GENE THERAPY FOR ALZHEIMER’S DISEASE:

Characterising lentivirus and adeno-associated virus spread from the adult mouse hippocampus

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Abstract
Alzheimer’s Disease is a neurodegenerative condition with progressively worsening memory and cognitive function, which ultimately results in death. The two major neuropathological hallmarks of AD are extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tau tangles (NFTs). Other pathways and neuronal mechanisms are also likely to be affected as AD is a complex disease resulting from anomalies in components of different pathways.
Currently, apart from a few FDA approved drugs which serve to delay symptom progression through slowing neurotransmitter breakdown (Cholinesterase inhibitors) and cell damage (NMDA-receptor antagonist), no effective treatment of the underlying causes of AD is available. One of the hypothesised underlying cause of AD is the formation of extracellular Aβ plaques. Current work in the Neural Development and Disease (NDD) lab have researched viral-mediated gene therapy as a means to introduce a neuroprotective secreted amyloid precursor protein α (sAPPα) gene intracranially.
This project’s primary aim was optimising and comparing the spread between lentiviral (LV) and adeno-associated viral (AAV) vectors; along with the effects of systemically delivering an osmotic agent, mannitol. Viral spread would be determined by the use of a GFP reporter gene, where expression indicated transduction and extent of viral spread. This project has showed a significant improvement in transduction efficiency when using an AAV vector to deliver a reporter gene. It efficiently achieved widespread transduction of the CNS from a unilateral injection at the hippocampus. However, while LV had a limited spread in comparison to AAV9, it would be useful for targeted delivery of treatment. Mannitol did not produce any significant effect on vector spread.
The secondary aim was to create a plasmid to visualise secretion of sAPPα from virally
transduced cells. Once packaged into a lentiviral vector, the plasmid was shown to successfully transduce primary neurons \textit{in vitro}, but expression of reporter genes were not observed \textit{in vivo}. 
Chapter 1: Introduction

1.1 Alzheimer’s Disease & Dementia

1.1.1 Epidemiology and statistics

Alzheimer’s Disease (AD) is an (as-yet) incurable neurodegenerative condition. AD is the most common form of dementia, thought to be responsible for 60-80% of all cases of dementia with over 48,000 (1.1%) of the population in New Zealand affected (Economics, 2011), and 36 million affected globally (Prince et al., 2014). Furthermore, about 40% of AD cases are thought to go undiagnosed or unreported, suggesting that numbers could be even higher (Prince et al., 2011).

AD is a disease which affects the elderly, with most diagnoses of AD (96%) at age 65 and above (Brookmeyer et al., 1998; Hebert et al., 2013). AD prevalence is expected to increase, a consequence of the aging population and projections have numbers in NZ expected to triple between the years 2011 to 2050 (Economics, 2011). Only 4% of AD patients are younger than 65, suffering from the much rarer early-onset AD (Hutton et al., 1996; Harvey et al., 2003). The incidence of AD rises significantly with age, with risk of developing AD doubling every 5 years after the age of 65; nearly half of the over-85 year old population will have developed AD (Prince et al., 2014).

AD has a typical progression of symptoms; onset is typically characterised by short term memory loss (or an inability to form new memories), cognitive decline and behaviour/personality changes with later stages of basic body function impairments and death.

In latter stages of AD, sufferers are no longer able to cope unaided, necessitating the need for caregivers to offer almost round-the-clock care. While this may come in the form of professional care, more often than not, family members may also give up time and income to provide some or all of the long-term personal care needed. This long-
term care, professional or familial, is a great financial burden on society; estimated to cost over US$600 billion worldwide. The smallest contributions towards this societal cost comes from direct medical care; while the greatest contributor is made up of long-term care and is especially pre-dominated by unpaid care provided by family (Alzheimer's Association, 2013).

Due to the rising prevalence of AD and its resultant rising costs, it is vital to develop novel and effective treatment for AD. Strategies which can target, prevent and/or rescue the fundamental causes, rather than treating the symptoms alone will be the most desirable. The challenges are the unknown mechanisms or molecular triggers which cause disease development and the cause-and-effect sequence of brain anomalies observed in AD patients.

1.1.2 Neuropathology of Alzheimer's

The biological and structural changes in a healthy ageing brain is vastly different from an AD affected brain. The normal process of ageing produces regional reduction in volume (Raz et al., 2005); this shrinkage has differential region-specific rates. Minimal change was observed in the entorhinal and visual cortices. Substantial shrinkage was observed in the cerebellum, hippocampus and association cortices; where rate of shrinkage in the cerebellum and hippocampus increased with age.

In contrast, the entorhinal cortex is one of the first regions shown to be affected in early/mild AD (Braak et al., 1993; Du et al., 2001), and shrinkage has been shown to be a predictor of memory decline (Raz & Rodrigue, 2006). Hippocampal volume reduction was shown to correlate with severity of cognitive impairment from normal, mild cognitive impairment and AD affected individuals (Pennanen et al., 2004). The same paper corroborated that entorhinal cortex atrophy precedes atrophy of the
hippocampus in AD individuals. Brain atrophy is simply one of the features in AD affected brains; however it is not a defining characteristic as distinguishing between normal age-related and AD brain atrophy is difficult. Diagnosis of AD is made through symptoms and cognitive tests; symptoms observed include: memory loss and worsening cognitive function; such as aphasia, object recognition, motor function and decision making (Prince et al., 2014). Further tests can be conducted to reject symptom-causing diseases. Research and clinical diagnosis employ many tests to assess cognitive impairment; one of which is the AD Assessment Scale-Cognitive Subscale (ADAS-Cog) (Cano et al., 2010). Typically an assessment is carried out with multiple other tests, such as the Clinical Dementia Rating (CDR) (Hughes et al., 1982; Tan, Strauss & Sherman, 2011) or Mini-Mental State Examination (MMSE) (Cockrell & Folstein, 2002). Utilisation of multiple tests will be useful in tracking changes from normal ageing to mild cognitive impairment (MCI) to AD. It is important to note the difference between MCI and early/mild AD as MCI may not progress into AD, while early/mild AD will almost certainly progress. Improved diagnostic accuracy is possible with the use of scans like magnetic resonance imaging (MRI) or positron emission tomography (PET) (McGeer et al., 1984; Albert et al., 2011); and potentially, biomarker testing (Humpel, 2011; Mapstone et al., 2014). But at the moment, confirmation of the diagnosis can only be made by post-mortem analysis of brain tissue. Primary hallmarks of AD that are looked for in brain tissue are neurofibrillary tangles and neuritic plaques (Wilcock & Esiri, 1982; Tiraboschi et al., 2004; Braskie et al., 2010). They were first characterised by A. Alzheimer in a case of “pre-senile dementia” (Alzheimer, 1907) and named Alzheimer’s Disease by E. Krapaelin in 1910 (Berrios, 1990). The accumulation of neuritic plaques and neurofibrillary tangles are noticeably
greater in AD sufferers than healthy aging individuals (Price et al., 1991).
The intracellular NFTs are made of hyperphosphorylated tau protein which aggregate to form insoluble tangles. NFTs alone are not diagnostic of AD as they are found in other disease related tauopathies and normal ageing (primary age-related tauopathy (PART); Crary et al., 2014). NFTs are observed as another age-related change to brain morphology. However while the density of NFTs may increase with age in a healthy individual, unlike an AD individual, they do not begin localising in specific regions and Aβ plaques are not a common feature seen across normal ageing brains (Price et al., 1991).

Thus in combination with other pathologies, Aβ plaque presence and load are a unique characteristic for AD diagnostic pathology. In healthy, unaffected individuals, Aβ is simultaneously formed and degraded which makes their accumulation an anomaly. The idea that Aβ plaques are the underlying cause of AD is known as the amyloid hypothesis. The presence of Aβ plaque in AD pathology will be discussed further (section 1.2.1) and is the current focus of the NDD lab’s efforts in AD treatment.

1.1.3 Affected neuroanatomy in Alzheimer’s

It was previously mentioned that cortical regions, the hippocampus and entorhinal cortex suffer from atrophy in individuals with AD. The entorhinal cortex has been shown to be one of the first region of the brain to be affected in early/mild-AD individuals (Du et al., 2001; Khan et al. 2014).

The hippocampus, which is the region of the brain responsible for learning, memory and spatial navigation (White & Gaskin, 2006; Winocur et al., 2006); is one of the major regions of the brain to suffer atrophy in AD (Pennanen et al., 2004). Brains from AD individuals show increased region-specific localisation of NFTs; especially in the
hippocampus, with tangles appearing within the cornus ammonis region 1 (CA1) in older individuals (Price et al., 1991; Rodriguez et al., 2013; Neuman et al., 2015). This correlates with early symptoms of learning and short-term memory failure. The CA1 and entorhinal cortex have a similar and related pathology in AD due to the shared network that they are components of.

Research into episodic memory has revealed that memory is primarily stored in the neocortex; with consolidation through interactions with the hippocampus. Hippocampus and neocortex interactions are largely mediated by the entorhinal cortex (Fyhn et al., 2004). The EC-hippocampus system processes multi-sensory information received by the association cortices in the formation of memory for storage (Burwell, 2000; Suh et al., 2011). The inputs and outputs to and from the entorhinal cortex and hippocampus allow for retrograde transport of virally delivered transgene products along the axonal projections between regions.

Unsurprisingly, this has made the hippocampus a well-studied region with regards to AD, especially in animal models. The hippocampus has been shown to exhibit discrete functional regions; the ventral hippocampus is implicated in anxiety-related behaviours while the dorsal hippocampus (of which the CA1 region is a part of) mediates memory and spatial memory (Strange et al., 2014). It is for these reasons that the CA1 region of the hippocampus was selected as a meaningful AD-related target site for viral vector delivery. This region of the hippocampus has been shown to be associated with learning and memory (Bartsch et al., 2011). Alterations in long-term potentiation (LTP) in this region resulted in impairments to spatial learning (Okada et al., 2003). While lesions to the CA1 and CA3 regions affect memory retrieval (Hasselmo, 2005; Ji and Maren, 2008; Bartsch et al, 2010).
1.2 Risk factors and associated causes of Alzheimer's

AD does not have a single definitive cause, rather it is a result of a myriad of heterogeneous factors that form the disease symptoms known as Alzheimer’s Disease. It does, however, have a few known risk and association factors. Old age, as mentioned before, is the major risk factor of AD. Other epidemiological risk factors that are linked to AD are cerebrovascular diseases (Jellinger, 2002), hypertension (Skoog & Gustafson, 2006) and head injuries (Mayeux et al., 1995; Nicoll et al., 1995).

In terms of causative factors, there are a number of proposed hypotheses such as the tau or cholinergic hypothesis. This project will focus on the amyloid hypothesis and how viral vector mediated gene therapy will seek to treat the formation of Aβ plaques.

1.2.1 Amyloid Hypothesis

The amyloid hypothesis proposes that the AD pathogenesis begins with accumulation of Aβ peptides which form neuritic plaques (Hardy & Higgins, 1992; Hardy, 2009; Sung et al., 2014) before symptom development and secondary pathologies (such as NFTs). MRI scans have shown that amyloid precursor protein (APP) expression within the entorhinal cortex drives tau spread and toxicity (Khan et al., 2014). Initiation of Aβ formation has been suggested to be due to cerebral capillary haemorrhaging (Stone, 2008). The hypothesis proposed that a capillary haemorrhage provides an ischaemic condition which promotes Aβ formation and haemoglobin oligomerises Aβ into plaques. This is supported by the fact that cerebrovascular disease is a risk factor for AD (Esiri et al., 1999; Jellinger, 2002) and the elderly have weaker capillary beds which are more prone to rupture. Furthermore, it would explain Aβ deposition following traumatic head injury (Nicoll et al., 1995).
Figure 1.1 – Processing of amyloid precursor protein by the amyloidogenic or non-amyloidogenic pathway with α-, β- and γ-secretases

Amyloid precursor protein (APP) can proceed down the amyloidogenic or non-amyloidogenic pathway by the initial cleavage of β- or α-secretase respectively. Subsequent cleavage by γ-secretase forms either the amyloid-β (responsible for neuritic plaques; under the amyloidogenic pathway) or neuroprotective sAPPα (under the non-amyloidogenic pathway).

Picture reproduced with permission from Eva Babusikova, Andrea Evinova, Jozef Hatok, Dusan Dobrota and Jana Jurecekova (2013). Oxidative Changes and Possible Effects of Polymorphism of Antioxidant Enzymes in Neurodegenerative Disease, Dr. Uday Kishore (Ed.)

Aβ is produced from the proteolytic pathway of the transmembrane amyloid precursor protein (APP). APP processing may proceed down either the amyloidogenic or non-amyloidogenic pathways, where APP may be initially cleaved by either β- or α-secretase, respectively. In the amyloidogenic pathway, APP is first cleaved with β-secretase. This produces a soluble N-terminal APP fragment (sAPPβ) and a C-
terminal fragment (β-CTF). The transmembrane β-CTF is further cleaved by γ-secretase to produce the extracellular Aβ and APP intracellular domain (AICD).

There are two main species of Aβ produced, Aβ\textsubscript{40} and Aβ\textsubscript{42}, due to different cleavage sites of γ-secretase producing different lengths of Aβ. Aβ\textsubscript{42} is the more toxic form; aggregating easily and making up the major component of neuritic plaques. The Aβ\textsubscript{42}/Aβ\textsubscript{40} ratio is a potential biomarker for AD.

Aβ was found to increase neuronal sensitivity to excitotoxicity and was neurotoxic in neuronal cultures (Hardy & Higgins, 1992). Aβ was found to be only neurotoxic to mature neurons; while having a neurotrophic effect (at low concentrations) to undifferentiated hippocampal neurons (Yankner et al., 1990). The neurotoxic mechanisms of Aβ in causing cell death have been suggested to be: interference with calcium ion homeostasis resulting in neuronal loss (Mark et al., 1995), endoplasmic reticulum (ER) stress, accumulation in autophagic vesicles resulting in lysosomal leakage (and subsequent cell death) and disruption of mitochondrial function to cause apoptosis (Umeda et al., 2011).

In the non-amyloidogenic pathway, APP is initially cleaved by α-secretase in the middle of the Aβ region. This produces a larger N-terminal APP fragment (sAPP\textalpha) and a αCTF. αCTF is then cleaved by γ-secretase. The larger sAPP\textalpha has been found to have neurotrophic and neuroprotective properties (Turner et al., 2003; Turner et al., 2007); being 100 times more effective than sAPPβ in conferring neuroprotection to hippocampal cells against excitotoxicity, Aβ neurotoxicity, and glucose deprivation (Furukawa et al., 1996).

Aβ levels in the brain are maintained by a constant cycle of generation and clearance; an imbalance will result in accumulation and formation of neuritic plaques. Thus, sAPP\textalpha is a therapeutic of interest and past work in the NDD lab had used lentiviral-
mediated delivery of a sAPPα transgene to both rescue and prevent AD symptoms.

1.2.2 Genetic risks

There are numerous AD associated genes which carry with them a small but nonetheless significant risk. Because Aβ formation is a major contributing factor to AD pathogenesis, the APP gene which expresses the precursor protein for Aβ is known to be directly linked to AD. There are three other genes linked to AD; Apolipoprotein E (APOE4 in particular), and Presenilin 1 and 2 (PSEN1 and PSEN2) (Tanzi & Bertram, 2005).

The APP gene is located on chromosome 21 and mutations within the Aβ region of the APP gene can result in hereditary susceptibility for AD; according to Tanzi and Bertram, 2005, there are 16 established mutations, most increasing the Aβ42/Aβ40 ratio and Aβ generation and aggregation. Three mutations at codon 717 of the APP gene cause familial AD (Hardy & Higgins, 1992), while another mutation of the transmembrane Aβ region resulted in an autosomal dominant inheritance of AD in family members (Murrell et al., 1991). Other familial AD have been reported with mutations found outside of the Aβ region which resulted in a rise in Aβ production (Citron et al., 1992). Furthermore, its increased expression from trisomy 21 has been linked as a likely cause of early-onset AD in individuals with Down Syndrome (Lott & Head, 2005; Shi et al., 2012).

The ApoE4 allele is found on chromosome 19 and encodes the apolipoprotein E4 variant; which has been linked to a number of diseases. In terms of its role in AD, the E4 isoform is linked to sporadic late-onset AD and thought to do so by tipping the Aβ balance by increasing aggregation and decreasing clearance.

The PSEN1/2 genes are part of a family of transmembrane proteins which form a
complex with γ-secretase. PSEN1 is found on chromosome 14 while PSEN2 is found on chromosome 1. They are both strongly linked to early-onset familial AD (Wragg et al., 1996). According to the Alzheimer Disease & Frontotemporal Dementia Mutation Database (Cruts et al., 2012), there are 196 known mutations in PSEN1 and 25 in PSEN2. A large number of the mutations cause an increase in the \( \text{Aβ}_{42}/\text{Aβ}_{40} \) ratio (Tanzi & Bertram, 2005).

To illustrate the mechanisms of these genes, a double transgenic (tg) mouse was created which carried mutant APP and PSEN1 transgene (Holcomb et al., 1998). An APP mutant tg mouse had hippocampal and cortical extracellular Aβ deposits while the PSEN1 mutant tg mouse had increased levels of Aβ\(_{42}\). The double tg mice produced from crossing the two mutants developed AD-like pathology and Aβ plaques much earlier and is a better model for AD therapeutic research as it has an additional facet of the disease which can be tested at the same time. In this case, increased levels of Aβ\(_{42}\) and high levels of plaque formations.

To summarise the significance of the amyloid hypothesis in AD pathogenesis; regardless of the mechanisms or pathways which result in the presence of neuritic plaques, the negative impact Aβ plaques have on the brain and cognitive function is undeniable (Selkoe, 2008). They can block cell-cell communication at synapses, resulting in impairments in learning and forming new memories and progressively, memory recall. Aβ contributes to cell death directly by inducing apoptosis and disrupting organelle function. Indirectly, Aβ may also activate an immune response to trigger inflammation and recruit immune cells which phagocytose non-functional cells, contributing to cell death. The resultant cell loss eliminates portions of signalling and synaptic pathways which result in an inability to form new memory or loss of memory.
1.3 Gene therapy

Gene therapy was developed as a technology to introduce a functional copy of a gene to specific sites as a therapeutic for genetic diseases (Rogers, 1970; Friedmann & Roblin, 1972). However, delivery of a gene into sites of interest requires a vector to carry the DNA and facilitate entry.

Gene therapy has the advantage of reducing (or in some cases, eliminating the need for multiple and sustained administration of the therapeutic. This is because it has been shown that there can be robust transgene expression up to 2 years after viral vector introduction (Tan, unpublished PhD research).

The vector types used are classed into methods which use viral vectors or non-viral methods. Examples of non-viral vectors include oligonucleotides, lipoplexes and inorganic nanoparticles.

1.3.1 Viral vectors

Treatment with viral mediated gene therapy utilises the inherent ‘invasive’ properties of viruses as a delivery mechanism; where viruses bind and introduce a therapeutic transgene instead of their genomes into host cells, transgene expression would rescue or prevent the effects of a disease causing mutant.

Following identification of a potential therapeutic gene, the wildtype viral genome is modified to create a construct with 1) a promoter to allow either constitutive or specific expression of 2) the therapeutic gene and/or 3) a reporter gene to allow for its detection and selection. To create the vector, the construct is then packaged into its viral particle before being introduced in vitro or in vivo.

Conceptually straightforward, it proved more challenging in practice; with selection of
the viral vector having to meet certain requirements before use; 1) modification to remove pathogenicity: usually by the deletion of replication and/or packaging genes (which serves a two-fold purpose of increasing transgene capacity), 2) tissue specificity: by virus choice and/or pseudotyping, 3) identification: insertion of a reporter gene such as antibiotic resistance.

Viral vectors commonly modified for use are lentivirus (LV) (Lundberg et al., 2008; Matrai et al., 2010) and adeno-associated virus (AAV) vectors (Warrington & Herzog, 2006).

1.3.2 Lentivirus vs. Adeno-associated virus

LV are retroviruses containing single-stranded, positive sense RNA, which have the ability to transduce both dividing and non-dividing cells. They have a relatively large genome capacity of approximately 9-10 kb, and elicit a minimal immune response which makes them ideal as a vector choice. This makes them a useful choice for gene therapy within the central nervous system (CNS), especially for their ability to transduce non-dividing cells such as neurons. Following cell entry, the LV genome is reverse transcribed and integrated randomly into the host genome, resulting in long term expression; even after cell replication, expression is sustained in daughter cells. The unpredictable integration was shown to have a tendency to cause mutagenesis at a low rate (Montini et al., 2006).

AAV are parovirus containing single-stranded DNA (ssDNA), also with the ability to transduce both dividing and non-dividing cells (Weinberg et al., 2012). AAV are also non-pathogenic and not known to be disease causing in humans, making them another suitable vector choice. Unlike LV vectors, AAV most often integrate predictably into the host cell genome; at human chromosome 19 (Kotin et al., 1990;
Surosky et al., 1997). However, due to a much smaller genome capacity; of approximately 4.7 kb, the genes responsible for this capability have typically been removed in AAV vectors to increase their transgene capacity. This causes either random integration or persistence in an episomal state (Flotte & Carter, 1995). However, episomal DNA is lost in subsequent generations of daughter cells as it is not replicated along with host DNA.

1.4 Enhancing transduction efficiency

Initially, most gene therapy trials had begun with the aim to treat monogenetic diseases such as cystic fibrosis (Hyde et al., 1993), haemophilia (Kay et al., 1992) and muscular dystrophy (Cox et al., 1993). However, there was much difficulty in achieving tissue specific targeting along with high levels of transduction efficiencies. Due to modifications to the wildtype viral genome, most vectors are rendered replication-deficient, therefore each vector introduced is required to be as “efficient” as possible at attaching and facilitating entry into a cell.

Gene therapy within the central nervous system (CNS) presents additional unique challenges such as; mature non-dividing cells, heterogeneous cell types and selective access (Costantini et al., 1999). The CNS is highly selective about macromolecule entry; which means novel methods to facilitate entry into the brain. Direct intracranial injections are more invasive but remove the need to bypass the blood-brain barrier; which is the main selective barrier that needs to be bypassed by most other routes of administration. Intracranial delivery of viral vectors still have certain hurdles such as the densely packed nature of the neuronal cells which prevent spread from the site of injection and poor transduction efficiencies of certain viral vectors to CNS cells.
1.4.1 Serotyping vs. Pseudotyping

Methods which were previously examined to improve the transduction efficiencies of AAV looked at the various serotypes and their natural tropism range; where tissue specificity was determined by capsid serotype (Grieger & Samulski, 2005; Zincarelli et al., 2008). AAV serotypes are assigned based on the different capsid proteins and the resulting affinity conferred to distinct host or tissue type receptors which serves to facilitate entry via receptor-mediated endocytosis (Yan et al., 2002).

The serotype used in this project; AAV9, has been shown to exhibit natural tropism towards the CNS and global bio-distribution (when introduced intravenously), it also shows rapid-onset and high expression levels (Zincarelli et al., 2008).

Altering the tropism of LV vectors can also be accomplished by pseudotyping, which is the replacement of natural viral envelope glycoproteins with foreign glycoproteins to produce a pseudotyped viral vector, with altered tropism. The viral vector then attains the host tropism of the virus from which the glycoprotein was originally derived, due to gaining an increased binding affinity (Cronin et al., 2006).

Prior work in the NDD lab has worked on pseudotyping surface glycoproteins on virus particles to improve transduction efficiencies. A glycoprotein used in the NDD lab takes advantage of the neural tropism of the family of rabies virus. The vesicular stomatitis virus glycoprotein G (VSV-G) is used due to its stable pseudotype and wide tropism of all cell types (Trono, 2000; Mazarakis et al., 2001).

1.4.2 Osmotic agents (Mannitol)

Apart from modifying the viral vectors, the tightly regulated and highly selective environment of the CNS, and brain in particular, can be subtly altered to assist with increasing distribution of viral vectors (or therapeutic macromolecules) throughout the
CNS. The ever-present difficulty of macromolecule delivery into the brain is the blood-brain barrier (BBB) which is a neuroprotective separation preventing diffusion between the blood and brain extracellular fluid; however, the BBB is only a problem when introducing viruses systemically with the goal of entry into the brain. However, with intracranial delivery of viral vectors, the difficulty is no longer the inability to cross the BBB to enter the brain but tightly packed neurons and glial cells which prevents the spread of viral vectors from a localised point of injection.

Osmotic agents are commonly used as a means of relieving intracranial pressure by decreasing extracellular fluid (ECF) volume (Mohanty et al., 2011). The osmotic agent, mannitol, is a commonly used BBB disruptor to achieve improved diffusion within the brain. Mannitol generates a positive osmotic pressure within neural cells which causes water to exit the cells along the osmotic gradient (Pardridge, 2001). Consequently, the cells shrink and move apart. This provides greater extracellular access and results in greater spread of macromolecules, such as viral vectors, from the point of introduction. The effect of mannitol has been found to peak approximately 4-5 hours post-administration and return to baseline levels by 24 hours post-administration (Burger et al., 2004).

Current research has used mannitol to enhance brain microcirculation but an explicit examination and determination of an optimised concentration and route of administration has not been conducted. However, the general consensus has reported improved transduction efficiency resulting from the use of mannitol treatment (Ghodsi et al., 1999; Mastakov et al., 2001; Carty et al., 2010).

The concentrations of mannitol used varied; from 6.7% to 25% (Burger et al., 2005; Fu et al., 2003), along with the volume delivered and the rate at which that volume was administered. Differing concentrations of mannitol was trialled by Fu et al., 2003,
with 12.5% mannitol having similar results to negative controls; with observably higher transduction efficiencies with 25% mannitol.

This project will use 25% w/v mannitol delivered systemically to observe the effect it has on viral vector spread

1.5 Visualising secretion of sAPPα from transduced cells

Previous studies in the Neural Development and Disease lab have utilised a LV to deliver sAPPα with a separate GFP in preventative and rescue studies. LV injection occurred pre- and post-symptom manifestation before conducting behavioural and post-mortem studies. The post-mortem observations were only able to observe the regions of virus spread through the GFP expressing transduced cells. But it was unknown where the secreted therapeutic sAPPα had travelled to. This was important to know as AD results in various regions of the brain being affected and expression is secondary to delivery of the therapeutic to regions of the brain where it was needed. The design of a plasmid containing a GFP-sAPPα-T2A-mCherry transgene enabled transduced cells to be observed with the mCherry reporter while cleavage at the T2A peptide allowed GFP-tagged sAPPα to be secreted and its extracellular spread can be visualised to track region of spread.
Aims

This project has 2 overall aims regarding viral vector mediated gene therapy for Alzheimer's Disease;

1) Primarily we are comparing the spread and distribution of lentivirus to adeno-associated virus mediated transduction of neuronal cells; main points of comparison are physical distance of spread and regions of reporter gene expression. We will also look how an osmotic agent, mannitol, is able to increase the spread of viral vector delivery through its hyperosmotic action.

2) Secondary to the first aim, design of a plasmid to visualise secretion of the therapeutic secreted amyloid precursor protein α. This is achieved with a sAPPα molecule with a GFP reporter tag and a mCherry reporter gene, other portions are bisected by a cleavage peptide.
Chapter 2: Materials and methods

Wildtype mice (strain C57BL/6), aged 3 to 4 months old, were injected unilaterally into the CA1 region of the hippocampus in the left hemisphere. One microlitre of either the lentivirus (LV) or adeno-associated virus serotype 9 (AAV9) was injected at an infusion rate of 150 nL/min. The viral vectors expressed a green fluorescent protein (GFP) reporter gene. The needle was left in place before being drawn up slowly to prevent backflow of the viral vectors. Incubation times varied from 7, 14 to 28 days; however, treatment groups used in subsequent comparison were all incubated for 28 days.

To compare the use of different viral vectors and the effect of mannitol, the mice were perfused at the end of the incubation period. The brains were fixed, cryoprotected and frozen prior to sectioning. Sections were imaged for GFP expression and mapping using fluorescent microscopy. The area of GFP expression was quantified to determine the spread and transport of viral vectors from the site of injection. The total distance of spread from anterior to posterior of the brain and distribution of GFP expression was calculated to compare the effect of using either LV or AAV9 vectors along with the effects of using the osmotic agent, mannitol.

Immunohistochemistry was also carried out on sections of tissue to determine cell types that were transduced. NeuN antibodies were used to determine if neurons were transduced while GFAP antibodies were used to stain for astrocytes (indicating an immune response to viral vector treatment). Anti-GFP staining was used in some cases to enhance weak GFP signal (or in some cases, confirm the absence of GFP) in some tissue.

To visualise the secretion of secreted amyloid precursor protein α (sAPPα) from lentiviral transduced neurons, a plasmid was designed to express a GFP-tagged sAPPα molecule and mCherry as a fused protein, bisected by a T2A peptide.
Expression was controlled by a synapsin promoter.

Upon cleavage at the T2A peptide, the mCherry reporter molecule would remain in the transduced neurons while the sAPPα molecule would be secreted extracellularly. The GFP tag would enable visualisation of sAPPα (green) while differentiating from transduced neurons (red).

2.1 Plasmid design and cloning of pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry

A plasmid was designed to contain a GFP-sAPPα-T2A-mCherry sequence which would allow for visualising sAPPα via a GFP tag while differentiating from transduced neurons with mCherry. The plasmid was created by linearising a pre-existing 9615 base-pair (bp) plasmid vector pCDH-pSyn-SP-GFP-sAPPα (generated by Dr Lucia Schweitzer using a sAPPα clone from Prof Warren Tate) by SalI restriction digest. The T2A-mCherry insert was amplified with In-Fusion® primers from a plasmid containing the region of interest [pCDH-pSyn floxed Kozak-T2A-mCherry], to produce an approximately 813 bp sequence with complementary 15-20 bp overhangs to the vector plasmid. An In-Fusion® reaction was carried out to ligate insert fragment to vector. Figure 2.1 shows the expected final plasmid.
Figure 2.1 – pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry plasmid

The plasmid shown has a GFP reporter tag (green), sAPPα gene (blue), T2A (purple triangle), mCherry reporter gene (red) and AmpR selection gene (yellow). Figure created using Geneious (version 8.1).

Competent cells were transformed and screened with colony PCR for positive colonies. Likely positive colonies were crude-mini-prepped for DNA isolation and sequencing. A sequence which was positively aligned with the desired insert present was midi-prepped for higher quality and quantity DNA for virus production.
2.1.1 In-Fusion® primer design

Primers used for amplifying and cloning of the T2A-mCherry sequence were designed with the aid of Geneious. The primers were designed to allow for an in-frame insertion of the T2A-mCherry sequence after the sAPPα gene in the vector plasmid, while removing the STOP codon to allow for expression under the same Synapsin promoter. The forward primer served to produce a complementary overhang with the 3’ end of sAPPα while removing its STOP codon. The reverse primer produced a complementary overhang with the plasmid vector while removing the SalI restriction cut site. These primers are shown in Table 1.1.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>618 InF mCherry rev</td>
<td>GAGGTTGATTGTGGTCTGACCTACTTGTACAGCAGCTGCTCCAT</td>
</tr>
<tr>
<td>619 InF sAPPα T2A fwd</td>
<td>AAGTTTCATCATCAAAAAAGTGAGGGGAGAGGAAAGT</td>
</tr>
</tbody>
</table>

Table 2.1 – In-Fusion® primer sequences

Green: vector-specific overhang; Red: complementary mCherry sequence; Yellow: complementary T2A sequence.

The expected insert from PCR amplification using the designed In-Fusion® primers is shown in Figure 2.2 below.

**Figure 2.2 – T2A-mCherry sequence**

The expected insert fragment (777 bp; 813 bp when including overhangs) from amplification using In-Fusion® primers (beige) with vector-specific complementary overhangs (green) flanking T2A (yellow) and mCherry gene (red).
2.1.2 Amplification of insert using In-Fusion® primers

PCR was carried out using brand new aliquots of all reagents (due to prior unsuccessful attempts leading to a conclusion of possible contamination in the PCR reagents). Each 50 μL reaction contained 5 μL of 10X buffer, 1.5 μL of 50 mM MgCl₂, 1 μl of Roche PCR Grade Nucleotide Mix (10mM each dNTP), 1μL of each forward and reverse primers (10 mM), 0.5 μL of Platinum Taq DNA Polymerase (Invitrogen, NZ) and either 30, 50, 100 or 300 ng of plasmid DNA; with the remaining volume consisting of sterile dH₂O. Each reaction of different DNA concentrations was prepared and carried out in duplicate, for a total of 8 reactions. An additional 2 reactions containing only 100 ng of plasmid DNA without primers were included as negative controls. PCR amplification was carried out in a BioRad C1000 thermal cycler.

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR parameters</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>618 InF mCherry rev</td>
<td>1. 95 °C – 3 minutes</td>
<td>813 bp</td>
</tr>
<tr>
<td>619 InF sAPPα T2A fwd</td>
<td>2. 95 °C – 30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. 55 °C – 1 minute</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. 72 °C – 1 minute</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. 35 cycles of 2-4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6. 72 °C – 5 minutes</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2 – PCR parameters for T2A-mCherry amplification using In-Fusion® primers

2.1.3 Agarose gel electrophoresis

All gel electrophoresis (unless otherwise stated) were carried out in a Thermo Scientific Easycast B1 tank with 50 mL of 1% UltraPure Agarose (Invitrogen) gels with
5 μL of 10 mg/mL ethidium bromide (Invitrogen NZ; 10488-085) immersed in 1x TAE buffer also with 5 μL of ethidium bromide pipetted into the gel tank at the positive terminal and electrophoresed using a BioRad PowerPac HC. 5 μL of each of the 8 experimental reactions and both negative controls (2.1.2) was mixed with 1 μL of loading dye before loading into wells before running out on a 1% agarose gel for 1 hour at 100 V (or until the loading dye had run at least halfway down the gel). TrackIt 1 kb Plus DNA Ladder (Invitrogen NZ; 10488-085) was used to determine the amplified fragment size. The gel was imaged and photographed on a BioRad Gel Doc imager using UV transillumination under the appropriate settings.

2.1.4 Purification of PCR product

Of the 8 experimental reactions, PCR purification was carried out on the sample which began with 300 ng of plasmid DNA. PCR purification was carried out according to instructions in Nucleospin Gel & PCR clean-up kit (Macherey-Nagel), section “5.1 PCR clean-up”.

For every 1 volume of sample, 2 volumes of Buffer NTI was added. The whole sample was then loaded onto a Nucleospin Gel and PCR Clean-up Column in a collection tube and centrifuged at 11,000 x g 30 seconds. The flow through was discarded. The membrane was washed by adding 700μL Buffer NT3 and centrifuged at 11,000 x g for 30 seconds. The flow through was discarded again and the wash step repeated. The flow through was discarded and the membrane dried of Buffer NT3 with an additional minute of centrifugation at 11,000 x g. The spin column was placed in a new collection tube. DNA was eluted by adding 15 μL Buffer NE, incubating at room temperature for 1 minute and centrifuging at 11,000 x g for 1 minute. Eluted DNA was stored at -20 °C.
The concentration of DNA was measured using a Nanodrop Technologies spectrophotometer, which was blanked using TE buffer.

2.1.5 Gel extraction and purification

For gel extraction and purification of PCR product, each remaining reaction (~45 μL) of the 30, 50 and 100 ng starting plasmid DNA concentration was mixed with 6 μL of loading dye and 50 μL of the mixture was electrophoresed on a 1% agarose gel for 1 hour at 100 V. To reduce prolonged UV damage to the DNA, the bands of amplified inserts were visualised using UV light on the Polaroid MP4 land camera and cut out using a sterile scalpel and placed into 1.5 mL microcentrifuge tubes.

The gel extraction protocol was carried out according to instructions for DNA extraction from agarose gels in Nucleospin Gel & PCR clean-up kit (Macherey-Nagel).

Following gel excision, the gel fragment was solubilised. For every 100 mg of agarose gel, 200 μL of Buffer NTI was added and the samples incubated at 50 °C, vortexing briefly every 2 minutes until melted. Subsequent steps are identical to those listed in (2.1.4) for purification of PCR product.

2.1.6 Restriction digest of vector plasmid

Restriction digest was used to linearise the vector plasmid. Restriction digest was carried out on pCDH-pSyn-SP-GFP-sAPPα using the restriction enzyme SalI which cut the vector plasmid at the 3’ end of sAPPα.

A 30 μL restriction digest consisted of 3 μL of SalI buffer, 1 μL of SalI restriction enzyme and 1 μg of vector plasmid DNA; with the remaining volume made up with sterile dH₂O. The restriction digest was incubated in a 37 °C waterbath for 1 hour.
2.1.7 In-Fusion® reaction

The In-Fusion® reaction was carried out according to instructions in the In-Fusion® HD Cloning Kit User Manual (Clontech® Laboratories, Inc), Section VI.B: In-Fusion® Cloning Procedures for Spin-column purified PCR fragment.

All reactions were made up to a total volume of 10 μL. The cloning reaction contained approximately 50 ng of purified insert DNA and 200 ng of linearised vector DNA, and 2 μL of 5x In-Fusion® HD Enzyme Premix (Clontech®); with the remaining volume made up with sterile dH₂O. The negative control consisted of no purified insert DNA, 1 μL of linearised vector DNA and 2 μL of 5x In-Fusion® HD Enzyme Premix (Clontech®); with the remaining volume made up with sterile dH₂O. The positive control consisted of 2 μL of 2 kb control insert (Clontech®), 1 μL of pUC19 control vector (Clontech®) and 2 μL of 5x In-Fusion® HD Enzyme Premix (Clontech®); with the remaining volume made up with sterile dH₂O. All samples were incubated at 50 °C for 15 minutes for the In-Fusion® reaction to occur. All reactions were stored at -20 °C until used for transformation of competent cells.

2.2 Transformation of competent E.coli

All E.coli use was approved by the Environmental Risk Management Authority (ERMA), now the Environmental Protection Agency (EPA) and is carried out under approval number GMD002848.

PC1 conditions were observed while conducting transformation of genetically modified E.coli. Stbl3 competent E.coli cells were used for transforming with the plasmid construct due to high transformation efficiency and high DNA yields.

The Stbl3 cells were thawed on ice and 2.5 μL of both the In-Fusion® only and In-Fusion® + SalI reactions were added to the competent cells. For controls, 2.5 μL of
the In-Fusion® positive and negative controls were also added to competent cells. Cells were incubated on ice for 30 minutes prior to heat shocking at 42 °C in a waterbath for 45 seconds for transformation to occur before returning to ice for 2 minutes. 250 μL of S.O.C media (Invitrogen) was added to each sample and incubated at 37 °C for an hour in an Innova 4300 Incubator Shaker (New Brunswick Scientific) shaker at approximately 225 rpm. All samples were spun down using a Thermo Scientific Heraeus Pico17 centrifuge at 6000 rpm for 5 minutes to concentrate cells. The supernatant was removed and the pellet resuspended in 100 μL of S.O.C media. All 100 μL of both experimental and both (positive and negative) controls were spread onto separate Lennox L agar (LB agar) plates (Invitrogen) containing 100 μg/mL of ampicillin (Sigma Aldrich) using ethanol and flame sterilised glass Pasteur pipettes (Volac). Plates were incubated agar-side up at 37 °C overnight.

2.3 Identifying positive transformants and isolation of plasmid DNA

2.3.1 Colony PCR

Screening for positive colonies of transformants containing the plasmid construct was achieved by performing colony PCR. This process can be a simple way of determining the presence of a desired insert DNA sequence within a plasmid construct. The presence of insert DNA is detected either using primers which will amplify the insert DNA to determine its presence or using primers flanking the insert region to determine its presence from the resulting fragment size.

In this case, a combination of both primer choices was used. A mCherry 3’ forward primer and WPRE N-terminus reverse primer were chosen (Table 2.3).
### Table 2.3 – Colony PCR primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>318 mCherry 3’ seq fwd</td>
<td>CCCGGCGCCTACAACGTCAA</td>
</tr>
<tr>
<td>379 WPRE rev Nterm</td>
<td>GCAGCGTATCCACATAGCG</td>
</tr>
</tbody>
</table>

Each 25 μL reaction was prepared with 2.5 μL of Taq 10X buffer (brand and details), 0.5 μL of dNTPs (10 mM each), 1 μL of forward primer (10 pmol), 1 μL of reverse primer (10 pmol), 0.2 μL of Taq polymerase and 19.8 μL of dH2O. Each 25 μL is aliquoted into separate 0.2 mL PCR strip tubes (Axygen). In-Fusion and In-Fusion + SalI colonies are picked off plates with a pipette tip and struck onto a new agar plate before dipping into the PCR strip tube, turned to mix and then discarded. PCR amplification was carried out in a BioRad C1000 thermal cycler.

#### Table 2.4 – PCR parameters for colony PCR of transformants

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR parameters</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>318 mCherry 3’ seq fwd</td>
<td>1. 95°C – 3 minutes</td>
<td>221 bp</td>
</tr>
<tr>
<td></td>
<td>2. 95°C – 30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. 60°C – 30 seconds</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. 72°C – 1 minute</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5. 35 cycles of 2-4</td>
<td></td>
</tr>
<tr>
<td>379 WPRE rev Nterm</td>
<td>6. 72°C – 5 minutes</td>
<td></td>
</tr>
</tbody>
</table>

Successful amplification by both primers will produce an approximately 200bp fragment as shown in Figure 2.3 below.
Figure 2.3 – Colony PCR product

The selected primers (dark and light green) will amplify through the 3’ end of the inserted mCherry sequence into the vector plasmid if the In-Fusion® reaction was successful.

PCR products are run on an agarose gel (2.1.3) to determine if the insert DNA has been successfully introduced into the vector plasmid by the presence of a band of the desired size.

2.3.2 Mini-prep isolation of plasmid DNA

Positive colonies which produce the expected PCR fragment will be inoculated in 5 mL of Luria broth (LB; Sigma Aldrich) containing 100 μg/μL ampicillin. All were incubated at 37 °C overnight to grow; along with an uninoculated control. Following the overnight incubation, the tubes were checked for signs of growth (cloudy LB broth) as well as ensuring that the uninoculated control was free of contamination (clear broth).

1.5 mL microcentrifuge tubes were than filled with the broth of overnight growth and centrifuged at 13000 rpm for 1 minute. The supernatant was discarded and the pellet resuspended in 300 μL of Qiagen buffer P1 (Resuspension Buffer) by vortexing. 300 μL of Qiagen buffer P2 was added and vortexed to lyse the bacteria before adding 300 μL of Qiagen buffer P3 (neutralisation buffer) and vortexed again. The entire mixture was centrifuged at 13000 rpm for 10 minutes. The supernatant (with plasmid DNA) was transferred to a fresh microcentrifuge tube while avoiding the pellet. 300 μL of Phenol:Chloroform:Isoamyl Alcohol (24:24:1) (Sigma Aldrich) was added and
vortexed before being centrifuged at 13000 rpm for 10 minutes. The top aqueous phase (with plasmid DNA) was again transferred to a fresh microcentrifuge tube and 500 μL of room temperature isopropanol was added and vortexed for DNA precipitation; and centrifuged at 13000 rpm for 10 minutes to pellet the DNA. The supernatant was discarded and the DNA pellet was washed with 500 μL of 70% ethanol and centrifuged at 13000 rpm for 2 minutes. The ethanol was discarded and the DNA pellet air dried.

Once dry, it was resuspended in 50 μL of TE buffer. The concentration of DNA was measured using a Nanodrop Technologies spectrophotometer, which was blanked using TE buffer.

2.3.3 Sequencing and aligning sequences on Geneious

All 10 mini-prep isolated plasmid DNA samples were prepared and sent for sequencing. In a 5 μL reaction, 200 ng of plasmid DNA and 3.3 pmol of the WPRE N-terminus reverse primer (2.3.1); with the remaining volume made up by sterile dH₂O. Samples were sent to the University of Otago, Genetic Analysis Services (GAS) for sequencing. The returned sequences were analysed for sequence alignment with the expected sequence by Geneious.

2.3.4 Midi-prep isolation of plasmid DNA (and re-sequencing for verification)

Sequence verified plasmid-containing E.coli were grown in a flask of 300 mL of LB containing 100 μg/mL of ampicillin and inoculated with a scraping of glycerol stock. The flask was incubated in a 37 °C shaker overnight. The midi-prep was carried out according to instructions in the NucleoBond® Xtra Midi Plus high-copy plasmid DNA purification kit.
The bacterial cells were harvested in centrifuge tubes by centrifugation of the broth of overnight growth at 6000 x g for 10 minutes at 4 °C in a Beckman Avanti™ J-25 centrifuge. The supernatant was discarded and the pellet resuspended by vortexing in 8 mL of Resuspension buffer RES + RNaseA. For cell lysis, 8 mL of Lysis buffer LYS was added and mixed by gently inverting the tube and incubating at room temperature for no more than 5 minutes. While incubating, a NucleoBond® Xtra Column and filter are equilibrated by applying Equilibration buffer EQU to the rim of the filter and allowing to empty. 8 mL of Neutralisation buffer NEU is added to the lysate and mixed by gently inverting the tube. Immediately before loading the lysate onto the equilibrated column, the suspension is homogenised by gentle inversion again. Once the column has emptied, it is washed by applying Equilibration buffer EQU to the rim of the filter and allowing to empty. The filter is discarded. The NucleoBond® Xtra Column is washed with Wash buffer WASH. The plasmid DNA is then eluted using 5 mL of Elution buffer ELU and collected in a fresh centrifuge tube and 3.5 mL of room temperature isopropanol was added and thoroughly vortexed for DNA precipitation. The precipitated plasmid DNA was centrifuged at 15000 x g for 30 minutes at 20 °C to pellet the DNA. The supernatant was discarded and the DNA pellet was washed with 2 mL of 70% ethanol and centrifuged at 15000 x g for 5 minutes. The ethanol was discarded and the DNA pellet air dried.

Once dry, it was resuspended in 150 μL of TE buffer. The concentration of DNA was measured using a Nanodrop Technologies spectrophotometer, which was blanked using TE buffer.

The midi-prep isolated plasmid DNA was prepared in the same way as stated in (2.3.3) and sent to the University of Otago, Genetic Analysis Services (GAS) for sequencing. The returned sequence was verified for sequence alignment with the expected
sequence by Geneious.

### 2.3.5 Glycerol stocks

Glycerol stocks of transformed *E.coli* were made. Using aseptic techniques, 900 μL of overnight bacterial growth from mini-preps was added to 300 μL of sterile (autoclaved) UltraPure glycerol (Invitrogen) in 1.8 mL Nunc Cryotube Vials (Thermo Scientific) and vortexed well. Glycerol stocks were kept at -80 °C for long term storage.

### 2.3.6 Viral constructs and packaging

Midi-prep isolated plasmid DNA was packaged into lentivirus by Hollie Wicky of the NDD lab using methods described in Linterman et al., 2011. Viral construct tested *in vitro* on primary neurons. 0.5 μL and 2 μL of the generated LV construct was administered to 2 wells of primary neurons. They were allowed to incubate overnight before fixing.

Immuochemistry was carried out with anti-GFP to enhance the fluorescence signal; protocol described in section (2.9).

### 2.4 Virus used

Use of lentivirus for *in vivo* mouse brain experiments was approved by the Environmental Risk Management Authority (ERMA), now the Environmental Protection Agency (EPA) under approval number GMD002851.

The LV-pRRLSIN-PGK-GFP vector was pseudotyped with Rb B19 envelope glycoprotein with the GFP reporter gene expressed under the constitutive yeast phosphoglycerate kinase (PGK) promoter (Hawley et al. 1994; Qin et al., 2010). It has a functional titre of 2.2 x 10^8 TU/mL. The LV-pCDH-pSyn-SP-GFP-sAPPα-T2A-
mCherry vector has a functional titre of $2 \times 10^9$ ssRNA/mL.

AAV use for in vivo mouse experiments was similarly approved by the EPA but under approval number GMD101582. The AAV9 vector was purchased and imported (under approval GMC100166) from the University of Pennsylvania. It has a genomic titre of $3.6 \times 10^{13}$ genomes/mL.

2.5 Animals and treatment groups

All animal procedures were reviewed and approved by the Animal Ethics Committee (AEC) of the University of Otago under AEC approval number: 106/11. Animal handling and Restricted Veterinary Medicine (RVM) drug usage and administration was taught and certified by the Animal Welfare Office (AWO) with a refresher course conducted (online) every 12 months.

A total of 41 male mice were used, all were between 3 to 4 months old at the time of vector injection and each weighing between 25-37 g. The animals used were wildtype mice (strain C57BL/6) sourced from the Hercus Taieri Resource Unit (HTRU). Mice from each surgery group were housed together until the day of surgery; when they were separated into individual cages. They were housed singly post-surgery (due to mutual removal of sutures observed in the first surgical group) and were monitored for 5 days post-surgery to ensure that none were experiencing adverse side effects from the surgery (eg. pain, dehydration, weight loss). Mice were fed and watered ad libitum for the stated number of days (Table 2.5) until euthanasia.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No virus control</td>
<td>1</td>
</tr>
<tr>
<td>5% w/v dextran (O/N)</td>
<td>4</td>
</tr>
<tr>
<td>LV (7 days)</td>
<td>1</td>
</tr>
<tr>
<td>LV (14 days)</td>
<td>3</td>
</tr>
<tr>
<td>LV (28 days)</td>
<td>12</td>
</tr>
<tr>
<td>AAV9 (28 days)</td>
<td>4</td>
</tr>
<tr>
<td>AAV9 – 1:10 dilution (28 days)</td>
<td>4</td>
</tr>
<tr>
<td>LV + mannitol (28 days)</td>
<td>4</td>
</tr>
<tr>
<td>AAV9 (1:10 dilution) + mannitol (28 days)</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 2.5 – Summary of treatment groups and animal numbers

2.6 Stereotaxic surgery

Animals were anaesthetised in preparation for surgery via a subcutaneous (SC) injection (into the loose skin at the hind legs) with an anaesthetic cocktail consisting of ketamine, domitor (both anaesthetics) and atropine (prevents lung congestion) (KDA) (refer to Appendix for drug concentrations, dosages and suppliers). Once anaesthetised, the mouse was tested for pain sensation with paw pinches, until no limb withdrawal reflex is observed, before proceeding. The dorsal region of the mouse head was shaved using an electronic clipper to expose the scalp. Another SC injection of lopaine (pre-op analgesic) was delivered into the dorsal region of the head, at the site of incision.

The stereotaxic frame and work areas are wiped down with 70% ethanol, stereotaxic surgery tools (scalpel, surgical scissors, tweezers and needle holder) are autoclaved prior to use, immersed in hibitane when not in use and dried on a clean paper towel
before use. The mouse was secured in a stereotaxic frame with blunt 45° ear bars 
(chosen to prevent tympanic rupture) and tooth bars (World Precision Instruments 
(WPI); item#: TAXIC-650). The exposed scalp was cleaned with a swab dipped in 
habitane before an incision was made sagittally using a sterile scalpel to reveal the 
skull. The reference point of Bregma (commonly used as a ‘zero-point’ landmark for 
stereotaxic surgery of the brain) where the coronal and sagittal sutures intersect 
perpendicularly was located and recorded; the coordinates for the injection were 
located at -2.2 mm anteroposterior (A-P) and +1.4 mm mediolateral (M-L) relative to 
Bregma (coordinates: A-P -2.2 mm/M-L +1.4 mm). A burr hole was carefully drilled in 
the skull at the appropriate stereotaxic coordinates.

The dorsoventral (D-V) coordinate of the brain surface was recorded as the depth at 
which the needle bevel was observed to make contact with the cortex. The injection 
D-V coordinate is calculated relative to this ‘zero-point’.

The infusate (5% w/v dextran, LV or AAV9) was administered using a NanoFil 10 μL 
syringe (WPI; #NANOFL) coupled with a 33 gauge (33G) beveled NanoFil needle 
(WPI; #NF33BV-2). The needle and syringe is affixed into the UltraMicroPump (WPI; 
#UMP3), which depresses the needle, attached to the stereotaxic frame. A Micro4™ 
MicroSyringe Pump Controller (WPI; #SYS-MICRO4) is connected to the 
microinfusion pump to control the infusion rate.

A unilateral injection is made by lowering the 33G needle slowly to the required depth 
into the dorsal hippocampus to a depth of 1.3 mm (coordinate: DV -1.3mm) (to target 
the CA1 hippocampus). The microinfusion pump controlled the delivery of the infusate 
was dispensed, the needle was left in place for a minimum of 5 minutes before being 
slowly withdrawn from the brain over 3 minutes.

The open head wound was then cleaned, closed and sutured. Norocarp (an anti-
inflammatory) and antisedan (for anaesthetic reversal) was then administered subcutaneously, along with 250 µL of 0.9% saline.

2.6.1 5% w/v dextran administration
The 5% w/v dextran solution was prepared on the day of surgery using fluorescently labelled Tetramethylrhodamine dextran of molecular weight 10 kDa (Molecular Probes) in sterile dH₂O. These surgeries served as practice surgeries to gain clearance from the AWO for solo surgeries and to determine accuracy of the injection coordinates at targeting the CA1 region of the hippocampus.

The dextran injections were carried out under PC1 conditions, in the small animal surgery room of the Hercus Animal Facility. Mice were prepared as previously described and injected with 200 µL of dextran. Perfusion and tissue collection took place the following day.

2.6.3 Mannitol pre-treatment
The mannitol solution was prepared on the day of the surgery by dissolving D-mannitol (Sigma-Aldrich; M4125-100G) in sterile water.

Once anaesthetised, the mice in the treatment group received an intraperitoneal (IP) injection of a 25% w/v mannitol solution at a single dose of 3 mL/100g of bodyweight (Carty et al., 2010) approximately 15-20 minutes prior to vector injection. Each mouse was observed for any signs of pain (such as twitching, arched back, writhing, staggering, flinching or pressing of the belly) resulting from mannitol administration. No signs of pain was observed.

Previous studies have used a control group which were given an IP injection of saline (along with the subsequent intracranial viral injection). However, due to the consensus
in the literature (which did not show any significant difference in transduction levels due to saline injection) and time constraints; the control used to compare transduction efficiencies of either LV or AAV vectors with mannitol treatment will be the mice injected with the respective vector alone.

2.6.4 Viral vector administration

All viral aliquots were collected the morning of surgeries and transported on dry ice from the Department of Biochemistry to the Hercus Animal Facility in accordance with containment controls outlined by HTRU Standard Operating Procedures; in an additional sealed and biohazard labelled container.

All viral injections were carried under PC2 conditions in the biobubble of the large animal surgery room (H408) of the Hercus Animal Facility. Mice were prepared as previously described and 1 µL of either LV or AAV9 vector was injected into the left dorsal hippocampus at a rate of 150 nL/min.

LV and AAV9 injections were always scheduled for different days. Different needles used for either LV or AAV9; and where possible, a different syringe was used for the different viral vectors. Otherwise, the syringe was cleaned out by flushing out with viraclean followed by a triple wash of sterile dH₂O prior to using a different viral vector.

2.7 Tissue collection

Mice were killed after the set incubation period. Euthanasia was achieved with an overdose of sodium pentobarbital. Immediately following euthanasia, the mouse had its chest cavity opened and a butterfly needle was inserted into the heart at the apex. A small snip was made using surgical scissors at the right atrium and the mouse was first perfused with 20 mL of 0.9% saline, followed by 20 mL of freshly prepared 4%
paraformaldehyde in 0.1 M PB (PFA).

The head was removed and the brain was carefully dissected out of the skull and immersed in approximately 10 mL of fresh 4% PFA in a foil wrapped (to prevent photo-bleaching) sterile universal for 24 hours. This is followed by floating the brain in 30% sucrose solution for cryoprotection until the brain has sunk (usually 36-48 hours later). Each brain was individually fixed in a Peel-A-Way® embedding molds (Polysciences, Inc.; 18646A-1) by freezing in Tissue-Tek® O.C.T™ (optimal cutting temperature) Compound (Sakura; 4583) using dry ice and isopropanol. The cryofixed brains were stored at -80 °C until sectioning.

Serial coronal sections were taken at 50 µm thickness using a Research Cryostat Leica CM3050 (-20 °C). Each section was stored in a well of 100 µL of cryoprotectant solution (Solutions appendix) in a 96-well plate. One brain typically required two 96-well plates. Plates were wrapped in foil and kept at -20 °C until required for imaging.

2.8 Imaging

Every sixth section was transferred into a well of 0.1 M PB in a 24-well plate to wash off any O.C.T and/or cryoprotectant solution overnight. Sections were mounted on slides with antifade to preserve fluorescence and viewed under an Olympus inverted fluorescence research microscope IX71 to locate GFP reporter gene expression in transduced cells.

Sequential images were captured of whole sections which were then merged together using Adobe® Photoshop® CS6 (Version 13.0 x64) to generate a composite image of the section. Using the composite image, regions of GFP expression were mapped against the Paxinos mouse atlas to determine the regions of the brains transduced.
2.9 Immunolabelling

2.9.1 Immunohistochemistry for cell type analysis

The indirect method of immunohistochemistry (IHC) was carried out on tissue sections for neuronal marker, NeuN, using a mouse monoclonal anti-NeuN (Millipore; MAB 377); astrocyte marker, GFAP, using a mouse monoclonal anti-GFAP (Sigma Aldrich; G3893); and in certain cases, for GFP, using a rabbit polyclonal anti-GFP (Abcam; Ab290). The labelled secondary antibody for anti-NeuN and anti-GFAP was Alexa Fluor 594 Goat anti-mouse IgG (Invitrogen NZ; A-11037). The secondary antibody for anti-GFP was Alexa Fluor 488 Goat anti-rabbit (Invitrogen NZ; A-11008). The table below summarises the antibodies and the dilutions used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
</tr>
<tr>
<td>Anti-GFAP</td>
<td>1:1000 (IHC)</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>1:1000 (IHC and ICC)</td>
</tr>
<tr>
<td>Anti-NeuN</td>
<td>1:1000 (IHC)</td>
</tr>
<tr>
<td><strong>Secondary antibodies</strong></td>
<td></td>
</tr>
<tr>
<td>Alexa-Fluor 488</td>
<td>1:1000 (IHC and ICC)</td>
</tr>
<tr>
<td>Alexa-Fluor 594</td>
<td>1:1000 (IHC)</td>
</tr>
</tbody>
</table>

Table 2.6 – antibodies and dilutions used in immunohistochemistry and immunocytochemistry

Sections were prepared for IHC by 3 washes of 30 minutes each in 1 mL of PBS in 24-well plates at room temperature (or an overnight wash in PBS at 4 °C if taken directly from cryoprotectant. Blocking, to prevent non-specific antibody binding, was carried out by incubating sections in blocking solution for 1 hour at room temperature. The rabbit polyclonal anti-GFP was highly non-specific, which meant it required an additional pre-block step to remove non-specific binding antibodies. This involved adding the required volume of primary antibody to 1 mL of blocking solution and adding
500 μL to two wells containing GFP absent tissue for non-GFP-specific antibodies to bind. This pre-block was incubated for 1 hour at room temperature (while tissue blocking is taking place). The 1 mL of primary antibody was removed from the wells (now free of non-GFP-specific binding antibodies) and topped up with blocking solution to the working dilution.

The primary antibody mixture was prepared to the desired dilution and the blocking solution was removed from the well before adding the primary antibody at 1 mL per well. The plate was parafilmed, foil wrapped and incubated at 4 °C for 2 days.

The primary antibody was removed and 3 washes of 30 minutes each with 1 mL of PBS-T to remove unbound and weakly bound primary antibody. The secondary antibody was prepared to the required dilution and 1 mL was added per well after removal of the third PBS-T wash. The plate was again parafilmed, foil wrapped and incubated for 4 hours at room temperature on a shaker.

The secondary antibody was removed and three 30 minutes washes with PBS-T was repeated to remove unbound and weakly bound secondary antibody. PBS was added after removal of the final wash.

A 1:10,000 dilution solution of DAPI (Sigma NZ; D9564) Nuclear Stain was prepared and 1 mL was added per well after removal of PBS. Incubation was 15 minutes at room temperature.

2.9.2 Immunocytochemistry of transduced cell for viral testing

Immunocytochemistry (ICC) of primary neurons transduced with LV-pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry used anti-GFP primary antibody and its respective secondary.

The ICC protocol with the rabbit polyclonal anti-GFP is identical to the IHC protocol as
listed above (2.9.1). It only differed in antibody incubation times; the primary antibody was incubated overnight at 4 °C while secondary antibody incubation was 90 minutes at room temperature. Cells were not DAPI stained before imaging.

2.10 Image analysis

2.10.1 Quantifying area of fluorescence

The composite images were then analysed using the program ImageJ (1.48v) to determine the area of fluorescence within each section. The original image is first converted into a binary image and the fluorescence appears black while all other colour is converted to white background. The analysis is then scaled according to known distances in the original image by using the scale bar. The program then calculates the black area to produce the area transduced within that section (Appendix). The area obtained is recorded relative to its mapped position in the mouse atlas.

2.10.2 Cell counting for determining viral titre

An experiment was designed to examine for a possible direct effect of mannitol presence (at different concentrations) on lentiviral titre. HT1080 cells were prepared in a 24-well plate by Hollie Wicky. The LV used in this experiment was the rabies B19 pseudotyped GFP expressing virus (LV-pRRLSIN-PGK-GFP) and 0.5 μL was added into the media of each well. Five concentrations were prepared; 5, 12.5, 15, 20 and 25% w/v mannitol, along with an untreated (LV only) control. Mannitol was administered at a relative concentration to be used for in vivo experiments of 3 mL/100mL of media. This translated to 3 μL of each concentration added to 1 mL of media per well.
Two methods of administration was used; a sequential and co-administration of mannitol with LV. The sequential method involved adding mannitol into the well before introducing the virus. Co-administration involved adding the 0.5 μL of LV into 30 μL of mannitol before adding the total volume into the media of each well. Transduction was allowed to occur overnight before fixing the cells for immunocytochemistry with GFP antibody (2.9.1). Primary antibody incubation was carried out overnight at 4 °C. Secondary antibody incubation was carried on the shaker for 90 minutes at room temperature. A functional titre was calculated by taking 5 frames of each well under a fluorescence microscope. The cell count in each frame was determined using ImageJ to carry out an automated cell count (Appendix C).

2.11 Statistical analysis

Statistical analysis of anterior-posterior distance of GFP expression spread was conducted in Microsoft Office Excel to carry out t-tests to generate p-values. To generate plots illustrating a scaled mean for each treatment group, code written by Dr Michael Black, of the Department of Biochemistry, was used. The raw data was entered into RStudio (Version 0.99.442) to generate a plot. That unscaled data was scaled against its mean to zero to generate a scaled plot. Using that scaled plot, a mean trendline with a 95% confidence interval of the each treatment group was generated.
Chapter 3: Results

Following the incubation period post-injection, all mice were perfused, with brains fixed and cryoprotected for analysis. All the following mouse brain images shown are of 50 μm coronal sections. Sections are oriented to display in a posterior to anterior direction of view (ie. The orientation is true to view; left and right hemispheres are on the left and right of the image shown). All microscopy images were taken on an Olympus inverted fluorescence research microscope IX71. Images of whole sections were taken and mapped to determine distance of spread and specific regions of GFP expression. Area of fluorescence was calculated to determine distribution across that distance.

3.1 Using 5% w/v Dextran to verify injection coordinates

These initial Dextran injections were conducted at the same stereotaxic coordinates (2.6) at a depth 1.00 mm ventral of the brain surface. Two hundred nanolitres of Dextran dye (molecular weight: 10,000 Da) was injected into the left dorsal hippocampus. Figure 3.1 shows the staining observed within the left hippocampus formation of a 50 um coronal brain section.
Figure 3.1 – 5% w/v Dextran stained mouse hippocampus

Labelled regions of Dextran staining – Or: oriens layer; LMo: lacunosum moleculare layer; Mo: molecular layer of the dentate gyrus (section at approximately 1.94 mm posterior to bregma). Animal ID: IC007; scale bar 500 μm.

The shallower injection results in strong staining in the oriens layer of the hippocampus dorsal to the CA1 and CA2 regions of the hippocampus, the molecular layer of the dentate gyrus (DG) and into the lacunosum moleculare layer of the hippocampus formation.

Due to the depth of injection mostly delivering the Dextran to the oriens layer of the hippocampus, it was decided to change the depth of injection to -1.3 mm dorsal-ventral (D-V). Proceeding forward, all viral vector injections were conducted at the new depth to improve targeting of the CA1 region of the hippocampus proper.

3.2 Determining ideal timeframe for viral vector incubation

An initial trial was conducted to determine the incubation times for future treatment groups; 7, 14 and 28 days were trialled. 7 and 14 days were insufficient time to produce
expression beyond the immediate injection time (results not shown). In the end, 28
days was chosen to allow ample time for transgene expression and as it provided
enough time for analysis between surgery groups.

3.3 Spread of lentivirus-mediated expression of GFP
One microliter of the rabies pseudotyped GFP expressing LV (titre: $2.2 \times 10^8$
(transducing units) TU/mL) was injected into the left dorsal hippocampus at a depth of
1.30 mm. Perfusion and analysis took place 28 days post-injection.

As observed in Figure 3.2, LV-mediated GFP expression was largely limited to the
hippocampal regions of the injection site.

Regions of the hippocampus with observed GFP expression at and posterior to the
injection site included the CA1, pyramidal cell layer, granular and polymorph layer of
the dentate gyrus, lacunosum moleculare layer and dorsal hippocampal commissure. (Figure 3.2A and 3.2B; magnified in Figure 3.3).

Figure 3.3 – Magnified mouse hippocampus at site of LV injection
Labelled regions of GFP expression – dhc: dorsal hippocampal commissure; Py: pyramidal cell layer; LMoI: lacunosum moleculare layer; PoDG: polyporph layer of the dentate gyrus (DG); GrDG: granular layer of the DG. Animal ID: IC028; scale bar 500μm.

There were fewer cells transduced anteriorly and those observed were found to be in the CA3, pyramidal cell layer and stratum lucidum of the hippocampus (Figure 3.4).
The stratum lucidum is mainly composed of densely packed mossy fibres and interneurons projecting to CA3 pyramidal cells or the stratum oriens below. The CA1, hilur regions and lacunosum molecule layer of the hippocampus also receive axonal projections from the interneurons of the stratum lucidum (Vida & Frotscher, 2000). These projections and connections within the hippocampus explain the spread of GFP expression to distal region from the injection site.

3.3.1 Distribution of spread from bregma achieved with LV injections

The area of fluorescence was determined using ImageJ (Appendix C) and saved into a spreadsheet. Using code written by Dr Michael Black of the Department of Biochemistry, the raw data was entered into RStudio (Version 0.99.442) to generate a plot. That unscaled data was scaled against its mean to zero to generate a scaled plot.
Using that scaled plot, a mean trendline of the treatment group was generated as shown below.

![Graph of scaled area of fluorescence and mean trendline of LV treated group](image)

Figure 3.5 – Graph of scaled area of fluorescence and mean trendline of LV treated group

Mean (in black line) and 95% confidence interval (CI; grey band)

Because the plot was generated with an x-axis representing Bregma, a single off-target injection site (animal ID: IC006) skewed the data set and the resulting mean trendline. However, the tight grouping and narrow spread is indicated on the plot indicating a very narrow distribution of GFP expression and thus LV spread. The left peak on the mean trendline where most of the on-target injections are centred is at
approximately -2.25 mm relative to bregma indicating that on average the injections and spread were close to the injection coordinate of -2.2 mm relative to bregma.

3.4 Spread of undiluted AAV9-mediated expression of GFP

One microlitre of AAV9 (titre: 3.6 x 10^{13} genomes/mL) was injected unilaterally into the left CA1 region of the hippocampus at a depth of 1.30 mm. Perfusion and analysis took place 28 days post-injection.

As observed in Figure 3.6, the undiluted AAV9 injection resulted in widespread and robust transduction (while not shown, GFP was observable with the naked eye in the cortex during dissection of the brain).

Posterior to the injection, GFP expression extended to large regions of the cerebellum. These included the 4\textsuperscript{th} and 5\textsuperscript{th} cerebellar lobules, simple lobule (not shown in magnified
image), inferior cerebellar peduncle, crus 2 and 1 of the ansiform lobule and paraflocculus (Figure 3.6A; magnified in Figure 3.7).

![Image of cerebellum with labels]

**Figure 3.7 – Magnified cerebellum of undiluted AAV9 injected mouse**

Labelled regions of GFP expression – 4&5Cb: 4th and 5th cerebellar lobules; icp: inferior cerebellar peduncle; Crus1/2: crus 1/2 (respectively) of the ansiform lobule; pfs: paraflocculus. Animal ID: IC017; scale bar 500 μm.

In the medulla, there is strong expression of the pyramidal tract (which travel to the brain stem or spinal cord) and raphe pallidus nucleus. Motor neuron fibres in the pyramidal tract originate from pyramidal cells in layer V of the cortex, which show high levels of transduction (Figure 3.6B; label “Ctx”). Projections from the reticular nuclei (which is visible as GFP fluorescent fibres in Figure 3.6A; magnified in Figure 3.8) terminate in the raphe pallidus nucleus.
The reticular formation resembles a ‘highway’ of neuronal projections from various parts of the brain. It is likely due to this diverse network of projections which resulted in the high level of transductions seen. It is split into 3 columns; of which the raphe pallidus nucleus forms the median column. The lateral column is known as the parvocellular reticular nucleus and is highly transduced (regions with GFP expression are the gigantocellular (Gi), Gi α-part, lateral paragigantocellular and intermediate reticular nuclei).

Around the injection site, there is almost complete transduction of all nearby structures (Figure 3.6B). This is a marked difference, even within the immediate site of injection, in the level of transduction and GFP expression in the contralateral hemisphere;
compared to the LV vector where most contralateral GFP expression came from the corpus callosum and the occasional cell in the subiculum.

The left injected hippocampus is almost completely transduced especially within the CA1 to CA3 region; with fewer cells transduced in the dentate gyrus (magnified in Figure 3.9). All regions of the cortex were transduced; although the secondary auditory cortex had lower GFP expression levels. The cortical regions immediately dorsal to the hippocampus, such as the retrosplenial and visual cortex, exhibit high levels of transduction. It is not known if this might be caused (to some extent) by a combination of backflow of virus during needle removal and/or greater transduction efficiency of AAV9. There was also very clear GFP expression in the entorhinal cortex due to retrograde transport along projections between the two structures. Superficial layer III of the entorhinal cortex projects to the CA1 region of the hippocampus and subiculum; all three structures have been implicated in AD neuropathology.
Anterior to the injection site, GFP expression was observed throughout the frontal association, prelimbic and orbital cortex of both the left and right hemisphere (Figure 3.6C; labelled in Figure 3.10). GFP expression is restricted to the dorsal and ventral tenia tecta, olfactory nucleus, and plexiform layer and cell layer of the olfactory bulb of the left hemisphere. However, the intrabulbar part of the anterior commissure expressed GFP in both left and right hemispheres.
Figure 3.10 – Most anterior GFP expressing section of undiluted AAV injected mouse

Labelled regions of GFP expression – AOD/L/M/V: anterior olfactory nucleus, dorsal/lateral/medial/ventral part; PrL: prelimbic cortex; FrA: frontal association cortex; M/V/L/DLO: medial/ventral/lateral/dorsolateral orbital cortex; aci: anterior commissure, intrabulbar part; VTT ventral tenia tecta. Animal ID: IC017; scale bar 500 μm.

From a visual inspection, the AAV9 vector demonstrated a greater ability to spread from the injection site to far distal regions of the brain compared to a rabies-pseudotyped LV vector, which was restricted to the injected hippocampal formation.
3.4.1 Distribution of spread from Bregma achieved with undiluted AAV9 injections

The plot produced from the area of fluorescence of undiluted AAV treated brains has a much wider spread as expected from the fluorescence microscopy images. The peak of the mean trendline (indicating largest area of fluorescence, and hence the injection site) is centred approximately -2.25 mm relative to bregma which is where it is expected to be; as the injection coordinates was -2.2 mm relative to bregma.
3.5 Spread of (1:10) diluted AAV 9-mediated expression of GFP

The dilution was prepared at the start of the day of surgery; a 5 μL aliquot of virus was thawed on ice, under PC2 conditions. Fifty microlitres of dPBS was added and pipetted gently to mix; before transporting on dry ice to the animal facility.

One microlitre of the diluted AAV9 (titre: 3.6 x 10^{12} genomes/mL) was injected unilaterally into the left CA1 region of the hippocampus at a depth of 1.30 mm. Perfusion and analysis took place 28 days post-injection.

As observed in Figure 3.12, there was widespread expression of GFP in distal regions from the hippocampus in brains transduced with the 1:10 diluted AAV9 aliquot. Visually, there were fewer cells transduced from the deceased intensity of GFP fluorescence. However similar regions were transduced when compared to the undiluted AAV9 injected brains.
Posterior to the injection site, GFP expression was limited to the pyramidal tract (Figure 3.12A; magnified in Figure 3.13) and did not exhibit the same transduction of the reticular formation as was seen in the medulla of the undiluted AAV9 injected brain (Figure 3.8). No GFP expression was observed in the cerebellum.

![Magnified medulla of diluted AAV9 injected mouse](image)

**Figure 3.13 – Magnified medulla of diluted AAV9 injected mouse**

Labelled regions of GFP expression – py: pyramidal tract. Animal ID: IC031; scale bar 500μm.

Similarities in transduction patterns to the undiluted AAV9 brains include widespread GFP expression within the left hippocampus with expression levels consistent through most regions of the hippocampus formation (Figure 3.12B). The CA1 to CA3 regions of the left hippocampus proper exhibit high levels of transduction along with the various layers cell layers of the dentate gyrus. The contralateral hippocampus formation is not as evenly transduced; the hippocampus proper is GFP expressing from CA1 to CA3 but the dentate gyrus lacked as many visibly transduced cells.
Cortical transduction was also limited to the retrosplenial, parietal association, primary and secondary somatosensory, secondary auditory and temporal auditory cortices. There is also some GFP expression in the entorhinal cortex. Contralateral cortical transduction is largely absent in comparison to the undiluted AAV9 injected mouse brains; although some spread along the corpus callosum is visible.

Ventral to the hippocampus, transduction is also limited to the ipsilateral hemisphere. Regions with observed GFP expression include the dorsal lateral geniculate nucleus, lateral posterior thalamic nucleus and reticular thalamic nucleus. Spread towards more ventral regions was observed along the stria terminalis, internal capsule and zona incerta. The fornix and perifornical nucleus is transduced. The contralateral fornix is also transduced.
In the anterior regions, expression is visibly reduced compared to the brains injected with undiluted AAV9. The regions are weakly transduced and limited to the prelimbic cortex and medial to ventral orbital cortex (Figure 3.15).

Figure 3.15 – Most anterior GFP section of diluted AAV9 injected mouse

Labelled regions of GFP expression – PrL: prelimbic cortex; VO: ventral orbital cortex. Animal ID: IC031; scale bar 500 μm.
3.5.1 Distribution of spread from bregma achieved with 1:10 diluted AAV9 injections

![Graph of scaled area of fluorescence and mean trendline of diluted AAV9 treated group](image)

**Figure 3.16** – Graph of scaled area of fluorescence and mean trendline of diluted AAV9 treated group

Mean (in black line) and 95% confidence interval (CI; grey band)

The mean trendline for the diluted AAV9 has a narrower distribution compared to the undiluted AAV9 treatment group. This indicates a relatively lower volume of GFP expression which is an expected outcome from a diluted/lower titre of virus. However, the spread anterior to posterior is not significantly different and the peak of the mean trendline is centred above the coordinate of the approximate injection site.
3.6 Effects of mannitol pre-treatment

3.6.1 Effects on lentiviral-mediated expression of GFP

The 25% w/v mannitol solution was administered systemically via an intraperitoneal injection after anaesthetising and before beginning surgery and viral vector injection. One microlitre of the same rabies B19 pseudotyped GFP expressing LV (titre: $2.2 \times 10^8$ TU/mL) was injected into the left dorsal hippocampus at a depth of 1.30mm. Perfusion and analysis took place 28 days post-injection.

![Systemic mannitol pre-treatment with LV injected mouse brain sections](image)

Figure 3.17 – Systemic mannitol pre-treatment with LV injected mouse brain sections

No GFP expression was found; sections chosen from the most posterior and anterior sections available along with the approximate injection site to illustrate no observable GFP. A: most posterior section (bregma -6.36mm); B: approximate injection site (bregma -2.1mm); C: most anterior section (bregma 2.10mm). Animal ID: IC021; scale 500μm.

No GFP expression was observed in any of the 4 brains from the LV + mannitol treatment group. It is unknown why there would fail to be any observable sign of GFP expression. An unsuccessful attempt to stain for GFP was also made in an effort to enhance a weak GFP signal.

The virus was identical to what was used in the first LV surgery and was known to be functional and all transport and handling procedures were identical in all other
injections. Virus was dispensed during the injection as the expected volume had been infused from the syringe after each animal.

This negative result prompted an *in vitro* experiment using primary neurons to test for any adverse effect that mannitol might be having on viral titre and thus transduction efficiencies (viii).

### 3.6.2 Effects of mannitol on (1:10) diluted adeno-associated virus serotype 9-mediated expression of GFP

The 25% w/v mannitol solution was administered systemically via an intraperitoneal injection after anaesthetising and before beginning surgery and viral vector injection. One microlitre of 1:10 diluted AAV9 (titre: $3.6 \times 10^{12}$ genomes/mL) was injected into the left dorsal hippocampus at a depth of 1.30 mm. Perfusion and analysis took place 28 days post-injection.
With mannitol pre-treatment, AAV-mediated GFP expression distribution and pattern resembled those found in brains injected with undiluted AAV9; except for GFP expression in the hippocampi all four brain was lacking which does not seem likely to have happened due to a missed injection site across all four mice.

Posterior to the injection site, the pyramidal tract and mossy fibres also strongly expressed GFP (Figure 3.18A; magnified in Figure 3.19), similar to what was observed in the undiluted AAV9 injected mouse brains. However, no GFP expression was observed in the cerebellum.
At the injection site, hippocampal transduction was markedly reduced compared to both diluted and undiluted AAV9 injected in all four mice. Some hippocampal neurons expressing GFP, towards the lateral region of CA1 and through CA2 and 3, are the extent of transduction; along with the subiculum (Figure 3.18B; magnified in Figure 3.20). The dentate gyrus has little expression of GFP; except within the polymorph layer.
Figure 3.20 – Magnified mouse hippocampus at site of diluted AAV9 injection with mannitol pretreatment

Labelled regions of GFP expression – RSG: retrosplenial granular cortex; RSA retrosplenial agranular cortex; M/LPtA: medial/lateral parietal association cortex; V2L: secondary visual cortex; Or: oriens layer; PoDG: polymorph layer of the dentate gyrus; S: subiculum. Animal ID: IC023; scale bar 500 μm.

The extent and regions of GFP expression in the cortex was similar to brains injected with undiluted AAV9, but at lower levels with fewer cells transduced. Regions of GFP expression included the retrosplenial granular and agranular cortices, medial and lateral parietal association cortices, secondary visual and primary somatosensory cortices (Figure 3.20). The temporal association cortex, entorhinal and perirhinal cortices are also strongly transduced (Figure 3.21).
Figure 3.21 – Lateral cortical region of section at the site of diluted AAV9 injection with mannitol pre-treatment

Labelled regions of GFP expression – TeA: temporal association cortex; Ect: entorhinal cortex; PRh: perirhinal cortex. Animal ID: IC023; scale bar 500 μm.

In the anterior regions, GFP expression was higher than that observed in diluted AAV9 injected brains. Particularly in the dorsal left hemisphere, where expression closely resembled brains injected with undiluted AAV9; all regions express GFP strongly except for the medial regions; such as the cingulate cortex, area 1, prelimbic cortex and medial orbital cortex, which are weakly transduced. Ventrally, weak GFP expression is observed mostly in the anterior olfactory nucleus. The contralateral hemisphere is weakly transduced (Figure 3.22).
Figure 3.22 – most anterior GFP expressing section of diluted AAV injected mouse brain with mannitol pre-treatment

Labelled regions of GFP expression – CG1: cingulate cortex, area 1; PrL: prelimbic cortex; M/V/LO: medial/ventral/lateral orbital cortex; AI: agranular insular cortex; M1/2: primary/secondary motor cortex; D/L/M/VO: dorsal/lateral/medial/ventral olfactory nucleus. Animal ID: IC023; scale bar 500μm.
3.6.3 Distribution of spread from bregma achieved mannitol pre-treatment on (1:10) diluted adeno-associated virus serotype 9 injections

Figure 3.23 – Graph of scaled area of fluorescence and mean trendline of diluted AAV9 with mannitol pre-treatment group

Mean (in black line) and 95% confidence interval (CI; grey band)

While the anterior to posterior distance of spread is still comparable to both undiluted and diluted AAV9 treatments, the curve is skewed towards the right; with the peak above bregma. This might either indicate a missed injection site or lower levels of expression around the injection coordinate or -2.2 mm relative to bregma due to the
lack of hippocampal expression; which is where a large proportion of GFP expression was observed in sections from diluted AAV9 injected brains.

3.6.4 Comparing anterior-posterior distance of spread

Firstly, the total anterior to posterior distance of spread was calculated from mapping the position of the most anterior and posterior sections where GFP expression was observed. The average distance within each treatment group was calculated and shown in the graph below (Figure 3.17); with the error bars indicating standard deviation within each treatment group. A 2-tailed t-test was applied in Excel to determine the p-value between treatment groups (Supplementary Figures). The null hypothesis was “the difference in spread between group 1 (array 1 of the t-test) vs. group 2 occurred by chance”. A p-value of less than 0.05 was deemed to be significant (ie. There was less than 0.05% chance of the null hypothesis being true; that difference in spread occurred by chance)

![Comparison of spread of GFP expression](image)

**Figure 3.24 – Graph comparing average total A-P distance of GFP expression across treatment groups**
The LV mannitol group was left out as no GFP expression was observed. The LV injected brains averaged 1.98 mm of spread with the AAV9 undiluted, diluted and mannitol pre-treatment averaged 9.67, 8.52 and 9.375 mm respectively.

The p-values calculated by comparing any one of the AAV9 treatment groups with another were all greater than 0.05; meaning AAV9 mediated GFP expression was not affected by viral titre or mannitol treatment.

However, the p-values obtained when comparing LV mediated spread with all AAV9 treatments were less than 0.05; indication that AAV9 produced a significantly greater anterior-posterior spread compared to LV.

3.7 Immunohistochemistry of transduced cell type analysis

3.7.1 Cell types transduced in lentivirus brains

IHC staining of LV transduced brain with GFAP and NeuN was carried out as described in the methods (2.9).
GFAP in an astrocyte marker and there is little co-localisation with GFP expressing cells and GFAP positive astrocytes (Figure 3.25B) indicates that the use of LV did not trigger an immune response.
Staining with NeuN allowed the visualisation of neuronal cells. The CA1 neurons of the LV transduced mouse brain are very distinctly expressing GFP. The GFP positive cell body are co-localised with NeuN staining (Figure 3.26) indicating that CA1 neurons were successfully transduced with LV injection into the hippocampus.

Figure 3.26 – LV injected mouse brain with anti-NeuN staining

A: CA1 hippocampal neurons expressing GFP and NeuN staining.
B: magnified hippocampal neuron; co-localisation of GFP neurons and NeuN positive cells.
C: same magnified view as B; overlaid with DAPI nuclear stain.
3.7.2 Cell types transduced in AAV9 brains

GFAP staining showed little to no co-localisation of transduced cells with astrocytes (Figure 3.27). This indicated that AAV had low immunogenicity within the brain, at least in this strain of mice. However, visually, it did seem that there was a higher level of GFAP staining in brain sections from AAV injected mice rather than LV treated mice. It would be difficult to say definitively without quantifying the levels of staining between the treatment groups.
As seen in Figure 3.28A, NeuN staining was highly co-localised with AAV transduced hippocampal neurons, especially within the CA3 region. Individual cell bodies in the magnified image (Figure 3.28B) are shown to be co-localised with NeuN staining (appearing yellow when images are overlaid).
3.7.3 Cell types transduced in AAV9 with mannitol pre-treated brains

Most of the AAV and mannitol pre-treatment brains had little to no GFP expression within the hippocampus. In the section stained for NeuN, there was a lone CA1 neuron transduced (Figure 3.29B) which was difficult to determine co-localisation with the NeuN staining surrounding it. However some neurons do not produce NeuN but based on the location and morphology, it strongly suggests that it is a neuron.

Images of GFAP staining on AAV and mannitol pre-treatment sections are unfortunately missing.
3.7.4 GFP staining in LV with mannitol brains

IHC staining with anti-GFP was attempted to enhance any possible weakly fluorescent signal in LV injected with mannitol pre-treatment brain section.

![Figure 3.30 – anti-GFP stained fluorescent cells in LV injected mouse brain with mannitol pre-treatment](image)

A: Fluorescent cell in the cortex.
B: Fluorescent cells in the left entorhinal cortex. Taken at the same magnification as A
C: Fluorescent cell around the hypothalamic nucleus

Even with anti-GFP staining to enhance the signal, the fluorescent cells observed in Figure 3.30 was the extent of any fluorescence indicative of likely LV transduction which had occurred in the LV and mannitol pre-treatment group.
3.8 Effect of mannitol concentration on lentiviral transduction

The experiment was designed to examine for a possible effect of mannitol on lentiviral titre, and thus subsequently the number of cells transduced, which might have resulted in the lack of GFP expression in vivo.

HT1080 cells were either sequentially administered or co-administered mannitol with lentivirus. Five different concentration of mannitol (up to the 25% w/v used for the in vivo experiments) were tested, together with a virus only control.

For each concentration of mannitol, five field-of-view images were captured at 20x magnification. An image from each concentration of mannitol co-administered with LV is shown in Figure 3.31 below.

![Figure 3.31 - LV transduced HT1080 cells at different concentrations of mannitol applied with virus treatment](image)

A: LV only control; B: 5% w/v mannitol; C: 12.5% w/v; D: 15% w/v; E: 20% w/v; F: 25% w/v. Scale bar 200 μm
The number of cells transduced per frame was counted using ImageJ and averaged. The average number of cells transduced per frame when co-administered with each concentration of mannitol is shown in the graph below.

![Graph showing average number of cells transduced when different concentrations of mannitol are co-administered with LV](image)

**Figure 3.32** – graph showing average number of cells transduced when different concentrations of mannitol are co-administered with LV

Error bars indicate standard deviation.

The average number of cells transduced did not show any difference following treatment with various mannitol concentrations (Figure 3.32).

For the sequential administration, 30 μL of each concentration of mannitol was added to each well of cells prior to LV administration.
Figure 3.33 – LV transduced HT1080 cells at different concentrations of mannitol applied prior to virus treatment

A: LV only control; B: 5% w/v mannitol; C: 12.5% w/v; D: 15% w/v; E: 20% w/v; F: 25% w/v. Scale bar 200 µm

The average number of cells transduced per frame when co-administered with each concentration of mannitol is shown below.
There is no significant effect of mannitol concentration on the number of cells transduced when administered sequentially (Figure 3.34; as was done during the stereotaxic surgeries).

3.9 Cloning

3.9.1 PCR amplification of T2A-mCherry insert

PCR amplification of the T2A-mCherry sequence with In-Fusion® primers produce an 813 bp fragment. Five microlitre of each PCR reactions was electrophoresed on a 1% agarose gel and stained with ethidium bromide to visualise DNA. The gel image obtained is shown below (Figure 3.35).
Figure 3.35 – Amplification of T2A-mCherry insert using In-Fusion® primers

T2A-mCherry PCR product from using In-Fusion® primers with different starting amounts of plasmid DNA carried out in duplicates. All PCR runs returned positive with a band just under 850 bp (against the ladder), which matches the expected fragment size of 813 bp. The no primer control did not produce a band.

All PCR runs returned positive with band on the gel of the expected amplicon size, just under 850 bp according to the DNA ladder. The remainder of the PCR products were purified before running out on a gel for gel excision and purification of only the required fragment size. The gel of purified PCR products was visualised with UV light for excision of the gel fragment containing the PCR product; it was imaged post-excision to show that the desired fragment had been precisely removed.
Figure 3.36 – Gel image post-excision of PCR products for gel purification

Gel image taken after excision of gel fragments containing PCR products (derived from starting amounts of 30, 50 and 100 ng of plasmid DNA) at around the 850 bp size for gel purification.

Nanodrop results of the gel purified PCR products came back with low yield and poor A260/A280 ratios which indicated poor purity of DNA. It was decided to proceed with the In-Fusion® cloning reaction using the purified PCR product from the 300 ng of starting plasmid DNA which had a 260/280 ratio of 1.98 and DNA yield of 51.1 ng/μL.

3.9.2 Restriction digest of vector plasmid

The vector plasmid was linearised using the restriction enzyme SalI. The restriction digest was electrophoresed on a 1% agarose gel (against an uncut vector) and stained with ethidium bromide. This enabled the verification of the digested plasmid size; being the same as the uncut. It also allowed for gel purification of the digested product.
The resulting image (Figure 3.37) shows that the digested plasmid was the same size as the uncut, approximately 9000 bp.

![Gel image showing restriction digested and undigested vector plasmid](image)

**Figure 3.37 – Gel image showing restriction digested and undigested vector plasmid**

(left – right) Sall digested and undigested vector plasmids are of the same size; streaking may be due to overloading of DNA sample into wells.

### 3.9.3 In-Fusion® reaction and transformation of competent cells

The *Sall* digested vector plasmid was used to carry out the In-Fusion® cloning protocol with the purified PCR product. Following the cloning protocol, Stbl3 competent *E.coli* cells were transformed with the cloning reaction and both positive and negative In-Fusion controls. LB agar are inoculated with transformed cells. The positive and
negative In-Fusion® controls produced expected results; with the positive control producing too many colonies to count, while the negative control had less than 10 colonies on the plate.

3.9.4 Screening for positive colonies

Colonies from the plate of In-Fusion® cloned plasmid were picked and inoculated onto a strike plate and used for colony PCR. Eight colonies from the plate of transformants were chosen for colony PCR using the selected primers (2.3.1). The In-Fusion cloning reaction was also screened with the colony PCR primers to verify that the cloning reaction was successful. Successful In-Fusion cloning will allow amplification through both primers which would produce a 213 bp fragment.

Colony PCR products from all 8 colonies and the In-Fusion® cloning reaction were electrophoresed on a 1% agarose gel; shown below.
Successful colony PCR would produce an approximately 200 bp fragment. The In-fusion cloning reaction has a clear band of the right size; indicating that the cloning was successful. Colonies 2 to 8 show signs of being positive of successful In-Fusion cloning.

**3.9.5 Sequencing and alignment**

Colony 2 and 3 were selected and mini-prepped for DNA isolation, to be sent for sequencing as verification of successful cloning. The returned sequences were uploaded onto Geneious and aligned with the expected pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry plasmid. The sequence alignment (Figure 3.39) for colony 2 indicated that it had been successfully cloned using In-Fusion primers.
Figure 3.39 – Alignment of sequencing results against expected plasmid

Sequencing results from GAS for colony 2 aligned against the sequence for pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry; green peaks show 100% consensus in alignment, which is observed through the majority of the good quality sequencing result of colony 2.

The glycerol stock of colony 2 was then used to midi-prep for higher yield DNA isolation to be used in virus packaging.

3.10 Virus production

3.10.1 Testing in primary neurons by immunocytochemistry

Upon completion of lentiviral packaging and production, the LV-pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry was tested in vitro on primary neurons. Two volumes (0.5 μL and 2 μL) of virus was administered and allowed to incubate overnight before fixing and ICC was carried out with anti-GFP.
Figure 3.40 – Primary neurons show co-localisation of GFP and mCherry from 0.5 μL of LV transduction

A-B: one frame of cells showing GFP and mCherry (respectively); C: overlay of A and B.
D-E: second frame of cells showing the same respective reporter genes; F: overlay of D and E.
C and F shows co-localisation of GFP and mCherry, indicating successful expression by the viral construct with GFP-sAPPα and mCherry. Scale bar 200 μm.

A second volume, increased to 2 μL, was also tested to examine if it would improve expression by increasing the number of viral particles in the media.
Figure 3.41 – Primary neurons show co-localisation of GFP and mCherry from 2 μL of LV transduction
A-B: one frame of cells showing GFP and mCherry (respectively); C: overlay of A and B.
D-E: second frame of cells showing the same respective reporter genes; F: overlay of D and E.
C and F shows co-localisation of GFP and mCherry, indicating successful expression by the viral construct with GFP-sAPPα and mCherry. Scale bar 200 μm.

The increased volume delivered (Figure 3.41) did not have a significant effect on the transduction efficiency of the virus (Figure 3.40); mCherry was still well-expressed and clearly localised to cells but GFP expression was still weak and had to be enhanced by ICC to be easily observed.

3.11 Spread of sAPPα from lentivirus-mediated transduced cells in the brain
It was decided to use the LV-pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry in vivo and inject 1 μL in the same manner as described for other stereotaxic surgeries.
Figure 3.42 – Detected fluorescence in LV-pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry injected mouse brain
A: Anti-GFP stained section with some visualised fluorescence; B: possible mCherry reporter gene expression; C: fluorescence co-localises

All four animals injected with this virus failed to show any sign of fluorescence; of either GFP or mCherry. Even with anti-GFP IHC carried out on tissue sections, fluorescence was only observed in the section shown in Figure 3.42 which suggests unsuccessful viral expression, and potentially a combination of auto-fluorescence and non-specific antibody binding from the IHC protocol.
Chapter 4: Discussion

4.1 Summary

The primary aim of this study was successfully achieved; viral vector spread between the AAV9 vector and rabies B19 pseudotyped LV vector was compared. AAV9 was found to be able to achieve significantly more widespread distribution compared to the LV. Distance of spread achieved by undiluted AAV9, a 1:10 diluted titre of AAV9 and AAV with mannitol pre-treatment was not significantly different. LV with mannitol pre-treatment was unsuccessful as no GFP expression was observable.

The pCDH-pSyn-SP-GFP-sAPPα-T2A-mCherry plasmid was packaged into a LV construct and tested successfully in vitro on primary neurons. In vivo use of the virus did not express either reporter genes.

4.2 Viral vector transduction efficiency

4.2.1 LV vs. AAV9

LV mediated transduction was shown to be localised to the immediate region of the injection. Distance of spread was approximately a millimetre either side (anterior-posterior) of the injection site. Retrograde transport to distal regions such as the entorhinal or association cortices was not observed. Retrograde transport to the subiculum in some mice hippocampi were faintly observable.

At the undiluted higher titre of AAV9, GFP expression was widespread and observed through the length of the entire brain; 1 cm of spread from cerebellum to the olfactory nuclei. Such a high level of GFP expression throughout each section meant that if any effect of mannitol pre-treatment were to be observed, a lower ‘baseline’ for an AAV9 spread would be required. Therefore, it was decided that another set of injections
would be carried out with a 1:10 diluted AAV9 titre so as to provide a comparable baseline for the mannitol pre-treatment.

The highly widespread distribution of the AAV9 vectors was noteworthy as a single unilateral injection at a higher viral titre was able to achieve transduction of a significant proportion of the cerebral volume. When using a LV vector, this was only possible with multiple injection sites in both hemispheres of the brain (as was done by NDD lab members; Fairuz Yahya and Valerie Tan, in unpublished PhD research). Their work used lentiviral mediated introduction and over-expression of sAPPα to either prevent or rescue the effects in transgenic AD mice. The studies have used a LV vector to deliver sAPPα with a separate GFP molecule to tag transduced cells. The APP/PSEN1 double mutant tg mice (1.2.2) were used. A total of 4 injections at 2 depths each (8 sites in total) into both the left and right hippocampus were used to achieve high level of transgene expression. LV administration was carried on groups of mice before and after AD-like symptoms develop to determine if sAPPα could prevent and/or rescue AD-like pathology.

The 1:10 diluted AAV9 injections performed just as well in terms of distance of spread. When area of fluorescence was quantified, the distribution of GFP expression was shown to have a very slightly narrower peak than that of the undiluted AAV9 group. It was also visually observable that fewer cells had been transduced, due to the presence of fewer viral particles in the same volume.

Furthermore, another aspect in which the AAV9 vectors outperformed the LV was the distal regions and structures that were GFP expressing from retrograde transport. Regions such as the entorhinal and association cortices were shown to be strongly transduced; the ability of the AAV9 vector to produce retrograde spread to these
structures both validates the suitability of AAV9 as a gene therapy vector in the CNS and the CA1 region as a good target for AD and other cognitive diseases.

Furthermore, the distance of GFP expression spread with AAV9 was shown to be approximately 1 cm, however that was only examining expression with in the brain. All AAV9 transduced brains showed signs of the pyramidal tract being strongly GFP expressing in the medulla (the spread of transgene expression is reliant on transport along neuronal projections and processes and so, a one centimetre spread in the mouse brain is translated to a relatively greater distance in the human CNS). The pyramidal tract conducts motor neurons and impulses from the brain to the spinal cord; this means that it was likely that AAV9 mediated transduction extends to farther regions of the CNS (spinal cords of all AAV9 injected mice were fixed and stored; and this may be confirmed in future).

AAV9 had already been shown to efficiently transduce CNS cells (Dayton et al., 2012) through other routes of administration, such as intravenous and systemic delivery (Manfredsson et al., 2009) but they did not achieve widespread transduction of the CNS through non-intracranial routes. This has implications for CNS diseases such as motor neuron disorders which will require widespread delivery of therapeutics throughout the CNS.

Reducing injection sites by having a viral vector with a much greater transduction efficiency would also improve behavioural tests conducted on affected animals delivered with a therapeutic. Especially when targeting the hippocampus which is the primary area of interest in AD and responsible for memory and learning. Multiple injections into the hippocampus to deliver a therapeutic transgene carrying viral vector may achieve the desired spread and delivery of the therapeutic, however, it would
serve little purpose if the trauma from multiple descending needles had damaged the hippocampus significantly to render results from behavioural studies unusable.

However, while not achieving the same level of transduction efficiency as the AAV9 vector, LV can still be used to deliver therapeutics within the CNS for disorders with localised affected neurocircuitry, such as targeting of the substantia nigra for treating Parkinson’s Disease (Fearnley & Lees, 1991).

This is relevant in terms of translating viral vector mediated gene therapy for human use in the future; where vector choice and administration routes into a patient would determine the safety and desirability of such a procedure.

4.2.2 Effect of 25% w/v mannitol

LV injections of mice, pre-treated with mannitol, failed to produce any GFP expression. The effect of mannitol on LV titre, and resulting transduction efficiency was tested in vitro. However, neither simultaneous nor sequential addition of mannitol followed by LV (mimicking the process in vivo), produced any significant changes to the number of transduced cells expressing GFP.

Diluted AAV9 with mannitol pre-treatment resulted in significantly improved distance of spread compared to LV; but not significantly different from either undiluted or diluted AAV9, which ruled out a mannitol effect on improved AAV9 versus LV spread. However, of note in the AAV9 with mannitol pre-treatment, was the lack of hippocampal expression of GFP. There were a few possible reasons; firstly human error (misidentifying bregma, misreading the stereotaxic frame coordinates, mistake in reading dorsal-ventral depth coordinates etc) during surgery resulting in either a shallow injection site or missing the hippocampus entirely. It seemed unlikely that it
would happen in four consecutive animals, especially after numerous successful surgeries on previous occasions. Furthermore, apart from the absence of GFP within the hippocampus, the pattern of expression closely matches that of the diluted AAV9 group; this suggests that distal expression of GFP arose from retrograde spread from a CA1 hippocampal administration of AAV9.

It is possible that both mannitol and AAV9 introduced together in mice might trigger an immune response which localised to the area of trauma (ie. injection site), preventing transgene expression and/or accumulation within the region.

4.2.3 Future use of mannitol with viral vectors

Mannitol as an osmotic agent is still useful as a means of opening intercellular space for dispersal of transport of therapeutics to and from an area. It might be worth considering administering mannitol after viral injection; this would give time for expression of the transgene and in the case of sAPPα, secretion into the extracellular space. Mannitol administration at this point would allow specific spread and dispersal of any secreted therapeutic. This would allow for much more specific targeting of transgene expression (especially if combined with using cell-specific promoters) with subsequent dispersal of the therapeutic.

Furthermore, it was recently reported that transient opening of tight junctions and the blood-brain barrier (similar actions of mannitol) with scanning ultrasound allowed recruitment of macrophages which reduced plaque formation and improved memory (Leinenga & Götz, 2015).
4.2.4 Visualising sAPPα in vivo

Unfortunately despite producing a working plasmid and virus which showed expression of both reporter (mCherry and GFP) genes in vitro, transgene expression was not visible in vivo across any of the four animals. There may have been insufficient time for sAPPα to express and/or accumulate (if sAPPα accumulates at all) to be visualised. sAPPα may have a short half-life, or its function involves a rapid turnover of the molecule. However, none of these reasons explain the absence of mCherry in the brains; mCherry was very strongly expressed when testing the virus in primary neurons after a 24 hour incubation period.

4.3 Limitations of techniques

4.3.1 Analysis of brain tissue

Fluorescence microscopy of the sections provided a simple way of visualising reporter gene expression however because only every sixth section was being imaged, it does mean that a large proportion of the transduction distribution is simply extrapolated from the imaged sections. Three-dimensional imaging may provide a better (or overall time-saving) method to visualise structures in the brain, especially fluorescently labelled cells. A method known as “clear lipid-exchanged, acrylamide-hybridised rigid, imaging/immunostaining tissue hydrogel” (CLARITY; Chung & Deisseroth, 2013), which replaces the lipids of the brain with a clear acrylamide to enable visualisation of proteins and structures of the brain while fixed ‘in situ’. The actual method of conducting CLARITY is not the biggest challenge; rather finding an adequate and cost-effective 3D imaging platform.
Another method of imaging, confocal microscopy would have to be carried out, in future, especially on IHC stained sections to improve visualisation of co-localisation of differentially labelled cells.

4.3.2 Statistical analysis

A direct comparison was not made with each treatment group due to personal limitations with the statistical program RStudio. The statistical code used for scaling and presenting the data was written in full by Dr Michael Black. However the data gathered had off-target injections which skewed certain plots, namely the mean trendline for the LV treatment group.

Improvements to the data analysis could be carried out with the area of fluorescence regrouped against the largest area (expected injection site) as the ‘zeroed’ coordinate (instead of against bregma; to account for off-target injections) to produce the trendline.

4.4 Conclusion and Future directions

This project has shown the marked improvement in transduction efficiency from using AAV9 vector to deliver a reporter gene. It efficiently achieves widespread transduction of the CNS from a unilateral injection at the hippocampus. However, AAV has a limited transgene capacity (4-5 kb), about half that of LV vectors. However, while LV has a limited spread, it would be useful for targeted delivery of treatment.

At the moment, AD research in the NDD lab is looking to utilise AAV9 as a vector to deliver truncated regions of the sAPPα peptides which retain the neuroprotective properties of the full-length protein.
While mannitol did not have any observable positive effect, future attempts can be made to determine if it does have a definitive negative effect on viral vectors; or utilising timepoints post-injection to administer the mannitol for improving secreted therapeutic spread rather than directly enhancing the spread of viral vectors.
References


Hasselmo, M. E. (2005). The role of hippocampal regions CA3 and CA1 in matching entorhinal input with retrieval of associations between objects and context: theoretical comment on Lee et al.(2005).


# Appendix A: Details of treatment groups in chronological order

<table>
<thead>
<tr>
<th>Group ID</th>
<th>Animal no.</th>
<th>Animal ID</th>
<th>Vector</th>
<th>Duration</th>
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<tbody>
<tr>
<td>1_30Apr</td>
<td>3</td>
<td>5081</td>
<td>LV</td>
<td>7 days</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5082</td>
<td>LV</td>
<td>31 days</td>
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<tr>
<td></td>
<td></td>
<td>5083</td>
<td>No virus control</td>
<td>7 days</td>
</tr>
<tr>
<td>2_5Jul</td>
<td>3</td>
<td>IC001 – IC003</td>
<td>LV</td>
<td>14 days</td>
</tr>
<tr>
<td>3_8Jul</td>
<td>3</td>
<td>IC004 – IC006</td>
<td>LV</td>
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</tr>
<tr>
<td>4_12Dec</td>
<td>3</td>
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<td>5% w/v dextran (MW 10,000)</td>
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<td>4</td>
<td>IC010 – IC013</td>
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<td>28 days</td>
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<tr>
<td>5.1_Jan</td>
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<td>IC014 – IC017</td>
<td>AAV9</td>
<td>28 days</td>
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<tr>
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<td>IC018</td>
<td>5% w/v dextran (MW 10,000)</td>
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<td>4</td>
<td>IC019 – IC022</td>
<td>LV +/mannitol</td>
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<tr>
<td>7.1_May</td>
<td>4</td>
<td>IC023 – IC026</td>
<td>1:10 AAV9 +/mannitol</td>
<td>28 days</td>
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<tr>
<td>8_July</td>
<td>4</td>
<td>IC027 – IC030</td>
<td>LV +/mannitol</td>
<td>28 days</td>
</tr>
<tr>
<td>8.1_July</td>
<td>4</td>
<td>IC031 – IC034</td>
<td>1:10 AAV9</td>
<td>28 days</td>
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<tr>
<td>9_Dec</td>
<td>4</td>
<td>IC035 – IC038</td>
<td>LV [GFP-sAPPα-T2A-mCherry]</td>
<td>28 days</td>
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### Appendix B: RVM drug concentrations and administration dosage

<table>
<thead>
<tr>
<th>Drug</th>
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<th>Vol. to weight</th>
<th>Supplier</th>
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<tr>
<td>Ketamine</td>
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<td>10 mg/ml</td>
<td>4μL/g</td>
<td>Parnell Laboratories NZ Ltd.</td>
</tr>
<tr>
<td>Domitor</td>
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<td>0.1 mg/ml</td>
<td>2.5μL/g</td>
<td>Novartis NZ Ltd.</td>
</tr>
<tr>
<td>Atropine</td>
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<td>0.0065 mg/ml</td>
<td>7.7μL/g</td>
<td>Baxter Healthcare Ltd.</td>
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<tr>
<td>KDA</td>
<td></td>
<td></td>
<td>14.2μL/g</td>
<td></td>
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<td>8μL/g</td>
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<td>0.5 mg/ml</td>
<td>2.5μL/g</td>
<td>Zoetis NZ Ltd</td>
</tr>
</tbody>
</table>
Appendix C: ImageJ functions used

Area of fluorescence processes
Open image

Image > Type > 8-bit

Edit > Invert

Image > Adjust > Threshold [settings: adjust visually; exclude on edge]

ImageJ – cell counting
Open image

Process > subtract background [settings: 50.0 pixels; no selection]

Image > adjust > threshold [settings: B&W; adjust as required]

Image > type > 8-bit

Process > binary > fill holes

Process > binary > convert to mask

Process > binary > watershed

Analyse > analyse particles [settings: 1000-infinity; exclude on edge]
Appendix D: Solutions and Buffers

Solutions for Gel Electrophoresis

25x TAE Buffer

- 800 mL dH2O
- 57.1 mL Glacial acetic acid
- 100 mL EDTA 0.5 M
- 242 g Tris

Mix and make up to 1000 mL with dH2O

1x TAE Buffer

- 40 mL TAE buffer 25x
- 960 mL dH2O

1% Agarose

- 3g Agarose Ultra-Pure Powder
- 300 mL TAE 1x

Microwave to dissolve agarose

- 5 μL of ethidium bromide to every 50 mL of agarose per gel casting

Solutions for tissue fixing and mounting

4% Paraformaldehyde

- 40 g paraformaldehyde (PFA)
- 500 mL 0.1 M PB

Stir and warm to 60°C until the PFA has dissolved

Fill to 1 L with 0.1 M PB

Filter and pH to 7.4
0.1M PB

Solution A

27.6 g sodium phosphate monobasic (NaH2PO4H2O)
Add 1 L dH2O

Solution B

53.56 g sodium phosphate dibasic (Na2HPO47H2O)
Add 1 L dH2O

Mix 280 mL of solution A with 720 mL of solution B
Dilute with dH2O to a volume of 2 L
pH to 7.2 by adding more solution A or solution B

Cryoprotectant

400 mL 0.1M PB
300 g Sucrose
300 mL Ethylene glycol

Antifade

20 mL of 0.1M PB
1.1 g of p-phenylenediamine dihydrochloride (PPD)
80 mL glycerol

Solutions for Immunolabelling

1x PBS

1 L 0.1M PB
9 g of sodium chloride (NaCl)

1x PBS + 0.2% Triton

500 mL of 1x PBS

1 mL of Triton X-100

Blocking solution

97 mL of 1x PBS-T + 0.2% Triton

3 mL of normal goat serum (NGS)

Primary or secondary antibody

X μL of primary antibody (depending on concentration required)

Make up to 1 mL with blocking solution