

Characterization of AAVrh.10 as a Vector to Deliver Long-Lasting Genetic Constructs into Neurons of Neonatal Mice Following Intravenous Injection

By

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Abstract

Prion diseases are a fatal neurodegenerative disease caused by the misfolding of the cellular prion protein, PrP^C into an infectious isoform, PrP^{Sc}. Accumulation of this infectious isoform leads to the damage and death of neurons, and the progression of the disease. The molecular events involved in the pathogenesis of this disease are poorly understood and uncovering them has proven challenging due to the difficulties of identifying and isolating degenerating neurons for analysis. Ultimately, the aim of this project is to characterize the use of AAVrh.10 to deliver genetic constructs into the neurons of mice *in vitro*, and *in vivo*. AAVrh.10 was compared to AAV8, and AAV9 to assess transduction efficiencies in primary hippocampal neuron cell cultures. All 3 AAV serotypes were found to transduce primary neurons with high efficiency, and low toxicity. Further work showed that intravenous injection of AAVrh.10 into neonatal mice via the superficial temporal vein was capable of inducing long-lasting transgene expression in neurons and astrocytes within the brain, throughout the entire course of prion disease. IHC analysis is performed to assess the distribution of the AAVrh.10 throughout multiple regions of mouse brain. In addition, the kinetics of the vector concentration in the brain over time will be described. This work is the basis for engineering vectors capable of targeting specific neurons in mouse brain tissue in future studies to understand prion pathogenesis and prion strains *in vivo*.

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List of Abbreviations

AAV	Adeno-associated virus	miRNA	micro RNA
AAVrh.10	Adeno-associated virus serotype rh10	MOI	Multiplicity of Infection
ACC	Canadian Council on Animal Care	NAPA	Nonadaptive prion amplification
ALS	Amyotrophic lateral sclerosis	N-CAM	neural cell adhesion molecule
ASO	Antisense Oligonucleotide	NHP	Non-Human Primates
AUD	Animal Use Document	NTC	No Template Control
BBB	Blood-Brain barrier	NWP	Normalized Water Permeability
BSA	Bovine Serum Albumin	pAV	Adeno-associated transgene-containing plasmid
CA1	Corno Ammonis Hippocampal layer	PBS	Phosphate-Buffered Saline
CJD	Cruetzfeldt-Jakob disease	PCR	Polymerase Chain Reaction
CNS	Central Nervous System	PEI	Polyethylenimine
CNS	Central Nervous System	PRNP	Prion Gene
CSCHAH	Canadian Science Centre for Human and Animal Health	prnp	prion protein gene
C _T	Threshold Cycle	PrPC	Cellular prion protein
CWD	Chronic Wasting Disease	PrPSc	Infectious prion protein
DAPI	4',6-diamidino-2-phenylindole	psig	pressure per square inch gauge
DIV	Days <i>in vitro</i>	RFP	Red Fluorescent Protein
DPI	Days Post-Infection	RIN	RNA Integrity Number
eGFP	Enhanced Green Fluorescent Protein	RISC	RNA-Induced Silencing Complex
FACS	Fluorescence-Activated Cell Sorting	RML	Rocky mountain laboratory
FBS	Fetal Bovine Serum	RNAi	RNA interference
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase	RT	Reverse Transcription
GFAP	Glial Fibrillary Acidic Protein	rtPCR	Real-time PCR
GFAP-luc	FVB/N-Tg(Gfap-luc)53Xen Mouse Strain	SEM	Standard Error of the Mean

GPI	Glycosylphosphatidylinositol anchor	TBST	Tris-Buffered Saline, Tween Solution
HCC	Hippocampus	TFF	Tangential flow filtration
HRP	Horse Radish Peroxidase	TSE	Transmissible Spongiform Encephalopathy
ITR	Inverted Tandem Repeat Sequence	UNG	Uracil;-N-Glycolase
LCM	Laser Capture Microdissection	vCJD	Variant Cruetzfeldt-Jakob disease
LDH	Lactate Dehydrogenase	VTs	Veterinary Technical Services
MAP2	Microtubule-Associated Protein 2		

Chapter 1. Introduction

1.1 Prion Disease

Prion diseases, or transmissible spongiform encephalopathies, are fatal neurodegenerative diseases that affect a variety of animal species, as well as humans ^{1,2}. Human prion diseases are characterized as having a long incubation times followed by a rapidly progressive dementia, leading to the inevitable death of the individual. Symptoms of the disease include muscle stiffness, difficulty walking, changes in gait, confusion, and ataxia. Death generally occurs within a year after the onset of symptoms ². The disease is associated with the accumulation of a misfolded isomer of the cellular prion protein, a ubiquitously expressed protein in mammals ³. It is currently understood that the misfolded protein may arise through spontaneous misfolding, genetic mutations or through exposure to exogenous misfolded prion proteins.

The documented history of prion diseases began with the first report of Scrapie, a naturally occurring prion disease found in sheep, goat, and mouflon, in Europe in the 16th century ⁴. However, it is likely that pottery images depicting a diseased sheep found from the early Chinese and Roman epochs are in reference to the disease ⁵. Although first postulated by J.S Griffith to be of protein origin in 1967 ⁶, prion diseases were believed to be caused by a “slow virus” ⁷ until the infectious agent was isolated and demonstrated to be of protein origin by Stanley Prusiner in 1982 ⁸. Due to the novel properties of this protein, which differentiated it from known viruses, plasmids, and viroids, the term “prion” was proposed to identify a small *proteinaceous infectious particle* ⁸. In 1985 Bruce Chesebro and Richard Race deduced an mRNA transcript encoding the protein sequence of the protease-resistant prion particle that was isolated by Prusiner ⁹. Surprising at the time, this mRNA transcript was found in both infected brain samples, and

samples of healthy control brains ⁹. Later that year, Prusiner and Charles Weissman discovered that a host cellular gene, *PRNP*, was the one responsible for encoding the prion protein ¹⁰. The locus containing the gene-encoding protein was then isolated by Stanley Prusiner and George Carlson ¹¹. The cellular prion protein gene, *PRNP*, was later demonstrated to be necessary for prion disease by inoculating scrapie-infected brain homogenate into mice lacking *PRNP*, which failed to induce disease ¹².

Originally, prion diseases were only known to be transmissible between animals ^{13,14}. However, with the release of a report in 1957 describing a transmissible neurodegenerative disease within a cannibalistic tribe of Papua New Guinea, the human prion disease now known as Kuru was first to be identified ¹⁵. Research of this disease led to the discovery that Kuru, Scrapie, and Creutzfeldt-Jakob disease (CJD) were distinct forms of the same type of disease ¹⁵. Further research demonstrated that Kuru could be transmitted from humans to chimpanzees ¹⁶, the first piece of evidence of prion transmissibility. The potential risks of other human prion diseases being transmissible were raised through reports describing the transmission of Creutzfeldt-Jacob disease through iatrogenic means ¹⁷. Beginning in the late 1980s and spanning for nearly 10 years, an outbreak of BSE occurred in British cattle ¹⁸. In 1995, cases of variant Creutzfeldt-Jakob disease (vCJD) were found in humans, with rates rising until their peak in 1999 ¹⁹. These two events were found to be correlated, because of the ingestion of prion-contaminated meat from BSE-infected cattle. Through molecular analysis it was discovered that this new variant of CJD disease had strain characteristics distinct from other types of CJD, which closely resemble those seen in experimental BSE transmission to mice and macaques ²⁰. Using transgenic mice expressing bovine cellular prion proteins it was demonstrated that injection of brain homogenate from BSE-infected cattle resulted in the mice developing neurological disease ²¹. It was also

demonstrated that injection of brain homogenate from patients with vCJD resulted in the transgenic mice developing the neurological disease, with the same incubation period of 250 days as seen in the BSE inoculation of the mice ²¹.

Today, the majority of human prion diseases are CJD, with approximately 90% of these cases occurring sporadically, affecting 1-1.5 people per million annually ²². Table 1 outlines the types of prion disease found in humans, their modes of occurrence and the rates of occurrence, if available.

Table 1. Human Prion Diseases

Etiology	Disease Names	Rates
Sporadic	Sporadic CJD (sCJD)	1-1.5/ million ²²
	Sporadic Fatal Insomnia (SFI)	
	Variably Protease-Sensitive Prionopathy (VPSPr)	1-2/100 million ²³
Genetic	Familial/ Genetic CJD (f/ gCJD)	10% of CJD Cases ²
	Gerstmann-Straussler-Scheinker disease (GSS)	1-10/ 100 million ²⁴
	Fatal Familial Insomnia (FFI)	
Acquired	Kuru	
	Iatrogenic CJD (iCJD)	
	Variant CJD (vCJD)	

Outline of human prion diseases, the etiology of disease, and the rate of occurrence.

Although work in mice has found several treatments or compounds that when used therapeutically, increases the survival length of prion-infected animals ²⁵⁻²⁷, there has yet to be a finding that has shown efficacy in humans. With the rates of neurodegenerative diseases projected to increase, largely due to the increasing lifespans of humans ²⁸, there is a need to better understand prion diseases as a whole. Changes to farming practices, initiation of surveillance systems, and improvements into the diagnosis of prion disease have managed to reduce the rates of human vCJD cases to near-zero, with the numbers of BSE cases worldwide

reduced to the single digits ¹⁹. However, Chronic Wasting Disease (CWD), a prion disease found in deer, elk, and moose, rates are currently increasing in both captive and wild animals, therefore there is a clear need for improved systems for prion research. Additionally, unlike BSE which is transmitted through the ingestion of contaminated food, CWD prions are found within the soil and on plants, and spread freely between captive and wild populations of cervids ²⁹. Due to the stability of the infectious agent, and the lack of information we have regarding inter-species transmission, there is a very real need for further research to better understand the pathogenesis and progression of the disease. Advances in uncovering the molecular mechanisms involved in the pathogenesis of prion disease will allow the identification of pathways which may be targeted in future therapeutics and may help assess the zoonotic risks of CWD transmission to humans. Ultimately, there is a need to further understand the mechanisms involved in the progression of prion disease as there needs to be significant improvements made in the diagnosis of the early stages of disease. This requires a more in-depth knowledge of how these infectious proteins cause infection.

1.2 Prion Protein

The infectious prion protein, PrP^{Sc} is the causative agent of prion disease. The infectious prion protein shares the same amino acid sequence as its cellular isoform, PrP^{C} , therefore infectivity is believed to be caused by the structural conformation of this protein ³⁰. PrP^{C} , the normal cellular form of the protein, is a soluble monomeric protein rich in α -helices, that is sensitive to protease K digestion. Conversely, the disease causing isomer, PrP^{Sc} is rich in β -sheets, forms insoluble aggregates, and shows resistance against protease K digestion ³¹. Although not supported by all, the protein-only hypothesis states that template-directed misfolding of prion protein is the cause of prion diseases.

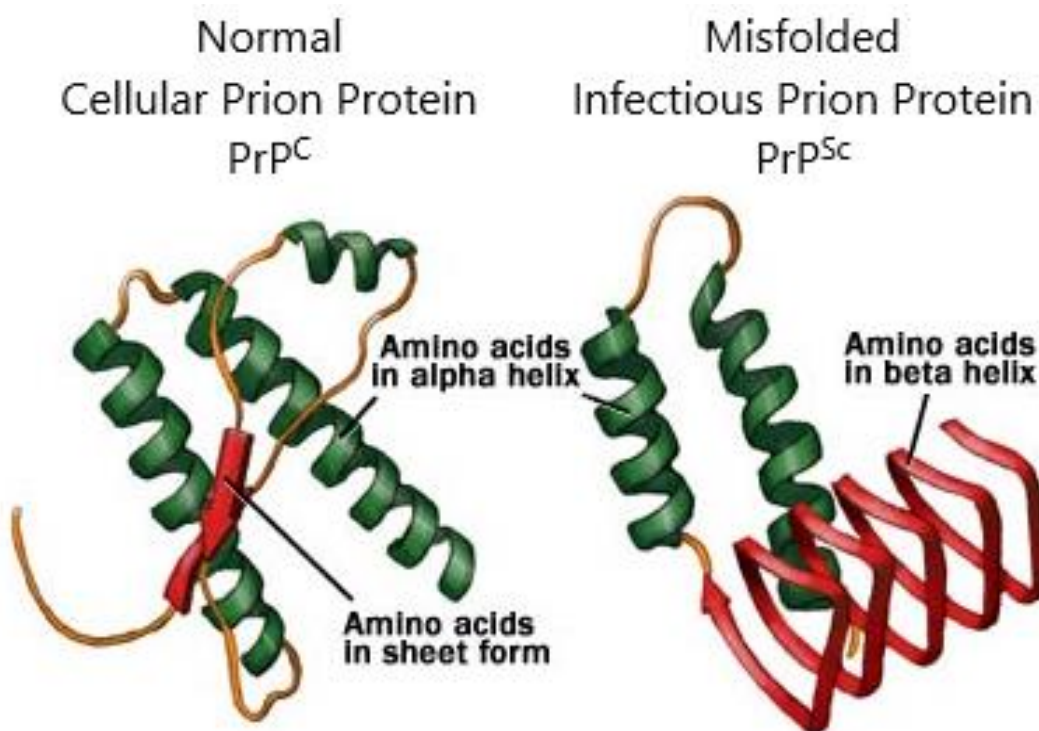


Figure 1. Structure of Normal PrP^{C} Protein and Misfolded PrP^{Sc} Protein

Normal cellular form of the prion protein, PrP^{C} and its misfolded disease-causing conformation, PrP^{Sc} . The normal prion protein is rich in α -helices, while the disease-causing form is rich in β -sheets. Figure used with permission from Mayo Clinic ³².

The infectious protein may arise through spontaneous misfolding, genetic mutation, or exposure to an exogenous protein. The misfolded proteins are then able to self-propagate their replication *in vivo* and *in vitro*. Two models are offered to explain the template-directed misfolding, and replication of the infectious prion proteins. First, in the template assistance model, PrP^{Sc} is believed to be a stable monomer that binds with PrP^C, inducing a conformational conversion³³, however there has yet to be experimental evidence for the existence of such a stable PrP^{Sc} monomer³¹. In the nucleation polymerization model, the nucleation phase is considered the rate limiting step, or the lag phase, for the formation of aggregation. Once the seeds have formed, the aggregates grow rapidly by the attachment of PrP monomers or oligomers in the elongation phase. Fragmentation of these structures appears to be a special and important feature of prion protein aggregation, as this generates more active nuclei and could be toxic to neuronal cells³⁴. This model proposes that the conformational transition of PrP^C and PrP^{Sc} is separated by a large energetic barrier, and that modifications such as mutations, or cofactors such as nucleic acids or lipids, lower this energy barrier by destabilizing the PrP^C protein³¹. This model is demonstrated in Figure 2. Although some cellular molecules such as glycosaminoglycans, nucleic acids, and lipids have been identified as cofactors, which promote the conversion of PrP^C to PrP^{Sc}^{1,35,36}, the molecular mechanisms of this conversion have yet to be defined.

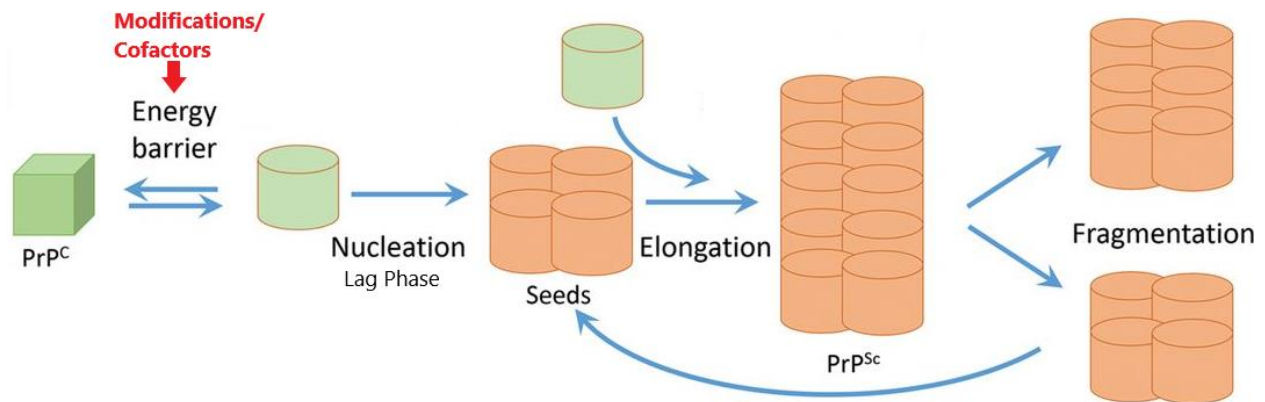


Figure 2. Prion Template Dependent Refolding Following the Nucleation Model

The nucleation polymerization model shows that PrP^{C} is destabilized by several factors including genetic mutations, and potential cofactors, lowering its energy barrier for the conformational transition to PrP^{Sc} . These proteins form aggregated seeds, which fragment to produce new seeds to propagate further spread of PrP^{Sc} . Figure used with permission of Oxford University Press ³¹.

The normal cellular form of the prion protein, PrP^{C} is found throughout the body, however the highest levels are found within neurons of the brain and spinal cord ³⁷. PrP^{C} is also expressed at lower levels on glial cells within the central nervous system (CNS) ³⁸. Within the peripheral systems of the organism PrP^{C} is expressed on a subset of cells including nerve cells within the skin, dendritic cells, as well as subpopulations of lymphocytes within the skin, gut, and bronchus ³⁹. Expression of PrP^{C} begins during early embryogenesis ⁴⁰, and continues to be expressed throughout the lifetime of the organism ³⁹. Due to the ubiquitous nature of this protein, and the high levels of evolutionary conservation within its sequence ⁴¹, there is an implied function of this protein. Although there has yet to be a defined function, there have been several proposed, including neuroprotection against apoptosis ⁴², resistance to oxidative stress ⁴³, and copper binding ⁴⁴. Since most PrP^{C} is expressed on the cell surfaces within lipid rafts, attached by a glycosylphosphatidylinositol (GPI) anchor, several studies have suggested PrP^{C} plays roles in transmembrane signaling ⁴⁵. Culturing neurons with increased PrP^{C} expression increases the levels of formation of axons and dendrites, and increases the numbers of synapses formed

between neurons, suggesting that PrP^C plays a role in synapse maintenance ⁴⁶. Due to the high levels of interactions PrP^C has with other proteins, including the neural cell adhesion molecule (N-CAM), it has also been proposed that PrP^C may play a role in cell adhesion ⁴⁷.

Due to the numerous proposed functions, it was believed that creating knockout strains of mice would enable identification of its true function. However, early research on strains of mice lacking PrP^C found there were no immunological defects, and development of the mice appeared normal ⁴⁸. Most significantly, it was found that prion knockout mice become resistant to prion disease ⁴⁹, and were unable to propagate infectious prions ⁵⁰. Continued research using prion knockout mice concluded that neuronal expression of PrP^C is required for prion-induced neurotoxicity ⁵¹. Neurografting brain tissue from wild-type mice onto brain tissues of knockout mice revealed that the PrP^{+/+} neurografted tissues were able to replicate the infectious prions, and demonstrated the expected neuronal damage, however the knockout mouse tissues remained unharmed, even in the presence of high levels of PrP^{Sc} ⁴⁹.

1.3 Disease Pathology

Spongiform degeneration, glial proliferation, neurodegeneration and deposition of aggregated PrP^{Sc} are common hallmarks of prion disease seen in the brain. The first signs of prion disease, during the early clinical phase, can be observed at the site of the dendritic spines, the smallest structural unit of neurons involved in forming synapses on the affected neurons⁵²⁻⁵⁴. Neuronal sub compartments heavily rely on one another to maintain the overall health of the neuronal system, therefore damage to one compartment often leads to the degeneration of the next⁵⁵. Reduction of synaptic spines progresses into the retraction of dendrites, and eventually the overall loss of dendrites from neurons. These effects progress into the degeneration and death of neurons, leading to the development of vacuoles throughout the brain, demonstrated in Figure 3-a. Reactive astrogliosis or the activation of astrocytes and glial cells, the immune cells throughout the brain, is a common process observed in prion disease,^{56,57}. This process includes several mechanisms including hypertrophy, increased proliferation, and significant upregulation of the glial fibrillary acidic protein (GFAP) expression⁵⁷. GFAP expression is detected using gene expression analysis, requiring the collection of a brain sample, or through imaging GFAP proteins once they assemble into fibrils⁵⁷⁻⁵⁹. Reactive astrocytes also promote inflammation through the release of cytokines, which may inadvertently result in the damaging of tissues⁶⁰. Figure 3-b demonstrates an increase in activated astroglia stained with GFAP in the thalamus of a CJD case.

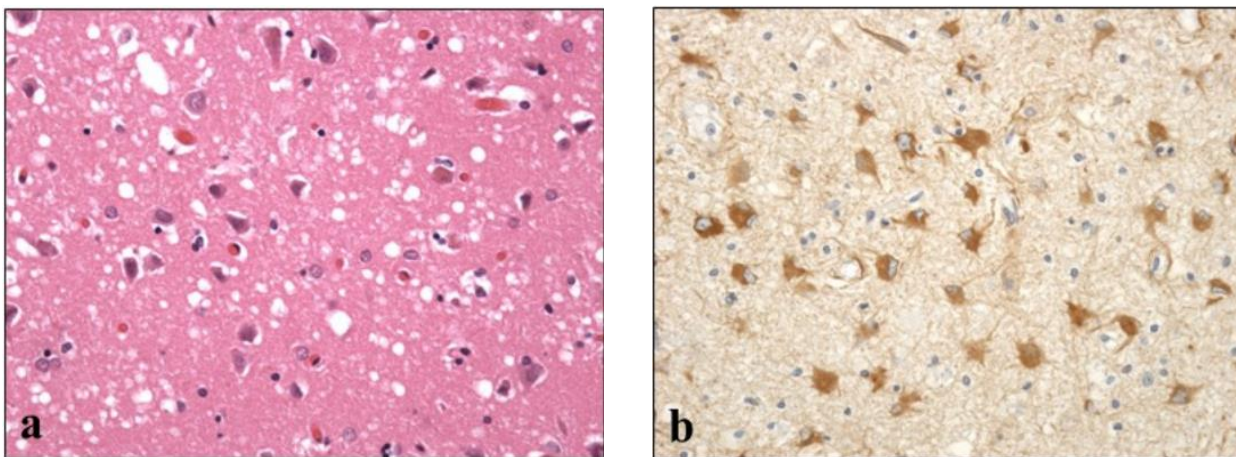


Figure 3. Neurodegeneration and Astrogliosis of Human CJD Prion Disease

A: End point neurodegeneration of the frontal cortex in sporadic CJD visualized with a haematoxylin and eosin stain). This figure demonstrates the vacuolation throughout the brain that occurs during transmission spongiform pathologies. B: Astrocytes immunolabelled for GFAP in the thalamus of a vCJD case. This figure demonstrates a high level of activated astrocytes, detected by GFAP expression. Figures used with permission from the National CJD Research & Surveillance Unit (NCJDRSU) ⁶¹.

Immunohistochemistry to identify the infectious prion protein, PrP^{Sc} has remained the most important tool to give definite diagnosis of prion disease, and has played a major role in our current pathogenic understanding of the disease ⁶². However, the amount and distribution of PrP^{Sc} deposits may not always correlate with type and severity of the neurodegeneration in those areas ⁶². Neuropathology is typically performed with use of a haematoxylin and eosin staining for evaluation of prion disease ⁶³.

In the mouse, pathology depends on the strain of mouse, and the strain of prion, making comparisons between each a lengthy topic. Different prion strains may be characterized based on the disease incubation period, histopathology, and variations in PrP^{Sc} deposition following serial passage in inbred strains of mice ⁶⁴, then further characterized based on biochemical properties ⁶⁵. Although there is a high level of overlap between many of them, there are features that remain distinct to each strain. Specifically, RML prion proteins injected into CD-1 mice results in

diffuse PrP^{Sc} deposition throughout the brain, with relatively weak levels of PrP^{Sc} deposition within areas such as the hippocampus, amygdala, and hypothalamus ⁶⁶. In the early stages of disease, PrP^{Sc} deposition can first be identified in the thalamus at 40-60 days following prion inoculation ⁶⁷. Neurodegeneration within the hippocampal CA1 region has been identified as early as 90 days following prion infection ⁶⁸. At the end point of disease, astrocytes and microglia are found to be more associated with the CA1 region, with high levels of astrocyte infiltration into the dense granular layer ⁶⁸.

1.4 Mechanisms of Disease

The mechanisms of prion disease pathogenesis have yet to be defined, however research supports the hypothesis that the conversion of PrP^C to PrP^{Sc} leads to neurotoxicity through the production of toxic intermediates ⁶⁹. This leads to the formation of insoluble protein aggregates throughout the brain ⁷⁰ and potentially, the overloading of protein clearance pathways ²⁷. The mechanism by which neurotoxicity occurs is unknown, however the fact that prion knockout mice are resistant to prion disease, even in the presence of a large amount of PrP^{Sc}, suggests that PrP^{Sc} itself is not toxic to neurons ⁷¹.

Cells within the brain are broadly classified as neurons, glia, endothelial cells, and pericytes ⁷², with neurons being highly outnumbered ⁶⁸. PrP^{Sc} deposition is only found within the parenchyma among regions containing neurons; PrP^{Sc} does not accumulate in areas of high glial scarring with high glial activity ⁶².

The role that activated astrocytes play, whether beneficial or detrimental to the infected individual, remains undefined. Intracellular aggregates in astrocytes are a common feature seen in neurodegenerative diseases; these aggregates are believed to interrupt normal astrocyte

function in ways that may be damaging to neurons ⁷³. Furthermore, although reactive astrogliosis is likely a beneficial process aimed at restoring the homeostasis and limiting tissue damage, persistent activation of these cells may be detrimental to the health of neurons ⁷⁴. In a healthy mammalian brain, astrocytes have several beneficial functions, such as in the restructuring of neuronal synapses, as well as their control and maintenance ⁷⁵⁻⁷⁷. Astrocytes also play a supportive role in the health of neurons by controlling the energy supply to neurons through the release of vasoactive compounds such as prostanoids, and by controlling the turnover of neurotransmitters ^{78,79}. Finally, astrocytes play a role in the development and maintenance of the blood-brain barrier ⁸⁰ and control blood flow throughout the CNS ⁸¹. It is clear that astrocytes play a major role in the maintenance of a healthy CNS, making them a focal point of neurodegenerative disease research.

Neuroinflammation is a process that is common to all neurodegenerative diseases, characterized by an increased number of glial cells, both astrocytes and microglia. These cells take on a reactive morphology, accompanied by the production of inflammatory mediators that are not found in healthy brain tissues ⁸². Glial scar formation within the central nervous during prion disease is thought to be mediated by microglia which drive the migration and proliferation of astrocytes ⁸³. Glial scars form physical compartments that work to contain neurodegeneration and prevent its spread by reducing the numbers of inflammatory cells that are able to enter the area ⁷³. However, this scar formation has also been shown to be detrimental to the recovery of the neurons isolated within, as molecules released by scar-forming astrocytes inhibits neuronal growth ⁷³. In animal models the activation of astrocytes, identified by the expression of GFAP, has been demonstrated to occur before neuronal loss ^{84,85}.

Many challenges exist to deciphering the pathogenesis of prion disease. One major challenge is the fact that prions generally do not replicate in immortalized cell cultures, limiting this research to the use of live animals. Next, the brain is an incredibly complex organ, with high levels of heterogeneity between cells even within the same region ⁶⁸, and this heterogeneity makes understanding the molecular pathobiology extremely challenging. Third, although strains of prion disease have defined patterns of infection throughout the brain, the individual cells affected within each region may differ between strains of prions; certain subsets of neurons have been shown to be more vulnerable to neurodegenerative events ⁸⁶.

Recently, techniques such as fluorescence-activated cell sorting (FACS) and laser capture microdissection (LCM) have emerged as tools that can isolate specific cell types, for example, microglia or neurons from the *Cornu Amonis* region, as a means to ‘cut-through’ this heterogeneity ^{40,68,87}. One contribution of this approach is to disentangling the temporal genetic alterations occurring specifically in neurons over the course of mouse-adapted scrapie disease progression that led to the identification of novel patterns of gene changes. This suggested that the transcriptome is directly altered in response to the progressive replication of prions in the brain ⁶⁸. Furthermore, it was shown that although strains of prions results in different patterns of PrP^{Sc} deposition, the altered gene expression was similar between all 3 strains tested, and any differences likely reflected the extent of neuroinflammation in these strains ⁶⁷.

Clearly, need for improved methods to target individual cells will further enable the elucidation of the molecular pathways that lead to disease, in particular those that mediate damage and death of neurons and ultimately the clinical phenotype. With this information, it is possible that therapeutic genes or pathway targets may be identified, which may lead to the development of drugs that are effective against prion disease.

1.5 Models of disease

Historically, non-human primates (NHP) have provided pivotal evidence of the transmissibility of human prion diseases, such as transmission of Kuru ¹⁶ and CJD by ingestion and surgical contamination⁸⁸. They were also seminal in the demonstration of zoonotic transmission of BSE to humans that resulted in the emergence of variant CJD ⁸⁹. Today, NHP continue to be used to assess the zoonotic potential of other prion strains, such as scrapie ⁹⁰, and CWD ⁹¹, or to assess the safety of using filtering devices to remove variant CJD causing prions from blood ⁹².

Cynomolgus macaque monkeys are the most commonly used primates in prion research due to their longevity, genetic similarity to humans, and their comparable digestive tract physiology ⁹³. NHP experimentation is associated with high costs, prolonged study lengths, and ethical concerns ⁹³, limiting their use.

Early prion disease research initially began in sheep to study scrapie, which was the first recognised prion infection ⁷, then goats ⁹⁴. Notably, goats were the first animal model in which the phenomenon of prion strains was identified and studied. In 1961, Pattison and Millson reported that infection of goats with the same batch of brain material from a sheep suffering from scrapie led to animals exhibiting one of two different clinical phenotypes. These were called “scratching” and “nervous”, based on the symptoms of disease, believed to be due to the genetic background differences between the hosts selecting for the preferential propagation of different strains ⁹⁴. Wild type mice were then used to repeatedly passage and select for scrapie strains with unique clinical and pathological phenotypes ⁶⁷. Host genetics was demonstrated to play a major role in the selection of strain type on passaging when a single nucleotide polymorphism in the mouse *PRNP* sequence was determined to be strongly linked to incubation period and pathological phenotype ⁹⁵. Current evidence suggests that the main difference between prion

strains is in the different conformational arrangements that PrP^{Sc} acquire ⁹⁶. The secondary structures of PrP^{Sc} aggregates are known to differ between some strains, and these structures appear to be maintained during templated replication of prions using a single primary PrP protein sequence ⁹⁷. This being said prion strains are routinely distinguished from each other based on parameters including incubation time, titration, and pathology, which are highly reproducible among strains in a given host ⁹⁸. A scoring system based on the localization and intensity of vacuolation in 9 regions of the brain has been developed to be an accurate typing system in trained hands ⁹⁹.

A number of naturally occurring scrapie isolates have been isolated and “cloned” through a process of limiting dilution and serial passage, to the point where only a single prion strain appears to be present. Further passage to cells or serial passage in the same species has occasionally led to the emergence of alternative strains ^{100,101}. These “mutations” are likely due to errors in fidelity of structural conversion between template PrP^{Sc} and substrate PrP^C ¹⁰².

Mice remain remarkable models of the prion disease due to their cost efficiency, and the availability of several transgenic mouse models. Of note, the prion knock-out mouse strain developed in 1992 was used to demonstrate that the cellular prion protein was necessary to propagate infectious prions ⁵⁰. Additionally, this strain of mice set the stage for the development of transgenic mice expressing cellular prion proteins of several different species ⁹³. However, transgenic mice are limited in their use to study interspecies transmission due to the phenomenon termed nonadaptive prion amplification (NAPA). In NAPA a mouse model expressing transgenic prion proteins from another species is capable of replicating the transgenic species’ infectious prion proteins, however the resulting amplified prion protein population fails to cause disease upon further passages to the other transgenic mice expressing the species’ prion protein ¹⁰³.

1.5.1 Cell Culture Models

Numerous immortalized cell lines of neuronal origin have been generated; however, all lines are extremely limited in the range of prion strains that they are capable of propagating ^{26,104}, making the comparison between different cell lines or between different cultured prion strains difficult.

Primary cell culturing uses tissues removed from a live animal to propagate cells without immortalization. Brain tissue can be isolated, treated to an enzymatic digestion, followed by mechanical dissociation and plating. Neurons can be selected for by the use of media enriched for factors that promote neuronal maintenance and growth and inhibit division of support cells such as astrocytes, microglia, and fibroblasts. Embryonic mice are most commonly used as the source of these cultures as neurons from embryos are less susceptible to damage during the procedure of dissection, dissociation of cells, and plating ¹⁰⁵ than those isolated from adult mice ^{106,107}. This is likely due to the still rapidly developing neuronal processes occurring in embryonic mice ¹⁰⁵. Due to this, primary neuron cultures harvested from embryonic mice are more robust, have a higher yield of healthy viable cells, and have a lower proportion of non-neuronal cells ¹⁰⁵. The longevity of the primary neuron cell cultures is limited to a few weeks, too short a period for the robust propagation of prions, however prion-infected cell cultures do demonstrate a progressive cell death and may be used to study the mechanisms related to prion-related neurodegeneration at the cellular level ¹⁰⁸.

Organotypic slice cultures are an adaptable culturing technique, allowing the maintenance of a 100- to 400- μm thick slice of brain grown on top of a semipermeable membrane in culture for up to 4 months ¹⁰⁹. These cultures give great insight on the 3-dimensional organization of cells, and have been proven to be very useful in molecular research, such as in studies of apoptosis ¹¹⁰, assaying of neuroprotective molecules ¹¹¹ and recapitulating molecular mechanisms of

Alzheimer's¹⁰⁹. Organotypic slice cultures generated from transgenic mice that overexpress prion protein have been adapted to allow for prion amplification for up to 8 weeks, showing a build up of PrP^{Sc} at 5 times the normal rate¹¹². This allows comparisons between uninfected and infected brains samples taken from the same animal.

Neuronal cell culturing techniques are extremely useful as they allow experimentation at a cellular level. Cell cultures can be used to assess the toxicity of potential therapeutics, as well as the mechanisms involved in disease and treatments. However, many of the promising therapeutic results found in neuronal cell cultures were not able to be replicated in a living animal system. As a result, live animals remain the superior model to research prion diseases, as other models fail to provide a 'complete picture' of prion disease progression, including neuropathology, biochemistry, and behavior changes⁹³.

1.5.2 Future Models

There are some potential methods or models that may drastically alter the ways that prion disease, or other neurodegenerative diseases are studied.

1.5.3 *In Vivo* Imaging

The ability to monitor the progression of a disease within a living organism, in real-time, has several advantages, for example the benefit of reducing the numbers needed to perform *in vivo* experiments. Instead of requiring several mice to be euthanized at the various time points in an experiment, it is possible to perform an experiment on a much smaller group of mice and follow the progression of the disease. Additionally, with the use of optical imaging, *in vivo* imaging may be performed non-invasively, and may be performed repeatedly as visible light photons are less harmful than the use of ionization radiation commonly used in other medical imaging techniques

¹¹³. Traditional imaging techniques are capable of producing high resolution images of neurons at a level that dendritic spines are visible ^{114,115}, however these methods require surgical intervention to remove or reduce the skull thickness, as well as the use of transgenic mice.

In 2009 the transgenic mouse strain Tg(*Gfap*-luc) was used to demonstrate prion disease progression. This system was used to detect bioluminescent signaling that is triggered when GFAP is expressed, demonstrating astrogliosis as early as ~55 days following inoculation of the mice with infectious prions, and ~62 days before neurologic deficits could be detected ¹¹⁶.

However, the use of the GFAP promoter to drive expression of luciferin limits the information that can be extracted from these experiments. As alluded to, GFAP expression is a hallmark of prion disease, as the expression of this gene is upregulated over a hundred-fold during reactive astrogliosis. However, this is an indirect marker of prion disease progression, as it has been demonstrated that prion disease can progress in mice lacking GFAP expression ¹¹⁷.

Unfortunately, there are no biomarkers of prion disease that allow direct observation of the progression of neuronal degeneration in prion disease. Therefore, an *in vivo* imaging system enabling the direct monitoring of neuronal cells would be a powerful tool that can be used to uncover the mysteries of prion disease progression and neurodegeneration throughout the brain. Uncovering genes specific to the degeneration of neurons may enable the development of a system that can be used to more-accurately monitor prion disease-associated neurodegeneration.

1.5.3.1 Mini Brains

Recently much progress has been made in the use of pluripotent stem cells originating from human skin cells to create a cerebral organoid system, named a Mini-brain. These organoid systems were able to be infected at five-months of age, with prions from two CJD subtypes.

These organoids were then monitored for 6 months following prion infection, seeding activity was identified, an indicator of infectious prion propagation, in both of the samples ¹¹⁸. Furthermore, these two subtypes exhibited different pathobiology within the organoids, confirming that the prion seed greatly influences the disease phenotype and that organoids present a robust system in which prion strains can be faithfully studied ¹¹⁸.

1.6 Genetic Manipulation of PRNP Expression in Cells and Animals

Given that ablation of PRNP expression in mice and other animals renders them resistant to prion diseases, gene silencing is a promising strategy for treatment of humans suffering from CJD ¹². Notably, silencing of the prion protein expression using a Cre-lox system in a prion-infected mouse has been demonstrated to not only reverse early spongiform deformities, but is also capable of preventing neuronal death, and halting progression of prion disease ⁷¹. However, as this system involved the development of chimeric mice with lentiviral transduction into embryonic stem cells, this system cannot be employed in a clinical setting. The Cre-lox system was used to silence prions only in the neurons of mice but retain PrP^C expression within astrocytes. This resulted in accumulation and deposition of PrP^{Sc}, from misfolding of the PrP^C expressed on astrocytes, yet the neurons remained immune ⁷¹. These results demonstrate that it may be possible to treat prion disease, if the disease can be diagnosed early.

It has been shown that reducing PrP^C by 50% with use of heterozygous knockout mice, that mice survive more than twice as long ⁵¹. The opposite holds true, where an increase in PrP^C expression results in acceleration of the disease process ¹¹⁹.

1.6.1 RNAi

RNA interference (RNAi) is a result of a long co-evolution between viruses and their hosts that has evolved as a defence against invading viruses and transposable elements ¹²⁰. Within the host, dsRNA is not naturally found. Therefore, during a viral infection the host protein, Dicer, is capable of recognizing dsRNA introduced via viral infection, which loads it into the RNA-induced silencing complex (RISC) to cleave the RNA to degrade the viral genetic material ¹²⁰. Previous research demonstrated a limited ability to utilize an antisense single-stranded RNA to

reduce gene expression ¹²¹. However, delivery of a double stranded RNA molecule resulted in much higher levels of gene downregulation, with increased specificity for the target gene ¹²². Since this discovery, the use of RNAi mechanisms has proven to be a powerful tool allowing the downregulation of a gene of interest, without modification of the hosts genome. There are several approaches to RNAi. The two most common methods of RNA interference are siRNAs, chemically or in-vitro synthesized double-stranded RNAs, and short hairpin RNAs, double stranded RNAs expressed from within the cell. ¹²³. The earlier forms of this system used transfection methods to introduce purified double-stranded RNA molecules into cells, or plasmids encoding a small hairpin RNA ¹²⁴. However, as is the case in the recently-approved first RNAi for human drug trials, named Onpatro, these effects are not long-lasting, and frequent repeated dosing for long-term effects are needed.

1.6.2 shRNA

Short hairpin RNAs can be encoded onto the genome of a virus and transduced into a specific target cell. The short hairpin RNA is encoded within the DNA genome of a cell. The DNA sequence consists of two small sequences of DNA of approximately 20 base pairs, attached by a loop sequence. When transcribed, these small RNA sequences form complimentary pairs, as the loop sequence allows the RNA structure to fold in on itself. This dsRNA is then converted into siRNAs using the same machinery that processed using the same complex of Dicer and RISC. From here, the silencing effects of siRNA and shRNA are identical. Attached to the RISC complex, the siRNA targets mRNA sequences based on complementary base pairing, usually requiring perfect homology ¹²⁵. This binding effect removes the bound mRNA sequence from the cytoplasm, acting as a form of gene knockdown, or this complementary binding may induce

cleavage of the RNA phosphate backbone by Ago-2, a member of the argonaute protein family¹²⁵.

Previous research has shown that long-term expression of a shRNA sequence in chimeric mice resulted in increased survival of chimeric mice with at least 50-70% of their cells expressing the shRNA sequence¹²⁶. Other research involving the use of viral-delivered anti-PrP^C shRNA focused on the delivery of either lentiviruses into the hippocampus¹²⁷, or AAVs into the thalamus¹²⁸, of prion-diseased mice. It was demonstrated that lentiviral-delivered shPrP administered intracranially into the hippocampal region of RML-infected mice was capable of increasing the lifespan of the diseased mouse by 24%, and promoted recovery of neurons leading to reversal of cognitive defects¹²⁷. Other research performed intracranial injection, using a convection-enhanced delivery method capable of delivering large volumes and quantities of molecules within the brain to areas up to 1 cm away from the injection site¹²⁸. This study injected mice in the thalamus, delivering approximately 4.2E10 AAV2 into the thalamus region on each side of the brain. Although the AAV2 injection resulted in an overall reduction of PrP^C in the thalamus by 74%, the treatment did not significantly increase the lifespan of the mice¹²⁸. However, the levels of neurodegeneration in the AAV2-shRNA treated mice were reported to be markedly reduced in the thalamus compared to control mice¹²⁸. This suggests that a global high level of PrP^C decline is required to see a significant increase in the lifespan of mice.

1.6.3 Antisense

Use of an antisense oligonucleotide (ASO) is also a promising method of manipulating prion disease. ASOs are short strands of DNA that are the reverse complement (antisense) of the target RNA sequence, so that the DNA strand binds the RNA. Once bound, the ASO targets the RNA

for degradation, resulting in a reduced amount of the target gene's expression ¹²⁹. Use of ASO's have shown promising results in treating genetic diseases of the CNS. In 2016, the first use of an ASO in humans was effective in modifying spinal muscular atrophy, a neurodegenerative disease, following delivery into the CSF via a lumbar puncture ¹³⁰. However, this ASO was designed to change the processing of its genetic target, rather than reduce the total amount of RNA as in the cause of ASO use in prion disease. Further use of an ASO in Huntington's disease, another neurodegenerative disease where lowering one specific gene's expression, in this case the Huntington gene, would result in increased survivability for the diseased individuals. In 2018 it was shown that an ASO used to target the Huntington gene to lower its expression, resulted in a decrease of mutant huntingtin gene RNA by 40% in the CSF at the highest dose ¹²⁹.

In prion diseases, the prophylactic use of an ASO has been shown to increase the lifespan of prion infected mice by 61-98%, and a single injection prior to the onset of clinical symptoms increased survival lengths by 55% ¹³¹. It has yet to be seen whether these results translate to human trials.

1.6.4 Indirect manipulation

Other methods of manipulating prion disease may not directly target either PrP^C or PrP^{Sc}, but may instead target gene-regulating miRNA (miRNA) as a potential therapeutic ¹³². miRNA are small, non-coding RNAs that regulate the expression of multiple genes ¹³³. Previous research has identified a number of these that play a role in fundamental neuronal processes such as neuronal differentiation, development, plasticity, and survival against prion disease ⁶⁸. Furthermore, links between the miRNA dysfunction and neurodegeneration has been shown in prion disease ^{134,135}.

A therapeutic using miRNA as a target to treat prion disease would aim to either upregulate miRNA associated with resistance and recovery of prion-associated neurodegeneration or downregulate those associated with the progression of the disease. Although targeting the disease-associated miRNA appears to be a promising therapeutic, their multimodal effects require that these molecules be further understood before safe clinical trials can be performed, as the nonspecific targeting raise concerns of unwanted side effects. Continued research into the molecular mechanisms that play roles in the pathogenesis of prion disease, or pathways related to the survival of neurons, may identify key pathways or regulatory molecules that can be used in the development of therapeutics.

1.6.5 Delivery Strategies for Brain Gene Therapies

A major hurdle in developing gene therapies for diseases affecting the brain is delivery across the blood-brain barrier. The blood brain barrier is a highly selective semipermeable border that separates the circulating blood from the brain and extracellular fluid in the central nervous system which serves to protect the brain from toxins and infection ¹³⁶. Once the blood brain barrier is crossed, further challenges include the targeting of the transgene to the affected region of the brain or a specific cell type ¹³⁷. This comes with additional risks, such as off-target effects that result in transgene-associated toxicity ^{138,139}

Non-viral vectors, such as liposomes, exosomes, polymeric nanoparticles, are considered due to their safety profile and simple production methods ¹⁴⁰. However, these methods mediate a transient effect and often show relatively low efficiency of successfully delivering their cargo, often requiring repeated administrations of the treatment ¹³⁷.

Viral vectors have been demonstrated to be very powerful agents that may be used to deliver genetic products into a target cell, tissue, or organ ^{135,137,141}. Viruses have evolved alongside organisms, so they are very efficiently able to deliver their genetic products into a very specific target, hijacking the cellular machinery to replicate the viral genomes, and produce proteinaceous viral particles to package the viral genomes into ¹⁴¹. Fortunately, research has identified ways where we may adapt viruses and use their efficient transducing capabilities to safely deliver a range of genetic products.

There are several commonly used viral vectors that have been used to deliver genetic products into cells, each bring their own set of benefits and downsides to their use. Common viral vectors include herpesvirus, adenovirus, adeno-associated virus (AAV), and lentivirus ¹⁴². A range of viral vectors allows selection of a specific virus depending on several factors, including the cell type to be transduced, the size of the genetic construct to be packaged into the virus, the mode of delivery of the viral vector, and the length of transgene expression required ¹²⁴.

Ideally, a virus should have tropism and highly efficient transduction of a target cell or tissue, with minimal off-target transductions of other cells, tissues, or organs. If the chosen virus transduces other cell types or organs there will be less viruses to transduce the intended cell type, resulting in lower levels of expression within the intended cell type. The ideal virus should express a transgene for long periods of times, and at levels needed for the maximal therapeutic impact. If the transgene is not expressed long enough, there may be a lack of therapeutic effect. Conversely, too high levels of expression may result in cytotoxicity of the transduced cell, through overburdening the cell, or by initiating an immune response against the cell ¹⁴³. There should be no side effects such as immune responses against the viral vector, and the virus vector should not be able to cause any vector-related pathologies. Finally, methods must be available to

allow for large scale production of the virus, while producing a high-quality virus sample that meets the guidelines for therapeutic use.

Specifically, for the purpose of inducing long-term expression of transgenes within the CNS, AAVs are very strong candidates. AAVs are capable of transducing mitotic and post-mitotic cells ^{137,139}. However, due to the AAV genome functioning as an episome in transduced cells, opposed to integrating into the host genome such as with lentiviruses, the episome is lost from dividing cells, through various potential mechanisms that are not clearly defined ¹³⁹. Therefore, the episome can persist in non-dividing cells, such as neurons, for long periods of time ¹³⁶. Additionally, some AAV serotypes have been shown to be capable of bypassing the blood-brain barrier (BBB), such as AAV9 and AAVrh.10 ¹³⁶.

1.7 Adeno-Associated Viruses

AAVs are single-stranded DNA viruses of the family *Parvoviridae*, in the genus *Dependovirus*.

The virus is unable to replicate without the presence of a helper virus, typically Adenovirus or Herpesvirus in natural infections ¹⁴⁴, so viral vectors can be administered without fear of the virus replicating within the receiving host. There are several serotypes available, each capable of transducing different regions of the body, or cell types, with higher tropism, with examples of some of the most common serotypes shown in Table 2.

Table 2 Commonly used AAV Serotypes and Tissue Tropism

AAV Serotype	Target Tissues
AAV2	Kidney, CNS
AAV6	Skeletal Muscle, Lung
AAV7	Skeletal Muscle, Liver
AAV8	Liver, Skeletal Muscle, Heart , Pancreas,
AAV9	Liver, Skeletal Muscle, Lung, Heart, Kidney, Brain
AAVrh.10	Heart, Brain

Outline of AAV serotypes and their tropisms. Table adapted from ^{145–148}.

There are several advantages that AAVs offer over other viruses used in gene transduction. Due to the safety of AAVs, they have been approved for use in animal studies ^{144,146}, as well as in human studies ^{149,150}. AAVs can be produced using methods resulting in the production of replication-defective viruses, as they lack the genes necessary for reproduction. After transduction of a cell, in the absence of the helper virus, the viral genome persists as an episome in a latent state as monomers and multimers in linear and circular forms ^{151,152}, allowing long-term expression in non-dividing cells. AAVs have been demonstrated to support long-term, non-toxic gene expression for over 8 years in the tissues of dogs ¹⁵³, and over 6 years in primates ¹⁵⁴. There are several different serotypes available, each with different sets of capsid proteins,

allowing them to target different receptors. This allows selection a serotype more suited for transducing the intended target cells or organs.

The major downside to the use of AAVs is that the virus is only able to package a sequence of DNA spanning approximately 4.5 kb. Additionally, transgene expression is dependent on second-strand synthesis, making it a limiting step in cell transduction ¹⁵⁵. The use of dual-vectors has allowed AAVs to be used to deliver transgenes up to 8 kb ^{156,157}, as each vector delivers a fragment of DNA which then undergoes recombination to reform the long sequence of DNA. The dual-vector approach has been useful in cell cultures, but the low rates of co-transduction of a single cell, and recombination of the DNA fragments greatly limit this approach ¹⁴³.

Although widely regarded as safe to use in various animal models and in humans, there have been an increasing number of publications identifying AAV-induced tumors ^{139,150,153}. In the presence of the Rep 78/68 proteins, typically expressed on the helper virus during infection with natural AAVs, integration of the genome occurs at a relatively low rate and has a preference for a specific site on chromosome 19 ¹⁵⁸, although integration of recombinant AAVs is extremely rare ¹⁵⁸. In the absence of these proteins, as is the case when using recombinant AAVs, the site specificity is lost and the integration rate is further reduced ¹⁵⁸. In a study covering over 600 mice there were no significant signs of tumorigenesis found when AAVs were directly injected to the mouse liver ¹⁵⁹. However, in more recent long-term studies using intravenous injection into neonatal mice, there was an increased risk of the mice to develop tumors in their lungs and livers ^{139,160}. Due to the limited number of publications focused on this topic ^{139,160}, there is a need to further assess the safety of administering these viruses into younger animals, and humans.

1.7.1 AAV use in the CNS

Delivery of genetic material into the CNS has proven to be a complicated task. The BBB, the collection of blood cells and membranes that act as a barrier between the bloodstream of the brain and that of the rest of the body, remains the main bottleneck against genetic therapeutic use in the central nervous system ¹⁶¹. In fact, all human clinical trials to date (outlined in Table 3) have used intracranial injections to bypass the BBB ¹⁴¹.

Table 3 Example of AAV Clinical Trials of the CNS

AAV Serotype	Disease	Transgene Product
AAV2	Alzheimer's	NGF
AAV2	Canavan	ASPA
AAV2	Parkinson's	NTN, GDNF, GAD
AAV2 AAVrh.10	Late infantile neuronal ceroid lipofuscinosis	Human CLN2
AAV5	Sanfilippo syndrome	Human α -N-acetylglucosaminidase

ASPA: Aspartoacylase, NGF: Nerve growth factor, Neurturin (NTN), Glial cell-derived neurotrophic factor (GDNF), Glutamate decarboxylase 1 (GAD). Table adapted from ¹⁴¹.

Early use of AAVs in the CNS began with AAV2, then moved onto AAV5 and AAV8, and required direct injection into the brain ^{144,162}. Intracranial injection, performed by inserting a flexible catheter through holes drilled in the brain, allows injections of AAVs into a specific site of the brain ¹⁶³. However, due to the limited diffusion of AAVs (1-3 mm) ¹⁶⁴, multiple injections are needed to treat a large portion of the CNS. It is estimated that between 50 to 350 injections would be needed to administer AAVs throughout a human infant's brain ¹⁴¹. This invasive procedure also increases the risk of hemorrhaging, edema, and infection ¹⁴¹.

Fortunately, AAV serotypes AAV9 ¹⁶⁵⁻¹⁶⁷ and the more recently-discovered AAVrh.10 ^{168,169} have been demonstrated to be capable of bypassing the blood brain barrier to transduce cells within the brain of adult cats, mice, and monkeys ^{166,167,170}. Discovery of AAV9 with its efficient

transduction of neurons and astrocytes, and its ability to efficiently cross the blood brain barrier following intravenous injections was momentous, as this allows minimally-invasive delivery of these viruses into the cells of the CNS ^{166,171}. Further research demonstrated that intravenous delivery of AAV9 had great potential as a genetic delivery vector, with potential to treat CNS diseases. Intravenous administration of AAV9 expressing heparan N-sulfatase in a Sanfilippo syndrome mouse model resulted in correction of the gene deficit throughout the entire mouse and resulted in significant increased lifespans of the affected mice ^{172,173}. However, these results showed that the age that the mouse receives treatment is crucial, as treatment of older mice failed to prolong their survival, despite the mice demonstrating improvements in behavior, and the reduction of pathological effects ¹⁷⁴. Finally, intravenous delivery of AAV9 into neonatal mice resulted in long-term, high levels of gene expression within the brain, as this region contains a high number of non-dividing cells ¹⁶⁵. Intravenous delivery of AAV9 into neonatal mice to express the *Hexb* cDNA to correct the neurodegenerative phenotype was shown to be capable of inducing long-term correction of Sandhoff Disease in mice for up to 43 weeks ¹⁶⁰.

The use of AAVrh.10 in neonatal gene therapy treatments is quite promising, with several papers demonstrating superior transgene delivery in neonatal mice when compared to other AAV serotypes. AAVrh.10 and AAV9 have near identical transduction profiles, but AAVrh.10 has a stronger expression level throughout the life of the mouse ^{119,120}. Additionally, it has been shown that when AAV9 and AAVrh.10 are delivered in the lower range of doses (3E10 viruses) AAVrh.10 has a higher transduction efficiency throughout the brain ¹⁶⁹. This is explained by the fact that AAV are able to infect the same cell multiple times, so at the higher ranges of virus doses the overall number of cells transduced does not significantly increase, however the number of viral genomes per cell increases ¹⁶⁹.

Although these vectors are known to cause high levels of transduction throughout the brain, there are risks associated with intravenous AAV delivery to neonatal mice. As demonstrated, 8 of the 10 mice that received intravenous AAV9 injection as neonates had signs of tumors in either their lungs or liver¹⁶⁰. These results show that AAVs may be very strong tools allowing wide-spread transduction of cells throughout the brain, and other organs, following intravenous injection, however further research may be necessary to assess the risk potential of intravenous injection into neonatal mice.

1.8 Further Customization of AAV Vectors

1.8.1 Modification of Transduction Tropism

Several receptors have already been found for AAV serotypes 1-9, and several advances have been made in developing novel capsids. For example, the liver-tropic AAV2 capsid binding genes were swapped for those of the muscle-tropic AAV8, resulting in a novel muscle-targeting capsid, with very low tropism for liver cells ¹⁷⁵. Further studies showed that even minor changes in the receptor binding footprint of the capsid had drastic effects on the AAV ¹⁷⁶. Previous research grafted a small section of the capsid surface footprint onto the AAV1 virus which resulted in the virus adapting a CNS-targeted transduction profile, while diminishing vascular and hepatic transduction through unknown mechanisms ¹⁶¹. These studies have demonstrated that through a rational design, AAV serotypes may be customized to improve transduction efficiencies of a target cell type or reduce transduction of others.

Opposed to rational design, a directed evolution approach makes use of random mutagenesis, followed with rounds of selective pressure to isolate variants with the desired properties ^{177,178}. Furthermore, DNA shuffling uses random digestion and ligation of pools of AAV serotype capsid sequences, which when followed by directed evolution, allows the isolation of a range of unique molecular footprints in one capsid ^{179,180}. These advances may allow customization of AAVs to do some amazing things, such as producing an AAV2 that targets only brain endothelial cells of diseased, but not healthy animals ¹⁸¹.

1.8.2 Selection of Promoters to Control Transgene Expression

The use of specific expression cassette elements has been shown to drive target-specific expression¹⁸², and may be used to further restrict expression to a smaller set of cells. For example, expression of a shRNA typically requires a Pol III promoter, such as U6 or CMV. These types of promoters are expressed in cells throughout the body. Pol II promoters are much more cell specific. Modifications to the shRNA sequence have been shown to allow Pol II promoters to drive expression of the shRNA.

Therefore, advances in either the cell-tropism of AAV serotypes, the transduction efficiencies, and of the internal expression vectors all may play a role in developing future novel AAV serotypes allowing CNS gene therapy treatment.

1.9 Production of AAV

Although the use of AAVs offers several avenues to be researched, production of the viruses remains a difficult task, with lengthy incubation times and several complex steps. Overall, the experiment requires, at minimum, 10 days to complete starting at the culturing of HEK293T cells and ending with a sample of viruses ready to be used in experiments. Although protocols found in the literature differ in the minor details, they do follow a general outline of AAV production^{164,183–185}. Each protocol begins with the triple transfection of HEK293T. The plasmids supply the genes necessary for the replication of the viral genome, and the genes for the determination of the serotype of virus. The final plasmid is supplied by the user, and it contains the genetic product that is to be packaged into the viruses. The use of three plasmids allows this process to be easily modified as one plasmid can easily be substituted to change the serotype of the produced AAVs. Modifications to the production and purification of AAV's have been reported that may contribute to the efficiency of production.

1.9.1 Upstream AAV Production

It is necessary to have high transduction efficiencies when introducing the expression vector, capsid plasmid, and the Adenovirus-derived helper plasmid into HEK293T cells. The common method of calcium phosphate transfection is typically used when cultures are grown on a plate, as this has shown high levels of transfection in HEK293T cells, with transfection rates of 80-100% commonly being reported in the literature^{186,187}. Other transfection methods, such as Polyethyleneimine (PEI) are commonly used when HEK293T cells are grown in suspension for AAV production, as this transfection typically does not require a media change following transfection¹⁸⁸. The transfected culture is maintained for typically 3 days before harvesting of AAV. The media is collected and separated from the cells, and the cell fraction is subject to

freeze-thaw rounds to release the virus for collection. Several protocols consider the processing of the collected media fraction to be an optional step ^{164,188}.

1.9.2 Downstream Purification of AAV

Downstream purification of the crude AAV harvest is required to reduce the number of protein contaminants, and remove portions of empty package AAVs. Serotype-independent methods include PEG precipitation and centrifugation filtration devices for small scale preps, or a choice between CsCl or iodixanol concentration gradients purification methods for larger scale production ¹⁸⁹. Affinity binding columns are available to purify specific AAV serotypes based on their capsid proteins ¹⁸⁵.

Although small-scale preparations using PEG precipitation or centrifugation devices enable small-scale experimentation, productions on this scale produce limited numbers of AAV particles. The numbers of viral particles required in animal studies or therapeutic scenarios is substantial, requiring a considerable scale-up in production methodologies. Furthermore, there are increasing reports showing that PEG precipitation may reduce the transduction efficiency, and effect cellular tropism ¹⁹⁰.

The two most commonly used methods of AAV purification are cesium chloride gradient ultracentrifugation ^{190–192}, and iodixanol gradient ultracentrifugation ^{188,189,193}. Although both of these methods use buffer density gradients to concentrate the AAVs into a tight band between two concentration layers, there are important differences between them. Most importantly, CsCl purification requires two successive 20-hour gradient ultracentrifugation steps, compared to one 2-hour gradient ultracentrifugation step performed in iodixanol purification. Both methods have been demonstrated to be capable of producing highly concentrated, purified viral samples, with

CsCl removing a larger portion of viral particles that do not contain the full genome^{189,193}.

However, there is a concern of traces of CsCl being introduced when administering the viruses, meanwhile iodixanol is chemically inert and has not shown any adverse effects when injected into animals in small amounts¹⁹³. Another system frequently used for AAV purification and concentration is the tangential flow filtration system. This system is a closed pump-filter system that continuously cycles the sample over and through a membrane consisting of pores with a 100 kDa molecular weight cut-off., This enables serotype-independent concentration of AAVs based on the molecular pore size of the filtration membrane of the system^{142,183,191,194–196}. AAVs with an average molecular weight of 3746 kDa¹⁹⁷ remain within the system, while media and contaminants are diafiltered out of the sample.

Chapter 2. Gaps in Knowledge, Hypothesis, and Objectives

2.1 Gaps in Knowledge

There are several key aspects of prion pathogenesis that remain unclear or understudied. This is largely due to the difficulties of researching the disease during early stages. Advances in the methods used to study prion disease are promising ¹¹³, such as the use of LCM to isolate small sections of brain ^{87,113,135}. Further improvements will be the development of further methods used to study neurodegeneration during early disease, when drugs may be effective in delaying or curing the disease. To understand the molecular pathways that direct neurodegeneration in these early stages and to study the effectiveness of drugs, improved methods to genetically manipulate neurons in the brain of living animals are essential.

In this study we chose to explore the use of AAV's as a delivery system for transgenes into the brain of living mice that could be used to genetically manipulate neurons as a way to study prion pathogenesis. The viral vector chosen was AAVrh.10 as it has been shown to result in higher levels of transduction in the brain, when compared to other AAV serotypes following intravenous injections into neonatal mice ^{168,198}. Furthermore, it has been shown to be capable of inducing transgene expression within the brains of mice, with transgene expression remaining detectable for at least 22 months ^{160,199}. The transgene expression of these viruses is long enough to enable prion disease studies in mice, which typically end 140 following prion-infection ¹³⁵. The transgene chosen in these exploratory studies was an anti-PrP^C shRNA sequence which shown to reduce PrP^C expression in mice following direct injection into the brain ^{127,128}, and to extend survival following infection ¹²⁷.

2.2 Hypothesis

Based on evidence that it is possible to introduce a genetic construct into the neurons of mice following intravenous injection of neonatal mice, it is hypothesized that with use of the AAVrh.10 vector an shRNA targeting the PrP^C sequence can be introduced into neurons to drive transgene expression throughout the course of prion disease. If transduction throughout the brain reaches approximately 50%, this molecule is believed to be capable of extending the lifespan of prion-infected mice.

2.3 Objectives

The three main objectives of this research are:

1. Development and optimization of the methodologies of AAV production, followed by the characterization of AAV serotypes as vectors for shRNA delivery to neurons, based on transduction efficiencies, cytotoxic effects, and levels of gene knockdown
2. Delivery of AAV into neonatal mice, to assess the specificity of neuronal expression of the transgene over time.
3. To determine effects of AAVrh.10 carrying the anti-PrP^C shRNA on the pathogenesis of prion disease in mice infected with mouse-adapted scrapie.

The goals of Objective 1 are as follows: to optimize production of AAVrh.10 to maximise the total yield of AAV particles in our laboratory, to determine the transduction efficiency of the construct into primary neuron cultures *in vitro* in comparison to the other commonly used serotypes AAV8, and AAV9, to determine the levels of gene knockdown as a result of the anti-PrP^C shRNA, and to ensure safety of use prior to work in live animals.

The goals of Objective 2 are as follows: To perform intravenous injection of AAVrh.10 into neonatal mice and to determine successful delivery into the brain, to determine the extent of neuronal targeting of the vector in mice, and to determine the longevity of expression from the vector over an 8-month period.

The goals of Objective 3 are as follows: To infect treated mice with the Rocky Mountain Laboratory (RML) strain of mouse adapted scrapie, to determine the effect of treatment on the incubation period of disease and specifically, whether AAVrh.10-anti-PrP^C provides resistance to neurodegeneration to transduced neurons *in vivo*.

Chapter 3. Materials and Methods

3.1 Ethical Use of Animals for Experimentation

All procedures involving live animals were approved by the Canadian Science Centre for Human and Animal Health - Animal Care Committee (CSCHAH-ACC) according to the guidelines set by the Canadian Council on Animal Care. All protocols were designed to minimize animal discomfort. The approval identifications for this study were animal use document (AUD) #'s H15-032 and H15-022.

3.1.1 Primary Neuronal Cultures

All animals used to produce primary neuron cell cultures were cared for as outlined in the Animal use Document (AUD) H15-032. Timed-pregnant CD1 female mice were first anesthetized and then cervically dislocated following CACC outlines. The uterus was then collected, and the embryos removed then decapitated using sharp surgical scissors. Pregnancy was timed to ensure that collections were performed at approximately embryonic day 18. Pregnant mice were delivered to the Public Health Agency of Canada from the University of Manitoba the day before collections were to be performed. These mice were provided housing and food and were cared for until collection. Veterinary Technical Services (VTS) staff within the Public Health Agency of Canada Biosafety Level 2 Laboratory performed these procedures.

3.1.2 AAV Delivery and Prion Infection of Mice

All animals were cared for as outlines in the Animal use Document (AUD) H15-022. Veterinary Technical Services (VTS) staff within the Public Health Agency of Canada Biosafety Level 2 Laboratory performed all procedures. Pregnant mice were delivered to the Public Health Agency of Canada from the University of Manitoba the day before collections were to be performed.

These mice were provided housing and food and were cared for until delivery of their litter. Following litter delivery, neonatal mice (1-2 days old) received injections of AAVrh.10 in PBS via intravenous injection into the superficial temporal vein. AAV vectors contained an anti-PrP^C shRNA sequence or an empty control vector, which also expresses mStrawberry RFP. Control mice received injections of PBS or no treatment. The intravenous delivery of our AAV was performed by Dr. Jagdeep Walia, KGH Research Institute, Kingston, Ontario, Canada. At 60 days following AAV injection, mice received RML infection by 5 µl of 10% brain homogenate of RML-infected brain, or a mock infection using 5 µl of 10% healthy brain homogenate. RML was administered intracranially with use of a Tri-Dek. Mice were then cared for as complete litters until weaned. Mice were caged in groups of 4 based on gender, and experimental group. Samples of brain, liver, lung, spleen, and blood were collected at DPI 0, 60, 90, and at end-point of disease, and stored in formalin for immunohistochemistry analysis. End-point of the disease is classified as a score of 5 on the TSE Clinical Scoring Chart in Appendix 1.

3.2 AAV Preparation

3.2.1 HEK Cell Maintenance

HEK293T cells are seeded onto a 15 cm Nunclon petri plate (ThermoFisher Scientific) at 5E6 cells per plate and grown at 37 °C with 8% CO₂ with 20 ml of DMEM + 1% Pen/Strep (ThermoFisher Scientific) + 10% Heat-inactivated Fetal Bovine Serum (FBS) (ThermoFisher Scientific). HEK293T cells are seeded at 5E6. Once near confluent, cultures are split 1:3 into new dishes so plates are near confluency the next day, and ready for triple transfection.

3.2.2 Upstream AAV Production

Small scale production was performed using 5-10 Nunclon 15 cm petri dishes (ThermoFisher Scientific). Large scale production was performed using 50 plates. The day before transfection confluent plates of HEK293T cells were split 1:3 to yield the desired number of plates. Media is changed in the morning. Triple transfection (plasmids used are explained in detail in Table 4) on 50 plates was performed by slowly adding Solution A (9.5 ml CaCl₂ 2.5 M, 650 µg cis plasmid (AAV ITR + transgene), 650 µg of trans plasmid (AAV rep + cap), 1250 µg of helper plasmid (Adenovirus genes: E1B, E4, and VARNA), sterile water to 75 ml) dropwise to 5 falcon tubes (ThermoFisher Scientific) each containing 15 ml of Solution B (2x HEPES Buffer: 16.4 g NaCl, 11.9 g HEPES, 0.21 g Na₂HPO₄, 1 L water, pH 7.05) while vortexing. This solution was left at room temperature for 20-30 minutes, then 3 ml was added to each 15 cm plate dropwise, while trying to spread the drops evenly. The media was changed 12 hours following transfections. Cultures were maintained for 4 days to yield optimum virus production of AAVrh.10, with addition of 10 ml of media at Day 3. The media fraction was collected by tilting the plates and carefully removing the media from the cell culture. HEK293T cells were then harvested using a

cell scraper (ThermoFisher Scientific), then centrifuged at 2500 g in a swinging bucket rotor. Supernatant was removed from the cell pellet and added to the media fraction. Cell and media fractions were either processed immediately, or frozen at -80 °C until processed. 20 ml resuspension buffer (50mM TrisCl per 200mM NaCl per 2mM MgCl₂, pH 8.0) was added to the tubes containing cell pellets.

Table 4. Plasmids used in the triple transfection of HEK293T

Plasmid	Role	Genes Supplied
Trans Plasmid	Replication of the AAV genome, genes encoding capsule serotype of the virus	Rep and Cap
Helper Plasmid	Contains Adenovirus genes required for packaging of the AAV	E1, E2A, E4, VARNA
Cis Plasmid	Contains transgene sequence flanked by two ITR	Transgene sequence, limited to 4.5kb

Description of the 3 plasmids supplied to HEK293T cells during triple transfection to produce AAVs. Trans, Helper, and Cis plasmids are supplied in equimolar amounts to provide the genes required for AAV production to the transfected HEK293T cells.

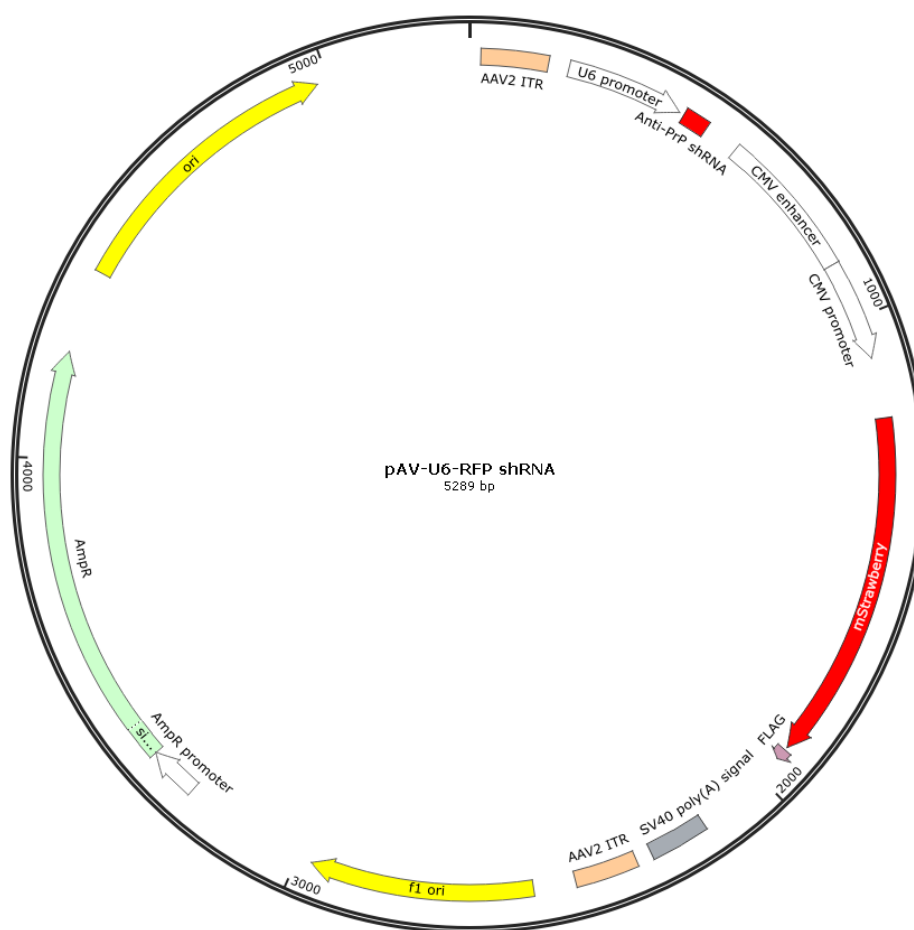


Figure 4. pAV-U6-RFP Plasmid Map

Plasmid backbone obtained from Vigene Biosciences. Vector contains either an anti-PrP^C shRNA sequence driven by the U6 promoter, or an empty sequence as a negative control vector. Vector also contains mStrawberry driven by the CMV promoter.

Table 5. Features of pAV-U6-RFP Plasmid

U6 Promoter	Ubiquitous RNA pol III promoter to drive expression of short hairpin RNA
shRNA / Empty Sequence	Short hairpin RNA sequence against prion protein gene / Empty sequence no shRNA insert)
CMV Promoter	Ubiquitous promoter commonly used in mammalian expression systems
mStrawberry	Bright monomeric red fluorescent protein
AAV2 ITR Regions	Sequence between these segments are packaged into viral progeny

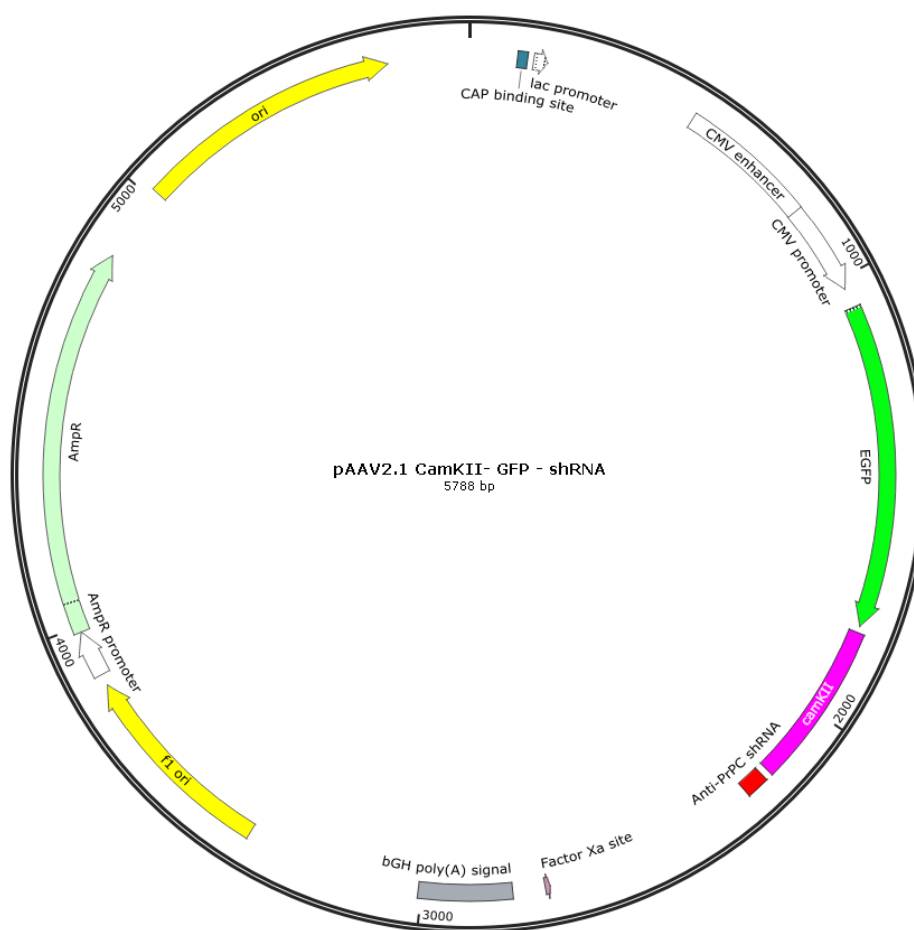


Figure 5. pAAV2.1 CamKII-GFP Plasmid Map

Vector contains either an anti-PrP^C shRNA sequence driven by the CamKII promoter, or an empty sequence as a negative control vector. Vector also contains eGFP expression driven by the CMV promoter.

Table 6. Features of pAAV2.1 CamKII- GFP Plasmid

CamKII Promoter	Dopaminergic neuron-specific RNA Pol II promoter
shRNA / Empty Sequence	Anti-PrP ^C shRNA sequence / Empty control sequence
CMV Promoter	Ubiquitous promoter commonly used in mammalian expression systems
eGFP	Enhanced Green Fluorescent Protein
AAV2 ITR Regions	Sequence between these segments are packaged into viral progeny
bGH poly(A) Signal	Region used to design probes against for qPCR quantification

3.2.3 Downstream Concentration of Crude AAV Harvest

If samples are frozen, the media and cell pellets were incubated at 37 °C until thawed. Cell were lysed by performing 3 rounds of freeze-thawing, either using a dry-ice ethanol bath or by incubating at -80 °C, followed by incubation at 37 °C. Cell lysate was clarified by centrifugation at 2500 g for 20 min. Supernatant was collected and moved to a new tube. The clarification step was repeated, and the supernatant was moved to a fresh 50 ml falcon tube. Lysate was incubated in 100U/ml Benzonase (ThermoFisher Scientific), or 5000 U DNase (ThermoFisher Scientific) for 1 hour at 37 °C. The final concentration of NaCl was brought up to 1M by adding 4.75ml of 5M NaCl. 1.5 ml of 10% Octyl β -D-glucopyranoside (Sigma) was added, to a final concentration of 0.5%. Lysis Buffer (50 ml 1 M tris (pH 8.0), 2 ml 1 M MgCl₂, 40 ml 5 M NaCl, 908 ml water) was used to fill the tube to 30 ml. Cell lysates and media was concentrated using the following methods:

1. Small preps: for concentrating smaller volumes, 40% PEG8000 (Sigma) and 2.5N NaCl was added to the combined cell lysate and cell culture supernatant to a final concentration of 8%. The solution was placed on ice for 2H, centrifuged at 2500 g for 30 min, and the supernatant was discarded as the pellet contains the virus. Preparations were resuspended in 10 ml of resuspension buffer, then either quantified for optimization procedures, or further purified for use *in vitro*.
2. Large preps: tangential flow filtration with a Centramate Cassette and Omega 100k MW Filter membrane (PALL) was used to concentrate large volumes of media and cell lysate. The system was cycled at 30 psig for at least one hour to concentrate the product to volumes below 30 μ m to allow further purification.

3.2.4 CsCl Ultracentrifugation Gradient Purification of AAVs

The CsCl gradient was prepared by first preparing 15 ml of 1.3 g/ml CsCl in Sw32Ti Polyallomer Centrifuge tubes (Beckman Coulter). 7.5 ml of 1.5g/ml CsCl was then underlaid gently using a serological pipette. 15 ml of AAV sample (media or cell lysate, or combination of both) was then gently pipette on top of the gradient. The tubes were spun at 29101 RPM (182000g) at 20 °C for 20 hours minimum in a Beckman Coulter Optima L-90K Ultracentrifuge.

The tube is then placed in a rack holder and fractions were collected by placing an 18G needle (BD Biosciences) approximately 1 cm up from the bottom of the tube. Fractions were collected using a 96-well plate, and then measured using a Leica Mark II Plus refractometer (Leica Microsystems). Bands between 1.374 and 1.366 were combined into one tube, and then brought up to 13 ml with 1.41 g/cm³ CsCl. The solution was then added to a Beckman Thin-walled Polypropylene Tube (Beckman Coulter), the top was sealed using a Cordless Tube Topper (Beckman Coulter) and centrifuged at 182000g (38392 RPM) for 20 hours at 20°C in a SW41 rotor (Beckman Coulter).

Two protein bands can be visualized following ultracentrifugation. The bottom protein band contains the packaged AAVs, while the top contained improperly assembled AAVs (viral particles without a full genome). The bottom band was collected by inserting an 18G needle slightly under the band and collecting flow through. 50 ml Final Formulation buffer (PBS +35 mM NaCl) was prepared, and 15 ml added to the flow through. The solution was centrifuged between 2000g and 4000g for 2 minutes using an Amicon 100K filter in a sorvall rotor. The time and speed were increased if the solution was flowing through slowly, to ensure that there was at least 1.5 ml of solution remaining in the tube following centrifugation. 15 ml of the Final Formulation Buffer was added at a time, and the sides of the Amicon filters rinsed with the

added solution to prevent buildup of virus on the membrane. This was continued until all 50 ml of Final Formulation buffer was added, and approximately 1.5ml of solution remains in the Amicon filter. Using a 200 µl pipette the sides of the filters were rinsed by pipetting up and down along the filters, taking care not to scrape them as this will puncture the filter. Glycerol was added to the collected viruses at a final concentration of 5% and viruses were aliquot and frozen at -80 °C.

3.2.5 Iodixanol Ultracentrifugation Gradient Purification of AAVs

The following iodixanol gradient concentrations were prepared by diluting iodixanol (Sigma) in Iodixanol Buffer (PBS containing 10 mM MgCl and 25 mM K) to achieve the final concentrations of iodixanol: 15%, 25%, 40%, 54%. Note that iodixanol is commonly supplied at a 60% stock concentration. In A SW32Ti tube (Beckman Coulter) successively underlay the following iodixanol gradients: 4 ml of 15%, 9 ml of 25%, 9 ml of 40%, 5 ml of 54%. 10 ml of AAV sample was overlaid on top of the gradients. Tubes were centrifuged at 350000g in a 70Ti rotor (Beckman Coulter) at 18 °C for 70 minutes. Fractions were collected using an 18 gauge needle (BD Biosciences) inserted approximately 1 cm from the bottom of the tube. 250 µl fractions were collected in a 96-well plate (ThermoFisher Scientific). Fractions were diluted 20x with water into a UV-transparent 96-well plate (ThermoFisher Scientific), and absorbance was measured at 340 nm using a plate reader. Absorbance values were plotted to find a spike at 340 nm, indicating the contaminating protein band. All fractions before this band were combined. 10 volumes of Final Formulation Buffer (PBS + 35 mM NaCl) was prepared and used to dilute the virus in an Amicon 100,000 MW filter (Millipore). The fractions were first put into the Amicon filter then Final Formulation Buffer added to fill the tube. The tube was centrifuged in a sorvall rotor for 2 minutes at 2000-4000 RPM. The speed and time was adjusted depending on the rate

of the solution flowing through the membrane, making sure there was at least 1.5 ml of solution remaining in the tube following centrifugation. This was continued until all Final Formulation Buffer was used, and ~1.5 ml of sample remained in the Amicon filter. The viruses were collected by rinsing the sides of the filter using a 200 µl pipette tip, making sure not to scrape the filter. Glycerol was added to the virus to a final concentration of 5% and samples were aliquot and froze at -80 °C.

3.3 Quantifying Viral Titres

Quantitative Real-time PCR (qPCR) was performed using a Viia7 machine (ThermoFisher Scientific) on viral samples to determine viral titres. Probe-based qPCR was performed to quantify viruses containing the pAAV2.1 CamKII-GFP vector, and SYBR Green qPCR was used to quantify viruses containing the pAV-U6-RFP vector.

Prior to either quantification method, 5 µl of virus was digested with Proteinase K (ThermoFisher) at a final concentration of 0.1 units of Proteinase K. The sample was gently mixed. The reaction was set up as follows: 5 µl virus, 1 µl Proteinase K, 44 µl ddH₂O. The tube was incubated at 37 °C for 30 minutes, followed by 95 °C for 10 minutes. A 10-fold serial dilution of the virus up to 1E6 dilution was made, using 45 µl Nuclease-free water and 5 µl of virus per dilution.

A standard curve of known plasmid concentrations, ranging from 5E6 copies/µl to 1 copy/µl was produced as follows. Plasmid samples containing either the bGH poly(A) signal or the ITR sequence were linearized with restriction enzymes, purified with the QIAquick PCR Purification Kit (Qiagen), with elution of the product in a minimal volume to ensure high concentration of the plasmid.

3.3.1 Probe-Based qPCR Determination of Viral Titres

The master mix for each well was prepared as follows: 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems), 0.27 µl 100 µM Forward Primer, 0.27 µl 100 µM Reverse Primer, 2.5 µl 2 µM Probe. 10 µl of DNA was loaded into each well. A no template control was included, containing only dH₂O and Master Mix. 15.5 µl of master mix was added to each well. Plates were covered with transparent film, centrifuged at 3000 rpm for 2 minutes. qPCR program

was set as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C.

Table 7. Primers and Probes used in Probe-based qPCR viral titration

Primer/ Probe	Sequences (5' – 3')	Concentration
Primer BGH FW	TCTAGTTGCCAGCCATCTGTTGT	100 µM
Primer BGH Rev	TGGGAGTGGCACCTTCCA	100 µM
Probe	6FAM – TCCCCCGTGCCTTCCTTGACC – TAMRA	2 µM

Primers and probes used in probe-based qPCR to determine titre of AAV samples.

3.3.2 SYBR Green qPCR Determination of Viral Titres

The master mix for each well was prepared as follows: 10 µl 2x Power SYBR Green Mix (ThermoFisher Scientific), 0.1 µl 100 µM Forward Primer, 0.1 µl 100 µM Reverse primer, 4.8 µl Nuclease free water. Plates were loaded as follows: 5 µl of sample was loaded into each well. A no template control was included, containing only dH₂O and Master Mix. 15 µl of master mix was added to each well. Plates were covered with transparent film, centrifuged at 3000 rpm for 2 minutes. qPCR program was set as follows: 1; Uracil-N-Glycolase (UNG) treatment at 50 °C for 2 min. 2; Initial denaturation step at 95 °C for 10 min. 3; 40 cycles of denaturation at 95 °C for 15 seconds followed by annealing/extension at 60 °C for 1 min. 4; Melt curve.

Table 8. Primers used in SYBR-Green qPCR viral titration

Primer	Sequence (5' – 3')	Concentration
ITR Forward	GGAACCCCTAGTGATGGAGTT	100 µM
ITR Reverse	CGGCCTCAGTGAGCGA	100 µM

Primers used in SYBR Green-based qPCR to determine titre of AAV samples.

3.4 Configuration of the Tangential Flow Filtration System

Tangential flow filtration system was assembled using the Minimate Tangential Flow Filtration System pump, reservoir, and reservoir stir plate (PALL). The Centramate LV cassette holder (PALL) contained the 100k MW T-Series Omega membrane (PALL). Hosing (Pharmed) was cut as short as possible to reduce the volume of liquid remaining in the system following concentration. Prior to the concentration of viruses, crude viral cell lysates and media were filtered through a 0.45-0.8 μ M vacuum-filter (PALL) to reduce cellular debris that may clog the membrane of the TFF.

Concentration was performed as per the manufacturers guidelines outlined in the User guide code USTR 2433b. Briefly, the system was first pre-rinsed with PBS containing 0.1% Pluronic F-68 for at least 15 minutes. The system was then rinsed for 5 minutes with PBS. Crude viral product was added to the feed tank, either all at once, or by adding portions every so often, depending on the total volume. An equal volume of high-salt PBS was added to the sample feed tank to reduce clogging. The system was run at 30 psig (as per the gauges) until the sample was near the minimum working volume. Then, the lines were redirected so the product could be collected into a falcon tube. To collect volumes of sample to measure total viruses remaining trapped within the system, the TFF system was carefully disassembled, and each hose was drained into a collective beaker. Following collection of the viral product, the system was rinsed with a 0.1 N NaOH for 30 minutes. The system was then ready for a rinse with Pluronic-F68, or rinsed with water prior to disassembly and storage.

3.5 Primary Hippocampal Neuron Culture

The method of culturing primary neurons from mice has been adapted following Kaech and Banker's paper outlining the protocol for establishing primary neuron cultures from rats²⁰⁰. During the dissection procedure it is important to work fast. The entire procedure from sample collection to plating should take under 2 hours.

3.5.1 Dissection of the Hippocampi

Embryonic mouse heads were collected in a tube containing 1X Dissecting Media (10X recipe: 10.15g of 100 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$, 0.946 g of 10 mM Kynurenic acid (Sigma), 11.92 g of 100 mM HEPES in 500 ml HBSS, diluted in (1X) Hank's Balanced Salt Solution (-Calcium Chloride)(-Magnesium Chloride)(-Magnesium Sulfate) (Life Technologies), pH 7.2), and were kept on ice until dissections began. Dissections were performed in 60 mm x 15 mm Polystyrene Petri Dishes (Fisher) on top of a cold block, using Fine Science tools Scissors and Forceps (DUMONT). Mouse heads were held still by inserting forceps at the eye sockets. The skin was peeled back by pinching the skin between the eyes and pulling back. The skull was cut along the superior sagittal sinus and along the symmetrical transverse sinuses to divide the skull into 4 pieces, which were then removed using forceps to expose the brain. Forceps are carefully inserted in under the brain and closed to detach the olfactory lobes. The brain is then removed from the skull by lifting up, and all brains were collected in a fresh dish containing 1X Dissection Media (chilled). Using forceps the left and right cerebral hemispheres were separated from the mid brain, and forceps were used to removed the meninges. Using fine scissors the cuts were made following the corpus callosum, which is directly above the hippocampus. The hippocampus is then removed, and excess tissue is removed using forceps and scissors.

Hippocampi were then collected in a 15 ml Falcon Tube (ThermoFisher Scientific) and kept on ice until all hippocampi have been dissected out.

3.5.2 Tissue Dissociation and Plating

All enzymes and dissociation media were prepared fresh right before use. Papain Dissociation Solution was prepared by adding 100 μ L of papain suspension (Cedarlane) and 25 μ L of 10 mg/ml Deoxyribonuclease I from bovine pancreas (Sigma) to 5 ml of 1X Dissection Media, then filter sterilizing using a 0.2 μ m filter (VWR). Excess 1x Dissection Media was removed from the hippocampi using a serological pipette or aspirator, and the Papain Dissociation Solution was added. Hippocampi were incubated at 37 °C for 4 minutes, with gentle tapping performed at 2 minutes to gently mix the hippocampi. The Papain Dissociation Solution was removed, then 5 ml of Trypsin Inhibitor (0.15g Trypsin Inhibitor from Chicken Egg White (Sigma) in 15 ml 1X DM, pH 8.5, and 75 μ L 10 mg/ml DNase I (Sigma), filter sterilized (0.2 μ m filter) and incubated for 3 minutes. This was repeated 2 more times, for a total of 15 ml Trypsin Inhibitor used. Trypsin Inhibitor was removed and 5 ml of NBActiv4 media (Brain Bits) without antibiotics was added. NBActiv4 media was then gently removed to not disrupt the cells, and 1 ml of NBActiv4 media was gently added. The cells were then triturated using a P1000 pipette set to 700 μ l to avoid creating bubbles, until a homogenous cell suspension was achieved. Cells were counted with a Bright-Line Hemocytometer (Hausser Scientific) using 10 μ l of cell suspension and 10 μ l of trypan blue (ThermoFisher Scientific). The top two rows and bottom two rows were counted on the hemocytometer, skipping the middle row. In total, 20 squares measuring 0.02cm x 0.02cm x 0.01cm were counted. The following equation was used to calculate the number of cells per ml:

$$\text{Cells/ml} = \text{cell count}/20 \times 2 \text{ (dilution factor)}(1\text{mL}/(0.02\text{mL}\times0.02\text{mL}\times0.01\text{mL}))$$

Cells were then diluted in NBActiv4 (BrainBits) and plated at 50,000 or 75,000 cells/well on a 24-well plate coated with Poly-D-Lysine (BD Biosciences). 300 μ L of NBActiv4 was added to the wells four days post-plating. Every 3 days following initial feeding, 300 μ L of media was removed and replaced with an equal amount of fresh NBActiv4 media. The media does not contain animal serum, as the removal of FBS from primary neuron cell cultures reduces or eliminates glia cells from the cultures ²⁰¹.

3.5.3 Transduction of Primary Neuron Cell Cultures

All virus preparations were diluted into PBS (Thermo) so that 20 μ l of sample delivered the total required number of viruses per well of a 24-well, or 100 μ l of sample for a 6-well plate. Prior to treating with viruses, half of the culture media was moved into a new plate and incubated at 37 °C. Viruses were delivered to each well dropwise, followed by a gentle rocking to ensure viruses are spread around the wells. Four hours following transduction, the entire volume of media is removed from each well and quickly replaced with the media that was removed prior to the transduction. NBActiv4 (BrainBits) is then used to fill each well up to its normal volume, and cultures are maintained following their regular schedule until samples are to be collected.

3.6 Fixing and Staining Primary Neuron Cell Cultures

3.6.1 Preparation of Coverslips and Plates

Coverslips (High Precision Microscope Cover Glasses – Deckgläser 12 mm or 13 mm Marienfeld-Superior (Zeiss)) were first washed with 1 M HCl to remove any contaminants. Coverslips were then incubated in 1M HCl heated to 50-60 °C overnight with gentle stir-bar agitation. Coverslips were then washed twice with distilled water, then twice with double-distilled water, with stirring of the coverslips to make sure to wash in between the stuck together coverslips. Coverslips were then rinsed with 100% ethanol, then dried between P8 Filter Papers (Fisher Scientific). Once dried, the coverslips were autoclaved, then kept sealed until use. Coverslips were carefully put into 24-well plates (ThermoFisher Scientific) using forceps. 1 mg/ml Poly-D-Lysine (BD Biosciences) dissolved in Borate Buffer (4.76 g Boric acid (EM Science), 2.54 g sodium borate (Sigma), 1 L ddH₂O, pH 8.4) at 0.02 mg/ml was then added to wells at 500 µL per well in 24-well plate, to give a final quantity of 10 µg of PDL/well. Plates were incubated at 37 °C overnight. Prior to the dissections, PDL was removed and wells were washed with 500 µL sterile milliQ water twice, then 300 µL NBActiv4 media was added per well.

At each desired timepoint, cells were fixed in 4% paraformaldehyde-PBS solution (Sigma-Aldrich) at room temperature for 10 minutes. After incubation, the paraformaldehyde solution was removed, and the slides were washed twice with PBS. The slides were then stored in PBS at 4 °C until all slides were collected, to allow bulk processing.

A range of antibodies were used depending on cells being stained. Astrocytes were immunostained with an antibody to detect glial fibrillary acidic protein (GFAP) (1:500 dilution) (Dako; Z0334) with a 1:200 dilution of a goat anti-rabbit secondary antibody conjugated to

Alexa Fluor 488 (Invitrogen; A-11034). Neurons were detected with antibody to microtubule-associated protein 2 (MAP2) (1:1000) (Abcam; ab5392) with a 1:200 dilution of a goat anti-chicken secondary antibody conjugated to Alexa Fluor 555 (Abcam; ab150174), or goat-anti-chicken antibody conjugated to Alexa Fluor 647 (Invitrogen; A32933. All antibodies were diluted in 1X PBS pH 7.4 (Life Technologies).

At the time of staining, the PBS was removed from the coverslips. Cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich) for ten minutes, followed by incubation with primary antibodies for 1 hour at room temperature. Antibody staining was performed by pipetting 20 μ l of the antibody solution onto a piece of parafilm (FisherScientific), then inverting the coverslip onto the droplet using forceps. Following antibody incubation coverslips were returned to the 24-well plate. Coverslips were then washed once with 0.1% Triton X-100 and then twice with PBS. Coverslips were incubated with secondary antibody for 1 hour at room temperature, while covered to protect them from light. The washes were repeated, once with 0.1% Triton X-100, twice with PBS, then the coverslips were mounted on slides using ProLong Gold Mounting Media containing DAPI (4',6-diamidino-2-phenylindole) (ThermoFisher Scientific), or with ProLong Diamond Mounting Media without DAPI (ThermoFisher Scientific). Prior to mounting with mounting media lacking DAPI, slides were incubated in 300 mM DAPI in PBS (ThermoFisher Scientific) for 5 minutes, then washed 3 times with PBS. Coverslips were flattened using forceps to ensure the removal of air bubbles.

3.7 Imaging of Cell Cultures

Immunostained slides were visualized on the MIRAX MIDI (Zeiss) scanner, or an LSM 700 confocal microscope (Zeiss). Panoramic Viewer (3DHISTECH) and Zen Blue (ZEISS) were used to analyze the images collected from scanning. The following procedure was used to optimize image collection, to allow consistent comparisons between cell cultures in different conditions.

3.7.1 Optimization of Imaging Method for Differentiation of Transduced Cells

Using a positive control slide images were taken using standard confocal settings (all lasers were first adjusted to 2% of total voltage, and optical gain was set to 650 (~median of the dynamic range), to collect a baseline image. Next, the laser intensities and Gain (Master) were changed to so that all stained markers could be visualized, and none of the signals were saturated. Next, the excitation and emission ranges of each fluorescent signal were reduced until there was no cross-talk between fluorophores of different wavelengths. Images were collected using one laser turned on at a time to ensure no signal cross-talk. Finally, negative control slides (slides stained with all but one fluorophore) were measured using the same settings used on the positive control. If each signal on its corresponding negative control could not be detected with the current settings, then images were collected to be counted and analyzed.

Images were collected using 4 tracks, each laser set to a separate track as this will reduce photobleaching and cross-talk between channels. Images were collected at 5x magnification, and 20x magnification using the Plan-Apochromat 20x/0.8 M27 objective. For 20x imaging a frame size of 2048x2048 was captured, using the Tile Scan setting to image 2x3 tiles (6 tiles total), with a pixel dwell of 0.64 μ sec. Images were averaged 4x to reduce background signal, and

images captured as a 12-bit image. Two sections from each Tile Scan image were selected, consisting of 200-300 cells on average to be counted. Finally, analysis of the images was performed using the ImageJ plugin for Zen 2.3. Images were changed to 8-bit, as ImageJ has difficulty working on images at 12-bit. Brightness and contrast were adjusted similarly between all images to make counting easier. The ImageJ Cell Counter plugin was used to count the images.

3.8 RNA Analysis

3.8.1 RNA Collection From Primary Neuron Cell Cultures

The Total RNA Purification Micro Kit (Norgen Biotek Corp.) was used to isolate the total RNA from primary hippocampal neuron cells growing in culture. For RNA studies, 350,000 cells were plated into a 6-well plate (ThermoFisher Scientific), subjected to experimental conditions, then RNA was collected at the specific timepoints, and stored at -80 °C until needed. Specifically, the protocol titled “Cell Lysate Preparation from Cells Growing in a Monolayer” was followed as per manufacturer guidelines. Media was removed from the cell culture, and 350 µL of Buffer RL was added to each well. Cells lysates were collected by scraping with a cell scraper while keeping the plate angled, and removed using a pipette. Lysates were collected in 1.5 ml Eppendorf tubes (FisherScientific). 200 µL of anhydrous ethyl alcohol (Commercial Alcohols) was added and the lysates were vortexed then transferred into a micro spin-column from the Norgen kit. The column was centrifuged for 1 minute at 3500 x g (Eppendorf Centrifuge 5417C) and flow-through discarded. 400 µL of Wash Solution A (containing anhydrous ethyl alcohol) was then put into the column, and centrifuged for 1 minute at 14,000 x g. This wash step was then repeated two more times, disposing the flowthrough after each step. After the final wash the empty column was centrifuged at 14,000 x g to dry the resin in the column. The column was placed into a new RNase-free tube and 20 µL of Elution Solution A was carefully pipetted into the centre of the column, making sure that the elution buffer was not administered onto the side of the tube. The RNA was eluted via centrifugation at 200 x g for 2 minutes, followed by 14,000 x g for 1 minute. Aliquots of the RNA samples were used to quantify the RNA concentration using a BioAnalyzer (Agilent Technologies). Samples were 10-fold serially diluted to 1000x dilution and loaded onto a Eukaryote Total RNA NanoChip (Agilent Technologies) in duplicate,

to be analyzed with the BioAnalyzer. RIN numbers were all determined to be between 7 and 10. Repeat BioAnalyzer tests on RNA samples collected at different timepoints yielded similar results, therefore it was safe to conclude that this method of isolating RNA was successful in isolating high quality RNA.

3.8.2 Reverse Transcription and cDNA Cleanup

Reverse transcription was performed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Reactions were run in duplicate in a 96-well plate. Reactions were setup as follows: 2 μ l 10X RT Buffer, 0.8 μ l 25X dNTP Mix (100mM), 2 μ l 10X RT Random Primers, 1 μ l MultiScribe Reverse Transcriptase, 4.2 μ l Nuclease-free H₂O, and 10 μ l of RNA sample diluted to equal 50ng RNA. After loading the reaction wells, plates were vortexed, then centrifuged in a Sorvall Legend RT Centrifuge (Thermo Electron Corporation) before being placed into the Research PTC-200 Peltier Thermal Cycler (Marshall Scientific). The following temperatures were used in the reverse transcription reactions: 30 minutes at 16 °C, 30 minutes at 42 °C, 5 minutes at 85 °C, followed by a hold at 4 °C.

CDNA was then cleaned up with the Invitrogen ChargeSwitch PCR Clean-Up Kit (Life Technologies). The ChargeSwitch PCR Clean-up Kit uses a magnetic bead-based technology that is charge dependent on the surrounding pH. Lowering the pH in the sample to positively charge the bead causes the bead to bind cDNA, and raising the pH releases the bound cDNA. Briefly, because the reverse transcription reaction is assumed to be a 1:1 reaction, the entire contents of the reverse transcription reactions were used (equivalent to 50 ng of input RNA in each well). Replicate wells were combined, and 40 μ L of the reverse transcription reactions were pipette into a 1.5 ml microcentrifuge tube. 10 μ L of ChargeSwitch magnetic beads were added

and pipetted gently to mix the samples. Tubes were then incubated for 1 minute at room temperature. The tubes were then incubated while on the MagnaRack for 1 more minute before the supernatant was removed and discarded. The tubes were then removed from the MagnaRack and washed in 150 μ L Wash Buffer (W12), with gentle pipetting to mix. The tubes were then incubated for 1 minute on the MagnaRack before the wash buffer was removed and discarded. The washing step was then repeated once more. The tubes containing the magnetic beads were removed from the MagnaRack and 12 μ L of elution buffer was added, then pipetted to mix. Tubes were incubated at room temperature for 1 minute, then in the MagnaRack for 1 minute. Finally, the buffer containing the purified cDNA samples was collected. The cDNA was stored at 4 °C if it was to be used within a day or stored at -20 °C for long term.

3.8.3 Quantitative Real-time PCR

Quantitative real-time PCR was performed in multiplex on a Viia7 machine (Applied Biosystems). No template controls (NTCs) were used for each probe. Endogenous control assays were used for comparison, using *GAPDH* gene expression as the housekeeping gene. 50 ng of RNA equivalent was used per 20 μ L reaction. The following amounts were used in each 20 μ L reaction: 6 μ L cDNA template (50 ng RNA equivalent), 10 μ L of TaqMan Universal PCR Master Mix II (2X) no UNG, 2 μ L of Primers (10x), 1 μ L Probes against *PRNP*, 1 μ L Probes against *GAPDH*.

Each reaction was performed in duplicate. Following setup of the reaction wells, the 96-well plate was sealed with MicroAmp Optical Adhesive Film (Applied Biosystems), vortexed, then centrifuged at 1000 rpm for 2 minutes at 4 °C in a Sorvall Legend RT Centrifuge (Thermo Electron Corporation), then loaded into the Viia7 machine. The thermal cycling conditions were as follows: 10 minutes at 95 °C for enzyme activation, followed by 40 cycles of 15 seconds at 95

°C for denaturation and 60 seconds at 60 °C for annealing and extension. The resulting data was analyzed using the $\Delta\Delta C_T$ method as outlined ²⁰², using Microsoft Excel. The $\Delta\Delta C_T$ method first compares the difference in gene expression of the gene of interest, *PRNP*, to a housekeeping gene, *GAPDH*, with stable gene expression. This value is the ΔC_T value. This comparison is performed using the rtPCR gene expression data collected from primary neuron cell cultures treated with the AAVrh.10 containing either an anti-PrP^C shRNA sequence or the Empty control vectors, and PBS-treated cultures, using the following formula:

$$\Delta C_T = C_T[\text{PRNP}] - C_T[\text{GAPDH}]$$

Next, to determine the $\Delta\Delta C_T$, the ΔC_T values of the anti-PrP^C shRNA treated cell cultures and the Empty control vector control vectors compared to determine the $\Delta\Delta C_T$ value, shown in the following formula:

$$\Delta\Delta C_T = \Delta C_T[\text{shRNA}] / \Delta C_T[\text{Empty}]$$

3.9 Protein Analysis

3.9.1 Protein Extraction

Extraction of proteins from HEK293T cell cultures and whole brain homogenate were performed using M-PER Mammalian Protein Extraction Reagent (ThermoScientific) or N-PER Neuronal Protein Extraction Reagent (ThermoScientific), respectively.

For HEK293T cells, media was removed from cultures of adherent cells. To each well of a 6-well plate, 200 μ l of M-PER reagent. Cell lysates were collected in a falcon tube with a cell scraper. Cell lysates were centrifuged at 14,000 x g for 10 minutes to pellet the cell debris, and the supernatant was transferred to a new tube. Aliquots were quickly made, and the protein samples were stored at -80 °C until used in experiments.

For whole brain homogenate protein collection, whole brains were transferred into a 15 ml falcon tube, then tissues were covered with 1 ml of N-PER reagent. Tissue homogenization was performed with an Omni Tissue Homogenizer (Omni International), using short intervals of grinding until sample seemed well-mixed. Tubes were then centrifuged at 14,000 x g to pellet the cell debris, and the supernatant containing the protein was collected, aliquot, and frozen at -80 °C until used.

3.9.2 Quantification of Proteins using BSA Method

Quantification of proteins prior to Western blotting was performed with use of the Pierce BCA Assay (ThermoScientific). Briefly, a standard curve of protein concentrations ranging from 25 – 2000 μ g/ml BSA concentration were added into a 96 well plate as per manufacturer guidelines. The same protein extraction reagent was used to dilute the BSA standard samples. The protein

samples were diluted serially until a 1:100 dilution. 25 µl of each protein sample or standard sample were loaded into a 96 well plate in duplicate. 200 µl of the working reagent was added to each well, then the plates were covered were mixed on a vortexed for 30 seconds. The plate was then centrifuged for 2 minutes in a Sorvall Legend RT Centrifuge (Thermo Electron Corporation), then incubated at 37 °C for 30 minutes. The plates were then cooled to room temperature, then absorbance was measured at a wavelength of 562 on a Promega GloMax Microplate Reader (Promega). The unknown sample values were plotted against the standard curve to calculate the protein concentrations of the sample.

3.9.3 Western Blotting

For western blotting, 50 µg of each protein sample was first boiled in Laemmli buffer, with the reaction set up as follows: 50 µg protein sample, 5 µl 4X Laemmli sample buffer (BioRad), water to 20 µl. Samples were gently pipette to mix and spun at 3,000 x g in a tabletop centrifuge at room temperature for 1 minute. Samples were then incubated for 5 minutes at 95 in a thermocycler, then cooled to room temperature before being spun again at 3,000 x g to bring the contents of the tube to the bottom.

Gel electrophoresis was performed in mini TGX precast gels (BioRad) in 1x TGS running buffer (BioRad). 5 µl of precision Plus Protein Dual Color Standard (BioRad) was added per gel, and each 20 µl reaction was loaded into the wells. 20 µl of Laemmli buffer in water was added to any empty wells. The gels were run at 300V for about 20 minutes. Gels were then transferred onto a nitrocellulose membrane using an iBlot Transfer system (ThermoFisher), as per manufacturers protocol, on setting 3: 20V for 7 minutes.

Membranes were first blocked in 5% milk powder in TBST for 1 hour at room temperature while shaking. The primary antibodies, either Anti-RFP (ThermoFisher R10367) or anti-mCherry (Abcam ab167453), and Anti- β -Actin (Abcam) was diluted to 1:1000 and incubated at 4 °C overnight while shaking. The membranes were then washed 3 times for 5 minutes each with TBST. Secondary antibodies, Goat anti-Rabbit-HRP were diluted (1:20,000) in 5% milk powder/TBST and incubated on the membrane for 1 hour at room temperature while shaking. Membranes were then washed 4 times with TBST, then 2 times with TBS for 5 minutes each, with shaking at room temperature. Enhanced chemiluminescence was used to visualize the protein bands using the Pierce ECL Western Blotting Reagents (ThermoFisher Scientific) by mixing equal parts of the two supplies reagents, then incubating on the membrane for 5 minutes at room temperature. Imaging of the membrane was performed using a ChemiDoc Imaging System (BioRad).

3.10 Cytotoxicity Analysis

The CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) was used to determine the cytotoxic effects of the various treatments to primary neuron cell cultures. Primary neuron cultures were grown in either 50,000 cells or 70,000 cells per well of a 24 well plate (ThermoFisher Scientific). Following treatment of Calcium Phosphate, AAVs, or Lipofectamine 2000, cultures were maintained for 3 days. Control wells included cells with no treatment, wells with no cells (for media background measurement), and cells treated with vehicle. After 3 days incubation at 37 °C media was collected, leaving 100 µl of media remaining in each well. Media was collected in a fresh 24 well plate and froze at -80 °C. Neuron cultures were lysed by freezing at -80 °C for approximately 20 minutes, then incubating at 37 °C for approximately 15 minutes. 50 µl of media and cell lysate from each well of tests and controls were transferred to a fresh 96-well flat clear bottom plate (ThermoFisher Scientific) in duplicate. A positive control cytotoxicity test (2 µl LDH positive control in 10 ml PBS +1% BSA) was included in duplicate on each 96 well plate. 50 µl of the CytoTox 96 reagent was added to each well, and the plate was vortexed for 10 seconds, then centrifuged for 2 minutes at 200g in a Sorvall Legend RT centrifuge (ThermoFisher Scientific). The plate was covered to protect it from light and incubated at room temperature for 30 minutes. After incubation, 50 µl of Stop Solution was added to each well. A needle (BD Biosciences) was used to pop any large bubbles in the wells. Absorbance at 490nm of each plate was measured using a Glomax Multi Detection System (Promega). The average values of the culture medium background (measured in a well containing no cells) was subtracted from all values of experimental wells. The percentage of cell death was calculated using the following equation:

$$\text{Percent Cytotoxicity} = 100 \times (\text{Medium OD490})/(\text{Cell OD490})$$

3.11 Administration of AAVs to Neonatal Mice

50 µl of adeno-associated virus (6E10 viruses per mouse; Vigene Bioscience) was delivered through the facial temporal vein of a day 1-2 old mouse. Dr. Jagdeep Walia performed all mouse injections. This procedure was performed by laying the mouse over a light facing upwards, illuminating the head to make the vein more visible. Using a 31G needle (BD Biosciences), 50 µl of PBS (ThermoFisher Scientific), or PBS containing the viruses was slowly delivered into the temporal facial vein of the mouse. During the injection the vein can be seen blanching, indicating that the solution is being properly injected into the vein of the mouse. Following injection, mice were warmed by holding them in a hand, then monitored for a few minutes to ensure recovery. Recovery of the mice occurred under one minute, on average. Following recovery, the neonatal mice were gently returned into the mother's cage. At 7 weeks of age, mice were administered RML infection or a mock-infection by administering 5 µl of 10% brain homogenate from either an RML-diseased mouse, or a healthy brain control. Infection was performed with use of a Tri-Dek syringe system to intracranially inject the brain homogenate into the brain of our mice. The day of RML-infection is counted as Day 0 in our experiment. At Days 0, 60, 90, and at end-point of the disease (a scoring of 5 on the Animal Scoring Chart; Appendix 1) mouse tissue and blood samples were collected. Mice were first anesthetized with use of isoflurane and sacrificed via spinal dislocation following the CACC guidelines. Brain, lung, spleen, and liver samples were collected. Tissues were either frozen using a dry ice-methanol bath by covering the tissue with Optimal Cutting Temperature Compound (ThermoFisher Scientific), or tissues were stored in formalin for at least 24 hours, prior to being prepared for immunohistochemistry. Blood samples were collected via cardiac puncture.

3.12 Imaging of Mouse Brain Slices

Imaging of mouse brain slices was performed on an Axio Scan Z1 scanning microscope (ZEISS) using brightfield imaging.

3.12.1 Brain and Organ Slice Preparations

Brains were harvested from mice and divided into the front/mid/hindbrain regions using a scalpel. Brains were fixed in 10% neutral buffered formalin prior to being paraffin-embedded. Organs were harvested and also fixed in 10% neutral buffered formalin prior to being paraffin-embedded. Sections (5 μ m) of brains and organs were prepared by slicing on a microtome (ThermoFisher Scientific). Slices were floated on top of a layer of cooled water before being lifted with a glass slide. Slides were baked at 37 °C overnight.

3.12.2 Immunohistochemistry of Brain and Organ Slices

Brain and organ slices were stained using the Polink-2 HRP Plus Rabbit DAB Detection System Immunohistochemistry kit (GBI Labs). A graded Xylene/Ethanol wash is performed by incubating the slides 3x in Xylene (Sigma) for 5 minutes each, followed by 3x of 100% ethanol for 3 minutes each, 2x of 95% ethanol for 3 minutes each, then 70% ethanol for 3 minutes. Slides are then washed using TBST 3x for 2 minutes each. Slides are then incubated in the peroxidase blocking reagent (3% H₂O₂ solution) for 10 minutes. The slides are then rinsed with distilled water. Antigen retrieval is performed by incubating the slides for 20 minutes at 121 °C, then the slides are washed 3x in TBST for 2 minutes at a time. Using a Pap pen, the tissue sections on the slides are circled. Primary antibodies (Anti-mCherry; Abcam ab167453) showed best results when diluted 1:800 in antibody dilution solution on brain tissues, and 1:800 to 1:1000 were ideal for organ slices. Tissues were covered with 2 drops (~100 μ l) of primary antibody solution used

to cover the tissue section, and slides are left overnight in a humidity chamber at 4 °C. Slides are washed 3x with TBST for 2 minutes at a time. 2 drops (~100 µl) of Rabbit Antibody Enhancer are then added to each slide and left to incubate for 30 minutes in a moist chamber at room temperature. Slides are then washed 3x with TBST for 2 minutes at a time. 2 drops (~100 µl) of Polymer-HRP secondary antibody solution is added to each section then incubated for 30 minutes in a moist chamber at room temperature. Slides are washed 3x in TBST for 2 minutes at a time. 1 drop of Reagent B was added to each 1 ml of Reagent A to form the DAB mixture. 2 drops of this (~100 µl) mixture were added to each slide and left at room temperature for approximately 1-1.5 minutes. Color development was monitored under the microscope to ensure proper levels of staining are achieved. Slides are then rinsed with distilled water for 2 minutes. Slides are then counter stained in Hematoxylin for 1.5 minutes, then washed under tap water for 2 minutes. Slides were then rinsed in Acid Alcohol to decolorize the slides, then rinsed in tap water for 2 minutes. A graded ethanol wash (70%, 2x 95%, 3x 100%) was performed by moving the slides from the more dilute wash towards the 100%, 3 minutes per ethanol wash. From here, slides were washed 3x in Xylene for 5 minutes at a time. Finally, coverslips were mounted using Permount and coverslips were pressed using forceps to ensure removal of air bubbles from the tissues. Mounted slides were visualized on an Axio Scan (Zeiss) scanning microscope using brightfield imaging.

Chapter 4. Results

4.1 AAV Production and Purification Rationale

Interest in the use of AAVs as a gene-therapy delivery platform is steadily increasing. A search of PubMed revealed 4848 publications containing the key word “AAV” in the last 10 years, compared to 2757 published prior to 2009. One challenge that has been identified is to produce sufficient virus particles for *in vivo* studies and therapeutic trials. A review of the current literature revealed a lack of consensus regarding the most efficient methodologies for laboratory scale AAV production. Factors such as media composition, length of incubation for optimal viral propagation, and the most efficient ways to harvest virus from media or lysed cells are rarely considered.

Although many published protocols describe methods for the preparation of all AAV serotypes^{203,204}, many are tailored to the production of a single serotype¹⁸³. It has been reported that in some instances the use of well-optimized protocols that produces large amounts of virus of one serotype, may produce suboptimal levels of other serotypes²⁰⁵. Optimization of AAV production requires a focus on two aspects of protocol development; the generation of virus particles in culture, and the downstream purification and concentration of these viral particles.

These factors were explored to optimise the production of the AAVrh.10 serotype in the laboratory.

4.1.1 Calcium Phosphate Triple Transfection of HEK293T Cells

Generation of AAV virus particles requires the transfection of three plasmids into the production cell line, HEK293T. These plasmids contain the expression vector and gene of interest, two

essential genes controlling the serotype of the virus, and the Adenovirus-derived helper plasmid. Efficient transfection is required to ensure the highest number possible of HEK293T cells receive all three of the plasmids required to produce AAVs. The calcium phosphate method of triple transfection was used to transfect HEK293T cell cultures at 70% confluency.

Transfection efficiency was determined by measuring the proportion of transfected cells expressing an enhanced green fluorescent protein (eGFP) located on the expression vector plasmid. The transfection efficiency was found to be very high, as shown in the representative image provided in Figure 6, as GFP expression is seen consistently throughout the culture.

The total cell area and total fluorescent signal area from each image was determined by manually outlining the sections, to calculate total image area in ImageJ, as shown in Figure 7. A comparison of these areas found that approximately 75% of the total cell areas contained fluorescent signal, seen in the bottom image of Figure 2. Overall, these images demonstrate that calcium phosphate transfection results in high levels of transfection of HEK293T cells.

Following triple transfection, HEK293T cells that express fluorescent proteins can be assumed to have received the other two plasmids needed for AAV production given the high transfection efficiency and the use of pooled plasmids in equimolar amounts. Other methods of transfection, for example lipofectamine, were not tested due to the initial success of the calcium phosphate method and the added expense to procure sufficient lipid-based transfection reagents for downstream applications.

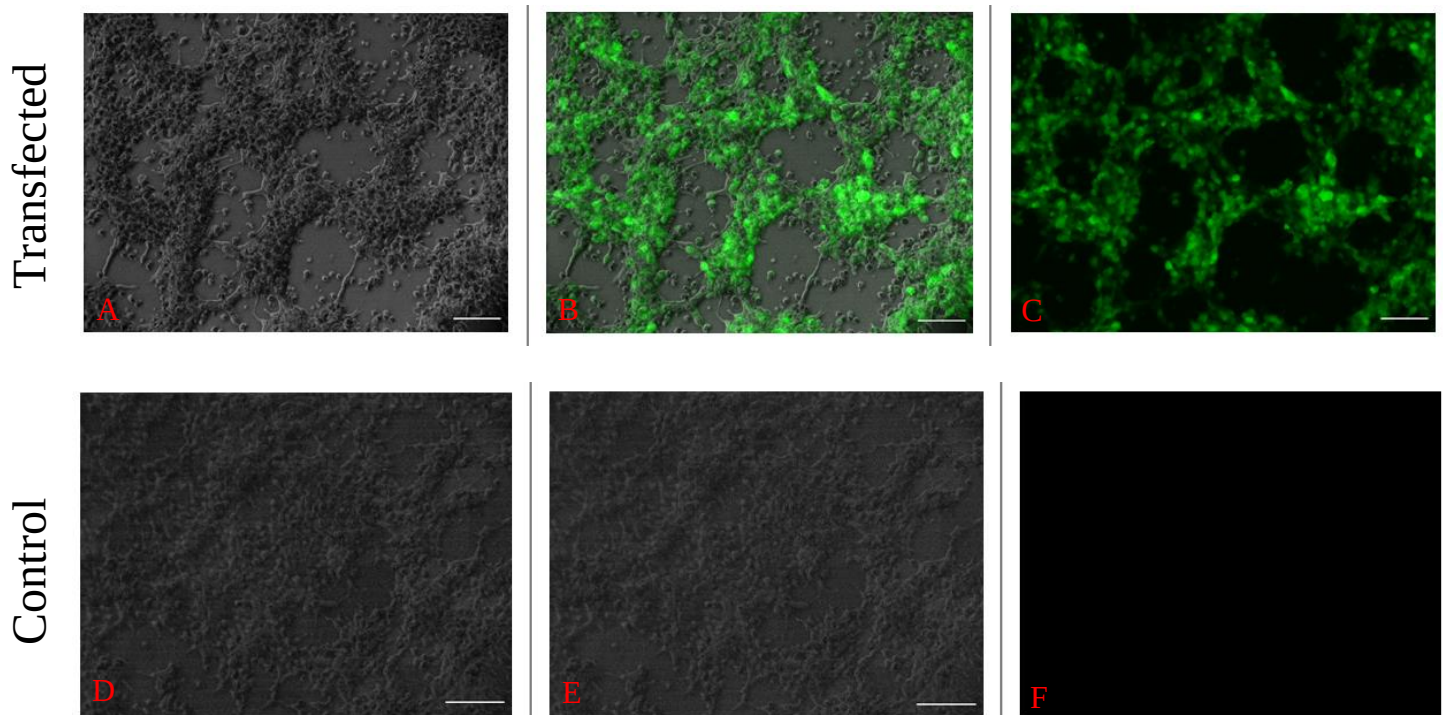
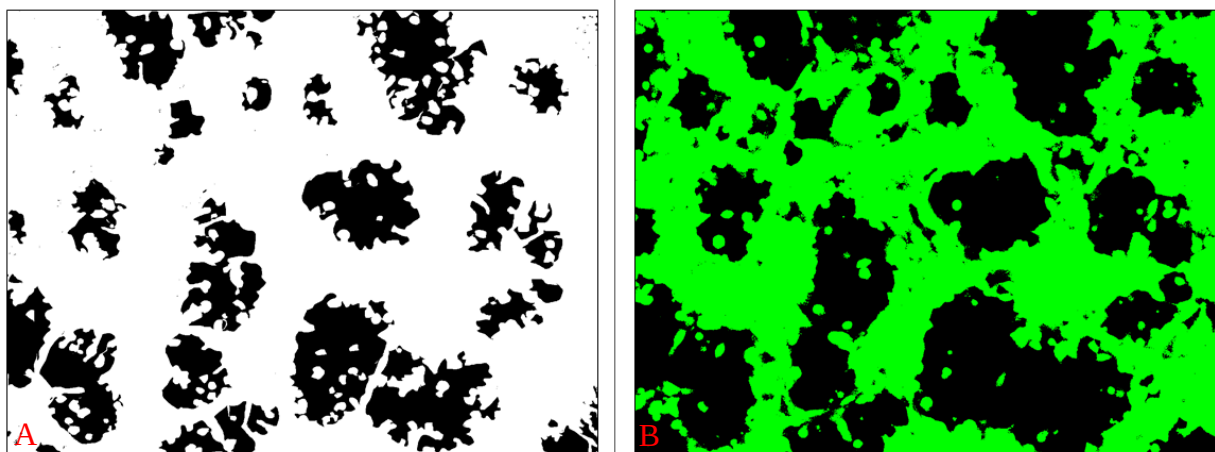


Figure 6. GFP Expression in Calcium Phosphate Triple Transfected HEK293T Cell Cultures Shows High Transfection Efficiency

Confluent plates of HEK293T cells were split 1:3 the day before performing triple transfection, then the triple transfection was performed the next day. After 24hr incubation cultures were visualized under fluorescent microscope to detect GFP. A) Transfected HEK293T culture visualized with brightfield microscope, C) Transfected culture visualized with fluorescent microscopy to detect GFP expression, B) Merging of images A and C to show levels of GFP expression within transfected culture. Images D, E, F, show non-transfected HEK293T cell culture images with same settings, merging of brightfield and fluorescence layers, and fluorescence microscopy of non-transfected HEK293T cell culture. Bars represent 50 μ M.



Total Area of Cell and Fluorescence

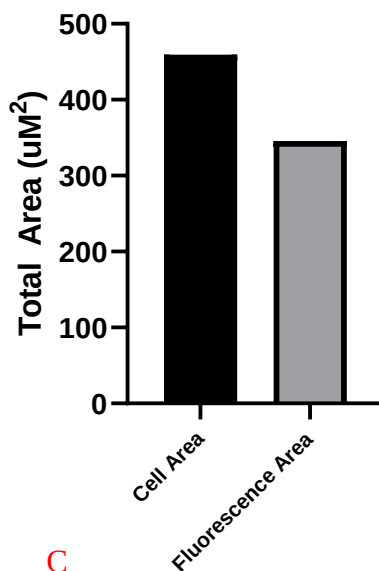


Figure 7. Transfection Efficiency of Calcium Phosphate Calculations Based on Cell and Fluorescent Areas

Images were outlined to determine the total areas covered by cells or fluorescent signal. A) Tracing of cells where black is the background and white is area covered by cells, B) Tracing of fluorescence signal where black is background and green is fluorescent signal, C) Total areas of each image are plotted on the bottom, demonstrating 75% of the total cell area contains fluorescent signal. n=1.

4.1.2 Removal of FBS From Growth Medium Following Transfection Reduces AAV Yield

Animal serum is ubiquitously used in mammalian cell cultures to provide a range of factors required for cell growth and proliferation, and for cell attachment²⁰⁶. However, the production of AAV in suspension using serum-free media has been demonstrated to be successful, and possible at a much larger scale than the conventional culturing production methods^{184,207}. Furthermore, serum-free media allows use of a chemically-defined media which can be modified to identify specific metabolic needs of the cell line, allowing specific optimization of media conditions to increase the production of viral vectors being produced²⁰⁸. Additionally, production of viruses in the absence of serum reduces a large portion of the protein contaminants usually collected in the crude viral harvest, which is advantageous for the following downstream processing of the crude lysate²⁰⁸. Previous research has demonstrated that removal of FBS from the growth media following triple transfection resulted in an increased level of HEK293T cell death, without affecting the total number of AAVs produced¹⁹¹.

During the production of AAVs the growth medium of HEK293T cells are changed prior to, and following, their triple transfections. To determine the effect of FBS on total viral yield, three FBS conditions were assessed; either growth medium containing FBS throughout the entire procedure, removal of FBS prior to and following transfection of a confluent culture, or transfection of the cultures in media containing FBS, with a media change to media lacking FBS 12 hours after transfection. Three groups of 3 confluent 15 cm plates of HEK293T cells were triple transfected, and AAVs were harvested after 3 days of incubation (n=3). Viruses were collected from the media and cell fractions using PEG precipitation and quantified using qPCR with probe-based qPCR against the bGH poly(A) signal on the viral vector, against a standard curve of known copy numbers.

The complete removal of FBS from the growth media led to a substantial decline in total AAV particles produced, as shown in Figure 8. Additionally, removal of FBS from the growth media 12 hours after triple transfection resulted in a significant decline of over 50% total viruses produced. Overall, these results demonstrate that the use of FBS in the cell growth media of HEK293T supports optimal AAV production when HEK293T cells are grown on tissue-culture plates, and that removal of FBS leads to reduced numbers of AAVs collected at time of harvest.

FBS Effect on AAV Production

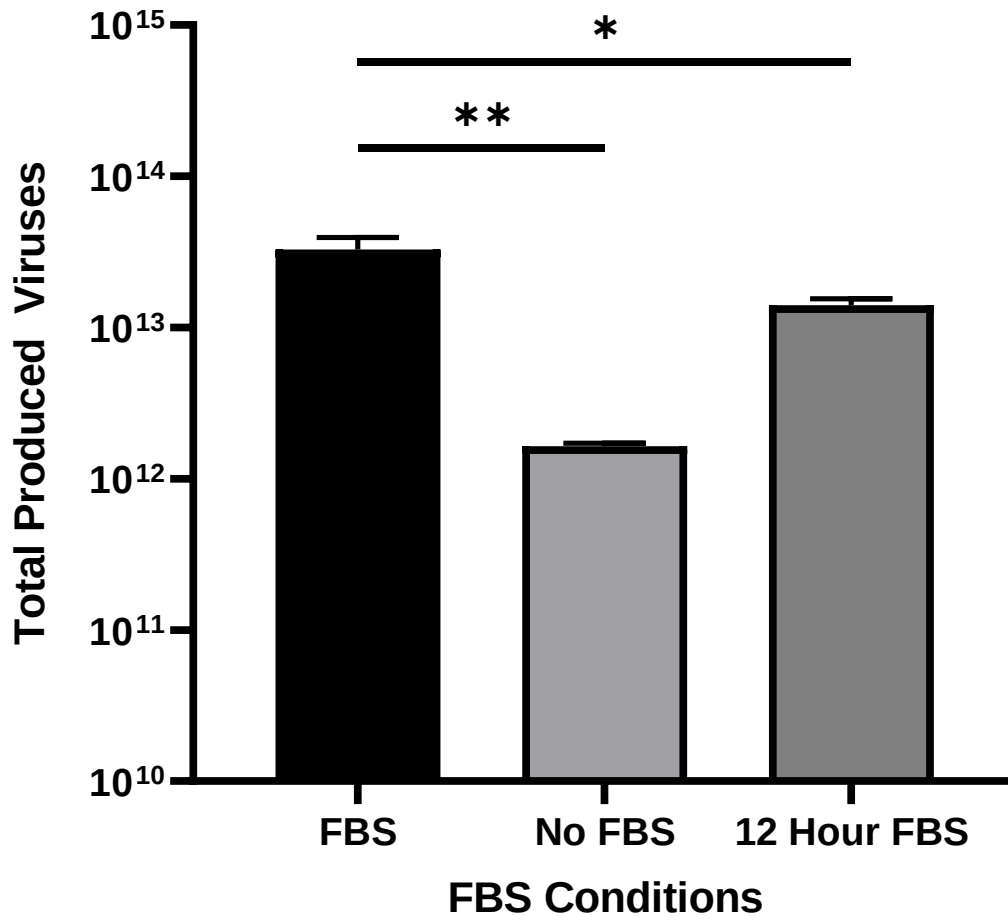


Figure 8. Effect of Removal of FBS from the Growth Media of HEK293T Cells on Total AAV Yield

AAVrh.10 virus titres collected from three pooled 150 mm petri plates for each condition. Media of each plate was changed prior to performing the triple transfection, then again 12 hours following triple transfection. AAV were harvested at Day 3 and quantified using probe-based qPCR against bGH poly(A) signal on the viral vector. FBS represents growth of cultures in DMEM with 10% FBS at all times. No FBS represents change of media to DMEM without FBS prior to transfection and following transfection. 12 Hour FBS represents transfection of the cultures in DMEM +10% FBS, then a change to DMEM without FBS 12 hours following transfection. Bars represent mean values +SEM (n=3). Student's t-test was used to analyze statistical significance: * = p-value ≤ 0.05 ; ** = p-value of ≤ 0.005 .

4.1.3 Adherence of HEK293T Cells to the Growth Surface Increases AAV Yield

Although FBS provides attachment factors to the HEK293T cell allowing cellular adherence to the growth surface²⁰⁶, growth of HEK293T cells in suspension has been successful in large-scale AAV production¹⁸⁴. In my experience, culturing HEK293T cells on untreated polystyrene produces a weakly-adhered culture, with large amounts of detached cells in the media. A literature search of gelatin pre-treatment of petri plates for mammalian cell culturing found that gelatin concentrations (w/v) of 0.1²⁰⁹ – 1%²¹⁰ are commonly used to support HEK293T growth.

To assess the effect of adherence on AAV production, HEK293T cell cultures were grown on untreated polystyrene plates, polystyrene plates with a 0.1% gelatin coating, or on ThermoFisher's Nunclon™ pre-treated plates. AAVs from the cell and media fractions were pooled, concentrated by PEG precipitation, and quantified with probe-based qPCR against the bGH poly(A) signal on the viral vector, against a standard curve of known copy numbers (n=3). Coating the untreated plates with a 0.1% gelatin solution significantly increased the total number of produced AAVs, as shown in Figure 9. Although not statistically significant, there is a trend for the Nunclon™-treated plated to yield higher number of AAVs (p=0.061). Overall, these results demonstrate that cellular adherence is necessary for optimal production of AAV's from tissue-culture plates.

Effect of Coating of Plates on AAV Production

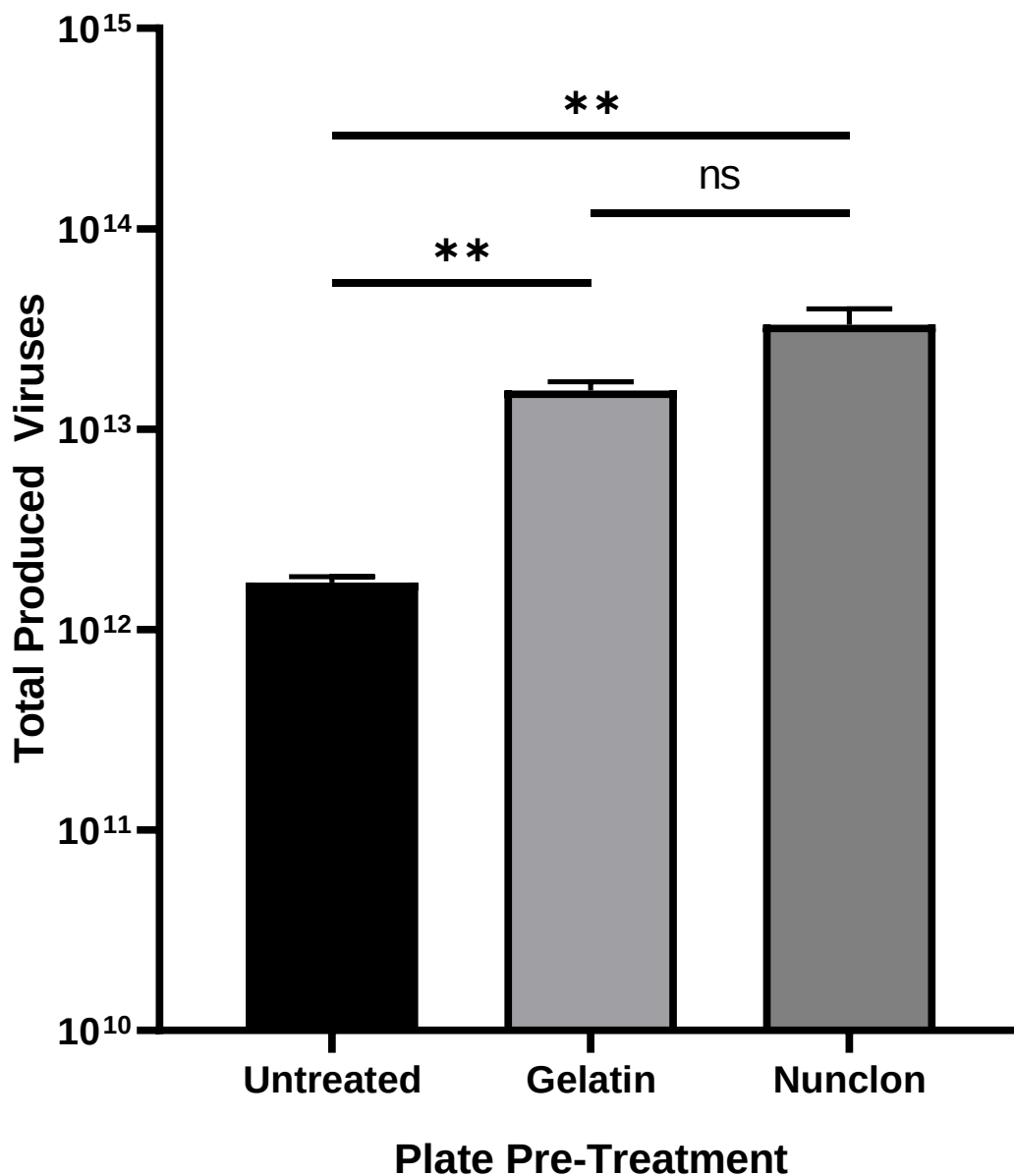


Figure 9. AAVrh.10 Yield from Tissue Culture-Treated Petri Plates

AAVrh.10 virus titres collected from three pooled 150 mm petri plates. AAV were harvested at Day 3 from plates containing DMEM 10% FBS, and quantified using probe-based qPCR targeting the bGH poly(A) signal on the viral vector. 150 mm petri plates were used untreated, or following pre-treatment with 0.1% Gelatin, or Nunclon™ treatment from Thermo. Bars represent mean values +SEM (n=3). Student's t-test was used to analyze statistical significance: ** = p-value of ≤ 0.005 ; ns = no significance (p-value > 0.05).

4.1.4 Increasing Incubation Time of HEK293T Cultures Increases AAVrh.10 Yield

Following the triple transfection of the HEK293T cell cultures, the cells were incubated at 37 °C to allow production of AAV's. A review of the current literature found a consensus of protocols agreeing on a three-day period of incubation prior to harvesting AAV's^{183,191,193,194,203,204}. Additionally, these procedures often focus on the downstream processing of the collected HEK293T cell fraction, with processing of the media fraction treated as optional step^{191,193,203}. However, Vandenberghe *et al*, and Lock *et al* have described a serotype-dependent release of AAVs into the media following a 72 hour¹⁹², or 120 hour incubation¹⁸⁸, respectively. Between these two groups, the release of over 50% of total produced AAVs into the media was demonstrated in AAV1, AAV 5, AAV 6, AAV7, AAV8, and AAV9. This effect was not observed in AAV2, as the viral particles remained closely associated with the cell fraction, presumably due to its heparin sulfate binding domain^{188,192}. Further research reproduced these findings in AAV8, and found that the proportion of total viruses found within the media increases as the length of incubation of the HEK293T cells is increased¹⁹¹. Additionally, it was found that increasing the length of incubation resulted in an increased number of total AAV8 produced, with the optimal AAV8 yield collected after 6 days of incubation¹⁹¹. The viral titres released into the media during AAVrh.10 production had previously not been assessed, and therefore remained a necessary step to perform to optimize AAVrh.10 production.

Total virus numbers found within each fraction was determined by harvesting the pooled cell and media fractions from three 150 mm HEK293T cell cultures every 24 hours, between day 3 and day 6 (n=3). Viruses were collected from the media and cell fractions using PEG precipitation, and viral titres were measured using probe-based qPCR targeting the bGH poly(A) signal on the viral vector, measured against a standard curve of known copy numbers.

Harvesting the cell cultures after 4 days (96 hours) of incubation increased the total number of viruses by 5×10^{13} (p value = 0.0397), compared to harvesting at day 3, and yielded the highest number of total produced viruses, as shown in Figure 10. Additionally, 83% of total AAVrh.10 viruses were found within the media fraction when harvesting is performed after 4 days of incubation. These results demonstrate that production of AAVrh.10 should include processing of the media fraction, as this fraction contains a large portion of the total viruses. Furthermore, increasing the length of incubation by 24 hours over the more commonly suggested 72 hours results in not only a significant increase in total AAVrh.10 yield, but also a significant increase of the portion of viral particles released to the media fraction.

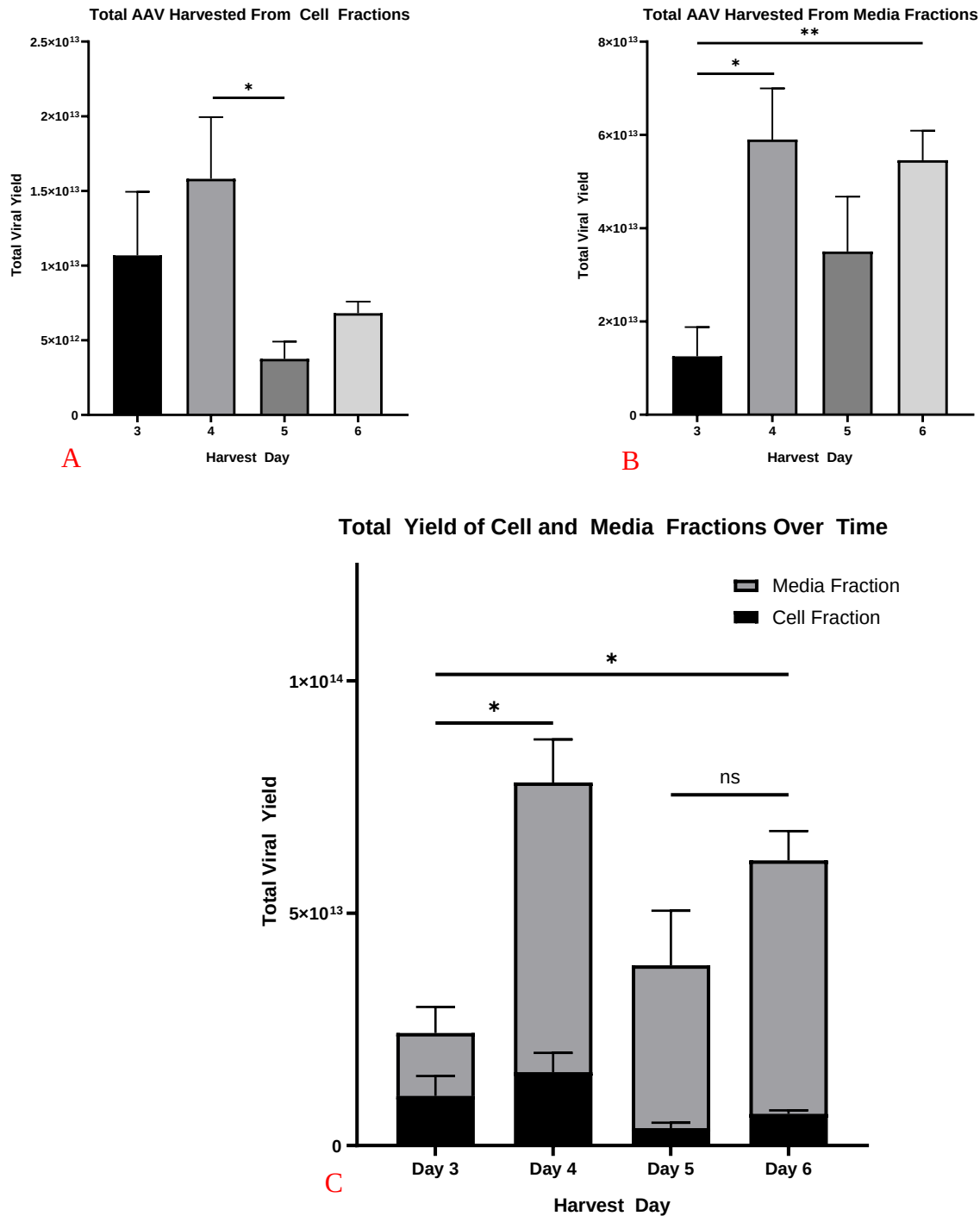


Figure 10. Total AAV Yield of Cell and Media Fractions from Cultures Harvested at 3-6 Days

HEK293T cells were triple transfected and incubated in DMEM +10% FBS for 3-6 days, with additional 10 ml media added at Day 3. At each timepoint cell (A) and media (B) fractions were collected from three 150 mm plates and pooled (C). Viral titre determined using probe-based qPCR for bGH poly(A) signal on the viral vector. Mean data +SEM is shown (n=3). Student's t-test was used to analyze statistical significance: * = p-value of ≤ 0.05 ; ** = p-value of ≤ 0.005 . Bars on bottom graph represent significant changes in the total AAVrh.10 yield of combined cell and media fractions.

4.1.5 Addition of NaCl to Cell Culture at Time of AAV Harvest

Prior to harvesting AAV's from HEK293T cells, NaCl is generally added to the cultures to increase the number of viral particles released to the media. The addition of NaCl to a final concentration of 500 mM for 2 hours prior to the harvesting of AAVs has previously been shown to successfully induce the release of AAV2 into the culture medium ¹⁹⁴, believed to be due to a mechanism involved in cellular stress ¹⁹⁴, or a reduced affinity for the cell fraction ¹⁸⁸. Further research demonstrated this effect in AAV6, and AAV9, with an increase of approximately 20% of the total viruses being found within the media following NaCl incubation, but did not notice a significant increase in AAV release to the media of AAV1, AAV7, or AAV8 ¹⁸⁸. The release of AAVrh.10 following NaCl addition to the media has not yet been assessed.

If the addition of NaCl to HEK293T cell cultures has a similar effect to those seen in cultures of AAV6 and AAV9 ¹⁸⁸ during the production of AAVrh.10, this method could reduce the numbers of steps required to perform downstream purification of AAVs. Since 83% of the total AAVrh.10 particles are already collected within the media fraction when harvesting is performed after a 4-day incubation period, the release of the majority of remaining AAVs into the culture media would allow further downstream purification to focus only on the media fraction.

Therefore, I assessed whether NaCl addition to the media at a final concentration of 500 mM 2 hours prior to harvesting AAVs resulted in a release of AAVrh.10 into the culture media. Three 150 mm petri plates of HEK293T cells were grown for 3 days, in triplicate. NaCl was added to a final concentration of 500 mM 2 hours prior to harvesting the viruses. At harvest, the three 150 mm petri plates for each biological replicate were pooled. Viruses were collected from the media and cell fractions using PEG precipitation, and viral titres were measured using qPCR with SYBR Green detection of PCR products amplified with primers targeting the inverted tandem

repeat sequence (ITR) of the viral backbone.

The addition of NaCl to HEK293T cell cultures did not induce a statistically significant release of AAVrh.10 particles into the media (p value = 0.2), as shown in Figure 11. Additionally, the increased NaCl concentration resulted in the dissociation of the HEK293T cells from the culture plates, which led to the collection of cells with the media fraction.

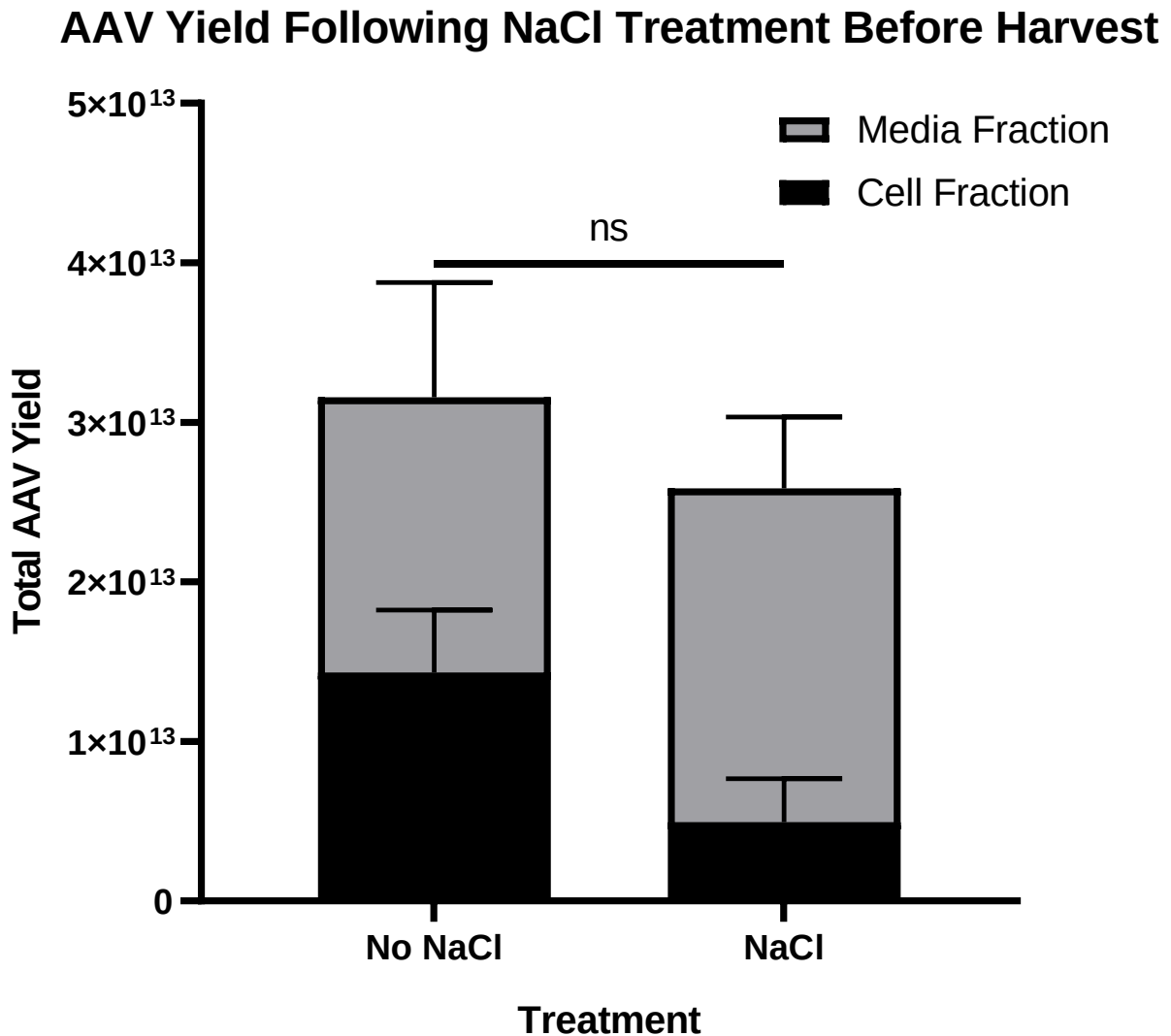


Figure 11. Effect of NaCl Pre-Treatment on AAV Yield at Harvest

HEK293T cells were triple transfected and incubated in DMEM +10% FBS for 3 days. NaCl pre-treated group was treated to NaCl and incubation at 37 °C for 2 hours prior to harvesting. Three 150 mm petri plates were pooled for each condition, and virus titres determined using SYBR Green qPCR with standard curve. Mean data +SEM is shown (n=2). Student's t-test was used to analyze statistical significance: ns = no significance (p-value > 0.05).

4.1.6 Assessment of Tangential Flow Filtration System

Following the production and harvest of AAV, the media and cell fractions must be concentrated into manageable volumes that can be further processed to purify viruses. A tangential flow filtration system (TFF) is commonly used, as this system concentrates AAVs and other molecules based on molecular weights, and therefore is applicable to all AAV serotypes^{142,183,191,194–196}. The TFF setup is shown in Figure 12. This system is a closed pump-filter system that continuously cycles the sample over and through a membrane consisting of pores with a 100 kDa molecular weight cut-off. AAV's, that have an average molecular weight of 3746 kDa,¹⁹⁷ remain within the system, while media and contaminants are diafiltered out of the sample. Prior to running the system, it is necessary to perform a calibration test to ensure that no viruses are being lost due to permeation of the virus through the membrane. Viruses loss in this system may also be due to binding to the system's hoses or membrane^{188,211}, or due to small volumes of the concentrated product remaining trapped within the system following collection of the product. To assess the proportion of viruses lost due to concentration with the TFF system, a concentration procedure was performed on a viral sample of known titre. The sample was diluted to 300 ml in PBS with additional NaCl to 500 mM, as the high salt concentration minimizes aggregation of the viral vectors²¹², which may increase total virus recovery during downstream purification¹⁸⁸. The sample was concentrated with the TFF, and the concentrated product was collected. A wash step was performed by cycling high-salt PBS through the system for an additional 15 minutes, to assess the levels of virus lost by remaining bound to hose or system. The system was then disassembled to collect the fraction of viruses that are lost due to being trapped within the system following a standard concentration cycle (Volume D, viruses lost to the TFF system is further explained in the materials and methods section).

The total viruses collected from each fraction is outlined in Table 9. These results show that overall 94% of the total input viruses can be recovered following concentration with the TFF system. Furthermore, the 94% of viruses can be collected in the first fraction, without the need of a second wash cycle that would further dilute the product. These results show that there are no detectable levels of viruses within the collected filtrate waste therefore no viruses are being lost due to permeation through the TFF membrane. Additionally, the highest percentage of viral particle loss was measured in the fraction that remain trapped within the TFF system following the concentration procedure, accounting for a loss of nearly 3% of the total input viruses. However, as some of this fraction was lost during disassembly of the system, the percentage loss is likely to be higher. The second wash step did not yield a significant number of viruses, and therefore is not necessary following a standard procedure.

Overall, these results demonstrate that the TFF system is an efficient option allowing the concentration of large volumes of crude AAVs into volumes that facilitates further downstream purification. Using this system, 94% of the total virus input can be collected as a concentrated viral product, without the need of a second wash step.

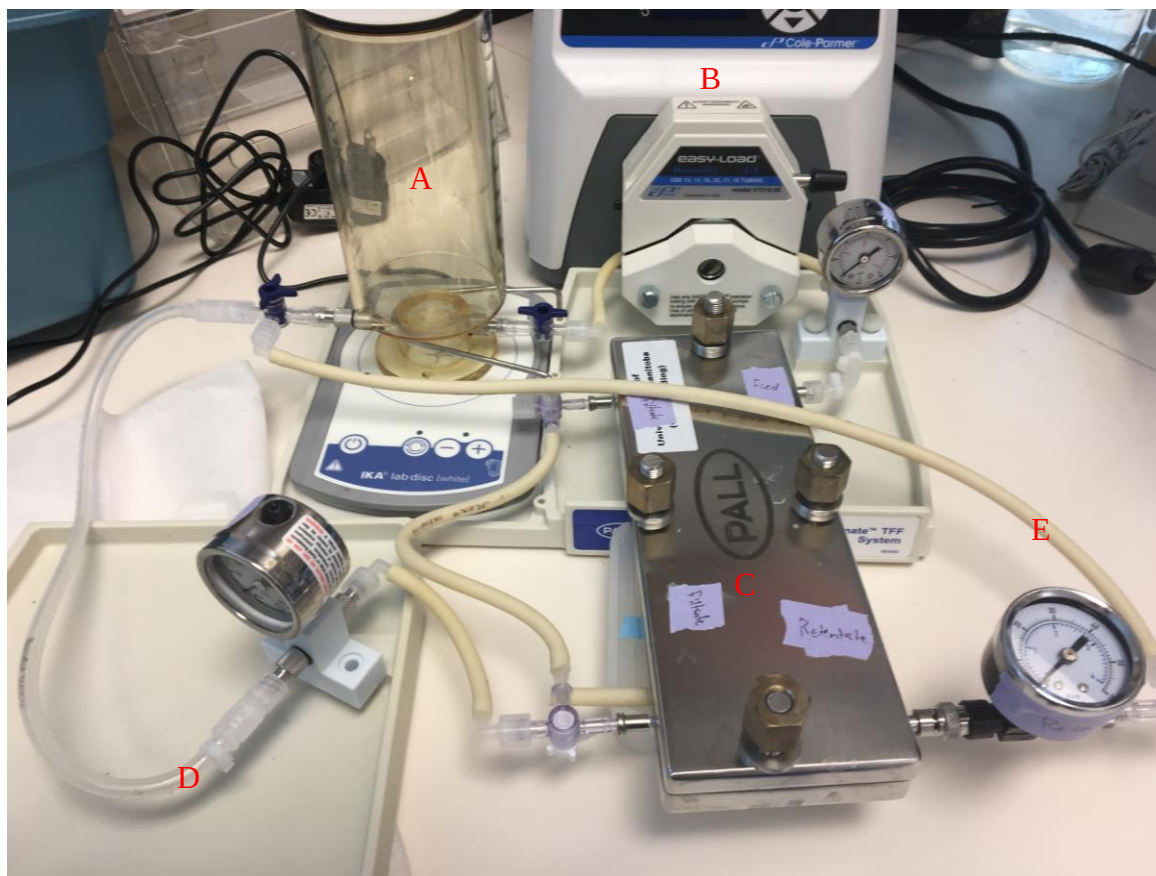


Figure 12. Tangential Flow Filtration System Setup

Fully-assembled tangential flow filtration system. Crude viral collections are transferred into the sample reservoir tank (A). The peristaltic pump (B) pumps the viral collections into the membrane compartment (C). This compartment contains a 100kDa MW cutoff membrane that prevents AAVs from bypassing the membrane. The filtrate that is passed through the membrane is pumped through hose (D), which is either routed back into the sample reservoir tank (as shown in this figure) or into a waste collection receptacle. The retentate fraction containing AAVs is pumped back into the sample reservoir tank through hose (E).

Table 9. Virus Titres Collected from Each Fraction Following TFF Concentration

Samples Taken During TFF	Total volume (ml)	Virus Concentration (GC/ml)	Total Virus	Percent of total AAVs
Input Viral Sample	300	7E10	2.1E12	100
Concentrated Product	31	6.38E10	1.98E12	94.3
Additional Wash Step	30	10660	3.2E5	0
Filtrate Waste of TFF	230	0	0	0
Volume D (Trapped Product)	3	1.91E10	5.73E10	2.72

Concentrated virus = concentrated product retrieved after tangential flow filtration, Concentrated virus following PBS wash = product retrieved following additional wash with PBS, Waste = product retrieved in the waste (flow through) of membrane, Volume D = product that cannot be retrieved from TFF

4.1.7 Comparison of Gradient Ultracentrifugation Purification Methods

Although the TFF system is capable of concentrating the crude harvested viruses, the volume that can be collected from the system is limited by the system's size, so viral products remain relatively dilute. Therefore, following harvesting and concentration with use of the TFF system, it is necessary to further purify the AAVs from the concentrated viral product. Further purification also aims to separate produced viruses from the contaminating proteins leftover from the cell culture, as well as to reduce the number of improperly packaged viral particles. The two most commonly used methods of AAV purification are cesium chloride gradient ultracentrifugation^{190–192}, and iodixanol gradient ultracentrifugation^{188,189,193}. Although both of these methods use buffer density gradients to concentrate the AAVs into a tight band between two concentration layers, there are important differences between, such as the total amount of time required to perform the procedures, and the number of ultracentrifugation steps involved in each.

Both of these methods were used during the production of AAV's, however the iodixanol method yielded much higher titres of viruses when used to purify viruses from the same number of HEK293T cell culture plates. Purification of AAVrh.10 was performed on the cell lysate and media fractions collected from 50 15 cm plates of triple transfected HEK293T cells incubated for 3 days. Viral titres from the collected samples were determined with qPCR, and the C_T values were plotted against a standard curve of known copy numbers.

The total number of viruses collected following the first two purifications using the CsCl or iodixanol method are shown in Figure 13. The first attempt of purifying AAVs using the cesium chloride method yielded AAV titres that were below the range of detection of the standard curve, however when extrapolating from the standard curve, yielded 53,000 viral

particles. The second attempt of using the CsCl purification method yielded 6E8 viral particles. Finally, the first and second attempts at using the iodixanol gradient ultracentrifugation method of purifying AAVs yielded 2E11, and 1.9E12 viral particles, respectively.

These results demonstrate that although CsCl gradient ultracentrifugation has been demonstrated to yield comparable viral titres as the iodixanol method ¹⁸⁹, the iodixanol method may be more easily-manipulated by beginners to this method.

Comparison of Purification Procedures

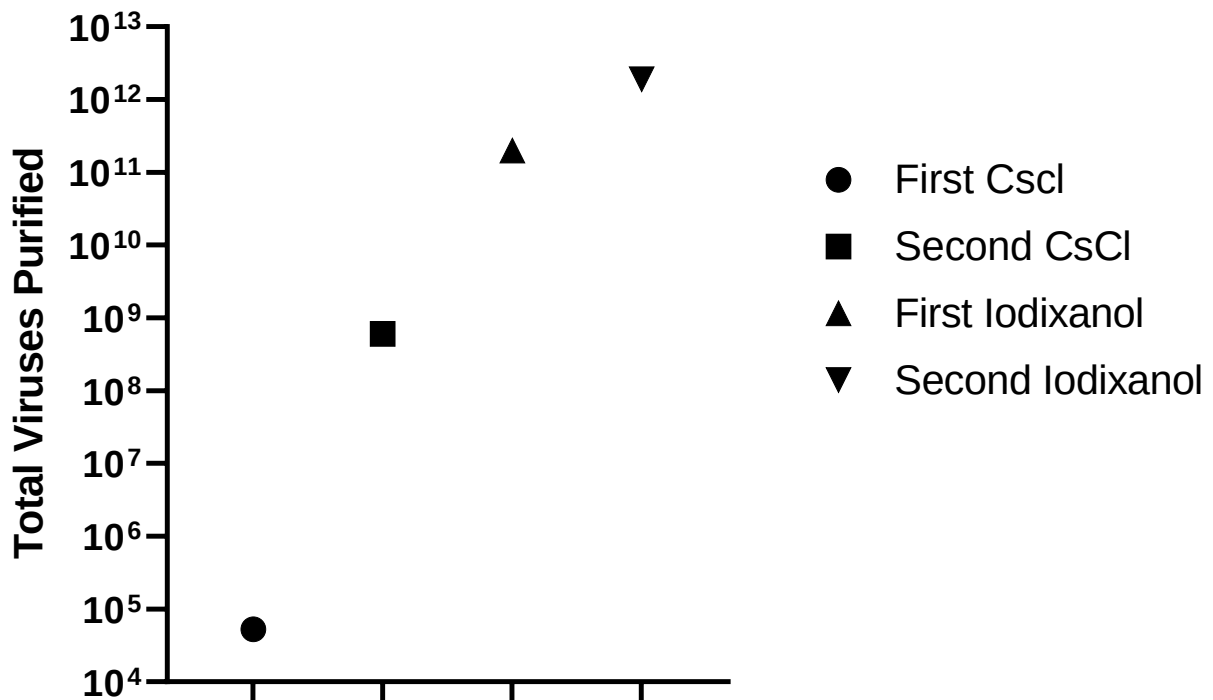


Figure 13. Total Viruses Produced with First Two CsCl and Iodixanol Ultracentrifugation AAV Purifications

Fifty 15 cm plates of HEK293T cells were harvested at day 3 of incubation, and AAVrh.10 viruses harvested from cell and media fractions. Viruses were concentrated using 100k MW centrifuge filters, then purified with either CsCl or iodixanol gradient ultracentrifugation. Virus titre was determined using real-time PCR with probes for the bGH poly(A) signal on the viral vector. (n=1)

4.1.8 Production of AAV8 and AAV9 with AAVrh.10-Optimized Protocol

Throughout the literature there are several examples of protocols adapted for a specific AAV serotype producing varying amounts of other serotypes, sometimes in suboptimal amounts^{203,204,213}. Due to the range of AAV serotypes available, and the often overlap of target cells and organs, it is common for research to require a number of different AAV serotypes. Therefore, having a single protocol efficient in the production of a number of AAV serotypes is desirable.

To assess the levels of AAV8 and AAV9 produced by the protocol adapted for optimized AAVrh.10-production, viruses were collected from the cell and media fractions of triple-transfected HEK293T cells after 4 days of incubation. Crude viruses were pooled from 10 15 cm plates, concentrated by TFF, then purified with iodixanol gradient ultracentrifugation (n=2). Viral titres of the purified 1 ml viral products were determined with SYBR green qPCR with primers designed against the ITR sequence of the viral vector.

These data show that although optimized for AAVrh.10 production, this protocol may be used to produce high titres of AAV8 and AAV9. Of all 3 AAV serotypes tested, production of AAVrh.10 was the highest, with a final purified AAV titre of 7.7E13 on average. Production of AAV8 and AAV9 yielded average purified titres of 1.5E12, and 7.8E12, respectively.

Overall, these data demonstrate that different serotypes are produced at different levels using the same AAV protocol. Although the protocol was adapted for optimal AAVrh.10 production, relatively high levels of AAV8 and AAV9 were also produced.

Comparison of AAV Serotype Production

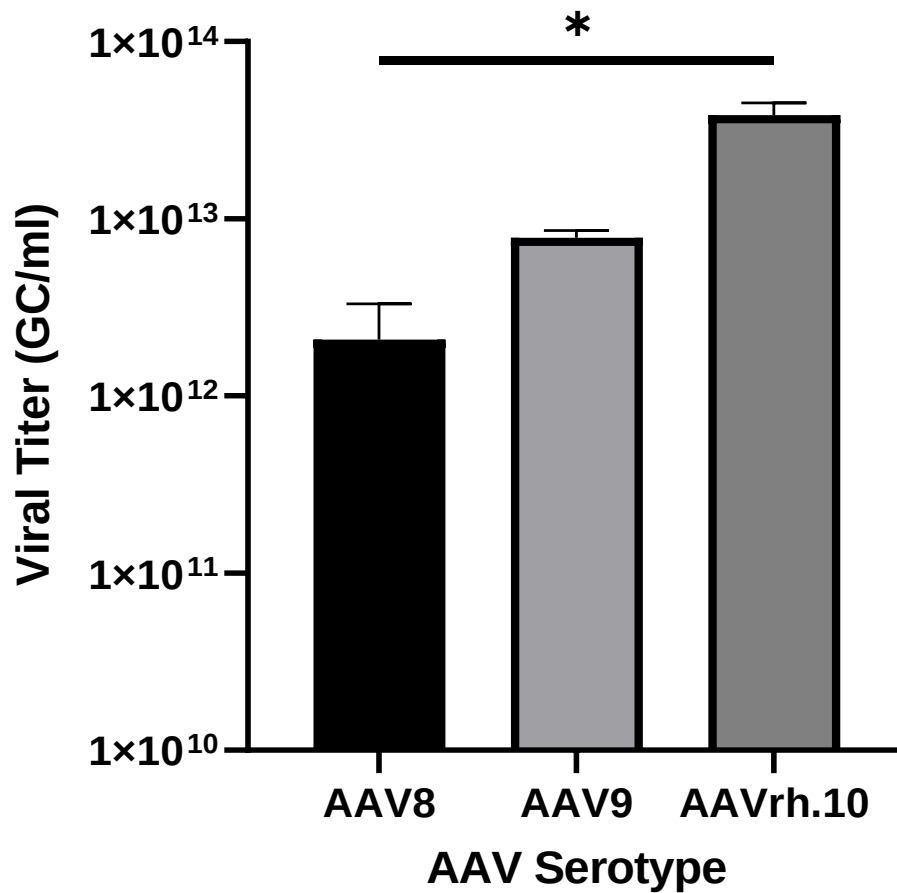


Figure 14. Differences in Produced AAV Serotypes

Total AAV viral titres collected from the protocol optimized for AAVrh.10 production. Pooled AAV collections from 10 15 cm plates grown for 4 days were concentrated by TFF, purified by iodixanol gradient ultracentrifugation, then quantified by SYBR green qPCR using primers against the ITR sequence of the viral vector. Average values +SEM of total viral titre (genome copies/ml) of the 1ml purified samples are shown (n=2). Student's t-test was used to analyze statistical significance: * = p-value ≤ 0.05 .

4.2 Characterization of Adeno-Associated Viruses and Genetic Constructs

Rationale

Prior to any live animal studies, characterization of the AAVs should be performed *in vitro*, using a comparable model. Although AAV is considered to have little to no cytotoxic effects^{169,190,214,215}, there have been reports of AAV-induced cytotoxicity due to high viral doses²¹³, shRNA over-expression¹³⁸, or expression of a fluorescent protein²¹³. Therefore, it is crucial to assess transduction efficiencies, cytotoxic effects, and the effects of transgene expression prior to the introduction of AAVs into a living organism.

Immortalized neuronal cell lines are available, however they often do not follow the typical stages of neurodegeneration as seen in living animal brain tissues and they fail to form well-defined axons, dendrites and synapses with other neurons¹⁰⁵. Furthermore, the specific requirements of each cell line^{104,216} makes comparisons to other cell lines *in vitro*, and cells *in vivo* difficult. However, primary hippocampal cultures can be highly enriched for neuronal cells and remain viable for 3-4 weeks^{105,200}, enabling characterization of AAV's in cells that closely resemble the *in vivo* environment^{205,213}.

4.2.1 Establishment of Primary Neuron Cell Cultures

Primary neurons were cultured using the techniques established by Kaech and Banker in 2006²⁰⁰, which remains one of the most well-established techniques to study neuronal cells *in vitro*, with Kaech and Banker's original paper currently sitting at over 500 citations. Primary neuronal cell cultures have been demonstrated to be transduced by AAV's with high efficiency, and with a low toxicity, allowing assessment of their transgene products^{213,217,218}. Therefore, I used this cell model to assess AAV characteristics of neuronal transduction, cytotoxicity, and effects of transgene expression. First, however, I assessed the characteristics of the primary neuron cell culture to ensure that high numbers of neurons could be maintained for an extended period of time.

The hippocampus from E18 embryonic mice was used as a starting tissue to culture primary neuron cells. The hippocampus consists of a high density of neurons²¹⁹, and this region of the brain is known to undergo significant neurodegeneration during prion disease²²⁰. Compared to other regions of a normal healthy brain which contains a variety of cells including neurons, astrocytes, and microglia, the hippocampus has been found to be mostly comprised of neurons²²¹. The primary neuron cell cultures were maintained for 18 days once plated, with images collected every 2 days, beginning at day *in vitro* (DIV) 4, using a Mirax slide scanning microscope. Immunohistochemistry for imaging was performed using antibodies against MAP2 to stain for neurons, and antibodies against GFAP to stain for astrocytes, and DAPI staining to identify nuclei. The established hippocampal primary neuron cell cultures were found to contain a high proportion of neurons (Red), with low levels of astrocytes (green), as shown in Figure 15. Additionally, cultures remained highly neuronal for at least 18 DIV.

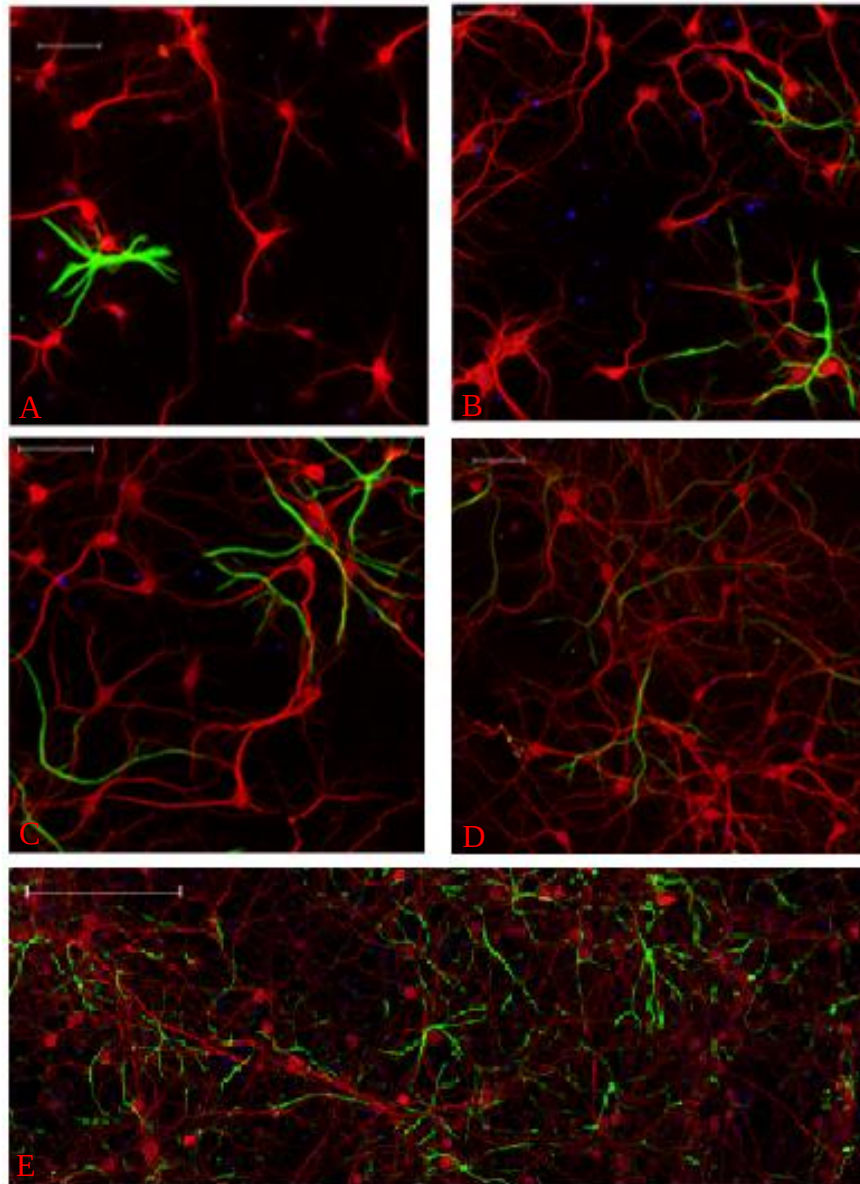


Figure 15. Primary Hippocampal Neuron Cell Culture Images at Various Stages of Growth.

Image of cultures at DIV 4 (A), 6 (B), 8 (C), 10 (D), 18 (E). Images collected with use of confocal microscope at 20x magnification. Cells were stained for MAP2 (Red) to identify neurons, GFAP (Green) to identify astrocytes, and with DAPI (Blue) to identify nuclei. Bars on A, B, C, D represent 100 μm ; Bar on E represents 200 μm .

4.2.2 Assessment of Culture Purity and Total Cells Over Time

To assess the culture purity and determine the proportions of neurons and astrocytes within the culture, cultures of primary hippocampal neurons were grown over a period of 18 days. At each timepoint, slides were collected and stained for the following cell markers: MAP2 = Neurons, GFAP = Astrocytes. Slides were imaged using a Mirax slide scanner and counted using the ImageJ plugin Cell Counter. Two different cultures were established for each timepoint, and one image consisting of approximately 100 cells was selected at random from each culture. The following table shows the total cells counted, total neurons counted, and total astrocytes counted from the primary neuron cell cultures at each timepoint.

Table 10. Results of Counting Cells of Primary Neuron Cell Cultures

DIV	# of Neurons		# of Astrocytes	
4	111	96	4	8
6	82	95	8	18
8	97	100	11	23
10	64	65	9	19
12	20	47	3	3
14	70	42	3	6
16	31	53	32	26
18	28	23	21	31

The total numbers of neurons and astrocytes counted in the cultures at each time point are shown in Figure 16. These results show that the cultures contain a large proportion of neurons throughout the 18 days that the cultures are maintained. Of note, a large decline in neuron counts is observed at DIV 10. To assess whether this is due to a neuronal death, or general death of all cells in relatively equal proportions, the percentages of neurons vs total cells were calculated using the following equation:

$$\text{\% of Neurons} = (\text{Counts of MAP2}) / (\text{Counts of MAP2} + \text{Counts of GFAP})$$

The percentage of neurons of the total cell counts in each culture are shown in Figure 17. These results show that the total proportion of neurons remains stable in cultures between DIV 4-14, suggesting that the large decline in neuronal cell counts seen at DIV 10 in Figure 16 is due to the death of neurons and astrocytes in relatively equal proportions. However, a decline in the proportion of neurons is observed at DIV 16 and 18 in Figure 17, corresponding to the higher astrocyte counts in Figure 16 at these timepoints.

In total, the primary neuron cell cultures have been shown to consist of approximately 90% neurons throughout the initial 14 days following plating of the cultures. Beyond this time the proportion of astrocytes in the culture increases, however the proportion of neurons does not drop below 50%.

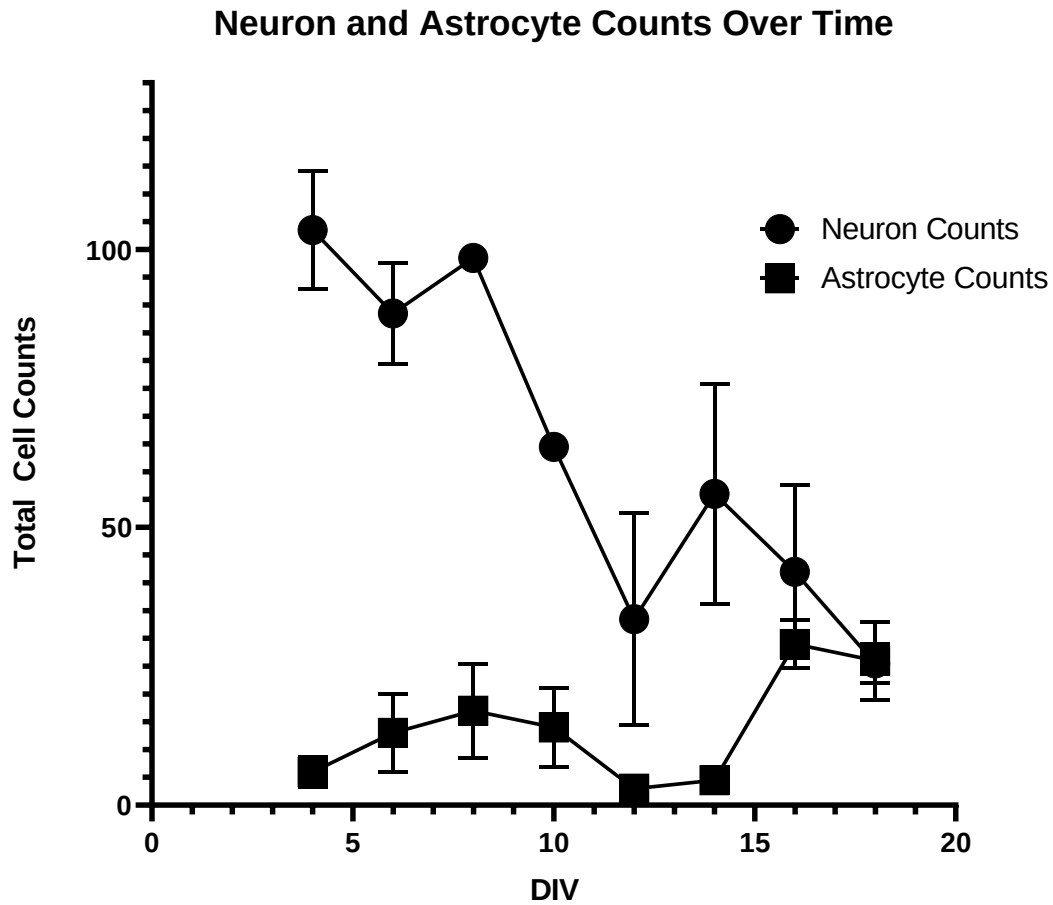


Figure 16. Total Number of Cells Counted in Primary Hippocampal Neuron Cultures at DIV 4-18

Primary hippocampal neuron cultures were imaged at the various timepoints (DIV 4-18). Cells were stained for MAP2 to detect neurons, and GFAP to detect astrocytes. ImageJ Cell Counter plugin was used to count cells in each image. Mean of counts are plotted, with bars representing SEM (n=2).

Fraction of Neurons vs Total Cells

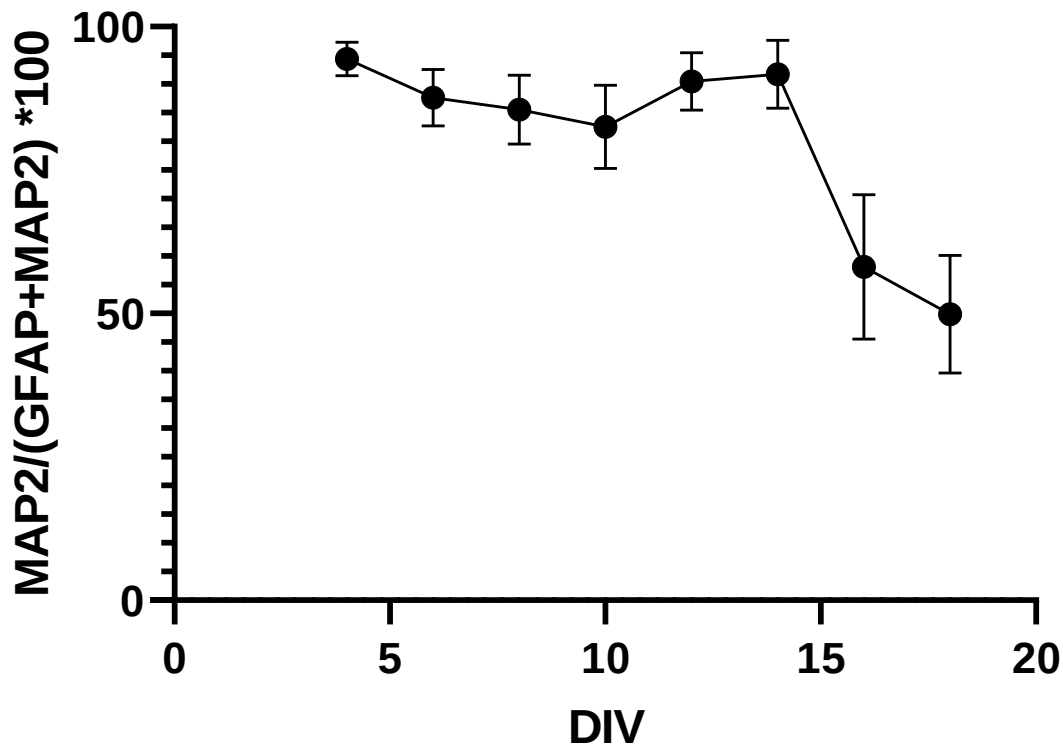


Figure 17. Total Percentage of Neurons in Primary Hippocampal Cell Culture at DIV 4-18
Proportion of neurons vs total cells counted in the primary hippocampal cell cultures at DIV 4-18. Cells were stained for MAP2 to detect neurons, and GFAP to detect astrocytes. ImageJ Cell Counter plugin was used to count cells in each image. Counts from neurons were divided by the total counts of neurons and astrocytes to determine proportion of neurons in each image. Mean of counts are plotted, with bars representing SEM (n=2).

4.2.3 Neuronal Transduction of AAV Serotypes *in Vitro*

Several AAVs have been shown to be very efficient in transducing primary neuron cell cultures, with transduction efficiencies of over 90%, at very low cytotoxicity^{201,213,222}. Shown below are reports of high neuronal transduction efficiencies of AAVs, and the MOI used in each study.

Table 11. AAVs Capable of Primary Hippocampal Neuron Transduction *in vitro*

AAV Serotypes	Neuronal Transduction Efficiency	MOI
AAV2, AAV 5	90-95	3-6E4 ²²³
AAV1, AAV8, AAV9	+90%	1.5E5 ²⁰¹
AAV8, AAV9	94.2 ± 1.9%, 79.5 ± 5.8% (in rats)	1.25E6 ²²⁴

Of the viruses listed in the previous table, AAV8 and AAV9 serotypes have been the most extensively-studied serotypes capable of neuron transduction *in vivo*^{136,169,190,211}. AAV8 is unable to efficiently bypass the blood-brain barrier, resulting in low levels of transduction following intravenous injection of mice¹⁵⁰. However, when intracranially injected into specific sites of the brain, AAV8 demonstrates high transduction efficiencies^{144,171,225}. It has been shown that AAV9 can cross the blood brain barrier and transduce both neurons and astrocytes following intravenous delivery in both neonatal and adult mice, with a higher neuronal specificity when administered to neonatal mice, and a higher specificity for astrocytes when injected into adult mice¹⁶⁶. One of the more recently described AAV serotypes, AAVrh.10 has also shown promising results *in vivo*. In particular, it was shown that AAV9 and AAVrh.10 have near identical transduction profiles, but AAVrh.10 results in a stronger expression level throughout the life of the mouse when intravenously injected into neonatal mice^{168,169}. In a comparison of AAV8, AAV9, AAVrh.10 transduction profiles in adult mice, it was shown that AAV9 and AAVrh.10 resulted in the largest amounts of transduced cells within the hippocampus¹⁷¹.

Furthermore, AAVrh.10 resulted in the highest levels of transduction in the thalamus, cortex, and striatum¹⁷¹. The extensive histories of AAV8 and AAV9 *in vitro* and *in vivo* make these viruses ideal standards when assessing the neuronal transduction efficiencies of other AAV serotypes. To assess the transduction efficiencies of AAV8, AAV9, and AAVrh.10, primary neuron cell cultures at DIV 10 were administered each AAV serotype at MOI 1E4. Each virus contained the eGFP gene, allowing identification of transduced cells. Cultures were harvested 3-7 days following transduction, then fixed and stained for MAP2 to mark neurons (Figure 18 and Figure 19– Red). Transduced cells were identified by eGFP expression (Figure 18 and Figure 19 – Green), and astrocytes were either unstained, shown in Figure 18 and Figure 19, or were stained for GFAP to allow accurate counting of astrocytes, with results shown in Figure 20. Expression of eGFP was seen throughout primary neuron cell cultures, as seen in the lower magnification images in Figure 18, and higher magnification images in Figure 19. These figures show a high level of neuronal transduction, even at the lowest tested MOI of 1E4. Additionally, as shown in the AAV8 and AAV9 images of Figure 19, GFP-positive, MAP2-negative cells are identified (White arrows), which are presumed to be astrocytes based on morphology. Two primary neuron cell cultures were produced for each experimental condition, and one image consisting of approximately 100 cells was selected from each (n=2). Within these images, the total number of neurons, total number of transduced neurons, and total number of transduced astrocytes was determined using the ImageJ CellCounter plugin, and the results are shown in the following table.

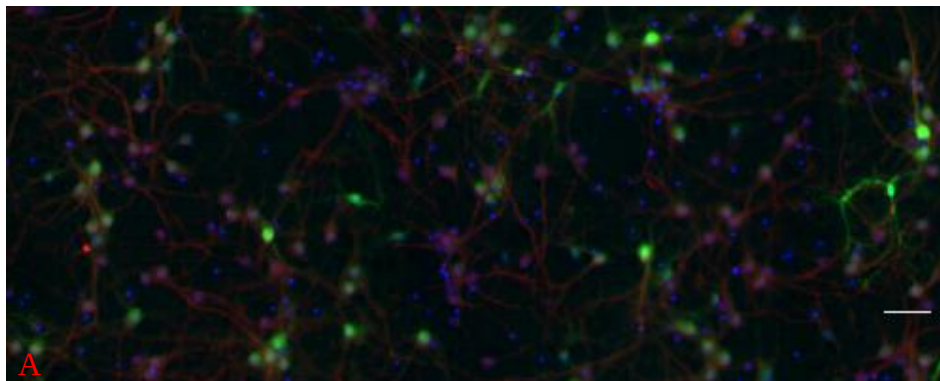
Table 12. Counting Results of Primary Neuron Transduction by AAV8, AAV9, and AAVrh.10

	AAV8						AAV9						AAVrh.10					
DIV	Total Neurons		Transduced Neurons		Transduced Astrocytes		Total Neurons		Transduced Neurons		Transduced Astrocytes		Total Neurons		Transduced Neurons		Transduced Astrocytes	
3	115	93	104	88	16	20	65	84	61	80	5	16	82	127	65	107	15	2
5	82	120	77	105	11	4	94	112	85	98	19	17	96	133	81	123	9	11
7	130	75	120	63	22	16	123	87	102	71	24	16	118	108	96	92	9	3

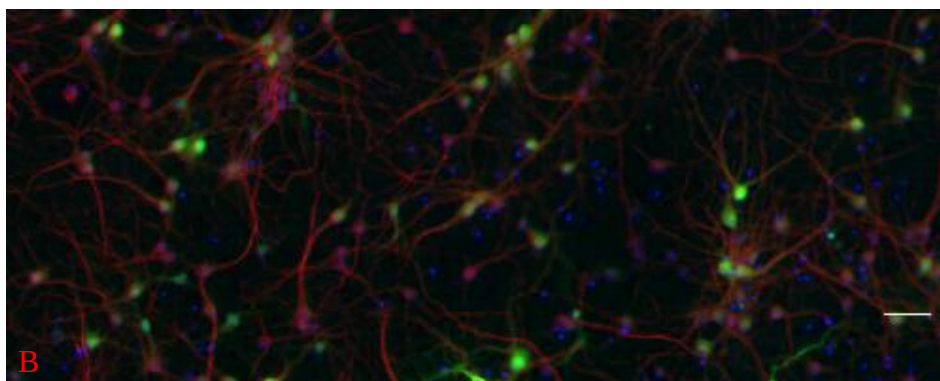
The neuronal transduction efficiencies at each MOI is shown in Figure 20, with transduction efficiencies consistently ranging between 80-90% for AAV8, AAV9, and AAVrh.10 at MOI 1E4, at all time points (DIV3-7). Additionally, the percentage of transduced astrocytes out of all transduced cells remained low, with overall transduction rates of astrocytes of approximately 5-20% for all serotypes at DIV3-7.

Overall, these results demonstrate that primary neuron cell cultures can be used to assess transduction efficiencies of various MOI, as these cultures can be efficiently transduced at DIV 10 to allow transgene expression for at least 7 days post-transduction.

AAV8



AAV9



AAVrh.10

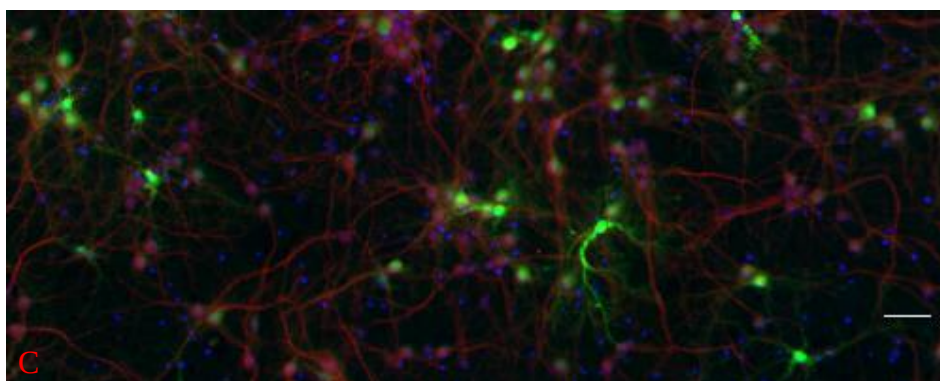


Figure 18. Scanning Microscope Images Showing Transduction of Cells in Primary Hippocampal Cell Cultures

Primary neuron cell cultures were transduced at a MOI of 1E4 and imaged after 5 days of incubation using a scanning microscope at 20x. eGFP expression in transduced cells was detected by fluorescent microscopy (Green), and neurons were stained with anti-MAP2 antibodies (Red). Cell nuclei were stained using DAPI (Blue). Scale bars represent 50 μ m.

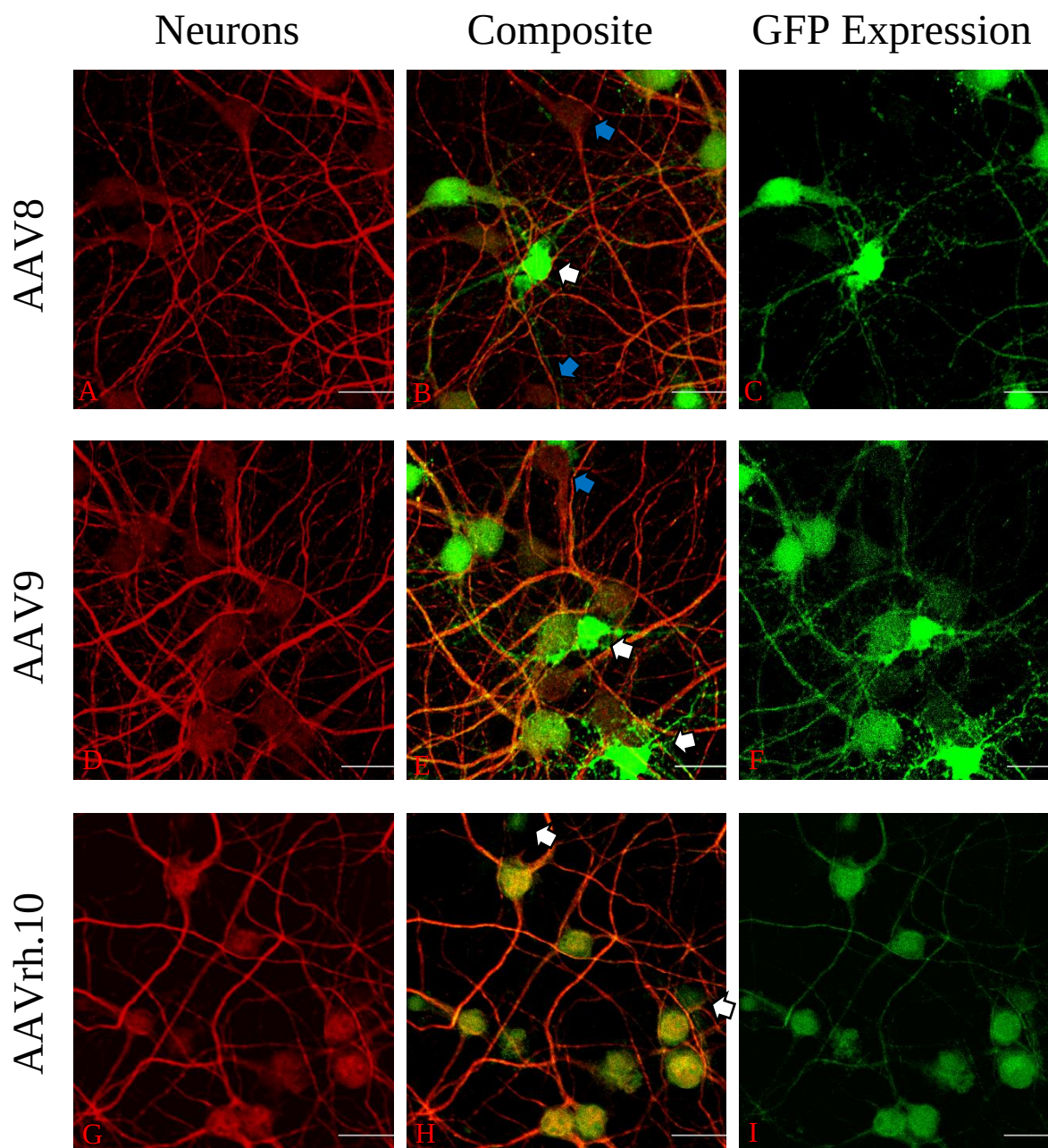


Figure 19. Confocal Images of GFP Expression in Primary Neuron Cell Cultures

Primary neuron cell cultures were transduced at a MOI of 1E4 and imaged after 5 days of incubation. eGFP expression in cells was detected by confocal microscopy (Green), and neurons were stained with anti-MAP2 antibodies (Red). Left images (A, D, G) show imaging for MAP2, a marker of neurons. Images on the right (C, F, I) show eGFP detection. Center column (B, F, H) shows an overlap of the two filters. White arrows indicate MAP2-negative transduced cells. Blue arrows indicate GFP-negative MAP2 stained cells. Scale bars represent 20 μ m.

Neuron Transduction Efficiency and Rate of Transduced Astrocytes at MOI 1E4

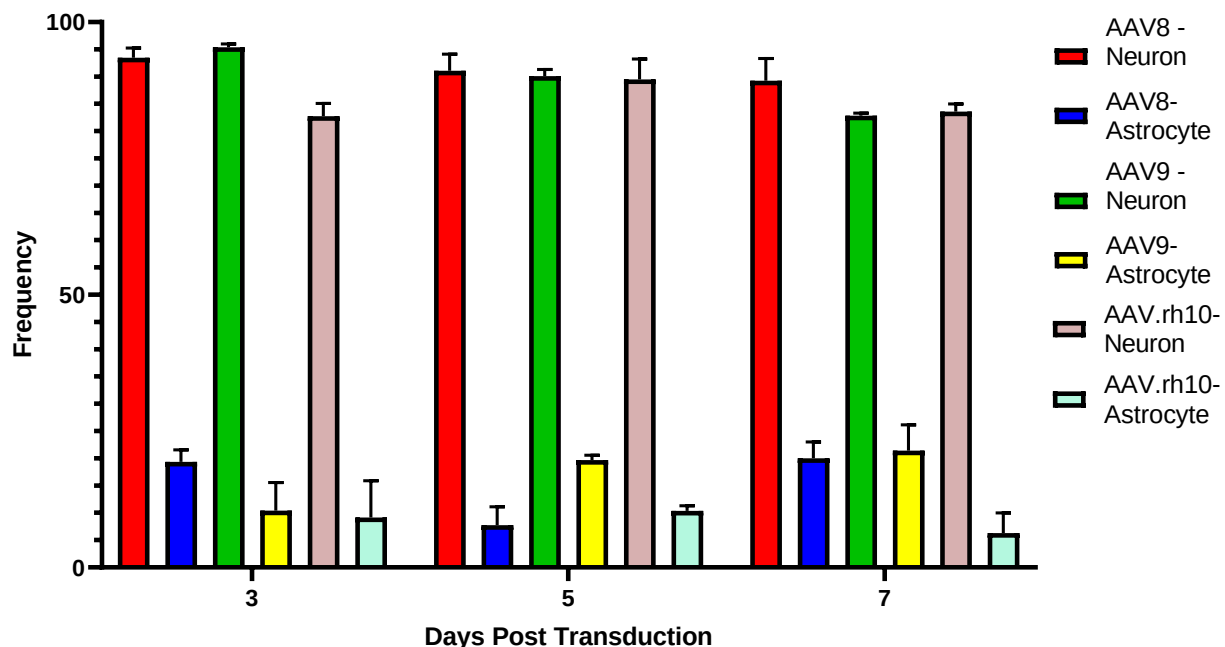


Figure 20. Transduction Efficacies of AAV8, AAV9, AAVrh.10 at MOI 1E4 at Day 3, 5, and 7.

Primary neuron cell cultures were administered AAV8, AAV9, AAVrh.10 at DIV 10 at a MOI of 1. 20 μ l of PBS containing AAVs were delivered to each well. Cell nuclei were stained with DAPI, and antibodies against MAP2 used to mark neurons, and antibodies against GFAP to mark astrocytes. eGFP expression was detected to identify transduced cells. Images counted using ImageJ Cell Counter plugin. Mean values of neuron transduction efficiencies (AAV8- Red, AAV9- Green, AAVrh.10 Pink) and rates of astrocyte transduction out of total cell numbers of transduced cells (AAV8- Blue, AAV9- Yellow, AAVrh.10- Teal), bars represent SEM (n=2).

4.2.4 Effect of Multiplicity of Infection on Neuron Transduction Efficiency

As biochemical experiments require cultures of high proportions of transduced cells ²²⁴, it is necessary to identify the MOI which results in the highest rates of transduction. AAVs have been shown to be capable of transducing the same cell multiple times, and therefore increasing the MOI of viruses delivered to the cells may not necessarily cause a significant increase in numbers of transduced cells, it may instead result in the increase of viral genomes per cell ¹⁶⁹.

To assess whether an increased MOI increases the transduction efficiencies in primary neuron cell cultures, AAVs were administered at a MOI of 1E4, 1E5, 1E6, and 1E7. Cultures were imaged at 3-days post-transduction, and at 5-days post-transduction, with staining for MAP2 to identify neurons and GFAP to identify astrocytes. eGFP expression located on the viral vector was used to identify transduced cells. The efficiencies of neuronal transduction were determined by counting the number of GFP-positive neurons and dividing the counts by the total number of neurons. Astrocyte transduction rates were determined by comparing the total number of transduced astrocytes to the total number of transduced cells.

Figure 21 shows fluorescent images of primary neuron cell cultures transduced with AAV8, AAV9, and AAVrh.10 at a MOI of 1E4 and 1E7. The images displayed here were collected at 3- and 5-days post-transduction. These figures show a very high proportion, ranging from 80-95% of transduced cells by all AAV serotypes at all MOIs assessed.

The results of counting images to determine neuronal transduction rates of each AAV serotype at each MOI, when imaged at 3- and 5-days post-transduction is shown in Figure 22. These results demonstrate that increasing the MOI beyond 1E4 does not yield a consistent statistically significant increase in the number of transduced neurons in primary cell cultures.

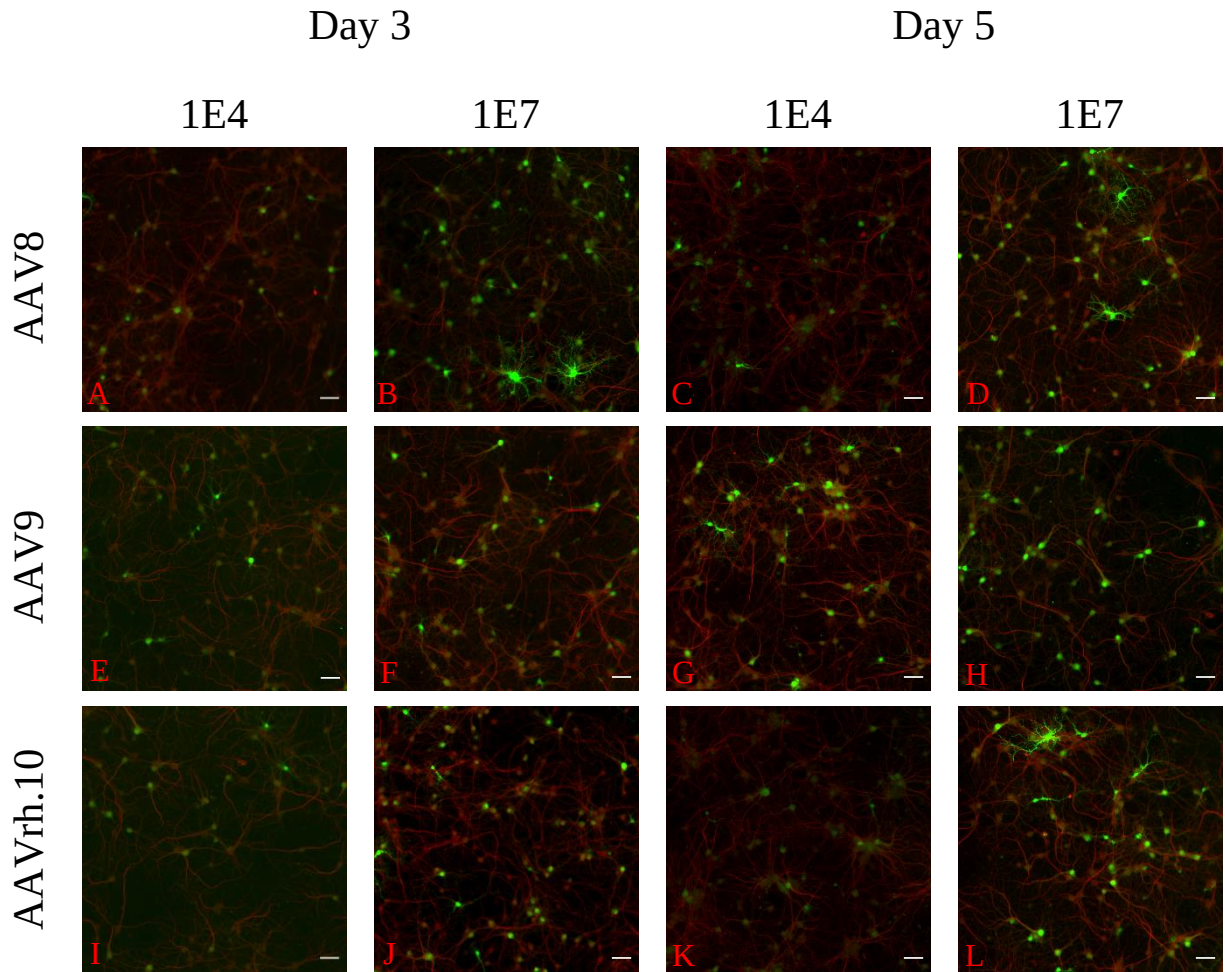
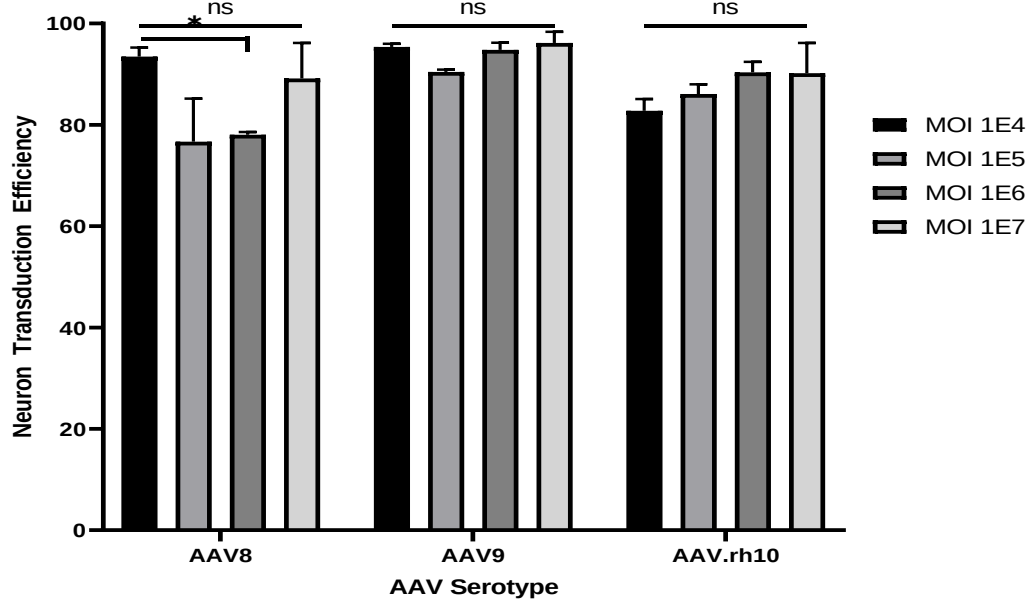


Figure 21. Fluorescent Images of Primary Neuron Cell Cultures Transfected at MOI 1E4 and 1E7 Imaged 3- and 5- Days Post-Transduction

Primary neuron cell cultures were transduced at DIV 10 with AAV8 (A, B, C, D), AAV9, (E, F, G, H), and AAVrh.10 (I, J, K, L). Cultures were transduced at a MOI of 1E4 or 1E7, and images were collected at 3 and 5-days post-transduction. Neurons were stained for MAP2 (Red), eGFP expression from transduced cells was detected using fluorescent microscopy (Green). Scale bars represent 50 μ m.

Neuron Transduction Efficiencies of MOIs at 3 Days Post-Transduction



Neuron Transduction Efficiencies of MOIs at 5 Days Post-Transduction

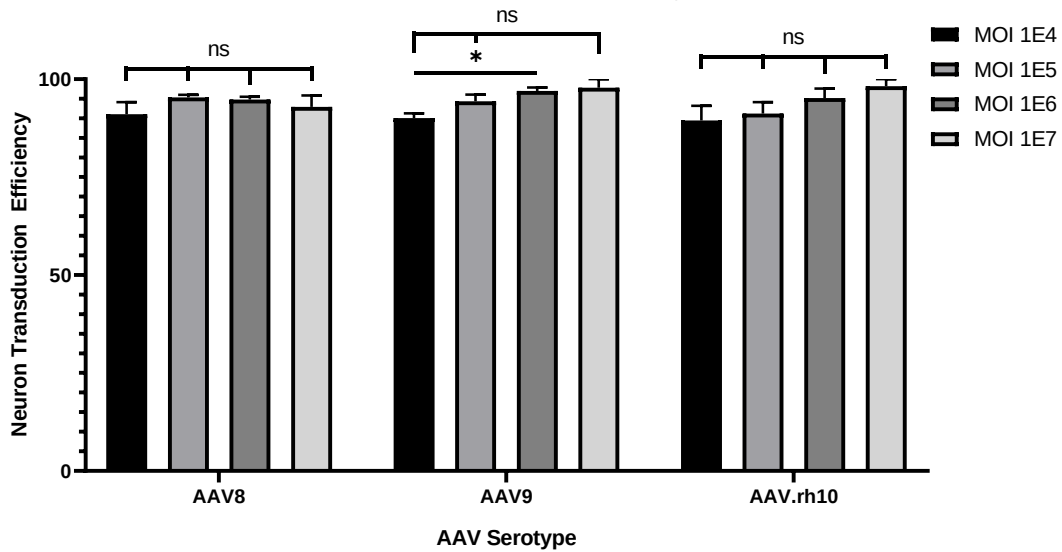


Figure 22. Transduction characteristics of AAV8, AAV9, AAVrh.10 at MOI 1E4, 1E5, 1E6, 1E7 at 3- and 5-Days Post-Transduction

Primary neuron cell cultures were administered AAV8, AAV9, AAVrh.10 at DIV 10 at each listed MOI. Cells were stained for MAP2 to mark neurons, and eGFP expression was detected to identify transduced cells. Images counted using ImageJ Cell Counter plugin. Mean values of neuron transduction efficiencies shown, with bars representing SEM (n=2). Student's t-test was used to analyze statistical significance: * = p-value of ≤ 0.05 ; ** = p-value of ≤ 0.005 ; ns = not significant (p-value > 0.05).

4.2.5 AAV Use in Primary Neuron Cell Cultures Does Not Induce Cell Death

The use of AAVs has been shown to be well-tolerated in various models, such as *in vivo* in mice¹⁶⁰, NHP^{164,167}, and humans^{214,226}, and *in vitro* in primary neuron cell cultures^{213,227}, and in immortalized embryonic stem cell cultures from humans and mice²²⁸. Although AAVs reportedly induce no, or little cytotoxic effects, there is an increasing number of publications reporting AAV toxicity due to specific AAV serotypes²²⁴, or due to transgene overexpression²¹³. Therefore, it is necessary to confirm that the use of AAVs is not toxic to the primary neuron cell cultures. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme, with a half-life of approximately 9 hours²²⁹, that when detected in the culture medium is used to measure plasma membrane integrity of cells in culture²¹⁷. Additionally, degenerating neurons²³⁰ and astrocytes²²⁹ are known to release LDH *in vitro* and *in vivo*.

Assessment of the cytotoxic effects was performed using the Promega CytoTox 96 kit, which uses an enzymatic reaction involving LDH to convert an introduced tetrazolium salt into a red formazan product, a light-emitting compound that can be detected using a plate reader.

Primary neuron cell cultures were plated at 50,000 cells per well in a total volume of 800 µl media volume in 24-well plate. Cultures at DIV 8 were administered either AAV8 or AAV9 containing CAMKII-shPrP or its -Empty control, or AAVrh.10 containing pAV-U6-RFP-shPrP, or the -Empty control vector, at a MOI of 1E4, in 20 µl total volume of PBS. Vehicle control wells were administered PBS + 5% glycerol to assess the cytotoxicity of the vehicle compound. Previous research has demonstrated that 5% glycerol in PBS in volumes up to 50 µl cause no adverse effects when administered to primary neuron cell cultures²²⁴, therefore all viruses were diluted so that 20 µl volume contained the required number of viruses for treatment. After incubation for 3 days, media was collected to detect LDH

released from cells, and cells lysed with a freeze-thaw cycles to determine maximum levels of LDH from each culture. Reactions were performed using in a 96-well plate and detected at an absorbance at a wavelength of 490nm. Percent cytotoxicity was calculated with the following equation:

$$\% \text{ Cytotoxicity} = \text{Media LDH} / \text{Cellular LDH}$$

As demonstrated in Figure 23, there are no significant differences in levels of cytotoxicity between any of the viral vectors and the control samples when AAVs were delivered at a MOI of 1E4. None of these treatments resulted in a significant increase in cytotoxicity when compared to vehicle-treated cells, or untreated cells, confirming that these viral particles and the vehicle used to deliver them do not induce cytotoxicity at this dose. Additionally, these results demonstrate that there is no significant difference in the cytotoxicity between shPrP-treated cells, and the empty control-treated cultures. Therefore, these results demonstrate that there are no detectable levels of increased toxicity induced in a primary neuron cell culture due to expression of the fluorescent protein, or from the expression of the shRNA sequence.

Next, to assess the cytotoxic effects of each AAV serotype at higher viral doses, AAVs were administered to primary neuron cell cultures at MOIs of 1E5 and 1E6. Demonstrated in Figure 24, an increase dose of AAV particles did not increase the levels of cell death found within each culture treated with any AAV at any MOI, as the percentages of cell death remain consistent around the levels of cell death seen in vehicle treated wells.

Overall, these results demonstrate that AAV8, AAV9, and AAVrh.10 are well-tolerated when administered to a primary neuron cell culture, even when the viruses are delivered at a high MOI of 1E6 in culture. Additionally, no cytotoxic effects were found in these cultures due to expression of either mStrawberry or eGFP, or from expression of the anti-PrP^C. Therefore, these viral particles provide a reliable, non-cytotoxic method of introducing genetic constructs into

primary neurons and astrocytes in primary neuron cell cultures.

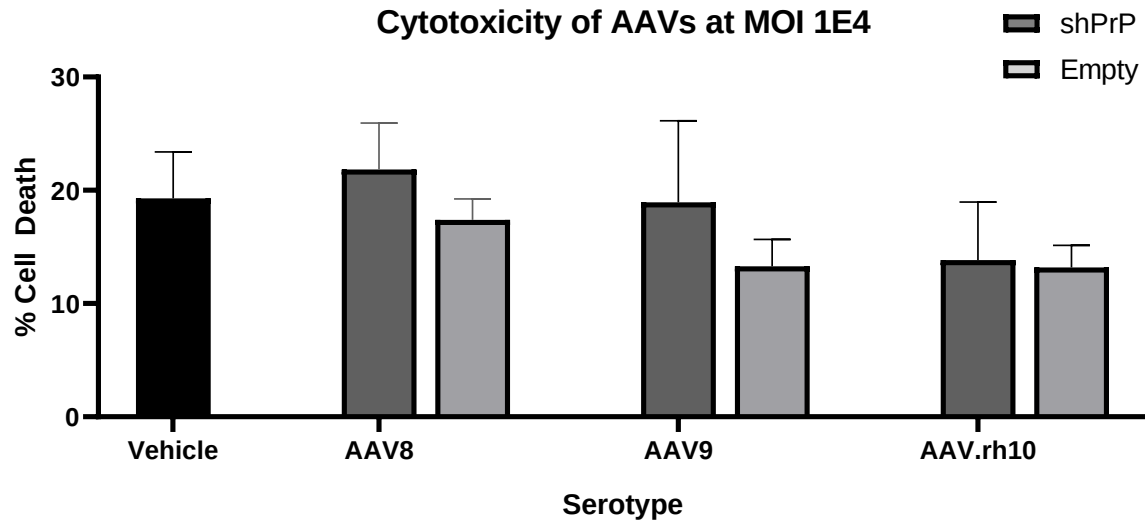


Figure 23. Cytotoxicity of AAV8, AAV9, AAVrh.10 at MOI 1E4

Primary neuron cell cultures at DIV 8 were administered AAV8, AAV9 containing the CamKII-shPrP vector or the -Empty control vector, or AAVrh.10 containing pAV-U6-RFP-shRNA, or the -Empty control vector, at MOI 1E4. Samples were collected 3 days following incubation, and levels of cell death were measured with the Promega CytoTox 96 kit. Mean values are plotted with bars representing SEM (n=2). Student's t-test was used to analyze statistical significance:

* = p-value of ≤ 0.05 ; ** = p-value of ≤ 0.005 , unmarked = p-value >0.05 .

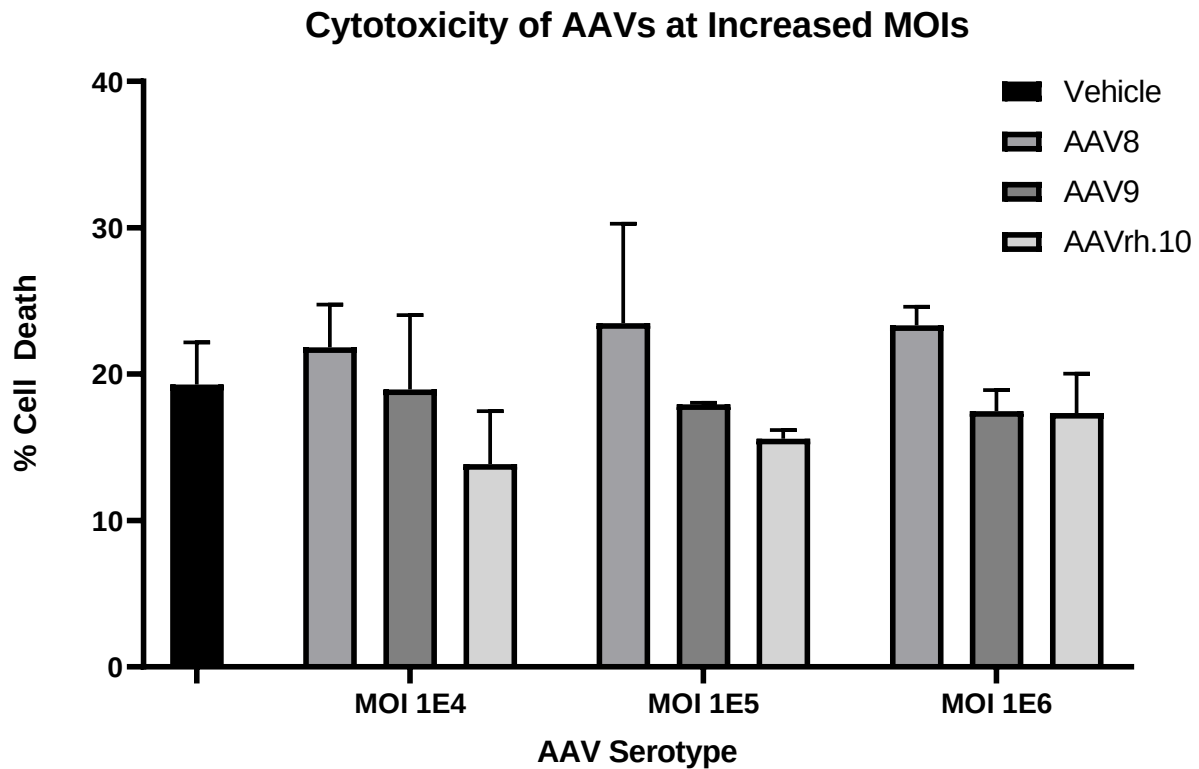


Figure 24. Cytotoxic Effects of AAVs at MOI 1E4, 1E5, and 1E6 in Primary Neuron Cell Cultures

Primary neuron cell cultures at DIV 8 were administered AAV8, AAV9 containing CamKII-shPrP, or the -Empty control vector, or AAVrh.10 containing pAV-U6-RFP-shRNA, or the -Empty control vector, at MOI 1E4, 1E5, and 1E6. Samples were collected 3 days following incubation, and levels of cell death were measured with the Promega Cytotox 96 kit. Mean values are plotted with bars representing SEM (n=2). Student's t-test was used to analyze statistical significance: * = p-value of ≤ 0.05 ; ** = p-value of ≤ 0.005 . unmarked = p-value > 0.05 .

4.2.6 Short Hairpin RNA Effects

To assess the levels of *PRNP* gene knockdown of an AAVrh.10-delivered anti-PrP^C shRNA sequence, primary neuronal cell cultures at DIV 8 were administered AAVrh.10 containing either pAV-U6-RFP-shRNA or the pAV-U6-RFP-Empty control vector at a MOI of 1E4. Control primary neuron cell cultures were administered the same volume of 5% glycerol in PBS vehicle, lacking any viruses. Cultures were maintained for 14, 18, 21 days, and RNA samples were collected at each timepoint using the Norgen Total RNA kit. Following reverse transcription, levels of *PRNP* and *GAPDH* gene expression were measured using rtPCR, with probes against *PRNP*, and *GAPDH*.

As shown in Figure 25, primary neuron cell cultures that received AAVrh.10 containing pAV-U6-RFP-shRNA showed a substantial increase in the threshold cycle (C_T) values for the *PRNP* gene, compared to cultures that received the pAV-U6-RFP-Empty control vector, or vehicle. Additionally, levels of *GAPDH* expression across all cultures remained fairly consistent, suggesting that the levels of *PRNP* gene knockdown is due to the short hairpin sequence.

The levels of gene knockdown are further demonstrated in Figure 26, which displays the ΔC_T values, comparing the levels of gene knockdown of AAVrh.10-shPrP, AAVrh.10-Empty control vector, and the vehicle treated cultures, relative to changes of *GAPDH* gene expression. A significant reduction of *PRNP* gene expression, relative to *GAPDH*, is seen in primary neuron cell cultures treated with AAVrh.10 containing an anti-PrP^C shRNA sequence.

Overall, these results demonstrate that an AAVrh.10-delivered anti-PrP^C sequence delivered at a MOI of 1E4, is capable of inducing high levels of gene knockdown, compared to control viral vectors, and vehicle treated cultures.

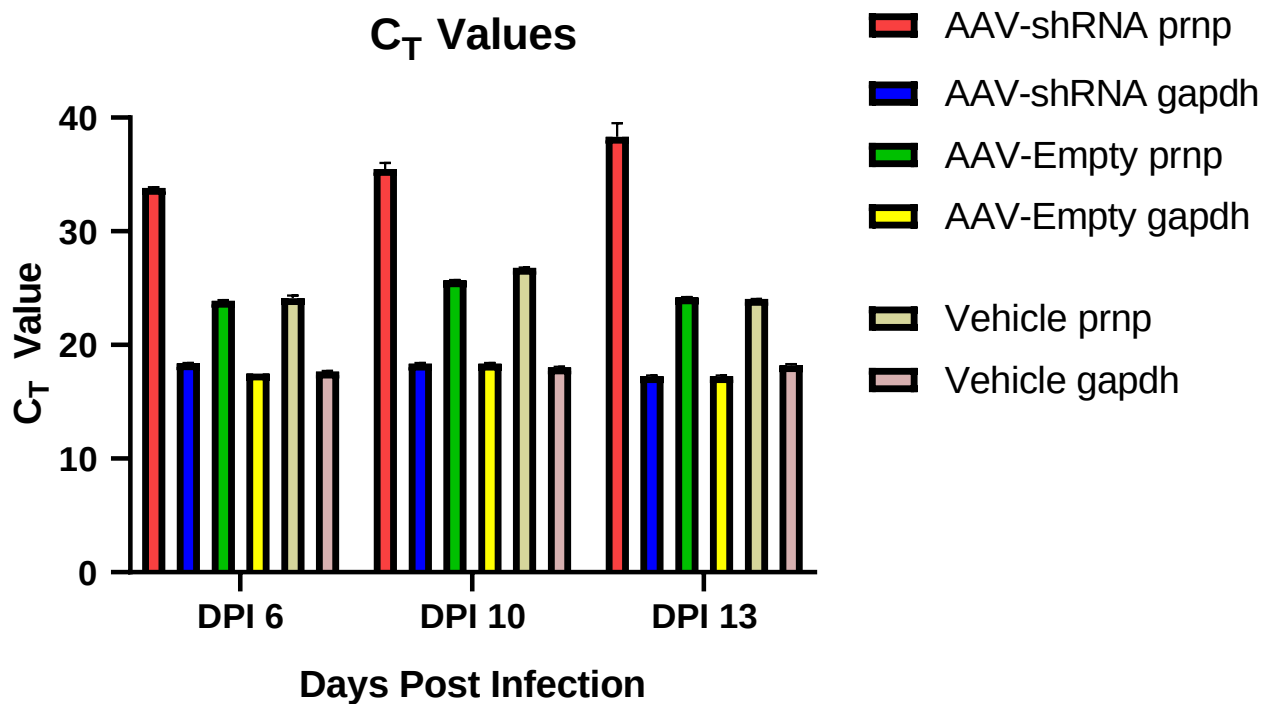


Figure 25. C_T Values of *PRNP* and *GAPDH* Gene Expression in Primary Hippocampal Neuron Cultures

C_T values of cellular prion gene expression following AAVrh.10-shRNA or -Empty control treatment of primary neuron cell cultures at MOI 1E4. Samples were collected at 6, 10, or 13-days post-transduction. Values determined using rtPCR to measure C_T values of *PRNP* in each culture, and *GAPDH* gene expression as an internal reference gene. Mean C_T values are plotted, with bars representing SEM (n=3).

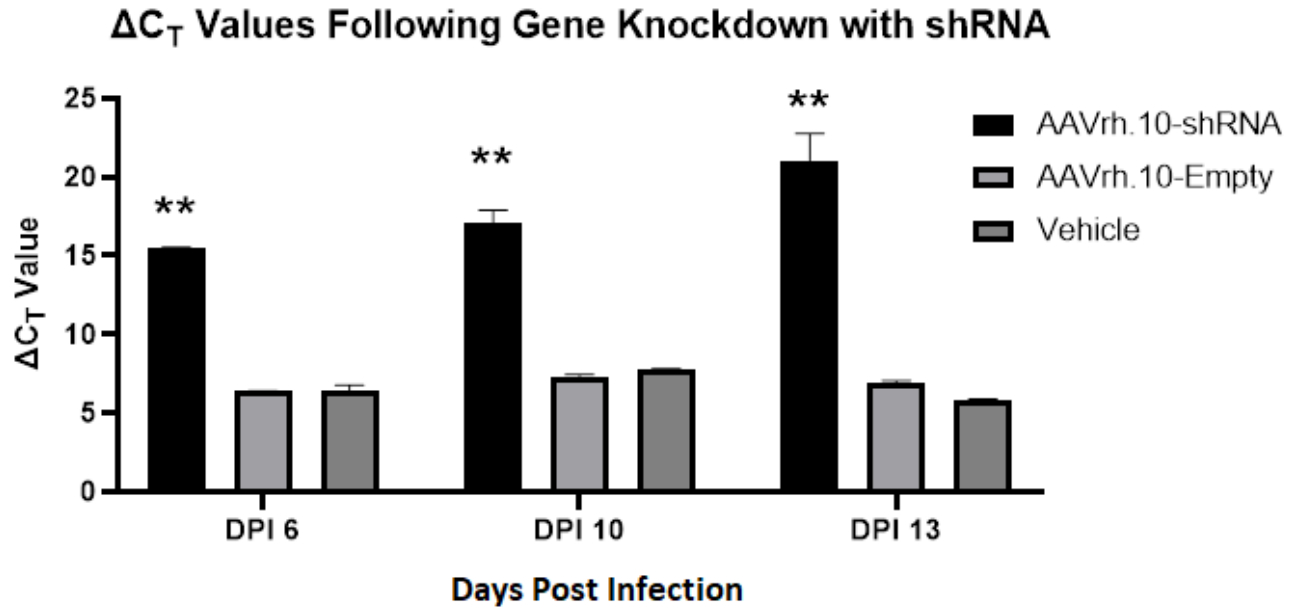


Figure 26. ΔC_T Values of AAVrh.10-shRNA *PRNP* Gene Knockdown

ΔC_T values shown at DPI 6, 10, and 13 is in comparison to the AAVrh.10-Empty negative control gene expression measurements, and vehicle treated wells. Average values calculated using the ΔC_T method following identification of C_T using probe-based rtPCR for *PRNP* gene expression, and *GAPDH* internal reference gene. Mean ΔC_T values are plotted, with bars representing SEM (n=3). Student's t-test was used to analyze statistical significance: ** = p-value of ≤ 0.005 compared to AAVrh.10-Empty.

4.3 Delivery of AAVrh.10 Via Neonatal Intravenous Injections Rationale

The discovery that certain AAV serotypes, specifically AAV9 and AAVrh.10¹³⁶, were capable of bypassing the blood brain barrier broadened the potential uses of AAVs, as this allowed the intravenous delivery of AAVs to transduce cells within the brain and other areas of the CNS^{137,166,198}. However, systemic delivery of AAVs also results in the transduction of cells and organs throughout the body of the animal¹⁹⁸. Therefore, to assess AAVrh.10 as a potential gene-therapy delivery vector it is necessary to demonstrate that AAVrh.10 is capable of establishing transduction in the brain following intravenous injection, and to assess the transduction of non-target cells in other organs. Once transduction of the brain and other organs has been established, it is necessary to monitor the transduced animals over an extended period of time to assess the transduction efficiencies over time, as this will allow assessment of the levels of long-lasting transgene expression in transduced cells.

The AAVrh.10 viral vectors used in this study contain an anti-PrP^C shRNA sequence designed to reduce the levels of prion protein expression in the transduced cells. Groups of mice that received neonatal intravenous injections of AAVrh.10 containing either an anti-PrP^C shRNA sequence or an Empty control sequence were infected with RML prions at 6 weeks of age to assess transduction efficiencies over time in prion-infected animals.

4.3.1 Experimental Outline of Neonatal Intravenous AAVrh.10 Injections

To assess the transduction efficiencies of AAVrh.10 following neonatal intravenous injection, groups of mice were delivered 6E10 AAVrh.10 viral genomes (vg) containing an anti-PrP^C shRNA sequence or an Empty control sequence. Both AAVrh.10 vectors express the mStrawberry protein under control of the CMV promoter. The negative control groups consisted of mice that were delivered 50 µl of PBS containing no viral vectors, or mice that received no injections at all. The following table shows the numbers of mice in each group in the following experiments.

Table 13. Table of Treatment of Intravenous AAV Injection

Treatment	AAVrh.10-shRNA	AAVrh.10-Empty	PBS Only	No Injection
Number of Mice	36	36	12	12

Table outlines the four treatment groups of mice in the AAVrh.10 injections. Mice received neonatal intravenous injection of AAVrh.10 containing either an anti-PrPC shRNA sequence or an Empty control sequence. Negative control groups received injections of PBS or no injections at all. The total number of mice used in study is 96.

Neonatal mice of 1-2 days of age received intravenous injections of AAVrh.10 via the superficial temporal vein. Following intravenous injections, the superficial temporal vein which is indicated by the white arrows in Figure 27 becomes blanched and difficult to see, as shown by the green arrow in Figure 27. Success of the injection procedure is indicated by the blanching of the superficial temporal vein, and a rapid recovery following the injection procedure. A total of two mice that received injections displayed complications and had to be euthanized due to ethical concerns. However, this was much less than the expected 15% error rate.

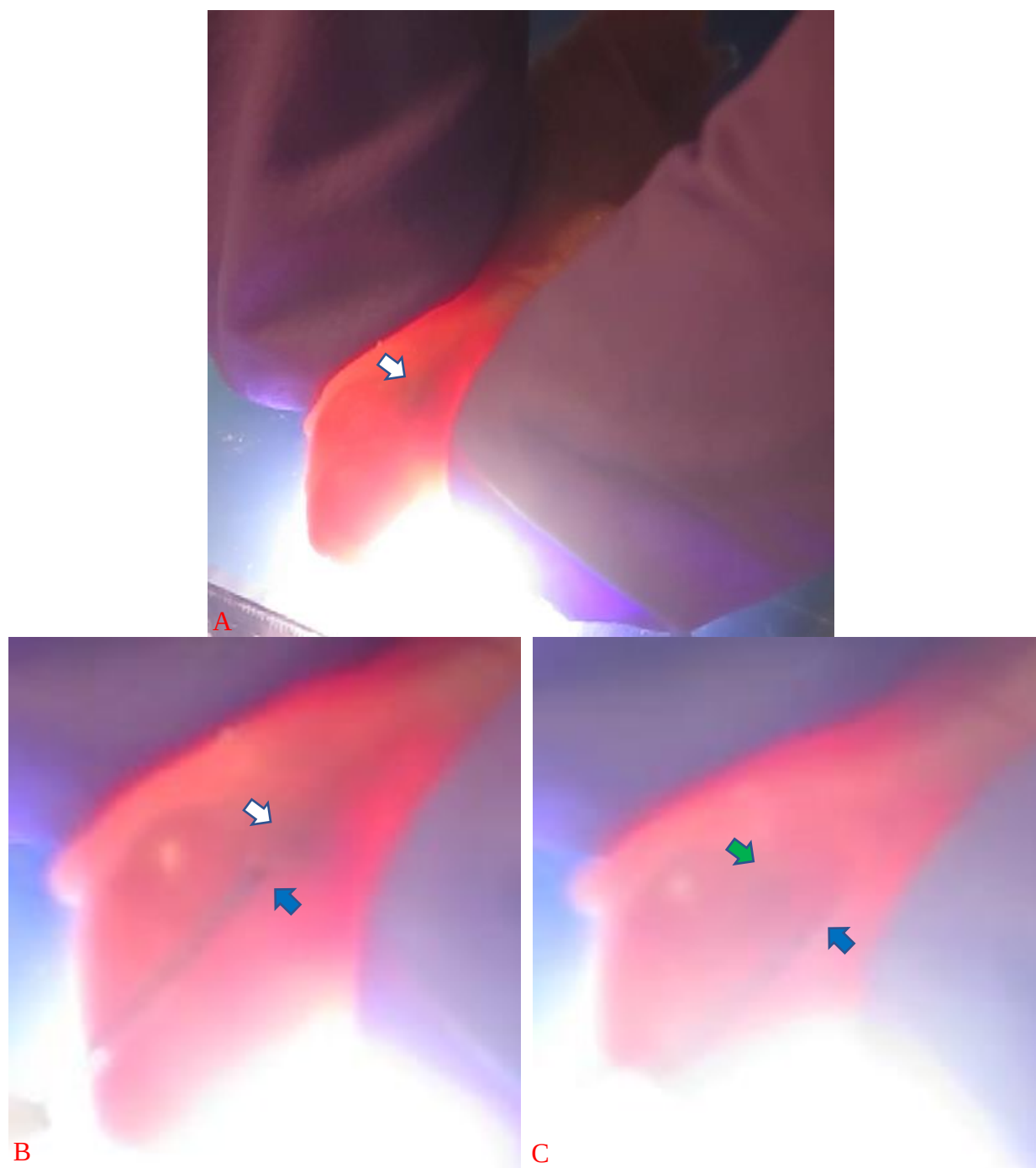


Figure 27. Images of Intravenous AAV Injection Procedure

Intravenous injection of neonatal mice. A) neonatal mouse is held with fingers on the top and bottom of the head. White arrow indicates temporal facial vein. B) Close-up image showing vein prior to receiving injection. White arrow indicates temporal facial vein, Blue arrow indicates needle tip. C) Close-up image showing vein immediately following injection. Green arrow indicates blanched vein, Blue arrow indicates needle tip.

4.3.2 Confirmation of Primary Antibody Specificity for Immunohistochemistry

Two primary antibodies were tested to compare the levels of specificity against mStrawberry, the red fluorescent protein expressed from the pAV-U6-RFP viral vector. Cultures of HEK293T cells were transfected with pAV-U6-RFP plasmids containing either the shRNA sequence, or the Empty control sequence. HEK293T cell cultures with no transfected plasmids were included as a negative control. Whole brain homogenate from embryonic mouse brains was used as a negative control to test the second antibody, after finding non-specific binding in the first antibody measured. β -Actin was included as a protein loading control.

Specific binding of the antibody should yield a single band at approximately 27 kDa, as mStrawberry has a molecular weight of 26.6 kDa. β -Actin has a molecular weight of 42 kDa. No bands for mStrawberry should be identified in the non-transfected cultures, or in the murine whole brain homogenates, as these samples should not contain mStrawberry.

As demonstrated in Figure 28, the antibody on the left appears to detect a high molecular weight protein of approximately 150 kDa in both transfected HEK293T cultures, and the non-transfected control cultures. However, the right side of Figure 28 demonstrates the specific binding of the second antibody tested, with a band found at approximately 27 kDa in only the transfected HEK293T cell cultures that should be expressing mStrawberry. No cross-reactivity is seen within the band containing the whole brain homogenate, therefore the second tested antibody, ab167453 from Abcam, can be reliably used for further immunohistochemical analysis.

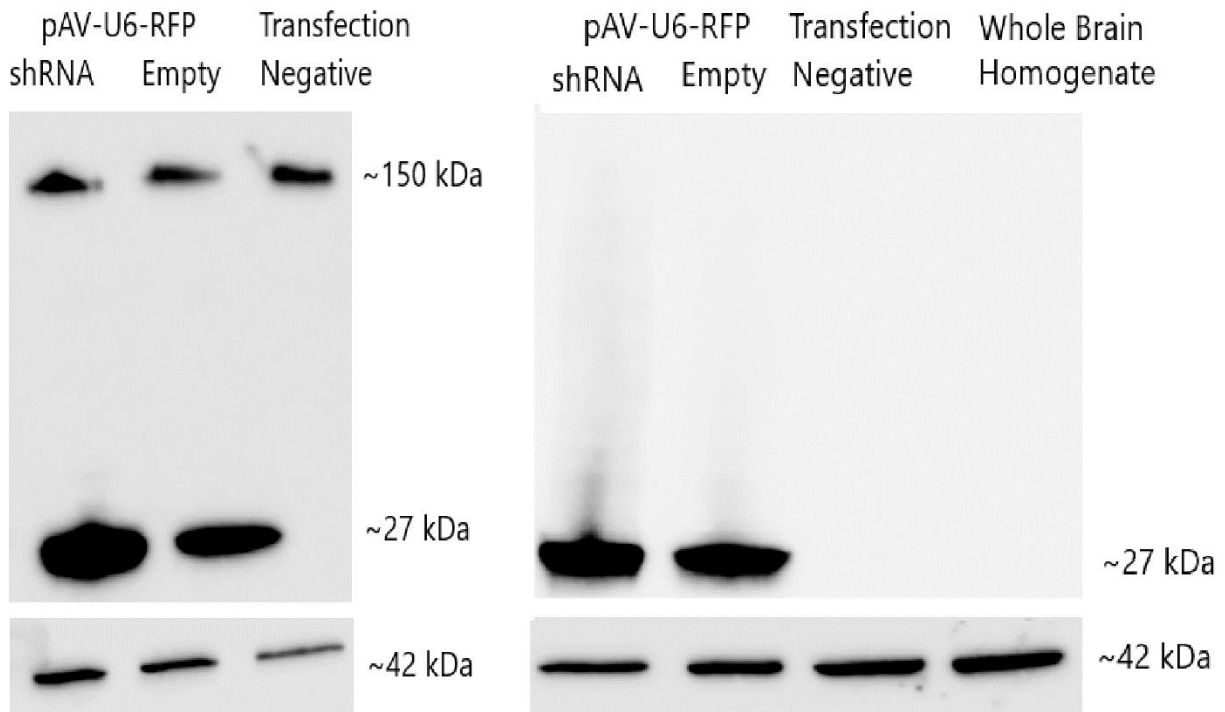


Figure 28. Western Blot Images of two Antibodies Tested for Specificity Against mStrawberry

Left: Image of anti-RFP (Fisher Scientific R10367) tested against proteins extracted from HEK293T cells transfected with pAV-U6-RFP containing either the shRNA sequence against the prion protein, or the Empty control vector, both expressing mStrawberry. Transfection Negative is protein extracted from non-transfected HEK293T cells. Right: Image of second antibody tested, ab167453 from Abcam, against protein extract from HEK293T cells transfected with pAV-U6-RFP containing either the shRNA sequence against the prion protein, or the Empty control vector. Transfection Negative is protein extracted from non-transfected HEK293T cell cultures. Whole Brain Homogenate is protein extracted from the homogenate of neonatal mouse brain. 150 kDa bands identifies non-specific binding of the antibody on the left, but not on the right. 27 kDa marks mStrawberry protein (26.6 kDa MW). 42 kDa marks β -Actin detection, detected as an internal loading control protein.

4.3.3 Immunohistochemical Staining Shows AAVrh.10 Crosses the Blood Brain Barrier and Infects Neurons and Astrocytes Following Neonatal Intravenous Injection

Confirmation of transduction was first established when mice were 60 days of age. Brain tissue and organ samples were collected from 2 mice that received AAVrh.10-shRNA and 2 mice that received AAVrh.10-Empty injections. Tissue samples from mice that did not receive AAV injections were used for negative controls. Transduction was visualized by performing DAB staining using primary antibodies targeting mStrawberry expression in the transduced cells. Mouse brain slices were stained using primary antibodies targeting mStrawberry, with DAB staining to identify transduced cells. Hematoxylin counterstain was performed to identify nuclei of cells to count total cell numbers. The ImageJ Cell Counter plugin was used to count numbers of transduced cells and cell nuclei.

Sections of the CA1 hippocampal region and the thalamus were counted to assess the levels of transduction in the brain. The CA1 hippocampal region is known to consist mainly of neurons²⁰⁰, and a range of cell types can be found within the thalamus²³¹. Additionally, previous research has demonstrated transduction of cells in these regions following intravenous AAVrh.10 and other AAV injections^{232,233}.

As demonstrated in Figure 29, transduction of cells can be seen in the brains of mice that received AAVrh.10-shRNA or AAVrh.10-Empty injections, and no staining of cells can be seen in the negative control brain tissue of mice that did not receive injections. Transduced neurons (Red arrows) and Astrocytes (Green arrows), based on morphology, can be identified 60 days following transduction.

To calculate the proportion of stained cells found within the CA1 hippocampal region, a 600 µm segment was selected. Within the thalamus, a 500 µm by 650 µm region was selected for imaging. Two images were collected, from different brains, to be counted. The total number of cells in each image were counted by counting the total number of cell nuclei. The total number of stained cells were counted by identifying cells expressing DAB staining. The total numbers of cells counted within each image is shown in the table below.

Table 14. Results of Counting Brain Slices at Day 60

	CA1 Hippocampal Region				Thalamus			
	Total Cells		Transduced Cells		Total Cells		Transduced Cells	
AAVrh.10-shRNA	196	155	7	3	461	622	5	8
AAVrh.10-Empty	178	153	7	2	563	544	7	2

The percentage of transduced cells was calculated by the following equation:

$$\% \text{ Transduced Cells} = ([\text{DAB-Positive Cells}] / [\text{Number of Cell Nuclei}]) * 100$$

The percentage of transduced cells counted within the CA1 hippocampal region and the thalamus are shown in Figure 30. These images show transduction rates of around 3% of cells within the CA1 hippocampal region, and around 1% transduction rate of cells found in the thalamus. Additionally, these images show no difference in the transduction rates of the AAVrh.10 vectors carrying either the anti-PrP^C shRNA or the Empty control sequence.

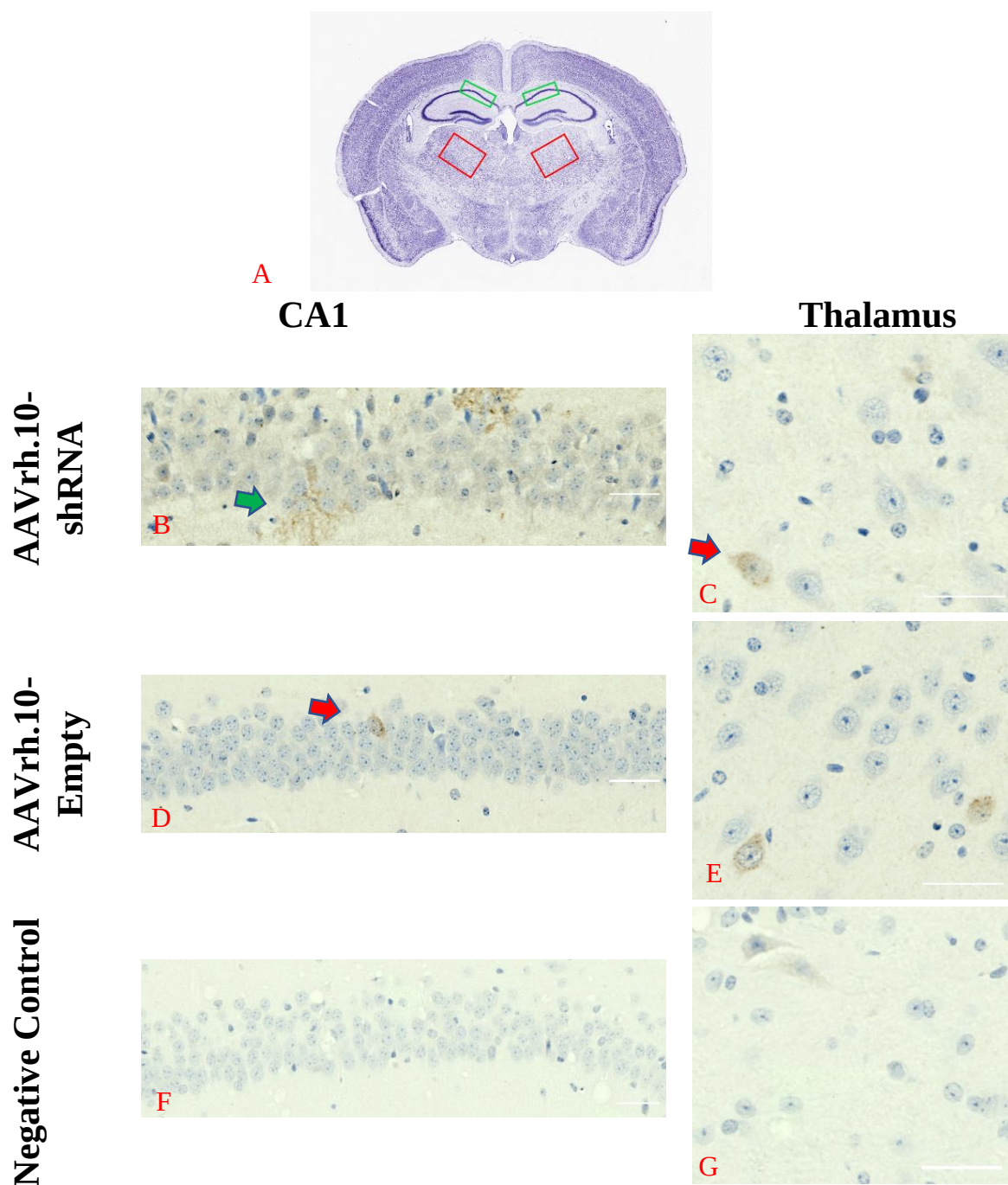


Figure 29. Immunological Staining Showing Transduction of Cells in the CA1 Hippocampal Region and Thalamus

DAB staining of brain tissues collected from mice 60 days following neonatal intravenous injections performed using primary antibody targeting mStrawberry expression of transduced cells within the CA1 region and thalamus. A) Reference image from Allen Brain Atlas outlining CA1 regions (Green Squares) and Thalamus regions (Red Squares). CA1 regions of mouse that received: B) AAVrh.10-shRNA, D) AAVrh.10-Empty, or F) No injections. Thalamus regions of mouse that received C) AAVrh.10-shRNA, E) AAVrh.10-Empty, or G) No injections. Red arrows indicate Neurons, Green arrows indicate Astrocytes. Scale bars represent 50 μm.

CA1 and Thalamus Transduction

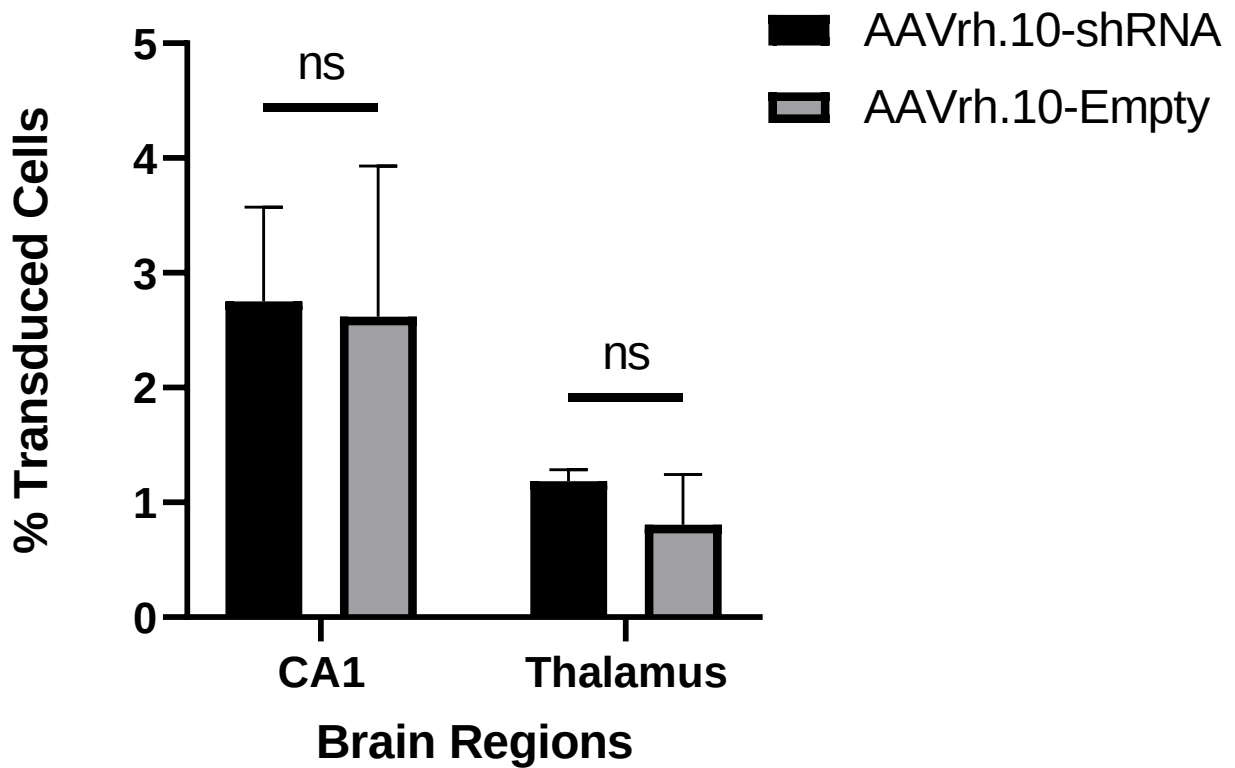


Figure 30. Transduction Rates of Cells in the CA1 Hippocampal and Thalamic Regions 60 Days Post-Transduction

Results of DAB staining for mStrawberry expression in the CA1 hippocampal and thalamic regions of brains from mice that received AAVrh.10 intravenous injection at day 1-2 old. AAVrh.10-shRNA are mice that received the anti-PrP^C shRNA; AAVrh.10-Empty are mice that received the Empty control sequence. Images were manually counted to determine transduction rates. Data of average transduction efficiencies is plotted with bars representing SEM (n= 2). Student's T-test was used to assess statistical significance. ns = no statistical significance between any of the results from each group.

4.3.4 Immunohistochemical Staining Shows Neonatal Intravenous Injection of AAVrh.10

Results in the Transduction of Cells Within the Liver, Lung, and Spleen of Mice

Although intravenous injection of AAVrh.10 into neonatal mice is known to induce strong transgene expression throughout the brain ^{169,198}, it is also known to transduce organs and cells throughout the body at varying levels, as shown in Table 15.

Table 15. Transduced Cells and Organs of AAVrh.10 Following Intravenous Injection into Neonatal Mice

Organ / Cells	Level of Transduction
Adipose tissue	High ²³⁴
Renal tubules of kidney	High ²³⁴
Pancreas	High ²³⁴
Skin	High ²³⁴
Liver	Low ^{168,234} , Moderate ²³⁵
Cardiac muscles of heart	Mild ²³⁴

Assessment of initial transduction within the livers, lungs, and spleens of mice was also performed when mice were at 60 days of age. DAB staining was performed using primary antibodies targeting the mStrawberry expression in the transduced cells.

The immunohistochemical analysis of the livers, lungs, and spleens of mice 60 days following the neonatal intravenous injection of AAVrh.10 can be seen in Figure 31. Transduction of the livers and lung of mice can be seen in mice that received AAVrh.10-shRNA and Empty, with no apparent differences in the transduction between these groups. No stained cells can be identified in the negative control tissues collected from mice that did not receive injections. Transduced cells cannot be found in the spleens of mice that did not receive AAVrh.10 injections, either PBS-injected mice or mice that received no injections. However, background staining is apparent within the negatively controlled spleen samples, presumably due to the high

levels of endogenous peroxidases found within the spleen which react with the DAB compound used to perform IHC of the tissues^{236,237}, leading to the appearance of staining within the tissues.

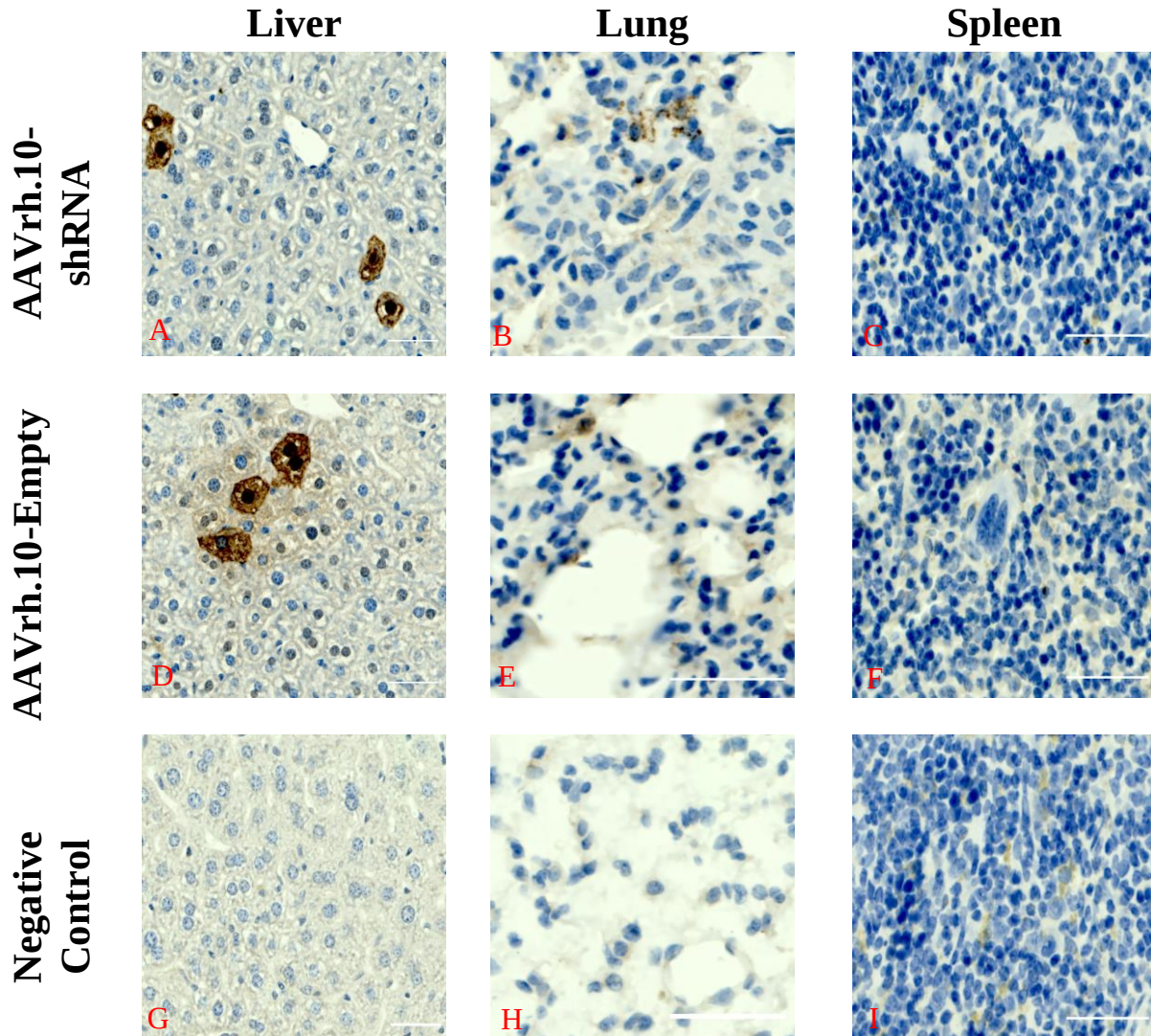


Figure 31. Transduction of Liver, Lung, and Spleen of Mice 60 days Following Intravenous Neonatal AAVrh.10 Injections

DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the Liver, Lungs, and Spleens of mice 60 days following neonatal intravenous injection of AAVrh.10 containing anti-PrP^C shRNA or an Empty control sequence, both driving expression of mStrawberry within transduced cells. Mice receiving PBS injections were used as negative controls. Images of liver transduction: mice that received A) AAVrh.10-shRNA, D) AAVrh.10-Empty, G) no injection. Lung images of mice that received: B) AAVrh.10-shRNA, E) AAVrh.10-Empty, H) no injection. Spleen images of mice that received: C) AAVrh.10-shRNA, F) AAVrh.10-Empty, I) no injection. Scale bars represent 50 μ m.

4.3.5 Outline of Tissue Collection Timepoints to Demonstrate Long-Term Transgene

Expression in Mice following Neonatal Intravenous AAVrh.10 Injection

Mice that received neonatal intravenous injections of AAVrh.10 containing an anti-PrP^C shRNA sequence or an Empty control sequence were monitored over a period of 200 days total. Tissue samples were collected at the following timepoints: 60 days post-transduction, 120 days, 150 days, and 200 days. The following table details the number of samples collected for histological analysis at each of the timepoints.

Table 16. Number of Histological Samples Collected at Each Timepoint

Treatments	Timepoints			
	60	120	150	200
AAVrh.10-shRNA	2	3	3	3
AAVrh.10-Empty	2	3	3	3
PBS Injected	1	1	1	1
Non-Injected	1	1	1	1

4.3.6 Neonatal Intravenous Injection of AAVrh.10 Results in Transduction and Long-Term Transgene Expression in Cells of the CA1 Hippocampal Region

The hippocampus is known to be a major area of disease pathology during prion disease ²³⁸. Specifically, the *Corno Ammonis* (CA1) layer of the hippocampus is of interest. This region of the hippocampus contains a population of neurons that are of relatively high homology, with low abundance of other cell types ⁶⁸. Furthermore, intravenous injection of AAVrh.10 has been shown to result in relatively high levels of transduction in the hippocampus, with higher levels of transgene expression compared to other areas of the brain ¹³⁶.

Mice received injections as outlined in section 2.14, and tissue samples of the CA1 hippocampal region were collected at 60, 120, 150, and 200 days post-transduction following neonatal intravenous injection of AAVrh.10-shRNA or AAVrh.10-Empty are shown in Figure 32. These images show that transduced cells are visible at all time points. The majority of cells appear to be neurons, however, transduced cells with the distinct appearance of astrocytes can be seen at both 60 days post-transduction, as indicated by the green arrows (Figure 32).

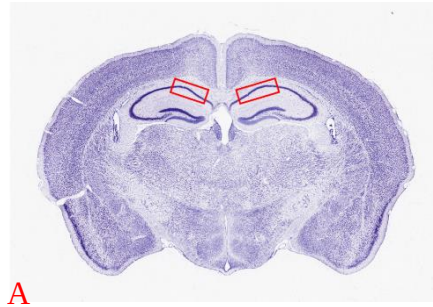
To calculate the percentage of transduced cells within the CA1 hippocampal region, a 600 μ m section was chosen for counting. Cell nuclei were used to count total cell numbers, and presence of DAB staining indicated stained cells. 2-3 images were chosen for each timepoint, each from a different mouse brain (n=2-3). The total number of cells and the total numbers of transduced cells collected from each timepoint are shown below.

Table 17. Counting Results of the CA1 Hippocampal Region 60, 120, 150, and 200 Days Post-Transduction

Days Post-Transduction	AAVrh.10- shRNA						AAVrh.10- Empty			
	Total Cells			Transduced Cells			Total Cells		Transduced Cells	
60	196		155	7		3	178	153	7	2
120	160	179	140	3	3	4	138	139	3	6
150	140		176	3		12	127	132	11	7
200	151		144	8		12	142	146	2	9

The transduction rates of cells in the CA1 hippocampal region at each time point are shown in Figure 33. The transduction efficiencies within this region remained relatively stable at 3-7% for all time points. Additionally, there is no significance difference between the transduction efficiencies of AAVrh.10 containing either the anti-PrP^C shRNA sequence or the Empty control sequence.

Overall, these results demonstrate that neuronal transduction can be identified in the brains of mice over a length of 200 days following neonatal intravenous injection of AAVrh.10.

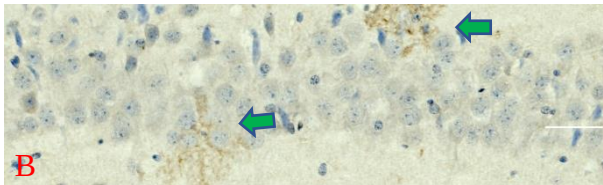


A

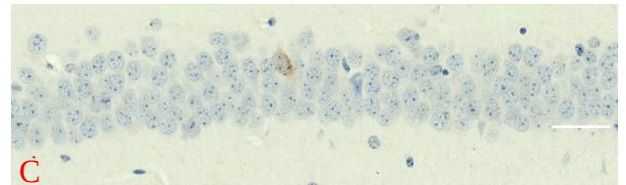
AAVrh.10-shRNA

AAVrh.10-Empty

60 Days

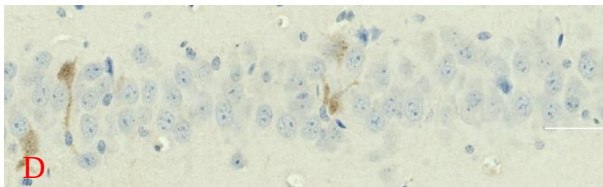


B

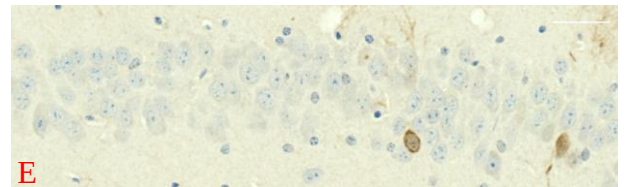


C

120 Days

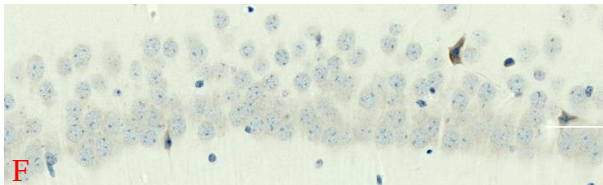


D

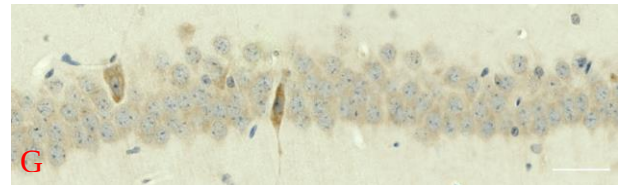


E

150 Days

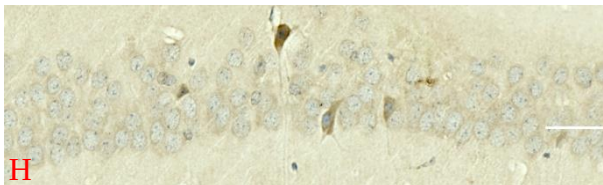


F

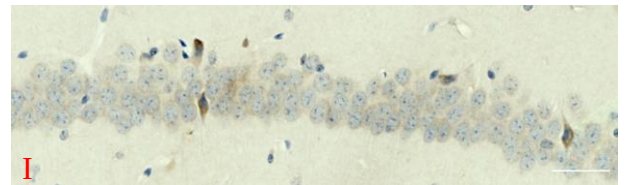


G

200 Days



H



I

Figure 32. Images of CA1 Regions of Mouse Hippocampi at 60, 120, 150, and 200 Days Post-Transduction

DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the CA1 hippocampal region in the brains of mice that received AAVrh.10 injections. Second row; mice 60 days after injections of B) AAVrh.10-shRNA, C) AAVrh.10- Empty. Third row; Mice 120 days following injection of D) AAVrh.10-shRNA, E) AAVrh.10-Empty. Fourth row; mice 150 days following injection of AAVrh.10-shRNA, G) AAVrh.10-Empty. Bottom row; mice 200 days following injection of H) AAVrh.10-shRNA, I) AAVrh.10-Empty. Green arrows identify transduced astrocytes. Scale bars represent 50 μ m.

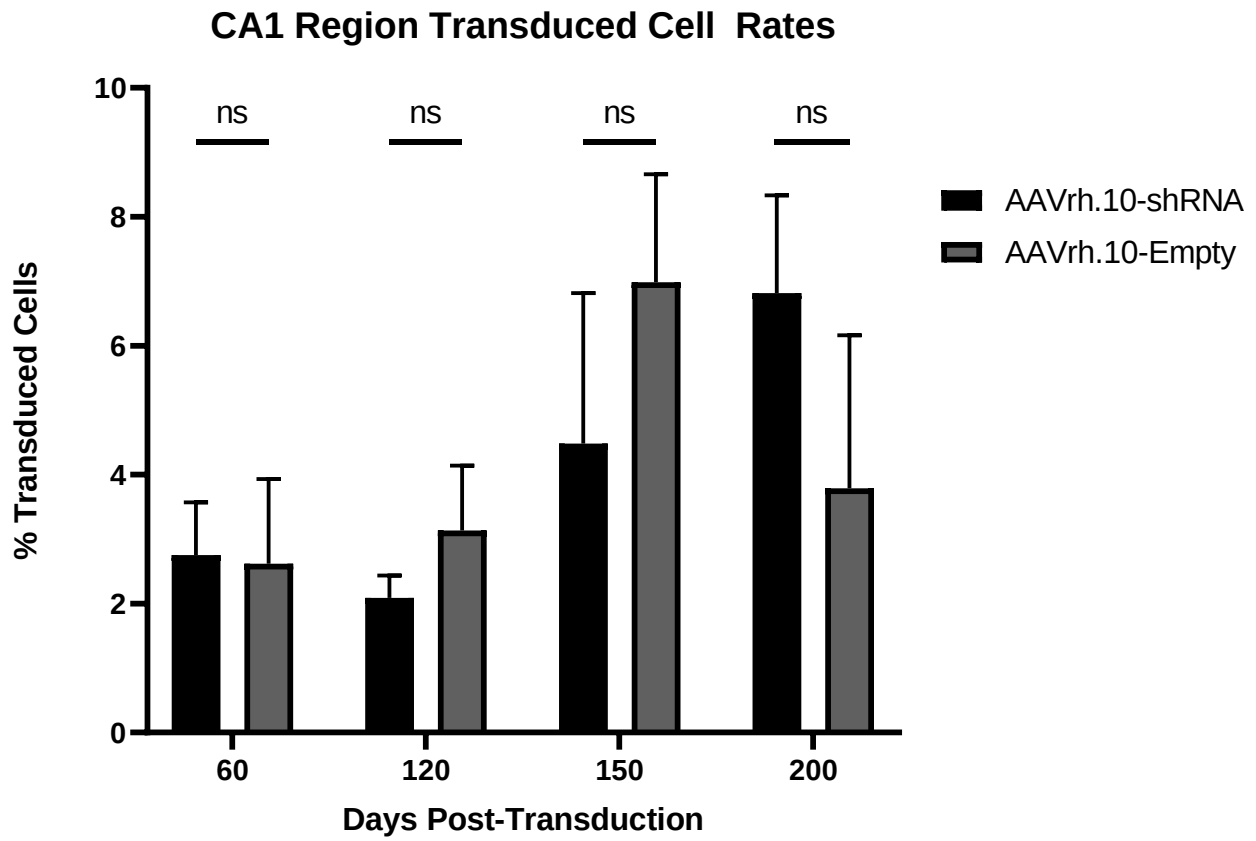


Figure 33. CA1 Hippocampal Region AAVrh.10 Transduction Rates Over Time

Results of DAB staining for mStrawberry expression in the CA1 hippocampal region of brains from mice that received AAVrh.10 intravenous injection at day 1-2 days of age. AAVrh.10-shRNA is mice receiving the ant-PrP^C shRNA; AAVrh.10-Empty is the empty control sequence. A 600 μ m section of the CA1 hippocampal region from each mouse were counted to determine the numbers of transduced cells, and total cell numbers to find the transduction rate. Data of average transduction efficiencies is plotted with bars representing SEM (n= 2-3). Student's T-test was used to assess statistical significance. ns = no statistical significance between any of the results from each group.

4.3.7 Neonatal Intravenous Injection of AAVrh.10 Causes Transduction and Long-Term Transgene Expression in the Thalamus of the Mouse Brain

Previous research has described AAVrh.10 as having a relatively low transduction rate of cells within the thalamus, with a preference for transducing neurons in this region^{136,168}. However, neuronal transduction within this region may be of interest for prion disease, as signs of prion disease are detectable within this region as early as 40 days following infection with prions⁶⁷.

Mice received injections as outlined in section 2.14, and tissue samples were collected at timepoints 60, 120, 150, and 200 days post-transduction. Images were collected from the central thalamic region, spanning the ventral posteromedial nucleus, posterior nucleus of thalamus, and nucleus ventralis lateralis regions of the thalamus, shown in Figure 34. These images show transduction of neuronal cells (Red arrows), and presumably astrocytic cells (Blue arrows), based on their morphologies up to 200 days post-transduction.

To calculate the percentage of transduced cells within the thalamus, a 600 μm x 500 μm section was chosen for counting. Cell nuclei were used to count total cell numbers, and presence of DAB staining indicated stained cells. 2-3 images were chosen for each timepoint, each from a different mouse brain (n=2-3). The total number of cells and the total numbers of transduced cells collected from each timepoint are shown below.

Table 18. Results of Counting Thalamus Transduction by AAVrh.10 60, 120, 150, and 200 Days Post-Transduction

Days Post-Transduction	AAVrh.10- shRNA						AAVrh.10- Empty			
	Total Cells			Transduced Cells			Total Cells		Transduced Cells	
60	461		622	5		8	563	544	7	2
120	572	401	602	3	4	6	590	614	5	3
150	329		536	10		6	514	430	5	6
200	574		551	5		15	809	680	6	6

The transduction rates of cells in the thalamus at 60, 120, and 200 days post-transduction are shown in Figure 35. These results demonstrate that mStrawberry expression can be identified in 1-3% of the cells in the thalamus over a period of 200 days post-transduction. Additionally, there were no significant differences between the transduction rates of AAVrh.10 containing either the anti-PrP^C shRNA, or the Empty control sequence at any of the timepoints.

Overall these results further demonstrate that neonatal intravenous injection results in transduction and long-term transgene expression within the mouse brain, with transgene expression lasting at least 200 days post-transduction.

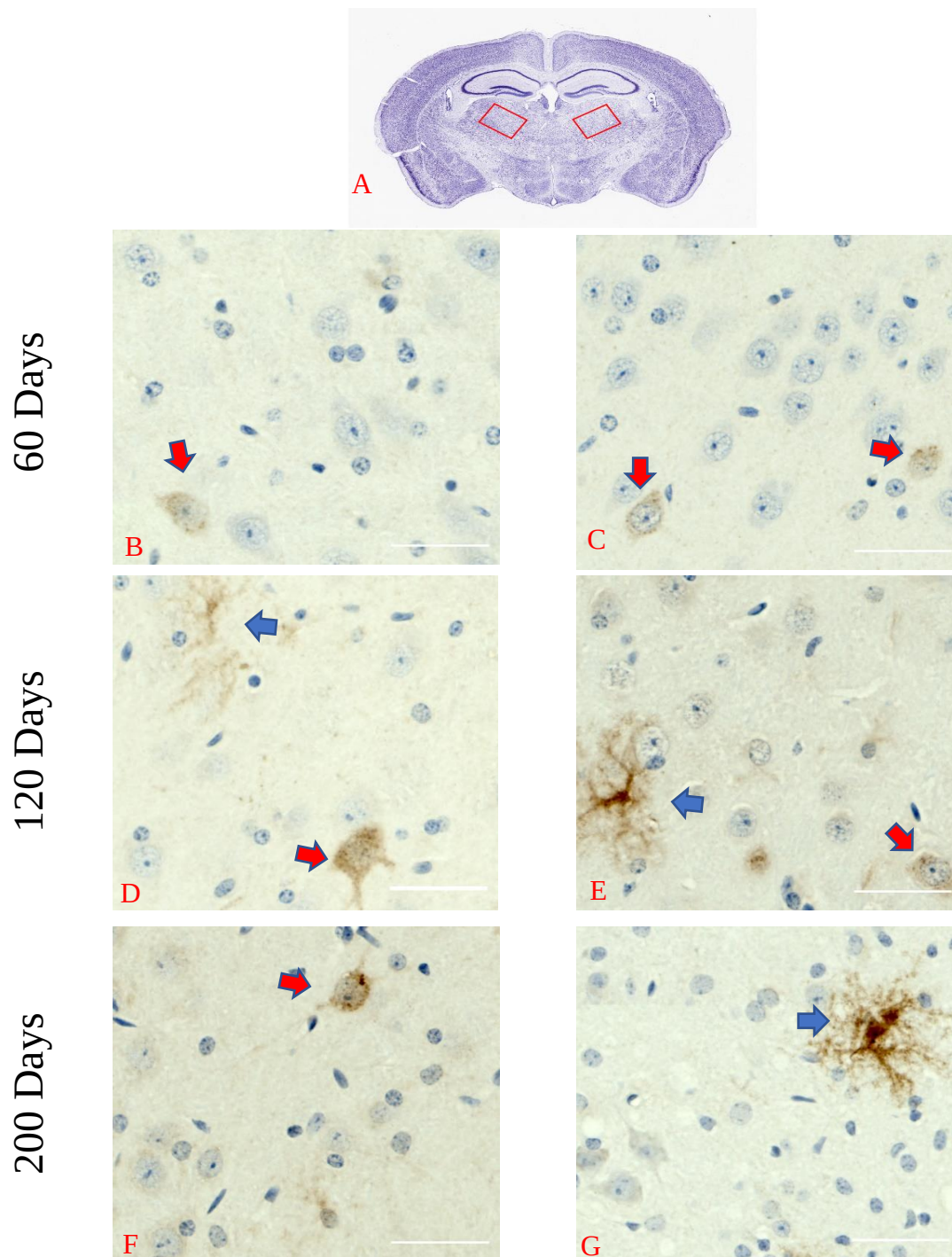


Figure 34. AAVrh.10 Transduction of the Thalamus at 60-, 120- and 200- Days Post-Transduction
DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the thalamus of mice that received AAVrh.10 injections. A) Reference image from Allen Brain Atlas outlining areas selected for imaging. Second row; mice 60 Days following transduction that received B) AAVrh.10-shRNA. C) AAVrh.10- Empty Third row; mice 120 Days following transduction of D) AAVrh.10 shRNA) E) AAVrh.10-Empty. Bottom; mice 200 Days following transduction with received F) AAVrh.10-shRNA G) AAVrh.10-Empty. Red arrows identify transduced neurons, blue arrows identify transduced astrocytes. Scale bars represent 50 μm.

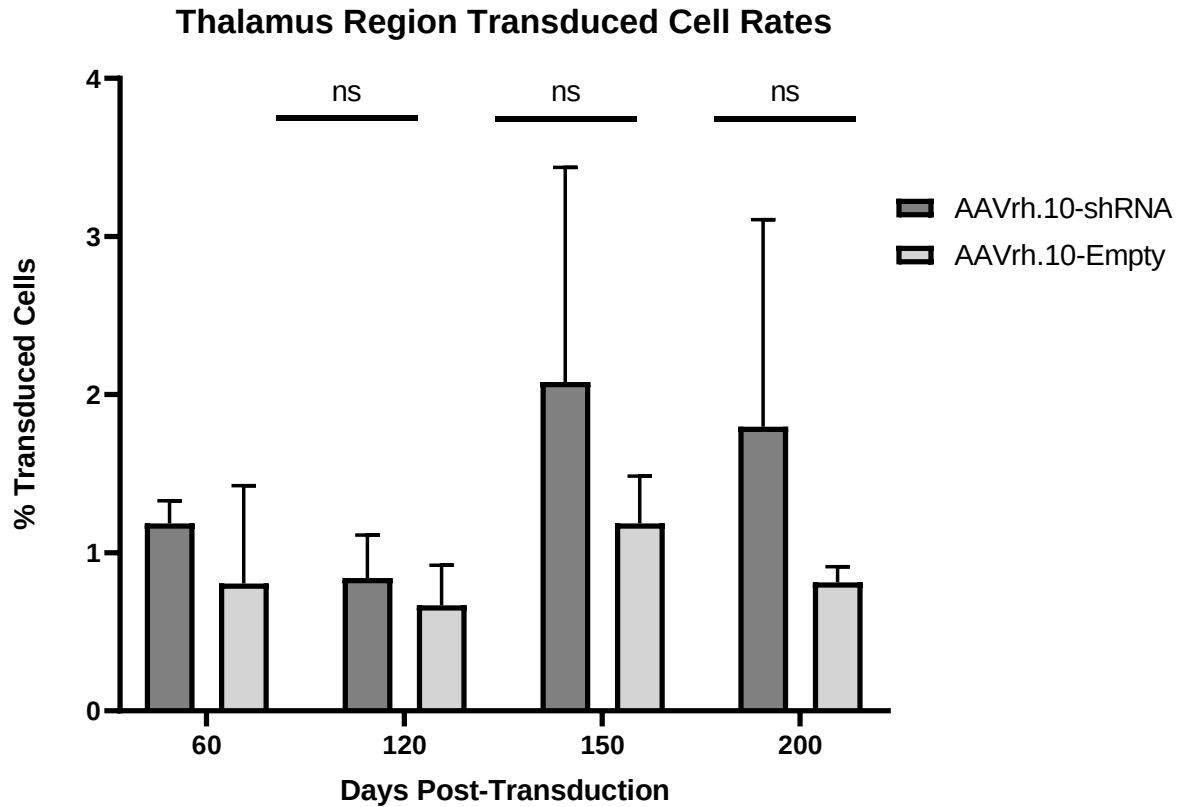


Figure 35. Thalamus Region Transduction Rates of AAVrh.10

Results of DAB staining using primary antibodies targeting mStrawberry expression of transduced cells in the thalamic region of brains from mice that received AAVrh.10 intravenous injection at day 1-2 old. AAVrh.10-shRNA is mice receiving the anti-PrP^C shRNA; Empty is the control sequence. Days Post-Transduction represents the number of days following neonatal intravenous injection of AAVrh.10. Images were manually counted to determine transduction rates. Data of average transduction efficiencies is plotted with bars representing SEM (n= 2-3). Student's T-test was used to assess statistical significance. ns = no statistical significance between any of the results from each group.

4.3.8 Regions of High Neuronal Transduction of AAVrh.10 Following Neonatal

Intravenous Injection

Identifying the unique transduction characteristics of the different AAV serotypes following neonatal intravenous AAV injection may allow selection of specific serotypes depending on transduction efficiencies of specific regions of the brain. Previous research has demonstrated that intravenous injection of AAVs results in a transduction gradient within the brain, with the areas around the ventricles having higher numbers of transduced cells, and a gradual decline in the number with an increase in distance away from the ventricles¹⁶⁸. Transduction of brain regions surrounding the ventricles, such as the lateral habenula, CA2 hippocampal region, and the deep cortex are commonly described in literature^{168,171,198}.

Areas of high transduction rates are identified in Figure 36. These images demonstrate relatively high rates of transduction of cells found within the CA2 region (Blue box, Images B-D), CA3 region (Green box, images E-G), and the lateral habenula (Red box, images H-J). The grey arrow identifies a continuation of the CA3 layer, which also contains relatively high levels of neuron transduction. The black arrow identifies the fasciola cinerea. The white arrow identifies the lateral posterior and dorsal nuclei of the thalamus, residing along the hippocampal region. This area contains relatively higher levels of cell transduction; however, a large portion of transduced cells appear as astrocytes (images not shown).

As shown in Images B-D, the CA2 region contains a relatively high number of transduced neurons compared to other regions of the brain. Furthermore, the CA3 region (Images E-F), contained relatively high levels of neuronal transduction, with high amounts of transduced cells found further down the CA3 region, identified by the grey arrow (Figure 36).

The region of the fasciola cinerea, identified by the black arrow (Figure 36) also contained notable high levels of transduced cells, presumably due to its close distance to the central ventricle.

The area that contained considerably high levels of neuronal transduction is the lateral habenular nucleus, identified by the red box (Images H-J, Figure 36). This area demonstrated consistently high levels of neuronal transduction at all timepoints examined.

Overall, these results demonstrate that although overall transduction of the brain remains low, there are regions of increased neuronal transduction. These regions represent areas of interest that may be used to collect tissue samples containing high proportions of transduced neurons for further studies.

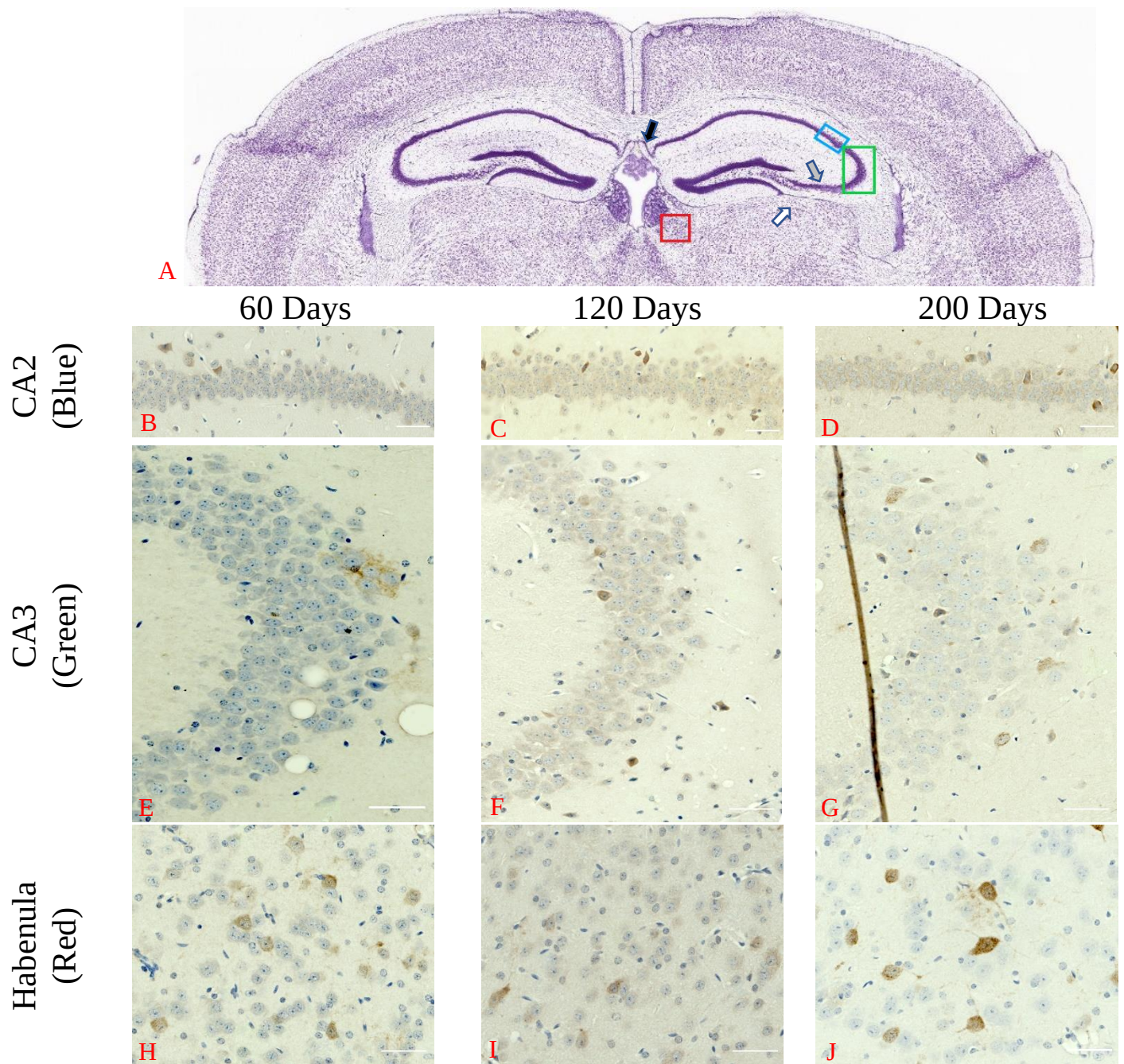


Figure 36. Areas of High AAVrh.10 Transduction Within Mouse Brain Following Neonatal Intravenous Injection of AAVrh.10

DAB staining to identify mStrawberry expression of transduced cells within the CA2 hippocampal region (Blue square; images B-D), CA3 hippocampal region (Green square; images E-G), and Lateral Habenula (Red square; images H-J) of mice that received AAVrh.10 injections. A) Reference image from Allen Brain Atlas outlining areas identifying regions where images were taken. Second row; CA2 regions from mice that received: B) AAVrh.10-Empty, C) AAVrh.10-shRNA, D) AAVrh.10-shRNA. Third row; CA3 regions from mice that received: E) AAVrh.10-Empty, F) AAVrh.10-shRNA, G) AAVrh.10-shRNA. Bottom row; Lateral Habenula regions from H) AAVrh.10-Empty, I) AAVrh.10-shRNA, J) AAVrh.10-shRNA. Black arrow identifies the fasciola cinerea; white arrow, posterior and dorsal nuclei of the thalamus; grey arrow identifies the CA3 region. Scale bars represent 50 μm .

4.3.9 Neonatal Intravenous Injection of AAVrh.10 Causes Transduction and Long-Term Transgene Expression in Cells of the Liver, Lungs, and Spleens of Mice

Opposed to site-specific injections, which typically limits the spread of viruses to 3 mm from the site of injection ¹²⁸, intravenous injection results in transduction of cells throughout the body. Due to the very high rate of cell turnover in the organs of neonatal mice, transgene expression is quickly lost during the early life stages of the mouse ¹³⁹. One study found that expression of GFP within the livers of neonatal mice intravenously injected with AAV2 lost detectable signal within 2 weeks ²³⁹. However, studies have found long-term expression remaining in relatively low amounts within organs of mice, compared to the brain, suggesting that integration of the AAV genome has occurred ^{139,160}. Furthermore, one study found that following intravenous injection of neonatal mice, 8/10 of them had developed either a lung or liver tumor over 43 weeks ¹⁶⁰. The topic of genome integration following intravenous AAV injection into neonatal mice is rather understudied ¹³⁹, yet it remains a significant topic to study to fully assess the health risks of AAV use.

Mice received injections as outlined in section 0, and tissue samples were collected at timepoints detailed in section 4.3.5. Images of the livers, lungs, and spleens, between 60 and 200 days post-transduction are shown in Figure 37, Figure 38, and Figure 39, respectively.

As shown in Figure 37, transduction of the liver remained fairly consistent at all time points, from 60 days- post-transduction to 200 days post-transduction. The strong DAB staining of the cells of liver appears to suggest that transgene expression is high in these cells. Furthermore, examples of clusters of transduced cells, which is a sign of genome integration ¹³⁹, was found in the samples at all timepoints.

As shown in Figure 38, transduction was found also found in the lungs at all time points, however at levels less than the liver.

As shown in Figure 39, transduction of the spleen appeared to show lower levels of transduction of the cells in this organ, with seemingly more pronounced transduction rates in the end point samples. However, similar DAB staining was found in the negative control samples from mice that did not receive AAV injection, showing that there is a high background signal in certain parts of the spleen. Therefore, cells within the spleen are most likely not transduced by AAVrh.10 at a detectable level when intravenously administered.

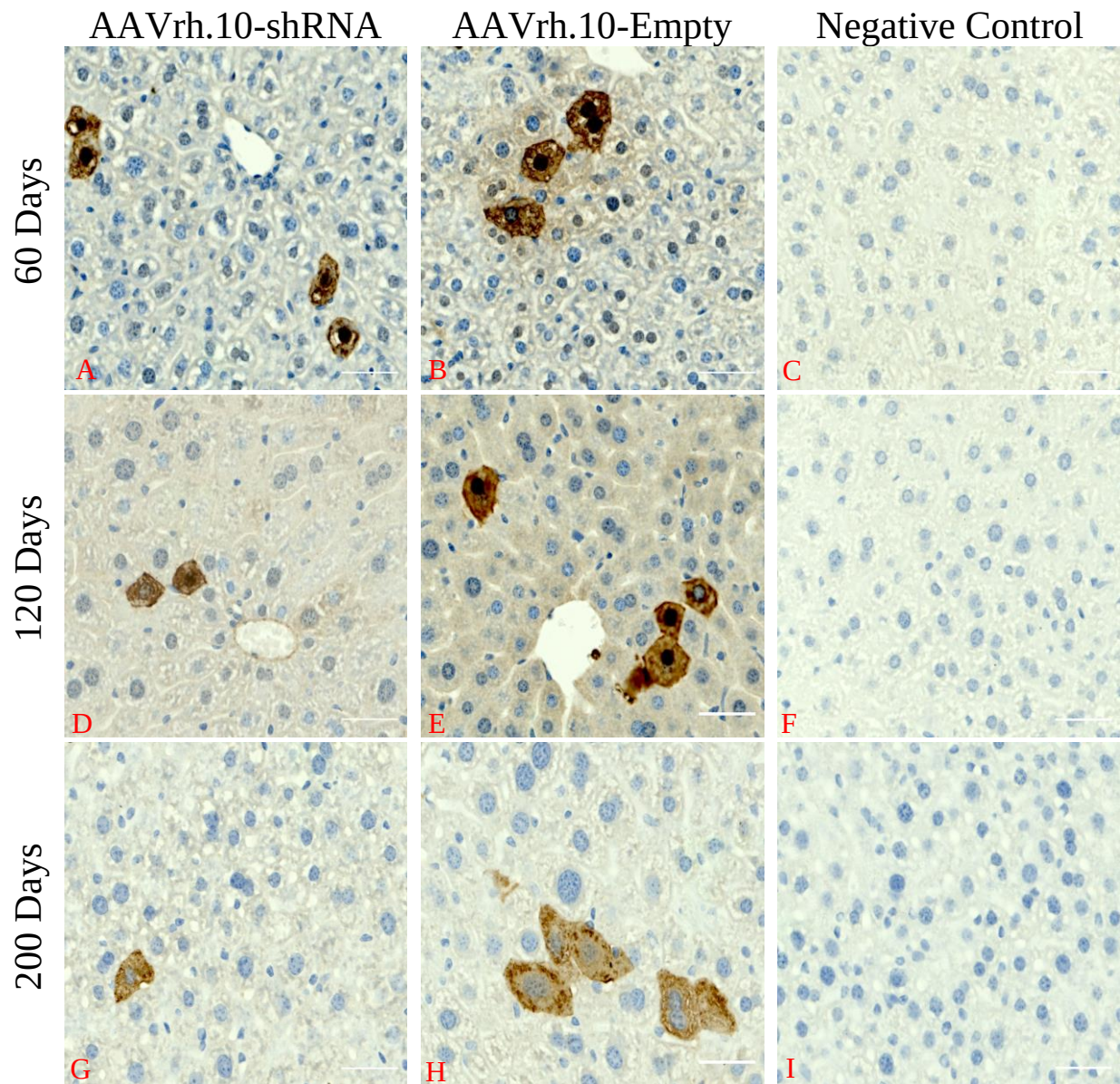


Figure 37. Transduction of Liver at 60-, 120-, and 200 Days Post-Transduction

DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the liver of mice that received AAVrh.10 injections. 60 Days Row; mice following the injection of A) AAVrh.10- shRNA, B) AAVrh.10-Empty, C) PBS. 120 Days row; mice 120 days following the injection of D) AAVrh.10-shRNA, E) AAVrh.10-Empty, F) PBS. 200 Days row; mice 200 days following the injection of G) AAVrh.10-shRNA, H) AAVrh.10-Empty, I) PBS. Scale bars represent 50 μ m.

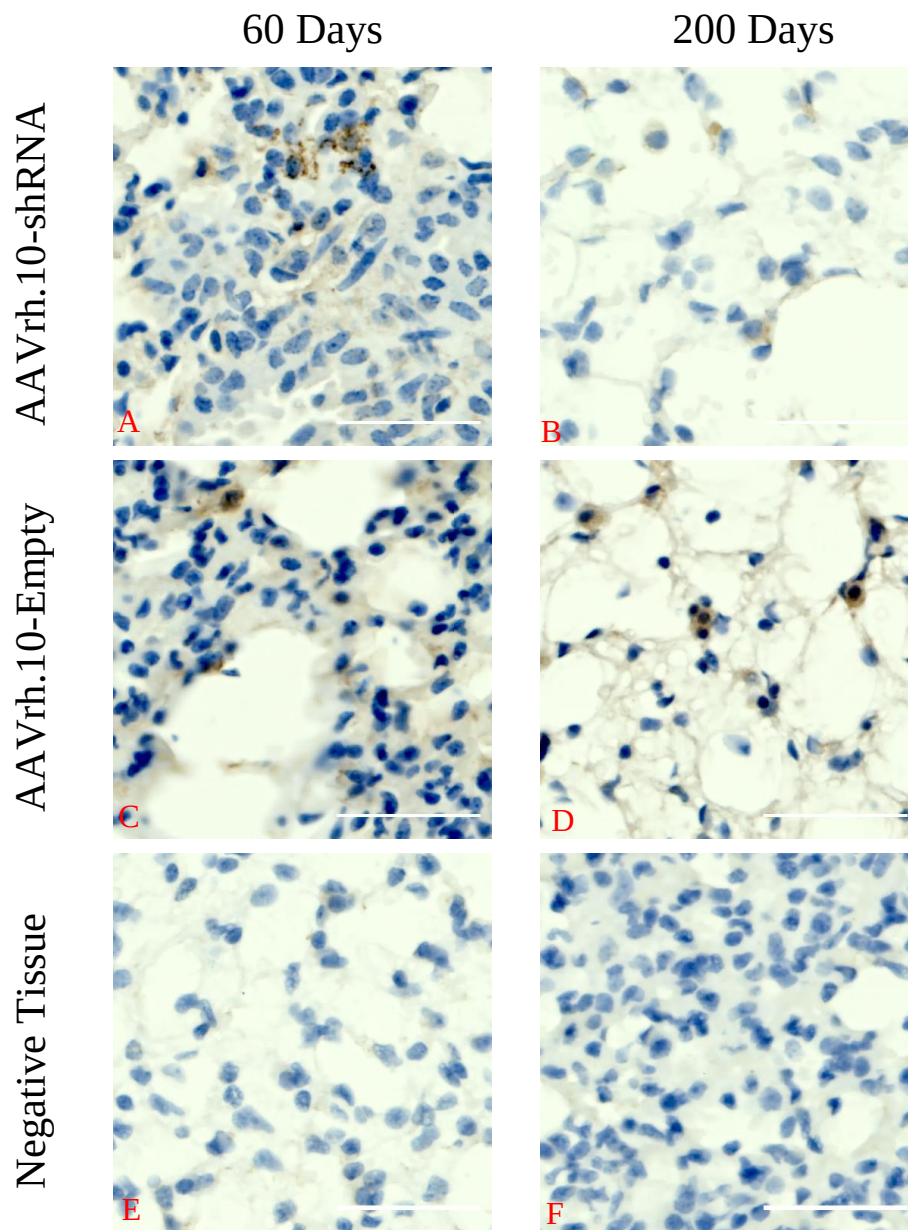


Figure 38. Transduction of Mouse Lungs

DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the lungs of mice that received AAVrh.10 injections. A) AAVrh.10-shRNA injected mice at 60 days post-transduction, B) AAVrh.10-shRNA injected mice at 60 days post-transduction, C) AAVrh.10-Empty injected mice at 60 days post-transduction, D) AAVrh.10-Empty injected mice at 200 days post-transduction, E) Negative control tissue at day 60, F) Negative control tissue at day 200. Scale bars represent 50 μ m.

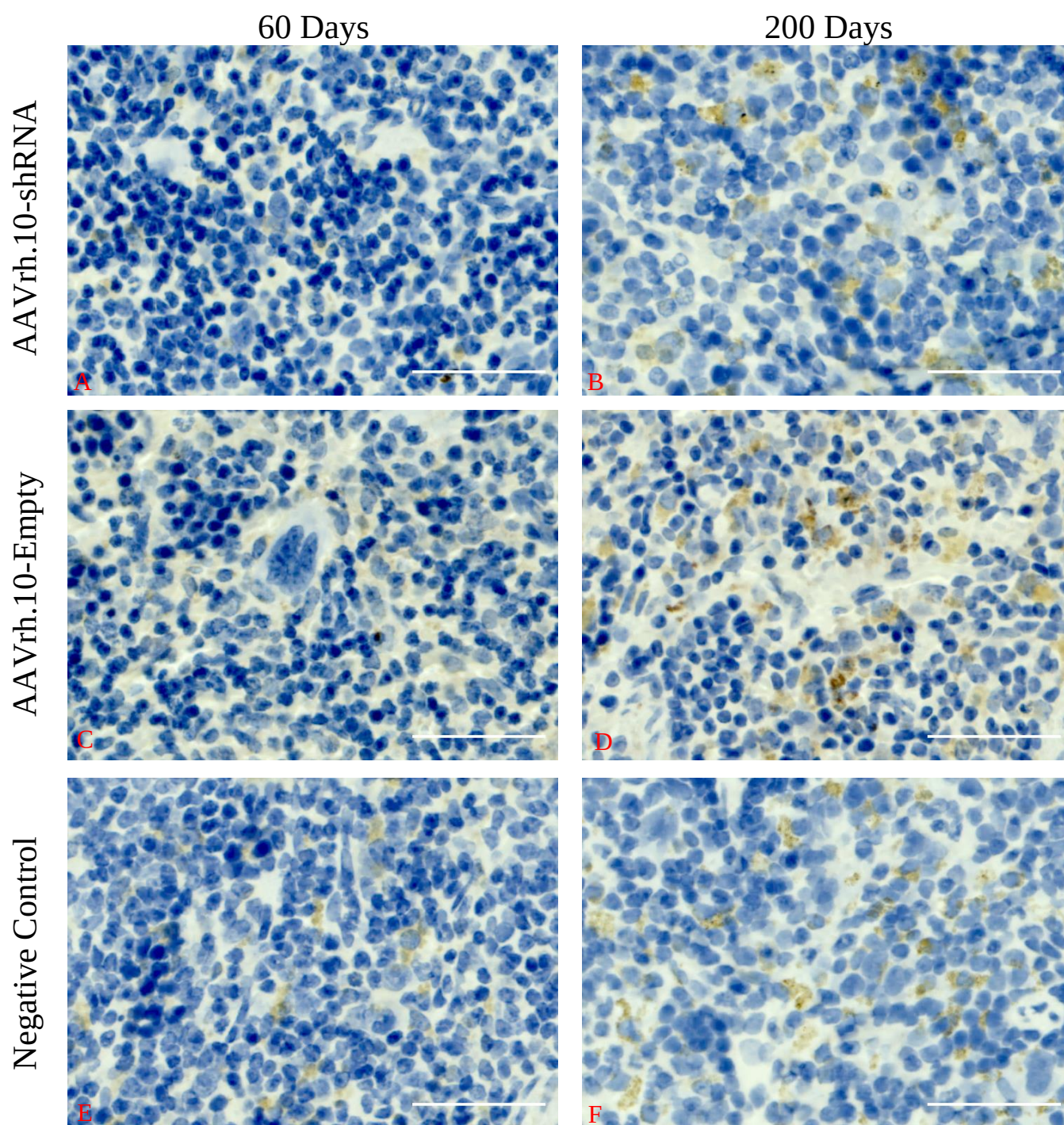


Figure 39. Transduction of Mouse Spleen

DAB staining using primary antibody targeting mStrawberry expression of transduced cells within the spleens of mice that received AAVrh.10 injections. A) AAVrh.10- shRNA injected mice at day 60. B) AAVrh.10- shRNA -injected mice at day 200, C) AAVrh.10-Empty injected mice at day 60, D) AAVrh.10-Empty injected mice at day 200, E) Non-injected mice at 60 days, F) Non-injected mice at day 200. Scale bars represent 50 μ m.

4.3.10 Neonatal Intravenous Injection of AAVrh.10 Delivering Anti-PrP^C shRNA Increases the Survival of Prion-Infected Mice

Previous research has demonstrated the successful use of an anti-PrP^C shRNA sequence prior to prion infection to prolong the lifespans of mice following prion infection. Specifically, Pfeifer et al demonstrated that mice with 50-70% of their neurons expressing the anti-PrP^C shRNA sequence showed a significantly increased lifespan following prion infection ¹²⁶. Intravenous use of AAVs, in particular AAVrh.10, has shown the transduction of cells located throughout the body, with high levels of transduction throughout the brain ^{168,171,198}. We have established that our AAVrh.10 viral vector resulted in long-term transduction of cells, and transgene expression in the transduced cells, agreeing with previous research ^{168,198}.

To assess the effects of an intravenously-administered AAVrh.10 particles carrying either an anti-PrP^C shRNA sequence, or an empty control sequence, day 1-2 old neonatal mice were administered a dose of 6E10 viral genomes in 50 µl of PBS via the superficial temporal vein. At 62 days of age, mice received RML prion infection, or mock-infection. Mice were monitored for signs of prion disease and were scored according to the disease score chart in Appendix A. Mice were euthanized upon reaching a score of 5.

As shown in Figure 40, treatment of neonatal mice with AAVrh.10 containing an anti-PrP^C shRNA sequence resulted in a statistically significant increase in lifespan of 10 days on average, compared to mice that received the empty control viral vector, PBS injection, or no injection. The total mouse numbers used for each group, and the average survival time of mice in each group are shown in Table 20.

The following table outlines the numbers of mice used and the numbers of mice following mortalities at each day.

Table 19. Mortality Table of AAVrh.10-Injected Mice Following Prion Infection

Days Post-Infection	RML - No Injection	RML - PBS	RML - shRNA	RML - Empty
0	5	6	15	15
115	5			
129	4			15
132	3	6		12
135			15	11
136		5	14	
140			12	6
143		2		
146		1		4
147	2		8	2
149			6	1

Neonatal mice received intravenous injection of AAVrh.10 containing an anti-PrP^C shRNA (RML - shRNA) or an Empty control sequence (RML - Empty). Groups of mice also received injection with PBS (RML – PBS) or no injections (RML – No Injection). At 60 days of age mice were infected with RML prion disease and the groups were monitored for signs of disease. Days Post-Infection counts the numbers of days of survival following infection with RML prion disease. Experimental group columns count number of mice remaining on each day, with a change of number indicating a loss of test subject.

Survival of RML-Infected Mice

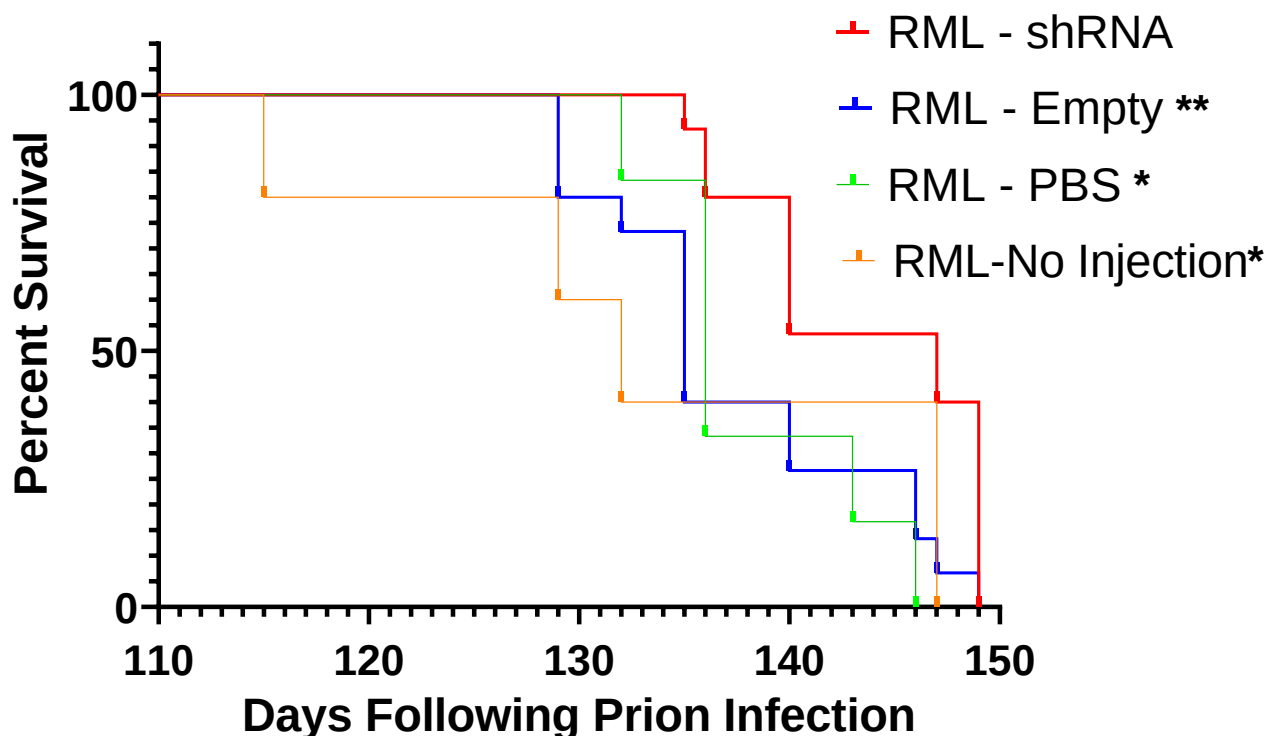


Figure 40. Survival Graph of RML-Infected Mice Following Neonatal Intravenous Injections of AAVrh.10

Day 1-2 old mice received intravenous injections via the superficial temporal vein of AAVrh.10-shRNA, AAVrh.10-Empty, PBS, or no injections. At 7 weeks of age mice were infected with the RML prion strain through intracranial injection of 5% RML-infected brain homogenate, or mice received mock-infections. Mice were monitored for the duration of the experiment, and received scoring based on prion disease symptoms (Appendix). The Mantel-Cox method was used to determine statistical significance of RML-shRNA mice compared to negative controls: * = p-value of ≤ 0.05 ; ** = p-value of ≤ 0.005 . Statistical significance of each treatment group is shown compared to the shRNA-treated mice.

Table 20. Outline of Mouse Numbers Used for *in vivo* Experiment

	RML-shRNA	RML-Empty	RML-No Injection	RML-PBS
Number of Mice	15	15	5	6
Median Survival	147	135	132	136

4.3.11 RML Infection Following Neonatal Intravenous Injection of AAVrh.10 Does Not Affect Levels of Transduced Cells

All previous images of AAVrh.10 transduction in live mouse tissue were collected from groups of mice that were mock-infected, to assess levels of transduction in groups of healthy animals. In order to characterize AAVrh.10 as a vector that may be used to administer genetic constructs that persist throughout prion disease in a live mouse model, it is necessary to assess whether prion infection of the mice 60 days following neonatal intravenous injection of AAVrh.10 resulted in a change of transduction efficiencies over time, compared to mock-infected mice.

Sections of the CA1 hippocampal region and the thalamus of mice that received neonatal intravenous injection of AAVrh.10-shRNA, then either RML-infected or mock-infected are shown in Figure 41. Images displayed were collected from mice at 60 days of age (prior to RML or mock-infection), 120 days (60 days post-prion infection), 150 days (90 days post-infection), or at 200 days (endpoint of prion disease). These figures show that there is no observable difference between the transductions in RML-infected or mock-infected mice.

To calculate the percentage of transduced cells within the CA1 hippocampal region, a 600 μm section was chosen for counting. To count the thalamus, a section of 600 μm x 500 μm was selected. Cell nuclei were used to count total cell numbers, and presence of DAB staining indicated stained cells. 2-3 images were chosen from the mouse tissues that received RML infection for each timepoint, each from a different mouse brain (n=2-3). The total number of cells and the total numbers of transduced cells within the CA1 hippocampal region collected from the RML-infected mouse tissues each timepoint are shown in Table 21. Non-infected counting results are shown in Table 17. Total cell counts collected from the thalamus region in RML-infected mice are shown in Table 22, and the results of the mock-infected mice are found in Table 18.

Table 21. Counting Results of Transduction of the CA1 Hippocampal Region by AAVrh.10 in RML-Infected Mice, Collected at 60- and 90-Days Post-Infection, and at Endpoint of Prion Disease

Days Post-Infection	AAVrh.10- shRNA						AAVrh.10- Empty					
	Total Cells			Transduced Cells			Total Cells			Transduced Cells		
60	141	132	170	4	7	5	176	147	180	5	10	11
90	122		228	8		8	171		167	5		2
Endpoint	220		161	7		3	151		212	12		5

Table 22. Counting Results of Transduction of the Thalamus by AAVrh.10 in RML-Infected Mouse Tissues Collected at 60- and 90- Days Post-Infection and at Endpoint of Prion Disease

Days Post-Transduction	AAVrh.10- shRNA				AAVrh.10- Empty			
	Total Cells		Transduced Cells		Total Cells		Transduced Cells	
60	662	369	14	2	602	771	8	16
90	581	744	11	6	639	516	6	4
Endpoint	561	311	6	8	699	686	5	11

Figure 41 also demonstrates one aspect of prion disease in the CA1 hippocampal region.

Irreversibly-apoptotic neuronal nuclei, which have the appearance of small, dense nuclei, as described by Yang et al ²⁴⁰, are visible in the RML-infected CA1 hippocampal regions in the images collected at the later stages of disease, 120 days post-infection and at endpoint of prion disease, as indicated by the Red arrow.

Endpoint samples collected from AAVrh.10-shRNA and AAVrh.10-Empty-injected mice, and control mice, and RML or mock-infected mice are shown in Figure 42. Similarly, endpoint images of the thalamus regions of mice are shown in Figure 44. These figures demonstrate that there is no easily detectable difference in the RML-mice that received AAVrh.10 injections containing either an anti-PrP^C shRNA sequence, or an empty control sequence.

The results of counting regions of the CA1 hippocampus and thalamus to identify numbers of transduced cells are shown in Figure 43 and Figure 45, respectively. Figure 43 demonstrates that RML-infection at 60 days of age does not have a significant effect on the number of transduced cells found within the CA1 hippocampal region or thalamus over a period of 200 days following neonatal intravenous injection of AAVrh.10. Similarly, RML-infection does not appear to significantly affect the number of transduced cells found within the thalamus, as shown in Figure 45.

Overall, these data demonstrate that there are no detectable differences of transduction in the CA1 hippocampal region or thalamus in RML-infected or mock-infected mice over time. Therefore, RML-infection of mice that received neonatal intravenous injections of AAVrh.10 does not significantly alter the numbers of transduced cells over time. Further experimentation to better identify neurodegeneration is needed to assess the levels of prion pathogenesis between experimental groups.

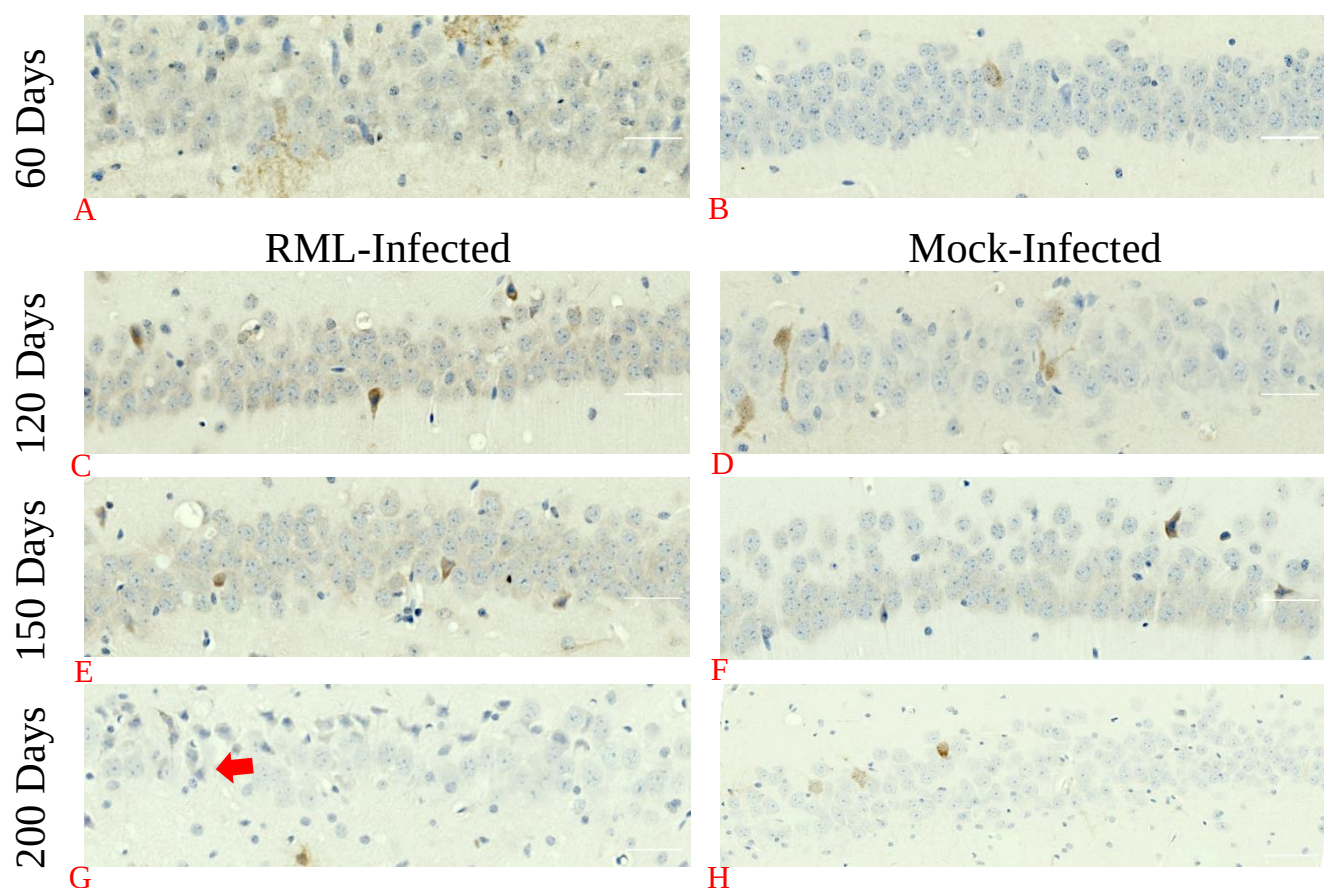


Figure 41. Immunohistological Staining of CA1 Hippocampal Region of AAVrh.10-shRNA-Injected Mice That Were RML-Infected or Mock-Infected at 60 Days of Age

DAB staining to identify mStrawberry expression in CA1 hippocampal regions in mock-infected and RML-infected mice, that received neonatal intravenous injections of AAVrh.10. 60 Days: Mice at 60 days of age that received A) AAVrh.10-shRNA, B) AAVrh.10-Empty. At 60 days of age mice were RML-infected or Mock-infected. 120 Days: Mice at 120 days of age (60 days post-prion infection) that received AAVrh.10-shRNA and then C) RML-Infected, D) Mock-infected. 150 Days: Mice at 150 days of age (90 days post-prion infection) that received AAVrh.10, E) RML-Infected, F) Mock-infected. 200 Days: Mice at 200 days of age (Endpoint, approximately 140 days post-prion infection) that received AAVrh.10-shRNA, G) RML-infected, H) Mock-infected. Red arrow marks the condensed, apoptotic nuclei, a hallmark of prion disease. Scale bars represent 50 μ m.

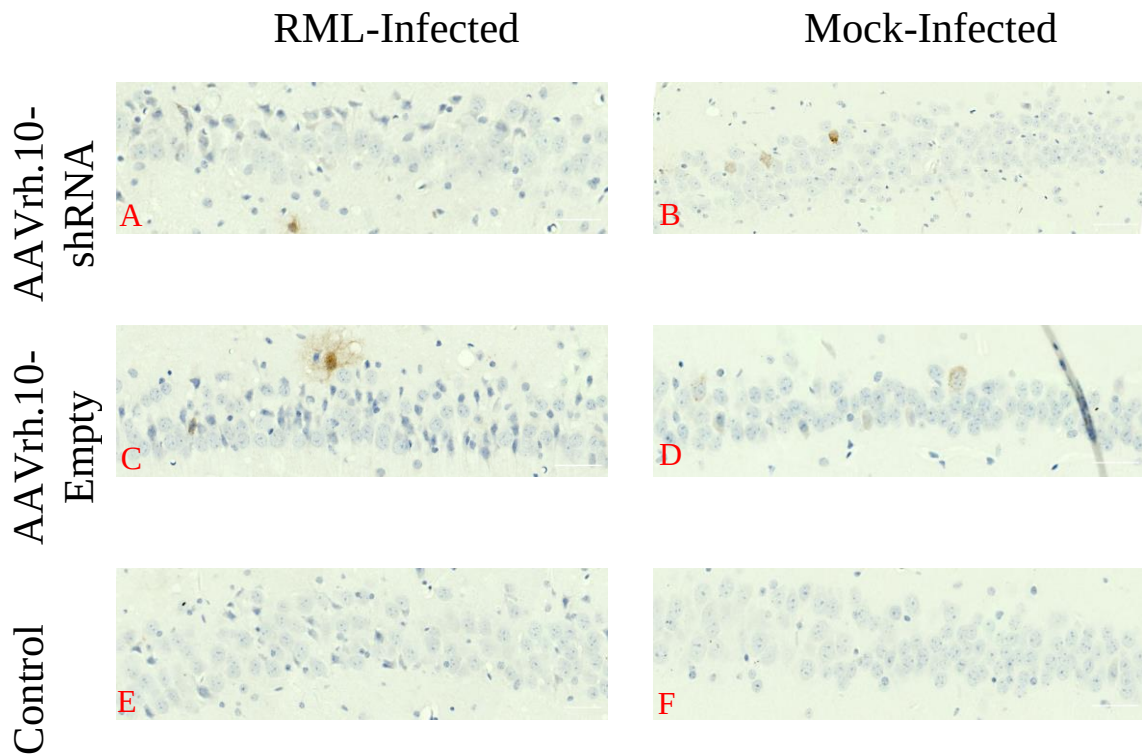


Figure 42. Immunohistological Staining of CA1 Hippocampal Region of Mice That Received Injection with AAVrh.10-shRNA and -Empty Control Vector at Endpoint of Prion Disease

Results of DAB staining for mStrawberry expression in the CA1 and thalamus regions of RML- and Mock-infected mice at Endpoint of RML disease. Tissues from Mock-infected mice were collected at the same timepoints as Endpoint for RML-infected mice. A) RML-infected AAVrh.10-shPrP injected mice, B) Mock-infected AAVrh.10-shPrP injected mice, C) RML-infected AAVrh.10-Empty injected mice, D) Mock-infected AAVrh.10-Empty injected mice, E) RML-infected PBS injected mice, F) Mock-infected PBS injected mice. Scale bars represent 50 μ m.

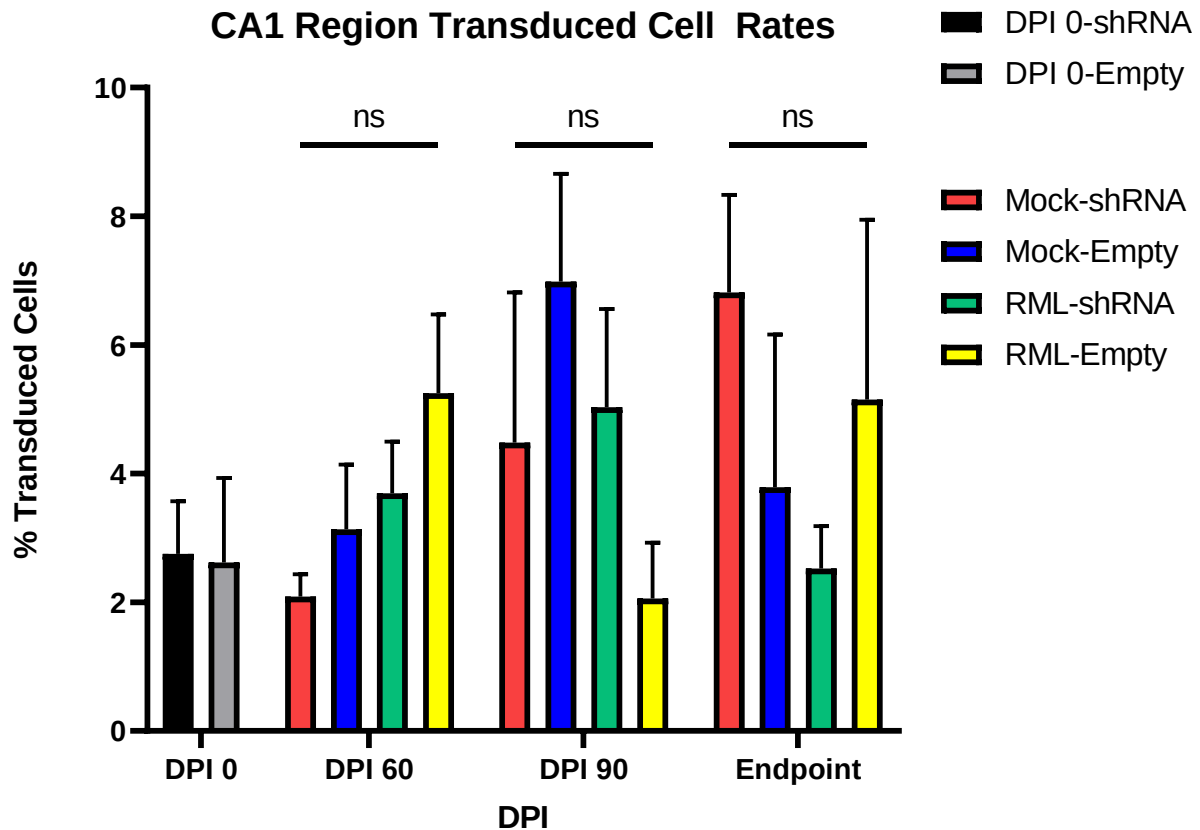


Figure 43. CA1 Hippocampal Region AAVrh.10 Transduction Rate in RML-Infected and Mock-Infected Mice

Results of DAB staining for mStrawberry expression in the CA1 hippocampal region in brains of mice that received AAVrh.10 intravenous injection at 1-2 days of age, then infected with RML prion disease or mock-infected at 60 days of age (DPI 0). Mock is mock-infected mice, RML is RML-infected mice. shRNA is the mice that received pAV-U6-RFP-shRNA injection, -Empty is mice that received the pAV-U6-RFP-Empty control vector. A 600 μ m section of the CA1 hippocampal region from each mouse were counted to determine the numbers of transduced cells, and total cell numbers to find the transduction rate. Data of average transduction efficiencies is plotted with bars representing SEM (n= 2-3). Student's T-test was used to assess statistical significance. ns = no statistical significance between any of the results from each group.

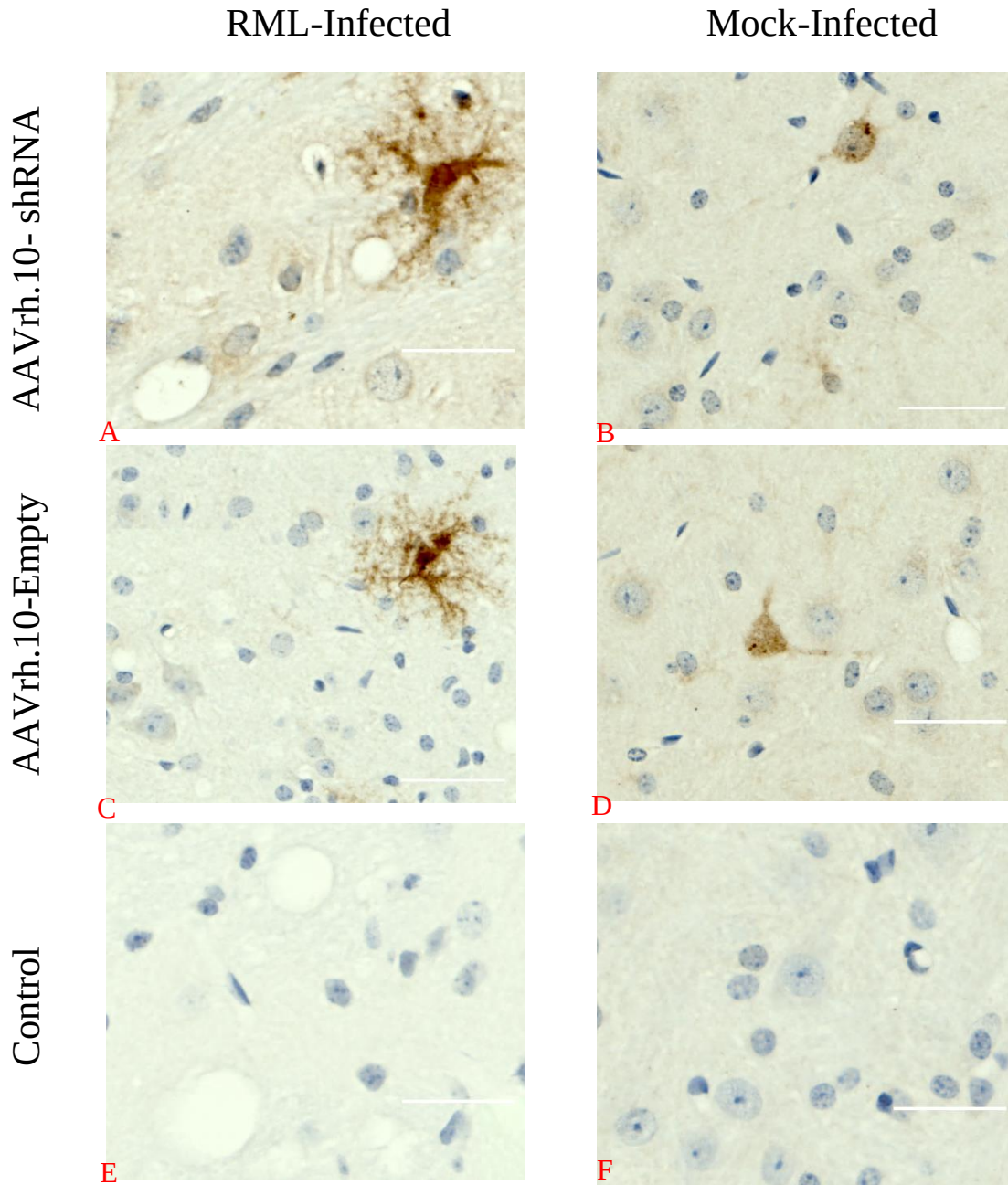


Figure 44. Immunohistological Staining of Thalamus of Mice That Received Injection with AAVrh.10-shRNA and -Empty Control Vector at Endpoint of Prion Disease

Results of DAB staining for mStrawberry expression in the thalamus regions of RML- and Mock-infected mice at Endpoint of RML disease. Tissues from Mock-infected mice were collected at the same timepoints as Endpoint for RML-infected mice. A) RML-infected AAVrh.10-shPrP injected mice, B) Mock-infected AAVrh.10-shPrP injected mice, C) RML-infected AAVrh.10-Empty injected mice, D) Mock-infected AAVrh.10-Empty injected mice, E) RML-infected non-injected, F) Mock-infected PBS injected mice. Scale bars represent 50 μ m.

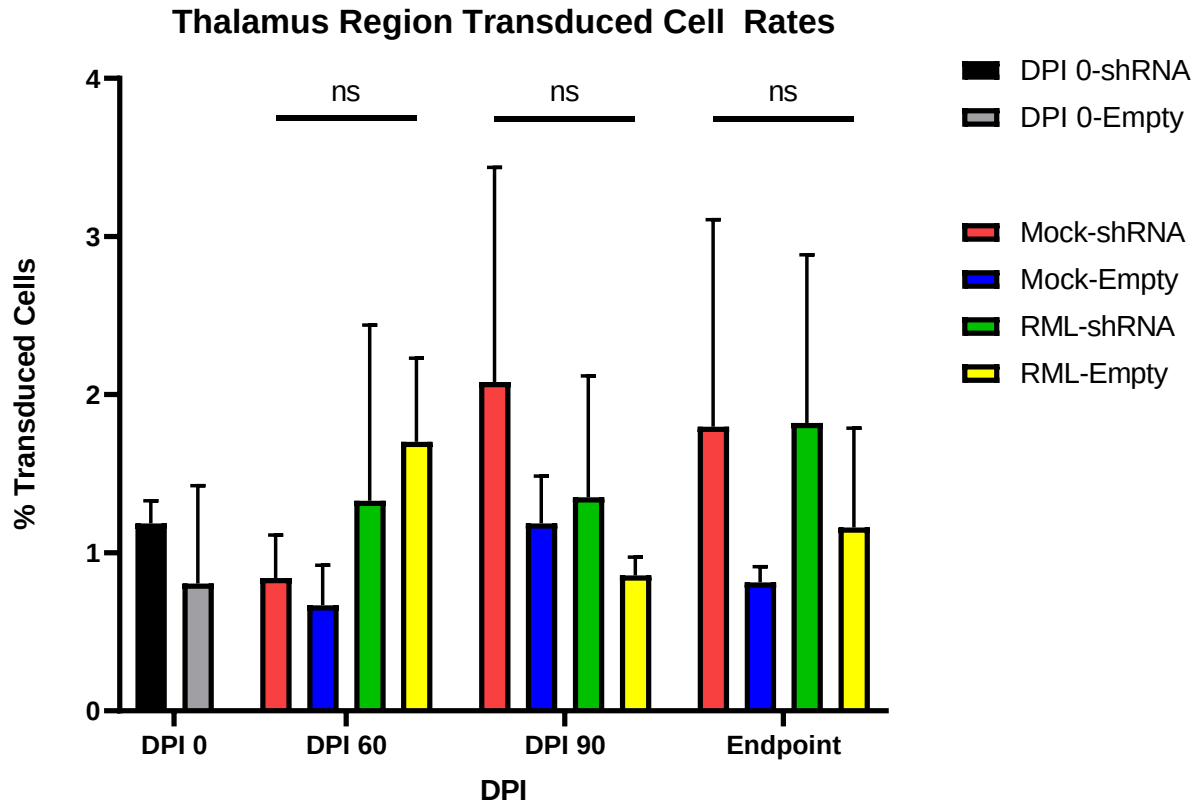


Figure 45. Thalamus Region Transduction Rates of AAVrh.10 in RML-Infected and Mock-Infected Mice

Results of DAB staining for mStrawberry expression in the thalamic region of brains from mice that received AAVrh.10 intravenous injection at 1-2 days of age, then infected with RML prion disease or mock-infected at 62 days of age (DPI 0). Mock is mock-infected mice, RML is RML-infected mice. shRNA is the mice that received pAV-U6-RFP-shRNA injection, -Empty is mice that received the pAV-U6-RFP-Empty control vector. Images were manually counted to determine transduction rates. Data of average transduction efficiencies is plotted with bars representing SEM (n= 2-3). Student's T-test was used to assess statistical significance. ns = no statistical significance between any of the results from each group.

Chapter 5. Discussion

This work has demonstrated several methods that may be adapted for optimization of AAV production, such as increasing the length of incubation of the HEK293T cell cultures and harvesting the viruses from the media fraction at later timepoints to increase overall viral yield. These viruses are capable of transducing neurons in primary cell cultures without causing significant cytotoxicity and are capable of significantly reducing the expression the prion gene with use of an anti-PrP^C shRNA sequence. Finally, neonatal intravenous injection of these viruses via the superficial temporal vein is capable of bypassing the blood brain barrier and transducing neurons and astrocytes within the brains of mice, causing transgene expression persisting for at least 200 days post-transduction. In a prion-infection model, neonatal intravenous injection of AAVrh.10 containing an anti-PrP^C shRNA sequence was shown to result in a statistically significant increase in the lifespan of prion-infected mice.

5.1 Optimization of AAV Generation

Shown here are several important aspects of laboratory-scale AAV production that are often not considered. A common theme throughout the results demonstrates the unique characteristics of each AAV serotype, such as the large differences in total viral numbers of each serotype produced following the same culturing conditions. Clearly, these results show that optimization of the protocols for each AAV serotype to be produced may be worthwhile, as minor changes in the protocol may significantly alter the yield of AAVs produced.

The most significant increase in total AAV yield was due to increasing the total incubation time following the triple transfection of HEK293T cells. Here I have demonstrated that the optimal production of AAVrh.10 occurs during a 4-day incubation period. Furthermore, I have demonstrated at this time point the majority of total AAVrh.10 particles are found within the media fraction. Therefore, processing the media fraction should not be treated as an optional step when large numbers of AAVs are required. The demonstrated increase in total virus production, and total viral release into the media due to an increased incubation length does agree with previous research that demonstrated these effects during production of AAV2 and AAV8^{194,241}. It was found that an optimal AAV2 titre could be collected at 5 days post-transfection of the HEK293T cell culture¹⁹⁴, and AAV8 at 6 days post-transfection²⁴¹. Lee et al have recommend harvesting AAVrh.10 producing cultures 3 days post-transfection, due to the diminishing returns in AAVs produced when increasing total incubation time¹⁸³. However, my results demonstrate that the total AAVrh.10 yield can be increased three-fold as a result of increasing the incubation time by 24 hours. Therefore, if large numbers of viruses are needed it may be advantageous to perform optimization of the production protocol to determine the optimal incubation length to increase total AAV production.

5.2 Optimization of AAV Purification

The tangential flow filtration system was demonstrated to be a highly effective method of concentrating large volumes of crude viruses collected in the cell lysate and media fractions of the HEK293T cell cultures. Overall, this system is capable of concentrating a large volume of crude viruses into volumes that can be subject to further ultra gradient centrifugation purification that is required to further separate contaminating proteins and empty capsids from properly packaged viral particles.

The successful use of cesium chloride and iodixanol concentration gradients has been demonstrated, with each resulting in comparable levels of virus purification^{189,193}. Therefore, the low viral titres that I collected after the attempted CsCl gradient purifications are undeniably due to human-error. However, the successful use of the iodixanol gradient ultracentrifugation to purify 2E11 and 1.9E12 viruses total, after the first and second attempt, respectively, suggests that the iodixanol method may be more accessible to individuals with no prior experience in performing these techniques.

5.3 AAV Use in Primary Neuron Cell Cultures Results in Efficient Transduction of Neurons and Astrocytes, and Stable Transgene Expression

The primary hippocampal neuron cell culture was demonstrated to be a valuable tool in analyzing the characteristics of AAV preparations prior to performing experiments in live animals. As demonstrated, primary neuron cell cultures can be maintained for at least 14 days without a significant change to the proportions of cells within the cultures. Beyond this point, astrocyte numbers are found to increase, however the cultures approximately 90% neurons up until DIV 14. These cultures can be treated with a high dose of AAV particles, up to $1E7$ viral genomes, without an increase in cell death. Furthermore, fluorescent protein expression can be detected within these cultures as early as 3 days. Finally, expression of an anti-PrP^C shRNA sequence in the primary neuron cell cultures using AAVrh.10 was found to result in significant reduction of gene expression, measured by rtPCR.

The transduction efficiencies of all AAV serotypes tested, AAV8, AAV9, and AAVrh.10 were very high at all MOIs tested between $1E4$ and $1E7$, with transduction efficiencies ranging between 80-95% on average. These transduction efficiencies compare with other publications reporting similar transduction efficiencies in primary hippocampal neuron cell cultures from mice ^{201,223}, and of primary hippocampal and cortical cell cultures from rats ²²⁴. Characterization of biochemical processes within transduced cell cultures requires a large transduction efficiency, so that minor effects are more easily detectable, and not masked by non-transduced cells. The high transduction efficiencies of all three AAV serotypes, AAV8, AAV9, and AAVrh.10 shows that these viral vectors are powerful tools that can be used to reliably transduce neurons *in vitro*.

5.4 Neonatal Intravenous AAVrh.10 Injection Results in Transduction of Neurons and Astrocytes Throughout the Brain

These results demonstrate that AAVrh.10 is capable of bypassing the blood brain barrier and transducing neurons and astrocytes throughout the brain. As demonstrated in previous research^{169,232}, it was found that transduction rates were higher in the areas surrounding the ventricles. Specifically, in this study, increased levels of AAVrh.10 transduction were found in the choroid plexus, hippocampus, deep cortex, caudate putamen, and in the lateral habenula of the mouse brain following intravenous injection of AAVrh.10.

Transduction throughout the thalamus was relatively low, as expected from previous research^{136,198}. In this study the transduction efficiency of cells within the thalamus was found to be 1-2% on average following a dose of 6E10 AAVrh.10 via the superficial temporal vein of day 1-2 old mice. However, there does seem to be areas of the thalamus that contain increased numbers of transduced cells, such as the posterior and dorsal nuclei of the thalamus, indicated by the white arrow in Figure 36. Opposed to previous research demonstrating a higher transduction rate of areas of the brain near the ventricles¹⁶⁸, these regions are not very close to the ventricles. It is important to note that transduced cells with the appearance of astrocytes are visible in the brain tissue samples collected at Day 150 and Day 200. It is believed that adeno-associated viruses are lost from dividing cells within the brain^{232,233}, therefore expression of the transgene within astrocytes at these timepoints may indicate that the viral genome has integrated into the genome of the astrocyte. This could explain the high variation in the proportion of transduced cell counts observed within the later timepoints, at Day 150 and Day 200, within the CA1 hippocampal region and the thalamus. Further analysis involving IHC to stain specifically for astrocytes and transduced cells will help determine if the proportion of transduced astrocytes remains consistent

over time, or if division of these cells are leading to the increase in the total numbers of transduced cells within the regions of the brain.

Transduction within the CA1 region of the hippocampus was also demonstrated in these results, showing that cells in this region are transduced more than those in the thalamus. Additionally, as indicated in Figure 36, a portion of the CA1 region that resides close to the central ventricle seemed to contain a higher number of transduced cells than other CA1 regions. This region may be of interest for future experiments, as it may allow isolation of a section of the CA1 hippocampal region containing higher rates of transduction than other sections of the CA1 hippocampal region.

The relatively high transduction in the lateral habenula, shown in Figure 36, may be a valuable finding. A search on PubMed for articles including the keywords “Habenula” and “Prion” found 2 results. This region may be of use for future studies as a large population of transduced neurons can be isolated from this region, allowing the assessment of various genetic constructs transduced into neurons. Additionally, the regions identified that contain relatively higher portions of transduced neurons represent potential areas of importance, where further analysis may help uncover molecular events related to prion disease progression, or the protection of neurons.

5.5 Neonatal Intravenous AAVrh.10 Injection Results in the Transduction of Cells in the Livers and Lungs of Mice

Although the focus of this research is on the brain, intravenous injection does result in deposition of the virus throughout the body. Several of the AAV serotypes are known to transduce organs in adult animals, however there are fewer studies examining long-term expression in transduced organs following neonatal mouse injections. In an adult mouse liver the cells turn over at a very low rate, approximately once every 180-400 days ²⁴², and it has been found that long-term expression following intravenous injection into adult mice is due to the persistence of episomes within the transduced cells ²⁴³. Alternatively, cells within the neonatal mouse liver are mitotically active, resulting in a decline of all AAV serotype expression in the liver by 2 logs during the first 10 weeks of life, as the liver grows from 0.05 grams to 1.18 grams ¹⁶⁹. It is believed that beyond this point, a very large percentage of any remaining expression is due to the integrated form of the AAV ¹⁶⁹.

Shown here are relatively low levels of transduction found within the liver, at all time points tested, between 60 days post-transduction with AAVrh.10, until approximately 200 days post-transduction. These results agree with previous research that demonstrated overall low levels of liver transduction following neonatal intravenous injection of AAVrh.10 ^{139,239,244}.

Previous research has demonstrated a initially high liver transduction rate for AAV2 when intravenously delivered into neonatal mice, however the majority of episomal vectors was almost completely lost by 2 weeks ²³⁹. Furthermore, this research did identify long-term gene expression in the liver following in a small percentage of hepatocytes, often in two- to eight-cell clusters, suggesting genome integration occurred ²³⁹. Indeed, transduction of the liver in our study also

demonstrated a clustering of transduced cells at 60 days post-transduction, up until 200 days post-transduction, with some examples shown in Figure 37.

Transduction within the lung occurred at a similar or lower rate than in the liver, with expression being detected at all time points, as shown in Figure 38. Previous research has found that similar to the liver, the number of transduced cells within the lung of a neonatal mouse rapidly declines until day 35 due to the rapid growth of the animal ¹⁶⁹. One study found that even after a high dose of 4E11 AAVrh.10 intravenously into a neonatal mouse, there was low levels of lung transduction when tissues were examined after 3 weeks ²³⁴. Therefore, these results are as expected, and may also suggest integration of the viral vector into the genomes of cells within the lung. Some papers have reported lung tumor growths in mice monitored up to 43 weeks following intravenous AAV injection into neonatal mice, however at 1/8th the rate of liver tumors ¹⁶⁰. However, due to the limited number of mice expressing lung tumors in the previous study (n=1) ¹⁶⁰, further analysis of the transduced cells of these regions may help identify risks associated with neonatal gene therapies. Although clinical human AAV trials are limited to adults ¹³⁷, understanding the potential risks of these viruses in young humans is important.

No obvious transduction was found in the spleen of any mice at any time points. Sections of this organ from negative control animals contained levels of background that were comparable to the levels of signals seen in the AAV-injected tissue samples. These results agree with previous studies comparing numerous AAV serotypes that have not reported on transduction of any AAVs within the spleen of mice ^{233,234}. The background staining found within the spleen are likely due to the improper inactivation of the endogenous peroxidases of the spleen, which are known to be relatively high ²³⁷.

Due to the limited number of publications describing liver genome integration of AAVs, further research to identify the levels of gene integration may be of significance in future long-term neonatal AAV injection experiments. Further analysis into cells likely containing an integrated viral genome may help shed light on the risk profiles of AAVs when administered intravenously into neonatal animals and may be of importance in determining safe methods of administering AAVs to humans in the future.

5.6 Neonatal Mice Receiving Intravenous AAVrh.10 Injection Survive Longer

Following Prion-Infection

The increase in survival length of the mice treated with AAVrh.10 containing an anti-PrP^C shRNA sequence was unexpected. Previous work with chimeric PrP^C knockdown mice produced by lentiviral infection of embryonic stem cells resulting in the insertion of the anti-PrP^C shRNA into the genome, demonstrated that only mice with 50-70% of cells containing an anti-PrP^C shRNA sequence showed significant increases in their lifespan when infected with prion disease¹²⁶. As demonstrated in my study, the transduction rate throughout the brain was significantly less than 50%, as the highest transduction rate documented was within the CA1 region with a 6% proportion of transduced cells. One major difference between this study and previous studies involving intravenous injections of AAV into neonatal mice is the dose size. In this study, a relatively small dose of 6E10 viral genome particles were delivered into each mouse to assess whether the superior transduction capabilities of this viral serotype when delivered in higher doses^{198,232}, are evident when delivered in low doses. To fully characterize the AAVrh.10 vector's capabilities to deliver genetic materials into the neurons of neonatal mice, it is important to understand the effects observed when administered in different dose ranges. The relatively low transduction rates of 1-6% throughout the brain observed in this study are likely due to the relatively low number of viruses administered to each animal, and the systemic spread of the viruses throughout the body of the mouse. Due to the lower levels of transduction, an increase in the length of survival of the mice involved in this study was not expected. However, we did observe a significant increase in the length of survival over the control mice used in this study, of approximately 10 days longer than the AAVrh.10-Empty mice, the PBS-injected mice, or mice that received no treatment.

One possible explanation of these results is the selective transduction of specific regions on the brain, resulting in high levels of localized transduction. In the previous chimeric mouse study, two mice with low levels (10-20%) of PrP^C-silenced cells did show markedly increased lifespans following prion infection, suggesting that there may be specific regions of a brain where a high concentration of PrP^C-silenced cells significantly increases the resistance of mice against prion disease ¹²⁶. It is possible that this effect was seen in our study, as regions of the brain, in particular the CA1, CA2 and CA3 hippocampal regions, and the lateral habenula, that were found to contain high concentrations of transduced cells compared to the thalamus and other regions of the brain. Further analysis of these regions to identify levels of PrP^C expression, deposition of PrP^{Sc}, and levels of neurodegeneration may help identify regions of the brain with markedly reduced signs of prion pathology.

Although the use of a viral-delivered shRNA cannot be performed on neonatal humans, due to ethical concerns ²⁴⁵ and potential risks involved ^{159,160,227}, the results demonstrated herein suggest that it may be possible to treat prion disease if the disease is detected within the early stages. Several advances in diagnostic procedures are required before prion diseases can be reliably diagnosed within the early stages of disease. A system allowing the detection of prion-resistant neurons within the brain of a prion-infected mouse has the potential to help uncover the mechanisms associated with progression of prion diseases, which may help identify key players that may be targeted for the treatment of prion disease. Furthermore, the increased survival of the prion-infected mice that received the anti-PrP^C shRNA in my experiments suggests that there may be specific regions of the brain that are essential for the survival against prion disease. Further analysis of the brain tissues may help identify these essential brain regions and performing similar experiments with use of different AAV serotypes may further uncover

regions of the brain that may be the target for treatment of prion disease. Creation of a cocktail of AAV serotypes that show preference for transducing different regions of the brain essential for the survival of prion disease may lead to the creation of a treatment that is at least capable of extending the lifespan of a prion-infected individual.

5.7 RML-Infection Does Not Alter Numbers of Transduced Cells in the CA1

Hippocampal Region or Thalamus

This work demonstrated that following neonatal intravenous injections of AAVrh.10, transduced cells remain detectable over 200 days. Additionally, infecting the neonatally-injected mice with RML prions at 60 days of age was shown to have no significant effect on the numbers of transduced cells counted in the CA1 and thalamic regions of the mouse brains.

Overall, these data show that AAVrh.10 is capable of bypassing the blood brain barrier and transducing neurons and astrocytes throughout the brain. The transduced cells continue to express the transgenes over a period of 200 days, and prion-infection does not significantly affect the portions of transduced cells. Therefore, AAVrh.10 remains a powerful viral vector that may be used to transduce and express specific genetic constructs throughout the lifespan of a prion-infected mouse model.

The effects of introducing an anti-PrP^C shRNA sequence prior to prion infection cannot be concluded from the data demonstrated in this thesis. Irreversibly apoptotic nuclei, a sign of neurodegeneration²⁴⁰, can be seen in the CA1 regions of tissue samples collected from mice at the later stages of prion disease, as shown in Figure 41. Further research using standard methods of detecting neurodegeneration, such as H&E staining to visualize prion spongiform pathogenesis, or Fluoro-Jade staining to label degenerating neurons, is required in order to assess whether the levels of neurodegeneration differ between experimental groups of mice.

Future Directions

This work has identified several potential future directions that can be followed.

First, this work has shown that AAVs may be intravenously injected into neonatal mice to drive long-term transgene expression in neurons of the brain. This platform may be adapted to be used in several experimental models, such as to deliver prion genes from other species to further study species transmission, or to manipulate prion disease with use of regulatory molecules such as miRNA. Silencing of other genes with use of a shRNA may also help identify key players in prion disease progression.

With respect to AAV production, these methods outline specific conditions that may be assessed to help improve the yield of industrial-scaled AAV productions that grow HEK293T cells in suspension. These modifications have the potential to improve AAV yield or improve the efficiency of the production.

As demonstrated, primary neuronal cell cultures are highly transduced with AAVs at a MOI of 1E4 or higher, and their transduction and transgene expression induced no significant increase in cell death of these cultures. Therefore, AAVs may be used to deliver a range of genetic constructs to assess the effects of genetic manipulation *in vitro* prior to their use *in vivo*.

The samples collected from the live mouse study may be further used to further study prion diseases. Specifically, I have shown that transduced neurons and astrocytes have been detected throughout the brain, with some regions containing higher levels of transduction. Using methods of LCM, as previously demonstrated ¹³⁵, it is possible to isolate regions containing high numbers of transduced cells, which most likely will reduce the background signal that is commonly seen when performing analysis on much larger sections of brain. Another promising option is the use

of a 10X genomics machine, which is capable of performing genomics on a single cell. Further analysis of these samples may help identify specific regions of the brain where a high level of PrP^C knockout neurons is associated with an increase in lifespan of the mouse following prion infection. These studies have the potential to identify specific regions of the brain that may be potential targets for the treatment of prion diseases in the future.

Further analysis of the collected tissues will help us better understand the transduction efficiencies of the viruses, and the effects of the anti-PrP^C shRNA. Identification of regions that are more highly transduced by each AAV serotype will help guide the development of AAV-based therapies of the CNS to target specific regions of the CNS.

Finally, further research into the potential integration of AAV genomes within the lung and liver may help assess the risks associated with intravenous delivery of AAVs into neonatal animals.

Conclusions

The hypothesis of this research was that AAVrh.10 could be used to deliver long-lasting transduction of a genetic construct via intravenous injection of a neonatal mouse. Shown here is the long-lasting transduction of cells throughout the brain, with higher concentrations of neurons within the hippocampal CA1, CA2, and CA3 regions, and the lateral habenula, compared to the rest of the brain. Transgene expression was found throughout the brain until at least 200 days post-transduction. Additionally, transgene expression was found within the liver and lungs at all time points, possibly suggesting integration of the viral vector into the genome of the host cells.

Another significant finding is that treatment of neonatal mice with a low dose of AAVrh.10 containing an anti-PrP^C shRNA sequence increased the lifespan of neonatal mice by 10 days on average, compared to empty vector, PBS treated, and untreated controls. Further analysis of PrP^C expression, PrP^{Sc} deposition, and levels of neurodegeneration may help identify specific regions of the brain where low levels of PrP^C expression correlates with the increased lifespans of mice following prion infection.

Finally, shown here are several factors that must be considered when producing AAVs, such as media composition, methods of harvesting AAVs, and of purifying the crude viral harvests.

Further shown in this work is the use of a hippocampal primary neuron cell culture to characterize transduction of AAVs to allow assessment of their transduction efficacies and cytotoxic effects prior to their *in vivo* use.

References

1. Fasano C, Campana V, Zurzolo C. Prions. *J Mol Neurosci*. 2006;29(3):195-214. doi:10.1385/JMN:29:3:195
2. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci*. 2001;24:519-550. doi:10.1146/annurev.neuro.24.1.519
3. Prion proteins: a biological role beyond prion diseases - Hu - 2007 - Acta Neurologica Scandinavica - Wiley Online Library. <https://onlinelibrary.wiley.com/doi/pdf/10.1111/j.1600-0404.2007.00868.x>. Accessed June 4, 2019.
4. Gaiger SH. Scrapie. *Journal of Comparative Pathology and Therapeutics*. 1924;37:259-277. doi:10.1016/S0368-1742(24)80046-2
5. Mathiason CK. Scrapie, CWD, and Transmissible Mink Encephalopathy. In: *Progress in Molecular Biology and Translational Science*. Vol 150. Elsevier; 2017:267-292. doi:10.1016/bs.pmbts.2017.07.009
6. Zabel MD, Reid C. A brief history of prions. *Pathog Dis*. 2015;73(9). doi:10.1093/femspd/ftv087
7. Cuille J, Chelle P. Investigations of scrapie in sheep. *Vet Med*. 1938;34:417-418.
8. Prusiner S. Novel proteinaceous infectious particles cause scrapie. *Science*. 1982;216(4542):136-144. doi:10.1126/science.6801762
9. Chesebro B, Race R, Wehrly K, et al. Identification of scrapie prion protein-specific mRNA in scrapie-infected and uninfected brain. *Nature*. 1985;315(6017):331-333. doi:10.1038/315331a0
10. Oesch B, Westaway D, Wälchli M, et al. A cellular gene encodes scrapie PrP 27-30 protein. *Cell*. 1985;40(4):735-746.
11. Carlson GL artist, Ebeling C, Torchia M, Westaway D, Prusiner SB. Delimiting the location of the scrapie prion incubation time gene on chromosome 2 of the mouse. *Genetics*. 1993;133(4):979-988.
12. Büeler H, Aguui A, Sailer A, Autenried P, Ague M, Weissmann' C. Mice Devoid of PrP Are Resistant to Scrapie. 1993:9.
13. Gordon W. Advances in veterinary research. *Vet Rec*. 1946;58:516-525.
14. Chandler RL. ENCEPHALOPATHY IN MICE PRODUCED BY INOCULATION WITH SCRAPIE BRAIN MATERIAL. *The Lancet*. 1961;277(7191):1378-1379. doi:10.1016/S0140-6736(61)92008-6

15. Gajdusek DC, Zigas V. Kuru: Clinical, pathological and epidemiological study of an acute progressive degenerative disease of the central nervous system among natives of the Eastern Highlands of New Guinea. *The American Journal of Medicine*. 1959;26(3):442-469. doi:10.1016/0002-9343(59)90251-7
16. Gajdusek DC, Gibbs CJ, Alpers M. Experimental Transmission of a Kuru-like Syndrome to Chimpanzees. *Nature*. 1966;209(5025):794. doi:10.1038/209794a0
17. Duffy P, Wolf J, Collins G, DeVoe AG, Streeten B, Cowen D. Letter: Possible person-to-person transmission of Creutzfeldt-Jakob disease. *N Engl J Med*. 1974;290(12):692-693.
18. Zou W-Q, Gambetti P. Variant Creutzfeldt-Jakob Disease. *Ann Neurol*. 2009;65(3):233-235. doi:10.1002/ana.21637
19. Facts about variant Creutzfeldt-Jakob disease. European Centre for Disease Prevention and Control. <http://ecdc.europa.eu/en/vcjd/facts>. Accessed June 4, 2019.
20. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of “new variant” CJD. *Nature*. 1996;383(6602):685-690. doi:10.1038/383685a0
21. Scott MR, Will R, Ironside J, et al. Compelling transgenic evidence for transmission of bovine spongiform encephalopathy prions to humans. *PNAS*. 1999;96(26):15137-15142. doi:10.1073/pnas.96.26.15137
22. Mead S. Balancing Selection at the Prion Protein Gene Consistent with Prehistoric Kurulike Epidemics. *Science*. 2003;300(5619):640-643. doi:10.1126/science.1083320
23. Variably Protease-Sensitive Prionopathy (VPSPr) - Neurologic Disorders - MSD Manual Professional Edition. <https://www.msdmanuals.com/professional/neurologic-disorders/prion-diseases/variably-protease-sensitive-prionopathy-vpspr>. Accessed August 8, 2019.
24. Gerstmann-Sträussler-Scheinker Disease. *NORD (National Organization for Rare Disorders)*. <https://rarediseases.org/rare-diseases/gerstmann-straussler-scheinker-disease/>. Accessed August 8, 2019.
25. Dohgu S, Yamauchi A, Takata F, et al. Uptake and Efflux of Quinacrine, a Candidate for the Treatment of Prion Diseases, at the Blood-Brain Barrier. *Cell Mol Neurobiol*. 2004;24(2):205-217. doi:10.1023/B:CEMN.0000018617.21378.95
26. Giles K, Olson SH, Prusiner SB. Developing Therapeutics for PrP Prion Diseases. *Cold Spring Harb Perspect Med*. 2017;7(4):a023747. doi:10.1101/cshperspect.a023747
27. Aguzzi A, Sigurdson CJ. Antiprion immunotherapy: to suppress or to stimulate? *Nature Reviews Immunology*. 2004;4(9):725-736. doi:10.1038/nri1437

28. Neurodegenerative diseases. <https://www.nature.com/collections/rgktdtljnv>. Accessed August 12, 2019.
29. Appleby BS, Rhoads DD, Mente K, Cohen ML. A Practical Primer on Prion Pathology. *Journal of Neuropathology & Experimental Neurology*. March 2018. doi:10.1093/jnen/nly019
30. Requena JR, Wille H. Chapter Fifteen - The Structure of the Infectious Prion Protein and Its Propagation. In: Legname G, Vanni S, eds. *Progress in Molecular Biology and Translational Science*. Vol 150. Prion Protein. Academic Press; 2017:341-359. doi:10.1016/bs.pmbts.2017.06.009
31. Zhou Z, Xiao G. Conformational conversion of prion protein in prion diseases. *Acta Biochim Biophys Sin (Shanghai)*. 2013;45(6):465-476. doi:10.1093/abbs/gmt027
32. How prions fold. Mayo Clinic. <https://www.mayoclinic.org/diseases-conditions/creutzfeldt-jakob-disease/multimedia/normal-and-diseased-prions/img-20007478>. Accessed August 10, 2019.
33. Prusiner SB. Molecular biology of prion diseases. *Science*. 1991;252(5012):1515-1522. doi:10.1126/science.1675487
34. Baskakov IV. Branched chain mechanism of polymerization and ultrastructure of prion protein amyloid fibrils. *FEBS J*. 2007;274(15):3756-3765. doi:10.1111/j.1742-4658.2007.05916.x
35. Gill AC, Agarwal S, Pinheiro TJT, Graham JF. Structural requirements for efficient prion protein conversion: cofactors may promote a conversion-competent structure for PrP(C). *Prion*. 2010;4(4):235-242. doi:10.4161/pri.4.4.13394
36. Surewicz WK, Apostol MI. Prion Protein and Its Conformational Conversion: A Structural Perspective. In: Tatzelt J, ed. *Prion Proteins*. Topics in Current Chemistry. Berlin, Heidelberg: Springer Berlin Heidelberg; 2011:135-167. doi:10.1007/128_2011_165
37. Harris DA, Lele P, Snider WD. Localization of the mRNA for a chicken prion protein by in situ hybridization. *Proc Natl Acad Sci U S A*. 1993;90(9):4309-4313.
38. Moser M, Colello RJ, Pott U, Oesch B. Developmental expression of the prion protein gene in glial cells. *Neuron*. 1995;14(3):509-517.
39. Ford MJ, Burton LJ, Morris RJ, Hall SM. Selective expression of prion protein in peripheral tissues of the adult mouse. *Neuroscience*. 2002;113(1):177-192.
40. Manson J, West JD, Thomson V, McBride P, Kaufman MH, Hope J. The prion protein gene: a role in mouse embryogenesis? *Development*. 1992;115(1):117-122.

41. Rivera-Milla E, Oidtmann B, Panagiotidis CH, et al. Disparate evolution of prion protein domains and the distinct origin of Doppel- and prion-related loci revealed by fish-to-mammal comparisons. *FASEB J*. 2006;20(2):317-319. doi:10.1096/fj.05-4279fje
42. Roucou X, LeBlanc AC. Cellular prion protein neuroprotective function: implications in prion diseases. *J Mol Med*. 2005;83(1):3-11. doi:10.1007/s00109-004-0605-5
43. Halliwell B. Oxidative stress and neurodegeneration: where are we now? *J Neurochem*. 2006;97(6):1634-1658. doi:10.1111/j.1471-4159.2006.03907.x
44. Vassallo N, Herms J. Cellular prion protein function in copper homeostasis and redox signalling at the synapse. *J Neurochem*. 2003;86(3):538-544.
45. Taylor DR, Hooper NM. The prion protein and lipid rafts. *Mol Membr Biol*. 2006;23(1):89-99. doi:10.1080/09687860500449994
46. Kanaani J, Prusiner SB, Diacovo J, Baekkeskov S, Legname G. Recombinant prion protein induces rapid polarization and development of synapses in embryonic rat hippocampal neurons in vitro. *J Neurochem*. 2005;95(5):1373-1386. doi:10.1111/j.1471-4159.2005.03469.x
47. Schmitt-Ulms G, Legname G, Baldwin MA, et al. Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J Mol Biol*. 2001;314(5):1209-1225. doi:10.1006/jmbi.2000.5183
48. Büeler H, Fischer M, Lang Y, et al. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. *Nature*. 1992;356(6370):577. doi:10.1038/356577a0
49. Brandner S, Isenmann S, Raeber A, et al. Normal host prion protein necessary for scrapie-induced neurotoxicity. *Nature*. 1996;379(6563):339. doi:10.1038/379339a0
50. Sailer A, Büeler H, Fischer M, Aguzzi A, Weissmann C. No propagation of prions in mice devoid of PrP. *Cell*. 1994;77(7):967-968.
51. Büeler H, Raeber A, Sailer A, Fischer M, Aguzzi A, Weissmann C. High prion and PrPSc levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. *Mol Med*. 1994;1(1):19-30.
52. Jeffrey M, Halliday WG, Bell J, et al. Synapse loss associated with abnormal PrP precedes neuronal degeneration in the scrapie-infected murine hippocampus. *Neuropathol Appl Neurobiol*. 2000;26(1):41-54.
53. Šišková Z, Page A, O'Connor V, Perry VH. Degenerating Synaptic Boutons in Prion Disease. *Am J Pathol*. 2009;175(4):1610-1621. doi:10.2353/ajpath.2009.090372
54. Fuhrmann M, Mitteregger G, Kretschmar H, Herms J. Dendritic Pathology in Prion Disease Starts at the Synaptic Spine. *Journal of Neuroscience*. 2007;27(23):6224-6233. doi:10.1523/JNEUROSCI.5062-06.2007

55. Conforti L, Adalbert R, Coleman MP. Neuronal death: where does the end begin? *Trends in Neurosciences*. 2007;30(4):159-166. doi:10.1016/j.tins.2007.02.004
56. Hafiz FB, Brown DR. A Model for the Mechanism of Astrogliosis in Prion Disease. *Molecular and Cellular Neuroscience*. 2000;16(3):221-232. doi:10.1006/mcne.2000.0868
57. Pekny M, Wilhelmsson U, Pekna M. The dual role of astrocyte activation and reactive gliosis. *Neuroscience Letters*. 2014;565:30-38. doi:10.1016/j.neulet.2013.12.071
58. Field EJ. The significance of astroglial hypertrophy in Scrapie, Kuru, Multiple Sclerosis and old age together with a note on the possible nature of the scrapie agent. *Deutsche Zeitschrift f Nervenheilkunde*. 1967;192(3):265-274. doi:10.1007/BF00244170
59. Manuelidis L, Tesin DM, Sklaviadis T, Manuelidis EE. Astrocyte gene expression in Creutzfeldt-Jakob disease. *Proc Natl Acad Sci USA*. 1987;84(16):5937-5941. doi:10.1073/pnas.84.16.5937
60. Colangelo AM, Alberghina L, Papa M. Astrogliosis as a therapeutic target for neurodegenerative diseases. *Neurosci Lett*. 2014;565:59-64. doi:10.1016/j.neulet.2014.01.014
61. neuropath.pdf. <https://www.cjd.ed.ac.uk/sites/default/files/neuropath.pdf>. Accessed August 10, 2019.
62. Budka H. Neuropathology of prion diseases. *Br Med Bull*. 2003;66(1):121-130. doi:10.1093/bmb/66.1.121
63. Legname G, Nguyen H-OB, Baskakov IV, Cohen FE, DeArmond SJ, Prusiner SB. Strain-specified characteristics of mouse synthetic prions. *Proc Natl Acad Sci U S A*. 2005;102(6):2168-2173. doi:10.1073/pnas.0409079102
64. Prion Biology and Diseases, Second Edition. https://www.cshlpress.com/default.tpl?cart=1565466064325790897&fromlink=T&linkaction=full&linksortby=oop_title&--eqSKUdatareq=454. Accessed August 10, 2019.
65. Thackray AM, Hopkins L, Klein MA, Bujdoso R. Mouse-Adapted Ovine Scrapie Prion Strains Are Characterized by Different Conformers of PrPSc. *Journal of Virology*. 2007;81(22):12119-12127. doi:10.1128/JVI.01434-07
66. DeArmond SJ, Sánchez H, Yehiely F, et al. Selective Neuronal Targeting in Prion Disease. *Neuron*. 1997;19(6):1337-1348. doi:10.1016/S0896-6273(00)80424-9
67. Carroll JA, Striebel JF, Rangel A, et al. Prion Strain Differences in Accumulation of PrPSc on Neurons and Glia Are Associated with Similar Expression Profiles of Neuroinflammatory Genes: Comparison of Three Prion Strains. *PLoS Pathog*. 2016;12(4). doi:10.1371/journal.ppat.1005551

68. Majer A, Medina SJ, Niu Y, et al. Early Mechanisms of Pathobiology Are Revealed by Transcriptional Temporal Dynamics in Hippocampal CA1 Neurons of Prion Infected Mice. Westaway D, ed. *PLoS Pathogens*. 2012;8(11):e1003002. doi:10.1371/journal.ppat.1003002
69. Sandberg MK, Al-Doujaily H, Sharps B, Clarke AR, Collinge J. Prion propagation and toxicity *in vivo* occur in two distinct mechanistic phases. *Nature*. 2011;470(7335):540-542. doi:10.1038/nature09768
70. Collinge J. Mammalian prions and their wider relevance in neurodegenerative diseases. *Nature*. 2016;539(7628):217-226. doi:10.1038/nature20415
71. Mallucci G, Dickinson A, Linehan J, Klöhn P-C, Brandner S, Collinge J. Depleting neuronal PrP in prion infection prevents disease and reverses spongiosis. *Science*. 2003;302(5646):871-874. doi:10.1126/science.1090187
72. Kandel: Principles of neural science - Google Scholar. https://scholar.google.com/scholar_lookup?author=E.+R.+Kandel+&publication_year=2013&title=Principles+of+Neural+Science. Accessed August 10, 2019.
73. Phatnani H, Maniatis T. Astrocytes in Neurodegenerative Disease. *Cold Spring Harb Perspect Biol*. 2015;7(6):a020628. doi:10.1101/cshperspect.a020628
74. Burda JE, Sofroniew MV. Reactive gliosis and the multicellular response to CNS damage and disease. *Neuron*. 2014;81(2):229-248. doi:10.1016/j.neuron.2013.12.034
75. Eroglu C, Barres BA. Regulation of synaptic connectivity by glia. *Nature*. 2010;468(7321):223-231. doi:10.1038/nature09612
76. Ullian EM, Sapperstein SK, Christopherson KS, Barres BA. Control of Synapse Number by Glia. *Science*. 2001;291(5504):657-661. doi:10.1126/science.291.5504.657
77. Chung W-S, Barres BA. The role of glial cells in synapse elimination. *Current Opinion in Neurobiology*. 2012;22(3):438-445. doi:10.1016/j.conb.2011.10.003
78. Pellerin L, Bouzier-Sore A-K, Aubert A, et al. Activity-dependent regulation of energy metabolism by astrocytes: An update. *Glia*. 2007;55(12):1251-1262. doi:10.1002/glia.20528
79. Choi HB, Gordon GRJ, Zhou N, et al. Metabolic Communication between Astrocytes and Neurons via Bicarbonate-Responsive Soluble Adenylyl Cyclase. *Neuron*. 2012;75(6):1094-1104. doi:10.1016/j.neuron.2012.08.032
80. Liebner S, Czupalla CJ, Wolburg H. Current concepts of blood-brain barrier development. *Int J Dev Biol*. 2011;55(4-5):467-476. doi:10.1387/ijdb.103224sl

81. Attwell D, Buchan AM, Charpak S, Lauritzen M, MacVicar BA, Newman EA. Glial and neuronal control of brain blood flow. *Nature*. 2010;468(7321):232-243. doi:10.1038/nature09613
82. Ransohoff RM. How neuroinflammation contributes to neurodegeneration. *Science*. 2016;353(6301):777-783. doi:10.1126/science.aag2590
83. Bardehle S, Krüger M, Buggenthin F, et al. Live imaging of astrocyte responses to acute injury reveals selective juxtavascular proliferation. *Nature Neuroscience*. 2013;16(5):580-586. doi:10.1038/nn.3371
84. Baker CA, Martin D, Manuelidis L. Microglia from Creutzfeldt-Jakob Disease-Infected Brains Are Infectious and Show Specific mRNA Activation Profiles. *Journal of Virology*. 2002;76(21):10905-10913. doi:10.1128/JVI.76.21.10905-10913.2002
85. Borner R, Bento-Torres J, Souza DR, et al. Early behavioral changes and quantitative analysis of neuropathological features in murine prion disease. *Prion*. 2011;5(3):215-227. doi:10.4161/pri.5.3.16936
86. Schaefer AM, Sanes JR, Lichtman JW. A compensatory subpopulation of motor neurons in a mouse model of amyotrophic lateral sclerosis. *J Comp Neurol*. 2005;490(3):209-219. doi:10.1002/cne.20620
87. Majer A, Medina SJ, Sorensen D, et al. The cell type resolved mouse transcriptome in neuron-enriched brain tissues from the hippocampus and cerebellum during prion disease. *Scientific Reports*. 2019;9(1). doi:10.1038/s41598-018-37715-z
88. Gibbs CJ, Gajdusek DC, Asher DM, et al. Creutzfeldt-Jakob disease (spongiform encephalopathy): transmission to the chimpanzee. *Science*. 1968;161(3839):388-389.
89. Lasmézas CI, Deslys JP, Demaimay R, et al. BSE transmission to macaques. *Nature*. 1996;381(6585):743-744. doi:10.1038/381743a0
90. Comoy EE, Mikol J, Luccantoni-Freire S, et al. Transmission of scrapie prions to primate after an extended silent incubation period. *Sci Rep*. 2015;5:11573. doi:10.1038/srep11573
91. Race B, Williams K, Orrú CD, Hughson AG, Lubke L, Chesebro B. Lack of Transmission of Chronic Wasting Disease to Cynomolgus Macaques. *Journal of Virology*. 2018;92(14):e00550-18. doi:10.1128/JVI.00550-18
92. Lescoutra-Etchegaray N, Jaffré N, Sumian C, et al. Evaluation of the protection of primates transfused with variant Creutzfeldt-Jakob disease-infected blood products filtered with prion removal devices: a 5-year update. *Transfusion*. 2015;55(6):1231-1241. doi:10.1111/trf.12999
93. Brandner S, Jaunmuktane Z. Prion disease: experimental models and reality. *Acta Neuropathologica*. 2017;133(2):197-222. doi:10.1007/s00401-017-1670-5

94. Pattison IH, Millson GC. Scrapie produced experimentally in goats with special reference to the clinical syndrome. *J Comp Pathol*. 1961;71:101-109.
95. N. van den Pol A, K.Ghosh P. Selective Neuronal Expression of Green Fluorescent Protein with Cytomegalovirus Promoter Reveals Entire Neuronal Arbor in Transgenic Mice | *Journal of Neuroscience*. 1998;18(24). <https://www.jneurosci.org/content/18/24/10640>. Accessed August 5, 2019.
96. Morales R, Abid K, Soto C. The prion strain phenomenon: Molecular basis and unprecedented features. *Biochim Biophys Acta*. 2007;1772(6):681-691. doi:10.1016/j.bbadis.2006.12.006
97. Caughey B, Raymond GJ, Bessen RA. Strain-dependent differences in beta-sheet conformations of abnormal prion protein. *J Biol Chem*. 1998;273(48):32230-32235. doi:10.1074/jbc.273.48.32230
98. Igel-Egalon A, Béringue V, Rezaei H, Sibille P. Prion Strains and Transmission Barrier Phenomena. *Pathogens*. 2018;7(1). doi:10.3390/pathogens7010005
99. Fraser H, Dickinson AG. Scrapie in mice. Agent-strain differences in the distribution and intensity of grey matter vacuolation. *J Comp Pathol*. 1973;83(1):29-40.
100. Bartz JC, Bessen RA, McKenzie D, Marsh RF, Aiken JM. Adaptation and selection of prion protein strain conformations following interspecies transmission of transmissible mink encephalopathy. *J Virol*. 2000;74(12):5542-5547. doi:10.1128/jvi.74.12.5542-5547.2000
101. Li J, Browning S, Mahal SP, Oelschlegel AM, Weissmann C. Darwinian evolution of prions in cell culture. *Science*. 2010;327(5967):869-872. doi:10.1126/science.1183218
102. Giles K, Glidden DV, Patel S, et al. Human prion strain selection in transgenic mice. *Ann Neurol*. 2010;68(2):151-161. doi:10.1002/ana.22104
103. Bian J, Khaychuk V, Angers RC, et al. Prion replication without host adaptation during interspecies transmissions. *PNAS*. 2017;114(5):1141-1146. doi:10.1073/pnas.1611891114
104. Nishida N, Harris DA, Vilette D, et al. Successful Transmission of Three Mouse-Adapted Scrapie Strains to Murine Neuroblastoma Cell Lines Overexpressing Wild-Type Mouse Prion Protein. *J Virol*. 2000;74(1):320-325.
105. Beaudoin GMJ, Lee S-H, Singh D, et al. Culturing pyramidal neurons from the early postnatal mouse hippocampus and cortex. *Nature Protocols*. 2012;7(9):1741-1754. doi:10.1038/nprot.2012.099
106. Brewer GJ, Torricelli JR. Isolation and culture of adult neurons and neurospheres. *Nature Protocols*. 2007;2(6):1490-1498. doi:10.1038/nprot.2007.207

107. Eide L, McMurray CT. Culture of adult mouse neurons. *BioTechniques*. 2005;38(1):99-104. doi:10.2144/05381RR02
108. Cronier S, Laude H, Peyrin J-M. Prions can infect primary cultured neurons and astrocytes and promote neuronal cell death. *Proc Natl Acad Sci U S A*. 2004;101(33):12271-12276. doi:10.1073/pnas.0402725101
109. Croft CL, Noble W. Preparation of organotypic brain slice cultures for the study of Alzheimer's disease. *F1000Res*. 2018;7. doi:10.12688/f1000research.14500.2
110. Gianinazzi C, Grandgirard D, Simon F, et al. Apoptosis of Hippocampal Neurons in Organotypic Slice Culture Models: Direct Effect of Bacteria Revisited. *J Neuropathol Exp Neurol*. 2004;63(6):610-617. doi:10.1093/jnen/63.6.610
111. Drexler B, Hentschke H, Antkowiak B, Grasshoff C. Organotypic Cultures as Tools for Testing Neuroactive Drugs - Link Between In-Vitro and In-Vivo Experiments. doi:info:doi/10.2174/092986710794183042
112. Falsig J, Aguzzi A. The prion organotypic slice culture assay—POSCA. *Nature Protocols*. 2008;3(4):555-562. doi:10.1038/nprot.2008.13
113. Patterson AP, Booth SA, Saba R. The Emerging Use of *In Vivo* Optical Imaging in the Study of Neurodegenerative Diseases. *BioMed Research International*. 2014;2014:1-14. doi:10.1155/2014/401306
114. Mizrahi A, Crowley JC, Shtoyerman E, Katz LC. High-Resolution In Vivo Imaging of Hippocampal Dendrites and Spines. *J Neurosci*. 2004;24(13):3147-3151. doi:10.1523/JNEUROSCI.5218-03.2004
115. Feng G, Mellor RH, Bernstein M, et al. Imaging Neuronal Subsets in Transgenic Mice Expressing Multiple Spectral Variants of GFP. *Neuron*. 2000;28(1):41-51. doi:10.1016/S0896-6273(00)00084-2
116. Tamgüney G, Francis KP, Giles K, Lemus A, DeArmond SJ, Prusiner SB. Measuring prions by bioluminescence imaging. *Proceedings of the National Academy of Sciences*. 2009;106(35):15002–15006.
117. Tatzelt J, Maeda N, Pekny M, et al. Scrapie in mice deficient in apolipoprotein E or glial fibrillary acidic protein. *Neurology*. 1996;47(2):449-453. doi:10.1212/wnl.47.2.449
118. Groveman BR, Foliaki ST, Orru CD, et al. Sporadic Creutzfeldt-Jakob disease prion infection of human cerebral organoids. *Acta Neuropathologica Communications*. 2019;7(1):90. doi:10.1186/s40478-019-0742-2
119. Fischer M, Rülcke T, Raeber A, et al. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J*. 1996;15(6):1255-1264.

120. Obbard DJ, Gordon KHJ, Buck AH, Jiggins FM. The evolution of RNAi as a defence against viruses and transposable elements. *Philos Trans R Soc Lond B Biol Sci*. 2009;364(1513):99-115. doi:10.1098/rstb.2008.0168
121. Izant JG, Weintraub H. Inhibition of thymidine kinase gene expression by anti-sense RNA: A molecular approach to genetic analysis. *Cell*. 1984;36(4):1007-1015. doi:10.1016/0092-8674(84)90050-3
122. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 1998;391(6669):806. doi:10.1038/35888
123. Paddison PJ, Cleary M, Silva JM, et al. Cloning of short hairpin RNAs for gene knockdown in mammalian cells. *Nature Methods*. 2004;1(2):163-167. doi:10.1038/nmeth1104-163
124. Tomar RS, Matta H, Chaudhary PM. Use of adeno-associated viral vector for delivery of small interfering RNA. *Oncogene*. 2003;22(36):5712-5715. doi:10.1038/sj.onc.1206733
125. O'Keefe EP. siRNAs and shRNAs: Tools for Protein Knockdown by Gene Silencing. *Materials and Methods*. May 2019. /method/siRNAs-and-shRNAs-Tools-for-Protein-Knockdown-by-Gene-Silencing.html. Accessed June 11, 2019.
126. Pfeifer A, Eigenbrod S, Al-Khadra S, et al. Lentivector-mediated RNAi efficiently suppresses prion protein and prolongs survival of scrapie-infected mice. *J Clin Invest*. 2006;116(12):3204-3210. doi:10.1172/JCI29236
127. White MD, Farmer M, Mirabile I, Brandner S, Collinge J, Mallucci GR. Single treatment with RNAi against prion protein rescues early neuronal dysfunction and prolongs survival in mice with prion disease. *Proceedings of the National Academy of Sciences*. 2008;105(29):10238-10243. doi:10.1073/pnas.0802759105
128. Ahn M, Bajsarowicz K, Oehler A, Lemus A, Bankiewicz K, DeArmond SJ. Convection-Enhanced Delivery of AAV2-PrPshRNA in Prion-Infected Mice. *PLoS One*. 2014;9(5). doi:10.1371/journal.pone.0098496
129. Developing an antisense drug for prion disease.
<http://www.prionalliance.org/2018/07/09/developing-an-antisense-drug-for-prion-disease/>. Accessed August 10, 2019.
130. Chiriboga CA, Swoboda KJ, Darras BT, et al. Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology*. 2016;86(10):890-897. doi:10.1212/WNL.0000000000002445
131. Raymond GJ, Zhao HT, Race B, et al. Antisense oligonucleotides extend survival of prion-infected mice. *JCI Insight*. 2019;4(16). doi:10.1172/jci.insight.131175
132. Hanna J, Hossain GS, Kocerha J. The Potential for microRNA Therapeutics and Clinical Research. *Front Genet*. 2019;10. doi:10.3389/fgene.2019.00478

133. Christopher AF, Kaur RP, Kaur G, Kaur A, Gupta V, Bansal P. MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspect Clin Res.* 2016;7(2):68-74. doi:10.4103/2229-3485.179431
134. Sonntag K-C. MicroRNAs and deregulated gene expression networks in neurodegeneration. *Brain Res.* 2010;1338:48-57. doi:10.1016/j.brainres.2010.03.106
135. Majer A, Boese AS, Booth SA. The Role of MicroRNAs in Neurodegenerative Diseases: Implications for Early Detection and Treatment. In: Mallick B, Ghosh Z, eds. *Regulatory RNAs: Basics, Methods and Applications*. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012:443-473. doi:10.1007/978-3-642-22517-8_18
136. Zhang H, Yang B, Mu X, et al. Several rAAV Vectors Efficiently Cross the Blood–brain Barrier and Transduce Neurons and Astrocytes in the Neonatal Mouse Central Nervous System. *Molecular Therapy.* 2011;19(8):1440-1448. doi:10.1038/mt.2011.98
137. Saraiva J, Nobre RJ, Pereira de Almeida L. Gene therapy for the CNS using AAVs: The impact of systemic delivery by AAV9. *Journal of Controlled Release.* 2016;241:94-109. doi:10.1016/j.jconrel.2016.09.011
138. Martin JN, Wolken N, Brown T, Dauer WT, Ehrlich ME, Gonzalez-Alegre P. Lethal toxicity caused by expression of shRNA in the mouse striatum: implications for therapeutic design. *Gene Ther.* 2011;18(7):666-673. doi:10.1038/gt.2011.10
139. Chandler RJ, LaFave MC, Varshney GK, Burgess SM, Venditti CP. Genotoxicity in Mice Following AAV Gene Delivery: A Safety Concern for Human Gene Therapy? *Molecular Therapy.* 2016;24(2):198-201. doi:10.1038/mt.2016.17
140. Conceição M, Mendonça L, Nóbrega C, et al. Intravenous administration of brain-targeted stable nucleic acid lipid particles alleviates Machado-Joseph disease neurological phenotype. *Biomaterials.* 2016;82:124-137. doi:10.1016/j.biomaterials.2015.12.021
141. Ojala DS, Amara DP, Schaffer DV. Adeno-Associated Virus Vectors and Neurological Gene Therapy. *Neuroscientist.* 2015;21(1):84-98. doi:10.1177/1073858414521870
142. van der Loo JCM, Wright JF. Progress and challenges in viral vector manufacturing. *Hum Mol Genet.* 2016;25(R1):R42-R52. doi:10.1093/hmg/ddv451
143. Lentz TB, Gray SJ, Samulski RJ. Viral Vectors for Gene Delivery to the Central Nervous System. *Neurobiol Dis.* 2012;48(2):179-188. doi:10.1016/j.nbd.2011.09.014
144. Aschauer DF, Kreuz S, Rumpel S. Analysis of Transduction Efficiency, Tropism and Axonal Transport of AAV Serotypes 1, 2, 5, 6, 8 and 9 in the Mouse Brain. Qiu J, ed. *PLoS ONE.* 2013;8(9):e76310. doi:10.1371/journal.pone.0076310
145. Takeda S, Takahashi M, Mizukami H, et al. Successful gene transfer using adeno-associated virus vectors into the kidney: comparison among adeno-associated virus serotype

- 1-5 vectors in vitro and in vivo. *Nephron Exp Nephrol*. 2004;96(4):e119-126. doi:10.1159/000077378
146. Wanisch K. Using AAV for neuronal tracing. <https://blog.addgene.org/using-aav-for-neuronal-tracing>. Accessed August 10, 2019.
147. Blankinship MJ, Gregorevic P, Allen JM, et al. Efficient transduction of skeletal muscle using vectors based on adeno-associated virus serotype 6. *Mol Ther*. 2004;10(4):671-678. doi:10.1016/j.ymthe.2004.07.016
148. Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. Unrestricted Hepatocyte Transduction with Adeno-Associated Virus Serotype 8 Vectors in Mice. *J Virol*. 2005;79(1):214-224. doi:10.1128/JVI.79.1.214-224.2005
149. Chandler RJ, LaFave MC, Varshney GK, et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J Clin Invest*. 2015;125(2):870-880. doi:10.1172/JCI79213
150. Lykken EA, Shyng C, Edwards RJ, Rozenberg A, Gray SJ. Recent progress and considerations for AAV gene therapies targeting the central nervous system. *J Neurodev Disord*. 2018;10. doi:10.1186/s11689-018-9234-0
151. Duan D, Sharma P, Yang J, et al. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol*. 1998;72(11):8568-8577.
152. Schnepf BC, Jensen RL, Chen C-L, Johnson PR, Clark KR. Characterization of adeno-associated virus genomes isolated from human tissues. *J Virol*. 2005;79(23):14793-14803. doi:10.1128/JVI.79.23.14793-14803.2005
153. Hadaczek P, Eberling JL, Pivrotto P, Bringas J, Forsayeth J, Bankiewicz KS. Eight Years of Clinical Improvement in MPTP-Lesioned Primates After Gene Therapy With AAV2-hAADC. *Mol Ther*. 2010;18(8):1458-1461. doi:10.1038/mt.2010.106
154. Rivera VM, Gao G, Grant RL, et al. Long-term pharmacologically regulated expression of erythropoietin in primates following AAV-mediated gene transfer. *Blood*. 2005;105(4):1424-1430. doi:10.1182/blood-2004-06-2501
155. Ferrari FK, Samulski T, Shenk T, Samulski RJ. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol*. 1996;70(5):3227-3234.
156. Duan D, Yue Y, Yan Z, Engelhardt JF. A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat Med*. 2000;6(5):595-598. doi:10.1038/75080

157. Ghosh A, Yue Y, Duan D. Efficient transgene reconstitution with hybrid dual AAV vectors carrying the minimized bridging sequences. *Hum Gene Ther.* 2011;22(1):77-83. doi:10.1089/hum.2010.122
158. McCarty DM, Young SM, Samulski RJ. Integration of adeno-associated virus (AAV) and recombinant AAV vectors. *Annu Rev Genet.* 2004;38:819-845. doi:10.1146/annurev.genet.37.110801.143717
159. Bell P, Wang L, Leberer C, et al. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol Ther.* 2005;12(2):299-306. doi:10.1016/j.ymthe.2005.03.020
160. Walia JS, Altaleb N, Bello A, et al. Long-Term Correction of Sandhoff Disease Following Intravenous Delivery of rAAV9 to Mouse Neonates. *Molecular Therapy.* 2015;23(3):414-422. doi:10.1038/mt.2014.240
161. Albright BH, Storey CM, Murlidharan G, et al. Mapping the Structural Determinants Required for AAVrh.10 Transport across the Blood-Brain Barrier. *Molecular Therapy.* 2018;26(2):510-523. doi:10.1016/j.ymthe.2017.10.017
162. Burger C, Gorbatyuk OS, Velardo MJ, et al. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther.* 2004;10(2):302-317. doi:10.1016/j.ymthe.2004.05.024
163. Lowery RL, Majewska AK. Intracranial Injection of Adeno-associated Viral Vectors. *J Vis Exp.* 2010;(45). doi:10.3791/2140
164. Cunningham J, Pivrotto P, Bringas J, et al. Biodistribution of Adeno-associated Virus Type-2 in Nonhuman Primates after Convection-enhanced Delivery to Brain. *Mol Ther.* 2008;16(7):1267-1275. doi:10.1038/mt.2008.111
165. Duque S, Joussemet B, Riviere C, et al. Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol Ther.* 2009;17(7):1187-1196. doi:10.1038/mt.2009.71
166. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal-neurons and adult-astrocytes in CNS. *Nat Biotechnol.* 2009;27(1):59-65. doi:10.1038/nbt.1515
167. Gray SJ, Matagne V, Bachaboina L, Yadav S, Ojeda SR, Samulski RJ. Preclinical differences of intravascular AAV9 delivery to neurons and glia: a comparative study of adult mice and nonhuman primates. *Mol Ther.* 2011;19(6):1058-1069. doi:10.1038/mt.2011.72
168. Tanguy Y, Biferi MG, Besse A, et al. Systemic AAVrh10 provides higher transgene expression than AAV9 in the brain and the spinal cord of neonatal mice. *Frontiers in Molecular Neuroscience.* 2015;8. doi:10.3389/fnmol.2015.00036

169. Hu C, Busuttil RW, Lipshutz GS. RH10 provides superior transgene expression in mice when compared with natural AAV serotypes for neonatal gene therapy. *J Gene Med.* 2010;12(9). doi:10.1002/jgm.1496
170. Cotugno G, Annunziata P, Tessitore A, et al. Long-term amelioration of feline Mucopolysaccharidosis VI after AAV-mediated liver gene transfer. *Mol Ther.* 2011;19(3):461-469. doi:10.1038/mt.2010.257
171. Cearley C, Wolfe J. Transduction characteristics of adeno-associated virus vectors expressing cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Molecular Therapy.* 2006;13(3):528-537. doi:10.1016/j.ymthe.2005.11.015
172. Haurigot V, Marcó S, Ribera A, et al. Whole body correction of mucopolysaccharidosis IIIA by intracerebrospinal fluid gene therapy. *J Clin Invest.* 2013;123(8):3254-3271. doi:10.1172/JCI66778
173. Ruza A, Marcó S, García M, et al. Correction of pathological accumulation of glycosaminoglycans in central nervous system and peripheral tissues of MPSIIIA mice through systemic AAV9 gene transfer. *Hum Gene Ther.* 2012;23(12):1237-1246. doi:10.1089/hum.2012.029
174. Fu H, Cataldi MP, Ware TA, et al. Functional correction of neurological and somatic disorders at later stages of disease in MPS IIIA mice by systemic scAAV9-hSGSH gene delivery. *Mol Ther Methods Clin Dev.* 2016;3:16036. doi:10.1038/mtm.2016.36
175. Asokan A, Conway JC, Phillips JL, et al. Reengineering a receptor footprint of adeno-associated virus enables selective and systemic gene transfer to muscle. *Nat Biotechnol.* 2010;28(1):79-82. doi:10.1038/nbt.1599
176. Tse LV, Klinc KA, Madigan VJ, et al. Structure-guided evolution of antigenically distinct adeno-associated virus variants for immune evasion. *Proc Natl Acad Sci USA.* 2017;114(24):E4812-E4821. doi:10.1073/pnas.1704766114
177. Schaffer DV, Maheshri N. Directed evolution of AAV mutants for enhanced gene delivery. *Conf Proc IEEE Eng Med Biol Soc.* 2004;5:3520-3523. doi:10.1109/IEMBS.2004.1403990
178. Marsic D, Zolotukhin S. Altering Tropism of rAAV by Directed Evolution. *Methods Mol Biol.* 2016;1382:151-173. doi:10.1007/978-1-4939-3271-9_11
179. Asokan A, Samulski RJ. AAV does the shuffle. *Nat Biotechnol.* 2006;24(2):158-160. doi:10.1038/nbt0206-158
180. Gray SJ, Blake BL, Criswell HE, et al. Directed evolution of a novel adeno-associated virus (AAV) vector that crosses the seizure-compromised blood-brain barrier (BBB). *Mol Ther.* 2010;18(3):570-578. doi:10.1038/mt.2009.292

181. Chen YH, Chang M, Davidson BL. Molecular signatures of disease brain endothelia provide new sites for CNS-directed enzyme therapy. *Nat Med*. 2009;15(10):1215-1218. doi:10.1038/nm.2025
182. Powell SK, Rivera-Soto R, Gray SJ. Viral expression cassette elements to enhance transgene target specificity and expression in gene therapy. *Discov Med*. 2015;19(102):49-57.
183. Lee H, Quach L, Fan F, et al. 309. Optimization of Production of AAVrh.10 Viral Vectors. *Molecular Therapy*. 2016;24:S124-S125. doi:10.1016/S1525-0016(16)33118-5
184. Durocher Y, Pham PL, St-Laurent G, et al. Scalable serum-free production of recombinant adeno-associated virus type 2 by transfection of 293 suspension cells. *J Virol Methods*. 2007;144(1-2):32-40. doi:10.1016/j.jviromet.2007.03.014
185. Clark KR. Recent advances in recombinant adeno-associated virus vector production. *Kidney Int*. 2002;61(1 Suppl):S9-15. doi:10.1046/j.1523-1755.2002.0610s1009.x
186. Chen Y. Calcium Phosphate Transfection of Eukaryotic Cells. *BIO-PROTOCOL*. 2012;2(3). doi:10.21769/BioProtoc.86
187. Goetze B, Grunewald B, Baldassa S, Kiebler M. Chemically controlled formation of a DNA/calcium phosphate coprecipitate: Application for transfection of mature hippocampal neurons. *Journal of Neurobiology*. 2004;60(4):517-525. doi:10.1002/neu.20073
188. Lock M, Alvira M, Vandenberghe LH, et al. Rapid, Simple, and Versatile Manufacturing of Recombinant Adeno-Associated Viral Vectors at Scale. *Human Gene Therapy*. 2010;21(10):1259-1271. doi:10.1089/hum.2010.055
189. Strobel B, Miller FD, Rist W, Lamla T. Comparative Analysis of Cesium Chloride- and Iodixanol-Based Purification of Recombinant Adeno-Associated Viral Vectors for Preclinical Applications. *Human Gene Therapy Methods*. 2015;26(4):147-157. doi:10.1089/hgtb.2015.051
190. Ayuso E, Mingozzi F, Montane J, et al. High AAV vector purity results in serotype- and tissue-independent enhancement of transduction efficiency. *Gene Therapy*. 2010;17(4):503-510. doi:10.1038/gt.2009.157
191. Piras BA, Drury JE, Morton CL, et al. Distribution of AAV8 particles in cell lysates and culture media changes with time and is dependent on the recombinant vector. *Mol Ther Methods Clin Dev*. 2016;3. doi:10.1038/mtm.2016.15
192. Vandenberghe LH, Xiao R, Lock M, Lin J, Korn M, Wilson JM. Efficient Serotype-Dependent Release of Functional Vector into the Culture Medium During Adeno-Associated Virus Manufacturing. *Human Gene Therapy*. 2010;21(10):1251-1257. doi:10.1089/hum.2010.107

193. Zolotukhin S, Byrne BJ, Mason E, et al. Recombinant adeno-associated virus purification using novel methods improves infectious titer and yield. *Gene Ther.* 1999;6(6):973-985. doi:10.1038/sj.gt.3300938
194. Atkinson EM, Takeya RK, Aranha IL. Methods for generating high titer helper-free preparations of released recombinant AAV vectors. May 2003. <https://patents.google.com/patent/US6566118B1/en>. Accessed June 28, 2019.
195. Sterjanaj E, Mall H, Legmann R, Dang J. Tangential flow filtration and scalability in viral vector purification. :19.
196. Buclez P-O, Florencio GD, Relizani K, Beley C, Garcia L, Benchaouir R. adeno-associated virus produced by baculovirus expression. *Molecular Therapy.* 2016;10.
197. signagen. rAAV Average Molecular Weight – SignaGen Blog. <https://signagen.com/blog/2018/01/08/raav-average-molecular-weight/>. Accessed July 6, 2019.
198. Yang B, Li S, Wang H, et al. Global CNS Transduction of Adult Mice by Intravenously Delivered rAAVrh.8 and rAAVrh.10 and Nonhuman Primates by rAAVrh.10. *Molecular Therapy.* 2014;22(7):1299-1309. doi:10.1038/mt.2014.68
199. Hu C, Lipshutz GS. AAV-based Neonatal Gene Therapy for Hemophilia A: Long-Term Correction and Avoidance of Immune Responses in Mice. *Gene Ther.* 2012;19(12):1166-1176. doi:10.1038/gt.2011.200
200. Kaech S, Banker G. Culturing hippocampal neurons. *Nature Protocols.* 2006;1(5):2406-2415. doi:10.1038/nprot.2006.356
201. Malik SZ, Maronski MA, Dichter MA, Watson DJ. The use of specific AAV serotypes to stably transduce primary CNS neuron cultures. *Methods Mol Biol.* 2012;846:305-319. doi:10.1007/978-1-61779-536-7_26
202. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2- $\Delta\Delta$ CT Method. *Methods.* 2001;25(4):402-408. doi:10.1006/meth.2001.1262
203. Arden E, Metzger JM. Inexpensive, serotype-independent protocol for native and bioengineered recombinant adeno-associated virus purification. *J Biol Methods.* 2016;3(2). doi:10.14440/jbm.2016.102
204. Nass SA, Mattingly MA, Woodcock DA, et al. Universal Method for the Purification of Recombinant AAV Vectors of Differing Serotypes. *Mol Ther Methods Clin Dev.* 2017;9:33-46. doi:10.1016/j.omtm.2017.12.004
205. Holehonnur R, Luong JA, Chaturvedi D, et al. Adeno-associated viral serotypes produce differing titers and differentially transduce neurons within the rat basal and lateral amygdala. *BMC Neurosci.* 2014;15:28. doi:10.1186/1471-2202-15-28

206. Brunner D. Serum-free cell culture: the serum-free media interactive online database. *ALTEX*. 2010;53-62. doi:10.14573/altex.2010.1.53
207. Beltran Paschoal JF, Patiño SS, Bernardino T, et al. Adaptation to serum-free culture of HEK 293T and Huh7.0 cells. *BMC Proc*. 2014;8(Suppl 4):P259. doi:10.1186/1753-6561-8-S4-P259
208. Merten O-W, Schweizer M, Chahal P, Kamen AA. Manufacturing of viral vectors for gene therapy: part I. Upstream processing. *Pharmaceutical Bioprocessing*. 2014;2(2):183-203. doi:10.4155/pbp.14.16
209. Wang L, Zhu H, Wu J, Li N, Hua J. Characterization of embryonic stem-like cells derived from HEK293T cells through miR302/367 expression and their potentiality to differentiate into germ-like cells. *Cytotechnology*. 2014;66(5):729-740. doi:10.1007/s10616-013-9639-2
210. Schmid J. *Methods with Mammalian Cells*. Medical University of Vienna <http://www.meduniwien.ac.at/user/johannes.schmid/mammalian.htm>.
211. Doria M, Ferrara A, Auricchio A. AAV2/8 Vectors Purified from Culture Medium with a Simple and Rapid Protocol Transduce Murine Liver, Muscle, and Retina Efficiently. *Hum Gene Ther Methods*. 2013;24(6):392-398. doi:10.1089/hgtb.2013.155
212. Wright JF, Le T, Prado J, et al. Identification of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its occurrence during vector purification and formulation. *Mol Ther*. 2005;12(1):171-178. doi:10.1016/j.ymthe.2005.02.021
213. Howard DB, Powers K, Wang Y, Harvey BK. Tropism and toxicity of adeno-associated viral vector serotypes 1, 2, 5, 6, 7, 8, and 9 in rat neurons and glia in vitro. *Virology*. 2008;372(1):24-34. doi:10.1016/j.virol.2007.10.007
214. Bennett J, Wellman J, Marshall KA, et al. Safety and durability of effect of contralateral-eye administration of AAV2 gene therapy in patients with childhood-onset blindness caused by RPE65 mutations: a follow-on phase 1 trial. *The Lancet*. 2016;388(10045):661-672. doi:10.1016/S0140-6736(16)30371-3
215. Bras AL. A look at AAV toxicity in the mouse. *Lab Animal*. 2019;48(5):142. doi:10.1038/s41684-019-0296-8
216. Bosque PJ, Prusiner SB. Cultured Cell Sublines Highly Susceptible to Prion Infection. *Journal of Virology*. 2000;74(9):4377-4386. doi:10.1128/JVI.74.9.4377-4386.2000
217. Aras MA, Hartnett KA, Aizenman E. Assessment of cell viability in primary neuronal cultures. *Curr Protoc Neurosci*. 2008;Chapter 7:Unit 7.18. doi:10.1002/0471142301.ns0718s44
218. Hammond SL, Leek AN, Richman EH, Tjalkens RB. Cellular selectivity of AAV serotypes for gene delivery in neurons and astrocytes by neonatal intracerebroventricular injection. *PLOS ONE*. 2017;12(12):e0188830. doi:10.1371/journal.pone.0188830

219. Hitti FL. Genetically targeted anatomical and behavioral characterization of the cornu ammonis 2 (CA2) subfield of the mouse hippocampus. :134.
220. Cunningham C, Deacon R, Wells H, et al. Synaptic changes characterize early behavioural signs in the ME7 model of murine prion disease. *Eur J Neurosci*. 2003;17(10):2147-2155.
221. Klausberger T, Somogyi P. Neuronal diversity and temporal dynamics: the unity of hippocampal circuit operations. *Science*. 2008;321(5885):53-57. doi:10.1126/science.1149381
222. Kügler S, Lingor P, Schöll U, Zolotukhin S, Bähr M. Differential transgene expression in brain cells in vivo and in vitro from AAV-2 vectors with small transcriptional control units. *Virology*. 2003;311(1):89-95. doi:10.1016/S0042-6822(03)00162-4
223. Shevtsova Z, Malik JMI, Michel U, Bähr M, Kügler S. Promoters and serotypes: targeting of adeno-associated virus vectors for gene transfer in the rat central nervous system in vitro and in vivo. *Experimental Physiology*. 2005;90(1):53-59. doi:10.1113/expphysiol.2004.028159
224. Royo NC, Vandenberghe LH, Ma J-Y, et al. Specific AAV serotypes stably transduce primary hippocampal and cortical cultures with high efficiency and low toxicity. *Brain research*. 2008;1190:15–22.
225. Klein RL, Dayton RD, Tatom JB, Henderson KM, Henning PP. AAV8, 9, Rh10, Rh43 Vector Gene Transfer in the Rat Brain: Effects of Serotype, Promoter and Purification Method. *Molecular Therapy*. 2008;16(1):89-96. doi:10.1038/sj.mt.6300331
226. Russell S, Bennett J, Wellman JA, et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *The Lancet*. 2017;390(10097):849-860. doi:10.1016/S0140-6736(17)31868-8
227. Castle MJ, Turunen HT, Vandenberghe LH, Wolfe JH. Controlling AAV Tropism in the Nervous System with Natural and Engineered Capsids. In: Manfredsson FP, ed. *Gene Therapy for Neurological Disorders*. Vol 1382. New York, NY: Springer New York; 2016:133-149. doi:10.1007/978-1-4939-3271-9_10
228. Smith-Arica JR, Thomson AJ, Ansell R, Chiorini J, Davidson B, McWhir J. Infection efficiency of human and mouse embryonic stem cells using adenoviral and adeno-associated viral vectors. *Cloning Stem Cells*. 2003;5(1):51-62. doi:10.1089/153623003321512166
229. Kaja S, Payne AJ, Naumchuk Y, Koulen P. Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes. *Curr Protoc Toxicol*. 2017;72:2.26.1-2.26.10. doi:10.1002/cptx.21

230. Lin H, Muramatsu R, Maedera N, et al. Extracellular Lactate Dehydrogenase A Release From Damaged Neurons Drives Central Nervous System Angiogenesis. *EBioMedicine*. 2017;27:71-85. doi:10.1016/j.ebiom.2017.10.033
231. TLAMSA AP, BRUMBERG JC. Organization and morphology of thalamocortical neurons of mouse ventral lateral thalamus. *Somatosens Mot Res*. 2010;27(1):34-43. doi:10.3109/08990221003646736
232. Tanguy Y, Biferi MG, Besse A, et al. Systemic AAVrh10 provides higher transgene expression than AAV9 in the brain and the spinal cord of neonatal mice. *Front Mol Neurosci*. 2015;8. doi:10.3389/fnmol.2015.00036
233. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV Serotypes 1–9 Mediated Gene Expression and Tropism in Mice After Systemic Injection. *Molecular Therapy*. 2008;16(6):1073-1080. doi:10.1038/mt.2008.76
234. Zhong L, Li M, Mu X, Boisvert C, Ratner D. Systematical Evaluation of Tropism of Different AAV Serotype Vectors in Adult and Neonatal Mice Following I.V. Injections. *Molecular Therapy*. 2011;19:S156-S157. doi:10.1016/S1525-0016(16)36976-3
235. Selot R, Arumugam S, Mary B, Cheemadan S, Jayandharan GR. Optimized AAV rh.10 Vectors That Partially Evade Neutralizing Antibodies during Hepatic Gene Transfer. *Front Pharmacol*. 2017;8. doi:10.3389/fphar.2017.00441
236. Buchwalow I, Samoilova V, Boecker W, Tiemann M. Non-specific binding of antibodies in immunohistochemistry: fallacies and facts. *Sci Rep*. 2011;1. doi:10.1038/srep00028
237. Ward JM, Rehg JE. Rodent Immunohistochemistry: Pitfalls and Troubleshooting. *Veterinary Pathology*. 2014;51(1):88-101. doi:10.1177/0300985813503571
238. Kaneko M, Sugiyama N, Sasayama D, et al. Prion disease causes less severe lesions in human hippocampus than other parts of brain. *Psychiatry Clin Neurosci*. 2008;62(3):264-270. doi:10.1111/j.1440-1819.2008.01792.x
239. Cunningham SC, Dane AP, Spinoulas A, Alexander IE. Gene Delivery to the Juvenile Mouse Liver Using AAV2/8 Vectors. *Molecular Therapy*. 2008;16(6):1081-1088. doi:10.1038/mt.2008.72
240. Yang M, Lu J, Miao J, et al. Alzheimer's disease and methanol toxicity (part 1): chronic methanol feeding led to memory impairments and tau hyperphosphorylation in mice. *J Alzheimers Dis*. 2014;41(4):1117-1129. doi:10.3233/JAD-131529
241. Piras BA, Drury JE, Morton CL, et al. Distribution of AAV8 particles in cell lysates and culture media changes with time and is dependent on the recombinant vector. *Molecular Therapy - Methods & Clinical Development*. 2016;3:16015. doi:10.1038/mtm.2016.15
242. Fausto N, Campbell JS. The role of hepatocytes and oval cells in liver regeneration and repopulation. *Mech Dev*. 2003;120(1):117-130.

243. Nakai H, Yant SR, Storm TA, Fuess S, Meuse L, Kay MA. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J Virol.* 2001;75(15):6969-6976. doi:10.1128/JVI.75.15.6969-6976.2001
244. Thomas CE, Storm TA, Huang Z, Kay MA. Rapid Uncoating of Vector Genomes Is the Key to Efficient Liver Transduction with Pseudotyped Adeno-Associated Virus Vectors. *J Virol.* 2004;78(6):3110-3122. doi:10.1128/JVI.78.6.3110-3122.2004
245. Berns KI, Muzyczka N. AAV: An Overview of Unanswered Questions. *Hum Gene Ther.* 2017;28(4):308-313. doi:10.1089/hum.2017.048

Appendix – Animal Scoring Chart

Clinical Signs (TSE)	Points Allotted		Score/ Animal ID									
Coat Condition	Normal	0										
	Ruffled or soiled, piloerection	1										
Posture	Normal	0										
	Hunched	1										
Weight loss (loss of appetite for several days without	0-5%	0										
	5-9%	1										
	10-14%	2										
	15-19%	3										
	20% or >	5										
Activity in cage (may indicate pain)	Normal	0										
	Doesn't move when coaxed - affected	1										
Nestlet Shredding	Shredding	0										
	Shredding, not full nest	1										
	Full nestlet not shredded, partial nest	2										
	Partial shredded, no nest	3										
	Less than half shredded, no nest	4										
	Not shredding, no nest	5										
Socialization	Normal	0										
	Animal segregated from cage mates	1										
Behaviour	Normal	0										
	Depressed	1										
Mobility	Normal movement	0										
	Circling	2										
	Paralysis, paresis, ataxia	5										
	Loss of righting reflex	5										
Skin Condition (can be urine scalding or dermatitis- check full body of animal)	Wetness of urogenital area	1										
	Erythema	1										
	Alopecia	1										
	Ulceration < 3mm	2										
	Ulceration > 3mm	3										
Splay leg (when moving)	Normal movement	0										
	Present	2										
	Gait incoordination	5										
Response to Stimuli (Reactivity to external stimulus- ie cage movement)	Normal	0										
	Hyperexcitable	1										
	Delayed	2										
	Unresponsive	3										
TOTAL												

Endpoint sentence: If a total of 5 is reached or any of the following signs are seen, immediately contact the AC veterinarian, PI and VTS staff for tissue collection:

- abolished righting reflex
- seizures
- weight loss of 20% Immediate euthanasia to be discussed with the AC Vet.
- paralysis (full or partial)
- bleeding ulcers >1cm (urine scalding)