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AAV-vector Mediated Gene Delivery for Huntington’s Disease: An Investigative Therapeutic Study

Adrian P. Kells

Abstract

Progressive degeneration in the central nervous system (CNS) of Huntington’s disease (HD) patients is a relentless debilitating process, resulting from the inheritance of a single gene mutation. With limited knowledge of the underlying pathological molecular mechanisms, pharmaceutical intervention has to-date not provided any effective clinical treatment strategies to attenuate or compensate the neuronal cell death. Attention has therefore turned to biotherapeutic molecules and novel treatment approaches to promote restoration and protection of selectively vulnerable populations of neurons in the HD brain. Rapid advances in vectorology and gene-based medicine over the past decade have opened the way for safe and efficient delivery of biotherapeutics to the CNS. With numerous factors known to regulate the development, plasticity and maintenance of the mammalian nervous system many proteins have emerged as potential therapeutic agents to alleviate HD progression. This investigative study utilised gene delivery vectors derived from the non-pathogenic adeno-associated virus (AAV) to direct high-level expression of brain-derived neurotrophic factor (BDNF), glial cell-line derived neurotrophic factor (GDNF), Bcl-xL or X-linked inhibitor of apoptosis protein (XIAP) within the rodent striatum. Maintenance of the basal ganglia and functional behaviour deficits were assessed following excitotoxic insult of the striatum by quinolinic acid (QA), a neurotoxic model of HD pathology.

Enhanced striatal expression of BDNF prior to QA-induced lesioning provided maintenance of the striosome-matrix organisation of the striatum, attenuating impairments of sensorimotor behaviour with a 36-38% increase in the maintenance of DARPP-32 / krox-24 expressing striatal neurons, reduced striatal atrophy and increased maintenance of striatonigral projections. Higher levels of BDNF however induced seizures and weight-loss highlighting the need to provide regulatable control over biotherapeutic protein expression. Continuous high-expression of BDNF or GDNF resulted in a downregulation of intracellular signal mediating proteins including DARPP-32, with AAV-GDNF not found to enhance the overall maintenance of striatal neurons. Neither of the anti-apoptotic factors provided significant protection of transduced striatal neurons but tended towards ameliorating QA-induced behavioural deficits, displaying behaviour – pathology correlations with the survival of parvalbumin-expressing neurons in the globus pallidus. The results of this thesis suggest BDNF as a promising putative biotherapeutic for HD, but emphasises the requirement to control expression following gene delivery, and for further elucidation of the physiological impact that enhanced expression of endogenous factors has on the host cells. Additionally the maintenance of neural networks beyond the caudate-putamen will be vital to ensuring efficient clinical outcomes for HD.
Acknowledgements

I wish to specifically convey my gratitude to a number of people who have assisted either directly or indirectly over the past four years to ensuring the completion of this thesis.

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Journal Publications

Research Articles


Abstracts


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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-NP</td>
<td>3-nitropropionic acid</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2-Azino-di-3-ethylbenzthiazoline sulfonate</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
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<td>ANOVA</td>
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<td>A-P, M-L, D-V</td>
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<tr>
<td>GFRα1</td>
<td>GDNF family receptor α-1</td>
</tr>
<tr>
<td>GPe</td>
<td>Globus pallidus external segment</td>
</tr>
<tr>
<td>GPi</td>
<td>Globus pallidus internal segment</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HD</td>
<td>Huntington’s disease</td>
</tr>
<tr>
<td>Hdh</td>
<td>Huntingtin gene</td>
</tr>
</tbody>
</table>
HEK293 Human embryonic kidney 293 cells
HIAP Human inhibitor of apoptosis
HSV Herpes simplex virus
HT-1080 Human osteosarcoma cells
IAP Inhibitor of apoptosis protein
IMDM Iscove’s Modified Dulbecco’s Media
ITR Inverted terminal repeats
LB Luria-Bertani broth
Luc Luciferase
LV Lentivirus
MAPK Mitogen-activated protein kinase
N171-82Q Transgenic mice with 171aa N-terminal fragment of Hdh with 82 CAG repeats
NADPHd Nicotinamide adenine diphosphate diaphorase
NAIP Neuronal apoptosis inhibitor protein
NEB New England Biolabs
NeuN Neuronal nuclei
NGF Nerve growth factor
NMDA N-methyl-D-aspartate
NOS Nitric oxide synthase
NR2B NMDA receptor 2B subunit
p75NTR p75 neurotrophin receptor
PBS Phosphate buffered saline
PCR Polymerase chain reaction
Pen Penicillin
PNS Peripheral nervous system
poly-Q poly-glutamine tract
QA Quinolinic acid
R6/2 Transgenic mice with exon 1 of Hdh containing ~150 CAG repeats
rh Recombinant human
RM ANOVA Repeated measures analysis of variance
SNc Substantia nigra pars compacta
SNr Substantia nigra pars reticulata
ssDNA Single-stranded DNA
Strep Streptomycin
STS Staurosporine
TE Tris-EDTA buffer
TH Tyrosine hydroxylase
WPRE Woodchuck hepatitis post-transcriptional regulatory element
XIAP X-linked inhibitor of apoptosis

XVII
Chapter 1

Review of Published Literature

1.1 Huntington’s Disease

Huntington’s disease (HD) is an autosomal dominant genetic disorder that causes relentless neurodegeneration resulting in a progressive decline of physical motor function, cognitive abilities, and psychiatric disturbances first described by George Huntington (1872). Affecting up to 1 in 10,000 people depending on geographical location, physical HD symptom onset is generally between 30 and 50 years of age. While genetic testing is widely available to “at risk” relatives of HD patients, no effective treatment exists to prevent the onset, slow the progression, or alleviate the symptoms of HD. Despite the identification of the causative genetic defect – a trinucleotide cytosine-adenine-guanine (CAG) repeat expansion (>36 repeats) in exon-1 of the *Hdh* gene encoding a 350kDa protein termed huntingtin (Huntington's Disease Collaborative Research Group 1993), it remains elusive how this single gene defect results in the complex but selective pattern of HD neurodegeneration (Borrell-Pages *et al.* 2006). Huntingtin protein is widely expressed within the central nervous system (CNS) with the disease form containing an elongated polyglutamine (poly-Q) tract within the N-terminal domain (Trottier *et al.* 1995). With the lack of a clear understanding as to the molecular mechanisms connecting the selective cell death with the genetic defect, research towards therapeutic treatment has been focused on pharmacological intervention in general cell death processes, restoration of lost brain function by cellular transplantation, and genetic intervention to reduced the susceptibility of affected neurons (for review see (Handley *et al.* 2006)). Neurodegeneration generally occurs via three main mechanisms; metabolic compromise, excitotoxicity, or oxidative stress, acting independently or cooperatively (Alexi *et al.* 1997). Interfering with these processes or increasing the resistance of the neurons using neurotrophic factors has shown encouraging results in improving behavioural function and preventing neurodegeneration in HD models, thus providing hope that effective therapeutic treatment will soon become a reality for HD patients and their families.
1.2 Huntington’s Disease: Neuropathology

HD pathology is evident at a gross anatomical level by severe bilateral atrophy of the striatum and neocortex. Microscopically, neurodegeneration is most pronounced within the striatum where GABAergic projection neurons are selectively lost while interneurons resist degeneration (Reiner et al. 1988; Glass et al. 2000). The striatum is a highly organised structure receiving glutamatergic innervations from all cortical regions in a topographical arrangement (McGeorge and Faull 1989), and sending GABAergic efferent projections to the globus pallidus and substantia nigra, influencing basal ganglia output, thereby making the striatum a vital processing centre coordinating mood and movement (Selemon and Goldman-Rakic 1985; Alexander et al. 1986; Penney and Young 1986; Brown et al. 1997; Smith et al. 1998). Disruption of striatal function, responsible for the characteristic HD choreiform movements, is also likely to be the cause of the non-motor cognitive and emotional symptoms of HD (Penney and Young 1986; Reiner et al. 1988; Mitchell 1990).

Vonsattel established a grading scale of end-point HD pathology based on post-mortem histopathological examination of the caudate nucleus and putamen (Vonsattel et al. 1985). This five-point grading scale provides a semi-quantitative assessment of the extent of neuronal cell loss in the caudate-putamen, and associated atrophy; with up to 30% neuronal loss but no apparent striatal atrophy in Grade 0, through to a 95% depletion of striatal neurons, severe atrophy and extensive gliosis in Grade 4.

Immunohistochemical studies of the striatum have identified a number of distinct populations of neurons distinguishable by various neurochemical markers. The highly vulnerable GABAergic medium spiny projection neurons, comprising up to 95% of all striatal neurons, are divided into two groups by their neuro-modulatory transmitters’ enkephalin and substance P – corresponding with different projection targets (Table 1-1). The vast majority of these GABAergic projection neurons also contain the calcium binding protein calbindin, which is commonly used to visualise the extent of neurodegeneration within post-mortem HD human brains and in animal models of HD. The high expression of dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) by dopaminergic neurons can also be used as a general marker of the medium spiny projection neurons and their terminal projections (Ouimet et al. 1984; Ouimet et al. 1998). In addition to the projection neurons, the striatum also contains a varied population of interneurons of which the majority are also GABAergic and the remaining neurons being large aspiny cholinergic interneurons expressing the neurochemical marker choline acetyltransferase (ChAT). Aspiny GABAergic interneurons are divided into three subpopulations determined by their expression of the neurochemical markers calretinin, parvalbumin, or the co-expression of nicotinamide adenine
diphosphate diaphorase (NADPHd), nitric oxide synthase (NOS), neuropeptide Y and somatostatin (Table 1-1; (Alexi et al. 2000)).

<table>
<thead>
<tr>
<th>Neurons</th>
<th>Transmitter</th>
<th>Neurochemical markers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Medium spiny projection neurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Striatopallidal (GPe)</td>
<td>GABA</td>
<td>Enkephalin, calbindin, DARPP-32</td>
</tr>
<tr>
<td>- Striatopenupenduncular (GPi) and Striatonigral (SNr)</td>
<td>GABA</td>
<td>Substance P, dynorphin, calbindin, DARPP-32</td>
</tr>
<tr>
<td>- Striatonigral (SNc)</td>
<td>GABA</td>
<td>Substance P, dynorphin, DARPP-32</td>
</tr>
<tr>
<td><strong>Aspiny interneurons</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Large aspiny</td>
<td>ACh</td>
<td>ChAT</td>
</tr>
<tr>
<td>- Medium aspiny</td>
<td>GABA</td>
<td>NADPHd, NOS, neuropeptide Y, somatostatin</td>
</tr>
<tr>
<td>- Small aspiny</td>
<td>GABA</td>
<td>Parvalbumin</td>
</tr>
</tbody>
</table>

Table 1-1 Neuronal phenotypes within the striatum

Table modified from (Alexi et al. 2000) Progress in Neurobiology. Abbreviations: GPe, globus pallidus external segment; GPi, globus pallidus internal segment; SNc, substantia nigra pars compacta; GABA, \(\gamma\)-aminobutyric acid; ACh, acetylcholine; ChAT choline acetyltransferase; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of 32 kDa; NADPHd, nicotinamide adenine diphosphate diaphorase; NOS nitric oxide synthase.

HD involves a complex pattern of striatal degeneration in which there is not uniform degeneration throughout the striatum, but a “wave of degeneration” spreading laterally and ventrally through the caudate nucleus, putamen and nucleus accumbens in succession (Vonsattel et al. 1985). Enkephalin positive GABAergic medium spiny projection neurons projecting to the external globus pallidus are usually the first to degenerate correlating with the appearance of choreiform movements and early cognitive and psychiatric symptoms and indicating the degeneration / dysfunction of neurons within the caudate nucleus, which are largely involved in cognitive functions as well as motor control (Alexander et al. 1986; Reiner et al. 1988; Glass et al. 2000). Subsequent degeneration of substance P / dynorphin positive GABAergic medium spiny projection neurons correlates more with the onset of muscle dystonia (Reiner et al. 1988; Glass et al. 2000). Striatal interneurons are the least vulnerable with several subpopulations relatively spared in HD, specifically the somatostatin, neuropeptide Y, NADPHd positive and cholinergic interneurons (Reiner et al. 1988; Glass et al. 2000). Cortical neurons in layer III, V and VI of the cerebral cortex are also seen to degenerate in HD (Vonsattel et al. 1985; Hedreen et al. 1991; Macdonald and Halliday 2002). With layer III and V pyramidal neurons projecting to the striatum it is possible that the neurons are undergoing
secondary neurodegeneration following the loss of striatal connections, however with layer VI projecting to non-degenerative regions it would appear that independent degeneration is occurring within the cortex (Hedreen et al. 1991). In more severe grades of HD, neuronal cell loss occurs in many regions of the brain including the globus pallidus, subthalamic nucleus, cerebellum and thalamus (Vonsattel and DiFiglia 1998).

This complex pattern of neurodegeneration does not reflect the expression pattern of huntingtin, nor the pattern of neuronal intranuclear inclusion or neuropil aggregate formation, suggesting that other

**Figure 1-1**  Potential mechanisms of mutant huntingtin induced cellular pathogenesis
Huntingtin is predominantly a cytoplasmic protein involved in numerous processes including vesicle transport of BDNF along microtubules through interaction with motor proteins which is inhibited by the expanded poly-Q tract causing abnormal protein folding. Mutant huntingtin also undergoes enhanced proteolytic cleavage with the N-terminal fragment containing the poly-Q repeat forming a β-sheet structure that can cause cellular disruption either as soluble monomers, oligomers or as insoluble aggregates. Toxicity in the cytoplasm may be induced via potentiation of NMDA glutamate receptors, mitochondrial effects, caspase activation or cellular disruption by perinuclear or neuritic aggregate formation. N-terminal fragments are also translocated to the nucleus where they form intranuclear inclusions can interfere with gene transcription of neuroprotective molecules including BDNF. (Reproduced from (Marx 2005), Science)
factors contribute to the selective vulnerability of the GABAergic medium spiny projection neurons (Saudou et al. 1998; Gutekunst et al. 1999). Although the molecular mechanism leading to HD neurodegeneration are not fully understood, the wild-type huntingtin protein has been shown to have clear roles in gene transcription, intracellular transport, and possesses anti-apoptotic properties; all of which are disrupted by the expanded poly-Q tract (Figure 1-1; (Borrell-Pages et al. 2006)). The disrupted regulation of transcription has been demonstrated by the reduced cytoplasmic sequestration of the transcription factor that inhibits BDNF transcription leading to a reduction in BDNF expression (Zuccato et al. 2001; Zuccato et al. 2003). Following N-terminal cleavage and translocation to the nucleus, a proposed requirement for HD neurodegeneration (Saudou et al. 1998), the poly-Q-huntingtin fragment can interfere with the regulation of transcription across a broad range of genes, inducing apoptosis (Luthi-Carter et al. 2000; Steffan et al. 2000; Nucifora et al. 2001). Intracellular microtubule-based vesicle transportation mediated by the binding of huntingtin with huntingtin-associated protein-1 and other components of the molecular motor machinery is disrupted by the expanded poly-Q tract causing a reduction in the vesicular transportation of BDNF (Engelender et al. 1997; Gauthier et al. 2004). Axonal transportation can also be physically blocked by the formation of N-terminal huntingtin aggregates within the axons (Li et al. 2000; Gunawardena et al. 2003).

1.3 Animal Models of Huntington’s Disease

Animal models of neurodegenerative diseases are vital for advancing the development of therapeutic treatment strategies aimed at both preventing the degenerative progression and replacing the lost neurons. The development of genetic models of HD since the identification of the causative CAG repeat expansion in Hdh has greatly aided elucidation of pathological mechanisms and offer alternative cellular and animal models of HD to the acute neurotoxin induced mimicking of pathological neurodegeneration. Here I review current rodent models of HD with particular focus on their suitability for investigation of preventative therapeutic strategies to attenuate HD-induced neurodegeneration. The excitotoxic QA lesion model was ultimately selected as the most suitable model readily available for this thesis neuroprotective investigation.

1.3.1 Transgenic models of Huntington’s disease

For HD, a single gene disorder, the possibility exists in theory that the exact neurodegenerative processes should be replicable in transgenic models. Numerous transgenic mice have been generated with either an N-terminal Hdh fragment containing an expanded CAG repeat in exon 1 (Mangiarini
et al. 1996; Schilling et al. 1999; Laforet et al. 2001), full-length Hdh (Reddy et al. 1998; Hodgson et al. 1999), or knock-in mice with additional CAG repeats inserted into the murine Hdh gene (Shelbourne et al. 1999; Wheeler et al. 2000; Lin et al. 2001). However to date these transgenic mice have not proven to completely replicate the specific neuronal cell death with only two lines showing significant striatal cell loss (Reddy et al. 1998; Laforet et al. 2001), although they do displayed some progressive phenotypic behaviours indicative of neurological impairment. Specifically the transgenic R6/2 mice progressively develop an irregular gait, resting tremor, stereotypic and abrupt irregularly timed movements, and epileptic seizures from nine weeks of age coinciding with a loss in body weight leading to death at 10-13 weeks (Mangiarini et al. 1996). Others show early hyperactivity followed by end stage hypoactivity (Reddy et al. 1998; Hodgson et al. 1999; Laforet et al. 2001). The restricted lifespan of these models may explain the absence of cell death despite neurological impairments and the presence of neuronal intranuclear inclusions, consisting of N-terminal fragments of the expanded huntingtin protein, similar to those found in the cortex and striatum of HD patients although these inclusions are more widespread in the transgenic mice (Morton et al. 2000). More recently a transgenic rat model of HD has been developed that better replicates the slow phenotypic progression of HD with emotional and cognitive impairments proceeding motor impairments (von Horsten et al. 2003). Specific loss of striatal neurons and intranuclear inclusions were evident from 12 months of age but not at six months, with no loss of cortical neurons never seen (Kantor et al. 2006). While transgenic models have become very valuable in elucidating the etiology of HD and the role of wild-type huntingtin, until recently they have been of restricted use for neuronal rescue / repair studies in which neuronal loss and function are the primary outcome measures. Overall it has become apparent that the more genetically accurate the model, the more subtle and variable the model becomes, and the greater it reflects the variability that is seen amongst HD patients (Hersch and Ferrante 2004).

1.3.2 Chemical neurotoxin models of Huntington’s disease

As a substitute to genetic modelling, animal models that closely replicate the neuronal cell loss of HD can be generated through either excitotoxic lesioning of the striatum or metabolic impairment via mitochondrial disruption. Early hypotheses of HD etiology suggested the involvement of excitotoxins, with numerous studies using various excitotoxins to try and mimic the selective neurodegeneration (Beal et al. 1986; Schwarcz et al. 1988; Bruyn and Stoof 1990; DiFiglia 1990; Beal 1992a; Beal 1992b). Quinolinic acid (QA), an endogenous tryptophan metabolite present in the human brain at low concentrations, has proved to be the most successful excitotoxin at reproducing the selective degeneration of GABAergic medium spiny striatal projection neurons, while selectively
sparing somatostatin / neuropeptide Y striatal interneurons and fibres of passage (Schwarcz and Kohler 1983; Schwarcz et al. 1984; Beal et al. 1991; Brickell et al. 1999). Other excitotoxins, kainic acid, ibotenic acid, and N-methy-D-aspartate (NMDA), do not produce the selective sparing observed with QA (Beal et al. 1986). The resistance of neonatal animals and the high vulnerability of GABAergic medium spiny striatal projection neurons to QA gives support to the QA excitotoxin hypothesis of HD (Schwarcz et al. 1984). Neurons containing somatostatin or neuropeptide Y also express NADPHd, which could conceivably contribute to their survival in HD by allowing these neurons to take-up and metabolise QA (Schwarcz et al. 1984). This hypothesis that QA contributes to HD pathogenesis was recently supported by van Horsten and colleagues (2003) suggesting from indirect neurochemical data that QA synthesis may potentially be increased in their HD transgenic rats, which also maintain susceptibility to the excitotoxicity of QA (Winkler et al. 2006). Similarly, the chronic exposure to very low concentrations of QA has been shown to induce neuronal cell death (Chiarugi et al. 2001), and just recently it has been reported that wild-type huntingtin protects striatal neurons from QA-induced excitotoxicity (Leavitt et al. 2006).

While the extent to which QA contributes to clinical HD is still to be conclusively determined, QA lesioned animals have been extensively used as models of HD pathology. Unfortunately while QA generates some of the behavioural and pathological changes observed in HD, the progressive nature of HD is not easily reproduced (Tobin and Signer 2000), with the majority of cell death occurring in the three days following intrastrial injection (Portera-Cailliau et al. 1995; Hughes et al. 1996), and no further loss of phenotypic neuronal markers after seven days (Bazzett et al. 1994). Two models of chronic QA administration – microdialytic pump (Bazzett et al. 1993; Bazzett et al. 1994), and gradual release from polymeric implants (Haik et al. 2000) – have been developed and produce a progressive lesion more characteristic of HD, however the acute intrastrial QA injection protocol is still widely utilized and readily produces consistent unilateral or bilateral striatal lesions.

The progressive nature of HD is slightly better mimicked by 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase that is commonly used as an alternative to excitotoxic animal models (Alexi et al. 1998). Inhibition of succinate dehydrogenase disrupts mitochondrial respiration and prevents ATP synthesis causing cell death by both apoptotic and necrotic mechanisms. The systemic administration of 3-NP via subcutaneously implanted minipumps causes the selective bilateral death of striatal GABAergic medium spiny projection neurons while relatively sparing the striatal interneurons and fibres of passage and thereby closely mimicking the pathology of HD (Borlongan et al. 1997). Systemic delivery allows for prolonged administration and therefore generates a prolonged insult, causing progressive neurodegeneration somewhat
reminiscent of the slow neuropathological progression of HD (Borlongan et al. 1997). This preferential loss of striatal projection neurons, over all other cell types following chronic systemic delivery of 3-NP, gives significant support to the hypothesis that energy impairment plays a major role in the etiology of HD. 3-NP does not however replicate the “wave of degeneration” or the sequential loss of subpopulations of striatal GABAergic projection neurons that occurs in HD (Sun et al. 2002). The striatal lesion is instead centred round the medial striatal artery and uniformly kills the projection neurons regardless of their target efferent nuclei (Nishino et al. 1995).

In addition to the pathological neurodegeneration, the excitotoxic (QA) and energy impaired (3-NP) animal models also show varying degrees of behavioural deficits (Shear et al. 1998). A range of motor skill and cognitive function tests have been developed in addition to general open field observational tests to quantify behavioural impairments following unilateral or bilateral striatal lesions. Unilateral striatal lesions generally cause contralateral impairments of motor and sensory function which are commonly assessed by forelimb akinesia (Olsson et al. 1995), “staircase” testing – a measure of lateralised forelimb reaching and dexterity (Montoya et al. 1990; Montoya et al. 1991; Abrous et al. 1993), spontaneous exploratory forelimb use (Schallert et al. 2000), and sensory neglect (Salzberg-Brenhouse et al. 2003; Dowd et al. 2005). Amphetamine and apomorphine induced ipsilateral rotational behaviour is also characteristic of unilateral lesions resulting from an imbalance in striatal projection neurons or more specifically their dopamine receptors (Ungerstedt 1971; Schwarcz et al. 1979). Bilateral lesions show greater impairments in tests of cognitive ability (e.g. alternation T-maze and Morris water maze) than unilateral lesions in which the unlesioned hemisphere is though to be largely compensatory against the loss in cognitive ability (Pisa et al. 1981; Isacson et al. 1986; Popoli et al. 1994; Emerich et al. 1997a).

### 1.4 Therapeutic Intervention for Huntington’s Disease

The development of efficient therapeutic treatment strategies for neurodegenerative diseases has been slow and relatively unsuccessful in generating long-term clinical benefits. The absence of long-term results for HD is thought to be related to the progressive nature of neurodegeneration, which is not addressed by any currently available pharmacological treatments that provide some symptomatic relief from the psychiatric disturbances, choreiform movements and cognitive deficits (Handley et al. 2006).

As the molecular mechanisms by which mutant huntingtin appears to elicit neurodegeneration are pieced together, novel therapeutic approaches that directly interfere with the processing and
abnormal function of mutant huntingtin are beginning to be investigated (Qin et al. 2005). However with excitotoxicity appearing to play an important role in the selective pathology of HD, treatment strategies directed at intervening in general processes of cell death – including caspase activation, mitochondrial dysfunction and oxidative stress – may well hold significant clinical benefits for preventative therapy (Leegwater-Kim and Cha 2004; Handley et al. 2006). Independent of the specific therapeutic compound, efficient treatment against HD neurodegeneration will clearly require continuous supply of the therapeutic to the vulnerable neurons. This poses challenges associated with the need to cross the blood brain barrier, while avoiding deleterious side effects resultant from interactions with non-targeted cells, effectively preventing systemic delivery of many potentially beneficial compounds (Barinaga 1994; Tan and Aebischer 1996). However a number of pharmaceutical drugs – caspase inhibitors, mitochondrial enhancers and glutamate receptor blockers – have now been examined in clinical trials, having previously shown sufficient efficacy in preclinical research (Bonelli et al. 2004; Handley et al. 2006). With direct intraparenchymal infusion of therapeutic agents limited by the physical requirements necessary to achieve long-term administration, attention has been directed to the use of novel gene transfer techniques and cellular transplantation to produce biotherapeutics in situ.

The use of encapsulated cells – ciliary neurotrophic factor (CNTF)-producing fibroblasts (Emerich et al. 1996; Emerich et al. 1997b; Mittoux et al. 2000) or choroid plexus cells (Borlongan et al. 2004; Emerich et al. 2006) – implanted into the striatum have provided protection against excitotoxic neuronal death and behavioural deficits in both rodent and primate studies. The in vitro manipulation of cells prior to implantation in semi-permeable capsules allows direct assessment of the secreted factors before implanting, with the encapsulation preventing host immune rejection and also allowing for graft recovery (Tan and Aebischer 1996; Emerich 2004b). Encapsulated cell implants do however suffer from limited diffusion and down-regulation of transgene protein expression (Emerich et al. 1996; Emerich and Winn 2004a). Alternatively direct transplantation of genetically modified cells (ex vivo gene therapy) can be performed with the potential for migration throughout the striatum, thereby reducing required diffusion distances (Martinez-Serrano and Bjorklund 1996). Transplantation of neuronal progenitor cells may also provide a method for replacing lost neurons in a potential joint protection-restoration treatment approach (Fallon et al. 2000; Hsich et al. 2002). However cell grafts cannot be recovered in the event of adverse reactions, although the incorporation of a suicide gene may allow for graft elimination (Garin et al. 2001). In vivo gene delivery is appealing in that it potentially avoids a number of problems associated with other methods of delivery, including the need for any permanent delivery device or the use of
imortalized cell-lines and their potential adverse effects (Hsich et al. 2002). However, in vivo gene delivery does rely on engineering the patients own host cells to drive production of the therapeutic proteins and is not without its own disadvantages, including potential adverse toxicity, immune responses, or oncogenic potential due to insertional mutagenesis (Monahan and Samulski 2000; Hsich et al. 2002). The ongoing development of in vivo vectors with improved safety, control and stability of transgene expression, and reduced pathogenicity / toxicity all increase the appeal of in vivo gene therapy for long-term delivery of therapeutic proteins directly to vulnerable neurons.

With both ex vivo and in vivo gene therapy techniques showing promise for delivering biotherapeutics to the CNS, I elected to employ a direct in vivo gene delivery technique allowing direct manipulation of the vulnerable striatal neurons and thereby enabling the investigation of both secreted and non-secreted intracellular acting therapeutic molecules using the same gene therapy protocols.

1.5 in vivo Gene Delivery Vectors

The main requirement of vectors used for in vivo gene delivery to the CNS is their ability to efficiently deliver transgenes to terminally differentiated neurons, generating stable protein expression while avoiding the stimulation of inflammation and immunogenic host responses (Hsich et al. 2002). Viral-derived vectors have become the vectors of choice for many neurological applications due to their natural inclination to transfer their genetic material to targeted cells (Bjorklund et al. 2000; Hsich et al. 2002). The most promising vectors for direct CNS gene delivery are derived from human viruses including herpes simplex virus (HSV), adenovirus (Ad), adeno-associated virus (AAV), and lentivirus (LV) (Thomas et al. 2003). Each of these vectors are briefly reviewed here with particular emphasise on the properties of AAV vectors which I utilised for in vivo transduction of the rodent striatal neurons in the neuroprotective investigations.

1.5.1 Herpes simplex and adeno viral vectors

First generation HSV and Ad vectors with large transgene capacities (HSV ~50kb, Ad 7.5kb) are efficient for gene delivery to many brain cell types, however the expression of viral proteins stimulate adverse immune reactions (Hsich et al. 2002). Elimination of viral genes has led to the development of HSV amplicon vectors with essentially no toxicity, and “gutless” Ad vectors with greatly reduced toxicity and immunogenicity (Thomas et al. 2000; Hsich et al. 2002). However, transgene expression following Ad-mediated gene delivery is not long-lasting and the presence of
neutralising antibodies against the Ad structural proteins block transduction following re-administration of the same serotype vector (Zoltick et al. 2001). Pre-existing immunity to HSV is also a limiting factor for HSV vectors (Lauterbach et al. 2005), which also only generate transient expression after elimination of the viral genes (Samaniego et al. 1997).

1.5.2 Adeno-associated viral vectors

AAV is a very small (25nm) non-pathogenic parvovirus dependent on a helper virus, usually adenovirus, to proliferate. The wild-type AAV genome contains two open-reading frames rep (genes required for replication) and cap (genes encoding the capsid proteins) flanked by 145 bp inverted terminal repeat sequences (ITR; (Srivastava et al. 1983)). However only the ITR sequences are required in cis for replication and encapsulation of single stranded DNA, up to ~4.7kb, into the icosahedral AAV virion particle of 60 capsid proteins (Monahan and Samulski 2000; Hsich et al. 2002). This allows for the complete removal of viral genes in recombinant AAV (rAAV) vectors eliminating the potential for an immune response against the expression of viral proteins (Jooss et al. 1998), although the production of neutralising antibodies against the capsid proteins is still a concern, particularly for repeat vector delivery (Halbert et al. 2006). Production of rAAV vectors is typically undertaken by DNA plasmid co-transfection of a packaging cell line, usually human embryonic kidney 293 cells (HEK293), with the AAV-rep and -cap genes plus the necessary Ad genes supplied on separate constructs in trans to the ITR-flanked AAV expression cassette (Figure 1-2; (Xiao et al. 1998)). With the characterisation of at least eight distinct serotypes of AAV showing different host cell tropisms, and recent identification of over 100 additional variants (Gao et al. 2004), the majority of attention for gene transfer has been given to serotype-2 as the first serotype fully characterised (AAV\textsubscript{2}; (Samulski et al. 1982; Srivastava et al. 1983); for current review see (Wu et al. 2006)). AAV\textsubscript{2} alone presents strong affinity for the heparan sulphate proteoglycan receptor which appears to mediate its binding-to and transduction of host cells (Summerford and Samulski 1998), and also allows heparin affinity column purification to produce high-titre recombinant vectors (Zolotukhin et al. 1999). The apparent clinical safety of AAV\textsubscript{2} as a gene delivery vector has now been well tested in clinical trials (Carter 2005). With different serotype capsid proteins conferring selective tissue and cellular tropism, attention has been directed to altering the tropism and transduction efficiency of AAV\textsubscript{2} and other AAV serotypes by either genetic alteration of the capsid proteins (Muzychka and Warrington 2005) or production of chimeric (also called mosaic) rAAV virions containing a mixture of different capsid proteins to combine the transduction properties of different serotypes (Hauck et al. 2003; Rabinowitz et al. 2004). In respect to the transduction of neurons in the CNS, serotype-1 and -5 rAAV vectors display higher transduction efficiencies than
AAV₂ vectors (Davidson et al. 2000; Burger et al. 2004). Recent production of chimeric vectors with serotype-1 and -2 capsid proteins (AAV₁/₂), combined in a 1:1 ratio and retaining the ability to be purified by heparin column, show enhanced neuronal transduction over standard AAV₂ vectors (Hauck et al. 2003; Richichi et al. 2004).

In general the serotype-2 ITR sequences flanking the expression cassette have been retained, as the *cis*-acting element to direct replication and packaging of the vector genome, irrespective of the packaging capsids. While wild-type serotype-2 AAV can integrate into the human genome at a specific site on chromosome 19 (Kotin et al. 1992), the rAAV vectors have been observed to integrate at non-specific sites (Linden et al. 1996; Ponnazhagan et al. 1997; Yang et al. 1997) however, the predominant persistence of the AAV genome *in vivo* is extrachromosomal as circular monomeric or multimeric structures with enhanced episomal stability (Clark et al. 1997; Duan et al. 1998; Duan et al. 1999; Schnepp et al. 2005). With the high stability of AAV genome in host cells the persistence of transgene expression following rAAV transduction appears dependent on the regulatory elements controlling transgene expression. Transgene expression from the hybrid CMV-

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**Figure 1-2  Co-transfection rAAV packaging**

Production of rAAV is typically undertaken by co-transfection of HEK293 cells with separate plasmids containing the recombinant AAV genome (pAAV) with the expression cassette flanked by ITRs (red), the AAV rep and cap genes (pHelper), and a plasmid containing the necessary Ad viral genes (pAd). The *trans* acting AAV *rep* and Ad genes direct replication of single stranded rAAV genome and encapsulation into AAV virions constructed following expression of the AAV capsid proteins (Capₓ). Vector serotype is determined by the *cap* genes in the pHelper plasmid (Modified from (Merten et al. 2005), Gene Therapy).
chicken-β-actin (CBA) promoter was still stable in the rat brain after 25 months (Klein et al. 2002), however the CMV promoter has shown susceptibility to silencing by hypermethylation in some cells (Prosch et al. 1996). A major limiting factor for rAAV vectors is the relatively small transgene capacity (~4.7 kb), restricting delivery of larger transgenes and the incorporation of regulatory elements that will be vital for controlling expression in some clinical applications (Monahan and Samulski 2000; Hsich et al. 2002).

Delivery of rAAV vectors to the CNS through direct intracranial injection allows targeting of specific populations of cells, however for larger structures the transduction efficiency can be hindered by limited vector spread, requiring multiple injection sites (Bjorklund et al. 2000). Ultraslow injections and co-infusion of mannitol or heparin have both been shown to increase transduction efficiency and distribution of transduced neurons by 200-300% (Mastakov et al. 2001; Nguyen et al. 2001). More recently, systemic delivery of mannitol has shown to provide even greater enhancement of rAAV vector spread and transduction efficiency (Burger et al. 2005).

1.5.3 Lentiviral vectors

LV vectors are retroviral vectors derived from the immunodeficiency viruses HIV (human) and FIV (feline) (Poeschla 2003; Wiznerowicz and Trono 2005). In contrast to other retroviral vectors, LV can stably integrate into non-dividing cells as well as dividing cells, therefore generating long-term transgene expression (Wong et al. 2006). Extensive use of LV vectors has been delayed due to safety issues surrounding the production and application of vectors derived from such a highly pathogenic virus. However these biosafety issues have been addressed with further elimination of viral genes and the development of a self-inactivating (SIN) version (Miyoshi et al. 1998; Zufferey et al. 1998). LV vectors have a transgene capacity of at least 8kb and have high affinity and transduction efficiency for fully differentiated neurons (Watson et al. 2002; Wong et al. 2004). Primate studies have reported the absence of toxicity or inflammatory responses (Kordower et al. 1999; Bjorklund et al. 2000; Kordower et al. 2000; Palfi et al. 2002). Thus, as safety concerns are minimized, LV becomes a very attractive vector for long-term transgene expression in the CNS.

1.6 Neurotrophic Factors as Biotherapeutic Agents for HD

Neurotrophic factors are proteins produced and secreted by neurons and glia that regulate the development, survival and function of neurons within the developing central and peripheral nervous system (Huang and Reichardt 2001). In addition, neurotrophic factors also contribute to activity-
dependant neuronal plasticity, biochemical function and survival of neurons within the mature adult
nervous systems (Sofroniew et al. 2001). Expressed heterogeneously within the CNS, neurotrophic
factors function through specific receptors differentially expressed by various neuronal and non-
neuronal populations of cells (Mufson et al. 1999). Target-derived support appears vital in directing
the development of neuronal circuits with growing axons expressing receptors that compete for
limited neurotrophic factor expression in the target nuclei, thereby promoting neurite growth,
synapse formation and regulating neuron survival to limit the number of neurons (Purves 1988;
Burke 2006). While target-derived support is essential in the developing CNS, the degree to which
mature, terminally differentiated neurons depend on target-derived trophic support is unclear with
autocrine / paracrine activities potentially more important, although the ability to retrogradely
transport neurotrophic factors is still maintained (Mufson et al. 1999; Murer et al. 2001).
Widespread CNS neurotrophic factor expression, in addition to regions of abundant expression, and
co-localization with neurotrophic factor receptors support proposed autocrine / paracrine interaction
in promoting neuronal function and survival (Connor and Dragunow 1998). Increases in
neurotrophic factor expression within adult animals following a neuronal degenerative event or
neuronal injury is indicative of the support role that neurotrophic factors play in the adult CNS where
they can act as anti-excitotoxins and antioxidants or can up-regulate calcium binding proteins and
anti-apoptotic signals (Alexi et al. 2000; Alberch et al. 2004). Neurotrophic factor deprivation has
also been shown to cause up-regulation of pro-apoptotic gene transcription, indicating the
neurotrophic factor dependence of some mature neurons to survive and maintain neuronal function
(Johnson et al. 1989; Oppenheim 1991).
The expression of neurotrophic factors in the striatum and their modified expression in response to
striatal lesioning (Marco et al. 2002), and in neurological diseases including HD (Ferrer et al. 2000),
has led to neurotrophic factors being viewed as potential therapeutic agents (Alexi et al. 2000;
Alberch et al. 2004). Administration of neurotrophic factors to the striatum in animal models of HD
have been investigated via three broad techniques: direct intracerebellar infusion, transplantation of
neurotrophic factor producing cells, or in vivo gene delivery. Four neurotrophic factors – the
neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), glial cell-
line derived neurotrophic factor (GDNF), and CNTF – have received significant attention in relation
to their potential therapeutic value as neuroprotective agents for HD (Alberch et al. 2004) and are
reviewed here with particular focus on BDNF and GDNF which I selected to investigate further with
in vivo AAV-mediated gene delivery prior to QA lesioning. Additionally many other neurotrophic
factors have been demonstrated to have neuroprotective actions on striatal neurons including
neurotrophin 4/5 (Alexi et al. 1997), neurturin (Perez-Navarro et al. 2000), transforming growth
factor-α (Alexi et al. 1997), basic fibroblast growth factor (Frim et al. 1993b; Kirschner et al. 1995) and activin-A (Hughes et al. 1999). The method of neurotrophic factor delivery to the striatal neurons has proven to be highly influential on the ultimate capacity to impart potent neuroprotection, with the prospect of efficient clinical treatment becoming reality, proved safe and efficient delivery procedures are developed.

1.6.1 Nerve growth factor

NGF, the first identified neurotrophic factor (Levi-Montalcini and Hamburger 1953), is widely expressed throughout the CNS with high expression in regions of the CNS innervated by cholinergic neurons of the basal forebrain, particularly the hippocampus, olfactory bulb and neocortex (Levi-Montalcini 1987; Mufson et al. 1994; Das et al. 2001). Expression of NGF in the developing striatum is seen to correlate with the differentiation of cholinergic interneurons which express the high-affinity NGF receptor TrkA and low-affinity p75 receptor (Mobley et al. 1989; Ringstedt et al. 1993; Steininger et al. 1993; Barbacid 1994). Co-localization of NGF and TrkA in the CNS (Barde 1989) and high NGF expression throughout adulthood, suggests NGF regulates maintenance and survival of adult neurons through autocrine / paracrine activity (Kokaia et al. 1993; Miranda et al. 1993). Enhanced striatal NGF levels following injection of glutamate receptor agonists further support the suggestion that NGF provides endogenous neuroprotection of the cholinergic neurons (Perez-Navarro et al. 1994; Strauss et al. 1994; Canals et al. 1998).

NGF has been investigated in several animal models of HD (Alberch et al. 2004) with significant neuronal protection observed when released from NGF-producing cells transplanted into the striatum or corpus callosum prior to ipsilateral QA (Schumacher et al. 1991; Frim et al. 1993a; Martinez-Serrano and Bjorklund 1996; Kordower et al. 1997), 3-NP (Frim et al. 1993c) or NMDA-agonist (Frim et al. 1993b) induced lesioning. These studies have demonstrated that locally produced NGF results in a significant reduction in striatal lesion volumes by 60-80% compared with untreated animals (Schumacher et al. 1991; Frim et al. 1993a; Frim et al. 1993b; Frim et al. 1993c; Martinez-Serrano and Bjorklund 1996; Kordower et al. 1997). Martinez-Serrano and Bjorklund (1996) also observed protection of striatal neurons and their efferent projections to the substantia nigra and globus pallidus. Complete sparing of ChAT-immunoreactive cholinergic interneurons and sparing of NADPH histochemically stained neurons was reported following transplantation of NGF-producing cells (Schumacher et al. 1991; Frim et al. 1993a; Martinez-Serrano and Bjorklund 1996; Kordower et al. 1997). In contrast, direct intrastriatal NGF protein injections (Davies and Beardsall 1992) or prolonged infusions (Davies and Beardsall 1992; Venero et al. 1994; Anderson et al. 1996) failed to
protect the vulnerable striatal GABAergic projection neurons, but selectively protected cholinergic interneurons (Altar et al. 1992a; Perez-Navarro et al. 1994). The lack of NGF receptor expression by the GABAergic medium spiny projection neurons suggests that the neuroprotection of striatal GABAergic neurons, reportedly provided by NGF-producing cells, is not directly due to the actions of NGF, but possibly related to other factors secreted by the grafted cells, such as basic fibroblast growth factor that has been demonstrated to provide significant neuroprotection (Frim et al. 1993b; Kirschner et al. 1995), or the antioxidant properties of the grafted cells (Galpern et al. 1996). However both Frim et al. (1993b) and Martinez-Serrano and Bjorklund (1996) reported considerably less protection of the medium spiny projection neurons in their parallel investigations of BDNF ex vivo gene therapy than was provided by NGF-secreting cellular grafts, indicating a differential effect of the NGF or BDNF transgenic protein secretion.

1.6.2 Brain derived neurotrophic factor

Closely related to NGF, BDNF is widely expressed throughout the CNS with expression increasing during fetal and early postnatal development, and is maintained throughout adult life (Friedman et al. 1991; Connor and Dragunow 1998). Increasing expression as CNS regions mature suggests BDNF plays a greater role in the maintenance and plasticity of the adult CNS than in development (Altar et al. 1997). Abundant BDNF expression in the hippocampus is thought to be important for maintaining a high degree of neuronal plasticity vital for the hippocampus’s role in learning and memory (Lindsay et al. 1994). Expression and secretion of BDNF is modified by numerous events including bioelectrical activity, neurotransmitters, hormones and cellular insults (Dragunow et al. 1993; Marty et al. 1997; Hughes et al. 1999; Murer et al. 2001). Activity-dependant release of BDNF from neurons (Allendoerfer et al. 1994) is proposed to be induced by depolarisation through a calcium-dependent mechanism – glutamate receptor agonists induce BDNF, while GABA A receptor agonists inhibit BDNF expression (Goodman et al. 1996; Marty et al. 1997). Cellular insults – hypoxia-ischemia and hypoglycaemic coma – have also been observed to increase both BDNF and TrkB receptor expression suggesting that endogenous BDNF plays a significant role in the general protection of the CNS (Merlio et al. 1993). More specifically related to HD, an intrastratal injection of QA was found to induce an up-regulation of BDNF in cortical layers II, III, V and VI due to damage in the striatal target area (Canals et al. 2001), but no BDNF increase in the striatum (Canals et al. 1998). The TrkB receptor was also up-regulated by cortical neurons following intrastratal QA injection (Checa et al. 2001), suggesting an endogenous mechanism to support cortical neurons following striatal damage.
High affinity TrkB receptors for BDNF are found throughout the CNS on both neurons and glia. Receptors are mostly localized to the axons, synaptic terminals and dendritic spines supporting the proposal that synapses are the main site at which BDNF functions in the CNS, with TrkB mediating synaptic plasticity and retrograde transport of BDNF (Drake et al. 1999; Watson et al. 1999; Aoki et al. 2000). It has been demonstrated that glial cells have the ability to internalise BDNF via truncated TrkB receptors lacking catalytic kinase activity, thus regulating BDNF availability and restricting diffusion (Yan et al. 1994; Biffo et al. 1995; Rubio 1997; Altar 1999). Although lacking catalytic kinase activity a truncated TrkB receptor isoform is the predominantly expressed isoform in the adult brain and is hypothesised to regulate BDNF signalling by impairing catalytic TrkB receptors and may also act as a cellular adhesion molecule regulating synapse plasticity and axonal outgrowth (Armanini et al. 1995; Murer et al. 2001). TrkB receptor expression appears to be regulated by mechanisms similar to those that also regulate BDNF expression, including bioelectrical activity and cellular insults – hypoxia-ischemia, hypoglycaemic coma and axotomy (Merlio et al. 1993). The dendritic targeting of BDNF and TrkB induced by bioelectrical activity suggests that autocrine activity is important in maintaining and regulating dendritic tree function (Tongiorgi et al. 1997). Intracellular TrkB receptors are also translocated to the plasma membrane in response to depolarisation, thus rapidly increasing responsiveness to BDNF potentially important for maintaining synapses (Meyer-Franke et al. 1998). Prolonged BDNF exposure however causes a down-regulation of catalytic TrkB receptors (Frank et al. 1996).

In addition to the high affinity TrkB receptor, BDNF also binds to p75 neurotrophin receptor (p75NTR) which has traditionally been viewed as a coreceptor for the Trk neurotrophic factor receptors facilitating Trk dimerisation to allow high-affinity neurotrophin binding and modulation of Trk signalling (Chao 1994; Chao and Hempstead 1995). More recently however, p75NTR, a member of the tumor necrosis factor receptor family, has been demonstrated to elicit biological functions following neurotrophic factor binding including the induction of apoptosis in the absence of Trk receptors (Dobrowsky et al. 1994; Bamji et al. 1998; Boyd and Gordon 2002; Troy et al. 2002).

As with all neurotrophins, BDNF is synthesised as a pro-neurotrophin which undergoes proteolytic cleavage by furin or proconvertases to produce the mature form of BDNF protein. However a number of recent studies have demonstrated that proneurotrophins are also physiologically active (Nykjaer et al. 2004), are endogenously secreted by neurons (Mowla et al. 2001; Chen et al. 2004; Teng et al. 2005), and can be cleaved extracellularly by plasmin and metalloproteinases (Lee et al. 2001). ProBDNF itself however does not stimulate TrkB phosphorylation or promote neurite outgrowth, but rather can induce apoptosis through activation of p75NTR and the coreceptor sortilin
The induction of apoptosis appears to be dependent on the presence of the high-affinity sortilin receptor, with extracellular proteolytic cleavage to mature BDNF occurring in the absence of sortilin (Nykjaer et al. 2004). These entirely opposing actions of pro- and mature forms of BDNF indicate the delicate role that this neurotrophic factor plays in facilitating the survival and plasticity of BDNF dependent neurons in the mature CNS, with an ability to respond rapidly to a wide range of stimuli.

In order to modulate neuronal plasticity BDNF acts in a localised synapse-specific manner that is thought to be regulated by the activity level of individual synapses potentially influencing the dendritic targeting of BDNF mRNA, the secretion of BDNF at active synapses, TrkB receptor expression and receptor-neurotrophin internalisation (for reviews see (Lu 2003; Kuipers and Bramham 2006)). Mature BDNF acting through the TrkB receptor appears to be a vital protein in the establishment of late-phase long-term potentiation (Pang et al. 2004) through the activation of the microtubule-associated protein (MAP) kinase cascade enhancing the transcription of several immediate early genes which are responsible for controlling specific neuronal function (Friedman and Greene 1999; Bolton et al. 2000). The synthesis of proteins within dendritic regions is activity-dependent with BDNF-TrkB signalling central to regulating the translation of mRNA through phosphorylation of the eukaryotic initiation factor 4E (eIF4E) and eukaryotic elongation factor 2 (eEF2; Figure 1-3; (Bramham and Messaoudi 2005; Soule et al. 2006)).

**Figure 1-3** TrkB signalling pathways controlling dendritic protein synthesis
BDNF binding to postsynaptic TrkB receptors activates a number of major signalling pathways which ultimately provide control over dendritic translation. BDNF-TrkB activation of PI3K and ERK cascades causes phosphorylation of eIF4E and enhances the initiation of cap-dependent mRNA translation. Bi-directional control over eEF2 phosphorylation state by TrkB signalling provides control over mRNA translation with phosphorylated eEF2 inhibiting peptide chain elongation. (Reproduced from (Soule et al. 2006))
BDNF has also been demonstrated to play an important role in the promotion of neurogenesis in the adult brain, enhancing neuron generation and the migration of neuroblasts from the sub ventricular zone to the striatum and olfactory bulb (Zigova et al. 1998; Benraiss et al. 2001; Chmielnicki et al. 2004; Chen et al. 2007; Henry et al. 2007). BDNF-stimulated promotion of neurogenesis appears to be mediated via \( p75^{NTR} \) signalling, with neurogenic precursor cells in the adult sub ventricular zone expressing the \( p75^{NTR} \) receptor (Young et al. 2007). Survival and maturation of neuroblasts by BDNF has been shown to occur through TrkB signalling (Benraiss et al. 2001), however neurogenic precursor cells do not express Trk receptors suggesting that BDNF interacts with \( p75^{NTR} \) to direct differentiation of precursor cells into neuroblasts by promoting withdrawal from the cell cycle and terminal differentiation (Cattaneo and McKay 1990; Ito et al. 2003; Chittka et al. 2004; Young et al. 2007). Although known primarily as a cell death inducing receptor, \( p75^{NTR} \) expression by precursor cells in the sub ventricular zone does not appear to affect survival of precursor cells in the presence or absence of BDNF (Young et al. 2007), but has been shown to stimulate cell death in neuroblasts which are prevented from migrating (Gascon et al. 2007). Within both the developing and adult striatum, medium spiny neurons rely on BDNF to maintain their phenotype, function and survival (Mizuno et al. 1994; Ivkovic and Ehrlich 1999; Baquet et al. 2004) through activation of TrkB mediated signalling (Hetman et al. 1999; Vaillant et al. 1999; Gavalda et al. 2004). In addition, BDNF also supports neuronal survival by up-regulating anti-apoptotic Bcl-2 expression via TrkB mediated Akt activation (Schabitz et al. 2000; Almeida et al. 2005; Perez-Navarro et al. 2005).

High levels of BDNF are present in the striatum with the striatal projection neurons expressing TrkB receptors (Costantini et al. 1999) and displaying a dependence on BDNF for survival (Saudou et al. 1998; Baquet et al. 2004). However with low levels of BDNF mRNA in the striatum, the majority of striatal BDNF is thought to be produced in the cortical neurons and anterogradely transported to the striatum ((Altar et al. 1997); Figure 1-4). Normal aged rats display a reduction by half in striatal BDNF levels (Katoh-Semba et al. 1998) suggesting a normal decline with aging that may increase the vulnerability of the striatal neurons. BDNF protein expression in HD has been investigated by Ferrer et al. (2000) using human post-mortem samples from grade-3 HD patients and age-matched non-neurological controls. BDNF protein expression was found to be reduced 53-82% in the caudate nucleus and putamen of the HD patients, but preserved in the parietal cortex, temporal cortex, and hippocampus. Most striatal BDNF is contained within the corticostriatal projections following anterograde transport, however the density of synaptic terminals in the HD striatum were unchanged and BDNF-positive fibres were still observed in HD patients (Ferrer et al. 2000). While this suggested a selective defect of striatal neurons and not a loss of cortical BDNF input, more recent investigation has shown mutant huntingtin both disrupts the transcription of BDNF (Zuccato et al.
2001; Zuccato et al. 2003) and reduces the axonal trafficking of BDNF vesicles along microtubules ((Gauthier et al. 2004); Figure 1-4). Therefore maintaining or increasing BDNF protein expression directly within the caudate nucleus and putamen of HD patients may potentially be of therapeutic benefit in preventing or slowing the progression of neurodegeneration in HD.

**Figure 1-4**  Mutant huntingtin disrupts transcription of BDNF and vesicle transportation from the cortex to the striatum.

A large proportion of striatal BDNF is produced by the cortical neurons and anterogradely transported via corticostriatal projections and released at synapses in the striatum. Wild-type huntingtin is crucial for the transcription and transportation of BDNF, both of which are impaired by the expanded poly-Q tract. BDNF expression is regulated by a neuron-restrictive silencer element (NRSE) in the promoter region that facilitates the blocking of transcription through association with a repressor complex. The normal sequestration of Repressor element 1-silencing transcription factor (REST) by huntingtin in the cytoplasm prevents repressor complex formation allowing BDNF transcription (A). Mutant huntingtin is less capable of preventing REST from entering the nucleus and silencing the expression of NRSE regulated genes (B). Huntingtin is also involved in controlling microtubule transportation of BDNF vesicles through association with motor protein complexes (C). Mutant huntingtin is thought to cause a conformational change through enhanced binding to huntingtin-associated protein 1 (HAP1) attenuating movement along microtubules (D). coREST, REST co-repressor; HDAC, histone deacetylase; SIN3A, SIN3 homologue A, a transcription regulator. (Modified from (Cattaneo et al. 2005), Nature Reviews: Neuroscience).
Review of Published Literature

In vitro studies of striatal neurons have shown BDNF to be a potent neurotrophic factor for GABAergic striatal neurons, promoting differentiation, increasing soma size, and increasing neurite branching (Mizuno et al. 1994; Widmer and Hefti 1994; Ventimiglia et al. 1995). BDNF has also been demonstrated in vitro to protect GABAergic striatal medium spiny projection neurons against excitotoxic stress and to inhibit apoptosis in striatal cells engineered to express huntingtin with an expanded CAG repeat sequence (Nakao et al. 1995; Saudou et al. 1998). Despite the promising neuroprotective actions of BDNF observed in vitro, direct infusion of BDNF protein into the rat striatum failed to provide any neuronal protection against QA lesioning (Anderson et al. 1996).

Transplantation of BDNF-producing fibroblasts to the corpus callosum failed to supply protection against excitotoxic insult of the striatum (Frim et al. 1993b), however similar transplantation into the striatum prior to QA injection resulted in a reduced lesion size and increased survival of the striatal projection neurons (Perez-Navarro et al. 2000a). Martinez and Bjorklund (1996) using BDNF-producing neural stem cells observed a partial protection of DARPP-32 expressing striatal projection neurons.

The use of direct in vivo gene transfer to direct the expression of BDNF in the striatum has also been previously investigated using an Ad-BDNF vector injected intrastriatally prior to QA lesioning (Bemelmans et al. 1999). They reported a 55% reduction in QA lesion size with an increase in the survival of DARPP-32 striatal projection neurons from 46% in control Ad-βGal treated animals to 64% survival in the Ad-BDNF animals. A slight shrinkage of the striatum was attributed to the toxicity of the first-generation adenoviral vector, which possibly restricted transgene expression, thus potentially limiting neuroprotection (Bemelmans et al. 1999). An earlier investigation conducted by my colleagues and I (Kells et al. 2004) showed AAV-BDNF mediated partial protection of striatal projection neurons and NOS expressing striatal interneurons against QA-induced excitotoxicity.

The spectrum of neuroprotection observed across the different BDNF delivery procedures highlights the importance of the delivery method in determining the ability for the neurotrophic factors to provide efficient neuronal protection. Together the previous studies suggest that continuous local production of BDNF is vital for this neurotrophic factor to provide neuronal protection, possibly explaining the lack of efficacy for direct BDNF injection (Anderson et al. 1996) and BDNF-producing fibroblast transplanted into the corpus callosum (Frim et al. 1993b). The necessity for high level, local production of BDNF is possibly explained by its limited penetration / diffusion of BDNF through the brain parenchyma or rapid clearance, most likely due to the high density of BDNF receptors in the striatum and glial cell uptake via truncated trkB receptors (Mufson et al. 1999).
1.6.3 **Glial cell-line derived neurotrophic factor**

A potent neurotrophic factor for dopaminergic neurons (Lin *et al.* 1993), GDNF is globally expressed throughout the CNS, with the highest expression occurring in regions receiving dense dopaminergic innervations including the developing striatum, ventral pallidum, olfactory tubercle, and piriform cortex (Bohn 1999). The developing striatum exhibits the highest level of GDNF with mRNA expression peaking at birth before rapidly declining and becoming undetectable by *in situ* hybridisation after 4 weeks (Stromberg *et al.* 1993; Choi-Lundberg and Bohn 1995). Sensitive RT-PCR assays have however reported that a low level of GDNF mRNA expression is maintained in the adult rat and human striatum (Schaar *et al.* 1993; Springer *et al.* 1994; Choi-Lundberg and Bohn 1995), with neuronal expression localised to large cholinergic interneurons (Pochon *et al.* 1997; Trupp *et al.* 1997). This expression pattern illustrates the target-derived role of GDNF in directing development of the nigrostriatal dopaminergic connections, with the dopaminergic neurons in the substantia nigra pars compacta (SNc) expressing high GDNF expression in the adult brain providing long-term autocrine / paracrine maintenance (Pochon *et al.* 1997; Marco *et al.* 2002). GDNF signalling is mediated by the ret receptor but requires the initial binding to the GDNF specific co-receptor GDNF family receptor α-1 (GFRα1) (Jing *et al.* 1996; Treanor *et al.* 1996). Both ret and GFRα1 show similar striatal localisation to GDNF, with low expression in the striatal projection neurons (Marco *et al.* 2002). However, following excitotoxic lesioning, GDNF and GFRα1 expression is largely localised to astrocytes with the suggestion that reactive astrocytes may present the GFRα1/GDNF complex to the striatal neurons thereby enhancing ret-mediated signalling to promote neuron survival (Trupp *et al.* 1997; Yu *et al.* 1998; Marco *et al.* 2002).

The therapeutic effects of GDNF have been extensively investigated in animal models of Parkinson’s disease and amyotrophic lateral sclerosis. GDNF delivery can both prevent the degeneration and promote the regeneration of dopaminergic neurons, and enhance survival of motoneurons (Bohn 1999; Bjorklund *et al.* 2000; Bohn *et al.* 2000a; Bohn *et al.* 2000b). Enhanced survival of motor neurons by GDNF has been shown to be dependent on anti-apoptotic caspase inhibitors ((Perrelet *et al.* 2002), refer Section 1.7.2). GDNF also shows promise for HD with both direct intraventricular GDNF protein infusions (Araujo and Hilt 1997) and striatal transplantation of GDNF-secreting fibroblasts (Perez-Navarro *et al.* 1996; Perez-Navarro *et al.* 1999) partially rescuing striatal medium spiny projection neurons from QA induced cell death. Only striatonigral projection neurons were maintained by the GDNF-producing fibroblasts, with the striatopallidal projection neurons and striatal parvalbumin-positive interneurons not surviving QA lesioning (Perez-Navarro *et al.* 1996; Perez-Navarro *et al.* 1999). Araujo and colleagues (1997) reported that in addition to partial
protection of medium spiny projection neurons, an intraventricular infusion of GDNF protein prior to QA lesioning significantly reduced amphetamine-induced rotational asymmetry and attenuated the numerous neurochemical deficits caused by the QA lesions. They also found that GDNF infusion provides protection against 3-NP induced neuronal cell loss in the striatum and associated locomotion deficits (Araujo and Hilt 1998). More recently, GDNF-derived support of striatal neurons was demonstrated following \textit{in vivo} AAV-GDNF gene delivery to the striatum prior to 3-NP administration (McBride \textit{et al.} 2003) and QA lesioning (Kells \textit{et al.} 2004). McBride and colleagues (2003) reported a 70\% increase in surviving NeuN immunopositive striatal neurons accompanied by significant preservation of motor performance following overexpression of GDNF in the striatum. This has been followed up with an investigation in a transgenic mouse model of HD (N171-82Q) where the presymptomatic mice received AAV-GDNF to enhance GDNF expression in the striatum, and showed significant protection of the striatal neurons and attenuated behavioural deficits (McBride \textit{et al.} 2006). In contrast, LV-GDNF delivery to the R6/2 mouse model of HD failed to protect against striatal cell loss or functional behaviour impairments (Popovic \textit{et al.} 2005). The conflicting results of these investigations were proposed to have arisen due to the greater severity of the R6/2 model or possibly the need for presymptomatic delivery of the GDNF vectors (McBride \textit{et al.} 2006).

### 1.6.4 Ciliary neurotrophic factor

Although beyond the focus of this thesis, CNTF has shown considerable promise for treating HD. While not vital for CNS development (Takahashi \textit{et al.} 1994; DeChiara \textit{et al.} 1995), CNTF expressed by glia enhances survival and differentiation of neurons in the CNS and PNS (Conover \textit{et al.} 1993; Helgren \textit{et al.} 1994; Sleeman \textit{et al.} 2000). CNTF deficiency is associated with motor neuron degeneration (Masu \textit{et al.} 1993) and is up-regulated in astrocytes following neuronal injury (Friedman \textit{et al.} 1992; Ip \textit{et al.} 1993; Rudge \textit{et al.} 1995). Therefore CNTF is proposed to play a critical role in maintaining and possibly restoring neuronal function following CNS injury (Rudge \textit{et al.} 1995). Abundant expression of CNTF receptors in the striatum (Ip \textit{et al.} 1993; Rudge \textit{et al.} 1995) suggest striatal neurons may rely on CNTF for survival (Choi 1988; Rudge \textit{et al.} 1994).

In contrast to most other neurotrophic factors, significant protection of GABAergic striatal neurons has been achieved by direct intrastratal protein infusion of CNTF starting 3-4 days prior to QA injection, with 69 ± 17\% maintenance up from 29 ± 11\% survival in untreated animals (Anderson \textit{et al.} 1996). Implantation of CNTF-secreting cells into the lateral ventricle in rat (Emerich \textit{et al.} 1996; Emerich \textit{et al.} 1997a) and primate (Emerich \textit{et al.} 1997b) models of HD have been extensively
investigated with the continuous supply of CNTF providing significant protection to striatal neurons (Emerich 1999). Behavioural studies undertaken in both unilaterally and bilaterally QA-lesioned rats with CNTF-secreting implants displayed improvement in both locomotor and cognitive functions to the extent that bilaterally lesioned rats with CNTF secreting implants were non-distinguishable from sham-lesioned animals (Emerich et al. 1996; Emerich et al. 1997a). Emerich and colleagues showed additional protection of layer V motor cortex neurons and maintenance of GABAergic innervations of the globus pallidus and substantia nigra pars reticulata using a primate model of HD (Emerich et al. 1997b). Mittoux et al. (2000) reported similar results using encapsulated CNTF-secreting cells implanted bilaterally into the striatum of a primate 3-NP model of HD, with secretion of CNTF beginning at symptom onset providing restoration of cognitive and motor functions associated with persistent neuronal loss or dysfunction (Mittoux et al. 2000).

Direct in vivo gene delivery to direct enhanced CNTF expression throughout the striatal parenchyma has also been investigated. LV-CNTF vector delivery prior to QA lesioning significantly prevented neurodegeneration of DARPP-32 striatal projection neurons, cholinergic neurons and NADPHd containing interneurons, significantly attenuating apomorphine-induced rotational behaviour (de Almeida et al. 2001). Assessment of Ad-CNTF gene delivery to the striatum demonstrated retrograde vector transport and anterograde CNTF transport resulting in widespread distribution of CNTF protecting the integrity of the cortical-striatal-pallidal connections from 3-NP induced cell death (Mittoux et al. 2002).

1.7 Apoptosis and Huntington’s Disease Neurodegeneration

With the molecular mechanisms underlying HD neurodegeneration not yet fully elucidated, the extent to which apoptotic mechanisms contribute to the selective neuronal cell loss remains to be conclusively determined. While the expression of mutant huntingtin – both in cell culture and in transgenic mice – induces apoptotic cell death (Reddy et al. 1998; Hodgson et al. 1999; Wang et al. 1999; Li et al. 2000; Rigamonti et al. 2000; Jana et al. 2001), the relative lack of clear apoptotic features in HD brains has caused controversy over whether the neurons die by apoptotic or necrotic cell death (Hickey and Chesselet 2003; Sawa et al. 2003).

Apoptosis is typically characterised by nuclear and cytoplasmic condensation, DNA fragmentation, membrane blebbing and finally the phagocytosis of membrane-bound apoptotic bodies (Kerr et al. 1972). This programmed cell death commonly occurs in isolated cells requiring the initiation of a cell death pathway to induce the expression and activation of pro-apoptotic proteins including
numerous proteases (e.g. caspases) and endonucleases (Martin and Green 1995; Sastry and Rao 2000). DNA fragmentation has been commonly used to study apoptosis by in situ TUNEL labelling of DNA fragments (Gavrieli et al. 1992), however random DNA cleavage is often observed in neurotic tissue making apoptotic and necrotic cell death indistinguishable by DNA fragment labelling alone (Charriaut-Marlangue and Ben-Ari 1995). Therefore while TUNEL labelling has been observed in the post-mortem striatum of HD patients (Dragunow et al. 1995; Portera-Cailliau et al. 1995; Thomas et al. 1995; Butterworth et al. 1998), the lack of typical apoptotic DNA laddering (Cohen et al. 1994) suggests these cells may not be undergoing apoptotic cell death (Dragunow et al. 1995; Portera-Cailliau et al. 1995). More recently the implication of apoptotic mechanisms contributing to HD has been indicated by an increase in the expression of pro-apoptotic proteins in post-mortem HD brains, including caspase-9, Bax, cytochrome c and poly(ADP-ribose) polymerase (Kiechle et al. 2002; Vis et al. 2005). Vis and colleagues (2005) suggested that the difficulties which have been encountered in positively identifying neurons exhibiting typical apoptotic morphology are due to the slow progressive nature of HD, meaning that only a few cells are likely to be undergoing cell death at any one time.

Apoptosis of striatal neurons has been found to occur in the excitotoxic QA lesion model of HD (Dure et al. 1995; Portera-Cailliau et al. 1995; Hughes et al. 1996; Qin et al. 1996; Bordelon et al. 1999; Nakai et al. 1999), and also in full-length transgenic animal models which display neuronal cell loss (Reddy et al. 1998; Hodgson et al. 1999). Following QA injection into the rat striatum both apoptotic and necrotic cell death is observed to occur, with the apoptotic cells observed mainly in the dorsomedial striatum (Bordelon et al. 1999). Portera-Cailliau and colleagues (1995) found typical apoptotic DNA laddering in the hours following QA lesioning but not at later time points suggesting that both apoptotic and necrotic mechanisms may contribute towards the QA induced cell death process. Investigations with genetic models of HD, including the YAC transgenic mice (full-length huntingtin with 46, 72 or 128 poly-Q repeats; (Hodgson et al. 1999)), have demonstrated greater vulnerability to excitotoxic death mediated by an enhancement of NMDA receptor activity resulting in increased intracellular calcium levels (Levine et al. 1999; Cepeda et al. 2001; Laforet et al. 2001; Zeron et al. 2002). More specifically the poly-Q huntingtin protein induces potentiation of the NR2B receptor subunit which is predominantly expressed by the selectively vulnerable striatal projection neurons (Landwehrmeyer et al. 1995; Testa et al. 1995; Chen et al. 1999; Sun et al. 2001; Zeron et al. 2001; Zeron et al. 2002). Increased calcium levels have been well established to induce apoptosis via the intrinsic, mitochondrial regulated apoptotic pathway (Figure 1-5; (Hajnoczky et al. 2003; Orrenius et al. 2003)).
Molecular mechanisms responsible for triggering apoptosis are dependant on the specific apoptotic stimuli with different cell death pathways being activated by different insults, although most ultimately lead to the activation of an “executioner” caspase (Adams 2003). The two most well understood pathways are the extrinsic death-receptor pathway and the intrinsic apoptotic pathway. The extrinsic pathway requires activation of death receptors, such as tissue necrosis factor or Fas, which in turn activate “initiator” caspases (caspase-8 and -10), subsequently leading to the activation of the executioner caspases-3 and / or -7 (Ashkenazi and Dixit 1998). Intrinsic apoptosis is initiated

![Figure 1-5 Potential involvement of Ca$^{2+}$ signalling in the induction of apoptosis in HD](image)

Excessive cytosolic Ca$^{2+}$ can lead to mitochondrial Ca$^{2+}$ overload and the release of apoptogenic factors, including cytochrome c, inducing intrinsic apoptosis. Striatal neurons abundantly express NDMA receptors (NMDAR) and mGluR5 which when stimulated by corticostriatal glutamate (Glu) release cause an increase in cytosolic Ca$^{2+}$ – NMDAR directly through allowing Ca$^{2+}$ entry, and mGluR5 via inositol 1,4,5-trisphosphate (InsP$_3$) signalled release of Ca$^{2+}$ from intracellular storage organelles. In HD, expanded huntingtin (Htt$^{exp}$) disrupts Ca$^{2+}$ signalling through potentiation of the 2B subunit of NMDAR, sensitisation of InsP$_3$ receptors (InsP$_3$R1), and destabilisation of mitochondrial handling. Therefore normal glutamate stimulation of striatal neurons can result in supranormal Ca$^{2+}$ responses contributing to the vulnerability of medium spiny neurons (MSN) to HD induced degeneration. Although not directly targeted at the disrupted Ca$^{2+}$ signalling, anti-apoptotic factors may potentially attenuate the induction of apoptosis of striatal neurons through maintaining mitochondrial membrane integrity – Bcl-x$_L$ – or inhibiting caspase activation – XIAP. Mito, mitochondria; MCU, mitochondrial Ca$^{2+}$ uniporter. (Modified from (Tang et al. 2005), PNAS).
internally by cellular stress causing increased free calcium uptake into the mitochondria and opening of the mitochondrial permeability transition pores allowing the release of pro-apoptotic factors, including cytochrome c, from the mitochondria (Figure 1-5 and 1-5; (Hajnoczky et al. 2003; Orrenius et al. 2003)). Pro-apoptotic Bcl-2 family proteins such as Bax and Bak also appear to be critical for inducing apoptosis through recruitment to the outer mitochondrial membrane inducing the release of apoptotic factors, although there is still considerable question over how this occurs (Figure 1-6; (Gross et al. 1999; Martinou and Green 2001; Newmeyer and Ferguson-Miller 2003)). Cytochrome c is a co-factor for apoptotic protease activating factor-1 which can bind procaspase-9 in the presence of cytochrome c allowing the formation of large apoptosomes and the activation of bound caspase-9 leading to the subsequent activation of caspases-3 and -7 (Zimmermann et al. 2001). Additional death-promoting factors released from the mitochondria include Smac/DIABLO

**Figure 1-6  Mitochondrial permeabilisation in intrinsic apoptosis**

In healthy cells the integrity of the mitochondrial outer membrane is maintained by anti-apoptotic Bcl-2-like proteins (Bcl-2) with caspases in the cytosol present as inactive pro-proteins. Apoptotic signalling causes release of sequested BH3-only proteins to interact with Bcl-2, allowing the recruitment of pro-apoptotic Bcl-2 family proteins (Bax, Bak) to the mitochondrial membrane facilitating release of apoptogenic proteins. Cytochrome c induces binding of Apaf-1 and procaspase-9 to form apoptosomes activating procaspase-9 (c9) and subsequently caspase-3 (c3). Cytosolic IAP are inhibited and degraded by the release of Smac/DIABLO (Diablo) and HtrA2/OMI (Omi) proteins, while further apoptogenic proteins (apoptosis inducing factor (AIF), endonuclease G (endoG)) can enter the nucleus inducing DNA degradation. (Reproduced from (Adams 2003), Genes and Development)
and HtrA2/OMI proteins that bind and degrade inhibitor of apoptosis proteins (IAP), preventing caspase sequestration and inhibition (Figure 1-6; (Srinivasula et al. 2003; Saelens et al. 2004)).

In addition to the potentiation of NMDA receptor signalling, the poly-Q huntingtin protein has been shown to directly impair mitochondrial function (Figure 1-5; (Sawa et al. 1999; Panov et al. 2002; Choo et al. 2004)) and initiate cytotoxic mechanisms following N-terminal caspase cleavage (Wellington et al. 2002) including transcriptional dysregulation, proteosome inhibition and the formation of aggregates and neuronal intranuclear inclusions (Figure 1-1; (Sawa et al. 2003)).

Rigamonti and colleagues have shown that wild-type huntingtin has pro-survival properties that protect neurons against apoptotic cell death at the level of procaspase-9 processing (Rigamonti et al. 2000; Rigamonti et al. 2001), suggesting that the expanded poly-Q tract not only has toxic “gain-of-function” consequences, but normally occurring anti-apoptotic functions are also impaired. A recent investigation found enhanced expression of full-length wild-type huntingtin provided neuroprotection against QA-induced excitotoxicity with reduced activation of caspase-3 (Leavitt et al. 2006). Activation of caspase-3 has been shown to be induced by mutant N-terminal huntingtin fragments suggesting a destructive feedback loop maybe initiated resulting in extensive activation of caspase-3 (Wellington et al. 2002; Gafni et al. 2004). Induced activation of caspase-3 in HD suggests that whether or not the striatal projection neurons die strictly by apoptosis, apoptotic mechanisms are likely to underlie the neurodegeneration by stressing normal cellular functions and increasing the neurons sensitivity to other potentially neurotoxic insults. Therefore an enhancement of endogenous anti-apoptotic factors or administration of apoptotic inhibitors could conceivably be of therapeutic benefit by preventing the caspase-mediated cleavage of huntingtin, thereby reducing the production of toxic N-terminal fragments and maintaining pro-survival function, or via general augmentation of the cells ability to withstand cellular stress.

With the pathogenesis of HD appearing to involve disruptions to numerous cellular processes involved in the induction of apoptotic related neurodegeneration, a number of targets have been identified for potential therapeutic intervention including NMDA receptor-mediated excitotoxicity, mitochondrial dysfunction, transcriptional dysregulation, and apoptotic executing caspases. A number of pharmaceuticals have shown efficacy in attenuating HD neuronal death in preclinical trials, with some clinical trials currently being conducted (Leegwater-Kim and Cha 2004; Handley et al. 2006). However a less studied approach is the use of gene delivery to enhance the expression of naturally occurring anti-apoptotic factors that may counteract pro-apoptotic mechanisms while additionally increasing the capacity of the neurons to handle neurotoxic insult (Mochizuki et al. 2002).
1.7.1 Anti-apoptotic Bcl-2 and Bcl-x\textsubscript{L} proteins

Bcl-2 family proteins are involved in regulating the release of mitochondrial pro-apoptotic factors, including cytochrome c, which is a key event in the initiation of the intrinsic apoptotic pathway (Figure 1-6; (Cory and Adams 2002)). There are three groups of Bcl-2-like proteins: anti-apoptotic proteins (e.g. Bcl-2, Bcl-x\textsubscript{L}), pro-apoptotic proteins (e.g. Bax, Bak) and BH3-only proteins (e.g. Bid, Bad). Anti-apoptotic Bcl-2 and Bcl-x\textsubscript{L} are integral membrane proteins located on the mitochondrial, endoplasmic reticulum and nuclear membranes that are thought to preserve the permeability of the membranes against the potential pore-forming pro-apoptotic proteins, although the actual molecular mechanisms remain controversial (Martinou and Green 2001; Kuwana \textit{et al.} 2002; Newmeyer and Ferguson-Miller 2003). The pro-apoptotic proteins appear to be recruited to the membranes following a cellular apoptotic stimulus to facilitate the release of pro-apoptotic factors (Figure 1-6). However in order for apoptosis to occur, the inhibitory effects of the anti-apoptotic proteins need to be overcome; this is thought to involve interactions with BH3-only proteins (Huang and Strasser 2000; Gross 2001). Over-expression of Bcl-2 or Bcl-x\textsubscript{L} can prevent apoptosis occurring following a variety of cytotoxic stimuli including neurotrophic deprivation, ionizing radiation and oxidative stress. There does not appear to be a significant difference in the protective effects of Bcl-2 and Bcl-x\textsubscript{L}, however endogenous expression of Bcl-2 is seen to peak during embryonic development while Bcl-x\textsubscript{L} expression continues to increase postnatally, reaching peak expression in the adult CNS (Gonzalez-Garcia \textit{et al.} 1994; Gonzalez-Garcia \textit{et al.} 1995).

The therapeutic potential for using Bcl-2 or Bcl-x\textsubscript{L} in neurodegenerative diseases has been shown by a few studies using viral vectors to cause overexpression in the CNS. HSV vector delivery of Bcl-2 to the substantia nigra prior to 6-hydroxydopamine lesioning doubled the survival rate of dopaminergic neurons (Yamada \textit{et al.} 1999). Similarly AAV-mediated overexpression of Bcl-2 in the CA1 hippocampal neurons attenuated cell death and reduced DNA fragmentation following ischemia even when AAV delivery was delayed until one hour after the ischemic insult (Shimazaki \textit{et al.} 2000). Motor neuron survival in a transgenic mouse model of amyotrophic lateral sclerosis was also increased by AAV mediated delivery of Bcl-2 which consequently delayed the onset of muscular deficits (Azzouz \textit{et al.} 2000). The LV vector mediated overexpression of Bcl-x\textsubscript{L} in septal cholinergic neurons was highly efficient in preventing axotomised cell death of transduced neurons (Blomer \textit{et al.} 1998), while AAV-Bcl-x\textsubscript{L} retrogradely delivered to entorhinal projection neurons also protected against axotomy-induced apoptosis (Kaspar \textit{et al.} 2002). A limitation of anti-apoptotic factor mediated protection is that only the individual transduced cells that are over-expressing the anti-apoptotic protein can be expected to show increased resistance to an apoptotic stimulus in
contrast to a secreted neuroprotective molecule that can have paracrine activity and therefore provide assistance to neighbouring cells. The simultaneous delivery of a neurotrophic factor and an anti-apoptotic factor as investigated by Natsume and colleagues (2001) using HSV vector delivery of both Bcl-2 and GDNF to the substantia nigra ensured that secreted GDNF may at least provide some protective support to those neurons not expressing the anti-apoptotic factor. Co-injection of HSV vectors encoding Bcl-2 and GDNF resulted in the additive survival rate of dopaminergic neurons following 6-hydroxydopamine induced degeneration (Natsume et al. 2001).

More recently neuroprotection against glutamate excitotoxicity has been reported following AAV-Bcl-xL transduction of neuroblastoma cells and primary motor neurons as a model of amyotrophic lateral sclerosis (Garrity-Moses et al. 2005). Similarly, LV-Bcl-2 delivery to the CA1 region of the hippocampus attenuated neuronal cell death following an excitotoxic NMDA injection (Wong et al. 2005). While these studies have indicated Bcl-2 / Bcl-xL is capable of imparting neuroprotection against excitotoxic insult, an earlier investigation of QA-induced cell death in the striatum of transgenic mice over-expressing Bcl-2 reported no attenuation of the loss of DARPP-32 expressing striatal projection neurons (Maciel et al. 2003).

1.7.2 Inhibitors of apoptosis

With caspases playing a vital role in the initiation and physical execution of apoptosis, they present a reasonably specific target for inhibiting apoptosis independent of the apoptotic pathway that is initiated. The function of inhibiting caspases is carried out in healthy cells by a family of proteins known as inhibitors of apoptosis (IAP) (Robertson et al. 2000). Six members of this family have been identified in humans: Neuronal apoptosis inhibitor protein (NAIP), X-chromosome-linked IAP (XIAP), Human IAP-1 (HIAP-1), HIAP-2, Survivin and Bruce (Deveraux and Reed 1999). The neuroprotective properties of these IAPs have been investigated with the most potent caspase inhibitor proving to be XIAP (Simons et al. 1999) which has two sites capable of inhibiting caspase activity – one site that is specific for caspase-3 and -7, and the other a specific inhibitor of caspase-9 (Deveraux et al. 1999). By inhibiting the initiator caspase-9, XIAP is capable of inhibiting both the activation and the activity of the executioner caspases-3 and -7.

Robertson and colleagues have reported that NAIP is up-regulated in cholinergic striatal interneurons following ischemic insult, suggesting that the neuronal resistance to ischemia and excitotoxicity may be related to the inhibition of caspases (Xu et al. 1997). Ad-vector mediated delivery of NAIP prior to 6-OHDA lesioning provided protection to nigrostriatal dopaminergic neurons, attenuating the amphetamine-induced motor deficits (Crocker et al. 2001). Over-expression of NAIP (Xu et al.
1997) or XIAP (Xu et al. 1999) in hippocampal CA1 neurons prevented ischemic-induced apoptosis by inhibiting caspase-3 activation. The Ad-XIAP delivery to CA1 neurons increased survival 5-6 fold and attenuated the loss of spatial memory as measured in the Morris water maze (Xu et al. 1999), while delivery to retinal ganglion cells after axotomy of the optic nerve prevented any secondary apoptotic cell death (Kugler et al. 2000). More recently a reduction in DNA methylation-induced apoptosis in photoreceptors has been shown following AAV-XIAP transduction (Petrin et al. 2003a; Petrin et al. 2003b).

Perrelet and colleagues have similarly shown that the adenoviral mediated overexpression of NAIP, HIAP-1 or HIAP-2 are each capable of attenuating the degeneration of motor neurons following sciatic nerve axotomy (Perrelet et al. 2000). In addition they have demonstrated by selective antisense inhibition of IAPs the absolute requirement of XIAP and NAIP to mediate the neuroprotective effects of GDNF, but not BDNF or CNTF (Perrelet et al. 2002). Only GDNF treatment upregulated XIAP and NAIP expression showing the stimulation of anti-apoptotic pathways in GDNF mediated neuroprotection (Perrelet et al. 2002). Ad-mediated over-expression of XIAP and GDNF have also been shown to act synergistically to protect nigrostriatal dopaminergic neurons against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced cell death and maintain striatal dopamine levels (Eberhardt et al. 2000). While XIAP alone was sufficient to rescue the nigrostriatal neurons from cell death, the dopaminergic striatal terminals degenerated in the absence of GDNF (Eberhardt et al. 2000). Recent evidence that XIAP and HIAP-1 undergo degradation in the HD caudate, following aberrant release of pro-apoptotic mitochondrial proteins Smac/DIABLO and HtrA2/OMI, further suggests upregulation of IAP may be therapeutically beneficial for HD (Goffredo et al. 2005).

1.8 Summary

The growing understanding of pathogenic processes initiated in HD by an expanded poly-Q tract in huntingtin, has provided an opportunity to develop therapeutic intervention tailored towards either the potentiation of an impaired cellular mechanism, or conversely the attenuation of acquired cytotoxic functions. With a large pre-symptomatic window-of-opportunity available through genetic testing for HD in “at-risk” individuals, preventative therapeutic intervention instigated prior to the initiation of neuronal dysfunction leading to selective neurodegeneration is a serious proposition for HD patients. Excitotoxicity appears to play a significant role in the ultimate death of the vulnerable striatal neurons with mutant huntingtin causing a reduction in vital neurotrophic support through interruption of BDNF transcription and transportation, and interference with the molecular
machinery of programmed cell death resulting in impaired ability to handle the naturally high corticostriatal glutamate stimulation.

Figure 1-7  Model of neurotrophic factor and anti-apoptotic factor supplied protection. AAV-mediated gene delivery to targeted striatal neurons and possibly adjacent glial cells results in stable biotherapeutic protein expression within the striatum. Non-secreted anti-apoptotic factors – Bcl-xL and XIAP – remain contained within the transduced cells acting in the cytosol and at the mitochondrial membrane to inhibit apoptotic mechanisms. Only directly transduced neurons are exposed to enhanced anti-apoptotic factor expression. Neurotrophic factors – BDNF and GDNF – are secreted following production within transduced cells providing autocrine and paracrine actions on the target striatal neurons through cell surface receptors and therefore providing trophic support to a greater number of neurons than actually transduced. The transduction of cells in the projection nuclei may additionally provide target-derived trophic support through retrograde transportation of the neurotrophic factors. (Modified from Blomer et al. 1998, PNAS)

From current research it appears that while neurotrophic factors and anti-apoptotic factors hold real potential as therapeutic agents, the need for a continuous supply across the entire striatum makes the mechanism of delivery vitally important for the success of these factors as effective therapeutic agents. Cellular transplantation and direct in vivo gene delivery techniques appear to be the most promising delivery methods for neurotrophic factors, as physiologically relevant concentrations can be continuously produced locally within the striatum directly supplying support to surrounding striatal neurons, while limiting any adverse effects resultant of activity in non-targeted regions. Non-secreted biotherapeutics, such as anti-apoptotic factors, require very efficient delivery techniques, generally requiring in vivo gene delivery vectors, as only cells directly expressing / receiving the
therapeutic agent will benefit from any effects. Therefore further research and development of effective gene delivery techniques are vital for pursuing the true potential of targeted biotherapeutics for attenuating the insidious progression of HD.

1.9 Thesis Objectives

This thesis set out to investigate if continuous neurotrophic factor or anti-apoptotic factor expression achieved via in vivo gene delivery can attenuate neuronal cell loss and behavioural deficits in the excitotoxic QA lesion animal model of HD.

Although neurotrophic factors have been previously demonstrated to partially prevent the death of striatal projection neurons, difficulties in achieving efficient delivery of neurotrophic factors to the striatum in vivo may limit the true potential that neurotrophic factors hold for counteracting the neurodegenerative process of HD. Ongoing improvement in non-toxic, non-immunogenic viral based vectors has allowed for the efficient delivery of therapeutic proteins to the CNS following in vivo gene transfer. In vivo gene delivery is suited to the continuous production of therapeutic proteins within a specific population of cells such as the neurons of the striatum, and this local production of neurotrophic factors at physiologically effective concentrations across the entire striatum may prove to be therapeutically beneficial at preventing or slowing the progression of HD. Construction of recombinant AAV vectors and the verification of functional transgenic protein expression is detailed in Chapter 2 (Kells et al. 2006).

With a large number of neuroprotective compounds currently under investigation following the ongoing elucidation of molecular mechanisms underlying HD, it has been recently suggested that preclinical studies of pharmaceutical and biotherapeutic agents for HD should be proven efficacious in transgenic models of HD, by two independent research groups, prior to moving forward into non-human primate and clinical trials (Bates and Hockly 2003). However the increasing evidence that excitotoxic mechanisms contribute to HD neurodegeneration ensure that the QA model is still a relevant research tool to investigate neuroprotective agents. Chapter 3 covers the optimisation and characterisation of a unilateral QA lesion rat model of HD.

Previous in vivo gene delivery mediated expression of neurotrophic factors in the striatum has demonstrated the protection of striatal neurons by BDNF, GDNF and CNTF (Bemelmans et al. 1999; de Almeida et al. 2001; Mittoux et al. 2002; McBride et al. 2003; Kells et al. 2004). While neurotrophic factors show promise in these studies, there is a need for further investigation of more efficient gene delivery vectors, assessment of functional behavioural protection and determination of
neuronal fibre protection in order to fully evaluate the most promising approaches to advance through to primate studies and clinical trials.  **Preventative therapeutic delivery of BDNF or GDNF prior to QA striatal lesioning is investigated in Chapter 5.**

Targeted delivery of anti-apoptotic factors to the striatum in an attempt to prevent striatal cell death has not previously been investigated as a potential treatment for HD, although *in vivo* gene delivery of Bcl-2-like proteins (Bcl-2 and Bcl-x\textsubscript{L}) and IAPs (XIAP and NAIP) have provided protection to other neurons following apoptotic stimulating insult.  With apoptotic mechanisms being induced in HD, anti-apoptotic factor expression may hold therapeutic potential to enhance the survival of striatal neurons.  **In Chapter 6 I investigate if enhanced Bcl-x\textsubscript{L} or XIAP expression can attenuate QA-induced excitotoxic neuronal cell death.**

Ultimately this thesis research aimed to investigate whether the use of chimeric AAV\textsubscript{1/2} vectors for the *in vivo* gene delivery of the neurotrophic factors BDNF or GDNF, or the anti-apoptotic factors Bcl-x\textsubscript{L} or XIAP could efficiently generate continuous production within the rat striatum at a level that is sufficient to maintain the functional integrity of the striatal neurons in the face of an acute excitotoxic insult.  This was achieved by assessment of neuropathological changes and functional behavioural impairments.
Chapter 2

**Adeno-Associated Viral Vectors:**

*Plasmid Cloning, Vector Packaging and In Vitro Functional Testing*

### 2.1 Overview

AAV derived recombinant vectors have become the vector of choice for many investigators studying potential clinical applications of gene delivery primarily due to their efficient stable transduction of post-mitotic cells and non-pathogenic viral origin (Peel and Klein 2000). To mediate gene transfer to striatal neurons I produced high-titre recombinant AAV vectors containing cDNA sequences for each of the potential biotherapeutic proteins. It is clearly essential for gene delivery applications that the protein products expressed following gene transfer into the target cells are correctly translated and processed into functionally active molecules capable of influencing cellular activity. Therefore it was important that I developed procedures to assess the functional impact of each transgenic protein following rAAV vector transduction. Given the well characterised functional activity of the neurotrophic factors and anti-apoptotic proteins under investigation, I devised efficient *in vitro* assays to demonstrate the expected functional impact of each protein.

### 2.2 AAV Vector Development and Production Procedures

Production of recombinant AAV vectors was undertaken *in vitro* by co-transfection into HEK293 cells of a plasmid containing my AAV vector expression cassette along with helper plasmids encoding AAV *rep* and *cap* proteins and adenoviral genes essential for AAV production. The use of separate helper plasmids supplying the necessary AAV replication and packaging proteins in *trans* ensures the production of replication deficient vectors with only the expression cassette DNA flanked by AAV specific inverted terminal repeat (ITR) sequences being packaged into the AAV particles.


Note: Production of the AAV-BDNF plasmid construct was kindly undertaken by Rebecca Henry, Department of Pharmacology, The University of Auckland. Functional testing of the AAV-BDNF vector was performed in conjunction with Rebecca Henry.*
Replication deficient viral particles were subsequently harvested from the HEK293 cells, purified by heparin affinity column and centrifuge concentrated to provide high titre stocks for *in vivo* gene delivery.

Development of rAAV vectors for the neuroprotective gene delivery investigations involved cloning the therapeutic genes-of-interest into a previously constructed AAV expression cassette (Klugmann *et al.* 2005), packaging and purifying the AAV vector particles, testing for efficient AAV transduction and assaying for functional transgenic protein expression *in vitro*.

### 2.2.1 Molecular biology protocols

#### 2.2.1.1 PCR Cloning

To facilitate ligation of the genes-of-interest into AAV expression cassettes I PCR amplified the cDNA sequences from source plasmids (Table 2-1) using customised primer pairs (Table 2-2) incorporating unique restriction sites to flank the cDNA sequences and complement the restriction sites at the insertion site of the AAV backbone plasmid. Direct replacement of the cDNA stop codons with either an *Eco*RI or *Bgl*II restriction site sequence allowed in-frame ligation with the downstream HA-tag sequence.

The PCR program to clone each gene-of-interest was tested using Red Hot® DNA polymerase (ABgene) to optimise the primer annealing temperature and cycle number required to produce a single clean DNA product. Each reaction was setup with 2.5µL Red Hot® PCR Reaction buffer (ABgene), 2.0µL MgCl₂ (25mM; ABgene), 1.0µL dNTPs (2.5mM; Invitrogen), 0.2µL forward primer (10µM; Invitrogen), 0.2µL reverse primer (10µM; Invitrogen), 1.0µL miniprep DNA plasmid template, 0.25µL Red Hot® DNA Polymerase (ABgene) and made up to 25.0µL with sterile dH₂O. Reactions were run in a GeneAmp® PCR System 9700 (Applied Biosystems) thermal cycler programmed for:

<table>
<thead>
<tr>
<th>Initial denaturing</th>
<th>95°C</th>
<th>5 min</th>
<th>1 cycle</th>
</tr>
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<tbody>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Annealing</td>
<td>56 / 60 / 65°C</td>
<td>30 sec</td>
<td>35 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>60 sec</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72°C</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
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</tbody>
</table>

PCR products were visualised on a 1% agarose gel against a 1kb Plus DNA ladder (Invitrogen).
Following optimisation of the PCR program with Red Hot® DNA polymerase a larger scale PCR was performed using an Expand High Fidelity PCR System (Roche). Each PCR was setup with 5.0µL Expand High Fidelity Buffer containing 15mM MgCl₂ (Roche), 4.0µL dNTPs (2.5mM; Invitrogen), 1.5µL forward primer (10µM; Invitrogen), 1.5µL reverse primer (10µM; Invitrogen), 1.0µL miniprep DNA plasmid template, 1.0µL Expand High Fidelity Enzyme mix (Roche) and made up to 50.0µL with sterile dH₂O. PCR program:

<table>
<thead>
<tr>
<th>Initial denaturing</th>
<th>94°C</th>
<th>2 min</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing</td>
<td>94°C</td>
<td>15 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>62°C</td>
<td>60 sec</td>
<td>30–35 cycles</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>90 sec</td>
<td></td>
</tr>
<tr>
<td>Final Elongation</td>
<td>72°C</td>
<td>7 min</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
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</tbody>
</table>

PCR products were visually checked by running a sample on a 1% agarose gel and then separated out by gel extraction or with a Wizard® SV Gel and PCR Clean-Up System kit (Promega).

### 2.2.1.2 Agarose Gel Extraction

The extraction of DNA fragments from agarose gel was performed to isolate a specific length DNA product following PCR or restriction digests. Reaction mixtures were run on a 1% low melting point agarose gel in TAE buffer. A single band containing the DNA product-of-interest was excised, transferred to a Costar Spin-X column (Corning) and frozen at -80°C for 5mins. The DNA was eluted through the column by centrifugation at 12,000rpm for 10mins (Heraeus Biofuge Stratos). Spin-X column was discarded and 600µL phenol:chloroform:isoamyl alcohol mixture (Sigma-Aldrich) added to separate out DNA by further centrifugation at 12,000rpm for 10mins. The top layer containing my DNA product was transferred to a new centrifuge tube and made up to 150µL with NaCl solution to give a final concentration of 200nM NaCl. DNA was precipitated and pelleted by adding 375µL 95% ethanol, centrifuging at 12,000rpm for 2min and carefully discarding supernatant. The DNA was washed with 70% ethanol and dried at room temperature before resuspending in 20µL sterile dH₂O and storing at -20°C.

The Wizard® SV Gel and PCR Clean-Up System (Promega) was also used as an alterative to the Costar Spin-X columns for agarose gel extraction or for direct PCR product purification. Briefly the gel band or PCR mixture was incubated with a Membrane Binding Solution and loaded onto a SV Minicolumn by centrifugation. The DNA bound to the SV Minicolumn was washed with an ethanol based Membrane Wash Solution and then eluted with 50µL sterile dH₂O.
2.2.1.3 p-GEM®-T Easy Vector
PCR products encoding single cDNA gene sequences flanked by unique restriction sites were ligated into pGEM®-T Easy TA cloning vectors (Promega) as per the suppliers’ instructions. Ligations were setup with 5.0µL Rapid Ligation Buffer, 1.0µL pGEM®-T Easy vector, 3.0µL PCR product, 1U T4 DNA ligase and incubated overnight at 4°C. Overnight ligation mixture was used to heatshock transform Escherichia coli (E. coli) competent cells (Section 2.2.1.4). Insertional disruption of the LacZ gene spanning the pGEM®-T Easy vector ligation site provides ready identification of colonies containing an insert by their white appearance when grown on LB agar (USB) plates containing ampicillin (amp; 50 µg/mL; Sigma-Aldrich) and X-gal (20 mg/mL; Sigma-Aldrich). A single transformed colony containing the correct insert was selected by colony PCR screening and used to inoculate overnight cultures for plasmid mini prep and for long-term storage as a 30% glycerol stock.

2.2.1.4 Heatshock Transformation
Transformation of E. coli cells was performed by mixing the plasmids with competent cells and providing a heatshock stimulus to induce plasmid uptake. A vial of DH5α E. coli Max Efficiency Competent cells (500µL; Invitrogen) was thawed on ice from -80°C and gently mixed with 0.1µL plasmid mini-prep. After 30mins incubation on ice the cells were heatshocked at 42°C for 60secs and placed back on ice for 1-2mins before transferring to a centrifuge tube with 450µL LB and incubating at 37°C for 1hr. The cells were then plated overnight at 37°C on LB agar containing ampicillin, with or without X-gal, to select for transformed E. coli colonies.

2.2.1.5 Colony PCR
Colony PCRs were used to screen E. coli colonies following heatshock transformation for the presence of a plasmid containing a cDNA sequence of the specific gene-of-interest. Individual colonies were picked off an LB agar / amp plate with a sterile tip, streaked gently across a fresh LB agar / amp plate and then suspended in 20µL LB. Using 2.5µL of the resuspended culture for the DNA plasmid template I performed PCRs to amplify the gene-of-interest using the Red Hot® DNA polymerase protocol as described in Section 2.2.1.1.

2.2.1.6 Restriction Enzyme Digestions
Complementary restriction digests were performed in preparation for transferring the genes-of-interest from the pGEM®-T Easy vectors into the AAV expression cassette. Digests were setup in 1.5mL eppendorf tubes using 2.0µL DNA plasmid prep, 2.0µL NEB Buffer (Table 2-4), 2.0µL BSA (NEB), 20U of each restriction endonuclease (Table 2-4), made up to 20µL with sterile dH2O and incubated at 37°C for 2-3hrs. Following restriction digestion the desired DNA fragment was gel
extracted on a low melting point 1% agarose gel using the Wizard® SV Gel and PCR Clean-Up System.

2.2.1.7 **Ligation into the AAV Expression Cassette**
Ligation of the gene-of-interest into the AAV expression cassette was facilitated by complimentary ssDNA “sticky” ends on the AAV vector and DNA inserts generated by the restriction digests. To restrict self ligation of the AAV backbone vector it was treated with calf intestinal phosphatase (CIP) to remove the 5’ phosphate groups. Following restriction digest of the AAV vector the restriction enzymes were inactivated by incubation at 65°C for 20mins, 1.0µL of CIP was added to the digestion reaction mix and incubated at 37°C for 30min. CIP was inactivated by incubation at 75°C for 30mins.

Ligation reactions were setup with 1.0µL CIP treated AAV expression cassette digest, 5.0µL insertion DNA, 1.0µL T4 ligase buffer (Invitrogen), 2.0µL dH₂O, 1.0µL T4 ligase enzyme (Invitrogen) and incubated overnight at 4°C.

2.2.1.8 **Plasmid Amplification and Purification**

**DNA plasmid mini-prep**

Small-scale plasmid purifications were undertaken with a High Pure Plasmid Isolation kit (Roche). E.coli cells containing the plasmid-of-interest were pelleted from a 3-4mL overnight LB / amp culture by centrifugation at 9,000rpm for 30secs. Cells were resuspended in 250µL Suspension Buffer containing RNase and lysed by the addition of 250µL Lysis Buffer. Chromosomal DNA and cellular debris was precipitated by adding chilled Binding Buffer and separated by centrifugation at 13,000rpm for 10min. Supernatant containing the DNA plasmid was transferred to a High Pure filter tube and centrifuged at 13,000rpm for 1min. The bound plasmids were washed twice by centrifugation with Wash Buffers before eluting the plasmid DNA with 100µL Elution Buffer (10mM Tris-HCl). Samples of the mini-preps were run on 1% agarose gel to check plasmid yield. DNA plasmid preps were stored at -20°C.

**DNA plasmid maxi-prep**

Large-scale plasmid purifications were performed using a QIAGEN® Plasmid Maxi Kit. An E.coli culture containing the plasmid to be amplified was used to inoculate 500mL LB / amp and incubated overnight at 37°C with vigorous shaking. The overnight culture was transferred to 250mL centrifuge bottles and the E.coli cells harvested by centrifugation at 4,300rpm for 15mins (Sorvael RT7Plus). The supernatant was discarded and the E. coli cells resuspended in buffer solution containing RNase (Buffer P1: 50mM Tris-Cl, 10mM EDTA, 100µg/mL RNase A, pH 8.0). Cells were lysed by
addition of lysis buffer (Buffer P2: 200mM NaOH, 1% SDS) and the cellular debris precipitated by neutralization buffer (Buffer P3: 3.0M potassium acetate). Following centrifugation the plasmid containing supernatant was transferred to an equilibrated QIAGEN-tip. The QIAGEN-tip was washed (Buffer QC: 1.0M NaCl, 50mM MOPS, 15% isopropanol, pH 7.0) and the plasmid eluted (Buffer QF: 1.25M NaCl, 50mM Tris-Cl, 15% isopropanol, pH 8.5). DNA was precipitated with isopropanol and pelleted by centrifugation at 11,000rpm for 30mins. The pelleted DNA was washed with 70% ethanol and air-dried before reconstituting in Tris-EDTA (TE) buffer and stored at -20°C. The plasmid concentration was measured by DNA spectrophotometry (Ultrospec™ 2100, Biochrom).

2.2.1.9 DNA Sequencing

All DNA sequencing was performed on a 3100 capillary sequencer (Applied Biosystems) by the Genomics Unit, Centre for Genomics and Proteomics, School of Biological Sciences, The University of Auckland.

2.2.2 AAV plasmid construction

DNA plasmids containing an empty AAV expression cassette, AAV rep and cap genes and the Ad helper genes required for AAV packaging were made available by Professor Matthew J. During and Dr Deborah Young, Department of Molecular Medicine and Pathology, FMHS, The University of Auckland (Table 2-1, Appendix A.2). The AAV backbone plasmid (Figure 2-1) contained an AAV expression cassette regulated by a strong constitutively active chicken-β-actin (CBA) / cytomegalovirus (CMV) hybrid enhancer-promoter to drive continuous stable expression in all transduced cells (Xu et al. 2001), a woodchuck post-transcriptional regulatory element (WPRE) to further enhance the transgene expression level (Loeb et al. 1999; Paterna et al. 2000) and a bovine growth hormone poly-A (bGH poly-A) tail sequence. Inverted terminal repeat (ITR) sequences from AAV serotype-2 flanked the expression cassette to direct ssDNA packaging into the vector particles. The multiple cloning site for transgene insertion was directly adjacent to a nine amino acid HA sequence that allowed in-frame fusion of an HA epitope-tag to the 3’-end of the transgene by direct replacement of the stop codon with either an EcoRI or BglII restriction enzyme recognition site. Incorporation of the short HA-tag at the C-terminal allowed for unequivocal immunocytochemical staining of the transgenic protein expression, distinct from endogenous neurotrophic factor or anti-apoptotic factor expression. A fully constructed AAV-Luciferase plasmid containing a Firefly Luciferase cDNA sequence with the same regulatory elements, but without an HA-tag, was also supplied by MJ During and D Young to be used as a control for AAV transduction and transgenic protein expression (Appendix A.2.2).
Table 2-1 **Source of cDNA sequences and AAV Plasmids**
cDNA sequences of the genes-of-interest were sourced from either expression cassette plasmids previously constructed by the MJ During and D Young laboratory group, Department of Molecular Medicine and Pathology, The University of Auckland; or commercially sourced from Science Reagents. The empty AAV expression cassette, control AAV-Luciferase and AAV helper packaging plasmids were all kindly supplied by MJ During and D Young.

* Full sequences in Appendix A.1. † Plasmid maps in Appendix A.2.
To investigate the neuroprotective potential of the four proteins-of-interest, BDNF, GDNF, Bcl-xL and XIAP, cDNA sequences for each were transferred into separate AAV expression cassettes. Each of the cDNA sequences were PCR cloned out of their source plasmids (Table 2-1; Appendix A.1) using customised primers (Table 2-2) to add unique restriction sites to each end of the cDNA sequence in preparation for ligation into the AAV expression cassette. All of the forward primers were constructed with a XhoI restriction site (CTCGAG) upstream of the ATG methionine start codon. Reverse primers were designed to allow fusion of the transgene protein with the C-terminal HA-tag by replacing the stop codons with a BglII restriction site (AGATCT) for the BDNF, GDNF and Bcl-xL cDNA sequences. Reverse primer for the XIAP sequence incorporated an EcoRI restriction sequence (GAATTC) due to the presence of a BglII recognition site within the XIAP cDNA sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’ → 3’</th>
<th>TM</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF – Forward</td>
<td>CAACTCGAGCACACGGTGAGAAGAGTGATGAC</td>
<td>82</td>
</tr>
<tr>
<td>– Reverse</td>
<td>CTAGATCTTTCCCTTAATGGTCAATG</td>
<td>75</td>
</tr>
<tr>
<td>GDNF – Forward</td>
<td>GAACTCGAGCAGGGACTCTAAGATGAAG</td>
<td>82</td>
</tr>
<tr>
<td>– Reverse</td>
<td>CAAGATCTGATACATCCACCCGTTTACG</td>
<td>78</td>
</tr>
<tr>
<td>Bcl-xL – Forward</td>
<td>GCCCTCGAGTTATAAAAAATGTCTCAGAGC</td>
<td>77</td>
</tr>
<tr>
<td>– Reverse</td>
<td>GAAGATCTGGTGAAGCGTTCCTGGCC</td>
<td>82</td>
</tr>
<tr>
<td>XIAP – Forward</td>
<td>GCGCTCGAGAATGACTTTTAACAGTTTTGAAGG</td>
<td>78</td>
</tr>
<tr>
<td>– Reverse</td>
<td>CGCAATTCCGAGACATAAAAAATTTTTTGTCTTG</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 2-2 PCR primer sequences
Customised primer pairs were designed to PCR amplify the cDNA sequences from plasmid templates. The forward primers incorporated a unique XhoI restriction site (underlined) upstream of the cDNA coding region (bold). Reverse primers complimentary to the 3’ end of the cDNA gene sequence contained a BglII or EcoRI restriction site (underlined) in place of the stop codon.

Optimisation of PCR programs to clone the cDNA sequences from source plasmid was performed using Red Hot DNA polymerase with annealing temperatures of 56, 60 and 65°C (Section 2.2.1.1). The cleanest PCR fragments were produced with the 65°C annealing temperature, however product yield was reduced compared with 60°C annealing. Reducing the number of cycles for XIAP from 35 to 30 and diluting the template DNA mini-prep 1:200 in dH2O resulted in a cleaner PCR product. After PCR program optimisation, High Fidelity DNA Polymerase was used to ensure accurate cloning of the cDNA sequences. High Fidelity PCRs were run with an annealing temperature of 62°C cycling 35 times for the BDNF, GDNF and Bcl-xL sequences and 30 times for XIAP.
Following PCR cloning of each cDNA sequence I ligated the PCR products into pGEM cloning vectors for long-term storage and easy handling (Section 2.2.1.3). The pGEM plasmids were heatshock transformed into competent *E. coli* cells (Section 2.2.1.4) and individual colonies screened by colony PCR with the same primers (Table 2-2) to confirm insertion of the correct gene-of-interest (Section 2.2.1.5). A single colony generating the correct length PCR product for each gene-of-interest was used to inoculate an LB culture for DNA plasmid mini-prep and storage as a glycerol stock (Section 2.2.1.8). DNA sequencing from plasmid mini-preps to confirm error-free cloning of each cDNA gene sequence was performed using T7 and Sp6 primers to sequence bidirectionally across the pGEM®-T Easy vector insertion site (Section 2.2.1.9). The cDNA sequences were each fully BLAST aligned with matching mRNA database sequences to confirm sequence homology (Table 2-3; Appendix A.1). The Bcl-xL sequence had a C-terminal truncation of the last 21 amino acids containing a transmembrane domain for mitochondrial targeting which has previously been shown to retain anti-apoptotic properties (Fang *et al.* 1994; Muchmore *et al.* 1996). BDNF, GDNF and XIAP cDNA sequences had full homology with database mRNA sequences (Table 2-3).

<table>
<thead>
<tr>
<th>Gene-of-Interest</th>
<th>Description</th>
<th>Accession Number</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>Homo sapiens BDNF mRNA</td>
<td>NM 170735.4</td>
<td>GI: 60218885</td>
</tr>
<tr>
<td>GDNF</td>
<td>Rattus norvegicus GDNF mRNA</td>
<td>NM 019139.1</td>
<td>GI: 9506720</td>
</tr>
<tr>
<td>Bcl-xL*</td>
<td>Homo sapiens Bcl-2-like 1 mRNA</td>
<td>NM 138578.1</td>
<td>GI: 20336334</td>
</tr>
<tr>
<td>XIAP</td>
<td>Homo sapiens BIRC4 mRNA</td>
<td>NM 001167.2</td>
<td>GI: 32528298</td>
</tr>
</tbody>
</table>

Table 2-3 Genes-of-interest

* The Bcl-xL sequence encoded only the first 212 amino acids with a truncation of the final 21 codons encoding a transmembrane domain.

Transfer of the cDNA sequences from pGEM cloning vectors into the AAV expression cassette was performed by excision of the cDNA sequences by restriction digestion (Section 2.2.1.6) followed by ligation into the AAV backbone plasmid (Section 2.2.1.7). Endonuclease digestion of the pGEM plasmids and the AAV backbone plasmid with the same pairs of restriction enzymes (Table 2-4) generated ssDNA overhangs on the excised cDNA sequences that complemented the ssDNA overhangs on the linearised AAV backbone plasmid.

<table>
<thead>
<tr>
<th>pGEM vector insert</th>
<th>Restriction Enzymes</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF / GDNF / Bcl-xL</td>
<td>XhoI / BglII</td>
<td>NEB #3</td>
</tr>
<tr>
<td>XIAP</td>
<td>XhoI / EcoRI</td>
<td>NEB EcoRI</td>
</tr>
</tbody>
</table>

Table 2-4 New England Biolabs (NEB) restriction enzymes and buffers
Digested cDNA sequence fragments were isolated by agarose gel extraction and the AAV backbone DNA CIP treated to prevent self-ligation. Complimentary pairing between the ssDNA overhangs directed orientation dependant ligation of cDNA gene sequences into the AAV expression cassette in-frame with the C-terminal HA-tag (Section 2.2.1.7). The ligated AAV expression cassette plasmids were heatshock transformed into competent *E. coli* cells and plated onto LB agar / amp (Section 2.2.1.4). Individual colonies were screened for the presence of my cDNA gene sequences by colony PCR (Section 2.2.1.5). An *E. coli* colony that generated the correct length PCR product was selected to inoculate an LB culture for DNA plasmid mini-prep and storage as a glycerol stock (Section 2.2.1.8). To check that the cDNA sequences were correctly inserted into the AAV expression cassette in-frame with the HA-tag sequence, I used a DNA primer complementary to the downstream WPRE sequence (5′→3′ CAAATTTTGTAATCCAGAGGTTC) to reverse sequence across the HA-tag and into the 3′ end of my inserted cDNA gene sequences (Section 2.2.1.9).

After confirming the insertion of each cDNA gene sequence into the AAV expression cassette I performed DNA plasmid maxi-preps to generate large quantities of each plasmid for rAAV vector packaging (Section 2.2.1.8). In addition to the AAV backbone plasmids, maxi-preps were performed for each of the helper plasmids (pRV1, pH21 and pFΔ6) required for rAAV packaging. The quantity of DNA in each maxi-prep was determined by spectrophotometry (Ultrospec™ 2100, Biochrom) and I performed restriction digests to check the purity of each plasmid by the length of digested DNA fragments on a 1% agarose gel (Appendix A.2.3).

### 2.2.3 Mammalian cell culture and analysis protocols

#### 2.2.3.1 HEK 293 and HT-1080 Cell Culture

Human embryonic kidney 293 cells (HEK 293) or human osteosarcoma cells (HT-1080) were used for rAAV vector packaging and checking transgene protein expression following plasmid transfection and AAV vector transduction. HT-1080 cells are more receptive to AAV transduction and were utilised for checking transgene protein expression and functional anti-apoptotic activity of AAV-mediated XIAP expression. BDNF, GDNF and Bcl-xL expression was verified using HEK293 cells. A vial containing ~1 × 10⁶ HEK293 or HT-1080 cells was removed from liquid nitrogen storage dewar, quickly thawed and transferred to cold Dulbecco’s Modified Eagle Media (DMEM; GIBCO®) containing 10% fetal bovine serum (FBS). To remove the storage media I pelleted the cells by centrifugation at 1000rpm for 5min (Heraeus Multifuge 3S-R), discarded the supernatant and resuspended the cells in 20mL DMEM / 10% FBS. The resuspended cells were transferred to a single T75 cell culture flask (Nunc) and incubated at 37°C, 5% CO₂ changing media with fresh
DMEM as required. When the cells reached ~70% confluence I passaged them by rinsing with sterile PBS and incubating with 1mL 0.05% trypsin / 0.53mM EDTA for 3-5mins until the cells lifted from the plate with gentle tapping. Once the cells had detached from the culture flask I added 10mL DMEM / 10% FBS media to inhibit the trypsin and stop the dissociation reaction before centrifuging at 1000rpm for 5mins. The cells were resuspended in fresh DMEM / 10% FBS and split into 5 new T75 flasks or counted using a standard hemocytometer (Appendix B.2.2) and seeded onto cell culture plates at the required density.

### 2.2.3.2 In Vitro Immunocytochemistry

Immunocytochemical staining was performed to visualise transgenic proteins and the expression of phenotypic markers following *in vitro* rAAV transduction. *In vitro* cell cultures were fixed by incubation with 4% paraformaldehyde for 10 mins and stored at 4°C in PBS. Endogenous peroxidase activity was blocked by adding a solution of 1% hydrogen peroxide / 50% methanol to the cells for 10 mins and then gently washing four times with PBS-Triton. The primary antibodies were diluted in PBS-Triton with 1% normal goat serum and incubated with the cells overnight at 4°C (Table 2-5). Secondary biotinylated antibodies raised against the primary antibody host species were diluted 1:500 in PBS-Triton with 1% normal goat serum and incubated with the cells for 2-3hrs at room temperature with gentle rocking following four washes of the cells with PBS-Triton. Goat serum was omitted when staining with goat anti-Luciferase primary antibody. Extravidin peroxidase was diluted 1:500 in PBS and incubated with the cells for 2-3hrs at room temperature before I gently washed the cells four times with PBS and added DAB or DAB-nickel solution for 10mins to visualise cells expressing the target protein.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-epitope</td>
<td>HA.11</td>
<td>1:1000</td>
<td>Covance</td>
</tr>
<tr>
<td>Luciferase</td>
<td>Luciferase</td>
<td>1:10000</td>
<td>Chemicon</td>
</tr>
<tr>
<td>GABAergic Neurons</td>
<td>Calbindin</td>
<td>1:5000</td>
<td>Swant</td>
</tr>
<tr>
<td>Dopaminergic Neurons</td>
<td>Tyrosine Hydroxylase</td>
<td>1:500</td>
<td>Chemicon</td>
</tr>
</tbody>
</table>

Table 2-5 Primary antibodies for *in vitro* immunocytochemistry

### 2.2.3.3 Hoechst Nuclear Staining

To visually assess apoptotic cell death *in vitro*, the cells were fixed with 4% paraformaldehyde and then incubated in 10 µg/mL Hoechst 33258 for 10 mins. Hoechst nuclear staining was visualised on an inverted Nikon Eclipse TE2000-U microscope with a UV filter.
### 2.2.4 AAV expression cassette transfection testing

Transfection of HEK293 or HT-1080 cells with my AAV expression plasmids was undertaken to confirm protein expression from cDNA gene sequences inserted into the AAV expression cassettes prior to vector packaging.

HEK293 / HT-1080 cells cultured in high glucose DMEM (GIBCO®) with 10% FBS (GIBCO®) were plated into 12-well poly-D-lysine (Sigma-Aldrich) coated cell culture plates (Nunc) at a seeding density of $2 \times 10^5$ cells/well. After overnight incubation the cells were ~70% confluent and I replaced the media with 1mL IMDM (GIBCO®), 5% FBS for 2hrs prior to calcium precipitate transfection with my AAV backbone plasmids. To form a DNA CaPO$_4$ precipitate I added 5µg of my AAV plasmid prep to 30µL 2.5M CaCl$_2$, made up to 250µL with dH$_2$O, filtered with a 0.2µm syringe filter (Pall) and transferred to 250µL 2× HeBS buffer (50mM HEPES, 280mM NaCl, 1.5mM NaHPO$_4$) while vortexing. After resting for 1min I added the finely precipitated DNA solution drop-wise to the HT-1080 cells media, swirled to mix and incubated for 4-8hrs before changing the media back to DMEM, 10% FBS. Following transfection the cells were incubated with DMEM media for three days before washing with sterile PBS and fixation with 4% paraformaldehyde. Immunocytochemistry was performed with an HA antibody to visualise the expression of the HA-tagged transgenic protein (Section 2.2.3.2).

### 2.2.5 AAV vector production

#### 2.2.5.1 Packaging and Purification of AAV Vector Particles

Production of chimeric rAAV vector particles was undertaken as previously described (During et al. 2003; Hauck et al. 2003) by co-transfection of HEK293 cells with the AAV expression cassette plasmid and AAV packaging plasmids containing the AAV Rep and Cap genes and Adenoviral E2A, E4 and VA genes required to assemble the AAV viral capsule and direct packaging (Matsushita et al. 1998).

HEK 293 cells grown in high glucose DMEM, 10% FBS were seeded into five 15cm cell culture plates (Nunc) at 2.0-2.2 $\times$ 10$^7$ cells per plate one day prior to transfection. With the HEK 293 cells ~70% confluent I replaced the media with 25mL IMDM, 5% FBS 2hrs prior to CaPO$_4$ transfection. A CaCl$_2$–DNA solution was prepared using 12.5µg AAV backbone plasmid, 25.0µg pFΔ6, 6.25µg pRV1, 6.25µg pH21, 330µL 2.5M CaCl$_2$ (final concentration of 0.3M), made up to 13.75mL with dH$_2$O and filter sterilised through a 0.2µm syringe filter (Pall). To precipitate the DNA for transfection I added 2.5mL 2× HeBS buffer to an equal volume of the CaCl$_2$-DNA solution while
vortexing. The transfection solution was then left for 50 secs to form a fine CaPO₄ precipitate before adding drop-wise to one 15cm plate of HEK 293 cells and swirling gently to mix. This was repeated for each of the 5 plates. After overnight incubation (8-15hrs) I replaced the media with 25mL DMEM, 10% FBS. 48-60hrs after transfection I harvested the HEK 293 cells by gently washing with PBS, adding another 25mL PBS to each plate and using a cell scraper to lift the cells. The harvested cells were pooled together by centrifugation at 200g for 5min, resuspended in 50mL of a 150mM NaCl 20mM Tris pH 8.0 solution, split into two 25mL lots and frozen overnight at -20°C.

Isolation of the rAAV vector particles from the harvested HEK 293 cells was performed by adding sodium deoxycholate (final conc. 0.5%) plus Benzonase® endonuclease (50U/mL) and incubating at 37°C for 30mins. Initial cellular debris was separated by centrifugation at 3000g for 15mins, 4°C. The supernatant was transferred to a new centrifuge tube and incubated at 56°C for 15mins. A freeze thaw cycle using a dry ice/ethanol bath and a 56°C water bath followed by centrifugation (3000g; 15mins; 4°C) to pellet cell debris was run three times before freezing supernatant overnight at -20°C.

HiTrap Heparin HP columns (Amersham Biosciences) were used to purify the rAAV vector particles by re-centrifuging the harvested rAAV after thawing and passing the supernatant through a HiTrap Heparin HP column pre-equilibrated with 10mL of a 150mM NaCl, 20mM Tris pH 8.0 solution using a Harvard infusion pump set at 1mL/min. The rAAV loaded column was washed with 20mL 100mM NaCl / 20mM Tris pH 8.0 solution before eluting the purified rAAV vector from the column using 1mL solutions with increasing concentrations of NaCl - 200, 300, 400, 450, 450 and 500mM NaCl buffered with 20mM Tris pH 8.0. All of the eluted factions were pooled and the rAAV particles concentrated using 100K MWCO Microsep™ Centrifugal Devices (Pall). The concentrators were centrifuged at 3000g, 4°C until there was ~300µL remaining. The concentrated rAAV vector was then loaded into a Slide-A-Lyzer dialysis cassette (Pierce) and suspended overnight at 4°C in a sterile container of PBS containing 1mM MgCl₂. Following overnight dialysis I filtered the rAAV vector through a 0.2µm syringe filter (Pall) and divided into 10 or 15µL aliquots for storage at -80°C.

2.2.5.2 Genomic Particle Titre
Concentrations of my rAAV vector stocks were determined by real time quantitative-PCR to measure the genomic particle titre for each vector preparation. The rAAV vector DNA was extracted in triplicate reactions from the rAAV vector particles using DNAseI (Invitrogen) and Proteinase K (Sigma-Aldrich). 2.0µL of the AAV vector was diluted in 10µL DNAseI reaction buffer (Invitrogen) plus 86.5µL dH₂O and incubated with 0.5µL DNAseI at 37°C for 30min before inactivating the DNase at 70°C for 10mins. 0.5µL Proteinase K was then added and incubated at 50°C for 1hr before inactivation at 95°C for 20mins. The extracted DNA was further diluted 1:50 in dH₂O for a total
vector dilution of 1:2500 and stored at -20°C. Standards with known genome copy number were prepared from a maxi-prep of the AAV-Luciferase backbone plasmid containing the WPRE sequence (Appendix B.3). The standard AAV backbone plasmid was diluted to $1 \times 10^7$ plasmid copies per µL and then ten-fold serial dilutions were done down to $1 \times 10^4$ copies/µL giving four plasmid DNA standards of known copy number. Quantitative-PCR was performed using primers to amplify the WPRE sequence from the expression cassette within each of my rAAV vectors DNA.

Each quantitative-PCR sample was run in triplicate on a 96 well plate using 12.5µL ABsolute™ QPCR SYBR Green mix (ABgene), 0.5µL forward WPRE primer (10µM; 5’→3’ GGCTCGGCTGT TGGGCACTGAC), 0.5µL reverse WPRE primer (10µM; 5’→3’ GGGCCGAAGGGACGTAGCA GAA), 6.5µL dH2O and 5.0µL of extracted vector DNA, standard WPRE plasmid dilution or dH2O as a no template control. The plate was sealed with an ABI Prism™ Optical Adhesive Cover (Applied Biosystems) and inserted into an ABI Prism® 7700 Sequence Detection System (v1.9; Applied Biosystems) programmed to run the following thermal cycle:

<table>
<thead>
<tr>
<th>Polymerase activation</th>
<th>95°C</th>
<th>10 min</th>
<th>1 cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing</td>
<td>95°C</td>
<td>15 sec</td>
<td>40 cycles</td>
</tr>
<tr>
<td>Annealing and Extension</td>
<td>60°C</td>
<td>60 sec</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72°C</td>
<td>10 min</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

Mean cycle time (Ct) values from triplicate reactions were used to calculate the number of genomic copies in each of the AAV vector stocks (Appendix B.3). A standard curve was generated from the Ct values of the known concentration standards plotted against the Log value of the absolute number of plasmid copies in the reaction mix. Using the standard curve Ct values for the rAAV samples were converted to Log input quantities. To calculate actual genomic titres these values were inverseLogged, multiplied by the extracted DNA dilution factor of 2500 and multiplied by 200 to convert from the 5µL used in the reactions to copies per mL. To account for the single stranded AAV genome in contrast to the double stranded plasmid DNA the concentrations calculated from the plasmid standards were multiplied by 2 to give the actual genomic particle titre of my rAAV vectors.

2.2.6 **In vitro transduction testing**

Each of the newly constructed rAAV vector stocks were tested on HEK 293 or HT-1080 cells to ensure they contained infectious particles. HEK 293 or HT-1080 cells were grown on poly-D-lysine coated plates (24-well, Nunc) to ~70% confluence in 1mL DMEM media with 10% FBS. The media
was replaced with fresh DMEM and 2.0µL of the rAAV stock was added to each well. Cells were incubated for one week refreshing the media four days after rAAV transduction. The final media from AAV-BDNF, AAV-GDNF and AAV-Luciferase transduced cultures was collected and stored at -80°C for ELISA quantification of secreted neurotrophic factors. Cells were then gently washed with sterile PBS and fixed with 4% paraformaldehyde.

Immunocytochemistry was performed with either HA (Covance) or Luciferase (Chemicon) antibodies to visualise transgene protein expression within successfully transduced cells (Section 2.2.3.2). Control immunostaining was performed with the same antibodies on non-transduced cultures.

Quantitative ELISAs were run on the collected media from the AAV-BDNF and AAV-GDNF transduced HEK293 cells to measure secreted BDNF and GDNF protein using Promega E_max ImmunoAssay kits (BDNF, G7610; GDNF, G7620). The conditioned culture media was diluted 1:2 and 1:4 in DMEM media and run in triplicate wells on the ELISA plate against the recommended eight-point standard series for each kit. Conditioned media collected from non-transduced HEK293 cells was also assessed on the same ELISA plates to provide controls for any endogenous neurotrophic factor expression. Optical absorbance was read at 450nm on a Spectra max plate reader (Molecular Devices) and the quantity of neurotrophic factor expression calculated from the standard curves.

2.3 **Functional AAV-Mediated Protein Expression Testing In Vitro**

Functional assays were performed with each rAAV vector construct to ensure that the encoded protein-of-interest was expressed in a functionally active form following AAV transduction. Differentiation assays with primary neuronal cultures were used to visualise neurotrophic factor assisted phenotypic differentiation. Anti-apoptotic protein function was assessed with a cell death assay kit to measure the level of apoptosis following the induction of apoptosis in rAAV transduced in vitro cultures.

2.3.1 **Isolating and culturing primary cells**

Primary neuronal cells were cultured from embryonic day 14 (E14) or day 15 (E15) Wistar rat embryos. Dopaminergic precursor neurons were isolated from the E14 ventral mesencephalon, GABAergic neuronal precursors from the E15 striatum and mixed cortical cultures from E15 cortex. Tissue from each brain region was isolated under a dissecting microscope as described by Barker and
Johnson (1995). Three independent parallel cultures were established for each region from separate timed pregnant Wistar dams, harvesting tissue from 8-12 embryos per dam. The tissue from each embryo was finely diced in chilled Leibovitz L15 media (GIBCO®) with added glucose and pen/strep, and pooled together. Treating each dam separately, the dissected tissue was centrifuged at 300g for 2min and the L15 media replaced with 2mL fresh chilled L15 media. Three fire-polished Pasteur pipettes of decreasing opening diameter were used sequentially to gently triturate the diced tissue into single cells. After letting the debris settle the cloudy supernatant containing single cells was removed and centrifuged at 1000g for 5min to pellet cells. The cells were then resuspended in Neurobasal media (GIBCO®) with added glucose, L-glutamine, pen/strep and 2% B27, and plated as required onto 24- or 96-well plates (Nunc) coated with 0.1mg/mL poly-D-lysine and pre-incubated with 10% FBS in PBS.

2.3.2 AAV-BDNF

The expression of functional BDNF was assessed by the presence of calbindin expressing neurons following AAV-BDNF transduction of primary striatal cultures. Primary striatal neurons isolated from E15 Wistar rat embryos from three independent Wistar dams were seeded onto 24-well plates at $2 \times 10^5$ cells per well (Section 2.3.1). AAV-BDNF or AAV-Luciferase vectors were added to the Neurobasal culture media 24hours after seeding at a concentration of 1,000 genomic particles per cell ($2 \times 10^8$ AAV genomic particles per well). Triplicate culture wells seeded from each independent dam received either AAV-BDNF, AAV-Luciferase or were left untreated as negative controls ($n = 9$ per treatment). The cultures were maintained for 21 days at 37°C, 5% CO$_2$ changing 50% of the Neurobasal media every three days before gently washing with PBS and fixation with 4% paraformaldehyde.

The differentiation of the primary striatal neurons was assessed by immunocytochemistry with anti-calbindin (1:5000, Swant) to visualise calbindin protein expression (Section 2.2.3.2). Cells expressing calbindin were quantified under a 40× objective (Nikon Eclipse TS100) in five randomly placed fields of view for each culture well.

2.3.3 AAV-GDNF

To verify functional GDNF protein expression I transduced embryonic ventral mesencephalic cultures with AAV-GDNF to induce maturation towards dopaminergic neurons. Primary E14 ventral mesencephalon cells from three independent isolations were seeded onto 24-well culture plates at $2 \times 10^5$ cells per well in Neurobasal media and incubated overnight (Section 2.3.1). Twenty-four hours
after seeding the cells, AAV-GDNF or AAV-Luciferase vectors were added to the Neurobasal culture media, at a concentration of 1,000 genomic copies per cell ($2 \times 10^8$ genomic copies per well), in triplicate wells for each culture. An additional three wells from each dam received 10ng/mL rhGDNF protein (R&D Systems) as a positive control and three wells were left untreated as a negative control. All cultures ($n = 9$ per treatment) were maintained at 37°C, 5% CO$_2$ changing 50% of the media every three days with fresh Neurobasal and supplementing the positive control wells with 10ng/mL rhGDNF protein. Three weeks after plating the cultures were gently washed with sterile PBS and fixed with 4% paraformaldehyde.

Dopaminergic neurons were visualised by immunocytochemistry with anti-tyrosine hydroxylase (anti-TH; 1:1000, Chemicon; Section 2.2.3.2) and quantified under a 40× objective (Nikon Eclipse TS100) in five randomly placed fields of view for each culture well.

### 2.3.4 AAV-Bcl-x$_L$

To test for functional Bcl-x$_L$ expression, primary cortical cultures were transduced with AAV-Bcl-x$_L$ and used to perform an apoptosis assay. Primary cortical cells from three independent cultures were plated onto 96-well plates at a density of $4 \times 10^4$ cells per well with 200µL Neurobasal media and incubated overnight at 37°C, 5% CO$_2$ (Section 2.3.1). Twenty-four hours after plating the cells were transduced with either AAV-Bcl-x$_L$ (six wells per culture), or AAV-Luciferase (three wells per culture). The rAAV vectors were added to the Neurobasal culture media at a concentration of 1,000 genomic copies per cell and maintained for three weeks replacing half of the Neurobasal media every two-or-three days with fresh media. Apoptosis was induced three weeks after transduction in half of the AAV-Bcl-x$_L$ transduced wells and all of the AAV-Luciferase transduced wells by incubating the cultures with Neurobasal media containing 0.5µM staurosporine (Sigma-Aldrich) for 48hrs ($n = 9$ per treatment). The remaining three AAV-Bcl-x$_L$ transduced wells from each culture received PBS as non-apoptosis induced controls.

A Cell Death Detection ELISA$^{PLUS}$ kit (Roche) was used to quantify the level of apoptosis occurring within each culture well by detecting apoptosis generated nucleosomes after 48hrs of staurosporine exposure. Briefly the culture media was carefully discarded and the cells lysed using the supplied lysis buffer and centrifuged to remove cellular debris and unfragmented DNA. Supernatant from each culture well was incubated in a streptavidin coated ELISA plate with immunoreagent solution containing both biotin labelled anti-histone and peroxidase conjugated anti-DNA antibodies. The quantity of bound nucleosomes was photometrically determined using ABTS (2,2-azino-di-3-ethylbenzthiazoline sulfonate) as a substrate for the bound peroxidase. Absorbance was read at...
405nm on a Spectra max plate reader (Molecular Devices) with the non-apoptosis induced control wells used to normalise data across the three independent cultures.

For visual analysis with immunocytochemical staining I cultured and treated a second set of primary cortical cells in parallel to the cultures used for the apoptosis quantification. Immunocytochemistry was undertaken with anti-HA (1:10000, Covance) and anti-Luciferase (1:10000, Chemicon) to visualise the transduced cells (Section 2.2.3.2). Hoechst 33258 nuclear staining allowed identification of cells undergoing nuclear condensation and fragmentation (Section 2.2.3.3).

### 2.3.5 AAV-XIAP

To assess functional XIAP expression I performed an apoptosis assay on HT-1080 cell cultures transduced with either AAV-XIAP or AAV-Luciferase. HT-1080 cells were plated onto 96-well plates at a low seeding density of $1 \times 10^4$ cells per well in DMEM, 10% FBS and incubated overnight at 37°C, 5% CO₂. AAV-XIAP or AAV-Luciferase were added to the culture media at a concentration of 10,000 genomic copies per cell, transducing 18 wells with each vector. Cells were grown for three days following AAV-XIAP transduction to ensure transgenic protein expression before incubating half of the wells from each group with DMEM containing 0.2µM staurosporine for 12hrs ($n = 9$ per treatment). Non-staurosporine induced wells served as controls.

A Cell Death Detection ELISAPLUS kit was used to quantify the level of apoptotic cell death in each well by measuring apoptosis generated nucleosomes (as described for AAV-Bcl-xL in Section 2.3.4).

Immunocytochemistry on parallel cultures was undertaken with either anti-HA or anti-Luciferase to visualise AAV-XIAP and AAV-Luciferase transduced cells (Section 2.2.3.2). Hoechst 33258 nuclear staining was performed to visually identify cells displaying apoptotic features of nuclear condensation and fragmentation (Section 2.2.3.3).

### 2.4 Results

The production of recombinant AAV vectors was divided into two distinct elements: AAV plasmid construction, and AAV vector packaging. This was then followed by functional testing of the newly constructed AAV vector particles.

#### 2.4.1 AAV plasmid construction

Construction of the AAV expression cassettes for the neuroprotective investigations was greatly aided by the availability of an empty AAV expression cassette containing all of the desired regulatory
elements, requiring only the insertion of a gene-of-interest. Following the insertion of cDNA sequences encoding BDNF, GDNF, Bcl-xL or XIAP into separate AAV expression cassettes I transfected HEK293 and/or HT-1080 cells with each newly constructed AAV plasmid. This was performed to check protein expression driven from the AAV expression cassette prior to AAV vector packaging. Immunostaining of the HEK293 or HT-1080 cells with an HA antibody confirmed the expression of transgenic proteins following transfection for each of my four AAV plasmids (Figure 2-2A-D). The fusion of a small HA-epitope to the transgenic proteins allowed easy identification of transgenic protein expression in transduced cells without potential staining of endogenously

**Figure 2-2 Transfection of AAV backbone plasmids**
Expression of HA-tagged proteins following AAV backbone plasmid transfection of HT-1080 cells visualised by immunocytochemistry with an HA antibody – (A) BDNF, (B) GDNF, (C) Bcl-xL, (D) XIAP and (E) non-transfected control culture. Scale bar = 25µm.
expressed protein that is likely to occur with antibodies against the neurotrophic factors and anti-apoptotic factors under investigation. All of the plasmids directed good expression of HA-tagged proteins with expression of BDNF and GDNF concentrated at the perimeter of the cells (Figure 2-2A,B) compared with the more evenly distributed anti-apoptotic factors (Figure 2-2C,D). No HA staining was detectable in any of the non-transfected culture wells (Figure 2-2E).

### 2.4.2 AAV vector production

After successful vector packaging, the concentration of each AAV vector stock was quantified by real-time PCR to determine its genomic titre. The AAV packaging and purification protocol produced reasonably consistent AAV titres of ~10^{12} AAV genomes per mL (Table 2-6) although the AAV-Bcl-x\textsubscript{L} was lower at 5.1 x 10^{11} genomes per mL and AAV-XIAP significantly higher than the other vectors at 1.9 x 10^{13} genomes per mL. To allow reasonable comparisons between the different vectors I diluted the AAV-XIAP 10-fold with sterile PBS prior to use.

<table>
<thead>
<tr>
<th>AAV vector</th>
<th>Genome Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-BDNF</td>
<td>2.0 x 10^{12}</td>
</tr>
<tr>
<td>AAV-GDNF</td>
<td>2.2 x 10^{12}</td>
</tr>
<tr>
<td>AAV-Bcl-x\textsubscript{L}</td>
<td>5.1 x 10^{11}</td>
</tr>
<tr>
<td>AAV-XIAP\textsuperscript{*}</td>
<td>1.9 x 10^{13}</td>
</tr>
<tr>
<td>AAV-Luciferase</td>
<td>4.6 x 10^{12}</td>
</tr>
</tbody>
</table>

Table 2-6 Genomic titres of AAV vector stocks

High-titre recombinant AAV vector stocks purified by heparin affinity chromatography ranged from 10^{11} – 10^{13} genomic particles per mL. The AAV-XIAP vector was diluted 10-fold to provide comparable vector titres of ~10^{12}.

### 2.4.3 AAV vector transduction

*In vitro* transduction of HEK293 or HT-1080 cells with each of the recombinant AAV vectors was confirmed by immunocytochemical staining with either HA or Luciferase antibodies (Figure 2-3). Immuno-positive cells were present in all culture wells that received one of the AAV vectors. Specificity of the antibodies to the transgenic proteins was confirmed by the lack of staining in the non-transduced cultures (Figure 2-3B,G). Transgenic protein expression following AAV-BDNF or AAV-GDNF transduction once again appeared localised to the cell surface in contrast to the whole cell accumulation of the anti-apoptotic factors and Luciferase. The HT-1080 cell line appeared more receptive to rAAV transduction than the HEK293 cells, used initially to test the AAV vectors, as evidenced by greater numbers of HA- and Luciferase-positive cells in the HT-1080 cultures.
Secretion of the neurotrophic factors into the culture media was quantified by ELISA to be approximately 350 pg/mL of BDNF and 600 pg/mL of GDNF.

Figure 2-3  AAV vector transduction in vitro
Successful AAV vector transduction of HEK293 or HT-1080 cells and the expression of transgenic proteins visualised by immunocytochemistry with (A,B) Luciferase or (C-G) HA antibodies: (A) AAV-Luciferase; (C) AAV-BDNF; (D) AAV-GDNF; (E) AAV-Bcl-xL; (F) AAV-XIAP; (B,G) non-transduced cultures. Scale bar = 25µm.
2.4.4 Functional protein expression

2.4.4.1 Brain Derived Neurotrophic Factor

AAV-BDNF mediated expression of functional BDNF was assessed following AAV-BDNF transduction of primary embryonic day-15 striatal cultures. BDNF enhances GABAergic neuronal differentiation and induces calbindin expression in the striatal neurons (Mizuno et al. 1994; Ventimiglia et al. 1995). Calbindin-positive cells were observed in all of the striatal cultures (Figure 2-4). To standardise the quantification of calbindin-positive neurons across the three independent cultures I calculated calbindin-positive cell counts as a ratio of the untreated control wells for each culture (Figure 2-5). Addition of AAV-BDNF resulted in a 96 ± 2% increase in the number of calbindin-positive cells over untreated wells (P < 0.001). AAV-Luciferase transduction did not significantly alter the number of calbindin-expressing cells compared with untreated cultures. Additionally, calbindin-positive neurons in the AAV-BDNF transduced cultures were observed to have a greatly enhanced dendritic morphology compared with the restricted number of projections in the control cultures (Figure 2-4).

2.4.4.2 Glial cell-line Derived Neurotrophic Factor

Transduction of undifferentiated ventral mesencephalon neuronal cultures isolated from embryonic day-14 rat embryos with AAV-GDNF was undertaken to verify the expression of functional GDNF (Figure 2-6). GDNF induces differentiation of neurons towards a dopaminergic fate in the developing mid-brain (Stromberg et al. 1993; Choi-Lundberg and Bohn 1995). Quantification of the TH-positive neurons was standardised to untreated control cultures to account for variations between the three independent source cultures (Figure 2-7). AAV-GDNF transduction induced a 62 ± 2% increase in TH-positive neurons compared with the number of TH-positive neurons in untreated cultures (P < 0.05). Supplementation of the culture media with rhGDNF as a positive control increased the number of TH-positive neurons by 46 ± 2%, however this failed to reach statistical significance (P > 0.05). The AAV-Luciferase vector control cultures were not significantly different from untreated cultures. TH-positive neurons within AAV-GDNF and rhGDNF treated cultures developed more extensive dendritic networks than the TH expressing cells in the AAV-Luciferase and untreated negative control cultures (Figure 2-6).
Figure 2-4  Phenotypic differentiation of primary striatal cultures
Representative images of calbindin-expressing neurons within embryonic striatal cultures three-weeks post AAV-BDNF or AAV-Luciferase transduction. (A,B) AAV-BDNF transduced cultures contained calbindin-positive neurons that developed an extensive dendritic morphology. (C,D) AAV-Luciferase and non-transduced control cultures had fewer calbindin-positive neurons which displayed restricted dendrite growth. Scale bar = 100µm for (A,C) and 50µm for (B,D).

Figure 2-5  AAV-BDNF induced calbindin expression in embryonic striatal cultures
Graph showing the increased number of calbindin-expressing neurons following AAV-BDNF transduction relative to the AAV-Luciferase transduced and un-treated cultures. Cell counts from untreated control wells were used to normalise data between the three independent cultures. One-way ANOVA P < 0.001 with Bonferroni’s post-hoc tests * P < 0.01, ** P < 0.001.
Figure 2-6 Phenotypic differentiation of primary ventral mesencephalon cultures
Representative images of TH-expressing neurons within embryonic ventral mesencephalon cultures three-weeks post (A) AAV-GDNF transduction, (B) supplementation with rhGDNF, (C) AAV-Luciferase transduction or (D) non-transduced cultures. AAV-GDNF and rhGDNF treated cultures contained numerous TH-positive neurons that developed into groups of extensively networked neurons. A smaller number of the ventral mesencephalon neurons in control cultures also differentiated into TH-expressing neurons. Scale bar = 100µm.

Figure 2-7 AAV-GDNF induced TH expression in ventral mesencephalon cultures
Graph showing the increased number of TH-expressing neurons following AAV-GDNF transduction, and the addition of rhGDNF protein, relative to AAV-Luciferase transduced and un-treated cultures. Cell counts from untreated controls wells were used to normalise data between the three independent cultures. One-way ANOVA P < 0.05 with Bonferroni’s post-hoc tests * P < 0.05.
2.4.4.3 Bcl-x<sub>L</sub>

Expression of functionally active Bcl-x<sub>L</sub> protein from the AAV-Bcl-x<sub>L</sub> vector was checked by measuring staurosporine induced apoptotic cell death in primary cortical cultures transduced with AAV-Bcl-x<sub>L</sub> (Figure 2-9). Apoptotic cell death across each of my three independent cortical cultures was assessed relative to the quantity of apoptotic nucleosomes detected in the AAV-Luciferase vector control wells following staurosporine exposure (Figure 2-8). The transgenic expression of Bcl-x<sub>L</sub> by cortical cells <em>in vitro</em>, following AAV-Bcl-x<sub>L</sub> transduction, reduced apoptotic nucleosomes formation by 19 ± 2% compared with my control AAV-Luciferase transduced cultures. Apoptosis in the control AAV-Bcl-x<sub>L</sub> transduced cultures not exposed to staurosporine was measured at a level equivalent to 45 ± 2% of apoptosis cell death occurring in the AAV-Luciferase transduced controls exposed to staurosporine.

Immunocytochemical staining with HA or Luciferase antibodies showed that only a small population of the cortical cells within each culture were successfully transduced (Figure 2-9A,C,E). Cells expressing transgenic Bcl-x<sub>L</sub> protein in the absence of staurosporine appeared healthy with extensive dendritic morphology (Figure 2-9A). Following the induction of apoptosis, AAV-Bcl-x<sub>L</sub> transduced

![Graph showing AAV-Bcl-x<sub>L</sub> mediated reduction in the level of apoptotic cell death occurring in primary cortical cultures following staurosporine exposure relative to the AAV-Luciferase transduced wells used to normalise data across the three independent cultures. A substantial level of non-induced apoptotic cell death was recorded in non-staurosporine exposed AAV-Bcl-x<sub>L</sub> treated cultures. Mann-Whitney analysis * P < 0.01, ** P < 0.001.](image)

Figure 2-8 AAV-Bcl-x<sub>L</sub> reduced staurosporine-induced apoptosis

Graph showing AAV-Bcl-x<sub>L</sub> mediated reduction in the level of apoptotic cell death occurring in primary cortical cultures following staurosporine exposure relative to the AAV-Luciferase transduced wells used to normalise data across the three independent cultures. A substantial level of non-induced apoptotic cell death was recorded in non-staurosporine exposed AAV-Bcl-x<sub>L</sub> treated cultures. Mann-Whitney analysis * P < 0.01, ** P < 0.001.
Figure 2-9  AAV-Bcl-x<sub>L</sub> transduced cortical cells following staurosporine-induced apoptosis
Representative images of primary cortical cultures transduced with (A-D) AAV-Bcl-x<sub>L</sub> or (E,F) AAV-Luciferase showing transgene expression and Hoechst nuclear staining.  (A) Cortical cells transduced by AAV-Bcl-x<sub>L</sub> express HA-tagged Bcl-x<sub>L</sub> protein as visualised by HA-positive neurons not exposed to staurosporine.  (C) After 48hrs of staurosporine exposure HA-positive cells were observed to resist cell death.  (E) Very few Luciferase-positive cells resisted staurosporine-induced apoptosis.  (B,D,F) Hoechst nuclear staining displays both large rounded healthy cells (arrowheads) and cells under going apoptosis induced nuclear fragmentation (arrows).  Scale bar = 50µm (DAB and Hoechst images are not matched).  Abbreviations: Luc, Luciferase; STS, Staurosporine.
cells were still present within the cortical cultures however they displayed condensed cell bodies with restricted dendritic projections (Figure 2-9C). Very few cells expressing Luciferase were seen to survive staurosporine induced cell death (Figure 2-9E). Hoechst staining showed widespread nuclear condensing and fragmentation within all staurosporine induced cultures (Figure 2-9D,F), in contrast to the predominantly large, rounded cells in the AAV-Bcl-xL transduced, non-staurosporine induced wells (Figure 2-9B).

2.4.4.4 X-linked Inhibitor of Apoptosis Protein
AAV-XIAP mediated expression of functional XIAP was verified by transducing HT-1080 cells with AAV-XIAP and assessing the level of apoptosis following staurosporine-induced apoptotic cell death (Figure 2-11). HT-1080 cells are readily transduced by AAV vectors providing a clean substrate for assessing the effect of non-cell dependent protein expression. Transduction with AAV-XIAP resulted in a 52 ± 2% reduction in apoptosis compared with the apoptosis induced by staurosporine in AAV-Luciferase cultures (Mann-Whitney P < 0.001; Figure 2-10). Non-staurosporine induced cultures transduced with either AAV-XIAP or AAV-Luciferase only had marginally detectable levels of apoptotic induced nucleosomes with no significant difference between the two groups.

Figure 2-10 AAV-XIAP reduced staurosporine-induced apoptosis
Graph showing AAV-XIAP mediated reduction in the level of apoptotic cell death occurring in HT-1080 cultures following staurosporine exposure relative to the AAV-Luciferase transduced wells used to normalise data across the three independent cultures. Negligible apoptotic cell death was detected in non-staurosporine exposed cultures. Mann-Whitney analysis * P < 0.001.
Visualisation of the transduced cells with immunocytochemical staining for HA or Luciferase showed a population of the HT-1080 cells were transduced and expressed transgenic XIAP or Luciferase (Figure 2-11). Staurosporine induced extensive cell death in all cultures however a number of HA stained cells survived in the AAV-XIAP transduced wells and exhibited a neuronal-like morphology (Figure 2-11C). A few AAV-Luciferase transduced cells were observed after staurosporine exposure however they were located within patches of cellular debris non-representative of the HT-1080 cells (Figure 2-11G). Co-staining of the transduced cells with Hoechst 33258 further showed the survival of non-fragmented HT-1080 cells in the AAV-XIAP transduced cultures including both the surviving HA-positive transduced cells (Hoechst staining is compromised by the DAB staining) and some additional non-transduced cells (Figure 2-11D). Cells displaying nuclear condensation and fragmentation were observed with Hoechst staining in the AAV-Luciferase control transduced wells and to a lesser extent within the AAV-XIAP transduced wells following staurosporine exposure, with the total number of HT-1080 cells remaining clearly reduced in comparison to the non-staurosporine treated cultures.

**Figure 2-11 Enhanced survival of AAV-XIAP transduced HT-1080 cells**
Representative images of HT-1080 cells transduced with (A-D) AAV-XIAP or (E-H) AAV-Luciferase showing transgene expression and Hoechst nuclear staining of the same cells (arrows; DAB staining reduces intensity of Hoechst staining). (A,E) HT-1080 cells are readily transduced by AAV vectors with individual XIAP expressing cells (C) surviving staurosporine exposure but with shrunken cytoplasm. (G) A few AAV-Luciferase transduced cells survived staurosporine treatment but were observed within clumps of cellular debris. Scale bar = 50µm. Abbreviations: Luc, Luciferase; STS, Staurosporine.
Figure 2-11 Enhanced survival of AAV-XIAP transduced HT-1080 cells
2.5 Discussion

2.5.1 AAV vector construction

AAV vectors were constructed to deliver DNA expression cassettes, encoding the potential biotherapeutic proteins BDNF, GDNF, Bcl-xL or XIAP, to targeted neuronal populations in the CNS. To direct transgenic protein expression I used an expression cassette containing the constitutive CBA/CMV promoter-enhancer hybrid shown to generate high level transgene expression (Klein et al. 2002) without down-regulation, ensuring continuous long-term protein production. A downstream WPRE sequence and poly-A tail help to further enhance transgene expression by stabilising the mRNA allowing greater protein translation (Loeb et al. 1999; Paterna et al. 2000; Xu et al. 2001). Each protein-of-interest was cloned into the expression cassette, in-frame with a nine amino acid C-terminal HA-tag epitope to allow clear immunocytochemical visualisation of transgenic protein expression following cellular transduction.

The expression cassettes were flanked by ITR sequences derived from serotype-2 AAV which have been extensively utilised in both preclinical research and clinical trials (Grieger and Samulski 2005). The ITR sequences are the only viral-derived DNA packaged into the rAAV vector particles which can hold up to ~4.7 kb of ITR flanked DNA. The expression cassettes I constructed ranged in length from ~2.9 for AAV-GDNF and AAV-Bcl-xL, ~3.0kb for AAV-BDNF, ~3.8kb for AAV-XIAP and ~4.1kb for AAV-Luciferase. Prior to rAAV packaging I confirmed protein expression from each AAV expression cassette by transforming the AAV-plasmids into in vitro cell cultures. HA-positive cells were present in all transformed cultures with the neurotrophic factors localising primarily to the cell surface and the anti-apoptotic factors appearing to disperse throughout the cytoplasm of the cells (Section 2.4.1).

The expression cassettes were packaged into chimeric rAAV particles consisting of serotype-1 and -2 capsid proteins in a 1:1 ratio. The capsid proteins influence cellular transduction with this combination of serotype-1/2 giving enhanced transduction efficiency of neuronal cells over either capsid -1 or -2 alone (Hauck et al. 2003; Richichi et al. 2004). Utilising the heparin affinity of the serotype-2 capsid proteins (Zolotukhin et al. 1999), AAV vectors were purified by heparin affinity column and concentrated to ~10^{12} genomic particles per mL. While I tested that the vector stocks contained infectious particles by transduction of cell-lines in vitro (Section 2.4.3), I did not undertake infectious particle titres. I would assume that following heparin purification all remaining genomic copies were packaged within AAV particles. However as AAV production requires capsid assembly followed by the rate-limiting encapsulation of the ssDNA genome a large proportion of empty virions...
are typically present within the final vector stocks and their theoretical impact cannot be discounted (Timpe et al. 2005). Empty capsids may compromise the gene delivery by unnecessarily increasing exposure of AAV particles which may influence transduction efficiency or enhance antibody generation, a potential concern for repeated AAV administration.

### 2.5.2 Functional protein expression

The production of functionally active biotherapeutic molecules following gene transfer is clearly essential for any gene therapy procedure. It was therefore important that I performed functional assays with each recombinant AAV vector to determine that the proteins-of-interest were correctly produced in a biologically active form following cellular transduction. Taking advantage of the well characterised properties of neurotrophic factors in assisting neuronal differentiation and anti-apoptotic factors in promoting cell survival, I designed and performed efficient phenotypic differentiation and cell-survival assays to confirm expected functional impact of the transgenic proteins following in vitro AAV vector transduction.

#### 2.5.2.1 Differentiation Assays to Confirm Neurotrophic Factor Activity

Neurotrophic factors are important for directing both phenotypic differentiation within the developing CNS, and maintaining cell plasticity and survival within the adult brain (Krieglstein 2004; Levy et al. 2005; Van Ooyen 2005). Neurotrophic factors are secreted proteins that signal via cell surface receptors and have both autocrine and paracrine activity. Therefore the biological effect of transgenic neurotrophic factor expression following gene delivery is expected to extend beyond the cells actually transduced by the viral vector. I developed functional assays for BDNF and GDNF using primary embryonic neuronal cultures isolated from gestation day 14 or 15 (E14 or E15) Wistar rat pups to visualise the effect of these neurotrophic factors on phenotypic differentiation following AAV-mediated gene transfer. Previous studies have shown that BDNF enhances the differentiation of GABAergic neurons (Mizuno et al. 1994; Ventimiglia et al. 1995), while GDNF plays a key role in the differentiation and maturation of dopaminergic neurons (Lin et al. 1993; Widmer et al. 2000). Primary embryonic cultures were chosen to verify transgenic neurotrophic factor functionality as immature embryonic neurons at E14/15 are naturally primed by their micro-environment and express appropriate cell surface receptors for neurotrophic factors which assist in directing their phenotypic differentiation and neurite outgrowth (Ernfors et al. 1992). Functional assessment of virally mediated neurotrophic factor expression has previously been undertaken by collecting conditioned media containing the transgenic protein from transduced cell lines, applying this media to non-transduced cultures or explants and assessing morphological changes (Martinez-Serrano et al. 1995; Watabe et
al. 2000; Sakamoto et al. 2003; Hu et al. 2005). The use of conditioned media is important for assessing any novel biological functions of secreted molecules as it avoids the potentially confounding involvement of physical AAV vector transduction or altered protein expression. However the functional assays I performed demonstrated that AAV-mediated expression of functional BDNF and GDNF can effectively be assessed in a readily reproducible protocol by directly transducing undifferentiated primary neuronal cultures.

**Brain Derived Neurotrophic Factor**

The biological activity of BDNF was assessed following AAV-BDNF transduction of primary embryonic striatal cultures isolated from E15 Wistar rat pups (Section 2.4.4.1). Embryonic striatal neurons were chosen as they predominantly differentiate into GABAergic medium spiny projection neurons *in vivo*, and express TrkB the high-affinity cell-surface receptor for BDNF (Ernfors et al. 1992; Mizuno et al. 1994). BDNF is vital for both striatal development and maintenance throughout adult life with mature striatal neurons both producing BDNF as well as receiving a significant supply via anterograde transport of BDNF from the cortex via corticostriatal axonal projections (Altar et al. 1997; Kokaia et al. 1998). Primary striatal cultures were transduced with AAV-BDNF and maintained *in vitro* for three weeks to ensure a stable level of transgenic BDNF expression and provide sufficient exposure of BDNF to the striatal cultures to enhance GABAergic differentiation and maturation. Functional activity of BDNF was assessed by the induction of calbindin expression. Calbindin is a calcium binding protein expressed by a population of striatal GABAergic neurons (Gerfen et al. 1985; DiFiglia et al. 1989; Mizuno et al. 1994) shown to be induced in striatal neurons by BDNF (Ventimiglia et al. 1995; Ivkovic and Ehrlich 1999). Three-weeks post AAV-BDNF transduction there were up to twice the number of calbindin-positive neurons in the AAV-BDNF transduced cultures compared with both AAV-Luciferase vector control and untreated cultures. This significant increase in the number of calbindin-positive neurons observed in AAV-BDNF transduced cultures indicates that the HA tagged BDNF transgenic protein produced was functionally active in influencing the GABAergic differentiation of E15 embryonic striatal neurons. In addition to the increased differentiation, it was observed that calbindin-positive neurons in the AAV-BDNF transduced cultures had enhanced dendritic morphology compared to calbindin-positive neurons within non-transduced cultures, suggesting that the transgenic protein expression has enhanced maturation of these striatal neurons. This enhanced neurite outgrowth and maturation is consistent with increased BDNF expression providing additional supporting evidence that the transgenic BDNF is being correctly produced in a biologically active form.
Glial cell-line Derived Neurotrophic Factor

Functional GDNF expression was verified following *in vitro* AAV-GDNF transduction of E14 ventral mesencephalon cultures. GDNF is a potent dopaminergic neurotrophic factor that assists with the differentiation and maturation of dopamine neurons. Neurogenesis and migration of dopaminergic precursor neurons into the ventral mesencephalon region occurs between gestation days E10 to E16 in rats, peaking around E14 (Altman and Bayer 1981; Prakash and Wurst 2006). The normal endogenous expression of GDNF, which peaks at birth in the CNS, assists in directing the dopaminergic precursor neurons to develop a dopaminergic phenotype (Stromberg *et al.* 1993; Choi-Lundberg and Bohn 1995). GDNF receptors expressed on the terminals of dopaminergic precursor neurons allow retrograde transport back to the substantia nigra to direct the axon projections towards high-level GDNF expression within the developing striatum (Lin *et al.* 1993; Tomac *et al.* 1995).

Three weeks after AAV transduction of E14 ventral mesencephalic cultures I performed immunocytochemical staining for TH, the rate-limiting enzyme in the production of dopamine, a phenotypic marker for dopaminergic neurons. With the E14 ventral mesencephalon cells already primed for a dopaminergic neuronal fate I observed a large number of TH-positive cells within all cultures. AAV-GDNF transduced wells however exhibited a significant (more than 50%) increase in TH-positive cells over AAV-Luciferase and untreated cultures with more extensive dendritic networking between the TH-positive neurons. Supplementation with rhGDNF failed to generate a statistically significant increase in TH-positive neurons despite showing an enhancement of TH-differentiation. This contrast with AAV-GDNF treatment was possibly due to the concentration of GDNF present in the cultures. AAV-GDNF transduced cells were likely to have been secreting a constant supply of GDNF that may well have led to significantly greater GDNF bioavailability than the 10 ng/mL rhGDNF added once daily to the E14 cells. AAV-Luciferase treatment did not alter the number of TH-expressing cells compared with untreated wells.

The impact of AAV-GDNF in influencing differentiation into TH-positive neurons, exceeding the influence of rhGDNF supplementation, is consistent with the known activity of endogenous GDNF in driving undifferentiated ventral mesencephalon neurons towards a dopaminergic fate *in vivo*, and is strongly indicative that the transgenic GDNF-HA is functional active. The additional observation of enhanced dendritic networking by the TH-positive neurons, possibly due to axonal targeting of GDNF expressing neurons and retrograde transportation of the GDNF, is further supportive of an increased bioavailability of functional GDNF assisting the maturation of dopaminergic neurons.
2.5.2.2 Cell-Survival Assays to Confirm Anti-Apoptotic Protein Function

Anti-apoptotic proteins play a vital role in ensuring cell survival by counteracting or inhibiting the function of pro-apoptotic caspases and mitochondrial proteins. Enhancing the expression level of anti-apoptotic factors is likely to shift the apoptosis balance towards cell survival and therefore increase the cells resistance to specific apoptotic induction cues (Simons et al. 1999; Xu et al. 1999; Yamada et al. 1999; Shimazaki et al. 2000; Perrelet et al. 2002; Miagkov et al. 2004; Malik et al. 2005). Apoptosis can be quantified by detecting changes in apoptotic parameters including the activation of apoptotic caspases, mitochondrial release of pro-apoptotic proteins or DNA fragmentation (Loo and Rillema 1998; Goldstein et al. 2005). To verify functional anti-apoptotic activity of the Bcl-xL and XIAP protein expression following AAV-mediated transduction I quantified the generation of cytoplasmic histone-associated-DNA-fragments (nucleosomes) following the induction of apoptosis. Apoptosis was induced following AAV-Bcl-xL and AAV-XIAP transduction by incubating the cultures with staurosporine, a potent kinase inhibitor that induces apoptotic cell death through both caspase-dependent and caspase-independent mechanisms (Zhang et al. 2004). Staurosporine provides a broad approach to apoptosis allowing the assessment of various anti-apoptotic factors in different cell lines independent of a specific pathological process. Bcl-xL is a pro-survival member of the Bcl-2 family of apoptosis regulating proteins involved in controlling both caspase-9 activation via the mitochondrial release of cytochrome c and other caspase-independent apoptogenic proteins (Kim 2005). XIAP is a potent anti-apoptotic factor that functions as an inhibitor of both initiator caspase-9 and the executioner caspases -3 and -7 through its dual caspase inhibiting sites (Deveraux and Reed 1999).

Bcl-xL

Functionally active Bcl-xL expression was confirmed by comparing staurosporine-induced apoptotic cell death following AAV-Bcl-xL or AAV-Luciferase transduction of rat E15 cortical cultures. Bcl-xL has been previously shown to prevent staurosporine-induced apoptotic cell death (Kaspar et al. 2002). Apoptotic generated nucleosomes were detected in all cultures, including the non-staurosporine exposed wells, possibly due to the high cell density or the age of the primary cultures with many cells observed to be undergoing nuclear fragmentation. However the staurosporine-induced apoptotic cell death in AAV-Bcl-xL transduced cultures was significantly lower than the AAV-Luciferase transduced control cultures. A population of HA-positive AAV-Bcl-xL transduced cells were observed to survive staurosporine induced apoptosis in contrast to the loss of most AAV-Luciferase transduced cells. This enhanced survival of Bcl-xL expressing cells reflects the 19% reduction in detected nucleosomes following AAV-Bcl-xL transduction. Assuming that non-staurosporine induced apoptosis occurred consistently in all cultures independent of treatment, the
actual reduction in staurosporine induced apoptosis following AAV-Bcl-xL transduction was ~35%. As Bcl-xL is not a secreted protein, only cells transduced by the AAV-Bcl-xL vector will be exposed to higher Bcl-xL levels and therefore have enhanced resistance against staurosporine-induced apoptosis. Given that I only transduced a proportion of the cultured cells the significant reduction in staurosporine induced apoptotic cell death of ~19% confirmed the expression of functionally active HA-tagged Bcl-xL protein.

**X-linked Inhibitor of Apoptosis Protein**

The functional activity of XIAP was assessed by inducing apoptotic death in HT-1080 cells cultures following AAV-XIAP and AAV-Luciferase transduction. Due to the highly permissive nature of the HT-1080 cell line towards AAV-mediated transduction, this cell line provides a reliable basis from which to assess functional expression from AAV vectors, and can be used as an alternative cell source when primary cells are not available or intrinsically necessary for assessing transgene function. The addition of staurosporine induced rapid cell loss and morphological changes in the HT-1080 cultures as visualised by light microscopy and Hoechst 33258 nuclear staining. However a population of AAV-XIAP transduced cells survived the staurosporine insult and the level of nucleosomes detected was halved by AAV-XIAP transduction. Only a minimal level of apoptosis was detectable within the non-staurosporine induced cultures. Given that I only transduced a restricted proportion of the cultured cells, the large reduction in staurosporine-induced apoptosis provided by AAV-XIAP transduction verified that the transgenic XIAP expressed is functionally active.

### 2.5.3 Summary

AAV vectors encoding each of my potential biotherapeutic molecules BDNF, GDNF, Bcl-xL or XIAP were successfully constructed and shown to direct the expression of functionally active proteins following cellular transduction. The use of efficient functional assays for known protein function provided reassurance that the therapeutic molecules were correctly translated and processed prior to proceeding with investigative *in vivo* gene transfer studies.

The functional assays I conducted to verify neurotrophic factor functionality offer an efficient reproducible method for direct assessment of phenotypic changes within AAV vector transduced cultures in which there is continuous expression and secretion of the neurotrophic factors. The use of conditioned media from transduced cell lines which is commonly used to assess the biological activity of secreted factors restricts functional assessment to the secreted proteins acting on un-modified cultures and results in a variable level of protein exposure as the neurotrophic factors are
used and degraded. In contrast, direct transduction assays assess the impact of continuous stable production and secretion of the transgenic proteins over an extended period of time in a cellular population containing both transduced and non-transduced cells. This ability to assess continuous stable expression would be of increased importance for vectors constructed to direct low level protein expression for long-term therapeutic application. While I could not exclude the possibility that physical transduction with AAV-BDNF or AAV-GDNF induced some phenotypic changes in the transduced cultures, I believe that it is the BDNF or GDNF secreted following AAV-mediated gene delivery that enhanced the differentiation of calbindin- and TH- expressing neurons by acting through cell surface receptors on both transduced and non-transduced cells.

Cell-survival assays in vitro using staurosporine to induce apoptosis confirm the expression of functional anti-apoptotic factors. The use of different culture systems – primary embryonic culture versus an engineered cell-line – for the anti-apoptotic factors demonstrated the potential for utilising different cells when the protein under consideration is not cell-type specific. For testing expression from AAV vectors the cells clearly need to be receptive to AAV transduction of which both neuronal cultures and the HT-1080 cells are, however the HT-1080 cells appeared to offer several advantages over primary cultures. Most noticeable were the homogeneity and stability of an immortalised cell-line with no confounding culture artefacts as observed along with the considerable level of apoptotic death occurring in the primary cultures possibly due to the high cell density or the length of time in culture.

While in vivo studies are required to conclusively determine the physiological activity and therapeutic potential of any expressed molecule, these initial in vitro assessments are important for verifying expected functional activity.
Chapter 3

**Rodent Model of Huntington’s Disease:**

*Quinolinic Acid Lesion*

### 3.1 Introduction

To investigate preventative *in vivo* gene therapy for Huntington’s disease I utilised the QA lesion model of striatal cell death. QA, an endogenous glutamate analogue, induces excitotoxic mechanisms following over-activation of the NMDA receptors expressed postsynaptically by the GABAergic medium spiny projection neurons in the striatum. Directly injected into the striatum, QA produces the most accurate neurotoxin-induced representation of HD striatal pathology in both rodents and non-human primates (Beal *et al.* 1986; Beal *et al.* 1989). While QA does not replicate the genetic mutation or the slow progressive characteristics of HD neurodegeneration, excitotoxic mechanisms – and possibly endogenous QA – are proposed to be a significant contributor to HD following a mutant huntingtin-mediated increase in the susceptibility of vulnerable neurons to excitatory inputs (Cowan and Raymond 2006).

Despite the extensive use of QA as a model of Huntington’s disease there has been no consensus within the relative literature regarding a delivery protocol for QA to the rodent striatum. It was therefore important that I briefly optimise a QA delivery protocol to generate a selective striatal lesion large enough to generate behavioural deficits without causing excessive mechanical damage or causing a physical hole at the site of injection.

### 3.2 Optimisation and Characterisation Procedures

#### 3.2.1 Animals and surgeries

Optimisation of the QA lesion model was undertaken in adult male Wistar rats (230 – 280g) with direct stereotaxic infusion of QA into the striatum on one side of the rodent brain. Stereotaxic injection of QA was performed as listed in Table 3-1 using surgical procedures outlined in Section 4.4. Based on my previous experience with QA administration I performed initial trials with 50 or 100nmol QA delivered in 500nL NaOH, pH 7.4. A second group of animals received either 30 or
50nmol QA dissolved in 400nL NaOH, pH 7.4. Behavioural deficits were analysed on a further cohort of animals that received 30nmol QA in 400nL before I undertook a more detailed behavioural and immunocytochemical analysis of the selected 50nmol QA in 400nL lesion. Later development of an additional behaviour task necessitated the use of a final cohort of rats to assess the level of sensorimotor neglect.

<table>
<thead>
<tr>
<th></th>
<th>QA</th>
<th>Coordinates from Bregma</th>
<th>No. rats</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial 1</strong></td>
<td>100nmol, 500nL; 50nmol, 500nL</td>
<td>+0.5 +2.8 -6.0</td>
<td>2</td>
</tr>
<tr>
<td><strong>Trial 2</strong></td>
<td>50nmol, 400nL; 30nmol, 400nL</td>
<td>+0.5 +2.7 -5.0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Trial 3: Behaviour Testing Setup</strong></td>
<td>30nmol, 400nL</td>
<td>+0.6 +2.6 -5.0</td>
<td>4</td>
</tr>
<tr>
<td><strong>Final Characterisation</strong></td>
<td>50nmol, 400nL</td>
<td>+0.5 ±2.7 -5.0</td>
<td>10</td>
</tr>
<tr>
<td><strong>Additional Behaviour</strong></td>
<td>50nmol, 400nL</td>
<td>+0.5 +2.7 -5.0</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3-1 QA lesion optimisation trials

Table detailing the intrastriatal injection of QA to produce unilateral lesioning of the striatum in the series of trials undertaken to optimise and characterise the neuropathological and behavioural changes. The anterior-posterior (A-P) and medial-lateral (M-L) coordinates were measured from Bregma and the dorsal-ventral (D-V) distance from the top of the dura with nose-tooth bar set at zero.

3.2.2 Behavioural assessment

It was necessary that the unilateral QA lesion generated enough of a performance imbalance between the two hemispheres of the basal ganglia that resultant functional motor deficits could be reliably detected by an imbalance in the rats left / right side behavioural activity. I setup an array of behavioural tests to assess QA induced deficits covering: non-induced motor control (spontaneous exploratory forelimb use); sensorimotor neglect (“corridor” task); and drug induced activity (apomorphine and amphetamine induced rotations). Detailed procedures for each behavioural assessment are provided in Section 4.5.

3.2.3 Neuropathological analysis

Rats were euthanised between seven and 20 days post-QA lesioning, fixed and processed for immunocytochemical assessment as described in Section 4.7.1. To analyse the extent of QA lesioning within the striatum, I used immunocytochemical techniques (Section 4.7.2) to visualise the
expression of the general neuronal marker NeuN (1:1000, Chemicon), and more specifically for GABAergic striatal neurons containing calbindin (1:5000, Swant) or DARPP-32 (1:750, Chemicon). Further characterisation of the final 50nmol QA lesion within the striatum was undertaken by immunocytochemistry against the interneuron marker proteins: calretinin (1:5000, Swant), parvalbumin (1:5000, Swant), choline acetyl-transferase (ChAT; 1:500, Chemicon) and NOS (1:10000, Piers Emson).

### 3.3 QA Lesion Optimisation Analysis

#### 3.3.1 Initial QA trial

As a starting point to optimise the QA lesion for the neuroprotective studies, I investigated 100 and 50nmol QA dissolved in 500nL 0.4M and 0.2M NaOH respectively, pH 7.4. These quantities of QA were selected based on both published literature (Qin et al. 1992; Arenas et al. 1993; Perez-Navarro et al. 1994; Perez-Navarro et al. 1996) and my own prior experience with QA ((Kells et al. 2004); unpublished data) in which I used these quantities but in significantly greater delivery volumes. With larger injection volumes it is common to observe physical damage to the striatum that can result in a hole at the site of injection.

Analysis of NeuN immunopositive cells seven days after QA injection showed both 100 and 50nmol QA injections generated large extensive lesions within the striatum and in some cases extending out into the adjacent cortical regions (Figure 3-1). The 100nmol QA injection resulted in extensive tissue loss in the striatum along the needle tract in both rats which was not observed with the 50nmol injection. Additionally, there appeared to be greater numbers of NeuN-immunopositive cells still present within the “transition zone” surrounding the core of the 50nmol QA lesion indicating the differential selectivity / vulnerability of different populations of striatal neurons to this protocol of QA delivery. Deciding to investigate a slightly smaller lesion with a lower delivery volume and quantity of QA, I did not further assess the specific populations of striatal neurons.
Figure 3-1  **Initial 100 and 50 nmol QA lesion assessment**
Representative images showing NeuN-positive neurons in the striatum following a unilateral injection of (A,B) 100nmol or (C,D) 50nmol QA (500nL) into the striatum. NeuN immunostaining displays extensive neuron loss within the injected striatum with physical damage surrounding the needle tract in the 100nmol QA injected striatum and loss of some adjacent cortical neurons. Surviving neurons were seen in the peripheral reaches of the lesion indicating a “transition zone” in which small populations of striatal neurons display resistance to the QA-induced excitotoxicity. Dotted lines show rough outline of the extent of QA lesioning in the striatum. Scale bar = 2mm for whole sections (A,C) and 500µm for higher magnification images (B,D).

### 3.3.2  **Trial 2: Lower QA quantity**

While the 50nmol QA lesion in my initial trial generated an acceptable lesion representative of late stage HD striatal pathology, I wanted to investigate a more restricted lesion that would potentially be more representative of earlier stage HD and possibly more applicable to the neuroprotective gene therapy research. For this trial I reduced the delivery volume by 20% to 400nL to limit the mechanical infusion, investigating injections of 50 and 30nmol QA.

Ten days post-QA the lower volume lesions appeared more contained than the 50nmol QA lesions in the initial trial, with both the 30 and 50nmol QA affecting ~50% of the striatum. The 30nmol QA looked to produce a slightly less severe lesion, with greater sparing of NeuN-positive striatal neurons.
throughout the lesion, compared with a small “transition zone” following the 50nmol QA injection (Figure 3-2A, C). Only a small population of calbindin-positive neurons were seen within the lesioned area of the striatum for either concentration of QA (Figure 3-2B, D), suggesting that the spared NeuN-positive cells are probably populations of interneurons and not the projection neurons which generally express calbindin (DiFiglia et al. 1989). Therefore I felt the lesion produced by the 30nmol QA injection was potentially more representative of the selective degeneration of HD, providing a less intensive model of excitotoxic cell death more suited to assessing protective effects following delivery of neuroprotective biotherapeutic molecules.

Figure 3-2  QA lesion testing: 50 and 30 nmol intrastriatal QA injections
Representative images showing surviving NeuN- and calbindin- immunopositive neurons following injection of (A,B) 50nmol or (C,D) 30nmol QA (400nL) into the striatum. Survival of striatal neurons within the lesioned striatum appeared greater in the lower concentration of QA (C), with some calbindin-positive neurons also seen to be maintained post-QA (D). Dotted lines show rough outline of the extent of QA lesioning in the striatum. Scale bar = 500µm
3.3.3 Trial 3: Behaviour testing – 30nmol QA

In order to later investigate functional protection against the development of motor impairments, I needed to ensure that the QA lesion was sufficient to generate behavioural deficits that could be readily observed and quantified by behaviour tests designed to assess imbalances in basal ganglia function following unilateral lesioning. Therefore to further assess the suitability of the less severe 400nL, 30nmol QA injection from the second QA lesion trial, I took another small cohort of rats (n = 4) and assessed their performance in behaviour tests seven days post-QA lesioning.

Following QA lesioning of the right striatum, all of the rats displayed preferential use of their right (ipsilateral) forelimb for spontaneous exploration of the cylinder, with overall asymmetry scores recording 54 – 71% ipsilateral forelimb use. However the administration of apomorphine or amphetamine only stimulated strong rotational behaviour in two of the four rats. The rats that did respond displayed contrasting rotational effects arising from the two drugs. Apomorphine induced the rats to rotate towards the lesioned striatum (94% ipsilateral), while amphetamine stimulated rotations away from the lesioned striatum (96% contralateral). While these results were not expected, previous assessment of striatal lesioning has shown similar results when the anterior portion of the striatum alone is lesioned (Norman et al. 1992; Fricker et al. 1996).

Overall these rats showed that the 30nmol QA lesion is severe enough to cause measurable behavioural deficits, however the lack of significant drug-induced rotational behaviour in half of the rats, and the differentially induced direction of rotation suggest that the ability to generate a lesion that gives consistent behavioural impairments over a large cohort of rats may be difficult with this quantity of QA. Larger lesions generally will provide greater consistency in overall behavioural performance between subjects, therefore I elected to not proceed with this 30nmol QA lesion but to use the higher concentration of 400nL, 50nmol QA for the neuroprotective investigations.

3.3.4 QA lesion model characterisation – 50nmol QA

After assessment of all the QA lesion trials I decided to proceed with 50nmol QA in 400nL NaOH, as this protocol generated a large striatal lesion without obvious mechanical damage, while sparing a population of non-calbindin containing neurons within the lesioned area suggestive of interneuron sparing. To more comprehensively characterise the neuropathological and behavioural changes occurring following the 50nmol QA injection I assessed the development of quantifiable functional behaviour deficits and performed immunocytochemical staining to positively identify sub-
populations of striatal neurons to determine more specifically which neurons are maintained in this particular QA lesion model.

3.3.4.1 Functional Behaviour Assessment of Unilateral QA Lesioned Rats

To ensure reliable assessment of the QA lesion and associated functional impairments I took a larger group of rats ($n = 10$) for investigation. Baseline measurements of exploratory forelimb use and amphetamine- / apomorphine-induced rotations were undertaken one week prior to stereotaxic QA injection. The rats were left for one week post QA injection to allow the acute lesion development to occur before proceeding with behavioural assessment. I analysed spontaneous exploratory forelimb use seven days post-QA lesioning followed directly by amphetamine-induced rotational assessment. Apomorphine-induced rotational behaviour was assessed ten days post-lesion before the rats were euthanised and processed for neuropathological analysis.

Functional impairments were measurable by behaviour tests one week following QA lesion. The unilateral QA lesion rats developed a significant $63\% \pm 3$ ipsilateral forelimb bias for exploratory behaviour compared with the $53 \pm 3\%$ baseline assessment (paired t-test $P < 0.05$; Figure 3-3C). Post-QA drug-induced rotational behaviour was preferentially towards the lesioned hemisphere with apomorphine-inducing $91\% \pm 5$ of rotations to be ipsilateral and amphetamine $82 \pm 9\%$ ipsilateral, showing a significant shift from the proportion of baseline ipsilateral rotations of only $48\% \pm 9$ and $34\% \pm 9$ respectively (paired t-tests $P < 0.05$; Figure 3-3A, B). The extent of drug-induced rotational behaviour following the QA lesion with apomorphine administration significantly increasing total rotational behaviour from $2.2 \pm 0.6$ rotations per minute to $4.7 \pm 0.7$ (paired t-test $P < 0.01$), however amphetamine only induced a rotation rate of $1.6 \pm 0.3$ rotations per minute that was non-significantly increased to $2.7 \pm 0.6$ following the QA lesion (paired t-test $P = 0.08$). The amphetamine induction of ipsilateral rotations was in contrast to that induced following the smaller 30nmol lesion which caused the rats to rotate contralaterally, suggesting the extent of striatal lesioning determines the direction of rotation (Norman et al. 1992).
3.3.4.1.1 Additional sensorimotor behavioural testing

The inclusion of the recently developed “corridor” task (Section 4.5.2; (Dowd et al. 2005)) to assess sensorimotor neglect in the neuroprotective investigations required a small group of rats \(n=4\) to facilitate the setting up of the corridor apparatus, determination of an assessment protocol, and to ensure that the 50nmol QA lesion induced a measurable sensorimotor deficit. Behavioural performance was observed on days one and two prior to QA lesion and on days 12 and 13 after injection.

Two of the rats displayed right side bias for sugar pellet retrieval in the baseline testing prior to the QA lesion indicating a dominant left hemisphere. Unfortunately the QA was administered to the right striatum of each rat, rather than their dominant side, resulting in an ipsilateral group bias prior to QA. Following QA, three of the four rats showed an increased preference for ipsilateral (right side) retrievals with an overall group shift from 59 ± 7% to 76 ± 7% ipsilateral retrievals (Figure 3-3D). This did not reach statistical significance (Wilcoxon signed rank test: \(P = 0.25\)), however this
was possibly due to the small group size and lesioning of the non-dominant hemisphere in half the subjects. Reanalysis of the data using the duplicate trail results for each rat in an unpaired t-test did indicate the significant development of sensorimotor neglect after QA lesion (P < 0.05).

3.3.4.2  **Neuropathological Assessment of QA Lesion**

Immunocytochemistry was performed on coronal sections through the striatum from each rat brain to assess the extent of the QA-induced striatal lesioning by NeuN-, calbindin- and DARPP-32-immunopositive staining. More specifically the surviving striatal interneurons were visually identified with antibodies directed against calretinin, parvalbumin, NOS and ChAT (Section 3.2.3). Using the general neuronal marker NeuN, the loss of neurons following the 50nmol QA intrastriatal injections was generally confined to the striatal region causing extensive neuronal loss without obvious structural damage ten-days post injection (Figure 3-4A). In some cases where the lesion was situated in the dorsal or lateral portion of the striatum, a loss of neurons was also observed in the deep layers of the cortex overlying the lesioned striatum. A number of striatal neurons within the lesioned area were however resistant to QA excitotoxicity (Figure 3-4C) as also observed in the earlier testing (Figure 3-2). While a scattering of DARPP-32-positive neurons (Figure 3-4F) and calbindin-positive neurons (not shown) were still observed within the QA lesioned area, the majority of the QA resistant neurons were assumed to be striatal interneurons which normally make up ~5% of the striatal neuron population. Partial sparing of striatal neurons was particularly enhanced in the dorsolateral portion of the striatum with the medial extent of the lesion usually showing a distinct border between the lesioned and non-lesioned striatum (Figure 3-4D, F). Immunocytochemistry for markers of subpopulations of the striatal interneurons showed that only the small aspiny calretinin-positive neurons (Figure 3-5C, D) and the large aspiny cholinergic ChAT-positive neurons (Figure 3-5G, H) were largely spared. The GABAergic parvalbumin (Figure 3-5A, B) and NOS (Figure 3-5E, F) immunopositive neurons were vulnerable to QA induced cell death.

I also observed that parvalbumin expressing neurons of the globus pallidus were affected by the QA lesion (Figure 3-4G, H) suggesting a potential process of secondary cell death in the striatal output nuclei which may have occurred following the loss of afferent projections from the striatum. No assessment was performed of the neurons in the substantia nigra following degeneration of the striatonigral projections.
Figure 3-4  Pathological characterisation of the 50nmol QA lesion
Immunocytochemical staining for (A,C,D) NeuN or (B,E,F) DARPP-32 positive neurons show the extent of the QA induced neuronal cell loss within the injected striatum compared to the intact contralateral hemisphere.  (G,H) Loss of parvalbumin positive cells in the globus pallidus indicates possible QA induced cell death outside of the striatum.  Dotted lines show a rough outline of the extent of QA lesioning in the striatum (D,F) and outline of the globus pallidus (G,H).  Scale bar = 3.5mm for whole sections (A,B), 500µm for (C-F) and 900µm for (G,H).
Figure 3-5  Striatal interneurons following QA lesioning
Representative images of the striatal interneurons within intact and QA lesioned striatum. GABAergic interneurons expressing (A,B) parvalbumin, (C,D) calretinin or (E,F) NOS are present in the striatum. The small population of calretinin neurons appeared to be fully maintained throughout the lesioned striatum, with the large ChAT-positive cholinergic interneurons also appearing to be relatively spared by QA. A few parvalbumin (B) and NOS (F) positive neurons were still present in the “transition zone” more distal from the QA injection site (right side of images). Scale bar = 200µm
3.4 **Discussion**

Optimisation and brief characterisation of the QA lesion model of HD was undertaken to ensure the model generated the major pathological neurodegeneration of striatal projection neurons representative of HD and produced quantifiable functional deficits in the rats’ behaviour. While the QA model cannot be claimed to replicate the slow progressive neurodegenerative mechanisms occurring in the HD brain, the selective vulnerability of striatal neurons is suggestive of excitotoxic involvement. By restricting the quantity of QA and volume injected into the striatum I was able to generate a partially selective lesion of the striatal neurons without inflicting gross structural changes of the brain tissue. While the lower quantity of 30nmol QA resulted in a slightly less intensive lesion and would have debatably provided a more realistic representation of excitotoxic insult for my preventative gene delivery studies, concern over the reliable level of measurable behavioural deficits meant that the 50nmol QA lesion protocol was ultimately selected.

3.4.1 **QA-induced neuropathology**

Previous investigations involving the intrastriatal injection of QA to model HD pathology in adult rats have been conducted with a diverse range of quantities and delivery volumes including: 300nmol, 1µL QA (Popoli *et al.* 1994; Scattoni *et al.* 2004); 225nmol, 1µL (Emerich *et al.* 1996; Borlongan *et al.* 2004; Emerich 2004b); 180nmol, 1µL (de Almeida *et al.* 2001; Escartin *et al.* 2004); 120nmol, 1µL (Dobrossy and Dunnett 2003); 120nmol, 0.5µL (Beal *et al.* 1986); 100nmol, 2µL (Alexi *et al.* 1999); 85nmol, 42.5nL (Brickell *et al.* 1999); 60nmol, 0.5µL (Bordelon *et al.* 1999); 50nmol, 2µL (Kells *et al.* 2004); 50nmol, 1µL (Figueroedo-Cardenas *et al.* 1998); 15-60nmol, 1µL (Ryu *et al.* 2003). In my personal experience the larger quantities of QA have generally resulted in physical damage to striatum, often causing a physical loss of striatal tissue at the site of injection as observed in the initial 100nmol QA trial (Figure 3-1). Although neuroprotection has been achieved against some of the larger doses of QA, the protracted but inevitable neurodegeneration induced by the poly-Q tract expansion in huntingtin would indicate a more subtle contribution of excitotoxicity to striatal neuronal death, better replicated by lower quantities of QA. It is possible that spreading the QA across multiple injection sites in the striatum as performed by Dobrossy *et al.* (2003) may further reduce the intensity of excitotoxicity directly surrounding the injection sites, and thereby avoid inducing such severe damage. However the lower quantities of QA are still capable of inducing neuronal cell death over a considerable portion of the striatum following a single intrastriatal delivery site, indicating that QA can readily diffuse through the striatal parenchyma. Multiple injection sites would enable a greater proportion of the striatum to be lesioned and reduce
the extent of QA concentration gradients presumably responsible for the development of a “transition zone” of partial neuron survival surrounding a core of total neuron loss (Brickell et al. 1999). A region of complete neuronal death proximal to the site of injection was often not clearly distinguishable following the selected 50nmol QA lesion although there was still a gradient of increasing neuron survival away from the injection site.

The selective sparing of small calretinin-positive interneurons and the large cholinergic neurons from QA-induced cell death is representative of their maintenance in the HD brain (Ferrante et al. 1987; Cicchetti and Parent 1996a; Cicchetti et al. 1996b) and in agreement with earlier QA lesion assessment (Figueroedo-Cardenas et al. 1994). However the apparent loss of medium aspiny NADPHd / NOS or parvalbumin containing striatal interneurons contrasts with their relative invulnerability to HD neurodegeneration (Ellison et al. 1987; Harrington and Kowall 1991), but has previously been reported following QA lesioning (Davies and Roberts 1987; Waldvogel et al. 1991; Figueredo-Cardenas et al. 1994); although others have detected sparing of these NADPHd / somatostatin / neuropeptide Y interneurons (Beal 1992a; Beal 1992b) possibly due to differences in QA injection protocols. The observed selectivity of striatal neuronal sparing of the 50nmol, 400nL QA injection concurs with the quantitative analysis of striatal interneurons I previously conducted following a larger volume 50nmol, 2µL QA lesion (Kells et al. 2004).

3.4.2 QA-induced behavioural impairments

A number of behavioural tests have been developed to assess functional deficits in rodent behaviour exhibited following excitotoxic lesioning of the striatum. While disturbances in cognitive function generally require bilateral lesioning, simple deficits in motor performance are readily induced by unilateral lesioning and can be detected by observing imbalances in lateralised behaviour (Nakao and Itakura 2000). Although it is difficult to correlate behavioural deficits in rodent models with HD symptoms, it is possible that some of the motor impairments are resultant from the same neuronal dysfunction responsible for the choreiform movements of HD patients. With symptomatic benefits being the most critical measure of any treatment strategy, the use of behavioural testing in animal-based investigations is a vital measure of therapeutic efficacy. Therefore to characterise the acquirement of motor deficits following the selected QA lesion I set-up four behaviour tests: drug-induced rotational behaviour, apomorphine and amphetamine; spontaneous forelimb use; and sensorimotor neglect “corridor” task.

Unilateral loss of striatal projection neurons creates a hemispherical imbalance in the populations of dopaminoceptive neurons. Administration of either a dopamine agonist (apomorphine) or dopamine-
releasing drug (amphetamine) following QA injection induced the majority of rats to rotate towards
the lesion side (ipsilateral) as previously reported for excitotoxic striatal lesions with the extent of
lesioning determining the degree of rotational asymmetry (Schwarcz et al. 1979; Dunnett et al. 1988;
Nakao et al. 1996; Nakao and Brundin 1997). The loss of neurons expressing dopamine receptors
would conceivably be expected to result in similar motor behaviour following drug induced
dopamine release or dopamine agonist administration through greater dopamine receptor stimulation
in the intact striatum. However, the brief assessment of a smaller 30nmol QA lesion suggested
contrasting effects with amphetamine-inducing rotations away from the lesion side suggesting that
smaller lesions may result in other more subtle changes to dopamine signalling, or may have been
related to the positioning of the lesion with anterior lesions having been shown to induce
contralateral rotational behaviour (Norman et al. 1992; Fricker et al. 1996). More traditionally used
for assessing the integrity of the dopaminergic nigrostriatal connections (Choi-Lundberg et al. 1998),
drug-induced rotational assessment can also provide some indication of striatal projection neuron
maintenance; although inconsistencies in the direction of induced rotation, especially when dealing
with small lesions, complicates assessment of deficit in relation to the degree of striatal lesioning.

A general assessment of lateralised brain injury causing motor function impairment, the spontaneous
forelimb use analysis following the QA lesion showed enhanced overall use of the ipsilateral
forelimb. Greater use of the forelimb on the same side as the lesion is induced due to impaired CNS
control of the contralateral forelimb, which is proportional to the severity of lateralised damage
(Schallert et al. 1997; Schallert et al. 2000). Recent development by Dowd et al. (2005) of the
“corridor” task, a simple test of lateralised response selection, to assess the development of
sensorimotor neglect following unilateral 6-hydroxydopamine lesioning of dopaminergic neurons
also showed contralateral side neglect following the unilateral QA striatal lesion. Although still an
assessment of spontaneous motor control, the lateralisated selection of food retrieval following
balanced left and right side external sensory cues possibly provides a more sensitive assessment of
basal ganglia control of sensorimotor coordination than the non-motivated exploratory forelimb use
analysis.

Overall, the non-drug induced assessments of sensorimotor function displayed significant
behavioural deficits following the 50nmol, 400nL QA injection without inducing purely unilateral
responses, which was often observed following drug-induced rotational behaviour. Sub-maximal
responses are more likely to allow for the detection of subtle changes potentially provided by
therapeutic agents against the neuropathological changes induced by QA lesioning.
Chapter 4

**Neuroprotective Study Methods**

4.1 Overview

To investigate AAV-mediated delivery of biotherapeutics as a preventative treatment strategy against striatal neurodegeneration and the onset of functional motor control impairments, I surgically injected AAV vectors unilaterally into the rats’ striatum prior to an excitotoxic insult with QA (Figure 4-1). To monitor the development of functional impairments I put each rat through a series of behavioural tests both before and after AAV vector delivery to determine baseline behaviour and then repeatedly for 6-7 weeks following QA lesion. On completion of behavioural testing the rats were euthanised, paraformaldehyde fixed and the brains processed for immunocytochemical analysis. To determine expression levels of the biotherapeutics within the striatum at the time of QA lesion, I injected additional cohorts of Wistar rats with the same AAV vectors, euthanised after three weeks and isolated the striatum for quantitative ELISA analysis.

![Figure 4-1 Neuroprotective study timeline](image)

**Figure 4-1 Neuroprotective study timeline**

Timeline of the *in vivo* investigative studies to assess the neuroprotective effects of AAV-mediated gene transfer and prevention of functional behavioural deficits associated with a striatal lesion. Numbers represent weeks prior and post QA injection with rats euthanised at various end-points post-QA.
4.2 Neuroprotective Investigations

4.2.1 In vivo expression testing

To test in vivo transduction and transgene expression for each of the AAV treatment vectors, prior to commencing the neuroprotective investigation, I injected 4.0 µL of each AAV vector unilaterally into the striatum at one or two stereotaxic sites (Table 4-1). The rats were housed for three weeks following AAV injection to let transgene expression stabilise in the transduced cells before euthanizing and processing for HA-immunostaining to visualise the spread and extent of transgenic protein expression.

<table>
<thead>
<tr>
<th>AAV vector delivery</th>
<th>Coordinates from Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A-P</td>
</tr>
<tr>
<td>First Investigation – Single site</td>
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</tr>
<tr>
<td>Second Investigation – Two-sites</td>
<td>+1.3</td>
</tr>
<tr>
<td></td>
<td>-0.2</td>
</tr>
</tbody>
</table>

Table 4-1 Stereotaxic injection coordinates for AAV vector delivery
Injection coordinates were determined from a rat brain atlas (Paxinos and Watson 1986), and in vivo testing with AAV-Luciferase (Section 4.4.2).

4.2.2 Initial investigations: AAV-BDNF (high-titre), AAV-GDNF, AAV-Bcl-xL

The first in vivo study I undertook investigated the protective effects of AAV-BDNF, AAV-GDNF and AAV-Bcl-xL administration. Wistar rats were randomly assigned into five groups to receive unilateral intrastriatal AAV vector or sham PBS injection (Table 4-4). Functional motor behaviour was assessed in the spontaneous forelimb use test and by drug-induced rotations (Section 4.5). Baseline behaviour was assessed to determine each rat’s naturally dominant hemisphere. Infusing directly into the dominant hemisphere using stereotaxic surgical procedures (Table 4-1; Section 4.4.1), each rat received 4.0 µL AAV-BDNF, AAV-GDNF, AAV-Bcl-xL, AAV-Luciferase or PBS, mixed with 1.0 µL 20% mannitol to increase the efficiency of transduction (Mastakov et al. 2001; Mastakov et al. 2002). Three weeks later all rats received an intrastriatal injection of QA at the same stereotaxic coordinates (Chapter 3). Functional motor behaviour was assessed weekly with the AAV-BDNF treated rats euthanised five weeks post-QA and the remaining groups seven weeks after QA (Table 4-2).
4.2.3 Follow-up investigations:  *AAV-BDNF* (diluted), *AAV-Bcl-x<sub>L</sub>, AAV-XIAP*

My second *in vivo* investigation followed-up on the initial results of the previous study to further investigate *AAV-BDNF* and *AAV-Bcl-x<sub>L</sub>* administration, in addition to assessing the newly constructed *AAV-XIAP* vector. Rats were randomly assigned to receive *AAV-BDNF*, *AAV-Bcl-x<sub>L</sub>* *AAV-XIAP*, *AAV-Luciferase* or PBS (Table 4-4). The dominant brain hemisphere was determined for each rat following baseline behaviour testing in the “corridor” task, spontaneous forelimb use and apomorphine-induced rotations (Section 4.5). Following baseline behaviour assessment, I injected the AAV vectors or PBS into the dominant striatum. In contrast to the first study (Section 4.2.2) I decided to split the vectors over two-injection sites within the striatum (Table 4-1), with each site receiving 4.0 µL vector or PBS plus 1.0 µL 20% mannitol, except the *AAV-BDNF* rats which only received 2.0 µL vector plus 1.0 µL 20% mannitol per site. The *AAV-BDNF* and *AAV-XIAP* vectors were each diluted 1:10 with sterile PBS prior to injection. QA was administered three weeks after vector delivery to all rats except five randomly chosen *AAV-BDNF* animals which provided non-lesioned *AAV-BDNF* treated controls. Rats were all euthanised eight weeks after QA lesion with behaviour assessed weekly or biweekly throughout (Table 4-3).
4.3 Animals

<table>
<thead>
<tr>
<th>QA Lesion Development</th>
<th>Number of Rats</th>
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<th>Functional Protection</th>
<th>Transgene Expression</th>
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<td>5</td>
</tr>
<tr>
<td>AAV-GDNF</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>AAV-Bcl-xL</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>AAV-XIAP</td>
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<tr>
<td>AAV-Luciferase</td>
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<th>Functional Protection</th>
<th>Transgene Expression</th>
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</thead>
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</tr>
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<td>5</td>
</tr>
<tr>
<td>AAV-Bcl-xL</td>
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<td>5</td>
</tr>
<tr>
<td>AAV-Luciferase</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>PBS</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<td>-</td>
</tr>
<tr>
<td>PBS</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4-4 Allocation of animals

Cohorts of male Wistar rats analysed for QA lesion development, AAV vector testing and neuroprotective gene transfer studies.

All of the *in vivo* studies were conducted in adult male Wistar rats (200 – 300g) supplied by the University of Auckland Animal Resources Unit with strict adherence to approvals granted by the University of Auckland Animal Ethics Committee in accordance with the NZ Animal Welfare Act (1999). Rats were housed in a temperature and humidity controlled environment on a 12hr light / 12hr dark regime. Depending on experimental design, housing was either in groups of 4-5 with food and water available *ab libitum*, or individually when dietary restriction was required to maintain the rats at 85% of their expected free feeding weight for incentive based behaviour testing – water was available *ab libitum*.
Following immunocytochemical analysis of the transgene expression and visualisation of the QA lesion in the main neuroprotective investigations I removed from the analysis any rats that lacked transgenic protein expression in the striatum, had no evidence of QA lesioning, or showed misplacement of either AAV vector or QA injection.

4.4 Stereotaxic Surgeries: AAV Vector Delivery and QA Injection

4.4.1 Operating procedure

Direct intrastriatal injection of the AAV vectors and QA was performed by stereotaxic surgery techniques using Kopf® small animal stereotaxic frames paired with a MicroSyringe Pump Controller (Micro 4; World Precision Instruments) to control the injections via Hamilton® syringes. Rats were systemically anaesthetised with an initial intraperitoneal (i.p.) injection of 60 mg/kg sodium pentobarbital (National Veterinary Supplies Ltd, NZ) plus additional dosing as required to maintain anaesthesia throughout the surgery. Marcain (AstraZeneca) was administered (0.5 mg/kg) by subcutaneous (s.c.) injection, directly above the rat’s skull, prior to beginning surgery to provide local analgesic at the site of incision. The rats were firmly secured into a Kopf® stereotaxic frame by nose/tooth bar and ear bars with the nose/tooth bar zeroed to hold the skull level. The skin above the skull was opened with a single longitudinal incision to expose the skull and positively identify Bregma from which the A-P and M-L stereotaxic injection site coordinates were measured. A burr hole was drilled with a 0.5mm tungsten carbide bit (Sunshine®-Dental) in the skull at the A-P, M-L injection coordinates through which a 5 or 10µL Hamilton® syringe (32G needle) was carefully lowered 1mm/min to the desired depth below the dura. Once the syringe was correctly positioned the MicroSyringe Pump Controller was programmed to slowly infuse either an AAV vector / PBS vehicle control at 150 nL/min or QA at 100 nL/min. After injection the syringe was left in place for an additional 5mins before withdrawing to minimise backflow up the needle tract while the syringe was slowly withdrawn. On completion of the stereotaxic injections the skin incision was sutured closed (Silk 4/0, Resorba) and Xylocaine® 2% Jelly (AstraZeneca) was topically applied to the wound. The rats were placed under a heat-lamp and monitored post-operatively until they recovered from the systemic anaesthesia before returning to their home cages.
To test the striatal coordinates and the required volume for AAV vector delivery I performed a small test using the AAV-Luciferase vector injecting either: 3.0 µL \((n = 3)\) or 4.5 µL \((n = 3)\) at a single site (A-P +0.5, M-L 2.7, D-V -5.0 mm); or 5.0 µL evenly split between two injection sites \((n = 3)\); Site 1: A-P +1.0, M-L 2.7, D-V -5.0; Site 2: A-P -0.2, M-L 3.0, D-V -5.0). All vectors were injected along with 1.0 µL 20% mannitol to increase the efficiency of transduction (Mastakov et al. 2001; Mastakov et al. 2002). The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson 1986) was used as a guide to determine initial stereotaxic coordinates for all injection sites.

Intrastriatal injection of AAV-Luciferase led to transduction of the targeted striatal cells plus pyramidal cortical neurons surrounding the needle tract, and pallidal neurons (Figure 4-2). The lower volume injection tended to result in transduction of striatal neurons in a broad band surrounding the needle tract in contrast to the more extensive spread of transduction throughout the striatum following the single 4.5 µL or two-site 5.0 µL injection protocols. Cortical neurons surrounding the needle tract were extensively transduced indicating some backflow of the vectors up the needle tract and also along the top of the corpus callosum. It is also possible that some of the cortical transduction was facilitated by retrograde transport of the AAV vectors as previously observed (Kaspar et al. 2002; Burger et al. 2004). Transduction of pallidal neurons within the globus pallidus, a target nucleus of the striatal projection neurons, is indicative of anterograde vector transport and was observed following all injection protocols. Transduction of the nigral neurons seen in the main investigation was not assessed in these initial AAV-Luciferase transduced animals. The anterograde transport and transduction of target nuclei may well be of therapeutic benefit for delivering biotherapeutics to the terminal axonal projection fields of the medium spiny neurons and supply target derived support of the neuronal projections against cellular insult.

Given the extensive spread of striatal transduction achieved following a single injection I decided to proceed initially with a single site 4.0 µL delivery protocol for my neuroprotective studies to minimise any surgical-related trauma associated with the vector delivery and generally restrict transduction to the striatum. The 4.0µL vector plus 1.0µL mannitol injection allowed the use of 5.0µL Hamilton syringes. In later investigations I elected to use a two-site injection to enhance the distribution of transduced neurons away from the direct site of QA injection.
Figure 4-2  Intrastriatal AAV vector delivery
(A) Visualisation of in vivo Luciferase expression following trials of various intrastriatal AAV-Luciferase injection protocols. Intrastriatal injection of AAV-Luciferase resulted in transduction of (B) cortical neurons, (C) striatal neurons, and (D) neurons within the globus pallidus. Scale bar = 2mm for whole sections (A), 100µm for (B) and 50µm for (C,D).
4.5 Functional Behavioural Analysis

To assess the physical impact of AAV-mediated treatment on the rat’s behaviour I observed their motor performance in a range of behavioural tests designed to show preferential biasness between the left and right side of the rats following a unilateral lesion in the basal ganglia. All assessment of behavioural testing was performed blinded to the specific AAV vector / PBS delivery and the treated hemisphere.

4.5.1 Spontaneous exploratory forelimb use

Spontaneous exploratory forelimb use assessed the development of a preference for the QA lesioned rat to use its forepaw ipsilateral to the lesioned hemisphere when exploring a novel environment (Schallert et al. 2000). Rats were transferred from their home cage to a clear acrylic cylinder (200mm internal diameter; 400mm high) where they were free to explore by rearing up on their hindpaws and using their forepaws to support themselves against the cylinder walls. Video recording of 5mins exploration was analysed to quantify left and right forepaw usage while rearing off the floor, initial placement on the cylinder wall and for landing back on the floor. A single asymmetry score was calculated by averaging the percentage of time each rat used its ipsilateral forepaw for rearing, initial cylinder wall placement and landing.

Figure 4-3 Spontaneous exploratory forelimb use

Each rat was placed into clear cylinder and video recorded for 5 mins for later analyse of left and right forelimb usage while exploring the cylinder. The placement of two-mirrors behind the cylinder allowed a full 360° view of the rats. Rat in (A) is shown initiating an exploratory rear off its right forelimb, and in (B) contracting the cylinder wall with its left forelimb.
4.5.2  “Corridor” task

The “Corridor” Task recently developed by Dunnett and colleagues to study 6-hydroxydopamine induced lesions assesses left / right preference for food retrieval (Dowd et al. 2005). Rats are placed into a long narrow corridor with caps containing sugar pellets located on each side of the corridor floor in evenly spaced adjacent pairs. The rat is free to explore up and down the corridor but when retrieving sugar pellets it is required to make a left or right selection or move onto the next pair of sugar pellet containing caps. Although not previously reported to assess QA lesions I choose to investigate using this test as it introduces an element of decision making and assesses the development of any sensorimotor neglect. To undertake this task the rats were diet restricted to 85% free feeding body weight in accordance with our ethical approval. In an effort to restrict exploratory behaviour during assessment the rats were habituated to the testing apparatus one day prior to testing by placing each animal into the corridor for 10mins with a few sugar pellets scattered along the floor of the corridor. Then immediately prior to testing the rats were placed into an empty corridor for 5mins before transferring directly into a corridor setup to run the task. Retrievals were scored whenever the rat placed its nose into a cap regardless of the number of sugar pellets actually retrieved. The first 20 retrievals (max 5mins) were recorded as being either from the left- or right-side. Rats were tested on two consecutive days with the percentages of retrievals ipsilateral to the lesion side averaged to ensure consistency.
Figure 4-4  Sensorimotor “corridor” task
Dietary restricted rats were allowed to freely explore up-and-down the corridor retrieving sugar pellets from evenly spaced containers along each wall of the corridor. A clear Perspex lid dissuaded the rats from vertical exploration. Corridor dimensions in mm.

4.5.3  Drug-induced rotational analysis

Drug-induced rotational behaviour analysis was used to measure bilateral imbalances in the dopamine system. Apomorphine – a dopamine agonist – and amphetamine – a dopamine releasing agent – are commonly used to assess functional performance in Parkinson’s disease models but have also been employed in QA lesion studies (Schwarcz et al. 1979; Dunnett et al. 1988). I investigated the use of both apomorphine (1mg/kg injected s.c.) and amphetamine (5mg/kg injected i.p.) dissolved in 0.9% saline. Following drug administration the rats were placed into rotational bowls and assessed for 1hr either by video analysis or in real time using an automated Rotomax Rotometer (AccuScan Instruments) system.
Figure 4-5   **Drug-induced rotational behaviour**
Rats were fitted with a harness, placed into clear rotational cylinder and tethered to a Rotomax Rotometer system used to quantify rotational behaviour for 1 hour following apomorphine or amphetamine administration.

### 4.6 Quantitative Transgene ELISA

Quantification of the transgene expression within the striatum following AAV vector gene delivery was undertaken to determine the level of biotherapeutic protein expression at the time of QA injection. Three weeks after AAV vector delivery the rats were deeply anesthetised with an overdose of sodium pentobarbital (~300mg/kg i.p.), decapitated and their brains immediately removed. The brains were split into two hemispheres down the mid-line and the striatal region from each hemisphere dissected out, snap-frozen in liquid nitrogen and stored at -80°C.

Homogenisation of the striatal tissue in preparation for ELISA quantification was performed in a 50mM Tris buffer pH 6.8 containing 0.5% Tween-20, 0.1% sodium azide, 1.5g/L EDTA, 5mg/L Pepstatin A and 10mg/L PMSF. Frozen striatal tissue was transferred to a 15mL tube along with 600µL of homogenisation buffer and mechanically homogenised using a motorised cylindrical Teflon pestle until tissue fragments were no longer visible. Homogenates were centrifuged at 3,000rpm for 15mins at 4°C. The protein containing supernatant was divided into aliquots and stored at -80°C to prevent protein degradation.

Quantitative ELISA assays were undertaken in accordance with the appropriate ELISA kit instructions. Promega $E_{\text{max}}^\circ$ ImmunoAssay Systems were used for BDNF (G7610) and GDNF (G7620), and R&D Systems Duoset® IC for XIAP (DYC822) and Bcl-xL (DYC894). To perform quantification the sample supernatants were each run in triplicate at two dilutions (Table 4-5) along with samples from AAV-Luciferase treated rats as AAV-vector transduction controls (1:10 and 1:50 dilutions) and each kits recommended eight-point standard series. Optical absorbance of the ELISA plate was read on a Spectra max plate reader (Molecular Devices). Protein concentrations calculated from the standard curve were subsequently standardised against total protein in each sample.
Total protein was measured using bicinchoninic acid (BCA; Sigma-Aldrich) and bovine serum albumin (BSA; Invitrogen) as a protein standard. A 4000 µg/mL BSA standard was prepared in 1N sodium hydroxide and serially diluted 1:2 to produce an eight point standard curve from 2000 – zero µg/mL. Sample supernatants were diluted 1:10, 1:20 and 1:40 and plated into a 96 well ELISA plate in duplicate alongside the BSA standards also plated in duplicate (50 µL/well). 100µL BCA reagent (1mL 4% copper sulphate solution per 50mL BCA) was added to each well and incubated at room temperature for 2-3 hours before reading on a Spectra max plate reader at 562nm. Total protein was calculated from the BSA standard curve.

<table>
<thead>
<tr>
<th>Sample Dilutions</th>
<th>Initial Investigations</th>
<th>Ipsilateral</th>
<th>Contralateral</th>
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<tbody>
<tr>
<td>AAV-BDNF</td>
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</tr>
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<tr>
<td>AAV-Bcl-xL</td>
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<td>1:10, 1:50</td>
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<tr>
<td>Follow-up Investigations</td>
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<td></td>
<td>AAV-XIAP</td>
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</table>

Table 4-5 Dilutions used for ELISA quantification of in vivo transgenic protein expression. Transgenic protein containing supernatants from the striatal tissue homogenates were diluted in supplied buffers to appropriate levels for ELISA quantification analysis. The ipsilateral hemisphere required greater dilution to fit standard curves.

### 4.7 Immunohistochemical Analysis

Immunocytochemistry was performed to visualise transgene expression following AAV gene delivery, analyse QA induced neurodegeneration and to correlate the attenuation of behavioural impairments with structural maintenance of brain anatomy.

#### 4.7.1 Brain tissue collection and processing

Paraformaldehyde fixation of the rat brains was used throughout the research. The rats were deeply anesthetised with an overdose of sodium pentobarbital (~300mg/kg i.p.) and perfused transcardially with 100mL chilled 0.9% saline followed by 500mL 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Following ~30min perfusion the brains were removed, post-fixed overnight in 4%
paraformaldehyde at 4°C and then cyroprotected for sectioning in 30% sucrose 0.1M phosphate buffered solution. A sliding microtome with freezing stage was used to cut 40µm coronal sections through the striatum from frozen brains. Eight sets of sections were collected from each brain (distance of 320µm between consecutive sections in each set) and stored in a cyroprotectant solution (30% sucrose, 30% ethylene glycol, 0.5M phosphate buffer) at -20°C.

### 4.7.2 DAB-staining immunocytochemistry protocol

The visualisation of specific cellular populations and / or structure is possible with immunocytochemistry using antibodies raised against specific proteins or peptides uniquely expressed by the cells of interest. I utilised a DAB (3,3’-diaminobenzidine tetrahydrochloride)-staining procedure to visualise various neuronal populations within the basal ganglia via the binding of primary antibodies raised against specific marker proteins (Table 4-6).

Immunocytochemical staining was performed on individual sets of free floating coronal sections spanning the region of interest. Sections were removed from the cyroprotectant solution and washed overnight in PBS. To block any endogenous peroxidase activity I first incubated the sections in a solution of 1% hydrogen peroxide, 50% methanol for 10mins and then washed four times in PBS-Triton (PBS with 0.2% Triton X-100). The primary antibodies (Table 4-6) were diluted in PBS-Triton, either with goat serum as a blocking agent or without any blocking serum. Goat serum (GIBCO®) was used as a blocking agent for all antibodies except for when the primary antibody was raised in goat. Sections were incubated in the diluted primary antibody overnight at room temperature with gentle agitation on a rocking table. Following overnight incubation the sections were washed four times in PBS-Triton and incubated with an appropriate secondary antibody diluted 1:500 in PBS-Triton with or without goat serum. All secondary antibodies were biotinylated antibodies raised against the host species of the primary antibody (Appendix B.4). The sections were incubated with the secondary antibodies for 2-3 hours at room temperature on a rocking table, washed four times with PBS-Triton and incubated for a further 2-3 hours with Extravidin peroxidise (Sigma-Aldrich) diluted 1:500 in PBS-Triton. Sections were washed four times in PBS before staining in DAB or DAB-nickel sulphate solution. After staining the sections were washed in PBS and mounted onto polysine-coated glass microscope slides (Biolab Scientific, NZ). The slides were dried overnight, serially dehydrated in 70%, 90% and 100% ethanol, delipidified in xylene and coverslipped with histomount mounting medium (DPX; Sigma-Aldrich).

<table>
<thead>
<tr>
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<th>Dilution</th>
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<td>Covance</td>
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Table 4-6 Primary antibodies for DAB-staining immunocytochemistry

Primary antibodies and working dilutions for immunocytochemical analysis of different neuronal populations and the localisation of transgene products in fixed free-floating brain sections. Additional antibody details in Appendix B.5.

4.7.3 Fluorescent immunocytochemistry analysis

Double labelling of striatal sections was performed by fluorescent immunocytochemistry to visualise the expression of cellular markers – NeuN, DARPP-32, krox-24 and GFAP – and the relative localisation of transgenic protein expression. Striatal sections were removed from the cryoprotectant solution and washed overnight in PBS. The sections were then incubated overnight at room temperature with pairs of primary antibodies diluted in PBS-Triton with 1% blocking serum (Table 4-7). Following overnight incubation the sections were washed four times in PBS-Triton and incubated in Alexa Fluor® secondary antibodies (Invitrogen) diluted 1:500 in PBS-Triton with 1% goat or donkey serum (Table 4-7). After washing in PBS the sections were mounted onto uncoated glass slides and coverslipped with Cytifluor (Agar Scientific) for analysis with confocal microscopy.
Table 4-7  Antibodies for co-immunofluorescent labelling
Pairs of primary antibodies used for co-labelling of neurons, working antibody dilutions, blocking serum (Goat, GIBCO®; Donkey, Sigma-Aldrich), secondary Alexa Fluor® conjugated antibodies raised in goat (Molecular Probes™, Invitrogen). Further antibody details in Appendix B.5.

4.7.4  Microscopy and stereology

Light microscopy analysis and stereological quantification was performed using a Nikon Eclipse E800 microscope coupled with StereoInvestigator® software (MicroBrightField) and MicroFire™ S99808 digital camera (Optronics). Staining intensity analysis was performed on captured images using ImageJ (National Institutes of Health, USA). Presented images were compiled in Adobe® Photoshop® (Adobe). Confocal analysis of NeuN, DARPP-32 and HA staining confirmed full penetration of the antibodies through the tissue slices (data not shown).

4.7.4.1  Striatal Neuron Stereology

DARPP-32 immunopositive neurons within the striatum were counted stereologically by unbiased optical fractionator sampling procedures. For the first investigation I assessed the striatum over twelve equispaced (320µm) coronal sections (sectioned at 40µm) extending from the head of the striatum at bregma +2.4 mm to -1.8 mm. The striatum was delineated on each section under a 2× objective using the lateral ventricle, corpus callosum, internal capsule and globus pallidus to help define the striatal borders. Medium spiny projection neurons expressing DARPP-32 were estimated by systematic sampling from a random starting point using a 60× objective lens. The optical fractionator was set to sample at intervals of \( x = 600\mu m, y = 700\mu m \) with a counting frame \( x = 90\mu m, y = 70\mu m, \) dissector height \( z = 7\mu m; \) giving a section sampling fraction (ssf) of 1/8, area sampling fraction (asf) of 1/67 and thickness sampling fraction (tsf) of 1/1.4. In the second investigation I restricted the striatal neuronal estimates to six serial coronal sections (40µm sections equispaced at 320µm) covering the site of AAV vector and QA striatal injection (bregma +1.8mm to -0.5mm). Optical fractionators were set with grid spacing \( x = 650\mu m, y = 650\mu m \) and counting frame \( x = 90\mu m, y = 70\mu m, z = 8\mu m \) (ssf = 1/8, asf = 1/67, tsf = 1/1.25). Coefficient of Error values were calculated using Gundersen equations, \( m = 1 \).

Subsequent stereological analysis of krox-24 expressing striatal neurons in the second investigation was undertaken over eight coronal sections (40µm sections equispaced at 320µm) through the striatum (bregma +2.1mm to -0.8mm). Optical fractionators were set with grid spacing \( x = 650\mu m, y = 650\mu m \) and counting frame \( x = 70\mu m, y = 70\mu m, z = 7\mu m \) (ssf = 1/8, asf = 1/86, tsf 1/1.4).
4.7.4.2  **Striatal Atrophy**
The extent of striatal atrophy was determined from the coronal section contours outlined for stereological counting of DARPP-32 neurons (Section 4.7.4.1). Total cross-sectional area of the ipsilateral striatum was calculated as a proportion of the contralateral striatal area.

4.7.4.3  **Striatal TH-Staining Intensity**
Maintenance of TH in the striatum was quantified by density measurements of TH-immunostained sections using ImageJ. Images of the ipsilateral and contralateral hemispheres from eight equispaced (320µm spacing) coronal sections through the striatum (bregma +2.1mm to -0.8mm) were captured under a 2× objective and the striatal regions outlined as above (Section 4.7.4.1). Integrated area-density measurements of TH-staining in the treated striatum relative to the adjacent contralateral striatum were averaged across the eight sections to give the relative maintenance of striatal TH expression.

4.7.4.4  **Maintenance of Striatonigral Projections**
Innervations of the substantia nigra by DARPP-32 containing fibres were also quantified by density measurements. Three equispaced (320µm spacing) coronal sections through the substantia nigra (bregma -4.9mm to -5.9mm) were photographed under a 4× objective and both ipsilateral and contralateral nigral regions outlined with ImageJ. Integrated area-density measurements of DARPP-32 staining in the treated nigra relative to the adjacent contralateral nigra were averaged across the three sections to determine relative maintenance of the DARPP-32 positive fibre innervations.

4.7.4.5  **Pallidal Neuron Stereology**
Parvalbumin expressing neurons in the globus pallidus were estimated by optical fractionator following delineation of the GP region on four equispaced (320µm spacing) coronal sections (sectioned at 40µm) between bregma -0.5mm to -1.8mm. Optical fractionator sampling parameters were set to grid spacing of $x = 200\mu m$, $y = 300\mu m$ and counting frame $x = 100\mu m$, $y = 100\mu m$, $z = 7\mu m$ (ssf = 1/8, asf = 1/6, tsf = 1/1.4).

4.7.4.6  **Confocal Microscopy**
To assess co-labelling of striatal cells following immunofluorescent staining I used a Leica TCS SP2 laser scanning confocal microscope (Biomedical Imaging Resource Unit, The University of Auckland) to captured single optical slice images of fluorescently stained striatal cells under a 63× objective lens.
4.8 **Statistical Analysis**

All statistics were performed with GraphPad Prism® (version 4). Unless otherwise stated all data presented represents group mean ± standard error of the mean (SEM).

Non-parametric Mann-Whitney tests were performed to assess the level of therapeutic transgenic protein expression following AAV-vector delivery relative to endogenous protein expression in AAV-Luciferase treated control striata and non-treated contralateral striata.

Data collected from behavioural testing was analysed by paired t-test to assess development of QA induced functional deficits relative to baseline testing (Section 3.3.4.1), or two-way repeated measures (RM) ANOVA to gauge the impact of therapeutic AAV vector delivery on QA-induced deficits over the post-QA assessment period of the studies relative to controls. Un-paired t-tests were used to assess any significant alterations imparted by AAV-vectors relative to controls prior to QA-lesioning.

Statistical analysis of all neuropathological changes – stereological cell counts, striatal atrophy, and immunostaining density – were all undertaken by un-paired t-tests between the treatment groups and control rats, thereby treating each individual vector as an independent investigation.

Correlations between functional behaviour and neuropathological alterations were performed by calculating each rat’s mean forelimb asymmetry score and “corridor” task retrieval score across all post-QA trials to give each rat an overall behavioural score for each test that could be correlated with the rats’ end-point pathological assessments. For apomorphine-induced rotational behaviour all individual post-QA trials were included in the correlation analysis. Pearson correlation coefficients were performed to determine any overall trends independent of treatment. Linear regression or Deming (Model II) linear regressions were calculated for the individual treatment groups to assess individual correlations and any significant alterations due to vector delivery.

In all assessments the AAV-Luciferase vector control and PBS vehicle control data was grouped for analysis provided they were not significantly different at the 5% level (P > 0.05).

One-way ANOVA with Bonferroni’s post-hoc tests or non-parametric Mann-Whitney analysis were used to analyse the earlier functional protein assays verifying transgenic protein expression following AAV-vector transduction.
Neurotrophic Factor Delivery: AAV-BDNF and AAV-GDNF

Chapter 5

Neurotrophic Factor Delivery: AAV-BDNF and AAV-GDNF

5.1 Overview

BDNF and GDNF were investigated as potential neuroprotective agents against excitotoxic insult by infusing an AAV vector encoding either BDNF or GDNF directly into the rodent striatum to enhance localised neurotrophic factor expression prior to a unilateral intrastratal injection of the excitotoxic glutamate analogue QA. Building on previous literature that has reported protective benefits associated with BDNF (Martinez-Serrano and Bjorklund 1996; Bemelmans et al. 1999; Perez-Navarro et al. 2000a; Kells et al. 2004) and GDNF (Perez-Navarro et al. 1996; Araujo and Hilt 1997; McBride et al. 2003; Kells et al. 2004), but which has indicated limitations with achieving efficient in vivo delivery procedures, I constructed AAV vectors with the intention of directing high level striatum targeted transgenic protein expression to support the striatal projection neurons and prevent associated functional deficits. Unfortunately following AAV-BDNF delivery some of the rats displayed detrimental behaviour, presumably due to excessive BDNF expression, resulting in early termination of this trial. Despite the adverse reaction I felt AAV-BDNF delivery still had sufficient merit as a therapeutic agent if these side effects could be avoided so conducted a second trial with a lower-titre of the AAV-BDNF vector. Continuous expression of the neurotrophic factors following AAV-mediated delivery showed some attenuation of the behavioural deficits resultant of QA lesioning however these functional benefits only had limited correlation with BDNF-derived maintenance of the basal ganglia. Overall these investigations demonstrated controlled delivery of neurotrophic factors could provide symptomatic relief without necessarily supplying significant neuroprotection to reduce the extent of neuronal cell death.

5.2 Procedures

Chimeric AAV1/2 vectors were constructed containing cDNA encoding BDNF or GDNF and were assessed with in vitro assays to verify they correctly directed the expression of biologically functional neurotrophic factors (Section 2.3; (Kells et al. 2006)) prior to checking in vivo...
transduction and transgene expression in the rodent striatum. Following the AAV vector testing I undertook two \textit{in vivo} studies, conducted as described (Chapter 4) to assess the influence of BDNF and GDNF expression on QA-induced striatal excitotoxicity and associated functional behavioural deficits. In the initial study I assessed the delivered of AAV-BDNF ($n = 7^*$) or AAV-GDNF ($n = 10$) to a single stereotaxic site within the rodent striatum prior to QA injection at the same stereotaxic coordinates. Following adverse reactions resulting from the AAV-BDNF delivery I conducted a second follow-up investigation with a ten-fold lower titre of AAV-BDNF ($n = 9$) delivered to two separate striatal sites flanking the QA injection site. An additional group of rats ($n = 5$) that received reduced-titre AAV-BDNF in this second study were assessed without QA lesioning to directly monitor the functional impact of AAV-mediated BDNF expression on sensorimotor performance. Control rats for each study received either AAV-Luciferase (Initial: $n = 8$; Follow-up: $n = 5$) or sterile PBS (Initial: $n = 10$; Follow-up: $n = 5$) prior to QA lesioning.

5.3 \textbf{Animal Welfare}

Throughout all \textit{in vivo} investigations the only rats that were observed to display any abnormal health issues were rats that received AAV-BDNF in the initial neuroprotective study. Starting five weeks after AAV-BDNF infusion – two-weeks post-QA – a number of these rats began to display excessive hyperactivity and aggression in their home cages, weight loss and seizure activity. In accordance with predetermined animal welfare guidelines the rats were euthanised if they had greater than 20\% weight loss or ongoing seizures. The AAV-BDNF study was prematurely terminated seven weeks post AAV delivery. No indication of any negative effects were noted during earlier \textit{in vivo} vector testing trials suggesting the health concerns were resultant of consistent long-term BDNF over-expression and therefore were not evident during the first three weeks post-AAV vector delivery in which I had conducted all prior assessment. It was not determined whether these welfare issues were due to the level of BDNF expression, the distribution within the brain, or a combined effect, but they nevertheless highlight the challenges associated with the administration of neurotrophic factors as therapeutic agents for neurological disorders. To avoid further welfare concerns but proceed with investigation of BDNF as a neuroprotective agent using the existing AAV-BDNF vector, I reduced the intensity of BDNF expression by lowering the vector titre 10-fold and splitting gene delivery over two separate stereotaxic sites within the striatum.

$^*$ \textit{n} values here represent the final number of rats included in the analysis of the investigations (Section 4.3).
5.4 *In Vivo AAV Vector Testing*

To assess the spread of *in vivo* AAV vector transduction and neurotrophic factor transgene expression prior to investigating the therapeutic efficacy of BDNF or GDNF expression, I injected naïve rats using the same stereotaxic surgical delivery procedures to be performed on the main cohorts of rats in the neuroprotective investigations. In the first instance, the surgical vector delivery protocol was determined from the earlier AAV-Luciferase delivery testing (Section 4.4.2), which I then modified for the second lower titre AAV-BDNF two-site injection study designed to reduce the expression level but maintain a wide distribution of transduced cells within the striatum. Three weeks post-AAV vector delivery transgenic protein expression was visualised by immunocytochemical staining against an HA-epitope attached to the C-terminal of the expressed neurotrophic factors.

![Figure 5-1 Spread of undiluted AAV-BDNF transduction and BDNF expression](image)

(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged BDNF expression three weeks after a single site 4.0 µL striatal injection of the undiluted AAV-BDNF vector. Cells expressing HA-tagged BDNF were largely contained within the injected striatum where a large population of the striatal neurons appeared to be transduced. Higher-power images of AAV-BDNF transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 100µm for higher magnification images (B-D).
Following the high-titre AAV vector injections, transduced cells were observed in an extensive but often irregularly dispersed distribution throughout the rostral-caudal extent of the striatum (Figure 5-1, Figure 5-2). A population of cells within the globus pallidus and substantia nigra pars compacta ipsilateral to the injected striatum were HA-positive indicating transportation of the AAV-vectors and transduction of cells in the striatal target nuclei. The ipsilateral substantia nigra pars reticulata also displayed HA-immunostaining, although this was mainly restricted to the striatonigral axonal fibres with very few identifiable HA-positive cell bodies.

Ten-fold dilution of the AAV-BDNF vector clearly restricted the extent and distribution of cellular transduction with the majority of HA-positive cells located close to the site of injection (Figure 5-3). The transduction of cells within the substantia nigra and globus pallidus was also diminished with only a few weakly HA-immunopositive cell bodies observed (Figure 5-3 C, D). A greater 100-fold

Figure 5-2 Spread of AAV-GDNF transduction and GDNF expression
(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged GDNF expression three weeks after a single site 4.0 µL striatal injection of the AAV-GDNF vector. Cells expressing HA-tagged GDNF were largely contained within the injected striatum where a large population of the striatal neurons appeared to be transduced. Higher-power images of AAV-GDNF transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 100µm for higher magnification images (B-D).
Figure 5-3 Spread of the reduced-titre AAV-BDNF transduction and BDNF expression
(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged BDNF expression three weeks after striatal injection of the AAV-BDNF vector diluted ten-fold. Extensive transduction of the striatal cells was seen in the proximity of the intrastratial injection site and directly adjacent to the corpus callosum. Higher-power images of AAV-BDNF transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 100µm for higher magnification images (B-D).

dilution of the AAV-BDNF vector almost completely eliminated striatal transduction detectable by HA-immunocytochemistry, with only a few isolated HA-positive cells visually detectable close to the site of injection.

5.5 BDNF and GDNF Expression Level

To quantify the level of BDNF and GDNF expression generated following AAV-mediated gene transfer, cohorts of rats were euthanised three weeks after AAV vector delivery and their ipsilateral and contralateral striatal tissue separately isolated for ELISA-based quantification of the total BDNF and GDNF protein. Expression was assessed three weeks post-AAV delivery to provide a measure of neurotrophic factor expression equivalent to that expected to be present in the neuroprotective
study group of rats at the time of QA injection. BDNF expression in the striatum following undiluted AAV-BDNF injection was enhanced ~1000 fold to $46 \pm 7$ ng/mg total protein compared with the $0.049 \pm 0.006$ ng/mg total protein detected in control AAV-Luciferase injected striatum ($P < 0.01$; Figure 5-4A). The contralateral striatum displayed a slight increase in BDNF expression to $0.12 \pm 0.02$ ng/mg total protein ($P < 0.05$ compared to AAV-Luciferase contralateral striatum $0.064 \pm 0.007$ ng/mg total protein) suggesting a small amount of transgene expression may have crossed to the non-injected hemisphere. The lower titre AAV-BDNF injections resulted in a ~150-fold increase to $4.2 \pm 0.7$ ng/mg total protein compared to $0.026 \pm 0.002$ ng/mg total protein in the contralateral striatum. AAV-GDNF administration induced a similar increase in GDNF expression with GDNF levels increased to $26 \pm 6$ ng/mg total protein in the injected striatum and $0.063 \pm 0.005$ ng/mg total protein in the contralateral striatum (Figure 5-4B). Striatal expression of GDNF protein within the control AAV-Luciferase treated rats was below the reliable detection limit of the ELISA assay.

**Figure 5-4** ELISA quantification of BDNF and GDNF expression in the striatum
The level of BDNF (A) or GDNF (B) protein expression in striatal tissue isolated three weeks after infusion of AAV-BDNF, AAV-GDNF or AAV-Luciferase was quantified by ELISA. Total protein in the samples was measured by BCA assay to normalise the individual samples. The GDNF expression in the AAV-Luciferase treated rats was considered to be below reliable detection levels. Mann-Whitney tests: comparison with AAV-Luciferase * $P < 0.05$, ** $P < 0.01$; comparison with high-titre AAV-BDNF ‡ $P < 0.01$. No statistics were performed on the GDNF expression levels.

### 5.6 Functional Behaviour Assessment

Throughout the neuroprotective studies I assessed the development of functional deficits by analysing the performance of each rat in behavioural tests designed to show hemispherical imbalances in brain function arising as a result of unilateral lesioning in the basal ganglia. The functional behaviour tests undertaken as described earlier (Section 4.5) involved spontaneous
forelimb use analysis, drug-induced rotational behaviour following either apomorphine or amphetamine dosing and preferential left / right side food selection. All the rats were assessed with baseline measurements taken both before and after AAV delivery and for 5-7 weeks post-QA injection (Table 4-2 and Table 4-3).

### 5.6.1 Spontaneous forelimb use

Deficits in functional motor performance were assessed by observing spontaneous forelimb usage during exploratory rearing behaviour. Delivery of the AAV vectors or PBS did not induce any significant alterations in baseline forelimb usage assessed prior to the QA injection, although both neurotrophic factor treated groups – AAV-BDNF and AAV-GDNF – were tending towards an increased preference to use their contralateral forelimb (Figure 5-5A,B). This slight contralateral preference resulted in a small divergence in forelimb use between the control and neurotrophic factor treated rats reaching statistical significance with lower-titre AAV-BDNF treated rats in the follow-up study (Unpaired t-test P < 0.05).

Following unilateral QA administration the AAV-Luciferase and PBS treated control rats showed an immediate shift towards preferentially using their ipsilateral forelimb for spontaneous exploration as expected from the earlier testing (Section 3.3.4). This ipsilateral bias was however only observed during the first two-weeks following QA injection in the initial study (Figure 5-5A), but maintained throughout the seven-week post-QA assessment period in the second study (Figure 5-5B). In contrast, the preferential forelimb use by AAV-BDNF or AAV-GDNF treated rats was not significantly affected by the QA injection in either investigation (Figure 5-5A,B). AAV-GDNF treated rats maintained baseline preferences of non-biased spontaneous forelimb use, significantly ameliorating the QA-induced ipsilateral bias (Two-way RM ANOVA P < 0.05; Figure 5-5A). Following QA injection the AAV-BDNF treated rats in the initial high expression study continued to develop a persistent contralateral forelimb preference in contrast to the immediate preferential ipsilateral forelimb shift in the control rats (Two-way RM ANOVA P < 0.001; Figure 5-5A). Subsequent BDNF investigation following ten-fold dilution of the AAV-BDNF vector eliminated the ongoing development of a contralateral forelimb bias beyond the initial divergence from the control group induced by AAV-BDNF transduction prior to QA injection (Figure 5-5B). Following QA injection into the striatum of reduced-titre AAV-BDNF treated rats, the acquirement of a slight contralateral forelimb use preference was reversed with a small shift towards greater ipsilateral forelimb usage, such that overall the group remained largely unbiased in contrast to the significant ipsilateral preference of control rats (Two-way RM ANOVA P < 0.05; Figure 5-5B). The extra
cohort of non-lesioned AAV-BDNF treated rats displayed a small shift towards contralateral forelimb use, but with no on-going changes the continuous induced expression of BDNF at this lower level did not appear to directly influence forelimb motor control (Figure 5-5B).

**Figure 5-5  Spontaneous ipsilateral forelimb use asymmetry score**
Plots displaying changes in group ipsilateral asymmetry scores quantified from forelimb use during exploratory rearing behaviour in (A) the initial AAV-BDNF / AAV-GDNF and (B) AAV-BDNF follow-up investigations. Ipsilateral asymmetry scores represent the combined usage of the ipsilateral forelimb, as a percentage of the total left and right forelimb use, for rearing, initial contact of the cylinder wall during an exploratory rear and landing. Un-paired t-test between treatment groups: * P < 0.05. Two-way RM ANOVA relative to the control group: † BDNF P < 0.001, GDNF P < 0.05; ‡ BDNF P < 0.05, BDNF – no QA P < 0.001.
In addition to the forelimb asymmetry scores, the level of exploratory activity in the cylinder was assessed showing general reduction in the number of exploratory rears made during the five minute observational period with sequential trials. Both AAV-BDNF and AAV-GDNF treated rats were observed to maintain a significantly higher level of exploratory activity following QA administration with the initial high-titre AAV-BDNF treated rats displaying full maintenance of exploratory behaviour (Figure 5-6A). A higher level of activity was recorded in the second study for both control and neurotrophic factor treated rats, which was likely to have been consequential of the dietary restriction regime in the second investigation or adjustment to the home cage environment which enhanced their level of exploratory behaviour (Figure 5-6B).

![Figure 5-6 Spontaneous exploratory rearing activity](image)

Plots displaying the number of exploratory rears in the assessment cylinder during the five minute analysis of spontaneous forelimb use in (A) the initial AAV-BDNF / AAV-GDNF and (B) AAV-BDNF follow-up investigations. No statistics were performed on the pre-QA baseline data. Two-way RM ANOVA relative to the control group: † GDNF P < 0.05, BDNF P < 0.01; ‡ BDNF P < 0.05, BDNF – no QA P < 0.01.

Overall the single administration of either AAV-BDNF or AAV-GDNF resulted in a significant amelioration of the ipsilateral forelimb use bias induced by a unilateral striatal lesion with high BDNF expression actually leading to predominant use of the contralateral forelimb. The apparent recovery of the control group towards un-biased forelimb use in the initial study was in contrast to the increasing bias in the second follow-up investigation. An explanation for this conflicting observation was not evident although the rats in the second study also exhibited a higher level of exploratory rearing behaviour, potentially due to the dietary restriction of the rats in the second study.
5.6.2 Drug-induced rotational behaviour

The extent of rotational behaviour induced by apomorphine or amphetamine was undertaken to assess imbalances in the dopamine system following unilateral intrastriatal QA injection and the subsequent loss of striatal neurons expressing dopamine receptors. Results of both the apomorphine and amphetamine induced rotational behaviour were difficult to interpret due to the inconsistency of rotational direction between individual rats within the treatment groups which was possibly resultant from slight variations in the positioning of the striatal lesion.

5.6.2.1 Apomorphine

Apomorphine administered subcutaneously acts as a non-specific dopamine receptor agonist stimulating dopaminergic neurons and thereby inducing rotational motor behaviour generally towards the lesioned striatum (Section 3.3.4). The prevalence of apomorphine induced rotational behaviour varied greatly between individual rats with rotation rates ranging from no rotational behaviour through to 20 rotations per minute. Within each group some rats displayed a contralateral rotational dominance in contrast to the majority of control treated rats that rotated towards the lesioned hemisphere (Figure 5-7). Although there were no significant differences between the treatment groups in terms of total rotations, I did observe an overall trend towards increased rotational behaviour after QA injection (Figure 5-8). The additional non-lesioned AAV-BDNF rats in the follow-up study also tended to display increased rotational behaviour in spite of not being lesioned and in contrast to the AAV-BDNF treated rats that were QA lesioned. For reliable assessment of rotational behaviour I restricted the analysis of ipsilateral rotation percentage to rats that had greater than 0.5 rotations per minute for apomorphine.

Having selectively treated the rats on their naturally dominant striatal hemisphere – determined following the baseline testing – the initial baseline rotational behaviour was generally in an ipsilateral direction less than 50% of the time (Figure 5-7). Apomorphine administration after AAV vector delivery but prior to QA injection resulted in a large distribution in the percentage of ipsilateral rotations for both neurotrophic factor – AAV-BDNF and AAV-GDNF – and the AAV-Luciferase or PBS control treated groups. The majority of AAV-BDNF treated rats were however observed to switch rotational direction following AAV-BDNF injection with the increase in striatal BDNF protein expression causing the rats to predominantly rotate towards their treated hemisphere. This change in rotational direction was particularly pronounced in the initial AAV-BDNF trial which had the highest BDNF expression levels (Figure 5-7A). The induction of ipsilateral rotational behaviour following BDNF expression was also observed in the smaller cohort of reduced-titre
AAV-BDNF treated rats in the follow-up study which were not selected for QA lesioning. However, five weeks following AAV-BDNF delivery the non-lesioned rats had reverted to a contralateral rotational dominance suggesting modifications in the dopamine system following continuous expression of BDNF in the striatum (Figure 5-7B). Following the QA lesion apomorphine-induced rotational behaviour became more uni-directional in all animals although it was not always in an ipsilateral direction. While the majority of the control treated rats exhibited persistent ipsilateral rotational behaviour, the AAV-BDNF and AAV-GDNF treated groups were more evenly divided between predominantly displaying contralateral or ipsilateral rotational behaviour. With the lack of a consistent rotational direction between similarly treated rats I did not perform any statistical analysis on the rotational data.

**Figure 5-7  Apomorphine-induced rotational behaviour**
Plots displaying each rats rotational behaviour towards the treated side of their brain as a percentage of total rotations following s.c. administration of 1mg/kg apomorphine in (A) the initial AAV-BDNF / AAV-GDNF and (B) AAV-BDNF follow-up investigations. Rats that had less than 0.5 rotations per minute were excluded from this analysis. Lines represent the median result for each group.
Figure 5-8  Total apomorphine-induced rotations
Plots displaying the mean rate of apomorphine-induced rotational behaviour for each group in (A) the initial AAV-BDNF / AAV-GDNF and (B) AAV-BDNF follow-up investigations following s.c. administration of 1mg/kg apomorphine. No significance was found between treatment groups and controls post-QA.

5.6.2.2  Amphetamine
Amphetamine was only used to induce rotational behaviour in the initial high-titre AAV-BDNF and AAV-GDNF study. Wide variation in the rotational response of individual rats was observed during baseline testing with the selection of the dominant hemisphere for treatment skewing this into an overall contralateral rotational preference (Figure 5-9A). The average rate of rotation also varied greatly with many rats not showing any induction of rotational behaviour, therefore I restricted the analysis of rotational direction to rats that displayed greater than an average 0.1 total rotations per minute over the 60 minute testing period. Following AAV-vector delivery, but prior to QA injection, the enhanced expression of BDNF and GDNF significantly increased the amphetamine induced rotation rate compared with the control treated rats (BDNF P < 0.05; GDNF P < 0.01; Figure 5-9B).

A majority of the AAV-BDNF and AAV-GDNF treated rats preferentially rotated towards their treated side following amphetamine administration, while control treated rats showed very little rotational behaviour. Post-QA injection the majority of rats from all treatment groups immediately displayed largely unidirectional rotational behaviour – predominantly in an ipsilateral direction although a few rats preferentially rotated away from their lesioned hemisphere. Over the sequential trials amphetamine-induced rotational behaviour of the control and AAV-GDNF treated rats became more polarised becoming purely unidirectional at week six. In contrast, AAV-BDNF treated rats displayed much greater variation in rotation direction than the control group with only a few rats acquiring complete unidirectional behaviour and displaying a return towards bidirectional rotational behaviour at four weeks post-QA. The BDNF rats were removed from this initial study five weeks after QA administration and amphetamine-induced rotations were not assessed in the lower AAV-BDNF follow-up study so I was unable to determine whether this observed return towards equal
directional rotational behaviour was enhanced or maintained at later time points. Statistical analysis was not performed on the directional rotation data due to the inconsistency in the direction of induced rotation following QA lesioning.

Figure 5-9 Amphetamine-induced rotational behaviour
Plots displaying the rotational behaviour induced following an i.p. injection of 5mg/kg of amphetamine in the initial AAV-BDNF / AAV-GDNF investigation. (A) Individual rats’ rotational behaviour towards the treated (ipsilateral) side of their brain as a percentage of total rotations. (B) Mean rate of rotational behaviour for each group. Rats that had less than 0.1 rotations per minute were excluded from the directional analysis. Lines in (A) represent the median result for each group at each time point. Post-AAV – Un-paired t-test: * P < 0.05, ** P < 0.001. Post-QA – Two-way RM ANOVA: † BDNF P < 0.05; GDNF P = 0.055.

5.6.3 Sensorimotor “corridor” task
The impact of AAV-BDNF delivery on the development of sensorimotor neglect was investigated in the second in vivo study by analysing the rats’ performance in the “corridor” task. During baseline assessment the rats displayed a fairly even distribution of sugar pellet retrievals from the left and right sides of the corridor with the selection of the dominant hemisphere for treatment skewing the group data slightly towards a preferential retrieval of pellets from the contralateral side of the
corridor (Figure 5-10). Delivery of the AAV vectors did not alter the percentage of ipsilateral retrievals for the control rats or the main group of reduced-titre AAV-BDNF treated rats. However, in contrast to the main treatment groups, the additional group of rats included to assess AAV-BDNF treatment in the absence of a QA lesion did display a small but significant increase in the retrieval of sugar pellets located on the ipsilateral side of the corridor post-AAV delivery (Paired t-test P < 0.05), although this shift did not result in a significant ipsilateral retrieval bias (Not significantly above 50%; Wilcoxon signed-rank test P > 0.05) and remained fairly consistent throughout the full testing period. Following QA administration the control rats rapidly acquired a strong ipsilateral retrieval bias that persisted throughout the seven week investigative period. In stark contrast the AAV-BDNF treated group did not show significant development of an ipsilateral retrieval preference post-QA remaining unchanged from baseline preferences and significantly separated from the control group (Two-way ANOVA post-QA P < 0.01).

![Figure 5-10](image)

**Figure 5-10  Preferential food selection in the sensorimotor “corridor” task**
Plot displaying group changes in the number of sugar pellet retrievals from the same side as the treated striatum as a percentage of the 20 retrievals per trial. Two-way RM ANOVA relative to the control group: † BDNF P < 0.01; BDNF – no QA P < 0.05.

### 5.7 Immunocytochemical Analysis

On completion of the functional behavioural assessment I analysed the post-mortem brain tissue to visualise and quantitatively determine the extent of any neuroprotection imparted by the AAV-BDNF or AAV-GDNF vector delivery prior to QA-induced excitotoxic insult. Immunocytochemical staining was performed on individual series of coronally sliced sections equispaced at 320µm intervals through the caudate-putamen portion of the striatum, the globus pallidus and substantia nigra to identify specific populations of basal ganglia neurons affected by the QA striatal lesion.
5.7.1 **AAV-mediated transgene expression and QA lesioning of the striatum**

To assess the level of neuroprotective support imparted by AAV-mediated neurotrophic factor expression against QA-induced excitotoxic insult, I initially visualised the distribution of transgene expression by HA or Luciferase immunocytochemical staining and quantified the extent of QA striatal lesioning using DARPP-32 as a marker for the GABAergic striatal projection neurons.

![Figure 5-11 High-titre AAV-BDNF transduction and striatal cell maintenance](image)

**Figure 5-11** High-titre AAV-BDNF transduction and striatal cell maintenance
Representative images from adjacent striatal sections showing AAV-mediated BDNF expression and neuronal cell loss five weeks post-QA. (A-C) HA-immunostaining displayed an irregular distribution of BDNF expression within the striatum resultant of undiluted AAV-BDNF transduction. Maintenance of striatal neurons as detected by (D-F) DARPP-32 and (G-I) NeuN-immunostaining was shown to inversely reflect the BDNF expression as identified by the arrows pointing to areas of high BDNF expression and asterisks in areas with no transgenic BDNF expression. Regions outlined by boxes are shown at higher magnification in figures immediately to the right. Scale bar = 1mm for (A,D,G), 200µm for (B,E,H), 50µm for (C,F,I).
5.7.1.1 Initial Study – AAV-BDNF and AAV-GDNF Delivery

Using adjacent sections to compare the distribution of neurotrophic factor expression and the maintenance of striatal projection neurons identified by their intense expression of DARPP-32, I observed an inverse relationship between areas of extensive HA-tagged neurotrophic factor expression and DARPP-32 positive neurons. This inverse correlation was particularly strong in the AAV-BDNF treated rats as shown in Figure 5-11 where a well-defined portion of striatum is devoid of HA-immunoreactivity but displays complete maintenance of DARPP-32 positive neurons, despite its close proximity to the site of QA injection. Although less apparent, the opposite was also seen.

Figure 5-12 AAV-GDNF transduction and striatal cell maintenance

Representative images from adjacent striatal sections showing AAV-mediated GDNF expression and neuronal cell loss seven weeks post-QA. (A-C) HA-immunostaining displayed the distribution of GDNF expression within the striatum resultant of AAV-GDNF transduction. Maintenance of striatal neurons was detected by (D-F) DARPP-32 and (G-I) NeuN-immunostaining. A large number of HA-positive GDNF expressing cells were observed within regions of the striatum that exhibited a complete loss of DARPP-32 staining (asterisks). Regions outlined by boxes are shown at higher magnification in figures immediately to the right. Scale bar = 1mm for (A,D,G), 200µm for (B,E,H), 50µm for (C,F,I).
with a reduction of DARPP-32 immunopositive cells in the dispersed areas of intensive BDNF expression, but more maintained in adjacent portions of the striatum raising the question of DARPP-32 down-regulation in AAV-BDNF transduced neurons. Overall this effect resulted in the AAV-BDNF treated rats not having a clearly defined “lesion” area based on the absence of DARPP-32 immunopositive cells as I observed with the PBS and AAV-Luciferase control rats (Figure 5-13). Immunostaining for NeuN in an adjacent section appeared to indicate that while there was a definite reduction in the number of neurons it was not to the extent indicated by DARPP-32.

Figure 5-13  AAV-Luciferase transduction and striatal cell maintenance
Representative images from adjacent striatal sections showing AAV-mediated Luciferase expression and neuronal cell loss seven weeks post-QA. (A-C) Immunostaining for Luciferase shows the striatal expression resultant of AAV-Luciferase transduction. Maintenance of striatal neurons was detected by (D-F) DARPP-32 and (G-I) NeuN-immunostaining showing a fairly well defined region of QA-induced lesioning. The vast majority of Luciferase expressing cells were located outside of the lesion borders within intact segments of the striatum. Regions outlined by boxes are shown at higher magnification in figures immediately to the right. Scale bar = 1mm for (A,D,G), 200µm for (B,E,H), 50µm for (C,F,I).
Similar to BDNF the AAV-GDNF treated rats displayed extensive HA-immunostaining of cells within areas of the striatum devoid of DARPP-32 cells, however the area of “lesion” was more clearly delineated and, in contrast to the BDNF rats, extended into regions of striatal tissue not showing any HA staining (Figure 5-12). Surprisingly, staining for the neuronal marker NeuN also displayed a lack of immunopositive neurons in the region of transgenic GDNF expression displaying a similar pattern of cell loss to DARPP-32. In general the maintenance of NeuN-positive cells appeared greater than DARPP-32-positive cells although I did not confirm this observation with stereological quantification. In AAV-Luciferase control treated rats the Luciferase expression was generally restricted to the areas of DARPP-32 staining although there was a scattering of Luciferase-positive cells remaining within the lesioned area that roughly correlated with the remaining NeuN-positive neurons (Figure 5-13). As with the PBS treated rats, AAV-Luciferase control rats displayed more uniformity in DARPP-32 striatal cell loss than the neurotrophic factor expressing rats.

5.7.1.2 Study 2 – Reduced Titre AAV-BDNF Delivery
The inverse correlation between HA-detected BDNF expression and DARPP-32 expression seen in the initial high-titre study was still observed following the 10-fold dilution of the AAV-BDNF vector

![Figure 5-14 Diluted AAV-BDNF administration and striatal cell maintenance](image)

Adjacent sections through the striatum showing AAV-mediated BDNF expression and the loss of striatal neurons eight weeks post-QA. (A,B) HA-immunostaining displayed the distribution of BDNF expression within the striatum resultant of reduced-titre AAV-BDNF transduction. Maintenance of striatal neurons was detected by (C,D) DARPP-32 and (E,F) NeuN-immunostaining. Regions outlined by boxes are shown at higher magnification in figures immediately below. Scale bar = 2mm for whole sections (A,C,E) and 200µm for higher magnifications (B,D,F).
and the two-site delivery procedure, although the correlation was less distinct with substantial areas of the striatum containing both HA-immunoreactive cells and DARPP-32 expressing neurons (Figure 5-14A-D). Additional staining for NeuN confirmed the presence of some neurons within areas of DARPP-32 loss further suggesting that the transduction with AAV-BDNF is leading to long-term down-regulation of DARPP-32 expression (Figure 5-14E, F). The control AAV-Luciferase and PBS treated rats continued to display a more regularly defined region of striatal lesioning, within which only a small population of DARPP-32 and NeuN-expressing cells remained (Figure 5-15).

**Figure 5-15 Two-site AAV-Luciferase injection and striatal cell maintenance**
Adjacent sections through the striatum showing AAV-mediated Luciferase expression and the loss of striatal neurons eight weeks post-QA. (A,B) Immunostaining for Luciferase shows the striatal distribution of Luciferase expression resultant of the AAV-Luciferase transduction following two-site delivery. Maintenance of striatal neurons was detected by (C,D) DARPP-32 and (E,F) NeuN-immunostaining, displaying a reasonably well defined region of QA lesioning. The majority of Luciferase expressing cells were located outside of the lesion borders within the intact segments of the striatum. Regions outlined by boxes are shown at higher magnification in figures immediately below. Scale bar = 2mm for whole sections (A,C,E) and 200µm for higher magnifications (B,D,F).

5.7.1.3 Stereological Analysis – DARPP-32
To quantify the striatal damage induced by QA lesioning following delivery of AAV vectors encoding BDNF, GDNF, Luciferase or PBS I employed stereological cell counting procedures to estimate the number of DARPP-32 immunopositive neurons remaining within both the treated striatum and the intact contralateral striatal hemisphere. In the initial high-titre AAV-BDNF and
AAV-GDNF investigations, optical fractionator probes estimated the number of DARPP-32 positive neurons in the sampled region of the caudate-putamen at 1,400,000 ± 120,000 in high-titre AAV-BDNF treated rats, 1,210,000 ± 90,000 in AAV-GDNF rats and 1,150,000 ± 120,000 in the AAV-Luciferase / PBS control rats compared with an overall average of 2,180,000 ± 50,000 DARPP-32 positive cells in the contralateral intact striatum (Figure 5-16A; Coefficient of Error:

**Initial High-titre Neurotrophic Factor Investigation**

![Graphs A and B](image)

**Follow-up Reduced-titre AAV-BDNF Investigation**

![Graphs C and D](image)

**Figure 5-16  Maintenance of DARPP-32 positive striatal projection neurons**

Graphs displaying the actual and relative numbers of DARPP-32 positive neurons in the striatum as estimated by optical fractionator stereological analysis of the treated (ipsilateral) and intact (contralateral) striatal hemispheres in (A,B) the initial AAV-BDNF and AAV-GDNF investigation and (C,D) reduced-titre AAV-BDNF follow-up study. Population estimates were determined from 12 sections spanning 3.56mm of the striatum for the initial study and 7 sections spanning 1.96mm of the anterior striatum for the follow-up study. Relative cell counts represent the number of DARPP-32 cells within the lesioned striatum as a proportion of cells present in the contralateral intact striatum. Statistical analysis revealed no significance differences between treatment groups at the 5% level (Unpaired t-test P > 0.05).
ipsilateral $\leq 0.04$, contralateral $\leq 0.02$). A smaller portion of the caudate-putamen centred around the QA lesion was analysed in the second investigation containing an estimated 1,530,000 ± 40,000 DARPP-32 positive neurons in the intact contralateral striatum that was reduced to 930,000 ± 100,000 in the reduced-titre AAV-BDNF treated striatum and 760,000 ± 60,000 in the AAV-Luciferase / PBS controls ($P = 0.18$; Figure 5-16C; Coefficient of Error: ipsilateral $\leq 0.06$, contralateral $\leq 0.03$).

Overall the proportion of cells expressing DARPP-32 in the QA lesioned striatum relative to the intact contralateral striatal hemisphere was reduced in control AAV-Luciferase / PBS treated rats to 52 ± 4 % in the initial study and 47 ± 4 % in the second follow-up investigation. Prior delivery of the AAV vectors to enhance neurotrophic factor expression at the time of QA lesioning was unsuccessful in significantly increasing the maintenance of DARPP-32 positive neurons. The proportion of DARPP-32 positive neurons remaining in the initial high-titre AAV-BDNF injected rats was estimated at 60 ± 4 % (t-test $P = 0.28$) and 58 ± 5 % in the AAV-GDNF treated group (t-test $P = 0.35$; Figure 5-16B). Dilution of the AAV-BDNF vector still failed to generate sufficient assistance to significantly protect the striatal neurons from QA-induced insult, despite increasing the relative maintenance of DARPP-32 neurons to 65 ± 8 % of the contralateral striatum (t-test $P = 0.054$; Figure 5-16D).

### 5.7.1.4 Striatal Atrophy

In addition to the loss of striatal neurons I observed an apparent atrophy of the striatum and enlargement of the adjacent lateral ventricle (e.g. Figure 5-15). By analysis of the striatal area using the same contours drawn to perform stereological cell estimates, I quantified the relative volume of the striatum in the control rats to be 73 ± 2% of the contralateral striatum in the initial study (Figure 5-17A) and 75 ± 3% in the second follow-up investigation (Figure 5-17C). AAV-BDNF treatment significantly prevented striatal atrophy with complete maintenance of the striatal area in the initial high-titre study (103 ± 3%, t-test $P < 0.0001$) and only a 9% reduction in the follow-up reduced-titre AAV-BDNF investigation (91 ± 6%, t-test $P < 0.05$). In contrast, AAV-GDNF delivery did not significantly influence QA-induced atrophy compared with the control rats (79 ± 2%, t-test $P = 0.12$). Comparison between the striatal atrophy and the loss of DARPP-32 expressing striatal projection neurons showed a direct correlation with the striatum contracting in proportion to the loss of striatal neurons (Figure 5-17B,D). The correlation was significantly shifted by AAV-BDNF delivery in the initial high-titre investigation, with the striatum maintaining its full volume despite DARPP-32 cell loss equivalent to some of the control and AAV-GDNF treated rats ($P < 0.001$).
Figure 5-17  Atrophy of the lesioned striatum
Graphs displaying the size of the lesioned striatum as a proportion of the intact contralateral striatal area, and plots correlating striatal atrophy with DARPP-32 positive cell loss in the striatum. (A,B) Initial high-titre AAV-BDNF and AAV-GDNF investigation and (C,D) the follow-up reduced-titre AAV-BDNF study. Cross-sectional striatal area was measured on 12 coronal sections for the initial study and 7 sections for the second study. Unpaired t-tests: * P < 0.05, ** P < 0.0001. Linear regression analysis: R values represent the overall Pearson correlation coefficient; Solid lines represent significant non-zero linear correlations for individual treatment groups (P < 0.05). Comparison with controls: (A) BDNF: slope P = 0.81, elevation P < 0.001 (†); GDNF: slope P = 0.43, elevation P = 0.12; (B) BDNF: slope P = 0.88, elevation P = 0.34.

5.7.2  Krox-24 expression
As an alternative to the DARPP-32 protein dependent analysis of neuronal survival, I also visualised striatal neurons in the reduced-titre AAV-BDNF follow-up investigation by their expression of krox-24 / early growth response protein 1 (Egr1) (Herdegen et al. 1995; MacGibbon et al. 1995). An early response transcription factor, krox-24 is induced in neurons following neurotransmitter or trophic factor activation of the mitogen-activated protein kinase (MAPK) signalling pathway, subsequently regulating the expression of a diverse range of neuronal proteins and thereby contributing to neuronal
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plasticity (Knapska and Kaczmarek 2004). Basal expression of krox-24 in rodent striatal neurons may provide a more reliable visualisation of surviving neurons than late-response gene products. Visual assessment of the normal rat striatum showed the vast majority of striatal neurons expressing NeuN also co-express both krox-24 and DARPP-32 (Figure 5-18). A late addition to the pathological analysis to complement DARPP-32 based quantification of striatal neurons, I did not retrospectively quantify krox-24 maintenance in the initial high-titre AAV-BDNF and AAV-GDNF investigations.

Figure 5-18  Double-immunofluorescent labelling of striatal neurons
Co-labelling of striatal neurons in the normal striatum with NeuN (red) and either (A) DARPP-32 or (B) krox-24 (green), demonstrated the vast majority of neurons expressing the general neuronal marker NeuN also express DARPP-32 and krox-24. All expression of DARPP-32 and krox-24 was localised to the NeuN-positive neurons. Scale bar = 30µm.

The distribution of krox-24 expression within the AAV-BDNF treated striatum appeared similar to DARPP-32 immunostaining, although as a nuclear protein krox-24 staining was more distinct and had less variability in DAB staining intensity (Figure 5-19). Quantification of krox-24 immunopositive cells was performed using the optical fractionator stereology probe on eight
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equispaced sections through the striatum (Figure 5-20A). An estimated 1,060,000 ± 110,000 krox-24 positive cells remained within the analysed region of the striatum in the control AAV-Luciferase / PBS treated rats, and 1,420,000 ± 180,000 in the reduced-titre AAV-BDNF treated rats (Coefficient of Error ≤ 0.05). The intact contralateral striatal regions contained an estimated 2,090,000 ± 60,000 krox-24 positive cells (Coefficient of Error ≤ 0.03). Overall I found similar maintenance of the krox-24 positive striatal cells to that quantified by DARPP-32 analysis (Section 5.7.1.3), however the survival of krox-24 expressing neurons following AAV-BDNF at 68 ± 8% did demonstrate a significant level of protection when compared with the 50 ± 6% krox-24 cell maintenance in AAV-Luciferase / PBS control rats (t-test P < 0.05; Figure 5-20B).

Correlation of the krox-24 expression with DARPP-32 cell maintenance demonstrated a parallel link between the maintenance of krox-24 expression and DARPP-32 expression following QA lesioning that was independent of the prior AAV-BDNF or control treatment (Figure 5-20C).

Figure 5-19  Krox-24 expression in the striatum
Representative images displaying the expression of krox-24 in striatal neurons following QA lesioning in (A,B) AAV-BDNF treated rats and (D,E) AAV-Luciferase treated rats. (C,F) Adjacent sections immunostained for DARPP-32 (copied from Figure 5-14D and Figure 5-15D) show the positive distribution correlation between krox-24 and DARPP-32 positive cells in the striatum. Regions outlined by boxes are shown at higher magnification in figures immediately to the right. Scale bar = 2mm for whole sections (A,D) and 200µm for higher magnifications (B,C,E,F).
**Figure 5-20  Maintenance of krox-24 expressing striatal neurons**

Graphs displaying (A) actual and (B) relative numbers of striatal neurons expressing krox-24 as estimated by optical fractionator stereological cell counting in the treated (ipsilateral) and intact (contralateral) striatal hemispheres in the follow-up, reduced-titre AAV-BDNF investigation. Relative cell counts represent the number of krox-24 expressing cells within the lesioned striatum as a proportion of krox-24 positive cells present in the contralateral striatum. Unpaired t-test: * P < 0.05. (C) Plot showing the correlation between the maintenance of krox-24 expressing neurons and DARPP-32 positive neurons in the striatum. Deming linear correlation: slope = 1.02 ± 0.10; r² = 0.87, P < 0.0001. R value represents the overall Pearson correlation coefficient.
5.7.3 Analysis of transduced striatal cells

To assess the phenotype of surviving AAV vector transduced cells within the striatum at the conclusion of the neuroprotective studies I performed double-immunofluorescent staining for transgene protein expression – Luciferase or HA – and either NeuN, DARPP-32, krox-24 or GFAP. Co-labelling of Luciferase and NeuN demonstrated that the vast majority of transduced cells were striatal neurons (Figure 5-21A) although a small amount of co-localisation was also observed with GFAP (Figure 5-21D), suggesting that while the AAV vectors preferentially transduced neurons they were capable of also transducing glial cells.

Despite the vast majority of AAV-Luciferase transduced striatal cells expressing the general neuronal marker NeuN, expression of specific markers of striatal neurons – DARPP-32 or krox-24 – by the AAV vector transduced cells varied with the different transgene being expressed. No DARPP-32 or krox-24 expression was observed within Luciferase expressing cells (Figure 5-21B,C). While the majority of AAV-BDNF transduced cells positively labelled for krox-24, the intensity of DARPP-32 expression appeared to be inversely related to the level of BDNF expression (Figure 5-22). A similar pattern was also observed for the GDNF expressing cells with very few maintaining expression of DARPP-32, and the majority of AAV-GDNF transduced cells also displaying a large reduction in detectable krox-24 expression (Figure 5-23).

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**Figure 5-21 Immunofluorescent analysis of AAV-Luciferase transduced cells**
Representative single-slice confocal images from an AAV-Luciferase treated rat from the second investigation showing AAV-Luciferase transduced cells (red) within the lesioned striatum and immunofluorescent staining for (A) NeuN-positive cells, (B) DARPP-32 expression, (C) krox-24 expression and (D) GFAP expression (all displayed in green). Few Luciferase-positive cells actually survived QA lesioning. (A) Co-labelling with NeuN was evident for most Luciferase expressing cells. AAV-Luciferase transduced cells very rarely co-expressed (B) DARPP-32 or (C) krox-24. (D) A few Luciferase-expressing cells appeared to co-localise with GFAP expression (arrows). Scale bar: 30µm

**Figure 5-22 Immunofluorescent analysis of AAV-BDNF transduced cells in the striatum**
Representative single-slice confocal images from AAV-BDNF treated rats showing HA-positive BDNF expressing cells within the striatum (red) together with immunofluorescent staining for (A-C) DARPP-32 or (D) krox-24 expression (both displayed in green). (A) Co-labelling with HA and DARPP-32 was only evident for some BDNF expressing cells in the undiluted AAV-BDNF study with many morphologically identical cells not appearing to express DARPP-32 (arrows). A greater proportion of HA-positive cells co-labelled in (B) the reduced-titre AAV-BDNF investigation, although DARPP-32 staining was still weaker for the HA-positive transduced cells than non-transduced cells, especially in (C) the non-QA lesioned AAV-BDNF treated rats. (D) Co-labelling for HA and krox-24 was evident for transduced cells in the reduced-titre AAV-BDNF study although not all HA-positive cells displayed krox-24 expression (arrowheads). Scale bar: 30µm
**Figure 5-21** Immunofluorescent analysis of AAV-Luciferase transduced cells
Figure 5-22  Immunofluorescent analysis of AAV-BDNF transduced cells in the striatum
Figure 5-23  Immunofluorescent analysis of AAV-GDNF transduced cells in the striatum
Representative single-slice confocal images from AAV-GDNF treated rats showing HA-positive GDNF expressing cells within the striatum (red) together with immunofluorescent staining for (A,B) DARPP-32 or (C) krox-24 expression (both displayed in green). (A) Co-labelling with HA and DARPP-32 was not evident in the QA-lesioned striatum despite overlapping distribution in some areas showing general maintenance of DARPP-32 positive neurons. (B) AAV-GDNF transduced cells were also found to survive within striatal areas largely devoid of DARPP-32 expressing cells. (C) Labelling of krox-24 revealed co-localisation with some HA-positive cells (arrows) although many of the AAV-GDNF transduced cells lacked krox-24 expression. Scale bar: 30µm
5.7.4 **Dopaminergic innervations of the striatum**

Using tyrosine hydroxylase (TH) as a marker to visualise the maintenance of dopaminergic input into the striatum I assessed TH-positive immunostained fibres within the treated striatum relative to the contralateral hemisphere. A clear loss of TH-positive fibres was visible in the striatum of control treated rats proximal to the site of QA injection, with significant disruption to the structural arrangement of TH fibres in a larger portion of the lesioned striatum surrounding the central lesion core (Figure 5-24A). QA-induced disruption of TH-fibres was less evident in the reduced-titre AAV-BDNF treated rats (Figure 5-24B) with a lesser extent of TH-expression within the lesion core (ii) and more extensive TH-positive matrix component of the striatum in the surrounding QA-lesioned striatum (i). AAV-GDNF delivery did not induce any obvious changes compared to controls (v, vi). Dashed lines indicate the extent of striatal disruption (middle images) and the TH-depleted core (bottom images). Scale bar = 1mm for top images and 200µm for higher magnification images.

**Figure 5-24  Tyrosine hydroxylase expression in the striatum**

Representative images displaying TH-immunoreactivity in the striatum following QA injection in (A) AAV-Luciferase, (B) reduced-titre AAV-BDNF, and (C) AAV-GDNF treated rats. The distribution of TH-positive fibres was greatly affected in the control treated striatum (A) with a loss of TH expression at the lesion centre (ii) and a condensing of the TH-positive matrix component of the striatum in the surrounding QA-lesioned striatum (i). AAV-BDNF treated rats displayed less disruption to the distribution of TH expression in the striatum (iii, iv), but AAV-GDNF delivery did not induce any obvious changes compared to controls (v, vi). Dashed lines indicate the extent of striatal disruption (middle images) and the TH-depleted core (bottom images). Scale bar = 1mm for top images and 200µm for higher magnification images.
AAV-BDNF treated rats with the injected striatum generally displaying greater maintenance of the normally dispersed arrangement of the TH-positive fibres within the striatal matrix, and less evident depletion of TH proximal to the site of injection (Figure 5-24B). TH-expression in AAV-GDNF treated rats from the initial investigation was also visually assessed given the potent trophic action of GDNF on dopaminergic neurons (Choi-Lundberg and Bohn 1995), however the localisation of striatal TH appeared to be disrupted by QA in a similar pattern and extent to that observed in the control treated rats (Figure 5-24C).

To quantify the maintenance of dopaminergic striatal innervations imparted by AAV-BDNF delivery, integrated density measurements of TH-positive staining in the treated striatum relative to the contralateral striatum were performed. Control rats displayed an 80 ± 3% maintenance of TH immunostaining density which was not significantly altered by the reduced-titre AAV-BDNF treatment at 82 ± 3% TH maintenance (Figure 5-25A). The loss of striatal TH innervations showed a significant correlation with the loss of DARPP-32 positive striatal neurons (Pearson r = 0.66, P < 0.01; Figure 5-25B). With no alteration in the overall level of TH-staining in the AAV-BDNF treated rats despite extensive maintenance of TH distribution, and no apparent maintenance of DARPP-32 neurons by AAV-GDNF, I did not undertake density quantification of TH-staining in the AAV-GDNF treated striatum.

Figure 5-25  Maintenance of tyrosine hydroxylase striatal innervations
(A) Graph showing the relative density of TH-positive fibre staining in the treated striatum compared with TH-staining intensity within the contralateral striatum. Maintenance of striatal TH was determined by measuring the integrated density of DAB immunoreactivity in the striatum averaged over eight equispaced coronal sections. (B) Plot displaying the correlation between the maintenance of TH expression in the striatum and relative maintenance of DARPP-32 expressing striatal neurons. Linear regression analysis: slope = 0.32 ± 0.09, P < 0.01. R value represents overall Pearson correlation coefficient.
5.7.5  

Cortical projection fibres in the striatum

Figure 5-26  SMI32 expression in the cortex and striatum
Representative images of SMI32 expression in the contralateral (left hemisphere) and ipsilateral (right hemisphere) cortex and striatum following QA injection in (A,E-G) AAV-Luciferase and (H-K) reduced-titre AAV-BDNF treated rats. (B,E,I) Pyramidal neurons predominantly in cortical layers III and V overlaying the striatum appeared unaffected by QA striatal lesioning. SMI32 expression in (C,D) the intact contralateral striatum and (F,G,J,K) the AAV vector and QA treated striatum. Regions outlined in boxes are shown at higher magnification in the figures immediately below. Scale bar = 1mm for whole sections (A,H), 100µm for (B,E,I), 200µm for (C,F,J), 50µm for (D,G,K).
Maintenance of cortical neuronal fibres within the striatum was visualised by immunostaining for SMI32 – a non-phosphorylated neurofilament protein – expressed by a subpopulation of pyramidal neurons (Campbell and Morrison 1989; Gerfen 1992), which are vulnerable to HD degeneration (Cudkowicz and Kowall 1990; Hedreen et al. 1991; Macdonald and Halliday 2002). The majority of SMI32 positive neurons are located in layers III and V with no apparent loss of neurons observed in the primary motor or sensory areas overlying the striatum following unilateral QA lesioning (Figure 5-26). Distribution of SMI32 expression in the striatum was however altered following QA lesioning. Within the intact striatum SMI32 appeared largely contained within fibres innervating the striosome-like patches which are irregularly dispersed throughout the striatum (Figure 5-26C,D). Following QA-lesioning of the control treated striatum, the striatal density of SMI32 staining increased with a greater proportion of the striatal area containing SMI32 consistent with a loss of striatal matrix and possible enlargement of the striosome compartments or fibre bundles (Figure 5-26F,G). This QA-induced alteration in striatal organisation is similar to the changes observed with TH staining (Section 5.7.4). The expression of SMI32 was also enhanced in AAV-BDNF treated rats in the striatum proximal to the site of QA injection with the fibres appearing larger than in the control rats, but was less extensively increased in the more distal surrounding striatum (Figure 5-26J,K). AAV-GDNF treated rats from the initial investigation were not assessed for SMI32 due to the lack of any apparent striatal neuroprotection.

5.7.6 Striatal projection nuclei

To investigate whether enhanced BDNF expression prior to QA lesioning of the striatum had any bearing on neuronal maintenance in the striatal projection nuclei I performed immunocytochemistry to identify neurons within the substantia nigra and globus pallidus. Analysis was only performed on the control and reduced-titre AAV-BDNF treated rats from the follow-up investigation with no evidence of any striatal neuroprotection being imparted by AAV-GDNF delivery.

5.7.6.1 Substantia Nigra

Dopaminergic neurons in the substantia nigra pars compacta region were visualised by TH immunocytochemical staining and showed no observable differences between brain hemispheres in either control (Figure 5-27) or AAV-BDNF treated rats (Figure 5-28). Similarly the parvalbumin-positive GABAergic neurons in the substantia nigra pars reticulata appeared to be equally unaffected by the striatal QA lesion with visually comparable expression in each hemisphere. With the nigral neurons not appearing to be influenced by the QA lesion I analysed the maintenance of striatonigral axonal innervations. The projection of DARPP-32 positive fibres, originating from the vulnerable
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DARPP-32 expressing striatal projection neurons, into the substantia nigra appeared to be partially depleted in the ipsilateral nigra (Figure 5-27C and Figure 5-28C). To quantify the loss of striatonigral innervations I measured the density of DARPP-32 immunoreactivity in the ipsilateral substantia nigra relative to the contralateral side of the brain (Figure 5-29A). Control rats displayed a 29 ± 3% loss of DARPP-32 immunostaining that was significantly reduced to 10 ± 5% in AAV-BDNF rats. Comparison with the relative maintenance of DARPP-32 positive neuronal cell bodies in the striatum showed a direct correlation (Pearson r = 0.75, P < 0.001) between striatal cell bodies and DARPP-32 immunoreactivity in the substantia nigra (Figure 5-29B).

Figure 5-27 Visual assessment of the substantia nigra in control AAV-Luciferase treated rats
Representative images of the substantia nigra in an AAV-Luciferase treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) TH-positive neurons in the pars compacta, (B) parvalbumin-positive neurons in the pars reticulata, and (C) DARPP-32 positive neurons and efferent fibres. Regions outlined by boxes are shown at higher magnification in figures to the right. Scale bar = 2mm for whole sections and 200µm for higher magnification images.
Figure 5-28  Visual assessment of the substantia nigra in reduced-titre AAV-BDNF treated rats
Representative images of the substantia nigra in a reduced-titre AAV-BDNF treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) TH-positive neurons in the pars compacta, (B) parvalbumin-positive neurons in the pars reticulata, and (C) DARPP-32 positive neurons and efferent fibres. Regions outlined by boxes are shown at higher magnification in figures to the right. Scale bar = 2mm for whole sections and 200µm for higher magnification images.
Figure 5-29  DARPP-32 innervations of the substantia nigra
(A) Graph showing the density of DARPP-32 immunoreactivity within the substantia nigra, ipsilateral to the treated striatum, as a proportion of DARPP-32 immunostaining in the contralateral substantia nigra. DARPP-32 density was measured on three coronal sections through the substantia nigra. (B) Plot displaying the correlation between DARPP-32 positive fibre innervations of the substantia nigra and maintenance of the DARPP-32 positive striatal neurons. * Un-paired t-test P < 0.01. Deming linear correlation: slope = 0.70 ± 0.15, P < 0.001. R value represents overall Pearson correlation coefficient.

5.7.6.2  Globus Pallidus
Pallidal neurons were visualised by immunostaining for parvalbumin and NeuN with a clear loss of neurons seen within the ipsilateral globus pallidus of AAV-Luciferase treated control rats (Figure 5-30A,B). AAV-BDNF treated rats did not generally have any obvious loss of pallidal neurons (Figure 5-31A,B). Additional staining for DARPP-32 showed the mostly intact caudal region of the striatum adjacent to the globus pallidus remained mostly intact, but displayed reduced DARPP-32 immunoreactivity in the globus pallidus compared with the contralateral hemisphere for both control (Figure 5-30C) and AAV-BDNF treated rats (Figure 5-31C).

With parvalbumin positive neurons in the globus pallidus clearly affected by the QA lesion in some rats, I performed stereological estimates over four equispaced coronal sections through the pallidus (Stereology coefficient of error ≤ 0.09). In contrast to all other analysis parvalbumin-positive neurons in the globus pallidus showed a significant difference between the AAV-Luciferase and PBS control groups (t-test P < 0.01; Figure 5-32A). AAV-Luciferase animals were massively depleted of parvalbumin neurons in the globus pallidus with only 15 ± 8 % maintained relative to the contralateral hemisphere while the PBS group had 87 ± 18 % maintenance. The parvalbumin-positive pallidal neurons were mostly maintained in the AAV-BDNF treated group (96 ± 10 %). There was no significant correlation between the survival of DARPP-32 positive striatal neurons and the parvalbumin expressing pallidal neurons (Pearson r = 0.42, P = 0.07; Figure 5-32B).
**Figure 5-30  Visual assessment of the globus pallidus following AAV-Luciferase treatment**
Representative images of the globus pallidus in a control AAV-Luciferase treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) parvalbumin-positive pallidal neurons, (B) NeuN-positive neurons, and (C) DARPP-32 immunoreactivity. Dashed lines indicate the boundary between the striatum and globus pallidus. Scale bar = 250µm.
Figure 5-31 Visual assessment of the globus pallidus following reduced-titre AAV-BDNF treatment
Representative images of the globus pallidus in a reduced-titre AAV-BDNF treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) parvalbumin-positive pallidal neurons, (B) NeuN-positive neurons, and (C) DARPP-32 immunoreactivity. Dashed lines indicate the boundary between the striatum and globus pallidus. Scale bar = 250µm.
Figure 5-32 Survival of parvalbumin-positive neurons in the globus pallidus
(A) Graph displaying the relative proportions of parvalbumin-immunopositive neurons surviving in the globus pallidus as estimated by optical fractionator stereology on four coronal sections through the globus pallidus. (B) Plot comparing the survival of parvalbumin-positive neurons relative to the maintenance of DARPP-32 positive striatal neurons. * Un-paired t-test P < 0.01. R value represents overall Pearson correlation coefficient, P = 0.07.

5.7.7 Pathological correlations with functional behaviour impairments

To assess whether there were significant correlations between the functional behavioural impairments and the loss of striatal neurons or pallidal neurons I analysed overall Pearson correlations and individual treatment group linear relationships for each of the behavioural tests undertaken in the follow-up, reduced-titre AAV-BDNF investigation. Due to the significant maintenance of parvalbumin-expressing pallidal neurons in PBS controls compared with the loss in AAV-Luciferase treated rats I have separately identified whether rats received PBS or AAV-Luciferase in the correlations against pallidal neurons but not for striatal DARPP-32 correlations where there was no significant difference between the groups. The PBS and AAV-Luciferase treated rats were still combined as a single control group to assess for linear relationships as they did not perform differently as two distinct groups in any of the functional behavioural assessments.

5.7.7.1 Spontaneous Ipsilateral Forelimb Use Bias

The proportion of exploratory behaviour undertaken by the rats with their ipsilateral forelimb following QA injection was found to be significantly correlated with the loss of DARPP-32 positive neurons in the striatum (Pearson r = -0.49, P < 0.05; Figure 5-33A). However, with the prior delivery of AAV-BDNF attenuating development of an ipsilateral forelimb use bias, independent
analysis for a linear trend between DARPP-32 maintenance and ipsilateral asymmetry score indicated that the extent of ipsilateral bias in AAV-BDNF treated rats was not greatly influenced by the loss of DARPP-32 positive striatal neurons. This was in contrast to the strong linear relationship between the loss of DARPP-32 striatal neurons and ipsilateral forelimb use in the control group (P < 0.001). Exploratory ipsilateral forelimb use in the AAV-BDNF treated rats did however appear to be strongly linked to the ipsilateral:contralateral ratio of parvalbumin-positive neurons in the globus pallidus (Linear regression P < 0.01) with a 1:1 ratio correlating with a non-biased 52 ± 2% ipsilateral asymmetry score (Figure 5-33B). Overall though the maintenance of parvalbumin-positive pallidal neurons did not significantly correlate with the ipsilateral asymmetry score (Pearson r = -0.39, P = 0.10) with the control rats displaying preferential ipsilateral forelimb use independent from the relative number of parvalbumin positive neurons in the globus pallidus (P = 0.518).

Figure 5-33  Preferential forelimb use correlation with striatum and globus pallidus cell loss
Plots correlating the extent of ipsilateral forelimb use bias with the relative proportion of (A) DARPP-32 and (B) parvalbumin-positive neurons remaining within the striatum and globus pallidus respectively. Data points represent individual rats’ average score over the seven post-QA trials. R values represent overall Pearson correlation coefficients independent of treatment. Solid lines represent significant non-zero linear correlations (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. Comparison with controls: (A) slope P = 0.14; elevation P < 0.001; (B) slope P = 0.001.

5.7.7.2  Contralateral Sensorimotor Neglect
Preferential retrieval of sugar pellets from the side of the corridor adjacent to the rats treated brain hemisphere was significantly correlated with the anatomical state of the globus pallidus (Pearson r = -0.68, P < 0.01) but not the extent of DARPP-32 striatal cell loss (Pearson r = -0.41, P = 0.08; Figure 5-34A). Maintenance of parvalbumin-positive pallidal neurons appeared to contribute towards
attenuating development of a contralateral sensorimotor neglect following QA injection. Individual AAV-BDNF and control group assessment for linear relationships indicated that this sensorimotor neglect correlation with pallidal neurons was stronger for AAV-BDNF treated rats (Linear trend slope = -63 ± 14, P < 0.0001) than the control rats (slope = -19 ± 5, P < 0.001; Figure 5-34B). While there was not overall a significant correlation with the DARPP-32 striatal neurons, the percentage of ipsilateral retrievals made by control rats did display a significant linear relationship with the loss of DARPP-32-positive striatal neurons (slope = -77 ± 19, P < 0.01; Figure 5-34A). AAV-BDNF treated rats did not show any relationship between attenuation of preferential ipsilateral retrievals and the gross maintenance of DARPP-32-positive striatal neurons.

Figure 5-34  Sensorimotor neglect correlation with striatal and pallidal neuronal cell loss
Plots correlating the percentage of ipsilateral sugar pellet retrievals made in the “corridor” task with the relative proportion of (A) DARPP-32 and (B) parvalbumin-positive neurons maintained within the striatum and globus pallidus respectively. Data points represent individual rats’ average retrieval preference over the four post-QA trials. R values represent overall Pearson correlation coefficients independent of treatment. Solid lines represent significant non-zero linear correlations (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. Comparison with controls: (A) slope P < 0.05; (B) slope P < 0.01.

5.7.7.3 Apomorphine-Induced Rotations
Apomorphine-induced rotational behaviour did not display any overall correlation with DARPP-32-positive striatal cell loss (Pearson r = -0.32, P = 0.19; Figure 5-35A). Separate linear trend analysis with the individual groups also failed to show any significant correlations for either the AAV-BDNF rats (P = 0.069) or control rats (P = 0.86). In contrast, comparison of apomorphine-induced rotations with parvalbumin-positive neurons in the globus pallidus did reveal a significant correlation with the maintenance of pallidal neurons (Pearson r = -0.66, P < 0.01; Figure 5-35B). This correlation was
very significant for the control treated rats (Linear trend P < 0.001), but not for the AAV-BDNF treated rats (P = 0.07) although both treatment groups showed similar trends. The direction of apomorphine-induced rotation also appeared to be strongly dependent on the integrity of the globus pallidus with a loss of pallidal neurons resulting in rotational behaviour towards the lesioned side, while apomorphine induced the rats to rotate contralaterally away from the lesioned brain hemisphere when the globus pallidus was fully maintained (Figure 5-35B).

Figure 5-35 Correlation of apomorphine-induced rotational behaviour with striatal and pallidal neuronal cell loss
Plots displaying the percentage of apomorphine-induced rotations in an ipsilateral direction in relation to the maintenance of (A) DARPP-32 and (B) parvalbumin-positive neurons within the striatum and globus pallidus respectively. Each data point represents the result of a single post-QA trial with three trials conducted per rat. Shaded boxes show the predominant contralateral rotational behaviour when the relative number of parvalbumin pallidal neurons is greater in the treated hemisphere and preferential ipsilateral rotations when the globus pallidus is compromised. R values represent overall Pearson correlation coefficients independent of treatment. Solid lines represent significant non-zero linear correlations (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. AAV-BDNF treatment did not significantly alter the linear correlations observed in the control rats.
5.8 Discussion

Through these preventative *in vivo* studies I investigated the pathological and functional impact of increasing the availability of neurotrophic factors using AAV vectors to establish continuous expression of either BDNF or GDNF within the striatum prior to challenging the striatal neurons with QA-administered excitotoxicity. While the protective effects of neurotrophic factors have been previously studied in models of Huntington’s disease with varying degrees of success (Section 1.6), the ongoing development and advances in gene transfer technologies make additional investigation necessary to enhance our understanding of both delivery vectors and potential gene-based therapeutics in an *in vivo* research setting. Stereotype-2 AAV vectors used in many early AAV gene transfer studies have significantly lower efficiency of neuronal transduction than the chimeric 1/2 serotype vectors used in these investigations. Using behaviour tests to measure functional motor deficits consequential of an interhemispherical imbalance in the integrity of motor and sensorimotor neuronal processing in unilaterally treated rats, I endeavoured to correlate the prevention of physical symptomatic impairments with the maintenance of basal ganglia anatomical organisation provided by the prior intrastrial delivery of the AAV-BDNF or AAV-GDNF vectors.

5.8.1 AAV-mediated BDNF or GDNF expression prior to QA

The AAV_{1/2} vectors I constructed – containing cDNA sequences for BDNF or GDNF under the control of a constitutive CBA promoter and WPRE sequence – provided an efficient mechanism for establishing high-level neurotrophic factor expression in the CNS. Spread of the AAV vector transduction throughout the rodent striatum following a single injection appeared reasonably extensive in the majority of cases although somewhat irregular in its penetration resulting in patches of intensive expression and other areas with few or no transduced cells. With the neurotrophic factors being secreted from transduced cells, the uneven spread of transduction was not immediately concerning as the non-transduced striatal neurons were still likely to be exposed to an increased level of BDNF or GDNF as the neurotrophic factors were released, diffusing away from the transduced cells.

5.8.1.1 Undiluted AAV-BDNF

Following the development of seizures, hyperactivity and continuous weight loss in some of the AAV-BDNF treated rats, evident five weeks after AAV-BDNF vector delivery and forcing early termination of the BDNF portion of the study, I had to reassess the delivery procedure for this vector.
No welfare concerns were raised with any of the other AAV vectors, and with the delayed onset the deleterious effects were most likely due specifically to the BDNF expression. BDNF has previously been implicated in seizure severity, thought to be modulated via BDNF activity in the entorhinal cortex and hippocampus (Scharfman 1997; Croll et al. 1999). Additionally BDNF can act as an appetite suppressor causing weight loss following ventricular infusion (Pelleymounter et al. 1995), and positively regulates locomotor activity (Kernie et al. 2000) which is consistent with the effects I observed. With the massive escalation in striatal BDNF levels following undiluted AAV-BDNF delivery, and with observed anterograde transport of the AAV-BDNF vector to striatal projection nuclei, it is probable that the availability of BDNF was also enhanced in other regions of the rodent brain including the entorhinal cortex which projects efferent fibres to the striatum. Retrograde transport of AAV-vectors to the entorhinal cortex has also been previously shown to occur (Kaspar et al. 2002).

Analysis of the transgenic BDNF expression in the striatum showed an inverse correlation with DARPP-32 expression suggesting the intensive BDNF expression was detrimentally impairing the functionality of transduced striatal neurons, but in direct contrast was supplying neuroprotective support to the neighbouring DARPP-32 positive neurons. While I did not conclusively determine whether this loss of DARPP-32 expression was due to a BDNF-induced down regulation of expression or an enhanced susceptibility of transduced neurons to QA-induced excitotoxicity, the persistence of HA-expressing cell bodies in the striatum and their co-labelling with krox-24 indicates survival – and presumed functional activity – of a population of BDNF expressing striatal neurons four weeks post-QA. Given the neuronal preference of the AAV_{1/2} vectors, and the normally high expression of DARPP-32 by striatal projection neurons (Ouimet et al. 1984; Ouimet et al. 1998), it is reasonable to assume that the vast majority of AAV-BDNF transduced striatal cells would have been expressing DARPP-32 at the time of transduction, prior to being challenged with QA. Striatal neurons transduced by AAV-GDNF or AAV-Luciferase also lacked DARPP-32 expression at the conclusion of the study. While some of the transduced neurons may well have been non-DARPP-32 expressing interneurons that were spared by QA-lesioning, the majority are likely to have been striatal projection neurons indicating a disruption of DARPP-32 expression, not specific to transgene expression, following AAV vector transduction. Given this apparent down-regulation of DARPP-32 expression in transduced striatal neurons, the use of DARPP-32 as a marker of striatal projection neurons to quantify BDNF and GDNF-derived support against QA-induced cell death will have lead to an underestimation of the actual number of surviving neurons. This may have contributed to the lack of a significant increase in the stereologically estimated striatal neurons maintained in AAV-BDNF treated rats relative to the control rats despite visually distinguishable areas of protected
neurons and the complete preservation of striatal volume (Section 5.7.1). However, functional implications of DARPP-32 down-regulation will need serious consideration given the central role of DARPP-32 in mediating intracellular signalling of both dopamine and glutamate in the dopaminoceptive striatal projection neurons (Greengard et al. 1999). A reduction in DARPP-32 has been reported in presymptomatic HD mice and may be a contributing factor to HD pathogenesis (Bibb et al. 2000).

Despite the non-significant maintenance of DARPP-32 positive neurons in the striatum, extensive BDNF expression in the initial investigation ameliorated the development of QA-induced ipsilateral forelimb use bias with the rats actually tending to favour their contralateral forelimb. The preservation / enhancement of forelimb motor control suggests a possible augmentation of brain function due to increased BDNF expression that was independent of actual neuronal cell maintenance in the striatum. Together these observations suggested that despite serious detrimental effects on the rats’ welfare and the potentially detrimental alterations to the function of transduced neurons, BDNF could still offer both neuroprotective support to striatal neurons against excitotoxic insult and prevent associated behavioural deficits. Provided BDNF delivery can be sufficiently regulated to limit the spread and control the level of expression it could well be of therapeutic benefit, and therefore I felt that AAV-mediated BDNF expression warranted further investigation.

5.8.1.2 AAV-GDNF

The delivery of AAV-GDNF and subsequent rise in striatal GDNF availability from normally very low expression levels in the adult striatum (Schaar et al. 1993; Springer et al. 1994; Choi-Lundberg and Bohn 1995) imparted significant attenuation of the ipsilateral forelimb use bias that developed in control rats following QA lesioning. The contrasting development of a contralateral forelimb use bias as seen in the AAV-BDNF treated rats was not however recorded in analysis of AAV-GDNF treated rats, suggesting that the maintenance of forelimb motor control provided by AAV-GDNF was probably mediated via a different signalling pathway than the AAV-BDNF effects. Apomorphine- and amphetamine-induced rotational behaviour did not appear greatly altered from the performance of control rats, with the AAV-GDNF treated rats preferentially displaying unidirectional rotations, usually towards the QA injected striatal hemisphere.

Despite the maintenance of forelimb motor control, AAV-GDNF delivery prior to QA did not enhance the resistance of DARPP-32 positive striatal projection neurons, supporting the observation of the drug-induced rotational testing. This lack of neuroprotection was in contrast to my earlier findings of partial protection of striatal neurons following transduction with a lower expression level AAV₂-GDNF vector (Kells et al. 2004) and other reports of AAV-mediated GDNF delivery in the 3-
NP mitochondrial toxin (McBride et al. 2003) and N171-82Q genetic models of HD (McBride et al. 2006), but in agreement with Popovic and colleagues (2005) who reported no attenuation of behavioural or neuropathological changes in R6/2 Huntington mice following LV vector-mediated GDNF expression. Given these previous results the lack of AAV-GDNF derived neuroprotection against QA-induced excitotoxicity was unexpected. I had hypothesised that the enhanced neuronal transduction efficiency of the new chimeric AAV1/2 vector, and higher CBA promoter driven GDNF expression, would increase GDNF-derived support of the striatal neurons. A common factor in the studies which have demonstrated GDNF expression-derived protection is the assessment of NeuN immunoreactivity (McBride et al. 2003; Kells et al. 2004; McBride et al. 2006), in contrast to the DARPP-32 expression used in both the LV-GDNF study (Popovic et al. 2005) and current analysis to quantify neuroprotection, raising the possibility that the neurons may survive but with altered phenotype / functionality. Alternatively the conflicting findings with GDNF may reflect the different processes of striatal cell loss between neurotoxins – excitotoxic QA versus mitochondrial inhibitor 3-NP – or genetic models – R6/2 (Hdh exon-1 with 150 CAG repeats) versus N171-82Q (Hdh N-terminal with 82 CAG repeats) – of HD. The 3-NP and N171-82Q models display a more protracted development of behavioural impairments and pathological changes suggesting a less intensive insult that may be better counteracted by GDNF. A further explanation of these differential GDNF findings could be the extent of transduction and / or the production of GDNF with the more efficient AAV1/2 and LV vectors potentially generating GDNF that exceeds therapeutically beneficial levels. Direct comparisons of the quantity of GDNF expression between studies was unfortunately not possible due to differences in the quantification protocols and in the reporting of transgene expression, however in my earlier investigations with AAV2-GDNF (Kells et al. 2004) the transgenic expression of GDNF was considerably lower [Unpublished data]. A recent study by Eslamboli et al. (2005) reported extensive protection of dopaminergic nigral neurons and attenuation of behavioural deficits following low-level GDNF expression in the primate striatum (three- to four-fold increase) prior to 6-hydroxydopamine modelling of Parkinson’s disease. High levels of striatal GDNF were shown to bilaterally increase TH and dopamine turnover causing a reduction in striatal dopamine (Eslamboli et al. 2005), and have also been reported to increase the loss of striatal neurons following ischemic stress (Arvidsson et al. 2003).

In reassessing the expression pattern of GDNF within the striatum it became apparent that large numbers of GDNF expressing cells were still present within the striatum following QA injection suggesting they had in fact received protection against QA-induced cell death. On correlating the location of these surviving AAV-GDNF transduced striatal cells to the area of QA-lesioning, defined by DARPP-32 immunoreactivity, it was evident that the majority of HA-positive cells resided in
areas largely devoid of DARPP-32 expression. Additional correlation with the general neuronal marker NeuN displayed a similar lack of immunopositive cells in the region of HA staining, although NeuN cells appeared more prevalent than DARPP-32 positive cells (Section 5.7.1.1). Furthermore, double-immunofluorescent labelling of the AAV-GDNF treated striatum revealed only minimal co-labelling of HA and DARPP-32 positive cells displaying a down-regulation of DARPP-32 in the HA-positive GDNF expressing cells (Section 5.7.3). The loss of DARPP-32 is highly likely to affect neurotransmitter signalling (Section 5.8.1.1 and 7.1). It therefore appeared that while many AAV-GDNF transduced neurons did actually survive QA-induced excitotoxicity, the expression of their usually dominant signalling proteins used as identification markers were severely compromised. How this alteration in protein expression influences the functionality of the striatal neurons was not specifically investigated but highlights the need for caution and assessment of host cells physiological functions following in vivo gene transfer to direct production of a bioactive therapeutic molecule. Stereological analysis of NeuN-immunopositive striatal neurons may possibly have better estimated the proportion of striatal neurons maintained, although from the lack of correlation in immunostaining it appeared that NeuN may also have been down-regulated in the AAV-GDNF transduced cells by the end-point of the investigation – 11-weeks post AAV-GDNF delivery. Although not quantified, TH staining in the striatum – indicative of dopamine innervations – following QA-lesioning did not appear to be altered by the enhanced GDNF expression. However the dopamine neurons in general are not affected by QA lesioning and therefore any TH-positive fibre sprouting induced by GDNF – as reported following striatal GDNF expression in animal models of Parkinson’s disease (Rosenblad et al. 2000; Kirik et al. 2000a; Georgievska et al. 2002) – may simply not be visibly discernable.

Therefore, although indirect evidence suggested that AAV-GDNF delivery imparted neuroprotective support to transduced striatal neurons, continuous high-level GDNF expression appeared to cause alterations in neuronal processes. Despite the fact that I was not aware of any adverse alterations in the rats behaviour during the extended period of functional behavioural testing – actually observing maintenance of forelimb motor control – the changes in protein expression patterns are likely to ultimately result in disruption to neuronal function, potentially negating any benefits of initial AAV-GDNF derived neuroprotective support. While better regulation of the GDNF expression may well circumvent some of these undesired effects, I opted against proceeding further with investigation of this AAV-GDNF vector in the QA lesion model of HD. However it is possible that a significantly lower level of GDNF expression maybe beneficial (Eslamboli et al. 2005), and that the extensive expression I induced exceeded tolerable quantities for a region that has a very low level of endogenous GDNF expression in adulthood (Springer et al. 1994; Choi-Lundberg and Bohn 1995).
5.8.2 Reduced-titre AAV-BDNF behavioural and pathological protection

Continuing investigation with delivery of the AAV-BDNF vector, I elected to shift the striatal injections site away from the same stereotaxic coordinates as the QA injection and split the vector delivery over two stereotaxic sites flanking the QA injection site in an attempt to even out the striatal distribution of transduced cells. To minimise any toxicity associated with AAV-BDNF transduction I also diluted the vector ten-fold to reduce the multiplicity of transduction and thereby decrease the intensity of BDNF expression per transduced cell. This reduced the enhanced expression level of BDNF in the striatum from ~1000-times endogenous expression to a ~150-fold increase at the time of QA administration, and prevented the previously observed weight-loss and seizure development. Visual assessment of AAV-BDNF transduced cells showed extensive transduction of neurons around the site of injection, but significantly less spread than the undiluted vector injections. To more fully assess the AAV-BDNF delivery on functional behaviour – without the confounding impact of a QA lesion – I additionally assessed a smaller cohort of reduced-titre AAV-BDNF treated rats for 11-weeks post-AAV vector delivery in parallel with the main neuroprotective investigation.

5.8.2.1 Behavioural Protection

Prior to QA-lesioning rats that received reduced-titre AAV-BDNF showed an enhanced tendency to use their contralateral forelimb for initiating exploratory movement and the induction of ipsilateral rotational behaviour following apomorphine administration, compared with unbiased lateralised behaviour of the control rats. While this behaviour was similar to that observed in the initial high-titre AAV-BDNF study, the slight preferential use of the contralateral forelimb did not develop into a strong contralateral bias in either the QA-lesioned or non-lesioned rats with both groups remaining insignificantly altered from baseline analysis in contrast to the ipsilateral bias in control rats and contralateral bias in the initial BDNF study (Section 5.6.1). While not significantly different, the two AAV-BDNF treated groups were separated after the main group received a QA injection inducing a small shift towards greater use of their ipsilateral forelimb, while the non-QA injected rats maintained slightly greater contralateral forelimb use, although neither AAV-BDNF treated group could be considered to have a significant forelimb use bias. These results suggested that while the expression of BDNF may provide some enhancement of basal ganglia coordinated motor control, the lack of a significant contralateral usage in the absence of striatal cell death implies AAV-BDNF derived amelioration of QA induced ipsilateral forelimb bias is unlikely to be solely attributed to
BDNF activity compensating for the loss of striatal cell loss, but involve the maintenance of functional striatal projection neuron circuitry by BDNF.

The transient display of apomorphine-induced ipsilateral rotational behaviour initially following AAV-BDNF delivery, before predominantly switching to induce contralateral rotations in both QA lesioned and non-QA lesioned rats (Section 5.6.2.1), suggests that BDNF expression causes opposing short and long-term actions. QA striatal lesioning of control rats caused a loss of dopaminoceptive projection neurons, generally resulting in ipsilateral rotations following either apomorphine or amphetamine administration. Therefore enhanced BDNF expression appeared to be acting long-term to enhance dopamine activity, but initially resulted in impairment to the striatal neurons ability to process dopamine signalling. With apomorphine directly stimulating both D1 and D2 dopamine receptors (Arnt and Hyttel 1985), rotational behaviour was most probably mediated through alterations in the expression of dopamine receptors by surviving striatal neurons, thereby making the results of drug-induced rotational testing difficult to conclusively assess in regards to any direct neuroprotective support provided by AAV-BDNF. However this does further highlight challenges facing the use of neurotrophic factors as therapeutic agents with widespread neurotrophic factor receptor expression and multiple signalling pathways creating a high potential for inducing non-targeted effects that clearly need consideration prior to any clinical investigation.

BDNF has previously been shown to enhance dopamine activity by increasing both the striatal release and turnover of dopamine (Altar et al. 1992b; Altar et al. 1994), and induces behavioural changes indicative of increased dopamine activity (Martin-Iverson et al. 1994; Martin-Iverson and Altar 1996). More recently though, BDNF has been shown to positively-regulate the expression of post-synaptic D3 dopamine receptors in the striatum (Guillin et al. 2001), suggesting that the unilateral delivery of BDNF may have resulted in sensitisation of the surviving striatal neurons to dopamine agonists, thereby counteracting any QA-induced deficiency in motor effector output. Therefore the initial dysfunction following AAV-BDNF delivery may have been consequential of a rapid increase in striatal BDNF levels causing functional disruption or desentisation of the dopamine receptors. Dopamine has been proposed to actually contribute to the degeneration of striatal neurons such that a sudden increase in BDNF-induced dopamine release, without a corresponding increase in dopamine uptake, may initially cause neuronal dysfunction (Filloux and Townsend 1993; Hastings et al. 1996; Hattori et al. 1998). Dopamine toxicity may also have been a contributing factor to the deleterious effects in the initial high-expression AAV-BDNF investigation. Another contributing factor may be the involvement of TrkB receptors which are likely to have been down-regulated at some stage following continuous enhancement of BDNF expression (Frank et al. 1996).
Additional evidence of AAV-BDNF delivery supplying neuroprotective support resulting in attenuation of functional impairments was observed in the “corridor” task assessment of sensorimotor neglect. The reduced-titre AAV-BDNF delivery completely prevented acquirement of the contralateral sensorimotor neglect observed following QA injection in control rats (Section 5.6.3). Although the non-lesioned and QA lesioned groups of AAV-BDNF treated rats did not significantly differ in their “corridor” task performance, the AAV-BDNF delivery alone did significantly change the non-lesioned rats’ preferential food retrieval from a slight baseline contralateral preference towards development of a slight contralateral neglect. This tended to suggest that AAV-BDNF delivery attenuated the acquirement of contralateral sensorimotor neglect through protection against QA-induced alterations and not via BDNF-induced enhancement of other compensatory mechanisms as suggested by the apomorphine-induced rotations.

5.8.2.2 Neuropathological Protection

Despite significant attenuation of behavioural deficits, analysis of neuronal survival following AAV-BDNF delivery revealed only limited enhancement of striatal maintenance, compared with the control treated rats. Maintenance of krox-24 expressing striatal neurons was significantly enhanced with a 36% increase in neuron survival following AAV-BDNF delivery; however the parallel analysis of DARPP-32 positive neurons did not quite reach significance despite a similar 38% average increase in the relative number of DARPP-32-positive cells. The lack of significance due to greater variation in the quantification of DARPP-32 neurons is likely to have arisen from the down-regulation of DARPP-32 or the less distinct immunostaining of cytoplasmic DARPP-32, compared with the krox-24 nuclear staining, making the striatal neurons more difficult to visually discern against the positive staining of dendritic processes. Co-labelling of the BDNF expressing transduced neurons with DARPP-32 and krox-24 still displayed inverse staining intensities, although krox-24 was detectable in most HA-positive cells and the majority also appeared to display some DARPP-32 expression.

While a small enhancement of cellular survival could potentially lead to an observable prevention of behavioural deficits in this QA-lesion model of HD, I felt that the quantified partial protection of striatal neurons supplied by AAV-BDNF was unlikely to have been solely responsible for the amelioration of functional impairments. Coupled with the maintenance of striatal projection neurons I observed that atrophy of the striatum was significantly reduced in the AAV-BDNF treated rats (Section 5.7.1.4), suggesting that the structural organisation of the striatum was protected against QA-induced excitotoxicity by BDNF, possibly to a greater extent than individual striatal neurons. Therefore I propose that the functionality of the striatum in regard to the assessed motor coordination
tasks is more reliant on structural organisation than the total number of striatal projection neurons, and that AAV-BDNF by enhancing the maintenance of this structural architecture, in addition to providing individual neurons with greater resistance against QA, was able to ameliorate the behavioural deficits. The striatum is topographically organised with the sensory and motor areas of the neocortex projecting to the striatum in a highly organised but overlapping longitudinal arrangement (McGeorge and Faull 1989). Therefore overall preservation of this structural arrangement, with a reduction in neuronal density but without areas of total projection neuron loss, may well attenuate the severity of behavioural deficits.

Further analysis revealed that preservation of substantia nigra innervations by striatonigral DARPP-32 positive fibres was significantly enhanced by AAV-BDNF, with the nigral neurons themselves not appearing to be greatly affected by the striatal lesion (Section 5.7.6.1). Maintenance of striatonigral projections was correlated with the maintenance of DARPP-32 positive neurons in the striatum. Despite no apparent loss of dopaminergic neurons in the substantia nigra pars compacta I assessed dopaminergic innervations of the striatum by measuring the density of TH-immunostaining to try and determine if maintenance of dopamine input may have been responsible for the reduced severity of behavioural impairments. While the integrated density measurements indicated equal loss of TH-positive fibres in the striatum averaging ~19% reduction relative to the contralateral striatum across all rats following QA lesioning, the control rats visually displayed intensification of the TH-positive fibres from the dispersed matrix into defined tracts interwoven around TH-negative patches (Section 5.7.4). Prior AAV-BDNF delivery attenuated this apparent atrophy of the striatal matrix, with TH-staining remaining diffusely distributed throughout the striatum, indicating a general maintenance of the striatal architecture by BDNF. Further evidence for preservation of the striosome-matrix arrangement following AAV-BDNF delivery was provided by visual assessment of the neurofilament protein SMI32 expression in the striatum – presumed to be contained within afferent projections of the SMI32-positive pyramidal neurons in the motor and sensory cortex (Section 5.7.5). Localised to striosome-like patches, SMI32 appeared to be greatly enhanced following QA lesioning possibly due to fibre sprouting or structural rearrangement occurring within these afferent corticostriatal fibres following the death of their target striatal neurons. While SMI32 expression was greatly enhanced across a substantial proportion of the striatum in control rats, with an accompanying increase in the density of striosomes, AAV-BDNF treated rats generally only displayed an increased intensity of SMI32 directly proximal to the QA injection site. A smaller enhancement of SMI32 following BDNF expression is possibly indicative of less fibre sprouting due to a greater maintenance or re-establishment of synaptic connections in the striatum following QA lesioning.
Pallidal neurons, in contrast to those in the substantia nigra, were subjected to QA induced neurotoxicity although this was highly variable with the two control groups displaying significantly contrasting levels of parvalbumin-positive neuronal maintenance (Section 5.7.6.2). A reason for these opposing results was undetermined with no correlation to the extent of DARPP-32 loss in the striatum. While it is possible that chance variation in the positioning of the QA lesion could have contributed to the opposing observations given the small group sizes, the influence of AAV-Luciferase transduction or Luciferase expression making these pallidal neurons more vulnerable to neurotoxic insult cannot be discounted. Negative impacts of marker proteins have previously been reported (Detrait et al. 2002; Krestel et al. 2004), possibly due to intensive expression of a foreign protein that may cause cellular stress through disruption of degradation mechanisms or accumulation to toxic levels – especially over extended periods of expression. An empty DNA expression cassette may provide a more valid control for AAV vector transduction. With only five rats in each control group and reasonably high amounts of variability with the QA lesion – demonstrated here with only one of the five PBS rats having extensive pallidal cell loss – I was unable to reliably draw any conclusions regarding the vulnerability of the pallidal neurons although it was important to consider their maintenance in respect to the striatal output via the globus pallidus and the impact that it has on motor control in the behavioural testing.

5.8.2.3 Pathological and Behavioural Correlations

To investigate whether there was any association between the quantified maintenance of the basal ganglia nuclei and the observed amelioration of functional deficits I performed correlation analysis between the results of each behavioural test and the relative maintenance of DARPP-32 striatal neurons and parvalbumin-positive pallidal neurons (Section 5.7.7). The loss of DARPP-32 striatal neurons showed significant correlation with reductions in the striatal area, TH striatal density and DARPP-32 fibre innervations of the substantia nigra, although none of these were reduced to the same extent as striatal neuron loss. Therefore I choose to directly compare the functional behaviour with the relative DARPP-32 positive cell maintenance, with the assumption that any correlations would also be reflected in these related structures. Separate correlations with parvalbumin-positive neurons in the globus pallidus were analysed due to the lack of any apparent correlation with the maintenance of striatal neurons (Section 5.7.6.2). As the behavioural tests are designed to assess an imbalance in functional control between the brains two hemispheres I performed all correlations against the ratio of neurons remaining in the treated hemisphere compared to the contralateral untreated hemisphere despite the possibility that the contralateral hemisphere may not be entirely unaffected by the AAV-vector delivery or QA lesion. I did not however observe any indications of
the contralateral hemisphere being influenced in any of the immunocytochemical analysis; although transduction of cortical neurons may have caused a small enhancement in striatal BDNF due to the bilateral projections of cortical neurons (McGeorge and Faull 1989).

Both the development of spontaneous ipsilateral forelimb use bias and contralateral sensorimotor neglect showed a positive correlation with the severity of striatal lesioning in the control rats as expected. However with only partial protection of the striatum, the reduced severity of behavioural deficits exhibited by the AAV-BDNF treated rats did not directly reflect maintenance of the striatum but instead correlated with the relative maintenance of parvalbumin expressing pallidal neurons. A correlation with the maintenance of the globus pallidus was also observed in the control rats development of sensorimotor neglect but not spontaneous ipsilateral forelimb use; possibly indicating the greater executive function required in processing the sensory information and making a left / right selection in the “corridor” task. AAV-BDNF treated rats that had a loss of parvalbumin-expressing pallidal neurons were observed to develop ipsilateral forelimb use equivalent to that seen in control rats with similar striatal cell loss, suggesting that the overall AAV-BDNF amelioration of the behavioural impairments was potentially the result of a dynamic interaction between maintenance of the striatal anatomical structure / organisation and striatal output via the globus pallidus. The inducement of rotational behaviour following apomorphine administration also reflected the integrity of the globus pallidus with rats that had no loss of parvalbumin-positive neurons preferentially rotating away from the lesioned striatum, while ipsilateral rotations were generally induced post-QA lesioning when the ipsilateral globus pallidus exhibited neuron loss.

Although I was unable to determine whether AAV-BDNF delivery provided any direct neuroprotective support to the pallidal neurons in this investigation, the significant correlations between the maintenance of striatal neurons and attenuation of behavioural deficits was indicative of BDNF-derived trophic support mitigating the extent of disruption to functional behaviour. Given the evidential requirement for pallidal neurons to facilitate normal sensorimotor behaviour, it is probable that efferent striatal projections to the globus pallidus, predominantly arising from the striatal matrix (Gerfen 1985), are better maintained in the AAV-BDNF treated rats independent of pallidal neuron survival. Assessment of DARPP-32 staining in the substantia nigra demonstrated BDNF-enhanced maintenance of the striatonigral projections, arising from both matrix and striosome compartments (Gerfen 1985), however DARPP-32 staining of the globus pallidus was less defined and definitive assessment of the striatopallidal projection survival should be undertaken with tracer studies.
5.8.3 Conclusion

Despite the vital role that neurotrophic factors, and specifically BDNF, play in maintaining the plasticity and functionality of the striatum, and the disruption of BDNF production / transportation in the HD brain, there has only been limited success to date in alleviating cell loss in the striatum through the administration of BDNF. While a low level of neuroprotection has been previously found (Bemelmans et al. 1999; Kells et al. 2004), these studies together with the current investigations show the significant impact that BDNF can impart on the coordination of functional behaviour and the ultimate need to be able to finely control the extent and localisation of enhanced BDNF expression. To my knowledge, all of the reported BDNF studies to date have used acute administration of neurotoxic chemicals to induce selective cell death in the vulnerable striatal projection neurons in which the cells are probably exposed to more intense insult than the actual neurons are in HD patients in which there is a slow progressive loss. Given the ability to provide protection in these studies, it is therefore conceivable that the continuous expression of neurotrophic factors would actually provide a greater level of support potentially with lower levels of exogenous expression being required; which will additionally limit any adverse effects of the transgene expression. Therefore the delivery of neurotrophic factors to transgenic models in which there is a progression of symptom onset and neuropathological changes, as has been reported for GDNF, may provide a truer reflection of the actual potential of neurotrophic factor based therapy for HD.

Ultimately the results of these neurotrophic factor investigations highlight the need to apply caution in the enhancement of endogenously expressed trophic factors, with excessive uncontrolled production potentially deleterious, while tightly controlled expression could be of significant therapeutic benefit. This is of particular importance given that protective treatments are likely to be of greatest efficacy when applied early in the degenerative disease process – potentially even prior to the onset of physical symptoms in HD. Consideration will also need to be given to areas of HD neuropathology beyond the caudate-putamen and whether neurotrophic factor delivery to the striatum is sufficient to attenuate HD symptoms, or if other areas of primary or secondary neurodegeneration are also suited to targeting with neurotrophic factor gene delivery.
Chapter 6

Anti-Apoptotic Factor Delivery: 

AAV-Bcl-x<sub>L</sub> and AAV-XIAP

6.1 Overview

The proposal that apoptotic cell death processes are implicated in Huntington’s disease neurodegeneration (Section 1.7) raises the question of whether enhancing the expression of anti-apoptotic factors in the vulnerable striatal projection neurons can reduced their susceptibility to neurotoxic processes occurring in the Huntington’s disease brain. In this investigation I constructed AAV vectors encoding either Bcl-x<sub>L</sub> or XIAP, using them to transduce striatal neurons prior to an intrastriatal injection of the glutamate analogue QA. Treated rats were observed in behavioural tests undertaken to assess whether the anti-apoptotic factor expression could provide any amelioration of motor impairments following unilateral QA-induced striatal lesioning. While there was some lessening of behavioural deficits in the AAV-Bcl-x<sub>L</sub> and AAV-XIAP treated rats, I found no quantified reduction of QA-induced pathology in assessed neuronal populations of the basal ganglia. Overall these investigations showed the ability to deliver anti-apoptotic factors to the central nervous system without causing any immediately apparent welfare concerns, however suggest that the enhancement of an isolated pro-survival protein is not sufficient to counteract acute excitotoxic insult of the striatal neurons.

6.2 Procedures

Chimeric AAV<sub>1/2</sub> vectors were constructed containing cDNA encoding Bcl-x<sub>L</sub> or XIAP and were assessed with in vitro assays to ensure they directed the expression of biologically functional anti-apoptotic factors (Chapter 2; Kells et al. 2006), prior to verifying in vivo transduction and expression in the rodent striatum. Following the AAV vector testing I undertook two in vivo studies, conducted as described (Chapter 4) to assess the influence of Bcl-x<sub>L</sub> and XIAP expression on QA induced striatal excitotoxicity and associated functional behavioural deficits. In the initial neuroprotective investigation I delivered 4.0µL AAV-Bcl-x<sub>L</sub> (n = 10<sup>3</sup>) to a single stereotaxic site within the rodent striatum prior to QA injection.
striatum prior to QA injection at the same stereotaxic coordinates. To further enhance the number of striatal neurons transduced, and their distribution within the striatum, I conducted a second investigation delivering 8.0µL of AAV-Bcl-xL ($n = 11$) or AAV-XIAP ($n = 9$) split between two stereotaxic sites in the striatum flanking the QA injection coordinates. These two neuroprotective investigations were undertaken in parallel with the previously described neurotrophic factors studies (Chapter 5) utilising the same groups of control rats which received either AAV-Luciferase (First study: $n = 8$; Second study: $n = 5$) or sterile PBS (First study: $n = 10$; Second study: $n = 5$) prior to QA lesioning as described (Table 4-2, Table 4-3).

6.3 In Vivo AAV Vector Testing

![Figure 6-1](image)

**Figure 6-1 Single delivery site for AAV-Bcl-xL transduction and subsequent Bcl-xL expression**

(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged Bcl-xL expression three weeks after a single 4.0µL striatal injection of the AAV-Bcl-xL vector. Cells expressing HA-tagged Bcl-xL were largely contained within the injected striatum where a large population of the striatal neurons appeared to be transduced. Higher power images of AAV-Bcl-xL transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 200µm for (B); 100µm for (C,D).
To assess the spread of in vivo AAV-Bcl-xL and AAV-XIAP transduction and transgene expression prior to investigating the preventative therapeutic efficacy of over-expressing anti-apoptotic factors in the striatum, I injected naïve rats using the same stereotaxic surgical protocols to be undertaken on the main cohorts of rats in the neuroprotective investigations. The initial single site delivery protocol was determined from the earlier AAV-Luciferase delivery testing (Section 4.4.2) which I then modified for the second two-site delivery study designed to increase the number of transduced cells and better distribute vector transduction around the QA-injection site. Three weeks post-AAV delivery the enhanced Bcl-xL (Single site: Figure 6-1; Two-site: Figure 6-2) and XIAP (Two-site: Figure 6-3) expression was visualised by immunocytochemical staining against the HA-epitope attached to the C-terminal of the anti-apoptotic factors. Both AAV-Bcl-xL and AAV-XIAP transduced cells were observed in an extensive but often irregularly dispersed distribution.

Figure 6-2  AAV-Bcl-xL transduction and Bcl-xL expression following two-site striatal delivery
(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged Bcl-xL expression three weeks after two 4.0µL striatal injections of the AAV-Bcl-xL vector. Cells expressing HA-tagged Bcl-xL were largely contained within the injected striatum where a large population of the striatal neurons appeared to be transduced. Higher power images of AAV-Bcl-xL transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 100µm for higher magnification images (B-D).
throughout the rostral-caudal extent of the striatum. Transgenic Bcl-\(x_L\) protein appeared to be distributed throughout the cytosol of transduced neurons (Figure 6-2B) making identification of individual transduced neurons less evident compared to the nuclear localisation of XIAP expression (Figure 6-3B). A population of cells within the globus pallidus and substantia nigra pars compacta ipsilateral to the injected striatum were also HA-positive indicating transportation of the AAV-vectors and transduction of cells within the striatal target nuclei. The ipsilateral substantia nigra pars reticulata also displayed HA-immunoreactivity although this was mainly restricted to the striatonigral axonal fibres with very few identifiable HA-positive cell bodies. I also detected the presence of HA-tagged proteins throughout fibres in the cerebral cortex ipsilateral to the two-site intrastriatal injection of AAV-Bcl-\(x_L\), suggesting either low level transduction of these cortical neurons – a potential effect of the larger volume of vector delivered – or retrograde transport of Bcl-\(x_L\) produced in the striatum.

Figure 6-3  Spread of AAV-XIAP transduction and subsequent XIAP expression
(A) Representative coronal sections of a rat brain showing the main regions of HA-tagged XIAP expression three weeks after two 4.0\(\mu\)L striatal injections of the AAV-XIAP vector (A). Cells expressing HA-tagged XIAP were largely contained within the injected striatum where a large population of the striatal neurons appeared to be transduced. Higher power images of AAV-XIAP transduced neurons in (B) the striatum, (C) globus pallidus, and (D) substantia nigra. Scale bar = 2mm for whole sections (A); 100\(\mu\)m for higher magnification images (B-D).
6.4 Bcl-xL and XIAP Expression Level

To determine the quantity of Bcl-xL and XIAP expression within the striatum following AAV-mediated gene delivery I injected additional cohorts of rats with the AAV-Bcl-xL or AAV-XIAP vectors using the same surgical protocol employed for the neuroprotective investigation. The rats were euthanised three weeks post-surgery and their striatal tissue separately isolated for ELISA-based quantification of the Bcl-xL or XIAP content (Section 4.6). Expression was assessed three weeks post-AAV delivery to provide a measure of the increased anti-apoptotic factor expression equivalent to the expression levels in the main neuroprotective study groups at the time of QA injection.

Bcl-xL expression in the striatum following the initial single site AAV-Bcl-xL injection was enhanced ~20-fold to 1200 ± 300 ng / mg total protein compared with the 49 ± 2 ng / mg total protein in the control AAV-Luciferase injected striatum (Mann-Whitney P < 0.01; Figure 6-4A). The contralateral striatum displayed a small increase in Bcl-xL expression at 66 ± 4 ng / mg total protein indicating a small amount of expression crossing over into the non-injected striatal hemisphere. The control AAV-Luciferase vector injections did not induce a significant alteration in Bcl-xL expression with the injected striatum having 49 ± 2 ng Bcl-xL / mg total protein and the non-injected striatum 39 ± 7 ng / mg total protein. Significantly higher Bcl-xL expression was generated following the 8.0µL two-site AAV-Bcl-xL injections with a ~2.5-fold increase over the single site injected striatum to 3100 ± 700 ng / mg total protein (~60-fold increase over controls; Mann-Whitney P < 0.05). A significant amount of transgenic Bcl-xL (120 ± 10 ng / mg total protein) was again detected in the non-injected contralateral striatum (Mann-Whitney P < 0.01).

AAV-XIAP delivery resulted in a much lower quantity of transgenic protein expression than the AAV-Bcl-xL delivery with the injected striatum containing 150 ± 20 ng XIAP / mg total protein, although this represented a comparable ~70-fold enhancement over the endogenous XIAP expression detected in the AAV-Luciferase injected control striatum (Mann-Whitney P < 0.01; Figure 6-4B). A small amount of hemispherical crossover of transgenic protein expression was again detected in the contralateral non-treated striatum with 4.3 ± 0.3 ng XIAP / mg total protein detected compared with the 1.7 ± 0.7 ng / mg total protein in the control rats (Mann-Whitney P < 0.05).
6.5 Functional Behaviour Assessment

Throughout the neuroprotective studies I assessed the development of functional deficits by analysing the performance of each rat in behavioural tests designed to show hemispherical imbalances in brain function arising as a result of a unilateral lesion in the basal ganglia. The functional behavioural tests undertaken as previously described (Section 4.5) involved spontaneous forelimb use analysis, drug-induced rotational behaviour following either apomorphine or amphetamine dosing, and preferential left / right side food selection. All the rats were assessed with baseline measurements taken both before and after AAV delivery and for 5-7 weeks post-QA injection (Table 4-2, Table 4-3).

6.5.1 Spontaneous forelimb use

Assessment of forelimb usage during spontaneous exploratory activity was undertaken by observing the rats rearing behaviour when placed into a clear cylinder (Section 4.5.1). Baseline assessment of the naïve rats prior to any treatment generally showed non-biased usage of both left and right forelimbs. AAV-Bcl-xL delivery in the initial single site delivery investigation did not induce any alterations in forelimb use following vector delivery (Figure 6-5A). Although initially appearing to attenuate development of the ipsilateral forelimb use bias seen in control rats, the AAV-Bcl-xL treated rats progressively acquired a strong preferential use of their ipsilateral forelimb while the...
Figure 6-5  Spontaneous ipsilateral forelimb use asymmetry scores
Plots displaying changes in group ipsilateral asymmetry scores as quantified during spontaneous exploration of the forelimb use assessment cylinder in (A) the first AAV-Bcl-xL and (B) second AAV-Bcl-xL / AAV-XIAP investigations. Ipsilateral asymmetry scores represent the combined use of the ipsilateral forelimb, as a percentage of the total left and right forelimb usage, for rearing, initial contact of the cylinder wall during an exploratory rear and landing. * Un-paired t-test between treatment groups: P < 0.05. Two-way RM ANOVA relative to the control group: † Bcl-xL P = 0.053, XIAP P < 0.001.
control rats appeared to undergo spontaneous recovery three-weeks post QA such that AAV-Bcl-xL provided no overall attenuation of the contralateral forelimb motor control deficit. In the second two-site vector delivery investigation the control rats developed and maintained a strong ipsilateral forelimb bias that tended to be restricted by the prior delivery of AAV-Bcl-xL but was not quite statistically significant (Two-way RM ANOVA P = 0.053; Figure 6-5B).

Delivery of AAV-XIAP in the second investigation induced a small but significant shift towards greater usage of the treated rats’ contralateral forelimb (44 ± 4% ipsilateral asymmetry) prior to QA lesioning, compared with the unbiased 53 ± 3% ipsilateral forelimb use in control rats (Unpaired t-test P < 0.05; Figure 6-5B). Following QA injection the AAV-XIAP treated rats showed an immediate shift back to non-biased forelimb usage with the over-expression of XIAP resulting in the complete attenuation of the ipsilateral forelimb use bias exhibited by the control treated rats (Two-way RM ANOVA P < 0.001; Figure 6-5B).

Comparison of total activity level between the treatment groups showed that while all rats have a reduction in the number of exploratory rears from initial baseline testing, the rats with enhanced anti-apoptotic factor expression overall showed higher levels of exploratory activity. With AAV-Bcl-xL treated rats in the first study and AAV-XIAP treated rats in the second study displaying significantly greater rearing behaviour than the control rats (Two-way RM ANOVA P < 0.05; Figure 6-6).

**Figure 6-6 Spontaneous exploratory rearing activity**
Plots displaying the number of exploratory rears in the cylinder during the five minute assessment of spontaneous forelimb use in (A) the first AAV-Bcl-xL and (B) second AAV-Bcl-xL / AAV-XIAP investigations. No statistics were performed on the pre-QA baseline data. Two-way RM ANOVA relative to the control group: † P < 0.05; ‡ Bcl-xL P = 0.11, XIAP P < 0.05.
6.5.2 **Drug-induced rotational behaviour**

Apomorphine- and amphetamine-induced rotational motor activity was analysed to assess alterations in striatal dopamine signalling following unilateral QA injection and the subsequent loss of dopamine receptive striatal neurons (Section 4.5.3). Analysis of the rotation data showed inconsistencies in rotational activity and direction between individual rats within the treatment.

![Graphs showing rotational behavior](image)

**Figure 6-7 Apomorphine-induced rotational behaviour**

Plots displaying each rat’s rotational behaviour towards the treated side of their brain as a percentage of total rotations following subcutaneous administration of 1mg/kg of apomorphine in (A) the first AAV-Bcl-xL and (B) second AAV-Bcl-xL / AAV-XIAP investigations. Rats that had less than 0.5 rotations per minute were excluded from this analysis. Lines represent the median result for each group at each time point.
groups – possibly resultant of minor variations in the actual positioning of the AAV-vector or QA injections – complicating the assessment of dopamine related functional motor impairment. Therefore statistical analysis was restricted to the assessment of total rotation rates post QA, independent from the direction of induced rotation.

6.5.2.1 Apomorphine
As expected from the earlier QA lesion testing (Section 3.3.4.1), the administration of apomorphine to rats with a unilateral striatal lesion tended to induce rotational motor behaviour towards the lesioned hemisphere (Figure 6-7), in contrast to a lower level of unbiased motor activity induced prior to QA lesioning (Figure 6-8). The extent of apomorphine-induced rotations varied significantly between individual rats in the two anti-apoptotic factor investigations with the rats showing up to 20 rotations per minute irrespective of their treatment, and with no significant overall differences in rotation rates between the groups in either investigation (Figure 6-8A,B). For reliable assessment of rotational behaviour I excluded trial data for rats that displayed less than 0.5 rotations per minute following apomorphine administration.

Baseline and post-AAV trials showed a wide spread in the extent and direction of rotational behaviour which was skewed towards a contralateral dominance by the selective treatment of the individual rats more dominant hemisphere (Figure 6-7A,B). The AAV vector delivery and transgene expression did not appear to be having any direct influence on the rotational behaviour prior to QA. Following QA injection the control rats predominantly had a strongly ipsilateral rotational bias while

Figure 6-8 Total apomorphine-induced rotations
Plots displaying the mean number of apomorphine-induced rotations for each group in (A) the first AAV-Bcl-xL and (B) second AAV-Bcl-xL / AAV-XIAP investigations following subcutaneous administration of 1mg/kg apomorphine. No significance was found between treatment groups and controls post-QA.
a number of anti-apoptotic factor expressing rats showed less unidirectional behaviour with a slight majority of AAV-Bcl-x<sub>L</sub> and AAV-XIAP treated rats in the second study actually displaying a greater preference towards contralateral rotations (Figure 6-7B). Although not statistically analysed, the observed changes to rotational behaviour in the second investigation were indicative of Bcl-x<sub>L</sub> and XIAP derived alterations to the QA-induced neuropathological disruptions.

**6.5.2.2 Amphetamine**

Rotational behaviour induced by amphetamine administration was only assessed in the initial AAV-Bcl-x<sub>L</sub> study where greater rotational behaviour was induced in AAV-Bcl-x<sub>L</sub> treated rats following

![Figure 6-9 Amphetamine-induced rotational behaviour](image)

Plots displaying the rotational behaviour induced following an i.p. injection of 5mg/kg of amphetamine in the first AAV-Bcl-x<sub>L</sub> investigation. (A) Individual rats’ rotational behaviour towards the treated (ipsilateral) side of their brain as a percentage of total rotations and (B) the total rotation rate for each group. Rats that had less than 0.1 rotations per minute were excluded from this analysis. Lines in (A) represent the median result for each group at each time point. No significance was found in rotation rates between AAV-Bcl-x<sub>L</sub> treatment rats and controls post-QA.
QA-injection than in the AAV-Luciferase / PBS treated control rats (Figure 6-9B). However the extent of this behaviour varied extensively between individual rats, with many not displaying any persistent rotational behaviour following amphetamine dosing and therefore being excluded from the assessment of rotation direction. Analysis of the number of ipsilateral rotations as a percentage of total rotational behaviour found that post-QA the majority of rats in both the AAV-Bcl-xL and control treated groups predominantly or solely performed unidirectional rotations, with the majority rotating towards the lesioned hemisphere (Figure 6-9A). The slight contralateral rotation preference observed in the pre-QA baseline trials was due to the selection of the dominant brain hemisphere for treatment. No statistical analysis was performed on the directional rotation data due to the lack of consistency between similarly treated rats in their extent or direction of rotation.

### 6.5.3 Sensorimotor “corridor” task

Inclusion of the “corridor” task in the second study provided an assessment of the impact that over-expressing anti-apoptotic factors had on the development of sensorimotor neglect following QA-lesioning. During baseline and post-AAV testing all treatment groups displayed a fairly even distribution of sugar pellet retrievals from both the left and right sides of the corridor with only the AAV-XIAP treated rats displaying an increase in the proportion of retrievals from the ipsilateral side.

![Figure 6-10 Preferential food selection in the sensorimotor “corridor” task](image)

Plot displaying group changes in the number of sugar pellet retrievals from the same side as the treated striatum as a percentage of the 20 retrievals per trial. No significance was found between treatment groups and controls post-QA.
prior to QA injection (paired t-test P < 0.05; Figure 6-10). The slight preference for contralateral retrievals in all groups during baseline testing is resultant of the intentional selection of each rat’s dominant brain hemisphere for treatment. Post-QA the development of contralateral sensorimotor neglect, resulting in preferential ipsilateral sugar pellet retrieval, was strongest in the control rats (77 ± 2%) but also observed in both the AAV-XIAP (73 ± 1%) and AAV-Bcl-xL (65 ± 1%) treated groups with no significant alterations between the different treatments (Two-way RM ANOVA post-QA P > 0.05).

6.6 Immunocytochemical Analysis

Immunocytochemical staining was undertaken to determine if AAV-vector mediated expression of the anti-apoptotic factors Bcl-xL or XIAP supplied any neuroprotective support against QA-induced excitotoxic stress in the striatum of adult rats. Following the completion of behavioural testing I processed the rat brains for immunocytochemical analysis, sectioning coronally through striatum, globus pallidus and substantia nigra for immunostaining against various neuronal makers.

6.6.1 AAV-mediated transgene expression and QA lesioning of the striatum

Initial assessment of the surviving AAV-mediated transgene expression was undertaken by visualising the HA-tagged anti-apoptotic factors or control Luciferase expression within the striatum. The extent of the QA-induced striatal lesioning was visually assessed by both DARPP-32 and NeuN immunostaining with stereological quantification to determine the maintenance of the vulnerable DARPP-32 positive striatal projection neurons.

6.6.1.1 Study 1 – AAV-Bcl-xL Delivery
In the first study AAV-Bcl-xL was delivered to the striatum at a single stereotaxic injection site prior to QA injection at the same coordinates. Using adjacent striatal sections individually immunostained for HA, DARPP-32 and NeuN to show the distribution of enhanced Bcl-xL expression and neuronal maintenance (Figure 6-11) I observed a generally positive correlation between immunoreactivity, although HA staining was less extensive than the neuronal markers. Intense HA staining of cellular processes in the striatum, suggesting intracellular accumulation of transgenic Bcl-xL, made identification of transduced neurons less distinct (Figure 6-11C). Despite a scattering of DARPP-32 and NeuN-positive cells within the area of striatal lesioning – particularly in the more distal “transition zone” – the extent of neuronal sparing varied considerably between individual AAV-Bcl-
Bcl-xL treated rats (Figure 6-11D-I) and was also observed in AAV-Luciferase / PBS treated control rats (Figure 5-13D-I). Overall there was no visually apparent evidence that prior AAV-Bcl-xL delivery reduced striatal cell loss.

Figure 6-11 Single injection site AAV-Bcl-xL transduction and striatal cell maintenance
Representative images from adjacent striatal sections showing AAV-mediated Bcl-xL expression and neuronal cell loss seven weeks post-QA. (A-C) HA-immunostaining displayed the distribution of Bcl-xL expression within the striatum resultant of AAV-Bcl-xL transduction prior to QA injection. Maintenance of striatal neurons was detected by (D-F) DARPP-32 and (G-I) NeuN immunostaining. Scale bar = 2mm for (A,D,G); 200µm for (B,E,H); 50µm for (C,F,I).
6.6.1.2 Study 2 – AAV-Bcl-xL and AAV-XIAP Delivery

The second investigation of anti-apoptotic factors in which AAV vectors encoding either Bcl-xL or XIAP were injected into two striatal sites flanking the QA injection coordinates showed some maintenance of transgene expression within the striatum. As with the previous AAV-Bcl-xL study, the striatal transgene expression was still only present in areas that also contained DARPP-32 and NeuN expression for both AAV-Bcl-xL (Figure 6-12) and AAV-XIAP (Figure 6-13) treated rats. With reasonably defined areas of striatal lesioning visualised with DARPP-32 or NeuN staining, the brains expressing enhanced levels of anti-apoptotic factors were not visually discernable from AAV-Luciferase / PBS treated control brains (Figure 5-15).

Figure 6-12 Dual injection site AAV-Bcl-xL transduction and striatal cell maintenance
Representative images from adjacent striatal sections showing AAV-mediated Bcl-xL expression and neuronal cell loss eight weeks post-QA. (A-C) HA-immunostaining displayed the distribution of Bcl-xL expression within the striatum resultant of AAV-Bcl-xL transduction prior to QA injection. Maintenance of striatal neurons was detected by (D-F) DARPP-32 and (G-I) NeuN immunostaining. Scale bar = 2mm for (A,D,G); 200µm for (B,E,H); 50µm for (C,F,I).
Figure 6-13 Dual injection site AAV-XIAP transduction and striatal cell maintenance
Representative images from adjacent striatal sections showing AAV-mediated XIAP expression and neuronal cell loss eight weeks post-QA. (A-C) HA-immunostaining displayed the distribution of Bcl-xL expression within the striatum resultant of AAV-XIAP transduction prior to QA injection. Maintenance of striatal neurons was detected by (D-F) DARPP-32 and (G-I) NeuN immunostaining. Scale bar = 2mm for (A,D,G); 200µm for (B,E,H); 50µm for (C,F,I).

6.6.1.3 Stereological Analysis – DARPP-32
Quantification of the DARPP-32 immunopositive neurons using optical fractionator stereology probes in the first AAV-Bcl-xL study estimated the number of DARPP-32 positive neurons remaining within the sampled region of the treated caudate-putamen at 1,300,000 ± 100,000 in the AAV-Bcl-xL treated rats compared with 1,150,000 ± 120,000 in the control AAV-Luciferase / PBS treated rats (Figure 6-14A; Coefficient of Error ≤ 0.04). The adjacent intact contralateral striatum contained an overall average across all rats of 2,200,000 ± 60,000 (Coefficient of Error ≤ 0.02). In the second investigation I limited the stereological analysis of DARPP-32 positive neurons to a smaller portion of the striatum centred around the QA lesion containing an estimated 820,000 ± 90,000 DARPP-32 positive neurons in AAV-Bcl-xL treated rats, 890,000 ± 100,000 in AAV-XIAP
treated rats and $760,000 \pm 60,000$ in control AAV-Luciferase / PBS treated rats (Figure 6-14C; Coefficient of Error $\leq 0.06$). The corresponding contralateral striatal region was estimated to contain an overall average across all treated rats of $1,530,000 \pm 30,000$ DARPP-32 positive neurons (Coefficient of Error $\leq 0.03$).

Figure 6-14  Maintenance of DARPP-32 striatal projection neurons
Graphs displaying the actual and relative numbers of DARPP-32 positive neurons in the striatum as estimated by optical fractionator stereological analysis of the treated (ipsilateral) and intact (contralateral) striatal hemispheres in (A,B) the first AAV-Bcl-xL and (C,D) second AAV-Bcl-xL / AAV-XIAP neuroprotective studies. Population estimates were determined from 12 sections spanning 3.56mm of the striatum for the first study and 7 sections spanning 1.96mm of the anterior striatum for the second study. Relative cell counts represent the number of DARPP-32 positive neurons within the lesioned striatum as a proportion of DARPP-32 expressing neurons in the contralateral intact striatum estimated independently for each rat. Statistical analysis revealed no significance differences between treatment groups at the 5% level ($P > 0.05$).
Analysis of the stereological estimates to assess the relative maintenance of DARPP-32 positive striatal neurons showed the control AAV-Luciferase / PBS treated rats to have 52 ± 4% maintenance in the initial study and 47 ± 4% in the second investigation. Over-expression of an anti-apoptotic factor – Bcl-xL or XIAP – in the striatum prior to QA injection did not significantly enhance the maintenance of DARPP-32 neurons. AAV-Bcl-xL treated rats had 57 ± 5% maintenance of DARPP-32 neurons in the first study (Figure 6-14B) and 52 ± 4% in the second (Figure 6-14D). Delivery of AAV-XIAP was estimated to have enhanced the maintenance of DARPP-32 positive neurons by ~23% to 58 ± 7% relative survival, although this failed to be statistically significance (P = 0.20; Figure 6-14D).

6.6.1.4 Striatal Atrophy

![Figure 6-15 Atrophy of the lesioned striatum](image)

Graphs displaying the size of the lesioned striatum as a proportion of the intact contralateral striatal area, and plots correlating striatal atrophy with DARPP-32 positive cell loss in the striatum. (A,B) First AAV-Bcl-xL study and (C,D) second AAV-Bcl-xL / AAV-XIAP neuroprotective investigation. Cross-sectional striatal area was measured on 12 coronal sections for the initial study and 7 sections for the second study. Combined correlation analysis between the striatal atrophy and the loss of DARPP-32 positive neurons displayed Deming linear regression correlations for both the first (B; slope = 0.51 ± 0.06, P < 0.001) and second (D; slope = 0.53 ± 0.08, P < 0.001) anti-apoptotic factor investigations. R values represent Pearson correlation coefficient independent of treatment.
As an alternative measure of the overall striatal maintenance I analysed the volume of the striatum which was observed to have significantly atrophied in most cases, with an associated enlargement of the adjacent lateral ventricle. By using contours drawn to perform stereological cell estimates, I calculated the striatal volume relative to the contralateral hemisphere for each rat. The AAV-mediated delivery of anti-apoptotic factors did not alter the extent of striatal atrophy following QA lesioning in either study. In the initial investigation the AAV-Bcl-\(x_L\) injected striatum contracted to 79 ± 2% of the contralateral volume compared with 73 ± 2% in control AAV-Luciferase / PBS treated rats (Figure 6-15A). Similarly, in the second study the striatum was reduced to 77 ± 2% of the contralateral striatal volume in AAV-Bcl-\(x_L\) treated rats, 76 ± 4% in AAV-XIAP treated rats and 75 ± 3% in the control group of rats (Figure 6-15C).

Analysis of the correlation between the relative striatal area and the maintenance of DARPP-32 positive striatal neurons showed a direct relationship with the loss of striatal neurons following QA injection ultimately resulting in a reduction in the overall striatal volume (Figure 6-15B, D). Neither AAV-Bcl-\(x_L\) nor AAV-XIAP delivery prior to the QA injection significantly altered this correlation.

### 6.6.2 Krox-24 expression

Stereological analysis of krox-24 expressing neurons was performed in the second investigation to further confirm the loss of striatal neurons using an alternative marker for the striatal neurons. The distribution of krox-24 positive neurons matched with the DARPP-32 immunostaining although being a transcription factor the krox-24 immunostaining was restricted to the nucleus resulting in more distinct staining (Figure 6-16). Quantification of the krox-24 positive neurons was performed using optical fractionator probes across eight equispaced striatal sections with the number of neurons estimated at 1,060,000 ± 100,000 in AAV-Bcl-\(x_L\) treated rats and 1,280,000 ± 150,000 following AAV-XIAP treatment compared with the 1,060,000 ± 110,000 krox-24 positive cells in AAV-Luciferase / PBS control rats (Figure 6-17A; Coefficient of Error ≤ 0.05). The corresponding contralateral striatal region contained an estimated average of 2,160,000 ± 40,000 krox-24 positive cells (Coefficient of Error ≤ 0.03). Overall the quantified maintenance of krox-24 positive neurons reflected the DARPP-32 analysis with 50 ± 5% relative maintenance of krox-24 neurons in the control rats that was not significantly enhanced by the prior delivery of either AAV-Bcl-\(x_L\) (50 ± 5%) or AAV-XIAP (58 ± 7%; Figure 6-17B).

Correlation of the krox-24 expression with DARPP-32 cell maintenance demonstrated a parallel link between the maintenance of krox-24 expression and DARPP-32 expression following QA lesioning.
that was independent of the prior AAV-mediated anti-apoptotic factor expression or control treatment (Figure 6-17C).

**Figure 6-16  Krox-24 expression in the striatum**
Representative images displaying the expression of krox-24 in striatal neurons following QA lesioning in (A,B) AAV-Bcl-xL, (D,E) AAV-XIAP, and (G,H) AAV-Luciferase treated rats. (C,F,I) Adjacent sections (copied from Figure 6-12E, Figure 6-13E and Figure 5-15D) immunostained for DARPP-32 show the distribution correlation between krox-24 and DARPP-32 positive cells in the striatum. Scale bar = 2mm for whole sections and 200µm for higher magnification images.
Figure 6-17  Maintenance of krox-24 expressing striatal neurons
Graphs displaying the (A) actual and (B) relative number of striatal neurons expressing krox-24 as estimated by optical fractionator stereological cell counting in the treated (ipsilateral) and intact (contralateral) striatal hemisphere in the second anti-apoptotic factor investigation. Relative cell counts represent the number of krox-24 expressing cells within the lesioned striatum as a proportion of krox-24 positive cells present in the contralateral striatum. (C) Plot showing the correlation between the relative maintenance of krox-24 positive neurons and DARPP-32 expressing neurons in the striatum. Trend line shows the overall Deming linear regression correlation across all treatment groups: slope = 1.05 ± 0.07, P < 0.001. R value represents overall Pearson correlation coefficient independent of treatment.

6.6.3  Analysis of transduced striatal cells
To further assess the state of AAV-vector transduced cells in the striatum at the conclusion of the investigations, I performed double-label immunofluorescent staining of the transgene expression with HA and either DARPP-32 or krox-24. Previous assessment of AAV-Luciferase transduced cells in the striatum showed co-expression of Luciferase with NeuN, but not with DARPP-32 or krox-24 (Figure 5-21). In contrast, for both AAV-Bcl-xL and AAV-XIAP treated rats I observed HA-positive cells co-labelling with DARPP-32 and krox-24 in the striatum (AAV-Bcl-xL: Figure
While the majority of HA-positive neurons in both AAV-Bcl-xL and AAV-XIAP treated rats co-expressed DARPP-32, krox-24 expression appeared highly variable with not all of the transduced HA-positive cells co-labelling. However this irregularity in krox-24 labelling intensity was also seen in non-transduced striatal neurons, so may not be related to the enhanced expression of the anti-apoptotic factors.

Figure 6-18  Immunofluorescent double-labelling of AAV-Bcl-xL transduced striatal neurons
Representative single-slice confocal images from an AAV-Bcl-xL treated rat from the second investigation showing AAV-Bcl-xL transduced cells (red) within the QA-injected striatum and immunofluorescent staining for (A) DARPP-32 expression and (B) krox-24 expression (both displayed in green). Co-labelling of the HA-positive cells with DARPP-32 or krox-24 was found in the striatum, although many of the AAV-Bcl-xL transduced cells only displayed weak krox-24 labelling (arrowheads). Scale bar: 30µm
Figure 6-19  Immunofluorescent double-labelling of AAV-XIAP transduced striatal neurons
Representative single-slice confocal images from an AAV-XIAP treated rat from the second investigation showing AAV-XIAP transduced cells (red) within the QA-injected striatum and immunofluorescent staining for (A) DARPP-32 expression and (B) krox-24 expression (both displayed in green). Co-labelling with DARPP-32 or krox-24 was evident for most HA-positive cells. However, a few HA-positive cells completely lacked DARPP-32 labelling (arrows) and krox-24 expression appeared reduced in a large proportion of AAV-XIAP transduced cells (arrowheads). Scale bar: 30µm

6.6.4  Dopaminergic innervations of the striatum

To visually assess the maintenance of dopaminergic input into the striatum I performed immunostaining for TH expression. The extent of TH-immunoreactivity appeared to be altered in the striatum of all treatment groups with a clear loss of TH proximal to the site of QA injection (Figure 6-20). Additionally, the structural organisation of TH-positive fibres appeared to be disrupted in a larger portion of the striatum surrounding the lesion centre with the normally diffuse TH labelling of the striatal matrix becoming condensed into tighter bundles surrounding what would appear to be an enlargement of striosome patches. Neither AAV-Bcl-xL nor AAV-XIAP delivery to the striatum prior to QA lesioning provided any attenuation of the disruption to the structural organisation of dopaminergic innervations.
Figure 6-20  Tyrosine hydroxylase expression in the striatum
Representative images displaying TH-immunoreactivity in the striatum following QA injection in (A) AAV-Luciferase, (B) AAV-Bcl-xL, and (C) AAV-XIAP treated rats. The distribution of TH-immunopositive fibres was affected in the striatum of all treatment groups with a reduction in TH-expression at the lesion core (bottom images), and a condensing of the TH-positive matrix component of the striatum in the surrounding QA-lesioned striatum (middle images). Dashed lines provide an approximate outline of the lesion core largely devoid of TH (bottom images), and the limits of the striatal alterations induced by QA (middle images). Scale bar = 1mm for top images and 200µm for higher magnification images.

6.6.5  Cortical projection fibres in the striatum
Corticostriatal projections were assessed by SMI32 expression in the striatum. Following QA lesioning there was an apparent increase in the staining of SMI32-positive fibres in the striatum relative to the intact contralateral striatum (Figure 6-21). SMI32 immunostaining was most intensely enhanced in the striatum proximal to the site of QA injection. Additionally the normally dispersed patches of SMI32 staining became a dominant feature filling the striatum and often appearing to be arranged into disjointed bands in the less severely damaged area surrounding the centre of the QA
lesion (Figure 6-21D). This alteration in cortical projections following QA lesioning did not appear to be affected by the prior enhancement of Bcl-xL (Figure 6-21F-H) or XIAP (Figure 6-21I-K) expression in the striatal neurons.

![Figure 6-21 SMI32 expression in the striatum](image)

**Figure 6-21  SMI32 expression in the striatum**
Representative images displaying SMI32-immunoreactivity in the contralateral intact striatum (left hemispheres and (B,C)) and following QA injection in (A,D,E) AAV-Luciferase, (F-H) AAV-Bcl-xL and (I-K) AAV-XIAP treated rats. The arrangement of SMI32-immunopositive fibres was affected in the striatum of all treatment groups with an increase in the density of SMI32 positive patches, particularly around the QA-lesion core. Scale bar = 1mm for whole sections; 200µm for middle column of images; 50µm for high magnification images on the right.
6.6.6  **Striatal projection nuclei**

With the AAV-vector transduction extending to cells in the target nuclei of the striatal axonal projections (Figure 6-2, Figure 6-3), I used immunocytochemical staining to assess whether the limited enhancement of Bcl-x\(_L\) or XIAP expression in the nigral or pallidal neurons had any bearing on their survival following QA lesioning of the striatum.

6.6.6.1  **Substantia Nigra**

Assessment of the substantia nigra was performed by immunocytochemical staining of the dopaminergic neurons in the pars compacta with TH, a population of GABA-receptive neurons in the pars reticulata using parvalbumin, and the maintenance of GABAergic innervations by striatonigral projection neurons by measuring the density of DARPP-32 immunostaining. I did not observe any apparent changes to the number of TH-positive neurons in either the controls (Figure 5-27), or anti-apoptotic factor treated rats (AAV-Bcl-x\(_L\): Figure 6-23A; AAV-XIAP: Figure 6-24A) with similar TH expression in each hemisphere. Similarly with the GABAergic neurons in the pars reticulata there was no apparent reduction in parvalbumin expressing neurons following QA striatal lesioning (Figure 6-23B, Figure 6-24B). While the nigral neurons appeared largely unaffected, the loss of GABAergic striatal projection neurons did reduce the intensity of DARPP-32 fibres innervating the substantia nigra (Figure 6-23C, Figure 6-24C). The reduced GABAergic innervations were quantitatively assessed by measuring the density of immunostaining in the ipsilateral substantia nigra.

![Figure 6-22  DARPP-32 innervations of the substantia nigra](image)

(A) Graph showing the density of DARPP-32 immunoreactivity within the substantia nigra, ipsilateral to the treated striatum, as a proportion of DARPP-32 immunostaining in the contralateral substantia nigra. DARPP-32 density was measured on three coronal sections through the substantia nigra. (B) Plot displaying the correlation between DARPP-32 positive fibre innervations of the substantia nigra and maintenance of the DARPP-32 positive neurons. Deming linear regression correlation across all treatment groups: slope = 0.67 ± 0.15, \(P < 0.001\). R value represents overall Pearson correlation coefficient independent of treatment.
relative to the contralateral hemisphere in the same coronal plane. The control rats displayed a 29 ± 3% reduction in the density of DARPP-32 fibre innervations that was not significantly altered by either AAV-Bcl-xL delivery (20 ± 3% reduction, P = 0.08) or AAV-XIAP treatment (20 ± 5% reduction, P = 0.15; Figure 6-22A). When compared with the maintenance of DARPP-32-positive striatal neurons, the reduction in DARPP-32 fibre immunostaining within the substantia nigra showed a strong, direct correlation with the loss of the striatal neurons (Pearson r = 0.65; Figure 6-22B).

**Figure 6-23 Visual assessment of the substantia nigra in AAV-Bcl-xL treated rats**
Representative images of the substantia nigra from an AAV-Bcl-xL treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) TH-positive neurons in the pars compacta, (B) parvalbumin-positive neurons in the pars reticulata, and (C) DARPP-32 positive neurons and efferent fibres. Scale bar = 2mm for whole sections and 200µm for higher magnification images.
Figure 6-24  Visual assessment of the substantia nigra in AAV-XIAP treated rats
Representative images of the substantia nigra from an AAV-XIAP treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) TH-positive neurons in the pars compacta, (B) parvalbumin-positive neurons in the pars reticulata, and (C) DARPP-32 positive neurons and efferent fibres. Scale bar = 2mm for whole sections and 200µm for higher magnification images.
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6.6.6.2  Globus Pallidus
Assessment of the globus pallidus was undertaken by immunostaining for parvalbumin and NeuN which displayed a loss of pallidal neurons in the ipsilateral hemisphere for AAV-Luciferase (refer previous chapter Figure 5-30A,B), AAV-Bcl-xL (Figure 6-26A,B) and AAV-XIAP (Figure 6-27A,B). Immunostaining for DARPP-32 generally showed a reduced density of DARPP-32 positive fibres within the globus pallidus consistent with the loss of striatopallidal projections post-QA for all treatment groups despite the maintenance of DARPP-32-positive cells in the striatum directly adjacent to the globus pallidus (AAV-Luciferase, Figure 5-30C; AAV-Bcl-xL, Figure 6-26C; AAV-XIAP, Figure 6-27C).

Neuronal maintenance in the globus pallidus was determined by quantifying the parvalbumin-positive neurons using stereological estimates of the ipsilateral and contralateral pallidal nuclei assessed over four equispaced coronal sections. As previously mentioned (Section 5.7.6.2), I found a significant split between the PBS vehicle and AAV-Luciferase control groups with no apparent alteration to the number of parvalbumin-positive pallidal neurons in the majority of PBS treated rats, in contrast to the extensive loss of parvalbumin expressing pallidal neurons in AAV-Luciferase treated rats. Maintenance of pallidal neurons containing parvalbumin in the AAV-Bcl-xL and AAV-XIAP treated rats varied extensively with the AAV-Bcl-xL group having 72 ± 10 % maintenance and

Figure 6-25  Survival of parvalbumin-positive neurons in the globus pallidus
(A) Graph displaying the proportions of parvalbumin-immunopositive neurons surviving in the globus pallidus as estimated by optical fractionator stereology on four coronal sections through the globus pallidus relative to the contralateral hemisphere. (B) Plot comparing the survival of parvalbumin-positive neurons relative to the maintenance of DARPP-32 positive striatal neurons. R value represents overall Pearson correlation coefficient independent of treatment (P < 0.05). Solid line represents significant non-zero linear correlation (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. No statistically significant differences were found between treatment groups.
AAV-XIAP rats showing only 55 ± 11% maintenance (Figure 6-25A). Overall there was a correlation between the loss of parvalbumin-positive pallidal neurons and the striatal projection neurons (Pearson r = 0.43, P < 0.05) although this correlation was only clearly seen with the AAV-XIAP treated group of rats (Figure 6-25B).

![Visual assessment of the globus pallidus following AAV-Bcl-XL treatment](image)

**Figure 6-26  Visual assessment of the globus pallidus following AAV-Bcl-XL treatment**  
Representative images of the globus pallidus of an AAV-Bcl-XL treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) parvalbumin-positive pallidal neurons, (B) NeuN-positive neurons, and (C) DARPP-32 immunostaining. Scale bar = 250µm.
Figure 6-27  Visual assessment of the globus pallidus following AAV-XIAP treatment
Representative images of the globus pallidus of an AAV-XIAP treated brain displaying the comparison between the treated (ipsilateral) and un-treated (contralateral) hemispheres. Immunocytochemical staining of (A) parvalbumin-positive pallidal neurons, (B) NeuN-positive neurons, and (C) DARPP-32 immunostaining. Scale bar = 250µm.
6.6.7 Pathological correlations with functional behaviour impairments

Without any clear indications from the pathological analysis of significant AAV-Bcl-xL or AAV-XIAP supplied neuroprotection I analysed data collected from individual rats to directly assess correlations between the quantified basal ganglia pathology and the extent of functional motor impairment for each of the behaviour tests in the second investigation. Due to the significant maintenance of parvalbumin-expressing pallidal neurons in PBS controls compared with the large loss in AAV-Luciferase treated rats I have separately identified whether rats received PBS or AAV-Luciferase in the correlations against pallidal neurons but not for striatal correlations where there was no significant difference between the groups. The PBS and AAV-Luciferase treated rats were however combined as a single control group to assess for any linear relationships as they did not perform differently as two distinct groups in any of the functional behavioural assessments.

6.6.7.1 Spontaneous Forelimb Use

The proportion of exploratory behaviour undertaken by the rats with their ipsilateral forelimb showed a direct correlation with the loss of DARPP-32 positive striatal neurons, with greater cell loss following the unilateral lesioning causing increased use of the rats ipsilateral forelimb (Pearson

![Figure 6-28 Preferential forelimb usage correlation with neuron loss in the striatum and globus pallidus](image)

Plots correlating the extent of ipsilateral forelimb use bias with the relative proportion of (A) DARPP-32 and (B) parvalbumin-positive neurons remaining within the striatum and globus pallidus respectively. Data points represent individual rats’ average score over the seven post-QA trials. R values represent overall Pearson correlation coefficient for combined data from all rats. Solid lines represent significant non-zero linear correlations (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. Comparison with controls: (A) Bcl-xL slope P = 0.26, elevation P < 0.01; XIAP slope P = 0.08, elevation P < 0.001; (B) Bcl-xL slope P < 0.05; XIAP slope P = 0.72, elevation P < 0.001.
r = -0.43, P < 0.05). However the analysis of individual linear trends for the AAV-Bcl-xL, AAV-XIAP and control treated groups found that only the AAV-Luciferase / PBS treated control rats actually displayed significant correlation between ipsilateral forelimb use and quantified striatal cell loss (P < 0.05, Figure 6-28A). Preferential usage of the rats ipsilateral forelimb did not show any overall correlation with the loss of pallidal neurons (Pearson r = -0.05, P = 0.79), with only AAV-Bcl-xL treatment showing a weak correlation (Figure 6-28B).

6.6.7.2 Sensorimotor Neglect

Development of sensorimotor neglect on the contralateral side following QA-lesioning resulted in a greater proportion of ipsilateral sugar pellet retrievals in the “corridor” task, and was significantly correlated with both DARPP-32-positive striatal cell loss (Pearson r = -0.39, P < 0.05) and the loss of parvalbumin-positive pallidal neurons (Pearson r = -0.68, P < 0.001; Figure 6-29). Individual assessment of linear trends for each treatment group showed a strong correlation for the AAV-XIAP and AAV-Luciferase / PBS control treated rats between the loss of DARPP-32 neurons and the extent of sensorimotor neglect, but not for the AAV-Bcl-xL treated rats which displayed a wide spread of preferential sugar pellet retrievals that were seemingly unrelated to the level of striatal lesioning. Separate linear comparisons with the pathological state of the globus pallidus revealed

Figure 6-29 Sensorimotor neglect correlation with striatal and pallidal neuronal cell loss
Plots correlating the percentage of ipsilateral sugar pellet retrievals made in the “corridor” task with the relative proportion of (A) DARPP-32 and (B) parvalbumin-positive neurons remaining within the striatum and globus pallidus respectively. Data points represent individual rats’ average retrieval preference over the four post-QA trials. R values represent overall Pearson correlation coefficient for combined data from all rats. Solid lines represent significant non-zero linear correlations (P < 0.05) and the dotted lines non-significant linear trends for individual treatment groups. Comparison with controls: (A) Bcl-xL slope P = 0.10, elevation P < 0.01; XIAP not-significant; (B) Bcl-xL slope P < 0.05; XIAP slope P = 0.001.
significant non-zero linear trends for each treatment group, although the correlations were weakened for the control and AAV-Bcl-xL groups by a few outliers that displayed strong ipsilateral bias despite no disruption of pallidal neurons. Significantly though, all rats that had extensive loss of pallidal neurons also displayed strong contralateral sensorimotor neglect.

### 6.6.7.3 Apomorphine-Induced Rotations

Comparisons between the proportion of apomorphine-induced ipsilateral rotations and the extent of DARPP-32 neuronal cell loss showed no overall correlation (Pearson $r = -0.22$, $P = 0.24$; Figure 6-30A). Individual treatment group analysis however demonstrated that the AAV-XIAP treated group did show a trend towards a greater proportion of contralateral rotations when the striatal lesion is smaller, but ipsilateral rotations when there is high loss of DARPP-32 expressing striatal cells. In contrast the proportion of rats preferentially rotating in a contralateral direction showed strong correlation with the maintenance of the globus pallidus independent of treatment (Pearson $r = -0.75$, $P < 0.001$; Figure 6-30B).

![Figure 6-30 Correlation of apomorphine-induced rotational behaviour with striatal and pallidal neuronal cell loss](image)

Plots displaying the percentage of rotations in an ipsilateral direction in relation to the maintenance of (A) DARPP-32 and (B) parvalbumin-positive neurons within the striatum and globus pallidus respectively. Each data point represents the result of a single post-QA trial with three trials conducted per rat. Shaded boxes show the predominant contralateral rotational behaviour when the relative number of parvalbumin pallidal neurons is greater in the treated hemisphere and preferential ipsilateral rotations when the globus pallidus is compromised. R values represent overall Pearson correlation coefficient for combined data from all rats. Solid lines represent significant non-zero linear correlations ($P < 0.05$) and the dotted lines non-significant linear trends for individual treatment groups. Neither AAV-Bcl-xL nor AAV-XIAP treatment significantly altered the linear correlations observed in the control rats.
6.7 Discussion

Through the delivery of AAV vectors encoding anti-apoptotic factors to the rodent striatum I investigated the potential for increasing the expression of Bcl-xL or XIAP within the striatal neurons with a view to enhancing their resistance against excitotoxic cell death processes that contribute to HD neurodegenerative disease processes. While apoptotic processes are likely to be major contributing factors in HD neurodegeneration, and other neurodegenerative disorders, until recently there has been little published literature surrounding the use of anti-apoptotic factors as therapeutic agents (Section 1.7). A major limiting factor for direct therapeutic administration of endogenous anti-apoptotic factors is their intracellular localisation and site-of-action requiring efficient and extensive targeting of the vulnerable neurons. The AAV<sub>1/2</sub> vectors I investigated provide an avenue to direct the continuous expression of a therapeutic protein within transduced neurons. By injecting either AAV-Bcl-xL or AAV-XIAP into the rodent striatum – inducing over-expression of Bcl-xL and XIAP respectively – and using behavioural tests to measure motor activity before and after an intrastriatal injection of QA I assessed whether these anti-apoptotic factors could provide any symptomatic or neuropathological relief from the striatal excitotoxic insult.

6.7.1 AAV-mediated Bcl-x<sub>L</sub> or XIAP expression prior to QA

The chimeric AAV<sub>1/2</sub> vectors constructed to enhance Bcl-x<sub>L</sub> or XIAP expression prior to QA administration appeared to readily transduce the striatal neurons with significant enhancement of anti-apoptotic factor expression following a single AAV-Bcl-x<sub>L</sub> injection in the first study, and the dual injections of either AAV-Bcl-x<sub>L</sub> or AAV-XIAP in the second investigation (Section 6.3). While the AAV vectors appeared capable of travelling through the striatal parenchyma transducing neurons a reasonable distance from the injection coordinates, the actual distribution of transduced striatal cells was not consistent. This irregular distribution of cells with enhanced anti-apoptotic factor expression – some parts of the striatum appearing to have the majority of neurons transduced but adjacent areas showing no visible HA-positive cells – did not appear to follow any structural pattern in the striatum, although many rats showed numerous transduced cells adjacent to white matter tracts suggesting the vectors can more readily diffuse or are transported along these tracts. Assuming the transgenic protein expression remained intracellular, any enhanced resistance against the primary excitotoxic insult would have been restricted to the transduced cells, making extensive transduction of the targeted striatal neurons of vital importance. However the presence of HA-tagged transgenic protein throughout the cerebral cortex following AAV-Bcl-x<sub>L</sub> delivery suggested that the Bcl-x<sub>L</sub>
construct lacking the C-terminal transmembrane domain may possibly have been transported away from the transduced neurons. While transportation to the cortex may possibly be advantageous to cortical neurons subjected to apoptotic cell death, the Bcl-xL protein did not appear to be transported to other striatal neurons. Bcl-xL accumulated to a much greater extent within the striatum than XIAP despite the same regulatory elements driving expression; however both Bcl-xL and XIAP were enhanced 60-70 times above detected endogenous expression levels suggesting the different concentrations maybe related to normal processing of these anti-apoptotic factors. Transduction of nigral and pallidal neurons indicated the ability for these AAV vectors to be anterogradely and retrogradely transported to transduce neurons in the target nuclei in agreement with previous reports (Kaspar et al. 2002).

6.7.1.1 Neuropathological Changes
In the first anti-apoptotic factor investigation I injected the AAV-Bcl-xL vector into the striatum at a single stereotaxic site prior to delivering QA to the same site three-weeks later. While the most extensive transduction of the striatal neurons were therefore likely to be found in close proximity to the QA injection site, the level of excitotoxic stress that these neurons were exposed to would also have been significantly greater than that in the more distal reaches of the QA-lesion. As a model of excitotoxic cell death that potentially replicates pathological mechanisms which contribute to HD neurodegeneration, I felt that the less intensive excitotoxic insult as the QA diffuses away from the injection site is possibly more representative of the challenges facing striatal neurons in HD patients than the lesion core, despite still being a very acute model. With striatal cells proximal to the site of QA injection facing very severe excitotoxicity, multiple forms of cell death pathways are likely to have been initiated including necrotic mechanisms (Portera-Cailliau et al. 1995; Bordelon et al. 1999), such that a single factor is unlikely to have any discernable impact. Therefore with this hypothesis of QA-induced cell loss and the lack of any neuroprotection in the initial AAV-Bcl-xL investigation I continued investigation of anti-apoptotic factors but increased AAV-vector delivery to two-sites within the striatum slightly removed from the site of QA injection. This enhanced the proportion of neurons transduced in the “transition zone” of the striatum surrounding the central core of the QA lesion where there is also greater survival of the less vulnerable striatal interneurons (Section 3.3.4.2).

The independent enhancement of Bcl-xL or XIAP expression in the striatum by AAV-mediated gene transfer was not found to confer any statistically significant increase in the resistance of transduced striatal neurons against QA-induced excitotoxic cell death, although AAV-XIAP treatment did result in a slightly higher average survival of striatal neurons (Section 6.6.1.3). The lesioned area within
Anti-Apoptotic Factor Delivery: AAV-Bcl-xL and AAV-XIAP

the treated striatum could be clearly delineated, despite the delivery of AAV-Bcl-xL or AAV-XIAP prior to QA administration, with no quantified maintenance of striatal neurons, and no apparent evidence of transduced neurons surviving within the confines of QA lesioning. Co-labelling of transduced neurons within the unlesioned portion of the striatum with HA and DARPP-32 or krox-24 indicated that expression of transgenic Bcl-xL or XIAP was not disrupting normal cellular processes to the extent seen with the continuous Luciferase expression in which the transduced neurons lost all expression of these marker proteins (Section 6.6.3). Although there was no evidence of Bcl-xL or XIAP supplied maintenance of the striatal neurons, I assessed other neuropathological changes in the basal ganglia to allow correlations with the changes in functional behaviour. While no significant differences were observed in the substantia nigra, parvalbumin-positive neurons in the globus pallidus showed remarkably different levels of maintenance between individual rats irrespective of treatment group (Section 6.6.6). Only AAV-XIAP rats exhibited a positive correlation with the extent of striatal lesioning, suggesting pallidal cell death is not solely consequential of the loss of afferent striatal projections, but may have involved direct QA exposure or be modulated by the AAV vector transduction. Contrasting results between AAV-Luciferase and PBS injected control rats prevented assessment regarding any potential neuroprotective action of the anti-apoptotic factors on maintenance of the globus pallidus, with Luciferase potentially enhancing degeneration of pallidal neurons as discussed previously (Section 5.8.2.2). Afferent innervations of the striatum by TH-positive dopaminergic neurons (Section 6.6.4) and SMI32-positive corticostriatal projections (Section 6.6.5) demonstrated the disruption of the striosome-matrical architecture of the striatum following QA lesioning, but neither AAV-Bcl-xL nor AAV-XIAP delivery provided any visibly discernable protection against the disruption of striatal structural arrangement evident eight-weeks following acute excitotoxic insult.

6.7.1.2 Behavioural Protection

Despite the lack of a significant reduction in any of the investigated pathological changes induced in the striatum by QA lesioning, I did observe some attenuation of functional behaviour impairments when compared with the AAV-Luciferase / PBS treated control rats. AAV-XIAP treated rats, while tending towards having enhanced striatal maintenance, displayed complete amelioration of an ipsilateral forelimb use bias in the spontaneous exploratory forelimb use assessment. AAV-Bcl-xL treated rats in the two-site delivery investigation also had a trend towards a reduction in the preferential ipsilateral forelimb use that was not quite significant and a similar trend towards reduced contralateral sensorimotor neglect. Across all treatment groups the extent of ipsilateral forelimb use bias was correlated with the loss of striatal neurons, while the severity of sensorimotor neglect
appeared to be more dependent on the maintenance of pallidal neurons. Therefore assuming the observed trends in functional behaviour for each treatment group reflected real effects of treatment, AAV-XIAP and AAV-Bcl-xL mediated prevention of a strong ipsilateral forelimb use bias would tend to indicate enhanced maintenance of striatal neuronal function, while reduced contralateral sensorimotor neglect in AAV-Bcl-xL treated rats is suggestive of greater globus pallidus functionality. Although this is circumstantial evidence, it is possible that the anti-apoptotic factors could have helped maintain the function of surviving neurons without significantly increasing overall survival of striatal neurons.

Greater variation in the apomorphine-induced rotation direction for both the AAV-Bcl-xL and AAV-XIAP treated rats, compared to the predominantly ipsilateral rotations in control rats, tended to also suggest a reduced severity of striatal lesioning or compensatory changes in the dopamine system specifically modulated by the enhanced anti-apoptotic factor expression; although I am not aware of any cellular pathway through which this could have occurred. In contrast, no attenuation of apomorphine- or amphetamine-induced rotational behaviour was seen in the initial single-site AAV-Bcl-xL delivery investigation suggesting a significant difference in the integrity of dopamine signalling or striatal efferent connections between AAV-Bcl-xL treated rats in the two studies. Although I only conducted limited overlapping neuropathological analysis of the two AAV-Bcl-xL studies I did not see any structural changes that would provide an explanation of these differential performances in apomorphine-induced behaviour. This therefore raises the question of other differences in the design of the two investigations. Besides vector delivery the only major design change was the dietary restriction of the rats in the second investigation required for the food retrieval based “corridor” task. Dietary restriction has been shown to induce an increase in endogenous BDNF expression (Mattson 2000; Duan et al. 2003) which could potentially have had an influence on the severity of QA lesioning and also more specifically on dopamine receptor expression. Without an evident reduction in the gross QA-induced striatal pathology in the dietary restricted study, and similar inducement of ipsilateral rotations in both groups of control rats, the prevention of ipsilateral rotational behaviour appeared to be a direct effect of the enhanced Bcl-xL expression – possibly in conjunction with a small increase in endogenous neurotrophic factor support. Alternatively, with the apomorphine-induced rotation direction correlating strongly with the maintenance of the globus pallidus, it could be that the pallidal neurons were better maintained in the second investigation – either by the greater Bcl-xL expression or chance positioning of the QA lesion causing differential loss of pallidal neurons between treatment groups. Overall though the sensorimotor neglect “corridor” task – which appeared to give the most sensitive measure of
functional behaviour in this QA lesion model – was not significantly attenuated by the prior delivery of AAV-Bcl-xL or AAV-XIAP.

### 6.7.2 Comparison with previous studies

The lack of any significant protection of striatal projection neurons by the viral vector delivery of Bcl-xL or XIAP prior to QA-induced excitotoxicity was in contrast to a number of previously reported situations in which pro-survival proteins have protected specific populations of neurons from various apoptosis promoting insults (Section 1.7.1). XIAP has also recently been reported to protect against the excitotoxic kainic acid insult of CA3 neurons following in vivo delivery of a XIAP-PTD (protein-transducing-domain) fusion protein (Li et al. 2006) and also glutamate-induced death of embryonic motor neurons and dorsal root ganglion cultures in vitro (Garrity-Moses et al. 2006). While these anti-apoptotic factors appear capable of attenuating cell death following apoptotic-inducing excitotoxic signals, a recent study of transgenic mice over-expressing Bcl-2 failed to display any reduction in QA-induced striatal cell loss (Maciel et al. 2003). These findings suggest that QA-induced cell death is not likely to be solely dependant on a single apoptotic mechanism, but may involve multiple mechanisms of cell death; including non-Bcl protein regulated mitochondrial permeability transition – commonly induced by high intracellular Ca\(^{2+}\) accumulation following over-excitation (Bordelon et al. 1998) – and neuronal necrosis. With conflicting data surrounding the actual process of QA-induced cell death, it is possible that QA can activate multiple pathways leading to cell death depending on experimental conditions. Therefore, while Bcl-2 / Bcl-xL may protect striatal neurons against mitochondrial dependent apoptotic mechanisms, these pathways may simply be bypassed through activation of alternative cell death mechanisms including non-caspase dependent processes. The failure of XIAP to provide any significant protection is strongly indicative of caspase-independent apoptosis or necrotic cell death pathways. While I verified that both AAV-vectors produced functionally active anti-apoptotic proteins capable of preventing the induction of apoptosis by staurosporine (Chapter 2, (Kells et al. 2006)), I did not specifically test for the attenuation of QA toxicity in vitro given uncertainties over the induction of apoptotic mechanisms.

### 6.7.3 Conclusion

The selective degeneration of specific neuronal populations in HD, despite ubiquitous expression of mutant huntingtin in the CNS, indicates the induction of cell death through mechanisms specifically related to physiological properties of vulnerable neurons. Excitotoxicity is proposed as a leading initiator of HD neurodegeneration given intensive glutamate input to the striatum, selective
vulnerability of striatal projection neurons to chemical excitotoxins, and direct potentiation of glutamate receptor signalling by mutant huntingtin. With the developing and juvenile CNS appearing remarkably resilient to the presence of mutant huntingtin, it is conceivable that sensitive apoptotic mechanisms maybe initiated in older neurons that have reduced capacity to handle potentially destructive cellular stress. Therefore while apoptotic mechanisms initiated via excessive NMDA receptor signalling may be a significant contributor to the slow progressive degeneration in the HD brain, the failure of either enhanced Bcl-x\textsubscript{L} or XIAP expression to significantly attenuate QA-induced neuronal death in the current investigations may well have been due to the acute, intensive insult simultaneously initiating multiple cell death pathways such that single factor intervention is insufficient to ultimately block neurodegeneration. Whether the same process occurs in the HD brain with cell death occurring via multiple apoptotic and necrotic mechanisms is at this stage unknown, although apoptotic hallmarks are present in post-mortem HD brains (Section 1.7).

The \textit{in vivo} gene delivery and overexpression of Bcl-x\textsubscript{L} or XIAP did not appear to disrupt neuronal function, but conversely showed a potential preservation of neuronal signalling given the tendency towards a reduction in the degree of behavioural impairments exhibited by the AAV-Bcl-x\textsubscript{L} and AAV-XIAP rats despite no overall enhancement of neuronal maintenance. Therefore these results suggest that while anti-apoptotic factor production may not be effective as a stand alone therapy, in conjunction with other more efficacious neuroprotective molecules anti-apoptotic factors may provide critical preservation of neuronal function in a complimentary fashion. Further investigation in a more subtle / progressive model of striatal degeneration may also reveal whether the enhancement of endogenously expressed pro-survival proteins can directly attenuate or slow the degeneration of striatal neurons.
Chapter 7

General Discussion

The prospect that neuroprotective agents which halt the relentless progression of neurodegenerative processes occurring in the CNS of HD patients, and other neurological disorders will one day be clinical reality was the ultimate goal driving this thesis research and other similar bodies of current neuroscience investigation.

7.1 AAV-mediated Gene Delivery

Utilising AAV vectors to mediate the transfer of genetic constructs to the rodent brain, I directed the enhanced expression of a neurotrophic factor – BDNF or GDNF – or anti-apoptotic factor – Bcl-xL or XIAP – within vulnerable striatal projection neurons. While AAV vectors themselves have been shown to be non-toxic, and are currently used extensively in both pre-clinical research and clinical trials, the spread of vector particles and expression of the encoded therapeutic molecules needs to be closely analysed on a use-by-use basis. Following intrastriatal injection of the AAV1/2 vectors I observed extensive transduction of striatal neurons as well as numerous neurons in the globus pallidus, substantia nigra pars compacta and pars reticulata, and cerebral cortex (Section 4.4.2). This spread of transduction to cells residing in nuclei connected to the striatum via afferent and efferent projections indicated the ability for the AAV1/2 vector to be retrogradely, anterogradely and possibly postsynaptically transported in agreement with previous assessment of AAV vector transportation (Burger et al. 2004). While this may be potentially advantageous for many applications including neuroprotection, through the production of a therapeutic agent at both the targeted cell body and its terminal projection fields, it may also result in undesired deleterious actions. The extensive overexpression of BDNF following high-titre AAV vector delivery to the striatum in the initial investigation resulted in seizure development, aggression, weight-loss and death (Section 5.3). These deleterious effects were likely to have been induced by enhanced BDNF activity outside the striatum due to transportation of the AAV-BDNF vector particles, or alternatively the direct transportation / diffusion of the overexpressed BDNF protein, presumably to the entorhinal cortex or hippocampus (Pellemounter et al. 1995; Naert et al. 2006).

Although beyond the scope of this thesis research, future consideration should be given to assessing the impact of enhanced expression of endogenous proteins – particularly secreted neurotrophic
factors – on processes of neurogenesis that occur in the subependymal layer bordering the caudate-putamen and appear to be modified in both the HD brain (Curtis et al. 2003; Curtis et al. 2005), and following QA lesioning (Tattersfield et al. 2004; Gordon et al. 2007). The generation of new neurons has been shown to occur in the striatum, however the normal physiological importance of this phenomenon is not clear. Significant changes in the striatal expression of neurotrophic factors vital for directing brain development are likely to effect the migration, maturation, and / or survival of subependyma-derived neuroblasts. Although the enhancement of neurotrophic factors prior to excitotoxic insult was undertaken to primarily investigate protection against neurodegenerative processes and subsequent functional deficits, without direct assessment of proliferative cell markers the possible restoration of the striatum via neurotrophic factor mediated neurogenesis / migration cannot be discounted. With the mammalian brain potentially containing a regenerative capacity, gene delivery may provide an avenue through which to promote both neuronal restoration and enhance long-term survival of new neurons in the HD brain despite the persistence of pathogenic genetic pressures.

The level of expression required to provide therapeutic intervention following gene transfer is also an area that needs to be assessed in vivo. The expression cassette I used for all of the AAV vectors was designed to generate high-level expression that was sustained throughout the entire investigation. The hybrid CBA promoter is not down-regulated with expression maintained indefinitely following AAV vector transduction (Klein et al. 2002). While the need for continuous long-term expression is presumed to be a necessity to counteract slow neurodegenerative processes, the required level of expression will be specific to the biotherapeutic molecule. Following the welfare issues with AAV-BDNF treatment I reduced the expression level by diluting the vector stock prior to delivery; however this did result in a discernable reduction in the number of neurons that were transduced. While this was not of great concern for secreted neurotrophic factors, this approach is clearly not suitable for non-secreted therapeutics in which extensive transduction is paramount. A potentially better approach would be the incorporation of lower expression or regulatable promoters allowing extensive transduction of targeted cells but control of the transgene expression levels (Eslamboli et al. 2005). Surprisingly, the AAV-GDNF transduced neurons in the striatum, while being observed to have survived QA-induced cell death, displayed complete loss of striatal projection neuron markers likely to indicate severely compromised function due to the high expression of GDNF protein (Section 5.7.3). This further highlights the concern with continuous expression of a transgene product in which the host cells function maybe compromised either by the physical production of the transgenic protein or via continuous exposure. While a similar loss of neuronal proteins was observed in the AAV-Luciferase transduced neurons, the high-expression of anti-apoptotic factors
did not induce the same disruption to normal protein expression, suggesting the alterations are specific to gene expression (Section 6.6.3). Significant changes to dopaminergic mechanisms in the striatum following enhanced GDNF expression have been previously quantified including increased dopamine synthesis and turnover (Hudson et al. 1995; Kirik et al. 2000b), with the indication that enhanced dopamine exacerbates neuronal cell death in the striatum (Cyr et al. 2003). While neurochemical alterations were not assessed in the current investigations, the reduction in DARPP-32 neuron expression following high expression of BDNF or GDNF indicate that biochemical signalling pathways would have been compromised. DARPP-32 has been referred to as a “molecular switch” modulating the integration of dopaminergic and glutamatergic signalling cascades through its potent inhibition of protein phosphatase-1 (Gould and Manji 2005); with the phosphorylation state of all physiological effectors in striatal dopaminoceptive neurons regulated by the DARPP-32 / protein phosphatase-1 cascade (Greengard et al. 1999). DARPP-32 knockout mice display significant reductions in their molecular, physiological and functional behaviour changes normally induced by dopamine and dopamine-mediated drugs of abuse (Greengard et al. 1999; Valjent et al. 2005). The use of regulatable promoters allowing the on / off switching of transgene expression would also provide the ability for pulsatile application of the therapeutic molecule (Jiang et al. 2004; Goverdhana et al. 2005), which could be more therapeutically beneficial than straight continuous expression that may lead to down-regulation of receptors and effector proteins.

7.2 Preventative Therapy for Huntington’s Disease

To investigate whether the continuous delivery of these neurotrophic factors or anti-apoptotic proteins could enhance the resistance of striatal projection neurons, which are selectively vulnerable to cell death processes in HD, I locally injected QA into the striatum as a model of HD pathological changes (Chapter 3). QA has been extensively used to model the selective loss of striatal neurons seen in HD through over excitation of NMDA receptors expressed by striatal projection neurons. While the actual mechanism of neurodegeneration that occurs in HD has not been fully elucidated, there is reasonable evidence to suggest that cell death pathways stimulated by excitotoxic insults are involved with expanded huntingtin causing potentiation of the NR2B subunit expressed by striatal neurons (Chen et al. 1999; Zeron et al. 2001). It is also conceivable that QA itself may contribute towards the neurodegeneration (Chiarugi et al. 2001), with an increase in QA being reported in the HD brain (Guidetti et al. 2004). Therefore while QA tends to replicate the striatal pathology of HD, the direct intrastriatial injection causes an acute, intense neurotoxic insult localised to the tissue surrounding the site of injection not representative of the slow progressive HD neurodegeneration.
With complete non-selective cell loss directly at the site of QA injection, neuroprotection is more likely to occur in the surrounding penumbra region where the lower QA concentrations have spared the less vulnerable interneurons. Given that the QA stimulation of excitotoxic mechanisms greatly exceeds any similar activation in the actual HD brain, I therefore conversely expect that any protection seen against QA-induced pathology would be considerably greater in the HD brain.

Of the four proteins assessed as potential therapeutic agents, only enhanced BDNF expression resulted in a significant quantified protection of striatal neurons against QA-induced excitotoxicity, displaying gross maintenance of the striatal cytoarchitecture in addition to the survival of individual striatal projection neurons (Section 5.7.1). While AAV-GDNF transduced cells survived within the penumbra of the lesioned striatum, they lacked DARPP-32 and krox-24 expression suggesting a loss of neuron functionality. Neither Bcl-xL nor XIAP overexpression provided any significant protection to the transduced striatal cells – visually apparent by the absence of HA-positive cells within the lesioned striatum. However given the spread of the QA lesion – covering approximately half of the striatum – and substantial variation in the extent of neuronal cell death, it is possible that small levels of neuroprotective support may have occurred in the peripheral reaches of the lesion but were below the resolving power of these in vivo studies.

Downregulation of marker proteins raises additional questions over the actual extent of neuronal cell death following QA, as against the loss of phenotypic markers. A phenomena that has previously been reported in other neurological models, such as the loss of TH expression following 6-hydroxydopamine lesioning despite the persistence of dopaminergic cell bodies and nigrostriatal projections (Reis et al. 1978; Shirao et al. 1992; Sauer and Oertel 1994). The use of chemical tracers or histological markers, not biochemically linked to the GABAergic phenotype of the striatal projection neurons, may have provided more definitive assessment of the striatal neurons fate and any maintenance of axonal projections following QA. However the loss of Luciferase-expressing cells within the lesioned striatum of AAV-Luciferase treated control rats suggest that in the absence of enhanced trophic support the striatal neurons do ultimately die in this 50nmol QA lesion model, although no time-course of neurodegeneration was determined. The functional state of the transduced neurons expressing high-levels of BDNF or GDNF but with significantly down-regulated DARPP-32 and krox-24 expression remains to be determined, with the possibility that attenuation of behavioural impairments is resultant of neurotrophic factor activity on other non-transduced striatal neurons or actions outside of the striatum.

The very limited protection of striatal neurons solely by BDNF, despite reports of enhanced neuronal survival with GDNF and anti-apoptotic factors, brings into question the suitability / relevance of the
QA lesion model for conducting neuroprotective investigations of the striatum. GDNF has been previously shown to protect striatal neurons against cell death induced by the mitochondrial toxin 3-NP (McBride et al. 2003) and also in a transgenic mice model of HD (McBride et al. 2006). The failure of both Bcl-xL and XIAP to provide protection against QA neurotoxicity suggests the inducement of neurotic cell death or possibly the activation of multiple apoptotic mechanisms such that a single factor is unable to ultimately prevent degeneration. Despite the apparent severity of QA-induced striatal degeneration, promising reductions in the extent of QA lesioning have been reported by transplantation of encapsulated choroid plexus cells secreting multiple neurotrophic factors (Borlongan et al. 2004; Emerich et al. 2006), and following the combined delivery of pharmacological agents pyruvate and minocycline (Ryu et al. 2006), suggesting that delivery of multiple factors can overcome the QA-induced excitotoxic insult. With BDNF gene transfer alone providing what appeared to be significant maintenance of the gross striatal architecture but minimal enhancement of neuron survival, it would possibly have been more advantageous to have investigated co-delivery of the anti-apoptotic factor vectors looking for any additional complementary physical and functional enhancement of the BDNF-mediated protection. It is conceivable that the overexpression of BDNF may have enhanced the striatal neurons capacity to handle an increase in NMDA receptor activity, but not to the extent of blocking the activation of apoptotic mechanisms. While QA has provided a readily available model to study excitotoxicity in the striatum, and more specifically the degeneration of the vulnerable projection neurons; the severity and acuteness with which QA induces neuronal cell death maybe preventing the true realisation of therapeutic potential when used to investigate possible neuroprotective agents for HD. With the development of new transgenic HD models showing progressive degeneration of striatal projection neurons reminiscent of HD, the reliance on neurotoxic chemical based modelling of HD neurodegeneration has effectively been eliminated. Additionally, the realisations that pathological changes in the HD brain are not restricted to the caudate-putamen, but are evident in numerous regions of the brain such as the neocortex, globus pallidus, substantia nigra and thalamus (Vonsattel and DiFiglia 1998) make it important that future research into preventative therapies for HD do not neglect to consider the impact of these regions.

The confirmation of BDNF as a potential biotherapeutic molecule to prevent or restrict the degeneration of striatal projection neurons following AAV vector mediated gene delivery to the striatum, and the significant amelioration of functional behavioural defects displayed in these investigations further highlights the importance of BDNF in the functional maintenance of the striatum. With the necessity of BDNF for the functional maintenance of the striatal GABAergic neurons (Nakao et al. 1995; Ventimiglia et al. 1995), the well documented reduction of BDNF levels
General Discussion

in HD patients (Ferrer et al. 2000; Zuccato et al. 2001) and transgenic mice (Zuccato et al. 2005),
and the impaired transport of BDNF by mutant huntingtin (Zuccato et al. 2001; Zuccato et al. 2003),
an enhancement of striatal BDNF appears promising as a therapeutic approach. The disruption of
intracellular transport by expanded huntingtin may limit the transportation of BDNF to the striatal
target nuclei following the transduction of striatal neurons that I observed in these investigations;
however this may also limit the spread to undesired CNS regions reducing the potential for
deleterious side effects.

Finally the attenuation of behavioural deficits was found to correlate with the survival of both striatal
and pallidal neurons, although the loss of parvalbumin-positive GABAergic neurons in the globus
pallidus showed considerable variation within each treatment group that was largely independent
from the extent of striatal degeneration. A specific cause for the degeneration of pallidal neurons
following intrastratal QA injection was undetermined, but potentially involved direct QA exposure,
secondary neurodegeneration, or an adverse consequence of AAV vector transduction. With
considerable variation in the extent of pallidal degeneration any maintenance following
overexpression of BDNF, Bcl-xL or XIAP was indeterminable. It was however interesting to observe
gross alterations in functional behaviour correlations with the integrity of both the striatum and
globus pallidus following striatal gene delivery. While the gross level of locomotor activity did not
appear to display large changes following striatal lesioning, the unilateral QA lesion did induce
lateralised imbalances in motor control with an apparent reduction in sensorimotor activity
contralateral to the lesioned striatum. Attenuation of contralateral forelimb use deficits by AAV-
mediated therapeutic expression, without maintenance of DARPP-32 striatal neurons, resulted in a
weaker correlation between preferential forelimb use and striatal integrity, with the AAV-BDNF
treated rats specifically acquiring a strong correlation to the extent of pallidal cell survival, which
was not exhibited by the control rats (Section 5.7.7.1). A similar pattern was observed for the
amelioration of sensorimotor neglect following AAV-BDNF delivery, with a reduced correlation to
the relative survival of striatal neurons, but enhanced correlation with pallidal neuronal maintenance.
The degree of contralateral neglect in the “corridor” task was found to correlate with the integrity of
the globus pallidus for all rats irrespective of treatment – presumably reflecting the enhanced sensory
complexity of this lateralised selection task over the spontaneous forelimb use analysis. In addition
to the reduced contralateral motor activity observed in these two non-drug induced behavioural
analyses, the administration of apomorphine following the unilateral striatal lesioning induced
rotational behaviour that was generally towards the lesioned striatum. However correlation analysis
with the relative survival of pallidal neurons showed a strong relationship between the integrity of
the globus pallidus and the direction of induced rotation following striatal lesioning – ipsilateral
rotations induced when the pallidus was disrupted but contralateral rotations if the pallidus was fully maintained. AAV-BDNF delivery weakened the directional correlation with end-point pallidal pathology, possibly due to direct BDNF-induced modification of dopamine processing / signalling (Section 5.8.2.1) and / or enhanced maintenance of striatal projection neurons that resulted in apomorphine-induced ipsilateral rotations in initial tests following QA injections but contralateral rotations in later trials. Variation in the extent of striatal lesioning alone appeared to have no discernable influence on the direction of the apomorphine-induced rotations in these neuroprotective investigations despite previous reports of the lesion size and position influencing drug-induced rotations (Dunnett et al. 1988; Norman et al. 1992; Fricker et al. 1996; Nakao et al. 1996; Nakao and Brundin 1997).

These behavioural deficit correlations with neuropathology changes highlight the importance of the globus pallidus as a major striatal output nucleus for facilitating motor control, with BDNF overexpression specifically appearing to have a much greater functional impact when the pallidal neurons are maintained. Assessment of the globus pallidus has not previously been reported in neuroprotective investigations following striatal lesioning, however lesioning of the GPe (equivalent to the rodent globus pallidus) has been proposed as a potential treatment to correct HD symptoms (Joel et al. 1998; Ayalon et al. 2004; Reiner 2004; Temel et al. 2006). The basic direct pathway / indirect pathway model of basal ganglia circuitry describes two major striatal output circuits; stimulation of the direct pathway leading to disinhibition of the thalamus via the G Pi and SNr, and the indirect striatopallidal projections increasing thalamic inhibition via the GPe, subthalamic nucleus and G Pi (Penney and Young 1986; Crossman 1987; Albin et al. 1995; Joel 2001). Although the direct G Pi / SNr pathway is generally considered to promote intended movements by enhancing thalamocortical activity, and the indirect GPe pathway suppressing movements, they are presumed to have complimentary roles in controlling desired locomotion (Penney and Young 1986; DeLong 1990). Ayalon et al. (2004) reported that bilateral QA lesioning of the rat globus pallidus resulted in attenuation of behavioural impairments acquired following QA lesioning of the striatum and that pallidal lesions alone did not generally induce severe impairments in motor and cognitive tasks; however a slight reduction in activity was observed in some of their functional assessments as predicted by the basal ganglia circuitry model following a lesion of the GPe. The current findings that contralateral hypokinesia and sensorimotor neglect are exhibited following striatal lesioning, and are potentially compounded by a loss of pallidal neurons, appear contradictory to the earlier studies but may be resultant of different behavioural assessments or the use of unilateral lesioning compared with the earlier bilateral lesions. Lesioning of the globus pallidus is suggested as a treatment strategy for attenuating the hyperkinetic chorea symptoms of HD generally exhibited in the earlier stages of
disease progression; however the implications raised in these investigations – that a loss of pallidal neurons following striatal degeneration will enhance hypokinesia – as predicted by basal ganglia circuitry – highlights the concern that pallidal lesioning may exuberate the late-stage HD akinesia / bradykinesia symptoms (Reiner 2004). With the correlations between non-drug induced behavioural impairments and the integrity of the globus pallidus being significantly enhanced in the AAV-BDNF treated rats, it is possible that the observed BDNF-mediated amelioration of motor deficits were resultant of BDNF attenuating or compensating for the loss of striatal neurons but having no direct effect on any pathological changes in the globus pallidus. Although not investigated in the current studies, the differential loss / maintenance of striatopallidal (direct pathway) and striatoentopenducular (indirect pathway) neurons, and the integrity of the entopenducular nucleus should also be considered to further elucidate the pathological basis of the behavioural deficits I observed. Lesioning of the GPi (equivalent to the rodent entopenducular nucleus; pallidotomy) is known to alleviate hypokinetic motor dysfunction in Parkinson’s disease patients through eliminating the over-inhibition of the thalamus and thereby allowing easier initiation of movement (Bergman and Deuschl 2002). Byler et al. (2006) recently demonstrated that unilateral electrolytic lesioning of the entopenducular nucleus in rats induced hyperlocomotion with a tendency for ipsilateral circling behaviour, but they also reported the development of sensorimotor asymmetries and contralateral forelimb motor deficits which they proposed were due to non-specific damage to fibres of passage within the internal capsule (Byler et al. 2006). This would suggest that hyperactive motor impairments normally induced by specific entopenducular nucleus lesioning can be modified by pathological disruption to other basal ganglia circuitry, and that pathological disruption of pallidal neurons in the direct pathway may not be evident from behavioural testing. With the primary pathology in the current investigations localised to the striatum, there exists the possibility that behavioural impairments could partially be attributed to an unequal loss / protection of striatopallidal neurons differentially projecting to the internal and external segments of the globus pallidus. However with the exception of AAV-BDNF treated rats that did not develop contralateral deficits, the lesioned striatum displayed fairly extensive loss of DARPP-32 neurons and provided no indication of differential vulnerabilities between the GABAergic projections as have been previously reported to occur following QA lesioning (Figueroedo-Cardenas et al. 1994) and in early stage HD (Reiner et al. 1988; Sapp et al. 1995). A more tenable explanation for the behavioural deficit correlations with neuropathology changes maybe that the striatal lesioning disrupts processing of sensory inputs from the cortex, thereby resulting in reduced ability to initiate contralateral motor activity response to sensory input, with disruption to the globus pallidus acting to exaggerate any impairment of striatal output signalling in comparison to the intact contralateral basal ganglia.
7.3 Conclusion

Overall this thesis highlights the vital importance to assess putative biotherapeutic agents using \textit{in vivo} model systems and the need to consider adverse complications that can arise from non-regulatable control of transgene expression following gene delivery. Utilisation of AAV\textsubscript{1/2} vectors to mediate gene transfer to the striatum led to efficient transduction of striatal neurons, directing high-level expression of biologically functional proteins within the striatum prior to QA-induced lesioning. Additional transduction of neurons in CNS regions directly connected to the striatum also occurred to a lesser extent, which could potentially be of therapeutic benefit for striatal protection though enhanced trophic support within the terminal-fields, but may also induce unintended consequences such as the observed induction of seizures by BDNF.

Maintenance of the striatum by BDNF, accompanied by an attenuation of the behavioural impairments exhibited following acute excitotoxic lesioning of the striatum, provided further evidence of the promising therapeutic potential that BDNF holds for preventing the progression of HD. Further investigation is required however to determine an optimal level of BDNF expression and fully elucidate the impact of continuous long-term overexpression. Enhancement of striatal GDNF did not replicate the previously reported neuroprotective support of striatal neurons, with substantial disruption to the striatal architecture and an apparent downregulation of both the intracellular dopamine and glutamate signal modulating protein DARPP-32, and the early response neuronal transcription factor krox-24. Similar to the enhanced BDNF expression, the disruption to normal physiological protein expression suggested large increases in the striatal expression of GDNF is detrimental to the functionality of dopaminoceptive striatal neurons, negating any trophic assistance GDNF may potentially provide against excitotoxic stress when available to striatal neurons at lower concentrations. Together these BDNF and GDNF investigations demonstrated the prospect neurotrophic factor based therapy holds for reducing the susceptibility of striatal neurons in neurodegenerative disease, although the imperative need for greater control over the location and quantity of endogenously expressed proteins is clearly manifested. To my knowledge this is the first study to demonstrate downregulation of DARPP-32 and krox-24 in striatal projection neurons following the overexpression of neurotrophic factors and QA lesioning.

Enhanced expression of a single anti-apoptotic protein by striatal projection neurons did not convey any reduction in their susceptibility to acute excitotoxic-induced neurodegeneration. However the overexpression of either Bcl-x\textsubscript{L} or XIAP in the striatum provided attenuation against the severity of behavioural impairments, suggesting enhanced functionality of the maintained neurons with no
apparent transgene induced disruption of normal host cell signalling pathways. As intracellular therapeutic agents, the high efficiency of transduction provided by the AAV\textsubscript{1/2} vectors, without any adverse effects in these studies, is vital to ensure extensive expression of the vulnerable striatal neurons. Therefore while these investigations suggest anti-apoptotic factors in isolation may not prevent excitotoxic-induced neurodegeneration, they may well be of significant therapeutic benefit in a combined treatment strategy to prevent unintended inducement of apoptotic death and enhance the maintenance of neuronal function.

This thesis investigation also demonstrates the critical involvement of basal ganglia neurons beyond the striatum in modulating sensorimotor behaviour following acute lesioning of the striatum, and the importance for preventative HD treatment strategies to also consider the impact of other primary or secondary areas of neurodegeneration on HD symptoms. Maintenance of the globus pallidus proved vital in attenuating the degree of sensorimotor deficits and reversing drug-induced effects on locomotor activity following QA lesioning. As more accurate transgenic models of HD become available and inducible promoters of transgene expression are incorporated into the gene expression cassettes, future investigation targeting \textit{in vivo} delivery of neurotrophic factors and other endogenously expressed pro-survival factors to areas of mutant huntingtin induced neurodegeneration may well display considerable enhancement of the pathological and behavioural protection I observed in these investigative therapeutic studies.

Ultimately the safe and efficient demonstration of chimeric AAV-vectors to deliver biotherapeutics to the striatal neurons and specifically the maintenance of striatal structure and amelioration of functional impairments following the enhancement of BDNF, known to be depleted in the HD brain, confirms the promising potential for \textit{in vivo} gene therapy strategies to prevent the onset or slow progression of neurodegeneration.
Appendix A: Gene Sequences and DNA Plasmids

A.1 cDNA Gene Sequences

Combined sequences from pGEM-T Easy cloning vectors and reverse sequencing of AAV-backbone vectors across the C-terminal HA-tag.

Initial **ATG** (methionine) start codon and the **TGA TAG TAA** stop codons are highlighted.

Restriction sites flanking the cDNA sequences are underlined.

HA-tag sequence: **TAT CCG TAT GAT G** **T** **C** **T** **T** **G** **T** **C** **T** **T** **G** **T**

A.1.1 Brain Derived Neurotrophic Factor

BDNF cDNA sequence PCR cloned out of pAM/CRE-BDNF-WPRE-bGHpA (M. During and D. Young, Department of Molecular Medicine and Pathology, The University of Auckland)

BLAST alignment with:
- Homo sapiens brain-derived neurotrophic factor (BDNF), transcript variant 1, mRNA.
  - Accession Number: NM_170735.4
  - Complete match with bases 563..1303
  - Signal peptide: 563..616
  - Pro-protein: 617..1303
  - Mature peptide: 947..1303

Protein Identity with Rattus norvegicus: 96% (positives 97%)

```
CTC GAG CAC CAG GTG AGA AGA GTG ATG ACC ATC CTT TTT CTT ACT ATG GTT
ATT TCA TAC TTT GGT TGC ATG AAG GCT GCC CCC ATG AAA GAA GCA AAC ATC
CGA GGA CAA GGT GGC TTT GCC TAC CCA GGT GTG CCG ACC CAT GGG ACT CTG
GAG AGC GTG AAT GGG CCC AAG GCA GGT TCA AGA GGC TTG ACA TCA TTG GCT
GAC ACT TTC GAA CAC GTG ATA GAA GAG CTG TTG GAT GAG GAC CAG AAA GTT
CGG CCC AAT GAA AAC AAT AAG GAC GCA GAC TTG TAC ACG TCC AGG GTG
ATG CTC AGT AGT CAA GTG CCT TTG GAG CCT CCT CTT CTC TTT CTG CTG GAG
GAA TAC AAA AAT TAC CTA GAT GCT GCA AAC ATG TCC ATG AGG GTC CGG CGC
CAC TCT GAC CCT GCC CGC CGA GGG GGG TGG GTA ACG GCG GCA GAC AAA AAG ACT
GAG ACT GAG GAC AAG ACT GCA GTG GAC ATG TCG GGC GGG
AGC GTC ACA GTC CTT GAA AAG GTC CCT GTA TCA AAA GGC CAA CTG AAG CAA
TAC TTC TAC GAG ACC AAG TGC AAT CCC ATG GGT TAC ACA AAA GAA GGC TGC
```
Appendix A: Gene Sequences and DNA Plasmids

A.1.2  **Glial cell-line Derived Neurotrophic Factor**

GDNF cDNA sequence PCR cloned out of AAV/NSE-GDNF-WPRE-bGHpA (M. During and D. Young, Department of Molecular Medicine and Pathology, The University of Auckland)

BLAST alignment with:
- Rattus norvegicus glial cell-line derived neurotrophic factor (GDNF), mRNA.
- Accession Number: NM_019139.1
- Complete match with bases 50..682
  - Signal peptide  50..106
  - Mature peptide  344..682

Protein Identity with Homo sapiens: 92% (positives 95%)
A.1.3  Bcl-x\textsubscript{L} 

Bcl-x\textsubscript{L} cDNA PCR cloned out of pcDNA3-HA-Bcl-xL (Science Reagents)

BLAST alignment with:
- Homo sapiens BCL2-like 1 (BCL2L1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.
- Accession Number: NM_138578.1
- Complete match with bases 367..1002
  - Gene 367-1068

Protein Identity with Rattus norvegicus: 97% (positives 98%)

\[
\begin{align*}
CT & \text{ CGA GTT ATA AAA ATG TCT CAG AGC AAC CGG GAG CTG GTG GTT GAC TTT} \\
CTC & \text{ TCC TAC AAG CTT TCC CAG AAA GGA TAC AGC TGG AGT CAG TTT AGT GAT} \\
GTT & \text{ GAA GAG AAC AGG ACT GAG GCC CCA GAA GGG ACT GAA TCG GAG ATG GAG} \\
AAG & \text{ CCC AGT GCC ATC AAT GGC AAC CCA TCC TGG CAC CTG GCA GAC AGC CCC} \\
GCG & \text{ GTG AAT GGA GCC ACT GCC CAC AGC AGC AGT TTG GAT GCC CGG GAG GTG} \\
ATC & \text{ CCC ATG GCA GCA GTA AAG CAA GCG CTG AGG GAG GCA GCC GGC GAC GAG TTT} \\
GAA & \text{ CTG CGG TAC CGG CGG GCA TTC AGT GAC CTG ACA TCC CAG CTC CAC ATC} \\
ACC & \text{ CCA GGG ACA GCA TAT CAG AGC TTT GAA CAG GTA GTG AAT GAA CTC TTC} \\
CGG & \text{ GAT GGG GTA AAC TGG GGT CGC ATT GTG GCC TTT TCC TCC TTC GCC GGG} \\
GCA & \text{ CTG TGC GTG GAA AGC GTA GAC AAG GAG ATG CAG GTA TTG GTG AGT CGG} \\
ATC & \text{ GCA GCT TGG ATG GCC ACT TAC CTG AAT GAC CAC CTA GAG CCT TGG ATC} \\
CAG & \text{ GAG AAC GGC GGC TGG GAT ACT TTT GTG GAA CTC TAT GGG AAC AAT GCA} \\
GCA & \text{ GCC GAG AGC CGA AAG GGC CAG GAA CGC TCC AAC CGC AGA TCT TAT CCG} \\
TAT & \text{ GAT GTT CCT GAT TAT GCT TGA TAG TAA}
\end{align*}
\]

A.1.4  X-linked Inhibitor of Apoptosis

XIAP cDNA PCR cloned out of pCI-neo-Flag-XIAP (Science Reagents)

- Homo sapiens Baculoviral IAP repeat-containing 4 (BIRC4), mRNA.
- Accession Number: NM_001167
- Complete match with bases 129..1619
  - Gene 129-1619

Protein Identity with Rattus norvegicus: 89% (positives 95%)

\[
\begin{align*}
\text{CTCG} & \text{ AGA ATG ACT TTT AAC AGT TTT GAA GGA TCT AAA ACT TGT GTA CCT} \\
GCA & \text{ GAC ATC AAT AAG GAA GAA TTT GTA GAA GAG TTT AAT AGA TTA AAA}
\end{align*}
\]
Appendix A: Gene Sequences and DNA Plasmids

ACT TTT GCT AAT TTT CCA AGT GGT AGT CCT GTT TCA GCA TCA ACA CTG GCA
CGA GCA GGG TTT CTT TAT ACT GGT GAA GGA GAT ACC GTG CGG TGC TTT AGT
TGT CAT GCA GCT GTA GAT AGG TGG CAA TAT GGA GAC TCA GCA GTT GGA AGA
CAC AGG AAA GTA TCC CCA AAT TGC AGA TTT ATC AAC GGC TTT TAT CTT GAA
AAT AGT GCC ACG CAG TCT ACA AAT TCT GGT ATC CAG AAT GGT CAG TAC AAA
GTT GAA AAC TAT CTG GGA AGC AGA GAT CAT TTT GCC TTA GAC AGG CCA TCT
GAG ACA CAT GCA GCT ATC TTG AGA ACT GTG CTT GTA GAT ATA TCA
GAC ACC ATA TAC CCG AGG AAC CCT GCC ATG TAT AGT GAA GAA GCT AGA TTA
AAG TCC TTT CAG AAC TGG CCA GAC TAT GCT CAC CTA ACC CCA AGA GAG TTA
GCA AGT GCT GGA CTC TAC TAC ACA GGT ATT GGT GAC CAA GTG CAG TGC TTT
TGT TGT GGT GGA AAA CTG AAA AAT TGG GAA CTC TGT GAT CTT GCC TGG TCA
GAA CAC AGG CGA CAC TTT CCT AAT TGC TTC TTT GTT TTG GGC CGG AAT CTT
AAT ATT CGA AGT GAA TCT GAT GCT GTG AGT TCT GAT AGG AAT TTA CCT AAT
TCA ACA AAT CTG CCA AGA AAT CCA TCC ATG GCA GAT TAT GAA GCA CGG ATC
TTT ACT TTT GGG ACA TGG ATA TAC TCA GTT AAC AAG GAG CAG GCT GCA AGA
GCT GGA TTT TAT GCT TTA GGT GAA GGT GAT AAA GTA AAG TGC TTT CAC TGT
GGA GGA GGG CTA ACT GAT TGG AAG CCC AGT GAA GAC CCT TGG GAA CAA CAT
GCT AAA TGG TAT CCA GGG TGC AAA TAT CTG TTA GAA CAG AAG GGA CAA GAA
TAT ATA AAC AAT ATT CAT TTA ACT CAT TCA TTT GAG GAG GTG GTA GGA AGA
ACT ACT GAG AAA ACA CCA TCA CTA ACT AGA AGA ATT GAT GAT ACC ATC TTC
CAA AAT CCT ATG GTA CAA GAA GCT ATA CGA ATG GGG TTC AGT TTC AAG GAC
ATT AAG AAA ATA ATG GAG GAA AAA ATT CAG ATA TCT GGG AGC AAC TAT AAA
TCA CTT GAG GTT CTG GTT GCA GAT CTA GTG ATG CAA GAT CAG AGT ATG
CAA GAT GAG TCA AGT CAG ACT TCA TTA CAG AAA GAG ATT AGT ACT GAA GAG
CAG CTA AGG CGC CTG CAA GAG GAG AAG CTT TGC AAA ATC TGT GAT GAT AGA
AAT ATT GCT ATC GTT TTT GTT CCT TGT GGA CAT CTA GTC ACT TGT AAA CAA
TGT GCT GAA GCA GTT GAC AAG TGT CCC ATG TGC TAC ACA GTC ATT ACT TTC
AAG CAA AAA ATT TTT ATG TCT CGC GAA TTC GGA AGA TCT TAT CCG TAT GAT

GTT CCT GAT TAT GCT TGA TAG TAA

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A.2 DNA Plasmid Maps

A.2.1 AAV Backbone Plasmid

Replacement of the insert DNA stop-codon with either a EcoRI or BglII restriction site allows in-frame C-terminal fusion with the nine amino acid HA epitope tag.

HA-tag sequence with 5’ EcoRI (GAATTC) and BglII (AGATCT) restriction sites and triplicate stop codons (TGA TAG TAA):

```
GAA TTC  GGA AGA TCT  TAT CCG TAT GAT GTT CCT GAT TAT GCT  TGA TAG TAA
```
A.2.2 **AAV-Luciferase**

AAV-expression cassette containing a Firefly Luciferase cDNA sequence supplied by M. During and D. Young
A.2.3 AAV Helper Plasmids

A.2.3.1 AAV rep and cap genes

Restriction digest with XbaI
2 bands:
- 7505bp
- 3867bp

Restriction digest with SacI
3 bands:
- 3953bp
- 2837bp
- 540bp

pRV1: Diagrammatic representation only – restriction sites are only representative of fragment lengths when digested with XbaI, actual location within plasmid was unknown.
A.2.3.2 Adenoviral packaging genes

Restriction digest with *HindIII*
5 bands:
- 5572bp
- 3011bp
- 2937bp
- 2381bp
- 1522bp

Or with *EcoRI*
3 bands:
- 6587bp
- 6490bp
- 2338bp
Appendix B: General Materials & Protocols

B.1 Molecular Biology

**Luria-Bertani broth (LB)**
20 g/L LB broth, ready-made powder (USB) containing casein peptone, yeast extract and NaCl.

**LB amp Agar plates**
20 g/L LB broth, ready-made powder (USB)
25 g/L Agar (USB)
Autoclave sterilised
Added 50 mg/L ampicillin
Poured 25 mL/plate

**Ampicillin (amp)**
50 mg/mL Ampicillin, sodium salt (Sigma-Aldrich) in sterile dH₂O
Added to LB or LB agar at 1:1000 (50 µg/mL)

**X-gal**
20 mg/mL X-gal (Sigma-Aldrich) in dimethysulfoxide (DMSO; Sigma-Aldrich)
Spread 40µL onto pre-poured LB amp agar plates and dried.

**1% Agarose Gel Electrophoresis**
250 mg SeaKem® LE Agarose (BioWhittaker Molecular Applications) was added to 25 mL 1× TAE buffer and microwaved to melt agarose. 1µL ethidium bromide (Invitrogen) added to agarose and poured into a gel casting tray to set.

DNA samples with 1× gel loading dye were transferred to wells in the gel alongside 5-10 µL 1kb⁺ DNA ladder (0.1µg/µL; Invitrogen) and gel electrophoresis run at 100 volts. DNA bands were visualised under short wave UV light using a Gel Documentation System.

**TAE buffer (50× stock)**
Tris base 2 M
EDTA 100 mM
Glacial Acetic acid 1 M
pH adjusted to 8.5

**Tris EDTA (TE) Buffer**
Tris-HCl (pH 7.5) 10 mM
Na₂EDTA (pH 8.0) 1 mM
Autoclaved sterilised

**Gel Loading Dye (10×)**
Bromophenol Blue 0.25% w/v
Xylene Cyanol 0.25% w/v
Sucrose 40.0% w/v
Appendix B: General Materials

B.2 Cell Culture

B.2.1 Tissue culture media

<table>
<thead>
<tr>
<th>Culture Media / Reagent</th>
<th>Supplier</th>
<th>Cat. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco’s Modified Eagle Media (DMEM)</td>
<td>GIBCO®</td>
<td>12100</td>
</tr>
<tr>
<td>Iscove’s Modified Dulbecco’s Media (IMDM)</td>
<td>GIBCO®</td>
<td>12200</td>
</tr>
<tr>
<td>Leibovitz’s L-15</td>
<td>GIBCO®</td>
<td>11415</td>
</tr>
<tr>
<td>Neurobasal™ Medium</td>
<td>GIBCO®</td>
<td>21103</td>
</tr>
<tr>
<td>100× L-glutamine</td>
<td>GIBCO®</td>
<td>25030</td>
</tr>
<tr>
<td>100× Sodium pyruvate</td>
<td>GIBCO®</td>
<td>11360</td>
</tr>
<tr>
<td>100× Penicillin-Streptomycin</td>
<td>GIBCO®</td>
<td>15140</td>
</tr>
<tr>
<td>100× Non-essential amino acids</td>
<td>GIBCO®</td>
<td>11140</td>
</tr>
<tr>
<td>B27</td>
<td>GIBCO®</td>
<td>17504</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>GIBCO®</td>
<td>10091</td>
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Dulbecco’s Modified Eagle Media (DMEM)

3.7g Sodium hydrogen carbonate
pH 7.1 with 10N NaOH
Added: Non-essential amino acids, Sodium pyruvate, Penicillin-Streptomycin

Iscove’s Modified Dulbecco’s Media (IMDM)

3.0g Sodium hydrogen carbonate

Leibovitz’s L15

Added: Penicillin-Streptomycin, D-glucose (3 g/L)

Neurobasal™ Medium

Added: L-glutamine, Penicillin-Streptomycin, D-glucose (3 g/L)

B.2.2 Cell counting

Hemocytometer Cell Counting

85μL serum-free media
10μL Trypan blue
5μL Cell suspension

The trypan blue / cell mixture was transferred to a hemocytometer and viable cells in the four corner squares counted under a Nikon TE200 inverted scope.

\[
\frac{\text{cell count}}{4} \times 10^4 \times 20 = \text{Cells/mL}
\]
B.3 AAV Vector Genomic Titering

Plasmid Standard Calculation

50µg/mL DNA of 1kb has $4.74 \times 10^{13}$ molecules per mL

$$\frac{4.74 \times 10^{13}}{\text{plasmid size (kb)}} \times \frac{\text{plasmid conc. (µg/mL)}}{5 \times 10^4} = \text{DNA copies/µL}$$

$$\log \text{template copies} = Ct \text{ value} - \frac{y \text{ intercept}}{\text{slope}}$$

$$\text{Genome copies/mL} = 10^{\log \text{template copies}} \times \text{dilution factor} \times \frac{1000}{\text{template volume used (µL)}} \times 2$$

B.4 Immunocytochemistry Buffers

10× Phosphate Buffered Saline (PBS)
- KH$_2$PO$_4$: 18 mM
- Na$_2$HPO$_4$: 82 mM
- NaCl: 1.4 M
- KCl: 27 mM

PBS-Triton
- 1× PBS plus 0.2% Triton X-100

0.4M PB
- NaH$_2$PO$_4$: 76 mM
- Na$_2$HPO$_4$: 324 mM
- pH adjusted to 7.2

0.1M Phosphate Buffer
- NaH$_2$PO$_4$: 28 mM
- Na$_2$HPO$_4$: 72 mM
- pH adjusted to 7.2

Cyroprotectant Solution
- Phosphate buffer: 0.5 M
- Sucrose: 30 % w/v
- Ethylene glycol: 30 % v/v

4% Paraformaldehyde
- Phosphate buffer: 0.1 M
- Paraformaldehyde: 4 % w/v
- heated to 60°C to dissolve
- pH adjusted to 7.4
DAB
3,3’-diaminobenzidine tetrahydrochloride (DAB) 0.5 mg/mL
Phosphate buffer 0.1 M
Hydrogen peroxide 0.01 % v/v
Nickel sulphide 0.04 % w/v (optional)

B.5 Antibodies

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<th>Primary Antibody</th>
<th>Host species</th>
<th>Dilution</th>
<th>Company</th>
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<td>MAB 377</td>
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during behaviorally effective infusions to the substantia nigra. Experimental Neurology 130(1): 31-40.


with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nature Genetics* 35(1): 76-83.
