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Neuroprotective potential of AAV-mediated NURR1 expression in a rat model of Parkinson’s disease

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Science, The University of Auckland, 2015.
Abstract

Parkinson’s disease (PD) is characterised by the progressive neurodegeneration of the nigrostriatal dopaminergic neurons that results in motor deficits including bradykinesia, rigidity and tremor. Currently available pharmacological therapies for PD provide only symptomatic relief, and hence lead to therapeutic failure in the advanced stages of the disease. Gene therapy that targets the underlying disease mechanisms is an appealing alternative strategy to ameliorate disease progression. Adeno-associated viral (AAV) vectors are currently the preferred gene delivery vehicles in clinical gene therapy applications. To date, clinically-approved AAV vectors for CNS gene therapy almost exclusively target neurons, and astrocytes that regulate CNS physiology and contribute to disease pathogenesis represent a largely unexplored therapeutic target. Therefore the aims of this thesis were to develop an AAV vector that selectively and efficiently target nigral astrocytes by coupling astrocyte-tropic AAV serotypes and DNA promoter elements, and subsequently utilise this vector to overexpress the therapeutic transcription factor NURR1 in a rat model of PD.

Recent evidence suggests that astrocytic molecular diversity may heterogeneously regulate transcriptional activity of the commonly used astrocyte-specific glial-fibrillary acidic protein (GFAP) promoter, prompting us to isolate putative promoter sequences from the recently characterised pan-astrocytic marker aldehyde dehydrogenase family 1, member L1 (ALDH1L1), to determine the ability of these promoters to efficiently regulate transgene expression in astrocytes. Four promoter sequences were generated and tested in the context of the astrocyte tropic AAV serotypes 5, 8 and 9 in the rat substantia nigra pars compacts (SNpc). Unexpectedly, these promoters mediated exclusive and efficient neuronal transgene expression, indicating the potential applicability of these promoters in neuronal-targeted gene therapy.

In a subsequent comparative analysis of AAV5 and AAV9, AAV5 that predominantly targeted astrocytes in the nigra with minimal dissemination of transgene expression to neighbouring midbrain regions was selected for further studies. Moreover, comparative analysis of the promoters, constitutively active cytomegalovirus (CMV), GFAP and shorter GFAP variant gfaABC1D in the context of AAV5 indicated that the GFAP promoter mediated the most efficient transgene expression predominantly in nigral astrocytes. Therefore, the GFAP promoter in the context of AAV5 was selected to mediate efficient
transgene expression in astrocytes. Using this vector construct, the multifaceted transcription factor NURR1 that regulates midbrain DA neuronal phenotype, and downregulates glia-driven inflammation was selectively expressed in astrocytes to determine the neuroprotective potential of astrocyte-targeted gene therapy. NURR1 overexpression promoted nigral DA neuronal survival, ameliorated astrocyte reactivity, and improved contralateral forepaw deficit, indicating therapeutic potential of astrocyte-targeted gene transfer. These results support the development of a dual cell targeting AAV vector in future studies for the expression of NURR1 in both neurons and astrocytes to target DA phenotypic dysfunction, chronic inflammation and neurodegeneration in PD.
Acknowledgements

Thank you to my principal supervisor Associate Professor Deborah Young for the opportunity to undertake this PhD. I am grateful for your guidance that has broadened my scientific thinking ‘outside the box’.

Thank you to my co-supervisor, Dr Alexander Mouravlev, the “cloning guru”, for contributing your expertise in molecular biology to this thesis, and willingly sharing your many cloning secrets.

I am immensely grateful to Dr Dahna Fong for being the ‘unofficial’ scientific advisor for this thesis, and contributing to its success.

It has been a privilege sharing this experience with my friends and fellow graduate students Jerusha Naidoo, Angela Wu, Tanya Wagner, Katharine Zhang, and Thomas Chen.

Jerusha and Angela, your friendship and support, especially in the last year of this PhD helped me through the many obstacles.

Thank you to Teckla Perera and Ernest Sirimanne for your help with the technical aspects of this thesis.

Thank you to Ms Daniela Von Heiber, the behaviour unit manager at the University of Auckland, for your help and advice in setting up the animal studies in this thesis in a timely manner.

Thank you to my parents, and siblings for your unwavering love and support. Thank you for being my personal cheer squad, sharing my victories and many failures, and constantly reassuring me that you are more than happy to support me through this PhD, and all other life decisions. I owe you a lifetime of gratitude.

This has been an immensely challenging and equally rewarding journey, a journey that has moulded me into a scientist, inspired me, taught me vital life skills, and given me lifelong friendships. I feel privileged to have been given the opportunity to be able to make a meaningful contribution to society in the coming years.
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Chapter 1. General Introduction
1.1. Outline

Astrocytes, traditionally considered to be the ‘glue’ that provided a structural scaffolding for neuronal networks are indeed active participants of synapses. It is increasingly evident that dynamic interactions between neurons and astrocytes regulate central nervous system (CNS) physiology, and detrimental phenotypic evolution of these cellular elements contributes to neurodegeneration in various neurodegenerative diseases. Currently, the available pharmacological and surgical therapies for neurodegenerative diseases such as Parkinson’s disease (PD) only offer short-term symptomatic relief. Genetic manipulation that targets the underlying pathological mechanisms in disease is a promising alternative strategy. Gene therapy based on the use of adeno-associated viral (AAV) vector systems to mediate transfer of a therapeutic gene demonstrates efficient transduction and disease-modifying potential in the CNS in the absence of adverse cytotoxicity. To date, clinically-approved AAV vectors for CNS gene therapy almost exclusively target neurons, and astrocytes represent a largely unexplored therapeutic target in the clinical setting.

Using PD as a prototype neurodegenerative disease, the aims of this thesis are to develop an AAV vector that selectively and efficiently targets astrocytes in the substantia nigra, and subsequently use this vector to overexpress the therapeutic transcription factor NURR1 in a 6-OHDA model of PD.

This introductory chapter will outline the functional significance of astrocytes in health, disease pathology and astrocytic mechanisms in the context of PD, astrocyte-targeted AAV vectors, and the novel therapeutic molecule NURR1.

1.2. Astrocytes in the central nervous system

Astrocytes, oligodendrocytes, microglia and ependymal glia constitute the morphologically and functionally diverse glial population in the CNS (Reichenbach & Wolburg, 2004). Astrocytes account for nearly one half of the cells in the human brain. Michael von Lenhossek coined the term astrocyte in 1893 to describe the ‘star-like’ cells in histological brain specimen (Figure 1-1). Since the late nineteenth century, it has been recognised that astrocytes are morphologically and biochemically heterogeneous (Miller & Raff, 1984; Reichenbach & Wolburg, 2004). The relative number of astrocytes increases considerably
with phylogeny and brain complexity. Neurons outnumber glia by 6:1 in *Caenorhabditis elegans* (Sulston, Schierenberg, White, & Thomson, 1983). In the cortex of lower mammals such as rats and mice, neuron to astrocyte ratio is 3:1, whereas in the human cortex there are approximately 1.4 astrocytes for every neuron (Norman, Helen, Alfred, & Caroline, 1971), suggesting that evolutionary pressure may have dictated a greater abundance of astrocytes for regulating complex synaptic networks in the human brain.

![Image of astrocyte morphology](image)

**Figure 1-1** ‘Star-like’ morphology of a GFAP-immunoreactive astrocyte in the rat brain

scale bar: 50µm

### 1.2.1. Astrocytes are organised into synaptic domains

Classic techniques predominantly based on immunostaining for the astrocyte-specific glial fibrillary acidic protein (GFAP) and metal-impregnation suggested that astrocytes and their extensively intermingled processes represented a structural framework for neuronal circuits (Distler, Dreher, & Stone, 1991; Eng, Vanderhaeghen, Bignami, & Gerstl, 1971; Rohlmann & Wolff, 1996). Analysis of fluorescent dye-filled astrocytes using advanced laser confocal and electron microscopy reveal that the extent of GFAP immunoreactivity is ~15% of the astrocytic volume, and the true astrocytic morphology indeed exhibits greater complexity with a profusion of distal processes that ramify the territory of astrocytes (Bushong, Martone, Jones, & Ellisman, 2002; Ogata & Kosaka, 2002).

Furthermore, recent studies show that astrocytes are organised into elaborate synaptic domains and it increasingly evident that astrocytic functional versatility may depend on this ordered architecture. Three dimensional reconstruction of astrocytes from rat or mouse brain
tissue revealed that protoplasmic astrocytes in the hippocampus and cortex are organised into non-overlapping spatial domains with limited interdigitation accounting for ~5% of the volume of an astrocyte, occurring only between the peripheral processes of adjacent astrocytes (Bushong et al., 2002; Halassa, Fellin, & Haydon, 2007; Halassa, Fellin, Takano, Dong, & Haydon, 2007; Ogata & Kosaka, 2002). It is estimated that a rodent astrocytic domain may envelop ~20,000 to 120,000 synapses, whereas a human astrocyte may contact ~270,000 to 2 million synapses (DeFelipe, Alonso-Nanclares, & Arellano, 2002; Oberheim et al., 2009). Moreover, almost all human protoplasmic astrocytes appear to contact vasculature, either through extension of long processes, or capillaries within its domain (Oberheim et al., 2009). These astrocytic end-feet processes extensively interdigitate and overlap to form a continuous vascular sheath (Kacem, Lacombe, Seylaz, & Bonvento, 1998; Mathiisen, Lehre, Danbolt, & Ottersen, 2010) that may play an important role in maintaining CNS homeostasis, and neurovascular and neurometabolic coupling as described in section 1.2.3.

1.2.2. Reciprocal neuronal-astrocyte communication regulates synapses

It is increasingly evident that contrary to the classic neurocentric view on synaptic activity, astrocytes are also integral participants of synapses. Unlike neurons, astrocytes are electrically non-excitable, and maintain their resting membrane potential at ~-85mV which only slightly fluctuates in response to stimuli (Kang, Jiang, Goldman, & Nedergaard, 1998). Thus, it was widely accepted that astrocytes were passive elements that nurture neurons and regulate CNS homeostasis.

Accumulating evidence now supports the existence of tripartite synapses, where reciprocal communication between neurons and astrocytes via a rich repertoire of signalling molecules and receptors regulate synaptic activity (Porter & McCarthy, 1997) (Figure 1-2). *In vitro* and *in vivo* studies implicate intracellular Ca$^{2+}$ signalling as the mechanism via which astrocytes integrate and modulate synaptic activity. Various neurotransmitters such as glutamate, ATP, GABA, serotonin, and nitric oxide released during synaptic activity have been shown to induce intracellular Ca$^{2+}$ elevations (Araque, Martin, Perea, Arellano, & Buno, 2002; Butt & Jason, 1994; Francisco, Alberto, & Carmen, 1995; Jalonen et al., 1997; Jourdain et al., 2007; Navarrete et al., 2012; Porter & McCarthy, 1996; Salter & Hicks, 1995). The ensuing Ca$^{2+}$-dependent release of a myriad of gliotransmitters such as glutamate, ATP, GABA, adenosine,
TNFα, and prostaglandins in turn modulate synaptic activity and plasticity (Evgeny & Leonard, 2008; Kang et al., 1998; Min, Melyan, & Kullmann, 1999; Stellwagen & Malenka, 2006). For example, in response to neuronal firing, Ca^{2+}-dependent release of astrocytic glutamate activates NR2B containing presynaptic NMDA receptors, leading to increased neurotransmission that facilitates synaptic potentiation (Jourdain et al., 2007).

Given that human astrocytes exhibit greater morphological complexity and heterogeneity than rodent astrocytes, much more intricate reciprocal neuron-astrocyte communication may occur in the human CNS (Oberheim et al., 2009). In the human neocortex, protoplasmic astrocytes are 2.6-fold larger in diameter and extend 10-fold more GFAP-immunoreactive primary processes than their rodent counterparts. In hippocampal and cortical slice cultures prepared from biopsies from drug-resistant TLE patients, in response to electrical stimulation of proximate neuronal axons or ATP-mediated activation of astrocytic purinergic receptors induced intracellular Ca^{2+} oscillations in astrocytes that were significantly more rapid than that exhibited by rodent astrocytes, suggesting that greater structural and physiological complexity may allow human astrocytes increased functional competence. Furthermore, glutamate released from stimulated astrocytes regulated excitability of neurons via postsynaptic NMDA receptor-dependent slow-inward currents, providing evidence for the existence of bidirectional communication between astrocytes and neurons in the human brain (Navarrete et al., 2012). Although extrapolating molecular mechanisms characterised in pathological specimen to the healthy CNS may not necessarily be accurate, given the morphological and functional alterations related to disease and any pharmacological interventions, the revelation that astrocytes are integral synaptic elements has important implications in the development of disease modifying therapeutic strategies as discussed in this thesis.

### 1.2.3. Astrocytes regulate CNS homeostasis and synaptic activity

Astrocytes play a vital role in maintaining CNS homeostasis and activity. Aquaporin 4 (AQ4) water channels predominantly expressed in astrocytic end-feet are vital molecular elements of the blood-brain barrier (BBB) that regulate brain water homeostasis (Satoh, Tabunoki, Yamamura, Arima, & Konno, 2007). Astrocytes express various ion transporters that regulate ion homeostasis essential for functional integrity of the CNS. For example, neuronal excitability is regulated by astrocytic Na^+/K^+ ATPase and inward-rectifying K^+
channels that maintain extracellular K\textsuperscript{+} (Howe, Feig, Osting, & Haberly, 2008; Newman, 1993) and the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger and Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter regulate intracellular Ca\textsuperscript{2+}-dependent gliotransmission (Girouard et al., 2010; Zorec et al., 2012). The two astrocyte-specific excitatory amino acid transporter 1 and 2 (EAAT1 and EAAT2) (GLAST and GLT-1, respectively in rodents) are primarily responsible for maintaining extracellular glutamate homeostasis (Lehre, Levy, Ottersen, Storm-Mathisen, & Danbolt, 1995; Milton et al., 1997), particularly EAAT2 that accounts for approximately 80% of glutamate clearance in the brain (Furness et al., 2008; Rothstein et al., 1996). Deficits in astrocytic glutamate transport that may promote neuronal excitotoxicity are characteristic of various neurodegenerative diseases (Lin, Kong, Cuny, & Glicksman, 2012). Supported by \textit{in vitro} studies and mathematical modelling of cerebral metabolism, it is hypothesised that neurons and astrocytes are metabolically coupled, a cooperation termed the astrocyte-neuron lactate shuttle, whereby astrocytes regulate glucose utilisation and neuronal delivery of energy metabolites in a manner dependent on synaptic activity (Bélanger, Allaman, & Magistretti, 2011; Morgello, Uson, Schwartz, & Haber, 1995; Pellerin & Magistretti, 1994; Tarczyluk et al., 2013). However, further characterisation of this pathway is required, as recent \textit{in vivo} studies suggest that direct neuronal utilisation of glucose may sustain synaptic activity (Lundgaard et al., 2015; Patel et al., 2014). Furthermore, in response to synaptic activity, Ca\textsuperscript{2+}-dependent release of vasoactive gliotransmitters such as prostaglandins, nitric oxide (NO), and arachidonic acid dynamically regulate vasodilation and blood flow (Schummers, Yu, & Sur, 2008; Takano et al., 2006; Zonta et al., 2003), and thereby astrocytes effectively couple synaptic activity and cerebral blood flow.

ROS homeostasis in the CNS is maintained by vital antioxidant mechanisms the include superoxide dismutase (SOD), catalase and glutathione peroxidase (Dringen, 2000). However, the activity of these enzymes in the brain is lower in comparison to that in the liver and kidney (Jenner, 2003), and hence detrimental perturbations in ROS generation or activity of antioxidant processes may disrupt ROS homeostasis leading to excessive oxidative stress that contribute to neurodegeneration. Compared to astrocytes, neurons exhibit weak intrinsic antioxidant mechanisms, and succumb rapidly to oxidative stress (Bolaños et al., 1997). The glutathione system, predominantly expressed in astrocytes, plays a crucial role in ROS detoxification in neurons via the ‘glutathione shuttle’ (Dringen, 2000; Dringen, Pfeiffer, & Hamprecht, 1999; Sagara, Miura, & Bannai, 1993; Sun et al., 2006). A recent study elucidated the mechanisms governing the exquisite metabolic coupling between astrocytes
and neurons that regulate neuronal antioxidant protection in a manner dependent on synaptic activity (Jimenez-Blasco, Santofimia-Castano, Gonzalez, Almeida, & Bolanos, 2015). Astrocytes stably express the master antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), while its constitutive destabilisation in neurons may account for the inefficient neuronal antioxidant defence (Jimenez-Blasco et al., 2015). During neuronal firing, synaptically released glutamate stimulates astrocytic NMDA receptors, leading to the expression of Nrf2-dependent antioxidant genes. While NMDA receptor stimulation in pure neuronal cultures had no effect on cellular glutathione, astrocytic NMDA receptor stimulation in neuronal-astrocyte co-cultures significantly increased levels of neuronal glutathione and attenuated excitotoxicity induced by excessive glutamate. These results suggest that neuronal regulation of the antioxidant capacity of astrocytes sustains the astrocyte-derived neuroprotection during high synaptic activity.

Furthermore, astrocytes express a range of neurotrophic and growth factors such as glial derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and activity-dependent neurotrophic factor (ADNF) that are critical for neuronal development, differentiation and survival (Baquet, Bickford, & Jones, 2005; Dougherty, Dreyfus, & Black, 2000; Furman et al., 2004; Pyka, Busse, Seidenbecher, Gundelfinger, & Faissner, 2011; Roussa & Krieglstein, 2004; Schaar, Sieber, Dreyfus, & Black, 1993; Winter, Saotome, Levison, & Hirsh, 1995; Zafra, Lindholm, Castren, Hartikka, & Thoenen, 1992). In response to pathological stimuli, in the ensuing acute inflammatory phase, enhanced expression of neurotrophic factors in reactive astrocytes has been shown to promote neuronal survival and regeneration (Dougherty et al., 2000; Iravani, Sadeghian, Leung, Jenner, & Rose, 2012; Sendtner, Kreutzberg, & Thoenen, 1990; Winter et al., 1995).

1.3. Neuroinflammation and reactive astrogliosis

Neuroinflammatory defence mechanisms in response to pathologies such as toxins, trauma, pathogens, and disease, were traditionally considered to be exclusively regulated by glia, the ‘resident’ immune cells of the CNS, and infiltrating leukocytes. In addition to directing CNS physiological processes, it is increasingly evident that dynamic interactions between neurons and glia orchestrate defence mechanisms against CNS insults of all forms and severities, which may range from subtle disturbances in tissue homeostasis to chronic pathologies such
as neurodegenerative disease. The myriad of molecular mediators of neuroinflammation released from neurons and glia include neurotransmitters, gliotransmitters, cytokines, chemokines, reactive oxygen and nitrogen species, and growth factors. While some of these factors exert neuroprotective and trophic effects that promote synaptic function and survival, others may simultaneously enhance detrimental pathways that contribute to neuronal death [reviewed in (Xanthos & Sandkühler, 2014)]; hence, the prevailing effect may depend on the context, and temporal progression of neuroinflammation. In the context of unresolved neuropathology, maladaptive phenotypic evolution of the cellular elements may lead to pathological neuroinflammation that contribute to neurodegeneration. Given that there is no unequivocal discrimination between homeostatic, physiological and pathological responses coordinated by interacting neurons and glia, an updated definition of neuroinflammation that encompasses all of these responses has been proposed (Xanthos & Sandkühler, 2014). For the purpose of this thesis, the role of astrocytes in modulating synaptic function and survival in neurodegeneration will be the main focus; description of microglial function in disease will be restricted to astrocyte-microglia interactions, and the potential myelin-independent mechanisms of oligodendrocytes in CNS physiology and pathology are not elaborated.

The molecular and functional alterations undergone by astrocytes in response to CNS perturbations are called reactive astrogliosis. The role of reactive astrocyte in neurodegeneration in the context of PD will be discussed in this thesis. Although PD is a complex multi-system neurodegenerative disorder involving both genetic and environmental factors (Lang & Obeso, 2004), the predominant vulnerability of the nigro-striatal dopaminergic (DA) pathway, and the availability of a range of disease models that replicate various aspects of disease pathology allow researchers to elucidate pathological cellular mechanisms that drive neurodegeneration, and assess the efficacy of disease modifying therapeutics.
Figure 1-2 Tripartite synapse

Astrocytes reciprocally communicate with neurons and microglia to regulate the health and activity of synapses.

1.4. Parkinson’s disease

PD predominantly affects the basal ganglia circuitry that regulates voluntary movement, cognition and reward learning (Schultz, 2007; Wise, 2004). It is the second most common neurodegenerative disorder affecting more than 1% of the population over 65 years of age (Thomas & Beal, 2007). The disease was first described by James Parkinson in 1817 in his renowned ‘Essay on the Shaking Palsy’. The cardinal symptoms of PD are tremor at rest, muscle rigidity, bradykinesia (slowness of movement), and postural instability. The early
symptomatic phase of the disease manifests following approximately 30-50% of nigral neuronal and 60-70% of striatal terminal loss (Cheng, Ulane, & Burke, 2010). It is estimated that by 2030, 9 million people worldwide will be afflicted with PD. The pathological hallmarks of PD are the progressive neurodegeneration of nigrostriatal DA neurons, and the accumulation of intracellular protein inclusions called Lewy bodies (LB) comprised of α-synuclein, parkin, ubiquitin and neurofilaments in the remaining neurons (Spillantini et al., 1997). Approximately 90-95% of PD cases are sporadic with no apparent genetic basis, while in 5-10% of familial PD patients, the disease is inherited (Lesage & Brice, 2009).

1.4.1. Mutations in key molecules

Genetic mutations that affect structural and functional integrity of cellular proteins, intracellular trafficking, and degradation pathways have been identified in familial PD, and to a lesser extent in sporadic PD. These include α-synuclein, a widely expressed protein in presynaptic nerve terminals that may regulate synaptic vesicle trafficking and dopamine release; leucine-rich repeat kinase 2 (LRRK2), a multidomain protein that may facilitate vesicle trafficking; ubiquitin carboxy-terminal hydrolase L1 (UCHL1) in the ubiquitin proteasomal degradation pathway; parkin (PARK2), and PTEN-induced kinase 1 (PINK1), molecular regulators of mitochondrial trafficking and degradation (Leroy et al., 1998; Mata, Wedemeyer, Farrer, Taylor, & Gallo, 2006; Stefanis, 2012; Valente et al., 2004; von Coelln, Dawson, & Dawson, 2004).

Supported by genome-wide association studies (GWAS), evidence to date suggest that in the more prominent idiopathic form of PD, potentially interacting genetic predispositions that include single nucleotide polymorphisms (SNPs) at risk loci, many of which are also implicated in familial PD (International Parkinson Disease Genomics, 2011; Nalls et al., 2014; Satake et al., 2009), may collaborate with epigenetic modifications, and environmental factors (Feng, Jankovic, & Wu, 2015) to drive neurodegeneration. Although the aetiology and exact molecular mechanisms of PD are not fully illuminated, in both forms of the disease, deficits in cellular protein processing, oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammation orchestrated by interacting neurons and glia appear to be key pathological mechanisms that drive the progressive demise of DA neurons.
During early stages of PD, it appears that the disease is characterised by a surviving, yet dysfunctional population of nigral DA neurons. In post-mortem tissue of early stage human PD brains, a significant number of the surviving DA neurons express reduced levels of tyrosine hydroxylase (TH) indicating deficits in neurotransmission (Fearnley & Lees, 1991).

Furthermore, microarray analysis of post-mortem SNpc of parkinsonian patients indicate aberrations in expression of a repertoire of cellular genes including those implicated in DA transmission, ubiquitin-proteasomal degradation, energy metabolism and oxidative stress, intracellular trafficking, inflammation, cell structural maintenance, signal transduction and transcription, revealing extensive cellular deficits characteristic of PD pathogenesis (Grunblatt et al., 2004).

1.4.2. The glial cell population in the substantia nigra pars compacta

The substantia nigra pars compacta (SNpc), the DA region most vulnerable to PD, contains the lowest density of astrocytes (271 astrocytes/mm² in the naïve human brain), indicating that this region is poorly primed against exposure to cellular stress. Whereas, DA neurons residing in regions that have a high density of astrocytes, such as the central gray substance (694 astrocytes/mm²) exhibit greater resistance to neurodegeneration with less than 5% neuronal loss in PD (Hirsch, Hunot, Damier, & Fauchex, 1998). The severely affected SNpc also displays the greatest increase in astrocytic glutathione peroxidise immunoreactivity, indicative of reactive gliosis.

Originating from a myeloid lineage, microglia, the resident immune cells in the CNS play a central role in regulating neuroinflammation (Gomez-Nicola & Perry, 2015). In comparison to other brain regions, the SNpc has a relatively abundant population of microglia (Kim et al., 2000; Lawson, Perry, Dri, & Gordon, 1990). Under basal conditions, microglia actively survey the brain microenvironment for disturbances in tissue homeostasis, and like astrocytes, become reactive in response to pathology (Hanisch & Kettenmann, 2007; Nimmerjahn, Kirchhoff, & Helmchen, 2005). Activated microglia express a plethora of cytokines, chemokines, and neurotrophins such as interleukin-1β (IL-1β), TNFα, macrophage inflammatory protein-1α and -1β, and TGFβ, and reactive oxygen and nitrogen species that coordinate a potent inflammatory response against the pathological insult (Long-Smith, Sullivan, & Nolan, 2009). High numbers of reactive microglia are present in the vicinity of
the degenerating DA neurons in the SNpc of post-mortem PD brains (McGeer, Itagaki, Boyes, & McGeer, 1988). As discussed in detail in section 1.4.4, a high density of microglia, and low density of astrocytes that regulate microglial activity may render the SNpc particularly vulnerable to neurodegeneration.

Moreover, oligodendrocytes, predominantly known for axonal myelination, are also known regulate axonal integrity and neuronal survival via myelination-independent mechanisms that include trophic factor release, metabolic support, and ROS detoxification (Kassmann et al., 2007; Lee et al., 2012; Wilkins, Chandran, & Compston, 2001; Wilkins, Majed, Layfield, Compston, & Chandran, 2003). Recent evidence has suggested a potential role of α-synuclein accumulation in oligodendrocytes in neuronal dysfunction and degeneration in various synucleopathies including PD, multiple system atrophy, and dementia with Lewy bodies has been implicated (Reyes et al., 2014).

1.4.3. Oxidative stress, deficits in energy metabolism and excitotoxicity

Midbrain DA neurons are autonomous pacemakers that generate action potentials in the absence of excitatory synaptic input (Hyland, Reynolds, Hay, Perk, & Miller, 2002), and hence represent a high metabolic and oxidative expenditure as evident by an overabundance in the naïve nigra of energy metabolism RNA transcripts implicated in glycolysis and mitochondrial oxidative phosphorylation (Greene, Dingledine, & Greenamyre, 2005, 2010). Long-range polymerase chain reaction (PCR) analysis of laser micro-dissected neurons from ageing and PD human brains reveal an increase in mitochondrial DNA (mtDNA) deletions associated with cytochrome c oxidase deficiency, indicative of respiratory chain dysfunction, with higher mtDNA deletions detected in nigral neurons from PD patients (Bender et al., 2006; Schapira et al., 1990). Interestingly, compared to the nigra, mtDNA deletions in cortical, hippocampal, and cerebellar neurons were negligible (Bender et al., 2006; Kraytsberg et al., 2006), suggesting that nigral neurons exhibit an increased vulnerability to oxidative stress. Furthermore, elevated levels of oxidised coenzyme Q-10 and 8-hydroxy-2'-deoxyguanosine, indicative of mitochondrial oxidative damage are measured in the cerebrospinal fluid (CSF) of PD patients (Isobe, Abe, & Terayama, 2010). Conditional deletion of the mitochondrial transcription factor A (Tfam) in midbrain DA neurons that regulates mtDNA transcription resulted in respiratory chain deficiency that led to progressive neuronal degeneration, and motor impairment in mice, further supporting a role for
mitochondrial dysfunction in the pathophysiology of PD (Ekstrand et al., 2007). *In vitro* and *in vivo* studies demonstrate that inhibitors of mitochondrial complex I, including MPTP and rotenone alter mitochondrial gene expression, compromise oxidative phosphorylation, enhance the production of ROS and mediate DA neuronal degeneration and motor deficits in animal models of PD, suggesting an intimate association between oxidative stress and mitochondrial dysfunction in disease pathogenesis (Burte, De Girolamo, Hargreaves, & Billett, 2011; Cannon et al., 2009; Piao, Kim, Oh, & Pak, 2012).

As discussed in section 1.2.3, antioxidant capacity of neurons is supported by astrocytes in the form of glutathione. Glutathione levels are downregulated in the post-mortem PD nigra, and its depletion appears to be an early biochemical event in disease pathogenesis (Dexter et al., 1994; Sian et al., 1994). Significant depletion of glutathione concentrations in the nigrostriatal circuitry following the irreversible inhibition of astrocytic γ-glutamyl cysteine synthetase had no effect on nigral neuronal survival or DA transmission (Toffa, Kunikowska, Zeng, Jenner, & Marsden, 1997), suggesting the glutathione depletion alone is insufficient to cause neurodegeneration. However, glutathione depletion potentiates 6-OHDA- and MPTP-mediated neurotoxicity *in vivo* (Pileblad, Magnusson, & Fornstedt, 1989; Wüllner et al., 1996), indicating that deficits in antioxidant mechanisms enhance the susceptibility of DA neurons to oxidative stress.

In addition to mitochondrial dysfunction in neurons that contribute to deficits in energy metabolism and oxidative stress, mitochondrial dysfunction in astrocytes may compromise astrocytic homeostatic functions that maintain neuronal activity. A recent study demonstrated that neuronal activity promotes the recruitment and retention of mitochondria to sites of glutamate transport in astrocytic processes via a yet unknown mechanism associated with astrocytic glutamate transport and the concomitant Na⁺/K⁺ ATPase activation (Jackson, O'Donnell, Takano, Coulter, & Robinson, 2014). Immobilisation of mitochondria at sites of glutamate uptake may facilitate a role of mitochondria in glutamate transport-associated oxidative phosphorylation (Eriksson, Peterson, Iverfeldt, & Walum, 1995; Pellerin & Magistretti, 1994), glutamate oxidation (Yudkoff, Nissim, & Pleasure, 1988), and maintenance of ion homeostasis (Bernardinelli, Azarias, & Chatton, 2006). Deregulation of mitochondrial proteins essential for maintenance of astrocytic functional integrity is evident in nigral astrocytes in PD (Cook, Hoekstra, Eaton, & Zhang, 2015; Hoekstra et al., 2015). For example, both nigral neuronal and astrocytic expression of mitochondrial dynamin-like protein 1 (dlp-1) is downregulated in post-mortem PD brains. Deletion of astrocytic dlp-1 *in
vitro disrupted intracellular Ca\(^{2+}\) homeostasis that regulate glutamate transport, and subsequent elevation in extracellular glutamate enhanced the susceptibility of DA neurons to excitotoxicity in neuron-astrocyte co-cultures (Hoekstra et al., 2015).

Furthermore, it is hypothesised that glutamatergic projections from the disinhibited subthalamic nucleus may excessively innervate the SNpc leading to excitotoxicity in PD (Rodriguez, Obeso, & Olanow, 1998). In the MPTP-induced mouse model of PD, elevated extracellular glutamate and DA neuronal death in the SNpc have been observed (Meredith, Totterdell, Beales, & Meshul, 2009). Downregulation of the astrocyte-specific glutamate transporter GLT-1 that maintains extracellular glutamate homeostasis has been reported in neurotoxin-induced rodent PD models (Chung, Chen, Chan, & Yung, 2008; Holmer, Keyghobadi, Moore, & Meshul, 2005), suggesting that deficits in astrocytic homeostatic functions may further enhance neurodegeneration.

### 1.4.4. Inflammation

Reactive astrogliosis is induced by various inflammatory mediators including cytokines such as TNF\(\alpha\), IL6, IL-1\(\beta\), growth factors, reactive oxygen and nitrogen species, pathogenic glycoproteins such as bacterial lipopolysaccharide (LPS), toxic protein aggregates associated with neurodegenerative diseases such as \(\alpha\)-synuclein, and small molecules such as ATP (Sofroniew, 2014; Sofroniew & Vinters, 2010). Astrocytes in turn express a plethora of chemokines, cytokines and growth factors that regulate inflammation.

Microarray profiling of reactive astrocytes reveal that reactive astrogliosis is characterised by extensive alterations in the cellular transcriptome (Hamby et al., 2012; Zamanian et al., 2012). The myriad of genes regulated include structural proteins, ion channels, transporters, receptors, enzymes, various molecular mediators of intracellular signal transduction, and most notably inflammatory mediators including cytokines, chemokines, and growth factors that are significantly upregulated (the reader is referred to a recent review by M. Sofroniew for an in depth analysis of reactive astrocytes in inflammation (Sofroniew, 2014)).

Furthermore, these phenotypic alterations are highly dynamic and heterogeneous. For example, combinatorial treatment of primary astrocytes with TGF-\(\beta\)1, LPS, and interferon-gamma (IFN\(\gamma\)) synergistically regulated over 380 astrocytic genes, an expression profile that was unpredictable by summing the gene regulatory effects of the individual inflammatory
molecules (Hamby et al., 2012). In vivo studies further confirm the diverse nature of reactive astrogliosis (Lichter-Konecki, Mangin, Gordish-Dressman, Hoffman, & Gallo, 2008; Zamanian et al., 2012). For example, reactive astrogliosis in ischemic stroke significantly upregulated neurotrophic factors such LIF, IL-6, and thrombospondins that may promote neuronal survival and synaptogenesis (Zamanian et al., 2012). In contrast, in response to LPS, reactive astrocytes upregulated a greater proportion of proinflammatory genes such as the complement cascade implicated in neurodegeneration. These results elucidate the potential for therapeutic regulation of phenotypic evolution of reactive astrocytes in a neuroprotective orientation. Furthermore, transcriptome analysis of reactive astrocytes in early and late PD may reveal molecular mechanisms that govern progressive neurodegeneration, and potential therapeutic targets.

The SN of post-mortem PD brains and disease models exhibit severe neurodegeneration, reactive gliosis, and robust inflammation (Forno, DeLanney, Irwin, Monte, & Langston, 1992; McGeer & McGeer, 2008; Miklossy et al., 2006). Molecular mediators of pro-inflammatory processes such as TNFα, IL-1β, IL-6 and interferon γ are upregulated in the post-mortem SN, and CSF of PD patients (Boka et al., 1994; Dobbs et al., 1999; Mogi et al., 1994; Stypula, Kunert-Radek, Stepien, Zylinska, & Pawlikowski, 1996). Genetic variations in neuroinflammatory genes are associated with an increased incidence of neurological diseases, and epidemiological studies suggest that regular intake of non-steroidal anti-inflammatory drugs is associated with a lower risk of PD (Chen, Zhang, Hernán, & et al., 2003), indicating a role of neuroinflammation in disease pathophysiology. Therapeutic efficacy of anti-inflammatory strategies in PD models further confirms that detrimental neuroinflammation contributes to disease progression. For example, overexpression of the glial anti-inflammatory cytokine IL-10 in the 6-OHDA-lesioned rat striatum attenuated DA neuronal death and associated behaviour impairments (Johnston et al., 2008). Viral vector-mediated downregulation of TNFα in nigral glia concurrently with striatal infusion of 6-OHDA, or two weeks post-6-OHDA lesion exerted equally robust neuroprotective effects against neurodegeneration (Harms et al., 2011), implicating a role for sustained inflammation in regulating progressive neurodegeneration.

In vitro studies reveal the potent pro-inflammatory capacity of astrocytes in response to inflammatory stimulation. In response to IFN-γ, reactive astrocytes released a potent cocktail of neurotoxic factors that included IL-6 and glutamate, and upregulated free radical generating NADPH oxidase and cyclooxygenase (COX) that generate prostaglandins (M.
Exposing differentiated human neuroblastoma cells to conditioned media (CM) from IFN-γ-stimulated astrocytes significantly reduced their viability. Individually inhibiting each of these proinflammatory factors in reactive astrocytes prior to exposing the CM to neuroblastoma cells was shown to increase cell viability. Combined inhibition of all inflammatory molecules resulted in a significant improvement in cell viability with a 91% reduction in CM-induced toxicity. These results suggest that a predominantly neurotoxic phenotype in severely reactive astrocytes may sustain chronic inflammation that contributes to disease progression. Therefore, in comparison to targeting a single cellular pathway, targeting multiple pathways implicated in neuroinflammation could potentially be a more effective therapeutic strategy in PD and other neurodegenerative diseases.

Nigral infusion of the inflammogen, LPS induced the expression of pro-inflammatory cytokines such as TNF-α and IL-1β in the nigra, and substantially elevated GDNF expression in astrocytes (Iravani et al., 2012). Additionally, in the context of neuroinflammation, reactive astrocytes have been shown to upregulate homeostatic functions that regulate glutamatergic neurotransmission and energy metabolism. In response to lentiviral-mediated overexpression of the cytokine, ciliary neurotrophic factor (CNTF) in rat striatal astrocytes, reactive astrocytes expressed highly glycosylated forms of GLAST and GLT-1 that were enriched into cholesterol-rich microdomains, known as lipid rafts in the cellular membrane that facilitate glutamate clearance (Butchbach, Tian, Guo, & Lin, 2004; Escartin et al., 2006). The subsequent striatal infusion of quinolinic acid (QA) that stimulates excessive presynaptic glutamate release induced excitotoxicity (Stone, 1993). However, in comparison to control animals, extracellular accumulation of glutamate was significantly lower in rats overexpressing astrocytic CNTF, indicating enhanced glutamate clearance by reactive astrocytes. Furthermore, CNTF-activated astrocytes in vivo and in vitro downregulated glycolytic flux, and sustained mitochondrial oxidative phosphorylation via upregulated β-oxidation of fatty acids and ketolysis (Escartin et al., 2007). This metabolic plasticity improved survival of neuron-astrocyte co-cultures in response to metabolic insults including inhibition of glycolysis, and prolonged exposure to the saturated fatty acid, palmitate, a precursor for de novo synthesis of ceramide (Blázquez, Galve-Roperh, & Guzmán, 2000), a lipid signalling molecule that promotes apoptosis (Kolesnick & Krönke, 1998). These results suggest that astrocyte can acquire highly diverse reactive phenotypes in a context-dependent
manner, and genetic manipulation may allow the potentiation of neuroprotective profiles, or attenuation of detrimental pathways.

However, the susceptibility of astrocytes for detrimental phenotypic evolution in the presence of microglia reveals the potential for pathological amplification of glia communication that contributes to neurodegeneration. Activation of the receptor CXCR4 on astrocytes by CXC-chemokine, stromal cell-derived factor 1 (SDF-1) or HIV-1 envelope glycoprotein gp120 induces the release of TNFα, which subsequently stimulates the Ca$^{2+}$-dependent autocrine release of glutamate from astrocytes (Bezzi et al., 2001). Reactive microglia dramatically potentiated TNFα and glutamate release from astrocytes in response to SDF-1 stimulation. In comparison to mixed astrocyte and microglia cultures under basal conditions, co-culturing astrocytes with reactive microglia that have been exposed to LPS prior to seeding significantly enhanced astrocytic TNFα and glutamate release. Interestingly, SDF-1-induced release of TNFα in pure cultures of reactive microglia was significantly lower in the absence of astrocytes. Furthermore, glutamate excitotoxicity-induced neuronal apoptosis in neuron-glial co-cultures following exposure to HIV-1 gp120 exclusively depended on the presence of reactive microglia, indicating that detrimental amplification of astrocyte-microglia communication may promote evolution of tightly regulated physiological processes into self-sustained neurotoxic events in the context of chronic pathology.

1.4.5. Deficits in astrocyte-derived neurotrophic support

In post-mortem PD brain nigral tissue, levels of neurotrophins are significantly decreased indicating that the loss of trophic support may further promote neurodegeneration (Chauhan, Siegel, & Lee, 2001; Mogi et al., 1999). Elevated expression of proinflammatory cytokines and decreased levels of neurotrophic factors are recapitulated in neurotoxin models of PD (Mogi et al., 1998). The high potency of astrocyte-derived GDNF in promoting DA neuronal survival and differentiation, its severe depletion in the PD post-mortem nigra, and the therapeutic efficacy of GDNF in enhancing DA transmission, neuronal survival, axonal regeneration and motor function in toxin-induced rodent and primate models of PD suggest that GDNF may play a crucial role in phenotypic maintenance and survival of DA neurons, and its depletion may contribute to neurodegeneration (Björklund et al., 2000; Hoffer et al., 1994; D. Kirik, Georgievska, & Bjorklund, 2004; Kordower et al., 2000; Lin, Doherty, Lile, Bektesh, & Collins, 1993; Scharr, Sieber, Dreyfus, & Black, 1993). Furthermore, GDNF in
astrocyte-conditioned media has been shown to downregulate microglia activity, suggesting a role for GDNF in the regulation of neuroinflammation (Rocha, Cristovão, Campos, Fonseca, & Baltazar, 2012). However, contrary to the remarkable preclinical results, clinical translation of GDNF therapy via direct cerebroventricular infusion or AAV-mediated expression in the basal ganglia failed to confer therapeutic efficacy in PD patients (Bartus et al., 2011; Lang et al., 2006; Nutt et al., 2003).

These results elucidate a major limitation of neurotoxin-induced PD models; the inability to replicate intracellular α-synuclein aggregation and associated pathological mechanisms characteristic of clinical PD. A recent study by Decressac et al. demonstrated that viral vector-mediated expression of GDNF in the nigra or striatum, or both sites failed to protect nigral DA neurons or their striatal terminals in a rat α-synuclein PD model (Decressac et al., 2011). While neurotoxin-induced acute pathogenesis replicates oxidative stress and mitochondrial dysfunction, α-synuclein overexpression was shown to replicate a spectrum of deleterious mechanisms that underlie cellular dysfunction and progressive neurodegeneration in PD. α-synuclein-induced deficits in cellular trafficking, impairment of axonal transport in surviving, yet dysfunctional DA neurons may compromise widespread nigrostriatal delivery of GDNF, resulting in therapeutic failure (Chung, Koprich, Siddiqi, & Isacson, 2009; Decressac et al., 2011). These results suggest that testing potential neurotherapeutics in multiple preclinical models that replicate various pathogenic mechanisms relevant to the human disease is necessary prior to clinical translation.

1.4.6. α-synuclein pathology

A key feature of the pathophysiology of PD is the intraneuronal aggregation of α-synuclein, a highly abundant presynaptic protein that regulates vesicle trafficking and dopamine release (Dauer & Przedborski, 2003; Masliah et al., 2000). A multitude of evidence implicates α-synuclein, the main component of LBs in the pathogenesis of PD (Spillantini et al., 1997). Mutations in the gene encoding α-synuclein (SNCA) are associated with familial cases of PD, and GWAS demonstrate that SNPs in the SNCA gene is the most frequently associated risk locus in sporadic PD (Anwar et al., 2011; Sidhu, Wersinger, & Vernier, 2004; Venda, Cragg, Buchman, & Wade-Martins, 2010). Recent evidence suggests that toxicity associated with the expression of aberrant proteins in both neurons and astrocytes may contribute to the pathogenesis of PD. Astrocytic α-synuclein accumulation has been identified in human post-
mortem PD brains, and the number of α-synuclein-positive astrocytes correlated with the severity of neuronal loss (Braak, Sastre, & Del Tredici, 2007; Wakabayashi, Hayashi, Yoshimoto, Kudo, & Takahashi, 2000). Selective expression of mutant α-synuclein in astrocytes led to widespread reactive astrogliosis, chronic inflammation, loss of DA neurons in the SNpc, and rapidly progressing paralysis in mice (Gu et al., 2010). The normal supportive roles of astrocytes were compromised as evident by BBB disruptions that resulted in cerebral microhaemorrhage, and down-regulation of astrocytic glutamate transporters, GLAST and GLT1 that may contribute to neuronal excitotoxicity (Lehre et al., 1995; Milton et al., 1997).

The formation of LB inclusions in healthy neuronal transplants in both human PD cases (Kordower, Chu, Hauser, Freeman, & Olanow, 2008) and animal models (Kordower et al., 2011) inferred the potential prion-like behaviour of α-synuclein inclusions in disease propagation. Widespread α-synuclein accumulation in the nigrostriatal circuitry, progressive loss of SNpc DA neurons and associated motor deficits in mice following striatal infusion of α-synuclein fibrils further support neurotoxicity of extracellular α-synuclein oligomeric species (Luk et al., 2012). Given that CNS synapses are tripartite in nature, localised cellular dysfunction has the capacity to be transmitted to other synaptic elements that may promote chronic neurodegeneration. Astrocytic α-synuclein aggregation following endocytosis of neuronal-derived α-synuclein has been demonstrated both in vitro and in vivo (Lee et al., 2010). The internalised α-synuclein co-localised with the lysosomal marker LAMP-2, and lysosomal inhibition with bafilomycin A1 amplified astrocytic accumulation of α-synuclein following exposure to media conditioned with α-synuclein (Lee et al., 2010), demonstrating that clearance of potentially toxic extracellular protein aggregates by astrocytes maintains homeostasis. However, the eventual saturation of astrocytic protein degradation machinery may lead to the formation of α-synuclein aggregates that may promote detrimental evolution of reactive astrogliosis. Astrocytic accumulation of α-synuclein has been shown to upregulate the expression of pro-inflammatory cytokines such as TNF-α, IL-1α, IL-1β, and IL-6, and chemokines such as CCL3 (Gu et al., 2010; Klegeris et al., 2006; Lee et al., 2010), with the magnitude of inflammation correlating with the extent of α-synuclein aggregation. Moreover, reactive astrocyte-derived pro-inflammatory signals recruited microglia that may further release various potentially neurotoxic molecules including IL-1β, NO, and ROS, leading to a self-sustained chronic inflammatory state that drive neurodegeneration (Hanisch & Kettenmann, 2007).
1.5. Treatments for Parkinson's disease

Despite a greater understanding of the molecular mechanisms that dictate PD pathogenesis, effective therapies that alter disease progression remain elusive. Currently available pharmacological therapies that replace the DA deficit to alleviate the cardinal symptoms of PD (bradykinesia, rigidity, rest tremor, and gait disturbance) are associated with side-effects, and therapeutic failure over time (Marsden & Parkes, 1976).

To date, the dopamine precursor L-3,4-dihydroxyphenylalanine (L-Dopa) remains the gold-standard PD therapy. The orally administered drug circumvents the BBB, and is converted to dopamine in DA neurons. In the early stages of the disease, L-Dopa effectively improves DA content in the denervated striatum. (Goetz, 1998). However, with disease advancement, patients develop progressively more severe fluctuations in motor disability including dyskinesia and gait dysfunction that inevitably lead to therapeutic failure (Marsden & Parkes, 1977).

The antiparkinsonian effect of DA agonists such as ropinirole and pramipexole is based on direct activation of pre- and post-synaptic DA receptors (Parkinson Study, 2000; Olivier Rascol et al., 2000). Although motor complications are less frequent, DA agonists are associated with severe non-motor side effects such as nausea, hallucinations, peripheral oedema, and sleep disturbances.

Monoamine oxidase B inhibitors such as selegiline and rasagiline prevent dopamine metabolism and enhance the concentration of both endogenous dopamine and dopamine produced from exogenously administered L-Dopa (Lees, 2005). However, they are less potent than the other therapeutic compounds, and hence are used as adjunct therapy to L-Dopa in the initial stages of the disease (O. Rascol et al., 2005).

A greater understanding of the basal ganglia circuitry has also allowed the development of surgical therapies for advanced PD that exhibits intractable tremor and drug-induced motor fluctuations or dyskinesias. Although stereotactic ablative techniques that lesion the overactive internal globus pallidus or the subthalamic nucleus (STN) were initially utilised, deep brain stimulation (DBS), wherein high frequency electrical stimulation of these structures dampen overactivation is now the most commonly used surgical procedure (Benabid, Chabardes, Mitrofanis, & Pollak, 2009; A. L. Benabid et al., 1988; Parkin et al., 2001). Despite the motor benefits, DBS is associated with significant non-motor side effects
including depression, psychosis, and cognitive deficits (Smith, Wichmann, Factor, & DeLong, 2012).

1.6. Gene therapy for Parkinson's disease

*In vivo* gene therapy comprises the delivery of nucleic acid sequences to overexpress transgenic counterparts of downregulated or defective genes, or suppress detrimental gene expression using RNA-based gene silencing in diseased cells to rescue their normal phenotype. An essential requirement of gene transfer for chronic neurodegenerative disease is the robust, long-term therapeutic gene expression in the absence of immunogenicity or pathogenicity. Gene therapy offers several advantages over traditional pharmacological based therapies including the ability to directly manipulate molecular mechanisms that dictate disease pathogenesis, selectively target disease-affected regions and cell types to minimise inappropriate dissemination of therapeutic molecules, and achieve long-term therapeutic protein expression in the absence of repeated administration of pharmacological agents. Presently, non-viral and viral vector systems are explored as potential gene delivery vehicles. These vector particles typically consist of a transgenic expression cassette encoding a therapeutic gene under the control of a strong promoter sequence packaged into viral or non-viral capsules. Thus far, CNS gene delivery predominantly targets degenerating neurons, and the vast majority of strategies have focused on manipulation of neuronal physiology; however a growing understanding of disease mechanisms including the role of glia in neurodegeneration, and characterisation of vector systems with novel tropisms, and superior transduction capacities are accelerating the ability to develop effective gene therapy strategies.

While the majority of gene therapy strategies utilise highly efficient viral vectors, nanoparticle-based polymer- or lipid-nucleotide complexes that offer large packaging capacities, low immunogenicity and ease of manufacture are appealing alternatives currently in development (Liu, Huang, & Jiang, 2011). Although these synthetic vectors exhibit potential for CNS gene delivery, including the ability to traverse the BBB, further refinement of the technology is required to improve transfection efficiency that is hindered by inefficient cellular entry and rapid DNA degradation, and minimise any potential toxicity associated with inefficient biodegradability of the synthetic elements (Alvarez-May, Navarro-Quiroga,
Currently, the most effective CNS gene transfer is achieved with vectors derived from viruses. These vectors exhibit various transduction properties and differ in the packaging capacity, tropism, efficacy of transgene expression, and immunogenicity and pathogenicity, and hence differ in their suitability for various applications. To date, recombinant viral vector systems based on adenoviruses (Ad), lentiviruses (LV), herpes simplex virus (HSV), and adeno-associated viruses (AAV) have been developed for various gene therapy applications.

1.6.1. Adenoviral vectors for CNS gene delivery

Ad viruses consist of a nonenveloped double-stranded DNA genome with a packaging capacity of up to ~36kb. Although first generation Ad vectors that have a packaging capacity of up to 8.2kb transduce both quiescent and terminally differentiated cells with high efficiency, de novo synthesis of viral proteins induces a significant host immune response (Akli et al., 1993; Bohn et al., 1999; Lowenstein & Castro, 2003). Deletions in genes required for viral replication expanded packaging capacity of second generation Ad vectors; however, residual viral gene expression maintains immunogenicity and toxicity in vivo (Hardy, Kitamura, Harris-Stansil, Dai, & Phipps, 1997; C. E. Thomas, Schiedner, Kochanek, Castro, & Lowenstein, 2001; Zhu, Huang, & Yang, 2007). Even the latest third generation "gutted" or 'helper-dependent' Ad vectors that have a packaging capacity of up to ~36kb, and are void of all viral genes, except for cis-acting elements necessary for DNA replication and packaging elicit immunogenicity. This inherent ability of Ad viruses to induce an innate immune response remains a major disadvantage for CNS gene delivery (Zhu et al., 2007).

1.6.2. Lentiviral vectors for CNS gene delivery

Lentiviral (LV) vectors mainly based on human immunodeficiency virus type 1 (HIV-1) are lipid-enveloped RNA retroviruses that transduce both dividing and non-dividing cells, and exhibit a packaging capacity of 8 to 10kb. The incorporation of a variety of envelope glycoproteins allows the development of pseudotyped LV vectors that selectively target CNS neurons and glia with low immunogenicity (Cannon, Sew, Montero, Burton, & Greenamyre,
The potential risk of insertional mutagenesis, proto-oncogene activation and occurrence of replication-competent pathogenic recombinants associated with preferential LV integration into the host genome (Bartholomae et al., 2011) are minimised with the development of integrase-deficient LV vectors that promote episomal maintenance of the vector genome (Liu et al., 2014; Zufferey et al., 1998).

1.6.3. Herpes simplex viral vectors for CNS gene delivery

Herpes simplex virus (HSV) of the Herpesviridae family possesses a large lipid-enveloped double-stranded DNA genome. Attenuated (‘replication-conditional’) HSV vectors are deficient in viral genes required for replication in non-dividing cells, but retain the ability to replicate in dividing cells (Wu, Watkins, Schaffer, & DeLuca, 1996). These highly toxic vectors have been used mainly for treatment of cancers such as glioblastoma multiforme (Markert et al., 2008). Recently developed helper function-dependent amplicon vectors that retain only cis-acting elements required for efficient vector packaging has a packaging capacity of up to 150kb, and lack viral genes that induce cytotoxicity (Marconi, Argnani, Epstein, & Manservigi, 2009). While vectors derived from wild-type HSV are neurotropic, selective glial transduction can be achieved with pseudo-typed vectors (Anderson, Laquerre, Goins, Cohen, & Glorioso, 2000; Wolfe, Deshmane, & Fraser, 1992). However, currently the lack of a production method that effectively eliminates the potential for helper viral contamination, and yields high-titre vector is a significant obstacle.

1.6.4. Adeno-associated viral vectors in CNS gene delivery

1.6.4.1. Biology of adeno-associated viruses

Presently, AAV vectors that exhibit efficient transduction capacity and long-term transgene expression in the absence of immunogenicity or pathogenicity are the preferred vehicles for CNS gene delivery in the clinical setting. AAV is a non-pathogenic virus with a 4.7kb single-stranded DNA genome belonging to the family Parvoviridae and genus Dependovirus. AAV requires the co-infection of a helper virus such as adenovirus or herpes virus for effective replication and packaging (Atchison, 1970). To date, 12 primate AAV serotypes
and over 110 viral variants have been identified (Gao, Vandenberghe, & Wilson, 2005), and efforts have been mainly focused on the development and characterisation of vectors derived from AAV serotypes 1-9. The viral genome consists of two 145bp inverted terminal repeats (ITRs) that flank two open reading frames, rep and cap, which through use of alternative splicing and start codons, generate seven proteins with partially overlapping sequences (Srivastava, Lusby, & Berns, 1983). The rep gene codes for four non-structural proteins (Rep78, Rep68, Rep52, and Rep40) involved in ITR-dependent viral replication, transcription, site-specific integration, and AAV genome encapsidation (Dubielzig, King, Weger, Kern, & Kleinschmidt, 1999; King, Dubielzig, Grimm, & Kleinschmidt, 2001; Smith & Kotin, 1998). The cap gene codes for structural proteins VP1, 2, and 3 that assemble at a ratio ~1:1:18 to form a 60-monomer viral capsid.

In the absence of helper viruses, wild type AAV primarily persists as episomal monomers and multimers in a latent state (Schnepp, Jensen, Chen, Johnson, & Clark, 2005). Integration preferentially at the AAVS1 locus on chromosome 19 (19q13.3-qter) occurs at a low frequency (Kotin, Linden, & Berns, 1992; Kotin, Menninger, Ward, & Berns, 1991). Subsequent superinfection with a helper virus rescues the integrated AAV genome and the virus enters the lytic phase of its life cycle, resulting in viral replication and generation of progeny virions. Recombinant AAV vectors lack the capacity to integrate and hence persist as extrachromosomal double-stranded circular monomers or concatemers following second strand synthesis (Schnepp, Jensen, Clark, & Johnson, 2009b). Recombinant AAV vectors are generated by replacing the viral rep and cap with a transgenic cassette of up to ~4.7kb, while the flanking ITR sequences that contain cis-acting elements required for vector genome packaging into AAV capsids remain intact. The excised rep and cap genes are provided in trans on an AAV helper plasmid. The standard procedure for generating AAV vector particles consists of co-transfecting human embryonic kidney 293 (HEK 293) cells with the AAV expression plasmid encoding the ITR-flanked transgenic cassette and AAV helper plasmids encoding rep and cap (Grimm, Kern, Rittner, & Kleinschmidt, 1998), and Ad helper functions (Xiao, Li, & Samulski, 1998) that facilitate efficient AAV vector production.

Despite the rapidly expanding knowledge on novel AAV serotypes, intracellular trafficking of AAV2 that effectively transduces widely used cell lines such as HeLa and HEK293 remains the best characterised thus far. Viral infection is a complex, multistep process consisting of receptor binding and internalisation of the viral particle via endocytosis, intracellular trafficking to the nucleus, and following viral uncoating, conversion of the
single-stranded vector genome to a stable double-stranded molecule. AAV serotypes may utilise proteoglycan conjugates as primary receptors for attachment, and proteinaceous coreceptors for internalisation. Heparan sulphate proteoglycan (HSPG) is the primary cellular receptor for AAV2 (Summerford & Samulski, 1998). Candidate primary receptors for other serotypes include O-linked 2,3-sialic acid for AAV4, N-linked 2,3-sialic acid for AAV1, AAV5 and AAV6, and N-linked galactose for AAV9 (Kaludov, Brown, Walters, Zabner, & Chiorini, 2001; Walters et al., 2001). Coreceptors that facilitate efficient binding and endocytosis include fibroblast growth factor receptor-1 (Qing et al., 1999), and α5β1 and α5β3 integrins (Asokan, Hamra, Govindasamy, Agbandje-McKenna, & Samulski, 2006; Summerford, Bartlett, & Samulski, 1999) for AAV2, epidermal growth factor receptor for AAV6, platelet-derived growth factor receptor for AAV5 (Kaludov et al., 2001; Pilz, Di Pasquale, Rzadzinska, Leppla, & Chiorini, 2012; Walters et al., 2001), and the 37/67kDa laminin receptor (LamR) for AAV serotypes 2, 3, 8, and 9 (Akache et al., 2006). It is increasingly evident that in addition to interactions at the capsid-cell surface interface, intracellular processing of the virion determines AAV serotype-specific tropism and transduction efficacy. Following endocytosis, endosomal trafficking through early and late endosomes, and retrograde transport to the Golgi apparatus induce AAV capsid conformational changes that facilitate endosomal escape and nuclear transport, potentially via the nuclear pore complex (Nicolson & Samulski, 2014). Upon direct injection into the cytoplasm, Alexa568-labeled AAV2 particles failed to accumulate in the nucleus (Ding, Zhang, Yan, & Engelhardt, 2005), suggesting that endosomal processing of the AAV2 capsid may regulate nuclear transport and/or uncoating. For example, endosomal acidification initiates conformation changes of the AAV2 viral capsid exposing regions such as the N-terminus of VP1 capsid subunit that contains a phospholipase A2 homology domain required for efficient transduction (Girod et al., 2002; Sonntag, Bleker, Leuchs, Fischer, & Kleinschmidt, 2006). Following nuclear transport, capsid disassembly releases the single-stranded vector genome, which is subsequently converted to a stable double-stranded molecule capable of transgene expression (Schnepp, Jensen, Clark, & Johnson, 2009a).

Although our knowledge of intracellular trafficking of AAV is in its infancy, recent evidence suggests that various aspects of vector transduction including route and efficiency of endosomal processing, and viral uncoating in the nucleus may exhibit serotype-specific heterogeneity leading to differences in tropism and transduction efficacy (for a comprehensive discussion on the AAV intracellular trafficking pathway, the reader is referred
to a recent review (Nonnenmacher & Weber, 2012)). For example, despite an 83% amino acid similarity between AAV8 and AAV2 serotypes, following intravenous vector administration into mice, AAV8-mediated liver transduction was 20-fold higher than that of AAV2 (Nam et al., 2007; C. E. Thomas, T. A. Storm, Z. Huang, & M. A. Kay, 2004). Although both serotypes were effectively transported to the nucleus, AAV2-based vector genomes pseudotyped with AAV8 capsid proteins rapidly uncoated in the nucleus, whereas a large proportion of AAV2 vector particles persist as encapsidated single-stranded molecules up to 6 weeks post-vector administration (C. E. Thomas et al., 2004). Thomas and colleagues postulated that rapid capsid disassembly may facilitate annealing of complementary single-stranded vector genomes to form stable, transcriptionally active double-stranded molecules. In contrast, the slow rate of uncoating of AAV2 capsids may limit the concentration of naked, complementary single-stranded vector genomes available for efficient annealing, resulting in rapid degradation of single-stranded vector genomes. Therefore, to maximise applicability of an expanding repertoire of AAV serotypes, it is essential to elucidate any capsid-specific differences in the infectious pathway in the relevant target organs and cell types.

1.6.5. AAV vectors in Parkinson’s disease gene therapy

The first infectious clone of AAV2 was isolated in 1982 (Samulski, Berns, Tan, & Muzyczka, 1982), and AAV2-based vectors have been used to effectively deliver transgenes to various mammalian organs including the brain, liver, heart and skeletal muscle (Ali et al., 1996; During et al., 1998; S. Song et al., 1998; X. Xiao, Li, & Samulski, 1996; Ye et al., 1999). In preclinical studies, AAV2 vector-mediated gene expression in the CNS has been shown to persist up to 25 months in rodents (Klein et al., 2002) and 6 years in non-human primates (Bankiewicz et al., 2006). Although its modest packaging capacity restricts the size of the vector genome to less than 5kb, AAV2 mediates robust, long-term CNS transgene expression in the absence of immunogenicity or pathogenicity, making them appealing gene delivery vehicles for various neurological gene therapy applications (Mastakov et al., 2002; Peel & Klein, 2000). Consequently, AAV2 vectors are the viral serotype largely approved for CNS gene therapy clinical trials.

AAV2-mediated restoration of enzymes that regulate DA transmission, modification of neuronal phenotype to restore basal ganglia function, and expression of neurotrophic factors that promote function and regeneration, promoted DA transmission in the nigrostriatal
pathway, significantly attenuated disease progression, and spontaneous and drug-induced motor deficits in rodent and primate disease models (Luo et al., 2002; S. Muramatsu et al., 2004; Sanchez-Pernaute, Harvey-White, Cunningham, & Bankiewicz, 2001). Clinical translation of these strategies included the expression of aromatic-L-amino-acid decarboxylase (AADC) in the putamen to improve dopamine synthesis from exogenous L-Dopa (Christine et al., 2009; Muramatsu et al., 2010), glutamic acid decarboxylase (GAD) which converts glutamate to inhibitory GABA to suppress pathological overactivation of the STN, and neurturin, a structural and functional analogue of GDNF to confer neuroprotection in the putamen (Marks Jr et al., 2010). Although AAV-mediated long-term therapeutic gene expression was maintained in the human PD brain with an impressive safety profile, only modest therapeutic efficacy has been achieved (Feigin et al., 2007; Kaplitt et al., 2007; Mittermeyer et al., 2012).

The discrepancies in therapeutic efficacy between preclinical studies and clinical trials emphasises the requirement for animal models that effectively replicate multiple pathological mechanisms characteristic of the clinical condition, recruitment of patients with less severely progressed PD, and therapeutic targeting of multiple cellular pathways that deteriorate in PD. Widely used rodent and non-human primate PD models based on 6-hydroxydopamine (6-OHDA)- or 1-methyl-4-phenyl-1,2,3,6-tetrahydroxypyridine (MPTP)-induced oxidative stress and mitochondrial dysfunction inadequately replicate the spectrum of pathological mechanisms characteristic of PD (discussed in section 1.4), which may inadvertently exaggerate efficacy of therapeutic strategies. A severely degenerated nigrostriatal pathway in the late-stage PD brain may no longer be permissive to therapeutic intervention, which may contribute to therapeutic inefficacy. Additionally, extensive phenotypic dysfunction may require therapeutic targeting of endogenous molecules that exhibit ‘master regulatory’ status to target multiple pathological pathways.

1.7. Nuclear receptor-related 1 protein

It is increasingly evident that complex intra- and inter-cellular mechanisms involving neurons and glia regulate CNS physiology, and extensive dysfunction in these molecular mechanisms governs neurodegeneration in pathology. As discussed in section 1.6.5, traditional gene therapy strategies are focused on intercepting one aspect of cellular dysfunction, which in the
clinical context have repeatedly exhibited insufficient efficacy (Christine et al., 2009; Feigin et al., 2007; Kaplitt et al., 2007; Marks Jr et al., 2010; Mittermeyer et al., 2012; Muramatsu et al., 2010). In contrast, accumulating evidence suggests that nuclear receptors that regulate a wide range of genes involved in developmental, physiological, and metabolic processes in the healthy CNS, may also have a role in neurodegenerative disease (Nolan, Sullivan, & Toulouse, 2013), and hence represent a novel category of therapeutic candidates. Nuclear receptor-related 1 (NURR1; also known as NR4A2) protein is one such ‘master regulator’ that has significant functional relevance in astrocytes and midbrain DA neurons as discussed below.

NURR1 belongs to the nuclear receptor superfamily that comprises a group of transcription factors that orchestrate developmental and physiological responses to a variety of signalling events. Members of this superfamily share a common structural organisation, including a widely conserved DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The highly variable N-terminal regions of some nuclear receptors harbour potent transcriptional activation motifs known as activation function-1 (AF-1). Like many other nuclear receptors, NURR1 is referred to as an ‘orphan receptor’ that lacks identified ligands, and is a member of the nerve growth factor-induced clone B (NGFI-B) family of orphan receptors (Law, Conneely, DeMayo, & O'Malley, 1992). NURR1, together with NUR77 (NR4A1), and Nor1 (NR4A3) constitute the nuclear receptor 4A (NR4A) subfamily of three highly homologous receptors. The DBD binds to highly specific cis-acting DNA recognition motifs, originally termed ‘hormone response elements’, located upstream of target genes, to regulate the rate of transcriptional initiation. Based on X-ray crystallography of the NGFI-B receptor, Meinke et al. characterised the stereochemical interactions between the DBD and its NGFI-B response element (NBRE), AAAGGTCA (Meinke & Sigler, 1999). The constitutively active NURR1 can bind to target genes as a monomer, homodimer, or heterodimer with retinoid X receptors (RXRs) (Gronemeyer, Gustafsson, & Laudet, 2004; Zetterström, Solomin, Mitsiadis, Olson, & Perlmann, 1996). The crystal structure of the NURR1 LBD suggests that a ligand-independent mechanism, wherein an AF-2 sequence maintains the architecture of the LBD in a transcriptionally active state may confer constitutive activity of NURR1 (Wang et al., 2003). A number of synthetic lipophilic agonists have also been shown to stimulate NURR1 activate via its LBD (De Miranda et al., 2015; Kim et al., 2015).
1.7.1. NURR1 regulates midbrain dopaminergic neuronal phenotype

During embryonic development, differentiation patterns regulated by specific transcription factors allow neurons to acquire specialised identities in the CNS. Many of these transcription factors, including NURR1 persist in the adult brain where they maintain phenotypic integrity of differentiated neurons. The early stages of PD is characterised by a surviving, yet dysfunctional population of nigral DA neurons (Fearnley & Lees, 1991). Increasingly, evidence suggests that NURR1 dysfunction may render DA neurons vulnerable to pathological process, and aggravate the progression and severity of the disease. NURR1 mutations and polymorphisms are associated with rare cases of PD (Grimes et al., 2006; Jacobsen et al., 2008; Weidong Le et al., 2003; Zheng, Heydari, & Simon, 2003), and NURR1 expression is significantly decreased in sporadic PD brains, specifically in the nigral neurons with α-synuclein inclusions (Le et al., 2008), implicating deficits in NURR1 expression as a potential risk factor for PD. Furthermore, age-related decline in NURR1-positive SN neurons highly correlated with TH-positive neuronal decline in the human brain (Chu, Kompoliti, Cochran, Mufson, & Kordower, 2002). Given the incidence of age-related decline of nigral DA neurons (Fearnley & Lees, 1991) and that ageing is one of the main risk factors for PD, age-related decrease in NURR1 expression may promote DA neuronal dysfunction in pathology.

NURR1 is critical for midbrain DA neuronal development and differentiation, and its expression persists in the postnatal and adult brain where it regulates the maintenance of a functional DA phenotype (Kadkhodaei et al., 2009; Zetterström et al., 1997; Zetterström, Williams, Perlmann, & Olson, 1996). Prior to the appearance of phenotypically distinct DA neurons at embryonic day 10.5 (E10.5), NURR1 mRNA expression appears at approximately E10.5 in the ventral aspect of the developing mouse midbrain (mesencephalic flexure), and persists in the adult brain (Zetterström et al., 1997; Zetterström, Williams, et al., 1996). In NURR1 deficient mice, midbrain DA neurons failed to develop, and the postnatal SN and ventral tegmental area (VTA) were devoid of DA neurons, and striatal dopamine was dramatically reduced by 98% (Le et al., 1999; Zetterström et al., 1997). However, DA neurons in other regions including the hypothalamus, and olfactory bulb were preserved (Le et al., 1999). Adult heterozygotes, although lack histological and behavioural impairments, exhibit deficits in DA neurotransmission in the nigrostriatal pathway, and are more susceptible to neurotoxins.
Conditional ablation of NURR1 in late embryogenesis was associated with a rapid loss of striatal DA and nigral neuronal degeneration (Kadkhodaei et al., 2013; Kadkhodaei et al., 2009). Several DA neuronal markers including tyrosine hydroxylase (TH), dopamine transporter (DAT), aromatic acid decarboxylase (AADC), and vesicular monoamine transporter 2 (VMAT2) were diminished in NURR1-deficient mice, and striatal innervation is absent indicating severe phenotypic deficiency. In contrast, nigral NURR1 ablation in adult transgenic mice carrying a floxed NURR1 allele induced by AAV-mediated expression of Cre recombinase resulted in progressive phenotypic dysfunction characterised by striatal DA loss, and axonal and dendritic dystrophy that preceded DA neuronal death, similar to that reported in early human PD, and late onset behaviour deficits over 3 to 4 months (Cheng et al., 2010; W Le, Conneely, He, Jankovic, & Stanley, 1999).

A more comprehensive NURR1 ablation is achieved exclusively in mature DA neurons by tamoxifen-induced NURR1 deletion in conditional NURR1 gene-targeted mice generated by crossing floxed NURR1 mice with mice harbouring a CreER\textsuperscript{T2} allele regulated by the dopamine transporter locus (Kadkhodaei et al., 2013). NURR1 ablation during embryogenesis (E13.5) resulted in a near complete loss of TH within the striatum and DA neuronal cell bodies within the SNpc. Deletion of NURR1 in mature DA neurons resulted in a modest decrease in expression of various DA phenotypic markers including TH, VMAT2 and DAT, while the DA neuronal cell bodies in the SNpc remained intact. However, the integrity of fibres extending to the striatum and SNpr were compromised as characterised by reduced fibre density and fragmented dendrites frequently interrupted by varicosities (Kadkhodaei et al., 2013). These phenotypic abnormalities were associated with significant deficits in striatal DA transmission and motor function. Of interest, profiling the DA neuronal transcriptome of laser microdissection captured SNpc and VTA by RNA sequencing indicated that a large number of nuclear-encoded mitochondrial genes that regulate oxidative phosphorylation were significantly downregulated, suggesting that NURR1 may regulate mitochondria in midbrain DA neurons, and deficits in NURR1 may contribute to mitochondrial dysfunction and oxidative stress associated with PD pathology, as discussed in section 1.4.3.

Although familial forms of PD attributed to NURR1 mutations are rare, post-mortem analysis of brains from neuropathologically verified sporadic PD cases reveal a significant reduction in NURR1 expression in the SN, specifically in DA neurons with α-synuclein inclusions
indicating that the loss of NURR1 expression is related to the evolution of the disease process. This phenomenon is replicated in transgenic mice that selectively express mutant α-synuclein in midbrain DA neurons, wherein cytosolic and axonal α-synuclein aggregation was associated with diminished levels NURR1 protein, but not mRNA expression (Lin, Parisiadou, et al., 2012). Expression of NURR1 target genes, TH, DAT, VMAT2 and tyrosine kinase Ret was decreased in neurons with α-synuclein aggregates. In contrast, nuclear NURR1 expression in DA neurons that lacked α-synuclein pathology was comparable to that in naïve animals. Furthermore, in midbrain neuronal cultures derived from mutant α-synuclein animals, the pan-protease inhibitor MG132 increased NURR1 protein expression and promoted survival of TH-positive DA neurons. These results implicate a dynamic α-synuclein-dependent pathogenic mechanism that suppresses NURR1 expression at the post-translational level, which subsequently may exacerbate PD pathology.

Collectively, the above studies suggest that NURR1 regulates development and phenotypic maintenance of midbrain DA neurons, and NURR1 dysfunction contributes to phenotypic deficits and neurodegeneration in the DA nigrostriatal circuitry.

1.7.2. Glial NURR1 downregulates inflammation

A recent study by Saijo et al. suggests that the role of NURR1 in the SN extends beyond phenotypic regulation of midbrain DA neurons, wherein NURR1 expression in astrocytes and microglia protects neurons against pathological neuroinflammation by suppressing the expression of inflammatory genes (Saijo et al., 2009).

NURR1 upregulation in the seizure-damaged hippocampus and 6-OHDA-lesioned nigra has been reported previously (Crispino, Tocco, Feldman, Herschman, & Baudry, 1998; Ojeda, Fuentealba, Galleguillos, & Andrés, 2003). LPS that is commonly used in animal models to replicate chronic nigral inflammation characteristic of PD also induced NURR1 expression in the mouse nigra (Saijo et al., 2009). Subsequent lentiviral-mediated NURR1 knockdown predominantly in nigral microglia and astrocytes exaggerated LPS-induced expression of inflammatory mediators such as TNFα, IL-1β, and inducible nitric oxide synthase (iNOS), and significantly increased DA neuronal death. Surviving neurons exhibited morphological deficits, as evident by altered neuronal size, and reduced or absent processes. Furthermore,
overexpression of mutant α-synuclein in the nigra coupled with glial NURR1 knockdown also dramatically increased inflammation and DA neuronal loss. These results suggested that unresolved microglia-astrocyte inflammatory communication has the potential to establish a self-sustaining inflammatory state that promotes neurodegeneration, and NURR1 plays a crucial role in regulating glial inflammatory processes.

NURR1 protein and mRNA expression was detected in human and mouse astrocytes and microglia in vitro, and inflammatory stimulation upregulated NURR1 mRNA transcripts. shRNA-mediated silencing of NURR1 expression was associated with elevated expression of inflammatory molecules in response to LPS, and LPS significantly increased the neurotoxicity of CM derived from NURR-deficient astrocyte or microglia cultures, as evident by exaggerated death of Neuro2A cells and TH-positive DA neurons differentiated from mouse neuronal stem cells.

In vitro cultures revealed that LPS was not effectively sensed by neurons and did not directly cause neuronal death, and microglia were orders of magnitude more responsive to LPS than astrocytes, as evident by a significant induction of inflammatory molecules in microglia. Therefore Saijo et al. limited the investigation of astrocytes in LPS-induced inflammation to their role in potential amplification of microglia-derived inflammatory signals. Sequential transfer of CM from LPS-stimulated naïve microglia to non-stimulated astrocytes prior to treating neuronal cultures significantly increased CM neurotoxicity, indicating that astrocytes amplify microglia-derived inflammation. NURR1 knockdown in either microglia or astrocytes increased the neurotoxicity of the sequentially conditioned media. Whether LPS-stimulated astrocytes induce a similar amplification of inflammatory responses in microglia warrants further characterisation. A role for microglia-astrocyte communication in orchestrating inflammation is further supported by the induction of NURR1 mRNA in primary astrocytes upon stimulation with TNFα or IL-1β, inflammatory molecules typically released by microglia. Moreover, NURR1 knockdown dramatically increased the expression of astrocytic pro-inflammatory genes such as iNOS, and neutrophil cytosolic factor 1 (Ncf1) that regulate NO and ROS generation, respectively, and granulocyte-monocyte colony stimulating factor 1 that supports microglia proliferation, in response to inflammatory stimulation. These results suggest that glial NURR1 exerts a neuroprotective effect by downregulating the expression of proinflammatory mediators that are implicated in pathological neuroinflammation in neurodegenerative diseases.
Saijo et al. proposed that NURR1 interacts with the CoREST complex to downregulate proinflammatory gene expression via a transrepression pathway that operates in a feedback manner, to restore expression of genes regulated by the transcription factor complex NF-κB-p65 to basal levels (Saijo et al., 2009). Utilising an array of *in vitro* assays that included chromatin immunoprecipitation and co-immunoprecipitation assays coupled with knockdown or mutagenesis of various candidate genes based on characterised pathways in nuclear receptor-dependent transcriptional regulation, the authors identified key players in NURR1-mediated transrepression. NURR1 is recruited to the NF-κB-p65 complex on inflammatory gene promoters in a manner dependent on GSK3β-mediated phosphorylation of p65 (Buss et al., 2004). NURR1 subsequently recruits the CoREST corepressor complex in a manner dependent on nemo-like kinase-mediated phosphorylation of NURR1. CoREST assembles various chromatin-modifying enzymes, including histone methyltransferase G9a, histone demethylase, lysine-specific demethylase and histone deacetylase 1 and 2 (Shi et al., 2003) that may regulate dissociation of the NF-κB-p65 complex from proinflammatory promoter, thereby effectively terminating transcription.

Inactive NF-κB is sequestered in the cytoplasm by an inhibitory subunit IκB under basal conditions (Hohmann, Remy, Scheidereit, & van Loon, 1991). Various inflammatory stimuli including IL-1, TNFα, bacterial glycoproteins, and oxidative stress regulate IκB phosphorylation that results in the dissociation the NF-κB/IκB complex. The activated NF-κB translocates to the nucleus, and enhances transcription of proinflammatory genes such as TNFα, IL-2, IL-6, IL-8, GM-CSF, and iNOS that contain a specific NF-κB consensus sequences in their promoter regions (Lawrence, 2009). In response to CNS insults, NF-κB is highly activated, and expression of NF-κB-dependent inflammatory genes is upregulated, implicating NF-κB in CNS pathology (Stephenson et al., 2000). Nuclear localisation of NF-κB, in both neurons and glia is increased in post-mortem brains afflicted with PD and other neurodegenerative diseases such as Huntington’s disease and Alzheimer’s disease (Hsiao, Chen, Chen, Tu, & Chern, 2013; Hunot et al., 1997; Kaltschmidt, Uherek, Volk, Baueuerle, & Kaltschmidt, 1997). This increase in NF-κB activity and a corresponding upregulation of inflammatory gene expression are recapitulated in disease models (Ghosh et al., 2007; Lee, Park, Ji, Lee, & Lee, 2014). MPTP intoxication has been shown to induce a marked increase in NF-κB nuclear translocation and transcriptional activity in the mouse nigra, predominantly in astrocytes and microglia (Aoki, Yano, Yokoyama, Kato, & Araki, 2009; Ghosh et al., 2007). Downregulation of nigral NF-κB activity via the inhibition of its activation complex,
IκB kinase attenuated MPTP-induced glial reactivity and expression of proinflammatory molecules, enhanced survival and function of the nigrostriatal pathway, and ameliorated motor deficits (Ghosh et al., 2007). Collectively, these results suggest that glial NURR1-mediated downregulation of excessive NF-κB activity may attenuate chronic inflammation and neurodegeneration in PD. Given that phenotypic dysfunction, neurodegeneration and chronic inflammation are cardinal pathological features in PD, and the multifaceted functional relevance of NURR1 in both neurons and astrocytes in targeting these molecular pathways, therapeutic overexpression of NURR1 in both these cellular elements may potentially yield substantial efficacy.

1.8. Astrocyte-targeting AAV vectors

As discussed in detail in section 1.4, both neuronal and astrocytic dysfunction contribute to neurodegeneration in various CNS disorders, and hence the ability to either selectively or collectively transduce neurons and astrocytes in a context-dependent manner is advantageous. At the commencement of this thesis, CNS therapeutic targeting strategies had been largely neurocentric; however, an increased appreciation of the role of astrocytes in health and disease has directed an interest in identifying astrocytic therapeutic targets, and developing astrocyte-tropic vectors. As reported in numerous studies, AAV2 directs transgene expression exclusively to neurons, and transduction is relatively confined to the immediate vicinity of the injection site (Bartlett, Samulski, & McCown, 1998; Markakis et al., 2010). Incorporation of the astrocyte-specific GFAP promoter failed to alter this serotype’s inherent neurotropism (Xu, Janson, Mastakov, Lawlor, Young, Mouravlev, Fitzsimons, Choi, Ma, & Dragunow, 2001). However, the characterisation of additional serotypes that exhibit novel tropisms and superior transduction efficacies, and exhibit strong potential for the treatment of PD and other neurodegeneration diseases is rapidly expanding the repertoire of AAV vectors available for clinical application (Broekman, Comer, Hyman, & Sena-Esteves, 2006; C. Burger et al., 2004; Taymans et al., 2007).

A number of alternative AAV serotypes including AAV5, 8 and 9 that initially appeared to be predominantly neurotropic, repeatedly exhibit propensity to transduce astrocytes in the rodent and non-human primate CNS. Exclusive neuronal transduction by all three serotypes has been observed in various CNS regions including the hippocampus, striatum, substantia nigra,
thalamus, and spinal cord (C. Burger et al., 2004; Cearley et al., 2008; Taymans et al., 2007; Van der Perren et al., 2011). Specifically, in the rat substantia nigra, AAV5, 8 and 9 selectively mediated cytomegaloviral (CMV) promoter-regulated eGFP expression in neurons, with serotypes 8 and 9 transducing over 70% of TH-immunoreactive DA neurons, while AAV5 transduced ~50% of the DA neuronal population (Van der Perren et al., 2011). However, numerous studies have shown that in the presence of the astrocyte-specific GFAP promoter, following infusion into the hippocampus or striatum, astrocyte-tropism of these AAV serotypes was significantly increased (Drinkut, Tereshchenko, Schulz, Bahr, & Kugler, 2012; Lawlor, Bland, Mouravlev, Young, & During, 2009). At the commencement of this thesis, astrocyte transduction efficacy of these vectors in the rat SNpc remained to be characterised.

In a previous study conducted in our laboratory, AAV8 coupled with a hybrid CMV early enhancer/ chicken β-actin (CAG) promoter-regulated eGFP transgenic cassette (AAV8-CAG-eGFP), in addition to transducing neurons in the hippocampus, striatum and SNpc with high efficiency, transduced a small number astrocytes, while an AAVrh43 vector mediated minimal, but exclusive astrocytic transduction (Lawlor et al., 2009). Limiting further characterisation to the striatum and hippocampus, increasing vector titre from 4.5 x 10^9 genomes to 3 x 10^10 genomes improved efficacy of astrocyte transduction; however, this was accompanied by a significant neuronal transduction. Subsequent incorporation of the GFAP promoter into the vector genomes markedly altered tropism of both AAV8 and AAVrh43. The GFAP promoter in the context of AAVrh43 directed transgene expression almost exclusively to astrocytes, and significantly enhanced astrocyte-tropism of AAV8 with up to 88% of the total transduced cells comprising of astrocytes (Lawlor et al., 2009).

Direct intraparenchymal infusion of AAV9 has been shown to exclusively transduce neurons in various brain regions including the cortex, hippocampus, thalamus, striatum and substantia nigra (Cearley et al., 2008; Foust et al., 2009; Taymans et al., 2007). While predominantly neuronal transduction was previously observed in our laboratory following intraparenchymal infusion of a CAG-regulated GFP expression cassette encapsidated in AAV9 (AAV9-CAG-GFP) into the rat hippocampus (unpublished data), substituting the CAG promoter with a GFAP promoter directed transgene expression predominantly to astrocytes (Young et al., 2014). Foust et al. showed that following intravenous administration, AAV9-CBA-GFP effectively traversed the BBB, and transduced astrocytes extensively in the adult mouse brain and gray matter of the spinal cord, indicating that the route of entry may determine AAV
transduction properties (Foust et al., 2009). In contrast, intravenously administered AAV9 predominantly transduced neurons in the neonatal CNS. In the adult brain, astrocytic endfeet that completely ensheath the CNS microvasculature may restrict diffusion of AAV9 to neuronal elements (Mathiisen et al., 2010). Furthermore, astrocytic endfeet may exhibit a distinct cell surface molecular profile (Abbott, Ronnback, & Hansson, 2006), and the vascular entry route may allow astrocytic transduction by exposing AAV9 to capsid-binding receptors selectively expressed on astrocytic endfeet (Abbott et al., 2006), whereas immature astrocytes that lack fully developed endfeet may evade transduction, and allow greater diffusion of AAV9 within the neonatal brain (Abbott et al., 2006).

In contrast to Foust et al, utilising constitutively active viral promoters, Gray et al. observed that following intravenous administration, AAV9 transduced neurons and astrocytes extensively throughout the adult mouse CNS, with transduction of neurons exceeding astrocytes by ~2:1 (Gray et al., 2011). The authors consistently observed the same pattern of cell transduction in the mouse CNS when different purification methods, vector doses, and promoters were used, presenting no explanation for the shift in vector transduction profile from that reported by Foust et al. In comparison to the adult mouse CNS, in the juvenile non-human primate CNS, transduction was predominantly astrocytic, and the overall transduction efficiency was considerably lower (Gray et al., 2011). Species-specific molecular and functional characteristics of CNS cellular elements that regulate BBB transport and subsequent vector infection may contribute to the differences in vector delivery, tropism and transduction efficacy. Furthermore, high transduction of peripheral organs including liver, kidney, heart, and skeletal muscle, and the prevalence of neutralising antibodies that minimise transduction efficacy warrant further investigation prior to clinical translation of intravenous gene therapy (Gray et al., 2011). Various tissue-detargeting strategies have been explored to minimise peripheral transduction including incorporation of target sequences of specific endogenous miRNA into the vector genome to suppress peripheral transgene expression, and random mutagenesis of capsids sequences that selectively abolish tropism in peripheral organs (Pulicherla et al., 2011; Xie et al., 2011).

AAV5 isolated from human tissue is another serotype that has been shown to successfully transduce astrocytes (Bantel-Schaal & Zur Hausen, 1984). In addition to transducing a large volume of tissue, AAV5 efficiently and stably transduced both neurons and astrocytes in the rodent and non-human primate brain (Davidson et al., 2000; Drinkut, Tereshchenko, Schulz, Bahr, & Kugler, 2011; Drinkut et al., 2012; Markakis et al., 2010). Comparative analysis of
AAV serotypes 1-6 demonstrated that AAV5-CMV-eGFP transduced a significantly larger volume in the non-human primate caudate nucleus and substantia nigra, and mediated transgene expression in neurons and astrocytes with equal efficiency (Markakis et al., 2010). In the mouse hippocampus AAV5 predominantly directed transgene expression to astrocytes (Ortinski et al., 2010). The percentage of astrocyte transduction achieved with the CMV and GFAP promoters in the context of AAV5 was 85% and 99%, respectively.

In the naïve rat hippocampus, efficient AAV5 transduction, and CBA promoter-regulated transgene expression were achieved predominantly in neurons (Weinberg, Blake, Samulski, & McCown, 2011). In the kainic acid-lesioned hippocampus, neuronal degeneration correlated with reduced cellular transduction. Interestingly, in the seizure-afflicted hippocampus, AAV5-mediated transgene expression was increasingly localised to astrocytes, suggesting that neuropathology-associated degeneration and phenotypic alterations may alter AAV vector tropism and transduction efficacy. For example, reactive astrocytes in the pilocarpine-lesioned hippocampus upregulate expression of α-5 integrin subunits (Fasen, Elger, & Lie, 2003). Given that αβ integrin facilitates AAV cellular endocytosis, their selective upregulation on reactive astrocytes may significantly enhance astrocyte tropism of AAV vectors (Summerford et al., 1999). Furthermore, small punctate accumulations of fluorescently-labelled AAV capsids in microglia within 24 hours of vector injection (Bartlett et al., 1998) suggest that in the context of neuropathology, reactive microglia that upregulate defensive mechanisms in pathology may potentially enhance viral vector clearance, and therefore may occlude efficient transduction.

AAV5-mediated exclusively neuronal or astrocytic transgene expression in the mouse striatum has been achieved via the use of neuronal-specific human synapsin 1 (SYN) promoter or astrocyte-specific GFAP promoter, respectively (Drinkut et al., 2012), suggesting that both neurons and astrocytes express high affinity cell surface receptors for AAV5 infection, and stringent cell-specific promoter elements in the context of AAV5 could regulate vector tropism. Following AAV5-mediated unilateral transduction of striatal astrocytes, transgenic GDNF expression was strictly confined to the nigrostriatal pathway, via retrograde GDNF transport (Leitner et al., 1999). In contrast, striatal neuronal transduction resulted in extensive delivery of GDNF to extra-nigral midbrain regions in both ipsilateral and contralateral hemispheres (Ciesielska et al., 2011). The detailed circuitry of the human brain is yet to be fully elucidated, and it is impossible to predict the extent of neuronal projections to remote nuclei in the CNS, posing a risk of potential detrimental
effects associated with delivery of potent neurotherapeutic molecules to off-target regions (Manfredsson, Okun, & Mandel, 2009). In comparison to neuronal GDNF, astrocytic GDNF was equally efficient in preserving the ipsilateral nigrostriatal pathway and striatal DA transmission in mice systemically administered with MPTP, and improved motor function in rats that received unilateral striatal 6-OHDA lesions. While neuron-derived GDNF exerted a neurotrophic effect in both ipsilateral and contralateral basal ganglia, astrocyte-derived GDNF bioactivity was restricted to ipsilateral nigrostriatal pathway, suggesting that astrocytic expression of neurotrophic molecules may minimise potential off-target effects without compromising therapeutic efficacy. Furthermore, a recently published study demonstrated CMV-regulated GFP expression primarily in astrocytes in the mouse and non-human primate cerebral cortex following infusion of AAV5, 8, and 9 vectors, with all serotypes mediating equivalently high levels of transgene expression (Watakabe et al., 2015).

Results pertaining to AAV serotype-specific tropisms and transduction efficiencies in the CNS are difficult to interpret due to differences in vector titres, purification methods, route of administration, DNA regulatory elements and transgenes, CNS region of interest, duration of transgene expression, and animal species in different studies. Therefore, for a given study, comparative analyses of all serotypes of interest are required to accurately determine serotype-specific differences in vector properties.

Furthermore, in vitro and in vivo studies reveal extensive astrocytic molecular heterogeneity in developmental, physiological and pathological processes that imply functional diversity. Astrocytic genes that are heterogeneously expressed include those that encode surface glycoproteins; membrane ion channels, transporters and receptors; various cytokines and chemokines; gap-junction proteins; and cellular enzymes that regulate neurotransmitter metabolism (Cai, Schools, & Kimelberg, 2000; Fitting et al., 2010; Karavanova, Vasudevan, Cheng, & Buonanno, 2007; Macnab & Pow, 2007; Regan et al., 2007; Reuss, Leung, Ohlemeyer, Kettenmann, & Unsicker, 2000; Tang, Taniguchi, & Kofuji, 2009; Walz & Lang, 1998; Yeh, Lee, Gianino, & Gutmann, 2009). We have previously noted that the GFAP promoter coupled with the astrocyte-tropic AAV serotype rh43 predominantly targeted astrocytes in the rat hippocampus; however this vector construct failed to mediate any transgene expression in the SNpc, suggesting that astrocyte heterogeneity may influence efficacy of transduction and transgene expression (unpublished data). Therefore, in addition to identifying astrocyte-tropic native serotypes, and engineering novel serotypes that include mosaics (von Jonquieres et al., 2013), and random mutation-diversified variants (directed
evolution) (Koerber et al., 2009) that exhibit astrocyte tropism, further efforts have focused on characterising astrocyte-specific promoters that exhibit efficient transcriptional activity in the CNS.

1.9. DNA regulatory elements in the vector genome

While AAV capsids determine the transduction profile of the virus, efficacy and specificity of transgene expression are influenced by elements in the vector-derived genome. Transcriptional inactivity of the vector genome could account for a lack of transgene expression in transduced cells. Elements such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and bovine growth hormone polyadenylation (BGH-pA) are routinely incorporated into vector genomes to enhance transgene expression levels, and confer stability of mRNA transcripts. (Huang & Yen, 1995; Pfarr et al., 1986; Xu, Janson, Mastakov, Lawlor, Young, Mouravlev, Fitzsimons, Choi, Ma, Dragunow, et al., 2001; Zufferey, Donello, Trono, & Hope, 1999).

Transgenes are typically placed under control of promoters that drive transgene expression. Constitutively active viral promoters such as the CMV or CAG mediate high levels of transgene expression in the CNS (Klein et al., 1998; Paterna, Moccetti, Mura, Feldon, & Bueler, 2000; Tenenbaum et al., 2004). However, viral promoters are susceptible to epigenetic modification such as promoter methylation that silences transcriptional activity over time (Brooks et al., 2004).

Most AAV serotypes characterised to date exhibit tropism for both neurons and glia with varying efficacies (Drinkut et al., 2012; Gray et al., 2011; Lawlor et al., 2009). To selectively dictate transgene expression to specific cell populations, and minimise expression in others, cell-specific transgenic promoters that are regulated by the same endogenous transcriptional factors as the corresponding endogenous gene are routinely utilised. Widely used neuronal-specific promoters such as neuron-specific enolase (NSE), platelet-derived growth factor-β chain (PDGF-β), synapsin 1, and TH, in the context of neurotropic AAV serotypes efficiently target CNS neurons (Klein et al., 1998; Nathanson, Yanagawa, Obata, & Callaway, 2009; Oh, Hong, Huh, & Kim, 2009; Paterna et al., 2000). Similarly, the myelin basic promoter MBP has been shown to direct transgene expression to oligodendrocytes following AAV8 vector-
mediated gene transfer (Lawlor et al., 2009). Table 1 summarises tropisms of commonly used AAV serotype and promoter combinations in various mammalian brain regions.
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Promoter</th>
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<th>Brain Region</th>
<th>Tropism</th>
<th>Reference</th>
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Table 1 Tropisms of commonly used AAV serotype and promoter combinations in various mammalian brain regions
1.9.1. Astrocyte-specific promoters

1.9.1.1. GFAP promoter

The astrocyte-specific intermediate filament protein, GFAP is considered a classic marker of astrocytes, and currently the most widely used promoter in astrocyte-selective gene transfer applications is the 2.2kb transgenic GFAP promoter. The intermediate filament (IF) network of the mature astrocytic cytoskeleton is composed of GFAP, vimentin, nestin and synemin. The initiation of GFAP expression is a defining element of astrocytic differentiation (Bignami & Dahl, 1974), and its upregulation is associated with reactive astrogliosis (Eng & Ghirnikar, 1994). Combined suppression of GFAP and vimentin in GFAP+/Vimentin−/− mice abolished the formation and upregulation of IF networks in reactive astrocytes (Pekny et al., 1999), and compromised astrocytic motility, proliferation, structural and functional maintenance of the BBB, and trafficking of vesicles and glutamate transporters (Hughes, Maguire, McMinn, Scholz, & Sutherland, 2004; Liedtke et al., 1996; Potokar et al., 2007; Rutka et al., 1994; Weinstein, Shelanski, & Liem, 1991), indicating that IF networks are essential cellular components that coordinate heightened cellular mechanisms in reactive astrocytes in response to pathological stimuli.

A 2.2kb 5′ sequence that span -2163bp to +47bp relative to the transcription start site of the human GFAP gene constitute the widely used GFAP promoter that selectively directs transgene expression to astrocytes (Besnard et al., 1991; Brenner, Kisseberth, Su, Besnard, & Messing, 1994b). Pioneering studies using transgenic mice expressing the LacZ reporter gene linked to the human GFAP promoter demonstrated that this promoter mediates efficient transgene expression specifically in CNS astrocytes (Brenner et al., 1994b). Furthermore, reactive astrogliosis and upregulation of endogenous GFAP in response to a stab injury was paralleled by enhanced expression of LacZ. Following lentiviral-mediated astrocyte transduction in the striatum, GFAP promoter-regulated increase in transgene expression in a manner dependent on the severity of ibotenic- or 6-OHDA-induced of reactive astrogliosis has also been observed (Jakobsson, Rosenqvist, Mårild, & Lundberg, 2006). These results suggest that the GFAP promoter contains one or more regulatory elements activated during reactive gliosis, a phenomenon that can be exploited to couple expression of therapeutic genes to the ‘reactivity’ of astrocytes, in the context of neurodegenerative diseases.
1.9.1.2. gfaABC1D promoter

Furthermore, the characterisation of sequence motifs in the endogenous GFAP promoter region that regulate its expression has facilitated the development of shorter transgenic promoters that exhibit equivalent or superior transcriptional activity to their 2.2kb predecessor in transgenic mice. These promoter variants exhibit specific patterns of astrocytic transcriptional activity in a region-dependent manner in the CNS, revealing that region-specific astrocytic regulatory mechanisms may specify GFAP expression (Lee, Messing, Su, & Brenner, 2008). Furthermore, relatively high transgene expression in certain neuronal populations following deletion of specific motifs in the promoter sequence indicates that neuronal transcriptional machinery may negatively regulate GFAP expression.

A recent study characterised the development of a transgenic mouse model that expressed an optimised 681bp GFAP promoter variant, gfaABC1D that retained only those regulatory sequences required to replicate transcriptional activity and astrocyte specificity of the 2.2kb GFAP promoter (Lee et al., 2008). This promoter retains sequence subregions that contain binding sites for several putative transcriptional factors expressed in the midbrain, and in astrocytes under physiological and pathological conditions. Furthermore, approximately twofold greater activity of the gfaABC1D promoter, particularly in the midbrain and hindbrain suggested that the removal of redundant sequences may improve accessibility of certain recognition motifs to transcription activators resulting in greater transcriptional efficiency.

1.9.1.3. Aldehyde dehydrogenase family 1, member 1 promoter

It is increasingly evident that astrocytes exhibit extensive molecular heterogeneity, and GFAP, traditionally considered a pan-astrocytic marker is one such molecule that is heterogeneously expressed in astrocytes (Cahoy et al., 2008; Lee et al., 2008; Walz & Lang, 1998; Yang et al., 2011). However, alternative astrocyte markers such as aldehyde dehydrogenase family 1, member 1 (ALDH1L1) that exhibit homogenous expression patterns throughout the CNS have also been identified (Cahoy et al., 2008). The ALDH superfamily of enzymes catalyse the NAD(P)⁺-dependent oxidation of a wide range of endogenous and exogenous aldehydes. Genome-wide transcriptional profiling showed that the ALDH1L1 isoform is selectively expressed in astrocytes throughout the brain in a pattern more
consistent with pan-astrocyte expression than the classic astrocyte marker, GFAP (Cahoy et al., 2008). ALDH1L1 catalyses the conversion of 10-formyltetrahydrofolate and NADP+ to tetrahydrofolate, and NADPH and hence plays an important role in various cellular pathways such as de novo nucleotide biosynthesis and regeneration of methionine (Krupenko, 2009). ALDH1L1 mRNA was shown to be expressed throughout the CNS, whereas GFAP mRNA is predominantly expressed in the white matter. Moreover, double labelling with GFAP and ALDH1L1 antibodies revealed that while GFAP is primarily expressed in the cell body and main processes of astrocytes, ALDH1L1 is expression extends to the finer astrocytic processes. Furthermore, ALDH1L1 labels both GFAP-positive and GFAP-negative astrocytes. A bacterial artificial chromosome (BAC) transgenic mouse that expresses eGFP under the control of the full-length genomic ALDH1L1 promoter replicated the astrocytic-specific pattern of expression of endogenous ALDH1L1 (Yang et al., 2011). Increased eGFP expression in reactive astrocytes in the striatum of BAC ALDH1L1 eGFP mice 7 days after an acute stab-lesion indicated that similar to GFAP, ALDH1L1 is upregulated in pathology. These results reveal the potential application of putative ALDH1L1 promoter sequences for the expression of transgenes in AAV-based gene delivery.
1.10. Outline of thesis

The main aims of this thesis are to generate an astrocyte-tropic AAV vector construct that selectively and efficiently targets astrocytes in the SNpc, and subsequently utilise this vector to evaluate the effect of astrocyte-specific overexpression of the therapeutic transcription factor NURR1 on the survival and phenotypic integrity of the nigrostriatal pathway in a rat 6-OHDA model of PD.

Chapter two describes the development and characterisation of four novel promoters derived from the astrocyte-specific ALDH1L1 gene. The transcriptional activity and cell-specificity of these promoters in the context of the astrocyte-tropic AAV serotypes AAV5, 8 and 9 were determined in the rat SNpc.

Chapter three describes the comparative analysis of the transduction properties of the astrocyte-tropic AAV serotypes AAV5 and AAV9 in primary astrocyte cultures and in the rat SNpc. The ability of the promoters, CMV, GFAP and GFAP variant gfaABC1D to regulate tropism and transgene expression of AAV-mediated gene transfer was also comparatively analysed *in vitro* and *in vivo*. Subsequently, vector-mediated expression of NURR1 and an additional therapeutic factor Nrf2 was analysed *in vitro* and *in vivo*. Prior to *in vivo* application in a PD model, *in vitro* transactivation and transrepression assays were performed to confirm functionality of the NURR1 protein.

Chapter four describes the investigation of the neuroprotective potential of astrocyte-targeted overexpression of NURR1 in a 6-OHDA-induced model of PD. Neuroprotective effects were assessed by behavioural tests and immunohistochemical analysis of brains.

Chapter five summarises and discusses findings of this thesis, conclusions and potential future directions.
Chapter 2. Characterisation of novel aldehyde dehydrogenase family 1, member 1 promoters
2.1. Introduction

Gene delivery vehicles that exhibit cell- and region-specificity, and mediate optimal levels of transgene expression in the context of disease are crucial for achieving therapeutic efficacy. To date, clinically-approved AAV vectors for CNS gene therapy almost exclusively target neurons, and astrocytes that regulate CNS physiology and contribute to disease pathogenesis represent a largely unexplored therapeutic target in the clinical setting. A rapidly expanding repertoire of novel AAV serotypes coupled with a greater understanding of molecular pathways that dictate disease pathogenesis have allowed the development of AAV vectors that exhibit diverse tropisms and efficient transduction. Effective astrocyte-targeting has been achieved by coupling astrocyte tropic AAV serotypes with the classic astrocyte-specific GFAP promoter. However, astrocytic phenotypic diversity, including heterogeneous expression of GFAP may influence the transduction efficacy of AAV vectors in a region-dependent manner. We have previously noted that the GFAP promoter in the context of the astrocyte-tropic AAV serotype rh43 predominantly targeted astrocytes in the rat hippocampus; however this vector construct failed to mediate any transgene expression in the SNpc, suggesting that astrocyte heterogeneity may influence efficacy of transduction and transgene expression (unpublished data).

Therefore, the aim of this chapter was to develop a novel AAV vector system that would selectively and efficiently target nigral astrocytes for therapeutic protein overexpression. ALDH1L1 is a recently characterised pan-astrocytic marker that appears to be more homogenously expressed than the classic astrocytic marker, GFAP [1]. In BAC transgenic mice, the full-length genomic ALDH1L1 promoter replicated the astrocyte-specific expression patterns of the endogenous ALDH1L1 gene under both physiological and pathological conditions. The aim of this chapter was to isolate and optimise putative ALDH1L1 promoter sequences that can potentially transcriptionally regulate AAV vector-delivered expression cassettes in astrocytes. The ability of these constructs to target astrocytes in the SNpc of rats was tested in the context of three AAV vector serotypes.
2.2. Materials and Methods

2.2.1. Restriction enzyme digestion

All AAV expression plasmids required for this thesis were generated by Dr Alexander Mouravlev. Vector NTI software (Informax, Frederick, MD, USA) was used to generate maps of plasmid constructs using DNA sequence information from Genbank sequences, DNA sequencing, and previously generated AAV expression plasmid sequences in our laboratory. Following DNA cloning, restriction enzyme digests were performed to determine structural integrity of the expression cassette. Restriction enzymes (RE) (New England Biolabs, Ipswich, MA) with known cleavage sites were used to cut the plasmid DNA to confirm the presence of transgenic expression cassette and other regulatory elements. The restriction enzyme reaction mixture was set up in 0.5mL microfuge tubes containing 0.4μg of plasmid DNA, 1μL of 10x reaction buffer, 0.5μL of the appropriate restriction enzyme(s), 1μL of 10x bovine serum albumin (BSA) if required, and sterile dH2O to a final volume of 10μL, and incubated at 37°C (except for SmaI digests which were incubated at 25°C) for 30 minutes. Subsequently, 1μL of 10x sample buffer was added to each RE digest and analysed by 1% TAE agarose gel electrophoresis.

2.2.2. Agarose gel electrophoresis:

Agarose gel electrophoresis was performed to separate DNA fragments by molecular size. 4% agarose (GibcoBRL) weight/volume (w/v) was prepared in 1 x Tris-Acetate EDTA (TAE) buffer in a microwave oven. 0.1μL of ethidium bromide (GibcoBRL, 100mg/mL) was added to the cooled gel and set in a casting tray. Subsequently, the gel was placed into an electrophoresis tank (BioRad, Hercules, CA) and submerged in 1 x TAE buffer. DNA samples containing 1 x sample buffer were loaded into wells adjacent to 3μL of DNA molecular mass standard 1kb+ ladder (GibcoBRL). 200V current was applied to separate the DNA fragments. The gel was exposed to UB light to visualise the DNA bands in a GeL Doc (BioRad).
2.2.3. Large-scale preparation of plasmid DNA

Following confirmation of sequence integrity of the cloned expression plasmids, large-scale plasmid DNA was prepared and purified. An aliquot (100µL) of DH5α E. coli cells stored at -80ºC was thawed on ice. 50ng of plasmid DNA was subsequently added to the cells and incubated for 30 minutes on ice. The cells were heat-shocked at 37ºC for 5 minute and cooled immediately on ice for 1 minute. 400µL Luria Broth (LB) was added to the cell and incubated at 37ºC for 60 minutes. 50µL of cells were streaked onto pre-warmed LB agar plates containing ampicillin and incubated overnight at 37ºC. The following day, a single colony was used to inoculate 100mL of LB containing 50μg/mL ampicillin and incubated at 37ºC in a shaker-incubator 18 hours. The plasmids were purified using MaxiPrep, a PureLinkTM HiPure plasmid DNA purification kit (Invitrogen) according to the manufacturer’s instructions.

2.2.4. Determining plasmid DNA concentration

The concentration of purified plasmid DNA was determined by UV spectrophotometry using a TE buffer-calibrated NanoDrop (Wilmington, DE). The absorbance of 1µL of purified plasmid DNA was measured at 260nm and 280nm. An A_{260/280} ratio of 2.0>1.8 indicated sample purity, where an A_{260/280} ratio <1.8 indicates the presence of protein contaminants, while an A_{260/280} ratio >2.0 indicates RNA contamination. The plasmid DNA was subsequently diluted in TE buffer to achieve a final concentration of 1µg/µL.

2.2.5. AAV vector production

AAV vector packaging and purification was kindly conducted by Dr Dahna Fong. AAV vector particles were generated by co-transfecting HEK 293 cells with AAV expression plasmids encoding the ITR-flanked transgenic cassette and AAV helper plasmids encoding rep and cap, and Ad helper functions (During, Young, Baer, Lawlor, & Klugmann, 2003). Packaged vector particles were subsequently iodixanol-gradient purified to yield high purity vector stock.
2.2.5.1. HEK293 cell transfection for rAAV vector packaging

For each AAV vector, HEK293 cells were seeded onto five 15cm tissue culture plates (Nunc) at a density of 2 x 10^7 cells in 25mL of DMEM/ 10% FBS media per plate and incubated at 37°C, 5% CO2. Three hours prior to transfection, the media was removed and replaced with 25mL of pre-warmed IMDM (Iscove’s Modified Dulbecco’s Medium; GibcoBRL, 5% FBS, 36mM NaHCO3).

For a batch of 5 plates, a transfection mixture consisting of 62.5μg AAV expression plasmid, 62.5μg AAV helper plasmid, 125μg of Ad helper plasmid (pFΔ6), 1.65mL CaCl2, and 12mL of sterile MilliQ water was prepared and filter sterilised through a 0.2μm Acrodisc filter (Pall, Ann Arbor, MI) into a 75cm^2 tissue culture flask (~13mL total volume). To precipitate the DNA for transfection, 13mL of sterile 2 x HeBs buffer (50mM HEPES, 280mM NaCl, 1.5mM Na2HPO4, pH 7.05) was added to the transfection mixture while vortexing for 15 seconds. The transfection mixture was incubated at room temperature for a further 1 minute and 45 seconds for a fine CaPO4 precipitate to form. Subsequently, 5mL of the transfection mixture was added drop-wise in a circular motion onto each plate, plates were swirled gently to evenly distribute the precipitate and incubated at 37°C. Sixteen hours post-transfection, the media was replaced with 25mL of fresh prewarmed DMEM.

2.2.5.2. Preparation of cell lysate containing rAAV vectors

Sixty hours post-transfection, the cells were carefully rinsed with 25mL of pre-warmed 1 x phosphate buffered saline (1 x PBS; 137 mM NaCl, 4.3 mM Na2HPO4, 2.7 mM KCl, 1.47 mM KH2PO4). The 1x PBS was replaced with an additional 25mL of 1x PBS, and the cell monolayer detached with a cell scraper. The detached cells were collected in sterile 50mL tissue culture tubes (~3 tubes per batch) and pelleted by centrifugation at 600g, 4°C for 35 minutes in a Multifuge 3S-R centrifuge (Heraeus, Hanau, Germany) equipped with a swing-out rotor. The supernatant was discarded and the cell pellets were resuspended in a total volume of 8mL of lysis buffer (150mM NaCl, 50mM Tris-HCl pH8.5) and stored overnight at -20°C.

The following day, the cell lysates were thawed at room temperature for ~20 minutes, and 400μL of 10% sodium deoxycholate (Sigma) (final concentration of 0.5% (w/v), and 1.6μL of 250U/μL benzonase (Sigma E1014) (final concentration of 50U/mL) were added to each
tube. Additionally, 8μL of pluronic acid per tube was added to a final concentration of 0.001% to prevent vectors adhering to the tubes. The cell lysates were incubated in a 37°C water bath for 60 minutes, and vortexed briefly every 15 minutes. Subsequently, the cell lysates were centrifuged at 3000g, 4°C for 30 minutes in a Multifuge 3S-R centrifuge equipped with a fixed angle rotor to pellet cellular debris. The supernatant was transferred to a fresh 50mL Falcon tube and stored overnight at -20°C.

2.2.5.3. Iodixanol gradient purification

The supernatants were thawed and centrifuged at 5000g, 4°C for 30 minutes to remove any residual cellular debris, and transferred to new 50mL tubes. Iodixanol gradients were assembled in an ultracentrifuge tube using a spinal needle attached to a 10mL syringe in the following order; 7.5-8.0mL cell lysate; 8.5mL 15% iodixanol; 6.0mL 25% iodixanol; 5.0mL 40% iodixanol; and 5.0mL 54% iodixanol. The ultracentrifuge tubes were sealed and centrifuged at 243,000g using a T865 rotor at 18°C for 90 minutes in an ultracentrifuge (Sorvall WX Ultra 100).

2.2.5.4. Retrieval and concentration of vector sample

Following centrifugation, the ultracentrifuge tubes were secured in a clamp stand, and an 18-gauge needle was inserted into the tube at the 40-54% interface containing the AAV vectors. An additional 21-gauge needle was inserted at the top of the tube to allow air flow. Approximately 3.5 mL of the vector was drawn and diluted with 10mL of 1x PBS containing 1mM MgCl₂ and 2.5mM KCl (MK), and 0.001% pluronic acid (PA), and subsequently transferred to a 15mL capacity 100kDA MWCO Amicon centrifugal filter unit (Millipore), and concentrated by centrifugation at 3000g at 4°C for 15 minutes in a Multifuge 3S-R centrifuge (Heraeus, Hanau, Germany) equipped with a swing-out rotor till ~200μL of vector is retained in the concentrator. The 1x PBS-MK, 0.001% pluronic acid solution was discarded and the vector concentrate resuspended in 13mL 1xPBS-MK and centrifuged for 5 minutes. This step was twice repeated to remove any residual iodixanol. Subsequently, 250μL of concentrated vector was collected from the concentrator and transferred into a 0.5mL microfuge tube. The concentrator was rinsed with an additional 100μL of 1x PBS-
MK, 0.001% pluronic acid to collect any residual vector. The AAV vector suspension (~350μL) was then sterile filtered through a 13mm, 0.2μm Acrodisc filter (Pall) into a fresh 0.5mL microfuge tube, and 20μL vector aliquots were stored at -80ºC. Vector genomic titre was determined by RT-qPCR (kindly conducted by Dr Dahna Fong).

2.2.5.5. Genomic titering of AAV vectors

The number of AAV vector genomes packaged within the virions was quantified using genomic titering as previously described (M. During, D. Young, K. Baer, P. Lawlor, & M. Klugmann, 2003) (kindly performed by Dr Dahna Fong). A real-time PCR was performed to determine the number of genomic copies per mL of the vector using primers specific to the WPRE sequence. The titres were determined from a standard curve produced from a reference plasmid of known molarity serially diluted over a range of 10^{2-7} copies per μL. A no-template control of PCR grade water was also included.

To remove any contaminating DNA not packaged within the virion, an aliquot of vector stock was incubated with 350 units (17.5μL) of DNase I and 80.5μL of buffer (50mM KCl, 10mM Tris-HCl) at 37°C for 30 minutes. The DNase was inactivated by incubation at 70ºC for 10 minutes. The vector aliquot was then incubated with 10μg of proteinase K at 50°C for 1 hour to digest the AAV capsids, which was followed by enzyme inactivation by incubation at 95°C for 20 minutes. The DNase I/proteinase K-treated vector aliquot was subsequently serially diluted by 5 x 10^{1-5} fold to ensure that the quantity of AAV vector genome amplicon generated was within the linear range of the standard curve. The PCR master mix consisted of 6.25μL of 2 x Platinum SYBR Green qPCR Supermix UDG with ROX (Invitrogen) and 1μL of 300μL of 300nM primers designed to the WPRE sequence in the vector genome.

5’-GGCTGTTGGGCACTGACAAT-3’

5’-CCGAAGGGACGTAGCAGAA-3’

The PCR reaction mix comprised of 7.5μL of PCR master mix and 5μL of vector sample or no-template control, in a total volume of 12.5μL of combined in a wells of a 384-well plate (Applied Biosystems), and duplicates of each reaction were performed using an ABI 7900HT Sequence Detection System (Applied Biosystems). The vector titre was calculated based on
the standard curve generated from the reference plasmid, and multiplied by two to generate the number of single stranded AAV genomes per mL.

2.2.6. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Coomassie Blue-stained SDS-PAGE was used to determine the purity of AAV vector stocks. The separating gel consisting of 12% (w/v) acrylamide (BioRad, Hercules, CA) in 375mM Tris-HCL, pH 8.8, containing 0.1% (w/v) SDS was prepared, and polymerisation was catalysed by the addition of 0.03% tetramethylethylenediamine (TEMED, BioRad) and 0.08% ammonium persulphate (BioRad). The separating gel was poured into a gel casting chamber and overlaid with 500μL of 50% isopropanol to level the interface between the separating and the stacking gels. Once the separating gel had set, the isopropanol was decanted. The stacking gel consisting of 5% (w/v) acrylamide, 0.13% bis-acrylamide, 125mM Tris pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.1% (v/v) TEomed was prepared and poured above the separating gel. A comb was inserted to form wells for samples. 10μL of sample buffer was added to 10μL aliquot of each AAV vector preparation and heated at 95°C for 10mins to denature the AAV capsid proteins. The heat-denatured samples were loaded into wells adjacent to 4μL of protein molecular weight standard (Broad Range, BioRad). The proteins were separated by electrophoresis using 180V current in an electrophoresis tank buffer (25mM Tris, 192mM glycine, 0.1% (w/v) SDS) until the bromophenol blue dye front reached the bottom of the gel. Following electrophoresis, the running gel was removed from the gel plates and fixed in 100mL of fixative solution (50% (v/v) methanol, 10% (v/v) acetic acid) for 30 minutes. To visualise the protein bands, the gel was stained with Coomassie blue stain (50% (v/v) methanol, 0.05% (v/v) Coomassie Brilliant Blue R-250 (BioRad), 10% (v/v) acetic acid) for 2 hours. The gel was destained in 5% (v/v) methanol, 7% acetic acid overnight to remove non-specific background staining.

2.2.7. Stereotaxic AAV infusion into SNpc or hippocampus

Adult male Sprague Dawley rats (250-300g; Vernon Jansen Unit, The University of Auckland) were used for the in vivo studies in compliance with The University of Auckland Animal Ethics Committee approval and the New Zealand Animal Welfare Act 1999. The
animals were housed in a humidity- and temperature-regulated containment facility kept on a 12-hour light/dark cycle with food and water available *ad libitum*.

Intraneuronal AAV vector infusions were performed by stereotaxic surgery using Kopf® stereotaxic frames paired with MicroSyringe Pump Controller and 10μL Hamilton syringe (26g gauge, Hamilton, Reno, NV). The rats were anaesthetised with an intraperitoneal (i.p.) dose (70mg/kg) of sodium pentobarbitone (Virbac Laboratories, Auckland, New Zealand). The anaesthetised rat was placed in a separate cage until it no longer responded to pinching of the toe. Marcain (0.5mg/kg) (AstraZeneca, Wellington, DE) was administered subcutaneously (s.c.) directly above the skull as a local analgesic prior to surgery. The fur on the animal’s head was trimmed and the exposed skin cleaned with 0.5% chlorhexidine, 70% alcohol (Riotane). The rat was firmly secured into a stereotaxic frame, and the ear bars and the nose bar were positioned to hold the skull level. A longitudinal incision was made with a scalpel to expose the skull, and the skull landmarks bregma and lambda positively identified. The skull position was adjusted so that bregma and lambda were at equal dorso-ventral (DV) height by adjusting the vertical position of the incision bar. The anterior-posterior (AP) and medial-lateral (ML) stereotaxic coordinates were determined with reference to a rat brain atlas (Paxinos and Watson, 1986) were measured from bregma to target either the SNpc (AP -5.3mm, ML -2.3mm, DV -7.6mm, bregma = 0), or hippocampus (AP -4.0, ML -2.1, DV – 5.3, bregma = 0). A bur hole was drilled using a high-speed rotary drill (Dremel, Mount Prospect, IL) at the desired AP and ML injection site marked with a permanent marker. Approximately 4μL of the vector was withdrawn into the Hamilton syringe and the needle positioned below the dural surface in the hole with the bevel of the needle level with the skull, and slowly lowered to the appropriate DV coordinate, as measured from the skull surface to the target brain structure. Once the needle had reached the target structure, 2μL of vector was infused at a rate of 70nL/min into the SNpc. Once the infusion was completed, the needle was left in place for 5 minutes and then withdrawn slowly to minimise backflow. Once the needle was removed, the incision was sutured closed (Silk 4/0, Resorba, Nuremberg, Germany), and Lignocaine Jelly (AstraZenca) was topically applied to the wound. The rat was then given a s.c. dose of 0.9% saline (10mL/kg), and placed on a heat-mat to aid recovery. The animal was monitored post-operatively until it regained consciousness before returning to the home cage. The rats were monitored consecutively for three days post-operatively, followed by once per week until the rats were sacrificed.
2.2.8. Fixation of brain tissue

For histological analyses, brain tissue was fixed with 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4 (4% PFA). Three weeks post-vector infusion, rats were euthanased with an i.p. overdose of pentobarbitone (300mg/kg). Once respiration had ceased, the chest cavity was opened using a pair of scissors. The membranes surrounding the heart were cleared, and an 18-gauge needle attached to a perfusion syringe was inserted into the left ventricle and firmly clamped with forceps. Using scissors, a narrow cut was made in the right atrium to release blood. The animal was then transcardially perfused with 100mL saline (0.9% w/v NaCl), followed by 100mL of 4% PFA. The animal was decapitated, brain carefully removed and post-fixed overnight in 4% PFA at 4°C, followed by cryoprotection in 30% sucrose (Univar) in 1 x PBS for 72 hours at 4°C.

2.2.9. Sectioning of brain tissue

Fixed brains were frozen to -20°C, and 40μm coronal sections were cut through the nigra or hippocampus on a cryostat (Leica Microsystems, Wetzlar, Germany). Free-floating sections were collected in a 24-well plate in serial order in 1 x PBS with 0.01% sodium azide (Labchem) and stored at 4°C until immunohistochemical analysis.

2.2.10. Immunohistochemistry

Brain sections were washed in 1 x PBS with 0.2% Triton X-100 (PBS-T) and endogenous peroxidase activity was blocked by incubating the sections in 1% (v/v) hydrogen peroxide in 50% methanol (Merck, Whitehouse Station, NJ) for 30 minutes, and washed in PBS-T (2 x 5 minutes). Sections were incubated overnight at room temperature with primary antibodies (Table 2-1) diluted in immunobuffer (IB) consisting of 1 x PBS, 4% horse serum (GibcoBRL), 0.2% Triton X-100 and 0.04% methiolate (BDH Laboratory Supplies). Subsequently, sections were washed in PBS-T, and incubated with 200μL of biotinylated secondary antibodies for three hours, followed by PBS-T washes and incubation with ExtrAvidin Peroxidase (1:250, Sigma) for two hours. Sections were washed in PBS-T and stained with 0.2mg/mL 3,3-diamino-benzidine (DAB), 0.01% hydrogen peroxide in 0.1M phosphate buffer for ~5 minutes. Negative controls were conducted for omitting the primary
antibody. The sections were once more washed in PBS-T twice and mounted onto poly-l-lysine coated slides (Esco Biolabs Scientific, Portsmouth, NH), air-dried for 24 hours in a fume hood, serially dehydrated in ascending ethanol; 70% (v/v) ethanol, 95% (v/v) ethanol, 100% (v/v) ethanol for 10 minutes per solution, and delipidified in xylene for 30 minutes. The slides were coverslipped with Cytoseal 60 mounting medium (Richard Allen Scientific, Kalamazoo, MI). Sections were visualised on an Olympus AX70 microscope (Olympus, Centre Valley, PA, USA) and images acquired using a QImaging200R digital camera and StereoInvestigator software (MicroBrightField In. Welliston, VT, USA).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Catalogue Number</th>
<th>Dilution</th>
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<td>Sigma</td>
<td>F3165</td>
<td>1:2000</td>
</tr>
<tr>
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<td>mouse</td>
<td>Sigma</td>
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<td>1:5000</td>
</tr>
<tr>
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<td>rabbit</td>
<td>Abcam</td>
<td>ab280</td>
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</tr>
<tr>
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<td>mouse</td>
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<td>MMS-101P</td>
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</tr>
<tr>
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<td>mouse</td>
<td>Molecular Probes</td>
<td>A-21271</td>
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</tr>
<tr>
<td>Iba1</td>
<td>goat</td>
<td>Abcam</td>
<td>ab5076</td>
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</tr>
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<td>TH</td>
<td>mouse</td>
<td>Chemicon</td>
<td>MAB318</td>
<td>1:1000</td>
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</tbody>
</table>

Table 2-1 Antibodies used for immunodetection

2.2.11. Immunofluorescence

For immunofluorescence labelling, 0.01% hydrogen peroxide treatment was omitted, and sections were incubated with rabbit anti-Luc (1:500; 70C-CR2029; Fitzgerald) in combination with either mouse anti-GFAP (1:50,000; G3893; Sigma Aldrich), anti-TH (1:500; MAB318; Chemicon) or goat anti-Iba1 (1:5000; ab5076; Abcam) diluted in IB for 48 hours at 4°C, and subsequently incubated with donkey anti-rabbit Alexa-488 secondary antibody (1:1000; Invitrogen) in combination with either donkey anti-mouse Alexa-594 (1:1000; Invitrogen) or biotinylated donkey anti-goat secondary antibody (1:1000; Jackson
ImmunoResearch) for 24 hours at 4°C. For Iba1 detection, sections were further incubated with streptavidin Alexa-594 (1:1000; Invitrogen) for 24 hours at 4°C. The sections were once again washed in PBS-T (2 x 5 minutes), mounted onto poly-l-lysine coated slides, and air-dried for ~30 minutes in a fumehood. Slides were coverslipped with a glycerol-based anti-fade mounting medium (AF1; Citifluor). Images were acquired on an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan) with an oil-immersion x60 objective and Olympus Fluoview version 3.0 software.

2.2.12. Cavalieri estimator

The volume of midbrain tissue transduced in the SNpc, SNpr, and VTA was estimated using the Cavalieri estimator probe in Stereo Investigator 7 (MBF Bioscience, Williston, VT). On every sixth 40 μm section (each 240μm apart), points were placed at a grid size of 50μm, and markers were placed on points that overlay transduced tissue. The programme estimates the volume of transduction based on the average area of transduction and rostro-caudal distance within tissue.

2.2.13. Statistical analysis

Following validation of parametric test assumptions, comparisons of volume transduction were performed by one-way ANOVA with Tukey post-hoc test for multiple group comparisons using SPSS statistics software (IBM SPSS Inc. version 21, Chicago, IL, USA). A p value of less than 0.05 was considered statistically significant.
2.3. Results

Yang et al. recently demonstrated the ability of the full-length genomic ALDH1L1 promoter to efficiently regulate astrocytic-specific transgene expression in a BAC transgenic mouse (Yang et al., 2011). We used this as a starting point to derive four putative promoter sequences to determine whether these sequences retain astrocyte-tropism and mediate efficient levels of transgene expression in the naïve rat SNpc. A short (S) 931bp or long (L) 1974bp 5’ region upstream of the transcription start site were cloned into an AAV expression plasmid to generate ALDH1L1(S) and ALDH1L1(L) promoter constructs respectively (Figure 2-1). Furthermore, Olienik et al. recently reported that the inclusion of the untranslated exon 1 of the ALDH1L1 gene, significantly enhanced transcriptional activity of ALDH1L1 promoter sequences that ranged from -300 to -1500bp immediately upstream of the transcription start site (Oleinik, Krupenko, & Krupenko, 2011). Therefore, two additional constructs were generated by cloning a 138bp sequence spanning exon 1 (ex1) of the ALDH1L1 gene into the above promoter constructs to generate ALDH1L1(S)ex1 and ALDH1L1(L)ex1 (Figure 2-1). Transgene reporter sequences coding for Luciferase (Luc) or enhanced green fluorescent protein (eGFP) were subsequently cloned into these transgenic cassettes.

![ALDH1L1 promoter sequences characterised in this study](image)

The lengths of the promoters and ex1 are relative to the transcription start site (0bp).
2.3.1. Restriction enzyme digest-analysis of AAV expression plasmids

Following large-scale DNA amplification and purification, structural integrity of the plasmids was analysed by restriction enzyme (RE) digestion. Briefly, the presence of AAV2 ITRs required for vector packaging was confirmed with a SmaI digest, and other plasmid-specific REs were used to confirm the presence and correct orientation of promoter and transgene elements of the expression cassette flanked by the ITRs. Additionally, all expression plasmids contained an ampicillin resistance gene (AmpR), origin of replication for propagation in E. coli (pUC19 Ori), a simian virus 40 large T antigen origin of replication for propagation in mammalian cells (SV40 Ori), and downstream woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and bovine growth hormone polyadenylation signal (BGHpA) sequences (Figure 2-2 a-f). The RE digest-generated fragments were consistent with fragment sizes calculated from predicted RE cleavage sites in the plasmid maps confirming the presence and correct orientation of the elements within each of the plasmids.

These AAV expression plasmids were then packaged into AAV serotype 5, 8 or 9, that have previously been shown to target astrocytes in various CNS regions in the rodent and non-primate (Drinkut et al., 2012; Lawlor et al., 2009; Markakis et al., 2010). To characterise these AAV vector stocks, a 10µL aliquot of each AAV vector was heat-denatured and separated on SDS-PAGE and stained with Coomassie blue to confirm the presence of AAV vector in each preparation and assess the purity of the vector stock. AAV capsid proteins VP1 (87kDa), VP2 (73kDa) and VP3 (62kDa) assemble in the nucleus in a 1:1:18 stoichiometry to form mature virions (Asokan, Schaffer, & Samulski, 2012). The molecular weight and intensity of three Coomassie-stained protein bands corresponded to the molecular weights and assembly ratio of the capsid proteins (Figure 2-3). A small number of non-specific bands in AAV8-ALDH1L1(L)-Luc (Figure 2-3 g) indicated the presence of some minor protein contaminants, whereas an absence of non-specific bands in others vector samples confirmed high purity of these vector stock. The genomic titre of AAV vectors were determined by real-time quantitative PCR. The purified vectors were of high titre, ranging from $2.7 \times 10^{12}$ to $1.7 \times 10^{13}$ viral genomes/mL (vg/mL) (Table 2-2).
Figure 2-2 Agarose gel electrophoresis of restriction enzyme digest fragments of AAV expression plasmids

The RE digest-generated fragments were consistent with fragment sizes calculated from predicted RE cleavage sites in the plasmid maps confirming plasmid integrity.
a) Diagram of recombinant plasmid pITLAL1 showing vector and insert restriction sites.

Legend:
- **AmpR (6387-7247)**
- **pUC19 Ori (5474-6261)**
- **SV40 Ori (5053-5388)**
- **ITR (4870-5052)**
- **BGHpA (4592-4860)**
- **WPRE (3981-4573)**
- **ALDH1L1 (1-Lac-WPRE-BGHpa)**
- **XR (1-183)**
- **BglII (457)**
- **BamHI (1239)**
- **NcoI (1336)**
- **KpnI (1939)**
- **SstI (2184)**
- **MfeI (2429)**
- **Mbol (2885)**
- **HindIII (3976)**
- **ClaI (3983)**
- **XhoI (190)**

**Restriction Enzymes and Sizes:**
1) **SmaI** 4960, 3193, 11, 11
2) **NcoI** 4354, 3821
3) **Asp718/ MfeI** 7729, 490, 456
4) **XhoI/ SacI** 6181, 1994
5) Smal 3915, 3193, 11, 11
6) NcoI 3821, 3309
7) Asp718/ MfeI 6184, 490, 456
8) XhoI/ SacI 6181, 949
1) XhoI/ SacI  5302, 949
2) NcoI  3309, 1918, 1024
3) SmaI  3193, 3036, 11, 11
4) XhoI, SacI  5302, 1994
5) NcoI  4354, 1918, 1024
6) SmaI  4081, 3193, 11, 11
1) Smal  4830, 3193, 11, 11
2) KpnI/ HindIII  6138, 1907
3) XhoI/ KpnI  6296, 1749
1) SmaI 3785, 3193, 11, 11
2) KpnI/ HindIII 5093, 1907
3) XhoI/ KpnI 6296, 704
Figure 2-3 SDS-PAGE of denatured AAV vector preparations

The molecular weight and intensity of three Coomassie-stained protein bands corresponded to the molecular weights and assembly ratio of the AAV capsid proteins, VP1, 2 and 3. A small number of non-specific bands in g) AAV8-ALDH1L1(L)-Luc indicated the presence of some minor protein contaminants, whereas the absence of non-specific bands in others vector samples confirmed high purity of these vector stock.
Table 2-2 Stock vector titres (vg/mL)

<table>
<thead>
<tr>
<th>Vector</th>
<th>Titre (vg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) AAV9-ALDH1L1(L)ex1-eGFP</td>
<td>2.7 x 10^{12}</td>
</tr>
<tr>
<td>b) AAV9-ALDH1L1(L)ex1-Luc</td>
<td>2.7 x 10^{12}</td>
</tr>
<tr>
<td>c) AAV9-ALDH1L1(S)ex1-eGFP</td>
<td>4.3 x 10^{12}</td>
</tr>
<tr>
<td>d) AAV9-ALDH1L1(S)-ex1-Luc</td>
<td>6.9 x 10^{12}</td>
</tr>
<tr>
<td>e) AAV9-ALDH1L1(L)-Luc</td>
<td>9.1 x 10^{12}</td>
</tr>
<tr>
<td>f) AAV9-ALDH1L1(S)-Luc</td>
<td>4.1 x 10^{12}</td>
</tr>
<tr>
<td>g) AAV8-ALDH1L1(L)-Luc</td>
<td>6.2 x 10^{12}</td>
</tr>
<tr>
<td>h) AAV5-ALDH1L1(L)-Luc</td>
<td>4.0 x 10^{13}</td>
</tr>
</tbody>
</table>
2.3.2. ALDH1L1 promoter variants coupled with AAV9 selectively target neurons in the SNpc

Astrocyte-tropism and transduction efficacy of ALDH1L1 promoter variants were firstly investigated in the context of the astrocyte-tropic AAV serotype 9, as previous studies in our laboratory had shown that this serotype can target transgene expression to astrocytes in the hippocampus (Lawlor et al., 2009). Titre-matched AAV9 (2 x 10^8 genomes) vectors expressing a Luc reporter gene were unilaterally injected into the SNpc of subgroups of rats (n = 3 per vector), and three weeks post-vector infusion, a time-point at which peak transgene expression is achieved, animals were euthanised and brain sections analysed by IHC. An anti-Luc antibody was used to examine transgene expression patterns in the injected brains. Unexpectedly, all four ALDH1L1 promoters in the context of AAV9 exclusively targeted Luc expression to neurons as determined by the morphology of transgene-expressing cells in the SNpc (Figure 2-4). Luc was expressed throughout neuronal cell bodies and their fibres (Figure 2-4 e-p), whilst there was no evidence of transgene expression in cells with astrocytic morphology. No transgene expression was observed in the contralateral non-injected hemisphere (Figure 2-4 a-d). In addition to the predominant transgene expression in the SNpc, Luc immunoreactivity spread into the midbrain reticular nucleus, ventral to the SNpc, and medially into the VTA. ALDH1L1(L)ex1 appeared to regulate the highest levels of Luc expression, followed by ALDH1L1(L), ALDH1L1(S)ex1, and ALDH1L1(S) that achieved the lowest levels of transgene expression. Exclusive neuronal transgene expression was confirmed by double label IHC using antibodies to the DA neuronal marker TH, and the astrocytic and microglial markers GFAP and ionised calcium binding adapter molecule 1 (Iba1), respectively (Figure 2-5). Luc-immunoreactivity exclusively co-localised with neuronal TH, while no co-localisation was detected with GFAP or Iba1. Based on the levels of transgene expression, the two promoters containing the 1974bp ALDH1L1(L) sequence appeared to mediate considerably higher transgene expression in comparison to shorter counterparts containing the 931bp ALDH1L1(S) sequence. Furthermore, the inclusion of the exon1 appeared to moderately increase transcriptional activity of both ALDH1L1(L) and ALDH1L1(S) promoters.

The volume of tissue transduction was quantified using the Cavelieri estimator probe. Following validation of parametric test assumptions, comparisons of volume transduction were performed by one-way ANOVA and Tukey post-hoc test. The volume of tissue transduced within the SNpc, SN pars reticulata (SNpr), and VTA was highest with
ALDH1L1(L)ex1 and ALDH1L1(L) (Figure 2-6). A significantly smaller volume of tissue within these regions was transduced by both ALDH1L1(S)ex1 and ALDH1L1(S); in comparison to ALDH1L1(S)ex1, or ALDH1L1(S), ALDH1L1(L)ex1 (One-way ANOVA; p < 0.001) and ALDH1L1(L) (p < 0.001) transduced significantly larger volumes of tissue. Inclusion of exon1 to the long or short promoters resulted in a subtle increase in volume transduction; ALDH1L1(L)ex1 in comparison to ALDH1L1(L) p = 0.94; and ALDH1L1(S)ex1 in comparison to ALDH1L1(S) p = 0.99.
Figure 2-4 Transgene expression regulated by the ALDH1L1 promoter variants in the context of AAV9 in the SNpc

Three weeks post-unilateral vector injection into the SNpc, (a-d) no Luc expression was detected in the uninjected contralateral hemispheres. (e-p) In the vector injected hemispheres, all four ALDH1L1 promoter variants mediated Luc expression predominantly in neuronal cell bodies and their fibres in the SNpc (shown at high magnification in m-p). (e-h) Some transgene expression also extended dorsally into the midbrain reticular nucleus and medially into the VTA. In a manner dependent on promoter length, e, i, m) ALDH1L1(L)ex1 appeared to regulate the highest level of Luc expression, followed by f, j, n) ALDH1L1(L), g, k, o) ALDH1L1(S)ex1, and h, l, p) ALDH1L1(S).

Scale bars: (a-h) 500µm, (i-l) 200µm, and (m-p) 100µm
Figure 2-5 Exclusive nigral neuronal transgene expression mediated by AAV9-ALDH1L1(L)ex1-Luc

Double-labelling with anti-Luc and neuronal or glial markers revealed exclusive co-localisation of Luc immunofluorescence within a) TH-immunoreactive DA cell bodies (arrow) and fibres (arrowhead) in the SNpc, while no co-localisation of immunofluorescence was detected within b) GFAP-immunoreactive astrocytes or c) Iba-immunoreactive microglia.

Scale bar: (a-c) 20µM
The mean volume of tissue transduced within the SNpc, SNpr, and VTA was highest with ALDHL1(L)ex1 and ALDH1L1(L). Both ALDH1L1(S)ex1 and ALDH1L1(S) targeted a significantly smaller volume of tissue within these regions. Inclusion of exon1 in the long or short promoters resulted in a subtle increase in volume transduction.

Values represent the mean ± SEM

* $p < 0.001$
2.3.3. Neuronal-specific activity of the ALDH1L1(L) promoter in the context of AAV serotypes 5, 8, and 9 in the SNpc

Transduction properties of the ALDH1L1(L) promoter in the context of two additional astrocyte-tropic serotypes, AAV5 and AAV8 were subsequently investigated to determine whether nigral tropism of the ALDH1L1 promoters could be modified in an AAV serotype-dependent manner. Similar to AAV9, these serotypes have previously exhibited astrocytic tropism in various regions in the rodent and non-human primate CNS (Drinkut et al., 2012; Lawlor et al., 2009; Markakis et al., 2010). Nigral neuronal tropism of the ALDH1L1(L) promoter persisted in the context of AAV8, and AAV5 (Figure 2-7). However, in comparison to AAV9 (Figure 2-7 d, g, j) AAV8 (Figure 2-7 e, h, k) and AAV5 (Figure 2-7 f, i, l) appeared to mediate lower levels of transgene expression, and transduced a significantly smaller volume of tissue (One-way ANOVA; p < 0.01) (Figure 2-8), with minimal spread into the adjacent VTA, and the midbrain reticular nucleus, ventral to the SNpc. Furthermore, both AAV5 and AAV8 transduced comparable volumes of tissue (p = 0.98).
Figure 2-7 Transgene expression regulated by the ALDH1L1(L) promoter in the context of three AAV serotypes in the SNpc

Following unilateral vector injection into the SNpc, (a-c) no Luc expression was detected in the uninjected contralateral hemispheres. In the vector injected hemispheres, the ALDH1L1(L) promoter maintained neuronal transgene expression in the context of (d, g, j) AAV9, (e, h, k) AAV8, and (f, i, l) AAV5, with Luc immunoreactivity detected in nigral neuronal cell bodies and fibres (shown at high magnification in j-l). Transgene expression was predominantly restricted to the SNpc. AAV9 appeared to mediate the highest levels of Luc expression with additional transgene expression extending dorsally into the midbrain reticular nucleus and medially into the VTA (d, g). The levels of transgene expression achieved with (e, h, k) AAV8 and (f, i, l) AAV5 appeared to be considerably lower than that of AAV9.

Scale bars: (a-f) 500µm, (g-i) 200µm, and (j-l) 100µm
Figure 2-8 AAV serotype-dependent volume of nigral tissue transduction

The mean volume of tissue transduced within the SNpc, SNpr, and VTA was highest with AAV9. In comparison to AAV9, both AAV8 and AAV5 transduced a significantly smaller volume of tissue.

Values represent the mean ± SEM

* $p < 0.01$
2.3.4. ALDH1L1(L)ex1 promoter regulates neuronal-specific transgene expression in the hippocampus and SNpc

To determine whether region-specific cellular heterogeneity accounted for the neurotropism of the ALDH1L1 promoter variants, or AAV serotypes, AAV9-ALDH1L1(L)ex1-Luc that exhibited the highest level of activity in the SNpc was subsequently infused into the hippocampus in an additional group of rats (n = 3). Transduction properties of the ALDH1L1(L)ex1 promoter was compared to that of the widely used 2.2kb GFAP promoter that efficiently targets hippocampal astrocytes in the context of AAV9 (Young et al., 2014). Homogenous Luc-immunoreactivity mainly in astrocytes was detected in the AAV9-GFAP-Luc transduced hippocampus with high astrocytic transgene expression in the astrocyte-abundant stratum moleculare adjacent to the dentate gyrus (Ogata & Kosaka, 2002) (Figure 2-9, e, g, i). The intricate ‘star-like’ morphology of the transduced astrocytes is visible in this region (Figure 2-9 i, arrow). Neuronal Luc immunoreactivity was mainly restricted to the hilar region (Figure 2-9 g, k), with additional transgene expressing neurons sparsely distributed throughout the CA1 region. ALDH1L1(L)ex1 replicated a similar pattern of exclusive neuronal expression predominantly in the hilus and sparsely throughout the CA1 region, as evident by distinctive neuronal morphology revealed by Luc-positive neuronal cell bodies and dendrites (Figure 2-9 f, h, l). A negligible number of cells exhibiting characteristic astrocytic morphology were detected in the stratum moleculare (Figure 2-9 j, arrow).

To determine whether the AAV serotype 9-ALDH1L1 promoter coupled vectors target a wider population of cells in the hippocampus and SNpc than that revealed by Luc expression, the Luc transgene in expression cassettes regulated by ALDH1L1(L)ex1 or ALDH1L1(S)ex1 were substituted with the coding sequence for the highly stable eGFP. IHC using a highly-sensitive anti-GFP antibody was conducted to examine transgene expression patterns. Indeed considerably more intense eGFP expression was detected in the hippocampal dentate hilar region, and granule neurons with both vector constructs (Figure 2-10 a-c and f-h, respectively). eGFP immunoreactivity was also detected in granule neuronal dendrites in the stratum moleculare and their axonal projections in the mossy fibre pathway (Figure 2-10 a, b and f, g, respectively). CA1 pyramidal neurons expressed eGFP to a lesser extent. In the uninjected contralateral hippocampus, eGFP-immunoreactive terminals of commissural fibres of ipsilateral dentate hilar neurons were also detected (Figure 2-10 d, e and i, j, respectively), confirming neuronal transduction. In the midbrain, eGFP immunoreactivity was detected in
the SNpc, SNpr, VTA, and in ventral regions of midbrain reticular nucleus and thalamus with both vector constructs (Figure 2-10 k-m and p-r, respectively). eGFP-positive neuronal fibres were detected in the contralateral midbrain structures (Figure 2-10 n, o and s, t, respectively) predominantly in brains unilaterally injected with AAV9-ALDH1L1(L)ex1-eGFP (Figure 2-10 n, o). In both the hippocampus and nigra, ALDH1L1(L)ex1 promoter sequence (Figure 2-10 a-e and k-o, respectively) exhibited superior activity than the shorter ALDH1L1(S)ex1 (Figure 2-10 f-j and p-t, respectively), as evident by the lower intensity of eGFP immunoreactivity in both the vector-injected and contralateral structures in the AAV9-ALDH1L1(S)ex1-eGFP-injected brains.
**Figure 2-9 Comparison of cell tropism of GFAP and ALDH1L1(L)ex1 promoters in the hippocampus**

**e-i)** The GFAP promoter targeted Luc expression predominantly to astrocytes throughout the hippocampus, with high levels of astrocytic Luc expression in the stratum moleculare (SM), and more diffuse expression in the stratum radiatum (SR); high power magnification shows Luc-immunoreactive ‘star-like’ astrocytes with extensively ramified processes visible in the stratum moleculare (**i, arrow**).

**(e, g, k)** Neuronal expression was mainly restricted to the dentate hilar (H) region, with additional transgene expressing neurons sparsely distributed throughout the CA1 region; **(k)** high power magnification shows Luc expression in hilar neuronal cell bodies and fibres.

**(f-l)** ALDH1L1 replicated a similar pattern of exclusive neuronal expression predominantly in the hilus (H) and sparsely throughout the CA1 region, with **(j; arrow)** negligible astrocyte transduction in the stratum moleculare.

Non-specific staining was detected in the **(a-d)** uninjected contralateral hemispheres and in a **(m)** GFP-expressing control hippocampal section.

stratum moleculare (SM); stratum radiatum (SR); hilus (H); granule cell layer (GCL)

Scale bars: **(c-f, m) 500µm, (a, b, g, h) 200µm, and (i-l) 100µm, respectively**
Figure 2-10 Transgene expression regulated by the ALDH1L1(L)ex1 or ALDH1L1(S)ex1 promoter in the hippocampus and nigra

Replacing the Luc gene in expression cassettes regulated by ALDH1L1(L)ex1 or ALDH1L1(S)ex1 with the coding sequence for the highly stable eGFP resulted in considerably higher expression levels in both the hippocampus and SNpc.

In the vector injected hippocampus, with both (a-c) ALDH1L1(L)ex1 and (f-h) ALDH1L1(S)ex1 promoters, high intensity eGFP immunoreactivity was detected in the dentate hilar (H) region, and granule neurons in the granule cell layer (GCL). High magnification indicates neuronal morphology of eGFP expressing cells (c and h respectively). (a and f respectively) eGFP expression was also detected in granule neuronal dendrites in the stratum moleculare (SM) and their axonal projections in the mossy fibre (MF) pathway, while CA1 pyramidal neurons expressed eGFP to a lesser extent.

In the uninjected contralateral hippocampus, eGFP-immunoreactivity in terminals of commissural fibres of ipsilateral dentate hilar (H) neurons was slightly higher with the (d, e) ALDH1L1(L)ex1 promoter than (i, j) ALDH1L1(S)ex1 promoter.

In the midbrain, both (k-m) ALDH1L1(L)ex1 and (p-r) ALDH1L1(S)ex1 promoters mediated eGFP expression in the SNpc, SNpr, VTA, and in ventral regions of midbrain reticular nucleus and thalamus. High magnification of the SNpc indicates neuronal morphology of eGFP expressing cells (m and r respectively).

eGFP-positive neuronal fibre immunoreactivity in the contralateral midbrain structures was more prominent with the (n, o) ALDH1L1(L)ex1 promoter compared to (s, t) ALDH1L1(S)ex1 promoter.

stratum moleculare (SM); stratum radiatum (SR); hilus (H); granule cell layer (GCL), mossy fibres (MF)

Scale bars: (a, d, f, i, k, n, p, s) 500µm, (b, e, g, j, l, o, q, t) 200µm, and (c, h, m, r) 100µm
2.4. Discussion

The objective of this chapter was to generate a pan-astrocytic DNA promoter that in the context of an astrocyte-tropic AAV serotype efficiently directs transgene expression to SNpc astrocytes. The full-length promoter sequence of the recently characterised astrocytic ALDH1L1 gene has been shown to exhibit astrocyte-specific transcriptional activity throughout the CNS, at a level that was superior to the classic 2.2kb GFAP promoter (Cahoy et al., 2008; Yang et al., 2011). To determine whether 5’ sequences immediately upstream of the ALDH1L1 transcription start site exhibit astrocyte tropism and efficient transcriptional activity, 931bp ALDH1L1(S) and 1974bp ALDH1L1(L) promoter sequences were cloned into an AAV expression plasmid encoding Luc. To determine the influence of exon 1 that may contain enhancer sequences (Oleinik et al., 2011), a 138bp sequence spanning exon 1 of the ALDH1L1 gene was cloned into the above promoter constructs to generate ALDH1L1(S)ex1 and ALDH1L1(L)ex. The capacity of these expression cassettes to transduce astrocytes in the context of the astrocyte-tropic AAV9, AAV8 and AAV5 was evaluated in the naïve rat SNpc.

2.4.1. ALDH1L1 promoters target transgene expression to neurons in the SNpc

The ~4.7kb AAV packaging capacity imposes a stringent size restriction on the transgenic expression cassette. Expression plasmids exceeding 5kb compromise stability of the vector capsids resulting in inefficient packaging and transduction (Lai, Yue, & Duan, 2009; Wu, Yang, & Colosi, 2009). Therefore, to accommodate relatively large therapeutic transgenes in the vector genome without compromising stability, short regulatory elements with high transcriptional activity are ideal. Computational analysis of a 1443bp sequence of the ALDH1L1 gene that span -525 to +918bp relative to the transcriptional start site consisting of an extensive 5’ CpG island revealed a -10 to -260bp putative promoter region that contains multiple binding sites for the transcription factor SP1 (Oleinik et al., 2011). Oleinik et al. generated multiple ALDH1L1 promoter sequences that ranged from 300 to 1500bp from the region immediately upstream of the transcription start site (Oleinik et al., 2011). Transfection into A549 cells of DNA expression constructs encoding Luc regulated by these ALDH1L1 promoter variants mediated levels of Luc expression that was comparable to that achieved with a promoterless construct. Inclusion of the entire exon 1 (~167bp) significantly increased transcriptional activity of all promoter sequences. Firstly, to determine whether 5’ sequences
upstream of the ALDH1L1 transcription start site exhibit efficient transcriptional activity \textit{in vivo} in the absence of exon 1. 931bp ALDH1L1(S) and 1974bp ALDH1L1(L) promote sequences were cloned into an AAV expression plasmid encoding Luc. To determine the influence of exon 1, a 138bp sequence spanning exon 1 of the ALDH1L1 gene was cloned into the above promoter constructs to generate ALDH1L1(S)ex1 and ALDH1L1(L)ex1. The ability of these promoter variants to transduce astrocytes in the context of AAV9 was evaluated in the naïve rat SNpc.

Unexpectedly, the four ALDH1L1 promoter variants generated in this study mediated exclusive neuronal transduction in the SNpc. The potential deficits of the promoter variants that may have contributed to the modified cell tropism are discussed in \textbf{section 2.4.3}. Of the four promoters, ALDH1L1(L)ex1 and ALDH1L1(L) mediated high levels of transgene expression, while the shorter ALDH1L1(S)ex1 and ALDH1L1(S) mediated comparably lower levels of Luc expression and transduced significantly smaller volumes of midbrain tissue, suggesting that the longer promoters contain a greater number of DNA elements that positively regulate transcriptional activity. Furthermore, exon 1 may contain elements that enhance transcriptional activity as its inclusion into both long and short promoters appeared to enhance transgene expression, and subtly increased in the volume of tissue transduction. Quantification of intensity of Luc-immunoreactivity may determine whether the longer promoter and inclusion of exon 1 significantly enhance transgene expression.

\textbf{2.4.2. AAV serotype-dependent transgene expression in the SNpc}

Of the native serotypes characterised thus far, AAV9, 8 and 5 are the most efficient serotypes for astrocyte transduction. We and others have shown that these serotypes exhibit astrocytic tropism in various regions in the rodent and non-human primate CNS (Drinkut et al., 2012; Lawlor et al., 2009; Markakis et al., 2010; Young et al., 2014). It is increasingly evident that astrocytes exhibit molecular and functional heterogeneity in physiology and pathology (Cai et al., 2000; Fitting et al., 2010; Karavanova et al., 2007; Macnab & Pow, 2007; Regan et al., 2007; Reuss et al., 2000; Tang et al., 2009; Walz & Lang, 1998; Yeh et al., 2009). Such diversity in astrocytic molecular expression may influence region-specific tropisms and transduction efficacies of viral vectors. To determine whether the observed neuronal tropism in the SNpc of ALDH1L1 promoter variants in the context of AAV9 was primarily due to inefficient AAV9-mediated nigral astrocyte transduction, not promoter inactivity, the
ALDH1L1(L) expression cassette was tested in the context of the alternative astrocyte-tropic serotypes AAV8 and AAV5. These serotypes also directed transgene expression exclusively to neurons, although with lower efficiency than AAV9 (Figures 2-7 and 2-8). Viral infection is a complex pathway governed by a multitude of interactions between capsids of the intact virion and cellular machinery, and hence the efficacy of transduction depends on the efficacy of viral entry, intracellular trafficking to the nucleus and capsid disassembly (Nonnenmacher & Weber, 2012). These results suggest that AAV9 may exhibit the most efficient neuronal transduction mechanisms in the rat SNpc, followed by AAV5, and AAV8 that transduced the lowest volume of tissue. Furthermore, it is possible that some or all three AAV serotypes may effectively transduce nigral astrocytes, but the transgenic ALDH1L1 promoter is transcriptionally inactive or inefficient in these cells, leading to an absence of astrocyte-specific Luc expression. The potential role of region-dependent astrocyte heterogeneity in determining a lack of ALDH1L1-regulated transgene expression in nigral astrocytes is discussed in section 2.4.3.

2.4.3. ALDH1L1 promoter targets transgene expression to neurons in the hippocampus

We previously demonstrated that AAV9 coupled with the widely used 2.2kb GFAP promoter mediated high levels of transgene expression in hippocampal astrocytes, indicating that hippocampal astrocytes are permissive to both AAV9 transduction, and GFAP promoter activity (Young et al., 2014). To determine whether astrocyte heterogeneity contributes to the midbrain-dependent neurotropism of the ALDH1L1 promoter variants in the context of AAV9, AAV9-ALDH1L1(L)ex1-Luc that mediated the highest level of transgene expression was tested in the hippocampus in comparison to titre-matched AAV9-GFAP-Luc. AAV9-GFAP-Luc mediated efficient hippocampal astrocytic transduction as reported previously (Young et al., 2014). In contrast, disregarding a negligible level of astrocyte transduction, AAV9-ALDH1L1(L)ex1-Luc exhibited almost exclusive neuronal tropism in the hippocampus. These results suggest that transcriptional inactivity of the ALDH1L1 promoter in astrocytes, and not inefficient AAV9-mediated astrocyte transduction that may restrict ALDH1L1 promoter-regulated transgene expression to neurons.
The main epigenetic modification of the human genome is methylation of cytosine residues in cytosine and guanine (CpG) dinucleotides (Robertson, 2001). Collections of CpG dinucleotides termed CpG islands are frequently located near 5’end of genes. The ALDH1L1 gene contains an extensive 5’ CpG island of 96 CpG pairs spanning a region -525 and +918bp relative to the transcriptional start site. Oleinik et al. demonstrated that ALDH1L1 expression is regulated by CpG island methylation, wherein hypermethylation silences gene expression (Oleinik et al., 2011). Therefore, following efficient astrocyte infection by AAV5, 8 or 9, one potential hypothesis for the lack of astrocytic Luc expression in vivo is that in the absence of sequence motifs that regulate methylation of the endogenous ALDH1L1 gene, hypermethylation of the transgenic ALDH1L1 promoters may contribute to transcriptional silencing in astrocytes. In a subsequent study in Chapter three, both AAV9 and AAV5 effectively mediated nigral astrocytic transduction and transgene expression, omitting inefficient astrocyte transduction as a potential explanation for the absence of astrocytic Luc expression. In future studies, an in vitro methyltransferase inhibition assay in primary astrocytes transduced with the ALDH1L1 promoter-regulated expression cassettes may determine whether methylation suppresses transcriptional activity of ALDH1L1 promoters in astrocytes.

Interestingly, ALDH1L1 promoter sequences corresponding to a region within -600bp of the transcription start site exhibited slightly higher transcriptional activity in A549 cells in vitro compared to a 1500bp promoter that included additional distal sequences (Oleinik et al., 2011). In contrast to these results, the 1974bp ALDH1L1(L) promoter analysed in this study mediated significantly higher transcriptional activity in the SNpc than the 931bp ALDH1L1(S) promoter. Phenotypic alterations associated with carcinomas and in vitro expansion, in addition to species- and cell type-specific differences in molecular expression profiles may account for contradictory findings between Oleinik et al. and this study. It may be of further interest to determine whether expression of the ~600bp putative promoter region identified by Oleinik et al. may promote astrocytic transgene expression via partial elimination of CpG dinucleotides in the excluded distal sequences. However, the significantly high levels of neuronal transgene expression mediated by ALH1L1(L) promoters, and the subtle transcriptional upregulation by the inclusion of exon 1 that contains additional CpG dinucleotides suggest that CpG methylation may not regulate activity of this promoter in neurons. It is possible that the exclusion of specific DNA motifs that dictate cell-specific gene expression may have completely modified cell-tropism of these promoters.
Deletion analysis of the ALDH1L1 promoter region may allow the identification of such regulatory sequences, and facilitate the development of astrocyte-specific ALDH1L1 promoters.

To determine whether ALDH1L1(L)ex1 and ALDH1(S)ex1 in the context of AAV9 may potentially regulate expression of low levels of transgene in astrocytes that was undetected with Luc immunoreactivity, Luc in the vector genome was substituted with the coding sequence for the highly stable eGFP reporter protein, and transgene expression in the hippocampus and SNpc following AAV9-mediated transduction was analysed. Luc has a half-life of 2 to 4 hours (Ignowski & Schaffer, 2004; Leclerc, Boockfor, Faught, & Frawley, 2000), whereas GFP has a half-life of ~26 hours leading to its accumulation and efficient immunodetection (Corish & Tyler-Smith, 1999). IHC analysis with a highly sensitive anti-GFP antibody indicated that in comparison to Luc, eGFP immunoreactivity was substantially higher in both regions; however, excessive levels of GFP immunoreactivity disguised morphology of transduced cells and any potential differences in transcriptional activity of the two promoter variants. A subsequent tenfold dilution of the antibody against GFP revealed that transgene expression remained exclusively neuronal, further supporting ALDH1L1 promoter inactivity in astrocytes. Moreover, in comparison to ALDH1L1(S)ex1, greater intensity of ALDH1L1(L)ex1-regulated eGFP expression indicated that ALDH1L1(L)ex1 may exhibit greater transcriptional activity as determined previously. The extensive dissemination of eGFP protein into ipsilateral and contralateral regions from the injected SNpc reiterates the potential risk of side effects associated with delivery of potent neurotherapeutic molecules to off-target regions (Drinkut et al., 2012; Manfredsson et al., 2009). The detailed circuitry of the human brain is yet to be fully elucidated, and presently it is impossible to predict the extent of neuronal projections to remote nuclei in the CNS. Unlike neurons that project axons over long distances, astrocytic processes do not extend beyond 20μm from the cell body; therefore, the ability to selectively target transgene expression to astrocytes within the target region may improve the safety profile of gene therapy applications that overexpress bio-active therapeutics such as growth factors.

Alternative astrocytic promoters could potentially be derived from the gene coding for the astrocyte-specific glutamate transporter, GLT1 (EAAT2 in human). Fluorescence activated cell sorting (FACS) analysis showed that the overall eGFP expression intensity in BAC mice that expressed eGFP regulated by a 45kb GLT1 promoter was considerably higher than that in BAC mice expressing ALDH1L1-regulated eGFP, suggesting that the GLT1 promoter may
have substantially higher activity than ALDH1L1 promoter (Yang et al., 2011). ALDH1L1- and GLT1-positive cells share a highly homologous astroglial molecular identity, and less than 20 of 15,000 astrocyte-enriched genes in a transcriptome database showed ~twofold differences in expression. Furthermore, GLT1 mRNA expression is ~fivefold higher than that of ALDH1L1 mRNA, indicating that GLT1 promoter activity is higher than that of ALDH1L1. Epigenetic regulation of GLT1 promoter activity via DNA methylation has been shown (Yang et al., 2009); suggesting that long-term characterisation of potential GLT1 promoter sequences is required to determine heterogeneity in temporal and spatial regulation of these promoters in the CNS. Furthermore, a previous study identified a 2.5kb putative promoter sequence of the human EAAT2 gene that was shown to be transcriptionally activated by the inflammatory transcription factor NF-κB. As discussed in section 1.7.2, chronic activation of NF-κB in CNS diseases contributes to neurodegeneration. Therefore, an EAAT2-derived promoter may offer the potential ability to upregulate the expression of neuroprotective transgenes in the context of pathological reactive gliosis to ameliorate chronic inflammation (Su et al., 2003).

Novel ALDH1L1 promoters that unexpectedly target DA neurons in the SNpc are presented in this chapter. Further characterisation of this promoter in comparison to the presently used DA neuronal-specific promoters, TH and neuron-specific enolase (NSE) may determine the potential applicability of these promoters in neuronal-targeted gene delivery in the SNpc.
Chapter 3. Characterisation of an astrocyte-tropic AAV vector construct for the selective and efficient transgene expression in nigral astrocytes *in vivo*
3.1. Introduction

To date, CNS gene therapy strategies are predominantly neurocentric. Given that dynamic communication between neurons and astrocytes maintains CNS physiology, and detrimental neuronal and astrocytic phenotypic evolution leads to pathological interactions that contribute to neurodegeneration in CNS disorders, the ability to therapeutically target both cell types may yield greater efficacy. Prior to investigating the efficacy of this dual cell targeting approach in PD, we aimed to first determine whether astrocyte-specific therapeutic manipulation confers substantial neuroprotection in disease by restricting therapeutic transgene expression predominantly to astrocytes. Therefore, the main aim of this chapter was to develop AAV vectors that exhibit efficient astrocyte tropism and transduction by coupling astrocyte-tropic AAV serotypes and DNA promoter elements, to overexpress the therapeutic transgenes NURR1, or Nrf2 in nigral astrocytes. Additionally, given that the ability to regulate vector tropism and levels of transgene expression is a beneficial characteristic of gene delivery vehicles, the capacity of three DNA promoter elements to regulate vector tropism was investigated.

As described in Chapter 2, promoter sequences derived from the astrocyte-specific ALDH1L1 gene unexpectedly directed transgene expression to DA neurons in the SNpc. Therefore, in this chapter the efficacy of nigral transduction by the widely used astrocyte-specific GFAP promoter in the context of astrocyte-tropic AAV serotypes 5 and 9 was investigated. The constitutively active CMV promoter or a recently characterised shorter GFAP promoter variant, gfaABC1D were investigated for their potential to either enhance transgene expression and/ or modify vector tropism.

Moreover, to enhance astrocyte transduction specificity in future in vivo applications, a preliminary study was conducted to quantify the efficacy of a previously characterised microRNA (miRNA)-based ‘detargeting’ strategy in silencing AAV5-mediated transgene expression in nigral neurons. miRNAs, non-coding RNA sequences of ~19-25 nucleotides, present in abundance in eukaryotic organisms, play an important role in post-transcriptional regulation of gene expression in developmental and physiological processes (Bartel, 2004). Various gene therapy strategies exploit this RNA interference-mediated mechanism to downregulate the expression of endogenous genes via the delivery of specific miRNA sequences (Boudreau, Rodríguez-Lebrón, & Davidson, 2011). More recently, an alternative ‘detargeting’ strategy incorporates target sequences of cell type-specific endogenous
miRNAs into the vector genome to post-transcriptionally silence transgene expression in off-target cells (Brown et al., 2007; Colin et al., 2009; Pulicherla et al., 2011). Following vector-mediated transduction and transcription of the transgenic cassette, complementary endogenous miRNA in cells that express them binds to its target sequence on the transgenic mRNA transcript promoting its degradation or translational inhibition. Whereas, in the absence of the complementary endogenous miRNA, transgene expression in the cell type of interest is unaffected.

Colin et al. enhanced the specificity of a lentiviral vector-mediated transgene overexpression in astrocytes by incorporating a target sequence of the neuronal-specific miRNA, miR124 into the vector genome to selectively direct miR124-directed degradation of the transgenic mRNA transcript in neurons (Figure 3-1). Four copies of a 21bp sequence derived from the miR124 target gene (denoted miR124T), integrin-β1 were incorporated at the 3’ end of a reporter gene regulated by the astrocyte-specific EAAT2 promoter. Following lentiviral-based delivery of this vector construct into the mouse striatum, neuronal transgene expression was significantly reduced (from 18% to 6%), while astrocytic transgene expression was unaffected. Incorporation of the miR124T in the transgenic cassette had no effect on miR124 or its endogenous target, integrin-β1.

![Figure 3-1 miR124-based neuronal ‘detargeting’ of transgene expression](image)

Figure 3-1 miR124-based neuronal ‘detargeting’ of transgene expression
3.2. Material and Methods

3.2.1. Maintenance of HEK293 cells

A vial of HEK293 cells was removed from liquid nitrogen storage and quickly defrosted in a 37°C waterbath. The defrosted cells were then transferred to a 50mL conical tube containing 10mL of pre-warmed Dulbecco’s Modified Eagle Media (DMEM, high glucose; GIBCO®) and 10% fetal bovine serum (FBS, GibcoBRL). The cells were pelleted by centrifugation at 180g for 5 minutes. The supernatant was discarded and cells resuspended in 15mL of media. The cell suspension was transferred to a 75cm² tissue culture flask and incubated at 37°C, 5% CO2 (Thermo Scientific, Hanau, Germany).

Once cells reached ~90% confluency three days after plating, cells were split and reseeded into new 75cm² flasks. The cells were rinsed with 10mL of pre-warmed 1 x PBS (137mM NaCl, 8.1mM Na2HPO4, 2.7mM KCl, 1.5mM KH2PO4, pH 7.4) and incubated with 3mL of TrypleTM Express (Gibco) at 37°C for 2 minutes to detach cells. An equal volume of DMEM/10% FBS was added to inhibit trypsinisation, and the cells were pelleted by centrifugation at 180g at 20°C for 5 minutes. The supernatant was discarded and cells were resuspended in 5mL of DMEM/10% FBS. 1mL of the cell suspension was seeded into a new 75cm² tissue culture flask containing 14mL of DMEM/10% FBS and incubated at 37°C, 5% CO2.

3.2.2. Primary rat cortical astrocyte cultures

Postnatal day 2 (P2) male Sprague Dawley (SD) rats (250-300g; Vernon Jansen Unit, The University of Auckland) were used to generate primary cortical astrocytes in compliance with the University of Auckland Animal Ethics Committee Guidelines and the New Zealand Animal Welfare Act 1999. Pups were gently wiped with 70% ethanol-soaked tissue and euthanised by decapitation. The head was placed on a 5cm tissue culture dish, and an anterior to posterior midline incision was made along the scalp using a scalpel to reveal the skull. Another incision was made along the cranium, and cranial flaps removed with flat tip forceps. The brain was carefully removed with a small spatula and suspended in 5mL of ice-cold 1 x Hank’s balanced salt solution buffer (9.5g 10x HBSS, Sigma; 20mM HEPES; pH 7.4) in a 5cm tissue culture dish. Under a stereomicroscope, using one set of dissecting...
forceps to anchor the brain by the cerebellum, the olfactory bulb was removed with a second set of dissecting forceps, and a midline incision was made to separate the two cortical hemispheres and gently peel the cortices from the brain. The meninges were peeled off, and the cortices were transferred into 5mL of fresh HBSS.

The cortices were then minced into approximately 1mm3 cubes with a scalpel blade and the tissue suspension was transferred to a 50mL conical tube containing 5mL of 0.25% trypsin, and incubated at 37°C for 15 minutes, with gentle agitation every 5 minutes. After 15 minutes, enzyme activity was deactivated with 10mL of DMEM/ 10%FBS. The supernatant was carefully aspirated with a 10mL serological pipette, and any residual supernatant removed with a 1mL pipette tip. The cortical tissue was re-suspended in 5mL of DMEM/ 10% FBS, and dissociated into a single cell suspension by vigorous trituration using a 10mL pipette. After allowing ~2 minutes for large, undissociated pieces of tissue to collect at the bottom of the conical tube, ~4.5mL of the cell suspension was carefully transferred to a new 50mL tube, and the volume adjusted to 6mL with DMEM/ 10%FBS. 3mL of the diluted cell suspension per T75cm² tissue culture flask was seeded into two flasks and incubated at 37ºC, 5% CO₂. Four hours after plating, media was replaced to remove any non-adhered cellular debris, and every two days flasks were vigorously rinsed four times with pre-warmed 1 x HBSS to dislodge neurons and microglia. Cultures were maintained for up to 10 days to promote differentiation.

Once cells reached ~90% confluency at 8-10 days, cells were split and seeded into multi-well tissue culture plates at the required density. The cells were rinsed well with 10mL of pre-warmed 1 x HBSS (3 x 5 minutes), and incubated with 3mL of TrypleTM Express (Gibco) at 37°C for 5-10 minutes to detach cells. An equal volume of DMEM/ 10% FBS was added to inhibit trypsinisation, and the cells were pelleted by centrifugation at 180g at 20ºC for 5 minutes. The supernatant was discarded and cells were resuspended in 5mL of DMEM/ 10% FBS, and 10µL of the cell suspension was removed to determine concentration.

3.2.3. Quantifying cell density and plating in multi-well tissue culture plates

To plate HEK293 cells or primary astrocytes at a specific cell density for tissue culture applications, cell density was quantified to determine the dilution required to achieve the required cell numbers in multi-well tissue culture plates. To count cells, 10µL of cell
suspension was diluted with 40μL of Trypan blue (0.4%, Gibco) for a 1:5 dilution. 10μL of the cell dilution was transferred to a coverslipped haemocytometer (Reichert, Buffalo NY) and cells counted in the four corner grids. The number of cells/mL was determined using the following formula.

\[
\text{Cells/mL} = \text{average cell count} \times \text{dilution factor} \times 10^4
\]

The dilution factor required to achieve the required cell density per well was determined by dividing the cell concentration (cells/ mL) by the cell density (cells/ well) required:

\[
\text{Dilution factor} = \frac{\text{cell concentration}}{\text{cell density}}
\]

Cell suspensions were diluted accordingly with DMEM/ 10%FBS and plated onto multi-well tissue culture plates of appropriate surface areas, and incubated at 37ºC, 5% CO\(_2\) for 24 hours prior to experimentation.

### 3.2.4. Plasmid DNA transfection into cells

HEK293 cells or primary astrocytes plated at a density of 1 x 10\(^5\) cells in 0.5mL of media per well in 24-well plates were transfected with AAV expression plasmids to confirm protein expression prior to vector packaging. A transfection mixture containing 0.5μg of plasmid DNA and 1μL of XtremeGENETM HP (Roche) transfection reagent in 25μL of Optimem (serum free media, GiboBRL) was prepared and incubated at room temperature for 15minutes, and subsequently added to each well. The cultures were returned to the 37ºC incubator, and fixed with 4% PFA 48 hours post-transfection.

### 3.2.5. AAV vector transductions into primary astrocytes

Primary astrocytes were plated onto non-coated 96-, 24-, or 6-well plates at a density of 2.4 x 10\(^4\) (in 100μL of media), 1 x 10\(^5\) (in 200μL), or 3 x 10\(^5\) cells (in 2mL) respectively. Twenty-four hours later, each well of 96-, 24-, or 6-well plates was transduced with 8 x 10\(^8\) genomes, 4 x 10\(^9\) genomes, or 1.6 x 10\(^10\) genomes of vector respectively. Plates were swirled gently to evenly distribute vector in the media and incubated at 37ºC. Seventy-two hours post vector
transduction cells were fixed with an equal volume of 4% PFA (section 3.2.6), or manipulated further as outlined below.

3.2.6. 4% PFA fixation of cells

Forty-eight hours and 72 hours post-transfection or -transduction, cells were fixed with 4% PFA. 0.5mL of 4% PFA per well was added and incubated at room temperature for 15 minutes. Subsequently, the 4% PFA was aspirated and 1x PBS containing 0.1% sodium azide was added to each well to prevent microbial growth. Parafilm-secured plates were stored at 4°C.

3.2.7. Immunocytochemistry

4% PFA-fixed cells were rinsed with PBS-T, and incubated with 1% (v/v) hydrogen peroxide/ 50% methanol for 5 minutes to block endogenous peroxidase. Cells were subsequently rinsed with PBS-T (2 x 5 minutes) and incubated with 200μL of primary antibodies diluted in IB (Table 2-1) with gentle agitation at room temperature. The following day, cells were rinsed twice with PBS-T and incubated with 200μL of biotinylated secondary antibodies for 3 hours at room temperature, and further incubated with 200μL ExtrAvidin Peroxidase (1:250, Sigma) for 2 hours. The cells were once again rinsed twice with PBS-T and incubated with PB buffered DAB solution for ~5 minutes. Negative controls were conducted by omitting primary antibodies. Images of immunostained cells were acquired using an inverted microscope (Nikon Eclipse TE200-S, Tokyo, Japan) equipped with a digital camera (Nikon CoolPix 4500).

For immunofluorescence labelling, 0.01% hydrogen peroxide treatment was omitted, and cells were incubated with 200μL of primary antibodies as above. The following day, cells were rinsed twice with PBS-T and incubated with 200μL of donkey anti-rabbit Alexa-488 secondary antibody (1:1000; Invitrogen), donkey anti-mouse Alexa-488. For Iba1 detection, cells were incubated with biotinylated donkey anti-goat secondary antibody (1:1000; Jackson ImmunoResearch) for 24 hours at 4°C, rinsed with PBS-T and further incubated with streptavidin Alexa-594 (1:1000; Invitrogen) for 24 hours at 4°C.
3.2.8. Quantification of cell-specific transgene expression using confocal laser scanning microscopy

An imaging protocol for confocal microscopy was generated for quantification of neuronal-specific transgene expression. For each brain, once the region of highest transgene expression in the nigra was identified, two 40μm coronal sections separated by 240μm on both rostral and caudal aspects of this area were selected. Each section was incubated with rabbit anti-GFP and mouse anti-HuCD diluted in IB for 48 hours at 4°C, and subsequently incubated with donkey anti-rabbit Alexa488 and donkey anti-mouse Alexa594 for 24 hours at 4°C, and post-processed as described in section 2.2.11.

On a FV1000 confocal scanning microscope, using a x10 objective, an area within the SNpc that contained transduced cells exhibiting distinct cell morphologies was selected. Subsequently, using a x60 oil immersion objective, and guided by the Olympus Fluoview software, a 2μM guard zone was set at the top of the section. Relative to the guard zone, twenty images (twenty planes at a step-size of 0.5μM) were acquired sequentially in each of the respective channels, within a tissue thickness of 10μM. The projected images were merged and neurons that were clearly co-labelled with eGFP and HuCD were manually quantified on each of the sections. Prior to the above image acquisition for quantitative analysis, an additional section per animal (consecutive to one of the above sections) was also immunofluorescently labelled, and an image of each section was acquired using a x60 oil immersion objective to determine optimal image acquisition settings.

3.2.9. Mechanical injury

Primary astrocytes were plated onto 6-well plates at a density of 3 x 105 cells per well and transduced with AAV5-GFAP-dYFP or AAV5-GFAP-HA-NURR1 vectors 24-hours after plating (n = 4 wells per vector). Seventy two hours post-vector transduction, two wells per treatment were gently bisected three times at an angle of ~60° with a 20μL pipette tip to simulate mechanical injury. Two additional wells served as uninjured controls. 24 post-injury, media was aspirated, and wells gently rinsed with 2mL of ice-cold 1 x PBS (2 x 5 minutes). 100μL of radioimmunoprecipitate (RIPA) buffer containing a protease inhibitor cocktail (Roche) per well was added and incubated on ice for 15-20 minutes with agitation every 5 minutes. Subsequently, cell lysates were collected into 1mL microfuge tubes and
centrifuged at 8000g at 4°C for 10 minutes to remove cellular debris (Eppendorf 5417R centrifuge). The supernatant containing soluble protein was transferred to 0.5mL microfuge tubes, and stored at -20°C. Using an aliquot of supernatant, protein concentration in each sample was quantified using a Pierce™ Bicinchoninic Assay (BCA) kit according to manufacturer’s instructions.

3.2.10. Western blot assay

RIPA buffer-solubilised protein samples were thawed on ice, and samples were concentration-matched with additional RIPA buffer. 5µL of 6 x Laemmli buffer was added to 20µL of each sample and heated at 95°C for 5 minutes to denature proteins. The denatured proteins (concentration matched to 10-15µg per sample) were separated by SDS-PAGE gel electrophoresis (section 2.2.6). Following electrophoresis, separated proteins were transferred onto a Hybond ECL nitrocellulose membrane (Amersham) using the TransBlot™ Turbo Blotting System (BioRad). The resolving gel, nitrocellulose membrane and two pieces of extra thick filter paper (BioRad) were equilibrated in transfer buffer (48mM Tris; 39mM glycine; 20% methanol) for 10 minutes with gentle agitation. A transfer ‘sandwich’ was assembled on the electroblotting cassette according to the manufacturer’s instructions and transferred at 25V for 40 minutes. Subsequently, the nitrocellulose membrane was stained with Ponceau S solution for 5 minutes to visualise transferred proteins and molecular weight standards. The molecular weight standards were marked with pen, and the membrane was rinsed with 1 x Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) (20mM Tris; 0.5M NaCl; 0.05% Tween 20; pH 7.4) (3 x 10 minutes).

To block non-specific antibody binding, the membrane was incubated in 5% (w/v) non-fat dry milk in TBS-T for 1 hour at room temperature with gentle agitation. The membrane was rinsed with TBS-T for 5 minutes, incubated with primary antibodies diluted in TBS-T containing 1% (w/v) milk overnight at 4°C. The following day, the membrane was rinsed with TBS-T (3 x 10 minutes), and incubated with horseradish peroxidase-conjugated secondary antibodies diluted in TBS-T at room temperature for 2 hours. The membrane was rinsed with TBS-T (3 x 10 minutes) and placed on a transparent plastic bag after removing excess buffer. Enhanced chemiluminescence (ECL) detection reagent (Amersham) prepared according to the manufacturer’s instructions was evenly pipetted onto the membrane and incubated for 5 minutes. Chemiluminescence was visualised and gel images acquired using a
ChemiDoc MP (BioRad) system equipped with a charge-coupled device (CCD) camera. Density of proteins of interest relative to the reference protein, β-actin was estimated using the Image Lab software (version 4.1; BioRad).

### 3.2.11. NF-κB translocation assay

Primary astrocytes were plated onto non-coated 96-well plates at a density of $2.4 \times 10^4$ (in 100µL of media), and 24 hours post-cell adhesion, wells was transduced with $8 \times 10^8$ genomes of vector. 72 hours post-transduction, cells were treated with LPS (5ng/mL or 50ng/mL), or 1 x HBSS (control) (n = 3 wells per treatment), and fixed with 4% PFA at 2, 5, 8, or 24 hours post-LPS treatment. For immunofluorescence, wells were incubated with rabbit anti-NF-κB for 24 hours at room temperature, and subsequently incubated with donkey anti-rabbit Alexa 594 (1:1000; Invitrogen) for 1 hour at room temperature. Subsequently, cell nuclei were counterstained with 300nM 4,6-diamidino-2-phenylindole (DAPI, D9542, Sigma) diluted in 1 x PBS for 20 minutes, and washed with PBST (2 x 10 minutes). Images were acquired at 4 sites within each well (each separated by 20µM) using an automated ImageXpress® Micro XLS microscope (Version 5.3.0.1, Molecular Devices, Sunnyvale, USA). Prior to analysis, images were manually examined, and those of poor quality were excluded. Using the Enhanced-Translocation function of MetaXpress software (Version 5.3, Molecular Devices, Sunnyvale, USA), nuclear co-localisation of NF-κB with DAPI was quantified based on the size of the nuclear compartment and a pre-determined threshold intensity to identify nuclear localisation of immunoreactivity.

### 3.2.12. Statistical analysis

Comparison of neuronal transgene expression was performed by independent sample t-test, and comparisons of LPS-induced percentage NF-κB nuclear translocation were performed by one-way ANOVA as described in section 2.2.13.
3.3. Results

3.3.1. Confirmation of structural integrity of AAV expression plasmids

All plasmid constructs depicted in Figure 3-2 were generated by Dr Alexander Muravlev. dYFP, HA-NURR1, HA-tNURR1, or FLAG-Nrf2 derived from NC16 pcDNA3.1-FLAG-NRF2 (36971; Addgene) were cloned into an empty AAV expression plasmid containing the 2.2kb GFAP promoter and a multiple cloning site, commonly known as a polylinker (pL). To generate CMV- or gfaABC1D-regulated therapeutic constructs, the GFAP promoter was replaced with CMV (Figure 3-2 f), or gfaABC1D promoters derived from pTFY-gfaABC1D-tTA (19977; Addgene) (Figure 3-2 h, i). Sequence and structural integrity of all plasmids generated were confirmed with DNA sequencing and RE digest analysis, respectively. Briefly, the presence of AAV ITRs required for vector packaging was confirmed with a SmaI digest, and other plasmid-specific REs were used to confirm the presence and correct orientation of promoters, transgenes, and WPRE2 and spA (shorter WPRE and BGHpA counterparts, respectively) sequences. The RE digest-generated fragments were consistent with fragment sizes calculated from predicted RE cleavage sites in the plasmid maps confirming the presence and correct orientation of the elements within each of the plasmids.

3.3.2. Assessing purity of vector preparations

Following RE-digest analysis of structural integrity, and confirmation of functionality by ICC analysis of transgene expression in plasmid-transfected primary rat cortical astrocytes or HEK293 (section 3.3.3), expression plasmids were packaged into AAV vector constructs depicted in (Figure 3-2).

To characterise these AAV vector stocks, a 10µL aliquot of each AAV vector was heat-denatured and separated on SDS-PAGE and stained with Coomassie blue to confirm the presence of AAV particles, and assess the purity of the vector preparations. AAV capsid proteins VP1 (87kDa), VP2 (73kDa) and VP3 (62kDa) assemble in the nucleus in a 1:1:18 stoichiometry to form mature virions (Wu, Asokan, & Samulski, 2006). The molecular weight and intensity of three Coomassie-stained protein bands corresponded to the molecular weights and assembly ratio of the capsid proteins in each vector sample (Figure 3-3). Faint, non-specific bands in AAV5-GFAP-FLAG-Nrf2, AAV5-CMV-HA-NURR1, and AAV5-
gfaABC1D-dYFP indicated that these vector preparations contained some minor contaminating proteins, whereas an absence of non-specific bands in others vector samples confirmed high purity of these vector stock. The genomic titre of the rAAV vector stocks were determined by real-time quantitative PCR. The purified vectors were of high titre, ranging from $4.56 \times 10^{12}$ to $4.88 \times 10^{14}$ viral genomes/mL (vg/mL) (Table 3-1).
Figure 3-2 Agarose gel electrophoresis of restriction enzyme digest fragments of AAV expression plasmids

The RE digest-generated fragments were consistent with fragment sizes calculated from predicted RE cleavage sites in the plasmid maps confirming plasmid integrity.
GFAP (195-2402)

AmpR (4424-5274)

ITR (1-183)

GFAP-pL-WPRE2-SpA
6202 bp

XhoI (1277)

KpnI (2150)

SacI (2229)

SmaI (2300)

BamHI (2404)

XhoI (2411)

SacI (2423)

NruI (2425)

SpeI (2428)

EcoRI (2440)

EcoRV (2448)

HindIII (2452)

BglII (2616)

WPRE2 (2457-2811)

SpA (2818-2868)

ITR (2883-3059)

SalI (2813)

SacI (2831)

ClaI (2840)

NruI (2871)

EcoRI (2878)

SmaI (3033)

SmaI (3044)

1) XhoI  5517, 1134
2) EcoRI  5764, 438
3) KpnI/ SalI  4428, 1950, 194, 79
4) SmaI  3193, 2254, 1182, 11, 11
b) Diagram showing restriction sites and gene locations:

- **AmpR (5246-6096)**
- **GFAP (190-2392)**
- **dYFP (2412-3257)**
- **WPRE2 (3297-3651)**

Restriction sites:
- **Sma1 (35)**
- **Sma1 (46)**
- **XhoI (1277)**
- **PvuII (1764)**
- **KpnI (2150)**
- **SacI (2229)**
- **Sma1 (2300)**
- **BamHI (2394)**
- **HindIII (3130)**
- **HindIII (3265)**
- **BgIII (3438)**

Gene locations:
- **ITR (3705-3881)**
- **spA (3658-3708)**

Southern blot images:

1) **Sma1**
   - Bands: 3193, 2254, 1155, 11, 11

2) **BamHI/ HindIII**
   - Bands: 6153, 736, 135
1) Smal: 3193, 2254, 1617, 720, 150, 62, 11, 11
2) EcoRI/HindIII: 7258, 426, 334
3) BamHI: 6881, 1137
4) XhoI: 6871, 1124, 23
d) 

**AmpR (6141-6991)**

**ITR (4600-4776)**

**SpA (4535-4585)**

**WPRE2 (4174-4528)**

**GFAP (190-2392)**

**HA-tag (2433-2468)**

**Nurr1(delta aa1-34) (2469-4173)**

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**1) SmaI**  
3193, 2254, 1617, 621, 150, 62, 11, 11

**2) XhoI/NdeI**  
7249, 1124, 431, 23

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107
1) BamHI/ EcoRI  5718, 1062, 832, 438
2) SacI      3571, 2450, 2029
3) HindIII  7171, 879
4) Smal    3193, 2581, 2254, 11, 11
f) Amplification of the genomic region around the CMV-HA-NURR1-WPRE2-SpA vector.

- **AmpR (4732-5582)**
- **CMV (234-821)**
- **HA-tag (925-960)**
  - **VdeI (1446)**
  - **SmaI (1512)**
  - **SmaI (1662)**
  - **SmaI (1724)**
- **BamHI (2023)**
- **mouse Nurr1 (962-2764)**
- **WPRE2 (2765-3119)**
  - **SalI (3121)**
  - **SacI (3139)**
  - **ClaI (3148)**
  - **NarI (3179)**
  - **EcoRI (3186)**

**Gel Analysis**

- Lane 1: SmaI fragment sizes: 3193, 1617, 1466, 150, 62, 11, 11
- Lane 2: XhoI/NdeI fragment sizes: 5261, 530, 429, 290
g)

AmpR (4810-5660)

CMV (234-821)

FLAG (943-969)

NRF2 (982-2799)

HindIII (1959)

EcoRI (1994)

WPRE2 (2843-3197)

SpA (3204-3254)

1) SmaI  3373, 3193, 11, 11
2) XhoI  4865, 1723
3) HindIII 4664, 1045, 879

1) 2) 3)
1) Smal 3193, 2066, 727, 707, 150, 62, 11, 11
Figure 3-3 SDS-PAGE of denatured AAV vector preparations

The molecular weight and intensity of three Coomassie-stained protein bands corresponded to the molecular weights and assembly ratio of the AAV capsid proteins, VP1, 2 and 3. Faint, non-specific bands in e) AAV5-GFAP-FLAG-Nrl2, f) AAV5-CMV-HA-NURR1, and g) AAV5-gfaABC1D-dYFP indicated that these vector preparations contained some minor protein contaminants, whereas an absence of non-specific bands in others vector samples confirmed high purity of these vector stock.
<table>
<thead>
<tr>
<th>Vector</th>
<th>Titre (vg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) AAV5-GFAP-pL</td>
<td>$3.54 \times 10^{13}$</td>
</tr>
<tr>
<td>b) AAV9-GFAP-dYFP</td>
<td>$4.56 \times 10^{12}$</td>
</tr>
<tr>
<td>c) AAV5-GFAP-dYFP</td>
<td>$4.13 \times 10^{13}$</td>
</tr>
<tr>
<td>d) AAV5-GFAP-NURR1</td>
<td>$2.45 \times 10^{13}$</td>
</tr>
<tr>
<td>e) AAV5-GFAP-NRF2</td>
<td>$5.93 \times 10^{12}$</td>
</tr>
<tr>
<td>f) AAV5-CMV-HA-NURR1</td>
<td>$2.04 \times 10^{14}$</td>
</tr>
<tr>
<td>g) AAV5-gfaABC3D-dYFP</td>
<td>$1.11 \times 10^{14}$</td>
</tr>
<tr>
<td>h) AAV5-gfaABC3D-NURR1</td>
<td>$4.88 \times 10^{14}$</td>
</tr>
<tr>
<td>i) AAV5-GFAP-eGFP</td>
<td>$8.0 \times 10^{13}$</td>
</tr>
<tr>
<td>j) AAV5-GFAP-eGFP-miR124Tx4</td>
<td>$5.92 \times 10^{13}$</td>
</tr>
</tbody>
</table>

Table 3-1 Stock vector titres (vg/mL)
3.3.3. Transgene expression in plasmid transfected primary astrocytes and HEK293 cells

Prior to vector packaging, the functional integrity of the AAV expression plasmids was confirmed by ICC analysis of transgene expression in primary rat cortical astrocytes and HEK293 cells transfected with each plasmid. Three plasmid (µg) to transfection reagent (XtremeGENE™ HP) (µL) ratios (1:1, 1:2, and 1:4) were tested, and subsequent experiments used a ratio of 1:2 that mediated detectable levels of transgene expression in the absence of toxicity was selected for astrocyte transfections. This transfection protocol was also utilised for HEK293 cell transfections. Twenty four hours after plating, cells were transfected with pAAV expression plasmids, fixed 48-hours post-transfection, and transgenic protein expression was confirmed by ICC using antibodies to the respective transgenic proteins.

3.3.3.1. Primary cortical astrocyte cultures

Primary cortical astrocyte cultures were isolated and differentiated in culture. To confirm that the primary cultures were enriched with differentiated astrocytes, the levels of expression of neuronal and glial markers were determined. The cultures consisted predominantly of astrocytes that exhibited a cuboidal or elongated morphology; GFAP-immunoreactivity revealed the elaborate cytoskeletal networks within the cell cytoplasm (Figure 3-4 a, d). Iba1-immunoreactivity revealed the presence of a small number of Iba1-positive microglia residing on top of the astrocyte monolayer (Figure 3-4 b, e). An absence of neuronal microtubule-associated protein 2 (MAP2) expression indicated a lack of neurons in these cultures (Figure 3-4 c, f). Staining specificity was confirmed by omitting primary antibodies from the immunocytochemical procedure (Figure 3-4 g, h).
Figure 3-4 Immunocytochemical characterisation of primary astrocyte cultures

Primary glia cultures consisted predominantly of (a, d) GFAP-positive astrocytes that exhibited a cuboidal morphology with extensive GFAP-positive cytoskeletal networks. GFAP immunoreactivity revealed the extensive cytoskeletal networks within the cell body. (b, e) A small number of Iba1-positive microglia were also detected. (c, f) No MAP2-positive neurons were present in the cultures. GFAP expression was efficiently detected using both Alexa488 and other fluorescent dye-conjugated secondary antibodies (Alexa594, cy2 and cy3), while Iba1 was detected only using Alexa594. No MAP2 expression using Alexa488 or Alexa594 was detected.

Staining specificity was confirmed by omitting the anti-GFAP (g) and anti-Iba1 (h) antibodies from the immunocytochemical protocol.

Scale bar: 100µm
no primary controls

anti-GFAP

a

d

g

anti-Iba1

b

e

h

anti-MAP2

c

f
3.3.3.2. In vitro and in vivo transduction properties of AAV serotypes 5 and 9

Firstly, in vitro and in vivo comparative analyses of the astrocyte-tropic AAV serotypes 5 and 9 were conducted to determine the serotype most suited for gene delivery to the rat SN. To determine the efficacy of coupling the GFAP promoter sequence with these AAV serotypes, a transgenic cassette encoding GFAP-regulated reporter dYFP (GFAP-dYFP) was generated, as visualisation of native fluorescence as well as ICC using a sensitive anti-GFP antibody could be used to detect expression. The expression plasmid was transfected into primary astrocytes, and transgene expression analysed by ICC to confirm its functionality. Forty-eight hours post-transfection, GFAP-regulated dYFP expression was detected in both the nucleus and throughout the cytoplasm (Figure 3-5). Interestingly, the morphology of the transfected astrocytes differed considerably to that of naïve astrocytes (Figure 3-4 a, d), and AAV vector transduced (Figure 3-6) astrocytes. Plasmid transfected astrocytes exhibited a more rounded cell body and extended multiple fine processes.

Once functionality of the transgenic cassette was confirmed in vitro, the GFAP-dYFP expression plasmid was packaged into AAV5 or AAV9. As AAV8 mediated the least effective nigral transduction (section 2.3.3), this serotype was excluded from further analysis. Prior to investigating nigral transduction in vivo, vector constructs were tested on primary astrocytes in vitro. AAV vectors (4 x 10^9 genomes) were applied onto primary astrocytes plated on 24-well plates, and fixed 72 hours post-transduction for ICC analysis. GFP immunoreactivity revealed that AAV5 mediated considerably higher levels of dYFP expression than AAV9 (Figure 3-6). With both vector serotypes, dYFP was expressed in the cell nucleus and throughout the cytoplasm, and the morphology of transduced astrocytes was comparable to that of naïve astrocytes.

Given that in vitro vector transduction properties may not accurately represent vector transduction in vivo, titre-matched AAV5 and AAV9 vectors (2 x 10^8 genomes) were then tested in the rat SNpc. A ten-fold lower titre than the standard titre used in the laboratory for SNpc vector infusions was used to assist clear discrimination of AAV serotype-specific differences in transduction. Three weeks post-vector infusion, IHC analysis of PFA-fixed brains showed that AAV5 appeared to predominantly target nigral astrocytes (Figure 3-7 a-c), while AAV9 targeted both nigral neurons and astrocytes (Figure 3-7 d-f); dYFP-immunoreactive neurons exhibited elongated cell bodies that tapered into fibres (Figure 3-7 f, arrowhead), and ‘star-like’ astrocytes exhibited highly ramified processes (Figure 3-7 c,
and f, arrow). In both neurons and astrocytes, dYFP was expressed throughout the nucleus and cytoplasm. Contrary to in vitro transduction, AAV9 mediated superior transduction with high expression in the SNpc, SNpr, and ventral regions of midbrain reticular nucleus and thalamus. AAV5-mediated transgene expression was relatively confined to the SNpc, with dorso-ventral dissemination more restricted to the ventral region of reticular nucleus immediately above the SNpc. As the preliminary aim of this study was to determine the efficacy of astrocyte-specific therapeutic manipulation in the degenerating SNpc, AAV serotype 5 that exhibited predominant astrocyte tropism and relative region-specificity was selected to deliver therapeutic cassettes to the rat SNpc.
Figure 3-5 Transgenic protein expression in plasmid-transfected primary rat astrocytes

dYFP expression was detected throughout the cell body and nucleus, confirming functionality of the expression cassette. In contrast to naïve astrocytes, transfected astrocytes exhibited a more rounded cell body and extended multiple fine processes.

Scale bars: (a) 100µm (b) 50µm

Figure 3-6 GFAP promoter-regulated dYFP expression in AAV5 and AAV9 transduced primary astrocytes.

a, b) AAV5-mediated considerably higher dYFP expression in astrocyte than c, d) AAV9. With both vector serotypes, dYFP was expressed thought the cell body and nucleus.

Scale bars: (a, c) 100µm (b, d) 50µm
Figure 3-7 dYFP expression in SNpc regulated by the GFAP promoter in the context of AAV5 and AAV9

(a, b) AAV5-mediated transgene expression was relatively confined to the SNpc, with dorso-ventral dissemination more restricted to the ventral region of reticular nucleus immediately above the SNpc. AAV5-mediated predominantly astrocytic dYFP expression in the SNpc (c; arrow); dYFP-immunoreactive cells exhibited the typical ‘star-like’ astrocytic morphology with highly ramified processes.  

(d, e) AAV9-mediated transduction was more widespread with dYFP expression detected in the SNpc, SNpr, and ventral regions of midbrain reticular nucleus and thalamus. Moreover, AAV9 targeted both nigral astrocytes (f, arrow) and neurons (f, arrowhead); in addition to ‘star-like’ dYFP-immunoreactive astrocytes, dYFP-positive neurons that exhibited elongated cell bodies and tapering fibres were detected.

Scale bars: (a, d) 500µm, (b, e) 200µm, and (c, f) 100µm
3.3.4. Optimisation of transcriptional regulation of therapeutic expression cassettes

3.3.4.1. Transgene expression in plasmid-transfected primary astrocytes

To optimise astrocyte tropism at the transcriptional level, comparative analysis of the GFAP, CMV and gfaABC1D promoters in the context of AAV5 was conducted in vitro and in vivo. Reporter and therapeutic expression cassettes regulated by the respective promoter elements (Figure 3-2) were generated and RE digest-analysed to confirm structural integrity (section 3.3.1). Prior to packaging these expression cassettes into AAV5 vectors for characterisation of transduction in vitro and in vivo, the plasmid constructs were subsequently transfected into primary astrocytes and HEK293 cells to determine functionality. As previously stated, GFAP promoter-regulated dYFP protein expression was detected in both the nucleus and throughout the cytoplasm in primary astrocytes (Figure 3-8 a, e). In contrast, transgenic HA-NURR1 expression was exclusively detected in the nucleus using an antibody against the HA tag (Figure 3-8 c, g). However, no immunoreactive signal that paralleled HA-immunoreactivity was detected when an anti-NURR1 antibody (not shown) was used. No GFAP-regulated FLAG-Nrf2 expression was detected in primary astrocytes when an anti-FLAG (Figure 3-8 b, f) or anti-Nrf2 antibody (not shown) was used.

We also generated a construct encoding a truncated NURR1 (tNURR1) variant by replicating a recently defined sequence deletion that diminishes the rate of proteasomal degradation of NURR1 without compromising functionality (Alvarez-Castelao, Losada, Ahicart, & Castaño, 2013), to determine whether this may prolong NURR1 protein expression in primary astrocytes in vitro and potentially in the SNpc in vivo. Contrary to its purported gain-of-function sequence deletion, the amount of GFAP-regulated HA-tNURR1 expression in astrocytes transfected with GFAP-HA-tNURR1 was lower in comparison to that mediated by the GFAP-HA-NURR1 construct at the same plasmid concentration (Figure 3-8 d, h).

Primary astrocytes transfected with low efficiency, and GFAP promoter-regulated therapeutic transgene expression in primary astrocytes was undetectable with anti-NURR1 and anti-Nrf2 antibodies. To confirm that HA-NURR1 and FLAG-Nrf2 are expressed, and to determine whether a highly active viral promoter may enhance expression levels, expression cassettes encoding CMV promoter regulated HA-NURR1 or FLAG-Nrf2 (Figure 3-1 f, g) were generated and transfected into primary astrocytes as above. The CMV promoter moderately
improved FLAG-Nrf2 expression in primary astrocytes as evident by nuclear-localised FLAG-immunoreactivity in a small number of cells (Figure 3-9 a). However, no Nrf2-immunoreactivity was detected using the anti-Nrf2 antibody (not shown). Although higher levels of HA-NURR1 expression were achieved with the CMV promoter compared to the GFAP promoter (Figure 3-9 e), NURR1 immunoreactivity was still undetectable using the anti-NURR1 antibody (not shown).
Figure 3-8 GFAP promoter-regulated transgenic protein expression in primary rat astrocytes transfected with AAV expression plasmids

Following plasmid transfection into primary astrocytes, (a, e) dYFP-immunoreactivity was detected throughout the cell body and nucleus. dYFP-positive astrocytes exhibited a rounded cell body, and extended multiple processes.

(b, f) No FLAG-Nrf2 expression was detected using an anti-FLAG antibody.

(c, g) Exclusively nuclear-localised HA-NURR1 expression was detected by HA-immunoreactivity.

(d, h) Expression of nuclear HA-tNURR1 was lower than that of the full length HA-NURR1. ICC was performed on non-transfected astrocytes to confirm staining specificity of the respective antibodies; (i) anti-GFP, (j) anti-FLAG and (k) anti-HA

Scale bars: (a-d, i-k) 100μm (e-h) 50μm
anti-GFP
GFAP-dYFP

anti-FLAG
GFAP-FLAG-Nrf2

anti-HA
GFAP-HA-NURR1

anti-HA
GFAP-HA-1NURR1

non-transfected
3.3.4.2. Therapeutic transgene expression in plasmid-transfected HEK293 cells

Due to the lack of detection of therapeutic protein expression using anti-NURR1 and anti-Nrf2 antibodies, the CMV promoter-regulated therapeutic cassettes were tested in HEK293 cells. We have previously noted that in HEK293 cells, while viral promoters are highly active, GFAP promoter activity is inefficient. Transfection of the CMV-FLAG-Nrf2 construct into HEK293 cells substantially increased detection of both FLAG- and Nrf2-immunoreactive cells (Figure 3-9 b and c, respectively). FLAG-Nrf2 was predominantly expressed in the nucleus, as evident by the darkly stained nuclei, while sparse cytoplasmic immunoreactivity indicates some cytoplasmic protein localisation. Moreover, high levels of both anti-HA and anti-NURR1 were detected in HEK293 cells transfected with CMV-HA-NURR1 (Figure 3-9 f and g, respectively). As per expression in primary astrocytes, HA-NURR1 expression was restricted to the nucleus in HEK293 cells. Therefore, in subsequent studies, FLAG and HA antibodies were used for therapeutic protein detection.

Non-transfected cells were stained with anti-NURR1 and anti-Nrf2 to confirm an absence of endogenous Nrf2 or NURR1 expression in HEK293 cells (Figure 3-9 d and h, respectively). Anti-FLAG and anti-HA also demonstrated an absence of non-specific staining (not shown).
Figure 3-9 CMV promoter-regulated therapeutic gene expression in primary rat astrocytes and HEK293 cells transfected with AAV expression plasmids

In primary rat astrocytes, CMV promoter moderately improved therapeutic protein expression; predominantly nuclear (a) FLAG-positive FLAG-Nrf2, and (e) HA-positive HA-NURR1 were detected.

In HEK293 cells, high levels of FLAG-Nrf2 predominantly in the nucleus with sparse cytoplasmic expression was detected with antibodies against (b) FLAG and (c) Nrf2.

High levels of exclusively nuclear HA-NURR1 expression was detected with antibodies against (f) HA and (g) NURR1.

ICC with (d) anti-Nrf2 and (h) anti-NURR1 antibodies performed on non-transfected HEK293 cells confirmed an absence of endogenous Nrf2 or NURR1 expression in HEK293 cells, respectively.

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3.3.5. Promoter-dependent transgene expression in the context of AAV5 in primary astrocytes

Once functionality of the AAV expression plasmids was confirmed by ICC detection of transgene expression in plasmid-transfected primary astrocytes and HEK293 cells, constructs were packaged into AAV5 to determine vector transduction efficacy in vitro and in vivo. Two additional expression constructs encoding dYFP reporter or HA-NURR1 regulated by gfaABC$_{1}$D (681bp), a truncated variant of the GFAP promoter were generated to determine its ability to retain astrocytic tropism and transcriptional activity in the nigra. The GFAP promoter-regulated expression cassettes are considerably large and approach the ~4.7kb packaging capacity of AAV. Given that large expression plasmids may compromise AAV vector stability (Lai et al., 2009; Wu et al., 2009), the additional therapeutic constructs regulated by CMV or gfaABC$_{1}$D were included to determine the capacity of these promoters to regulate AAV5 vector tropism and/or efficacy of transgene expression. Excluding GFAP-HA-tNURR1 and CMV-FLAG-Nrf2, all other plasmid constructs were packaged into AAV5, and tested on primary astrocytes as previously.

AAV vectors (4 x 10$^{9}$ genomes) were applied onto primary astrocytes plated on 24-well plates, and fixed 72-hours post-transduction for ICC analysis. Efficient astrocytic transgene expression was achieved with both GFAP and gfaABC$_{1}$D promoters, as evident by the high levels of dYFP-immunoreactivity (Figure 3-10 a and b, respectively). AAV5-mediated delivery, or the promoter elements did not alter the sub-cellular localisation of dYFP and HA-NURR1 expression; dYFP was expressed in the nucleus and throughout the cytoplasm (Figure 3-10 a, b), while HA-NURR1 expression was localised to the nucleus (Figure 3-10 d-f). In the context of AAV5, the gfaABC$_{1}$D promoter replicated the transcriptional properties of the parental GFAP promoter. While the CMV promoter activity was higher than that of the GFAP promoter in plasmid transfected astrocytes, AAV5-CMV-HA-NURR1-mediated HA-NURR1 expression (Figure 3-10 f) was considerably lower than that achieved with AAV5-GFAP-HA-NURR1 (Figure 3-10 d). AAV5-GFAP-FLAG-Nrf2 vector failed to mediate any detectable levels of transgene expression (Figure 3-10 c). Non-transduced cells were stained with each of the primary antibodies to confirm antibody specificity (Figure 3-10 g-i).

In addition to the characteristic astrocyte morphology revealed by cell-wide expression of dYFP, co-localisation of GFAP promoter-regulated dYFP or nuclear-localised HA-NURR1
with astrocytic GFAP confirmed astrocyte-specific transgene expression \textit{in vitro} (Figure 3-11).
Figure 3-10 AAV5 vector-mediated transgene expression in primary astrocytes

(a) GFAP- and (b) gfaABC₁D-regulated dYFP expression was detected throughout the cell body and nucleus.
(c) No FLAG-Nrf2 expression was detected with antibodies against FLAG
HA-immunoreactivity indicated exclusively nuclear expression of (d) GFAP-, (e) gfaABC₁D- and (f) CMV-regulated HA-NURR1.
ICC performed on non-transduced primary astrocytes with (g) anti-GFP, (h) anti-HA, and (i) anti-FLAG confirmed antibody specificity.

Scale bar: 100µm
Figure 3-11 AAV5 directs transgene expression to primary astrocytes

(a-c) dYFP (green) co-localisation with GFAP (red) indicates transgene expression throughout the cell body and in the nucleus (arrow).

(d-f) HA-NURR1 (red) exclusively expressed in the nucleus co-localised with the cytoplasmic GFAP (green) (arrow).

Scale bar: 100µM
3.3.6. AAV5-mediated transgene expression in the naïve rat SNpc

3.3.6.1. Promoter-dependent transgene expression

In section 3.3.3.2, we determined that AAV5-GFAP-dYFP that mediated predominant astrocytic transgene expression that was relatively confined to the SNpc was more suited for selectively targeting nigral astrocytes than AAV9-GFAP-dYFP, which transduced a wider region within the midbrain, and targeted both neurons and astrocytes. To clearly distinguish serotype-dependent transduction properties, AAV5 and AAV9 vectors were previously infused into the nigra at a ten-fold lower titre (2 x 10^8 genomes). In the context of AAV5, GFAP-, gfaABC1D-, and CMV-dependent cell tropisms and efficacy of transgene expression in the SNpc were characterised next in this study. Each of the vector constructs was infused into the rat SNpc (n = 3) at 2 x 10^9 genomes, and IHC analyses were performed three weeks post-vector transduction.

The ten-fold increase in vector titre resulted in a marked increase in nigral transduction by AAV5-GFAP-dYFP (Figure 3-12 b-d and Figure 3-13). Transgene expression remained predominantly confined to the SNpc, and extended minimally to the VTA. Dorso-ventral transgene expression in the regions immediately adjacent to the needle tract was detected in the ventral regions of the midbrain reticular nucleus, and SNpr. While transgene expression in the SNpc was almost exclusively astrocytic at the lower titre (Figure 3-13 c arrow), in addition to enhanced astrocytic transduction, neuronal transgene expression was observed at the higher titre (Figure 3-13 f, arrowhead).

Of the three promoters, the GFAP promoter mediated the highest level of both dYFP and HA-NURR1 expression in the nigra (Figure 3-12 b-d and j-l, respectively). Based on the morphology of transgene expressing cells located in the SNpc periphery, GFAP promoter-regulated dYFP immunoreactivity was detected in intricate ‘star-like’ astrocytes with highly ramified processes (Figure 3-12 d arrow), and neurons and their fibres (Figure 3-12 d arrowhead), indicating efficient nigral astrocytic and neuronal transduction. Small, rounded nuclear-like HA-immunoreactivity indicated that HA-NURR1 was expressed exclusively in the nucleus (Figure 3-12 l, arrow). Although the shorter gfaABC1D promoter directed transgene expression to astrocytes and neurons similar to the GFAP promoter, it exhibited weaker activity in comparison to its predecessor, as evident by the weaker dYFP- and HA-NURR1 immunoreactivity (Figure 3-12 f-h and n-p, respectively). AAV5-CMV-HA-
NURR1 failed to mediate any transgene expression (Figure 3-12 r-t). No detectable levels of FLAG-Nrf2 were achieved with AAV5-GFAP-FLAG-Nrf2 (Figure 3-12 v-x). Based on these results, AAV5-GFAP-HA-NURR1 therapeutic vector and AAV5-GFAP-dYFP reporter construct were selected for further characterisation.

3.3.6.2. Rostro-caudal distribution of transgene expression

Based on GFAP promoter-regulated dYFP expression, at a vector titre of 2 x 10⁹ genomes, widespread nigral transgene expression along the rostro-caudal axis was detected 3 weeks post vector-infusion with a mean distribution of ~2.16mm (Figure 3-13 d). Whereas rostral transgene expression was predominantly confined to the SNpc with minimal spread into the VTA, considerable dorso-ventral spread of transgene expression is seen in the caudal SNpc with expression in the SNpr, and ventral regions of the midbrain reticular nucleus. While both dYFP and NURR1 exhibited similar rostro-caudal and dorso-ventral distribution, the levels of expression differed considerably for the two proteins. Furthermore, in comparison to the relatively homogenous expression of high levels of dYFP amongst AAV5-GFAP-dYFP injected animals, relatively low and variable expression levels were detected in AAV5-GFAP-HA-NURR1 injected animals (not shown).
Figure 3-12 Promoter-dependent transgene expression in the naïve rat SNpc transduced with AAV5 vectors

Three weeks post-unilateral vector injection into the SNpc, (b-d) GFP immunoreactivity indicated that the 2.2kb GFAP promoter mediated high levels of dYFP expression throughout the cell body and nucleus of nigral neurons (d, arrowhead), and astrocytes that exhibited a ‘star-like’ morphology with highly ramified processes (d, arrow).

(f-h) The 681bp gfaABC₁D promoter replicated a similar pattern of dYFP in the nigra; however dYFP expression levels were considerably lower than its predecessor, GFAP.

(j-l) The GFAP promoter mediated moderate levels of HA-NURR1 expression that was exclusively localised to the nucleus, as detected with an anti-HA antibody (l, arrow).

(n-p) The gfaABC₁D promoter mediated the expression of substantially lower levels of nuclear-localised HA-NURR1 expression (p, arrow).

(r-t) The CMV promoter failed to mediate detectable levels of HA-NURR1 expression.

(v-x) No GFAP promoter-regulated FLAG-Nrf2 was detected with an anti-FLAG antibody.

(a, e, i, m, q, u) Minimal non-specific staining was detected in the respective contralateral hemispheres of vector-transduced brains.

Scale bars: (a, b, e, f, i, j, m, n, q, r, u, v) 500µm, (c, g, k, o, s, w) 200µm, and (d, h, l, p, t, x) 100µm
Figure 3-13 Vector titre-dependent transduction properties of AAV5 in the SNpc

(a-c) Transgene expression that was relatively confined to the SNpc, and almost exclusively targeted to nigral astrocytes was achieved with AAV5-GFAP-dYFP at a titre of $2 \times 10^8$ genomes (c, arrow).

(d-f) The tenfold increase in vector titre to $2 \times 10^9$ genomes resulted in a marked increase in nigral transduction, and greater dissemination of transgene expression along the dorso-ventral axis. In addition to enhanced astrocytic transgene expression (f, arrow), neuronal tropism was observed at the higher titre (f, arrowhead).

Scale bars: (a, d) 500µm, (b, e) 200µm, and (c, f) 100µm
3.3.6.3. Nigral cell tropism of AAV5-GFAP-dYFP

To determine cell tropism, dYFP expressing nigral sections were double labelled with antibodies against dYFP and the DA neuronal TH, astrocytic GFAP or microglial Iba1 (Figure 3-14). Co-localisation of dYFP with TH and GFAP immunoreactive cells confirmed that AAV5 transduces nigral neurons and astrocytes, respectively (Figure 3-14 a-c and d-f, respectively); dYFP and TH co-expressing neurons exhibited elongated cell bodies that tapered into fibres, and dYFP and GFAP co-expressing ‘star-like’ astrocytes exhibited highly ramified processes. High intensity dYFP immunoreactivity was predominantly detected in astrocytes. Interestingly, transduced neurons within the SNpc that exhibited high intensity dYFP immunoreactivity did not co-localise with TH. Iba1-positive microglia that exhibited a rounded cell body and multiple branched processes did not co-localise with dYFP, indicating an absence of microglial transgene expression (Figure 3-14 g-i). Fluorescent labelling of anti-HA was inefficient, and yielded high levels of non-specificity and suboptimal HA staining. Experimental protocols for optimising immunofluorescence included two different anti-HA primary antibodies (mouse anti-HA, MMS-101P, Covance and rabbit anti-HA, ab13834, Abcam); various fluorescent dye-labelled secondary antibodies and concentrations; biotin-labelled secondary antibodies and streptavidin-conjugated Alexa488 or Alexa595; various buffers (horse, or goat serum containing immunobuffer, or serum-free 1 x PBS); and antibody incubation times and temperatures. However, none of these procedures improved detection.
Figure 3-14 GFAP promoter in the context of AAV5 directs transgene expression to neurons and astrocytes in the SNpc

dYFP immunoreactivity co-localised with the (a-c) DA neuronal marker TH (c, arrowhead), and (d-f) astrocytic GFAP (f, arrow), confirming vector-mediated transgene expression in nigral neurons and astrocytes, respectively. A lack of transgene expression in microglia was indicated by an absence of co-localisation of dYFP immunoreactivity with the (g-i) microglia marker Iba1.

Scale bar: (a-i) 20µM
3.3.6.4. miRNA-based silencing of transgene expression in nigral neurons

To determine whether astrocyte-specific therapeutic manipulation confers substantial neuroprotection in disease, the main aim of this chapter was to develop AAV vectors that exhibit selective astrocyte tropism. Although AAV5-GFAP-dYFP mediated efficient nigral transduction at $2 \times 10^9$ genomes, transgene expression was detected in both astrocytes and neurons at this higher vector titre. Following completion of the *in vivo* study that characterised the neuroprotective efficacy of HA-NURR1 expression predominantly in astrocytes in a PD model in Chapter 4, we revisited strategies to manipulate AAV vector transduction properties.

We replicated the miRNA-based neuronal ‘detargeting’ strategy employed by Colin et al., whereby integration of a complementary sequence of the neuronal-specific miRNA, miR124 (denoted miR124T) into the vector genome directed the miR124-regulated degradation of the transgenic mRNA transcript selectively in neurons (Colin et al., 2009), without affecting its expression in astrocytes. AAV5 vectors were coupled with either a transgenic reporter cassette containing an integrin-β1-derived target sequence of miR124 (miR124T), or a corresponding control cassette. In order to clearly distinguish morphology of transduced cells, AAV vector titre was reduced tenfold to $2 \times 10^8$ genomes. To facilitate IHC detection, a reporter sequence encoding the highly stable eGFP protein was cloned into the transgenic cassettes. A control GFAP-eGFP plasmid was generated, and four copies of miR124T (miR124Tx4) were incorporated at the 3’ end of the reporter cassette to generate the neuronal-detargeting GFAP-eGFP-miR124Tx4 construct (Colin et al., 2009). Following RE-digest analysis of plasmid structural integrity (*Figure 3-1 j, k*), these constructs were packaged into AAV5 and infused into the rat SNpc (n = 3 per vector) as previously described.

IHC analysis of fixed nigral sections using an anti-GFP antibody three weeks post-vector infusion revealed that incorporation of miR124Tx4 substantially downregulated neuronal transgene expression (*Figure 3-15*). While both neuronal and astrocytic eGFP expression was detected in control animals (*Figure 3-15 b, c, e, f*), eGFP expression was predominantly localised to nigral astrocytes in eGFP-miR124Tx4 expressing animals (*Figure 3-15 h, i, k, l*). Using confocal laser scanning microscopy, eGFP co-localisation with the neuronal marker HuCD was quantified to determine the efficacy of miR124-directed transgene silencing in neurons. While HuCD-immunoreactive neurons expressed high intensity eGFP in the control vector-transduced nigra (*Figure 3-16 a-c*), co-localisation of eGFP in HuCD-positive
neurons in the AAV5-GFAP-eGFP-miR124Tx4-tranduced nigra (Figure 3-16 d-f) was considerably lower. Quantitative analysis revealed that, in control animals, 76.0% of HuCD positive SNpc neurons expressed eGFP, whereas neuronal eGFP expression was significantly downregulated to 41.9% in eGFP-miR124Tx4 expressing animals (Independent sample t-test; \( p = 0.01 \)) (Figure 3-16 g).
Figure 3-15 Neuronal-specific miR124-based ‘detargeting’ of transgene expression in nigral neurons

In the nigra unilaterally transduced with the control (a-f) AAV5-GFAP-eGFP vector, eGFP expression, as revealed by an anti-GFP antibody, was observed throughout the rostral and caudal aspects of the nigra (b, e), with eGFP immunoreactivity detected in both neurons (c, arrowhead) and astrocytes (f, arrow).

In (g-l) eGFP-miR124Tx4 expressing animals, eGFP expression also extended along the rostral-caudal axis of the nigra (h, k); however, eGFP expression was predominantly localised to nigral astrocytes (i, arrow), and neuronal eGFP expression was considerably lower (l, arrowhead).

(a, d, g, j) Non-specific staining was absent in the respective uninjected contralateral hemispheres.

Scale bars: (a, b, d, e, g, h, j, k) 500µm and (c, f, i, l) 100µm
Figure 3-16 miR124-directed downregulation of neuronal transgene expression in the SNpc

Co-localisation of eGFP immunoreactivity with the neuronal marker HuCD was assessed to determine the efficacy of miR124-directed downregulation of neuronal transgene expression. In the nigra transduced with the control (a-c) AAV5-GFAP-eGFP vector, high levels of eGFP and HuCD colocalisation were detected (c, arrowhead). Fewer HuCD-positive neurons co-localised with eGFP-immunoreactivity in (d-f) eGFP-miR124Tx4-expressing animals (f, arrowhead). An example of an eGFP-negative HuCD-expressing neuron is indicated with an arrow (f).

(g) In control animals, 76.0% of HuCD positive SNpc neurons expressed eGFP, whereas neuronal eGFP expression was significantly downregulated to 41.9% in eGFP-miR124Tx4 expressing animals (Independent sample t-test; p = 0.01).

*p = 0.01

Scale bar: (a-f) 20µm
AAV5-GFAP-eGFP-miR124Tx4

% GFP/HuCD co-localisation in the nigra

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* Statistical significance
3.3.7. Upregulation of GFAP promoter-regulated transgene expression in primary astrocytes in response to 6-OHDA or scratch injury

Previous studies have shown that mechanical injury- or neurotoxin-induced upregulation of GFAP in reactive astrocytes was paralleled by a corresponding increase in transgenic GFAP promoter activity, suggesting that the transgenic promoter contains motifs that are regulated during reactive gliosis (Brenner, Kisseberth, Su, Besnard, & Messing, 1994a; Jakobsson et al., 2006), a phenomenon that can be exploited to couple expression of therapeutic genes to the ‘reactivity’ of astrocytes in the context of neurodegenerative diseases. To determine whether the transgenic GFAP promoter is upregulated in response to a pathological stimulus, transgene expression in response to 6-OHDA-induced oxidative stress was analysed in vitro.

Although astrocytes in culture exhibit greater resistance to 6-OHDA-induced oxidative stress than neurons, 6-OHDA has been shown to induce astrocytic morphological alterations and death in a dose-dependent manner (Račević, Mladenović, Perović, et al., 2005; Račević, Mladenović, Perović, Miljković, & Trajković, 2005). In a preliminary experiment, primary astrocytes plated in 24-well plates at 1 x 10^5 cells per well were transduced with AAV5-GFAP-dYFP or AAV5-GFAP-HA-NURR1, or treated with 1 x PBS-MK-PA (vehicle control) (n = 8 wells per vector). Seventy-two hours post-vector transduction, two wells per vector were treated with saline (vehicle), 20µM, 50µM or 100µM 6-OHDA. Twenty-four hours post-6-OHDA, cells were fixed with 4%PFA, and the expression of GFAP, dYFP and HA-NURR1 was analysed with anti-GFAP, anti-GFP or anti-HA antibodies, respectively.

Morphological alterations in 6-OHDA-treated naïve astrocytes were assessed by anti-GFAP immunoreactivity. Under physiological conditions, astrocytes exhibited a predominantly cuboidal morphology, whereas in response to 6-OHDA, astrocytes adopted a more elongated morphology with the appearance of convoluted fine processes (Figure 3-17 a-d), indicative of activation of primary astrocytes in culture (Garwood, Pooler, Atherton, Hanger, & Noble, 2011). Furthermore, wells treated with 100µM 6-OHDA also contained higher levels of cellular debris 24-hours post-toxin treatment, and density of GFAP-immunoreactive astrocytes appeared lower, which may indicate cell death. However, there were no apparent toxin-dependent alterations in levels of GFAP expression. Although there was no clear indication of 6-OHDA dose-dependent alterations in transgene expression with the AAV5-GFAP-dYFP (Figure 3-17 e-h) or AAV5-GFAP-HA-NURR1 vectors (Figure 3-17 i-l), in comparison to saline-treated astrocytes (Figure 3-17 e and i, respectively), 100µM 6-OHDA-treated astrocytes appear to express higher levels of dYFP or HA-NURR1 (Figure 3-17 h and
l, respectively). Based on these preliminary ICC analyses, the assay was repeated, and cellular proteins harvested for western blot analysis. Twenty-four hours after plating, primary astrocytes plated in 6-well plates at a density of 3 x 10^5 cells per well were transduced with AAV5-GFAP-pL, AAV5-GFAP-dYFP or AAV5-GFAP-HA-NURR1 vectors (n = 4 wells per vector). Seventy-two hours post vector-transduction, two wells per vector were treated with saline (vehicle) or 100µM 6-OHDA. Twenty-four hours post-6-OHDA, cellular proteins were harvested in RIPA lysis buffer, and analysed by western blot using anti-GFAP, anti-β-actin antibodies to determine endogenous gene expression, and anti-GFP or anti-HA antibodies were used to detect reporter and therapeutic protein expression, respectively. The molecular weights of protein bands detected with anti-GFP and anti-HA antibodies corresponded to that of the dYFP (~36kDa) and HA-NURR1 (~68kDa) respectively (Figure 3-18). While no non-specific bands were detected in empty or dYFP vector-transduced cells, HA antibody detected two prominent non-specific bands below 66kDa in both empty and HA-NURR1 vector-transduced cells (not shown due to overexposure). 6-OHDA-induced alterations in endogenous GFAP, and transgenic dYFP or HA-NURR1 expression were highly variable between three experiments, with no correlations between levels of GFAP expression and transgenic protein expression. Moreover, repeating the experimental assay with a lower concentration of 6-OHDA (50µM) yielded similar results. To determine whether HA-NURR1 confers protection against 6-OHDA toxicity, a thiazolyl blue tetrazolium bromide (MTT) cell viability assay was performed on vector-transduced primary astrocytes plated on 96 wells plates. Although 6-OHDA induced cell death, inter-assay variability was considerably high (data not shown).

Given that the results generated in the 6-OHDA toxicity assay were inconclusive, a scratch assay was subsequently conducted to determine transcriptional regulation of the GFAP promoter in primary astrocytes in response to mechanical injury. The scratch assay is commonly used to investigate astrocyte proliferation and migration in response to a scratch wound (Liang, Park, & Guan, 2007; Nishio et al., 2005). In response to a scratch wound, GFAP and other intermediate filament proteins that regulate cell morphology and motility are upregulated. As previously, primary astrocytes plated in 6-well or 24-well plates were transduced with AAV5-GFAP-pL, AAV5-GFAP-dYFP or AAV5-GFAP-HA-NURR1, or treated with 1 x PBS-MK-PA (n = 4 wells per vector). To simulate mechanical injury, 72-hours post-vector transduction, two wells per treatment in 6-well or 24-well plates were gently bisected three times at an angle of ~60° with a pipette tip. Two additional non-treated
wells per vector served as controls. Twenty-four hours post-injury, 24-well plates were fixed with 4% PFA, and cellular proteins were harvested in RIPA lysis buffer from 6-well plates. ICC and western blot analyses were conducted using an anti-GFAP antibody to determine GFAP expression levels, and anti-GFP or anti-HA were used to detect reporter and therapeutic protein expression, respectively. ICC revealed no clear evidence of injury-induced alterations in endogenous or transgenic gene expression or astrocytic morphology in cells that had undergone mechanical injury compared to controls (Figure 3-19). Although percentage increase in protein density in injured sampled varied greatly between three experiments, densitometry analysis of protein bands on western blots confirmed an upregulation of endogenous GFAP and a corresponding increase in dYFP and HA-NURR1 expression in injured astrocytes, in comparison to controls (Figure 3-20).
Morphological alterations in 6-OHDA-treated (20, 50, and 100μM) naïve astrocytes were assessed by anti-GFAP immunoreactivity. (a) Under physiological conditions, GFAP-immunoreactive astrocytes exhibited a predominantly cuboidal morphology, whereas (b-d) in response to 6-OHDA, astrocytes adopted a more elongated morphology with the appearance of convoluted fine processes, indicative of activation of primary astrocytes in culture. However, there were no apparent toxin-induced alterations in levels of GFAP expression. Although there was no clear indication of 6-OHDA dose-dependent alterations in either (e-h) dYFP or (i-l) HA-NURR1 expression, in comparison to (e, i) saline-treated, vector-transduced astrocytes, 100μM 6-OHDA-treated astrocytes appear to express higher levels of (h) dYFP or (l) HA-NURR1.

Scale bar: 100μm
Figure 3-18 No apparent 6-OHDA-induced alterations in GFAP promoter-regulated transgene expression in AAV5 vector-transduced primary astrocytes

Protein bands corresponding to the molecular weights of (c, d) dYFP (~36kDa) and (e, f) HA-NURR1 (~68kDa) were detected using anti-GFP and anti-HA antibodies in both control and 6-OHDA-treated primary astrocytes. While non-specific bands were absent in (a, b) empty or (c, d) dYFP vector-transduced cells, HA antibody detected two prominent non-specific bands below 66kDa in both (e, f) HA-NURR1 and (g, h) empty vector-transduced cells (not shown due to overexposure). Densitometry analysis of immunoblots from three independent experiments indicated that 6-OHDA-induced alterations in endogenous GFAP and transgenic protein expression were highly variable.
Figure 3-19 Mechanical injury-induced alterations in gene expression in AAV5 vector-transduced primary astrocyte

ICC against GFAP revealed no clear evidence of GFAP upregulation or alterations in astrocytic morphology in (a, b) vehicle treated or (c, d) empty vector-transduced controls in response to injury. Injury-induced alterations in transgenic (e, f) dYFP or (g, h) HA-NURR1 expression in AAV5-vector transduced astrocytes were also not detected.

Scale bar: 100µm
Densitometry indicated that in response to a scratch injury, an upregulation of endogenous GFAP expression was paralleled by an increase in (a, b) dYFP and (c, d) HA-NURR1 expression in AAV5 vector transduced primary astrocytes. While no non-specific bands were detected in empty (not shown) or (a, b) dYFP vector-transduced cells, HA antibody detected two non-specific bands below 66kDa in both empty (not shown) and (c, d) HA-NURR1 vector-transduced cells.
3.3.8. HA-NURR1 transgenic protein transcriptionally regulates a TH promoter sequence

DNA sequence data confirmed the integrity of the NURR1 coding region of the GFAP-HA-NURR1 expression plasmid. However, to further validate that incorporating the HA tag does not disrupt the structural and functional integrity of the transgenic HA-NURR1 protein, a co-transfection assay in HEK293 cells was performed to determine the ability of the therapeutic protein to transcriptionally regulate a TH promoter sequence that contains a NURR1 recognition sequence (TGGCCTTTA). As we have previously noted, while the transcriptional activity of the GFAP promoter is inefficient in HEK293 cells, the CMV promoter is highly active in these cells; therefore the CMV-HA-NURR1 construct was utilised for this study. HEK293 cells were co-transfected with a plasmid encoding TH promoter-regulated dYFP and the above plasmid encoding CMV-HA-NURR1 or a control CMV-Luc reporter construct. Forty-eight hours post-transfection, cells were either fixed or harvested for ICC and western blot analyses, respectively. In comparison to cells co-transfected with the CMV-Luc control plasmid (Figure 3-21 a, b, h), higher levels of dYFP expression was detected in cells co-transfected with the CMV-HA-NURR1 plasmid (Figure 3-21 c, d, i). A highly active CBA-dYFP plasmid transfected into HEK293 cells served as a positive control to identify the protein band corresponding to dYFP among the non-specific bands on western blots (Figure 3-21 e, f, j). Due to oversaturation of chemiluminescence, this sample (~4µg) run on the same gel was developed separately.
Figure 3-21 HA-NURR1 protein transcriptionally regulates a TH promoter containing a NURR1 recognition motif

In comparison to (a, b, h) basal levels of dYFP expression in HEK293 cells co-transfected with CMV-Luc and TH-dYFP, (c, d, i) HA-NURR1 expressed from a CMV-HA-NURR1 plasmid upregulated transcriptional activity of the TH promoter on a second expression plasmid, leading to increased TH promoter-regulated dYFP expression. (e, f, j) dYFP expressed from a CBA-dYFP plasmid served as a positive control for transgene expression.

Scale bar: 100µm
3.3.9. HA-NURR1 regulation of NF-κB

It was previously shown that NURR1 exerts an anti-inflammatory effect in glia by downregulating the inflammatory transcription factor complex, NF-κB (Saijo et al., 2009). Under basal conditions, NF-κB resides in the cytoplasm as an inactive multisubunit complex associated with an inhibitory subunit IκB (O’Neill & Kaltenschmidt, 1997). Various inflammatory stimuli including IL-1, TNFα, bacterial LPS, and oxidative stress regulate IκB phosphorylation that leads to dissociation of the NF-κB/ IκB complex promoter regions (Lawrence, 2009). NF-κB subsequently translocates to the nucleus, where it induces inflammatory gene expression by binding to a specific NF-κB consensus sequences in the promoter regions of inflammatory genes such as TNFα, IL2, IL6, granulocyte-monocyte colony stimulating factor (GM-CSF), and iNOS. To determine whether AAV5-mediated HA-NURR1 expression may influence NF-κB activation, LPS-induced NF-κB nuclear translocation was quantified in primary astrocytes transduced with AAV vectors.

Firstly, LPS-induced nuclear translocation of NF-κB in naïve primary astrocytes was confirmed by immunocytochemically analysing cellular localisation of NF-κB in response to 5, 10, 50, 100, or 500ng/mL LPS treatment for 2 hours using an anti-NF-κB antibody. Under basal conditions (HBSS-treated), NF-κB-immunoreactivity appeared relatively homogenous expressed throughout the cell body and nucleus, whereas LPS induced a robust nuclear translocation of NF-κB, as evident from the intensely immunostained cell nuclei (Figure 3-22 a and b, respectively). Subsequently, nuclear translocation of NF-κB in AAV vector-transduced primary astrocytes in response to two LPS concentrations (5ng/mL or 50ng/mL) (n = 3 per treatment) was analysed at four time-points (2, 5, 8, or 24 hours post-LPS treatment) in multiple preliminary experiments as outlined. Example images and quantitative analysis of co-localisation of NF-κB and DAPI nuclear counter-stain are shown for naïve and vector-transduced primary astrocytes treated with 5ng/mL LPS for 5 hours. A high level of NF-κB and DAPI co-localisation with some variability in the intensity of co-localisation was observed in all LPS-treated naïve and vector-transduced cells (Figure 3-23 a). Whereas under basal conditions, NF-κB appeared to be homogenously expressed throughout the cell, and co-localised with DAPI with considerably lower intensity (Figure 3-23 b). Quantification of NF-κB colocalisation with DAPI indicated that at any given time-point, 20-30% of control (1 x PBS-MK-PA) or vector-transduced cells treated with 1 x HBSS (vehicle) exhibited NF-κB nuclear translocalisation (AAV5-GFAP-dYFP is shown as an example in Figure 3-23 b) (quantitative data not shown). LPS treatment (5ng/mL or 50ng/mL) induced
a mean NF-κB translocation of 85-90% in control and all vector-transduced groups. In the example shown, a predominant nuclear localisation of NF-κB in all naïve and vector-transduced cells was observed; 86.7% PBS; 87.75% AAV5-GFAP-pL; 86.78% AAV5-GFAP-dYFP; 85.60% AAV5-GFAP-HA-NURR1; One-way ANOVA; p = 0.219) (Figure 3-23 c).
Figure 3-22 LPS-induced nuclear translocation of the inflammatory transcription factor NF-κB in primary astrocytes

(a) NF-κB-immunoreactivity in HBSS (vehicle)-treated primary astrocytes appeared to be expressed relatively homogenously throughout the cell body and nucleus.

(b) Predominantly nuclear expression of NF-κB in primary astrocytes treated with LPS (5ng/mL) for 2 hours confirmed LPS-induced nuclear translocation of NF-κB.

Scale bar: 100µm
Figure 3-23 AAV5-mediated HA-NURR1 expression has no effect on LPS-induced NF-κB nuclear translocation in primary astrocytes

The example images and quantitative analysis show cellular localisation of NF-κB in response to exposure to LPS (5ng/mL) for 5 hours.

(a) Naïve and AAV5 vector-transduced cells exhibited comparable levels of predominantly nuclear localisation of NF-κB in response to 5 hours post-LPS (5ng/mL) treatment.

(b) Under basal conditions (HBSS-treated), NF-κB was more homogeneously expressed throughout the cell in naïve and vector-transduced primary astrocytes with ~20-30% nuclear localisation (AAV5-GFAP-dYFP-transduced astrocytes are shown as an example; quantitative data not shown).

(c) Quantification of nuclear co-localisation of NF-κB and DAPI nuclear counter-stain confirmed high levels of nuclear translocation in all naïve and vector-transduced cells; PBS, 86.7%; AAV5-GFAP-pL, 87.75%; AAV5-GFAP-dYFP, 86.78%; AAV5-GFAP-HA-NURR1, 85.60% (One-way ANOVA; p = 0.219).
b) NF-κB  DAPI  Overlay

AAV5-GFAP-dYFP

c) % NF-κB nuclear translocation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% NF-κB nuclear translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>50</td>
</tr>
<tr>
<td>AAV5-GFAP-pL</td>
<td>70</td>
</tr>
<tr>
<td>AAV5-GFAP-dYFP</td>
<td>80</td>
</tr>
<tr>
<td>AAV5-GFAP-HA-NURR1</td>
<td>85</td>
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3.4. Discussion

The main aim of this chapter was to develop AAV vectors that exhibit efficient astrocyte tropism and transgene expression in the rat SNpc by coupling astrocyte-tropic AAV serotypes and DNA promoter elements. We firstly investigated the tropism and transduction efficacy of the astrocyte-tropic AAV serotypes 5 and 9 in the nigra. Furthermore, given that the capacity to regulate vector transduction properties is a beneficial characteristic of gene delivery vehicles, GFAP, CMV and gfaABC1D promoters were comparatively analysed for their potential ability to modify vector tropism and efficacy of transgene expression.

3.4.1. Comparative analysis of AAV5 and AAV9 in the rat SNpc

Of the AAV serotypes characterised in the CNS thus far, AAV5, 8 and 9 exhibit the highest propensity for astrocyte transduction in various CNS regions (Davidson et al., 2000; Drinkut et al., 2012; Lawlor et al., 2009). However, potential astrocyte tropism of these AAV vectors in the rat SNpc is only modestly characterised (Lawlor et al., 2009). Therefore, nigral astrocyte transduction efficacy of AAV5 or AAV9 coupled with the widely used 2.2kb GFAP promoter was evaluated. As AAV8 mediated the least effective nigral transduction in section 2.3.3, this serotype was excluded.

Owing to their highly stable structure, GFP and related variants possess a lengthy half-life, and therefore accumulate throughout the nucleus, cytoplasm and fine processes to reveal distinct morphologies of GFP expressing cells. To exploit this characteristic of GFP to determine tropisms of AAV5 and AAV9, an expression cassette encoding GFAP-regulated dYFP was generated and packaged into AAV5 or AAV9. Following transduction of primary astrocytes in vitro, AAV5 mediated considerably higher levels of dYFP expression than AAV9. Given that vector transduction properties in an isolated culture may not accurately represent vector transduction in vivo, the vectors were subsequently tested in the rat SNpc prior to interpretation of in vitro results. The vectors were infused into the SNpc at a titre tenfold lower (2 x 10⁸ genomes) than used previously in Chapter one to minimise excessive dYFP expression that could potentially disguise any AAV serotype-specific differences in transduction properties.

At this titre, AAV5 almost exclusively transduced SNpc astrocytes, whereas AAV9 transduced both nigral neurons and astrocytes. AAV9 mediated superior transduction with
high expression in the SNpc, SNpr, and ventral regions of midbrain reticular nucleus and thalamus, whereas off-target AAV5-mediated transgene expression was restricted to the dorso-ventral regions immediately adjacent to the needle tract. Given that inappropriate vector diffusion increases the potential risk of detrimental effects associated with transgene expression in off-target structures, AAV5 that mediated predominant astrocyte transduction in the SNpc and minimal transgene expression in neighbouring midbrain regions was selected for therapeutic gene delivery. The differences in transduction properties in vitro and in vivo are discussed in section 3.4.5. Quantification of the proportion of nigral astrocytes and neurons transduced by each AAV vector is required to accurately determine whether AAV5 exhibit greater astrocytic transduction in comparison to AAV9. As discussed in section 1.6.4.1, differences in serotype-dependent binding affinities to specific cell surface receptors and intracellular trafficking may determine differences in cell tropism and transduction efficacy of AAV5 and AAV9. Amongst AAV serotypes 1 to 10, AAV5 is the most divergent, sharing ~55% sequence homology with other serotypes (Sen et al., 2013), and hence may exhibit unique transduction properties. Additionally, AAV5 is immunologically distinct by virtue of their low seroprevalence and minimal cross reactivity against pre-existing AAV2 neutralising antibodies (Halbert et al., 2006). Comparative analysis of AAV serotypes 1-6 demonstrated that AAV5-CMV-eGFP transduced a significantly large volume of tissue in the non-human primate caudate nucleus and substantia nigra, and mediated transgene expression in neurons and astrocytes with equal efficiency (Markakis et al., 2010). Furthermore, a recent study also reported that AAV5 or AAV9 coupled with the CMV promoter predominantly targeted astrocytes in the non-human primate cortex, and transduced comparable volumes of tissue (Watakabe et al., 2015). Collectively, these results reveal the potential applicability of AAV serotype 5 in the human brain to achieve target-specificity and efficient transgene expression.

3.4.2. Functionality of AAV expression plasmids in vitro

As discussed in section 1.6.4.1, the ~4.7kb packaging capacity of AAV imposes a sequence length restriction on the regulatory elements and therapeutic genes that can be cloned into the expression cassette, as larger expression plasmids compromise the stability of the vector capsids leading to inefficient assembly and transduction (Grieger & Samulski, 2005; Wu et al., 2009). Although the widely used 2.2kb GFAP promoter effectively directs transgene
expression to astrocytes, it is a relatively large promoter that occupies almost half of the packaging capacity. Given that it is desirable to utilise smaller promoters that significantly ease the GFAP promoter-dependent length restriction on therapeutic sequences, we tested the ability of the constitutively active CMV promoter, and a recently characterised shorter GFAP promoter, gfaABC1D that lack redundant sequence motifs to regulate AAV tropism and transgene expression.

Firstly, expression plasmids encoding GFAP- or CMV-regulated HA-NURR1 or FLAG-Nrf2 were generated, and the GFAP-regulated dYFP served as the control construct. gfaABC1D-regulated expression constructs were developed subsequently in section 3.3.5. To determine functionality of promoter sequences, these plasmids were transfected into primary astrocytes, and transgene expression was analysed 48 hours post-transfection by ICC. dYFP expression throughout the cytoplasm and nucleus, and HA-NURR1 expression in the nucleus confirmed functionality of the GFAP promoter in astrocytes in vitro. It has previously been reported that NURR1 is mainly localised to the nucleus (García-Yagüe, Rada, Rojo, Lastres-Becker, & Cuadrado, 2013). In comparison to the GFAP promoter, the CMV promoter substantially enhanced expression of both HA-NURR1 and FLAG-Nrf2. In contrast, following AAV5-mediated delivery of the expression cassettes into primary astrocytes, the GFAP promoter mediated higher levels of HA-NURR1 expression than the CMV promoter (discussed in section 3.4.4), suggesting that plasmid size-dependent efficacy of transfection, rather than promoter-dependent transcriptional activity may have contributed to the higher CMV-regulated transgene expression in plasmid transfected astrocytes. The total length of the expression construct encoding the 587bp CMV promoter is 6510bp, whereas the 2.2kb GFAP promoter increases the plasmid length to 8018bp. Given that the size of the plasmid construct inversely affects efficacy of transgene expression in transfection assays (Yin, Xiang, & Li, 2005), the higher levels of HA-NURR1 expression achieved with the CMV promoter compared to the GFAP promoter may correspond to a greater transfection efficiency, rather than superior transcriptional activity. In comparison to primary astrocytes, high levels of CMV-regulated therapeutic gene expression were achieved in transfected HEK293 cells, indicating that HEK293 cells are more permissive to transfection than primary astrocytes. Immunoreactivity against both NURR1 and Nrf2 in transfected HEK293 cells confirmed therapeutic protein expression.
3.4.3. Increasing NURR1 half-life

In the context of chronic neurodegeneration, it is desirable to have a readily available cellular reservoir of therapeutic proteins. Both endogenous and transgenic NURR1 has a half-life of ~3-4 hours in vitro in PC12 and HeLa cells (Alvarez-Castelao et al., 2013); hence, transcription and translation of an optimal dose of NURR1 protein may exert a rate limiting effect on therapeutic efficacy. Proteasome inhibitors extended the duration of NURR1 expression in vitro, suggesting that NURR1 is subjected to degradation via the ubiquitin proteasomal pathway. The deletion of a 31 amino acid sequence from the N-terminal region of NURR1 extended its half-life by disrupting proteasomal degradation. The sequence deletion did not affect functionality of the mutant NURR1, as its ability to transactivate a promoter sequence containing a NURR1 recognition motif, or transrepress NF-κB-dependent iNOS expression in a co-transfection assay was comparable to that of full-length NURR1 in neuronal PC12 and non-neuronal HeLa cells (Alvarez-Castelao et al., 2013). However, due to its diminished degradation, greater levels of transcriptional activity were achieved with mutant NURR1. The ubiquitin-proteasomal degradation pathway is a critical regulator of protein activity in a cell, where the covalent addition of multiple molecules of ubiquitin, a 76aa polypeptide to form a polyubitiuinated tract targets various cellular proteins to the proteasome for degradation. The authors suggest that the sequence deletion may disrupt ubiquitination, or the poly-ubiquitylated mutant NURR1 may be inefficiently recognised by the 19S proteasomal complex or undergo inadequate translocation to the catalytic chamber of the 20S proteasomal complex for degradation.

We replicated this sequence deletion to generate a GFAP-HA-tNURR1 plasmid construct, and analysed the expression of this truncated NURR1 (tNURR1) in transfected primary astrocytes. Although primary astrocytes transfected inefficiently, the expression of HA-tNURR1 was expected to be higher than that of the full length HA-NURR1 at an equal plasmid concentration. Unexpectedly, HA-tNURR1 expression was substantially lower than full-length HA-NURR1 expression, suggesting that the sequence deletion may compromise expression of tNURR1 in astrocytes. Widely used cell lines including PC12 and HeLa derived from tumours may continue to undergo phenotypic drift in culture (Gazdar, Gao, & Minna, 2010; Masters, 2000); hence these cells may exhibit deficits in gene regulation and other physiological processes that could potentially contribute to inaccuracies in experimental data, whereas primary astrocytes derived from the naïve rat brain may more accurately
represent the physiology of in vivo astrocytes. Since HA-tNURR1 was less efficient than the full-length HA-NURR1, HA-NURR1 was selected for subsequent in vivo studies.

3.4.4. AAV5-mediated transgene expression in primary astrocytes

As discussed in section 1.6.4.1, cell entry and intracellular processing in a manner dependent on capsid protein sequences determine transduction efficacy of AAV vectors. Following stabilisation of the vector genome via second strand synthesis in the nucleus, DNA promoters and other regulatory elements in the transgenic cassette determine the efficacy of transgene expression (Thomas, Storm, Huang, & Kay, 2004). Astrocyte-specific transcriptional activity of GFAP, gfaABC1D, and CMV promoters in the context of AAV5 was analysed in primary astrocytes. In the context of AAV5, gfaABC1D promoter mediated transgene expression was comparable to that of its predecessor, GFAP, indicating that the sequence modifications did not compromise activity of the shorter promoter in vitro. Efficient levels of dYFP- and HA-immunoreactivity indicated that coupling GFAP or gfaABC1D promoter with AAV5 serotype generates delivery vectors that mediate efficient astrocytic transduction and transgene expression. No transgenic NURR1-immunoreactivity as detected by anti-NURR1 antibody was observed in HA-NURR1 transfected astrocytes, suggesting inadequate NURR1 antibody sensitivity, or that the antibody was not suitable for this application. Surprisingly, in comparison to the GFAP promoter, CMV promoter activity in the context of AAV5 was considerably lower, indicating inefficient CMV promoter function in astrocytes (discussed in section 3.4.5.4).

No GFAP-regulated FLAG-Nrf2 expression was detected in primary astrocytes. Given that the GFAP promoter is transcriptionally active in primary astrocytes, and CMV-regulated FLAG-Nrf2 expression in HEK293 cells confirmed functional integrity of the transgenic DNA sequence, low levels of expression or rapid turnover of Nrf2 may account for the lack of detection in primary astrocytes. In human hepatoma cells, Stewart et al. showed that endogenous Nrf2 exhibited a half-life of ~13 minutes, whereas Sekhar et al. reported a half-life of 3 hours for transgenic Nrf2 (Sekhar, Yan, & Freeman, 2002; Stewart, Killeen, Naquin, Alam, & Alam, 2003). Given that the phenotype of the same cell type may differ considerably amongst cultures, and laboratory-dependent variances in cell culture maintenance and experimental procedures affect study outcome, these findings cannot be extrapolated to our in vitro or in vivo FLAG-Nrf2 expression studies. Furthermore, the N-
terminal incorporation of a FLAG tag and primary rat astrocyte-specific cellular dynamics may affect expression and intracellular processing of FLAG-Nrf2 protein in astrocytes. Nrf2 is degraded via the ubiquitin proteasomal pathway, and proteasomal inhibitors such as MG-132 or lactacystin have been shown to promote Nrf2 accumulation in vitro (Nguyen, Sherratt, Huang, Yang, & Pickett, 2003; Sekhar et al., 2002; Stewart et al., 2003). Such proteasomal inhibition and quantification of FLAG-Nrf2 mRNA may have allowed us to determine whether rapid proteasomal degradation accounts for an inability to detect FLAG-Nrf2 expression in primary astrocytes by ICC.

3.4.5. AAV5-mediated transduction in the rat SNpc

3.4.5.1. Transgene expression in the SNpc

As discussed in section 2.4.2, heterogeneity in astrocytic molecular expression profiles in a manner dependent on the CNS region, cell subtype, and physiological or pathological states may influence tropism and transduction efficacy of AAV vectors in vivo (Cai et al., 2000; Fitting et al., 2010; Karavanova et al., 2007; Macnab & Pow, 2007; Regan et al., 2007; Reuss et al., 2000; Tang et al., 2009; Walz & Lang, 1998; Yeh et al., 2009). Furthermore, AAV transduction dynamics in an isolated in vitro culture may not accurately represent vector transduction properties in a more complex in vivo environment. Therefore, all the AAV5 vector constructs were tested in the naïve rat SNpc at 2 x 10⁹ genomes to determine the promoter that mediates optimal therapeutic transgene expression in the nigra.

The 10-fold increase in AAV5-GFAP-dYFP titre resulted in a significant increase in nigral transduction. In comparison to the almost exclusive astrocytic tropism at the lower titre, increased astrocytic and neuronal transduction were seen at the higher titre. Vector titre-dependent specificity of cell transduction has been observed previously (Lawlor et al., 2009). AAV5 capsid sequences may bind to astrocytic cell surface receptors with high affinity, resulting in selective astrocytic transduction at low vector titre, whereas inefficient capsid-neuronal receptor binding kinetics may improve with greater availability of viral particles, enhancing neuronal transduction at high titre. Additionally, neuronal transgene expression at high vector titre indicates that the transgenic GFAP promoter may exhibit weak transcriptional activity in neurons. Greater neuronal transduction may favour higher levels of GFAP promoter-regulated dYFP expression. At the high titre, GFAP-regulated dYFP
expression remained relatively confined to the SNpc which may limit any undesirable transgene expression in neighbouring regions. Although expression was considerably lower than dYFP, the pattern of nuclear-localised HA-NURR1 expression in the midbrain was comparable to that of dYFP. Wild-type GFP is estimated to have a half-life of ~26 hours in vitro (Corish & Tyler-Smith, 1999), and GFP variants such as dYFP and enhanced GFP may share this property. In contrast, both endogenous and transgenic NURR1 have a half-life of about 3-4 hours (Alvarez-Castelao et al., 2013). Although in vitro cellular dynamics may not accurately represent in vivo physiology, the levels of reporter and therapeutic transgene expression in vivo may represent their rates of degradation, and suggest that dYFP is considerably more stable than HA-NURR1. No detectable levels of FLAG-Nrf2 in the SNpc were achieved with AAV5-GFAP-FLAG-Nrf2. Given that we have used the anti-FLAG antibody in previous in vivo studies to successfully detect AAV-mediated transgene expression in the CNS, and absence of FLAG-Nrf2 expression suggested that this therapeutic molecule may exhibit a rapid rate of degradation in vivo (discussed in 3.4.4). Relative to PBS or empty vector control brains, for both HA-NURR1 and FLAG-Nrf2, immunoblot analysis of nigral tissue using anti-NURR1 and anti-Nrf2 antibodies respectively, may have confirmed expression of full length proteins, and allowed quantification of protein expression relative to endogenous levels. Furthermore, quantitative-PCR may have further allowed the quantification of transgene expression at the mRNA level.

Furthermore, co-localisation of fluorescently-labelled dYFP with TH and GFAP, respectively, indicated that the AAV5 vector construct targets nigral neurons and astrocytes respectively. dYFP-expressing astrocytic processes (native or immunohistochemically detected) frequently disguised neighbouring cells; therefore, accurately quantifying the proportions of transgene expressing neurons and astrocytes was not possible. High intensity dYFP immunoreactivity was predominantly detected in astrocytes, suggesting that the GFAP promoter may exhibit higher transcriptional activity in astrocytes. A number of neurons that exhibited high levels of dYFP-immunoreactivity were not TH-positive. Co-labelling with a pan-neuronal marker such as HuCD may determine whether AAV5 vectors target other non-DA neurons in the SNpc, or that high levels dYFP expression may either occlude binding of anti-TH antibodies to cellular TH or downregulate expression of neuronal markers, which may suggest potential toxicity associated with dYFP overexpression, as reported in a previous study (Watakabe et al., 2015). Stereological quantification of DA neurons may indicate potential neuronal loss associated with dYFP overexpression. An absence of dYFP
co-localisation with the microglia marker, Iba1 indicates either an inability of AAV5 to transduce microglia and/or inactivity of the GFAP promoter in microglia. Double-labelling with an oligodendrocyte marker such myelin basic protein may have determined whether the AAV5 vector directs transgene expression to oligodendrocytes. Moreover, given that cell-specific activity of the GFAP promoter may not necessarily represent tropism of AAV serotype in the SNpc, IHC analysis of colocalisation of AAV5 capsid proteins with cell-specific markers, or IHC in combination with in situ detection of the vector genome may reveal AAV5 transduction profile in the SNpc.

3.4.5.2. GFAP promoter activity in the nigra

GFAP promoter-regulated transgene expression was detected in both nigral astrocytes and neurons in a serotype- and titre-dependent manner. In the naïve brain, GFAP expression is predominantly astrocytic; however aberrant expression of a splice variant of GFAP in hippocampal neurons in post-mortem brains of AD and Down syndrome patients has been reported (Hol et al., 2003). The presence of neuronal transgene expression suggest that certain sequence motifs in the endogenous GFAP gene that suppress transcription in neurons are lacking in the 2.2kb transgenic GFAP promoter.

The viral ITRs, cis-acting elements essential for replication and packaging of recombinant vector genome have intrinsic transcriptional activity that may facilitate residual transgene expression, even in the absence of a promoter (Flotte et al., 1993). However, the lack of CMV-regulated transgene expression suggests negligible levels of heterologous promoter ‘leakiness’ that may have contributed to neuronal transgene expression. Hence, GFAP promoter activity in neurons may account for the neuronal transgene expression.

3.4.5.3. gfaABC1D promoter activity is less efficient than its predecessor

A recently characterised shorter GFAP promoter variant, gfaABC1D that has been shown to replicate transcriptional activity and astrocyte specificity of the 2.2kb GFAP promoter in a transgenic mouse model (Lee et al., 2008), was investigated for its capacity to retain these characteristics in AAV-based gene delivery. Although the gfaABC1D promoter-regulated transgene expression levels were comparable to that of the GFAP promoter in cultured
primary astrocytes, it mediated considerably weaker expression in the rat SNpc. Double labelling with the respective cell markers to determine the ratio of astrocyte to neuronal transduction may accurately determine whether the shorter promoter retains tropism of the GFAP promoter, even though its activity is diminished. It is possible that differences between AAV-mediated transgene expression and direct genomic modification, and potential species-specific differences in the regulation of GFAP expression, and activity of the gfaABC₁D promoter may account for the contradictory findings.

3.4.5.4. CMV promoter inactivity in the context of AAV5 in the SNpc

The CMV promoter in the context of AAV5 and other astrocyte-tropic AAV serotypes has been shown to efficiently transduce astrocytes in the nigra, striatum and cortex of the non-human primate brain, and hippocampal astrocytes in the mouse (Markakis et al., 2010; Ortninski et al., 2010; Watakabe et al., 2015). However, this promoter in the context of AAV5 failed to mediate any detectable levels of transgene expression in the rat SNpc. It is possible that species-specific and regional heterogeneity in astrocytes may determine CMV promoter inactivity in the rat SNpc. Moreover, transcriptional activity of viral promoters appears to be short-lived, as epigenetic modifications such as methylation inhibits activity over time (Brooks et al., 2004), further supporting the use of cell-specific promoter sequences that imitate transcriptional properties of the corresponding endogenous genes. IHC analysis of cell-specific localisation of AAV5 capsid proteins or in situ detection of the vector genome may confirm that the lack of transgene expression is due to promoter inactivity. The alternative constitutively active CMV early enhancer/ chicken β-actin (CAG) promoter coupled to various other AAV serotypes have been used to target neurons in the rat SNpc with high efficiency (Decressac, Kadkhodaei, et al., 2012; Decressac, Mattsson, Lundblad, et al., 2012; Lawlor et al., 2009). Whether such promoters may modify tropism and/or transduction efficacy of AAV5 warrants further investigation. As per gfaABC₁D promoter activity in vitro, different in vitro and in vivo transduction patterns mediated by the CMV promoter further emphasises the phenotypic differences between in vitro differentiated primary cortical astrocytes and mature nigral astrocytes in vivo.
3.4.5.5. miRNA-based silencing of transgene expression in nigral neurons

To enhance astrocyte specificity of AAV-mediated transduction in the SNpc, a miRNA-based neuronal ‘detargeting’ mechanism was investigated. Four copies of a target sequence of the neuronal-specific miR124 was incorporated into a transgenic cassette to post-transcriptionally silence AAV vector-mediated neuronal transgene expression (Colin et al., 2009). While both neuronal and astrocytic eGFP expression was detected in control animals, with 76.0% of HuCD positive SNpc neurons expressing eGFP, eGFP expression was predominantly localised to nigral astrocytes in eGFP-miR124Tx4 expressing animals, with 41.9% of HuCD-positive neurons expressing eGFP. In comparison to the control animals, intensity of neuronal eGFP immunoreactivity was considerably lower in eGFP-miR124Tx4 expressing animals, further supporting downregulation of neuronal transgene expression. Given the vital role of astrocytes in CNS health and disease, this strategy may further facilitate the ability to elucidate astrocyte-specific molecular mechanisms that govern their functional significance; therapeutically target astrocyte-specific defective pathways in pathology; and selectively target expression of bio-active molecules such as GDNF to astrocytes to restrict therapeutic activity to the target region, and minimise potential detrimental effects associated with neuronal delivery of transgenic proteins to off-target projection regions (Drinkut et al., 2012).

In two separate experiments, AAV5-GFAP-dYFP (section 3.3.3.2) and the above AAV5-GFAP-eGFP (section 3.3.6.4) control constructs were infused into the SNpc at 2 x 10^8 genomes, and transgene expression was analysed at three weeks post-vector infusion. Interestingly, while dYFP expression was almost exclusively astrocytic, 76.0% of HuCD-positive nigral neurons expressed eGFP. These results suggest that eGFP may exhibit greater structural stability than dYFP, and hence its accumulation may effectively improve immunodetection in neurons that express the transgenic cassette less efficiently than astrocytes.

3.4.6. In vitro and in vivo astrocytic phenotypic differences

Due to the ease of culture generation and maintenance, and wide availability of optimised culture protocols, primary cortical astrocytes from early postnatal rats were used for preliminary vector screening (Saura, 2007). The phenotype of primary cortical astrocytes derived from postnatal tissue differentiated in culture may differ significantly from that of
mature nigral astrocytes in vivo, contributing to the different transduction patterns achieved in vitro and in vivo. Investigating AAV serotype- and promoter element-dependent transduction profiles in primary nigral astrocytes or midbrain-wide astrocytes derived from the mature brain may have provided a more relevant in vitro representation of nigral astrocytes in the adult brain in vivo.

Furthermore, AAV delivery of this construct into other brain regions such as the striatum and hippocampus may reveal regional heterogeneity in astrocyte transduction. In vitro and in vivo studies reveal extensive astrocytic molecular heterogeneity in developmental, physiological and pathological processes that imply functional diversity. Heterogeneously expressed astrocytic genes include those that encode surface glycoproteins; membrane ion channels, transporters and receptors; inflammatory mediators; gap-junction proteins; and cellular enzymes that regulate neurotransmitter metabolism (Cai et al., 2000; Fitting et al., 2010; Karavanova et al., 2007; Macnab & Pow, 2007; Regan et al., 2007; Reuss et al., 2000; Tang et al., 2009; Walz & Lang, 1998; Yeh et al., 2009). Further elucidation of astrocytic molecular and functional diversity may facilitate the development of AAV vectors that achieve efficient astrocyte transduction, and express functionally relevant therapeutic genes in a context-dependent manner.

3.4.7. The transgenic GFAP promoter is upregulated in response to pathological stimulation

Previous studies have shown that injury-induced upregulation of endogenous GFAP in reactive astrocytes was paralleled by a corresponding increase in transgenic GFAP promoter activity (Brenner et al., 1994a; Jakobsson et al., 2006), suggesting that the transgenic promoter activity is enhanced by endogenous transcription factors upregulated in reactive gliosis, a phenomenon that can be exploited to couple expression of therapeutic genes to the ‘reactivity’ of astrocytes in the context of neurodegenerative diseases. To determine whether the GFAP promoter-regulated transgenic cassettes respond to pathology-dependent transcriptional regulation, alterations in endogenous GFAP and transgene expression in response to 6-OHDA or mechanical injury were assessed in cell lysates of AAV vector transduced primary astrocytes by western blot. The 6-OHDA toxicity assay consistently generated highly variable results that were inconclusive. It is possible that 6-OHDA induced
reactive gliosis may not exhibit the characteristic upregulation of GFAP expression, and hence may not influence transcription of the transgenic cassette. Furthermore, 6-OHDA-induced oxidative stress has been shown to induce extracellular signal-regulated kinase (ERK)-dependent apoptosis in astrocytes (Raićević, Mladenović, Perović, et al., 2005). An MTT assay performed to determine whether HA-NURR1 expression enhanced the anti-inflammatory capacity of primary astrocytes against 6-OHDA-induced toxicity generated highly variable results. Although the MTT assay confirmed 6-OHDA-induced cell death in all vector groups, the results were highly variable between experiments, suggesting that either primary astrocytes may not efficiently sense 6-OHDA, leading to variations in susceptibility to oxidative stress, or the toxicity assay may require further optimisation. Further characterisation of 6-OHDA-dependent phenotype of reactive astrogliosis is required to more accurately interpret these results.

The scratch assay that upregulates GFAP and other intermediate filament proteins that regulate astrocytic morphology and motility is commonly used to investigate astrocyte proliferation and migration in response to a scratch wound (Liang et al., 2007; Nishio et al., 2005). Injury-induced upregulation of endogenous GFAP and a corresponding increase in dYFP and HA-NURR1 expression in astrocytes in comparison to controls suggested that similar to the endogenous GFAP gene, the transgenic GFAP promoter may be regulated by transcription factors upregulated in response to the scratch injury.

3.4.8. HA-NURR1 transcriptionally regulates target genes

NURR1 dictates midbrain DA neuronal development and maintenance via regulation of various DA-specific genes that include TH, VMAT and DAT (discussed in section 1.7.1). Using a gel mobility shift assay, wherein recombinant NURR1 protein was probed with radiolabelled oligonucleotides corresponding to various regions of the TH promoter, a previous study identified a proximal NURR1 recognition sequence, 5’TGGCCTTT3’ that matches 7 of the 8 nucleotides of the complementary sequence of the NGFI-B response element (Iwawaki, Kohno, & Kobayashi, 2000; Kim et al., 2003). To confirm conformational and functional integrity of the HA-NURR1 transgenic protein, a co-transfection assay in which the ability of HA-NURR1 protein transcribed from the CMV-HA-NURR1 plasmid to regulate a DNA sequence encoding a TH promoter containing the above recognition motif was analysed. A co-transfection assay demonstrated that the transgenic
HA-NURR1 protein enhanced the expression of reporter dYFP from a TH promoter-regulated dYFP transgenic cassette. These results confirmed that the transgenic HA-NURR1 protein is transcriptionally functional.

3.4.9. HA-NURR1 exerts no effect on LPS-induced NF-κB nuclear translocation

The chronically activated inflammatory transcription factor NF-κB sustains glial expression of proinflammatory genes in CNS injury and disease (Ghosh et al., 2007; Hsiao et al., 2013; Hunot et al., 1997; Kalschmidt et al., 1997; Lee et al., 2014). Inflammatory stimulation induces the activation and nuclear translocation of NF-κB, which under basal conditions is sequestered in the cytoplasm by an inhibitory IκB subunit (Hohmann et al., 1991; O'Neill & Kalschmidt, 1997). As discussed in section 1.7.2, NURR1 attenuates pro-inflammatory gene expression by negatively regulating NF-κB activity. We aimed to determine whether AAV5-mediated HA-NURR1 expression in primary astrocytes may downregulate NF-κB activity in response to LPS. Treating primary astrocytes with LPS at different concentrations (5, 10, 25, 50, and 100ng/mL) for 1 or 2 hours induced nuclear translocation of NF-κB that was predominantly cytoplasmic under basal conditions. Active NF-κB has been shown to exhibit a half-life of ~30 minutes in vitro, while inactive NF-κB sequestered in the cytoplasm is more stable (Hohmann et al., 1991; O'Neill & Kalschmidt, 1997). As previously reported, in response to persistent inflammatory stimulation, de novo synthesis of NF-κB may have maintained levels of activated NF-κB in the nucleus up to 24 hours (data not shown) (Hohmann et al., 1991).

In AAV5 vector transduced primary astrocytes, therapeutic HA-NURR1 expression had no effect on nuclear localisation of NF-κB in response to 5ng/mL or 50ng/mL LPS concentrations. NURR1 regulates the dissociation of the NF-κB complex on promoters of pro-inflammatory genes (Saijo et al., 2009). Hence, NF-κB nuclear localisation may not necessarily correlate with the extent of NURR1-regulated anti-inflammatory effects, as NF-κB dissociated from DNA may remain in the nucleus. Alternatively, as planned in further studies, quantification of secreted pro-inflammatory molecules such as TNFα, IL-6 and IL-1β in the media using a cytometric bead array (CBA) assay, and quantification of the NO oxidation product, nitrite (NO$_2^-$) that provides a measure of activity of the NO producing
inflammatory enzyme iNOS, using a Griess assay may provide a more accurate indication of HA-NURR1 anti-inflammatory efficacy.

Based on these results the GFAP promoter in the context of AAV5 was selected to overexpress the HA-NURR1 therapeutic cassette in a neurotoxin model of PD in Chapter 4.
Chapter 4. Neuroprotective effects of astrocyte-targeted overexpression of NURR1 in a 6-OHDA-rat model of Parkinson’s disease
4.1. Introduction

In chapter 3 we coupled a GFAP promoter-regulated transgenic cassette with the AAV serotype 5 to predominantly transduce astrocytes in the rat SNpc. In this chapter, we utilised this vector construct to overexpress the transcription factor NURR1 in astrocytes in the naïve or 6-OHDA-lesioned SNpc, to determine whether AAV5 vector transduction and long-term transgene expression are associated with inflammatory responses in the naïve brain; and investigate the neuroprotective efficacy of astrocyte-targeted therapeutic gene expression in the PD brain, respectively.
4.2. Materials and Methods

4.2.1. AAV vector infusion to the SNpc

Stereotaxic infusion of AAV vectors (2 x 10^9 viral genomes) into the SNpc (AAV5-GFAP-pL n = 5; AAV5-GFAP-dYFP n = 8; and AAV5-GFAP-HA-NURR1 n = 22) was performed as outlined in section 2.2.7.

4.2.2. Striatal 6-OHDA infusion

To induce retrograde degeneration of the nigrostriatal pathway, three weeks post-vector infusion, all of the AAV5-GFAP-pL (n = 5), and half of each of AAV5-GFAP-dYFP (n = 4) and AAV5-GFAP-HA-NURR1 (n = 11) vector groups were unilaterally infused with 20µg of 6-OHDA (H116; Sigma) into the ipsilateral striatum (AP +1.0mm, ML +3.0mm, DV – 5.0mm, bregma = 0) (Figure 4-1). The remaining AAV5-GFAP-dYFP (n = 4) and AAV5-GFAP-HA-NURR1 (n = 11) animals served as controls and received a striatal saline (3 µL) injection. Four and eight weeks post-striatal toxin infusion, all subgroups were subjected to the cylinder test and amphetamine-induced rotation to measure motor function.
4.2.3. Cylinder test

One week pre- (baseline), and four and eight weeks post-striatal infusions, to measure forepaw asymmetry, the animals were placed inside a transparent cylinder (19cm diameter x 29cm height) and recorded for 10 minutes. A mirror folded at 90° was placed behind the cylinder to record forelimb movement when the animal was turned away from the camera. The recorded behaviour was analysed (VLC media player version 2.2.1) in slow motion to determine either simultaneous or independent use of the left and right forepaw for contacting the cylinder wall during a full rear, or to regain balance during lateral exploration in a vertical posture. Behaviour deficit was quantified as the use of the impaired contralateral forepaw as a percentage of the total forepaw use.
4.2.4. Amphetamine-induced asymmetric rotational behaviour

Four and eight weeks post-6-OHDA infusion, animals were given a subcutaneous (s.c.) injection of dexamphetamine sulphate (2.5mg/kg, and placed in a cylinder (30cm diameter x 33cm height) to quantify drug-induced rotation. 30 minutes following drug administration, the animals were recorded for a total of 90 minutes with a camera placed directly above the cylinder. The number of full ipsilateral and contralateral rotations was recorded over 60 minutes. A rotation was defined as a tight head-to-tail turn in a circle equal to and over 270°. The net ipsilateral rotations were determined by subtracting the total number of contralateral rotations from the total number of ipsilateral rotations. The results are presented as net ipsilateral rotations per minute.

4.2.5. IHC on 4% PFA-fixed coronal brain sections

Following completion of behaviour tests, animals were euthanised, and perfused with 4% PFA as outlined in section 2.2.8. Forty micron coronal sections were cut on a cryostat, and sections at the level of the SNpc were immunohistochemically analysed to examine expression of transgene, TH, HuCD, GFAP, and Iba1 as described in sections 2.2.9 and 2.2.10.

4.2.6. Fluoro-Jade B staining

Fluoro-Jade B, an anionic fluorescein derivative that selectively labels degenerating cell bodies and neurites was used to characterise neurodegeneration in the nigrostriatal pathway (Schmued & Hopkins, 2000). Coronal sections were washed and mounted onto poly-l-lysine coated slides in distilled water and dried overnight. Subsequently, slides were dehydrated in 100% (3 minutes) and 75% ethanol (1 minute), immersed in 0.06% potassium permanganate (KMnO₄) for 15 minutes, washed in distilled water for 1 minute, and immersed in 0.001% Fluoro-Jade B staining solution (Histo Chem Inc., Jefferson, AR, USA) for 30 minutes. Sections were then washed in distilled water (3 x 1 minute), and dried overnight. The following day, slides were immersed in Xylene, coverslipped with mounting medium, and viewed on an Olympus AX70 microscope using a Fluorescein isothiocyanate (FITC) filter.
4.2.7. Stereological quantification of TH-positive neurons in the SNpc

To quantify survival of DA neurons in the SNpc, the number of TH-positive neurons within the ipsilateral and contralateral SNpc was quantified using unbiased stereology by an investigator blinded to the experimental groups. Six nigral sections (starting at bregma - 4.70mm, every 6\textsuperscript{th} 40\textmu m section) per animal were analysed.

Using the optical fractionator probe in Stereoinvestigator 7 (MBF Bioscience, Willeston, VT), SNpc TH-positive neurons were counted at 40x magnification, within a frame of 80 x 80\textmu m placed over a grid size of 200 x 100\textmu m. Z-axis guard zones of 2\textmu m were specified at the top and the bottom of the tissue.

4.2.8. Statistical analysis

To test parametric test assumptions, the data were first analysed using the Shapiro-Wilk test of normality, Levene’s test of homogeneity of variance, and Mauchly’s test of sphericity using SPSS statistics software (IBM SPSS Inc. version 21, Chicago, IL, USA). The stereological data were analysed using an independent samples t-test or one-way ANOVA, and behaviour data were subjected to a mixed-design ANOVA with Tukey’s post-hoc comparisons with a p-value of less than 0.05 considered statistically significant.
4.3. Results

GFAP promoter-coupled AAV5 vector constructs were used to selectively overexpress the transcription factor NURR1 in astrocytes in the naïve or 6-OHDA-lesioned SNpc, to determine whether long-term AAV5 vector transduction is associated with inflammatory responses in the naïve brain, and to investigate the neuroprotective efficacy of astrocyte-targeted therapeutic gene expression in the PD brain, respectively. Control and therapeutic AAV5 vectors (2 x 10⁹ viral genomes) were unilaterally injected into the SNpc (AAV5-GFAP-pL n = 5; AAV5-GFAP-dYFP n = 8; and AAV5-GFAP-HA-NURR1 n = 22). To induce retrograde degeneration of the nigrostriatal pathway, three weeks post-vector infusion, all of the AAV5-GFAP-pL (n = 5), and half of each of AAV5-GFAP-dYFP (n = 4) and AAV5-GFAP-HA-NURR1 (n = 11) vector groups were unilaterally injected with 20µg of 6-OHDA into the ipsilateral striatum. The remaining AAV5-GFAP-dYFP (n = 4) and AAV5-GFAP-HA-NURR1 (n = 11) animals served as controls and received a striatal saline (3 µL) injection. Four and eight weeks post-striatal toxin infusion, all subgroups were subjected to the cylinder test and amphetamine-induced rotation to measure motor function.

4.3.1. AAV5-mediated transgene expression in the SNpc under physiological conditions

4.3.1.1. Transgene expression in the control nigra persists over time

To characterise long-term transgene expression under physiological conditions, AAV5-mediated dYFP or HA-NURR1 expression in the control SNpc was analysed eleven weeks post-vector infusion. Similar to that detected at three weeks post-vector infusion, efficient dYFP expression was detected in the SNpc at eleven weeks post-vector infusion (Figure 4-2 a-c). While dYFP expression minimally extended to the VTA, relatively high levels of expression were detected in the ventral regions of the midbrain reticular nucleus, and immediately adjacent to the needle tract along the dorso-ventral axis (Figure 4-2 a). Similar to the tropism of transgene expression seen at three weeks post-vector infusion, dYFP expression was detected in both neurons (Figure 4-2 c, arrowhead) and astrocytes (Figure 4-2 c, arrow). However, there was no detectable dYFP immunoreactivity in nigral neuronal terminals in the ipsilateral striatum (Figure 4-2 d, e). As expected, HA-NURR1 was exclusively expressed in the nucleus (Figure 2-1 f-h). Although expression was considerably
lower than dYFP, the pattern of HA-NURR1 expression in the midbrain was comparable to that of dYFP.
Figure 4-2 AAV5 vector-mediated transgene expression in the non-lesioned SNpc

Similar to the levels of dYFP expression at three weeks post-vector infusion, AAV5-mediated (a-c) dYFP persisted in the non-lesioned SNpc eleven weeks post-vector injection. Based on dYFP-immunoreactivity, (a) in addition to efficient transduction in the SNpc, relatively high levels of dYFP expression were detected in the ventral regions of the midbrain reticular nucleus, and immediately adjacent to the needle tract along the dorso-ventral axis, with minimal spread to the VTA. High magnification indicates dYFP expression in neurons (c, arrowhead) and astrocytes (c, arrow).

(d, e) Relative to the contralateral hemisphere, no dYFP-immunoreactivity was detected in the terminal fields of nigral neurons in the ipsilateral striatum.

(f-h) A pattern of HA-NURR1 expression similar to that of dYFP was detected with an anti-HA antibody, albeit at considerably lower levels. High magnification indicates exclusively nuclear expression of HA-NURR1 (h, arrow).

Scale bars: (a, d, e, f) 500µm, (b, g) 200µm, and (c, h) 100µm
4.3.1.2. Effects of AAV5-mediated transgene expression on DA neuronal survival

Following the eight week post-lesion time-point, to determine potential immunogenicity of AAV5 capsid proteins, or toxicity associated with long-term transgene overexpression under physiological conditions, TH-positive DA neurons in the SNpc were stereologically quantified, and presented as a percentage of the contralateral SNpc.

In HA-NURR1 expressing control animals, TH-immunoreactivity of DA neuronal cell bodies in the SNpc and their fibres in the SNpr, and the number of TH-positive nigral neurons (102.1% of the contralateral SNpc) was comparable to that in the uninjected contralateral hemisphere (Figure 4-3 g-l and Figure 4-4). In comparison to the therapeutic vector group, a loss of TH-immunoreactivity in the nigra, and a significant reduction in the percentage of DA neurons were evident in the dYFP expressing brains (85.0%; Independent sample t-test, p = 0.022) (Figure 4-3 a-f and Figure 4-4). To ensure that potential downregulation of TH expression associated with AAV5-GFAP-dYFP transduction was not interpreted as neuronal degeneration, nigral sections were immunostained for the neuronal marker HuCD. While TH- and HuCD-immunoreactivity in the HA-NURR1 expressing nigra was comparable to the contralateral side (Figure 4-3 q-t), the loss of TH-immunoreactive neurons and fibres in the dYFP expressing nigra was paralleled by a corresponding decrease in HuCD immunoreactivity in consecutive sections (Figure 4-3 m-p). To characterise integrity of nigral neuronal terminals in the ipsilateral striatum, striatal TH-immunoreactivity was assessed. In both dYFP- and HA-NURR1- expressing animals, striatal TH density appeared comparable to that in the contralateral hemisphere (Figure 4-5). Furthermore, Fluoro-Jade B staining did not reveal any degenerating neurons in the vector-transduced SNpc, or ipsilateral striatum of both vector groups (not shown).
Figure 4-3 DA neuronal integrity in the vector-transduced control SNpc

Eleven weeks post-vector injection, relative to the uninjected contralateral SNpc (a-c), a loss of TH immunoreactivity in the ipsilateral nigral neurons and fibres (d-f) was seen in the dYFP-expressing control animals.

In the HA-NURR1-expressing animals, neuronal and fibre integrity in the contralateral (g-i) and ipsilateral (j-l) nigrae appears comparable.

High magnification images indicate a loss of TH-immunoreactive fibre density in dYFP-expressing nigra (f) relative to the contralateral hemisphere (c), while fibre density in the HA-NURR1-expressing nigra (l) appears comparable to that in the contralateral nigra (i).

In comparison to the contralateral hemisphere (m, n), a decrease in HuCD-immunoreactivity in the vector-injected nigra (o, p) paralleled the loss of TH-positive neurons in the dYFP expressing animals.

Similar to TH-immunoreactivity, HuCD-immunoreactivity in the contralateral (q, r) and HA-NURR1-expressing (s, t) nigrae appeared comparable.

Scale bars: (a, d, g, j, m, o, q, s) 500µm, (b, e, h, k, n, p, r, t) 200µm, (c, f, i, l) 50µm
Figure 4-4 Stereological quantification of DA neurons in the vector-transduced control SNpc

The number of TH immunoreactive neurons in the vector injected SNpc is presented as a percentage of that in the uninjected contralateral SNpc. TH-positive cells in the dYFP-expressing SNpc ranged from 69.6 to 100.4% and from 84.7 to 117.0% in the HA-NURR1-expressing SNpc. The mean percentage of nigral TH-positive neurons in dYFP vector-injected (85.0%) animals was significantly lower than that in the HA-NURR1 vector-injected (102.1%) animals (independent samples t-test, \( p = 0.022 \)).

\* \( p < 0.05 \)
Figure 4-5 Striatal TH expression in nigral vector-injected control animals

Eleven weeks post-nigral vector injection, TH immunoreactivity in the striatum ipsilateral to the vector-transduced SNpc was comparable to that in the contralateral striatum in both (a-d) dYFP- and (e-h) HA-NURR1-expressing animals.

Scale bars: (a, c, e, g) 500µm, (b, d, f, h) 200µm
4.3.1.3. Effects of AAV5-mediated transgene expression on nigral inflammation

The resident CNS immune cells, astrocytes and microglia undergo molecular, morphological, and functional alterations in response to inflammatory stimuli in a process termed reactive gliosis. Upregulation of certain glial markers and cellular hypertrophy are classic phenotypic adaptations in reactive glia. To determine whether vector transduction induced an inflammatory response in the naïve nigra, potential glial reactivity was analysed by IHC using antibodies against the astrocyte marker, GFAP and microglia marker Iba1. GFAP, a key intermediate filament protein of the astrocytic cytoskeleton is upregulated in reactive astrocytes (Eng & Ghirnikar, 1994). The intensity of GFAP-immunoreactivity and ‘star-like’ astrocytic morphology in the HA-NURR1-expressing nigra appeared comparable to that in the contralateral side (Figure 4-6 e-h). In contrast, in the dYFP-expressing nigra, GFAP expression and the density of GFAP-positive processes were increased, indicative of cellular hypertrophy (Figure 4-6 a-d).

Iba1 that may play a role in regulating microglial mobility and phagocytosis is upregulated in reactive microglia (Ito, Tanaka, Suzuki, Dembo, & Fukuuchi, 2001; Ohsawa, Imai, Kanazawa, Sasaki, & Kohsaka, 2000). Under physiological conditions, microglia exhibit highly ramified processes, whereas reactive microglia acquire an amoeboid morphology and appear distended with retracted processes (Karperien, Ahammer, & Jelinek, 2013). The intensity of Iba1 immunoreactivity, size of the microglial cell bodies and branching of processes in both dYFP- (Figure 4-7 a-d) and HA-NURR1- (Figure 4-7 e-h) expressing nigra appear comparable to that in the contralateral hemispheres.
Figure 4-6 Characterisation of potential astrocyte reactivity in the vector-transduced SNpc

Relative to the contralateral SNpc (a, b), GFAP upregulation and increased numbers of GFAP-positive processes characteristic of astrocyte reactivity were detected in the dYFP vector-injected SNpc (c, d).

In contrast, GFAP-immunoreactivity and astrocytic morphology in the HA-NURR1 vector-injected SNpc (g, h) appeared comparable to that in the contralateral SNpc (e, f).

High magnification indicates hypertrophy of GFAP-positive astrocytic processes in the dYFP-expressing SNpc (d), in comparison to the contralateral SNpc (b). Morphology of astrocytes in the HA-NURR1-expressing SNpc (h) appeared comparable to that in the contralateral SNpc (f).

Scale bars: (a, c, g) 500µm, (b, d, f, h) 50µm
Figure 4-7 Characterisation of potential microglial reactivity in the vector-transduced control SNpc

In both (c, d) dYFP and (g, h) HA-NURR1 vector-injected SNpc, Iba1-immunoreactivity and morphology of microglia appeared comparable to that in the contralateral SNpc (a, b and e, f, respectively).

High magnification indicates that the size of cell bodies and branching of microglial processes in the (d) dYFP and (h) HA-NURR1 vector-injected SNpc were comparable to that in the contralateral SNpc (b, and f, respectively).

Scale bars: (a, c, e, g) 500µm, (b, d, f, h) 50µm
4.3.1.4. Effects of AAV5-mediated transgene expression on motor function

Unilateral deficits in the DA nigrostriatal pathway lead to asymmetrical striatal DA transmission between the lesioned and intact striati that translates into asymmetrical motor function. In this study, the cylinder test and amphetamine-induced rotation were used to quantify motor function in vector-treated control and PD animals. The behaviour data were analysed with a mixed-design ANOVA.

To determine whether AAV5 vector transduction and long-term transgene expression influence function of the nigrostriatal pathway, forelimb asymmetry and amphetamine-induced rotation in vector-treated control animals were assessed. The cylinder test quantifies the preference in a unilaterally-lesioned rat for its ipsilateral, non-impaired paw for contacting the cylinder wall during vertical exploration. Forelimb deficits were quantified as the use of the lesion-affected contralateral forepaw as a percentage of total forepaw wall contacts. A subtle decline in contralateral forepaw use was seen in dYFP-expressing control animals at 4 weeks that was maintained at 8 weeks (46.0%, 35.6%, and 35.1% at baseline, 4 weeks and 8 weeks post-striatal saline infusion, respectively); whereas an unexpected increase at a similar rate, in preference for the contralateral forepaw in HA-NURR1-expressing naïve animals (43.3%, 53.1%, and 58.4% at baseline, 4 weeks and 8 weeks post striatal infusions, respectively) was detected (Figure 4-8). However, there were no vector treatment- or time-dependent effects on forepaw bias (mixed-design ANOVA, p = 0.13).

Amphetamine, a competitive agonist for the DA transporter (DAT), inhibits extracellular dopamine uptake into DA terminals, and stimulate DAT-mediated reverse transport of DA into the synaptic cleft (Fleckenstein, Volz, Riddle, Gibb, & Hanson, 2007). It may also act as a weak base and increase the pH in cellular vesicles to promote DA release into the cytoplasm. The asymmetry in amphetamine-induced increase in DA neurotransmission between the lesioned and non-lesioned striati in unilaterally lesioned animals results in rotation in the ipsilateral direction (Glick, Jerussi, Waters, & Green, 1974). The total number of amphetamine-induced ipsilateral and contralateral rotations was counted over one hour period, and the net ipsilateral rotations per minute were determined. No drug-induced rotation bias was detected in dYFP (-1.56 and -0.81 net ipsiversive rotations per minute at four and eight weeks, respectively) and HA-NURR1 (-0.43 and -0.86 net ipsiversive rotations per minute at four and eight weeks, respectively) expressing animals at either time-point (mixed-design ANOVA, p = 0.23) (Figure 4-9).
A subtle decline in contralateral forepaw use was seen in dYFP expressing control animals (46.0%, 35.6%, and 35.1% at baseline, 4 weeks and 8 weeks post striatal saline infusion, respectively); while an increase at a similar rate in contralateral forepaw use in HA-NURR1 animals was detected (43.3%, 53.1%, and 58.4% at baseline, 4 weeks and 8 weeks post striatal saline infusion, respectively). However, there were no vector treatment- or time-dependent effects on forepaw bias (mixed-design ANOVA, p = 0.13).
Figure 4-9 Effect of AAV-mediated transgene expression on amphetamine-induced rotation in control animals

No drug-induced rotation bias was detected in dYFP (-1.56 and -0.81 net ipsilateral rotations per minute at 4 and 8 weeks, respectively) or HA-NURR1 (-0.43 and -0.86 net ipsilateral rotations per minute at 4 and 8 weeks, respectively) expressing control animals (mixed-design ANOVA, p = 0.23).
4.3.2. AAV5-mediated transgene expression in the SNpc in PD

4.3.2.1. Transgene expression in the 6-OHDA-lesioned SNpc

AAV5-mediated dYFP or HA-NURR1 expression was analysed eleven weeks post-vector infusion in the 6-OHDA-lesioned SNpc to determine whether transgene expression persists under pathological conditions. Similar to that under physiological conditions, relatively high levels of dYFP expression were detected in the ventral regions of the midbrain reticular nucleus, and immediately adjacent to the needle tract along the dorso-ventral axis, with minimal dissemination of transgene expression to the VTA (Figure 4-10 a, b). Furthermore, similar to that detected in non-lesioned control animals at three, and eleven weeks post-vector infusion, efficient dYFP expression in both neurons Figure 4-10 c, arrowhead) and astrocytes (Figure 4-10 c, arrow) was detected in the lesioned SNpc. Similar to that under physiological conditions, HA-NURR1 expression remained exclusively nuclear in the lesioned brain (Figure 4-10 d-f). Although expression was considerably lower than dYFP, the pattern of HA-NURR1 expression in the midbrain in PD animals was comparable to that of dYFP.

4.3.2.2. HA-NURR1 expression promotes survival of nigral DA neurons in PD

In empty vector-treated PD animals, TH-immunoreactivity revealed a severe loss of neuronal cell bodies and fibres in the nigra ipsilateral to the lesioned striatum (Figure 4-11 a-f). In comparison to the respective contralateral hemispheres, HA-NURR1-expressing (Figure 4-11 m-r) PD animals appeared to exhibit a slightly higher neuronal density in the lesioned SNpc than the dYFP vector-treated (Figure 4-11 g-l) PD group. Levels of HuCD-immunoreactivity were consistent with the loss of TH immunoreactivity in each vector group (Figure 4-12). Stereological quantification of TH-positive neurons further indicated that NURR1 overexpression resulted in a greater preservation of nigral DA neurons with a mean neuronal survival of 53.7%, in comparison to 38.9% and 44.4% surviving neurons in the empty-, and dYFP vector-treated PD groups, respectively (one-way ANOVA, p = 0.093) (Figure 4-13 a). There appeared to be a substantial loss of striatal TH-immunoreactivity in all vector groups that indicate 6-OHDA-induced degeneration of DA neuronal terminals in the striatum (Figure 4-14); Furthermore, TH-immunoreactivity and 6-OHDA lesion size appeared highly variable amongst the PD animals in all vector groups, and did not seem to
correlate with nigral DA neuronal survival or behaviour scores. Fluoro-Jade B staining in the 6-OHDA-lesioned striatum or SNpc did not reveal any degenerating neurons in any of the vector groups (Figure 4-14 a-f). Fluoro-Jade B stained degenerating neurons were detected in the positive control that expressed an AAV-mediated toxic mRNA sequence in the hippocampus (Figure 4-14 g, h).

To strengthen statistical power, the AAV5-GFAP-pL (n = 5) and AAV5-GFAP-dYFP (n = 4) PD groups were pooled into a single control group. In comparison to the merged empty and dYFP group (mean neuronal survival of 41.3%), a significantly higher percentage of surviving neurons were present in the NURR1 therapeutic group (Independent sample t-test, p = 0.036) (Figure 4-13 b).
Figure 4-10 AAV vector-mediated transgene expression in the 6-OHDA-lesioned SNpc

Based on dYFP-immunoreactivity, (a-c) in addition to efficient transgene expression in the lesioned SNpc, relatively high levels of dYFP expression were detected in the ventral regions of the midbrain reticular nucleus, and immediately adjacent to the needle tract along the dorso-ventral axis, with minimal spread to the VTA. High magnification shows dYFP expression in neurons (c, arrowhead) and astrocytes (c, arrow). (d-f) HA-immunoreactivity revealed a pattern of HA-NURR1 similar to that of dYFP in the lesioned nigra, albeit at considerably lower levels. Similar to that under basal conditions, HA-NURR1 expression was exclusively nuclear (f, arrow).

Scale bars: (a, d) 500µm, (b, e) 200µm, and (c, f) 100µm
Figure 4-11 HA-NURR1 expression may promote preservation of nigral DA neurons in the PD SNpc

(a-c) Relative to the contralateral hemisphere, a severe loss of TH-immunoreactive nigral neurons and fibres was seen in (d-f) empty vector-injected 6-OHDA-lesioned nigra. In comparison, relative to the respective contralateral hemispheres (dYFP, g-i; HA-NURR, m-o), a moderate loss of neuronal TH-immunoreactivity was detected in both (j-l) dYFP and (p-r) HA-NURR1 expressing PD nigra; however, HA-NURR1 vector-transduced animals appeared to exhibit a slightly greater density of TH-immunoreactive neurons and fibres.

Scale bars: (a, d, g, j, m, p) 500µm, (b, e, h, k, n, q) 200µm, (c, f, i, l, o, r) 50µm
Figure 4-12 Loss of HuCD immunoreactivity parallels the loss of TH-immunoreactive DA neurons in the lesioned SNpc

The loss of HuCD immunoreactivity corresponded to the severity of the loss of TH-immunoreactive neurons in all three vector groups; (a-d) empty, (e-h) dYFP, and (i-l) HA-NURR1.

Scale bars: (a, c, e, g, i, k) 500μm, (b, d, f, h, j, l) 50μm
Figure 4-13 HA-NURR1 expression may promote DA neuronal survival in the PD SNpc

(a) HA-NURR1 overexpression promoted a greater survival of TH-immunoreactive neurons with a mean neuronal survival of 53.7% in the 6-OHDA-lesioned nigra, in comparison to 38.9% and 44.4% surviving neurons in the empty, and dYFP vector groups, respectively (one-way ANOVA; p = 0.093).

(b) To increase statistical power, the empty and dYFP groups were pooled into a single control group. A statistically significant neuronal survival in HA-NURR1 expressing PD animals was seen in comparison to the 41.4% surviving neurons in the combined empty and dYFP control group (Independent sample t-test; p = 0.036).

*p < 0.05
Figure 4-14 6-OHDA induced a severe loss of TH-immunoreactive striatal dopaminergic terminals

In comparison to the respective intact contralateral hemispheres (empty, a, b; dYFP, e, f; HA-NURR1, i, j), a robust loss of TH immunoreactivity in the 6-OHDA-lesioned striatum was detected 8 weeks post-6-OHDA in c, d) empty, g, h) dYFP, and k, l) HA-NURR1 vector groups, with no apparent vector treatment-dependent differences.

Scale bars: (a, c, e, g, i, k) 500µm, (b, d, f, h, j, l) 50µm
Figure 4-15 Fluoro-Jade B staining in the 6-OHDA-lesioned striatum and SNpc

Fluoro-Jade B staining was not detected in the 6-OHDA-lesioned striatum or ipsilateral vector-transduced SNpc, eight weeks post-lesion in a, b) empty, c, d) dYFP, or e, f) HA-NURR1 groups.

g, h) Fluoro-Jade B staining revealed degenerating neurons in the hippocampus three weeks-post injection of an AAV vector that expressed a toxic miRNA sequence (positive control).

Scale bars: (a-g) 500µm, (h) 200µm
4.3.2.3. HA-NURR1 expression may downregulate inflammation in PD

In all PD vector groups, an upregulation of GFAP expression, and increase in the number of highly ramified GFAP-positive processes coupled with hypertrophy of the main processes indicated distinctive nigral reactive astroglisis eight weeks post-6-OHDA infusion (Figure 4-16). However, in the HA-NURR1 (Figure 4-16 i-l) expressing PD animals, these characteristic morphological adaptations in the lesioned nigra appeared less severe than that in the empty (Figure 4-16 a-d) and dYFP (Figure 4-16 e-h) groups.

In all PD vector groups, Iba1-immunoreactive microglia in the 6-OHDA lesioned nigra did not appear to exhibit a reactive phenotype (Figure 4-17). The intensity of Iba1 immunoreactivity, size of their cell bodies and branching of processes in all vector groups appeared comparable to that in the respective contralateral hemispheres.
Figure 4-16 HA-NURR1 expression may downregulate reactive astrogliosis in the PD SNpc

In comparison to the respective contralateral hemispheres (empty, a, b; dYFP, e, f; and HA-NURR1, i, j), an upregulation of GFAP expression, and increase in the number of GFAP-positive processes, characteristic of reactive astrocytes were detected in the (c, d) empty, (g, h) dYFP, and (k, l) HA-NURR1 expressing 6-OHDA-lesioned SNpc.

High magnification indicates distinctive hypertrophy of main astrocytic processes in the PD SNpc expressing (d) empty and (h) dYFP relative to the respective contralateral hemispheres (b and f, respectively). Relative to the contralateral hemisphere (j), in the (l) HA-NURR1 expressing PD SNpc, these characteristic morphological adaptations appeared less severe.

Scale bars: (a, c, e, g, i, k) 500µm, (b, d, f, h, j, l) 50µm
Figure 4-17 An absence of microglial reactivity in the PD SNpc

Relative to the respective contralateral hemispheres (empty, a, b; dYFP, e, f; and HA-NURR1, i, j), no discernible upregulation of Iba1 expression or morphological adaptations indicative of microglial reactivity were detected in the (c, d) empty, (g, h) dYFP, or (k, l) HA-NURR1 expressing 6-OHDA-lesioned SNpc.

High magnification indicates that the size of microglial cell bodies and branching of processes in the (d) empty, (h) dYFP, and (l) HA-NURR1 vector-injected PN SNpc were comparable to that in the respective contralateral SNpc (b, f, and j respectively).

Scale bars: (a, c, e, g, i, k) 500µm, (b, d, f, h, j, l) 50µm
4.3.2.4. HA-NURR1 expression may ameliorate motor deficits in PD

Relative to baseline, PD animals in all three vector groups had a decline in contralateral forepaw use at four weeks (42.9% to 16.0% in empty; 37.6% to 14.4% in dYFP; and 45.2% to 24.4% in HA-NURR1 groups), indicating 6-OHDA-induced motor impairment. At eight weeks, while forepaw bias remained stable in the empty vector group (15.7%), a subtle improvement in the contralateral forepaw use was seen in the dYFP group (23.5%) (Figure 4-18 a). In contrast, HA-NURR1-expressing animals completely recovered contralateral forepaw use to baseline levels (45.3%) at this time-point. However, there were no vector treatment or time-dependent differences in forepaw bias (mixed-design ANOVA, p = 0.38). Furthermore, in comparison to the pooled empty and dYFP control group (40.5%, 13.4%, and 19.2% at baseline, 4 weeks and 8 weeks, respectively), HA-NURR1 exhibited no differences in forepaw bias (mixed-design ANOVA, p = 0.16) (Figure 4-18 b).

All PD animals had an increase in amphetamine-induced ipsilateral rotations at both four (9.7, 9.1, and 10.4 ipsilateral turns per minute in empty, dYFP, and HA-NURR1 groups, respectively), and eight weeks (8.2, 7.3, and 8.5 ipsilateral turns per minute in empty, dYFP, and HA-NURR1 groups, respectively) (Figure 4-19 a). There was a negligible decrease in the number of ipsiversive rotations in each treatment group at eight weeks, with considerable variation in rotation within all groups. However, there were no vector treatment or time-dependent effects on drug-induced rotation (mixed-design ANOVA, p = 0.99). Comparing the therapeutic group to the combined controls at four weeks (9.4 ipsilateral turns per minute) and eight weeks (7.7 ipsilateral turns per minute) yielded a similar overall increase in net ipsilateral rotations in both groups (mixed-design ANOVA, p = 0.90) (Figure 4-19 b).
Figure 4-18 Long-term HA-NURR1 overexpression ameliorates 6-OHDA-induced forepaw asymmetry

(a) Baseline contralateral forepaw use in the empty (42.9%), dYFP (37.6%) and HA-NURR1 (45.2%) groups declined to 12.6%, 14.4%, and 24.4%, respectively, at 4 weeks. At 8 weeks, forepaw deficits remained stable in the empty vector group at 15.67%, while a subtle increase in the use of the impaired forepaw was seen in dYFP animals (23.5%). In contrast, although not statistically significant, a complete recovery in contralateral forepaw use to baseline levels was seen in HA-NURR1 animals at 8 weeks with 45.3% (mixed-design ANOVA, p = 0.38).

Furthermore, (b) in comparison to the combined empty and dYFP group (40.5%, 13.4%, and 19.2% at baseline, 4 weeks and 8 weeks, respectively), HA-NURR1 exhibited no differences in forepaw bias (mixed-design ANOVA, p = 0.16).
Figure 4-19 Absence of an HA-NURR1-mediated effect on amphetamine-induced rotation in PD rats

(a) 6-OHDA-lesioned animals in all three vector groups exhibited an increase in the net ipsilateral rotations at 4 weeks (9.7, 9.1, and 10.4 turns per minute in empty, dYFP, and HA-NURR1 groups, respectively), and 8 weeks (8.2, 7.3, and 8.5 turns per minute in empty, dYFP, and HA-NURR1 groups, respectively (mixed-design ANOVA, p = 0.99). A negligible decrease in the net ipsilateral rotations was detected in each treatment group at 8 weeks with considerable variation in rotation among all groups.

(b) Comparing the therapeutic group to the combined empty and dYFP group (9.4 and 7.7 ipsilateral turns per minute at 4 and 8 weeks, respectively) yielded a similar overall increase in net ipsilateral rotations in both groups, with a negligible decline in ipsilateral rotation at 8 weeks (mixed-design ANOVA, p = 0.90).
4.4. Discussion

In this study, we utilised an astrocyte-tropic AAV5 vector construct to overexpress the transcription factor NURR1 predominantly in astrocytes in the naïve or 6-OHDA-lesioned SNpc, to determine whether vector transduction and transgene expression are associated with inflammatory responses in the naïve brain, and investigate the neuroprotective efficacy of astrocyte-targeted therapeutic gene expression in the PD brain, respectively.

4.4.1. DA neuronal death and nigral inflammation related to AAV5 capsids or dYFP expression under physiological conditions

Eleven weeks post-vector and eight weeks post-saline injections into the SNpc, and ipsilateral striatum, respectively, nigral dYFP and HA-NURR1 expression persisted in nigral neurons and astrocytes at levels comparable to that observed at three weeks post-vector infusion, indicating that the AAV5 vector construct mediated robust, transgene expression for the duration of the study. An absence of dYFP-immunoreactivity in DA neuronal terminals in the striatum suggested that the levels of transgene expression in neurons are low and below the limits of detection and/or the numbers of transduced neurons are low, as typically, transgene expression can be detected in the striatal terminals when transgene expression is driven by strong promoters such as the CBA promoter. However, quantification of the proportion of transgene expressing neurons and astrocytes may have accurately defined the nigral tropism of the AAV5-GFAP vector construct.

The number of DA neurons in the HA-NURR1 expressing naïve SNpc was comparable to the uninjected contralateral hemisphere (102.1% of the contralateral SNpc). In contrast, dYFP expressing brains exhibited a significant reduction in the percentage of TH-immunoreactive DA neurons in the SNpc (85%; p = 0.025) that was paralleled by a loss of HuCD-immunoreactive nigral neurons, indicating neurotoxicity in this vector group. However, a corresponding decrease in striatal TH-immunoreactivity was not apparent, suggesting that a 15% loss of DA neuronal cell bodies in the nigra may not lead to a substantial loss of TH activity in their terminal fields in the striatum (Kirik, Rosenblad, & Bjorklund, 1998), as the largely remaining neurons may maintain phenotypic integrity of the nigrostriatal pathway. Quantification of striatal TH density may further confirm maintenance of striatal TH activity in dYFP expressing naïve animals.
Glia cell markers were used to characterise inflammation in the vector transduced nigra. Increased GFAP expression and cellular hypertrophy are classic phenotypic alterations of reactive astroglia. In naïve vector-treated animals, astrocytic and microglial morphology in the HA-NURR1 vector infused nigra appears comparable to those in the contralateral side indicating an absence of inflammation associated with nigral manipulation. Whereas, increased GFAP expression and cellular hypertrophy characteristic of astrocyte reactivity, and DA neuronal death in the dYFP vector infused nigra indicated inflammation and neurodegeneration in this vector group. Unexpectedly, Iba1 immunoreactivity indicated a lack of reactive microglia in the dYFP-expressing nigra. Functional significance of glial reactivity is discussed in detail in section 4.4.4.

Since the naïve group lacked an empty control vector, it is not possible to conclude that neuronal toxicity in AAV5-GFAP-dYFP animals is directly related to dYFP overexpression, and not due to toxicity mediated by AAV5 capsids. The inclusion of empty vector and vehicle (1 x PBS) controls to determine potential AAV5 capsid-related toxicity and stereotaxic injection-related tissue injury, respectively, may have allowed a more accurate interpretation of these results. Previous studies have reported that AAV5 capsids at high vector titre (over 1 x 10^{10} genomes) induced toxicity in both neurons and astrocytes in vitro and in vivo (Ortinski et al., 2010; Royo et al., 2008). If the toxicity were related to viral capsid proteins in this study, a lack of nigral neuronal death in the therapeutic vector group suggests a protective effect of HA-NURR1 against viral capsid-induced inflammation.

Furthermore, toxicity related to GFP expression in vivo has been reported previously, suggesting that the detrimental accumulation of heterologous reporter proteins may initiate stress responses that could compromise cell function and survival. In the primate cortex, AAV5-, 8- or 9-mediated expression of GFP in neurons was associated with downregulation of neuronal NeuN, and infiltration of glia (Watakabe et al., 2015). At high titre (3 x 10^{10} genomes), AAV5-mediated GFP expression in hippocampal astrocytes initiated selective astrocyte dysfunction that contributed to electrophysiological deficits in neuronal circuits (Ortinski et al., 2010). The wild-type GFP cloned from the jellyfish Aequorea victoria folds into a distinct, compact β-can structure that is resistant to chemical denaturation, and is stably expressed in most cells (Yang, Moss, & Phillips, 1996). It is estimated to have a half-life of ~26 hours (Corish & Tyler-Smith, 1999), rendering GFP and variants of GFP such as dYFP ideal reporters for a wide range of biological applications such as tracking gene expression profiles in development, and elucidating molecular pathways. In contrast, in vitro data
suggests that both endogenous and transgenic NURR1 have a half-life of about 3-4 hours (Alvarez-Castelao et al., 2013). Therefore, for the purpose of imitating a dynamic process such as endogenous gene expression to determine such aspects as temporal patterns and efficacy of a transgene delivery technique, a reporter that is degraded at a similar rate to that of the endogenous would be more appropriate.

4.4.2. AAV5-mediated transgene expression did not affect motor function in naïve animals

The asymmetry in forelimb use in the cylinder test and amphetamine-induced rotation bias associated with DA imbalances between the unilaterally lesioned striatum and intact hemisphere are behaviour paradigms widely used to investigate DA mechanisms and efficacy of therapeutics targeting the nigrostriatal pathway. While a steady decline in contralateral forepaw use that could potentially be attributed to neuronal toxicity was seen in dYFP expressing naïve animals, an unexpected increase in preference for the contralateral forepaw use in HA-NURR1 expressing control group was detected. We could speculate that the apparent increase in contralateral forelimb use in the HA-NURR1 group may have been due to a unilateral enhancement of nigrostriatal DA transmission associated with HA-NURR1 expression. The analysis of additional DA neuronal phenotypic markers such as DAT and VMAT, and quantification of striatal DA content may indicate whether there is a potential HA-NURR1-dependent upregulation in nigrostriatal DA transmission under physiological conditions. However, given that the group sizes are small, it is possible that sampling variation may have attributed to an apparent HA-NURR1-mediated physiological effect.

A rotation rate equal to or greater than 5-6 turns per minute is the widely accepted threshold for drug-induced behaviour deficits that correspond to a level of DA depletion that causes hemi-parkinsonism in unilaterally 6-OHDA-lesioned rats (Decressac, Mattsson, Lundblad, et al., 2012; Kirik et al., 1998). Saline-injected dYFP or HA-NURR1 animals did not exhibit a drug-induced rotation bias at either time point. Given that amphetamine-induced rotation is only detected following 30-50% loss of nigral DA cell bodies (Kirik et al., 1998), and the morphological and neurochemical adaptability of the denervated DA circuitry (discussed in section 4.4.5), a 15% neuronal depletion in the dYFP-injected nigra is unlikely to generate a DA deficit.
4.4.3. HA-NURR1 expression may confer neuroprotection in the SNpc in PD

The wide spectrum of NURR1 target genes range from those that dictate phenotypic identity and functionality of DA neurons, to those that downregulate inflammation in astrocytes and microglia. Its ‘master regulator’ status and functional relevance in both neurons and astrocytes, renders NURR1 an ideal therapeutic molecule in PD.

Nurr1 is critical for DA neuronal development and differentiation, and its persistent expression in the postnatal and adult brain maintains DA neuronal phenotype (Hou, Cohen, & Mytilineou, 1997; Kadkhodaei et al., 2009; Le et al., 1999; Zetterström et al., 1997; Zetterström, Williams, et al., 1996). NURR1 mutations and polymorphisms are associated with rare cases of familial PD, and its expression is downregulated in neurons with α-synuclein inclusions (Zheng et al., 2003). Conditional neuronal knockdown of NURR1 compromises phenotypic integrity of the nigrostriatal DA circuitry and enhances susceptibility to neurotoxins and α-synuclein pathology (Decressac, Kadkhodaei, et al., 2012). Conversely, AAV-mediated NURR1 overexpression in neurons promoted survival and ameliorated motor deficits in an α-synuclein-induced model of PD. Moreover, Saijo et al. recently characterised a role of glial NURR1 in downregulating expression of inflammatory genes (Saijo et al., 2009). Lentiviral-mediated Nurr1 knockdown predominantly in microglia and astrocytes in the SN increased the expression of inflammatory mediators such as TNFα, IL-1β, and iNOS, and significantly exaggerated DA neuronal loss in response to LPS-induced inflammation. Surviving TH neurons exhibited pathological morphology with altered size, shape and decreased processes. Furthermore, glial NURR1 knockdown coupled with mutant α-synuclein overexpression also resulted in a dramatic increase in inflammatory gene expression, and DA neuronal loss. These studies prompted us to firstly determine whether predominantly astrocyte-specific HA-NURR1 expression may confer substantial neuroprotection in a 6-OHDA-induced PD model, prior to investigating the efficacy of dual NURR1 expression in both neurons and astrocytes in a subsequent study.

In PD animals, HA-NURR1 expression resulted in a greater preservation of TH-positive DA neurons with a mean neuronal survival of 53.74% in the lesioned nigra, in comparison to 38.90% and 44.38% surviving neurons in the empty- and dYFP-treated control groups, respectively. Although, it was not possible to attribute neurotoxicity observed in the dYFP control animals to either the viral capsids and/ or dYFP protein, given that dYFP vector-
injected PD animals did not exhibit neuropathology that was significantly worse than the empty vector-injected PD animals (discussed in section 4.4.8), the empty and dYFP groups were merged to strengthen statistical power. Merging the empty and dYFP animals into a single control group resulted in a significantly higher neuronal survival in the HA-NURR1 therapeutic group (p = 0.036). Although our results suggest that predominant targeting of NURR1 expression to astrocytes in the SNpc may exert a neuroprotective effect in a 6-OHDA-induced PD model, due to differences in viral vector species or serotypes, vector titres, and animal models, no direct comparisons between the above studies and our results can be made. Further characterisation of phenotypic integrity of surviving DA neurons including quantification of striatal DA content, and analysis of additional DA neuronal markers such VMAT2 and DAT may more accurately define the therapeutic efficacy of astrocyte-targeted NURR1 expression. Furthermore, quantification of NURR1 protein and mRNA expression may allow the quantification of a potential dose-dependent therapeutic efficacy.

Moreover, DA dysfunction and axonal degeneration that appear to precede death of neuronal cell bodies in the SNpc is a characteristic pathological feature in PD, and therefore it is essential that therapeutic molecules exert a neuroprotective effect on both neuronal cell bodies and their axons (Cheng et al., 2010). A recent study by Decressac et al. demonstrated that viral vector-mediated expression of GDNF in the nigra or striatum, or both sites failed to protect nigral DA neurons or their striatal terminals in a rat α-synuclein PD model (Decressac et al., 2011), suggesting that axonal degeneration and deficits in axonal transport may account for the failure of GDNF therapy in PD patients (Bartus et al., 2011; Lang et al., 2006; Nutt et al., 2003). The subsequent AAV6-mediated overexpression of NURR1 selectively in nigral neurons promoted significant phenotypic preservation and survival of the nigrostriatal pathway, as evident by restoration of retrograde transport of both endogenous and exogenous GDNF, and amelioration of motor deficits (Decressac, Kadkhodaei, et al., 2012). The use of a neuronal retrograde tracer such as Fluoro-Gold in future studies to characterise phenotypic integrity of nigral neuronal fibres extending to the striatum may reveal whether astrocyte-targeted HA-NURR1 confers a potential axonal-protective effect in the lesioned nigrostriatal pathway. Furthermore, our study compliments findings by Decressac et al. and support dual targeting of NURR1 to both neurons and astrocytes to target phenotypic dysfunction and degeneration of DA neurons, and chronic glia-driven inflammation that are cardinal pathological mechanisms in PD.
4.4.4. HA-NURR1 expression may downregulate nigral inflammation in PD

In the transection-denervated or chronic seizure-affected hippocampus, the morphology and domain organisation of reactive astrocytes were altered in a manner dependent on the severity and duration of pathology (Oberheim et al., 2008; Wilhelmsson et al., 2006). In moderate reactive astrogliosis, astrocytes remain within their unique domains while increasing the thickness of their main cellular processes, whereas in the context of chronic pathology, reactive astrocytes lost the domain organisation, and overlap between neighbouring astrocyte processes increased by 10-fold, suggesting that the severity of structural alterations may reflect the severity of astrocyte reactivity. An upregulation of GFAP expression, and increase in the number of GFAP-positive processes characteristic of reactive astrogliosis were seen in PD animals in all three vector groups. Distinctive hypertrophy of main astrocytic processes relative to the contralateral nigra was evident in the empty and dYFP PD animals. In the HA-NURR1 expressing lesioned nigra, these morphological adaptations appeared less severe, which may reflect HA-NURR1-mediated downregulation of pathological reactive astrogliosis that contribute to chronic inflammation and neurodegeneration. Saijo et al. demonstrated that glial NURR1 appears to downregulate inflammation by inhibiting the activity of the inflammatory transcription complex NF-κB (Saijo et al., 2009). Analysis of expression of NF-κB-dependent cytokines such as TNFα, IL-1β, and IL-6 by quantifying inflammatory mRNA transcripts and proteins in nigral lysates may more accurately confirm an anti-inflammatory effect of HA-NURR1.

Persistent microgliosis and astrogliosis up to two weeks post-6-OHDA have been previously reported (Walsh, Finn, & Dowd, 2011); however, characterisation of the long-term temporal relationship between neurodegeneration and neuroinflammation in PD models is lacking. At eight weeks post-lesion, while GFAP-immunoreactivity indicated reactive astrogliosis, reactive microgliosis was not evident based on Iba1 immunoreactivity. It is possible that at this time-point, microgliosis may have resolved. However, mRNA and protein quantification of markers upregulated in reactive gliosis and inflammatory mediators that coordinate astrocyte-microglia communication in the lesioned nigra may more accurately reveal the extent of inflammation, and neuroprotective efficacy of astrocyte-targeted expression of HA-NURR1.

As described in section 4.4.3, NURR1 plays a critical role in regulating GDNF signalling in nigral DA neurons (Decressac, Kadkhodaei, et al., 2012). Infusion of the glia-selective
inflammogen LPS that mediate non-cell autonomous neurodegeneration in the nigra induced GDNF expression in reactive astrocytes that was detectible at the protein level (Iravani et al., 2012). In contrast, 6-OHDA and MPTP that mediate direct neurotoxicity via oxidative stress and mitochondrial dysfunction, although induced reactive astrogliosis, failed to upregulate GDNF expression (Inoue et al., 1999; Song et al., 2009). Indeed, a marked reduction in GDNF immunoreactivity following 6-OHDA treatment has been reported (Anastasia, de Erausquin, Wojnacki, & Masco, 2007). These results indicate that insult-specific mechanisms of neurodegeneration may dictate the inflammatory profile of reactive astrocytes, and therefore it is essential to test potential therapeutic strategies in multiple preclinical models to accurately determine efficacy. In our study, IHC failed to reveal GDNF expression in the nigra, suggesting that the analysis of endogenous molecules may require quantitative PCR to detect changes in expression at the mRNA level.

4.4.5. HA-NURR1 expression may ameliorate motor function in PD

PD animals in all three vector groups had a decline in contralateral forepaw use at four weeks, indicating 6-OHDA-induced motor impairment. At eight weeks, while forepaw bias remained stable in the empty vector group, an improvement in the contralateral forepaw use was seen in the dYFP group, emphasising the role of time-dependent morphological and neurochemical plasticity of the nigrostriatal DA pathway in partial restoration of function. Compensatory mechanisms that include enhanced TH expression, and biosynthesis and release of DA from surviving nerve terminals, and postsynaptic DA receptor supersensitivity, coupled with lesion-associated loss of DA reuptake sites may maintain DA neurotransmission to a certain extent (Altar, Marien, & Marshall, 1987; Blanchard et al., 1995; Narang & Wamsley, 1995; Ungerstedt, 1971; Zigmond, Acheson, Stachowiak, & Strickerm, 1984). Furthermore, intracerebral microdialysis 3-4 weeks post-6-OHDA lesions has indicated significantly higher striatal extracellular DA levels in comparison to that measured 4 days post-lesion (Robinson, Mocsary, Camp, & Whishaw, 1994; Robinson & Whishaw, 1988), suggesting that compensatory DA mechanisms post-nigrostriatal denervation are a progressive phenomenon, and hence must be taken into account when interpreting time-dependent recovery of function in experimental groups (Wolterink, Van Zanten, Kamsteeg, Radhakishun, & Van Ree, 1990). At eight weeks post-6-OHDA lesion, HA-NURR1-expressing animals recovered contralateral forepaw use to baseline levels, indicating that
astrocyte-targeted HA-NURR1 expression may rescue and maintain DA phenotypic integrity resulting in elevated neurotransmission and functional improvement.

At both time-points, all PD animals had an increase of over 5 turns per minute in ipsilateral rotations as previously reported by Kirik et al, indicating unilateral deficits in striatal DA (Kirik et al., 1998). However, there were no significant differences between vector treatments at either time point. In all vector groups, a subtle decrease in rotation bias with considerable variability was seen at eight weeks, similar to that noted by Kirik et al. at 6-weeks in an identical 6-OHDA terminal lesion model, implicating a role of time-dependent plasticity of the nigrostriatal pathway in partial restoration of function (Kirik et al., 1998).

Furthermore, there was no correlation between nigral neuronal loss and severity of behaviour deficits (data not shown). Striatal TH-immunoreactivity and 6-OHDA lesion size appeared highly variable amongst the PD animals in all vector groups, and did not seem to correlate with nigral DA neuronal survival or behaviour scores. However, quantification of striatal TH density may have more accurately confirmed potential vector treatment-dependent differences in striatal TH activity. In both 6-OHDA and α-synuclein models of PD, equally high rates of rotation (4-10 turns per minute) were observed over a wide range of nigral neuronal loss and striatal denervation, and not all animals with significant nigrostriatal degeneration exhibit impairment in behaviour paradigms (Decressac, Mattsson, Lundblad, et al., 2012; Decressac, Mattsson, & Björklund, 2012; Kirik et al., 1998), suggesting that the severity of behaviour deficits may not necessarily correspond to the lesion severity.

### 4.4.6. Route of drug administration may affect amphetamine-induced rotation

Additionally, the route of drug administration may be an additional confounding factor in rotation bias. Although rotation rates ranged from 7-10 turns per minute, similar to that reported by Kirik et al following a 2.5mg/kg dose of intraperitoneally administered amphetamine (Kirik et al., 1998), it is possible that the same dose administered subcutaneously may have resulted in suboptimal CNS bioavailability of the drug, leading to a less than maximal stimulation of DA transmission. The drug dose and route of administration were based on published work on the 6-OHDA rat model (Decressac, Mattsson, & Björklund, 2012; Hudson et al., 1993; Kirik et al., 1998; Sahin et al., 2014) and a trial experiment in which 2.5mg/kg of amphetamine was administered subcutaneously to three randomly
selected 6-OHDA-lesioned animals to determine whether this dose resulted in a net rotation of at least 5-6 turns per minute. A subcutaneous route was preferred for the ease of drug administration with greater accuracy than an intraperitoneal route. However, an unequivocal DA imbalance between the two hemispheres may not be produced if drug-induced DA increase in the intact striatum was not substantially higher than that produced by the surviving dopamine terminals in the lesioned striatum, which may result in similar levels of ipsilateral rotation bias in all PD vector groups regardless of any treatment-related differences in neuronal terminal preservation.

4.4.7. Striatal heterogeneity may influence 6-OHDA-induced PD phenotype

Moreover, given the functional heterogeneity of the striatum, and subregion specific regulation of different aspects of behaviour, functional deficits induced by intrastriatal 6-OHDA lesions depend not only on the toxin dose, but also the site of injection. Although functional overlap may exist between subregions, lesions predominantly affecting the dorsomedial striatum appear to have broad effects on locomotion and drug-induced rotation, whereas lesions in the ventrolateral striatum have pronounced effects on movement initiation, sensorimotor orientation and skilled motor behaviour (Carli, Evenden, & Robbins, 1985; Cousins, Sokolowski, & Salamone, 1993; Dunnett & Iversen, 1982; Sabol, Neill, Wages, Church, & Justice, 1985). With respect to amphetamine-induced rotation, various studies indicate that the amplitude of ipsilateral rotation is influenced by the extent of dorsoventral striatal denervation (Brundin, Strecker, Londos, & Björklund, 1987; Kelly & Roberts, 1983; Koob, Stinus, & Moal, 1981). Categorising striatal 6-OHDA lesions into exclusively dorsal versus dorsal and ventral was not implemented given the small group sizes, lack of distinct histological and immunohistochemical borders and wide variation in the spread of the lesion. Therefore, incorporating larger group sizes to minimise variation associated with stereotaxic targeting of multiple brain regions and inter-animal variability, and multiple tests that span akinesia (stepping test), drug-induced rotation, forelimb asymmetry, and sensorimotor orientation may allow a more comprehensive evaluation of therapeutic efficacy of HA-NURR1 overexpression in future studies.
4.4.8. Short-term dYFP accumulation may induce beneficial reactive astrogliosis

Eleven weeks post vector infusion, neurotoxicity and glial inflammation were evident in dYFP-expressing naïve animals. Based on these results, a cumulative toxic effect of dYFP and 6-OHDA was expected in the PD animals. Surprisingly, a slightly greater DA neuronal survival, and less severe loss of TH-immunoreactivity in neuronal cell bodies and fibres were seen in the dYFP expressing SNpc in comparison to the empty vector-treated SNpc. Given the small group sizes, although it is likely that variability contributed to these results, an alternative hypothesis may suggest that dYFP expression may confer an apparent neuroprotective effect in the context of pathology. As discussed in section 1.3, the phenotypic evolution of astrocytes involves a spectrum of molecular, cellular and functional changes in a context-dependent manner. These cellular adaptations may determine the overall functional status of reactive astrocytes that range from predominantly neuroprotective in mild inflammation to detrimental in severe neurodegeneration. For example, Saura et al. previously demonstrated that selective reactive astrogliosis (in the absence of microgliosis) in the SNpc induced by IL-1β five days prior to striatal 6-OHDA infusion conferred a significant neuroprotective effect, and improved motor deficits in the PD model (Saura et al., 2003), suggesting that the induction of beneficial reactive astrogliosis could potentially be protective against an imminent insult.

Although, no direct comparisons with other studies can be made, our results suggest that dYFP accumulation at three weeks may induce a state of mild astrocyte reactivity, characterised by enhanced expression of neuroprotective functions that may attenuate 6-OHDA-induced oxidative stress. These ‘pre-conditioned’ astrocytes may shield the SNpc against 6-OHDA-induced oxidative stress at this time point. 6-OHDA-induced oxidative stress is a key pathological mechanism in PD (Tabner, Turnbull, El-Agnaf, & Allsop, 2002). This model is characterised an acute retrograde degeneration of nigral neurons over 1-2 weeks post-toxin injection, and long-term progressive neurodegeneration (Kirik et al., 1998; Rosenblad, Kirik, & Bjorklund, 2000). Attenuating its deleterious effects in the acute phase may ameliorate the intensity of the progressive neurodegeneration, which may lead to an apparent protective effect of dYFP in comparison to the empty vector. However, to determine that this is a genuine dYFP-related protective effect, larger group sizes and the inclusion of appropriate empty vector and 1 x PBS control groups are required, and neuronal survival and astrocyte reactivity characterised three weeks post-vector infusion prior to 6-OHDA injection. Furthermore, long term dYFP accumulation in both neurons and astrocytes
may ultimately compromise cellular physiology and induce cell stress and apoptosis as seen in the naïve dYFP expressing SNpc. Stereological quantification of GFAP-positive astrocytes in the naïve SNpc transduced with AAV5-GFAP-dYFP or AAV5-GFAP-pL is required to determine whether in addition to neuronal death, dYFP accumulation leads to astrocytic death, or severe astrogliosis including astrocyte proliferation.

4.4.9. Investigating the therapeutic efficacy of astrocyte-targeted NURR1 expression in a genetic PD model

The ability to generate effective animal models of human neurodegenerative diseases is critical for elucidating pathological mechanisms and developing therapeutic strategies. Precisely replicating a progressive and age-dependent pathophysiology with early presymptomatic and late symptomatic stages of the disease remains a crucial challenge. To date, the well-established 6-OHDA rat model, and mouse and non-human primate MPTP models have been extensively used and have allowed us to gain valuable insight into the midbrain DA neuronal physiology and pathological processes that lead to cell degeneration.

6-OHDA induces nigrostriatal dopaminergic neuronal death via the generation of reactive oxygen species and mitochondrial damage (Choi et al., 1999). Depending on the dose and site of injection, striatal 6-OHDA mediates an acute retrograde degeneration of nigral neurons over 1-2 weeks post-toxin injection, and establishes long-term progressive neurodegeneration (Kirik et al., 1998; Rosenblad et al., 2000). However, this model fails to replicate the wide spectrum of pathological mechanisms in human PD that involve α-synuclein aggregation. In contrast, in α-synuclein overexpression models, the accumulation of α-synuclein is associated with inflammation, impaired proteasomal and lysosomal pathways, and deficits in intracellular trafficking and synaptic function characteristic of human PD. Decressac et al. demonstrated that AAV6-mediated overexpression of wild-type α-synuclein exhibited different temporal and neuropathological characteristics to the striatal 6-OHDA model. While the levels of nigral DA neuronal loss were comparable between the two models at 8 weeks post-manipulation, behaviour analysis at 3 and 8 weeks post-manipulation indicated that the threshold for significant behaviour deficits was reached with approximately 15-20% more surviving DA neurons and terminals in the α-synuclein model compared to the 6-OHDA model. In the α-synuclein overexpression model, severe axonal
degeneration, characterised by dystrophic axons and axonal aggregation of α-synuclein, preceded neuronal death (Decressac, Mattsson, Lundblad, et al., 2012; Lundblad, Decressac, Mattsson, & Bjorklund, 2012), reminiscent of disease progression in human PD. Axonal dysfunction was coupled with deficits in synaptic transmission as evident by decreased release and re-uptake of striatal DA as measured via in vivo amperometry, and downregulation of enzymes and transporters involved in DA neurotransmission, including TH AADC, VMAT2, and DAT. Therefore, the therapeutic efficacy of astrocyte-specific, and subsequent neuronal and astrocyte-targeted NURR1 overexpression may be more accurately tested in the α-synuclein model that replicates a wide spectrum of the pathological processes of human PD. However, given that no single model to date efficiently replicates the multifaceted pathogenesis of PD, evaluation of potential therapeutics in multiple neurotoxin and genetic models that may collectively reproduce disease pathological features is required.

The results presented in this chapter suggest that overexpression of the therapeutic factor NURR1 predominantly in astrocytes exhibit neuroprotective potential in PD, and support the development of AAV vectors in subsequent studies for the dual targeting of nigral neurons and astrocytes.
Chapter 5. Conclusions and Summary
Although the aetiology and exact molecular mechanisms of Parkinson’s disease are not fully illuminated, deficits in cellular protein processing, oxidative stress, mitochondrial dysfunction, excitotoxicity, and inflammation orchestrated by interacting neurons and glia appear to be key pathological mechanisms that drive the progressive demise of DA neurons. The early symptomatic phase of the disease manifests following the loss of approximately 30-50% of nigral neurons and 60-70% striatal terminals (Cheng et al., 2010). Currently available pharmacological and surgical therapies for PD provide only symptomatic relief and do not alter disease pathogenesis. Furthermore, many of these therapeutic strategies are associated with debilitating side-effects, and the efficacy of pharmacological agents is largely restricted to the early stages of the disease. Given the prevalence of a substantial number of surviving DA neurons at the time of diagnosis, the degenerating nigrostriatal circuitry may favourably respond to genetic manipulation strategies aimed at restoring phenotypic integrity of dysfunctional neurons and glia, which may potentially ameliorate disease progression.

Although its modest packaging capacity restricts the size of the vector genome to less than 5kb, AAV vectors that mediate robust, long-term CNS transgene expression in the absence of immunogenicity and pathogenicity are appealing gene delivery vehicles for various neurological gene therapy applications. The exclusively neurotropic AAV serotype 2 was the first AAV serotype to be isolated and extensively characterised, and the only serotype currently approved for CNS clinical trials. Although AAV2 vector-mediated therapeutic gene expression confers significant neuroprotection in experimental PD models, only modest therapeutic efficacy has been achieved in the clinical setting (Feigin et al., 2007; Kaplitt et al., 2007; Mittermeyer et al., 2012). A greater understanding of the dynamic molecular mechanisms that govern PD pathology, and the characterisation of novel AAV serotypes with unique transduction properties are advancing the development of significantly more efficient AAV vector systems for CNS gene delivery.

It is increasingly evident that dynamic interactions between neurons and glia regulate physiological processes such as synaptic activity and plasticity, and in the context of unresolved neuropathology, maladaptive phenotypic evolution of these cellular elements may contribute to neurodegeneration. Therefore, contrary to the traditional neurocentric therapeutic approach, the ability to additionally target astrocytes may prove clinically advantageous. Prior to investigating the efficacy of this dual cell targeting approach in PD, in this thesis we aimed to determine whether astrocyte-specific therapeutic manipulation confers
substantial neuroprotection in disease by restricting therapeutic transgene expression predominantly to astrocytes.

The first objective of this thesis was to develop an AAV vector that targets nigral astrocytes with high efficiency by coupling astrocyte-tropic AAV serotypes and DNA promoter elements. The characterisation of additional AAV serotypes that exhibit novel tropisms and superior transduction efficacies than AAV2 is rapidly expanding the repertoire of AAV vectors available for clinical application (Broekman et al., 2006; Markakis et al., 2010; Taymans et al., 2007; Watakabe et al., 2015). In the CNS, alternative vector serotypes such as AAV5, 8 and 9 that exhibit significantly greater transduction efficacy and tropic range than AAV2 in various regions are emerging as strong candidates for the clinical setting. At the commencement of this thesis, studies had shown that the incorporation of the widely used astrocyte-tropic 2.2kb GFAP promoter into the vector genome significantly enhanced astrocyte-tropism of AAV vectors derived from these serotypes in the rodent and non-human primate striatum and hippocampus (Lawlor et al., 2009; Markakis et al., 2010). However, astrocyte transduction efficacy of these vectors in the SNpc remained to be characterised.

It is increasingly evident that astrocyte exhibit extensive molecular and functional diversity, and GFAP, classically considered to be a pan-astrocytic marker is indeed heterogeneously expressed (Cahoy et al., 2008; Lee et al., 2008; Walz & Lang, 1998; Yang et al., 2011). ALDH1L1 is a recently characterised astrocyte-specific marker that appears to be more homogenously and extensively expressed than GFAP (Cahoy et al., 2008). The full-length genomic ALDH1L1 promoter has been shown to replicate the astrocyte-specific expression patterns of the endogenous ALDH1L1 gene in BAC transgenic mice, under both physiological and pathological conditions. In Chapter one, we isolated four putative ALDH1L1 promoter sequences that could potentially transcriptionally regulate AAV vector-delivered transgenic expression cassettes in nigral astrocytes. A short (S) 931bp or long (L) 1974bp 5' region immediately upstream of the transcription start site were cloned into an AAV expression plasmid to generate ALDH1L1(S) and ALDH1L1(L) promoter constructs respectively. To determine the influence of exon 1 that may contain enhancer sequences (Oleinik et al., 2011), two additional constructs were generated by cloning a 138bp sequence spanning exon 1 (ex1) of the ALDH1L1 gene into the above promoter constructs to generate ALDH1L1(S)ex1 and ALDH1L1(L)ex1. Contrary to the predicted astrocyte-specific transgene expression, these promoters in the context of AAV5, 8, and 9 exclusively targeted transgene expression to neurons in the SNpc. The neuronal transduction efficacy of AAV9
was considerably higher than that of AAV5 and AAV8, and promoter length positively influenced transgene expression. It is likely that the exclusion of specific DNA motifs that dictate cell-specific ALDH1L1 expression may have completely modified cell-tropism of the promoters derived from this gene. Deletion analysis of the ALDH1L1 promoter region may allow the identification of such regulatory motifs, and facilitate the development of astrocyte-specific promoters. The sequence length- and AAV serotype-dependent transcriptional activity of these novel neuronal-tropic ALDH1L1 promoter variants may allow the ability to regulate the expression of therapeutic genes based on their potency and the therapeutic requirements of SNpc neurons in future studies.

At the commencement of this thesis, the efficacy of nigral astrocyte transduction by the widely used GFAP promoter in the context of astrocyte-tropic AAV serotypes had not been characterised. Therefore, in Chapter 3, a GFAP promoter regulated dYFP expression cassette was coupled with AAV5 and AAV9, and the resulting vectors were tested in the rat SNpc to determine efficacy of nigral astrocyte transduction. While the AAV5 vector mediated predominantly astrocyte transduction that was relatively confined to the SNpc, AAV9 transduced a greater region within the midbrain, and targeted both neurons and astrocytes. Given that astrocyte-specific transduction was desired, with minimal inappropriate vector diffusion into neighbouring midbrain regions, AAV5 was selected for the nigral delivery of the transcription factors NURR1 and Nrf2 that exert anti-inflammatory and antioxidant effects in astrocytes, respectively. The 2.2kb GFAP promoter occupies almost half of the ~4.7kb packaging capacity of AAV vectors, and hence imposes a significant size restriction on the therapeutic gene in the expression cassette. Therefore, we additionally tested the ability of the constitutively active CMV promoter (578bp), and a recently characterised shorter GFAP promoter variant, gfaABC1D (681bp) to regulate AAV5 tropism and transgene expression in vivo. These AAV5 vector constructs were infused into the SNpc at a titre ten-fold higher than that used in the initial AAV5 and AAV9 comparison. The GFAP promoter exhibited the most efficient transcriptional activity in the SNpc and targeted both neurons and astrocytes at the higher titre. While efficient NURR1 expression was achieved with the GFAP promoter, Nrf2 expression appeared inefficient. The levels of protein expression may represent the different rates of intracellular degradation of these molecules.

Therefore, the AAV5-GFAP-HA-NURR1 therapeutic vector was selected for further characterisation in a PD model in Chapter four. An in vitro scratch assay confirmed that the GFAP promoter activity parallels the expression of the endogenous GFAP gene, and is
regulated by transcription factors upregulated in pathology. Moreover, in a co-transfection assay, the NURR1 transgenic protein transactivated a target TH promoter containing a NURR1 recognition motif confirming functionality of the therapeutic protein. In a transrepression assay that quantified the NURR1-dependent downregulation of NF-κB activity, NURR1 exerted no effect on LPS-induced NF-κB nuclear localisation, suggesting that NF-κB nuclear localisation may not necessarily correlate with the extent of NURR1-dependent anti-inflammatory mechanisms, as NF-κB dissociated from pro-inflammatory genes may remain in the nucleus. Quantification of NF-κB-regulated pro-inflammatory mediators such as TNFα, IL-6 and IL-1β and ROS may provide a more accurate indication of NURR1 anti-inflammatory efficacy.

Furthermore, the increased neuronal targeting by AAV5 at high titre prompted us investigate a previously described miRNA-based neuronal ‘detargeting’ mechanism to silence vector-mediated transgene expression in nigral neurons. This strategy resulted in a significant downregulation of neuronal transgene expression, and enhanced astrocyte-specificity of AAV5. Our results support the application of this technology in studies that aim to elucidate astrocyte-specific molecular mechanisms that govern their functional significance; therapeutically target astrocyte-specific defective pathways in pathology; and selectively express bio-active molecules in astrocytes to minimise potential detrimental effects associated with neuronal delivery of transgenic proteins to off-target projection regions is advantageous.

In Chapter four, AAV5-mediated expression of NURR1 in the SNpc three weeks prior to a striatal 6-OHDA lesion was shown to preserve TH-immunoreactive DA nigral neurons. Astrocyte reactivity in the therapeutic protein expressing PD animals was lower than that in the control groups, indicative of downregulation of inflammation. Quantification of pro-inflammatory mRNA transcripts and proteins in nigral lysates may more accurately confirm a neuroprotective effect of HA-NURR1. Amelioration of forepaw deficits in the cylinder test eight weeks post-lesion indicated that NURR1 expression may promote DA transmission in the nigrostriatal pathway. Further characterisation of phenotypic integrity of surviving DA neurons and terminals including quantification of striatal DA content, and analysis of additional DA neuronal markers such as VMAT2 and DAT may more accurately define the therapeutic efficacy of NURR1.
DA neuronal loss and reactive astrogliosis in saline-injected control animals expressing AAV5-mediated dYFP in the nigra indicated potential toxicity associated with dYFP expression or AAV5 capsids. The inclusion of empty vector and vehicle (1 x PBS) controls to determine potential AAV5 capsid-related toxicity and stereotaxic injection-related tissue injury, respectively, may have allowed a more accurate interpretation of these results. Furthermore, the use of larger group sizes to strengthen statistical power, and multiple behaviour paradigms that span akinesia (stepping test), drug-induced rotation, forelimb asymmetry, and sensorimotor orientation may allow a more comprehensive evaluation of the therapeutic efficacy of nigral NURR1 overexpression in future studies.

Nrf2 that was only characterised at the protein level in Chapter three is a ‘master regulator’ of over 200 cytoprotective genes that regulates the antioxidant capacity of astrocytes. Its potent antioxidant potential and downregulation in PD and other neurodegenerative diseases that may contribute to oxidative stress, renders Nrf2 an ideal therapeutic candidate. Although FLAG-Nrf2 was undetectable at the protein level in vivo, it is possible that a therapeutically relevant amount of FLAG-Nrf2 may still be expressed. Quantification of FLAG-Nrf2 mRNA transcripts may have confirmed transcriptionally activity of the expression cassette in vivo. Furthermore, quantification of its downstream targets such as hemeoxygenase-1 (HO-1) and the glutathione system in response to 6-OHDA may have provided a more accurate measure of its functionality. Amplified ROS-detoxification following aberrant upregulation of Nrf2 expression that subsequently modified the intracellular redox state has been shown to promote oncoregenesis in vitro (DeNicola et al., 2011), suggesting that therapeutic overexpression of this gene may require stringent regulation.

The wide spectrum of NURR1 target genes range from those that dictate phenotypic identity and functionality of DA neurons, to those that downregulate inflammation in astrocytes and microglia. Its ‘master regulator’ status and functional relevance in both neurons and astrocytes, renders NURR1 an ideal therapeutic molecule in PD, a multifaceted neurodegenerative disease. The results from chapter four suggested that overexpression of NURR1 predominantly in astrocytes exhibits neuroprotective potential in PD. Furthermore, these findings complement a recent study that demonstrated that AAV6-mediated NURR1 expression selectively in nigral neurons significantly promoted the survival and function of the nigrostriatal pathway, and ameliorated motor deficits in an α-synuclein PD model. Collectively, these results support the development of AAV vectors for the dual targeting of NURR1 expression to both neurons and astrocytes in the SNpc. AAV9 that targeted both
nigral neurons and astrocyte with higher efficiency than AAV5 can potentially be utilised in these subsequent studies. Moreover, the lack of astrocyte-specificity of the GFAP promoter that additionally targets transgene expression to neurons may be advantageous in this application.

Various AAV-mediated therapeutic strategies that conferred significant neuroprotection and functional recovery in animal models of PD have thus far achieved only modest therapeutic efficacy in the clinical setting (Feigin et al., 2007; Kaplitt et al., 2007; Mittermeyer et al., 2012). The commonly used 6-OHDA- and MPTP-induced PD models that mainly replicate oxidative stress and mitochondrial dysfunction of PD pathogenesis have allowed us to gain valuable insight into the physiological and pathological mechanisms of the nigrostriatal pathway, and investigate potential therapeutics (Burte et al., 2011; Piao et al., 2012; Pileblad et al., 1989; Wüllner et al., 1996). However, the inability to effectively replicate intracellular α-synuclein aggregation and the multifaceted pathological mechanisms characteristic of clinical PD is a significant limitation of these models that may lead to inadvertent exaggeration of efficacy of potential therapeutic strategies. A recent study by Decressac et al. demonstrated that AAV-mediated α-synuclein overexpression that replicated a range of α-synuclein-associated pathology including axonal dysfunction, inflammation, impaired protein degradation, and deficits in intracellular trafficking may represent a more accurate PD model (Decressac, Mattsson, & Björklund, 2012). However, given that no individual model replicates the full spectrum of PD pathology, it may be necessary to investigate efficacy of potential therapeutic strategies in multiple pre-clinical models.

In conclusion, we have generated novel ALDH1L1 promoters that exclusively and efficiently transduce neurons in the SNpc and hippocampus. The potential applicability of these promoters in neuronal-targeted gene therapy warrants further investigation. We also showed that the AAV5 vector serotype coupled with the GFAP promoter efficiently targets astrocytes in the SNpc. The anti-inflammatory and neuroprotective efficacy achieved with predominantly astrocyte-specific overexpression of NURR1 in an animal model of PD suggests that astrocytes are an effective therapeutic target. Furthermore, our results support the development of dual cell targeting AAV vectors to exploit the ‘master regulator’ status of NURR1 to target DA phenotypic dysfunction, chronic inflammation and neurodegeneration in PD.


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