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Preclinical evaluation of a novel AAV-GDF5 vector in a 6-OHDA model of Parkinson's disease and evaluation of the effect of 6-OHDA on the expression of HDACs.

Thesis presented by

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Declaration

The following thesis is my own work and has not been submitted for another degree, either at University College Cork or elsewhere. The live phase of the animal study was carried out by Dr. Ruth Concannon, with culls and perfusions carried out by Dr. Ruth Concannon, Ms. Tara Foley and Ms. Susan Goulding.

List of abbreviations

AAV	Adeno-associated virus	MFB	Medial forebrain bundle
BDNF	Brain-derived neurotrophic factor	MPP^+	1-methyl-4-phenylpyridinium ion
BMP	Bone morphogenic protein	MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
COMT	Catechol-O-methyltransferase	NET	Noradrenalin transporter
DA	Dopamine	NGF	Nerve growth factor
DAB	3,3'-Diaminobenzidine	PD	Parkinson's disease
DAT	Dopamine transporter	rAAV	Recombinant AAV
GDF	Growth differentiation factor	ROS	Reactive oxygen species
GDNF	Glial cell-derived neurotrophic factor	SAHA	suberoylanilide hydroxamic acid
HAT	Histone acetyltransferase	SN	Substantia nigra
HDAC	Histone deacetylase	SNc	Substantia nigra pars compacta
HDACI	HDAC inhibitor	SNCA	Synuclein gene
ICV	Intracerebroventricular	STR	Striatum
L-AAAD	L-acidic amino acid decarboxylase	TBA	Tubastatin A
L-dopa	levodopa	TGF-β	Transforming growth factor
LPS	Lipopolysaccharide	TH	Tyrosine hydroxylase
MAO	Monoamine oxidase	6-OHDA	6-hydroxy-dopamine

1. Introduction

1.1 Parkinson's disease

1.1.1 Clinical presentation and pathology

Parkinson's Disease (PD) is the second most common neurodegenerative disease after Alzheimer's Disease. A meta-analysis of epidemiological studies, published in 2018, found that the condition affects an estimated 6.1million individuals world-wide and that the prevalence of the condition has nearly doubled between 1990 and 2016 (Ray et al., 2018). PD is characterised by a progressive decline in motor functions, accompanied by several non-motor disorders, such as loss of smell, sleep disturbances, autonomic dysfunctions and changes in cognitive functions, for review see (Rizek et al., 2016; Tibar et al., 2018). A trio of motor disorders; tremor, bradykinesia and rigidity, are the cardinal features of PD and together are referred to as Parkinsonism. The tremor is often the initial symptom and is usually a resting tremor. It usually begins unilaterally and progresses to bilateral involvement. Bradykinesia is a slowness of voluntary movement. This is the most common clinical feature of PD and is required for a diagnosis. Rigidity results from a tension in the muscle and causes a resistance to passive movement. The rigidity seen in PD is sometimes called a 'cogwheel rigidity' as the muscles have a 'ratchet-like' motion when moved passively. A fourth common symptom, which is sometimes included under the term Parkinsonism, is postural instability. This can cause a loss of balance and increase the risk of falls, for review see (Massano and Bhatia, 2012; Sveinbjornsdottir, 2016; Przedborski, 2017). PD involves the progressive loss of dopamine (DA)-producing neurons from the nigrostriatal pathway of the midbrain.

This pathway begins in the substantia nigra (SN) and projects to the striatum (STR). These two nuclei form part of the basal ganglia, a group of nuclei found deep within the cerebral hemispheres which, among other functions, are involved in the control of voluntary movement (DeLong, 1972). There are two pathways of the basal ganglia which help to control the level of input to the motor cortex from the motor nuclei of the thalamus and thereby promote or inhibit movement. These are the direct pathway, which promotes movement, and the indirect pathway, which supresses it (Kravitz et al., 2010; Freeze et al., 2013). The balance between these two pathways is modulated by cholinergic transmission from striatal interneurons (Maurice et al., 2015) and dopaminergic transmission from the substantia nigra pars compacta (SNc). Cholinergic transmission favours the indirect pathway while dopaminergic transmission favours the direct pathway (Planert et al., 2013). In this way, dopaminergic transmission helps stimulate movement. In the case of PD, a large portion of this dopamine is lost, resulting in decreased stimulation of the direct pathway. This, coupled with the now unopposed action of the striatal cholinergic interneurons, results in decreased signalling from the motor thalamus to the motor cortex, causing difficulties initiating movement and resulting in decreased movement amplitude. These changes in signalling strength in the Parkinsonian state are illustrated in figure 1.1. For review, see (Lanciego et al., 2012).

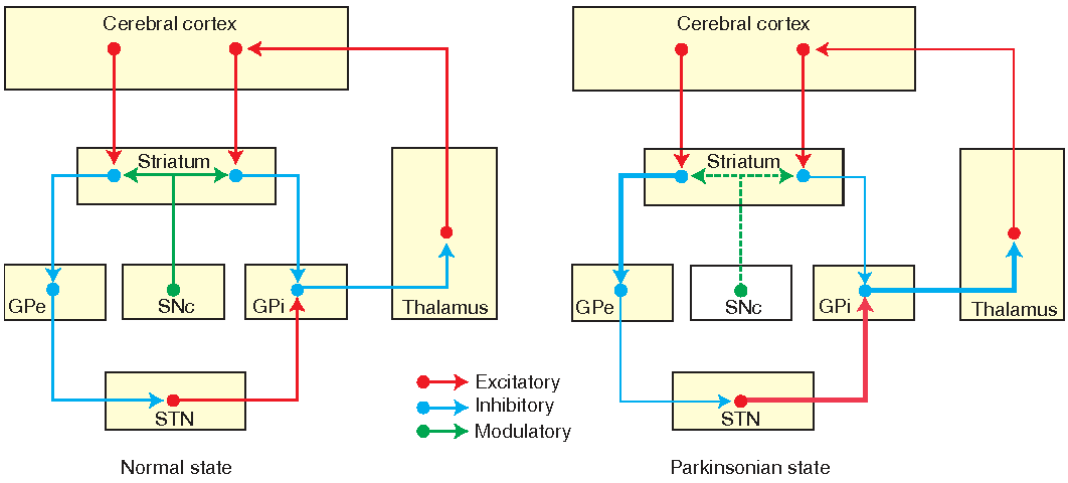


Figure1.1 Schematic diagram of the basal ganglia pathways regulating movement in both health and a Parkinsonian state. Thin lines represent hypoactivity, thick lines represent hyperactivity. Figure from Lanciego et al., 2012.

While it is unknown what event triggers an individual to develop PD, there are several processes at the cellular and molecular level that are known to contribute to the toxicity and neurodegeneration seen in the disease. A hallmark of PD pathology is the appearance of protein aggregates in the form of Lewy bodies or Lewy neurites. These are thought to begin in the dorsal motor nucleus of the glossopharyngeal and vagal nerves and the anterior olfactory nucleus and from here they spread in an ascending manner, showing little inter-individual differences (Braak et al., 2003). Limbic and motor systems appear to be particularly susceptible to developing these lesions (Braak and Braak, 2000). The main component of these aggregates has been shown to be α-synuclein (Spillantini et al., 1997). Mutations in the gene that encodes the α-synuclein protein which result in missense mutations (Polymeropoulos et al., 1997; Krüger et al., 1998; Zarranz et al., 2004), gene duplications (Nishioka et al., 2006) and gene triplications (Singleton et al., 2003) are all associated with autosomal dominant forms of PD. Similarly, polymorphisms in the transcriptional regulatory

region of the α-synuclein gene are associated with the development of sporadic PD (Farrer et al., 2001).

From this it can be seen that α-synuclein plays a key role in PD pathology. The exact functions of α-synuclein under normal physiological conditions is not yet known. However, it is known that α-synuclein protein is localised at the pre-synapse throughout the brain, and so may play a role in synaptic functions (Kahle et al., 2000; Fortin et al., 2004; Cheng et al., 2011). There is also evidence that it can influence mitochondrial function via interaction with ATP-synthase (Ludtmann et al., 2016). It also appears to interact with tubulin and in this way regulate microtubule dynamics (Zhou et al., 2010). In fact, missence mutations in the α-synuclein gene can alter cytoskeletal motility (Prots et al., 2013). In this way, α-synuclein is implicated in the mitochondrial dysfunction and transport alterations seen in PD. For review, see (Rocha et al., 2018). While a fibrillar form of α-synuclein is found in Lewy bodies, it is the soluble oligomeric form of the protein that is generally regarded to be the toxic species (Winner et al., 2011; Choi et al., 2013; Roberts et al., 2015). α-synuclein is subject to many forms of post-translational modifications, many of which seem to favour oligomerisation (Kim et al., 2011; Xiang et al., 2013; Brahmachari et al., 2016), reviewed by (Barrett and Timothy Greenamyre, 2015).

Several risk factors have been identified, both genetic and environmental, and it is thought that the majority of cases are brought about by the interaction of the two (Lesage and Brice, 2009; DeMaagd and Philip, 2015b; Bellou et al., 2016). This form of the disease is referred to as ‘sporadic’ or ‘idiopathic’ Parkinson’s Disease. However, a minority of cases, estimated at 5%-10%, are familial forms of the disease

caused by monogenic mutations. A number of genes have been identified from case studies of families which show inheritance of the disease. These genes include α-synuclein, UCHL1, LRRK2 (Khan et al., 2005), Omi/Htra2 (Unal Gulsuner et al., 2014) (resulting in autosomal dominant forms of the disease), Parkin (Lücking et al., 2000), PINK1 (Valente et al., 2004), DJ-1 (van Duijn et al., 2001) and ATP13A2 (Ramirez et al., 2006) (resulting in autosomal recessive forms of the disease). For review, see (Lesage and Brice, 2009; Bekris et al., 2010; Kalinderi et al., 2016).

The genome itself is not the only focus of attention. Epigenetic dysregulation has also been linked to neurodegenerative disorders and other complex disease states, such as cancer (Sanchez-Mut et al., 2016), for review see (Qureshi and Mehler, 2011). The term 'epigenetics' refers to those mechanisms which can alter gene expression or function without making any changes to the sequence of the DNA. This allows for the appearance of several phenotypes arising from the one genotype. These mechanisms include DNA methylation, post-translational modification of histone proteins and nucleosome positioning, reviewed by (Portela and Esteller, 2010; Kagohara et al., 2018; Morgado-Pascual et al., 2018). Altered DNA methylation has been observed in PD patients (Masliah et al., 2013). Particular focus has been given to the hypomethylation at the intron 1 promotor region of the α-synuclein gene (SNCA) (Ai et al., 2014; Pihlstrøm et al., 2015; Jakubowski and Labrie, 2017; Navarro-Sánchez et al., 2018). As demethylation of DNA is associated with gene activation (Pedanou et al., 2016; Lang et al., 2017), it is thought that this hypomethylation of the SNCA gene contributes to the overexpression of α-synuclein seen in PD (Jowaed et al., 2010; Matsumoto et al., 2010). Dysregulation of histone modifications is also seen in PD (Feng et al., 2015). Chromatin structure can be remodelled through histone

acetylation/deacetylation. Acetylated chromatin becomes less condensed and therefore is more accessible to transcription factors (Hamam et al., 2019). Deacetylation causes condensation of chromatin, which results in a reduction in gene transcription (Ranganathan et al., 2016). In healthy cells, histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). In neurodegenerative disorders this becomes unbalanced in favour of deacetylation (Rouaux et al., 2003; Sancho-Pelluz et al., 2010; McFarland et al., 2012), for review see (Saha and Pahan, 2006). A 2006 study found that α-synuclein associates with histones, inhibiting histone acetylation and that aggregation of α-synuclein in the nucleus promotes cell death in SH-SY5Y cells and *drosophila* and that this could be prevented by treatment with HDAC inhibitors (HDACIs) (Kontopoulos et al., 2006). This study indicates that aggregation of α-synuclein can disrupt the balance between HAT and HDAC activity, resulting in neurodegeneration, and that treatment with HDACIs can restore this balance, resulting in neuroprotection. A 2018 study showed that the incubation of N27 mesencephalic dopaminergic neurons with lactacystin (a proteasome inhibitor) results in significantly decreased levels of histone acetylation (Harrison et al., 2018). As epigenetic markers are subject to change by environmental factors (Turner, 2009), they may serve as the point of interaction between genetic and environmental risk factors in complex diseases such as PD.

1.1.2 Current treatments

There is, as of yet, no treatment available that is capable of slowing or halting the progress of PD. Current treatments focus solely on alleviation of symptoms, particularly through dopamine replacement or dopamine receptor agonism. These

approaches tend to provide effective symptomatic relief for the first few years after diagnosis, however, their efficacy begins to wear off over time. For reviews, see (Pezzoli and Zini, 2010; DeMaagd and Philip, 2015a).

Introduced in the 1960's, levodopa (L-dopa) is still the 'gold standard' for the treatment of PD-related motor symptoms (Pezzoli and Zini, 2010; Poewe et al., 2010). L-dopa is used as a dopamine replacement therapy. It is a precursor to dopamine and is converted to dopamine in dopaminergic neurons, by L-aromatic amino acid decarboxylase (L-AAAD) (Molinoff and Axelrod, 1971). This precursor is administered orally as dopamine itself cannot cross the blood brain barrier. As a treatment, L-dopa is effective against bradykinesia and rigidity, though its effect against tremor can be variable (DeMaagd and Philip, 2015a). Response to L-dopa is one requirement for the diagnosis of PD.

While treatment with L-dopa can initially significantly improve symptoms for most patients, its usage has its limits. L-dopa is not only converted to dopamine in the brain, but in the periphery also. For this reason, it is common to prescribe a peripherally acting inhibitor of dopamine decarboxylase, such as carbidopa, in order to increase the amount of L-dopa that reaches the brain. The two can be prescribed as a single formulation. L-dopa is also subject to high first-pass metabolism and has a short half-life (0.7-1.5hrs), all of which means that the amount of L-dopa actually reaching the brain can vary between doses. For a review of L-dopa pharmacokinetics and its co-administration with peripherally-acting enzyme inhibitors see (Contin and Martinelli, 2010; Gershanik, 2015). Treatment with L-dopa is most effective for the first three to five years. Following this, a number of motor complications begin to

develop (Schrag and Quinn, 2000; Ahlskog and Muenter, 2001). At this point, most patients develop levodopa-induced dyskinesia or dystonia (Barbeau, 1974), for review see (Calabresi et al., 2010). These can be painful and debilitating. It is thought that the short half-life and the continued loss of dopaminergic neurons due to the disease progression contribute to the change in response to L-dopa treatment (Fieblinger et al., 2014). When this begins, the individual often requires the addition of adjunct treatments to their prescription. These can include any combination of dopamine agonists, COMT inhibitors, MAO-B inhibitors or amantadine. For a review of PD pharmacotherapy see (DeMaagd and Philip, 2015a). Aside from L-dopa-induced motor disorders, there is also a decrease in L-dopa's efficacy in treating existing motor problems. "Wearing-off" is the phenomenon whereby motor symptoms re-emerge before the next scheduled dose of L-dopa. Fluctuations in motor symptoms have been reported to be present in 60-90% of patients after 5-10 years of treatment with L-dopa, reviewed by (Pahwa and Lyons, 2009; Mark, 2010; DeMaagd and Philip, 2015a).

Clearly the pharmacotherapy of PD is complex. The side-effects of treatment with L-dopa can begin to outweigh the benefits of the drug. This, coupled with the continued progression of the disease, can impact the comfort and quality of life of both patients and carers alike. For this reason, it is important to continue to search for new treatments, with the ability to alter the course of disease progression.

1.2 Next generation of treatments

1.2.1 Neurotrophic factors

Neurotrophic factors are biomolecules which are involved in the growth, differentiation and survival of neurons in the developing nervous system (Ernfors et al., 1994; Liu et al., 1995; Gouin et al., 1996) and continue to support the function and survival of neurons in the mature nervous system (Dreyfus et al., 1999; Gordon, 2010) . Due to their roles in the development and survival of neurons, neurotrophic factors offer a promising alternative to the purely symptom-managing treatments currently in use for neurodegenerative disorders. For reviews see (Sullivan and Toulouse, 2011; Hegarty et al., 2014a; Sullivan and O'Keeffe, 2016; Sampaio et al., 2017). There is hope that their supportive functions can be exploited to enhance the survival of the remaining neurons not yet killed by the disease or to encourage the integration of tissue grafts transplanted to replace lost cells (Wang et al., 2016; Moriarty et al., 2019). In these ways, neurotrophic factors have the potential to be used in the development of treatments that alter the progression of neurodegenerative disorders instead of simply acting to manage symptoms.

The first neurotrophic factor discovered and characterised was nerve growth factor (NGF) by Levi-Montalcini and Hamburger in the 1940's and 1950's, for review see (Levi-Montalcini, 1987; Aloe, 2004). Since then, numerous other neurotrophic factors have been discovered which have been categorised into 'families' based on their sequence homology and functions, for reviews, see (Itoh and Ornitz, 2011; Skaper, 2012; Weiss and Attisano, 2013). Of these families, the transforming growth factor β (TGF-β) superfamily is the one that is particularly associated with the growth and

maintenance of midbrain dopaminergic neurons (Krieglstein et al., 1995a; Farkas et al., 2003; Roussa et al., 2004), for review see (Hegarty et al., 2014b; Luo and Huang, 2016). This superfamily consists of a large number of evolutionarily conserved cytokines which can be broken into a number of subfamilies: TGF-β's, bone morphogenic proteins (BMPs), activins, inhibins, nodals and growth/differentiation factors (GDFs) (Massagué, 1998; Wrana, 2013). The members of this superfamily act as either hetero- or homodimers. They contain a characteristic cysteine knot motif created by intramolecular disulphide bonds. Signalling by the members of this group requires the involvement of two types of ligand-specific serine/threonine kinase receptors (Type I and Type II) (Massagué, 1998). When the ligand binds the constitutively active Type II receptor, the receptor phosphorylates the Type I receptor. The Type I receptor then phosphorylates Smad proteins. This initiates a cascade reaction, referred to as the 'canonical pathway', that results in Smad protein complex translocation into the nucleus and the regulation of gene transcription (Shi and Massagué, 2003). Members of the TGF-β superfamily can also signal through Smad-independent pathways, known as 'non-canonical pathways (Jung et al., 2017).

1.2.2 Clinical trials of neurotrophic factor therapy

Several clinical trials have taken place to assess the effects of neurotrophic factors in PD patients. These trials have focused on glial cell-derived neurotrophic factor (GDNF) and neurturin, for review see (Sullivan and Toulouse, 2011; Sullivan and O'Keeffe, 2016).

The results of these clinical trials have been mixed. A randomised, double-blind, placebo-controlled study which administered GDNF via intracerebroventricular (ICV)

catheter found no improvement in disease symptoms (Nutt et al., 2003). Patients experienced a number of adverse effects including nausea, anorexia and weight-loss (Nutt et al., 2003). A subsequent, open-label trial administered GDNF via intraputaminal infusion (Gill et al., 2003). This study did not find any adverse events and did report an improvement in both motor and non-motor symptoms (Gill et al., 2003). Furthermore, a follow-up study found that the treatment was safe and well tolerated two years following the GDNF treatment (Patel et al., 2005). A second open-label study which administered GDNF via intraputaminal infusion also reported an improvement in motor symptoms and found the treatment to be safe (Slevin et al., 2005). However, despite these promising results, a randomised, double-blind, placebo-controlled study, which also used intraputaminal infusion as the method of GDNF delivery, did not find improvements in motor symptoms by the primary end-point (Lang et al., 2006). This study also reported that several participants developed antibodies to GDNF (Lang et al., 2006), which raises questions about the safety of the treatment method.

The case of neurturin is somewhat similar to that of GNDF. An initial open-label trial, which administered AAV2-neurturin intraputaminally found the treatment to be safe, and observed improvements in disease symptoms (Marks et al., 2008). However, a subsequent randomised double-blind controlled study, also administering AAV2-neurturin intraputaminally, found no significant improvement in the primary outcome measures 12 months following treatment (Marks et al., 2010). This second study did find a modest improvement in primary outcome measures at 18 months following treatment, suggesting a delayed effect (Marks et al., 2010). Clinical trials for GDNF and neurturin therapy in PD patients have not proven to be

as successful as pre-clinical research had suggested they would. In light of this, it is important to continue the search for other neurotrophic factors that will work in the human context.

1.2.3 Growth differentiation factor 5 (GDF5)

GDF5 is a member of the BMP subfamily of the TGF-β superfamily of neurotrophic factors, for review, see (Bragdon et al., 2011). It plays an important role in embryonic and foetal development (Shwartz et al., 2016). It is particularly associated with the development of the skeleton and joints (Hatakeyama et al., 2004; Kiapour et al., 2018; Pregizer et al., 2018) with mutations in this gene being associated with osteochondrodysplagias (Farooq et al., 2013; Khan et al., 2018). GDF5 is also involved in the development and survival of dopaminergic neurons (Krieglstein et al., 1995b; O'Keeffe et al., 2004). Importantly, its signalling mechanism is RET-independent, unlike GDNF and neurturin. GDF5 has been shown to signal via BMP receptor 1 on SH-SY5Y cells, which then activates Smad 1/5/8 signalling (Hegarty et al., 2013). This is important as RET has been shown to be down-regulated by over-expression of human wild-type α-synuclein in rats via adeno-associated viral (AAV) vector injected into the SN (Decressac et al., 2012b). This may explain the disappointing results of the GDNF clinical trials in PD patients. This is because GDNF signals via the RET receptor (Durbec et al., 1996) and if this receptor is down-regulated by α-synuclein then GDNF will not be able to enact its neurotrophic effects on dopaminergic neurons in PD patients.

1.2.4 Gene therapy

Gene therapy is a field of research that has proved promising for a number of different disorders, with many early-stage clinical trials demonstrating the feasibility of genome editing/gene delivery, as well as the safety of doing so in human patients, reviewed by (Naldini, 2015; Ginn et al., 2018). In regard to neurotrophic factor therapy for PD, the introduction of the gene encoding the neurotrophic factor, instead of the protein itself, would allow for continued expression of the protein locally and thereby prolong the beneficial effects of the therapy. The use of viral vectors to deliver genes encoding neurotrophic factors is currently an area of great interest, in particular, research into the use of AAV vectors, for review, see (Vandenberghe et al., 2009; Kotterman and Schaffer, 2014; Kelly et al., 2015).

The AAV genome consists of single-stranded DNA (Rose et al., 1969), which in the wild-type virus integrates into the human host genome in a site-specific manner, at a site known as AAVS1 on human chromosome 19 (Kotin et al., 1992). In the absence of helper viruses (adeno virus for example), AAV enters a lysogenic lifecycle (Leonard and Berns, 1994). There are several factors which make AAV vectors attractive for gene therapy. The virus is not currently associated with disease in humans and elicits only a mild immune response (Naso et al., 2017), it can infect both dividing and non-dividing cells (Chen et al., 2018), the variety of serotypes with different tropisms allows for the targeting of specific cell populations (Castle et al., 2016; Hickey et al., 2017), and while recombinant AAV (rAAV) usually cannot insert into the host cell genome it can exist in an extrachromosomal form for extended periods of time (Choi

et al., 2006). However, the usefulness of AAV as a vector for gene therapy is limited by its small genome carrying capacity.

1.2.5 Histone deacetylase inhibitors (HDACIs)

The HDACs are an enzyme superfamily which can be broken up into several classes based on sequence homology: Class I (HDAC1,2,3,8), Class IIa (HDAC4,5,7,9), Class IIb (HDAC6,10), and Class IV (HDAC11) (Gregoretti et al., 2004). Sirtuins, a group of NAD-dependent enzymes are sometimes called Class III HDACs (Tanner et al., 2000). They are, however, unrelated to other classes. HDACs play a role in gene transcription regulation via deacetylation of histone proteins, though they interact with non-histone proteins also (Glozak et al., 2005). Some HDACs have the ability to shuttle between the nucleus and the cytosol, (Chawla et al., 2003).

Our group is particularly interested in Class IIa HDACS as the expression of two of these, HDAC5 and HDAC9, has been shown by our group to positively correlate with the expression of three different markers of dopaminergic neurons in the SN of both humans and mice (Mazzocchi et al., 2019). For this reason, this study will focus on these two HDACs. We will also look at HDAC3 as an example of another class of HDAC. In the case of HDAC5, we will examine its location within the cell as it undergoes this shuttling between the nucleus and cytosol.

As has been mentioned previously, there is an imbalance in HAT/HDAC activity in favour of deacetylation in neurodegenerative disorders, reviewed by (Saha and Pahan, 2006). One possible explanation for this imbalance is the overexpression of α-synuclein, which may 'mask' histones from the actions of histone acetylases. It has been reported that α-synuclein aggregation had a greater cytotoxic effect when the

protein was sequestered in the nucleus rather than the cytosol and that the A53T and A30P mutations (associated with familial forms of PD) had a greater tendency to localise in the nucleus. Cell loss induced by overexpression of either wild type α-synuclein or a variant which was targeted primarily to the nucleus, was reduced in both SH-SY5Y cells and *drosophila* when treated with the HDAC inhibitors (HDACIs) sodium butyrate or suberoylanilide hydroxamic acid (SAHA) (Kontopoulos et al., 2006).

HDACIs have been shown to reduce cell death in toxin-induced degenerative models of PD both *in vitro* and *in vivo*. The HDACIs sodium butyrate, valproate and SAHA have been reported to improve the survival of dopaminergic cells from 1-methy-phenypyridinium ion (MPP^+)-induced cytotoxicity *in vitro* (Kidd and Schneider, 2010). MPP^+ is a cation that arises from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Valproate has also been shown to be neuroprotective in mice after MPTP-induced degeneration of the nigrostriatal pathway (Kidd and Schneider, 2011). In this case, there was a greater neuroprotective effect in the SN than in the striatum, however the authors speculate that this may be due to the short study period preventing cell bodies from re-establishing connections in the striatum. Two other HDACIs, MS-275 (a selective inhibitor of HDAC1) and tubastatin A (TBA) (a selective inhibitor of HDAC6) have also been shown to reduce the MPP^+-induced reduction in diencephalic tyrosine hydroxylase (TH) positive neurons in zebrafish (Pinho et al., 2016).

HDACIs offer a possible avenue for the development of disease-altering treatment strategies for PD. Valproic acid has been shown to increase expression of the

neurotrophic factors GDNF and BDNF in astroglial primary cultures (Chen et al., 2006; Yasuda et al., 2009). Sodium butyrate has been shown to reduce inflammatory response in primary microglial cultures treated with lipopolysaccharide (LPS) (Huuskonen et al., 2004), while pre-treatment with valproic acid has been shown to protect dopaminergic neurons in rat primary midbrain cultures from LPS-induced cell death (Peng et al., 2005). HDACIs have also been shown to reduce oxidative stress-induced cell death in primary neuronal cultures exposed to high concentrations of glutamate (Langley et al., 2008). As inflammation and oxidative stress are both involved in the pathophysiology of PD (Fischer and Maier, 2015), these features of HDACIs add to their potential as a treatment.

1.3 Modelling Parkinson's disease

An important element in the research of any disease is the development of suitable models which can be used to elaborate on the underlying mechanisms of the disease or to test the efficacy of potential new treatments. Accurate models are a valuable resource as they allow for investigations that would not be possible in human patients. However, creating models which faithfully represent a human disease state provides its own challenges. In the case of PD, the condition has not been reported to occur naturally in any other species apart from humans. As such, animal models must be generated by artificial means, using neurotoxins for example. These artificially generated models will, of course, differ from the real-life disease in certain aspects, which adds a level of complexity to interpreting results obtained from their use. Therefore, it is important to understand the characteristics of the commonly used models as well as possible. That being said, there are a number of PD models

which are able to mimic a number of aspects of the disease. These are generally grouped into two categories: neurotoxic models and genetic models. Neurotoxic models utilise compounds which are capable of causing selective degeneration of dopaminergic neurons. Such compounds include 6-hydroxydopamine (6-OHDA) (Jing et al., 2016; Jalewa et al., 2017; Casarrubea et al., 2019), MPTP (Muñoz-Manchado et al., 2016; Bové et al., 2017), rotenone (De Miranda et al., 2019; Tapias et al., 2019) and paraquat (Cristóvão et al., 2020), reviewed by (Tieu, 2011; Blesa et al., 2012; Zeng et al., 2018). Genetic models of PD usually either overexpress those genes associated with PD in humans (α-synuclein for example) in either their native form or in mutated forms (Decressac et al., 2012a; Van der Perren et al., 2015), or these models knock out disease-associated genes in the animal model (Kuldip et al., 2014; Villeneuve et al., 2016). For reviews of the use of the models used in Parkinson's disease research, please see (Dawson et al., 2010; Blesa et al., 2012; Lee et al., 2012).

1.3.1 6-OHDA model

6-OHDA was the first neurotoxin used to induce neurodegeneration specifically in catecholaminergic neurons (Ungerstedt, 1968) and is still commonly used to generate animal models of PD. 6-OHDA is a neurotoxic compound whose structure closely resembles that of dopamine. Due to this similarity in structure, 6-OHDA can be taken up into catecholaminergic neurons via the dopamine transporter (DAT) or noradrenaline transporter (NET), thus making it selectively toxic to this population of neurons. This selectivity makes it a useful tool in the modelling of PD in animals and it is commonly used to induce neurodegeneration in the nigrostriatal system in

rodent and primate models, for reviews, see (Mitra et al., 2015; Santana et al., 2015; Ren et al., 2017).

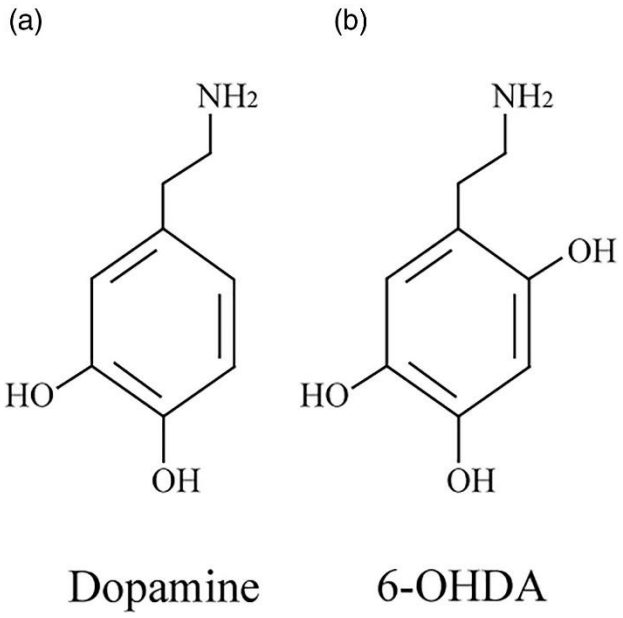


Figure1.3: The structural formulae of dopamine and 6-hydroxydopamine (6-OHDA), showing their similarity. Figure modified from Zeng et al., 2018.

The toxicity of 6-OHDA results from the overproduction of reactive oxygen species (ROS) and oxidative stress, for review, see (Hernandez-Baltazar et al., 2017). Several mechanisms have been proposed to be involved. 6-OHDA is highly oxidisable and so the production of ROS may stem from the auto-oxidation of 6-OHDA (Hanrott et al., 2005) or from the oxidation of 6-OHDA by the action of monoamine oxidase (MAO) enzyme, which is present in catecholaminergic neurons. The increased production of ROS may also stem from 6-OHDA-induced disruption of mitochondrial function (Marques et al., 2019). Dopaminergic neurons are particularly susceptible to oxidative stress. This is due to the natural presence of a larger number of ROS in

dopaminergic neurons than in other neuronal populations, as the normal metabolism of dopamine produces hydrogen peroxide (Meiser et al., 2013). This may be why 6-OHDA is so effective at inducing degeneration in dopaminergic neurons. It is important to note that oxidative stress is known to play a role in the neurodegenerative process in PD patients, for review, see (Burbulla et al., 2017).

There are several variations of the 6-OHDA model of PD, differing in where along the nigrostriatal pathway the lesion is made and whether the lesion is unilateral or bilateral, for review, see (Duty and Jenner, 2011; Bové and Perier, 2012). Due to the small size of relevant brain structures, these lesions are more commonly performed on rats than mice for ease of locating the injection site more accurately. The lesion may be made in the SNc, the medial forebrain bundle (MFB) or in the striatum. While each of these lesions produces loss of dopaminergic neurons, resulting in motor deficits, there are subtle differences in the degree and rate of degeneration and in the behaviour of the animals, for reviews, see (Deumens et al., 2002; Tieu, 2011). Lesions of the MFB can result in near complete degeneration of A9 (nigrostriatal pathway) and A10 (mesolimbic pathway) dopaminergic neurons, although this is more extensive than the degeneration seen in humans with PD. However, protocols for lesions more specific to the A9 group also exist and provide a more accurate model (Perese et al., 1989). Rats with these lesions not only show deficits in motor functions on the affected side, they also show deficits in cognitive function as tested by the Morris water maze (Whishaw and Dunnett, 1985). Lesions of the SNc result in more moderate degeneration. There is a degree of sparing of cells in the medial SN, which mimics the pattern of degeneration in human patients, which is mostly lateral (German et al., 1989). There is evidence to suggest that unilateral lesions of the SNc

can result in bilateral deficits in limb usage (Whishaw et al., 1992). Finally, lesions of the striatum can give different results based on the region within the striatum targeted and the number of doses administered. For example, lesions to the dorsomedial part of the striatum tend to result in a deficit in locomotion and in drug-induced turning behaviour, while lesions of the ventrolateral part of the striatum tend to result in deficits in movement initiation, sensorimotor orientation and skilled motor behaviours (Deumens et al., 2002). Injection of increasing doses of 6-OHDA to the striatum can be used to induce increasing levels of degeneration and, in this way, be used to model varying degrees of severity of PD (Przedborski et al., 1995).

While 6-OHDA lesioned animals reproduce some aspects of PD, there are several limitations to this approach. One of these is the timescale on which the degeneration occurs. Degeneration of dopaminergic neurons in humans with PD is a slow and progressive process. However, the degeneration induced by neurotoxins generally occurs more rapidly than is the case with the human disease. Administration of 6-OHDA to either the SNc or the MFB results in rapid degeneration within a few days, though administration to the striatum results in a slower, retrograde degeneration occurring over the course of 3-4 weeks (Dauer and Przedborski, 2003; Shimohama et al., 2003). Despite these limitations, the 6-OHDA model of PD is a well-established and characterised animal model and is commonly used in the search for improved therapies for the disease. For example, this model can reproduce the L-DOPA-induced dyskinesia observed in PD patients who have been using the drug for a prolonged period of time, and so may be used to develop our understanding of the phenomenon. For review, see (Tronci and Francardo, 2018). This model has also recently been used to assess the efficacy of such novel treatments as intranasal

insulin (Pang et al., 2016), glucose-dependant insulinotropic polypeptide (Yu-Wen et al., 2018) and a small molecule SERCA activator (Dahl, 2017).

1.3.2 The 6-OHDA model and HDAC expression

In 2007, Broide *et al.* carried out a study to map the expression of HDAC isoforms 1-11 in the normal rat brain (Broide et al., 2007). The study found that the isoforms each had a distinct expression profile within the brain, though there were overlaps between isoforms. HDAC expression mostly occurred in neurons, though certain isoforms, including HDAC3 and HDAC5, were found to be expressed at lower levels in oligodendrocytes also. Both HDAC3 and HDAC5 were found to have a moderate level of expression in the striatum. The expression of these two isoforms was slightly higher in the SNc. HDAC9 was found to be expressed in both the striatum and SNc, though at much lower levels than either HDAC3 or HDAC5.

As has been previously discussed, there is an alteration in HDAC activity seen in PD (Saha and Pahan, 2006) and HDACIs have shown promise as a neuroprotective strategy, for review, see (Coppedè, 2014). However, there have been no reports on the effects of 6-OHDA lesions on the expression of HDACs in the rat brain. That being said, there is evidence that the expression of other genes and proteins is altered following administration of 6-OHDA. Microarray analysis of striatal and nigral tissue from 6-OHDA lesioned rats found that, up to four weeks following 6-OHDA administration, a large number of genes were upregulated in both the striatum and SN, with a smaller number of genes being downregulated in the SN (Na et al., 2010). These genes were mostly associated with immune response and cell signalling. Expression of D_1 dopamine receptors has been found to be reduced in response to

6-OHDA (Gerfen and Engber, 1990; Qin et al., 1994). One study found that 6-OHDA-induced dopamine depletion in neonatal rats resulted in elevated levels of GAD65 and GAD67 mRNA in the striatum of these animals in adulthood, along with elevated levels of preproenkephalin and preprodynorphin mRNA (Laprade and Soghomonian, 1999). Another study found increased levels of GAD65 and enkephalin mRNA and a decrease in dynorphin mRNA levels in the striatum of 6-OHDA lesioned rats (Carta et al., 2001).

1.4 Aims

The first aim of this study is to assess the neuroprotective and neurorestorative efficacy of an AAV vector in delivering GDF5 to the SN, in adult rats with intrastriatal 6-OHDA lesions. The delivery of neurotrophic factor genes via viral vectors, rather than administration of the proteins themselves, is preferable for the treatment of PD due to the longer lasting effects of the intervention. If the protein were administered on its own, it would be degraded over time. This would require the patient to undergo repeated surgeries and therefore repeatedly expose them to the risks associated with surgery (infection etc.). With the delivery of the gene instead, the neurotrophic factor can be expressed in the patient's brain for an extended period of time. For this reason, it is important to examine the efficacy of viral vectors in delivering neurotrophic factors.

The second aim of this study is to examine the expression of HDAC3, 5 and 9 in the adult rat striatum and SN at 21 days and 28 days after intrastriatal administration of 6-OHDA. As HDACs have emerged as a promising target for PD treatment it is crucial to characterise the effects of common PD modelling techniques on their expression.

1.5 Objectives

- To assess the effect of intranigral AAV-GDF5, administered at the same time as intrastriatal 6-OHDA, on 6-OHDA-induced degeneration of nigrostriatal dopaminergic neurons, using immunohistochemical staining for TH in the striatum and the SN.

- To assess the effect of intranigral AAV-GDF5, administered 2 weeks prior to intrastriatal 6-OHDA, on 6-OHDA-induced degeneration of nigrostriatal dopaminergic neurons, using immunohistochemical staining for TH in the striatum and the SN.

- To assess the effects of intrastriatal 6-OHDA on the expression of HDAC3 and HDAC9 in the striatum and the SN, at 21 and 28 days after surgery, using immunohistochemistry.

- To examine the cellular location of HDAC5 expression in the SN, at 21 and 28 days after intrastriatal 6-OHDA, using immunohistochemical staining for HDAC5 and TH.

2. Materials and methods

2.1 Animals

Sprague-Dawley rats were group-housed with food and water *ad libitum* in a temperature-controlled room (21°C± 2°C) and a 12-h light/dark cycle (lights on at 08:00h). Animals were randomly assigned to one of six groups (Table 2.1): Vector Control (saline and AAV2/5-null) (n=7), AAV-GDF5 (saline and AAV-GDF5) (n=7),

Neurorestoration Control (6-OHDA and AAV2/5-null) (n=7), Neurorestoration (6-OHDA and AAV-GDF5) (n=7), Neuroprotection Control (AAV2/5-null followed two weeks later by 6-OHDA) (n=7), Neuroprotection (AAV-GDF5 followed two weeks later by 6-OHDA) (n=7). A further group of rats (Control, n=4) received no treatment.

All procedures were approved by the Animal Ethics Committee of University College Cork in accordance with the European Directive 2010/63/EU, and under an authorisation issued by the Health Products Regulatory Authority Ireland. HPRA project: AE19130/P057. All animal work was carried out by Dr. Ruth Concannon.

Experiment 1 – Neuroprotective and neurorestorative effects of intranigral AAV-GDF5 against intrastriatal 6-OHDA lesion in adult rats

Group	**n**	**Name**	**Surgery 1**	**Time between surgeries**	**Surgery 2**
1	7	Vector Control	Saline (STR)	none	AAV2/5-null (SN)
2	7	AAV2/5-GDF5	Saline (STR)	none	AAV2/5-GDF5 (SN)
3	7	Neurorestoration Control	6-OHDA (STR)	none	AAV2/5-null (SN)
4	7	Neurorestoration	6-OHDA (STR)	none	AAV2/5-GDF5 (SN)
5	7	Neuroprotection Control	AAV2/5-null (SN)	2 weeks	6-OHDA (STR)
6	7	Neuroprotection	AAV2/5-GDF5 (SN)	2 weeks	6-OHDA (STR)

Table 2.1: Treatment groups in experiment 1. STR=striatum, SN=substantia nigra

Experiment 2: Effects of intrastriatal 6-OHDA lesion on HDAC expression in the adult rat brain

Group	n	Name	Treatment	Time between lesion and culling
7	4	Control	none	N/A
5	7	Neuroprotection Control	6-OHDA (STR)	21 days
3	7	Neurorestoration Control	6-OHDA (STR)	28 days

Table 2.2: Treatment groups in experiment 2.

2.2 Stereotaxic surgery

Animals were anaesthetised using isoflurane (4-5% for induction and 2-3% for maintenance of anaesthesia). A stereotaxic guide was used to locate the regions of interest (striatum and substantia nigra). The co-ordinates of the injection in the substantia nigra were as follows: A.P.-5.3, M.L.+/-2.0, D.V.-7.2. The co-ordinates of the striatal injection were as follows: A.P. +/-0.0, M.L.+/-3.7, D.V. -5. All co-ordinates are relative to bregma. Saline, AAV-null, AAV-GDF5 and/or 6-OHDA solutions were injected via infusion at a rate of 0.5-1µl/min. The total volume of AAV-null or AAV-GDF5 administered was 1µl. The total volume of saline or 6-OHDA administered was 3µL. Viral vectors were obtained from Vector Laboratories. Following injection, the cannulae were allowed to remain in place for a further 2-3 min to reduce reflux and were then slowly withdrawn. Animals in groups 1-4 (Table 2.1) underwent surgery once, while animals in groups 5 and 6 underwent two surgeries. Following surgery,

animals were placed singly into cages on a heating pad and their condition monitored. Animals were allowed to recover for one week prior to further testing.

2.3 Tissue preparation

2.3.1 Transcardial perfusion

The tissue was fixed via transcardial perfusion. First, a lethal dose of pentobarbitone (200mg/kg) was administered via intraperitoneal injection. Toe-pinch reflex was used to assess whether the animal had been fully euthanised. Once it was established that the reflex was absent, the animal was placed in a dorsal recumbent position and the abdomen opened. A catheter was inserted into the aorta. The animal was injected with PBS followed by 4% paraformaldehyde (PFA). Once the entire body had been perfused the tissue was collected. Following removal from the skull, the brains were stored in PFA at 4°C overnight. Then the brains were transferred to 10% sucrose solution and stored at 4°C until they were sectioned. Perfusions were carried out by Dr. Ruth Concannon, Ms. Tara Foley and Ms. Susan Goulding.

2.3.2 Tissue sectioning

Sections were taken on a Bright 8000 Sliding Sledge Microtome. Section thickness was set to 40 µm. A one in six series of sections were taken. Sections were stored in 0.1% TBS azide at 4°C.

2.4 Immunostaining

2.4.1 Tyrosine hydroxylase immunostaining

Both striatal and nigral sections underwent immunohistochemical staining for TH. In both cases, a 1:6 series of free-floating sections was used. Sections were placed in

pots covered in a square of gauze to allow for the addition and draining of solutions. For all washing steps and incubations, the pots were left sit on a rocker set at 27osc/min at room temperature. Once transferred to the pots, the sections were washed three times with TBS for 5 min each. The tissue was then quenched (methanol: 2mL, H_2O_2: 2mL, distilled water: 16mL) for 5 min. This was followed by repetition of the previous washing step. Non-specific binding was then blocked by incubation with 3% normal horse serum in 0.2% Triton X-100 in TBS (TXTBS) for 1 h. The sections were then incubated overnight at room temperature in primary antibody solution at a concentration of 1:1000 in 1% normal horse serum in TXTBS (mouse anti-TH, MAB318, Merck). After removal of the primary antibody from the pots, the sections were washed three times with TBS for 10 min each. The sections were then incubated in a biotinylated horse anti-mouse secondary antibody at a concentration of 1:200 in 1% normal horse serum in TBS for 3 h (Biotinylated Horse-anti-mouse secondary, BA-2001, Vector.). This was followed by a repeat of the previous washing step. In order to amplify the signal, the sections were incubated in an avidin-biotin complex (ABC) for 2 h (Vectastain ABC Kit, PK-4000, Vector Laboratories Inc.). This was followed by another washing step. The sections were then removed from the rocker and left in TNS overnight at 4^{o}C. The staining was then visualised using 3,3'Diaminobenzidine (DAB peroxidase substrate kit, SK-4100, Vector Laboratories) as per the manufacturer's protocol. The sections were then mounted onto gelatine-coated slides and dehydrated before coverslipping.

2.4.2 HDAC3 and HDAC9 immunostaining

Both striatal and nigral sections underwent immunohistochemical staining for HDAC3 and HDAC9. Prior to staining, sections were mounted onto gelatine-coated slides. Once dried, a hydrophobic pen was used to draw around the sections (ImmEdge™ pen, Vector Laboratories Inc.). The sections were first washed in TBS for 5 min. 200µl of quench solution (4ml dist. H_2O, 500µl methanol, 500µl H_2O_2) were then added to each slide for 5 min. The sections were then washed three times in TBS, 5 min per wash. Non-specific binding was blocked by incubation with 3% normal horse serum in TXTBS for 1 h (200µl per slide). The sections were incubated overnight at room temperature with the primary antibody at a concentration of 1:50 in 1% normal horse serum in TXTBS (Anti-HDAC3, sc-376857, Santa Cruz or Anti-HDAC9, sc-398003, Santa Cruz). Following this incubation, the sections were washed three times in TBS, 10 min per wash. The sections were then incubated in a biotinylated horse anti-mouse secondary antibody solution at a concentration of 1:200 in 1% normal horse serum in TBS for 3 h (Biotinylated Horse-anti-mouse secondary, BA-2001, Vector Laboratories Inc.). This was followed by a repeat of the previous washing step. In order to amplify the signal, the sections were incubated in an avidin-biotin complex (ABC) for 2 h (Vectastain ABC Kit, PK-4000, Vector Laboratories Inc.). This was followed by another washing step. The sections were then allowed to sit in TNS overnight at 4°C. A humid incubation chamber was used to prevent evaporation of solutions during all incubations. The staining was visualised using 3,3'Diaminobenzidine (DAB peroxidase substrate kit, SK-4100, Vector Laboratories) as per the manufacturer's protocol. The sections were then dehydrated and the slides coverslipped.

2.4.3 HDAC5 immunostaining

Nigral sections underwent immunohistochemical staining for both HDAC5 and TH. This staining was done sequentially. Prior to staining, sections were mounted onto gelatine-coated slides. Once dried, a hydrophobic pen was used to draw around the sections (ImmEdge™ pen, Vector Laboratories Inc.). The sections were first washed in TBS for 5 min. 200µl of quench solution (4ml dist. H_2O, 500µl methanol, 500µl H_2O_2) were then added to each slide for 5 min. The sections were then washed three times in TBS, 5 min per wash. Non-specific binding was blocked by incubation with 3% normal goat serum in TXTBS for 1 h (200µl per slide). The sections were incubated overnight at room temperature with the anti-HDAC5 primary antibody at a concentration of 1:50 in 1% normal goat serum in TXTBS (Anti-HDAC5, sc-133106, Santa Cruz). Following this incubation, the sections were washed three times in TBS, 10 min per wash. The sections were then incubated in a goat anti-mouse fluorescent secondary antibody (Alexa Fluor 488, A11001, ThermoFisher Scientific) solution at a concentration of 1:500 in 1% normal goat serum in TBS for 2 h. Following this, the sections were washed three times in TBS, 10 min per wash. To prepare for the TH immunostaining, the tissue was re-blocked with 3% normal goat serum in TXTBS for 1 h. The sections were then incubated over night at room temperature with the anti-TH primary antibody at a concentration of 1:1000 in 1% normal goat serum (mouse anti-TH, MAB318, Merck). The sections were washed three times in TBS, 10 min per wash before incubation with a goat anti-mouse fluorescent secondary antibody (Alexa Fluor 594, A11005, ThermoFisher). For 2 h. Nuclei were visualised using DAPI staining. A humid incubation chamber was used to prevent evaporation of solutions during all incubations.

2.5 Microscopy

2.5.1 Brightfield microscopy

DAB-stained sections were imaged using brightfield microscopy on an Olympus BX53 Upright Microscope. In the case of those sections stained for TH, striatal sections were imaged at 2x magnification and nigral sections were imaged at 10x magnification (SN sections were imaged and analysed by Dr Ruth Concannon). In the case of those sections stained for HDAC3 and HDAC9, both striatal and nigral sections were imaged at 2x magnification. For striatal sections, both sides of the brain were included in the one image where possible, where this was not possible, separate images were taken of the left and right. For nigral sections taken at 2x magnification, one image was taken of the whole section. For nigral sections taken at 10x magnification, separate images were taken of the left and right of the brain. Images were taken of three sections for each animal.

2.5.2 Confocal microscopy

HDAC5 and TH co-stained sections were imaged on an Olympus FV1000 Confocal Laser Scanning Microscope. Sections were imaged at 60 x magnification. One image was taken on the lesioned side of the brain and one image was taken on the non-lesioned side of the brain per section and three sections were imaged per animal. The lasers used and their settings were as follows; 405 nm (HV=446, Gain=1.75, Offset=9, laser power=10.0%), 488 nm (HV=383, Gain=1.375, Offset=8, laser power=5.0%) and 543 nm (HV=588, Gain=2, Offset=9, laser power=29.0%).

2.6 Image analysis

2.6.1 Analysis of TH, HDAC3 and HDAC9 immunostianing

All analysis was carried out using Image J software. For striatal sections, the same method was used to analyse sections stained for TH, HDAC3 and HDAC9. Images were converted to 16-bit greyscale and the region of the striatum selected and the mean grey value (MGV) measured. The average MGV (AVMGV) for three sections per animal was then used to calculate the optical density, using the formula Log10(255/AVMGV). For nigral sections stained for TH, the cell bodies of TH^+ cells were counted and averaged across three sections per animal. For nigral sections stained for HDAC3 and HDAC9, images were converted to 16-bit greyscale and a region encompassing the SN was selected and the MGV measured. The average MGV (AVMGV) for three sections per animal was then used to calculate the optical density, using the formula Log10(255/AVMGV).

2.6.2 Analysis of HDAC5 immunostaining

Cytoplasmic and nuclear localisation of HDAC5 in dopaminergic neurons were analysed using Image J software. Three sections were analysed per animal with images being taken of both the left and the right side of each section. Three cells were analysed per image. The nuclei of individual neurons, as indicated by DAPI staining, were traced, and the intensity of the fluorescence given off by the HDAC5 immunostaining within this boundary was measured. The same process was repeated for cell bodies, as indicated by the TH immunostaining. In order to determine the intensity of the fluorescence of the cytoplasmic localisation of HDAC5,

the value obtained from the nuclear measurement was subtracted from the total measurement obtained from the cell body.

2.7 Statistical analysis

All data were analysed using GraphPad Prism 5 (GraphPad Software, USA). In both experiment 1 and experiment 2, one-way analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test was used to assess differences in staining on the lesioned side of the brain between experimental groups. For experiment 2, one-way ANOVA was also used to assess differences in HDAC5 localisation in TH immunopositive neurons between experimental groups. For experiment 2, two-way ANOVA followed by a Bonferroni *post hoc* test was used to assess whether changes in HDAC3 and HDAC5 expression were present on both lesioned and non-lesioned sides of the brain and how this changed over the timecourse of the experiment.

3. Results

3.1 Treatment with AAV-GDF5 did not prevent 6-OHDA-induced degeneration of striatal dopaminergic terminals

In order to assess the integrity of striatal dopaminergic terminals following intrastriatal injections of 6-OHDA and/or treatment with AAV-GDF5, striatal cryosections were immunostained for TH and the optical density of this staining was analysed (Figure 3.1). One-way ANOVA analysis showed that there was a significant difference between groups ($F(5,39)=14.20$, $P=0.0158$). Bonferroni post-hoc testing revealed that there was significantly lower intensity of immunostaining in each of the neurorestoration control, neuroprotection control, neurorestoration and neuroprotection groups compared to the Vector control group. Each of these four groups also exhibited significantly less TH immunostaining than that in the AAV2/5-GDF5 group. There was no significant difference between the Vector control group and the AAV2/5-GDF5 groups. There was also no significant difference between the neurorestoration control group and the neurorestoration group, or between the neuroprotection control group and the neuroprotection group.

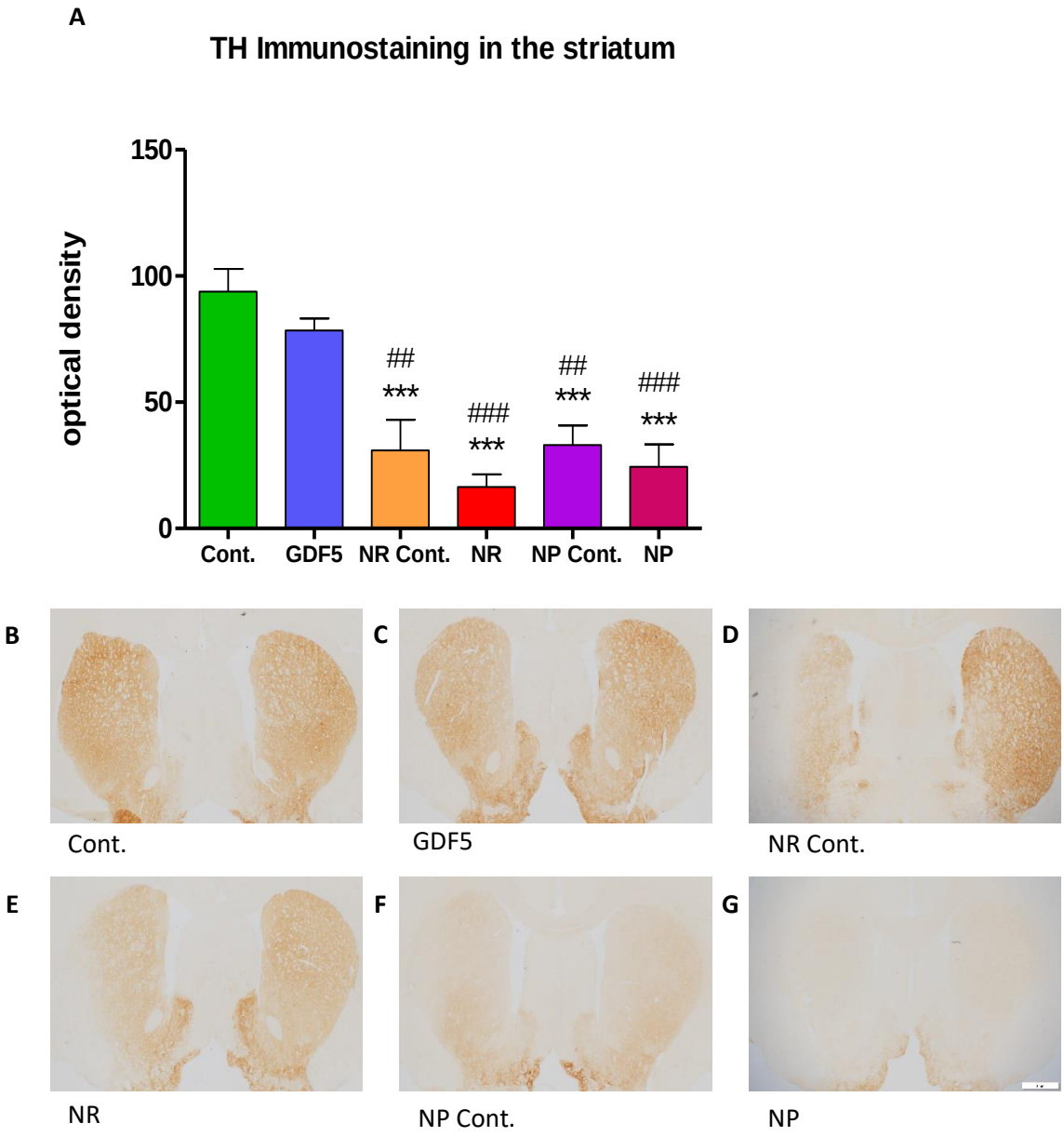


Figure 3.1: TH immunostaining in the striatum.

(A) Intensity of TH immunostaining, expressed as mean optical density, for each of the six experimental groups, as indicated. (B) – (G): Representitive photomicrographs of each of the six experimental groups. Cont. = Vector control, GDF5 = AAV2/5-GDF5, NR = neurorestoration, NP = neuroprotection. *** = $p<0.001$ compared to Vector control; ## = $p<0.01$, ### = $p<0.001$ compared to AAV2/5-GDF5; One-way ANOVA with Bonferroni post-hoc test. Values are expressed as mean ± SEM (n = 7). Scale bar = 1mm.

3.2 Treatment with AAV-GDF5 did not prevent 6-OHDA-induced degeneration of dopaminergic neurons in the SN

In order to assess dopaminergic cell survival within the SN the number of TH-immunopositive cell bodies were counted (Figure 3.2). One-way ANOVA analysis showed that there was a significant difference between groups ($F(5,35)=4.106$, $P=0.0049$). Bonferroni post-hoc testing revealed that there were significantly less TH-positive cell bodies in the neurorestoration control, neuroprotection control, neurorestoration and neuroprotection groups when compared to that in the control group. There were no significant differences between any other groups. There was a trend for TH staining to be lower in the AAV-GDF5 group than in the control group, though this difference did not reach significance ($P=0.1063$).

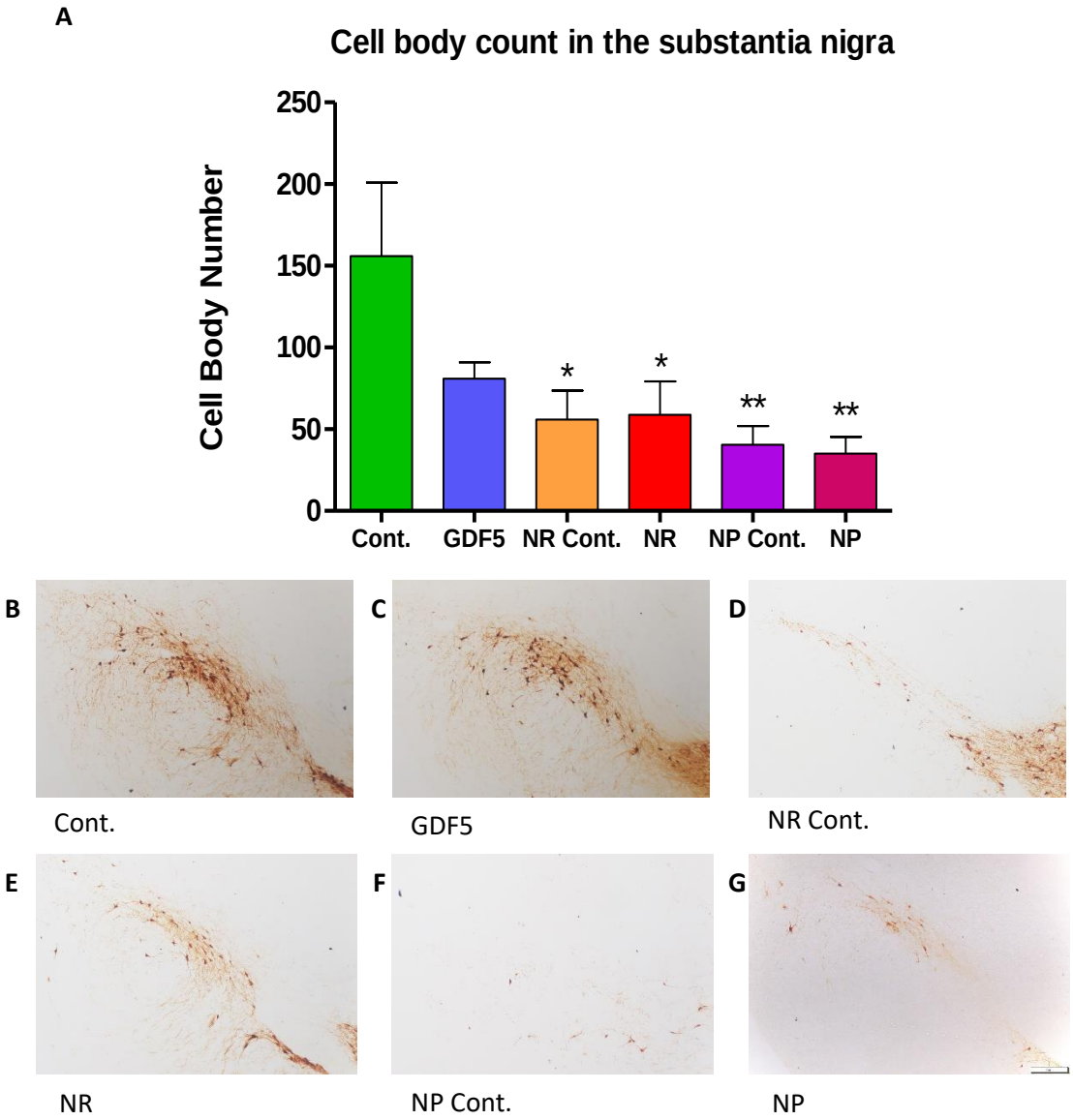


Figure 3.2: The number of TH-immunopositive cell bodies in the SN.

(A) Numbers of TH-positive cell bodies in of each of the six experimental groups, as indicated. (B) – (G): Representative photomicrographs taken from each of the six experimental groups. Cont. = Vector control, GDF5 = AAV2/5-GDF5, NR = neurorestoration, NP = neuroprotection. * = $P<0.05$, ** = $P<0.01$ compared to Vector control; One-way ANOVA with Bonferroni post-hoc test. Values are expressed as mean ± SEM (n = 7). Scale bar = 1mm.

3.3 HDAC3 is upregulated in the striatum bilaterally following unilateral administration of 6-OHDA

To assess the effect of 6-OHDA administration on HDAC3 expression in the striatum, striatal cryosections were immunostained for HDAC3 and the optical density of this staining was analysed (Figure 3.3). One-way ANOVA analysis showed that there was a significant difference between groups on the lesioned side of the brain ($F(2,15)=10.08$, $P=0.0017$). Bonferroni post-hoc testing showed significantly more intense immunostaining in the 21-day and 28-day groups compared to the Control group. As similar results were observed on the non-lesioned side of the brain, two-way ANOVA analysis was carried out to assess whether there was any interaction effect between the side of the brain and time. There was no significant effect of side on staining intensity ($F(1,30)=0.13$, $P=0.7200$). There was a significant effect of time on staining intensity $F(2,30)=22.03$, $P<0.0001$). There was no significant interaction between time and side ($F(2,30)=0.15$, $P=0.8625$).

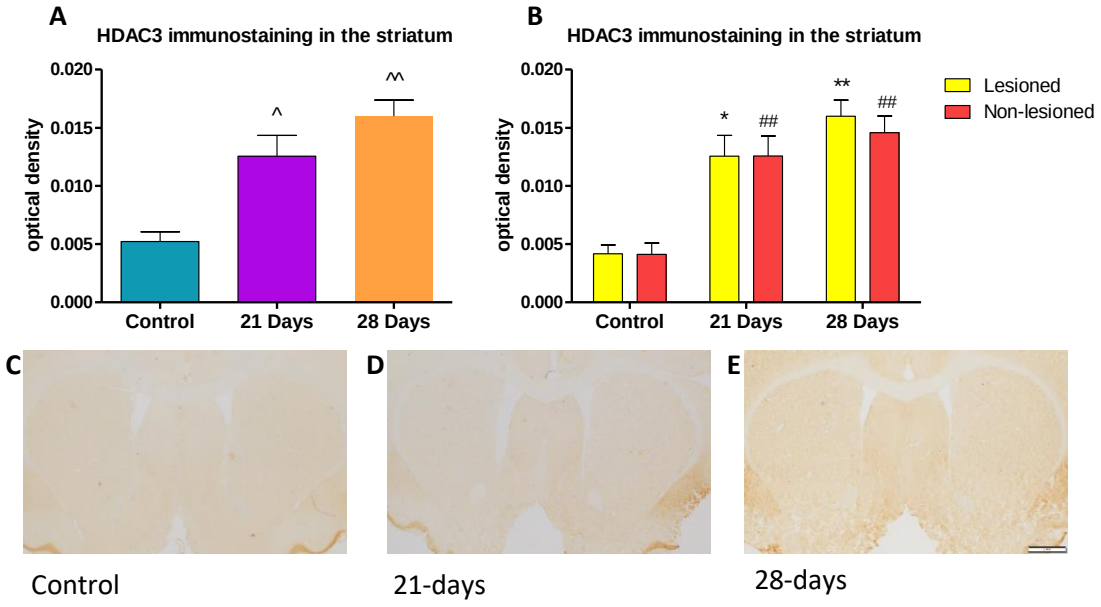


Figure 3.3: HDAC3 immunostaining in the striatum.

(A) Intensity of HDAC3 immunostaining on lesioned side of the brain, expressed as mean optical density for each of the three experimental groups, as indicated. (B) Intensity of HDAC3 immunostaining on the lesioned and non-lesioned sides of the brain, expressed as mean optical density for each of the three groups, as indicated. (C)-(E) Representative photomicrographs of each of the three groups, as indicated. ^ = P<0.05, ^^ = P<0.05 compared to control; * = P<0.05, ** = P<0.01, compared to control (lesioned side). ## = P<0.01, compared to control (non-lesioned side). One-way ANOVA with Bonferroni post-hoc test and two-way ANOVA with Bonferroni post hoc test. Values are expressed as mean ± SEM (n = 4 for control group; n = 7 for 21-day and 28-day groups). Scale bar = 1mm.

3.4 HDAC3 expression in the midbrain was not affected by unilateral administration of 6-OHDA

To assess the effect of 6-OHDA administration on HDAC3 expression in the midbrain, immunohistochemical staining against HDAC3 was carried out and the optical density of the staining was analysed (Figure 3.4). One-way ANOVA analysis showed that there was no significant difference in staining intensity on the lesioned side of the brain between the three groups ($F(2,11)=1.761$, $P=0.2161$). Two-way ANOVA analysis showed that there was a significant effect of time on staining intensity ($F(2,22)=3.69$, $P=0.0415$).

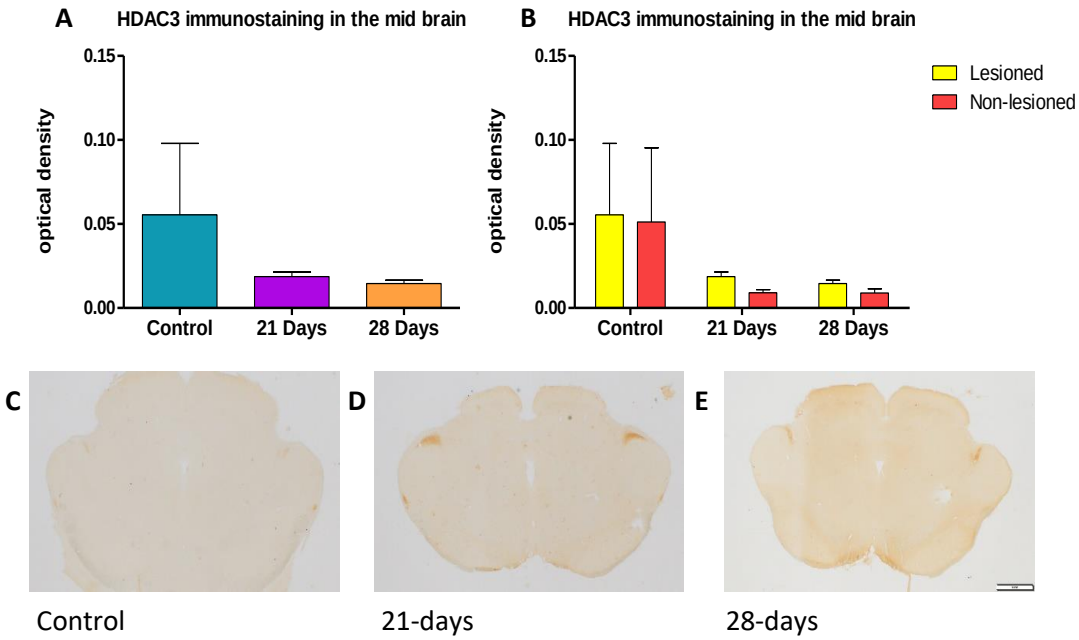


Figure 3.4: HDAC3 immunostaining in the midbrain.

(A) Intensity of HDAC3 immunostaining on the lesioned side of the brain, expressed as mean optical density for each of the three experimental groups, as indicated. (B) Intensity of HDAC3 immunostaining in the lesioned and non-lesioned sides of the brain, expressed as mean optical density for each of the three experimental groups. (C)-(E) Representative photomicrographs of midbrain sections from each of the three experimental groups, as indicated. One-way ANOVA with Bonferroni post-hoc test and two-way ANOVA with Bonferroni post hoc test. Values are expressed as mean ± SEM, n = 4 for control group; n = 7 for 21-day and 28-day groups. Scale bar = 1mm.

3.5 HDAC9 is upregulated in the striatum bilaterally following unilateral administration of 6-OHDA

To assess the effect of 6-OHDA administration on HDAC9 expression in the striatum, immunostaining against HDAC9 was carried out and the optical density of the staining was analysed (Figure 3.5). One-way ANOVA analysis showed that there was a significant difference between groups in HDAC9 expression on the lesioned side of the brain ($F(2,15)=5.776$, $P=0.0138$). Bonferroni post-hoc testing showed that there was significantly higher intensity of staining in the 28-day group compared to control groups (Figure 3.3 D). Once again, as similar results were found on the non-lesioned side of the brain also, a two-way ANOVA was carried out to assess whether there was any interaction between side and time. There was no significant effect of side on staining intensity ($F(1,30)=0.77$, $P=0.3863$). There was a significant effect of time on staining intensity ($F(2,30)=11.26$, $P=0.0002$). There was no significant interaction effect ($F(2,30)=0.04$, $P=0.9649$).

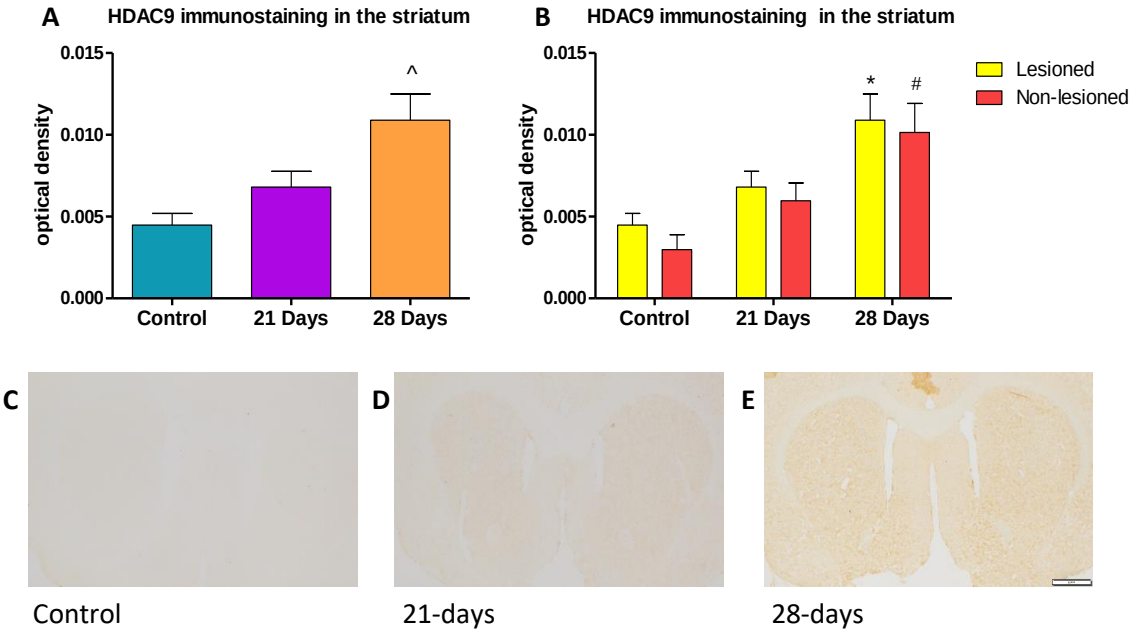


Figure 3.5: HDAC9 immunostaining in the striatum.

(A) Intensity of HDAC9 immunostaining on lesioned side of the brain, expressed as mean optical density for each of the three groups, as indicated. (B) Intensity of HDAC9 immunostaining in both the lesioned and non-lesioned sides of the brain, expressed as mean optical density for each of the three experimental groups. (C)-(E) Representative photomicrographs of sections from each of the three groups, as indicated. ^ = P<0.05 compared to control; * = P<0.05 compared to control (lesioned side). # = P<0.05 compared to Vector control (non-lesioned side); One-way ANOVA with post-hoc Bonferroni test and two-way ANOVA with post-hoc Bonferroni test. Values are expressed as mean ± SEM (n = 4 for Vector control group; n = 7 for 21-day and 28-day groups). Scale bar = 1mm.

3.6 HDAC9 is unilaterally upregulated in the midbrain following unilateral administration of 6-OHDA

To assess the effect of 6-OHDA administration on HDAC9 expression in the midbrain, immunostaining against HDAC9 was carried out and the optical density of the staining was analysed (Figure 3.6). One-way ANOVA analysis showed that there was a significant difference in the staining intensity between groups on the lesioned side of the brain ($F(2,15)=6.412$, $P=0.0097$). Bonferroni post hoc testing revealed that there was significantly more intense staining in the 28-day group compared to control group (Figure 3.6). Again, a two-way ANOVA was carried out. No effect of side was found ($F(1,30)=3.13$, $P=0.0873$). Time was found to have a significant effect on staining intensity ($F(2,30)=5.45$, $P=0.0096$). No interaction effect was found ($F(2,30)=0.28$, $P=0.7606$).

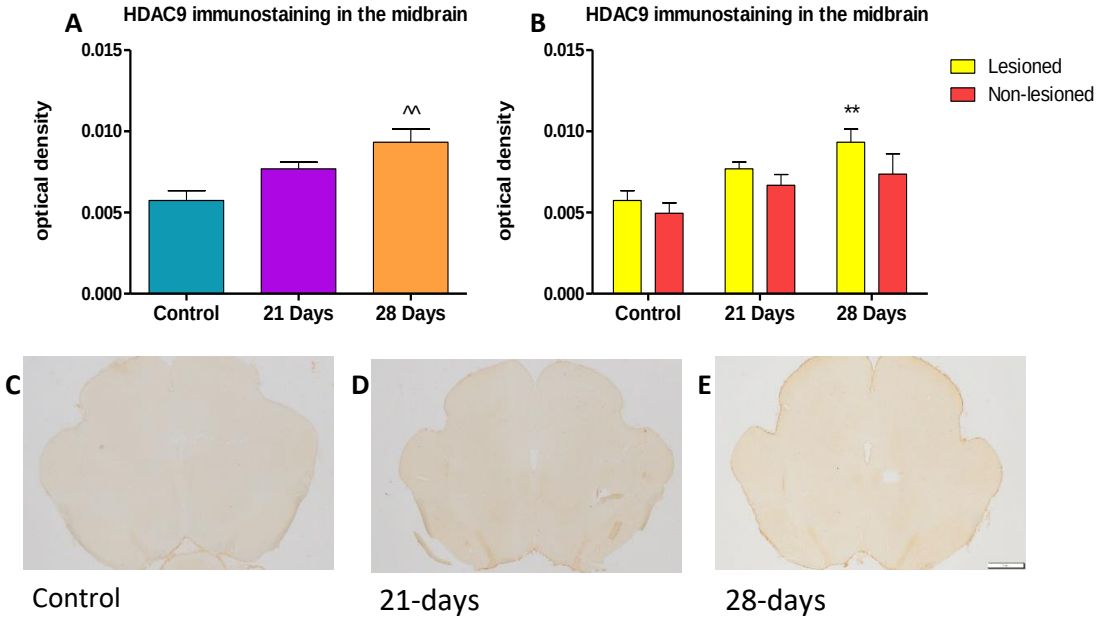
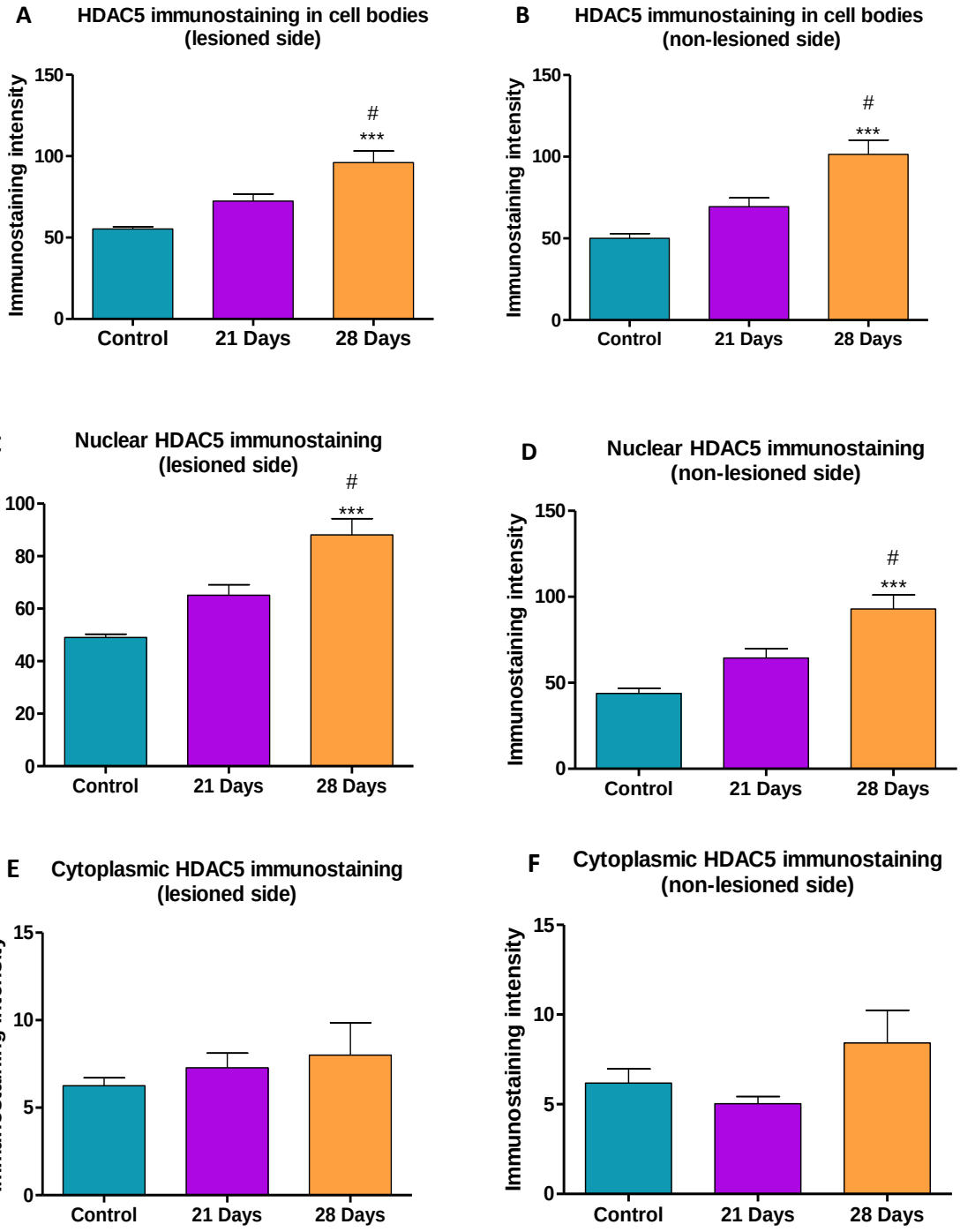


Figure 3.6: HDAC9 immunostaining in the midbrain.

(A) Intensity of HDAC9 immunostaining on the lesioned side of the brain, expressed as mean optical density for each of the three experimental groups, as indicated. (B) Intensity of HDAC9 immunostaining in both the lesioned and non-lesioned sides of the brain, expressed as mean optical density across the three groups, as indicated. (C)-(E) representative photomicrographs of each of the three experimental groups. ^^ = P<0.01 compared to control group; ** = P<0.01, compared to control group (lesioned side); One-way ANOVA with Bonferroni post-hoc test and two-way ANOVA with Bonferroni post hoc test. Values are expressed as mean ± SEM (n = 4) (control group), (n = 7) (21 days and 28 days groups). Scale bar = 1mm.

3.7 HDAC5 is upregulated bilaterally in midbrain dopaminergic neurons following unilateral administration of 6-OHDA

To assess the effect of 6-OHDA administration on HDAC5 expression in midbrain dopaminergic neurons, midbrain cryosections were immunostained for TH and HDAC5. Following this, the intensity of HDAC5 immunostaining in TH-immunopositive cell bodies was measured and analysed (Figure 3.7). One-way ANOVA analysis showed that there was a significant difference between groups on both the lesioned side of the brain ($F(2,15)=11.72$, $P=0.0009$) and the non-lesioned side of the brain ($F(2,15)=12.29$, $P=0.0007$). Bonferroni post-hoc testing revealed that there was significantly greater intensity of immunostaining in the 28-day group compared to both the control and 21-day groups. This was seen for both the lesioned and non-lesioned sides of the brain. Further analysis was carried out to assess the effect of 6-OHDA administration on the expression of HDAC5 in the nucleus and in the cytoplasm of TH immunopositive cells in the midbrain. In the nucleus, one-way ANOVA analysis showed that there was a significant difference between groups on both the lesioned side of the brain ($F(2,15)=13.79$, $P=0.0004$) and the non-lesioned side of the brain ($F(2,15)=12.03$, $P=0.0008$). Bonferroni post-hoc testing revealed that there was significantly greater intensity of immunostaining in the 28-day group compared to both the control and 21-day groups. This was seen for both the lesioned and non-lesioned sides of the brain. In the cytoplasm, one-way ANOVA analysis showed that there was no difference in immunostaining intensity between groups on both the lesioned side of the brain ($F(2,15)=0.3337$, $P=0.7214$) and the non-lesioned side of the brain ($F(2,15)=2.024$, $P=0.1667$).


A
HDAC5 immunostaining in cell bodies (lesioned side)
Immunostaining intensity
150
100
50
0
#
***
Control
21 Days
28 Days
B
HDAC5 immunostaining in cell bodies (non-lesioned side)
Immunostaining intensity
150
100
50
0
#
***
Control
21 Days
28 Days
C
Nuclear HDAC5 immunostaining (lesioned side)
Immunostaining intensity
100
80
60
40
20
0
#
***
Control
21 Days
28 Days
D
Nuclear HDAC5 immunostaining (non-lesioned side)
Immunostaining intensity
150
100
50
0
#
***
Control
21 Days
28 Days
E
Cytoplasmic HDAC5 immunostaining (lesioned side)
Immunostaining intensity
15
10
5
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Control
21 Days
28 Days
F
Cytoplasmic HDAC5 immunostaining (non-lesioned side)
Immunostaining intensity
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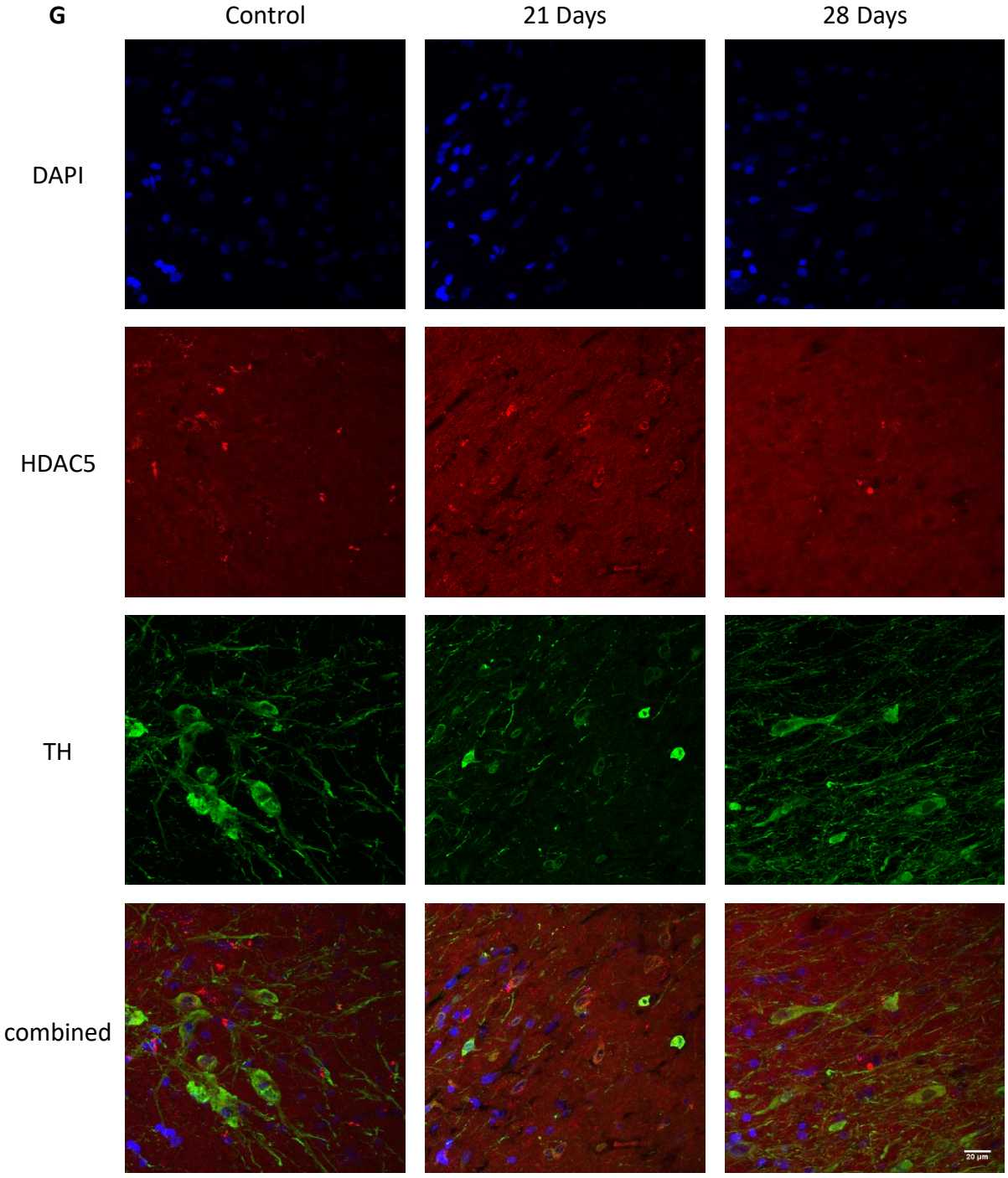
0
Control
21 Days
28 Days


G
Control
21 Days
28 Days
DAPI
HDAC5
TH
combined
20 µm

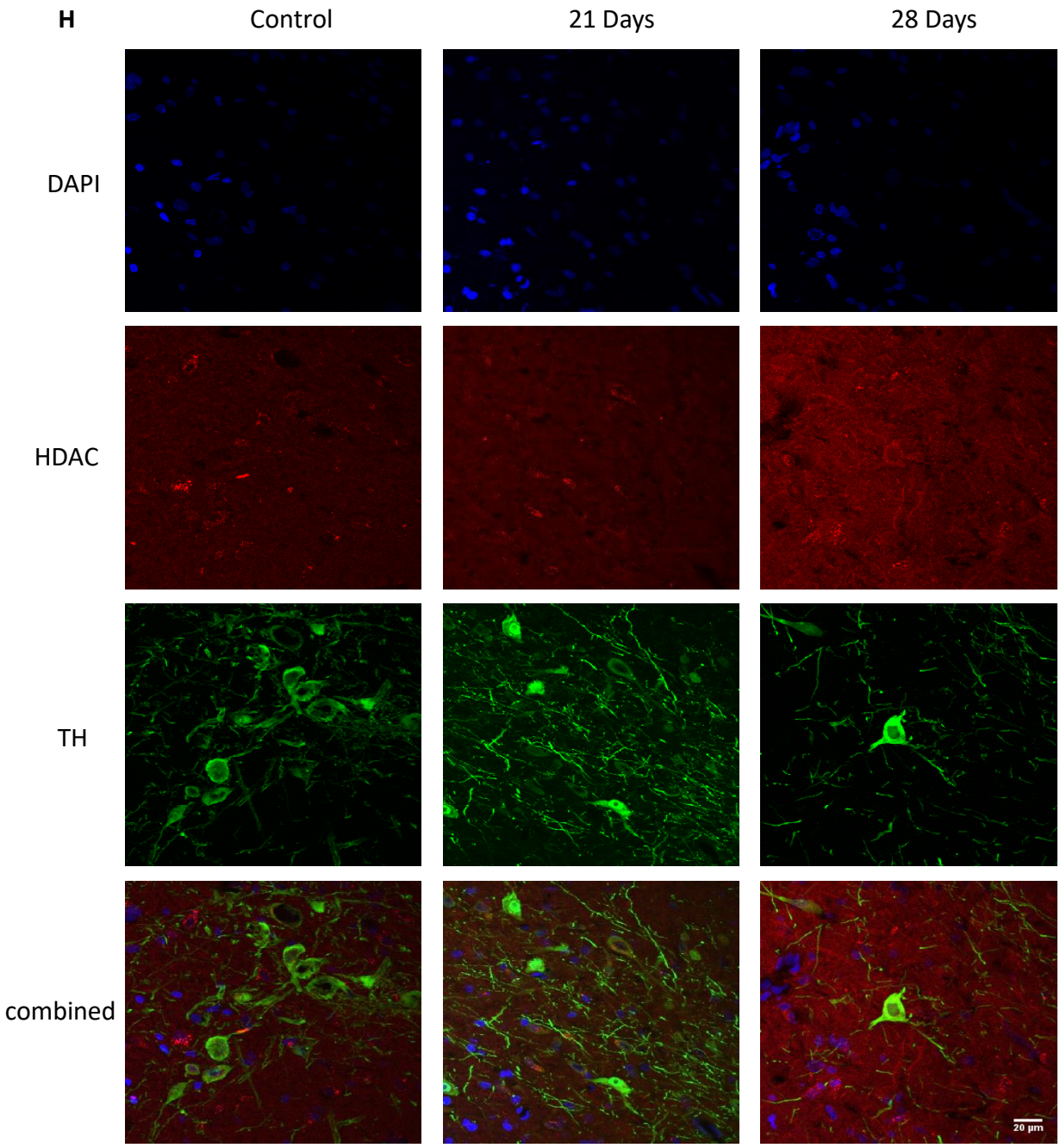

H
Control
21 Days
28 Days
DAPI
HDAC
TH
combined
20 µm

Figure 3.7: HDAC5 immunostaining in midbrain dopaminergic neurons.

(A) and (B) Intensity of HDAC5 immunostaining in TH-immunopositive cell bodies. (C) and (D) Intensity of HDAC5 immunostaining in the nucleus of TH-immunopositive cells. (E) and (F) Intensity of HDAC5 immunostaining in the cytoplasm of TH-immunpositive cells. (G) Representative photomicrographs of the lesioned side of the brain for each of the three experimental groups, as indicated. (H) Representative photomicrographs of the non-lesioned side of the brain for each of the three experimental groups, as indicated. # = $P<0.05$ compared to the 21-day group, *** = $P<0.001$ compared to control group; One-way ANOVA with Bonferroni post-hoc test. Values are expressed as mean ± SEM (n=4 for Vector control; n=7 for 21-day and 28-day groups). Scale bar = 20µm.

4. Discussion

In the first section of this study we examined both the potential neurorestorative and neuroprotective effects of AAV2/5-GDF5 treatment in a striatal 6-OHDA lesion model. The intensity of striatal TH immunostaining of the neurorestoration and neuroprotection groups was similar to that of the neurorestoration control and neuroprotection control groups, which means that those groups which had received AAV2/5-GDF5 treatment had similar levels of degradation of the striatum as those groups which had only received 6-OHDA. Similar results were seen in the substantia nigra, where treatment with AAV2/5-GDF5 did not affect the loss of TH immunopositive cells caused by 6-OHDA administration. This lack of a protective or restorative effect is surprising. GDF5 treatment has been shown in several studies conducted by our group to be effective in preventing 6-OHDA-induced degeneration of the nigrostriatal system. However, this was our first time using an AAV vector to introduce the GDF5 treatment. Previous studies have shown that GDF5 injected into either the striatum, SN or a combination of the two regions is capable of sparing striatal dopamine levels as well as TH immunopositive cells in the SN in rats which had also been injected with 6-OHDA (Sullivan et al., 1997; Sullivan et al., 1999). Another study found that GDF5 injected into either the SN or the striatum one week following 6-OHDA administration resulted in protection of TH immunopositive cell bodies in the SN, though no significant protection of the striatum was seen (Hurley et al., 2004). It is possible that the protection seen in these studies was due to the protein itself being administered. However, the present study was not the first to be conducted in our group which aimed to overexpress GDF5 *in vivo* instead of administering the protein. One study used transplanted CHO cells to overexpress

GDF5 *in vivo*. This study found that transplantation of GDF5-overexpressing CHO cells conferred both neuroprotective and neurorestorative effects on midbrain dopaminergic neurons in rats which had also been injected with 6-OHDA (Costello et al., 2012). This shows that overexpression of GDF5 can also protect against 6-OHDA-induced degeneration of the nigrostriatal system and that administration of the GDF5 protein itself is not required. This would suggest that perhaps the vector used may be the reason for the lack of protective effects seen in the present study. Previous studies have shown that AAV vectors are effective in delivering neurotrophic factors to dopaminergic neurons in both rodents (Wang et al., 2002; Ren et al., 2013) and non-human primates (Eslamboli et al., 2003; Redmond et al., 2013) however, there is the possibility that in the present study the virus failed to effectively increase the level of GDF5 expression. There are currently no ELISA kits available to detect secreted GDF5 in rats *in vivo* and so we were not able to verify alterations in GDF5 expression due to treatment. Future studies should develop western blotting or immunohistochemical methods for the detection of secreted GDF5. Having such a method could potentially shed light on the surprising results seen in this study. Another possible explanation is that the treatment itself contributed to cell death. In the substantia nigra the administration of AAV2/5-GDF5 in the absence of 6-OHDA resulted in a slight decrease in TH immunopositive cell numbers, though this was not statistically significant. This could suggest that either the virus had a toxic effect on midbrain dopaminergic neurons or that the overexpression of GDF5 resulted in a degree of cytotoxicity. It has been shown in yeast that overexpression of proteins results in toxicity (Vavouri et al., 2009; Makanae et al., 2013). If the level of GDF5 expressed was sufficiently high, it may have had a toxic effect. Once again, without a

method to measure GDF5 expression it is not possible to say for certain if high protein levels contributed to the cell death seen.

It should also be noted that the data presented here is based on one lesion type, a striatal 6-OHDA lesion. There are several regions of the nigrostriatal pathway that may be lesioned when modelling PD: the striatum, the substantia nigra and the medial forebrain bundle. Slight differences in pathology are seen between lesions of each of these regions (Bové and Perier, 2012). However, it seems unlikely that the location of the lesion in this study could be the reason behind the lack of protection seen. GDF5 has been shown to be effective against striatal 6-OHDA lesions in some of the above studies (Hurley et al., 2004; Costello et al., 2012).

In the second section of this study we examined the effect of unilateral administration of 6-OHDA on the expression of HDAC3, HDAC5 and HDAC9 in both the striatum and the midbrain. With HDACIs gaining attention as potential disease-modifying treatments for PD it is important that HDAC response to common PD modelling techniques be characterised.

In the striatum, there were significantly higher levels of HDAC3 immunostaining compared to controls at 21 days and 28 days after 6-OHDA administration. The level of HDAC9 immunostaining was found to be significantly increased at 28 days following administration of 6-OHDA. This increase in immunostaining was observed on both ipsilateral and contralateral sides of the brain to 6-OHDA administration.

This bilateral increase was not seen in the midbrain. No significant changes in HDAC3 immunostaining were observed on either the ipsilateral side or contralateral side to 6-OHDA administration. A significant increase in HDAC9 immunostaining was seen 28

days following 6-OHDA administration on the side ipsilateral to administration, but not on the contralateral side and no significant increase in staining was seen on either side of the brain 21 days following 6-OHDA administration. This difference in results between the striatum and midbrain may be an indication that HDAC response to 6-OHDA administration varies by brain region and the difference in results between HDAC3 and HDAC9 may indicate that each HDAC responds differently to such a challenge. In the healthy rat brain, HDAC expression varies across brain regions and each HDAC isoform has its own unique pattern of expression (Broide et al., 2007). The response elicited by a given challenge, such as 6-OHDA, may also have its own unique pattern. In order to explore this further, the expression of a number of HDACs in a wider range of brain regions could be examined following administration of 6-OHDA.

HDAC5 expression was significantly increased in midbrain dopaminergic cell bodies on both ipsilateral and contralateral sides of the brain at 21 days after 6-OHDA administration. There was a further significant increase at the 28-day timepoint. When the location of HDAC5 in the cell was examined, it was found that this increase was limited to the nucleus. There were no significant changes in the levels of HDAC5 in the cytoplasm. As a class IIa HDAC, HDAC5 may be transported out of the nucleus and carry out its de-acetylating functions on non-histone proteins in the cytoplasm of the cell. Similar nuclear accumulation has been reported for another class II HDAC, HDAC4, both *in vitro* and *in vivo*. In one study, ventral mesencephalic neurons from both wild type mice and A53T transgenic mice were treated with MMP^+ and it was found that, in the case of the A53T-expressing cells, MMP^+ treatment resulted in nuclear accumulation of HDAC4. The researchers reported this same phenomenon

when MPTP was administered to both wild type mice and A53T transgenic mice. The researchers reported no such nuclear accumulation of HDAC5 (Wu et al., 2017). This lack of nuclear accumulation of HDAC5 may be a result of different toxins being used. It would be interesting to further investigate this differential response of HDAC5 to 6-OHDA and MPP^+/MPTP. This aside, the study by Wu et al. supports the idea that exposure to neurotoxins can alter the distribution of class II HDACs within the cell. Future studies should examine what role, if any, this altered distribution plays in toxin-induced cell death and whether this nuclear accumulation results in changes in histone acetylation.

Upregulation of HDACs in the brain following the administration of neurotoxins has also been observed in other studies. One group reported greater HDAC immunostaining in the rat hippocampus and temporal cortex following a unilateral striatal injection of 6-OHDA (Ximenes et al., 2015). However, it was not specified which HDAC was stained for and there is no mention of HDAC levels in either the striatum or subtantia nigra being assessed. Another group reported greater HDAC immunostaining in the CA1 and CA3 regions of the hippocampus and the striatum following administration of 6-OHDA to the striatum in rats (Machado-Filho et al., 2014). Once again, it was not specified which HDAC was analysed. Without knowing which HDACs were involved it is not possible to make a direct comparison to the present study. These two studies do, however, agree with our finding that exposure of the striatum to 6-OHDA can lead to elevated levels of HDAC expression in the striatum as well as other regions of the brain. 6-OHDA does not seem to be the only neurotoxin which can induce an increase in HDAC expression. One group reported increases in HDAC5 and HDAC9 mRNA expression following administration of kainic

acid to the CA1 region of the dorsal hippocampus in mice (Jagirdar et al., 2016). This increase was found to occur both ipsilaterally and contralaterally to the kainic acid administration and, in the case of HDAC5, this bilateral increase was maintained in the granule cell layer up to 28 days following injection. From these studies, it can be seen that the administration of neurotoxins, including 6-OHDA, to the brain can induce increases in the expression levels of HDACs and that this increase can occur bilaterally. Not only this, but there is support for this study's finding that HDAC expression can remain elevated for an extended period of time following neurotoxin administration.

It should be noted, however, that this increase in HDAC expression may be a protective response to exposure to 6-OHDA and not a detrimental one. It has been shown *in vitro* in several dopaminergic cell lines, namely rat N27, mouse MN9D and human SH-SY5Y cells, that treatment with the HDACI TSA can reduce the viability of dopaminergic cells and that pretreatment of these cells with TSA can increase the neurotoxic effects of MPP^+ and rotenone (Wang et al., 2009). If the inhibition of HDACs can increase the vulnerability of dopaminergic cells to neurotoxin-induced cell death, then perhaps the increase in HDAC expression seen in the present study is a protective response against 6-OHDA. As HDACIs are currently undergoing research as neuroprotective agents against neurodegenerative disorders, including PD, the role HDACs play, if any, in the degenerative process of dopaminergic neurons should be further elucidated.

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