal routes. Therefore, research should be directed towards developing systems that could be delivered *via* intravenous or intranasal routes.

Age, weight, gender, and genetics of the examined species and the route of administration determine the NPs or viral vector dosing strategy. The transition from *in vitro* to *in vivo* studies and from mice to non-human primates (NHPs) is difficult as the delivery system design and dosing paradigms vary significantly between models.[168] Additionally, there are logistical concerns over bulk production and handling. Thorough dose-time kinetics experiments will be required to establish the therapeutic window of gene delivery for individual NDD. For instance, gene therapies for stroke would need a rapid onset of expression, or be prophylactically administered to those with a significant risk. Potential palliative measures for would require dose concentrations and frequencies tailored to particular patients based on relative risk and symptoms. Taken together, it can be discerned that the route of administration issue can be partly attributed to low BBB permeability (**Section 3.3.1**) and complex brain structure (**Section 3.3.2**); while, an optimal administration method may overcome the BBB and the brain heterogeneity challenges.

3.4 Selecting an ideal CNS cellular target for NDD therapy

Neuroinflammation and neurodegeneration are hallmarks of NDDs.[83,194-197] These hallmarks and intracellular processes that precede or succeed them involve a crosstalk

between neurons, astrocytes, microglia and peripheral blood monocytes.[198] Thus, all of these cell types could potentially serve as cellular targets for NDDs treatment.

Gene delivery challenges discussed in **Section 3.3** could be attributed to physical properties of the vehicle, *i.e.* viral vectors or NPs. Structural or physical modifications of vehicles could overcome the challenges to offer a feasible and efficient gene delivery. On the other hand, an equally important question would be which cell type would make an ideal target? In this section, we discuss some principal types of CNS cells in the context of their functions and their role in NDD pathogenesis to ascertain suitable cellular targets. A schematic of key underlying processes during NDDs and all discussed cell types along with their functional relevance is depicted in **Figure 3.1**.

3.4.1 Neurons

Structurally and functionally neurons remain the most investigated CNS cell type and therefore neuronal changes and biomarkers associated with NDDs have been well characterized. Neuronal numbers reduce in healthy aging and there is a rapid loss of certain brain region neurons during NDDs. For instance, loss of neurons in the frontal cortex, SN, and striatum occurs during AD,[199] PD,[200] and HD,[201] respectively.

Clinically, neuronal death is associated with dendritic loss, synaptic reduction and cognitive and/or motor impairment.[199] Synaptic transmission, the principal function of neurons, is altered during AD and PD.[202] Neuritic plaques, containing the 43-amino acid peptide amyloid-β (Aβ), and neurofibrillary tangles, containing tau protein, are pathological hallmarks of AD.[203] Accumulation of interneuronal cytoplasmic 'Lewy' bodies, composed of α-synuclein, parkin, ubiquitin, and neurofilaments, remain a diagnostic criterion for PD, in addition to dopaminergic neuron loss.[204] In HD, huntingtin

(htt) protein aggregates build up inside neurons.[201] Despite precise neuropathogenesis of every NDD, intracellular processes preceding or succeeding neuronal loss may overlap including mitochondrial and oxidative stress,[204] synaptic transmission dysfunction,[202] altered neurotransmitter levels[205] and changes in stimuli preventing or promoting neuronal cell death.[204] Disrupted synaptic transmission of the excitatory neurotransmitter glutamate leading to excitotoxicity has been implicated in ischemic stroke, epilepsy, MS, and HAND.[25,206] Axonal damage and subsequent impairment of axonal transport is linked to ALS[207] and MS[208] pathologies. Importantly, astrocyte or microglia or oligodendrocyte dysfunctions can cause neuronal damage by inducing the same processes listed above. Additionally, reduced expression of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF), is also implicated in NDDs and replenishing their levels is considered a potential therapeutic strategy, discussed in detail in **Section 3.5.1**.

Neuroprotection remains the ultimate aim of all therapeutic strategies either by preventing or reversing damage. Currently available palliative NDD treatments involve drugs for AD (*e.g.* acetylcholinesterase inhibitors[209] and PD (*e.g.* dopamine agonists[210], immunosuppressive therapy for MS[211], blood thinners for stroke (*e.g.* tissue plasminogen activator[173], rehabilitative care and physical therapy. Additionally, neural stem cell-based transplantations and other surgical procedures are also included in the NDD treatment repertoire. Unfortunately, these treatments are inadequate making gene therapy a promising alternative.

Neuronal gene delivery investigations have shown varying degrees of success to reverse pathogenesis.[212,213] However, targeting neurons still presents certain limitations.

The neuronal population is always reducing since they die during healthy aging, in addition to rapid loss during disease. Synaptic transmission is fastidious and must be tightly regulated. As pointed out by Drinkut and colleagues, if neurons secrete exogenous biomolecules from various cell parts, including the soma and synaptic sites, it could produce off-target, undesirable effects since axonal endings terminate on other neurons or peripheral organs.[128] Since NDDs are not diffuse or random but involve damage to a precise subset of neurons, neuronal gene delivery has always been challenging.[214] However, if gene expression could be restricted to a brain region and a strong, neuron-specific promoter is used, a low dose of exogenous DNA for neuronal expression may prove beneficial.

3.4.2 Microglia

Comprising ~10 to 15% of the total brain cells, microglia are the resident immune cells of CNS.[198] Resting microglia perform normal brain surveillance. [215,216] In acute conditions, activated microglia provide neuroprotection by releasing neurotrophic and anti-inflammatory factors including NGF, BDNF, neurotrophin-3 and GDNF, providing innate immunity, facilitating repair through guided migration of stem cells[217,218] and phagocytizing dead neurons and debris.[216]

During normal aging, microglia produce a chronic, mild inflammatory environment by secreting inflammatory cytokines interleukin (IL)-1β, tumor necrosis factor (TNF)-α, CCL2, CXCL8 and reactive nitrogen species such as nitric oxide, a potential risk factor for NDDs.[219] Triggers include extracellular adenosine triphosphate, serum factors (*e.g.* plasma thrombins), microbial agents (*e.g.* lipopolysaccharides) and pathological proteins (*e.g.* Aβ) induce microglial activation.[218] Microgliosis is another key process in chronic

inflammation, which is attributed to extravasation of circulating monocytes post-BBB disruption.[220] Neurons could inhibit the microglial activation *via* receptor-ligand interaction; an example is neuronal CD200 and its receptor on microglia;[215] however, disrupting such interactions could lead to chronic neuroinflammation.

Microglia cause neurotoxicity either as a response to external pro-inflammatory stimuli or through activation post-neuronal injury, eventually eliciting neurodegeneration by secretion of neurotoxic substances.[217] Since their responses differ drastically during mild, acute injury *versus* chronic inflammation, microglia can act as friend or foe depending on the stage and extent of injury.[215] Therefore, an ideal therapeutic approach would consist of weakening microglial responses instead of silencing them entirely.

Microglia cleared Aβ plaques in AD immunotherapy preclinical trials.[221] Non-steroidal anti-inflammatory drugs such as cyclooxygenase-2 inhibitors are being investigated to alleviate microglial activation.[222] Few studies reported that genes were successfully delivered to neurons, astrocytes, and oligodendrocytes as well as microglia.[223,224]

The window of opportunity to target microglia is very unpredictable, especially in chronic NDDs,[216] and would require timely diagnosis of particular disease and degree of microglial involvement. The extent of peripheral extravasation of monocytes during injury would be necessary to examine before targeting microglia. [220] Early detection of microglial activation will be essential to inhibit their neurotoxic effects and subsequent neuronal loss. To achieve this, sophisticated techniques such as positron emission tomography will be essential.[217]

3.4.3 Oligodendrocytes

Oligodendrocytes, a type of glial cells, are responsible for myelination of axons in the brain and spinal cord.[225] They originate as pre-progenitors in the subventricular zone (SVZ) and then migrate, proliferate, mature and start producing myelin sheaths. These processes are regulated by platelet-derived growth factors (PDGF), fibroblast growth factor 1 and 2, insulin-like growth factor-1 (IGF-1), transforming growth factor-β, neurotrophin-3, and ciliary neurotrophic factor (CNTF).[226] Oligodendrocytes electrically insulate sodium channel-clustering axons, which ensures saltatory nerve conduction.[227]

Metabolic changes in oligodendrocytes attributed to genetic defects, infections, or toxins lead to flawed myelination or demyelination.[228] Remyelination, *i.e.* formation of new myelin sheaths by oligodendrocytes, remains the intrinsic response. Remyelination does not occur in MS due to unknown causes.[229] Further, a variable loss of oligodendrocytes occurs in all MS subtypes, which includes apoptotic death in type III and non-apoptotic death in type IV. The underlying pathways linked to oligodendrocyte loss include oxidative and mitochondrial stress,[227] proinflammatory cytokine signaling, free radicals, and complement-facilitated injury.[230] Clinically, lack of myelin associated protein and 2,3-cyclic nucleotide 3-phosphodiesterase indicate oligodendropathy.[231] Oligodendrocytes are implicated in other demyelinating diseases including Marburg disease and Devic's disease.[231]

Preventing oligodendrocyte loss and increasing myelination are the two possible approaches to treat dysfunctional oligodendrocytes. Currently investigated MS treatments including immune-, cell-based-, and gene- therapies have direct implications/effects in oligodendrocytes.[232] Proposed strategies to prevent

oligodendropathy include upregulating growth factor gene expression, which regulate their maturation. However, a single growth factor is not involved in that process, posing a potential multiple gene delivery challenge. Oligodendrocyte-targeted gene delivery has been tested in studies investigating SCIs.[223,233] Overall, demyelination diseases constitute a small fraction of NDDs spectrum and oligodendrocytes are one of the least abundant cell types in the brain. Hence, they may not be the best choice to attain global CNS gene delivery.

3.4.4 Astrocytes

Astrocytes, principal glial cells of the CNS,[12] are structural components of tight junctions of the BBB and form 'tripartite' synapses with pre- and post-synaptic neurons.[234] Astrocytes produce neurotrophic factors, including BDNF, NGF, GDNF[235] and tissue inhibitor of metalloproteinases-1.[86,132] They regulate neurotransmission and synaptic activity by sequestering synaptic potassium and neurotransmitters, including glutamate. Astrocytes communicate with neighboring astrocytes *via* calcium waves and gap junctions,[236] and also by secreting a number of cytokines and chemokines including CCL2,[237] CXCL8,[238] IL-1β and TNF-α[127] that play critical roles in NDD pathogenesis. The astrocyte neurotrophic repertoire also includes antioxidant defense and metabolic support.

Neuroinflammation induces reactive gliosis whereby reactive astrocytes proliferate and migrate towards injury, leading to glial scar formation.[198,239-241] While this response is an attempt to repair the initial damage, both astrocyte and neuronal functions are compromised by chronic inflammation. Similar to microglia, astrocytes secrete neuromodulatory molecules, which can be either protective or damaging depending on

the stage of injury, making astrogliosis a 'double-edged' sword.[240] Other astrocyte intracellular processes that contribute to NDD pathologies include oxidative and mitochondrial stress,[118] reduced excitatory amino acid transporter (EAAT)2 levels that lead to excitotoxicity,[126] calcium dysregulation[114] and attenuated neurotrophin secretion.[125]

Astrocytes could be central targets for NDD gene therapies as they interact with other cell types including neurons, microglia, BMVECs, and ependymal cells throughout the brain contributing to the disease and recovery processes.[12] Their foot processes are present at the interface between the periphery and the brain, giving them preferential access to therapeutics delivered *via* vascular route. A phosphodiesterase inhibitor, Ibudilast, prevents glial activation; and clinical trials testing its potential for chronic migraine, and substance abuse disorders are underway.[242] Simultaneously, preclinical studies have been performed to develop astrocyte-directed gene delivery systems.[168,169] Clinical trials have been conducted in which astrocytes are cellular targets (**Section 3.8**). The majority of these preclinical and clinical trials intended to overexpress neurotrophin genes with or without astrocyte-specific promoter thereby mimicking inherent astrocyte neuroprotective function.

Before, focusing on astrocytes as a suitable cellular target for global CNS gene delivery, some potential issues must be discussed. Genes delivered *via* viral vectors, for example AAV, and NPs remain episomal. During chronic inflammation, these delivered genes could get diluted or lost due to astrocyte proliferation presenting astrogliosis as a "self-limiting" factor.[128] Additionally, abundant interconnected astrocytes may not be ideal for PD and stroke therapy where site-restricted gene expression is required.

Nevertheless, the fundamentally neuroprotective functions of astrocytes make them relevant. Considering the limitations stated in **Section 3.4.1** for neurons, targeting astrocytes might prove a beneficial strategy.

3.5 Gene delivery modalities

Gene delivery strategies for NDD therapies are categorized into three approaches: 1. Augmentation 2. Silencing and 3. Editing. The goal of such therapies would be to alter specific gene expression and correct disrupted CNS homeostasis. Increasing neuroprotective measures, for instance, secreted neurotrophins, or reversing neurotoxic mechanisms, such as excitotoxicity, could restore CNS homeostasis. Thus, an ideal CNS-targeted gene delivery method would increase neurotrophin levels or reduce proinflammatory biomarkers. Hereditary NDDs, such as HD, depict distinct gene mutations and require therapies to permanently correct the mutated gene, which can be achieved by gene editing. Though the names of gene therapy modalities are self-explanatory, it is essential to compare and contrast between these methods to determine their applicability in the context of NDDs.

3.5.1 Gene augmentation

Gene augmentation would be applicable when an insertion or missense mutation produces non-functional protein or when certain genes are downregulated owing to disease pathology. For CNS diseases, it has been employed for replenishing neurotrophin levels, for example BDNF, GDNF, and NGF. All neurotrophins, except BDNF, are constitutively expressed in the brain. Neurotrophin levels are reduced in NDDs and exogenous administration is not possible since they cannot cross the BBB.[243] Therefore, a gene augmentation modality could be used to increase or maintain

neurotrophin levels. Neuroprotective and neurorestorative effects of this approach have been reported in AD,[244] PD,[245] and HD.[125] Many gene therapy trials for neurodegeneration are testing neurotrophin augmentation **(Section 3.8)**.

A notable exception to the above theme includes the delivery of 7ND, a dominant negative analogue of CCL2. Overexpression of 7ND competitively inhibited CCL2-facilitated proinflammatory effects in an amyloid precursor protein/presenilin-1 rodent model of AD.[237] Additionally, genes of neurotoxic or neuroinflammatory proteins have been augmented to mimic disease pathology in preclinical studies. Examples of such studies include mutated htt gene delivery to study downstream effects on astrocyte function in HD. Mutated htt overexpression led to downregulation of astrocyte-BDNF indicating neurotrophin levels are critical to HD pathogenesis.[246] Similarly, inflammatory cytokines IL-1β[247] and TNF-α[248] were overexpressed in rat brains to determine subsequent effects in demyelination and PD, respectively. Gene augmentation is the oldest and principally tested gene delivery modality.

3.5.2 Gene silencing

Gene silencing can be useful for nonsense or repeat mutations that produce abnormally functioning proteins or to reduce levels of neurotoxic proteins that increase neuroinflammation. Gene silencing alters gene expression by post-transcriptional regulation of a gene by the RNAi technique. It has been employed for NDD-associated studies where small interfering RNA (siRNA),[249] microRNA (miRNA),[233] and short hairpin RNA (shRNA)[250] specific to the gene of interest were delivered successfully to CNS cells.

Gene silencing inquiries involve studying disease mechanisms, evaluating cellular or protein function, and testing therapeutic potential. An early work testing RNAi in brain

cells investigated the function of astrocyte-aquaporin (AQP) 4 water channels using siRNA. Reduced AQP4 resulted in reduced membrane water permeability, subsequently altering astrocyte morphology to maintain surface-volume ratio and water influx. Knocking down AQP4 also reduced ischemia-related protein expression indicating a possible therapeutic option.[251] Therapeutic potential of shRNA for glial fibrillary acidic protein (GFAP) and vimentin was determined in primary astrocyte cultures. Both GFAP and vimentin are markers astrocyte markers activated during glial inflammation and their knockdown expression resulted in reduced glial activation and migration.[250]

Lack of cell-specific restriction is a potential pitfall of using RNAi for therapy. Detrimental off-target effects could result from miRNA binding to multiple messenger RNAs. Exogenously produced siRNA and shRNA bind with single mRNAs; however, careful assessments must be performed during preclinical studies to avoid any off-target effects.

3.5.3 Gene editing

Gene editing is the least explored delivery modality due to its relatively novel nature compared to previously discussed modalities. Gene or genome editing is done with engineered nucleases composed of sequence-specific DNA-binding domains fused to a non-specific DNA cleavage module. These nucleases are capable of modifying DNA precisely and efficiently by inducing precise DNA double-strand breaks, which stimulates cellular DNA repair mechanisms. Such endonucleases include zinc-finger nucleases, transcription activator-like effector nucleases, and clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR-associated system 9 (Cas9) (CRISPR-Cas9) RNA guided system.[252] Genome editing components are delivered to the target cell with

overexpression vectors and have the same delivery and specificity limitations. In HD, a mutated htt gene contains increased CAG repeats at the 5' end of the gene, making it an ideal NDD for gene editing. Since normal htt functions are not well established, knocking it down may not be a suitable approach. The number of CAG repeats is linked to disease severity, thus, gene editing methods that precisely remove excessive CAG repeats may attenuate disease pathology.[253] Recently, CRISPR-Cas9 successfully eradicated HIV-1 proviral DNA from latently infected human CD4+ T cells and cells derived from microglia, monocytes, and T cells. [254,255] During HAND, neurons and oligodendrocytes are not infected, 2 to 20% of astrocytes are nonproductively infected, and microglia are productively infected with HIV-1.[256] Though anti-retroviral therapy reduces productive infection, HIV-1 proviral DNA is incorporated into microglia and astrocyte genome. These latently infected cells, astrocytes and microglia, become viral reservoirs in the brain, continually releasing viral proteins that cause neurotoxicity and neurocognitive decline. CRISPR-Cas9 technology could prove useful in eliminating latent infection in brain cells.[254] Since the complete inactivation or removal of proviral DNA from infected cells has been a critical hurdle for HIV therapies, this approach is a giant leap toward a cure and for therapeutic gene editing in general.

3.6 Delivery systems

Development of sophisticated systems has enabled successful gene delivery to CNS cells. Widely used gene delivery systems include, but are not limited to, viral vectors **(Tables 3.1, 3.2, and 3.3)**, and NPs **(Table 3.4).** Based on the nature of the gene, delivery system investigations can be categorized into 'proof of concept' and 'therapy-based' inquiries. Reporter genes are used for 'proof-of concept' experiments that establish and

validate delivery system's efficiency, for example, evaluating AAV9 vector's brain gene delivery potential using green fluorescent protein (GFP) reporter.[177] Therapy-based studies deliver genes to alleviate disease progression based on NDD pathogenesis. For example, low BDNF levels during disease led to testing BDNF gene therapy for HD.[183] The same gene and/or delivery system could result in varied outcomes in two studies based on differences in gene delivery route, targeted cell type, and *in vitro* or *in vivo* models.

3.6.1 Viral vectors

Viral vectors are preferred for gene delivery to brain cells as well as other cell types including muscles,[257] cardiac cells,[258] and cancer cells.[259] Viral vectors exploit the ability of a virus to infect mammalian cells and use of host machinery to produce viral proteins. Viral vector construction involves replacing immunogenic viral genome segments with the gene of interest. Structural viral proteins required for viral capsid and host genome integration are included as *trans*-acting factors. Literature on viral vectors derived from adenoviruses (AVs) (**Table 3.1**), AAVs (**Table 3.2**), and lentiviruses (LVs) (**Table 3.3**), major research breakthroughs and failures for each vector type are discussed here.

Adenovirus

In 2012, About 23% of gene clinical trials utilize AVs;[260] despite a setback in 1999 due to the death of a patient in an AV clinical trial.[261] Since then, replication-restricted or deficient AVs have been developed by deleting non-essential viral DNA.[178,261,262] Consistent AV-mediated brain gene expression has been found at three through thirty days, and was detectable at six months in Sprague-Dawley (SD) rats.[263] Adenoviruses do not integrate in the host genome and have been shown to preferentially transduce

neurons *in vivo*[182] and astrocytes *in vitro*.[264] When AV-LacZ was injected into the dorsal side spinal cord of SD rats, predominantly astrocytes were transduced on the dorsal side while neurons were transduced on ventral side.[265]

In ischemia therapy, AV-GDNF and AV-CNTF were delivered *via* intrastriatal injections a week prior to middle cerebral artery occlusion (MCAO)-induced ischemia in rats. These neurotrophins were primarily expressed in astrocytes and prevented neuronal damage compared to controls. At sites distant to the injection site, GDNF effects were stronger than CNTF.[266] Apart from neurotrophins, an astrocyte-specific molecule, secretory leukocyte protease inhibitor, expression led to neuroprotection indicated by reduced ischemic lesion size and lowered neurological deficits.[267] Similarly, adrenomedullin expression reduced apoptosis and astrocyte migration to the ischemic core *in vivo*. Compromised BBB could enhance the vector penetration.[172] AV-delivered heme oxygenase-1 expression increased astrocyte, but not neuronal survival, during hemin-induced apoptosis, post-hemorrhagic injury[187,268] indicating that cell-type-associated protective mechanisms must be studied while designing of cell-specific therapies.

Neurotrophin genes delivered *via* AV have been effective in certain NDD rodent models. Adenoviral vector-mediated GDNF gene delivery was tested in 6-hydoxydopamine- (6-OHDA) and cold-lesioned rats, modeling PD and brain trauma, respectively.[178,269] Lesions and apoptotic cells reduced in the brain trauma model [269], and dopaminergic neuronal loss decreased and motor function improved in the PD study.[178] When AV delivered BDNF to excitotoxic rats, quinolinic acid-induced lesion size was reduced and striatal neuron survival increased.[270] In transgenic HD mice,[183] AV-BDNF

delivery improved behavior tests and motor phenotype appearance.[183] Thus, neurotrophin expression alleviated disease outcomes despite the differences in targeted cell-type, associated disease, and species models reaffirming their applicability as NDD therapeutics.

In addition to high transduction efficiency, first generation AVs elicited an inflammatory response and caused striatal shrinkage at injection sites.[270,271] Subsequent peripheral AV immunizations led to reduced CNS gene expression, increased macrophage and T cell infiltration into the brain, microglial activation, and demyelination.[263,272,273] Second and third generation AV with deleted early regions (E1, E3, and E4) depicted low immunogenicity.[178] When Bellini and coworkers delivered IGF-1 to rat spinal cords, the resulting mild inflammatory response was attributed to IGF-1 reducing AV immunogenicity.[274] Taken together, certain segments of the AV genome, peripheral AV immunizations and gene of interest alter AV immunogenicity. Though AVs remain an efficient viral gene delivery system, they are not being tested in NDD clinical trials due to immunogenic potential and subsequent inflammatory outcomes.

Adeno-associated virus (AAV)

Low immunogenicity of AAV vectors and ability to transduce terminally differentiated neurons as well as dividing astrocytes, make them suitable for brain gene delivery.[275] Larger size gene constructs could significantly reduce uptake and the kinetics of gene delivery.[276] The smaller AAV genome compared to adenoviruses (4.7 kilobase (kb) *versus* 36 kb) facilitates gene uptake and delivery. CNS-targeted AAV vector studies can be divided into three main groups based on the serotype they investigate 1. AAV2,[277,278] 2. AAV9,[168,177] and 3. Other AAVs, which include AAV4, 6, 8, rh10, rh39, rh43

(AAV43), *etc*.[279,280] While AAV2 remains the prototype vector, some AAV serotypes are generated by pseudotyping, *i.e.* an AAV2 genome is packaged with a different capsid sequence such as AAV9. The majority of serotypes, other than AAV2, tested in the cited literature are designed this way. Contradicting results are documented on transduction efficiencies of self-complimentary AAVs (scAAV) and classical single-stranded AAVs (ssAAV). Smaller size scAAV (2.3 kb *versus* 4.7 kb) transduced effectively compared to ssAAV;[168,186] yet, the inverse may also be true.[281]

AAV2

Early studies demonstrated an AAV2-mediated immediate neuronal transduction. However, astrocytes were not transduced and microglial transduction was short-lived (24 hours).[277] However, in a later study, cytomegalovirus (CMV) or human (h) synapsin (Syn) promoter-driven AAV2 specifically transduced astrocytes and neurons, respectively.[278] Higher AAV2 infectivity and binding was reported in human astrocytes *in vitro* and was attributed to stronger interactions between AAV2 and secondary protein receptors on astrocytes facilitating endocytosis.[282] When AAV2 and AAV5 were compared, AAV2 preferentially transduced neurons and AAV5 transduced astrocytes.[185] While, kainate-induced limbic seizure activity reduced transduction efficiency, preferential cell type-specific expression persisted for both serotypes. Further, AAV5 transduced more efficiently than AAV2, potentially due to astrocyte activation, dying neurons or AAV2 neutralizing antibodies (NAbs).[283] One study also reported an exclusive neuronal transduction.[284] Recently preferential neuronal transduction by AAV2 and astrocyte activation was documented.[285] When tested in oligodendrocytes, AAV2 encoding myelin basic protein (MBP) promoter led to exclusive oligodendrocyte transduction *in vitro* and

in vivo with rat brain cells and mouse model, respectively, indicating therapeutic potential.[228] Similarly for microglia, *in vitro* and *in vivo* targeting was achieved by including murine F4/80 promoter in AAV2 and AAV5 in a rat model.[286]

Therapy-based enquiries for ischemia,[287] PD,[288] and AD,[244] have employed AAV2. In a cerebral focal ischemia rodent model, AAV2-mediated B-cell lymphoma-w (Bcl-w) expression in neurons, astrocytes and endothelial cells reduced infarct size, and improved neurological function.[287] Literature indicates neurons are the preferred target of AAV2, but potential expression in other cell types and effects on the disease prognosis remain to be seen. Additionally, the presence of anti-AAV2 NAbs in a significant percentage (~72%) of the human population[289] could become a key factor affecting therapeutic efficacy.

AAV9

Attention shifted from AAV2 to AAV9 for CNS gene delivery after its BBB crossing ability was reported.[177] Faust and colleagues first documented AAV9 neuronal transduction in neonatal mice and astrocyte transduction in adult mice.[177] Later reports found that AAV9 tropism shifted from neurons in fetal mice to astrocytes in neonatal mice [281], from astrocytes in neonatal mice to neurons in young mice,[169] and exhibited astrocyte tropism in NHPs of all ages.[164] Thus, literature presented conflicting data regarding AAV9-mediated preferential transduction of astrocytes or neurons. It can be discerned that, age of tested animals could be critical contributor of AAV9 cell-tropism. [169,177,281]

Similarly, variations in route of administration,[168,186] and tested species[164,168] have shown changes in AAV9 cell-tropism. In adult mice, intramuscular injection led to reporter expression in spinal cord neurons and astrocytes,[290] and intravenous administration

showed higher neuronal transduction in brain.[168] A study in NHPs showed brain parenchymal injection led to exclusive neuronal expression and intravenous injection resulted in preferential astrocyte transduction. Better access to astrocytes, potential AAV9-blood protein interactions, and presence of NAbs were attributed as contributing factors affecting gene expression post-intravenous administration.[168] With ICV injection, AAV9 was effective for neurons. [186] Thus, Gray and Aschauer study findings consistently showed AAV9-mediated neuronal expression when injected in the brain. In contrast, intracerebral scAAV9 injection showed a dose-dependent increase in neuron and astrocyte transduction efficiency without astrogliosis in mice.[291] Literature also showed the highest transduction efficiency and astrocyte tropism of AAV9. Despite widespread neuronal transduction, astrocytes were the predominantly transduced cell type[280] and reporter gene expression remained for over 18 months. [292]

Therapeutically, AAV9 has been used to deliver human erythropoietin (hEPO),[179] EAAT2, glutamine synthase (GS), miRNA against adenosine kinase,[293] and β-galactoside.[294] In an experimental PD model, a single intrastriatal dose of AAV9-hEPO was preceded by an additional intrastriatal or intramuscular injection to evaluate effects on immunogenicity and transduction efficiency. Prior intramuscular injection reduced transduction, increased major histocompatibility complex (MHC) class I and II expression, CD4 and CD8 T cells infiltration in the brain, and circulating NAbs for both AAV9 and hEPO in the blood. Alternatively, prior intrastriatal injection did not cause severe inflammation or reduced transduction efficiency, but increased peripheral blood cells.[179] Though intrastriatal or intramuscular might not be preferred routes of administration, these findings highlight the importance of assessing dosing route and frequency. AAV9-

EAAT2 and -GS delivery to rat hippocampal astrocytes did not alter kainate-induced seizures, while AAV9-miRNA against adenosine kinase reduced seizure duration suggesting a possible therapeutic usage.[293] Intravascular injection of AAV9-β-galactoside led to CNS and peripheral organ transduction. It partially reduced GM1-gangliosidosis by improving reactive astrogliosis in a rodent disease model.[294]

Since high peripheral organ transduction has been reported for AAV9 when injected intravenously, consequent studies investigating AAV9 immunogenicity in the CNS may have used intrastriatal or intrathecal routes. Immunogenic potential of AAV9 has been analyzed in comparison with AAV2. Human aromatic L-amino acid decarboxylase (AADC) was injected intrastriatally using AAV2 and AAV9 vectors and their effects on immune response and antigen-presenting cell activation were tested. In addition to higher brain expression compared to AAV2, AAV9 led to immune activation over time. Glia transduced by AAV9 revealed increased MHC class II expression within few days of infusion, which was absent with exclusively neuron-transducing AAV2. [295] A follow-up paper found AAV9 encoding GFP (foreign-protein) elicited a stronger immune response compared to AAV9 encoding aromatic L-AADC (self-protein) in NHPs suggesting need for syngeneic and non-functional reporter protein testing. Intriguingly, AAV2-GFP did not induce a significant immune response.[193] When AAV9 was delivered *via* intrathecal and intravenous routes, there was no global immune response, but higher reporter-expressing cell nuclei lacked Nissl bodies, an indication of neurotoxicity.[296] It is possible to avoid off-target effects by including brain-cell specific promoters to restrict expression, which is discussed in detail in **Section 3.7**. Since ongoing AAV9-based Phase I clinical trials involve intravenous or intrathecal injections (**Table 3.5**), findings

about AAV9 immunogenicity and peripheral expression are clinically relevant and must be taken into considerations for future trials.

Other AAV

Intrastriatal AAV1 injections resulted in preferential and higher neuronal expression than AAV2 control.[297] When AAV1 delivered galactocerebrosidase (GALC) to a twitcher mouse model of globoid cell leukodystrophy, the GALC gene was predominantly expressed in neurons and some astrocyte processes. Though oligodendrocyte precursors were effectively transduced *in vitro*, this was not reproduced *in vivo*. Leukodystrophy symptoms reduced, while increasing myelination and lifespan on GALC expression.[298]

Astrocytes in the SVZ and rostral migratory stream were transduced when AAV4 was delivered directly to the SVZ.[299] In another study, AAV4 showed a higher astrocyte binding but low transduction efficiency highlighting the importance of post-binding barriers that play a key role in effective transduction. The same study also generated AAV2 and AAV6 variants by engineered peptide loop replacement that presented increased specificity and transduction efficiency in astrocytes and Muller glial of the retina.[282] Neurons and astrocytes were successfully transduced by AAV5 encoding a reporter gene driven by hSyn or CMV promoter, respectively. However, *in vitro* results were reproduced only for neurons *in vivo.*[284]

Adeno-associated virus serotype 6 was shown to transduce only neurons in rats and also astrocytes in NHPs with higher MHC I and II expression.[300] Astrocyte restricted gene expression was tested using AAV8 and AAV43 containing GFAP promoters.[279] After being injected in sciatic nerve, AAV8 was found to be Schwann cells-tropic in mice and

AAV8-CNTF delivery led to increased levels of myelin protein P0 and PMP22 indicating its therapeutic potential.[301] Recently, AAV8 encoding GFAP promoter-driven achaete-scute complex homolog-like 1 converted dorsal midbrain astrocytes into functional neurons *in vitro* and *in vivo* in mice.[302] Neuron and oligodendrocyte transduction has been shown with AAVrh8[303] and AAVrh10[303,304] with an efficiency comparable to AAV9.[280,292] Both AAVrh8 and AAVrh10 crossed the BBB when injected intravenously in mice with minimal peripheral tropism as compared to AAV9.[303] Currently, AAVrh10 is under investigation in a Phase I clinical trial (**Table 3.5**).

Few AAV serotypes have successfully transitioned into clinical trials for NDD therapy. While inclusion of cell-specific promoters restricted AAV gene expression, mechanisms responsible for intrinsic cell tropisms of AAV serotypes have not been investigated well. Some AAV receptors are known, for example, PDGF receptor has a role in AAV5 transduction.[186] We infer that abundance of such AAV receptors on cell surface must have a key role in uptake, endocytosis and expression efficiency of AAV-mediated transduction. Yet, due to availability of alternatives and ease of manipulation, the overall focus has been on testing serotypes that present higher efficiency and intrinsic tropism for intended cellular target instead of elucidating AAV serotype transduction mechanism.

Lentivirus

Lentiviruses, from the *retroviridae* family, integrate in the host genome, unlike adenovirus and AAV. Therefore, LVs are capable of producing longer, more stable gene expression in diverse cell types.[305,306] Lai and Brody depicted LV-mediated gene expression in neurons and astrocytes.[307] When injected in rat dorsal funiculus, LVs

transduced astrocytes, oligodendrocytes, and their progenitors in spinal cord white matter.[223] During SCI in rats, LV injected at a lesion site led to high peripheral expression in astrocytes with lower transduction of neurons, oligodendrocytes precursors, and microglia.[224] When compared with AV and AAV, early investigations involving intrastriatal and intrahippocampal injections of LV showed comparable CNS transduction efficiency at two weeks and higher efficiency from six to twenty four weeks in mice. About 90% of the transduced cells were terminally differentiated neurons[308] indicating intrinsic neuronal tropism. Two decades later, a conflicting report demonstrated preferential astrocyte tropism of LV compared to AAV in NHPs.[285]

Most investigated LVs are HIV-1-derived and are pseudotyped, a process by which viral genome is packaged with glycoproteins derived from a nonpathogenic virus such as vesicular stomatitis virus-G (VSV-G). Pseudotyping is performed to increase transduction efficiency and specificity *i.e.* cell-specific tropism. Vectors packaged with envelope proteins of murine leukemia virus (muLV), lymphocytic choriomeningitis virus (LCMV), rabies-related Mokola virus, and VSV-G transduced striatum, thalamus, and white matter in mice brains. Additionally, both VSV-G- and Mokola-LV were effective in oligodendrocytes and their precursors *in vitro*. In mixed neuroglial cultures, VSV-G-LV-facilitated gene expression was higher in astrocytes.[309] In contrast, VSV-G-LV has been shown to preferentially transduce neurons in adult rats and embryonic mice, while glycoprotein 64-enveloped-LV mediated expression was seen in astrocytes of adult rats.[310] Also, astrocyte preference of LCMV- and muLV-pseudotyped LV was depicted post- infusion into SN.[311] On the other hand, an earlier study demonstrated VSV-G-LV-

mediated exclusive neuronal or astrocyte-transduction including cell-specific promoters depicting promoter dictated restriction and not pseudotyping.[312]

To deliver therapeutic genes *via* LV, neurons were targeted by LV-CNTF in embryonic neuroglial cultures and astrocytes became activated with altered membrane distribution of highly glycosylated forms of glutamate transporter (GLAST) and glutamate transporter-1. Improved astrocyte glutamate clearance ability in these studies suggested translation potential in excitotoxicity-associated disease.[313] Lentiviral vector encoding GDNF cDNA downstream of the GFAP or CMV promoters showed similar neuroprotection indicating the GFAP promoter, which produced only 1/10 the GDNF of the CMV promoter, was enough to impart neuroprotection.[184]

For RNAi investigations, LV has been the vector of choice. Compared to AV and AAV, more studies combined LV and RNAi. Colin and colleagues adapted a unique gene silencing approach to target astrocytes. They designed a LV construct pseudotyped with Mokola-G-LV and encoding miR124T to exclusively silence the 'off-target' neuronal gene expression. GLAST gene or miRNA regulating GLAST encoded downstream of miR124T in the above construct was delivered exclusively to astrocytes *in vitro*. Specificity of this system was shown *in vivo* using a LacZ reporter.[314] In a follow-up paper, the LV construct described above was modified further to include an astrocyte-specific GS1 promoter, an additional sequence of miR124T and a regulatory tetracycline response element to increase specificity and add a regulatory component.[315] High complexity of this construct could limit its therapeutic applications, although certain vector modification approaches tested in this study can be utilized to improve targeted gene delivery.

Other RNAi-associated studies employed LVs to silence astrocyte marker molecules GFAP and vimentin,[250] chondroitin polymerizing factor (CPF) and chondroitin synthase-1 (CS-1),[316] as well as miR145.[317] Astrocyte intermediate filaments, GFAP and vimentin, are upregulated during inflammation adversely affecting axonal and neurite regeneration post-injury. Thus, LV-shGFAP and shVimentin delivery decreased astrogliosis, astrocyte migration in scratch assay and improved neuronal survival in co-cultures.[250] Astrocyte-secreted chondroitin sulfate proteoglycans were activated by LV-miCPF and -miCS-1 delivery during CNS injury, which increased the neurite outgrowth in neuroglial cultures.[316] A negative regulator of reactive astrogliosis, miR145, delivered with LV reduced astrocyte activation, proliferation, and migration in an *in vitro* SCI model.[317]

In contrast to adenoviruses, LV-administration did not elicit significant immune response.[305,306] Microglial and macrophage accumulation at the injection site was attributed to injection toxicity and not LV.[223] Research later reported that prior peripheral LV immunization elicited an immune response to a subsequent CNS-injected LV only when both LVs encoded the same transgene.[318] Since LVs are integration proficient, there's a risk of insertional mutagenesis, and therefore integration-deficient vectors have been used as well.[319] Recently, a first-ever LV-based Phase I PD clinical trial was completed (**Table 3.5**). The trial results are not only encouraging for its transition into Phase II and III clinical trials, but also for the promise of increased LV therapies in the future (discussed in **Section 3.8**).

3.6.2 Polymeric nanoparticles

Research and clinical applications of nanotechnology have increased in recent years. Formulations <100 nm in at least one dimension, which may enable crossing BBB,

qualify as NPs. Effects of metallic NPs containing iron,[320,321] silver[322] or silica[323] have been evaluated in brain cells, but non-metallic polymeric NPs have been predominantly studied for gene delivery.[110] Types of NPs including polymeric, solid-lipid, nanoemulsion, and liposomes have been well documented in the literature along with strategies to optimize delivery and expression efficiency.[110,324-326] Investigations associated with CNS gene delivery have used neuronal or neural stem cell lines such as PC12,[327] C17.2,[328] and Neuro2a.[329] There are fewer studies, which utilize primary brain cells or animal models. Also, microglia and oligodendrocytes were less frequently targeted with NP-mediated gene delivery compared to neurons and astrocytes.

Cationic polyethylenimine (PEI)-DNA ‘polyplexes’ are avidly used for gene delivery.[330,331] Polyamidoamines (PAMAM) and poly(lactic-co-glycolic) acid (PLGA) have also been frequently examined. The positive charge on NPs allows electrostatic interaction with a negatively charged cell membrane and exogenous DNA to improve delivery and prevent systemic degradation of plasmid DNA (pDNA).[332] After cellular uptake, titratable amine groups on PEI and PAMAM increase chloride concentration leading to endosomal swelling and higher endosomal escape. Therefore, PEI and PAMAM have better transfection efficiencies compared to other positively charged polymers.[333]

Gene delivery inquiries involving NPs revolve around increasing transfection efficiency and reducing NP toxicity. Complexing NP with polyethylene glycol (PEG), or PEGylation, increased transfection efficiencies, reduced toxicity, and imparted "stealth" properties.[165,334-336] Convection-enhanced delivery also increased efficiency of NP administration.[181] Arginine addition enhanced the cellular uptake and delivery across the

BBB,[165,337] and tagging liposome NPs with transferrin receptor ligand facilitated transcytosis across the BBB and endocytosis into CNS cells.[338]

Lipid containing substances are frequently used for CNS delivery.[331] Early investigations showed liposome-mediated gene delivery, *i.e.* lipofection, was more efficient than calcium phosphate transfection in fetal human and rat astrocytes, and was comparable to that of modified vaccinia Ankara viral transduction in fetal spinal cord astrocytes,[339] and PEI in primary rat astrocytes.[331] Neutrally charged, 120-150 nm liposomal NPs containing palmitic acid, vitamin A, or vitamin E preferentially transfected astrocytes close to the injection site after ICV delivery. *In vitro*, apolipoprotein E (ApoE) co-treatment increased uptake and transfection efficiency lipid NP derived from vitamin A and E in astrocyte-derived cells.[340]

For mammalian cell transfections, PEI is considered the gold standard. But it has exhibited some toxicity.[341] Therefore, polymers with lower toxicity and comparable efficiency including C2-(dimethylamino)ethyl methacrylate-based cyclized knot polymer,[342] arginine-modified PEI derivative,[165,341] heavily PEGylated PEI,[181] arginine-modified PAMAM,[249] and PLGA[343] are preferred. In our collaborative work, arginine-PEI-PEG polyplexes formulated with luciferase-expressing plasmid DNA transfected rat primary astrocytes and neurons *in vitro* and *in vivo* following intravenous injection.[165]

As a PD therapy, 8-11 nm diameter PEGylated poly-l-lysine nanorods delivered the GDNF gene to rats *via* intracerebral injections. Neurons and glia were transfected and sustained expression for over 11 weeks.[192] Transfection efficiency was higher in denervated striatum of 6-OHDA-induced mice and old mice, highlighting their disease sensitivity and age as a factor in gene delivery.[336] A cyclized-knot polymer delivered

GDNF to primary rat astrocytes and the astrocyte cell line Neu7 that led to functional effects in co-cultured dorsal root ganglion cells.[342] As a PD therapy option, GFAP-promoter driven-tyrosine hydroxylase (TH) gene-loaded liposomes were delivered intrastriatally[344] or intravenously[345] to a 6-OHDA-induced rat model. Rotational behavior was improved in both studies, but TH expression was restricted to either astrocytes[344] or nigrastriatal neurons.[345]

For SCI, IL-10[190] and vascular endothelial growth factor (VEGF)[343] gene therapies were evaluated using PLGA NPs. Anti-inflammatory cytokine IL-10 could reduce gliosis during neuroinflammation due to astrocyte-specific receptor expression. Dose of IL-10 pDNA reduced significantly when delivered *via* NPs compared to naked pDNA to achieve similar expression.[190] Cholesterol-modified PLGA NPs efficiently transfected the VEGF gene in both neurons and astrocytes post spinal cord injection.[343]

Gene silencing strategies have been reported using polymeric NPs.[249,335,346] Carbosilane dendrimers successfully delivered siRNA specific to HIV-1 negative regulatory factor (Nef) to primary astrocytes and related cell lines.[346,347] An arginine-modified PAMAM derivative delivered high mobility group protein 1 siRNA to cultured primary mouse cortical cells and led to a reduction in infarct volume in rat brains post-ischemia.[337] Rungta and coworkers synthesized distearoylphosphatidylcholine/cholesterol/PEG-DMG containing lipid NPs to deliver siRNA targeting the neuronal GluN1 subunit of the N-methyl-D-aspartate (NMDA) receptor, reducing NMDA-based currents, but not α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-induced currents.[335] These NPs could be used to treat neurons, while co-delivering an astrocyte-specific gene.

Polymeric NPs are non-metallic and non-pathogenic. Their synthesis process can be better controlled than producing viral vectors in bulk. They can be optimized to increase transfection efficiency and BBB penetrability. Currently, PLGA is approved by the food and drug administration for therapeutic use indicating polymeric NPs are better accepted as delivery vehicles than viral vectors.

3.7 CNS-targeted gene delivery using cell-specific promoters

Low BBB permeability and brain structure complexity have already been discussed as major challenges in designing a delivery system for any neural cell type. In theory, cell-specific promoters could be used to target gene delivery to particular cells. Well-known cell-specific markers are neuron-specific enolase (NSE) for neurons, GFAP for astrocytes, F4/80 for microglia, and MBP for oligodendrocytes; which in turn represent the widely used methodology of restricting exogenous gene expression to specific cell types.

As discussed earlier in the review, neurons and astrocytes are targeted more frequently than microglia and oligodendrocytes. Targeting a precise neuronal subtype is essential during NDD treatment since that subtype often expresses genes linked to a disease; for instance, dopaminergic neurons express TH gene in PD. Yet few neuron-focused papers discuss gene vector design to improve expression specificity. Our observations suggest that neuronal targeting has been a tacit approach for numerous CNS gene delivery studies and implications of off-target gene expression in astrocytes or oligodendrocytes were not discussed. Nevertheless, NSE, hSyn, and PDGF are the frequently reported neuron-specific promoters.

When NSE and PDGF promoter-driven GFP encoding AAV2 were injected into the mid-cervical region of rat spinal cords, exclusive neuronal expression was obtained with at 15 and 45 weeks post-injection.[348] Later studies reported that inclusion of NSE promoter achieved relatively precise but not exclusive neuronal expression.[278,349] Among uncommon promoters, LV encoding a 1.6 kb segment of 'homeobox 9' promoter was used for motor neuron targeting in mice spinal cord.[350] Also, Thy1.2 and α-CAMKII promoter were shown to be specific for pyramidal neurons.[351] An AV, encoding multiple copies of a cis-regulatory element of human dopaminergic β-hydroxylase promoter, selectively transduced noradrenergic neurons.[352]

Macrophage-specific promoter sequences, such as human CD11b, CD68, and murine F4/80, were evaluated for *in vitro* and *in vivo* microglial targeting employing AAV constructs. Murine F4/80 offered the highest expression efficiency and restriction in SD rats.[286] Oligodendrocytes and Schwann cells in the CNS abundantly express MBP. An AAV2 vector encoding an MBP promoter precisely targeted oligodendrocytes *in vitro* and *in vivo,* in rat cells and mice, respectively.[228] Inclusion of the MBP promoter in LV-GFP also led to exclusive oligodendrocytes expression.[353]

Contrary to neurons, astrocyte-targeted studies have focused on one promoter, *i.e.* GFAP, and modified it to reduce size and increase efficiency.[354-356] Aldehyde dehydrogenase 1 L1 and GLAST/EAAT1 are other astrocyte-specific markers, but the GFAP promoter remains the 'promoter of choice' in gene delivery approaches.[355,357,358] Brenner and colleagues identified an essential 2.1 kb promoter segment (gfa2), in the 5' region of GFAP promoter capable of driving gene expression.[354] It has been utilized to restrict gene expression,[244,338] in transgenic models,[32] and to study astrocyte

function.[32,125,359] This promoter has also been used with viral vectors[360] and NPs.[338] In spite of the widespread acceptance for gfa2, some reports have debated over the promoter specificity. An early report documented that using only 5' flanking promoter sequence produced tissue restricted expression while both 5' and 3' flanking sequences were essential for cell-specific expression.[361] In agreement with the findings, GFAP-driven TH was expressed exclusively in nigrastriatal neurons and not in astrocytes.[345]

Immunogenicity is a major problem with viral vectors, especially AVs. Gerdes and colleagues showed that using a strong promoter like major immediate early murine CMV reduced vector dose and immunogenicity.[362] However, this approach is not suitable for cell-specific targeting. On the other hand, gfa2 promoter has been considered a weak promoter that resulted in lower expression[187] and its efficiency changed with age *in vivo*.[360] Thus, gfa2 has been modified over a decade to improve transgene expression. Recently, a shorter, 1740 kb gfa2 promoter driven AAV-GFP was delivered to cortical astrocytes.[356] Stronger promoter sequences, such as CMV and/or inverted terminal repeat sequences were appended upstream of the gfa2 promoter to increase expression. These modifications led to higher expression as compared to CMV promoter *in vitro* and *in vivo*, when injected intrastriatally.[363] Brenner laboratory followed up their original work on gfa2 promoter by concluding that a 681 kb segment of the GFAP promoter, gfaBC1D, is optimal to obtain promoter-driven gene expression. They suggested that gfaBC1D enhanced the expression efficiency due to its smaller size.[355] Since then, GfaBC1D was used to drive expression of the pleckstrin homology domain of phospholipase C-like protein p130 using AAV2/5.[364] Other astrocyte-specific promoters; aldehyde

dehydrogenase 1L1, which is active in all mature astrocytes with broader expression pattern and GLAST/EAAT1; have been utilized to a lesser extent.[236,357]

In addition to promoters, certain cellular receptors could be used for targeting. For instance, the rabies virus enters neurons *via* nicotinic acetylcholine receptor and a bacterial protein, namely tetanus toxin, binds with neurons through the triasialoganglioside receptor, GT1b.[175] Hence rabies virus glycoprotein or tetanus toxin could thus be tagged to exogenous DNA-vector complex to improve endocytosis. Additionally, a 12 amino acid peptide, Tet-1, has been identified and has been successfully used to target neurons.[175] Similarly, DNA-vehicle complex was tagged with neurotensin and NGF fragments for neuron-specific delivery.[365]

Another way to increase cell-targeting specificity is by including elements that respond to a biomarker characteristic of the neuropathology. This concept was utilized by flanking hypoxia response element with the VEGF gene sequence as a therapy for focal ischemia. Since hypoxia-inducible factor-1 binds to hypoxia response element, VEGF expression was enhanced during stroke.[366] Uptake and transfection efficiency of lipid NP uptake and transfection efficiency increased in the presence of ApoE suggesting that it could be utilized for astrocyte targeting.[340]

The perpetual process of designing and optimizing CNS-directed gene delivery system has remained a translational research priority. Though literature agrees on inclusion of a cell-specific promoter, standard constructs for each cell type have not been established. Going forward, comprehensive efforts must be directed toward delivery system design to enable BBB permeability, cell-specific targeting, and increasing expression of weak promoters.

3.8 Bench to bedside translation

One of the major highlights in gene delivery research has been its transition into clinical trials. Several viral vectors are currently being investigated in Phase I/II clinical trials (**Table 3.5**) for treatment of NDDs including AD, PD, and lysosomal storage disorders. Phase I clinical trial for AD treatment involved stereotactic injections of AAV2-NGF to the basal forebrain region of containing the nucleus basalis of Meynert. The study was conducted in ten patients with three different doses of AAV2-NGF to determine the highest safe dose for a Phase II study. Patients were monitored for over two years to ascertain treatment safety. All patients survived the procedure with few adverse events reported. AAV2-NGF therapy was evaluated to be safe and well tolerated.[367] Currently, a multicenter Phase II study evaluating the treatment efficacy is underway.

Several clinical trials have been completed or being carried out for PD treatment administering genes associated with either enzymes involved in dopamine synthesis, such as AADC, or neurotrophic factors, such as neurturin (NTRN) and GDNF. Most of these studies have successfully completed Phase I trials and have proven safety.[288,368] Phase II clinical trials evaluating efficacy are currently ongoing and have shown mixed results thus far. AAV-glutamate decarboxylase trial has been terminated due to financial constraints despite improvements observed during Phase II.[369] On the other hand, AAV-NTRN Phase II study results promised long-term safety but conclusive results on efficacy were not obtained.[370] Additionally, results from first-ever clinical trial employing a LV have been reported. It involved stereotactic injections of a tri-cistronic vector expressing genes for TH, AADC, and cyclohydrolase-1 with the intention of stimulating non-dopaminergic, striatal neurons to secrete dopamine, replenishing levels. Motor improvements and long-

term tolerability four years post-injection was established.[371] Currently, methods to increase effectiveness of this therapy are being investigated.

In vitro and preclinical investigations conducted so far have reported that presence of preexisting NAbs could be responsible for varied gene delivery efficiencies and immune responses between rodent models, NHPs, and humans. A study conducted with over 200 human serum samples indicated presence of AAV-NAbs in 40% to 70% samples for various AAV subtypes. In a preclinical study, presence of pre-existing NAbs reduced AAV9 transduction efficiency in NHPs.[168] Consistent with these findings, completed clinical trials discussed above have also reported presence of AAV antibodies or VSV-G antibodies for AAV- and LV- associated trials, respectively. While none of the published data report adverse events or immune responses; it must be noted that the primary endpoint of Phase I trials is safety and are conducted with small (<100) patient cohorts. It will be important to evaluate if presence of NAbs will reduce efficacy in larger patient cohorts. According to Palfi *et al. the* presence of NAbs should not interfere with LV transduction efficacy since NAbs were detected in some patients three months post-gene delivery; and thus, LVs would be integrated by the time NAbs appear.[371]

An ongoing clinical trial involves intravenous injections of an AAV9 encoding survival motor neuron gene to six to nine month old infants diagnosed with spinal muscular atrophy. The study involves monthly monitoring until death or continuous respiratory support for over 16 hours. The final results of the study are not available; however, preliminary data presented at scientific conferences and provided on the trial sponsor's website indicates safety and some motor function improvements.[372]

NPs are another major system being evaluated as gene delivery vehicles. Special characteristics of NPs must be considered before transitioning to preclinical or clinical trials. NPs have higher surface area resulting in higher toxicity *in vitro* compared to *in vivo* studies, leading to inconsistent results, a concept known as 'nanomaterial paradox'.[373] NPs that are toxic at low doses *in vitro* could be delivered at relatively higher dose *in vivo* without toxicity. Therefore, it is essential to perform *in vivo* experiments with NPs, even if *in vitro* findings indicate toxicity. Currently, no ongoing or completed clinical trials have used polymeric NPs for NDDs. It must be noted that polymeric NPs are being used in cancer drug delivery studies and can soon be expected to transition into clinical trials for gene delivery based on the translational data available in literature.[374-376] All gene delivery systems require careful and comprehensive analyses for route, dose, and frequency of administration, in addition to immunological profiling of the animal models to improve the transition from *in vitro* to *in vivo* and further into clinical trials.

3.9 Conclusions and future prospects

In this review, we appraised key elements of the ever-growing field of CNS-targeted gene delivery focusing on the past two decades. Challenges encountered in therapeutic strategies targeting the brain, such as low BBB permeability and brain structure complexity were carefully analyzed, specifically in the context of gene delivery. Furthermore**,** we critically evaluated functional aspects of principal CNS cell types implicated in NDD pathogenesis. Taken together, we infer that targeting neurons and astrocytes will have significantly broader implications in the context of CNS gene delivery, than microglia and oligodendrocytes. Ultimately, astrocytes will emerge as the most appealing therapeutic targets as compared to terminally differentiated neurons, with their

complex structure and fastidious synaptic transmission. We thus anticipate robust development of astrocyte-focused delivery systems in the near future.

Of all the delivery systems investigated thus far, there are pros and cons to each, and clearly, the quest for an ideal gene delivery system continues. Among the viral gene delivery approaches, higher immunogenicity limits the application of AVs despite their better transduction efficiency compared to other viral vectors; AAVs have an edge over other approaches with their successful progression into clinical trials and consistent results over the years; whereas LVs require further comprehensive investigations for clinical translation. A parallel line of investigation comprises of concurrent development of polymeric NPs to successfully tackle the BBB and target specific CNS cell types. The non-viral nature of NPs renders these formulations far more amenable for acceptance by the general population. All together, the sheer diversity of viral vectors and NPs ensures that suitable vehicles for CNS cellular targets will soon be available despite issues such as varying gene expression efficiency, distinct cell tropisms and immunogenicity. Considering these significant challenges and caveats encountered, NDD gene therapy has made impressive strides. **Fig. 3.2** summarizes the milestones of this exciting journey over the past two decades.

The litmus test for any therapeutic strategy is its success in the bench to bedside translation. In this regard, we have included viral vector- and NP-associated clinical trials for NDDs in our review (**Table 3.5**).[377] However, the enigma of the brain as the ultimate challenge is reflected in the clinical trial numbers as well. In 2012, only 2% of the clinical trials were associated with NDDs as compared to 64.4% for cancer, 10% for monogenic disease and 7.5% for cardiovascular diseases.[260] Without a doubt, safety of gene therapy

for NDDs is well established given the results of multiple successful Phase I clinical trials. However, based on the literature available thus far, no studies have provided conclusive data substantiating improved efficacy of gene therapy over current treatments. Thus, although gene therapy has shown long-term safety, transitions into Phase II to evaluate efficacy and fine-tuning the route, dose and frequency of administration will be critical.

The majority of the gene therapy investigations thus far have focused on validating the concept of gene delivery in attempts to overcome diverse multifactorial challenges. Consistent with this notion, most clinical trials that spearheaded these investigations employed classically known disease biomarker genes for first line investigations. One notable example would be clinical trial testing of L-AADC, which converts L-DOPA to dopamine, as a therapy for PD.[378] Going forward, emerging trends both in discovery of novel biomarkers and early diagnostic techniques, will pave the way for the next generation gene therapy approaches.

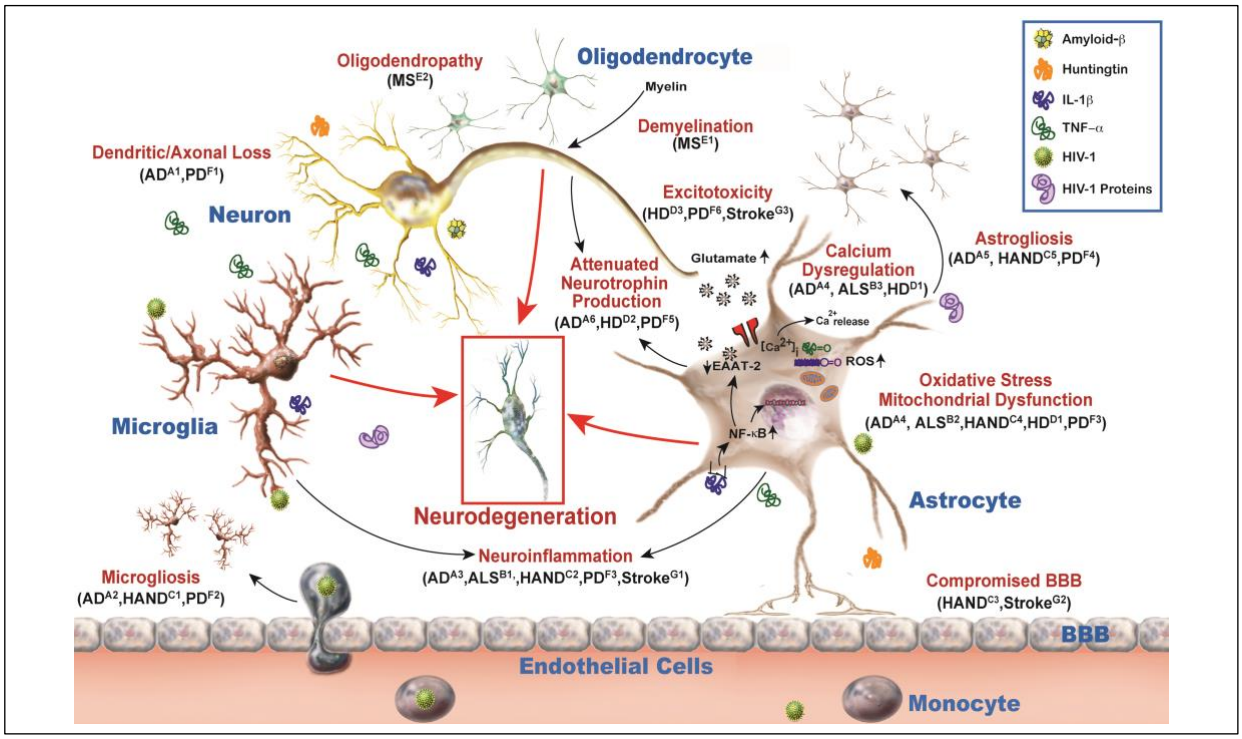
While significant research and resources have been invested in the last few decades establishing the basics of gene augmentation and silencing, these specific modalities may soon become obsolete with development of genome editing technology. Regardless, key challenges facing successful delivery of these technologies to the brain will remain the same, and past lessons learnt will thus help guide strategies for successful translation of novel gene editing techniques, such as CRISPR-Cas9, to the clinic.

The overall progress in gene delivery approaches promotes a brighter future for NDD management and a strong potential for the development of effective, personalized gene therapy. In conclusion, gene therapy has come of age with bits of both successes and failures thus far, yet, the lessons from past and current research promise that cell-

and disease-specific, safe and effective CNS gene delivery will be a reality in the not too distant future.

3.10 Figures and Figure Legends

Figure 3.1. Common cellular mechanisms implicated in neurological diseases and disorders (NDDs) NDDs are associated with cellular dysfunctions of principal CNS cells including neurons, microglia, oligodendrocytes, and astrocytes. Neuronal damage includes dendritic and axonal loss, and reduced neurotrophin secretion. Microglial activation occurs during brain injury and resultant proinflammatory cytokines induce neuroinflammation along with microgliosis due to extravasation of peripheral monocytes. Oligodendrocyte dysfunctions include demyelination and oligodendropathy, *i.e.* death of oligodendrocytes. During injury, astrocytes contribute to excitotoxicity, and neuroinflammation by reduced glutamate uptake and increased release of proinflammatory cytokines, respectively. They likely undergo oxidative stress, mitochondrial dysfunction, calcium dysregulation, attenuated neurotrophin production and astrogliosis. Additionally, altered BBB permeability can increase neuroinflammation and contribute to disease. These mechanisms precede or succeed neurodegeneration and overlap in diseases such as Alzheimer's disease (AD) [A1[199], A2 [220], A3 [113], A4 [114], A5 [116], A6 [379]], Amyotrophic lateral sclerosis (ALS) [B1 [117], B2 [118], B3 [119]], HIV-associated neurocognitive disorders (HAND) [C1 [380], C2 [120], C3 [381], C4 [121], C5 [122]], Huntington's disease (HD) [D1 [124], D2 [125], D3 [123]], Multiple Sclerosis (MS) [E1 [229], E2 [231]], Parkinson's disease (PD) [F1 [202], F2 [382], F3 [129], F4 [127], F5 [128], F6 [126]], and stroke [G1 [131], G2 [172], G3 [130]].


Amyloid-β
Huntingtin
IL-1β
TNF-α
HIV-1
HIV-1 Proteins
Oligodendropathy
(MS^E2)
Oligodendrocyte
Myelin
Demyelination
(MS^E1)
Dendritic/Axonal Loss
(AD^A1,PD^F1)
Neuron
Excitotoxicity
(HD^D3,PD^F6,Stroke^G3)
Glutamate
Attenuated
Neurotrophin
Production
(AD^A6,HD^D2,PD^F5)
Calcium
Dysregulation
(AD^A4, ALS^B3,HD^D1)
Astrogliosis
(AD^A5, HAND^C5,PD^F4)
Ca release
EAAT-2
ROS
Oxidative Stress
Mitochondrial Dysfunction
(AD^A4, ALS^B2,HAND^C4,HD^D1,PD^F3)
Microglia
NF-κB
Astrocyte
Neurodegeneration
Neuroinflammation
(AD^A3,ALS^B1,HAND^C2,PD^F3,Stroke^G1)
Microgliosis
(AD^A2,HAND^C1,PD^F2)
Compromised BBB
(HAND^C3,Stroke^G2)
BBB
Endothelial Cells
Monocyte

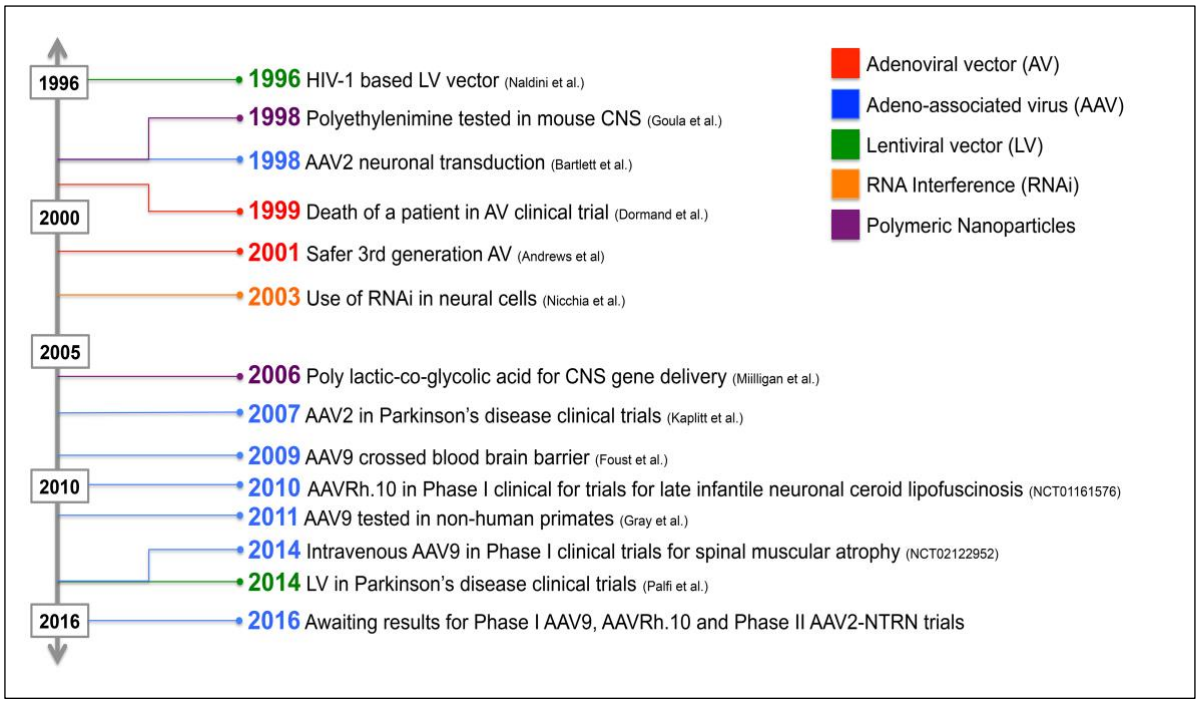


Figure 3.2 Milestones and breakthroughs in gene delivery over two decades.

Significant progress was made in the last twenty years of gene therapy research. Considering the time taken by any therapy to transition from bench to bedside, we can expect additional clinical gene therapy applications in the coming years with the help of cutting-edge technology.

3.11 Tables

Table 3.1. Adenoviral vectors for brain-targeted gene delivery

Gene	Disease/ Mechanism	Transduced Cell Types	*In Vitro Cell Source*	*In Vivo*	Result	Reference
Adrenomedullin	Stroke/ ischemia	Neurons, Astrocytes	Sprague -Dawley (SD) rats	Middle cerebral artery occlusion (MCAO) in SD rats	*In vitro:* reduced apoptosis, increased migration, nitric oxide, B-cell lymphoma (Bcl)-2 levels, and Akt, GSK-3β phosphorylation; *In vivo*: decreased infarct size and astrocyte migration to ischemic core	172
Brain-derived neuro-trophic factor (BDNF)	Huntington's disease (HD)	Astrocytes		R6/2 mice	Sustained expression, improved behavior, induced astrocyte motor phenotype	183
BDNF	HD	Not specified (N/S)	SD rats	Quinolinic acid (QA)-induced SD rats	Improved protection of striatal neurons	270
Ciliary neuro-trophic factor (CNTF)	Astrogliosis	Neurons, Astrocytes	Chicken embryo neural cells	SD rats	Hypertrophied astrocytes expressing gene for over 5 weeks	182
CNTF, Glial cell line-derived neuro-trophic factor (GDNF)	Stroke/ Ischemia	Astrocytes		B6 mice	Prior intrastriatal delivery increased viable neurons post-ischemia, Reduced injured cells and DNA fragmentation, GDNF-induced effects better than CNTF	266

Genetically encoded, fluorescent calcium indicator protein (G-CaMP2)		Astrocytes		Mice type N/S	Calcium signaling recorded in Bergmann glia and protoplasmic astrocyte	[264]
GDNF	Focal cortical trauma	Astrocytes		Cold injured SD rats	Reduced lesions attributed to reduced inducible nitric oxide synthase, caspase 3, and DNA fragmentation	[269]
GDNF	Parkinson's disease (PD)	Astrocytes	SD rats	6-hydroxyd-opamine (OHDA)-treated SD rats	Improved nigral dopamine (DA) neuron survival and behavior	[178]
Heme oxygenase-1	Oxidative injury	Astrocytes	BALB/c x 129/Sv mice		Reduced cell death post Hemin exposure	[187]
Insulin-like growth factor-1	Glial inflammation	Astrocytes	Caesarean derived (CD)-1 mice		Reduced toll-like receptor 4 expression, reduced NF-κB translocation	[274]
LacZ		Neurons, Astrocytes, Microglia		SD rats	Astrocytes and other cells expressed reporter	[271]
LacZ	Spinal cord injury (SCI)	Neurons, Astrocytes		Rats type N/S	Dorsal side astrocytes and ventral side neurons transduced, temperature-sensitive vector-mediated expression remained longer than controls	[265]

LacZ		Neurons, Astrocytes	SD rats	High-capacity vector maintained gene expression over 6 months, prevented immune cells infiltration in the brain post-peripheral injection	[263]
Secretory leukocyte protease inhibitor	Focal stroke	Neurons, Astrocytes	MCAO-in Hypertensive rats, SD rats	Reduced ischemic lesion size with neuroglial gene expression peak after 2 days	[267]

Table 3.2: Adeno-associated viral vectors for brain-targeted gene delivery

Type	Gene	Disease/ Mechanism	Transduced Cell Types	*In Vitro Cell Source*	*In Vivo*	Result	Reference
AAV1	Galactocere-brosidase (GALC)		Neurons, Astrocytes, Oligodendrocytes	Humans, mice	Twitcher (Twi) mice	Stable expression *in vivo*, improved myelination, symptom reduction, and increased life span	298
AAV1 AAV2	Green fluorescent protein (GFP)	Neuro-degenerative diseases and disorders (NDDs)	Neurons, Astrocytes, Oligodendrocytes, Microglia		C3H/HeJ mice	AAV1 transduced multiple cell types, higher efficiency and better distribution than AAV2	297
AAV1 AAV2 AAV8	CNTF	Myelin disorders	Sensory neurons, Schwann cells		CD-1 mice	AAV8: preferential Schwann cells transduction, lower immunogenicity, increased myelin and neuronal proteins associated with axonal regeneration	301
AAV1 AAV5 AAV9 AAVrh 10	GFP	SCI	Neurons, Astrocytes Oligodendrocytes		SD rats	AAVrh10: Neuronal transduction efficiency was similar to other vectors but also transduced other cell types	304
AAV1 AAV8 AAV9	GFP		Neurons, Astrocytes, Purkinje Cells		B6 mice	AAV9 most effective and sustained global expression for 18 months	292

AAV2	B-cell lymphoma2 -like 2 (*a.k.a.* Bcl-w)	Cerebral ischemia	Neurons, Astrocytes, Ependymal Cells		MCAO in SD rats	Global expression, infarct size reduction, improved neurological function	[287]
	GFP		Neurons, Microglia		SD rats	Neuronal expression 3 days post-infusion	[277]
	GFP		Neurons, Astrocytes	N/S	Wistar rats	Human synapsin promoter: neuronal expression *in vitro*, *in vivo* Cytomegalovirus promoter: glial expression *in vitro*, thalamic neurons *in vivo*	[278]
	GFP		Neurons, Astrocytes		Cynomolgus monkeys	Neuronal expression and astrocyte activation	[285]
	GFP		Oligodendrocytes	Rats type N/S	B6 mice	Expression in white matter *in vivo*, oligodendrocytes *in vitro* due to inclusion of myelin basic protein promoter	[228]
AAV2 AAV5	GFP, Luc	NDDs	Neurons, Astrocytes		Kainate-treated SD rats	Kainate-induction reduced transduction efficiency; AAV5 better than AAV2; AAV5 glial transduction in seizured brains	[283]

	LacZ		Neurons, Astrocytes	Wistar rats	Gerbils	*In vitro:* AAV2 neuronal tropism, AAV5 astrocyte tropism; *In vivo:* higher AAV5-mediated hippocampal expression	185
	LacZ, Red fluorescent protein (RFP)	NDDs	Microglia	Wistar rats	SD rats	F4/80 promoter provided the best specificity compared to CD11b, CD68 promoters	286
AAV2, AAV6	GFP		Astrocytes, Muller cells	Humans, rats	Fischer 344 rats	Higher efficiency *in vitro*, astrocyte, muller glia transduction ability *in vivo* of AAV variants generated by loop replacement	282
	GFP		Astrocytes		Human retinas, Crb1-/- mice	Astrocyte-expression with glial fibrillary acidic protein (GFAP) promoter	189
AAV2 AAV9	GFP, L-amino acid de-carboxylase		Astrocytes		SD rats, Cynomolgus monkeys	AAV2, but not AAV9, mediated protein expression without immune response	193,295
AAV4	GFP, LacZ		Astrocytes, Neuronal progenitor cells		C57/BL 6 mice	Expression in ependymal cells, astrocytes of subventricular zone, rostral migratory stream	299

	GDNF	PD	Astrocytes	MPTP-induced B6 mice, 6-OHDA-treated Wistar rats	Expression specific to the injected hemisphere, no off-target effects; same efficacy as neuron-derived GDNF; increased DA synthesis, behavioral improvement	128
AAV5	VIVIT, a peptide that interferes with the calcineurin/ nuclear factor of activated T-cells (NFAT) signaling pathway	Alzheimer's disease (AD)	Astrocytes	Amyloid precursor protein/ presenilin-1 mice	Reduced glial activation, amyloid levels, improved cognitive and synaptic function	244
AAV8 AAVcy5 AAVrh20 AAVrh39 AAVrh43	GFP		Neurons, Astrocytes, Oligodendrocytes	SD rats	Preferential astrocyte and oligodendrocyte transduction with AAV8 and AAVrh43 using cell-specific promoters	279
AAV8, AAV9	GFP, mCherry		Neurons, Astrocytes	B6 mice	AAV8 astrocyte tropism and AAV9 neuronal tropism; single complementary (sc) AAV better than single strand (ss) AAV	186
AAV9	Erythropoietin (EPO)	PD	Neurons, Astrocytes	6-OHDA treated SD rats	Prior intramuscular injection reduced intrastriatal transduction efficiency due to circulating antibodies to AAV and EPO	179

GFP	Amyotrophic lateral sclerosis	Neurons, Astrocytes	B6 mice	Crosses blood-brain barrier, neonatal mice: neuronal tropism, adult mice: astrocyte tropism	177
GFP	Spinal muscular atrophy (SMA)	Motor neurons, Astrocytes	Survival motor neurons Δ7 and B6 mice	Intramuscular injection transduced spinal cord motor neurons, astrocytes; increased median lifespan	290
GFP	SMA	Neurons, Astrocytes, Oligodendrocytes	Cynomolgus monkeys	Global CNS expression at different ages; selective CNS expression by cerebrospinal fluid delivery	164
GFP		Neurons, Astrocytes	BALB/C mice, Rhesus macaques	Higher neuronal expression in rodent, higher glial expression in NHPs, scAAV efficient than ssAAV	168
GFP		Neurons, Astrocytes	MF1 mice	Neuronal tropism in fetal mice, astrocyte tropism in neonatal mice; ssAAV efficient than scAAV	281
GFP		Neurons, Astrocytes	B6 mice	Neonatal mice: astrocyte tropism; young mice: neuronal tropism	(Gholizadeh et al., 2013)
GFP		Neurons, Astrocytes	B6 mice	Intrathecal delivery efficient than intravenous delivery	296

	GFP		Neurons, Astrocytes	SD rats, farm mice	Dose-dependent increase in global transduction, no astrogliosis	291
	Glutamine synthetase, excitatory amino acid transporter, micro RNA targeting adenosine kinase (miADK)	Temporal lobe epilepsy	Astrocytes	Kainate-treated SD rats	miADK reduced seizure duration	293
	β-galactosidase	GM1-gangliosidosis	N/S	GM1 mice	Reduced astrogliosis, improved behavior and median survival, moderate CNS expression	294
AAV9 AAVrh10	GFP		Neurons, Astrocytes	B6 mice	Efficient transduction without inflammatory response	280
AAVrh8 AAVhu32 AAVhu37 AAVpi2AAVhu48R3	GFP		Neurons, Astrocytes, Oligodendrocytes	C3H/HeOuJ mice	Neuronal transduction by all serotypes, astrocyte and oligodendrocyte transduction by certain serotypes	383

Table 3.3: Lentiviral vectors for brain-targeted gene delivery

Gene	Disease/ Mechanism	Transduced Cell Types	*In Vitro Cell Source*	*In Vivo*	Result	Reference
CNTF	Excitotoxicity	Neurons	SD rats	Quinolinic acid (QA)-induced Lewis rats	Hypertrophied astrocytes with increased glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) expression, improved glutamate handling	313
GDNF	NDDs	Neurons, Astrocytes	Mouse N2a neural cells, ventral mesencephalon cells		Gene expression for 3+ weeks, neuroprotection from 6-OHDA insult mediated by GDNF and glutathione together	184
GDNF	PD	Astrocytes		6-OHDA treated SD rats	Efficient, sustained expression, improved DA neuronal survival, improved behavior	319
GFP	PD	Neurons, Astrocytes		Lewis rats	Astrocyte tropism by lymphocytic choriomeningitis virus (LCMV)- and Moloney murine leukemia virus (muLV)-enveloped	311
GFP	Neural scar formation	Neurons, Astrocytes, Oligodendrocytes precursors, Microglia	Wistar rats	Wistar rats	*In vitro* astrocyte and meningeal cells transduction, lack of transduction at injury/injection site *in vivo* with peripheral transduction of several cell types except meningeal cells	224
GFP		Neurons, Astrocytes		B6 mice	Astrocytes transduced with neuron promoter	307
GFP		Neurons, Astrocytes		SD rats	Vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped vector transduces glia and neurons, promoter-dependent selectivity	312
GFP		Neurons, Astrocytes		SD rats, MF1 mice	Astrocyte tropism by gp64-enveloped	310

GFP		Neurons, Astrocytes		Cyno-molgus monkeys	Higher astrocyte transduction without activation over AAV2	285
LacZ	Demyelination	Astrocytes, Oligodendro-cytes, Oligodendro-cyte progenitors		SD rats	Expressed in glial cells of spinal cord white matter, microglia and macrophages at injection site	223
LacZ		Neurons, Astrocytes, Microglia	SD rats	C3H mice	Different brain regions transduced by muLV, LCMV, Mokola virus, VSV-G enveloped, lack of transduction with Ebola virus enveloped	309
LacZ		Neurons		Fisher rats	Neurons ~90% of transduced cells at the injection site	308
miR124, shRNA GLAST, dsRednuc, mCherry		Astrocytes		BAC-GLT1-eGFP, BAC-GLAST-DsRed, B6 mice	Neuron-specific silencing, used other miRNAs; astrocyte-specific promoters and tetracycline-inducible systems for astrocyte-targeting and regulation	315
miR124T, GLAST, LacZ		Neurons, Astrocytes	SD rats	B6 mice	miR124T blocked neuronal expression and reporter expressed in astrocytes	314
miRNA chondroitin polymerizing factor or chondroitin synthase-1		Astrocytes	Neu7 rats, chicken embryo dorsal root ganglia		Reduced chondroitin sulphate proteoglycans and neurite outgrowth inhibition	316
Neurotrophin-3, BDNF	SCI	Astrocytes, Schwann cells		Long-Evans rats	Transduced multiple cell types, increased axonal growth and myelination	233
Small hairpin RNA (shRNA) GFAP, vimentin	SCI	Astrocytes	B6 mice neuroglia		Reduced glial scarring, improved neuronal survival; axonal growth increased or not affected by shGFAP	250

Table 3.4: Polymeric nanoparticles targeting different cell types in central nervous system

Pomeric Nanoparticle	Gene	Disease/ Mechanism	Transfected Cell Types	*In Vitro* Cell Source	*In Vivo*	Result	Reference
Arg-polyamido-amine (PAMAM) dendrimer	Green fluorescent protein (GFP), luciferase (Luc), small interfering RNA (siRNA) (High mobility group box 1 protein, HMGB1)		Neurons, Astrocytes, Oligodendrocytes, Microglia	Mice		Lower toxicity compared to polylactic acid or lipofectamine, transfected multiple cell types	337
	siRNA (HMGB1)	Stroke/ ischemia	Neurons, Astrocytes	Mouse neuroglial cells	Sprague-Dawley (SD) rats	*In vitro*: siHMGB1 reduced H_2O_2- and N-methyl-D-aspartate-induced apoptosis; *In vivo*: decreased infarct volume	249
Carbo-silane dendrimers	siRNA (HIV-1 p24 and Nef)	HIV-associated neurocognitive disorders	Astrocytes	Humans, U87 glioma		Sustained siRNA release, transcytosis across blood brain barrier *in vitro*, lower viral replication	346
DMAP-BLP:DSPC: cholesterol:PEG-DMG [3-(dimethylamino)propyl (12Z,15Z)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl] henicosa-12,15-dienoate distearoyl phosphati	siRNA (glutamate receptor, ionotropic, N-Methyl D-aspartate 1, GRIN1; phosphatase and tensin homolog, PTEN)		Neurons	SD rats	SD rats	*In vitro* and *in vivo* testing, apolipoprotein E (ApoE) regulated NP uptake, knocked down both targets (GRIN1, PTEN)	335

dyl-choline: cholesterol: polyethylene glycol-(1,2-dimyristoyl-sn-glycerol)]							
Lipid nanoparticle-ss-pH-activated, lipid-like materials (LNPssPalm) vitamin A (A), vitamin E (E), myristic acid (M)	Luc, LacZ, mCherry		Astrocytes	KT-5 cells	ICR/HaJ Mice	*In vitro*: ApoE3-dependent uptake; *In vivo*: higher efficiency with LNPssPalmA, LNPssPalmE	340
Lipofect-amine®	LacZ, HIV Nef		Astrocytes	Humans, Rats, U87 glioma		Lipofection effective than modified vaccinia Ankara-vector and calcium phosphate precipitation	339
	Tyrosine hydroxylase	Parkinson's disease (PD)	Astrocytes		6-hydroxydopamine (OHDA)-treated SD rats	Expression in astrocytes, behavioral recovery	344
Organically-modified silica (ORMOSIL)	$(CAG)_{20}$ or $(CAG)_{127}$ fused to human influenza hemagglutinin-tag	Huntington's disease (HD)	Neurons		R6 mice, Wistar rats	$(CAG)_{127}$ delivery activated astrocytes in mice and rats	323
PEG-polyethyneinamine (PEI)	GFP, Luc	Neuro-degenerative disorders	N/S	Rats, Rabbits type N/S	Fischer rats	Higher efficiency and viability than PEI	181
Arg-PEI-PEG	Luc		Neurons	Rats type N/S	Nude mice	Low cytotoxicity and hemotoxicity compared to PEI. Crosses BBB	165
PEG L-cysteinyl-poly-l-	Glial cell line-derived neurotrop	Aging, PD	Astrocytes	SD rats	6-OHDA treated SD rats	Higher expression in the denervated striatum, in older	336

lysine (PLL)	hic factor (GDNF)					animals due to increased gliosis	
PEG-Liposomes	Luc, LacZ		Astrocytes		BALB/c mice	Astrocyte-specific expression with GFAP promoter	338
PEI, Lipofectamine®	GFP, Luc		Astrocytes, Schwann cells	Lewis rats, Fisher rats		Comparable efficacy *in vitro*, decreased viability with PEI	331
	LacZ		Astrocytes	Rat glioma, Astrocytes, Hippocampal progenitors		Lipofectamine-higher number of cells transfected, PEI- more gene expression per cell	384
PEI-methacrylate knot polymer	GDNF	PD	Astrocytes	Rats type N/S		Lower cytotoxicity and higher expression than PEI; increased neurite outgrowth in neuronal co-cultures	342
pFastBac1, (with PEI for *in vitro*)	Luc		Astrocytes	Wistar rats	Wistar rats	With modified promoter sequences: higher expression *in vitro*, improved astrocyte-specific expression *in vivo*	363
PEI, poly-lactic-glycolic-acid (PLGA)	Interleukin (IL)-10	Spinal cord injury (SCI)	Astrocytes		SD rats	Low intrathecal dose required compared to naked pDNA	190
PEI, cholesterol-PLGA	Luc, vascular endothelial growth factor	SCI	Neurons, Astrocytes	Mouse neural stem cells	SD rats	Lower *in vitro*, *in vivo* cytotoxicity than PEI, higher efficiency *in vivo* with angiogenesis, motor function recovery	343

Table 3.5: Gene delivery- and NP-based clinical trials for neurological diseases and disorders (NDDs)

Disease	Phase	Therapeutic Intervention	Institution/ Company	ClinicalTrials.gov Identifier
Alzheimer's Disease	I	Autologous intracerebral transplantation of skin fibroblasts genetically modified to produce nerve growth factor (NGF)	The Shiley Family Trust, California, USA	NCT00017940
	I, II	Bilateral stereotactic injections CERE-110 (Adeno-associated virus (AAV) 2-β-NGF) to basal forebrain region containing the nucleus basalis of Meynert (NBM)	Ceregene, San Diego, CA, USA	NCT00087789, NCT00876863
Batten Disease	I, II	Intrathecal single dose self-complementary AAV9 encoding ceroid-lipofuscinosis neuronal (CLN) 6 gene (scAVV9.CB.CLN6)	Nationwide Children's Hospital, Columbus, OH, USA	NCT02725580
Giant Axonal Neuropathy	I	Intrathecal administration of self-complementary AAV9 encoding gene for gigaxonin (scAAV9/JeT-GAN)	National Institute of Neurological Disorders and Stroke (NINDS), Bethesda, MD, USA	NCT02362438
Leber Hereditary Optic Neuropathy	Not available	Single intravitreal injection of recombinant AAV2-nicotinamide adenine dinucleotide phosphate dehydrogenase, subunit 4 (complex I) (rAAV2-ND4)	Bin Li, Huazhong University of Science and Technology, Wuhan, Hubei, China	NCT01267422
Late Infantile Neuronal Ceroid Lipofuscinosis	I	Brain delivery of an AAV2 encoding human CLN2 cDNA (AAV2CUhCLN2)	Weill Medical College of Cornell University, Ithaca, NY, USA	NCT00151216 NCT01414985 NCT01161576
	I, II	Brain delivery of an AAV2 encoding human CLN2 cDNA (AAV2CUCLN2)		
	I	Brain delivery of an AAVrh.10 encoding human CLN2 cDNA (AAVRh.10CUhCLN2) in two separate doses		
Meta-chromatic Leuko-dystrophy	I, II	Multiple intracerebral doses of AAVrh.10 encoding arylsulfatase A (ARSA) enzyme (AAVrh.10cuARSA)	Institut National de la Santé Et de la Recherche Médicale, Paris, France	NCT01801709
Parkinson's Disease	I	Convection enhanced delivery of AAV2-Glial Cell-Line Derived Neurotropic Factor (AAV2-GDNF)	NINDS Bethesda, MD, USA	NCT01621581
	I, II	Stereotactic intrastriatal injection of ProSavin delivering genes of three key enzymes involved in the synthesis of dopamine	Oxford BioMedica, Oxford, United Kingdom	NCT00627588

	I	Continuously infused intracerebral recombinant-methionyl GDNF (r-metHuGDNF)	National Center for Research Resources (NCRR) Bethesda, MD, USA	NCT00115427, NCT00111982, NCT00006488, NCT00148369
	I, II	Bilateral surgical infusion of AAV-mediated glutamic acid decarboxylase (AAV-GAD) gene transfer to subthalamic nuclei	Neurologix, Inc., Fort Lee, NJ, USA	NCT00643890, NCT00195143, NCT01301573
	I, II	Intraputaminal/striatal infusion of AAV-human aromatic L-amino acid decarboxylase	Voyager Therapeutics & Genzyme, Cambridge, MA, USA; Jichi Medical University, Shimotsuke, Tochigi, Japan	NCT01973543, NCT00229736, NCT02418598
	I, II	Intraputaminal and intranigral administration of CERE-120 (AAV2-Neurturin)	Ceregene, San Diego, CA, USA	NCT00985517, NCT00400634, NCT00252850
Progressive Supranuclear Palsy	I	Continuously infused intracerebral r-metHuGDNF	NINDS, Bethesda, MD, USA	NCT00005903
Schizophrenia, Cognition	I, II	Curcumin nanoparticles to improve cognitive dysfunction	VA Greater Los Angeles Healthcare System, Los Angeles, CA, USA	NCT02104752
Spinal Muscular Atrophy Type 1	I	Intravenous delivery of AVXS-101 *i.e.* self-complementary AAV9 encoding the spinal motor neuron gene under the control of a hybrid CMV enhancer/chicken-β-actin promoter	AveXis, Inc, Bannockburn, IL, USA	NCT02122952

CHAPTER 4

REACHING FOR THE STARS IN THE BRAIN: POLYMER-MEDIATED TIMP-1 GENE DELIVERY TO HUMAN ASTROCYTES

PARTS FORMATTED AND PUBLISHED AS

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4.1 Abstract

Astrocytes, the ‘star-shaped’ glial cells, are appealing gene delivery targets to treat neurological diseases due to their diverse roles in brain homeostasis and disease. Cationic polymers have successfully delivered genes to mammalian cells, hence present a viable, non-immunogenic alternative to widely used viral vectors. In this study, we investigated the gene delivery potential of a series of arginine- and polyethylene glycol-modified, siloxane-based polyethylenimine analogues in primary cultured human neural cells (neurons and astrocytes) and in mice. Plasmid DNAs encoding luciferase reporter were used to measure gene expression. We hypothesized that polyplexes with arginine would help in cellular transport of the DNA, including across the blood-brain barrier, polyethylene glycol will stabilize polyethylenimine and reduce its toxicity; while maintaining its DNA condensing ability. Polyplexes were non-toxic to human neural cells and red blood cells. Cellular uptake of polyplexes and sustained gene expression was seen in human astrocytes as well as in mouse brains post-intravenous injections. The polyplexes also delivered and expressed genes driven by astrocyte-restricted glial fibrillary acidic protein promoters, which are weaker than viral promoters. Subsequently, GFAP promoters were modified to enhance their activity and were used to drive luciferase and tissue inhibitor of metalloproteinases (TIMP)-1 expression in human astrocytes. To our knowledge, the presented work validates a biocompatible and effective polymer-facilitated gene delivery system for both human brain cells and mice for the first time.

4.2 Introduction

Gene-based therapies can address the growing prevalence of neurological diseases and disorders (NDDs). A single, effective gene delivery system could be used

to treat multiple NDDs by altering gene expression of one or more targets, thereby reversing damage and restoring function.[385] Astrocytes, the 'star-shaped' glial cells in the brain, are an attractive target for treating NDDs. During homeostasis conditions, astrocytes regulate neurotransmission and synaptic activity by sequestering potassium and neurotransmitters, including glutamate.[236] They have preferential access to therapeutics delivered *via* the vascular route since their foot-processes are the integral components of the blood-brain barrier (BBB).[234] Reactive astrogliosis is a hallmark of chronic inflammation characterized by subtle changes to astrocyte structure and function.[386] Due to such diverse structural and functional characteristics, modulating astrocyte gene expression could benefit a broad spectrum of NDDs. Further, astrocytes secrete several neurotrophic[132,235] and neuroinflammatory mediators.[127,237,387] Thus, an astrocyte-targeted gene therapy could be used to upregulate neurotrophic factor expression and/or silence that of toxic mediators. To avoid off-target effects on other brain cells, targeted gene delivery can be implemented by using astrocyte-restrictive promoters such as glial fibrillary acidic protein (GFAP)[355], glutamate transporter-1, excitatory amino acid transporter 2[357], and aldehyde dehydrogenase 1 L1.[358]

Gene delivery to astrocytes or any other cell type in the brain is contingent upon availability of an effective yet biocompatible gene delivery system. Few viral vector-based therapies for Alzheimer's and Parkinson's diseases that transitioned into clinical trials, did not show adequate effectiveness in early phases.[367,370] On the other hand, greater versatility, ability to modulate the polymer composition, and lack of immunogenicity make polymer-based gene delivery systems more appealing than viral vectors for NDD therapies. Among non-viral gene delivery systems, cationic polymers and lipids[249,330,343]

have been extensively tested. They bind with the negatively charged DNA and deliver it across negatively charged cellular membrane. Amongst cationic polymers, polyethylenimine (PEI) is considered as a 'gold standard' for transfection but its use is restricted due to its cytotoxicity caused by its high cationic charge, disrupting cell membrane integrity.[341,388] Further, PEI interacts with serum proteins *in vivo* and also causes red blood cells (RBCs) aggregation and lysis. Therefore, substantial efforts are required to mitigate PEI toxicity that offsets its transfection and DNA condensing abilities. We synthesized six siloxane-based PEI analogues, modified by arginine (A), and stabilized with polyethylene glycol (PEG)(P), collectively referred as A_nP_n, in which n denotes molar equivalent (eq) of A or P per PEI in the composition. Six different polymers were generated that differed in the molar ratio of arginine to PEI used while synthesizing these polymers. In addition, S1 series of polymers denotes where the reaction was carried out without the pH adjustment and S2 series where the pH of the reaction buffers were pre-adjusted (**Table 1**).

Choosing the most relevant *in vitro* or *in vivo* models to test gene delivery systems is crucial for proof-of-concept studies. Thus far, gene delivery investigations have predominantly used rodent-derived primary cells and rodent models,[165,169,177,331,343] while primary human brain cells have been seldom tested successfully. Gray and colleagues reported adeno-associated virus 9-based vector preferentially transducing neurons in mice and glia in non-human primates (NHPs).[168] Similar findings were also depicted in NHPs of all ages and the difference in transduced cell types in rodents versus NHPs was attributed to the difference in timing of gliogenesis of these species.[164] Another study testing adeno-associated virus-based delivery using rodent models reported gene

promoter dependent changes in transduced cell types.[169] These findings led us to believe that species of tested models and gene promoters are critical in achieving translational pre-clinical data. Therefore, in this study, we utilized primary human neurons and astrocytes for *in vitro* testing; before validating brain transfection potential of polyplexes in mice following intravenous (i.v.) injections. We also tested and modified astrocyte specific promoters to restrict gene expression to astrocytes. Plasmids encoding luciferase reporter (pLuc) and tissue inhibitor of metalloproteinases (TIMP)-1 were used to determine gene expression using different polyplexes.

We hypothesized that a balanced composition of the polymer with PEI condensing the DNA, arginine helping the DNA transport across plasma membrane, and PEG providing the stability to the polyplex would be effective in neural cell transfection and *in vivo* brain gene delivery. First, thorough *in vitro* studies were performed to assess transfection levels, bio- and hemocompatibility, cellular uptake, and sustained expression in primary cultures of human neurons and astrocytes using pLuc. The most suitable polymer, which showed highest transfection levels and biocompatibility, was then used to validate *in vivo* brain delivery in mice. We also confirmed polymer-mediated, GFAP promoter-driven gene delivery using reporter and TIMP-1 plasmids in human astrocytes. Our data convincingly demonstrate an effective, non-viral gene delivery system for human neural cells as well as rodent models and offer novel opportunities for preclinical and clinical options for treating neurodegeneration.

4.3 Materials and Methods

Synthesis of A_nP_n polymers

3-(2-Aminoethylamino) propyl-methyl-dimethoxysilane was purchased from Fluka (Sigma–Aldrich, St. Louis, MO), Polyethylenimine, branched, Mw; 25-kDa (PEI), N-hydroxysuccinimide (NHS), polyethylene glycol bis(carboxymethyl)ether, Mw; 600Da (PEG), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), Nα-(tert-Butoxycarbonyl)-Aspartic acid (Boc-Asp), 2-(N-morpholino) ethanesulfonic acid (MES), and L-arginine were purchased from Sigma–Aldrich (St. Louis, MO).

Oligo-(alkylaminosiloxane) was prepared as described previously[389]. Briefly, 1 eq of 1 N NaOH solution (32 µL) was added to 1.8 mmol (0.371g, 1eq) of 3-(2-aminoethylamino) propyl-methyl-dimethoxysilane and stirred for 20 h at RT. The removal of residual solvent and small volatiles gives Oligo-(alkylaminosiloxane). The schematic of this reaction and polymer compositions have been described previously.[390] Supplementary Figure 4.1 provides a schematic of A_nP_{10} polymers used in this study.

Next, L-arginine, PEI and PEG were conjugated in consecutive steps onto oligo-(alkylamino siloxane) by a four-step reaction using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide chemistry[23]. For every conjugation reaction, carboxylic acid activation and the reaction between amine and activated carboxylic acid were carried out in MES and phosphate buffer saline (PBS), respectively. Two types of polymers were synthesized. The first type were synthesized by not adjusting the pH (S1) and the second type by adjusting the pH of MES (6.5) and PBS (7.2) buffers, using HCl and NaOH, after adding all the reactants (S2). The conjugation efficiency is expected to be higher when the activation of carboxylic acid is performed at pH 6.5 and the reaction

between activated carboxylic acid and amine is performed at pH 7.2. All polymers where dialyzed extensively against milli Q water to remove untreated monomers and freeze-dried.

Plasmids, sub-cloning and amplification

All gene delivery experiments were carried out using cytomegalovirus promoter-driven luciferase as a reporter plasmid vector (pLuc) unless specified otherwise. Untagged human TIMP-1 open reading frame (ORF) clone was purchased from Origene (Catalog no. SC118082). All GFAP promoter-encoding constructs gfa2-lacZ (Catalog no. 53126), gfa28-LacZ (Catalog no. 53130), and gfaABC1D-LacZ (Catalog no. 53131) were obtained from Addgene (courtesy of Dr. Michael Brenner).[354,355] All plasmids were amplified by transforming *Escherichia coli* DH5α competent cells and isolated using endotoxin-free pDNA isolation kits according to manufacturer's instructions (Qiagen, Valencia, CA). Subcloning Luc or TIMP-1 was carried out using restriction digest, followed by DNA ligation. All reagents were purchased from New England Biolabs (Ipswich, MA) unless otherwise specified. Luciferase ORF and TIMP-1 ORF were excised from cytomegalovirus (CMV) promoter driven CMV-Luc and TIMP-1 ORF clone, respectively. The GFAP promoter constructs were digested to remove LacZ ORF. Restriction digests were run on an agarose gel, and DNA gel extraction Kit was used to isolate required fragments (Qiagen, Valencia, CA). Short oligonucleotide sequences were ordered from Sigma (St. Louis, MO). The oligos were designed such that two oligo strands formed double-stranded DNA inserts with 5' sticky ends for the segments of gfa, Luc, and TIMP-1. Equimolar concentrations of complimentary oligonucleotides were mixed and incubated at 85^{o}C for 5 minutes and cooled down to RT. The fragments (Luciferase ORF

or TIMP-1 ORF, GFAP promoter plasmids without LacZ ORF, double-stranded oligos) were mixed with DNA ligase as per manufacturer's instructions. Subsequently, DH5α competent cells were transformed with ligated products. Appropriately ligated clones were obtained after a series of plasmid minipreps and restriction digests, which were then isolated using endotoxin-free pDNA isolation kits (Qiagen, Valencia, CA).

Characterization of A_nP_n: pDNA polyplexes

Polymers and pDNA were dissolved in nuclease-free water at a concentration of 1 mg/ml. Polyplexes were prepared in 5 mM PBS (pH 7.4) by mixing pDNA with different amounts of polymer and medium. The pDNA-polymer solutions were mixed well by triturating, vortexed, and then incubated for 15–20 min at RT. Polyplex size and zeta potential were determined using quasi-elastic light scattering (PSS/NICOMP 380/ZLS Particle Sizing Systems, Santa Barbara, CA).

Cell culture

Primary human neurons and astrocytes were harvested from first- and early second-trimester fetal specimens, obtained from the Birth Defects Research Laboratory, University of Washington (Seattle, WA), in full compliance with the ethical guidelines of the National Institutes of Health (Bethesda, MD), University of Washington and University of North Texas Health Science Center, Fort Worth, TX. The Birth Defects Research Laboratory obtained written consent from all tissue donors. Human neurons and astrocytes were isolated as previously described.[99] Human neurons were cultured on poly-d-lysine treated plates in neurobasal medium with 1X B27 supplement for 2-4 weeks before treatments. Astrocytes were cultured in 1:1 v/v DMEM:F12 medium with 10% fetal bovine serum (Peak Serum, Lot no. 17C161) for 20-24 h before treatments. Both media

contained 1X penicillin-streptomycin-neomycin (PSN) (Catalogue No. P4083, Sigma Aldrich, St. Louis, MO) and Fungizone (Catalogue No. A9528, Sigma Aldrich, St. Louis, MO). Neurons were plated at a density of 100,000 cells/well and astrocytes at 150,000 cells/well in 48-well plates. All reagents for cell culture media were Gibco brand (Fisher Scientific, Waltham, MA) unless otherwise specified.

Transfecting with polyplexes in vitro

All transfection experiments were carried out with cells cultured in 48 well plates. Polymers and pDNA were dissolved in culture medium at a concentration of 1 mg/ml. The polyplexes were prepared by mixing appropriate amounts of pDNA and polymer in culture media to make a final volume of 15 µl/well. The mixtures were incubated for 15-20 min at RT for polyplex formation. More culture medium was added to the polyplexes before adding to cells and final concentration of the pDNA in each well was 1 µg/well. Unless otherwise specified, cells were treated with polymer:pDNA for three hours, polyplexes were then 'washed' after three h, fresh culture medium was added, and end-point assays were carried out 48 h post-treatment. For A_nP_n-pDNA uptake experiments, an additional treatment paradigm was used in which cells were left 'unwashed' at three h, and media was not replaced prior to protein expression and cytotoxicity evaluations.

Determination of luciferase activity in vitro

The culture media was removed from wells and cells were lysed with 65 µL of 1X Reporter Lysis Buffer. Then, lysates were loaded in 96-well white-colored plates (20 µL/well) and Luciferase activity was measured according to manufacturer's instructions (Promega, Madison, WI).

Determination of cytotoxicity

Metabolic activity of neural cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay as described previously.[391] Absorbance was measured at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Cell supernatants were collected and lactate dehydrogenase (LDH) activity was evaluated using Cytotoxicity Detection Kit^{PLUS} according to manufacturer's instructions (Sigma Aldrich, St. Louis, MO). The data was then normalized to untreated controls and plotted as % increase or decrease as compared to controls.

Determination of TIMP-1 levels

Levels of TIMP-1 were measured in cell supernatants using an enzyme linked immunosorbent assay (ELISA) (R and D Systems, Minneapolis, MN) as per manufacturer' instructions. After collection of supernatants, metabolic activity of cells was measured using MTT assay as described above. The MTT values were normalized against control for fold change in metabolic activity. The TIMP-1 levels were presented as TIMP-1/unit MTT by normalizing TIMP-1 levels against its MTT activity. Respective luciferase plasmid served as negative control.

Evaluation of hemocompatibility

RBC lysis in the presence of polyplexes was evaluated to determine their hemocompatibility. Blood was collected from healthy human volunteers in tubes containing potassium EDTA (Sigma–Aldrich). Collected whole blood was centrifuged at 1500 g for 10 min at RT (Sorvall Legend RT; Thermo Scientific, Waltham, MA) and RBCs were washed three times with PBS. To determine hemolysis, RBCs were diluted six times with PBS and incubated at RT with polyplexes (polymer:pDNA ratio 2:1 weight/weight,

w/w) for three h. The RBC-polyplex mixture was centrifuged for 10 min, and the supernatant (50 µl) was dissolved in 150 µl of 40:1 (v/v) ethanol: HCl mixture in a Nunc™ 96-well polypropylene MicroWell™ plate (Thermo Scientific, Waltham, MA). The absorbance was measured at 399 nm. RBCs incubated with deionized water were used as the positive control for complete lysis, and RBCs incubated with PBS served as no lysis control.

Animals and in vivo transfection

Male, athymic nude (nu/nu) mice (nu/nu, 5–6 weeks old) were purchased (Envigo, Allison Pointe Blvd, IN), housed in a temperature- and light-controlled room on a 12:12 h light: dark cycle with *ad libitum* water and food. Cleveland Clinic's institutional animal care and use committee approved all animal procedures, and these were carried out according to federal and internal guidelines. Mice were anesthetized with 2% isoflurane in oxygen and maintained in anaesthetized state during tail vein injections using 1% isoflurane in oxygen *via* nose cone. Polyplexes containing 50 µg of pDNA (2:1 w/w A_nP_n:pDNA) in 300 µl saline were injected *via* tail vein on 3 consecutive days at 24 h intervals. Animals not injected with polyplexes or DNA were used as controls to check for the background signal. At 24, 48, and 72 h following the last injection, intraperitoneal injection of luciferin (200 mg/kg, VivoGlo Luciferin, Promega, Madison, WI) was administered to mouse, anaesthetized, and euthanized by cervical dislocation. Animals were perfused with normal saline following cardiac puncture to wash out blood. Brains and livers were collected to determine the extent of protein expression. All organs following dissection were rinsed with saline, cut into small pieces, incubated with VivoGlo Luciferin (Promega) (1.5 µg per 5 mg tissue weight) for 40 min in 24 well plates (Becton Dickinson Labware,

Franklin Lakes, NJ) and imaged with IVIS® Lumina II (PerkinElmer, Hopkinton, MA). The bioluminescence signal intensity (photons per square centimeter steradian [p/s/cm^2/sr] of the organs from the injected and uninjected mice were plotted. Each treatment group consisted of 3-4 mice and cumulative data has been shown.

Immunocytochemistry and immunohistochemistry

For immunocytochemistry, neural cells were fixed in acetone: methanol (1:1) for 15-20 min at −20 °C. After 30 min incubation with blocking buffer, the cells were probed with primary antibodies and incubated overnight at 4°C. Human astrocytes were probed with primary antibody against GFAP (produced in rabbit, 1:700, Catalogue no. Z0334, Dako, Carpinteria, CA) and neuroglial cultures were probed with antibodies against Microtubule-associated protein (MAP)-2 (produced in chicken, 1:2000, Catalogue no. Ab5392, Abcam, Cambridge, UK) and GFAP (produced in rabbit, 1:1000, Dako, Carpinteria, CA). Then, cells were washed three times in PBS and labeled according to primary antibodies with anti-chicken and/or -rabbit (488 nm, green or 594 nm, red) Alexa Fluor secondary antibodies (1:100, Thermo Fisher Scientific, Waltham, MA), for 1.5 h at RT. Further, cells were washed thrice with PBS and 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) was added for 3 min at RT to visualize nuclei (1:1000, blue, Thermo Fisher Scientific, Waltham, MA). Post-DAPI staining, images were taken at 100-200x magnification on Nikon Eclipse and processed by NIS-Element BR 3.2 software (Nikon Inc., Melville, NY).

For immunohistochemical analysis, brains were harvested after perfusion of animals with saline. The brains were then fixed by immersing them into with 5 ml of 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) diluted in 1X PBS. The

harvested brains were cryopreserved in a 30% sucrose (Sigma–Aldrich) solution in 1X PBS at 4 °C overnight. The brains were then frozen in the cryotome cryostat using Tissue-Tek embedding medium (Sakura Finetek USA, Inc., Torrance, CA) at −20 °C. Frozen coronal sections of the brain of 40 µm thickness were cut (CM 1900, Leica, Buffalo Grove, IL). After blocking (3% horse serum, 0.3% Triton X-100 in 1X PBS), sections were incubated at 4 °C overnight with anti-luciferase antibody (produced in rabbit, 1:500, Abcam, Cambridge, UK) and GFAP antibody (produced in chicken, 1:1000, catalogue no. Poly28294, San Diego, CA). After washing the brain sections with PBS, they were stained appropriately with anti-chicken (488 nm, green) and -rabbit (488 nm, green) Alexa Fluor secondary antibodies (1:1000). Finally, nuclear staining was done with DAPI. Images were taken at 100X magnification on Zeiss LSM 510 (Carl Zeiss AG, Oberkochen, Germany).

Statistical analyses

All numerical data were taken as mean ± SEM for analyses and statistical analysis was performed using GraphPad Prism 7.0. In cytotoxicity analyses, data were normalized with untreated controls and raw data were used in luciferase assay analysis. All data were analyzed using a one-way (cytotoxicity assays) or a two-way analysis of variance (ANOVA)(luciferase assays) with Tukey or Fisher's least significant difference post-hoc tests for pair-wise comparisons. Differences were considered statistically significant with $p \leq 0.05$.

4.4 Results

Characterization of A_nP_n polymers

Synthesized A_nP_{10} polymers consisted of different molar amount of arginine used per PEI equivalent (A_1, A_5, and A_7). Polymers were synthesized by not adjusting (series 1, S1) or adjusting (series 2, S2) the pH of MES/PBS at 6.5 and 7.2, respectively. MES buffer was used to carboxylic acid activation and PBS buffer was used for reaction between amino group and activated carboxylic acid. The polymers and pLuc were mixed in 2:1 w/w ratio to form polyplexes. The zeta potential (23-29 mV) and particle size (50-90 nm) of all polyplexes were in a narrow range irrespective of the synthesis method or the number of arginine residues (**Table 4.1**).

A specific polymer:pDNA ratio achieves optimal gene expression and biocompatibility in primary human fetal astrocytes

Human astrocytes were transfected with different w/w ratios of one of the A_nP_n polymers, specifically S2 A_5P_{10}, and pLuc to evaluate the optimized polymer to pDNA ratio that is biocompatible and yet effective in achieving high level of transfection (**Figure 4.1**). Unmodified PEI was used as a positive control of transfection. Both S2 A_5P_{10} and PEI were complexed with pLuc at w/w ratios of 1:1, 2:1, 4:1, and 8:1 keeping concentration of pLuc constant at 2.5 µg/ml to form polyplexes. Luciferase expression was significantly higher in cells treated with 2:1 w/w ratio than other ratios for both polymer and PEI, respectively (*$p<0.05$, **$p<0.01$). Also, S2 A_5P_{10} 2:1 w/w transfection led to higher luciferase activity compared to PEI 2:1 w/w transfection ($^{\#\#}p<0.01$) (**Figure 4.1A**). In parallel, LDH activity increased in 4:1 and 8:1 S2 A_5P_{10}-treated cells compared to 1:1 S2 A_5P_{10}-treated cells (*$p<0.05$, **$p<0.01$). Though LDH activity did not change with

increasing polyplex ratio in PEI-treated cells, it was higher in 2:1 PEI compared to 2:1 A_nP_n-treated cells ($^{\#}P<0.05$) (**Figure 4.1B**). Metabolic activity, measured as MTT absorbance, did not change in S2 A_5P_{10} polyplex-treated cells with increasing ratios, and decreased in 4:1 and 8:1 PEI treated cells compared to 1:1 PEI-treated cells (**p<0.01, ***p<0.001). Cells treated with PEI showed lower MTT compared to S2 A_5P_{10} at 2:1, 4:1, and 8:1 ratios ($^{\#}p<0.05$, $^{\#\#}p<0.01$), respectively (**Figure 4.1C**). Since 2:1 w/w polyplexes provide higher transfection levels and low cytotoxicity compared to other compositions, further experiments were conducted using this ratio.

Physicochemical/pH differences during polyplex formulations alter the levels of gene expression and biocompatibility in human neural cells.

Polyplexes generated from six polymers (S1 A_1P_{10}, S1 A_5P_{10}, S1 A_7P_{10}, S2 A_1P_{10}, S2 A_5P_{10}, and A_7P_{10}) (details in **Table 4.1**) were tested for their transfection and biocompatibility in human neural cells. Transfection levels were evaluated by measuring luciferase activity while biocompatibility was assessed with LDH and metabolic assays. Polymer effects on cell morphology were determined as an additional indicator of biocompatibility.

In astrocytes, higher gene expression was observed in S2 A_1P_{10} and A_5P_{10} compared to S1 A_5P_{10}, S1 A_7P_{10}, and S2 A_7P_{10} (*p<0.05). A two-way ANOVA revealed that synthesis method, but not the number of arginine residues, affected transfection levels (*p<0.05 for S1 v/s S2) in astrocytes (**Figure 4.2A**). None of the six A_nP_n analogues increased LDH activity compared to controls (**Figure 4.2B**). Metabolic activity was reduced (7-21%) across polyplex treatments (*p<0.05, ***P<0.001). S1 A_1P_{10} and A_7P_{10} were the most cytotoxic with 21% and 15% decrease in metabolic activity, respectively.

S2 polymers presented better biocompatibility as indicated by 7-12% decrease in metabolic activity (**Figure 4.2C**). All polyplexes, with the exception of S1 A_1P_{10}, did not alter glial morphology in comparison to untreated controls as per GFAP staining (**Figures 4.2D-2J**). A DNA intercalating dye DAPI stained nuclei and pLuc in the cytoplasm. The cytoplasmic DNA staining indicated that polyplexes were in the cytoplasm (**Figures 4.2E-2J**). The cytoplasmic DAPI staining was most distinct in S1 A_1P_{10} -treated astrocytes (arrows in Figure 2E).

In neurons, S2 A_5P_{10} transfection led to the highest luciferase expression compared to all other polyplexes except S2 A_1P_{10} (*$p<0.05$). Buffer pH or the proportion of arginine residues used during synthesis did not affect the transfection levels in human neurons (**Figure 4.3A**). In neurons, S1 A_1P_{10} was the most cytotoxic compared to the other polymers (***$p<0.001$) as measured with LDH and MTT activities, respectively (**Figures 4.3B** and **4.3C**). Except S1 A_1P_{10}, none of the polyplexes altered neuronal morphology compared to controls as characterized by microtubule associated protein 2 (MAP-2) staining (**Figures 4.3D, 4.3F-3J**). However, S1 A_1P_{10}-treated neurons showed loss of processes and constricted cell bodies (arrows in **Figure 4.3E**). Co-staining with GFAP indicated small number of astrocytes in the neuronal cultures, which were similar in all treatments.

Overall, S2 polymers led to higher luciferase activity and better biocompatibility compared to their S1 counterparts in both astrocytes and neurons. Therefore, only S2 polymers were used in the follow-up studies.

Time-based kinetics reveals a rapid uptake of S2 polyplexes and sustained gene expression in primary human astrocytes

To evaluate the uptake pattern of polyplexes in primary human astrocytes, two different transfection paradigms were implemented. In the first method, consistent with the previous experiments, cells were treated with polyplexes for 3 h, then polyplexes were 'washed' and fresh media was replenished (**Figures 4.4A**-**4C**). In the second paradigm, cells were left 'unwashed' (**Figures 4.4D**-**4F**). Luciferase activity was measured at 3, 8, 24, and 48 h post-transfection (**Figures 4.4A** and **4.4D**). In both the treatment paradigms, luciferase expression was undetectable at 3h, detectable at 8 h and increased at 24 and 48 h in A_1P_{10} (red line, squares) and A_5P_{10} (blue line, triangles) treated cells. A_1P_{10}- and A_5P_{10}-mediated luciferase expression was higher compared to A_7P_{10} (green line, inverted triangles) at 24 and 48 h in both the paradigms ($^{\$}p<0.05$) (**Figures 4.4A** and **4.4D**). A_1P_{10}-facilitated protein expression in washed cells was also considerably higher than A_5P_{10} at 48 h ($^{\#}p<0.05$) (**Figure 4.4A**). Simultaneous cytotoxicity assays depicted no change in LDH activity in cells treated for 3 h (**Figure 4.4B**), and it increased by 8-15% at 48 h in continuous treatment (***p<0.001) (**Figure 4.4E**). The metabolic activity recovered in both the paradigms after an initial reduction at 8 h (**p<0.01) and was comparable to untreated controls (**Figures 4.4C** and **4.4F**). Collectively, these results indicate that optimal polyplex uptake occurs in a few minutes to hours, while cytotoxicity assays demonstrate that polyplexes are biocompatible even following a prolonged exposure to cells.

After delineating cellular uptake pattern, we evaluated sustained luciferase expression obtained with S2 analogues. To delineate this, human astrocytes were treated

with polyplexes for 3 h, and luciferase expression was assessed at time points starting at 8 h through 10 d. (**Figure 4.5A**). Consistent with the previous data in **Figure 4.4**, luciferase activity was detectable at 8 h and peaked at 2 d post-treatment. Expression dropped at 4 d (~75% from day 2) and then remained stable until 7 d. Both A_1P_{10} (red line, squares) and A_5P_{10} (blue line, triangles) consistently depicted higher expression compared to A_7P_{10} (green line, inverted triangles) at 2 d, 4 d, and 7 d, respectively ($^{\$\$\$}p<0.001$). At 7 d, luciferase expression was higher in A_1P_{10} polyplex-treated cells than A_5P_{10} polyplex-treated cells ($^{\#\#\#}p<0.001$) (**Figure 4.5A**). Beyond 7 d, luciferase expression, while detectable, dropped inconsistently up to 10 d in astrocytes derived from multiple brain tissues (data not shown). In parallel, LDH and MTT activities were measured at 4 d and 10 d (**Figures 4.5B** and **4.5C**). LDH activity increased for A_1P_{10} and A_5P_{10} at 4 d (***p<0.001); however, it recovered and was comparable to untreated controls at 10 d (**Figure 4.5B**). Metabolic activity decreased in A_7P_{10}-polyplex treated cells at 4 d and in all treatments at 10 d (***p<0.001, ~11-18%) (**Figure 4.5C**), which was comparable to the drop in metabolic activity seen at 2 d (**Figure 4.2C**).

Taken together, these results depict that cellular uptake of polyplexes with a short uptake can successfully sustain gene expression over longer time periods.

Hemocompatibility of A_nP_n polyplexes and polyplex-mediated gene delivery to mouse brains

Prior to confirming delivery to mouse brains, it was critical to confirm the hemocompatibility of polyplexes. Human RBCs were isolated from healthy human donors and were incubated with polyplexes for 3 h at room temperature (RT). Deionized water was used as a positive control. Lysis of RBCs incubated with all analogues, except S1

A_1P_{10} and S2 A_7P_{10} was ≥ 0.25% or undetectable indicating good hemocompatibility (**Figure 4.6A**). With S2 A_7P_{10}, RBC lysis was marginally higher ~2.2%). Significant RBC lysis was observed with S1 A_1P_{10} (~5.5%) when compared to the negative control (1X phosphate buffer saline). Additionally, none of the polyplexes induced RBC aggregation (data not shown).

After confirming hemocompatibility in human RBCs, we wanted to validate *in vivo* polymer-mediated i.v. gene delivery. For this, S2 A_5P_{10}-pLuc polyplexes were injected *via* tail vein to athymic nude (nu/nu) mice. Luciferase expression was evaluated in the liver and brain tissues (**Figure 4.6B**) at 24, 48, and 72 h following three injections. Liver tissue isolates depicted little to no luciferase expression compared to baseline controls. For brain isolates, the highest luciferase activity was measured at 48 h while brains isolated at 24 h and 72 h showed marginally higher luciferase activity than the control brain isolates. Immunohistochemical analyses were performed to detect luciferase localization in the mice brains. Confocal micrographs showed luciferase expression in the cortical brain regions in astrocytes (arrowheads in panels **4.6D4** and **4.6E4**) as well as other cells as indicated by luciferase-GFAP co-staining **(Figure 4.6C-4.6E)** (D Con, E 24 h, F 48h). We anticipate that using constructs with GFAP promoters would restrict the expression to astrocytes and eliminate off-target expression. In summary, polyplexes effectively crossed the BBB and delivered pDNA to brain cells, which underscores a broader gene delivery potential of our polyplex-based gene delivery system.

Polymer-mediated, GFAP promoter-driven gene delivery to human astrocytes

Brenner and colleagues have reported a 2.1 kilo-bases region of the GFAP promoter *i.e.* gfa2, and truncated portions of gfa2, *i.e.* gfa28 and gfaABC1D are

responsible for restricting gene expression to either a specific brain region or to astrocytes.[354,355] A luciferase reporter gene was sub-cloned downstream of these GFAP promoters (**Figure 4.7A**). Next, we tested delivery of GFAP promoter-driven luciferase plasmids using A_nP_n. Due to their highest transfection levels, we complexed A_1P_{10} and A_5P_{10} with each GFAP promoter construct (gfa-Luc) at 2:1 w/w ratio. Astrocytes were treated with polyplexes for 3 h and luciferase levels were measured at 48 h. While weak luciferase expression was measured in cells transfected with gfa2-Luc with both the polyplexes, gfa28-Luc and gfaABC1D-Luc led to higher luciferase activity **(Figure 4.7B)**. In A_1P_{10}-mediated transfections, gfaABC1D-driven gene expression was higher as compared to gfa2-driven (*$p<0.05$). In parallel, A_5P_{10}-mediated gfa28-Luc transfection resulted in higher expression compared to gfa2-luc (*$p<0.05$) **(Figure 4.7B)**. Next, A_nP_n-mediated gfa28-TIMP-1 and gfaABC1D-TIMP-1 delivery did not result in TIMP-1 overexpression (data not shown). Overall lower luciferase activity levels and lack of TIMP-1 overexpression were attributed to weaker GFAP promoter activity. However, luciferase expression results confirmed the translational feasibility for astrocyte-targeted gene delivery.

In order to increase the activity of GFAP promoter, a CMV enhancer was cloned upstream of gfa28-Luc and gfaABC1D-Luc (**Figure 4.8A**). Next, we evaluated the S2 A_5P_{10}-mediated delivery of gfa28-Luc and gfaABC1D-Luc in the presence or absence of CMV enhancer (**Figure 4.8B**). We complexed A_5P_{10} with GFAP promoter-driven luciferase constructs at 2:1 w/w ratio. Astrocytes were treated with polyplexes for 3 h and luciferase levels were measured at 48 h. Luciferase expression in astrocytes transfected with CMVe-gfaABC1D-Luc was higher than gfaABC1D transfection alone. Similarly,

CMVe-gfa28-Luc transfection led to robust luciferase expression compared to gfa28-Luc (**p<0.01). In parallel studies, CMV enhancer sequence was also cloned upstream of gfa28-TIMP-1 and gfaABC1D-TIMP-1 plasmids (**Figure 4.8C**). Next, A_5P_{10} was complexed with GFAP promoter-driven luciferase or TIMP-1 constructs at 2:1 w/w ratio. Astrocytes were treated with polyplexes for 3 h and TIMP-1 levels were measured at 48 h. Both CMVe-gfa28-TIMP-1 and CMVe-gfaABC1D-TIMP-1 increased TIMP-1 expression in astrocytes when compared to normalized TIMP-1 levels in CMVe-gfa28-Luc and CMVe-gfaABC1D-Luc, respectively (*p<0.05). These data indicate that GFAP promoter constructs could be modified to improve their activity and polymer-mediated GFAP promoter-driven gene delivery of therapeutic genes such as TIMP-1 is possible.

4.5 Discussion and Conclusions

In the past few years, CNS-targeted therapeutics have been in great need owing to increasing global prevalence of NDDs and lack of effective treatment options. Astrocytes have emerged as critical functional components of the CNS. Our review of the current literature[385] suggested that astrocyte-directed CNS therapies are predestined in the near future. This study is a first-step in that direction, testing a gene-therapy approach using biocompatible and hemocompatible polyplexes that demonstrated cellular uptake and sustained gene expression in primary neural cells and *in vivo* in mice (**Supplementary Figure 4.2**).

Selecting the appropriate *in vitro* model is a critical first step for evaluating translational potential of human therapies. Prior studies have successfully targeted astrocytes using viral or non-viral gene delivery systems in rodent disease models including for neuropathic pain,[190] in Alzheimer's disease,[244] Parkinson's disease,[344] and

Huntington's disease.[125,392] Results from these studies validate the utility of targeting astrocytes for the CNS therapeutics. However, human astrocytes have diverse morphologies and distinct electrophysiological properties as compared to rodent astrocytes.[393,394] Furthermore, gene expression profiles of human and rodent astrocytes are markedly different.[395] These findings suggest that the source species of astrocytes could impact their ability to express a gene or functional effects of therapeutic gene expression. Few viral vector-based studies have also highlighted that species of tested animals to be a critical aspect in determining cell-type specific gene expression patterns.[164,168] In this context, primary neural cells from over a dozen independent human tissues were used in our study. To our knowledge, this is the first proof-of-concept study simultaneously depicting polymer-mediated gene delivery to primary human brain cells and capable of traversing the BBB in rodent models.

Polyplex-based transfections are influenced by several factors including DNA condensation, cellular uptake, cytoplasmic delivery and DNA release from polyplexes for nuclear localization. Important factors also include, stability and aggregation in the presence of serum proteins, biocompatibility and hemocompatibility. The presented study addressed each of these factors by implementing step-wise, deliberate investigations to depict successful gene delivery to primary neural cells. First, we determined optimal polyplex ratio, which would condense pDNA, would not affect plasma membrane interactions, and facilitate their endosomal escape. A 2:1 w/w ratio was found to be more suitable than other combinations with respect to transfection levels and biocompatibility (**Figure 4.1**). The gel electrophoresis data also show DNA condensation at this polymer to DNA ratio (data not shown). More importantly, the higher viability of A_nP_n-transfected

cells compared to PEI transfections indicated that the PEG modifications reduced PEI toxicity, while maintaining the same or higher transfection levels (**Figures 4.1A** and **4.1C**).

Changes in pH could rearrange polymer components, ultimately affecting its DNA binding and delivery efficiency. To understand pH-associated changes, the pH of MES/PBS buffers were not adjusted (Series 1, S1) or pre-adjusted (Series 2, S2). The physicochemical characteristics of all the polymers were comparable and no specific changes were observed in the context of the buffer pH adjustments (**Table 4.1**). However, both astrocytes and neurons transfected with S2 polymers showed an overall higher luciferase expression, indicating increased transfection (**Figure 4.2A** and **4.3A**). The difference in luciferase expression with S1 and S2 polymers could be due to effects of altered buffer pH on arginine, PEG, and PEI conjugation efficiency in the overall composition. Alternately, the reactants in the S2 formulation could have formed stable structures, owing to higher conjugation efficacy at the pre-adjusted buffer pH, leading to higher transfection. Further investigations are necessary to delineate the underlying physicochemical changes, which could influence transfection levels. Nonetheless, luciferase activity observed with all polyplexes established their utility in transfection studies.

Some aspects of intracellular gene delivery mechanisms of PEI have been well demonstrated. First, endocytic vesicles internalize PEI-based polyplexes. Highly protonable amines of PEI bind H+ ions, reduce pH, and destabilize lysosomal enzymes, causing osmotic swelling and subsequent endosomal escape of polyplexes by vacuole disruption.[396,397] Further mechanisms of cytoplasmic release and nuclear membrane crossing of pDNA are unclear.[398] We observed luciferase expression at 48 h indicating

the nuclear entry of pDNA, and subsequent transcription and translation within that time frame post-transfection. However, gene expression kinetics was not well understood. Therefore, cellular uptake and sustained gene expression of A_nP_n polyplexes were evaluated. It was observed that uptake occurred with a short exposure of a few minutes to hours, and expression sustained for over a week in primary astrocyte cultures without cytotoxicity (**Figures 4.4** and **4.5**). It is unclear if there is a retrograde polyplex release and reuptake by cells from the culture media or if the polyplexes persist in cytoplasm without being metabolized and release pDNA over time. Regardless of the mechanism, these results are promising since this non-viral delivery system can be used in disease models where a transient, yet stable gene expression is required without the risk of genomic DNA integration observed with viral vectors. It must be noted that our *in vivo* experiments did not indicate a sustained expression pattern (**Figure 4.6B**); and these studies are in incipient stages and thorough investigations are warranted to delineate the *in vivo* expression kinetics.

It has been shown previously that PEI- or its derivatives mediate *in vitro* or *in vivo* neuronal gene delivery in rats,[342] mice,[330] or brain-derived primary cells, and cancer cell lines.[384,399] We also showed A_nP_n-mediated luciferase expression in primary neuronal cultures (**Figure 4.3A**); however, our objective was to validate a gene delivery system capable of astrocyte targeting. Hence, neuronal A_nP_n –polyplex treatments were predominantly focused on ruling out an off-target neuronal cytotoxicity (**Figures 4.3B** and **3C**). Concurrent luciferase activity in neuronal cultures was approximately a 1000-fold lower than astrocytes (**Figures 4.2A** and **4.3A**). This could be due to cell culture conditions, which are strikingly different for neurons versus astrocytes. Primary human

neurons were cultured in serum free medium to restrict the growth of astrocytes after isolation from human brain tissues. We obtained over 90-95% neuronal purity with a small number of astrocytes in cultures based on MAP-2 and GFAP staining (**Figures 4.3D-4.3J**). It is unclear if the luciferase activity seen in these cultures is coming from neurons or astrocytes. The polyplexes may possess an intrinsic glial tropism leading to more polyplex molecules binding to astrocytes, resulting in higher transfection levels. Alternately, astrocytes may take up the polyplexes more efficiently during proliferating phase in culture, which could subsequently increase gene expression compared to terminally differentiated neurons (**Figures 4.2A** and **4.3A**). Though the underlying mechanisms are not well understood at this time, these results are encouraging from a glia-targeting perspective.

For CNS-targeted therapies, a significant obstacle exists in the form of BBB. In our studies, S2 A_5P_{10} successfully crossed the BBB, and reporter expression was detected in the brain after i.v. tail vein injection in mice (**Figures 4.6B** and **4.6C**). It was surprising that there was little to no luciferase expression detected in the liver at any tested time points. Nonetheless, these results are consistent with previous findings that investigated gene delivery with PEI derivatives.[400,401] We speculate that either polyplexes get metabolized rapidly prior to pDNA release or can excrete out from liver *via* biliary duct to gut. Alternately, luciferase could be metabolized immediately post-translation in hepatocytes. Brain-targeted gene therapies have adopted other methods circumvent or facilitate entry through BBB, such as intrastriatal[402] or ventricular injections,[403] and focused ultrasound.[181] Considering the invasiveness or complexity of these methods, i.v. delivery is preferable.

A more direct approach to restrict off-target effects of gene delivery is to use a promoter restricted to a specific target cell type. The gfa2 segment in the 5' region of the GFAP promoter was identified to be capable of restricting gene expression to astrocytes.[354] Since then, the gfa2 promoter has been used in astrocyte targeting studies,[244,338] in transgenic rodent models,[32] and to study astrocyte function.[125,359] Subsequent experiments showed that segments within gfa2, namely gfa28 restrict gene expression to a specific brain region and gfaABC1D restrict it to astrocytes throughout the brain[355] (**Figure 4.7A**). We successfully used S2 A_1P_{10} and S2 A_5P_{10} to transfect astrocytes with GFAP promoter-driven constructs (**Figure 4.7B**). The gfa28 promoter is not specific to astrocytes, restricting expression to dorsal and caudal cortical regions, hippocampus, and caudal vermis of the cerebellum.[404] On the other hand, gfaABC1D promoter expresses ubiquitously through the brain and is restricted to astrocytes.[355] These differences in expression patterns can be used in targeting astrocytes for specific disease conditions, for e.g., a gfa28 promoter may not be used for designing therapeutics for Parkinson's disease, in which substantia nigra located in the midbrain is the primary affected area[370]; however, it can be used for treating HIV-associated neurocognitive disorders in which white matter loss in the corpus callosum region is observed.[9] The weaker promoter activity of GFAP compared to viral CMV and SV40 promoters is known. Therefore, additional manipulations of the promoter sequence, such as upstream insertion of a CMV enhancer or inverted terminal repeats[363,405] were suggested to increase gene expression. Consistent with this, upstream insertion of CMV enhancer led to significant increase in GFAP promoter-driven luciferase expression in our studies (**Figure 4.8B**). Though these results are encouraging, additional investigations are

warranted to rule out potential off-target effects as observed in some studies with such modifications.

The increase in TIMP-1 expression with polymer-mediated gene delivery would be considered a major milestone in the context of these studies (**Figure 4.8D**). In the brain, TIMP-1 is produced by astrocytes in response to injury and inflammation.[77,132] As TIMP-1 is downregulated during chronic inflammation, restoring TIMP-1 could be useful in chronic neuroinflammatory conditions owing to its MMP-inhibitory and -independent neuroprotective functions.[86,101] While gfa28 and gfaABC1D promoters by themselves were not strong enough to drive TIMP-1 gene expression (data not shown), upstream insertion of CMV enhancer in these constructs led to TIMP-1 overexpression in human astrocytes. The magnitude of overexpression was as high as seen in reporter expression assays. Nonetheless, ability to deliver and express TIMP-1 presents novel therapeutic opportunities for treating NDDs.

Viral vectors have thrived and transitioned better in preclinical brain-targeted gene delivery studies and all current gene therapy clinical trials for NDDs use viral vectors.[406] Some of these studies have reported issues such as immunogenicity and lack of efficacy in early phase clinical trials.[370,371] In comparison, investigations testing non-viral gene delivery for NDDs have been fickle due to lack of biocompatibility, efficacy, and inability to cross BBB. Our study is well timed in this aspect as we address the unmet need of a biocompatible and effective gene delivery system.

Conclusion

While neurons are the ultimate therapeutic cellular destination for treating neurodegeneration, first reaching the ‘stars’ *i.e.* astrocytes will be more practical and

effective. Gene delivery systems including viral vectors, inorganic nanoparticles, and lipomers are being tested for targeting gene therapy to the brain. Each of these systems has its own set of shortcomings that include but are not limited to immunogenicity, toxicity, and inadequate efficacy. Our current study convincingly shows that we have a non-viral, biocompatible and effective delivery system for targeting genes such as TIMP-1 to primary human neural cells and mouse brains. We propose that this approach will provide a powerful tool for delivering therapeutic genes not only to the brain, but also other difficult cell targets.

4.6 Figures and Figure Legends

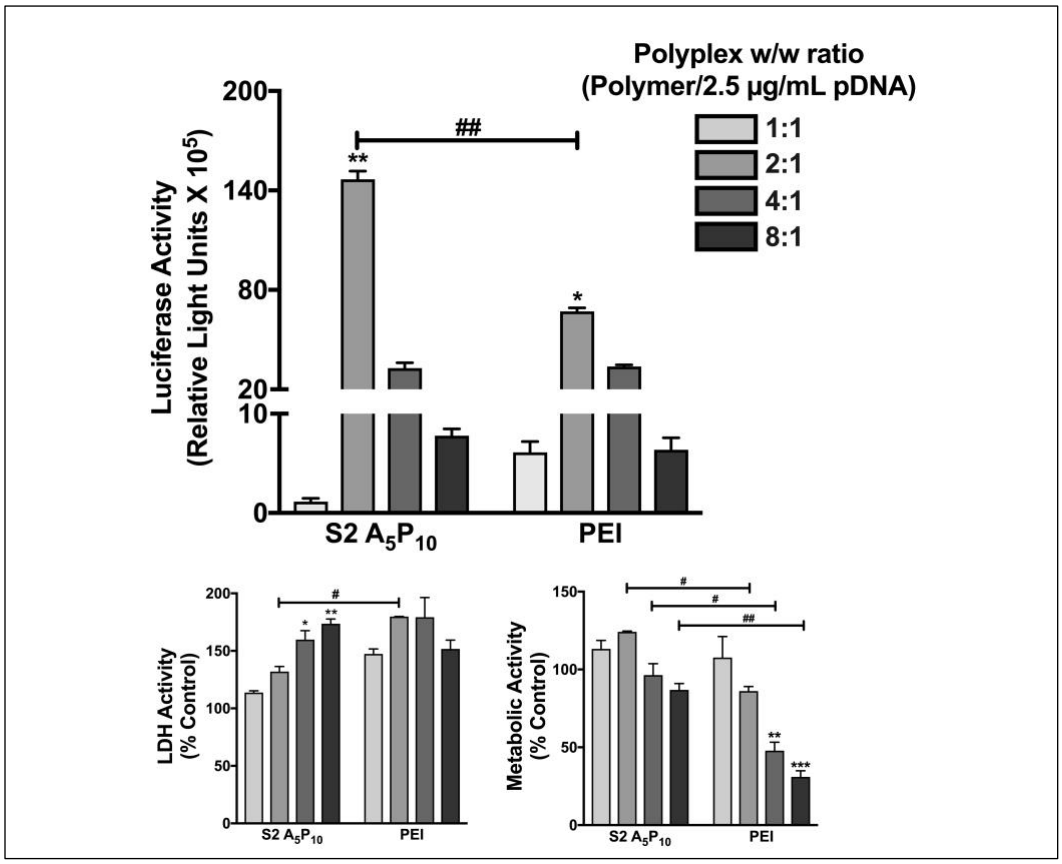


Figure 4.1 A specific polyplex ratio achieves optimal gene expression and biocompatibility in primary astrocytes. An A_nP_n analogue S2 A_5P_{10} or polyethylenimine (PEI) were mixed with 2.5 µg/mL luciferase plasmid in different weight/weight (w/w) ratios (1:1, 2:1, 4:1, and 8:1). Astrocytes (150,000/well) were treated with polyplexes for three hours. The polyplex containing-media was removed and fresh culture medium was added. The **(A)** luciferase, **(B)** lactate dehydrogenase (LDH), and **(C)** metabolic activities were measured 48 h post-treatment. Data represents mean ± SEM for two donors with a minimum of triplicate determinations/donor (*p<0.05, **p<0.01, and ***p<0.001 for 1:1 w/w v/s other ratios for S2 A_5P_{10} or PEI, $^{\#}$p<0.05, $^{\#\#}$p<0.01 for S2 A_5P_{10} v/s PEI at same w/w).

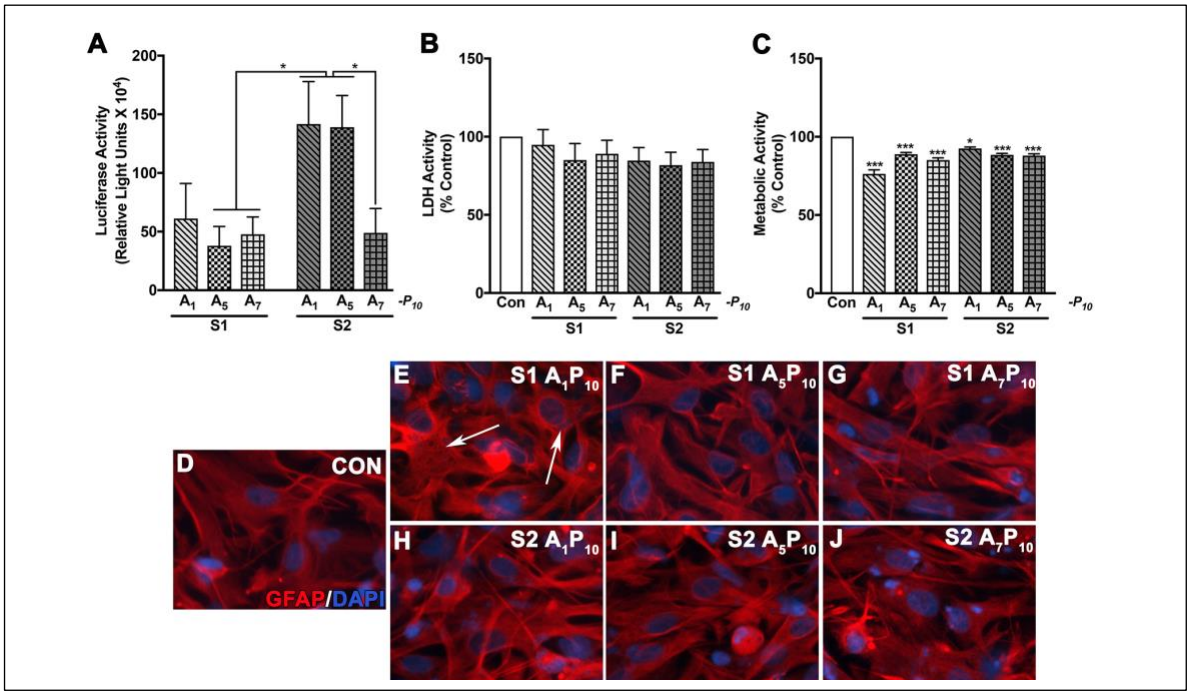
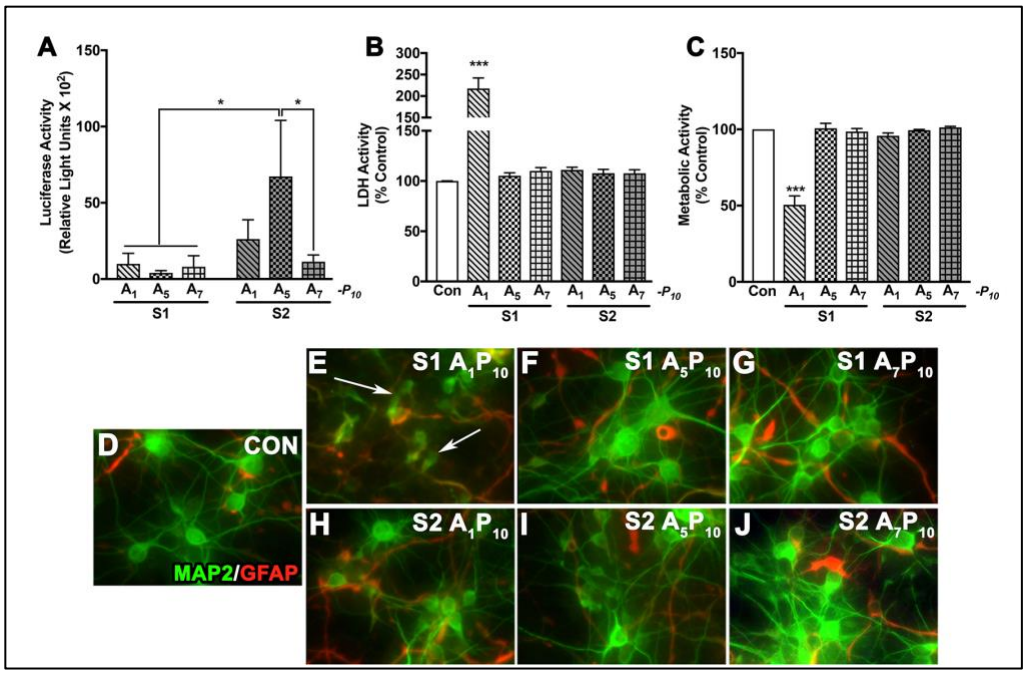


Figure 4.2 The transfection levels and biocompatibility of A_nP_n in human astrocytes are affected by pH and number of arginine residues. Arginine-modified PEI polymer-derivatives synthesized by method S1 or S2, containing 1, 5, or 7 arginine residues (A_1, A_5, A_7) and 10 polyethylene glycol (PEG) (P_{10}) residues/ PEI molecule, were mixed with pLuc in 2:1 ratio. Human astrocytes (150,000/well) were treated with polymer:pDNA polyplexes. After three hours, polyplex containing-media was removed and fresh culture medium was added. **(A)** Luciferase, **(B)** LDH, and **(C)** metabolic activities were measured 48 h post-treatment. Data represents mean ± SEM for three donors with a minimum of triplicate determinations/donor (*$p<0.05$, ***$p<0.001$). In parallel, Astrocytes were immunostained for astrocyte specific glial fibrillary acidic protein (GFAP, red) and nuclear dye DAPI (blue) (**D** untreated control, **E** S1-A_1P_{10}, **F** S1-A_5P_{10}, **G** S1-A_7P_{10}, **H** S2-A_1P_{10}, **I** S2-A_5P_{10}, and **J** S2-A_7P_{10}). Arrows indicate astrocytes with distinct cytoplasmic pDNA staining in endosomes. Representative donor images from four independent donors are shown. Original magnification x200.

Figure 4.3 A_nP_n polyplexes express reporter gene and are biocompatible in primary human neurons. Arginine-modified PEI polymer-derivatives synthesized by method S1 or S2, containing 1, 5, or 7 arginine residues (A_1, A_5, A_7) and 10 PEG (P_{10}) residues/ PEI molecule, were mixed with pLuc in 2:1 w/w ratio. Human neurons (100,000/well) were treated with polymer:pDNA polyplexes for three hours. The polyplex containing-media was removed and fresh culture medium was added. After 48 h, **(A)** Luciferase, **(B)** LDH, and **(C)** metabolic activities were measured. Data represents mean ± SEM for three to five human donors with a minimum of triplicate determinations/donor (*$p<0.05$, ***$p<0.001$). In parallel, neurons were immunostained for neuron-specific microtubule associated protein-2 (MAP-2, green). GFAP (red) staining indicated presence of astrocytes in the neuronal cultures (**D** untreated control, **E** S1-A_1P_{10}, **F** S1-A_5P_{10}, **G** S1-A_7P_{10}, **H** S2-A_1P_{10}, **I** S2-A_5P_{10}, and **J** S2-A_7P_{10}). Arrows indicate neurons without well-defined processes and shrunken cell bodies. Representative images from three individual donors are shown. Original magnification x200.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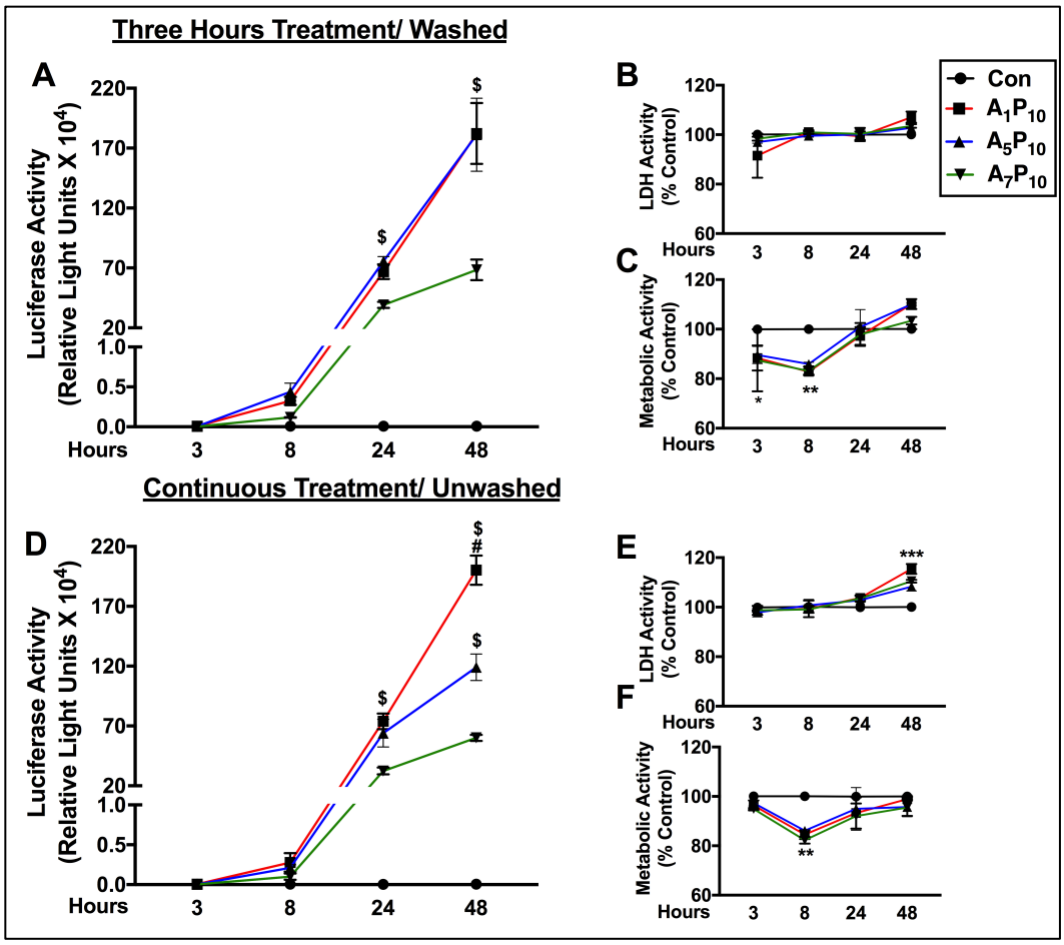


Figure 4.4 Polyplex exposure duration does not contribute to efficacy of gene expression and biocompatibility in astrocytes. Human astrocytes (150,000/well) were treated with S2 polymer:pDNA polyplexes **(A-C)** for three hours and washed or **(D-F)** left unwashed prior to testing. **(A, D)** Luciferase activity, **(B, E)** LDH activity, and **(C, F)** metabolic activity was measured at 3, 8, 24, and 48 h post-treatment (A_1P_{10} red line, A_5P_{10} blue line, A_7P_{10} green line). Data represents mean ± SEM for two donors with a minimum of triplicate determinations/donor ($^{\$}p<0.05$ for comparison to A_7P_{10}; $^{\#}p<0.05$ for comparison to A_5P_{10}, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ v/s controls at a specific time point).

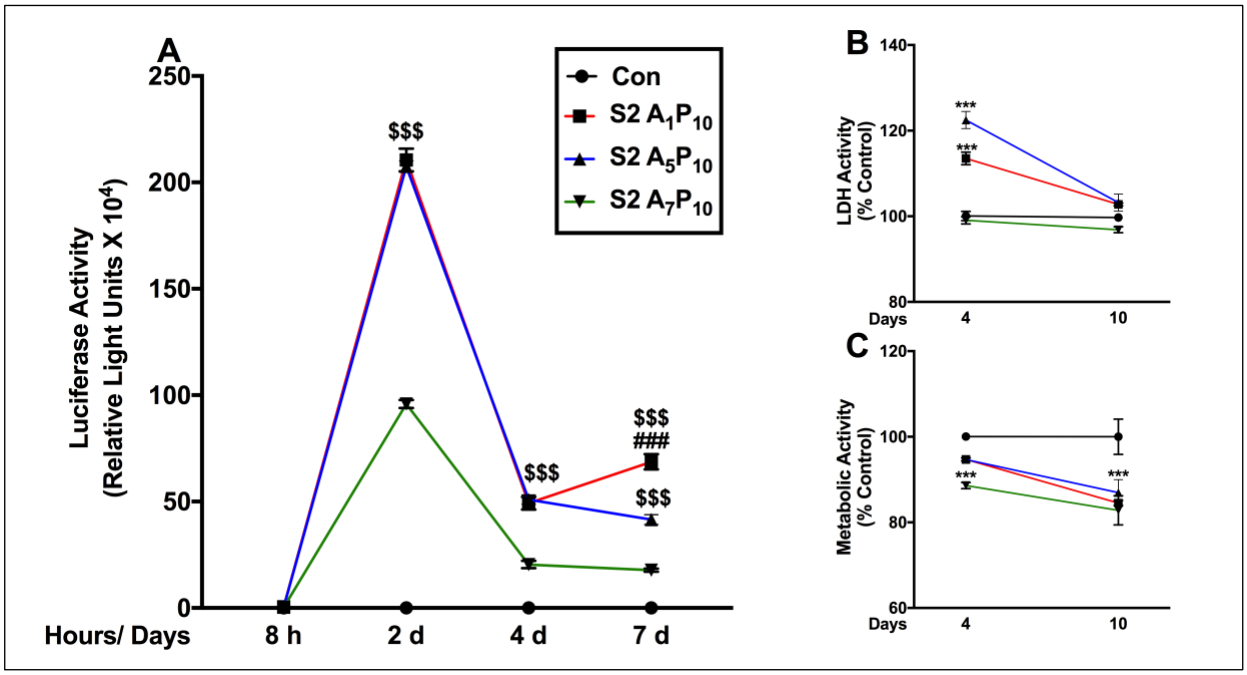
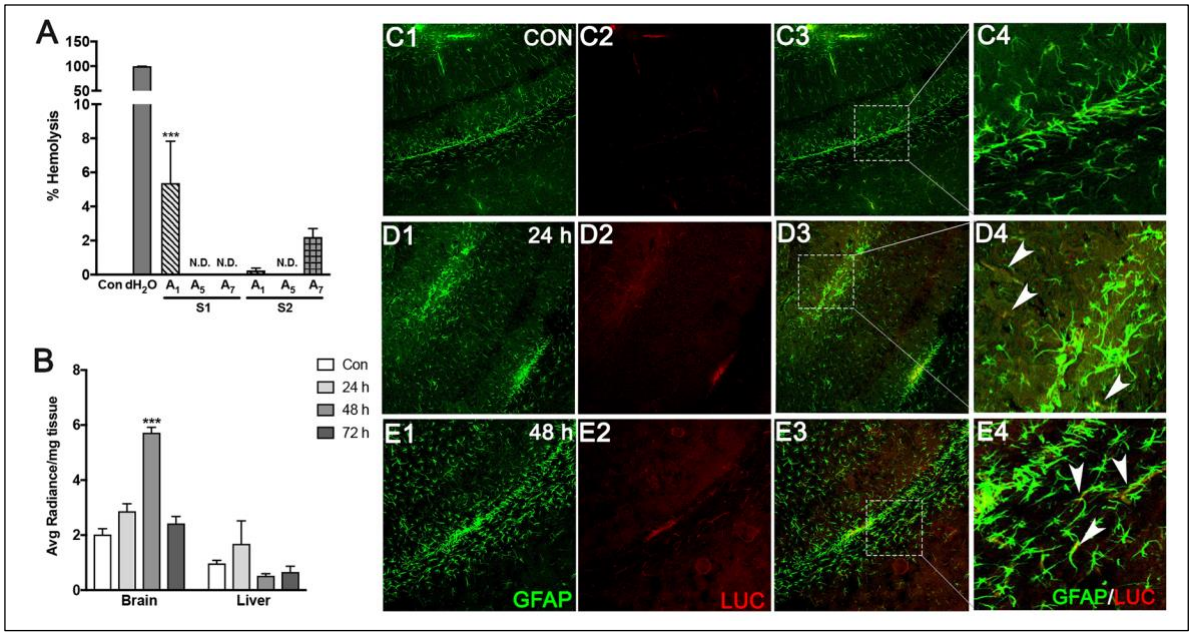


Figure 4.5 Polyplex-mediated gene expression is sustained for over a week in primary human astrocytes. Human astrocytes (150,000/well) were treated with S2 polymer:pDNA polyplexes for 3 h and washed. **(A)** Luciferase activity was measured at 8 hours (h), 2 days (d), 4 d, and 7 d post-treatment. **(B)** LDH and **(C)** metabolic activities were measured 4 d, and 10 d post-treatment (A_1P_{10} red line, A_5P_{10} blue line, A_7P_{10} green line). Data represents mean ± SEM for representative data from five donors with a minimum of triplicate determinations/donor ($^{\$\$\$}p<0.001$ for A_1P_{10} and A_5P_{10} v/s A_7P_{10}; $^{\#\#\#}p<0.001$, A_1P_{10} v/s A_5P_{10}, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ v/s controls; at a specific timepoint).

Figure 4.6 Polyplexes induce little to no hemolysis and mediate reporter gene expression in mouse brains. (A) Whole human blood was centrifuged at 2000 rpm. Pelleted red blood cells were mixed in saline at 1:6 ratio and incubated at 37°C with polyplexes (2:1 ratio) for 3 h. Phosphate buffered saline (1X) and deionized water were used as negative and positive controls, respectively. Post-incubation, supernatants were analyzed to measure % hemolysis compared to controls. Data shown represent mean ± SEM from three donors with a minimum of triplicate determinations/donor (***$p<0.001$). **(B)** Athymic nude mice were injected intravenously with S2 A_5P_{10}:pLuc polyplexes (100 µg polymer and 50 µg pDNA/dose) for 3 days. Brains and livers were cut into small pieces and incubated with vivo glo-d-luciferin in well plates**.** Luciferase activity was assessed in these tissue isolates harvested at 24, 48, and 72 h after last injection. Data shown represent mean ± SEM for 3 to 4 mice per condition (***$p<0.001$). (**C1-E4**) In a parallel experiment, brains harvested at 24 and 48 h were fixed with 4% paraformaldehyde and 40 µM frozen coronal sections were cut immunostained for GFAP (green) and luc (red) (**C** Control, **D** 24h, **E** 48 h). Arrowheads represent cells co-stained for luciferase and GFAP. n=3-4 mice per condition. Each image was captured at 8-15 'Z' sections using a confocal microscope. Representative images from the cortical regions of brain are shown. Original magnification 100X.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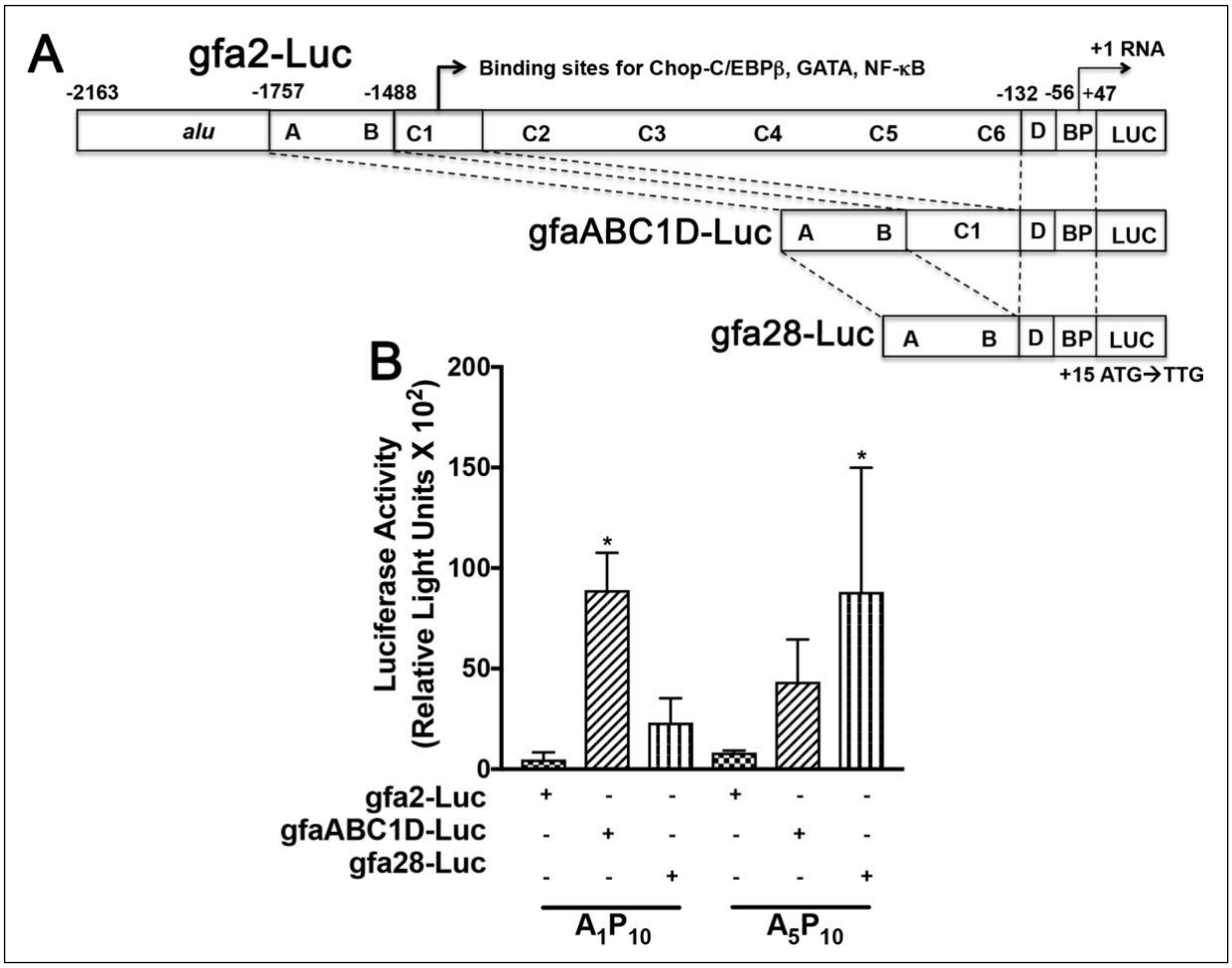


Figure 4.7 Select S2 polymers successfully deliver GFAP promoter fragment driven-luciferase expression in human astrocytes.

(A) Plasmids with GFAP promoter segments (gfa2, gfa28, and gfaABC1D) were used to drive luciferase (Luc). These promoter regions have been reported to restrict gene expression to a region and/or astrocytes in the brain. **(B)** Human astrocytes (150,000/well) were transfected with select S2 A_1P_{10} and A_5P_{10}:pgfa-Luc plasmids for 3 h and washed. Luciferase activity was measured 48 h post-treatment. Data represents mean ± SEM for two donors with a minimum of triplicate determinations/donor (*$p<0.05$ v/s gfa2-Luc for the same polyplex).

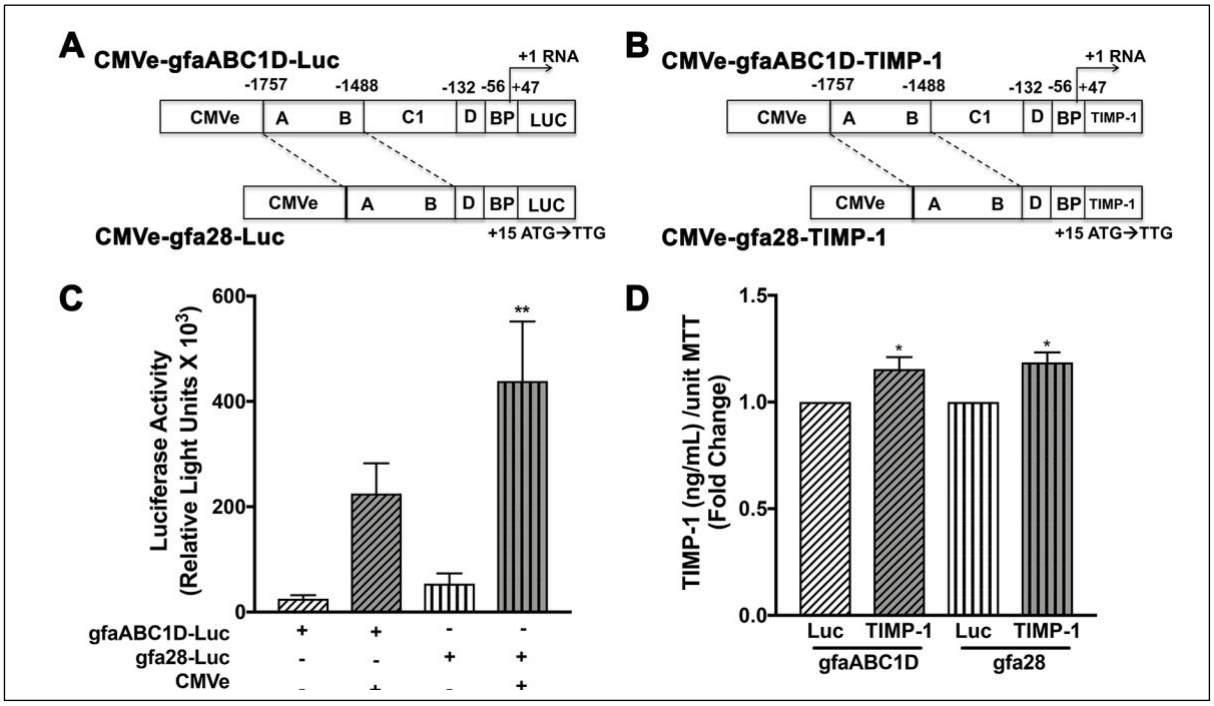
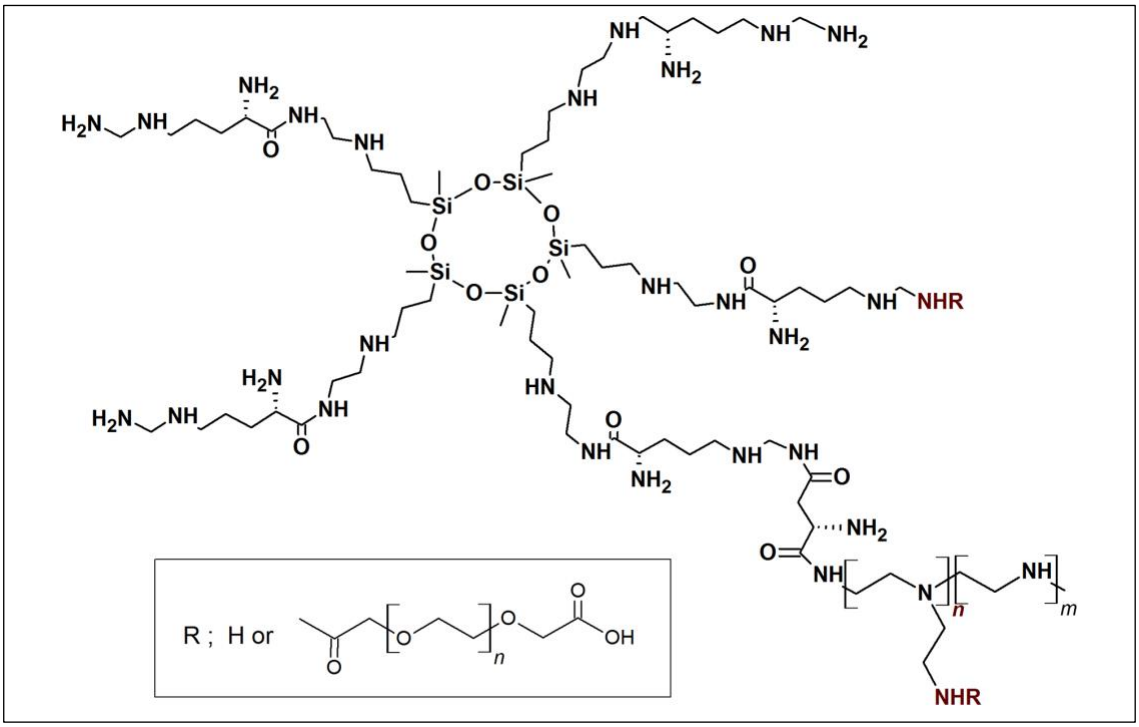
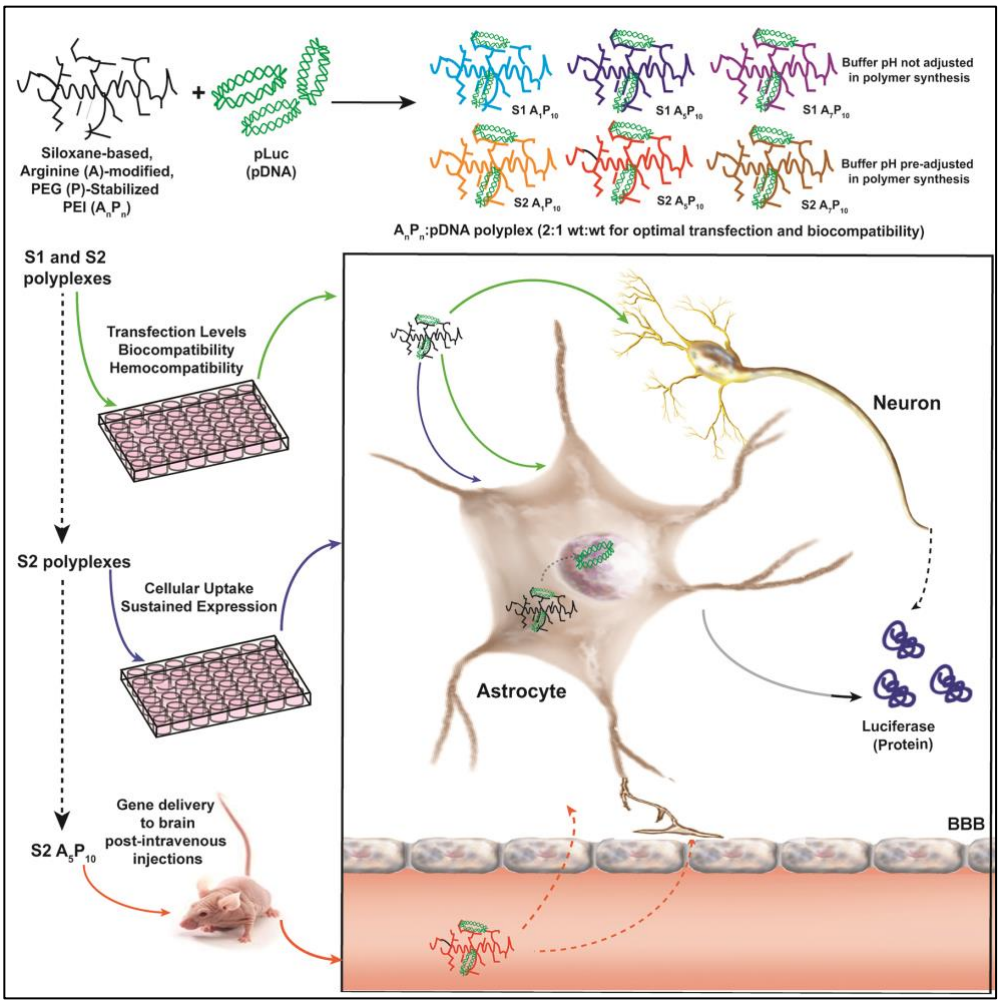


Figure 4.8 Polymer-mediated Luciferase and TIMP-1 gene delivery driven by modified GFAP promoters. (**A**) A CMV enhancer was sub-cloned upstream of gfa28 and gfaABC1D to increase promoter activity of truncated GFAP promoters. **(B)** Human astrocytes (150,000/well) were transfected with S2 A_5P_{10}:CMVe-gfa-Luc plasmids for 3 h and washed. Luciferase activity was measured 48 h post-treatment. Data represents mean ± SEM for three donors with a minimum of triplicate determinations/donor (**p<0.01 v/s same gfa-Luc without CMVe). (**C**) Similar to Luc plasmids, a CMV enhancer was sub-cloned upstream of gfa28 and gfaABC1D in plasmids containing GFAP promoter-driven TIMP-1 ORF.(**D**) Human astrocytes (150,000/well) were transfected with S2 A_5P_{10}:CMVe-gfa-Luc and S2 A_5P_{10}:CMVe-gfa-TIMP-1 plasmids for 3 h and washed. TIMP-1 levels and metabolic activity were measured 48 h post-treatment. Data represents mean ± SEM for two donors with a minimum of triplicate determinations/donor (*p<0.05 v/s respective gfa-Luc control).



Supplementary Figure 4.1 Schematic representation of A_nP_{10} polymers composition (Illustration Credit: Manju Saraswathy, adapted from[390])



Supplementary Figure 4.2 Summary of A_nP_n-mediated gene delivery. We investigated six arginine-modified, polyethylene glycol-stabilized polyethylenimine (A_nP_n) polymers (Series 1, S1, and Series 2, S2) for their biocompatibility, hemocompatibility, and gene delivery potential in human astrocytes and neurons using luciferase reporter plasmids (pLuc). Due to higher efficacy and better biocompatibility, S2 polymers were further tested for cellular uptake patterns and sustained expression profiles. Ultimately, S2 A_5P_{10} was selected for *in vivo* experiments and it successfully delivered pLuc across the blood-brain barrier in athymic nude mice leading to luciferase expression in mouse brains.

Table 4.1 Physicochemical characteristics of A_nP_n polyplexes

Polymer Name	MES/NHS Buffer pH	PEI Eq	Arginine Eq	PEG Eq	Polyplex Particle Size (nm)	Variance	Zeta potential (mV)
S1 A_1P_{10}	Not adjusted	1	1	10	55.33 ± 0.80	0.249 ± 0.02	28.29 ± 0.16
S1 A_5P_{10}	Not adjusted	1	5	10	60.00 ± 1.64	0.297 ± 0.03	23.82 ± 0.29
S1 A_7P_{10}	Not adjusted	1	7	10	61.97 ± 1.07	0.214 ± 0.02	27.21 ± 1.45
S2 A_1P_{10}	Pre-adjusted	1	1	10	56.8 ± 0.43	0.211 ± 0.01	27.08 ± 1.32
S2 A_5P_{10}	Pre-adjusted	1	5	10	60.43 ± 0.05	0.252 ± 0.01	27.9 ± 0.86
S2 A_7P_{10}	Pre-adjusted	1	7	10	85.77 ± 7.55	0.332 ± 0.02	24.60 ± 3.12

Polyplex (polymer: pDNA) 2:1 w/w, MES: 2-(*N*-morpholino)ethanesulfonic acid, PBS: phosphate buffer saline, PEI: polyethylenimine, PEG: polyethylene glycol, Eq: Equilvalents. Values represented as Mean ± S.E.M

CHAPTER 5

PUTTING THE TIMPUZZLE TOGETHER

AUTHOR

CHAITANYA R JOSHI

5.1 Summary, limitations, and ongoing studies

Despite ART, HAND prevalence remains high, affecting 30-70% of all HIV-infected individuals.[6] Yet, pharmaceutical companies have been reluctant to invest in developing HAND therapeutics since it is not perceived as a treatable condition along with other factors such as size of affected population and monetary benefits.[4] A potential question raised in this aspect for the academic researchers is- how *in vitro* and *in vivo* models of the disease could be used to discover and develop novel therapeutic agents? Our research program has actively investigated TIMP-1 as a therapeutic agent in the context of HAND and neuroinflammation for nearly two decades.[83,86,101,104,132,160,407] This dissertation attempted to take the oeuvre forward. We combined the diverse disciplines of cell biology, neuroscience, pharmacology, and nanotechnology to put this 'TIMPuzzle' together.

Here, we demonstrated a plausible HAND behavioral phenotype using the HIV-1 Tat-transgenic mouse model associated with TIMP-1 during chronic Tat exposure. In parallel, a polymer-mediated, astrocyte-targeted TIMP-1 gene delivery system was optimized to restore/elevate TIMP-1 levels (**Figure 5.1**). Although uncovering all intricacies associated with TIMP-1 regulation in the brain was beyond the scope of this work, we demonstrated the TIMP-1 differential expression paradox with acute versus prolonged astrocyte Tat expression in the GT-Tg mouse model. The changes in TIMP-1 and MMP-2 were consistent with our previous data in primary human astrocytes and HIV-infected brain tissue samples (**Figure 5.2**).[83,99,101] In parallel, acute Tat induction did not show cognitive decline, but prolonged Tat induction led to behavioral deficits in GT-Tg mice. TIMP-1 is a known neuronal plasticity regulatory protein, which affects memory and

cognitive behavior.[142,143] Based on this, we postulated that reduced TIMP-1 expression contributed to behavioral deficits observed in GT-Tg mice with chronic Tat expression. Thus, TIMP-1 restoration or replenishment to homeostatic levels would reverse behavioral deficits. However, a safe and effective delivery system was needed to accomplish these goals. Therefore, we next focused our efforts on optimizing a TIMP-1 delivery system.

Reactive astrocytes during CNS inflammation and injury are primary endogenous producers of TIMP-1 in the brain.[77,93,98] Hence; astrocytes were the ideal cell targets for TIMP-1 delivery. Multiple positively-charged polymers were tested and successful gene delivery to primary human neural cells (neurons and astrocytes) was depicted using luciferase reporter plasmids.[160] Lastly, human TIMP-1 cDNA was cloned into plasmids downstream of GFAP promoter(s) and polymer-mediated TIMP-1 gene delivery to astrocytes was achieved.

The results of this dissertation are constrained by certain limitations. First**,** The GT-Tg mouse model produces only one viral protein, *i.e.* HIV-1 Tat, in a specific cell type, *i.e.* astrocytes. Therefore, it is not possible to mimic HAND in its entirety or even complex aspects of HAND such as the HIV-associated dementia (HAD) phenotype in this model. A potential solution could be to use a simian immunodeficiency virus (SIV) model in macaques. Both SIV and HIV are closely related in terms of mode of infection, cellular targets, and disease progression with SIV causing AIDS in macaques as reviewed by Lackner and Veazey.[408] Thus, an SIV-infected macaque model could be used to simulate mild to severe stages of HAND and would be most suitable for translational studies such as ours. However, there are logistical and economical challenges associated with using

larger animal models such as SIV-macaque without prior proof-of-concept studies in smaller rodent models. Thus, our findings are an initial step towards validation in large species such as primates before TIMP-1 therapy is translated in humans. Since primate behavior studies were beyond the scope of the presented work, alternate strategies were not evaluated. Second**,** multiple polymorphisms in MMPs and TIMPs are documented and correlated with pathogenesis of abdominal aortic aneurysm,[409] lung cancer,[410] and asthma.[411] However, little to no information is available related to MMP or TIMP polymorphisms in relation to HAND or other neurodegenerative conditions. A study showed no impact of TIMP-1, -2, -3 polymorphisms on intracranial aneurysms.[412] Thus, it is hard to predict if there are any TIMP-1 polymorphisms associated with HAND neuropathogenesis and if TIMP-1 overexpression with gene delivery would be impacted directly or indirectly by it. Such investigation would be interesting but would be too broad in its scope, and thus was addressed in this work.

The ongoing and future studies are aimed as answering following questions:

1. Which pathways involved in Tat-mediated regulation of the MMP/TIMP balance?
2. Are changes in MMP/TIMP mRNA expression consistent with their proteins levels and activity?
3. Does TIMP-1 gene delivery result in sustained and effective protein expression *in vitro* and *in vivo*?
4. What is the specificity of GFAP promoter in targeting astrocytes after insertion of CMV enhancer?
5. Can polymer-mediated TIMP-1 gene delivery reverse the behavioral impairments in GT-Tg mouse model?

5.2 Overall impact and future implications

In 2015, the global burden of NDDs exceeded ischemic heart disease, respiratory diseases and neoplasms and will continue to increase significantly until 2030 and beyond.[413] These NDDs range from dementias such as Alzheimer's disease (AD) to infectious poliomyelitis, from impaired motor function in Parkinson's disease (PD) to HAND. Despite the high prevalence and awareness of NDDs, adequate therapeutic strategies are not available to cure or treat NDDs.[326] Such a conundrum exists due multiple reasons including incomplete understanding of suitable therapeutic targets and imperfect delivery systems. In the presented astrocyte TIMP-1-centric investigation, we have successfully addressed aspects of these two limitations. These findings set the stage for future experiments in which the therapeutic potential of TIMP-1 gene delivery can be investigated. The impact and implications of this work are discussed in the following section.

Over the last two decades, TIMP-1 has emerged as an important biomarker in the spectrum of NDDs including AD,[414-416] Huntington's disease,[414] PD,[414,417] multiple sclerosis (MS),[418,419] depression,[420] bipolar disorder,[421] schizophrenia,[420] traumatic brain injury,[422] and HAND.[90,101] Some of these investigations were establishing biomarkers for diseases or assessing TIMP-1 levels in the context of MMP expression and activity. Interestingly, TIMP-1 levels in the CSF, plasma, and/or tissues did not show consistent patterns across NDDs or within an NDD. For example, TIMP-1 levels in the CSF of AD patients were lower,[415] not different,[416] or higher[414] compared to controls. We reported lower TIMP-1 levels in HIV encephalitis CSF and brain tissues [101] while a recent study documented higher TIMP-1 in the CSF of HAND patients.[90] TIMP-1 levels were higher in

the CSF of HD patients,[414] higher in the plasma of depression and schizophrenia patients, [420] and lower in the CSF of progressive MS patients.[419] Such discrepancies can be partially addressed by taking into account mean age of patients, disease status, and medications. For example, the discrepancy in TIMP-1 levels in CSF of HIV infected patients in two studies[90,101] could be due to different ART medications used by patients in both studies.

These conflicting findings raise the question '*to TIMP, or not to TIMP?'* To answer this question, some facts must be reviewed. Elevated MMPs are implicated in most NDDs. MMPs degrade the ECM and the BBB, subsequently increasing the infiltration of immune cells in the brain causing neuroinflammation and other neurotoxic effects. Therefore, most literature infers that TIMP-1 is critical to inhibit MMPs and regulate their toxic effects. Additionally, the MMP-independent, growth factor-like effects of TIMP-1 makes it a therapeutically double-edged sword. Studies by our group and others have shown the neuroprotective effects of TIMP-1 *in vitro* or *in vivo*.[86,87,159] Constitutive, GFAP-restrictive TIMP-1 overexpression in a mouse model completely suppressed MMP activity in the brain without morphological and behavioral deficits indicating sustained TIMP-1 expression is not detrimental.[108] However, some studies showed the adverse effects of TIMP-1 as well. Some reports discussed damaging effects of TIMP-1 including interfering angiogenesis and tumor growth,[423] or increased CD4+ T cell migration across the BBB in viral encephalomyelitis.[424] Yet, the answer to the question '*to TIMP, or not to TIMP?*' is conditionally affirmative. We infer that TIMP-1 could be restored or overexpressed transiently, in a cell- or tissue- dependent manner, for specific diseases.

That brings us to the next question, ‘How to TIMP effectively in a transient, cell- or tissue- dependent manner for specific diseases?’

1. Cell-specific expression: GFAP is most abundantly expressed in the brain compared to other tissues and is a cellular marker of astrocytes. Further, specific segments of the GFAP promoter have been used to achieve astrocyte-specific expression.[355] Thus, a GFAP promoter-driven TIMP-1 gene would restrict the protein expression to astrocytes.

2. Transient versus prolonged gene expression: Though MMPs are considered the bad guys during inflammation, they are essential in homeostasis. They play important functions during neurogenesis, vascular remodeling after injury, and ECM-related signaling.[159] Hence, TIMP-1 therapy could be implemented transiently. Transient TIMP-1 expression can be achieved using nanoparticle-based delivery compared to viral vectors. Viral vectors such as lentiviral vectors are known for their capacity to integrate with genomic DNA,[306] and it could lead to constitutive, long-term expression. With nanoparticles, there is little to no risk of integration. The frequency of dosing can be adjusted to achieve expression for specific duration.

3. TIMP-1 restoration for specific diseases: Existing literature and biomarker studies related to various NDDs could help point the therapeutic development in the right direction. Plasma, serum, and/or CSF TIMP-1 levels can be correlated with neuropsychological testing, levels of MMPs and other inflammatory mediators, and/or overall neuropathology. One or more biomarker study/s may establish a correlation between lower TIMP-1 levels and disease pathology for a specific NDD. However, further preclinical studies would be critical to assess if TIMP-1 overexpression or restoration would ameliorate disease pathology. As the specifics of TIMP-1 gene delivery get fine-

tuned in preclinical studies, data could be collected simultaneously from observational and/or longitudinal NDD biomarker studies to assess if TIMP-1 gene delivery could be a suitable option. These studies could also provide essential information on the absolute TIMP-1 protein levels, changes observed during the disease, and degree of restoration required.

Some additional factors and/or limitations have to be considered. For example, if TIMP-1 overexpression or the nanoparticles could alter astrocyte function. Thus far, MMP-independent effects of astrocyte TIMP-1 on oligodendrocytes[425] and neurons[108] are known. These effects are potentially mediated by cell surface receptors such as tetraspanin CD63,[426] or LRP-mediated endocytosis.[427] It remains to be seen if there is TIMP-1 autocrine and/or paracrine signaling in astrocytes *via* tetraspanin, LRP or any other receptors. Thorough analysis of NDDs-related biomarker studies demonstrate the dynamic nature of MMP/TIMP axis, which could change with age, sex and disease status.[90,100,406,407,408,409,410,411,412] Therefore, continued efforts will be necessary to delineate subtle TIMP modulations before, during, and beyond the clinical translation of TIMP-1 gene delivery. Lastly, we acknowledge that an astrocyte-TIMP-centric approach alone would be inadequate to address the enigma of HIV neuropathogenesis. More rounded approaches will be in order which combine one or more other therapeutic targets in conjunction with TIMP-1.

Overall, it is evident that there are broader implications of our work with respect to HAND treatment and the NDDs spectrum. Successful TIMP-1 gene delivery to the brain would allow researchers to investigate its therapeutic potential in other NDDs where chronic neuroinflammation is a recurring theme. The delivery system itself could be used

for genes of other neurotrophic factors such as BDNF or anti-oxidant enzymes such as catalase to improve cellular function and/or reverse pathophysiology. Further, such systems could be used independently or in combination to increase the likelihood of successful therapy.

The future work related to this project will investigate novel avenues associated with astrocyte-TIMP-1 and contribute to the noble task of treatment and/or cure for HAND, as well as other NDDs. We are excited to put some pieces of the '*TIMPuzzle*' together and will keep striving to reach the 'star-shaped' light at the end of this tunnel.

5.3 Figures and Figure Legends

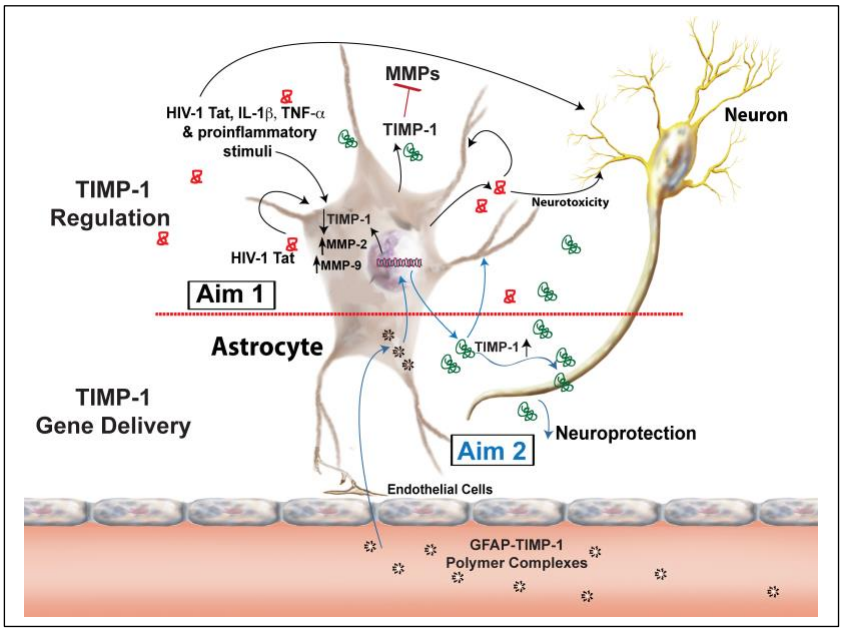


Figure 5.1 HIV-1 Tat-mediated regulation of MMP/TIMP balance and polymer mediated gene delivery to astrocytes. This dissertation investigated two aspects of astrocyte TIMP-1 *i.e.* regulation in a HIV-1 Tat transgenic model and gene delivery using polymeric nanoparticles. We wanted to delineate if TIMP-1 expression was altered in a HIV-1 Tat transgenic model and if changes in TIMP-1 were associated with a behavioral phenotype. The other goal of the project was to design and optimize an astrocyte-targeted gene delivery system for astrocyte TIMP-1 restoration. Our data showed that Tat expression led to behavioral impairments in a transgenic mouse model and altered TIMP-1 levels depending on acute or prolonged Tat expression. We also demonstrated polymer-mediated TIMP-1 gene delivery to human astrocytes. Future studies will combine these aspects and work towards TIMP-1 restoration as potential HAND therapy.

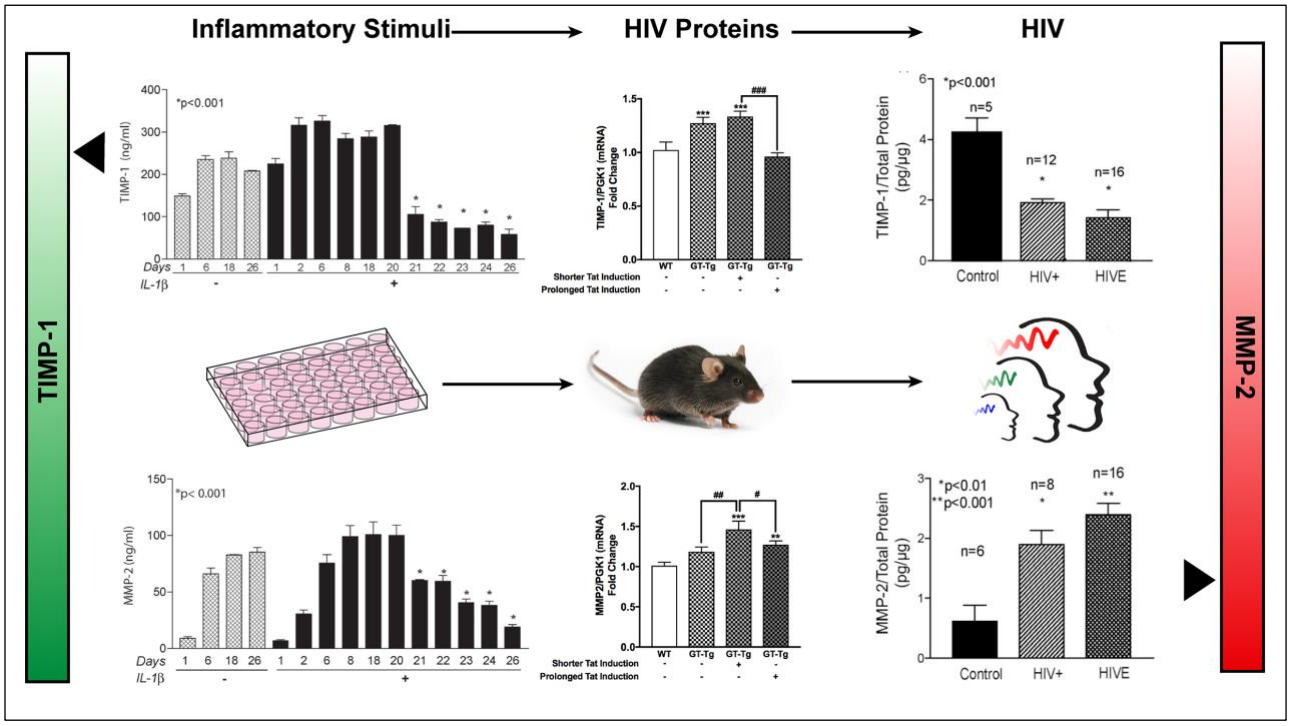


Figure 5.2 Dysregulation of the TIMP-MMP balance across multiple HAND-relevant disease models. (Left) Primary human astrocytes were cultured for several days in media with or without 20 ng/ml IL-1β. TIMP-1 and MMP-2 profiles were compared over time.[101] (Middle) The effects of shorter (intraperitoneal injections 7x @ 100 mg/kg over 3 weeks) and prolonged (1250 mg/Kg chow over 6 months) DOX-induced HIV Tat expression on TIMP-1 and MMP-2 mRNA levels in brains from GT-Tg and C57BL/6 (WT) mice. (Right) Protein extracts and total RNA isolated from human autopsy brain tissue obtained from control donors, HIV+ (seropositive) individuals without cognitive impairment and HIVE patients were analyzed for TIMP-1 and MMP-2 by ELISA.[101] The levels of TIMP-1 were reduced in human astrocytes, mouse brains, and HIV-infected brain tissues in the presence of prolonged inflammation, viral protein expression, and infection, respectively. In parallel, MMP-2 levels were elevated in the same samples indicating that MMP/TIMP balance was tipped towards MMPs.

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