The Neuroprotective and Behavioural Properties of NLX-112 in Models of Parkinson's Disease

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Abstract

Parkinson's disease (PD) is the second most common neurodegenerative disorder world-wide. The loss of dopamine (DA) producing neurons in the substantia-nigra is considered to be the primary cause of disease pathogenesis with the concomitant loss of neural DA leading to a vast array of debilitating -motor and non-motor symptoms. Therefore, preventing DAergic neuronal degeneration is key to halting the advancement of PD. There is evidence for serotonin 5-HT_{1A} agonists having neuroprotective effects on DA neurons but so far, the most promising results have never extended beyond pre-clinical investigation. NLX-112 is a potent and selective 5-HT_{1A} biased agonist that has had recent success in a phase 2a (Ph2a) clinical trial as a novel therapy for alleviating parkinsonism and levodopa induced dyskinesia. Here, the first evidence is shown indicating that NLX-112 acts as a neuroprotective agent in a neuroblastoma cell line and in C57b/6 mouse (C57b/6J and C57b/Ola-HSD strains). In vitro, modest but significant protection (+10%) of SH-SY5Y cells was achieved with low concentrations of NLX-112 (100μM approx.) against MPP^+ and MG132 toxic challenge. In an MPTP mouse model, NLX-112 almost completely reversed the effects of an MPTP-induced 40% SN DA lesion. Further still, this was likely achieved through NLX-112's tempering of reactive gliosis in the SNpr and striatum, with NLX-112 significantly attenuating microgliosis and astrocytosis. The standout finding from this thesis is the effect that NLX-112 has on enhancing endogenous glial derived neurotrophic factor (GDNF) secretion from astrocytes, which was associated with its neuroprotective property. This is a very important finding, as GDNF is recognised as one of the more promising avenues for neuroprotective treatments in PD. Taken together, the behavioural, biochemical and immunohistochemical data presented in this thesis provides evidence that NLX-112 is neuroprotective against DAergic neurodegeneration in the cellular and mouse models of PD.

Keywords: Neuroprotection, Parkinson's-disease, 5-HT_{1A}, NLX-112 (Befiradol), GDNF, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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Acronyms

5HIAA 5-hydroxyindoleacetic acid
5-HT 5-hydroxytryptamine
5-HTP 5-hydroxytryptophan
6-OHDA 6-hydroxydopamine
8-OH-DPAT 7-(Dipropylamino)-5,6,7,8-tetrahydronaphthalen-1-ol
AADC L-amino acid decarboxylase
AD Alzheimer's disease
ADR adrenaline
AMPAR a-amino-3- hydroxy-5- methyl-4-isoxazolepropionic acid
ATP adenosine-triphosphate
BDNF brain-derived neurotropic factor
bFGF basic fibroblast growth factor
BP binding potential
BLA basolateral amygdala
cAMP cyclic adenosine monophosphate
CCL5 chemokine (C-C motif) ligand 5
CNS central nervous system
CRP c-reactive protein
CSF cerebrospinal fluid
DA dopamine
DAT dopamine active transporter
DJ-1 Protein deglycase DJ-1
DOPAC 3,4-Dihydroxyphenylacetic acid
DMSO dimethyl sulfoxide
EPM elevated plus maze
EOPD Early onset Parkinson's disease
GABA gamma-aminobutyric acid
GDNF glial cell-derived neurotrophic factor
GFAP glial fibrillary acidic protein
GID graft induced dyskinesia
GPCR G-protein coupled receptor
GP globus pallidus
GPe external globus pallidus
GPi internal globus pallidus
iPScs induced pluripotent stem cells
IP-10 interferon gamma-induced protein 10
i.p. intraperitoneal
Iba1 ionised calcium-binding adaptor molecule 1
NGF nerve growth factor
IFN-γ interferon-gamma
iNOS inducible nitric oxide synthase
IL1β interleukin1β
IL6 interleukin 6
LAAD L-aromatic amino-acid decarboxylase
LOPD late onset Parkinson's disase
L-dopa L-3,4-dihydroxyphenylalanine

LB Lewy bodies
LID levodopa-induced dyskinesia
LPS lipopolysaccharide
LRRK2 Leucine-rich repeat kinase 2
MIP1α Macrophage Inflammatory Protein 1^{α}
MAO-A monoamine oxidase A
MAO-B monoamine oxidase B.
MG132 N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
mPFC medial prefrontal cortex
MPPP desmethylprodine
MPP^{+} 1-methyl-4-phenylpyridinium
MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.
MSN medium spiny neurons
mtDNA mitochondrial DNA
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NA noradrenaline
NAc nucleus accumbens
NMDA N-Methyl-D-aspartate
NRF2 Nuclear factor erythroid 2-related factor 2
NRM nucleus raphe magnus
NSAID non-steroidal, anti-inflammatory
OFT open field test
PARK7 Parkinson's disease protein 7
PBS phosphate-buffered saline
PD Parkinson's disease
PDP Parkinson's disease psychosis
PET positron emission tomography
PINK1 PTEN-induced kinase 1
PNS peripheral nervous system
RN raphe nucleus
ROS reactive oxygen species
s.c. subcutaneous
SEM standard error of the mean
SCN supra-chiasmic nucleus
SERT 5-HT transporter
SGZ sub granular zone
SN substantia nigra
SNpc substantia nigra pars compacta
SNpr substantia nigra pars reticulata
SSRI selective 5-HT reuptake inhibitor
STN subthalamic nucleus.
TH tyrosine hydroxylase
TNFα tumour necrosis factor α
Trx1 Thioredoxin-1
UPP ubiquitin-proteasome pathway
VMAT2 vesicular monoamine transporter 2
VTA ventral tegmental area

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Preface

As a species, our understanding of the human central nervous system and its corresponding disorders has been advanced considerably through pioneering medical techniques and ground-breaking research involving other anatomically similar mammalian species, notably non-human primates and rodents. This progress is not limited to recent times; it encompasses gains made during the Enlightenment and preceding centuries. However, it is the last two centuries that have witnessed the most comprehensive understanding of various CNS disorders, leading to their effective management and, in many cases, cures, thanks in part to animal research.

In 1840, the average life expectancy in the U.K. was 42 years old. Fast forward to 2016, and it has almost doubled to 82 years old. While improvements in diet, sanitation and working conditions have undoubtedly contributed to this significant increase in life span, animal research remains a considerable factor that has propelled advancements in scientific and medical inquiry.

With that in mind, this thesis will describe Parkinson's disease and its underlying aetiology based on human investigation but also delve into research conducted with animals. Furthermore, the aetiological descriptions and analyses presented in the following chapters gain additional relevance as the author conducted their research, almost exclusively, with mice.

"The most important criterion for knowledge is objective necessity"

- Aristotle

Publications and Conference Presentations

Journal articles:

Powell, W.H., Annett, L.E., Depoortere, R., Newman-Tancredi, A. and Iravani, M.M., 2022. The selective 5-HT1A receptor agonist NLX-112 displays anxiolytic-like activity in mice. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *395*(2), pp.149-157.

Powell, W.H., Ghita, A., Newman-Tancredi, A. and Iravani, M.M., 2025. The behavioural and biochemical characteristics of normal and spontaneously α-synuclein deficient mice treated with MPTP (*submitted to Journal of Neurochemistry*)

Powell, W.H., Annett, L.E., Depoortere, R., Varney, M., Newman-Tancredi, A. and Iravani, M.M., 2025. The neuroprotective properties of the 5-HT_{1A} receptor agonist, NLX-112, in MPTP-treated mice by prevention on inflammation and promotion of GDNF (*submitted to Journal of Experimental Neurology*)

Oral presentations:

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Conference abstracts:

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Chapter 1

Introduction

1.1. Parkinson's Disease

Parkinson's disease (PD) is an age-related neurodegenerative disorder, for which there is currently no known cure. As of 2020, the global incidence of PD was 9.4 million, compared to 2.5 million in 1990 (Maserejian et al., 2020). This rate of increase over 30 years far exceeds the rate of global population growth, which is likely due to global ageing populations. This indicates that the incidence of PD will continue to rise exponentially. An epidemiological study by Tarolli et al., (2020) predicts that by the year 2040, the number of people living with PD will have increased to 12.9 million. Therefore, it is critically important to develop effective therapies to mitigate the deleterious effects of this debilitating disorder.

First identified by James Parkinson in 1817, were the distinct and unusual overt physiological characteristics exhibited by individuals with what is now understood to be PD. These physical manifestations were in fact the consequence of severe maladies originating within the CNS and physically presented as postural instability, tremor, rigidity, paralysis and bradykinesia. It was not until almost 100 years later, that Konstantin Tretiakoff revealed that the crucial area of the brain affected was a structure in the basal ganglia called the substantia-nigra (SN) (Goedert et al., 2013) (Figure 1). Additionally, in 1912 Frederic Lewy noted the accumulation of irregular intracellular protein aggregations in the SN, which were subsequently referred to as Lewy bodies (LB) (Figure 2). Over the next several decades, other key neurological features of PD were revealed; the biochemical 3-hydroxytyramine (dopamine, DA) was shown to be the primary neurotransmitter affected by the loss of SN neurons, (Hornykiewicz, 1964) and in 1997 Spillantini and colleagues identified that the key component of Lewy bodies was the synapse regulating protein, α-synuclein (Spillantini et al., 1997)

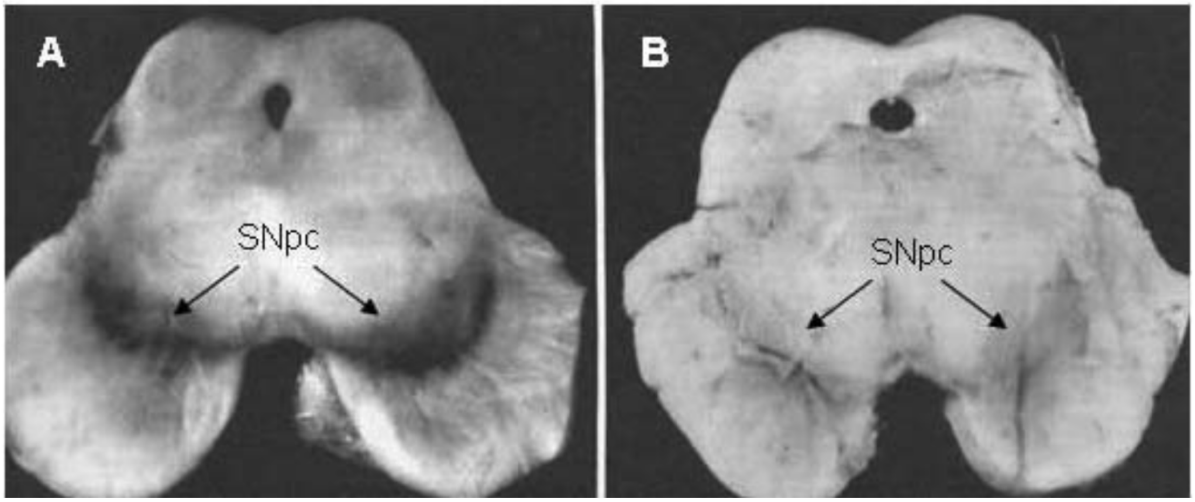

Figure 1. Dopamine neuron loss of the substantia nigra in the healthy A) and PD B) human brain. Note the dark colour of the DA neuronal cell group, hence the name 'nigra'. Adapted from Lima et al., (2012).

1.2. Pathology

The cause of PD is considered to be either familial in origin (genetic inheritance), or idiopathic/sporadic (of an unknown cause(s)). Scientific consensus has determined that the aetiological development of idiopathic PD is likely due to the combined interaction of genetic susceptibility, increased sedentary behaviour (reduced circulatory blood flow), environmental stressors, and the life-course exposure to toxins, including manganese, carbon monoxide and trichloroethylene, amongst others (Liou et al., 1997; Tsai et al., 2002; Simon-Sanchez et al., 2009; Zhang et al., 2014; Ascherio et al., 2016).

This multifactorial aetiology influences the timing of disease onset, with idiopathic PD typically occurring in individuals usually no earlier than 50 years of age, whereas familial forms of the disease often present early on in life in individuals who, prior to diagnosis, are often otherwise fit and healthy; and the incidence of those cases contributes to approximately 5-10% of all PD cases. The outcome for all of those who develop the disorder however is very similar, presenting with extensive loss of DAergic neurons in the SN and the subsequent reduction of DA content throughout the nigrostriatal and mesocortical pathways.

Interestingly, a reduced risk for developing PD is linked to smoking cigarettes, drinking caffeinated beverages and prescription of angiotensin receptor blockers

(Ross and Petrovic, 2001; Lin et al., 2022). Conversely, those who have higher urate concentrations in blood serum have an increased risk of developing PD (Shen et al., 2013), all factors which still confound the mechanistic understanding of the disease pathology.

1.3. Gut-brain axis

There is increasing evidence that idiopathic PD may originate within the gastrointestinal system and that PD propagation in the gut is a possible route for PD pathogenesis in the brain (Santos et al., 2019). This is certainly evidenced in mice that have had intragastric administration of the pesticide rotenone and then subsequently develop many of the neuropathological features of PD (Pan-Montojo et al., 2010).

Perhaps one of the best-known descriptions of how idiopathic PD pathology develops was provided in by Heiko Braak and colleagues in 2003. Their seminal investigation found that the progressive nature of Lewy pathology is likely commenced via the ingestion of a foreign agent that initiates a cascade of protein accumulation (and the various compositions of α-synuclein protein filaments) that aggregate in the gastrointestinal tract, which then progresses to the enteric-, peripheral-, and central nervous -system. Throughout its manifestation from the gut to the brain, the smaller components of Lewy bodies are thought to target vulnerable un-myelinated neurons culminating in the destruction of DAergic neurons in the SN and eventually increasing their presence throughout the neo-cortex (Braak et al., 2003). Heikko Braak was an anatomist and carefully analysed the post-mortem brain of healthy and parkinsonian individuals; from his work, he theorised that the Lewy body staging could be distinguished into 6 junctures:

1. Not yet Lewy body clumps, Lewy neurites and α-synuclein filaments present in the lower brain stem including the dorsal motor nucleus of the vagus-nerve and olfactory nervous system.
2. Accumulation of Lewy neurites still outnumber Lewy bodies at this stage and have now migrated to the RN and locus-ceruleus.

3. The structures in stage 1 and 2 continue being overcome with Lewy bodies but also progress into the SN pars compacta (SNpc) and cholinergic neurons in the forebrain.
4. Complete destruction of the SNpc. Lewy bodies have also formed in the basolateral amygdala (BLA) and sub-thalamic nucleus (STN).
5. Lewy body accumulation has now progressed into the parietal and temporal lobes (mesocortex, allocortex).
6. Including the cerebellum, Lewy bodies have lastly amassed throughout the frontal cortex and neo cortex.

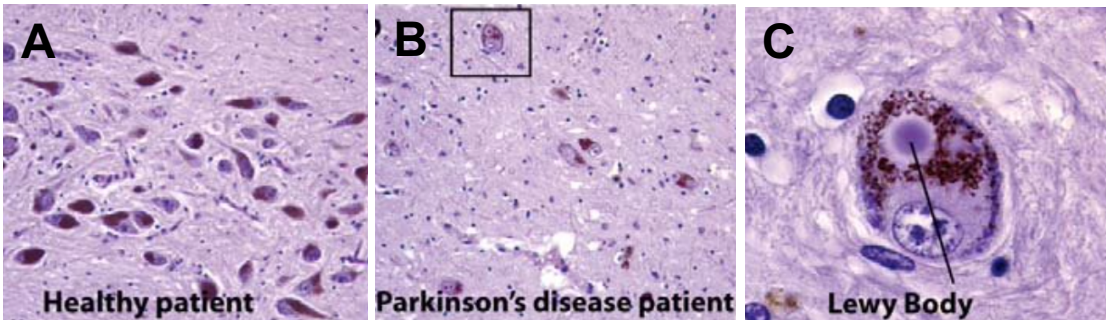

Figure 2. Histological comparison of the substantia nigra in a healthy patient and a PD patient. A) The substantia nigra from a healthy brain shows normal DAergic neuron density and morphology. B) In the PD brain, most DAergic neurons have degenerated, and many of the remaining neurons contain cytoplasmic inclusions (boxed). C) A higher magnification image reveals a Lewy body, a cytoplasmic aggregate primarily composed of α-synuclein. The red-brown pigmentation reflects melanin-containing granules within the cytosol. Adapted from Agamanolis (2006).

1.4. Inheritance

Dysfunction of several genes are known to be associated with having a heritable risk for developing PD. The first gene identified and known to be a causative factor of PD was discovered to be a mutation of the *SNCA* gene which codes for α-synuclein; a mutation rendering SNCA more susceptible for misfolding and facilitating the aggregation of α-synuclein (Polymeropoulos et al., 1997). Additionally, there are a wide array of genetic abnormalities responsible for causing mitochondrial dysfunction; a key mechanism of DAergic cell death in PD pathology. For example, PINK1 (encoded by *PINK1*, a serine/threonine protein kinase and Parkin (encoded by *PARK2*), a ubiquitin ligase, each work together in tandem as regulators of mitophagy and mitochondrial apoptosis (Jin and Youle 2012). Dysfunction of DJ-1 (encoded by *PARK7*), involved in the breakdown of

peptides, inhibits aggregation of α-synuclein, and mitochondrial calcium flux, protecting neurons against oxidative stress (Xu et al., 2018). These mutations described are strongly linked to the development of early onset PD (EOPD) whereas mutations of LRRK2 (encoded by *LRRK2*), a kinase enzyme, is more often associated with the development of late onset PD (LOPD) (Kumari et al., 2009). Due to the clinical phenotype of those with the LRRK2 mutation being similar to that of those with idiopathic PD, consequently makes accurate clinical diagnosis of the hereditary cause more difficult and is often misreported (Chen and Wu, 2022).

This genetic predisposition not only influences the motor symptoms typically associated with PD but also impacts the onset of non-motor symptoms in the early stages. Prior to the development of reduced motor function, during the early stages of the disease, one or several non-motor symptoms may begin to occur, such as loss of smell (Haehner et al., 2009), constipation (Adams-Carr et al., 2019), sleep dysfunction (Comella, 2008), frequent urination (Yeo et al., 2012), hyperalgesia (Sung et al., 2018) and various affective disorders including depression (Reijnders et al., 2008), anxiety (Prediger et al., 2012) and psychosis (Fénelon and Alves, 2010). Why these additional disorders often manifest, as SN neurons begin to slowly perish, is due to the ubiquitous role that the basal ganglia play in modulating, not only motor function but also the cognitive and affective faculties of the mammalian CNS (Graybiel, 2000).

1.5. Aetiology

1.5.1. The basal-ganglia

The basal ganglia have a vital role as a sub-cortical brain structure comprising of various nuclei including the caudate nucleus, putamen, globus pallidus (GP), thalamus, subthalamic nucleus (STN), and SN, which are essential for regulating sensorimotor coordination. Dopamine serves as the critical neurotransmitter facilitating communication among these regions. Progressive loss of DA-producing neurons in the SNpc disrupts crucial neural connections between cortical and midbrain areas, essential for normal neuronal function and communication.

This disruption underscores the importance of DA synthesis within DAergic nigral neurons, a process carried out through a series of enzymatic reactions. The precursor of DA, the amino-acid tyrosine, is taken up into DAergic neurons where it is converted into L-dopa (L-3,4-dihydroxyphenylalanine) by the enzyme tyrosine hydroxylase (TH). This stage is rate-limiting and therefore controls the net amount of DA produced. The final step of synthesis involves the enzyme L-amino acid decarboxylase (AADC), which removes the carboxyl group from L-dopa. The newly synthesised DA is then stored in vesicles within the DAergic neurons until required for release into the synaptic cleft.

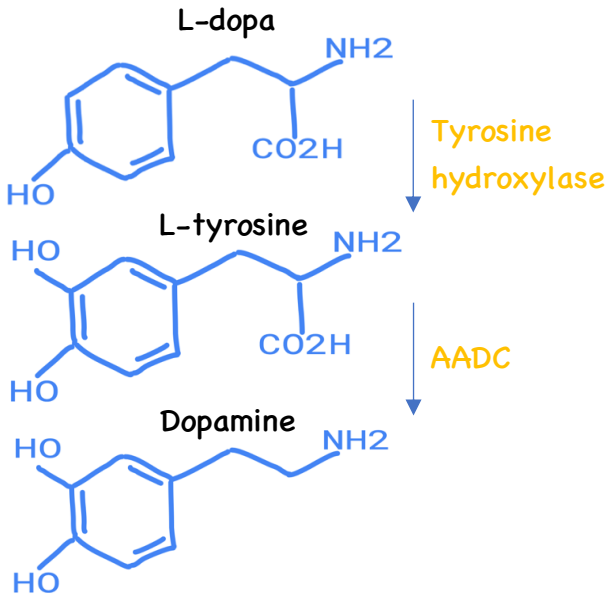

Figure 3. Enzymatic controlled synthesis of L-tyrosine into DA. L-dopa is converted to DA by the enzyme aromatic L-amino acid decarboxylase (AADC), which removes a carboxyl group from L-dopa, transforming it into DA.

Highly implicated in modulating motor control, the dorsal striatum is known to also play a discrete role in reward, motivation and decision making. Similarly, the medial striatum facilitates the convergence of several different brain regions involved in emotional processing and further executive functions in the mPFC and orbital frontal cortex (Balleine et al., 2007). The basal ganglia have widespread heterogenous organisation of cholinergic, glutamatergic and GABAergic neurons which aid to its facilitation of motor function (Hegeman et al., 2016). By exerting glutamatergic excitation to the SNpc and the internal globus pallidus (GPi), the STN plays a key role in aiding the execution of fine motor skills (Tewari et al., 2016). The SNpc is the ‘hub’ of DA synthesis within the basal ganglia, innervated

by various neurotransmitters from several brain regions that combine to modulate the firing rate of its neurons (Guatteo et al., 2009). It is the convergence, balance and fine tuning of these brain regions that facilitate the circuit of voluntary motor function (Barter et al., 2014). Regulation of this circuit can be distinguished into two DA regulating pathways, the “direct” and “indirect” pathways, which are triggered through activation of DA receptors on medium spiny neurons (MSN) within the striatum with each pathway being regulated by their respective DA receptors, D1R and D2R (Figure 4). Agonism of D1R (a Gαs coupled receptor) causes an increase in the second messenger, cAMP, resulting in a net increase in neuronal excitability; therefore, innervation of the nigrostriatal pathway, via D1R, regulates the direct pathway and any deliberate voluntary movement. Tempering of the direct pathway is mediated by the activation of the indirect pathway and carried out by D2R, a Gαi coupled receptor, on striatal MSN’s, which when activated, inhibits cAMP thus eliciting an overall decrease in neuronal excitability (Keeler et al., 2014).

When there is an intention to carry out any voluntary movement, the cortex sends an excitatory signal to the striatum which in turn sends an inhibitory signal to the GPi. Inhibition of the GPi releases the thalamus from a resting inhibited state, allowing it to send signals back to the cortex. The enhanced input of the cortex helps aid the execution of the desired voluntary movement (Kimura et al., 1990). In contrast the in-direct pathway serves to inhibit and control any non-deliberate movement. As with the direct pathway, initialisation of the in-direct pathway begins with the cortex which sends excitatory signals to the striatum. The striatum then sends an inhibitory signal to the external globus pallidus (GPe) which then inhibits the STN which then sends excitatory signals to the GPi; the increase in GPi excitation leads to greater inhibition of the thalamus thus reducing excitatory input to the cortex (McCormick et al., 2015). In the parkinsonian brain, the loss of DA neurons in the SNpc, and the reduction in DAergic striatal innervation, has a major impact on the normal function of the basal ganglia (Zhai et al., 2019) (Figure 4).

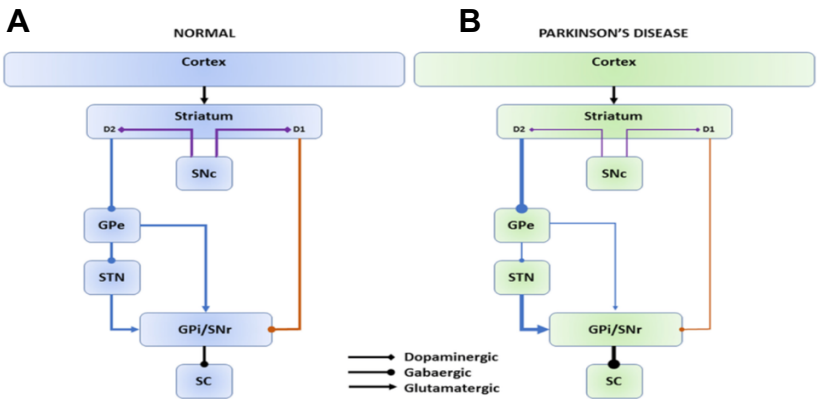

Figure 4. In a healthy brain (A), the direct pathway promotes movement by reducing inhibition on the thalamus, while the indirect pathway inhibits movement by increasing thalamic inhibition; in a Parkinsonian brain (B), DA loss weakens the direct pathway and overactivates the indirect pathway, leading to reduced movement. Adapted from Pretegiani and Optican (2017).

In PD pathogenesis there is a very long prodromal stage and subtle overt symptoms of the disorder typically begin only after approximately 40-60% of DA neurons in the SN have perished (Fearnley and Lees 1991; Greffard et al., 2006; Schapira et al., 2017). Upon the progressive and selective death of nigrostriatal DAergic neurons in PD, a functional change will occur in the remaining neurons that are still releasing DA (Blesa et al., 2017). A process known as synaptic compensation has been shown to modify the structural plasticity in surviving nigrostriatal DAergic neuronal synapses. These changes include an increase in DA neurotransmitter, an increase in postsynaptic GABAergic D1/D2 receptors in the striatum, and a reduced rate of DA inactivation (Zigmond et al., 1984; Zigmond, 1997). These changes allow for deficits in DA availability to be compensated to a such an extent, that during the progression of the disease an individual is likely to remain unaware of having the disorder. In late-stage PD however, when cell loss in the SN can no longer be compensated, the facilitation of neuronal DA from one synapse to another becomes delayed or unavailable altogether, which then leads to a succession of physiological and neurological incapacities (Perez et al., 2009). This progressive neuronal loss and the inability to compensate in later stages are compounded by mechanisms of neuronal cell death, including inflammation.

1.6. Mechanisms of neuronal cell death

1.6.1. Inflammation

Multi-cellular organisms protect themselves from microorganisms and other pathogens via innate and adaptive immunity. A key physiological component for

survival, innate immunity is found in all metazoans, whereas adaptive immunity is only expressed by vertebrates (Litman et al., 2010). The immune system is a finely tuned physiological protective barrier that can distinguish between the body itself, and any potential harmful foreign entities. A major factor in the aetiology of PD is when the adaptive immune system becomes dysregulated and as a consequence helps facilitate the destruction of SN DAergic neurons (Phani et al., 2012). Various inflammatory biomarkers, including C-reactive protein (CRP) and serum amyloid-A (SAA) (markers of autoinflammation (Legger et al., 2022)) are found to be markedly upregulated in the cerebral spinal fluid (CSF) of PD patients, compared to that analysed from healthy study participants (Hall et al., 2018).

1.6.1.1. Astrocytosis

As the most numerous cell type in the brain, astrocytes play a major role in maintaining neuronal function and overall neural homeostasis. Astrocytes facilitate the transport of vital molecules to neurons via the blood brain barrier (BBB) (Abbott et al., 2008). Including the brain's chief excitatory neurotransmitter glutamate, astrocytes also supply neurons with the neurotrophic factors, nerve growth factor (NGF), glial derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF), all of which are reduced in striatum depleted of DA (Nakagawa and Schwartz, 2004). When required to, astrocytes also remove excess extracellular glutamate (Mahmoud et al., 2019), therefore playing a key role in reducing the risk of neuronal excitotoxicity.

The key role that astrocytes play in PD pathology is seen when they undergo gliosis after stimulation by a toxic insult or a foreign agent (Pekny and Pekna, 2016). Reactive astrogliosis leads to the release of cytokines which build up in the extracellular space causing toxicity to nearby neurons, which then leads to a greater number of astrocytes releasing an even greater number of cytokines thus, resulting in an excessive auto-immune cycle (Agulohn et al., 2013). Some key components of this 'cytokine storm' are expressed by human astrocytes in culture, whereby stimulation with interleukin1β (IL1β) and tumour necrosis factor α (TNF-α) causes a greater release of interleukin1β (IL1β) and TNF-α, and also release of a wider variety of cytokines including interferon gamma-induced protein 10 (IP-10),

macrophage inflammatory protein (MIP1α), and chemokine (C-C motif) ligand 5 (CCL5), which are not shown to be expressed by resting unstimulated astrocytes (Choi et al., 2014). Importantly, astrocytes may also play a key role in the facilitation of α-synuclein transport throughout the brain, as it has been demonstrated that as well as being transmissible from neuron to neuron, α-synuclein is also transmissible from neuron to astrocyte (Lee et al., 2010).

1.6.1.2. Microgliosis

Considered the resident macrophage of the CNS, microglia make up to 15% of the cells in the brain, and are responsible for maintaining CNS homeostasis through the clearance of dying neurons, removal of foreign pathogens and aiding in neural tissue repair (Michell-Robinson et al., 2015). Microglia will mobilise upon an insult to the CNS and can switch between a resting ramified state and an activated ameboid state (Lynch, 2009). Such is the refinement of the mammalian adaptive and innate immune response, an acute moderate insult to the CNS can be tempered and ultimately eliminated, supported by activated microglia, without the host ever having a cognisant awareness of such processes occurring. However, more severe, chronic ailments such as stroke, ischemia, or viral infection can lead to a lengthier activation of microglia which can themselves then compromise optimal function of neurons (Goldmann and Prinz, 2013).

Upon neuronal damage due to internal (i.e. necrosis) or external, (i.e. traumatic head injury) factors, microglia will localise in the region of the brain where provision is needed to negate any further injurious insult or damage to the surrounding tissue (Bruce-Keller et al., 1999). Moreover, microglia are activated in particularly high number in neurodegenerative disorders, including PD (Ouchi et al., 1999). As such, there is a higher density of microglia in the SN compared to other brain regions, which may explain the greater vulnerability of SN DAergic neurons for succumbing to cell death compared to other monoaminergic neurons (Liu & Hong, 2003; Joh et al., 2006; Glass et al., 2010).

1.6.2. Mitochondrial dysfunction

As the powerhouse of the cell, mitochondria produce approximately 93% of the brain's ATP (Harris et al., 2012) thus making mitochondrial homeostasis vital for maintaining cellular energy metabolism but also for regulating cell death. Mitochondrial dysfunction inhibits complex-I of the electron transport chain thereby leading to an increase in oxidative stress. Excessive mitochondrial oxygen metabolism increases reactive oxygen species (ROS) and a subsequent build-up of pro-apoptotic proteins in the cytosol thus hindering optimal cellular function and inducing cell death (Ott et al., 2007). It is this pathway which is suspected to be primarily responsible for the loss of DAergic neurons in the SNpc, as with previous observations made in the post-mortem brain of PD patients exhibiting a marked deficit in mitochondrial complex-I (Schapira et al., 1990). However, there are numerous genetic and environmental factors that can exacerbate distinct features of this deleterious cascade such as the identification of mitochondrial dysfunction being attributed to the PD linked genes PINK1 and *parkin* (Jenner, 2001; Abou-Sleiman et al., 2006; Narnedra et al., 2012). In addition to mitochondrial dysfunction, another key pathological hallmark of PD is the presence of Lewy bodies in DA neurons.

1.6.3. Lewy toxicity

The accumulation of intracellular nigral Lewy bodies observed upon post-mortem investigation of the PD brain act as confirmation of PD pathology and satisfy one of the criteria for clinical diagnosis of UK Parkinson's Disease Society Brain Bank. Made up of many different fibrillar proteins, Lewy bodies main constituent is of an α-synuclein and ubiquitin oligomer and protofibril type. Additionally, many of the molecules making up these proteins are expressed by a variety of PD linked genes, including: DJ-1, LRRK2, *parkin*, and PINK1 (Wakabayashi et al., 2007). Interestingly, more contemporaneous theories surrounding the role of Lewy body pathology suggest that it is the initial non-fibrillar α-synuclein filament type that are the purveyors of cytotoxicity and that the fibrillar aggregates are a residual oligomer that may harbour neuroprotective properties (Wakabayashi et al., 2013). Given this, the role of the ubiquitin-proteasome system (UPS) in protein degradation becomes crucial.

1.6.4. Ubiquitin proteasome system

Degradation of intracellular proteins is vital for cell survival and the optimum function of the cell life cycle, including differentiation and apoptosis. The ubiquitin-proteasome pathway (UPP) is responsible for mediating this process through proteolysis. Dysregulation of the UPP leads to protein misfolding, aggregation and accumulation in neurons, and therefore a fundamental contributing factor in PD pathology. There is a marked reduction in proteasomal function in the SNpc of idiopathic PD patients (Mc'Naught and Jenner, 2001). Experiments that have inhibited the UPP in cell culture and in vivo animal models show diminished neuronal connectivity, increases in cytotoxicity, and formation of Lewy body inclusions (Lee at l., 2011; Raichur et al., 2006). This vulnerability is particularly pronounced in the dopamine-producing neurons of the substantia nigra.

1.6.5. SN dopamine neuronal sensitivity

Numbering approximately 800 thousand to one million neurons, the dopamine-producing cells in the human substantia nigra pars compacta (SNpc) are notably more vulnerable to oxidative stress than other neuronal cell types, including other dopamine-expressing neurons like those in the ventral tegmental area (VTA) (Brichta and Greengard et al., 2014). For many years, the reasons for this increased vulnerability remained largely unknown. However, recent evidence suggests that this may be due to the nigral DA neurons having a higher metabolic rate compared to other neurons. This elevated metabolic rate, attributed to the important role these relatively few neurons play in controlling motor functions and behavioural faculties, results in a higher turnover of ATP, making them more susceptible to oxidative stress and subsequent impairment of complex I in the mitochondrial respiratory chain (Nomoto et al., 2000; Surmeier, 2018). This higher metabolic turnover may be influenced by specific transcription factors in the SN, which were previously believed to only be involved in the differentiation and maintenance of midbrain nigral progenitor cells but are now also shown to regulate metabolic processes (Ni and Ernst, 2022).

1.6.6. Excitotoxicity

Another deleterious mechanism of PD pathology by which SN neurons perish is the excessive stimulation of neurons in the SN via the brain's chief excitatory

neurotransmitter, glutamate (Beal, 2000). This process of over stimulation leads to an overload of intracellular calcium, excessive oxidative stress and eventually, if not arrested, apoptosis (Ambrosi et al., 2014). And upon intracerebral perfusion of glutamate via microdialysis, there is an increased formation of hydroxyl radicals, resulting in an extensive excitotoxic lesion (Boisvert, 1992; Yang et al., 1995). Further still excitotoxicity relies on the second messenger Jnk3 to be functional, as mice with Jnk3 gene deletion are protected against excitotoxic induced apoptosis (Yang et al., 1997).

Glutamate activates neurons both pre and post-synaptically, on almost all neuronal types including DA neurons, via two major families of receptors: G protein metabotropic receptors (mGluR) that work in tandem with ionic ligand gated receptors including N-methyl-D-aspartate (NMDAR) and a-amino-3- hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), which when activated depolarise the neuronal membrane. Moreover, these receptors are also involved in long-term potentiation (LTP) (Anwyl, 2009), with LTP playing a role in the process of excitotoxicity that can also contribute to the host neurons' over stimulation (Gonçalves-Ribeiro et al., 2019). Further still, excitotoxicity does not exclusively depend on excessive quantities of glutamate in the synaptic cleft. Indirectly, excitotoxicity, through hyperactivation of NMDAR's can be induced without requirement of glutamate, and still lead to a compromised membrane potential which is more often associated with endogenous or exogenous induced mitochondrial impairment (Mira and Cerpa, 2021). These mechanisms however are not thought to be primary to the demise of DA neurons but an ancillary effect elicited through other types of insult and subsequently free to exert said effects with greater potency due to the intrinsic vulnerability of DA neurons, per se.

1.6.7. Iron accumulation

As the most abundant transition metal in the body, it is unsurprising that dysfunction and accumulation of iron is found to play a role in PD pathology (Mochizuki and Yasuda, 2012). A 65-year-old study unearthed a unique distribution of iron throughout the brain, one which revealed a diverse range of iron levels at only 1.4mg/100g fresh weight in the medulla oblongata and markedly

greater levels in the DA rich regions of the GP at 21.3mg/100g fresh weight and in the SN at 18.5mg/100g fresh weight (Hallgren and Sourander et al., 1958). Subsequent studies revealed that iron levels in the SN were greater in those with PD compared to healthy controls (Sofic, 1988; Dexter et al., 1989). Neuromelanin, the dark pigment that gives the SN its distinctive colour happens to be a major source of neuronal iron due to its capacity to bind to high levels of iron at two sites (Double et al., 2003). Furthermore, the neuromelanin in PD patients is known to have substantially more melanin bound iron compared to healthy controls (Jellinger et al., 1992; 1993). Iron may also play a role in stabilising α-synuclein to its non-toxic locality at neuronal presynaptic membranes, with other studies showing that α-synuclein aggregates in the presence of iron (Ostrerova-Golts et al., 2000). As PD progresses, and SN DAergic neurons begin to perish, the excess levels of melanin sourced iron may further exacerbate DA neuronal cell death through its capacity to facilitate the aggregation of α-synuclein. Given the complexity of PD pathology involving factors such as iron accumulation, accurate animal models are crucial for replicating all neuropathological features and neurochemical markers associated with the condition.

1.7. Animal models of Parkinson's disease

An accurate model of PD would replicate all neuropathological features and neurochemical markers associated with the condition. Depending on the study's objectives, this would encompass motor dysfunction, extensive cell death in the SN, depletion of DA content in the striatum and cortex, microglia-mediated neuroinflammation in the striatum and SN, proteasome dysfunction, mitochondrial impairment, calcium dysregulation, and the accumulation of cytoplasmic Lewy neurites in the SN, immunoreactive for α-synuclein. However, replicating all aspects of Parkinson's in one model is not always necessary or useful. Instead, a model can serve its purpose by simulating a specific aspect of Parkinson's relevant to the research focus.

For instance, if a study's aims are made up of determining whether a particular drug or cell transplantation therapy can restore motor function by increasing DA levels then a model which can partially deplete DA is necessary. Conversely, if the

study aims to evaluate whether a potential treatment can diminish the accumulation of α-synuclein in Lewy bodies, a model inducing the formation of Lewy bodies is required. The following paragraphs will detail various animal models and the efforts of researchers to replicate the neurophysiological hallmarks of PD.

1.7.1. 6-OHDA

Ungerstedt, (1968) was one of the first researchers known to have injected 6-hydroxydopamine (6-OHDA) into the striatum of an animal and subsequently found there to be anterograde cell death of the entire DAergic nigrostriatal system, the results of which were found to be similar in pathogenesis to what has been observed in human post mortem PD brain (Anglade et al., 1995). Furthermore, there is a reduction in total striatum DA concentration, presynaptic DA reuptake, and a greater relative rate of DA synthesis in residual striatal DA nerve terminals after injection with 6-OHDA into the lateral cerebroventricles of both neonatal and adult male Sprague-Dawley rats (Stachowiak et al., 1987). 6-OHDA inhibits mitochondrial function leading to DAergic cell loss, in-part through an increase in ROS reactivity and ERK activation (Kulich et al., 2007).The functional neurotoxic mechanism of 6-OHDA has been shown to attack the cell bodies and fibres of DA neurons which results in the degeneration of synaptic nerve terminals and its neurotoxic effects inhibiting mitochondrial respiratory enzymes which ultimately lead to: oxidative stress, neuronal metabolic deficits and eventual cell death (Blum et al., 2001). Due to being highly reproducible, 6-OHDA has continued to be used extensively in research for replicating robust models of PD pathology.

1.7.2. Rotenone

Rotenone is a lipophilic crystalline isoflavone and is broadly used as a commercial pesticide. The fact that it is also used to model PD in animals supports the theory that human exposure to environmental toxins may contribute to the development of idiopathic PD. Not utilised in research as much as MPTP and 6-OHDA due to the high variability in levels of fibre loss in the striatum and neuron loss in the SN. Its mechanisms of action for inducing cell death in DA neurons is similar to MPTP, whereby it causes cell death by interfering with the mitochondrial transport chain

leadimg to the subsequent reduction in ATP production, and eventual cell death (Hasan et al., 2020). Interestingly rotenone's toxicity, may increase the vulnerability of DA neurons by interfering with the integrity of the cytoskeleton (Roy et al., 2023), a concerning finding especially considering the fact that many agricultural communities around the globe still actively use the pesticide.

1.7.3. MPTP

The discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was made by a chemistry graduate in 1976 who synthesised desmethylprodine (MPPP), an opioid analgesic drug, into MPTP. He subsequently injected the compound into himself, and several years later so too did four others, all of whom developed parkinsonian characteristics as a result. These comparable parkinsonian symptoms were shown to be reversed upon levodopa treatment, and with some further investigation by J. William Langston the MPTP PD model was born (Langston and Palfreman, 2013).

The mitochondrial inhibitor MPP^+ induces the selective destruction of SN DAergic neurons via the depletion of ATP. MPP^+ is metabolically derived from MPTP (which in itself is not toxic) by astroglial monoamine-oxidase-B (MAO-B) enzyme. A mechanism which is highlighted by the fact that MAO-B knockout mice are resistant to MPTP neurotoxicity (Shih and Chen, 2004). MPP^+ is then taken up in to DA neurons by the DA transporter (DAT) where it inhibits the normal function of the mitochondrial electron transport chain resulting in cell death and the build-up of ROS which furthers cellular destruction. Mice that do not express DAT are protected from MPTP toxicity (Bezard et al., 1999). As a consequence, the high affinity that MPP^+ has for DAT makes DA neurons highly vulnerable to MPP^+ toxicity (Langston et al., 1984; Przedborski et al., 2004).

Since the 1980's the MPTP PD model has been investigated in several animal species, including; mice, cats, dogs, marmosets and macaques. The MPTP animal model is still widely utilised today due to several important factors; by administering specific doses of MPTP, the lesion in the SN can be moderated to whatever extent is required, therefore allowing for an early or late stage-like PD model. Unlike the

progressive development of parkinsonian symptoms exhibited by transgenic and knockout animals, which can take many months and even years to develop, MPTP administration can develop the required lesion within days, thus allowing for complex investigations, such as neuroprotection studies to be undertaken in a timely manner; the economic benefits are also a factor as transgenic models are significantly more expensive to run than the conventional neurotoxin models.

The PD characteristics most associated with MPTP administration are a loss of DA neurons in the SN, an overall loss of DA in the nigrostriatal pathway, and varying degrees of motor dysfunction (extensive in marmosets and macaques), which subsequently make the non-human MPTP primate model excellent for studying PD motor dysfunction including LID. There have been several studies that have shown chronic administration of MPTP can induce α-synuclein accumulation in the SN, however this is often only achieved with minipump administration via i.p. and s.c routes as opposed to bolus injections (Fornai et al., 2005). There are disparities however, in how different mammals present behaviourally upon MPTP dosing, for example marmosets develop extensive catalepsy (Iravani et al., 2005) whereas MPTP treated mice often exhibit no long-term motor deficits (Meredith and Kang, 2006; Meredith and Rademacher, 2011) and in instances of chronic MPTP dosing there is an increase in locomotor activity (Luchtman et al., 2009). It is likely that as the animal becomes more sentient MPTP treatment exerts its neurotoxic effects with greater potency; for example, in marmoset's doses of 1.0 mg/kg can produce a significant partial lesion as well as causing severe motor dysfunction (Iravani et al., 2005), whereas in mice a similar lesion is only achieved at doses closer to 25 mg/kg, and even at that dose is often not able to cause any ailments to normal motor function (Kurosaki et al., 2004).

Table 1. Advantages and disadvantages of the classical neurotoxin PD animal models. The 6-OHDA model causes DA loss and rotational behaviour without α-synuclein aggregation, useful for symptom studies. Rotenone induces DA loss with α-synuclein but is labour-intensive. MPTP causes DA loss but lacks α-synuclein aggregation (Adapted from Blesa et al., 2012).

Model	Behavioural symptoms	Nigrostriatal damage	a-synuclein aggregation/Lewy body formation	Model applications	Disadvantages
6-OHDA	Rotational behaviour after unilateral lesion	Loss of DA innervation at injection site (striatum)	No inclusions	Screen therapies that may improve PD symptoms. Study mechanism of cell death	Requires intracerebral injection, very little synuclein involvements
Rotenone	Reports of decreased motor activity in rodents	Loss of DA neurons accompanied by reduced DA innervation in striatum	Synuclein aggregation in DA neurons	Test neuroprotective compounds	Substantial morbidity and mortality. Labour and time intensive
MPTP	Motor impairments in primates Less obvious motor impairments in acute rodent models	Loss of DA neurons dependent on dosing regimen, reaching 95% in acute high-dose conditions. Reduced DA levels in striatum concurrent with midbrain DA neuron loss	Inclusions not prevalent. Few cases of synuclein aggregation in nonhuman primates, as well as increased synuclein immunoreactivity in rodents.	Screen therapies that may improve PD symptoms. Study mechanisms of cell death	Non-progressive model of cell death. Inclusion are rare

1.7.4. Transgenic animal models

The MPTP model is therefore not faultless, and discrepancies between the different mammalian models is still an area of modest contention and remain open to further investigation. In light of these limitations, researchers have also studied various genetic mutations associated with familial PD, including loss of function mutations to PINK1, Parkin, and DJ-1, as well as gain of function mutations in LRRK2 and α-synuclein, through knockout and transgenic mouse models to deepen our understanding of the disorder's mechanisms

Knockout and transgenic models provide specific but not all pathological hallmarks of PD; for example, knockout of Pink1 in mice results in a 25 % loss of TH reactive cells in the SN, deficits in ATP respiration, but no loss of DA or presence of Lewy bodies (Gopert et al., 2009; Moisoi et al., 2013). Knockout of the Parkin gene results in mitochondrial DNA (mtDNA) to undergo double stranded breaks and recombination in DAergic cells with an overall net increase in recombinant mtDNA (Pinto et al., 2018); whereas in MPTP treated mice the upregulation of the Parkin gene prevents motor deficits and DA cell loss (Yasuda et al., 2011). Equally, DJ-1

knockout augments DAergic neuron loss and neuroinflammation in MPTP treated mice, promotes inflammation in microglia and regulates the NLRP3 gene (expressed in macrophages and aids in the detection of damaged cells and extracellular ATP) via the transcription factors, Nuclear factor erythroid 2-related factor 2 (Nrf2) and Thioredoxin-1 (Trx1) (Ji et al., 2020). Mice carrying the A30P and A53T mutations of human α-synuclein have reduced levels of DA and increased levels of α-synuclein in the striatum and SN but exhibit no long-term changes in locomotor activity (Kilpelainen et al., 2019). Interestingly, DA neurons originated from LRRK2 iPScs (induced pluripotent stem cells) have reduced neurite morphology, impaired mitochondrial function and accumulation of α-synuclein and Tau protein (Daniel and Moore, 2015), but in LRRK2 knockout mouse models these findings are not replicated. Among the many attempts of modelling LRRK2 mouse knockout models there has been no notable DAergic neurodegenerative changes (Xiong et al., 2017). Moreover, the nigrostriatal pathway in LRRK2 knockout mice remains intact even at 2 years of age and nor do they exhibit any extra sensitivity to MPTP (Andres-Mateos et al., 2009; Hinkle et al., 2012). However, upregulation of LRRK2 in mice has been shown to perhaps best reflect PD like nigrostriatal neurodegeneration. Of the two known studies to have achieved this each has used the PDGF-β promotor to generate mutant LRRK2 mouse. One of the studies found 20% loss of DA in the SN (Ramonet et al., 2011) and the other noted 50% loss in the SN (Chen et al., 2012). However, these findings are rarely replicated making this model somewhat precarious for the accurate modelling of PD pathology and/or subsequent determination of PD associated therapies.

Each of the aforementioned transgene and gene-knockout models can provide vital information to aid in understanding the molecular mechanisms involved in PD pathogenesis, particularly those regulating the mitochondrial respiratory chain. However, there are few reports of cell loss in the SN or impairments in motor function. Depending on the aims of the study, a researcher investigating the potential neuroprotective properties of a novel drug would consider whether use of a traditional neurotoxin, or genetic animal model is most appropriate. Given that transgene models result in little to no loss of SN DA neurons or striatal DA nerve

fibers, utilising the MPTP model is arguably a more suitable approach for neuroprotective animal PD modeling due to the construct, predictive, and face validity a classic neurotoxin model offers.

Such considerations are crucial as researchers explore neuroprotection in the context of ageing and neurodegenerative diseases, including PD and AD, where the need for effective interventions is pressing.

1.8. Investigations of neuroprotection using models of Parkinsons disease

Metabolic slowing, the shortening of telomeres or the life exposure to stress and environmental toxins are all known contributing factors to ageing (Blaszczyk et al., 2020; Rackova et al., 2021; Verma et al., 2022). The brain is not exempt from these factors and for many of us it will often deteriorate at a faster rate than the rest of the body (Pluvinage and Wyss-Coray, 2020). The ageing brain is afflicted by the progressive loss of neurons often leading to deleterious and severely debilitating disorders, the two most common being PD and AD. If cell loss in the brain can be halted or even slowed, then many thousands of individuals would be able to extend their lives considerably as well as improve the quality of their life, and equally have an indirect but concomitant effect on reducing the burden on the health care system and that experienced also by caregivers. For this notion to become a reality there would have to be a major behavioural, environmental or pharmacological interjection. The point at which a clinical diagnosis is made for either PD or AD is almost always at a stage of considerable neuronal deterioration – therefore, if the factors responsible for cell loss in the brain are due to an environmental toxin or unknown injury, then, even if those factors can be identified, countering them to the point of negating neurodegeneration would be an extremely difficult and unlikely feat. Hence, what is coveted most by clinicians and patients alike, is the discovery of a pharmacological agent that can slow or even prevent cell death in the brain. Researchers have invested considerable time and resources in exploring potential neuroprotective drugs for PD. Whether it is an undiscovered organic extract sourced from the depths of the Amazon rainforest or a newly developed synthetic compound, the dedication to this area of research is substantial.

The field of neuroprotective research is vast, however no clinically available drug is known to halt neuronal cell death in a patient living with PD (Jankovic and Tan, 2020; Church, 2021; McFarthing et al., 2022). Though, there are countless synthetic and organic compounds which have shown exceptional promise in animal models of neurodegenerative disorders, which offer invaluable insights into the mechanisms controlling cell death and cell survival in PD aetiology.

1.9. Overview of contemperanous literature of MPTP mouse neuroprotection studies

The following is a strategy for providing an overview of key articles that highlight which drugs are proven effective in mitigating nigrostriatal DAergic degeneration in MPTP mouse models of PD. A comprehensive literature search was conducted using both PubMed and Google Scholar. The search terms "#Neuroprotection," "#Mice," and "#MPTP" were entered into the search engines of each platform, yielding a total of 50,101 results. Subsequently, the search criteria were to identify contemporary literature so only those published in the last 10 years were used, excluding studies published before 2013, which led to the retrieval of 19,361 results. Further refinement involved selecting studies exclusively published in the prestigious journal "Nature" and its affiliated journals, including but not limited to "Nature Reports," "Acta Pharmacologica," "Experimental Molecular Medicine," "Scientific Reports," and "Cell Death and Disease," resulting in 1,052 pertinent studies. To ensure a focused investigation, studies using exclusively knockout and transgene models, experiments incorporating composite drug treatments, and those not involving MPTP were excluded. This meticulous screening process led to the compilation of a final selection of 39 studies, offering a concise overview of some of the most promising current neuroprotective compounds derived from endogenous sources as assessed in an MPTP mouse model of PD.

Study selection

1. # Neuroprotection # Mice # MPTP – Years (*any*), source (*any*) = Scholar: 48,300 results; Pubmed: 1,801 results

2. # Neuroprotection # Mice # MPTP – Years (*2013-present*), source (*any*) = Scholar:18,300 results; Pubmed: 1061 results

3. # Neuroprotection # Mice # MPTP – Years (*2013-present*), source (*Nature*) = Scholar: 1010 results; Pubmed: 42 results

4. # Neuroprotection # Mice # MPTP – Years (*2013-present*), source (*Nature*), Abstract screening: exclude knockout and transgene models (except for those that have also used MPTP); exclude experiments using composite drugs; exclude studies that have not used MPTP = Scholar: 30; Pubmed: 19 (excluding duplicate search results = 10). Total: 39 Studies

Table 2. Pre-clinical models of PD that have utilised the neurotoxic compound MPTP for the purpose of investigating potentially neuroprotective compounds. Various compounds were tested in MPTP mice for Parkinson's neuroprotection: NSA, irisin, and 4A7C-301 protect DA neurons, while sulforaphane, melatonin, and GM1 ganglioside enhance DA levels. Natural products (e.g., Corynoxine B, withaferin A) and pharmaceutical agents (e.g., reboxetine, pramipexole) show anti-inflammatory and neuroprotective effects, each targeting different pathways.

Author	Drug	Target/action	Model	Outcome
Yea Hyun Leem 2023	Necrosulfonamide (NSA)	Mixed lineage kinase domain like pseudokinase (MLKL) inhibitor	MPTP mice	NSA effectively inhibits α-synuclein oligomerisation and phosphorylation within the SN of MPTP-treated mice. Inhibition occurred through the suppression of glycogen synthase kinase 3β and matrix metalloproteinase-3 activity.
Zhang et al., 2023	Irisin	Myokine	MPTP mice	Irisin provides neuroprotection by reducing apoptosis and oxidative stress, preventing mitochondrial fragmentation, and enhancing mitochondrial respiration and biogenesis.
Kim et al., 2023	4A7C-301	Nurr1 agonist	MPTP mice	4A7C-301 demonstrates protective effects on midbrain dopamine neurons.
Wang et al., 2023	(+)-desdimethylpinoresinol 03A10	Present in the fruit of *Vernicia fordii* (Euphorbiaceae)	MPTP mice	Oral administration of 03A10 at a dose of 5 mg/kg per day over a span of 4 months, reduces olfactory deficits, aggregated α-synuclein accumulation (but not monomeric α-synuclein).
Zhu et al., 2022	Corynoxine B derivative (CB6)	Natural alkaloid (autophagy inducer)	MPTP mice	Oral administration of CB6 (at doses of 10 and 20 mg/kg per day for a duration of 21 days) results in an improvement in motor dysfunction and prevents loss of DAergic neurons in the striatum and SNpc.
Li et al., 2022	Melatonin	Hormone of the pineal gland	MPTP mice	Treatment with melatonin, administered i.p. at a dosage of 20 mg/kg per day for a duration of 7 days, leads to marked protection of DA neurons. Protection was accompanied by reduced inflammation and an increase in the anti-inflammatory M2-like phenotype within microglial cells.
Li et al., 2022	Sulforaphane	Nrf2 activator	MPTP mice	Multiple administrations of sulforaphane, effectively mitigated DAergic neurotoxicity in mice treated with MPTP, primarily through activation of the neurotrophic factor, BDNF.
Zhao et al., 2021	Withaferin A	Steroidal lactone	MPTP mice	Administration of withaferin at doses of 20, 200, and 2000 μg/kg per day effectively alleviates loss of DAergic neurons in the SN.
Liu et al., 2021	Lovastatin	Inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase	MPTP mice	Lovastatin mitigates behavioural impairment and the loss of DAergic neurons by activating SHP2/Parkin-mediated mitophagy.
Feng et al., 2021	MXenzyme	Nano-enzyme	MPTP mice	MXenzyme restores redox homeostasis without disrupting the body's natural antioxidant defenses and alleviates damage caused by reactive oxygen species (ROS) with gentle therapeutic effects *in vivo*.
Guo et al., 2021	GM1 ganglioside	Ganglioside containing one sialic acid residue	MPTP mice	Following a 2-week period of i.p administration of GM1 ganglioside at doses of 25 and 50 mg/kg per day, it was shown that treatment ameliorated the behavioural disturbances induced by MPTP. Furthermore, GM1 ganglioside administration leads to a notable increase in the levels of DA and its metabolites within striatal tissue.
Rajan et al., 2020	Prostaglandin E1 (PGE1) and metabolite, PGA1	Stimulates Nurr1actiavtion	MPTP mice	PGE1 and PGA1 demonstrate neuroprotective effects in a manner dependent on Nurr1, increasing the expression of Nurr1 target genes in midbrain DA neurons.
Lin et al., 2020	Necrostatin-1	allosteric inhibitor of RIPK1 kinase activity	MPTP mice	The application of necrostatin-1, which blocks the necroptosis pathway, significantly elevates DA levels

				and rescues the loss of DAergic neurons which is shown to be independent of the apoptotic pathway.
Wei et al., 2019	Pyroxidine	An alcohol derived from pyridine	MPTP mice	Pyridoxine has the capability to induce the dimerisation of PKM2, facilitating the promotion of GSH biosynthesis. Experimental evidence suggests that supplementing with pyridoxine enhances the resilience of nigral DAergic neurons.
Zhou et al., 2019	Nicotinamide adenine dinucleotide phosphate (NADPH)	Co-factor used in anabolic reactions	MPTP mice	NADPH, crucial for maintaining glutathione levels, successfully shields DAergic neurons from MPTP neurotoxicity.
Ren et al., 2019	Ganoderrna lucidum extract (GLE)	Lingzhi mushroom	MPTP mice	Administration of GLE at a dosage of 400 mg/kg per day via oral gavage for a duration of 4 weeks improves locomotor performance. Additionally, there is a substantial increase in TH expression in the SNpc.
Kreiner et al., 2019	Reboxetine	Norepinephrine reuptake inhibitor	MPTP mice	Reboxetine ameliorates the parkinsonian phenotype and delays the progression of DAergic neurodegeneration in the SN and VTA. This treatment also leads to higher DA content in the striatum.
Ji et al., 2019	BDNF	Neurotrophin growth factor	MPTP mice	Focused ultrasound-enhanced intranasal delivery of BDNF induced significantly greater neuroprotection, highlighting the potential importance of enhancing BDNF induced neuroprotection through a specific treatment route.
Li et al., 2019	Imidazolone-morphinan (Compound 8)	Similar to in structure and activity to 3-hydroxymorphinan (inhibits glutamate release)	MPTP mice	Compound 8 effectively enhances motor behaviour, as evidenced by improvements in the rotarod test and pole test results, along with an increase in the number of TH+ve neurons.
Nicholatos et al., 2018	Nicotine	Receptor agonist at nicotinic acetylcholine receptors	MPTP mice	SIRT6 depletion in the brain protects against MPTP while SIRT6 overexpression exacerbates the condition. This implies that SIRT6 contributes to PD pathology and that nicotine can provide neuroprotection by promoting its degradation.
Zhang et al., 2018	Jia-Jian-Di-Huang-Yin-Zi (JJDHYZ) Classical prescription of Traditional Chinese medicine	Decoction (a method of extracting chemicals from herbal or plant materials by boiling them, often involving stems, roots, bark, and rhisomes to dissolve their constituents)	MPTP mice	Administration of a high dose of JJDHYZ (34 g/kg/day) effectively mitigates the loss of DAergic neurons, reverses DA depletion, and enhances the expression of glial-derived neurotrophic factor (GDNF) in comparison to the untreated model group.
Jeong et al., 2018	DA-9805 Extract	Angelica Dahurica root	MPTP mice	Oral administration of DA-9805 successfully restores DA content.
Castro-Hernandez et al., 2017	Pramipexole	D3 Dopamine agonist	MPTP mice	Administration of the preferential D3 receptor agonist, pramipexole, partially restores normal synaptic transmission and Long-Term Potentiation.
Chung et al., 2017	Capsaicin	Chili pepper extract vanilloid subtype 1 (TRPV1) agonist	MPTP mice	Activation of TRPV1 by capsaicin has a rescuing effect on nigrostriatal DA neurons, improves striatal DA functions, and enhances behavioural recovery.
Pan et al., 2017	*Coeloglossum viride*var. Bracteatum extract	Iranian poppy	MPTP mice	CE exhibits neuroprotective effects in both *in vitro* and *in vivo* settings.
Zhang et al., 2017	Rapamycin	Inhibits IL-2 and other cytokine receptor-dependent signal transduction mechanisms, via action on mTOR	MPTP mice	Rapamycin increases the expression of interleukin-6 (IL-6), which is linked to reduced expression of inflammatory cytokines, suggesting anti-inflammatory properties of IL-6.
Shen et al., 2017	Nesfatin-1	Neuropeptide produced in the hypothalamus	MPTP mice	Nesfatin-1 effectively mitigates the loss of nigral DAergic neurons in an MPTP mouse model of PD.

Gu et al., 2017	P7C3	Aminopropyl carbazole	MPTP mice	P7C3 successfully suppresses the activation of GSK3β and p53 in the midbrain, consequently preventing the loss of DAergic neurons in the SN.
Cheng et al., 2016	Copper ATSM	It is thought to function by delivering copper to damaged mitochondria within cells	MPTP mice	MPTP lesioning influenced a total of 143 genes associated with biological processes related to brain and cognitive development, DA synthesis, and disrupted synaptic neurotransmission. Following ATSM treatment, the expression of 40 genes involved in promoting DA synthesis, calcium signaling, and synaptic plasticity was restored.
Chung et al., 2016	JWH-133	CB2 receptor agonist	MPTP mice	Administration of JWH-133 at a dosage of 10 μg/kg through i.p injection effectively prevents the degeneration of DA neurons in the substantia nigra (SN) and their corresponding fibres in the striatum.
Filichia et al., 2016	P110	Inhibits the mitochondrial fission regulator Drp1	MPTP mice	Inhibition of Drp1 hyperactivation by the Drp1 peptide inhibitor, P110, demonstrates neuroprotective effects.
Ren et al., 2016	Dihydromyricetin	Flavanol form Japanese raisin tree	MPTP mice	Dihydromyricetin substantially mitigates the behavioural impairments and loss of DAergic neurons induced by MPTP in mice.
Drinkut et al., 2016	GDNF	Neurotrophic factor	MPTP and Ret gene knockout	The complete absence of Ret eliminates GDNF's neuroprotective and regenerative impact on the midbrain DAergic system. This underscores the essential requirement of Ret signaling for GDNF's ability to prevent and compensate for DAergic system degeneration, highlighting Ret activation as one of the primary targets of GDNF therapy in PD.
Ellet et al., 2016	Cu(II) (ATSM)	Copper complex	MPTP mice	The oral administration of ATSM improves stool frequency and also correlates with the restoration of neuronal subpopulations in the myenteric plexus.
Liu et al., 2015	Tiagabine	GABA re-uptake inhibitor	MPTP mice	Pre-treatment with tiagabine effectively reduces microglial activation, offers partial protection to the nigrostriatal axis, and ameliorates motor deficits.
Hong et al., 2015	NE100 Ro25-6981	Sigma 1 receptor (σ1R) agonist NR2B inhibitor	MPTP mice	The σ1R antagonist NE100 and the NR2B inhibitor Ro25-6981 are both effective at alleviating motor deficits and preventing the loss of DAergic neurons in MPTP mice.
Karuppagounder et al., 2014	Nilotinib	c-Abl inhibitor	MPTP mice	The administration of nilotinib reduces c-Abl activation and lowers the levels of PARIS (a substrate of parkin). This results in prevention of DA neuron loss and amelioration of behavioural deficits.
Park et al., 2013	6-Shogaol	Ginger constituent	MPTP mice	6-shogaol reverses the MPTP-induced reductions in the number of TH+ve cells in the SNpc and the intensity of TH immunoreactive fibers in the striatum. Furthermore, 6-shogaol significantly inhibits microglial activation induced by MPTP and reduced the levels of TNF-α, NO, iNOS, and COX-2 in both the SNpc and striatum.
Cartelli et al., 2013	Epothilone D	Microtubule stabiliser	MPTP mice	Daily administrations of the microtubule stabiliser Epothilone D not only rescues microtubule defects but also mitigates the degeneration of the nigrostriatal pathway.

The literature search has revealed a diverse range of chemical agents, including but not limited to; naturally occurring flavones, ACh agonists, and nano-enzymes, all of which have successfully exhibited neuroprotective properties in an MPTP model of PD (Table 2). The fact that researchers in the field of neuroprotection are

exploring such a wide spectrum of chemically structured compounds implies that recovery or preservation of damaged or dying DA neurons can potentially be achieved through a variety of molecular mechanisms. It should be noted however that these results and the mechanisms responsible for achieving murine neuroprotection may not fully translate to human PD pathology, though they do emphasise the breadth of pharmacological approaches carried out in the pursuit of finding a neuroprotective drug to counter PD pathogenesis.

1.10. Neuroprotection in Parkinson's disease

As previously described, there are many different drugs which are investigated preclinically with the ultimate ambition for that drug of becoming an efficacious treatment for a neurodegenerative disease. However, only 10-12% of drugs which have been shown to exhibit promise pre-clinically go on to achieve success following Phase3A clinical trials (Biotechnology Innovation Organisation (BIO), in collaboration with Informa and the Clinical Trials Transformation Initiative (CTTI) 2016). Several factors contribute to why only a small percentage of drugs become successful clinical treatments, despite their considerable pre-clinical success. These factors include the doses of the study drug used, insensitive clinical endpoints, and the patient population selected for study. For example, one clinical trial aimed to determine whether Omigapil (TCH346), an apoptotic inhibitor, could halt the progressive loss of DAergic neurons in human PD patients, but was ultimately unsuccessful (Olanow et al., 2006). Indeed, the drug had shown exceptional promise pre-clinically (Andringa et al., 2003), as rhesus monkeys bilaterally lesioned with MPTP were significantly neuroprotected with TCH346. The failure of the clinical trial, despite the success of the pre-clinical study, may be due to one or several of the factors already mentioned. Another possible reason is a mechanistic / pharmacological disconnect between MPTP-induced neurotoxicity of the DAergic nigral pathway in animal models, and the progressive physiological loss of nigral DA neurons in humans. TCH346 is structurally similar to selegiline, a known MAO-B inhibitor. However, TCH346 itself does not inhibit MAO-B, which suggests that the pharmacological profile of TCH346 does not account for its failure in human clinical trials, but still may be due to some other unknown pharmacological quality that inhibits MPTP's neurotoxic effects.

Similarly, serotonin (5-HT), a hormone and monoamine neurotransmitter, has been extensively studied for its potential neuroprotective effects (Chilmonczyk et al., 2017; Nykamp et al., 2022) underscoring its importance beyond its well-known role in modulating homeostatic and physiological functions of the mammalian CNS. The following paragraphs will discuss how and why serotonin is an important neurotransmitter and modulator of PD pathology.

1.11. Serotonin

Serotonin (5-HT) is a hormone and monoamine neurotransmitter, highly involved in modulating homeostatic and physiological functions of the mammalian CNS. Known for over a century as having vasoconstrictive properties, 5-HT was first isolated and characterised in 1948 (Rapport et al., 1948) and subsequently identified in 1953 by Twarog and Page (1953) as being one of the chief amines, amongst adrenaline (ADR), noradrenaline (NA), DA and histamine, modulating the CNS. The majority of 5-HT utilised in the brain is synthesised and stored primarily in the RN (Andén et al., 1966), and a smaller amount in the reticular formation. A much greater amount (90-95% of total) is stored by enterochromaffin cells in the gastrointestinal tract where it is used to regulate gut contractility. Serotonergic neurons from the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) project distinct serotonergic fibers via the medial forebrain bundle (MFB) to frontal and cerebellar/spinal regions, respectively. The DRN receives projections from the hypothalamus, medulla, cortex, and amygdala, while the MRN is influenced by projections from the amygdala, mPFC, and other cortical areas, with contributions from the hypothalamus and midbrain (Bang et al., 2012; de Aguiar et al., 2021). This highlights the widespread nature of serotonergic connectivity in the mammalian brain.

Production of 5-HT begins with the essential amino acid, tryptophan, being hydroxylated by tryptophan hydroxylase into 5-hydroxytryptophan (5-HTP). 5-HTP is then decarboxylated by L-aromatic amino-acid decarboxylase (AADC) to form 5-HT (Figure 5). Serotonin is synthesised and stored in the pre-synaptic serotonergic neurons in cell bodies of the pons, the largest nuclei being the RN. it is therefore key in regulating the serotonergic pathway, and has extensive nerve

fibres projecting into the frontal cortex and descending fibres into the medulla and spinal cord.

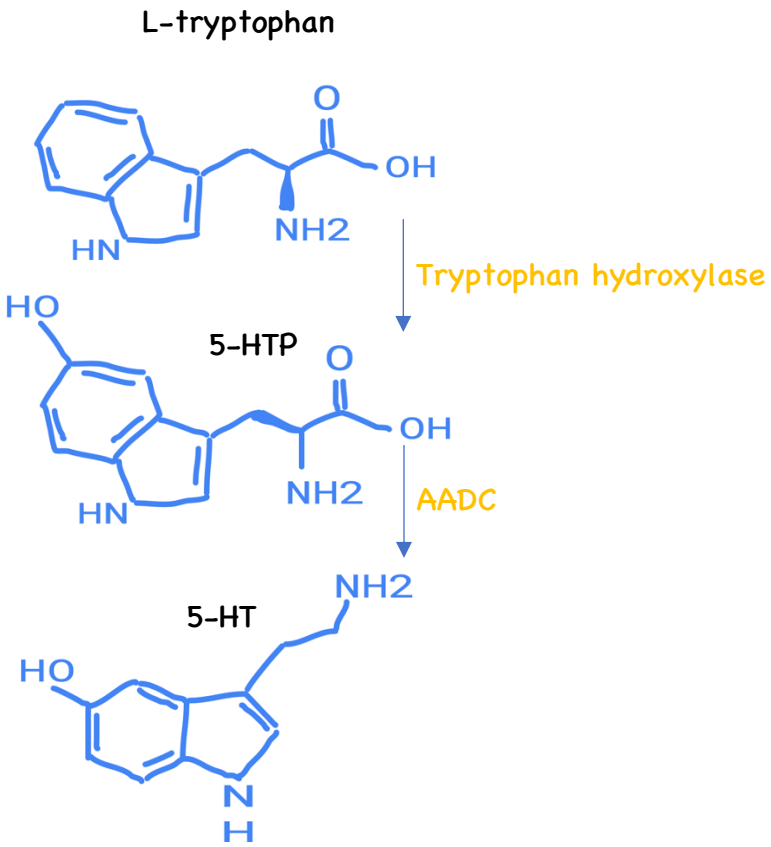

Figure 5. Enzymatic controlled synthesis of L-tryptophan into serotonin. L-tryptophan is converted to serotonin in two steps: first, tryptophan hydroxylase converts L-tryptophan to 5-hydroxytryptophan (5-HTP), and then aromatic L-amino acid decarboxylase (AADC) converts 5-HTP to serotonin.

The concentration of intracellular 5-HT is dependent upon its synthesis, metabolism and re-uptake by presynaptic terminals. Metabolism of 5-HT is carried out by monoamine oxidase A (MAO-A) which can also metabolise DA, ADR, NA and histamine. Metabolism of 5-HT by MAO-A produces the metabolite 5-hydroxyindoleacetic acid (5HIAA), the majority of which is excreted through the urinary tract.

Serotonin mediated inter-neuronal communication is carried out via cellular depolarisation of pre-synaptic neurons resulting in the rapid release of 5-HT into the synaptic cleft, where it is either received by 5-HT receptors on post-synaptic nerve terminals or taken back into the pre-synaptic terminals via the autoreceptor SERT. Re-uptake of 5-HT by SERT inhibits further release of 5-HT.

Each with their own distinct roles and second messenger mechanisms, there are fourteen types of 5-HT receptor currently known which can be divided into seven families: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6 and 5-HT_7. Apart from the 5-HT3 receptor which is activated via ion-gated channels, all other 5-HT receptor subtypes are G-protein couple receptors.

1.11.1. 5-HT_{1A}

The 5-HT_{1A} receptor is a G-protein coupled receptor made up of 422 amino acids which is ubiquitous throughout the CNS and abundant within the limbic system. It can be found both as an auto-receptor in the 5-HT nuclei of the RN and as a post-synaptic hetero-receptors in the hippocampal, hypothalamic and cortical regions of the brain (Lesch & Gutknecht, 2004; Sharp et al., 2007). The 5-HT_{1A} receptor is a Gi/o coupled receptor and initiates signalling of target neurons via inhibition of adenylyl cyclase. Upon activation, 5-HT_{1A} auto-receptors in dorsal RN (DRN) projecting to frontal cortex desensitise by inhibiting extracellular protein kinase (ERK) activity, whereas hetero-receptors in the hippocampus do not, therefore implying that the sensitivity of 5-HT auto-receptors is reflective of their enhanced role in mediating synaptic 5-HT neurotransmission via DRN 5-HT neurons (Riad et al., 2001). Moreover, administration of SSRI's induces a similar response, whereby 5-HT_{1A} auto-receptors in the DRN rapidly desensitise and internalise from the cell membrane (Descarries & Riad, 2012). It is this conformational change that is thought to allow for a greater amount of 5-HT availability at post-synaptic receptors, thus leading to increased serotonergic activity in the forebrain cortical regions (Celada et al., 2013).

Not only have 5-HT_{1A} receptors been implicated in mediating mood, cognitive proficiency and social interaction, they have also been shown to play a role in developmental neuronal migration and synaptic formation. Research utilising rat models shown that treatment with the 5-HT_{1A} partial agonist, buspirone reverses the negative effects of prenatal exposure to alcohol (Kim et al., 1997), therefore suggesting that 5-HT_{1A} receptor function to be a likely mediator in aiding cerebral

growth during foetal development and suggesting its involvement in supporting neuronal morphogenesis over the life course.

1.11.2. SERT

Homeostatic regulation of neuronal 5-HT is understood to rely on a feedback loop using 5-HT, and a coupled transporter protein, from within the synaptic cleft. Located within the presynaptic membrane is the 5-HT transporter (SERT). SERT is part of a carrier family of transport proteins and is comprised of a string of amino acids that passes in and out of the presynaptic cell membrane. Upon signalling, 5-HT is released by presynaptic transmembrane vesicles into the synaptic cleft where it is taken up via postsynaptic 5-HT receptors, once an optimum amount of neurotransmitter has been received by a post-synaptic receptor, 5-HT is then recycled out of the synaptic cleft by SERT (Fuller et al., 1991).

Due to one of its primary roles in mediating the transmission of action potential throughout the serotonergic pathway, SERT has subsequently become a recognised therapeutic target for treating different types of psychiatric disorder, whereby selective 5-HT reuptake inhibitors (SSRI's) functionally block SERT activity (Stahl, 1998). The blocking of SERT via SSRI's leads to an increased concentration of extracellular 5-HT in the synaptic cleft, thus enabling postsynaptic 5-HT receptors to access greater quantities of available neurotransmitter (Schloss & Williams, 1998). The underlying mechanisms of SERT's functional localisation, however, remain largely unknown, with current research still trying to uncover many of the undetermined molecular mechanisms of reuptake inhibitor psychiatric therapy (Fakhoury et al., 2016).

1.11.3. *HTR1A* and *SLC6A4*

The 5-HT_{1A} receptor is encoded by the *HTR1A* gene. Disruptions to its optimal function, such as promotor polymorphisms or environmentally induced DNA methylation have been shown to cause dysregulations in its transcription (Xu et al., 2022). Moreover, changes to *HTR1A*'s functional sequence have been shown to associate with a multitude of neuropsychiatric and affective disorders in various human populations (Drago et al., 2008; Le François et al., 2008).

SLC6A4 encodes the transmembrane 5-HT transporter SERT, with several variants and polymorphisms of the gene being documented as eliciting structural and functional changes in SERT activity. Whether in rodents with *SLC6A4* knockout or in humans with a psychiatric disorder, current literature indicates that the dysfunction of SERT severely affects the structural and functional activity of transmembrane 5-HT receptors, including 5-HT_{1A} (Fabre et al., 2000; Arias et al., 2005).

Dysfunctions of the serotonergic genes *HTR1A* and *SLC6A4* are more often implicated in the development of psychiatric disorders. The 5-HTTLPR polymorphism of *SLC6A4* however, is shown to be associated with an increased risk for developing PD (Zhang et al., 2014). A recent study by Redenšek et al., (2022) found that the genetic variability in *SLC6A4* and *HTR1A* was associated with the efficacy and tolerance of DA replacement therapy administered to PD patients, and that individual gene to gene interactions of various alleles were shown to influence the extent of impulse control and motor function. This is a major finding, considering that levodopa induced dyskinesia (LID) can be offset by various serotonergic drug therapies (Corsi et al., 2021; Newman-Tancredi et al., 2022), and therefore suggests that the likelihood of 5-HT treatment efficacy in those experiencing LID is partly dependent upon serotonergic gene variability.

1.12. Serotonin and Parkinson's disease

Serotonin has been extensively and successfully used as a key target for modulating human neurochemistry. As a neurochemical, its endogenous manipulation has had particular success in tempering neuropsychiatric disorders, however, there is less known regarding 5-HT's multi-faceted role in the Parkinsonian brain. A revived interest within the field of PD research is focusing its attention on targeting 5-HT as a multicellular neuroprotective agent. Newly developed therapeutic compounds are currently being investigated that are capable of targeting specific subpopulations of 5-HT receptors in select regions of the mammalian brain. The specificity of these modern compounds means that they can elicit precise neurophysiological responses without interference to other vital neural mechanisms, typically hindered by other prototypical PD targeting drugs.

1.13. Motor function and 5-HT in Parkinson's disease

Dopaminergic cell death leads to a loss of neural DA and therefore the breakdown of nervous system motor circuits. Serotonin may modulate the extent at which these circuits are maintained and protected within the aetiology of PD. This is certainly indicated in PD patients experiencing LID. For example, the tracers ^{11}C-DASB and ^{11}C-raclopride PET have been used to measure SERT and DA binding potential (BP) in the globus-pallidus (GP). One study has shown LID severity corresponds with a decrease in ^{11}C-raclopride binding potential (BP) in the GP but a marked increase in ^{11}C-DASB BP in the GP, suggesting that an increase in serotonergic innervation of the GP as a key marker of LID severity (Smith et al., 2015).

In order to alleviate motor dysfunction, DA replacement therapy with levodopa has proved to be the gold standard therapeutic for those living with PD. Unfortunately, side effects are not uncommon, with the more typical of these manifesting as non-deliberate movements known as dyskinesias. These side-effects induced by levodopa therapy become progressively worse after several years, and can only be tempered by reducing the amount of levodopa taken, or by co-administration of other drugs such as amantadine; an NMDA antagonist with contentious views on its efficacy (Crosby et al., 2003; Sawada et al., 2010; Wolf et al., 2010). However, the question still remains, why is levodopa causing dyskinesia and why does it worsen over time? Studies have shown that post-synaptic serotonergic nerve terminals in the striatum are able to take up and convert exogenous levodopa into DA and liberate it as a false neurotransmitter into the locality of the DA innervated striatum (Tanaka et al., 1999; Maeda et al., 2005). This is likely due to 5-HT raphe-striatal neuron post-synaptic terminals containing aromatic L-amino acid decarboxylase (AADC) which renders them capable of metabolising levodopa into DA (Arai et al., 1996). Just as it is required for the synthesis of L-dopa into DA by AADC, so too is the requirement for synthesis of 5-HTP into 5-HT (Figure 3 and 5). Both DA and 5-HT neuronal projections innervate the striatum from the SN and RN, respectively (Ungerstedt, 1971; Kale et al., 1989). The striatum acts as a neural relay and has an abundant mass of DAergic projections being received from, and extending out to the cortical regions of the mammalian brain (Gerfen, 2000; Costa et al., 2006). Researchers have investigated whether 5-HTR

expressing medium spiny neurons (MSN) in the striatum are hijacking the uptake of levodopa, either due to a loss of striatal DA terminals or due to 5-HT neurons' inclined affinity for levodopa uptake, or possibly, a combination of the two. It is thought that due to the lack of autoregulatory feedback control, such as reuptake channel mediated synaptic inhibition, 5-HT innervated neurons are therefore unable to regulate the timed release of DA. Thus, the surge in stimulation of striatal DA release, via 5-HT innervation is considered to signal the motor cortex, leading to an over-activation of motor units in skeletal muscle, i.e. LID (Santiago et al., 1998; Carta et al., 2007). That LID becomes more prominent over time is almost certainly due to the age-related progressive cell death of DA neurons and a subsequent compensatory response of 5-HT neurons to facilitate the conversion of levodopa to DA (Nicholson & Brotchie, 2002). Conversely, the reduced levels of 5-HT commonly observed in the human and rodent PD modelled brain may be due to the competition between levodopa and 5-HT for vesicle storage in serotonergic expressing neurons (Borah et al., 2007); particularly so, as the vesicular monoamine transporter 2 (VMAT2) is utilised by both DA and 5-HT neurons (Lohr et al., 2014). Additionally, chronic levodopa treatment has been shown to decrease 5-HT nerve fibre density, however this was carried out on intact non-lesioned animals (Nevalainen et al., 2014). Further still, in MPTP lesioned marmosets, chronic levodopa treatment is associated with distinct hypertrophy of 5-HT nerve fibre varicosities in the striatum and GP, compared to vehicle treated controls (Zeng et al., 2006).

Table 3. Pre-clinical research over the last twenty years of 5-HT_{1A} drugs being trialled as modes of therapy for treating AIMS/LID. Various serotonin-targeting compounds (5-HT agonists, SSRIs) tested in Parkinson's models primarily reduced levodopa-induced dyskinesia (LID), with agents like NLX-112 and eltoprazine consistently showing strong anti-dyskinetic effects without compromising motor benefits. Some compounds (e.g., 8-OH-DPAT) reduced LID but worsened parkinsonian symptoms.

Author & Year	Model	Drug(s)	Receptor(s)	Outcome
Bibbiani 2001	6-OHDA Rat, MPTP Monkey	Sarizotan	5-HT_{1A} agonist, D_2 antagonist, SERT SSRI	Reversed shortening of L-dopa induced motor response in rats and reduced LID dyskinesia in monkey.
Tomiyama 2005	6-OHDA Rat	8-OH-DPAT	5-HT_{1A} agonist, 5-HT_7 agonist	Inhibited development of L-dopa induced rotational behaviour.
Iravani 2006	MPTP Marmoset	8-OH-DPAT	5-HT_{1A} agonist, 5-HT_7 agonist	Reduction of LID but with worsening of parkinsonian features.
Eskow 2007	6-OHDA Rat	Buspirone	5-HT_{1A} agonist, $D_{2/3/4}$ antagonist	Dose dependent reduction of LID with an improvement in motor performance.
Munoz 2008	MPTP Macaque	8-OH-DPAT CP-94253	5-HT_{1A} agonist, 5-HT_7 agonist 5-HT_{1B} agonist	Drug combination completely suppressed LID at sub threshold individual doses.
Tani 2010	6-OHDA Rat	Piclozotan	5-HT_{1A} agonist	Reduced L-dopa induced forelimb hyperkinesia.
Ko & Bezard 2014	MPTP Macaque	L-tryptophan	5-HT precursor	Abolished LID but corresponded with a worsening of levodopa's therapeutic inducing action
Huot 2015	MPTP Macaque	NLX-101	5-HT_{1A} agonist	Reduced the severity of peak dose LID without interfering with L-dopa's therapeutic action.
Iderberg 2015a	6-OHDA Rat	NLX-101 F13714 Tandospirone	5-HT_{1A} agonist	F13714 completely abolished LID. NLX-101 did so more weakly and tandospirone only partially.. F13714 elicits ipsilateral rotation behaviour.
Iderberg 2015b	6-OHDA Rat	NLX-112	5-HT_{1A} agonist	Completely abolished LID and eliminated stress induced ultrasonic vocalisation and immobility in forced swim test. Elicits ipsilateral rotation behaviour.
Kucinski 2016	6-OHDA Rat	Donepezil Idalopirdine	Acetylcholinesterase inhibitor 5-HT_6 antagonist	Co-administration reduced falls in rats with impaired control of complex movements.
McCreary 2016	6-OHDA Rat	NLX-112	5-HT_{1A} agonist	Completely and sustainably abolished LID and when NLX-112 administration ceased LID returned.
Park 2016	6-OHDA Mouse	Palmitoyl-5-HT	FAAH antagonist	Attenuated development of LID.
Pinna 2016	MPTP Rat & Macaque	Eltoprazine Preladenant	5-$HT_{1A/2B}$ agonist, 5-HT_{2C} antagonist A2A antagonist	Co-administration prevented dyskinetic like behaviour without impairing motor activity.
Brys 2018	6-OHDA Rat	NLX-112 NLX-101 F13714	5-HT_{1A} agonist	Dyskinetic symptoms suppressed by all three compounds with NLX-112 exhibiting the greatest potency.
Aboulghasemi 2019	6-OHDA Rat	Ondansetron	5-HT_3 antagonist	Reduction in LID with dyskinetic features reappearing upon drug discontinuation.
Wang 2019	6-OHDA Rat	Eltoprazine	5-$HT_{1A/2B}$ agonist, 5-HT_{2C} antagonist	Co-administration robustly reduced LID.
Fisher et al., 2020	MPTP Marmoset	NLX-112	5-HT_{1A} agonist	Reduces levodopa induced dyskinesia and parkinsonian features. No interference with L-dopa's therapeutic action.
Depoortere et al., 2020	MPTP Macaque	NLX-112	5-HT_{1A} agonist	Reduces levodopa induced dyskinesia. No interference with L-dopa's therapeutic action.
Altwal et al., 2020	6-OHDA Rat	Vilazodone	5-HT_{1A} partial agonist SERT SSRI	Supressed all subtypes (axial, limb and orolingual) of AIMS

This understanding of the aforementioned neuronal mechanisms has paved the way for contemporary researchers to investigate further the role of 5-HT as a therapeutic target for modulating movement dysfunctions in PD. The targeting of 5-HT_{1A} receptors with agonist compounds has shown to be particularly successful in alleviating LID with analogous outcomes occurring across dissimilar mammalian genus' that have been rendered parkinsonian (Table 3). Consistency between earlier research however has been periodic as previous studies have intermittently shown that although the 5-HT_{1A} targeted compounds being used were alleviating LID, they were also reducing the capacity of levodopa to re-establish normal motor function (Iravani et al., 2006; Ko and Bezard, 2014). However, these results suggested that activation of the serotonergic pathway as playing an important modulatory role in PD associated movement disorders (or motor function in itself). More recently, the pre-clinical success of novel biased agonists, such as the fully selective 5-HT_{1A} agonist, NLX-112 (befiradol, F-13,640) have demonstrated greater levels of efficacy, likely due to the specificity of their action on pre and post-synaptic receptors in certain brain regions, but also because they do not interact with other monoaminergic receptors (Huot et al., 2015; Ideberg et al., 2015; Meadows et al., 2017; Levigeroux et al., 2019).

The pioneering technique of transplanting foetal nigral DA neurons directly into the striatum has been attempted in humans with PD, and was once considered one of the more encouraging solutions for halting or even curing the disorder. This technique presented conflicting results as transplantation of DA neurons in human PD patients was shown to lead to upsurges in night time dyskinesia during levodopa withdrawal. However, upon post-mortem examination the survival rate of transplanted DA neurons was shown to be considerably robust when compared to matched controls (Olanow et al., 2009). One explanation for this is that the grafts themselves, during the transplantation procedure, cause an increase in dyskinetic symptoms, commonly known as graft induced dyskinesia (GID). Similarly, a case study examination of two PD patients who had developed an increase in dyskinesia after receiving foetal grafts were shown, via PET imaging, as having a reduced decay in residual DAergic neurons. Both PD patients were prescribed the 5-HT_{1A} agonist buspirone due to the identification by the investigators of an excessive 5-

HT neuronal dysfunction having developed following the graft procedure. Indeed, buspirone eliminated GID in each of the patients (Politis et al., 2010). Nevertheless, the long-term effectiveness of 5-HT_{1A} agonists in alleviating GID is still disputed, as one case study showed that prescription of buspirone in a female PD patient following GID, after receiving foetal mesencephalic graft surgery, did not alleviate dyskinesia to any degree, although the patient did report an improved affective state; however, this is likely due to buspirone's anxiolytic or anti-depressant properties (Beaulieu-Boire, & Fasano, 2015). The balance between DAergic and serotonergic mechanisms in GID has been investigated further in a rodent model of PD. Levodopa primed 6-OHDA lesioned rats were transplanted with either DA neurons, 5-HT neurons, or DA neurons plus 5-HT neurons into the striatum. The group with 5-HT neuron transplants did not present with GID, however, the other two conditions did. Similar to what was observed by Politis et al (2010), buspirone administration was found to completely arrest GID. Moreover, the 5-HT_{1A} agonist 8-OH-DPAT markedly reduced GID which was reversed with the 5-HT releasing agent fenfluramine, thus highlighting the requirement of DA foetal transplants *per se*, to induce GID; the extent of which being likely modulated, in part, via the neuronal serotonergic pathway (Shin et al., 2012; 2012). Equally, GID may be exacerbated due to DA foetal transplants containing an unknown quantity of 5-HT neurons, with the scale in dyskinetic symptoms being possibly dependent upon the number of 5-HT neurons relative to DA neurons contained within the graft, as opposed to being due to the absolute number of 5-HT neurons transplanted (Carlsson et al, 2007; Carlsson et al., 2008).

This highlights the complexity of the serotonergic system's role in managing dyskinesia and suggests that further investigation into the interactions between DAergic and serotonergic systems is crucial for developing effective treatments.

1.14. Serotonin and non-motor symptoms of Parkinson's disease

1.14.1. Affective disorders

This intricate interplay between serotonergic dysfunction and dyskinesia in PD underscores the importance of understanding serotonergic contributions to other non-motor symptoms, which are often overshadowed by the primary motor

symptoms of the disease. Those who suffer with PD consistently present with marked reductions in neuronal 5-HT (Politis et al.,2012; Politis and Niccolini, 2015). Some of these patients suffer with depression and some do not. However, the general clinical understanding of depression and anxiety, as stand-alone pathologies, attributes dysfunctions of the serotonergic pathway, particularly in the RN and frontal cortex, as being primarily responsible for modulating the affective state (Hajós, et al., 1998). In PD however, the co-morbid manifestation of mood disorders is being understood to a greater extent via the combined deficits of the interconnected monoaminergic pathways. Ballanger et al., (2012) found reduced binding of the 5-HT_{1A} tracer ^{18}FMPPF throughout the brain in depressed patients with PD. Further still, these observations are similarly found in the frontal cortex in the post mortem PD depressed brain (Sharp et al., 2008).

Depression is a common complaint amongst those with PD and often precedes the official PD diagnosis, it is therefore difficult to ascertain to what extent depression is attributed to the pathological development of PD, or due to some other underlying neurological factor(s). As such, 5-HT levels are shown to be markedly reduced in the cerebral spinal fluid (CSF) of those with PD and depression, compared to those with PD and without depression (Mayeux et al., 1984). Similarly, PET scanning with the 5-HT transporter (SERT) binding radioligand [^{11}C]DASB negatively correlates with depressive symptoms but not PD disease severity (Boileau et al., 2008), suggesting that 5-HT dysregulation is a symptomatic component of depression but not of PD. When investigated further however, a different picture emerges implying that the depression experienced by the parkinsonian brain may be exacerbated by the combined deficits of both DA and 5-HT. For example, activation of 5-HT_6 receptors with the agonist WAY208466 in the hippocampus (HIP) of 6-OHDA forebrain bundle injected rats induces antidepressant effects but also increases DA concentration levels in the HIP and mPFC. However, when WAY208466 was administered to non-lesioned rats it had the opposite effect and induced a depressive state. Likewise, these observations are reversed when the experiment is carried out with the 5-HT_6 antagonist SB258585; blockade leads to a decreased depressive state in intact rats, and an increased depressive state in lesioned rats, suggesting that

the direction of 5-HT_6 receptor signalling in the HIP is dependent upon the peak function of residual DA neurons (Liu et al., 2015). Furthermore, when the same experiment was carried out by Zhang et al., (2016), alongside the inducement of parkinsonian and depressive states, intact and lesioned rats were shown to have an increase in DA and NA neurotransmission in the mPFC, HIP and BLA.

1.14.2.Cognition and memory

An imaging study of human PD patients found that an increase in global amyloid plaque deposition correlates with a decrease in serotonergic innervation in the frontal cortex, but does not correspond with deficits in working memory recall (Smith et al., 2020). Interestingly, in subjects that have taken SSRI's for longer than 6 months prior to imaging, they are shown to have significantly lower levels of amyloid plaque deposition compared to those who are SSRI naïve. Moreover, 5-HT cortico-striatal denervation is shown to negatively correspond with cortical and striatal amyloidopathy in PD patients (Kotagal et al., 2012). Therefore, the abundance of 5-HT concentration levels brought on by SSRI therapy may help protect against amyloid plaque deposition and thus the possibility of developing amyloid related cognitive decline, archetypally seen in late stage neurodegeneration (Van der Schyf et al., 2006; Burack et al., 2010; Kotagal et al., 2018).

In MPTP lesioned mice, associative memory is significantly reduced after 30 days of lesion as quantified via the food preference test. This change corresponds with a marked depletion in 5-HT and DA concentration levels in the BLA, mPFC and striatum. It could be argued that depletion of neural DA and, in particular 5-HT, merely induces an apathetic and depressive response, and that the observed deficits in predicted food preference are not due to memory loss but in fact due to an altered affective state (Kaji & Hirata, 2011). To counter this issue the researchers performed a set of behavioural tests measuring for anxiety and depression which were consistently observed as stable throughout the experiment (Vučković et al., 2008), suggesting that any loss of vital monoamines is likely a causative factor for depressed behaviour in PD pathology.

Broca's complex (BC) in the frontal lobe is typically associated with speech and language comprehension (Caplan, 2006). However, it also connects to the HIP through GABAergic neurons expressing 5-HT_{2A} receptors (Alreja, 1996; Luttgen et al., 2004; Li et al., 2015). In rats lesioned with 6-OHDA, there is an increase in working memory deficits, a decline in firing rates of GABAergic neurons in BC, reductions in HIP theta rhythm, and an overall decrease in DA concentration levels in the HIP and mPFC. Remarkably, injecting the 5-HT_{2A} biased agonist, TCB-2, directly into BC results in notable improvements: increased working memory, heightened firing rates in BC GABAergic neurons, elevated DA levels in the mPFC and HIP, normalised theta rhythm, and increased interstitial 5-HT levels in the HIP (Li et al., 2015). When rats have been intra-cranially injected with 6-OHDA there is an almost complete loss of TH immunoreactivity in the striatum and SNpc, but also a decrease in cell proliferation in the sub granular zone (SGZ) of the hippocampus as seen via stereological quantification of sparsely populated BrdU-positive cells. And when treated with the SSRI fluoxetine there is an amelioration of progressive cell loss in the SGZ which corresponded with diminished escape latency during the Morris water maze task (Suzuki et al., 2010). This may be attributed to fluoxetine's therapeutic properties for inducing greater levels of 5-HT within the raphe-cortical pathway, rather than negating working memory deficits. Regardless, this study shows that DAergic denervation corresponds with cell loss in the SGZ which is then slowed by treatment with the serotonergic therapeutic compound fluoxetine, thereby suggesting that the SGZ region of the HIP is partly innervated via nigral DAergic projections and that 5-HT appears to play a primary role in modulating the extent of DAergic projection depleted induced SGZ cell loss.

1.14.3. Neuropathic pain

Disruption to the nociceptive pathways, leading to hyperalgesia, affects over two thirds of those living with PD (Broen et al., 2012). Loss of DA in PD has been shown to correspond with and, in part, mediate the extent of hyperalgesia via an increase in striatal modulated nociceptive innervation and upregulation of striatal D_2 receptors (Hagelberg et al., 2004; Mylius et al., 2009). However, due to the physiological location of the RN, dorsal to the brain stem, 5-HT has also been suspected to modulate the nociceptive pathways in PD associated hyperalgesia,

with disruption of the RN formation being potentially attributed to Lewy body accumulation during the early stages of PD (Braak et al., 2003). HPLC measured plasma levels of 5-HT are shown to be lower in PD patients compared to age matched controls. Interestingly, as measured with the visual analog for pain score (VAS), the reduction in plasma 5-HT levels negatively corresponds with pain intensity, implying that even as a peripheral marker, 5-HT depletion associates with the magnitude of PD induced hyperalgesia (Tong et al., 2015).

Given that the neural inflammatory response is strongly implicated in PD pathology and also critical for modulating the extent in pain sensitivity (Inoue et al., 2004). Microglial and astroglial proliferation have therefore been subject to investigation in rodent PD chronic pain models. 6-OHDA lesioned rats pre-treated with 5-HT and NA reuptake inhibitors (RI), citalopram and desipramine, respectively, have a reduced state of hyperalgesia compared to untreated lesioned rats. Further still, activation of dorsal spinal astroglia and microglia is increased in lesioned rats but completely reversed upon SSRI and SNRI pre-treatment. Moreover, SSRI and SNRI treatment prevents the loss of noradrenergic TH+ve neurons in the locus coeruleus (LC) and completely arrests 5-HT neuronal loss in the nucleus raphe magnus (NRM), compared to vehicle treated controls (Domenici et al., 2019). It appears that elevations in sensitivity in the descending pain pathways, induced via nigro-striatal lesioning, are protected by nociceptive 5-HT and NA upregulation. However, in this instance, further investigation is warranted into what extent analgesia is attributed to 5-HT or NA modulated neuroprotection. Furthermore, rats lesioned with 6-OHDA show a marked tendency for increased pain sensitivity which corresponds with a decrease in 5-HT fibres and 5-HT neurons in the rostral ventromedial medulla of the spinal cord. This is markedly tempered by citalopram administration suggesting that the serotonergic pathway does indeed modulate analgesia in mammalian PD pathology (Wang et al., 2017).

The targeting of 5-HT_{1A} with the full agonist NLX-112 (Befiradol), has shown considerable promise as a therapeutic modulator of analgesia in rodent pain models. As heat is amplified mice subjected to a thermal hot plate increase their rate in paw licking, however mice who had been orally administered NLX-112 had

a significant reduction in repeated paw licking which was subsequently reversed with the 5-HT_{1A} antagonist WAY100635 (Salat et al., 2017). Furthermore, in rats injected intrathecally with NLX-112 in the L5 and L6 regions of the spine are shown to have significant reductions in tonic pain in the formalin test, as observed via reduced paw licking compared to controls (Newman-Tancredi et al., 2018). The 5-HT_{1A} agonist NLX-112 certainly appears to have analgesic effects in both acute and tonic pain states. Although the aforementioned studies did not investigate a PD-like dopamine-depleted state, they did subject the animals to various states of pain threshold.

1.14.4. Psychosis

In many cases, visual disturbances and hallucinations become part of the overall affliction for those living with PD. Visual processing has shown to be mediated, in part, by serotonergic transmission via 5-HT_{1A} and 5-HT_{2A} receptors throughout the cerebral cortex and ventral visual pathway (Chen et al., 1998; Kravitz et al., 2013).

The function of 5-HT_{2A} receptors in particular has been implicated in a host of psychiatric disorders, including schizophrenia, bi-polar disorder, dementia, and drug addiction (Lopez-Figueroa et al., 2004; Moreno et al., 2016; Cummings et al., 2018; Sholler et al., 2019) - but also, in PD psychosis (PDP), where 5-HT_{2A} is shown to be upregulated in the cerebral and ventral visual regions (Vijverman et al., 2016; Creese et al., 2017). Why it is upregulated in those with PDP is thought to be due to Lewy body accumulation in the cerebral cortex, thereby provoking degeneration of serotonergic pyramidal neurons, thus leading to synaptic compensation via upregulation of 5-HT_{2A} receptor number and affinity in residual pyramidal neurons (Ballanger et al., 2010). This theory of Lewy body accumulation and 5-HT_{2A} overexpression has shown to be true, at least, when observed in the frontal cortex of the human post-mortem PD brain; α-synuclein the major constituent of Lewy bodies is shown to be increased in parallel to 5-HT_{2A} overexpression. However, when 5-HT_{2A} and α-synuclein was investigated in transgenic mice overexpressing α-synuclein, 5-HT_{2A} binding was found to be decreased (Rasmussen et al., 2016). It has previously been investigated whether 5-HT_{2A} dysregulation and the concomitant visual hallucinations in those with PDP

may be due to a genetic polymorphism in the 5-HT_{2A} receptor gene itself, however, this was shown not to be the case (Kiferle et al., 2010), further supporting the Lewy body – 5-HT_{2A} mechanism of action hypothesis in human PDP patients.

The selective 5-HT_{2A} inverse agonist pimavanserin (Nuplazid®) has had greater success in alleviating PDP to compared to other prototypical PDP antipsychotic drugs. Commonly prescribed antipsychotics such as Clozapine, a 5-HT_{2A} antagonist, $GABA_B$ antagonist, and D_2 antagonist (Canton et al., 1994; Kaster et al., 2015) and Quetiapine, a D_2 antagonist, 5-HT_{1A} partial agonist, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_3, 5-HT_6, and 5-HT_7 antagonist (Croissant et al, 2006; Vaishnavi et al., 2007; Montebello & Brett, 2007), have each been shown to alleviate psychosis in PDP but with adverse side effects including prolonged sedation, hypersalivation, hypotension and in some cases, reduced motor function; likely due to their inhibitory action on D_2 receptor signalling (Motsiner et al., 2003; Stahl, 2016). Pimavanserin alleviates PDP but with only mild sedation and without inducing hypotension, salivation or attenuated motor function (Sahli & Terazi, 2018). This is likely due to the selective and inhibitive action of pimavanserin on 5-HT_{2A} receptor signalling, whilst at the same time not interfering with DA receptor function or other serotonergic receptor mediated pathways. Moreover, inhibition of 5-HT_{2A} with the highly selective antagonist EMD-281,014 is found to significantly reduce psychosis like behaviour in parkinsonian monkeys (Hamadjida et al., 2018).

1.14.5. Sleep, fatigue and circadian rhythm

Serotonin plays an important role in regulating the supra-chiasmic nucleus (SCN) via the raphe-superchiasmatic pathway (Glass et al., 2003), and dysfunction of this pathway may be causing severe disruptions to the homeostatic balance of regular sleep-wake patterns in those with PD. Interruption of serotonergic activity in PD sleep dysfunction has been investigated via the quantification of SERT binding in several brain regions of interest (ROI), as calculated with PET and the SERT tracer [^{11}C]DASB. SERT binding potential (BP) was significantly lower in those with PD sleep dysfunction in several ROI's including the caudate, putamen, striatum and RN with the greatest reductions occurring in the hypothalamus, when compared to those with PD but non-sleep disturbed (Wilson et al., 2018). Similarly, fatigue is

also an issue reported by those living with PD and is likely attributed to dysfunction of the raphe-superchiasmatic pathway. Dysregulation of SERT was investigated in PD patients suffering with fatigue but without a history of sleep disturbance. Similar to the sleep deprived group previously mentioned, those with PD and fatigue had significantly lower levels in SERT BP in the caudate, putamen, striatum and hypothalamus (Pavese et al., 2010). Both of the aforementioned studies have each demonstrated there to be marked reductions in SERT BP in identical ROI's (except for the RN) in PD patients complaining of either disturbed sleep or day time fatigue. Although each of these studies required participants to self-report their sleep disturbance and fatigue states, serotonergic dysfunction does appear to be a key factor in mediating the sleep-wake cycle in the human PD brain. Serotonergic dysfunction has also been investigated in those with PD who also suffer with sleep disordered breathing (SDB) (Lelieveld et al., 2012), however there was no corresponding effect between PD and SDB, with SDB incidence in PD patients being potentially attributed to other factors such as excessive weight or cardiopulmonary complications (Bixler et al., 2000; Young et al., 2005).

Mattam & Jagota, (2015) investigated whether various components of 5-HT metabolism influence the SCN circadian rhythm in a rotenone lesioned rat model of PD. Compared to control, rotenone induced an increase in α-synuclein immunoreactivity as well as a decrease in TH immunoreactivity. Differences were observed in 5-HT daily pulses, mean 24hr 5-HT levels, tryptophan daily pulses and mean 24hr tryptophan levels. Interestingly, the changes in each metabolic measure of 5-HT corresponded with a decrease in melatonin daily pulses and mean 24hr melatonin levels. This is a particularly important finding, not only because melatonin is derived from 5-HT, but also because as a pineal gland secreted hormone, it is vital for mediating the sleep wake cycle (Auld et al., 2017).

1.15. Serotonin and dopamine neuronal homeostasis: a balanced relationship

The interplay between 5-HT and DA demonstrates importance, evidenced by the depletion of serotonergic nerve fibers in intact non-lesioned rats. Removing these fibers reduces levodopa-induced striatal DA release by a third (Nevelainen et al., 2011), suggesting that 5-HT pathway destruction might precede and contribute to

DA dysfunction in levodopa-treated brains. However, these findings, not involving DA-targeted neurotoxins in rats, lack direct relevance to human PD aetiology. In contrast, in lesioned female cynomolgus monkeys, MPTP administration induces both DA denervation in the striatum and an increase in DA and 5-HT axonal sprouting in the pallidum (Gagnon et al., 2018). These results hint at an interaction between striatal DA cell loss and a mechanistic response from 5-HT and DA-positive axons. Yet, this may signify an early compensatory mechanism triggered by MPTP's impact on nigral DA cells, possibly temporary as DA cell loss progresses. Longitudinal monitoring of monoaminergic axonal sprouting post-MPTP lesioning would be valuable, revealing whether such sprouting represents a compensatory mechanism evolving or diminishing over time in PD brains.

The firing patterns between certain brain regions can be used to quantify the extent of neuronal activity in monoaminergic depleted rats. Monoaminergic interaction was investigated in 6-OHDA rats using the NA selective drug sDSP, and the 5-HT inhibitor pCPA. DA depletion increased the amount of irregular firing patterns in the pallidum and SN which was attenuated upon NA depletion. There was a marked interaction between 5-HT and DA depletion on the firing rate of SN neurons. Individually, NA did not alter pallidum or SN neuronal firing, whilst 5-HT depletion significantly decreased pallidum and SN neuronal firing rates, thus suggesting a modulatory role for both 5-HT and NA on signal transduction on the DA depleted brain (Delaville et al., 2012).

The effects of MPP^+ on DA and 5-HT expressing neurons in mouse embryonic cell lines has previously been shown to significantly reduce mitochondrial number, and change the conformational state of SERT and DAT receptors. Administration of MPP^+ induces an augmentation of cell surface DAT molecules but an internalisation of SERT molecules (Marti et al., 2017). Although the 5-HT receptor targeted, in this instance was SERT, the results highlight the limitations of using MPP^+ when investigating serotonergic function in rodent models of PD, as read outs may be interpreted incorrectly due to the MPP^+ induced changes in receptor conformation. Moreover, there is a marked reduction in DA releasing vesicles but no change in 5-HT releasing vesicles. The importance of DA vesicular transport in

PD pathogenesis is noted by Lohr et al., (2015) who shows that transgenic mice overexpressing VMAT2;Slc18a2 to have extensive improvements in extracellular DA availability and DA release in the striatum as well exhibiting increased DA terminal neuroprotection upon insult with MPTP. Evidence suggests that 5-HT and DA receptors, at their mutually localised striatal terminal sites, each compete for levodopa in the DA depleted levodopa treated mammalian brain (Nevalainen et al., 2011). Enhancements in DA vesicular transport could therefore prove significant in improving levodopa induced dyskinesias and also enhance striatal DAergic-nerve neuroprotection.

1.16. Serotonin modulates apoptotic pathways

1.16.1. Inflammation and oxidative stress

A large body of literature implicates systemic and localised CNS inflammation as exacerbating neuronal homeostasis in PD pathogenesis (McGeer & McGeer, 2004). As such, elevated numbers of activated microglia are found in the SN in both idiopathic and familial PD patients (Croisier et al., 2005; Russo et al., 2014). Moreover, the presence of inflammatory cytokines including tumour necrosis factor-alpha (TNF-α) and interleukin-1 β (IL-1β) are elevated in the SN of human PD patients (Leal et al., 2013). TNF-α and IL-1β presence has been observed via glial cell immunoreactivity in the SN in the human PD brain (Boka et al., 1994), thus indicating glia as mediating release of inflammatory cytokines in DAergic rich regions. Similarly, IL-6 positive transgenic mice present with a progressive increase in cerebral TNF-α mediated inflammation and a decreased cerebellum volume which corresponds with a significant reduction in motor function (Gyengesi et al., 2019). Further still, mice treated with MPTP have decreased levels of NA and DA in both the striatum and midbrain but also decreased levels of 5-HT in the frontal cortex. These changes also corresponded with marked increases in striatal IL-1β, TNF-α and interferon-gamma (IFN-γ) in the frontal cortex (Luchtman et al., 2009).

Serotonin has been shown in vitro to stimulate the release of pro-inflammatory cytokines via $5\text{-}HT_{2a,b}$ and $5\text{-}HT_4$ receptors on microglia (Glebov et al., 2015). Contrariwise, in gerbils subjected to hippocampal ischemia, pre-treatment with

duloxetine, a 5-HT/NA reuptake inhibitor, reduces microglial and astroglial proliferation in the CA1 region of the HIP (Lee et al., 2016). Moreover, the SSRI fluoxetine exerts an anti-inflammatory effect on microglial mediated liberation of TNF-α, IL-1β, and IL-6 upon oxygen glucose deprivation/reoxygenation (OGD/R), in vitro (Tian et al., 2019). Fluoxetine also has further reaching neuroprotective effects as observed in a rodent model of spinal cord injury. Mice subjected to laminectomy and nerve ligation treated with fluoxetine prevents oligodendrocyte cell death through the inhibition of microglial, p38-MAPK and pro-NGF activation (Lee et al., 2015). Even further, fluoxetine treatment has unearthed the differences of 5-HTs mediatory effect on the activation of microglia. Microglia M1 activation is typically involved with secreting pro-inflammatory cytokines and initiating neurotoxic cascades whereas microglia M2 activation is more closely involved with promoting tissue regeneration (Colton & Wilcock, 2010). Su et al., (2015) shows that fluoxetine, in combination with citalopram inhibits M1 mediated microglial activation whilst promoting M2 mediated microglial activation. Thus suggesting, that when attempting to quantify any neural inflammatory response via IHC specific to microglia, any conclusions should be carefully considered, as the microglial activity being observed may be exerting its function both neuroprotectivley and/or neurotoxically. Furthermore, rats injected in the SN with the endotoxin liposaccharide (LPS) have an increase in microglial NADPH oxidase activation and an upregulation in inducible nitric oxide synthase (iNOS) which are both markedly suppressed upon treatment with fluoxetine (Chung et al., 2010). Further supporting this observation, fluoxetine, co-administered with the monoaminergic tricyclic antidepressant imipramine, negates the LPS inducement of TNF-α and IL-1β activation in rat glial cell culture (Obuchowicz et al., 2012). Likewise, LPS induces 5-HT metabolism in the mPFC of male wild type rats but not in rats with SERT knock out (Korte-Bouws et al., 2018).

Serotonin may also play a modulatory role in axonal and dendritic growth via microglial mediated synaptic maturation and elimination. By examining the cross-regulation between 5-HT and microglia, Kolodziejczak et al., (2015) was able to determine that in mice neonates, microglia had an increased affinity for exogenous 5-HT. Using confocal microscopy, serotonergic varicosities and microglial

processes were shown to have an extremely close proximity to each other when observed in the tissue from normal expressing 5-HT_{2B} heterozygotes, with 5-HT appearing to regulate microglial process growth. Interestingly, in 5-HT_{2B} double knockout mice this was shown not to be the case, with a marked reduction in 5-HT–microglial growth interaction, suggesting 5-HT_{2B} to be an important functional microglial receptor, possibly aligned with early development neurogenesis. Although these mice were not subjected to DA neurotoxic lesioning, the importance of these interactions are no less noteworthy, especially as 5-HT_{2B} is significantly upregulated in patients with PDP and that microglial inflammation is also a known catalyst of DA cell death in the PD brain (Croisier et al., 2005).

Dysregulation in serotonergic function corresponds with the extent of neural inflammation in rodent models of PD. Mice treated with MPTP leads to degeneration of nigrostriatal DA neurons and increases in NADPH oxidase, ROS production, astroglial-myeloperoxidase, TNF-α and IL-1β and upon treatment with the SSRI paroxetine DA neurodegeneration becomes markedly reduced which corresponds with the suppression of TNF-α and IL-1β (Chung et al., 2010). Similarly, nigrostriatal lesioning with 6-OHDA in rats induces an inflammatory response in the SN, HIP and striatum, as quantified by microglial proliferation. Levels of DA and 5-HT in the striatum measured over several weeks reveal DA to remain consistently depleted, however, 5-HT levels in the striatum remain elevated but only in the first week, further supporting the notion that disruption to serotonergic homeostasis is an early mechanistic response in the progression of PD pathogenesis (Silva et al., 2016). Imipramine and amitriptyline are tricyclic antidepressants that block the reuptake of NA and 5-HT. Individual administration of these compounds on rotenone lesioned rats result in a marked enhancement of TNF-α activation, suppression of iNOS reactivity and restoration of BDNF in the striatum. Moreover, both of the compounds reverse rotenone induced depletions in SN TH immunoreactivity, thereby suggesting amitriptyline and imipramine to each be neuroprotective toward expiring DA neurons (Kandil et al., 2016).

Other 5-HT targeting compounds have produced similar results in animal models of PD. The SSRI fluvoxamine was examined in rats administered with 6-OHDA

that were subjected to a model of early maternal separation (modelled to replicate PD associated depression). Fluvoxamine suppressed IL-1β, IL-6 and TNF-α mRNA expression in the lesioned striatum in 6-OHDA treated rats whilst also increasing striatal levels of transforming growth factor β (TGF- β). Interestingly, lipid peroxidation was also down-regulated, suggesting that 5-HT is suppressing the neurotoxically induced ROS mediated apoptotic cascade (Dallé et al., 2017); a neural-process considered fundamental to the facilitation of cell death in PD pathogenesis.

1.16.2.Mitochondria, oxidative stress and cell death

Serotonin may protect mitochondria during axonal transport. In rat cortical cell cultures administration with the 5-HT_{1A} antagonist WAY100635 inhibits mitochondrial axonal movement whereas administration with fluoxetine increases mitochondrial axonal movement (Chen et al., 2007). Further still, in SH-SY5Y cell culture mitochondrial deficiency was induced via treatment with rotenone thereby inhibiting mitochondrial complex-I which led to significant increases in monoaminergic metabolites DOPAC and 5-HIAA (de la Fuente et al., 2017). Furthermore, targeting of neuronal 5-HT protects and attenuates DAergic cell loss in several different pre-clinical models of PD. For example, rats treated with 6-OHDA develop parkinsonian symptoms but after the combined supplementation of 5-HT, bone marrow cells, and gamma-aminobutyric acid (GABA) there is a marked reduction in 6-OHDA induced lipid peroxidation (Kuruvilla et al., 2013). MPTP has been shown to selectively inhibit the mitochondrial respiratory chain throughout of nigral DA cells in the brain in conscious rhesus monkeys. Marked reductions of nigro-striatal DAT and DA synthesis are subsequently observed but not D2 receptor binding. Interestingly, MPTP treatment induces a loss of SERT binding not only in the striatum but also in the frontal and temporal cortex with MC-I activity correlating with cortical SERT deficits. No changes in SERT binding activity was observed in the RN with 5-HT_{1A} receptor binding also remaining stable throughout the brain upon MPTP treatment (Kanazawa et al., 2017).

1.17. 5-HT$_{1A}$ and Parkinson disease

1.17.1. 5-HT$_{1A}$: A regulator of neuronal homeostasis

Neuronal 5-HT$_{1A}$ receptors are highly expressed in both invertebrate and vertebrate brains. From moths to humans, they are vital for modulating key aspects of the CNS, including cognition and motor function (Xiong et al., 2019). The 5-HT$_{1A}$ receptor has held a pivotal position in research for several decades due to its significant role in modulating complex animal behaviour. However, the extensive body of literature on the 5-HT$_{1A}$ receptor and its therapeutic effects has primarily focused on alleviating neuropsychiatric disorders such as anxiety and depression (Akimova et al., 2009).

Symptomatic treatment with drugs with either a direct (5-HT$_{1A}$ agonist) or indirect (SSRIs) modulation of the 5-HT$_{1A}$ receptor, result in changes in serotonergic activity throughout the brain. More recent evidence however, has shown that utility of 5-HT$_{1A}$ is also highly important for other neuro-physiological functions and associated pathologies including circadian rhythm and sleep (Boutrel et al., 2002), psychosis (Drago et al., 2008), PTSD (Lewis et al., 2020), cognition and memory (Ogren et al., 2008), movement disorders including PD (Bara-Jimenez et al., 2005) and Machado Joseph disease (Pereira-Sousa et al., 2021). The abundant facility that the 5-HT$_{1A}$ receptor has in modulating these pathologies is likely attributed to the fact that the 5-HT$_{1A}$ receptor couples with multiple intracellular signalling pathways via G-protein coupled receptor (GPCR) Gα variants (*i, o*) compared to other 5-HT receptor subtypes (Rojas and Fiedler, 2016). Moreover, the 5-HT$_{1A}$ receptor is ubiquitous throughout the brain and one of the most widely expressed (Albert et al., 1990); located pre-synaptically and post-synaptically, 5-HT$_{1A}$ autoreceptors are located on the cell body and dendrites of 5-HT expressing neurons in the RN (Riad et al., 2000), whereas 5-HT$_{1A}$ heteroreceptors are located typically on cortical and limbic pyramidal and interneurons expressing GABA (Halasy et al., 1995) choline (Cassell and Jeltsch, 1995) and glutamate (Ciranna, 2006). Further still, activation of 5-HT$_{1A}$ on presynaptic or postsynaptic receptors, with endogenous neurochemicals or exogenous compounds, can manifest varying degrees of 5-HT release throughout the brain. For example, activation of 5-HT$_{1A}$ autoreceptors in the RN inhibits transmitter synthesis which supresses 5-HT

release in the forebrain (Meller et al., 1990; Sharp and Hjorth, 1990). Whereas activation of 5-HT_{1A} heteroreceptors in pyramidal and cortical neurons increases release of other neurotransmitters including DA and glutamate within those same brain regions (Llado-pelfort et al., 2010).

1.17.2. 5-HT_{1A} activation and neuroprotection

Not only does 5-HT_{1A} targeted therapy alleviate psychiatric and physiological disorders but it has also been shown to modulate neuroprotection in a variety of pre-clinical animal and cellular models of neuronal cell death.

As discussed earlier, excessive excitatory levels of glutamate via *N*-methyl-d-aspartate (NMDA) are considered to be one of the key causes of cell death in PD. Axonal density is preserved with pre-treatment of 8-OH-DPAT prior to injections of NMDA into rat magnocellular nucleus basalis (Oosterink et al., 1998) and also protects against H_2O_2 induced neuronal cell death in rat cortical neurons, possibly via inhibition of Na^+ and Ca^{2+} influx, thereby inhibiting glutamate release (Melena et al., 2000; Lee et al., 2005). Similarly, 8-OH-DPAT almost completely abolishes NMDA induced caspase 3 activity and DNA fragmentation, which is reversed upon pre-treatment with WAY100635 (Madhaven et al., 2003). These mechanisms of 5-HT_{1A} activated neuroprotection may be regulated by increased phosphorylation of the transcription factor STAT-3 as observed in Neuro 2A cells transfected with 5-HT_{1A} receptors which corresponds with an increase in neurite outgrowth (Fricker et al., 2005). 8-OH-DPAT, as well as the partial 5-HT_{1A} agonists, buspirone and ipsapirone are likely inducing their protective effects via activation of the intracellular enzymes PI-3K and MAPKK (Druse et al., 2005). Moreover, the 5-HT_{1A} agonist repinotan (Bayx3702) inhibits serum-deprived induced apoptosis in chick embryonic neurons via the nerve growth factor signalling pathway (NGF) (Ahlemeyer et al., 1999). Furthermore, both 8-OH-DPAT and Bayx3702 block H_2O_2 induced cell death in HN2-5 mouse hippocampal neurons via activation of MAPK resulting in suppression of caspase 3 (Adayev et al., 2003).

The neuroprotective properties of 5-HT_{1A} agonists are also thought to be extensively controlled by the activity of astroglia (Miyazaki et al., 2020). Mice pre-

treated with 8-OH-DPAT then exposed to non-lethal doses of sarin (an organophosphate nerve agent that is an acetylcholine inhibitor, and when exposed to causes respiratory paralysis and brain damage (Abu-Qare, et al., 2002)) have markedly reduced glial fibrillary acidic protein (GFAP)+ve cells in the hippocampus which corresponds with the reduction of IL-1β in the BLA (Garrett et al., 2013). Furthermore, 8-OH-DPAT protects rat mesencephalic neurons against 6-OHDA toxicity by upregulating metallothionein (a regulator of oxidative stress (Kumari et al., 1998)) in striatal astrocytes (Miyazaki et al., 2013), with 8-OH-DPAT induced secretion of metallothionein from astrocytes directly protecting DAergic neurons from rotigotine toxicity (Isooka et al., 2020).

Given the primary hallmark of PD is a loss of DA neurons in the SNpc, it is therefore vital to determine whether certain drugs/compounds may offer protection against DAergic neuronal cell death. Putative neuroprotective drugs would therefore have to target one of, or several pathways involved in DA cell loss, this includes: inhibitors of DA metabolism including MAO inhibitors and DA agonists; inhibitors of α-synuclein formation, anti-inflammatory agents; NMDA receptor antagonists, and neurotrophic factors including glial derived neurotrophic factor (GDNF) and BDNF (Yacoubian and Standaert, 2009). However, between BDNF and GDNF, GDNF is known to be the more effective of the two at protecting against DAergic cell death (Sun et al., 2005). TH+ve cells in the SN of MPTP treated macaques are preserved upon pre-treatment with the 5-HT_{1A} agonist BAY639044 (Bezard et al., 2006), however, there is very little by way of previous research that has investigated further the neuroprotective properties of 5-HT_{1A} agonists in animal MPTP PD models.

1.18. Introduction summary

Taken together the studies described in the preceding paragraphs highlight the importance of 5-HT in PD aetiology and pathology. Parkinson's disease and its various motor and non-motor symptomology is evidently not just due to the loss of DA in the nirgo-striatal pathway but also to the dysregulation in serotonergic function, including important modulatory roles for the 5-HT_{1A} receptor, amongst many other 5-HTR subtypes. Clearly, 5-HT pathways intersect with DA networks

which has been extensively highlighted through research carried out with cell culture and in vivo PD models. Understanding the intricate interplay between DA and 5-HT systems is therefore becoming increasingly recognised as crucial for understanding PD's complexity and has led to the development of more comprehensive therapeutic strategies, than just those seeking to merely replace DA neurotransmitter. Thus, targeting 5-HT pathways alongside traditional DA-based treatments might offer promising avenues for managing of both motor and non-motor symptoms in PD.

1.19. NLX-112

This growing recognition of the role of 5-HT in PD has spurred the development of drugs targeting this pathway, such as NLX-112. For the last decade, Neurolixis, a biotechnology company specifying in developing drugs for various CNS disorders, has extensively investigated the 5-HT_{1A} agonist compounds NLX-112 (Befiradol) and NLX-101 (amongst several other similar drugs). Each has been shown to exert potent therapeutic effects in a multitude of different pre-clinical models. For example, NLX-112 is an exceptionally selective 5-HT_{1A} agonist with nanomolar affinity and a biased agonist which is a preferential Gαo activator (Colpaert et al., 2002; Newman-Tancredi et al., 2017; 2019); it does not bind to any other monoaminergic, opioid, GABAergic or 5-HT receptor subtypes (Colpaert et al., 2002) and is reported as having a selective efficacy at the 5-HT_{1A} receptor similar to that of 5-HT, and a markedly greater selectivity than other 5-HT_{1A} agonists including 8-OH-DPAT or buspirone (Newman-Tancredi et al., 2017). Considerable pre-clinical evidence has demonstrated NLX-112 to be highly efficacious in negating levodopa induced abnormal involuntary movements (AIMS) in rats and non-human primates (marmosets and macaques) (Iderberg et al., 2015; McCreary et al., 2016; Fisher et al., 2020 and Depoortere et al., 2020), which has led to the clinical development of NLX-112 for the treatment of levodopa-induced dyskinesia (LID) in PD. A recent human double blind placebo-controlled phase IIa trial has been completed with exceptionally promising reports that NLX-112 has successfully reduced both LID and parkinsonism in patients with advanced PD (https://www.parkinsons.org.uk/news/early-phase-clinical-trial-results-offer-new-hope-dyskinesia-treatment). Moreover, NLX-112 has also shown as having anti-

nociceptive (Salat et al., 2017), anti-depressive (Newman-Tancredi et al., 2018; Depoortere et al., 2020) and anti-aggressive (Peeters et al., 2019) properties in rodent models. NLX-112 completely inhibits electrical activity in the RN (Lladó-Pelfort et al., 2012), which suggests that its therapeutic properties on motor dysfunction may be principally mediated by presynaptic autoreceptors in the RN.

Unlike many other 5-HT_{1A} agonists which are, in effect, only partial agonists such as buspirone, 8-OH-DPAT, and aripiprazole, NLX-112 is a full biased agonist with no off-target interactions, whereas, 8-OH-DPAT has some binding affinity for D2, D3 and the adrenergic receptors α1 and α2, and buspirone is more multimodal, having efficacy for several other 5-HT receptor subtypes, as well as D2, D3 and D4 (Figure 6 A).

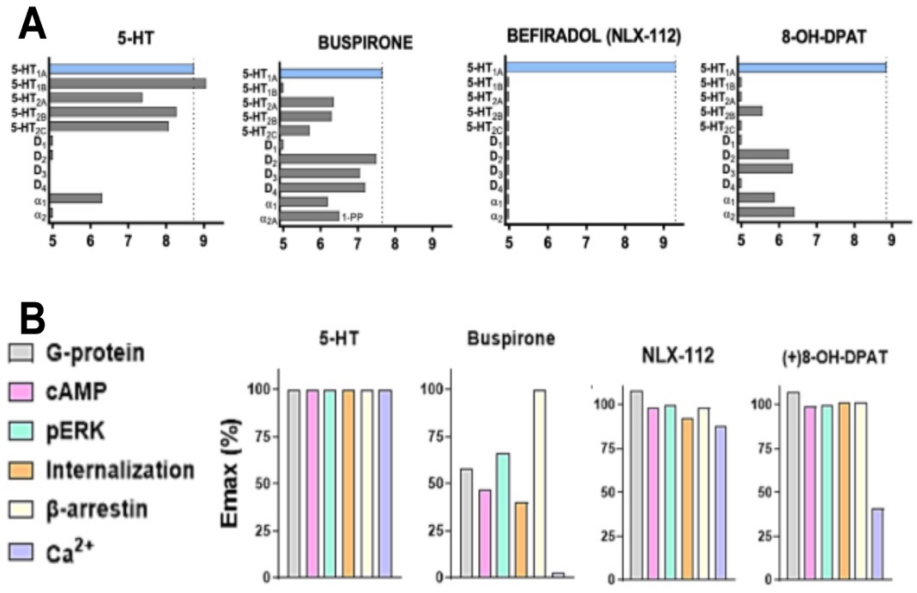

Figure 6. A) Receptor binding profiles of serotonergic agonists. Receptor affinity (expressed as pKi) was determined in competition binding experiments at 5-HT_{1A} (in blue) and other receptors using membranes prepared from rat brain tissue or from cell lines expressing recombinant human receptors. B) Agonist efficacy (Emax) was determined in vitro for six signal transduction responses in recombinant cell lines expressing human 5-HT_{1A} receptors. G-protein activation was determined by [^{35}S]GTPγS binding on 'total G-proteins' in HeLa cells, adenylyl cyclase (cAMP) inhibition was measured in HeLa cells, ERK1/2 phosphorylation (pERK) was measured in CHO cells and receptor internalisation was measured in HEK293 cells. β-arrestin recruitment was measured in a U2OS cell line construct and calcium (Ca^{2+}) mobilisation was determined in CHO cells. Emax values are expressed as a percentage of the stimulation elicited by 5-HT (10 μM) tested in parallel. Adapted from Newman-Tancredi et al., (2022).

1.20. Biased agonism

Also known as functional selectivity or ligand bias, biased agonism is a pharmacological phenomenon where a single receptor can activate multiple signalling pathways in response to the binding of different ligands. Biased agonism refers to the concept that not all agonists for a particular receptor elicit the same biological response, even though they bind to the same receptor. For example, when activated, receptors such as GPCR's which are the largest group of cell surface receptors in humans (Alberts et al., 2002), are associated with many intracellular signal transduction pathways and second messengers, which can lead to a variety of modalities such as the activation of β-arrestins, various enzymes, changes in gene expression, or alterations to cell membrane properties thus leading to distinct physiological responses (Kenakin, 2009). This is important because biased agonists can stabilise receptor conformation by preferentially stimulating one of these pathways therefore allowing a more targeted modulation of cellular function and treatment of disease. Compared to the prototypical 5-HT_{1A} partial agonists, buspirone and 8-OH-DPAT, NLX-112 has signal transduction properties much more aligned in profile to that of 5-HT neurotransmitter itself, including Emax values for G-protein activation, cAMP inhibition and ERK1/2 phosphorylation (Figure 6 B. Newman-Tancredi et al., 2022).

1.21. Thesis Aims

There is very good evidence to suggest that targeting the serotonergic pathway via 5-HT_{1A} receptor agonism will provide neuroprotection of various neuronal sub-types, including DA neurons.

NLX-112 is a compound which is fast becoming distinguished from other prototypical drugs as a potential treatment therapy capable of alleviating a diverse range of neurophysiological and neuro-psychiatric conditions. This is likely due to its exceptional selectivity at the 5-HT_{1A} receptor, but also the fact that it indirectly modulates the ubiquitous and highly important monoaminergic neuro-chemical, 5-HT. Considering the high levels of pre-clinical success NLX-112 has already exhibited, its recent success as a phase IIa trial drug, the mechanisms at which other 5-HT_{1A} agonists have previously been shown to protect against various forms

of exogenously induced cell death, it is reasonable to suggest that NLX-112 may possess neuroprotective properties, and could therefore be a promising candidate to be investigated for aforesaid therapeutic property in diverse in vitro and in vivo models of PD.

1.22. Thesis Hypotheses

This research aims to test this hypothesis by investigating whether the 5-HT_{1A} agonist, NLX-112 is neuroprotective toward DA neurons in DA cell-death models of PD. Specifically, these aims are:

1. Using the catecholaminergic SH-SY5Y cell line, cell death will be induced via the proteasome inhibitor MG132 and the mitochondrial inhibitor MPP^+.
2. Administering the mitochondrial inhibitor MPTP to two different sub-strains of C57b mouse to induce DAergic lesioning of the nigro-striatal pathway.
3. Determine whether NLX-112 is neuroprotective in the models described above.
4. Assess the kinetic and behavioural effects of NLX-112 before and after treatment with MPTP.

Chapter 2

Materials and Model Development

2.1. Introduction

For the first aim of this thesis, the SH-SY5Y cell line was chosen to investigate NLX-112's potential neuroprotective properties in vitro (rationale discussed in Chapter 3).

In vitro neuroprotection studies have conventionally taken the approach of preventative measures, in that a putative therapeutic compound will be incubated on the cell culture for several hours prior to a toxic insult. However, in PD pathogenesis, cell death in the SN has started to take hold many years before clinical diagnosis of the disorder has been made. Therefore, any in vitro or in vivo neuroprotection study should also consider inducing cell death prior to administering a putative therapeutic drug. Thus, any drug that can halt cell death or even aid in cell recovery after the cell death cascade has begun, is a mechanism of neuroprotection worth investigating, particularly so if face validity is to be fastidiously observed.

An effective in vitro neuroprotection model seeks to improve cell loss from a point at which cells are either capable of recovering or shielded from an insult altogether, this is typically 50% (EC50). The following assays sought to determine the effective dose of both MPP^+ and MG132 in achieving 50% cell loss over a 24hr period. Once this had been accomplished then varying types of timed neuroprotection assays can take place. For example, the putative neuroprotective compound NLX-112 can be added before, with, or after toxic drug administration. Variations all of which offer an insight into a drugs pharmacological and mechanistic neuroprotective properties.

2.2. Growing SH-SY5Y cells

SH-SY5Y cells were purchased from LGC Standards, grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with heat inactivated Foetal Bovine Serum (FBS), grown to 80-90% confluence in an incubation chamber with an atmosphere of CO_2 (5 %) and relative humidity (95 %), lifted and stored in liquid nitrogen vapour phase in cryotubes each containing approximately $10^{\wedge 6}$ number of cells. On the day of experiments cells were thawed from liquid

nitrogen cryopreservation and grown in a T75 flask containing culture medium made up from DMEM with 10% FBS, supplemented with non-essential amino-acids and glutamate. Cells were lifted from the flask once they had reached 80-90% confluency. Cell lifting and cell counting was carried out using a haemocytometer and trypan blue. This was achieved by performing the following steps: remove culture medium and wash once with PBS(1X), remove PBS(1X) and add 2mL of the proteolytic enzyme trypsin (0.05%), incubate for 2 minutes at 37C or until cells have begun to lift (plate detachment), add 3mL of DMEM to stop trypsin/cell reaction, the medium was removed and added to a 15mL falcon tube, spin the cells in a centrifuge at 1000G for 5 minutes. Once the centrifuge had stopped spinning the excess medium was removed leaving a pellet at the bottom of the falcon tube, add 5mL fresh complete medium and mix in the pellet with 200mL, and pipette until cells are widely dispersed in the medium. For cell counting an approximation of the number cells present, the following steps were carried out:

With the cell medium well mixed, 50uL was removed with a 200uL pipette and added to a 0.5mL Eppendorf, 50uL of trypan blue was then added and pipetted up and down several times until well mixed. 10uL of cell medium + trypan blue mix was added to the haemocytometer between the adjoining coverslip. The haemocytometer was placed under a microscope and the camera was focused until all cells were visible in each four corner quadrants. Cells were counted in each of the four quadrants, and the value for each quadrant was counted along. Any cell coloured blue was not be counted.

To calculate the total number of cells lifted from the plate the following formula was used:

$$N = (\Sigma / 4) \times 10\text{^}4 \times v^i \times df$$

Calculate the estimated number of cells (N) where Σ is the sum of all cell counted divided by the number of quadrants (4) on the haemocytometer multiplied by the volume of square on the haemocytometer ($0.1mm^3$) multiplied by the initial volume (V^i) of the cell medium and multiplied by trypan blue dilution (dilution factor / df).

Cells were cultured in a minimum of triplicates in a 96 well plate, seeded at 5x10^4 cells/well and cultured until 80-90% confluency (approx. 24 hours). Cells were then treated to either MPP^+ or MG132, and incremental concentrations of NLX-112. Neuroprotection assays were carried out using different combinations of NLX-112 with MG132 or MPP^+ treatment.

2.3. MG132

Degradation of intracellular proteins is vital for cell survival as well as maintaining the optimum function of the cell life cycle, including differentiation and apoptosis. The ubiquitin-proteasome pathway (UPP) is responsible for mediating this process through proteolysis. One compound that targets this pathway is the synthetic peptide aldehyde proteasome inhibitor, MG132 (carbobenzoxyl-L-leucyl-L-leucyl-L-leucine). MG132 induces the apoptotic cascade via the formation of reactive oxygen species, the extent of which relies on the activation of caspase cysteine proteases. Impairments to the UPP is considered to be a primary hallmark of PD aetiology (McNaught and Jenner, 2001), and the presence of Lewy bodies in post-mortem PD brain is thought to be attributed to dysfunctions of the UPP (Quinn et al., 2012). Thus, any therapeutic compounds that may ward off the deleterious cascade of proteasome dysfunction, and the concomitant build-up of intracellular toxic Lewy bodies, is an area of research considered very important.

2.4. MPP^+

Already described in detail in Chapter 1, the toxic metabolite derived of the neurotoxin MPTP is MPP^+. MPP^+ cannot cross the blood brain barrier (BBB). It is therefore highly utilised in in vitro research settings for modelling PD associated cellular toxicity. MPP^+ is an effective toxic agent as it inhibits mitochondrial respiration, supressing ATP production and leading to cell death.

2.5. Preparation of MG132

MG132 was purchased from Sigma.

Serial dilutions of MG132 were prepared using the following steps.

i. Prepare 10mM stock solution of MG132 (475.6g/mol) 0.0047g in 1mL of deionised water.
ii. Make serial dilutions of 1mM/mL, 100μM/mL, 10μM/mL, 1μM/mL and 0.1uM/mL in seperate Eppendorfs.
iii. For 100μL wells treated to 100μM of MG132, add 10μL of 1mM serial.
iv. For 100μL wells treated to 10μM of MG132, add 10μL of 100μM serial.
v. For 100μL wells treated to 5μM of MG132, add 5μL of 100μM serial.
vi. For 100μL wells treated to 1μM of MG132, add 10μL of 10μM serial.
vii. For 100μL wells treated to 0.1μM of MG132, add 10μL of 1μM serial.
viii. For 100μL wells treated to 0.01μM of MG132, add 10μL of 0.1μM serial.

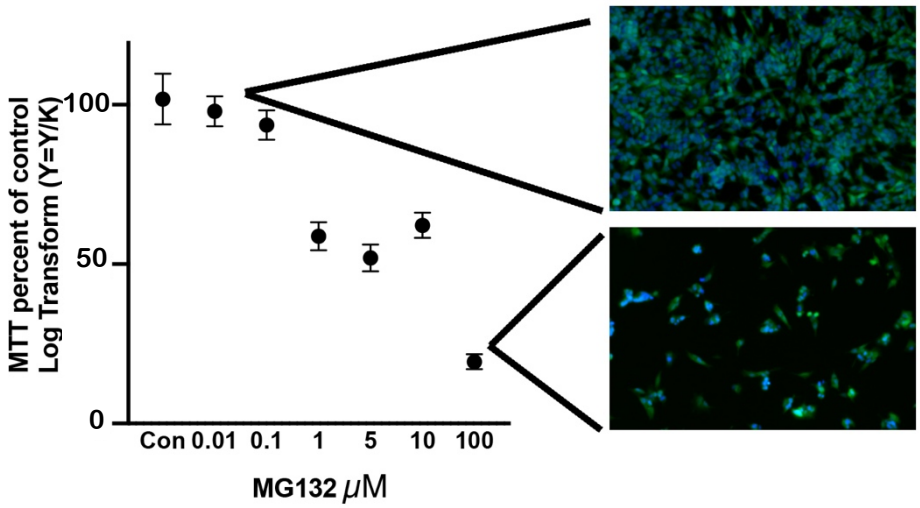

Figure 7. SH-SY5Y cells were treated with MG132 at concentrations of 10nM, 100nM, 1μM, 5μM, 10μM, and 100μM for 24 hours. Cell viability was assessed using the MTT assay. A significant reduction in viability was observed in a dose-dependent manner, with a 50% decrease in cell number occurring between 1μM and 5μM MG132 treatment.
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2.6. Preparation of MPP^+

Prepare serial dilutions of MPP^+, purchased from Sigma.

i. Prepare 10mM stock solution of MPP^+ (170.25g/mol) 0.00170g in 1mL of deionised water.

ii. Make serial dilutions of 1mM/mL 100µM/mL, 10µM/mL, 1µM/mL and 0.1uM/mL in seperate eppendorfs.
iii. For 100µL wells treated to 1mM of MPP^+, add 10µL of 10mM serial.
iv. For 100µL wells treated to 100µM of MPP^+, add 10µL of 1mM serial.
v. For 100µL wells treated to 50µM of MPP^+, add 5µL of 1mM serial.
vi. For 100µL wells treated to 10µM of MPP^+, add 10µL of 100µM serial.

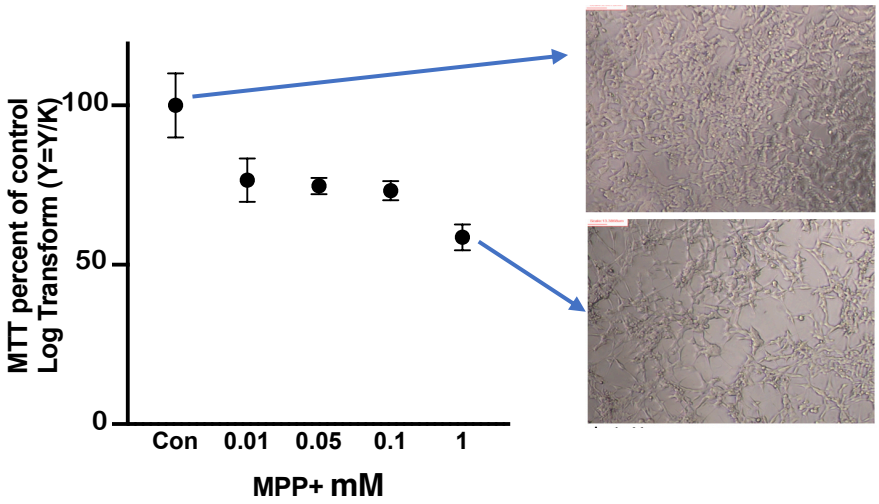


Figure 8. SH-SY5Y cells were exposed to MPP^+ at concentrations of 10µM, 50µM, 100µM, and 1mM for 21 hours. Cell viability was evaluated using the MTT assay. A dose-dependent decrease in cell viability was observed, with approximately 42% cell loss occurring at the highest concentration of 1mM MPP^+.
.

2.7. MPTP model development

In the early stages of the preliminary investigations, it came to light that approximately 10% of C57b/6J (Jax) sub-strain mice experienced cardiac arrest and ultimately perished after the initial dose of MPTP (21mg/kg • free base). Ensuring that our animal experiments adhere to a rigorous ethical framework, one that prioritises the welfare of all animals involved, the risk of losing 10% of mice due to the acute peripheral toxicity of MPTP was deemed too considerable to proceed with the in vivo investigations. Prior research has indicated that the C57b/Ola-HSD strain of mice exhibited notably higher levels of resistance toward the acute toxicity of MPTP (unpublished observations). Building on preliminary investigations, 21 mg/kg of MPTP freebase was administered to six C57b/Ola-HSD mice. None of these mice experienced cardiac arrest or premature death as a result of the treatment. These findings align with the aforementioned unpublished observations and confirm that C57b/Ola-HSD mice do indeed exhibit considerably greater resilience to the acute toxic effects of MPTP.

Development of the MPTP model was carried out using C57b/Ola-HSD mouse type only, and not C57b/6J. During these early stages of model development, the aim was to determine the appropriate MPTP dosing regimen while minimising the risk of losing animals to MPTP acute toxicity. This approach allowed for the identification of key behavioural traits exhibited both before and after MPTP treatment.

As with the in vitro neuroprotection assays, determination of whether NLX-112 is neuroprotective can be made only once a substantial loss of 40-60% DA neurons in the SN has been achieved following the optimal MPTP dosing regimen. Previous studies have employed variations of MPTP dosing regimen to induce SN DA cell loss. However, if the dosing regimen of MPTP is not carefully considered, then its acute effects on noradrenergic receptors in cardiac and vascular tissue, resulting in vasoconstriction and hypothermia which can then result in heart failure and death of the animal. Considering the agreed conditions between the Home-Office and the experimental protocol stipulation laid out in the project licence; 'no more than 10% of mice can succumb to death as a result from treatment with MPTP [sic]', then development and execution of the MPTP mouse model must be extremely precise. Indeed, the amount, frequency and route of MPTP dose administration must be sizeable enough to induce an optimal lesion of the nigro-striatal pathway, but that same dosing regimen must also not result in the death of the animal.

As our primary hypothesis is to investigate whether NLX-112 is neuroprotective, it is therefore not necessary to achieve a severe late-stage PD-like lesion (>75% TH +ve cell loss in SN). Previous MPTP studies have shown that the results (lesion severity) are not always consistent and can vary by considerable margins depending on dosing regimen used. It has previously been shown that 15 mg/kg of MPTP·HCl once a day for fourteen days results in almost no loss of striatal DA levels (µg/g tisue), whereas 30mg/kg of MPTP·HCl twice a day for five consecutive days results in 70% loss of striatal DA levels (Kurosaki et al., 2004). Further still, Jackson and Przedborski (2007), in their MPTP method validation paper, show that an acute MPTP dosing regimen ranging from 4 x 14 and up to 4 x 20 mg/kg

single doses of MPTP every 2 hrs can result in anywhere from a 40% to a 90% lesion in the SN, whereas a sub-acute dosing MPTP regimen of 1 x 30mg/kg once a day for 5 days will result in a 40-50% lesion in the SN. Thus, prior to determining NLX-112's potential neuroprotective properties, the model validation of achieving a 50% cell loss in the SN must first be carried out.

2.8. Serotonergic assessment of the MPTP model

As well as having a destructive property on the DAergic nigrostriatal pathway, MPTP lesioning also has a deleterious effect on the serotonergic pathway, in some, but not all areas of the brain. For example, mice treated with MPTP show a marked reduction 5-HT content in the hippocampus (Santiago et al., 2010; Lesemann et al., 2012) and in the striatum, mPFC and BLA (Vučković et al., 2008). Conversely, a study carried out by Melamed et al., (1985) found that mice were protected against the neurotoxicity of MPTP when co-treated with the DA reuptake inhibitor, desipramine but not when co-treated with the 5-HT inhibitors, clomipramine or fluoxetine. Unlike the loss of DA neurons and DA fibres in the SN and striatum, respectively, MPTP treatment has not been reported, to date, of causing a loss of 5-HT neurons in the RN. However, MPTP treatment in marmosets has been shown to cause serotonergic fibres in the striatum and globus pallidus to undergo hypertrophy whilst also exhibiting newly developed globulus varicosities (Zeng et al., 2010).

MPTP clearly has an adverse effect on the normal serotonergic function in the brain, however, the changes elicited by MPTP treatment are distinctly different to that which occurs to normal DAergic function. Given the specific serotonergic changes caused by MPTP, it is therefore important to ascertain whether our MPTP model has any effect on any number of the important components making up the serotonergic pathway, such as; changes to 5-HT cell bodies in the RN or hypertrophy of 5-HT fibres in the striatum and cortex.

2.9. Method

2.9.1. Behaviour

Analysis of mouse behaviour and locomotor activity will also be carried out before (1 day prior to MPTP treatment) and after dosing (6 days post MPTP treatment). Analysis of behavioural assays will be carried out by subjecting mice to two different maze paradigms, the open field and elevated plus maze.

2.9.2. Ethical approval

The experiments reported herein complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. All procedures and protocols were approved by the Animal Welfare and Ethical Review Board (AWERB) at the University of Hertfordshire, which were carried out under the U.K. Home Office approved Project Licence PD7255B4.

2.9.3. Animals

Ten-week-old male mice were purchased from Envigo laboratories and housed individually in 30cm x 40cm^2 stainless steel and Perspex cages. Mice had access to a wide array of environment enrichment (sc) (see Table 4 for MPTP dosing regimen).

2.9.4. MPTP preparation

Due to MPTP being a highly toxic lipophilic agent, extreme precaution was taken when dosing the animals. On the day of experiment mice were moved into a disposable plastic cage on top of a stainless-steel heating bench which had a 50/50 heating element beneath the surface, which then heated the plastic cage on one half to 28-31C and the other half remained at ambient temperature (23-25C). For administering MPTP, experimenters donned full Tyvek suits, goggles, respirator, and were double gloved. All MPTP administration, and to maintain construct validity, all saline administration, was carried out under a fume hood. All syringes (29g insulin syringes) were aspirated with bleach after being used for MPTP administration. Once dosing was complete, mice were then placed back into their disposable cages. After MPTP dosing, mice could choose to regulate their own

body temperature thus reducing the possibility of succumbing to MPTP induced hypothermia (Przedborski et al., 2001). The experimental group received a sub-chronic dosing regimen of MPTP (s.c), over 5 days (7x 15.4mg/kg of MPTP freebase) (n=10). The sham group received the same number of injections to that of the MPTP group but with saline (vehicle, 150uL), over the same period (n=8).

Table 4. Sub-chronic dosing regimen calculated as MPTP freebase and administered as daily single bolus injections SC. This MPTP dosing regimen involves administering 15.4mg/kg of MPTP once daily for four days (Day 1 to Day 4), followed by three doses of 15.4mg/kg on Day 5, totaling seven doses.

Day 1	Day 2	Day 3	Day 4	Day 5
1 x 15.4mg/kg	1 x 15.4mg/kg	1 x 15.4mg/kg	1 x 15.4mg/kg	3 x 15.4mg/kg

To calculate the required MPTP dose, the following equations were applied:

$$\text{drug (mg)} / \text{vol. veh (mL)} = \text{conc. (mg/mL)}$$

$$\text{dose (mg.kg}^{-1}\text{)} / \text{conc. (mg/mL)} = \text{drug conc./veh (mg.kg}^{-1}\text{/mL)}$$

$$\text{MFD} = \text{mass (kg)} \times \text{drug conc./veh (mg.kg}^{-1}\text{/mL)} \times 100$$

Divide the initial drug (MPTP) weight (drug) by the volume of vehicle (Vol. of veh) intended to use for diluting the drug, then divide the dose (that which has been chosen to administer to the animal: 10mg/kg, 20mg/kg etc) by the concentration thus giving the resultant drug concentration (Drug Conc.). The diluted MPTP freebase dose (MFD) can be then calculated by dividing the mouse mass (0.020kg, 0.025kg etc) by the drug concentration and multiplying that value by 100.

MPTP hydrochloride was purchased from Sigma. Hydrochloride (HCl) has a molar mass of 35.4 and makes up 17% of MPTP. Therefore, for a given amount of MPTP· HCl, its weight was divided by 1.17. e.g: 18 mg/kg of MPTP· HCl / 1.17 = 15.4 mg/kg MPTP· freebase.

The sham group were also housed in plastic cages on top of the 50/50 heating bench. After the last dose of MPTP or saline, mice were then left in their semi-

heated disposable plastic cages for a 5-day washout period to allow for all MPTP to be metabolised or excreted. Mice were then placed back into their original cages and subjected to post-treatment behavioural experiments in the open field and elevated plus maze.

2.9.5. Immunohistochemistry

Mice were culled via exposure to CO_2, trans-cardially perfused with ice cold PBS, decapitated, brains fixed in formalin (10% buffered) for 48 hours and stored in PBS plus sucrose (30%) and stored at 4C. Preparation for IHC analysis was carried out only once brains had sunk to the bottom of Eppendorf ensuring that all formalin had been displaced by the PBS sucrose solution thus rendering it cryoprotected. Brains were sectioned coronally in 30 µm thick sections and analysed for IHC and immunofluorescence (IF) using the following antibodies: tyrosine-hydroxylase (TH) anti-sheep, 1:500 Invitrogen®, tryptophan hydroxylase-2 (Tph2) anti-rabbit 1:60 Novus-bio®, glial fibrillary associated protein (GFAP) anti-chicken 1:1000 Invitrogen® and ionised calcium-binding adapter molecule 1 (Iba1) anti-rabbit 1:500 abcam®. Free floating or slide fixed sections were washed in PBS (1X), triton X-100 (0.1%), non-specific binding was inhibited with non-animal protein blocker (Vector®) and incubated overnight in primary antibody at 4°C. The following day, sections were washed and incubated in secondary antibody for one hour at room temp. Tissue was mounted on slides and images were taken on a Zeiss light microscope.

Calculation of TH+ve cell number in the SNpc was performed using a standardisd method. Using ImageJ software, only those SNpc sections that have the oculomotor nerve (MA3) dorsal to it, the medial terminal nucleus (MT) running through it and the presence of the medial tier of the SNpc (SNCM) and lateral portion of the SN (SNL), were used for TH+ve cell count comparison (Figure 9). With these criteria, only sections from Bregma -3.05 to Bregma -3.15 were analysed; a region no thicker than 100µm. TH+ve cells from the VTA were omitted from cell counting.

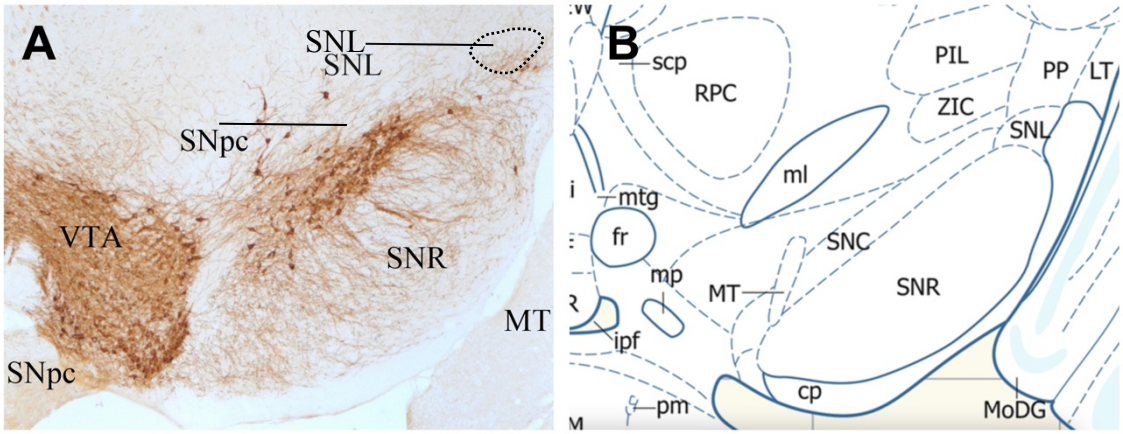


Figure 9. A) Representative TH-immunostained coronal section showing the anatomical location of the substantia nigra (SN), including the pars compacta (SNpc), pars reticulata (SNR), and pars lateralis (SNL), as well as the ventral tegmental area (VTA) and medial terminal nucleus (MT). B) Corresponding section from *Paxinos and Franklin's 'The Mouse Brain in Stereotaxic Coordinates', Fifth Edition* used to verify stereotaxic coordinates and delineate SN subregions for TH-positive cell quantification.

Cell counting was carried out using ImageJ software. Images went through a series of filters, such as converting to 8-bit greyscale and removal of the background and VTA (Figure 10). Only the TH+ve cells from the SNC, SNCM and SNL were counted using the ImageJ software. The following steps were carried out for each mounted TH stained tissue section.

1) Save image as JPEG
2) Drag image into ImageJ toolbar
3) Digitally remove VTA, leaving only the SNC, SNCM and SNL visible
4) Convert image to 8-bit greyscale
5) In the tool bar go to process → subtract background, set to 50.0 pixels and select light background
6) Set threshold, go to Image → adjust → threshold, set threshold to 180/255. Select 'Default', 'B&W' and 'Dark background'
7) Reverse the B&W contrast by going to Process → binary → convert to mask
8) Distinguish overlapping cells by going to Process → binary → watershed
9) Analyse → 'analyse particles'. For pixel size (pixel^2) set at 75-infinity; circularity to 0.00-1.00. Tick 'Display results', 'Clear results' and 'Summarise'

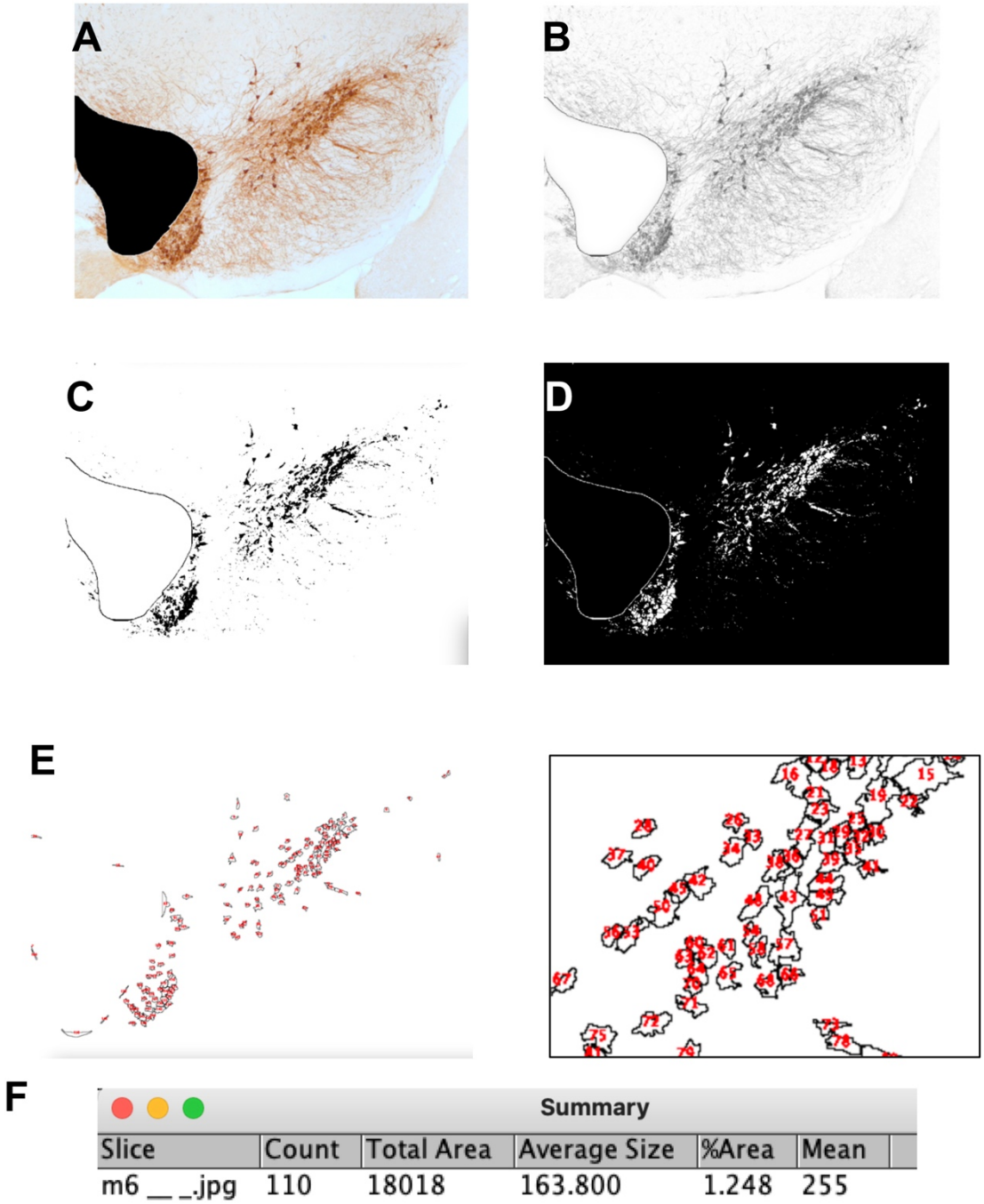


Figure 10. A) Representative image showing TH-immunostained tissue with the ventral tegmental area (VTA) manually excluded, leaving the substantia nigra pars compacta (SNpc), pars lateralis (SNL), and SNc medial (SNCM) for analysis. B) Image converted to 8-bit grayscale with background removed. C) Threshold set at 180/255 to isolate positive signal. D) Image converted to a binary mask and processed with the watershed function to separate touching cells. E) Output of the particle analysis with TH^+ cells marked and numbered. F) Example of the data output from ImageJ, showing total cell count (as "Count"), total area, average particle size, percent area, and intensity mean for a standardised image.

2.9.6. Apparatus

The open field arena consisted of four 40cm^3 open top black, infra-red translucent Perspex boxes (Dubovický et al., 1999) placed on top of an infra-red LED light box. The EPM was made entirely out of black Perspex and consisted of four arms each measuring 30cm in length and 5cm wide, the walls of the closed arm were 15cm in height (Lister, 1987). The EPM was raised 50cm from the ground and had an infrared LED light system fixed into all four arms of the apparatus. All OFT and EPM experiments were captured using a Basler infrared camera (Basler AG, Ahrensburg, Germany) fixed directly above the OFT and EPM experimental arena, with all kinetic and non-kinetic parameters analysed using Noldus EthovisionXT tracking software (Tracksys, Nottingham, UK).

2.9.7. Open field

To determine the extent of thigmotaxis*, a 30 cm^2 central zone that sat inside the centre of the arena was digitally created with the Ethovision-XT software, thus allowing a 5 cm wide channel to run around the perimeter of the OFT. The following parameters were measured electronically using the Ethovision-XT software: Total distance travelled (cm); distance travelled, duration (time spent in s) and mean velocity (cm/s) in the centre area or perimeter and number of entries into centre. * Thigmotaxis is the basal behaviour adopted by mice when in an open space. They will not venture into open ground and will remain close to proximal walls. This behaviour is exploited in the open field test.

2.9.8. Elevated plus maze

Mice were placed onto the centre square of the EPM facing toward the open arms. To determine when mice crossed into the different arms of the EPM five separate zones were digitally created; two for the closed arms, two for the open arms and one for the centre square where each four arms cross. Duration (time spent in s) in the open arms, closed arms and centre square, and entries into the open arms and closed arms, were calculated using the Ethovision-XT software

2.9.9. RotaRod

Mice were trained on the RotaRod (Ugo-Basile 47650) to become familiarised with the apparatus before any motor activity was recorded. Five mice were randomly

picked from each group to trial the RotaRod. The test consisted of three trials. One trial in the morning (which was considered as the training trial), and two experimental trials in the afternoon, with each trial separated by one hour. Mice were placed on the RotaRod one by one at a speed of 4 RPM. Mice traversed the RotaRod with the speed increasing incrementally every 2 seconds by 1 RPM, culminating to 35 RPM and continued to maintain at that speed until all mice had fallen off. The duration that mice stayed on the RotaRod was recorded, and a mean value of the two afternoon trials was used as the final result. All trial lasted no longer than 150 seconds.

2.9.10. Statistical analysis

All data analysis was performed using GraphPad prism software. Comparisons were made using either one-way ANOVA, two-way ANOVA or students T-test and data are presented as mean ± SEM (bars). Statistical significance was set at 95%.

2.10. Results of MPTP model development in C57b/Ola-HSD mice

MPTP was well tolerated by C57b/Ola-HSD mice and only led to significant weight loss after the first daily dose of 15.4mg/kg [$F(2.079\ (7, 72) = 0.3939\ p = 0.0567$] (Figure 11 A), after which MPTP mice gained weight in the same manner as saline treated mice [$F(0.6187\ (7, 56) = 0.6187\ p = 0.7382$] (Figure 11 B). At this point it should also be noted that it is the first dose of MPTP that appears to cause the greatest level of acute peripheral toxicity and that sequential daily doses of MPTP seem to have less of an impact on the recovery time of the animal. This effect may be due MPTP's property to impair the autonomic cardiovascular system via the desensitisation of vascular adrenergic receptors, elevate sympathetic signalling, increase tachycardia, and suppress parasympathetic tone (Ren et al., 2004; Liu et al., 2020). This phenomenon is a crucial finding and will aid in the dosing design of the forthcoming in vivo experiments.

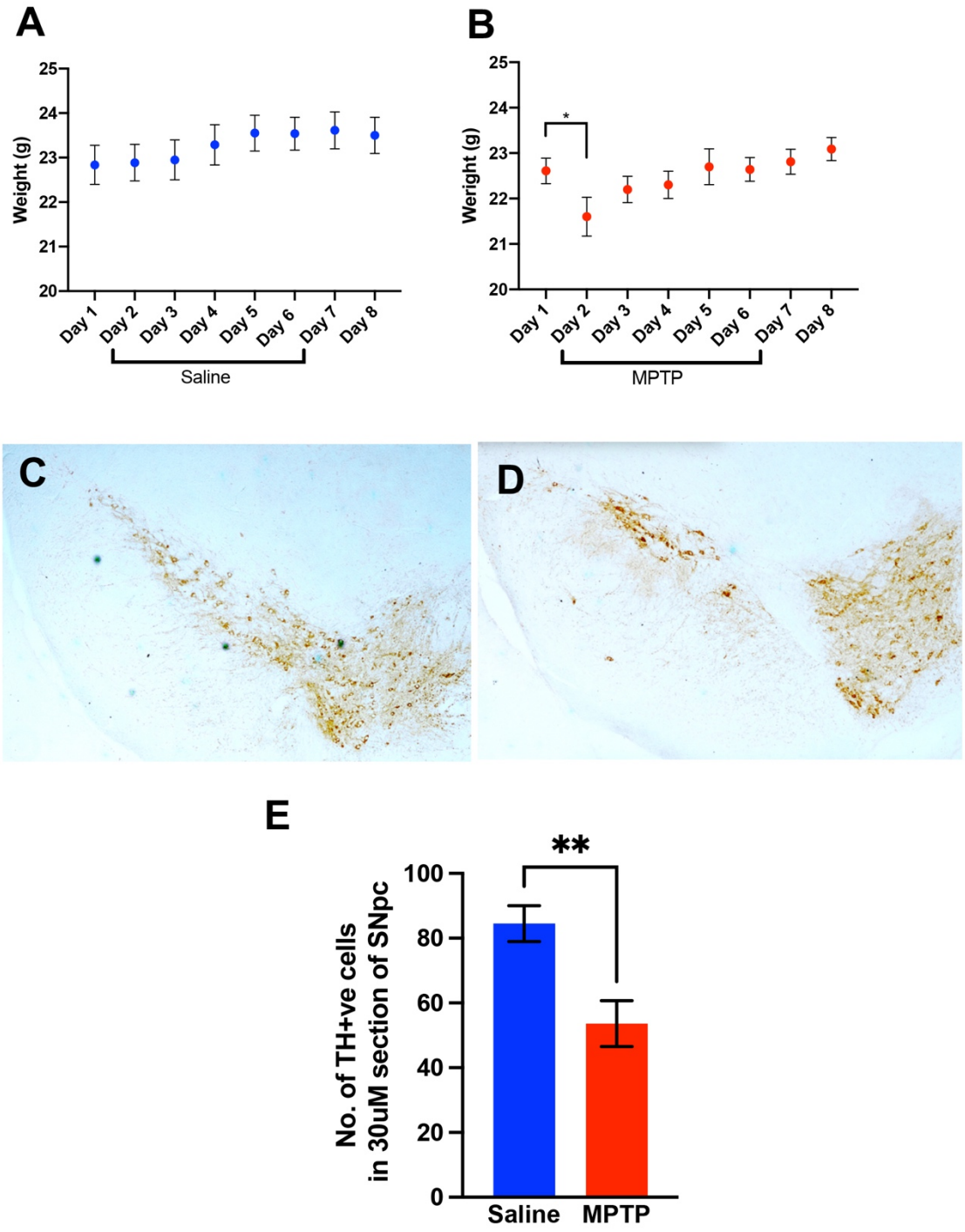


Figure 11. Body weight of A) sham and B) MPTP treated mice. Day 1 is body weight prior to first dose of vehicle or MPTP and days 2 to 6 are during treatment. Error bars denote SEM. One-way ANOVA followed by Fishers LSD post-hoc test. Representative immunohistochemical analyses of Tyrosine Hydroxylase reactivity in the substantia nigra of C) sham and D) MPTP treated mice. E) Cell count of total number of TH positive cells on 30µm mounted sections (Bregma -3.15). Students t-test. Error bars denote SEM. Sham (n=7), MPTP (n=9). (scale bar, 100 µM), *p<0.05, **p<0.01

The MPTP dose regimen used resulted in a significant loss of TH in the SN [t(3.680 (14) p = 0.0012] (Figure 11D, E), which corresponded with an increase of nigral GFAP+ve activation [t(2.635 (12) p = 0.0109] (Figure 12 A, C) and Iba1+ve activation [t(2.554 (12) p = 0.0092] (Figure 12 B, D).

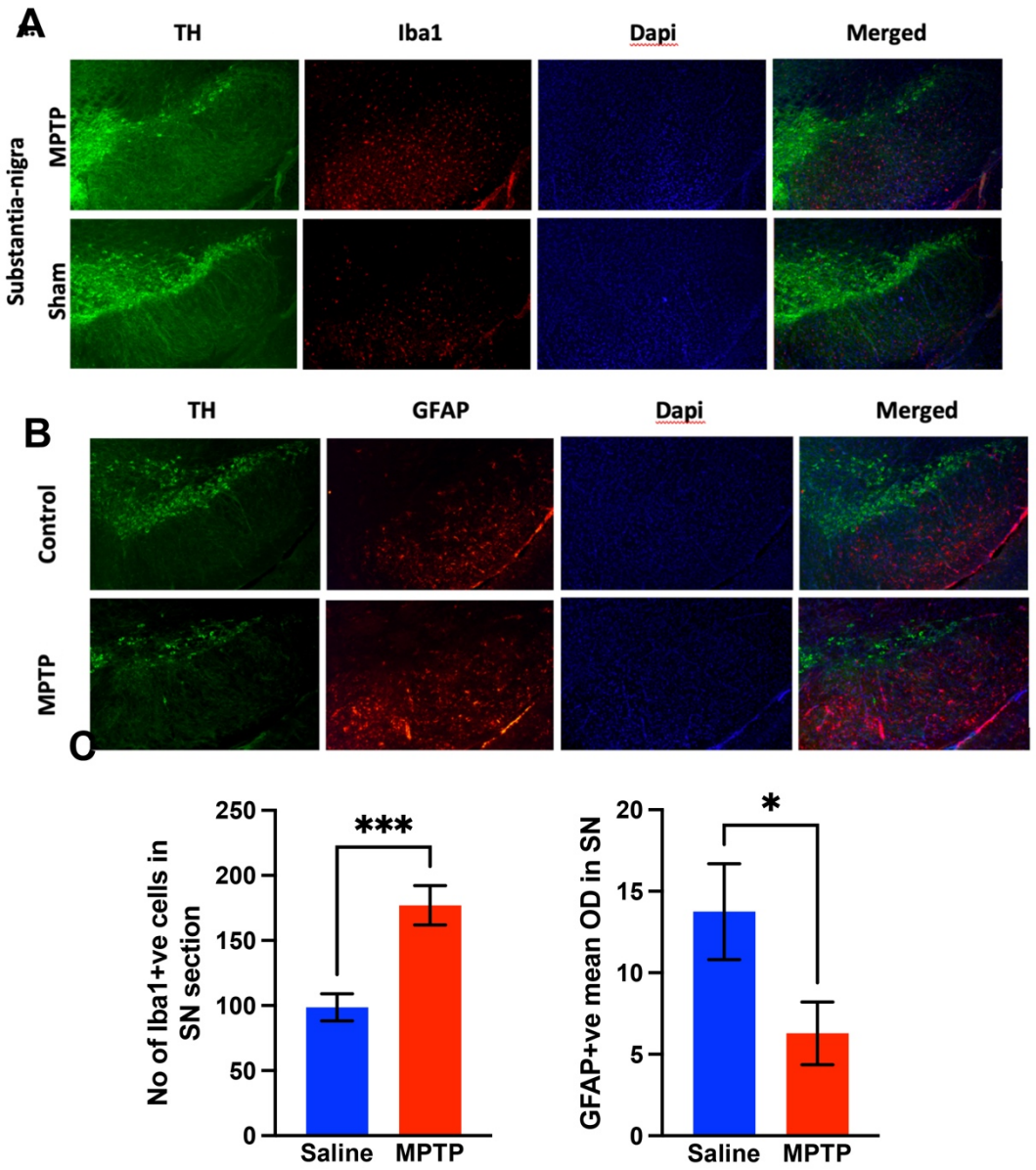


Figure 12 A) Representative images demonstrate the presence of DA neurons and microglia in the substantia nigra and striatum of saline- and MPTP-treated mice, using fluorescently labeled antibodies against tyrosine hydroxylase (TH) and Iba1. Additional representative images show the presence of DA neurons and astroglia in the substantia nigra of both treatment groups, labeled with TH and GFAP antibodies. Quantitative analysis was performed to assess the number of Iba1-positive cells and the mean grayscale fluorescence intensity of GFAP in the substantia nigra on 30 µm mounted brain sections. Statistical significance was determined using Student's *t*-test. Error bars represent the standard error of the mean (SEM). n= 6 - 8. $p < 0.05$ and $p < 0.01$.

MPTP also induced serotonergic changes in mice upon MPTP treatment. Compared to control, MPTP treatment led to a significant decrease in RN soma cell body area [t(2.743 (17) p = 0.0069] (Figure 13 E), an increase in RN axon thickness [t(2.370 (19) p = 0.0143] (Figure 13 D), an increased 5-HT fibre thickness in the mPFC [t(1.875 (10) p = 0.0451] (Figure 13 G), and an increased 5-HT fibre thickness in the dorsal striatum [t(1.887 (10) p = 0.0443] (Figure 13 F).

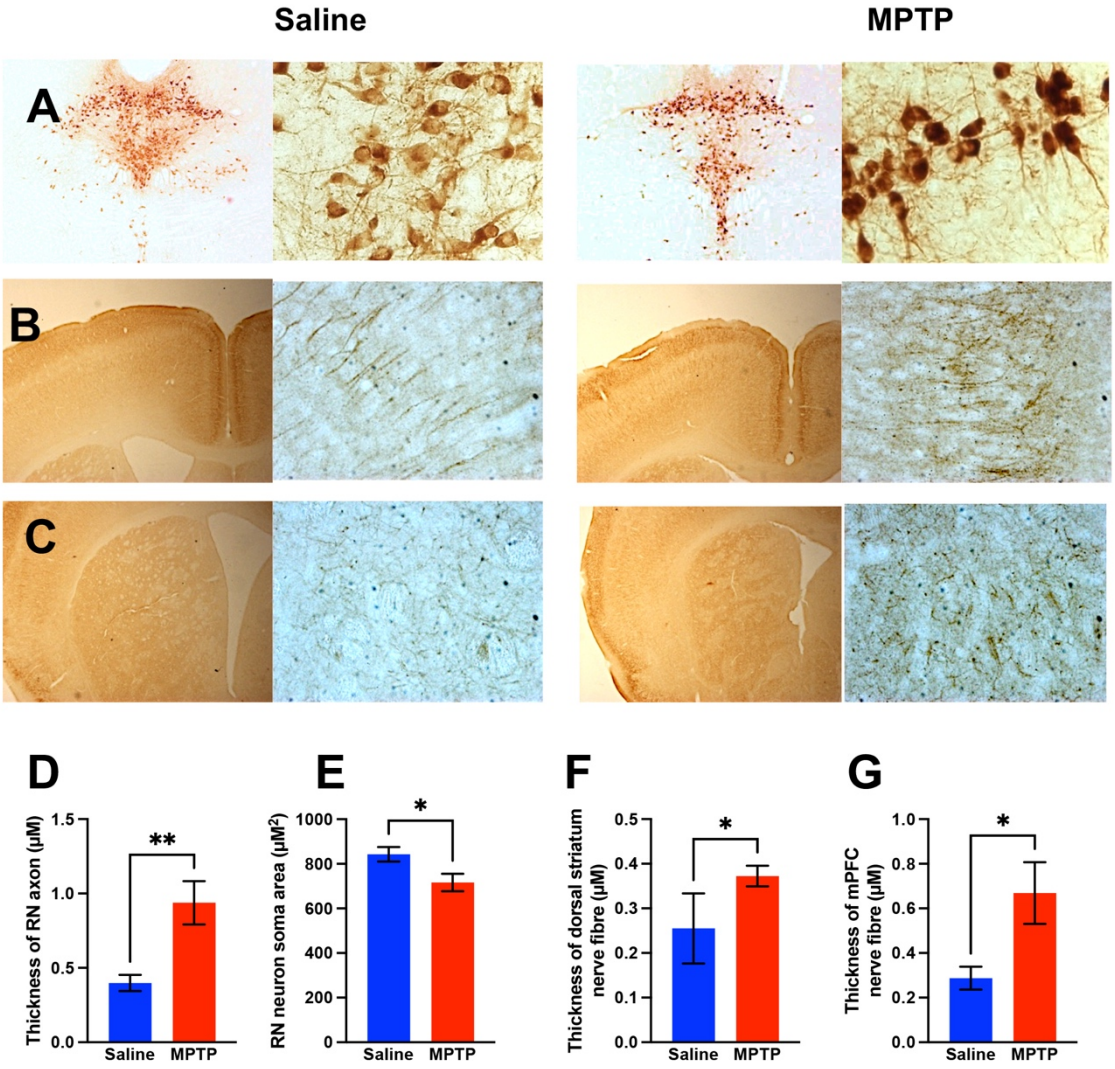


Figure 13. Tph2 immunoreactivity in the RN, mPFC, and mid-Cpu following saline or MPTP treatment. A) Representative images of Tph2-positive neurons in the raphe nuclei (RN) from saline- and MPTP-treated mice, with higher magnification images shown on the right. B) Tph2 immunoreactivity in the medial prefrontal cortex (mPFC). C) Tph2 immunoreactivity in the mid-caudate putamen. D) RN axon thickness, (E) RN neuronal soma area, (F) dorsal striatum fiber thickness, and (G) mPFC fiber thickness. Data are presented as mean ± SEM (n = 5–10 per group). Statistical analysis was performed using Student's *t*-test. $p < 0.05$, $p < 0.01$.

In depth behavioural assessments were carried out in saline and MPTP mice before and after experimental treatments. Compared to before experimental treatments, the distance travelled was significantly less following treatment with saline [t(7.238 (7) p = 0.0001] (Figure 14 A) and MPTP [t(4.304 (9) p = 0.001] (Figure 14 B). However, MPTP treatment led to mice spending more active time in the perimeter of the OFT [t(2.970 (9) p = 0.0078] (Figure 14 D) whereas there was no change in the locality of OFT activity in saline treated mice [t(0.3893 (7) p = 0.2921] (Figure 14 C).

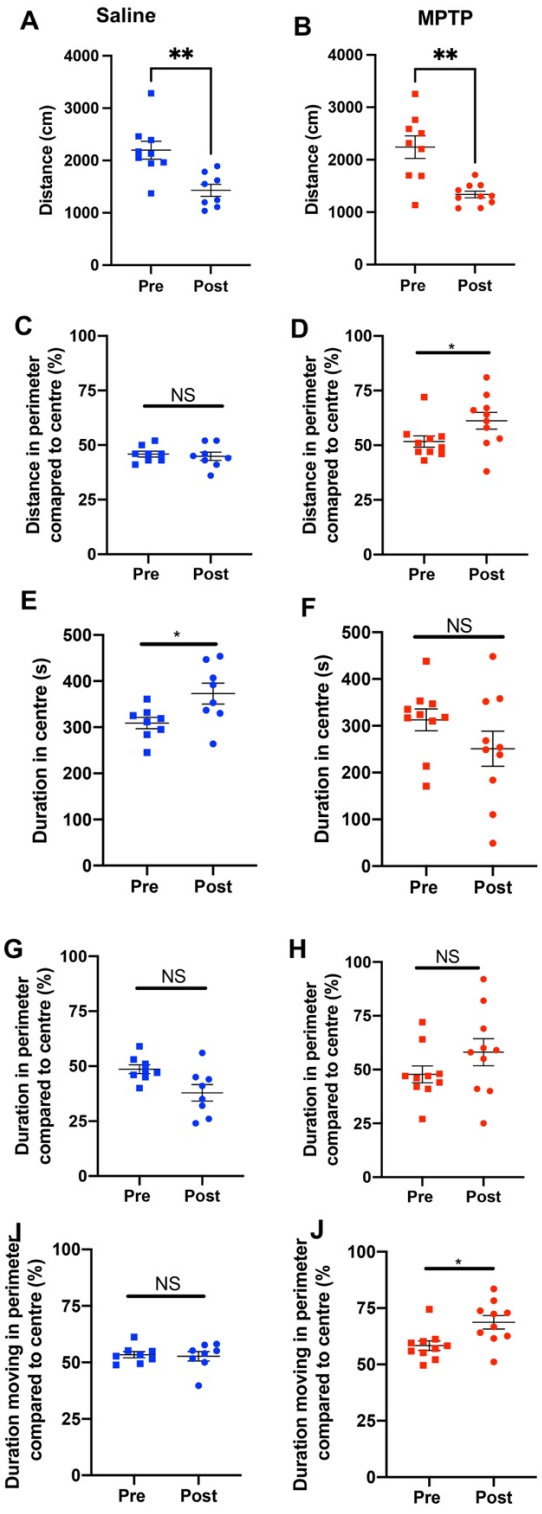


Figure 14. Behavioural assessment in the OFT before and after treatment (saline or MPTP). Pre-treatment behavioural assessment was carried out 48 hrs prior to test. Post-treatment behavioural assessment was carried our 6 days after final dose of either saline or MPTP. a) Distance travelled by sham. b) distance travelled by MPTP. c) distance travelled in perimeter compared to centre by sham. d) distance travelled in perimeter compared to centre by MPTP.

When both groups were compared following treatment, it was found that there was no difference in the distance travelled in the OFT [t(0.7432 (16) p = 0.2341], but duration spent in the centre was significantly reduced in the MPTP group compared to saline [t(2.579 (16) p = 0.01] (Figure 15 G), which corresponded with a marked reduction of entries made into the centre of the open field arena [t(1.708 (16) p = 0.0505] (Figure 15).

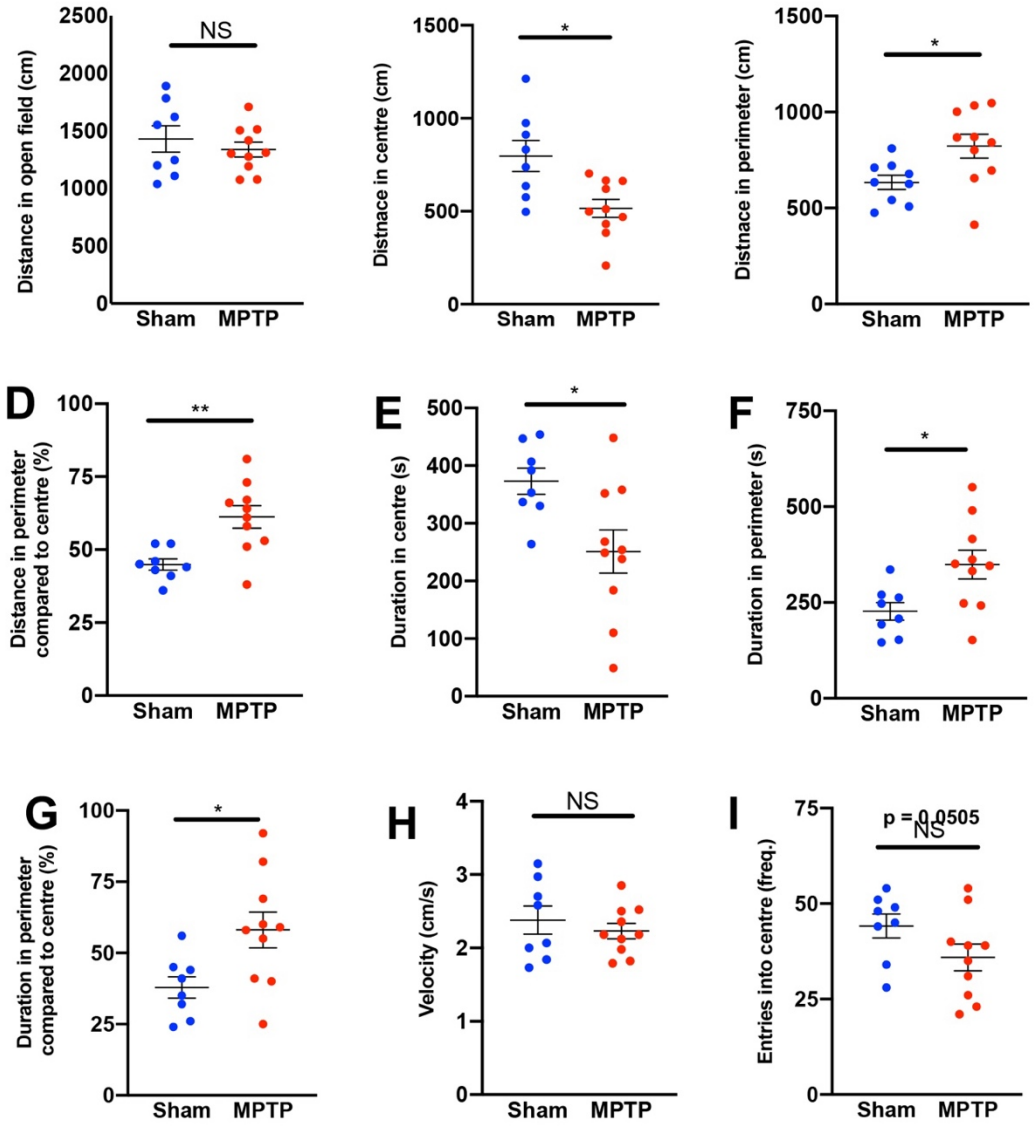


Figure 15. Behavioural assessment over 10 mins in the open field, 6 days after final *s.c.* dose of vehicle or MPTP. A) The distance travelled in the open field arena. B) The distance travelled in the centre of the open field. C) The travelled in the perimeter of the open field. D) The percent of distance travelled in perimeter compared to centre (%). E) The duration spent in the centre of the open field. F) The duration spent In the perimeter of the open field. g) The percent of duration spent in the perimeter compared to the centre (%). h) The average velocity achieved by mice over 10 mins in the open field. i) The number of entries made from the perimeter into the centre. n = 10. Students t-test, *p<0.05, **p<0.001 error bars denote SEM.

To test whether MPTP treatment induced a greater level of anxiety-like behaviour, mice were examined in the elevated plus maze (EPM), an apparatus designed to assess the innate anxiety-like state of rodents.

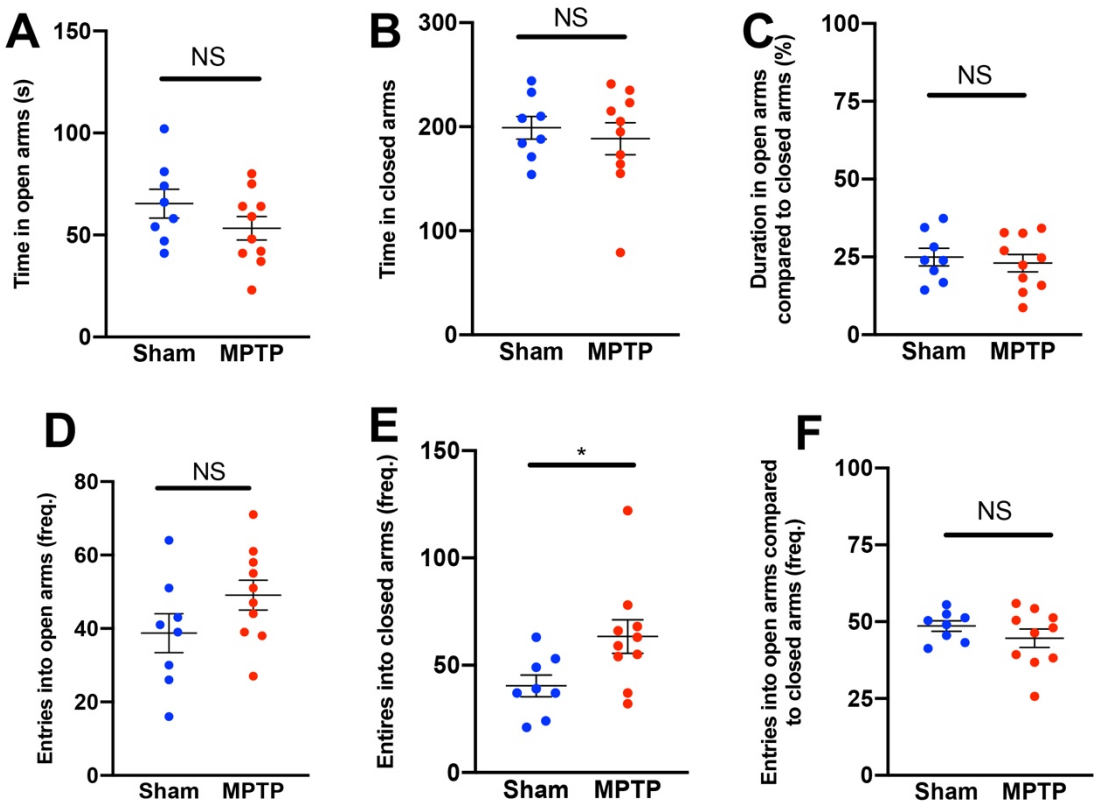


Figure 16. Assessment of anxiety-like behaviour in the elevated plus maze over 5 mins, 7 days after final *s.c.* dose of vehicle or MPTP. a) The time spent in the open arms. b) The time spent in the closed arms. c) The duration spent in the open arms compared to closed arms (%). d) The entries made into the open arms. e) The entries made into the closed arms. f) The entries made into the open arms compared to the closed arms. n = 10. Students t-test, *p<0.05, error bars denote SEM.

However, the EPM revealed very little in terms of any changed behaviour upon MPTP treatment, apart from MPTP leading to a greater exploratory behaviour as noted by a marked increased number of entries into the open arms [t(4.304 (9) p = 0.0] (Figure 16 E), and a significant increase in entries into the closed arms of the EPM, compared to saline treatment only.

2.11. Discussion

2.11.1. In vitro models

The in vitro model development assays provided the necessary molecular concentrations required to achieve 50% (Ec50) cell death in 24hrs, when using the proteasome inhibitor MG132 and the mitochondrial inhibitor MPP^+, at 5uM and 1mM, respectively (Figure 7, Figure 8).

2.11.2. In vivo model

Within the first hour post MPTP treatment, C57b/Ola-HSD mice presented with piloerection and marked reductions in active behaviour. With each sequential daily dose of MPTP, these overt features of MPTP treatment displayed by the mice, diminished considerably, suggesting an increase in MPTP tolerability upon each successive dose. Mice utilised the 50/50 heated bench and were able to regulate their body temperature of their own volition. After MPTP dosing, mice would localise to the heated side of the bench and splay their belly onto the floor of the plastic cage, displacing all of their bedding in what appeared to be so they could be as close as possible to the heat. There was a slight reduction in body weight (< 5%) after the first dose but this trend was short lived and not replicated upon treatment with any of the following daily doses. No mice died as a result of MPTP treatment. The fact that mice could ward off MPTP induced hypothermia, by laying on the heated bench, is likely a key factor to achieving a 100% survival rate.

After careful analysis using the image J software, this MPTP dosing regimen caused a 34% loss of TH+ve cells in the SNpc. To achieve a viable neuroprotection model, whereby the lesion achieved is closer to 50% cell loss, the dosing regimen will have to be increased and/or lengthened.

Additionally, from a resting state, there was an activation of Iba1+ve cells (approximately +80%) and GFAP+ve cells (+130%) in the SNpc of MPTP treated mice, indicating a microglial and astroglial associated inflammatory response upon MPTP lesioning. This finding enhances the face-validity of our model considering that inflammation, as a marker of PD pathogenesis (Teismann et al., 2003), is also observed in other studies utilising the MPTP mouse model (Martin et al., 2016).

Our research group has previously found that C57b/Ola-HSD mice tolerate 2 x single doses of 25mg/kg of MPTP freebase on consecutive days, with treated mice exhibiting a rapid recovery (unpublished observations). In order to achieve a viable neuroprotection model, it is therefore necessitated that the subsequent MPTP neuroprotection experiments subject mice to an increased dosing regimen of MPTP.

In addition to its harmful effects on the DAergic pathway, a sub-chronic dosing regimen of MPTP treatment was found to induce changes in serotonergic morphology. Measurement of soma area and axon thickness was carried out in the RN of Tph2+ve neurons, as well as measurement of fibre thickness in the mPFC and Cpu. In the MRN Tph2+ve soma size soma was significantly reduced and axon thickness was significantly increased. As well as Zeng et al., (2006) noting the hypertrophic response of serotonergic fibres in striatum and GP, a more recent study has shed light into 5-HT's role upon the depletion of DA in the brain and concomitant neural connectivity between key brain regions. Chaib et al., (2023) performed a multimodal imaging study on hemi-Parkinson rats that had been treated with L-dopa and/or NLX-112. In non-dyskinetic rats, L-dopa caused hypermetabolism in the motor regions, the cerebellum, brainstem and mesencephalon and hypometabolism in the cortical regions. In L-dopa treated dyskinetic rats there was the addition of hypermetabolism in the RN and hypometabolism in the hippocampus and striatum. NLX-112 treatment attenuated L-dopa induced hypermetabolism in the RN and hypometabolism in the cingulate cortex, suggesting that 5-HT_{1A} activation of pre-synaptic receptors in the RN acts as a modulator for reversing drug inflicted irregularities to the brains metabolic profile.

It is therefore important to acknowledge, and especially consider throughout the description of these experiments raised in this thesis that the MPTP model and its neurotoxic effects not only alter the DAergic pathway, but also the serotonergic.

Locomotor activity was not affected after treatment with MPTP, however in the second open-field assessments, after treatment had finished, both the saline and

MPTP treated mice had a significant reduction in locomotor activity; the cause being due to a well-known phenomenon in etho-behavioural maze assays, known as environmental habituation. The anxiety-like behaviour exhibited by MPTP treated mice in the OFT was not replicated in the EPM (a maze designed specifically to model anxiety-like behaviour). Why MPTP mice exhibit reduced anxiety-like behaviour in the OFT but not in the EPM is conflicting and needs to be investigated further. One fundamental difference between the two maze paradigms, is that with the open field the mice are exposed to the open space of the apparatus without anywhere to retreat, except close to the edge (thigmotaxis); whereas in the EPM, mice are able to stay within the confines of the closed arms without ever having to expose themselves to the open arms (if they so choose). This environmental variance is likely sequestering different behavioural responses from the mice, at least so when they are exposed to a stressful unfamiliar environment. Nonetheless, both the OFT and EPM will continue to be employed in the following experiments as accurate behavioural characterisation of MPTP, NLX-112 and their potential interaction must be quantified to the best possible measure. Both compounds (NLX-112 and MPTP) target specific components of the monoaminergic pathways, and any possible synergistic interactions could have profound effects on the mouse's behaviour. The behavioural characterisation of NLX-112 in mice is therefore paramount, and will be described in detail throughout the following Chapters.

Chapter 3

In vitro Investigation of NLX-112 in SH-SY5Y cells

3.1. Introduction

As it is the demise of DAergic neurons in the SN that underlie the motor symptoms of PD, in vitro cell models consequently aim to utilise cells of an exact or analogous phenotype. It is very difficult to obtain the expiring DA neurons of a PD patient; therefore, researchers have sought to design cell models as closely aligned to PD neuronal aetiology as possible. Dopaminergic cell death in the SN leading to a loss of striatal nerve terminals neural DA is known to be the primary cause of motor dysfunction in PD. It is therefore necessary to closely examine how DA neurons function in cell models of PD aetiology. One way that this can be achieved is to study the neurons of other mammalian species similar in monoaminergic neural circuitry. Rodent embryonic cells from the ventral mesencephalon (VM) are highly differentiable making them ideal for growing in culture. This then provides an ideal model for studying the neuronal pathogenesis of PD in vitro.

Researchers employing in vitro models have typically used embryonic mesencephalic mixed cell cultures to model experiments relevant to those with monoaminergic neuronal characteristics, however the yield of monoaminergic expressing neurons is often very low and thus the depth of investigations is restricted by this limitation. Primary cells taken from the pre-natal rodent ventral mesencephalon (VM) offer a good model capable of exhibiting characteristics akin to the cells of the human midbrain. When isolated and matured, the DAergic neurons of embryonic rodents can be investigated with a variety of techniques capable of reflecting DA neuronal characteristics (Gaven et al., 2014). Therefore, treatment with neurotoxins specific to DA expressing cells can provide a good in vitro model of PD neurodegeneration. Subsequent treatment with a therapeutic compound of interest can then be administered to the dying neurons to see whether it offers any attenuation of cell death but they can also be used to investigate the pharmacokinetics of a drug in relation to PD aetiology. The benefits of using rodent primary cells is that they are mammalian, this means that they behave in a similar way to the cells inside the human brain. But it also means that they are mixed with other cell types, such as astroglia and microglia which can either impede or benefit the scientific outcome of the assay, and depending on what the initial experimental limitations and aims were to begin with, may have to

be removed, which can often be very expensive and time consuming. Moreover, primary cells often take weeks to grow to optimal maturity and are easily perishable when the growing conditions are not quite right, such as the composition of the media or the exact O_2/CO_2 concentration ratio inside the incubation chamber.

The longevity of cell lines on the other hand, offer a more robust model (Ferrari et al., 2020); however, they do not overly express the characteristics of one singular monoaminergic pathway and may have to be differentiated with certain chemical agents to induce the expression of a desired phenotype. Unlike primary cells, cell lines offer an arguably more robust in vitro model. This is because unlike primary cells they have been mutated so as to evade the normal timed stages of cell death and as a consequence can continue to differentiate for considerable periods of time thus allowing for many series of passages to be carried out. This then allows for a substantial reduction in error when having to repeat the same experimental design. That there is typically only one cell type within a cell line further allows for greater experimental design specificity. Cell lines have therefore been used for in vitro PD models, the human neuroblastoma cell line SH-SY5Y is one of the most popular cell lines used (Xie et al., 2010). However, there is contention as to whether cell lines offer an accurate reflection of human neuronal biology due to the oncogenic characteristics that they exhibit (Falkenburger et al., 2006).

SH-SY5Y cells are a versatile and widely used cell line originally derived from a human neuroblastoma tumour. They possess the ability to differentiate into neuron-like or DAergic phenotypes, making them valuable for studying neurobiology, neurodegenerative diseases, and drug screening. The SH-SY5Y cell line is a subline of the SK-N-SH cell line which was immortalised in 1970 from a metastatic neuroblastoma cell biopsy of a 4-year-old female. The SH-SY5Y neuroblastoma cell line exhibits neuronal features that are catecholaminergic in phenotype but when serum starved and treated with retinoic acid they have been shown to develop DAergic characteristics including presence of VMAT, TH, D2, D3 and the DA transporter (DAT) (Presgraves et al., 2004; Lopes et al., 2010).

Previous research has shown that the SH-SY5Y cell line is receptive to a variety of compounds targeting the 5-HT_{1A} receptor, but any direct evidence for 5-HT_{1A}

presence on SH-SY5Y cells have seldom, if at all, been reported. The presence of other serotonergic functional components has however been identified in the SH-SY5Y cell line including MAO-A (Ugun-Klusek et al., 2019) and SERT (Nyarko et al., 2018) (Figure 17).

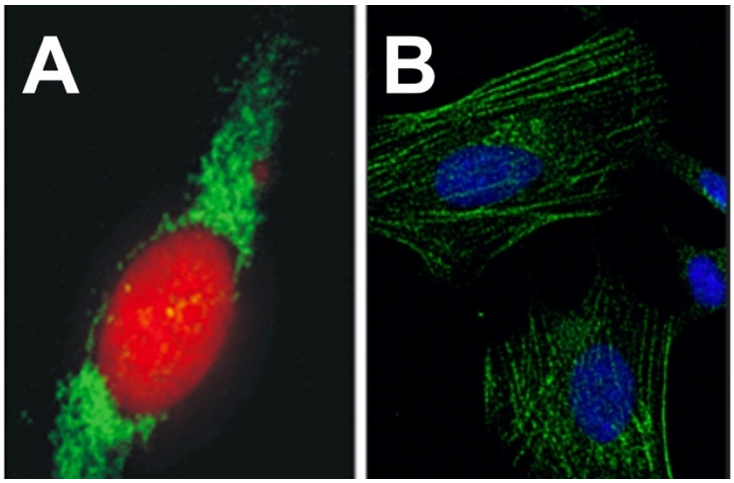


Figure 17. Immunohistochemical identification of MAO-A (green IF, panel A) and SERT (green IF, panel B) in SH-SY5Y cell line. Image adapted from Ugun-Klusek et al., (2019|) and Nykaro et al., (2018). In addition to 5-HT receptors, other key components of the serotonergic system have been identified in the SH-SY5Y cell line. Notably, this includes MAO-A, an enzyme responsible for serotonin degradation, and SERT, which regulates serotonin reuptake, further supporting the utility of SH-SY5Y cells in studying serotonergic signaling and metabolism.

The presence of the 5-HT_{1A} receptor in the SH-SY5Y cell line has been indicated, but via indirect means. For example, the partial 5-HT_{1A} agonist aripiprazole increases BDNF in SH-SY5Y cells by 85% compared to control, whereas the D2 inverse agonist haloperidol, has no effect on BDNF upregulation in the same cell line (Park et al., 2009). The most widely expressed receptor on SH-SY5Y cells is the *u*-opioid receptor (MOR); Levitt and colleagues found that adenyl-cyclase inhibition in SH-SY5Y cells by 8-OH-DPAT is halted by the MOR agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5- ol]-enkephalin (DAMGO), suggesting that the MOR and 5-HT_{1A} receptors are competing for the same adenyl-cyclase enzymes, but also that 5-HT_{1A} are expressed by the SH-SY5Y cell line (Levitt et al., 2011). Furthermore, in SH-SY5Y cells, the platelet derived growth factor-beta (PDGF-β) receptor is transactivated by 5-HT and 8-OH-DPAT which is blocked with the 5-HT_{1A} antagonist WAY100135 (Kruk et al., 2013), and knockdown of the regulator G-protein signalling protein 19 (RGS19) by small hairpin RNA allows 8-OH-DPAT to

dose-dependently increase ERK1/2 phosphorylation (Wang et al., 2014). Additionally, in SH-SY5Y cells damaged with Aβ, the polypeptide Tβ4, which has previously shown to have extensive neuroprotective properties, halts cell death through the inhibition of the ERK pathway following knockdown of HTR1A (Zhang et al., 2013).

These observations provide in-direct evidence that the SH-SY5Y cell line almost certainly expresses the 5-HT_{1A} receptor. The SH-SY5Y cell line is therefore likely a good model for investigating the efficacy of compounds such as NLX-112, which is already known to be highly selective for 5-HT_{1A}.

Using the SH-SY5Y cell line, this experiment sought to determine whether NLX-112 at varying concentrations is protective toward SH-SY5Y cells. A positive control condition using 8-OH-DPAT was also assessed and an assay to determine NLX-112's activity at the 5-HT_{1A} receptor by using the antagonist WAY100635 to see whether NLX-112 neuroprotective effect could be blocked (see Table 5 for treatment protocol).

3.2. Methods

SH-SY5Y cells were cultured in MEM supplemented with 10% FBS, non-essential amino-acids and GlutaMAX (refer to 'Methods' for a detailed composition). The cells were seeded in triplicates in a 96 well plate at a density of 50,000 per well and allowed to grow until 80-90% confluency which typically took approximately 24 hours. Subsequently, the cells were subjected to variuos concentrations of MPP^+ and incubated for approximately one day. To assess cell viability and infer MPP^+ toxicity, the MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay was performed, which provides insights into cell death by measuring mitochondrial activity.

3.2.1. MTT assay

The MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) assay was performed on the SH-SY5Y cells in order to infer cell death via the presence of total mitochondria. The formazan crystals were disassociated with isopropanol and although the crystals were solubilised after one hour of shaking there remained a white residue at the bottom of the well.

Table 5. The different chronological steps beginning with cell growth, drug administration and cell-viability -death assays. The neuroprotective potential of NLX-112 in SH-SY5Y cells exposed to toxins (MG132 or MPP^+). The study includes six experimental conditions: cells are either pre-treated, post-treated, or co-treated with NLX-112, with protection assessed using MTT or CellTiter-Glo assays to measure cell viability. Condition 1 tests the direct effect of NLX-112 alone. Condition 2 examines NLX-112's ability to prevent toxicity when administered before toxin exposure. Condition 3 assesses its rescue effects when applied after toxin exposure. Condition 4 evaluates simultaneous exposure to NLX-112 and MPP+, focusing on mitochondrial health using the CMX Ros MitoX red assay. Condition 5 explores a similar protective approach using 8-OH-DPAT, another $5\text{-}HT_{1A}$ agonist. Finally, Condition 6 investigates the role of $5\text{-}HT_{1A}$ receptor involvement by blocking it with WAY100635 before NLX-112 treatment.

Condition	DIV 1 (0 hrs)	DIV 2 (24 hrs)	DIV 2 (28hrs)	DIV 2 (29 hrs)	DIV 3 (53 hrs)
1. NLX-112 only	Seed cells 50k/well	Treat with NLX-112	-	-	MTT assay
2. NLX-112 Pre-protection	Seed cells 50k/well	Treat with NLX-112	-	Treat with MG132 or MPP+	MTT assay
3. NLX-112 Post-protection	Seed cells 50k/well	Treat with MG132 or MPP+	-	Treat with NLX-112	MTT assay
4. NLX-112 Post-protection	Seed cells 50k/well	Treat with MPP+ and NLX-112	-	-	CMX Ros MitoX red assay
5.8-OH-DPAT post protection	Seed cells 50k/well	Treat with MG132 or MPP+		Treat with NLX-112	MTT assay
6. NLX-112 post-protection - block with WAY100635	Seed cells 50k/well	Treat with MG132	Treat with WAY100635	Treat with NLX-112	CellTiter-Glo assay

This therefore meant that the light refraction used to determine mitochondrial presence was hindered. This issue was overcome by centrifuging the plate for one minute, removing 50μL from each well and re-plating. The read out after the protocol amendment showed a positive association between an increase in MPP^+ concentration and cellular toxicity ($R^2 = 0.992$). Cell loss of approximately 42% was achieved with 1mM of MPP^+. Dimethyl sulfoxide (DMSO) has since been used as the solubilising agent instead of isopropanol, as it does not leave any residue at the bottom of the plate. The aim is to achieve 50% cell loss. As the cells were subjected to the MTT assay post 21 hours MPP^+ incubation, it is likely that 50% cell loss can be achieved if MPP^+ is left to incubate on the cells for a longer period of time, perhaps 24 hours.

3.2.2. ATP assay (CellTiter-Glo)

The CellTiter-Glo assay is a luminescent reagent used for determining cell viability through the measurement of total ATP. ATP is an organic compound that provides energy for all life. ATP is a good measure of cell viability because it is crucial to optimal cellular function, providing energy through the conversion of glucose to pyruvate via the Krebs-cycle. Through mitochondrial respiration, each molecule of glucose produces about 30-fold increase in ATP. Any assay that can determine ATP content is therefore a good measure of cell viability.

The CellTiter-Glo assay was carried out using the following steps. Seed cells in a 96 well plate at 5x10^4, carry out culture protocol, equilibrate the 96 well plate to room temperature for approximately 30 minutes, add CellTiter-Glo reagent (100uL) to the 100uL well, induce cell lysis by mixing contents of well plate on a shaker for 2 minutes, allow to incubate for 10 minutes and record luminescence.

3.2.3. CMXros Red assay

The CMXros Red assay is a method employed to assess mitochondrial function and health by gauging the state of mitochondrial membrane potential and integrity. This assay utilises a fluorescent red dye, which secretively labels active and healthy mitochondria in proportion with their individual membrane potentials. The intensity of the red fluorescent signal observed within the cytosol directly reflects the degree of mitochondrial polarisation. A stronger red signal indicates higher mitochondrial polarisation, while a weaker signal suggests mitochondrial depolarisation or damage. This mechanism of action makes the CMXros Red assay a valuable tool for evaluating cell health and qualifying the effectiveness of potential therapeutic compound in a neuroprotection assay. Plate read at Excitation F:482-16, Dichroic 539.5, Emission 599-20.

3.2.4. NLX-112 effective dose concentration

The following assay was carried out so as to understand the effect of NLX-112 on untreated cells only. Newman-Tancredi et al., (2018) have already shown the signal transduction profile of NLX-112 elicits cell signalling at the low nanomolar range (G-protein activation EC50 = 7.37nM). This dose concentration may or may not translate to an effective dose sought to achieving neuroprotection in mice, or

any other higher species. Besides, there may also be differences in G-protein activation, as well as second messenger signalling, between different cell lines (HeLa vs SH-SY5Y for example). Given this, it is therefore important to qualify an effective dose range of NLX-112 treatment on SH-SY5Y cells in order to determine NLX-112 dose-toxicity. The following range was used: 1nM to 100uM. NLX-112 has the molecular weight 393.63g/mol and was prepared as a 10mM stock (0.00394g/mL) dissolved in DMSO. DMSO has previously been reported to exert neuroprotective properties (Di Giorgio et al., 2008; Sanmartín-Suárez et al., 2011). Before carrying out an NLX-112 dose-toxicity assay, a cell viability assay was first performed using DMEM plus incremental ratios of DMSO only. See table 6 below.

Table 6. Preparation of NLX-112 compound concentrations in a DMSO/DMEM media mixture for cell culture experiments. Starting from a 10mM stock solution in 100% DMSO (stored at -20°C), serial dilutions are prepared, ranging from 1mM to 1nM. At each dilution, the DMSO percentage is adjusted, with higher dilutions containing more DMEM and less DMSO, ensuring safe and consistent solvent exposure for cells.

NLX-112 compound concentration	DMSO/DMEM media
10mM (stock 1mL, stored in DMSO at -20°C)	100/0
1mM	10/90
100μM	1/99
10μM	0.1/99.9
1μM	0.01/99.99
100nM	0.001/99.999
10nM	0.0001/99.9999
1nM	0.00001/99.99999

3.3. Results

Figure 18 indicates DMSO as having neurotoxic properties at concentrations from 10% and above [F(47.62 (4, 10) = 0.5837 p < 0.0001].

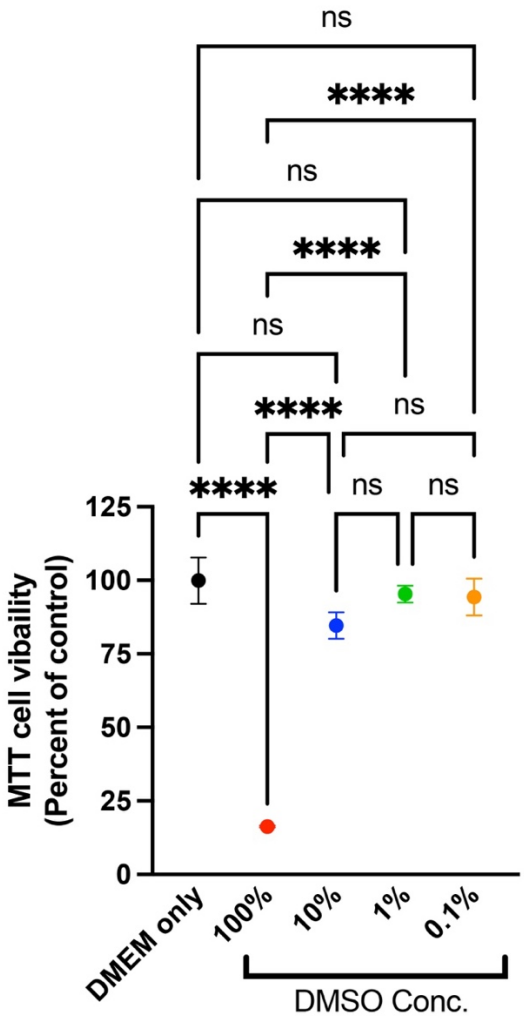


Figure 18. SH-SY5Y cells treated with decreasing concentrations of DMSO (100%-0.1%). Effects of different DMSO concentrations (100%, 10%, 1%, and 0.1%) on cell viability, measured by MTT assay, with results expressed as a percentage of the control (DMEM only). Cells exposed to 100% DMSO show a drastic reduction in viability (red dot), while 10%, 1%, and 0.1% DMSO maintain significantly higher viability (blue, green, and orange dots, respectively). n = 3, errors bars denote standard error of the mean (SEM). One way ANOVA followed by Bonfferoni's multiple comparison test. ****p<0.0001.

When NLX-112 was examined as drug only on SH-SY5Y cells there was increase in cellular proliferation at 1μM compared to vehicle treated cells [F(4.847 (6, 31) = 0.9493 p = 0.0014] (Figure 19 A, B).

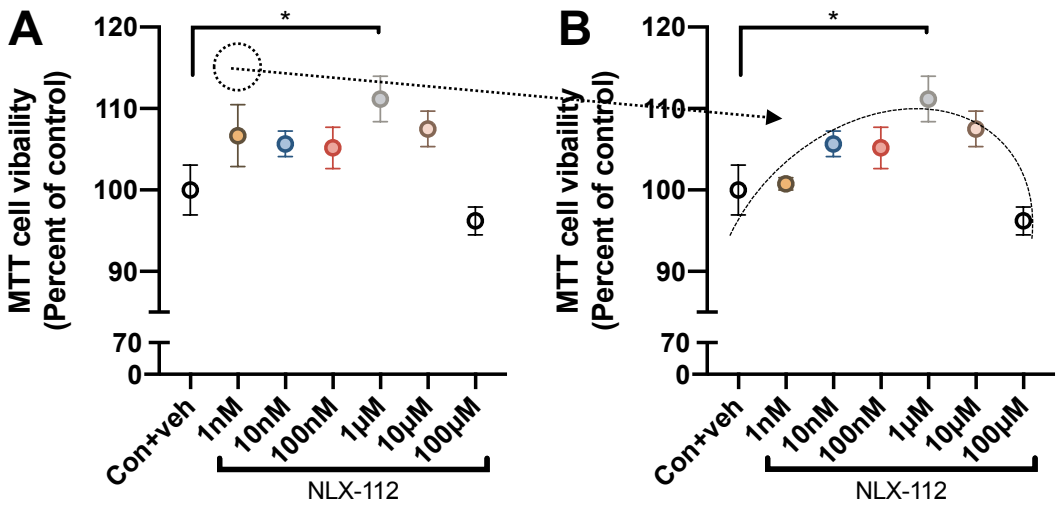


Figure 19. A) Dose-toxicity assay. SH-SY5Y cells were treated with NLX-112 at concentrations from 1nM to 100μM. B) furthest data point from the mean removed in 1nM reveal dose-cell proliferation curve. n = 6, errors bars denote standard error of the mean (SEM). One way ANOVA fowlled by Bonfferoni's multiple comparsion test. *p<0.05.

Inhibition of mitochondrial respiration in SH-SY5Y cells, in order to determine the protective properties of NLX-112, was investigated using MPP^+, and quantified two ways; the first, was carried out using the MTT assay which uncovered that NLX-112 induced modest, but significant cellular protection at 10nM (+5%), 100nm (+10%), and 1uM (+5%) in SH-SY5Y cells that had been treated 5 hours after MPP^+ toxicity had been induced [F(11.61 (3, 8) = 0.9493 p = 0.0027] (Figure 20 A).

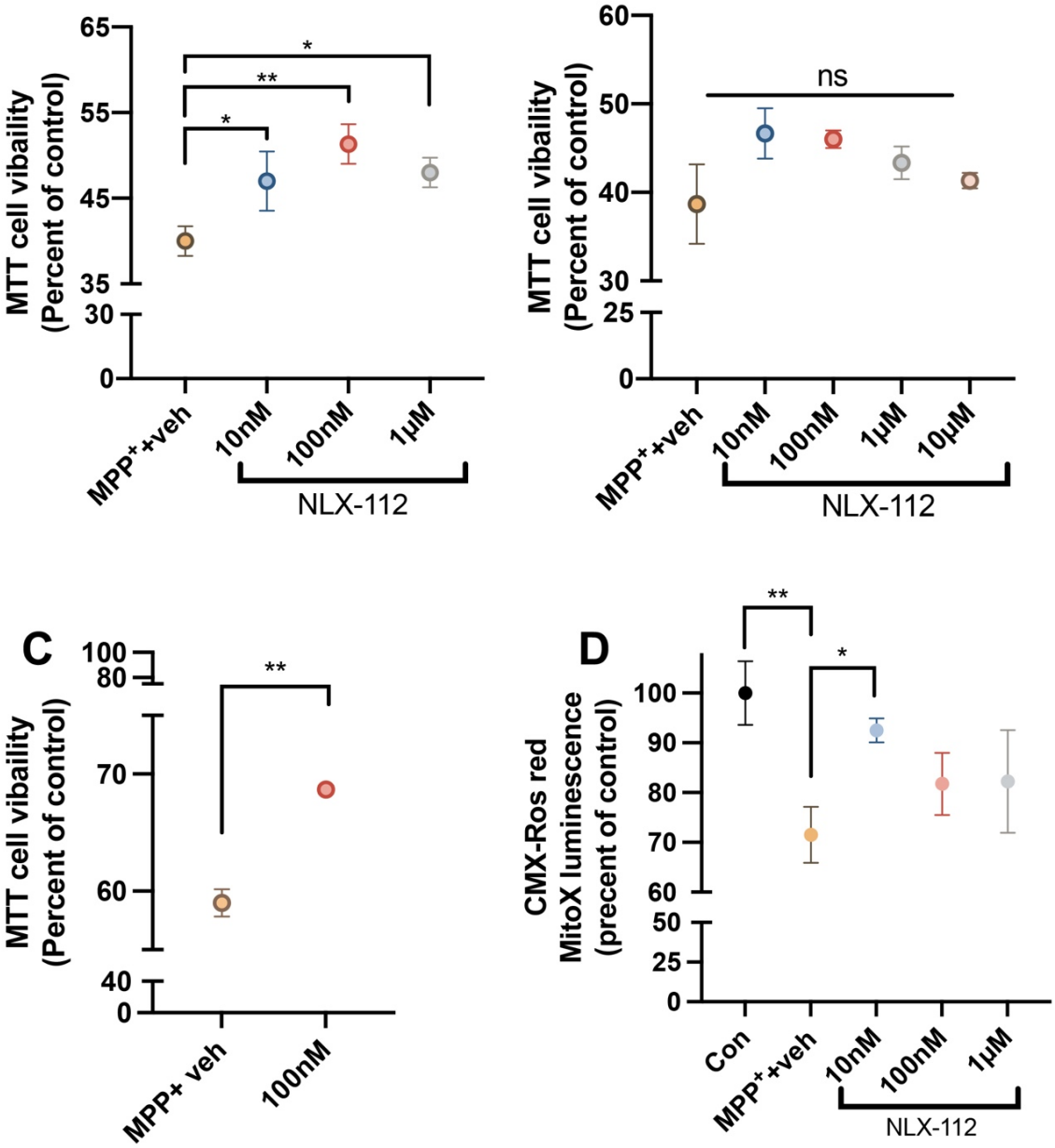


Figure 20. NLX-112 protection against MPP^+. SH-SY5Y cells were seeded at 50k cells/well in a 96 well plate and cultured for 24 hours. A) NLX-112 post-protection. SH-SY5Y cells were treated with MPP^+ (1mM), and 5 hrs later treated with NLX-112 at concentrations from 10nM to 1µM and incubated for a further 24 hrs at 37°C. B) NLX-112 pre-protection. SH-SY5Y cells were treated with NLX-112 at concentrations from 10nM to 1µM and 5 hrs later treated with MPP^+ (1mM), and incubated for a further 24 hrs at 37°C. C) Repeat of the post-protection assay at 100nM only. D) CMXRos Red assay. After 24 hours incubation, cells were treated with CMXRos red at 170nM concentration in serum free media. Error bars denote standard error of the mean (SEM). One way ANOVA followed by Fishers LSD test for differences. n= 3-6 *$p<0.05$, **$p<0.005$.

A reversal of this experiment was performed whereby SH-SY5Y cells were treated with NLX-112 and 5 hours later treated with MPP^+ and left for 24hrs until MTT assay. This chronological series of steps in administering NLX-112 then MPP^+ did not result in protection of SH-SY5Y cells [F(1.641 (4, 10) = 0.6162 p = 0.2391] (Figure 20 B). The second method for quantifying MPP^+ toxicity was carried out using the CMXrosRed Mitotracker which gives an indirect measure of mitochondrial polarisation and overall mitochondrial health. This assay was performed following the NLX-112 post protection protocol, as it had been the protocol to previously garner a successful result. CMXrosRed assay revealed NLX-112 to have a positive outcome on mitochondrial health at 10nM concentration, but not 100nM or 1μM [F(2.672 (4, 15) = 2.149 p = 0.0729] (Figure 20 D). The previous assays assessed NLX-112's protective property toward SH-SY5Y cells when mitochondrial respiration was severely compromised by the mitochondrial inhibitor MPP^+.

Mitochondrial dysfunction is but one of many pathways known to be key in accelerating the demise of DAergic neurons in PD pathology. Proteasome inhibition leading to the toxic build-up of un-degraded proteins is another pathway also known to be involved in DAergic cell death. MG132 administration on SH-SY5Y cells can offer some insight into whether NLX-112 exerts its property at the cytosolic proteasome complex. To determine if NLX-112 could inhibit or protect against proteasome inhibition, several neuroprotective assays using NLX-112, and the proteasome inhibitor MG132, were carried out. Treating cells with MG132, 5 hours later with NLX-112 resulted in modest but still significant neuroprotection when asessed 24hrs later using the MTT assay [F(4.681 (5, 29) = 0.2086 p = 0.0030]. (Figures 21 A, C, E and 22).

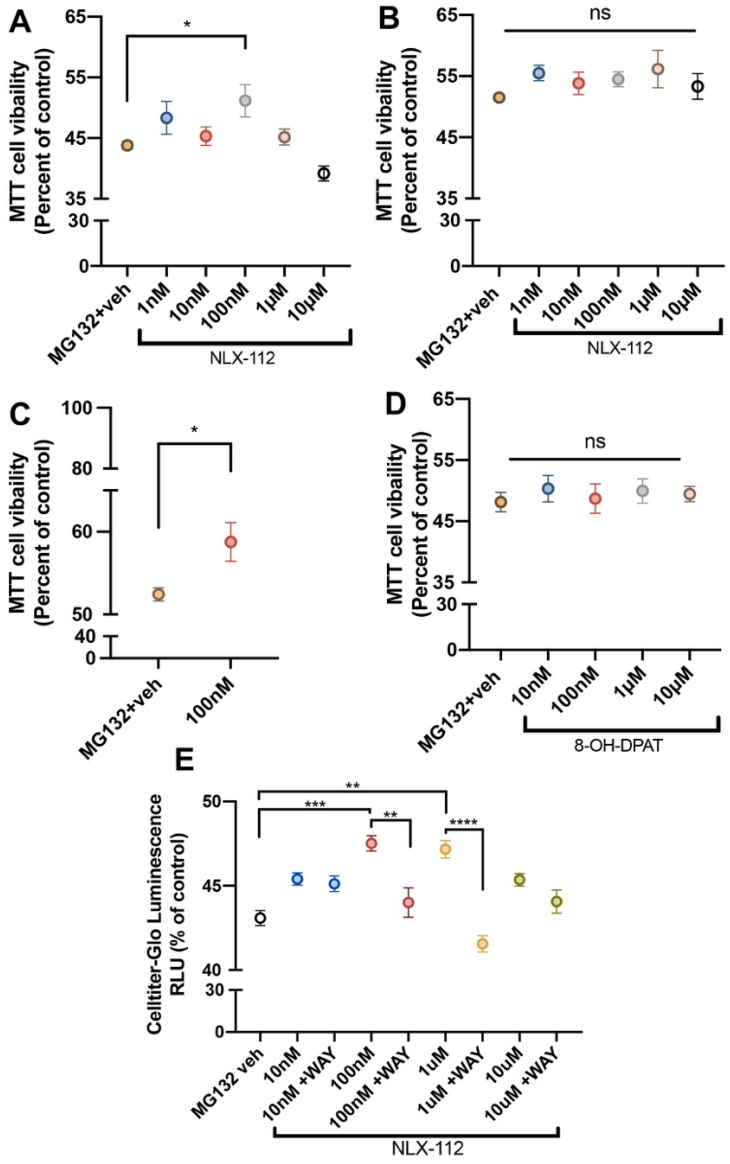


Figure 21. NLX-112 protection against MG132. SH-SY5Y cells were seeded at 50k cells/well in a 96 well plate and cultured for 24 hours. A) NLX-112 post-protection. SH-SY5Y cells were treated with MG132 (2.5μM), and 5 hrs later treated with NLX-112 at concentrations from 1nM to 10μM and incubated for a further 24 hrs at 37°C. B) NLX-112 pre-protection. SH-SY5Y cells were treated with NLX-112 at concentrations from 1nM to 10μM and 5 hrs later treated with MG132 (2.5μM), and incubated for a further 24 hrs at 37°C. C) Repeat of the post-protection assay at 100nM only. D) 8-OH-DPAT post-protection. SH-SY5Y cells were treated with MG132 (2.5μM), and 5 hrs later treated with 8-OH-DPAT at concentrations from 10nM to 10μM and incubated for a further 24 hrs at 37°C. E) Blocking of NLX-112 with WAY100635 measured by total ATP content. Cells were treated with MG132 then 2 hours later treated with WAY100635 (300nM) followed by NLX-112 treatment 1 hour later at concentrations ranging from 10nM to 10μM. Error bars denote standard error of the mean (SEM). One way ANOVA followed by Fishers LSD test for differences. n= 3-6 *p<0.05, **p<0.005., ***p<0.0005, ****p<0.0001

As with the MPP^+ assay's, seeking to determine whether NLX-112 had 'pre-protective property', this was also shown not to be case when NLX-112 had been pre-incubated on SH-SY5Y cells 5 hours prior to MG132 treatment [F(0.6300 (5, 28) = 0.8730 p = 0.6784] (Figure 21 B and 23).

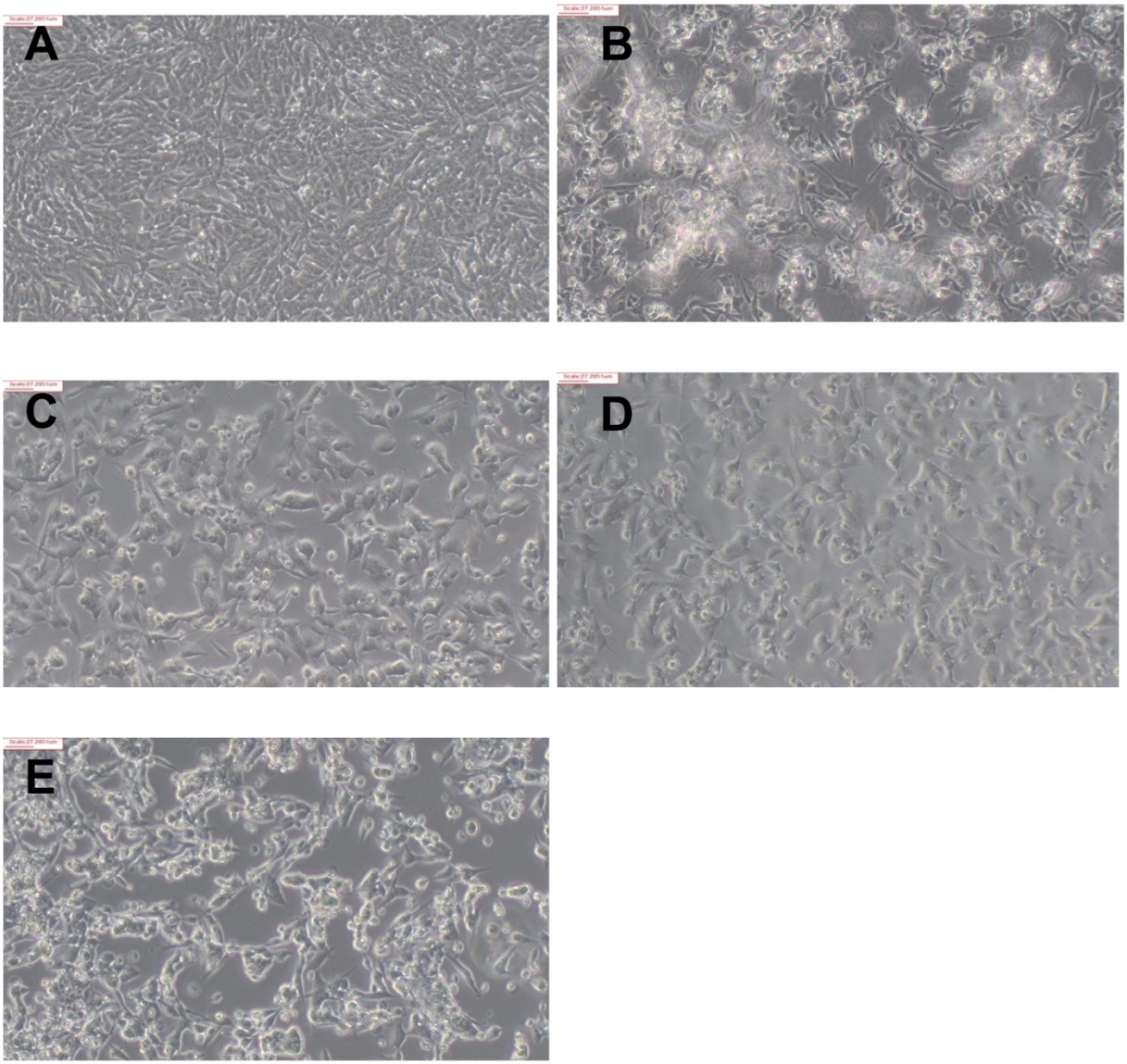


Figure 22. Representative images of SH-SY5Y cells pre-treated with NLX-112 at concentrations ranging from 10nM to 1μM for 5 hours, followed by treatment with the proteasome inhibitor MG132 at 2.5μM for 24 hours. In panel A, cells treated with vehicle only appear healthy, serving as the control. Panel B shows cells exposed to MG132 alone, demonstrating notable toxicity and morphological changes. Panel C, D, and E display cells pre-treated with NLX-112 at 10nM, 100nM, and 1μM, respectively, revealing a dose-dependent protective effect against MG132-induced damage. Images were taken 24 hours after MG132 exposure.

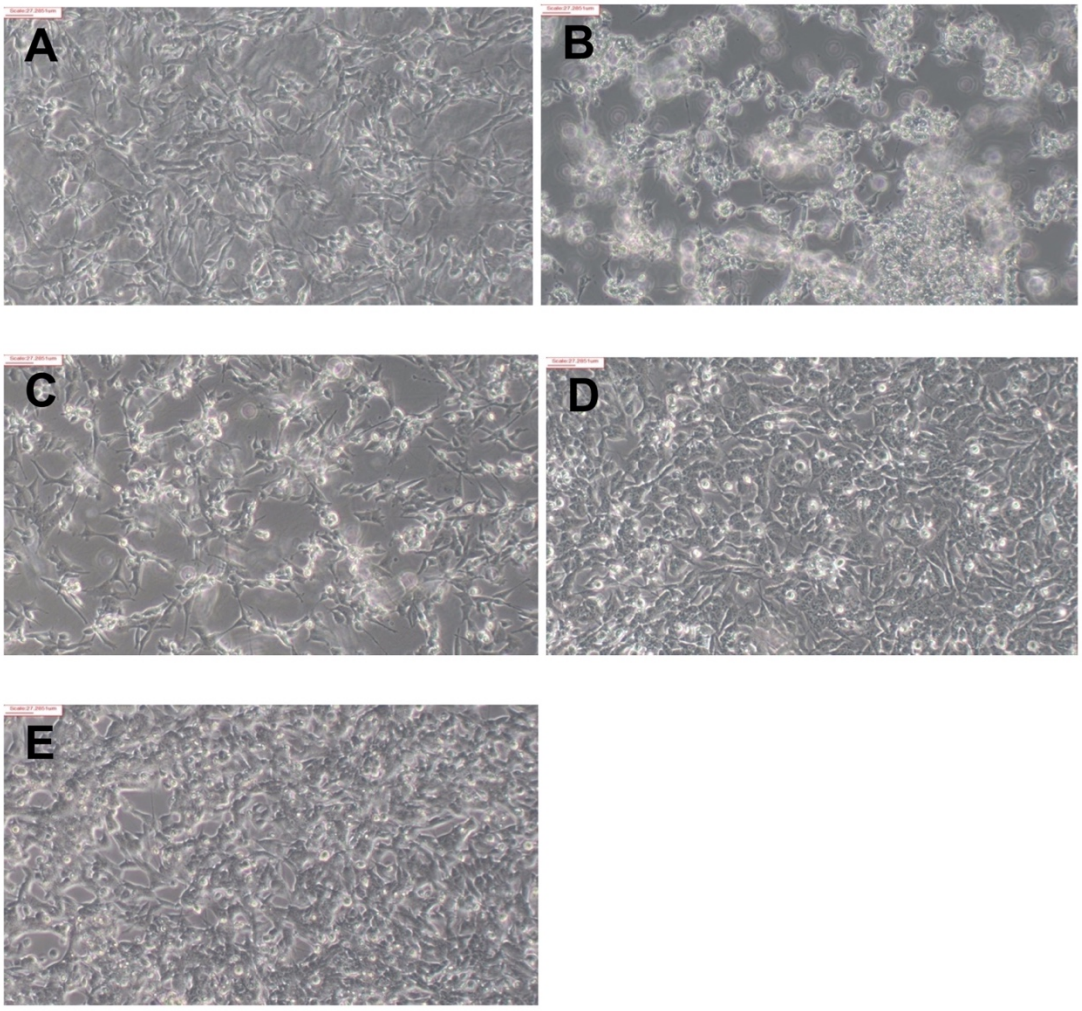


Figure 23. Representitive images of SH-SY5Y cells that were pre-treated with MG132 (2.5μM) for 5 hours, followed by treatment with incremental concentrations of NLX-112, ranging from 10nM to 1μM, for a 24-hour period. Group A, which was treated with vehicle only, served as the control; group B received MG132 treatment only, without subsequent NLX-112 exposure; group C was treated with 10nM NLX-112; group D was treated with 100nM NLX-112; and group E was treated with 1μM NLX-112. Images were taken 24 hours after the NLX-112 treatment.

At 100nM and 1μM, NLX-112's selective property for 5-HT_{1A} was blocked using the 5-HT_{1A} antagonist WAY100635, indicating that its post-protective property in SH-SY5Y cells is reliant on its selective binding for 5-HT_{1A} remaining functionally available [$F(12.23\ (8, 50) = 0.5120\ p < 0.0001$] (Figure 21 E). This was investigated further using the relatively selective 5-HT_{1A} agonist, 8-OH-DPAT as a positive control, however, from 10nM up to 10μM, and in contrast to the results observed with NLX-112 treatment, post-protection against MG132 toxicity was not observed with 8-OH-DPAT treatment [$F(0.1660\ (4, 22) = 0.4464\ p = 0.9534$] (Figure 21 D). To further confirm that NLX-112 was exhibiting neuroprotection after cellular necrosis had been induced using MPP^+ or MG132, the experiments were repeated

again but only using the most effective dose of NLX-112 which was recurrently found to be a concentration of 100nM. Indeed, this dose was again shown to have neuroprotective properties toward SH-SY5Y cells after 5 hours post toxic insult, which was also the case for MPP^+ induced toxicity [t(8.043 (4) p = 0.0013] (Figure 20 C) as well as MG132 toxicity [t(2.570 (4) p = 0.0310] (Figure 21 C).

3.4. Discussion

3.4.1. DMSO vehicle is not confounding experimental outcomes

Zhang et al., (2017) deduce from their experiments with rat primary mixed neuronal cell cultures that DMSO treatment of less than 0.25% obviates any spurious vehicle effects. Given that neuroblastoma cell lines are arguably much more robust that primary cell cultures, and that all NLX-112 neuroprotective assays were carried out using concentrations ranging from 1nM to 1uM, equivalent to diluted concentrations of 0.00001% to 0.01% DMSO, it is therefore likely that DMSO as a vehicle for NLX-112, will have little to no effect in interfering with the outcome of the neuroprotective assays.

3.4.2. NLX-112 induces cellular proliferation in untreated SH-SY5Y cells

A remarkable finding from this in vitro investigation was uncovered in the NLX-112 dose toxicity assays whereby NLX-112 treatment alone appeared to induce cellular proliferation of the SH-SY5Y cells, at a concentration of approximately 1μM (Figure 19 A and B). The cellular proliferative property of NLX-112 seemed to follow a concentration curve starting with little to no effect at 1nM and 10nM, and the mean number of cells, compared to control, climbing higher as the concentration was increased; peaking at 1uM (+10%) and reducing again when moving towards 100μM; a molecular concentration almost certainly as having a deleterious effect on cell viability. The proliferative response of the SH-SY5Y cells to NLX-112 treatment suggests NLX-112 may comprise neurotrophic properties, a finding which appears to be first of its kind.

3.4.3. NLX-112 is protective after MG132/MPP^+ toxicity, but not before

Pre-incubation with a range of NLX-112 concentrations for 5 hours appears to have had no effect on cellular protection upon a further 24 hours of MG132 (Figure 22)

or MPP^+ incubation. However, 5 hours of pre-incubation with MG132 (Figure 23) or MPP^+ prior to treatment with NLX-112 presents a different picture. Protection with NLX-112 follows a dose-dependent curve which becomes significantly greater than toxic compound treatment only at 100nM of NLX-112 treatment. When NLX-112 is assessed against MPP^+ toxicity it appears to have greater levels of protection (+10%) than when it is assessed against MG132 toxicity (+6%). This is also reflected by the CMXRosRed assay which shows a 21% increase mitochondrial polarisation in cells that have been treated with 10nM of NLX-112 compared to MPP^+ treatment alone.

3.4.4. NLX-112 acts via 5-HT_{1A} on SH-SY5Y cells

Confirmation of whether NLX-112's protective effects were regulated through activation of the 5-HT_{1A} receptor were further confirmed by blocking its activity with the 5-HT_{1A} antagonist WAY100635 (300nM). Further investigation looked to determine whether these findings could be replicated in the same model using the 5-HT_{1A} agonist 8-OH-DPAT, but was found to have no protective effect toward SH-SY5Y cells pre-treated with MG132, which is surprising because Levtit et al., (2001) found that 8-OH-DPAT prevents adenyl cyclase inhibition in SH-SY5Y cells. The difference between the two 5-HT_{1A} agonists may be due to NLX-112 having greater total G protein activation compared to 8-OH-DPAT (Newman-Tancredi et al., 2022), which may explain why NLX-112 does, but 8-OH-DPAT does not exhibit protection against MG132 induced neurotoxicity. Moreover, this supposition is supported by the fact that 8-OH-DPAT has activity at SERT which is likely further inhibiting excitation of the SH-SY5Y cell (Larsson et al., 1990; Sprouse et al., 2004).

3.4.5. NLX-112: a possible free radical scavenger

Another possible mechanism through which NLX-112 may exert its protective properties is by scavenging free radicals. Both MG132 and MPP^+ are known to trigger the accumulation of oxygen-derived free radicals, such as, nitric oxide, superoxide anion (O_2^{*-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (*OH). The inhibition of free radical formation can be achieved through various direct or in-direct mechanisms, including the removal of oxidising species, the inhibition of

oxidising radical formation, or the suppression of lipid peroxidase propagation. From a chemical perspective, an antioxidant supplemented from an endogenous ligand can prevent the loss of an electron from another compound, thereby halting the deleterious effects of free radicals. Investigating how NLX-112 may clear or inhibit the accumulation of toxic free radicals will undoubtedly require further research. Such theories could explain why Pramipexole, a D2 agonist, is able to halt cell death not only in SH-SY5Y cells but also in non-DAergic JK cells. This could be due to Pramipexole possessing antioxidant properties, as it has previously been found to reduce hydrogen peroxide toxicity in MES cells through free radical scavenging (Zou et al., 1999) and decrease superoxide production in SH-SY5Y cells (Cassarino et al., 1998).

3.5. Summary and conclusion

These results are somewhat conflicting, NLX-112 protects against MPP^+ and MG1342 toxicity, and its protective effects are blocked by the antagonist WAY100635. But protection is not observed with the positive control assay using 8-OH-DPAT. A distinguishing functional difference between NLX-112 and 8-OH-DPAT is that NLX-112 has a Ca^{2+} mobilisation Emax equal to 5-HT, whereas 8-OH-DPAT Ca^{2+} Emax is only half of that (Newman-Tancredi et al., 2022). NLX-112 could be exerting its neuroprotective effects through the efficient mobilisation of Ca^{2+} signalling. If NLX-112 is exerting its protective effects via Ca^{2+} signalling this could be very critical considering that intracellular free calcium is an important second messenger that regulates complex neuronal signalling. The utility of Ca^{2+} as key functional component of cell survival has been previously examined for its neuroprotective properties. For example, neuroprotection of midbrain DA neurons by nicotine is only achieved when Ca^{2+} levels are chronically elevated by concurrent depolarisation (Toulorge et al., 2011). And long-term neuroprotection of hippocampal neurons is still achieved even when synaptic activity has ceased but remains dependent on nuclear calcium signalling, as opposed to short-term evoked neuroprotection which is achieved solely by activation of the phosphatidylinositol 3-kinase/Akt pathway (Papadia et al., 2005). Moreover, dysregulation of Ca^{2+} is known to be associated with increased α-synuclein aggregation (Suremeier et al., 2016).

Further in vitro investigations should be conducted to ascertain the extent of NLX-112's neuroprotective properties via the pathways mentioned: as a free radical scavenger; a facilitator of Ca2+ mobilisation; and/or an agonist at 5-HT_{1A} receptors.

Chapter 4

The effect of NLX-112 on motor activity and behaviour in middle aged C57b/6J mice

4.1. Introduction

Anxiety and depression represent some of the commonest psychiatric ailments in the world and drugs used to treat these disorders are among the most widely prescribed. Anxiety and depression also represent important co-morbidities with several neurodegenerative disorders such as Alzheimer's disease and PD amongst others (Menza et al. 1993; Teri et al. 1999). Not only has NLX-112 had success in alleviating LID in human PD patients, but is has also been shown pre-clinically to have robust affective, analgesic and behavioural modulating properties (Iderberg et al., 2015; Newman-Tancredi et al., 2018; Peeters et al., 2019; Depoortere et al., 2021). This chapter will therefore address an important aspect of NLX-112's therapeutic properties; whether it is able to offset the trait level of anxiety-like behaviour in mice. The primary aim of this thesis is to determine whether NLX-112 is neuroprotective. If it is also found to alleviate significant co-morbid non-motor symptoms of PD, such as anxiety, the therapeutic value of NLX-112 would be further enhanced.

4.2. 5-HT_{1A} and anxiety

There is now a considerable body of evidence indicating the importance of 5-HT_{1A} receptors in anxiety (Akimova et al. 2009). Although the role of 5-HT_{1A} receptors in anxiety has been well established, the majority of supporting data has been provided by experiments using relatively non-selective 5-HT_{1A} receptor agonists such as buspirone and aripiprazole, amongst other ligands (Buitelaar and van der Hoeven 1998; Pae et al. 2008). As well as being a partial 5-HT_{1A} agonist, buspirone, which has been used as an anxiolytic agent, also acts as an antagonist on DA D_2, D_3 and D_4 receptors (Bergman et al. 2013), and like tandospirone, gepirone and ipsapirone, it is metabolised to the α_2-adrenergic receptor antagonist, 1-(2-pyrimidinyl)- piperazine (1-PP) (Bianchi et al. 1988; Gobert et al. 1999). Therefore, at least some of the observed results of these agents may be attributed to effects on DAergic or noradrenergic pathways. In contrast, and already mentioned throughout this thesis, NLX-112 is a 5-HT_{1A} agonist with nanomolar affinity; it is a biased agonist which is a preferential Gαo activator (Colpaert et al. 2002; Newman-Tancredi et al. 2017, 2019). NLX-112 is exceptionally selective for 5-HT_{1A} receptors, and, even at micromolar concentrations, it does not bind to other

monoaminergic, opioid or GABAergic receptors (Colpaert et al. 2002). Moreover, contrary to buspirone-like compounds, NLX-112 does not produce pharmacologically active metabolites (unpublished observations). Substantial pre-clinical evidence has demonstrated NLX-112 to be highly efficacious in negating levodopa-induced abnormal involuntary movements (AIMS) in rats and non-human primates (marmosets and macaques) (Iderberg et al. 2015, McCreary et al. 2016; Depoortere et al. 2020; Fisher et al. 2020), research which ultimately paved the way toward its successful Ph2a trial. However, limited information is available concerning its potential anxiolytic properties which are also relevant to PD because mood deficits, and particularly anxiety disorders, affect those with PD at a much higher rate than the general population (Richard et al. 2001; Dubovický et al. 1999; Walsh and Bennett 2001).

The aim of the study reported in this chapter was to therefore investigate the effect of acute systemic treatment with NLX-112 at different doses in in the open field test (OFT) and the elevated plus maze (EPM) in order to assess its potential anxiolytic properties. Importantly, since anxiety is a prominent non-motor feature of PD, this study was carried out using middle-aged C57b/6J mice.

The true potential of an anxiolytic drug on mammalian behaviour has to, ideally, be found consistently and repeatedly efficacious, regardless of the environment. A notable flaw in the modelling of anxiety in rodent maze paradigms is the occurrence of “one-trial tolerance”, in that the rodent will be receptive to the novelty of the maze environment once only, and that any attempts to reintroduce the rodent to the same maze will result in an apathetic or learned response (Walsh and Cummins, 1976). However, the majority of studies that have observed this phenomenon have predominantly used benzodiazepines (File et al., 1993; File and Zangrossi, 1993; Rodgers and Shepard, 1993; Treit et al., 1993). In contrast, in a second trial of the EPM, rats treated with the 5-HT_{1A} agonist 8-OH-DPAT had reduced anxiety-like behaviour (File and Gonzalez, 1996). Further still, previous treatment with buspirone supresses the one-trial tolerance in rats treated with chlordiazepoxide in the EPM also (Escarbajal et al., 2003). Considering that NLX-112 is a highly potent and selective 5-HT_{1A} agonist, this experiment therefore

subjected the same group of mice to three different doses of NLX-112 in order to determine whether NLX-112 negates the one-trial tolerance phenomenon, but also to determine which dose of NLX-112 is the most efficacious for inducing anxiolytic behaviour.

4.3. Method

4.3.1. Animals

The experiments reported herein complied with the ARRIVE guidelines and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments. All procedures and protocols were approved by the Animal Welfare and Ethical Review Board (AWERB) at the University of Hertfordshire, which were carried out under the UK Home Office approved Project Licence PD7255B4. Young adult C57b/6J male mice were purchased from Charles River Laboratories (UK) and kept until maturity (~ 6 months; 30–40 g). Room temperature was maintained at 22–24 °C, with an automated 12-h (light):12-h (dark) cycle. All experiments were carried out between 13:00 and 19:00 h at the ambient light intensity of 100 Lux at the normal light cycle.

4.3.2. Apparatus

For full details of apparatus setup, see Chapter 2.

4.3.3. The open field test

Nine mice were subjected to the OFT four times each, for 15 min per condition. Mice were divided into trials with each mouse of a particular group in each trial receiving either vehicle or NLX-112 at 0.1, 0.3 or 1 mg/kg subcutaneous (s.c.) on test days. A randomised dosing design was adopted so that by the end of the trials all animals received all four treatments, vehicle and 3 drug doses, with 48 h between each test day. In this study all animals underwent 12 different trials at different dates. To determine the extent of thigmotaxis a30-cm central zone that sat inside the centre of the arena was digitally created with the Ethovision-XT software, thus allowing a 5-cm- wide channel to run around the perimeter of the OFT. The following parameters were measured electronically using the Ethovision-

XT software: total distance travelled (cm); distance travelled, duration (time spent in s) and mean velocity (cm/s) in the centre area or perimeter; and number of entries in and latency time to enter the central area.

4.3.4. The elevated plus maze

Mice that had been assessed in the OFT previously were then subjected to the EPM and were each treated with vehicle, 0.1 or 0.3 mg/kg of NLX-112 in a cross-over design, with each test lasting for 15 min. Immediately after receiving either vehicle (s.c.) or NLX-112 (s.c.), mice were placed onto the centre square of the EPM facing toward the open arms. To determine when mice crossed into the different arms of the EPM, five separate zones were digitally created: two for the closed arms, two for the open arms and one for the centre square where each four arms cross. Duration (time spent in s) in the open arms, closed arms and centre square and entries into the open arms and closed arms were calculated using the Ethovision-XT software.

4.3.5. Drugs

NLX-112((3-chloro-4-fluorophenyl-[4-fluoro-4-([(5-meth-ylpyridin-2-yl) methylamino] methyl) piperidin-1-yl] methanone, fumarate salt) was provided by Neurolixis SAS (Castres, France). Freshly made solution of NLX-112 dissolved in 10% DMSO was diluted in saline to produce 1.0 mg/mL stock in saline, and this was further diluted in saline for preparation of 0.1, 0.3 and 1.0 mg/kg NLX-112. For the final DMSO content, a DMSO:saline ratio of 1:10 was used which was further diluted down with saline to make a final working concentration depending on the dose each mouse received. For those receiving 1 mg/kg, the final DMSO concentration was 0.18%, for 0.3 mg/kg, it was 0.054%, and for 0.1 mg/kg, it was 0.018%. There was at least a 48-h wash-out period between each dose of NLX-112 or vehicle.

4.3.6. Statistical analysis

Each parameter for the OFT and the EPM was analysed using a mixed effects model one-way ANOVA for repeated measures followed when appropriate with post-hoc Fishers LSD tests. Differences between group means were considered

statistically significant when $p < 0.05$. Statistical analyses were carried out using the GraphPad Prism v8.

4.4. Results

At all doses, NLX-112 reduced thigmotaxis and increased locomotor activity in the centre area in the OFT. In contrast, vehicle treated mice displayed extensive thigmotaxis, remaining mainly close to the perimeter wall and rarely venturing out into the centre.

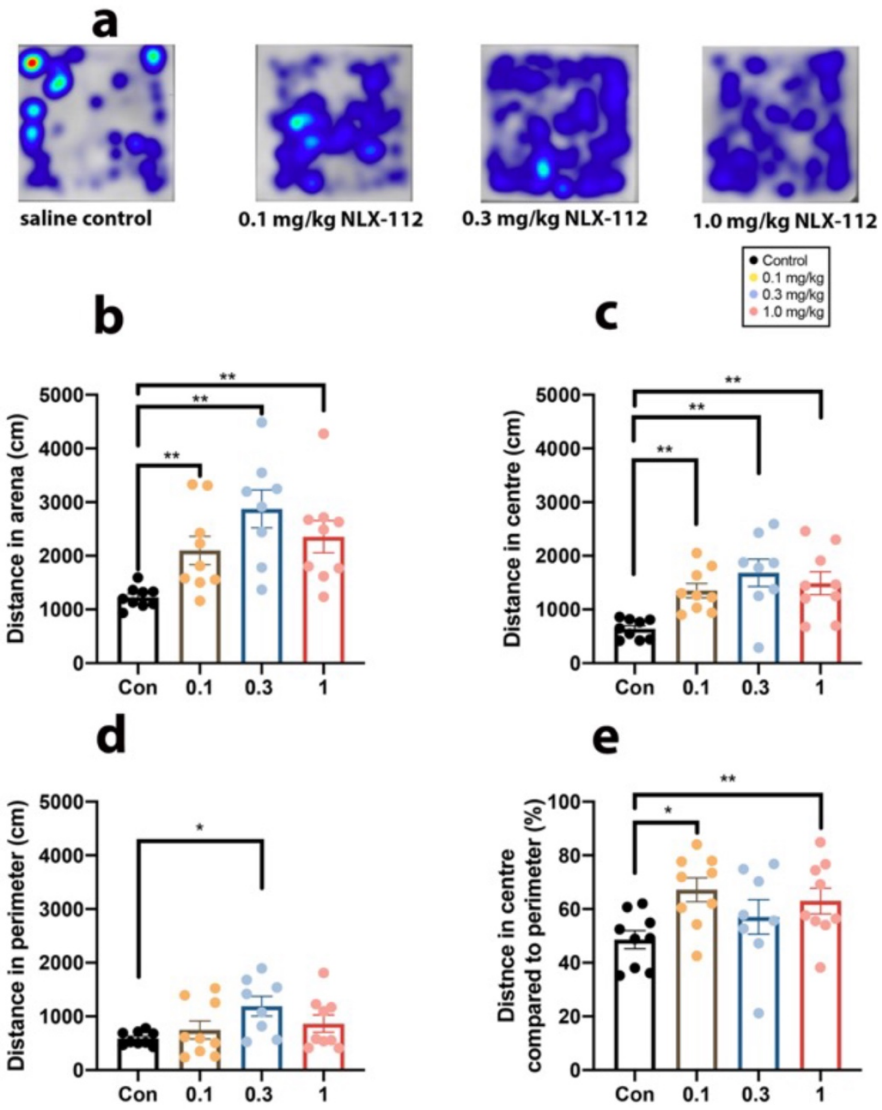


Figure 24. Effects of NLX-112 on distances travelled in the different compartments of the OFT. A) Representative heat tracking maps of mice treated with either vehicle control or NLX-112. B) Total distance travelled in the arena. C) Distance travelled in the centre of the arena. D) Distance travelled in the perimeter of the arena. E) Distance travelled in the centre compared to the

distance travelled in the perimeter of the arena (%). n = 9 mice per group. Results are presented as individual values (dots) and as mean ± SEM (bars), *p < 0.05, **p < 0.01, as compared to control (Con), Fishers LSD post-hoc test following significant mixed effects model one-way ANOVA for repeated measures

Administration of NLX-112 increased the distribution of movement more evenly throughout the open field arena with a dose-dependent trend (Figure 24). More specifically, NLX-112 significantly increased the total distance travelled overall throughout the arena compared to the vehicle control [$F(2.204, 16.90) = 8.815$ $p < 0.005$]. The distance travelled in the centre area increased significantly for all three doses [$F(2.145, 16.44) = 7.230$ $p < 0.005$] (Figure 24 C and 27, 28), with peak increase (164% above control level) observed at 0.3 mg/kg, while the distance travelled in the perimeter increased significantly only at 0.3 mg/kg [$F(2.191, 16.79) = 4.421$ $p < 0.05$] (Figure 24 D and 27, 28). Comparison of the ratio of the distance travelled in the perimeter versus the centre of the arena showed mice treated with 0.1 and 1.0 mg/kg (but not 0.3 mg/kg) NLX- 112 travelled further in the centre of the open field [$F(2.084, 15.98) = 3.781$ $p < 0.05$] and displayed reduced thigmotaxis (Figure 24 E and 27, 28). There was also significant and seemingly dose- related increases in the average velocity in the centre of the arena [$F(2.126, 16.30) = 9.729$ $p < 0.005$] (Figure 25 A) following NLX-112, but not in the perimeter [$F(1.440, 11.04) = 0.968$ $p = 0.381$] (Figure 25 B). This was also reflected in the finding that the actual time spent in the perimeter and in the centre was not increased by NLX-112, save only at the low (0.1 mg/kg) dose. Furthermore, the latency time to enter the centre of the arena was significantly decreased [$F(1.932, 12.24) = 4.136$ $p < 0.05$] (Figure 25 F), and the number of entries in the centre of the open field arena was significantly increased [$F(1.636, 12.54) = 11.18$ $p < 0.005$] (Figure 25 G).

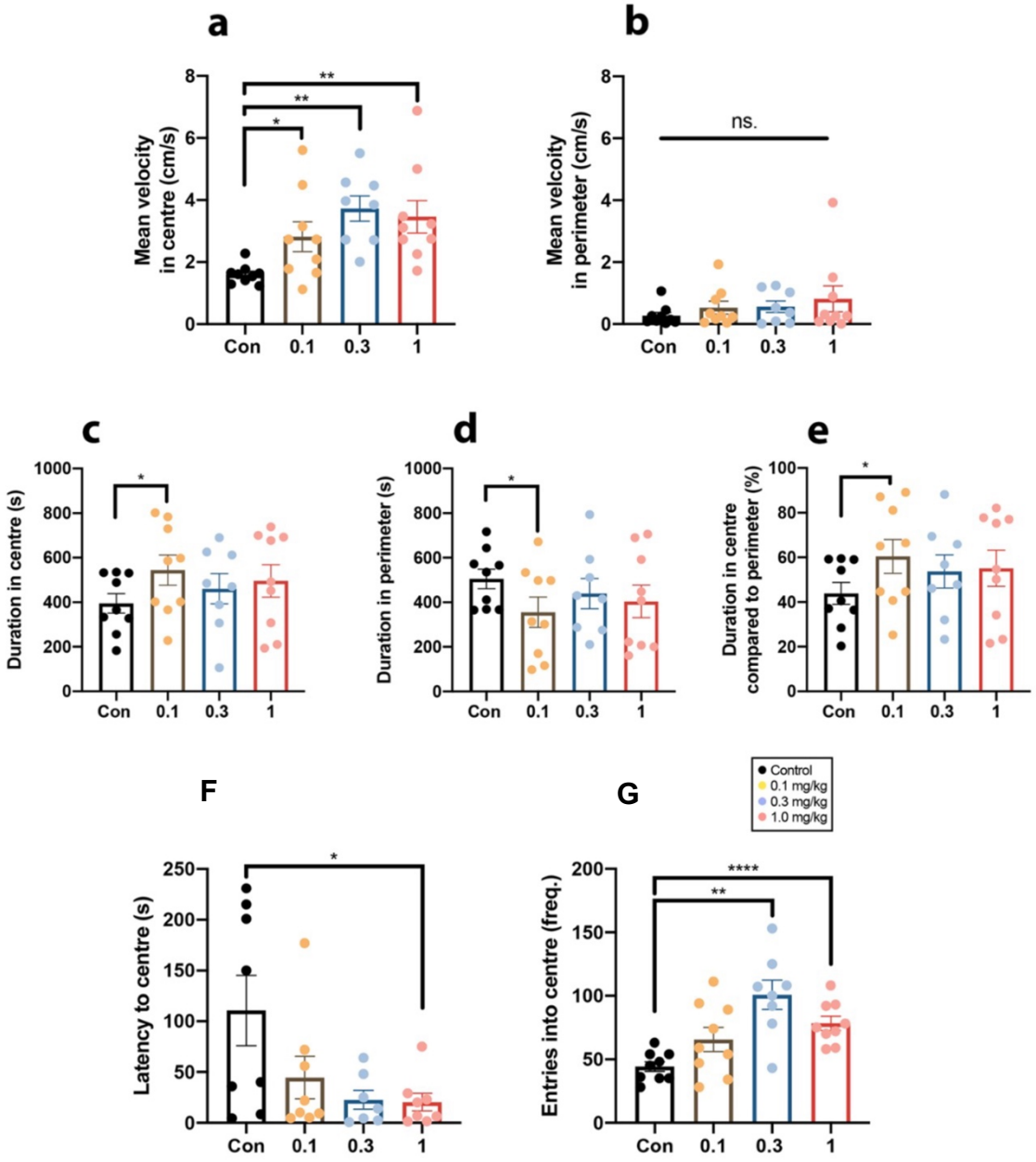


Figure 25. Effects of NLX-112 on additional parameters of the OFT. A) Average velocity in the centre of the arena. B) Average velocity in the perimeter of the arena. C) Duration (time spent in s) in the centre of the arena. D) The duration spent in the perimeter of the arena. E) The duration spent in the centre compared to the duration spent in the perimeter of the arena (%). F) The latency taken to move from the perimeter into the centre of the open field arena. G) The number of entries made into the centre of the arena from the perimeter. n = 9 mice per group. Results are presented as individual values (dots) and as mean ± SEM (bars). *p < 0.05, **p < 0.01, ****p < 0.0001, compared to vehicle control (Con), Fishers LSD post-hoc test following significant mixed effects model one-way ANOVA for repeated measures.

NLX-112 increases the percentage of time spent in the open arms in the EPM. Compared to vehicle treatment, NLX-112-treated mice dis- played a trend towards dose-dependent increase in motor activity in the open arms of the EPM as shown by the heat maps. The duration of time spent in the closed arms was significantly reduced [$F(1.372, 10.98) = 8.632$ $p < 0.005$] (Figure 26 B), while the time spent in the open arms was significantly increased [$F(1.273, 10.18) = 4.784$ $p < 0.05$] with peak increase (118% above control level) observed at 0.3 mg/kg (Figure 26 C). Furthermore, mice treated with NLX-112 spent significantly more time in the centre of the maze [$F(1.167, 9.338) = 6.488$ $p < 0.05$] (Figure 26 E), and when the duration of time spent in the open arms was compared to that in the closed arms, there was a significant increase as a percentage of time spent in the open arms also [$F(1.346, 10.77) = 5.110$ $p < 0.05$] (Figure 26 D). In mice treated with 0.1 mg/kg of NLX- 112, the frequency of entries into the open arms was almost doubled [$F(1.306, 10.450 = 7.927$ $p < 0.05$] (Figure 26 F), but no significant difference was seen in the frequency of entries into the closed arms. Subsequent comparison of the percentage of entries into the closed arms compared to the open arms was significantly decreased for both doses tested compared to vehicle-treated mice [$F(1.691, 13.53) = 8.295$ $p < 0.005$] (Figure 26 H).

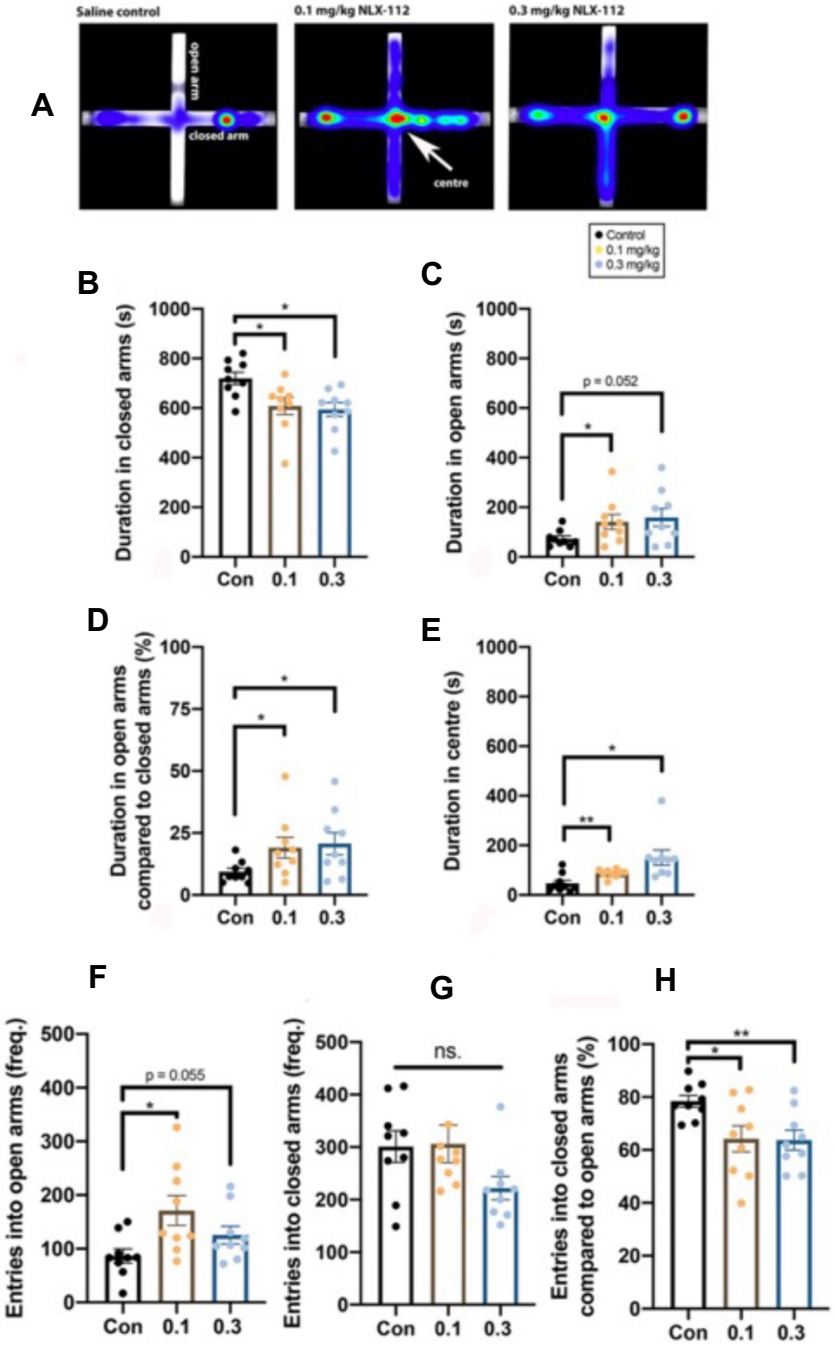


Figure 26. Effects of NLX-112 on time spent in diverse compartments of the EPM. A) Representative heat tracking maps of mice treated with either vehicle control or NLX-112. B) Time spent in the closed arms. C) Time spent in the open arms. d Time spent in the open arms compared to the closed arms (%). E) Time spent in the centre of the arms. *n*=9 mice per group. F) Number of entries into open arms. G) Number of entries into closed arms. h Number of entries into closed arms compared to open arms entries (%). Results are presented as individual values (dots) and as mean ± SEM (bars). **p* < 0.05, ***p* < 0.01 compared to control (Con), Fishers LSD post-hoc test following significant mixed effects model one-way ANOVA for repeated measures

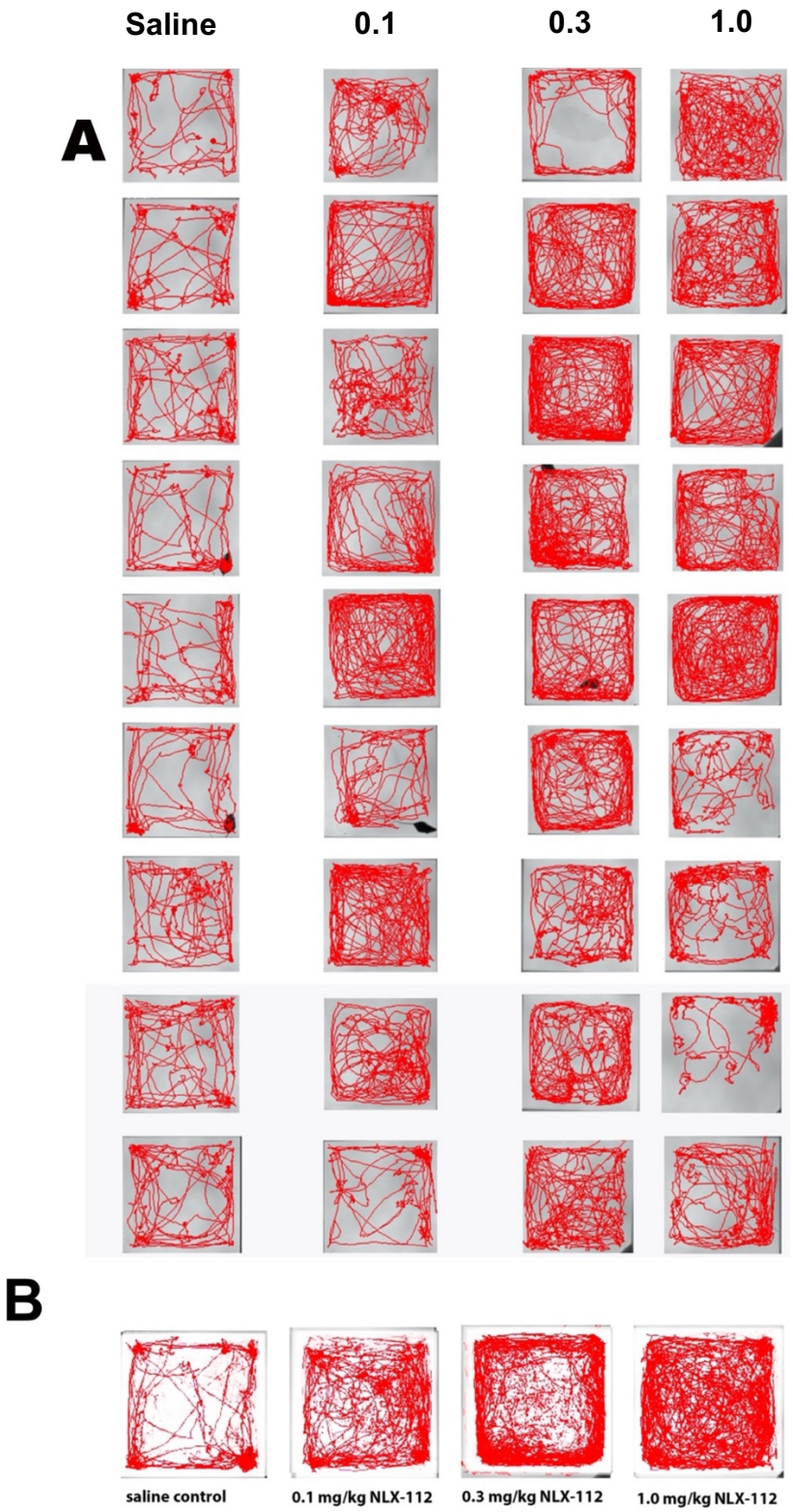


Figure 27. Panel A shows the tracking trail signature representing the distance traveled by mice throughout the open field arena over a 15-minute period. The figure highlights a notable difference between the saline treatment and all acute NLX-112 treatments (0.1, 0.3, and 1.0mg/kg), with the latter showing distinct variations in activity. Panel B presents the individual tracking signatures from each mouse, compiled into a single overlaid panel to provide a comparative view of the movement patterns across different treatments.

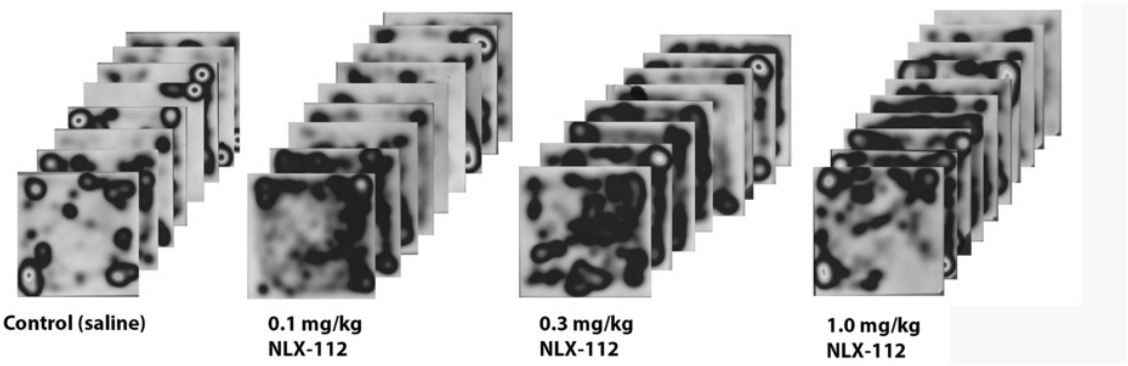


Figure 28. Overlaid heatmap images of mice in the OFT arena from 0-15 minutes. Distribution of heat signature is distinctly different between each of the conditions with the saline treated group clearly localising in the perimeter corners of the open field arena and 0.1 and 0.3mg/kg NLX-112 treated groups having a more evenly distributed movement pattern.

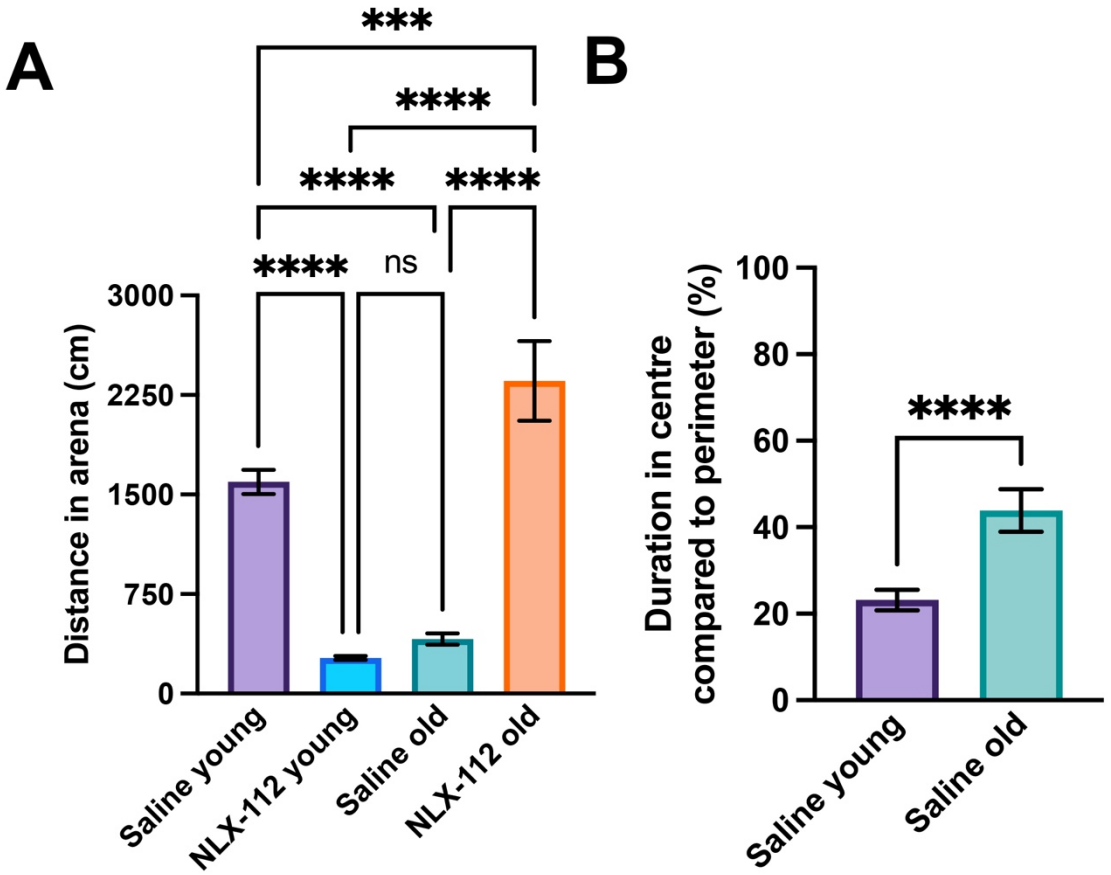


Figure 29. Compiled data from experiments in Chapter 5 and the current chapter, Chapter 4 of A) the distance travelled by saline and NLX-112 treated young and old mice and B) duration in the centre of the open field by saline treated young and old in the open field over 5 minutes. B) One-way ANOVA followed by Fishers LSD. $p < 0.05$, n= 9-24, error bars denote SEM.

4.5. Discussion

The present study is the first to report anxiolytic-like effects of acute treatment with NLX-112 in mice using two well established methods of anxiety assessment: the OFT and the EPM. There was a clear and distinct change in behaviour in a range of parameters measured in these experiments between mice treated with NLX-112 and those treated with vehicle, which corresponds to data from previous studies investigating 5-HT_{1A} agonists in murine models of anxiety (File and Gonzalez 1996; Vicente et al. 2008; Toth 2003).

The results of this study show that NLX-112 reduced thigmotaxis and increased locomotor activity in the centre of an open field arena. While there is some evidence for the role of 5-HT_{1B} agonists (Cheetham and Heal 1993) in increasing locomotor activity in mice, evidence for 5-HT_{1A} agonists is lacking. Indeed, in non-human primates, the reference 5-HT_{1A} agonist, 8-OH-DPAT (but not NLX-112), was shown to reduce locomotor activity (Iravani et al. 2006; Fisher et al. 2020). Therefore, the observed increase in the locomotor activity of mice by NLX-112, particularly in the centre of the open field arena, is highly suggestive of anxiolytic-like properties. Moreover, the present data on the elevated plus maze further supports this notion.

These results are somewhat similar to those of previous studies investigating the partial 5-HT_{1A} agonist buspirone which found reductions in thigmotaxis for both mice and rats in the OFT, but this was only achieved using higher doses of buspirone ranging from 2.5 to 15 mg/kg (Simon et al. 1994; Angrini et al. 1998). The same studies, however, observed an overall reduction in locomotor activity which is in marked contrast to the present investigation and likely due to buspirone's known DA D_2 receptor antagonist property (Ahlenius et al. 1993). In the EPM, NLX-112-treated mice spent longer time in the open arms and closed arms, entries were reduced relative to open arms entries, thus excluding locomotor activity as a factor of increased open arm duration which is consistent with the NLX-112-induced reduction of anxiety-like behaviour.

It has been shown that 5-HT_{1A} agonists are as effective as the prototypical benzodiazepine, diazepam, at reducing anxiety-like behaviours in the EPM

paradigm (Dunn et al. 1989). Prut and Belzung (2003) suggested that in approximately 68% of the studies that had used buspirone and 8-OH-DPAT (the prototypical 5-HT_{1A} receptor agonist), anxiolytic activity was demonstrated clearly compared to 63% with benzodiazepines. Moreover, the important role that the 5-HT_{1A} receptors play in modulating anxiety-like behaviour has been further confirmed in transgenic 5-HT_{1A} models where 5-HT_{1A} receptor knockout mice exhibited a greater level of thigmotaxis as well as marked deficits in exploratory behaviour in the OFT, EPM and elevated zero maze tests compared to wild-type controls (Heisler et al. 1998; Ramboz et al. 1998; Toth 2003).

The clinically approved non-benzodiazepine anxiolytic buspirone has supported the concept that the 5-HT_{1A} receptor is involved in modulating the affective states. However, it has not been fully established to what extent the anxiolytic benefits are attributed to 5-HT_{1A} mediated neurotransmission per se, as buspirone has been shown to bind (directly or indirectly via its metabolites) to a range of receptors including 5-HT_2, DAergic D_1 and D_2 as well as noradrenergic α and β receptor subtypes (Rodgers et al. 1994; Bonhaus et al. 1997) which have also been demonstrated to display anxiolytic properties (Azevedo et al. 2019; Mishra et al. 2019). As NLX-112 has been shown to be highly selective for the 5-HT_{1A} receptor with no known interaction for other monoaminergic, opioid or GABAergic receptors at the doses used (Colpaert et al. 2002), the data presented here suggests that its effects on anxiety-like behaviour in the C57b/6J middle-aged male mouse are indeed mediated exclusively by 5-HT_{1A} receptor mechanisms. To what extent these serotonergic mechanisms are presynaptic or postsynaptic is more difficult to ascertain due to the activity of NLX-112 being shown to occur in several cortical and midbrain regions (Levigoureux et al. 2018; Vidal et al. 2020). Indeed, the pre- and post-synaptic 5-HT_{1A}-mediated anxiolysis is com- plex as WAY-100135; a selective 5-HT_{1A} antagonist has been shown to reduce anxiety in the murine EPM (Rodgers and Cole 1994).

In an earlier study, a chemically related compound, F13714 with preferential biased agonist activity at somatodendritic 5-HT_{1A} receptors, potently and efficaciously reduced anxiety-related behaviour in a rat social interaction model (Depoortere et

al. 2019). Considering the wide body of evidence demonstrating that anxiety-like behaviours are mediated by 5-HT_{1A} neuronal presynaptic receptors in the RN (File and Gonzalez 1996; Cervo et al. 2000; Vicente et al. 2008; Akimova et al. 2009) and that activation of 5-HT_{1A} presynaptic receptors with NLX-112 in the rat induces complete inhibition of electrical activity in the RN (Lladó-Pelfort et al. 2012), it is likely that NLX- 112 is exerting its anxiolytic-like benefits via activation of presynaptic autoreceptors.

4.5.1. Comparison to young C57b/6J mice

Earlier generated data shows an acute dose of NLX-112 at 1mg/kg in young (12-week-old mice) has no effect at reducing basal levels of trait anxiety like-behaviour or increasing locomotor activity – in fact, it appears that at that given dose, young C57b/6J mice succumb to excess physiological 5-HT as shown by the marked levels of reduced locomotor activity (Figure 29 A and B). This is in stark contrast to what was observed in older C57b/6J mice as shown in the current experiment whereby 1.0mg/kg increases locomotor activity as exemplified through the various kinetic parameters including enhancements to velocity, entries into centre, distance travelled and reduced latency into the centre of the arena, when compared to vehicle treated mice.

The pharmacodynamic property of any drug can be very quickly assessed through one of the most basic behavioural assays using the open field. When mice are treated with vehicle or drug, the ensuing effects that they have on locomotor activity, and where that locomotor activity is localised, give an indication as to what neurological pathway(s) is (are) being targeted.

Not only does drug treatment with NLX-112 induce a change in locomotor activity compared to control, but that the effects are in fact reversed between young and old C57b/6J mice. Clues are therefore provided as to the property of NLX-112 in the young and old brain. Locomotor activity of saline treated mice shows that older C57b/6J mice are much less active than younger mice, an effect that knowingly translates to how energy levels are understood in younger and older humans, as well as most other mammalian species. By treating the two different age groups with NLX-112 it has been revealed that, as a result of potent biased agonist binding,

5-HT_{1A} receptors are dissimilarly equipped at facilitating 5-HT within the young and old brain.

Furthermore, middle-aged C57b/6J mice at 12 months of age exhibit significantly diminished levels of anxiety-like behaviour in contrast to their younger 3-month-old counterparts (Table 7, Li et al., 2015); a finding which is similarly observed from the research in this thesis and others (Shoji et al., 2016). This variation in affective behaviour is also shown to correspond to marked downregulation in the expression levels of 22 specific genes in the hippocampus of older mice compared to their younger counterparts. Among these genes are key regulators encompassing transcription and translation factors (Nfkb1, Fos, Creb1, and Fmr1), signal transduction associated genes (Ntrk2, Camk2a, Jak2, Prkca, and Gsk3b), genes associated with neuroplasticity (Arc, Shank1, Nlgn2, Homer1, Dlg4, and Ncam1) and those also associated with neurotransmission (Scg3, Syp, Snap25, Vamp1, Htr1d, and Grin1).

Interestingly, chronic administration of vortioxetine, a compound acting as a SERT inhibitor, 5-HT_{1D}, 5-HT_3, and 5-HT_7 antagonist, 5-HT_{1B} partial agonist, and a 5-HT_{1A} full agonist (Bang-Andersen et al., 2017), leads to an upregulation of several of the same genes in older mice, including Nfkb1, Fos, Fmr1, Arc, Shank1, Nlgn2, Camk2a, Rab3a, and DAT) (see Table 7), results which were not replicated in older C57b/6J mice treated with the SSRI fluoxetine. This suggests that vortioxetine's specific pharmacological properties, particularly its chronic engagement with other 5-HT receptors, including 5-HT_{1A} agonism (Li et al., 2015), but not its activity at SERT, potentially underlie its observed property for genetic upregulation. These findings add support to the notion that chronic NLX-112 treatment, leading to LTP, is having a positive effect on neural plasticity, possibly through the upregulation of important cell signalling and signal transduction pathways.

Table 7. Hippocampal gene expression levels are reduced in middle-aged mice. Hippocampal gene expression levels measured by qPCR and normalised using 3-month-old mice as 100. ↓: significant reduction. a* Indicates gene expression levels also changed through chronic treatment with vortioxetine. This table compares the expression levels of various genes and proteins involved in transcription, signal transduction, synaptic plasticity, and neurotransmission between young and middle-aged mice. The data shows a general decline in expression in middle-aged mice across many of these factors, with notable reductions in transcription factors, synaptic plasticity proteins, and neurotransmission components, indicating potential age-related changes in cellular function. Adapted from Li et al., (2015).

Gene	Protein	Function	Middle-aged vs young	Young	Middle aged
Transcription and translation factors					
Nfkb1[a]	Nuclear factor NF-κB p105 subunit	Part of NF-κB transcription factor	↓	100 ± 7	83 ± 3
Fos[a] (c-Fos)	FBJ murine osteosarcoma viral oncogene homolog	Part of transcription factor complex	↓	100 ± 15	44 ± 6
Fmr1[a]	Fragile X mental retardation protein	Jun/AP-1 Transport mRNA	↓	100 ± 5	88 ± 4
Creb1	cAMP responsive element binding protein 1	Phosphorylation-dependent transcription factor	↓	100 ± 3	86 ± 3
Signal transduction					
Ntrk2 (TrkB)	Neurotrophic tyrosine receptor kinase	Receptor for neurotrophic factors (BDNF, ↓neurotrophin-3 and neurotrophin-4)	↓	100 ± 5	83 ± 3
Camk2a[a]	Calcium/calmodulin-dependent protein kinase II α	Serine/threonine kinase required for hippocampal long-term potentiation (LTP) and spatial learning	↓	100 ± 7	83 ± 3
Prkca	Protein kinase C-α	Serine/threonine kinase in signal transduction pathway	↓	100 ± 5	83 ± 3
Jak2	Janus kinase 2	Tyrosine kinase in signal transduction pathway	↓	100 ± 3	85 ± 3
Gsk3b	Glycogen synthase kinase 3β	Serine/threonine kinase in signal transduction pathway	↓	100 ± 4	88 ± 3
Synaptic plasticity					
Arc[a]	Activity-regulated cytoskeleton-associated protein	Required for synaptic plasticity and memory formation	↓	100 ± 12	68 ± 5
Shank1[a]	SH3 and multiple ankyrin repeat domains 1	Adapter protein in the postsynaptic density of excitatory synapses	↓	100 ± 8	80 ± 2
Nlgn2[a]	Neuroligin 2	Cell adhesion molecule, binding partner of neurexins	↓	100 ± 6	80 ± 4
Homer1	Homer protein homolog 1	Postsynaptic density scaffolding protein	↓	100 ± 3	84 ± 3
Ncam1	Neural cell adhesion molecule 1	Cell adhesion molecule	↓	100 ± 4	88 ± 4
Dlg4 (Psd-95)	Postsynaptic density protein 95	Postsynaptic density scaffolding protein	↓	100 ± 7	85 ± 3
Neurotransmission					
Syp	Synaptophysin	Small synaptic vesicles membrane protein	↓	100 ± 4	81 ± 3
Scg3	Secretogranin-3	Protein located in secretory vesicles	↓	100 ± 4	87 ± 3
Snap25	Synaptosomal-associated protein 25	Docking of synaptic vesicles with the presynaptic plasma membrane	↓	100 ± 4	88 ± 3
Vamp1	Vesicle-associated membrane protein 1, Synaptobrevin 1	Docking of synaptic vesicles with the presynaptic plasma membrane	↓	100 ± 4	88 ± 3
Htr1d	5-HT receptor 1D	G-protein (Gi/Go) coupled 5-HT receptor	↓	100 ± 11	71 ± 6
Grin1 (Nmdar1)	Glutamate Receptor Ionotropic, NMDA 1	Ligand-gated cation channel	↓	100 ± 4	85 ± 3

The relevance of these findings draws upon a contemporary contentious debate concerning the difference between young and old depressed populations and the safe use of SSRI's – which will be addressed briefly. Some clinicians and health professionals have sought to have warnings of SSRI's amended due to the capcity that the drug has on enhancing depressive behaviour and even suicide ideation, phenomena which has predominantly affected those who are under 25 years of

age (Christainen et al., 2016; Li et al., 2022; Durbrall et al., 2023). This difference in SRRI efficacy and the different effect that NLX-112 has on older and younger mice in our study is likely due to brain being at distinctive developmental stages. Cortical structures are known to not be fully developed in humans until approximately 30 years of age and functional connectivity is continually changing across the life course (Edde et al., 2021), including decrements to brain modulating utilities such as neurotrophic factors (Shetty et al., 2005; Bimonte-Nelson et al., 2008), cognition and plasticity (Kumar, 2011; Balietti et al., 2012). Of course, SSRI treatment increases the release of 5-HT through reuptake inhibition at SERT receptors, and NLX-112 has the converse effect of inhibiting 5-HT release through its agonist property at presynaptic receptors in the RN. However, it does highlight that the pharmacological intervention of 5-HT release is differentially harnessed by the young and aged brain.

4.5.2. Caveats

This study suggests that NLX-112 induces anxiolytic-like effects in middle-aged C57b/6J male mice exposed to OFT and EPM maze paradigms. This study may also offer as a good control comparator for a mouse model of PD which might induce greater levels of anxiety-like behaviour. NLX-112 may therefore potentially diminish anxiety in man including those with co-morbid disorders such as PD and PD associated anxiety. However, there are several caveats. In this study, we did not use comparator compounds acting on the 5-HT_{1A} receptor; therefore, it is not clear whether other less selective drugs such buspirone or drugs with less pre- to post-synaptic bias, such as 8-OH-DPAT, would behave similarly to NLX- 112 or not. The priority in the present study was to keep the design of the investigation relatively simple and to use a relatively small number of age-matched older animals that might be used in models of PD and adhere to the NC3R principle; indeed, although both buspirone and 8-OH-DPAT have shown efficacy in anxiety models, they have not been investigated in older mice. Moreover, use of a comparator drug would have made it impossible to keep the number of animals low without the confounding effect of repetitive dosing in these animals. It is known exposure of the animals to EPM or OFT repetitively would have reduced the anxiety like behaviour and at the same time reduce the anxiolytic potential of the test compounds (File et al. 1990; Holmes and Rodgers 1999).

It should be noted that the choice of doses was informed by the extensive pre-clinical studies carried out with NLX- 112. For example, previous investigations have shown NLX- 112 to be effective at reducing immobility in the forced swim test in rats at 0.16 and 0.63 mg/kg (Iderberg et al. 2015), reducing levodopa-induced abnormal involuntary movements (AIMs model of dyskinesia) in the rat at 0.16 mg/kg (McCreary et al. 2016), reducing paw licking in the formalin pain test in mice at 0.1 and 0.5 mg/kg (Sałat et al. 2017) and reducing LID scores in MPTP-treated marmosets at 0.1 and 0.4 mg/kg (Fisher et al. 2020). The doses tested here were therefore consistent with those shown to be active in therapeutically relevant models. In addition, in our recent unpublished investigation de novo administration of 1.0 mg/ kg NLX-112 in young mice of a different strain (C57b/Ola-HSD), we observed significantly reduced motor activity in the OFT, which clearly points towards an inverted U-shaped dose response curve, as is often seen with other 5-HT_{1A} agonists such as buspirone and 8-OH-DPAT and that can constitute a confounding element (O'Neill and Conwa 2001; Vaidya et al. 2005; Bergman et al. 2013; Gluch- Lutwin et al. 2021).

There is already evidence that the anxiety levels in mice change with ageing; thus, a relatively narrow age difference could lead to significant behavioural differences during adulthood (Shoji et al. 2016). Therefore, in order to gain a better insight into the potential anxiolytic properties of NLX-112, the effect of NLX-112 may be investigated in animals of different age ranges (young and old) or perhaps on the behaviour of transgenic mice whose expression of 5-HT_{1A} receptors has been modified. Nevertheless, this present study proves some clues as to its potential value in providing anxiolysis in age-matched models of PD.

4.6. Summary and conclusion

NLX-112 inhibits dyskinesia in rats, in non-human primates and in a clinical trial. The reduction of anxiety-like behaviours shown in the present study suggests that subjects with PD who present with anxiety at a disproportionately higher rate than the rest of the population may also benefit not only from the NLX-112's mitigating effect on LID but also its potential anxiolytic properties. These affective properties exerted by NLX-112 may have a greater effect in the older, rather than younger brain.

Chapter 5

MPTP Model in C57b/Ola-HSD and C57b/6J Mice

5.1. Introduction

MPTP, a neurotoxic compound which selectively destroys DA producing neurons in the substantia nigra (SN) can be administered to mice, thus producing an animal model that can be used to identify drugs that possess putative neuroprotective properties. Described in detail in Chapter 1, metabolically derived from MPTP by MAO-B, the mitochondrial inhibitor MPP^+ induces the selective destruction of SN DAergic neurons via the depletion of ATP, inhibiting the normal function of the mitochondrial electron transport chain resulting in cell death and the build-up of ROS, furthering neuronal destruction. The high affinity that DAT has for MPP^+ makes SN DA neurons particularly vulnerable to MPP^+ toxicity, and consequently striatal DAergic nerve fibres, also (Langston et al., 1984; Przedborski et al., 2004). Adjusting the dose regimen, drug quantity and/or longevity of the study, will alter the extent of the nigrostriatal lesion as well as modification of the animal's behaviour.

The laboratory mouse most commonly used for these studies is of the C57b variety. In the United Kingdom (UK), there are several establishments that breed the C57b mouse and, as such, after many decades of inbreeding within each laboratory, genetic divergence between each respective laboratory's C57b mouse sub-strain has been detected. For example, the C57b/Ola-HSD strain, bred by Envigo laboratories has developed the spontaneous chromosomal deletion of the *Snca*1 gene, which encodes for α-synuclein (Specht and Schoepfer 2001; 2004). Unlike their C57b/Ola-HSD counterparts however, C57b/6J mice, bred by Charles River laboratories, have maintained normal expression of *Snca*1. This substantive difference between each mouse sub-strain for either expressing or not expressing this important synaptic regulating protein is shown to be associated with other major neuro-physiological differences. For example, there is reduced motor impulsivity in C57b/Ola-HSD compared to C57b/6J mice (Pena-Oliver et al., 2010), C57b/Ola-HSD mice express low levels of DAT and have increased levels of Syn-3 (a vesicle regulating synapsin) in the striatum compared to C57b/6J and human transgene (SYN120 tg) mice (Faustini et al., 2020), C57b/Ola-HSD mice have a significantly lower trabecular bone mass compared to C57b/6J mice (Li et al., 2005), and after peripheral nerve damage, C57b/Ola-HSD mice have greater levels

of preserved myelin and macrophage number compared to normal wildtype α-synuclein expressing controls (Siebert et al., 2010).

In the pathology of PD, α-synuclein plays a pivotal role as a primary constituent of toxic protein aggregates known as Lewy bodies. Investigating its pathogenesis in vivo has been accomplished through various murine models, one of which involves the complete chromosomal absence of α-synuclein in C57b/Ola-HSD mice, as previously described. The A53p and A30p mutations represent two of several mutations in the α-synuclein gene associated with familial forms of PD (Blandini et al., 2012). In A53p and A30p transgenic mice, the mutation is introduced into the mouse genome, resulting in the overexpression of the mutant α-synuclein protein in the mouse's neurons (Sommer et al., 2000). The most recent version of the α-synuclein mouse model involves the introduction of human recombinant α-synuclein pre-formed fibrils into various organs, leading to widespread α-synuclein presence in the central nervous system (Chung et al., 2019). These models facilitate the comprehension of α-synuclein's role in PD pathology through both its overexpression and its complete absence. However, what remains less understood is the role that α-synuclein plays under normal conditions.

Considering the extensive utilisation of the C57b mouse in MPTP studies and the significant differences between the sub-strains mentioned above, it was decided that differences in behaviour, MPTP lesion severity, and MPTP-induced nigrostriatal inflammation were investigated. Furthermore, we employed micro Raman spectroscopy to gain additional insights into the chemical composition of tissue sections collected from the substantia nigra of the two different sub-strains. There is a compelling argument in favor of using Raman spectroscopy. It does not require sample preparation, is non-invasive to the cell, and employs appropriate laser wavelengths and irradiance levels. Raman spectroscopy has a well-established reputation as a method utilised for histological analysis in various clinical or fundamental biology research settings (Manoharan et al., 1996). This method assists in the detection of vibrations associated with chemical bonds within molecules. The wealth of chemical information contained within a Raman spectrum provides valuable insights into biochemical imbalances inherent to various diseases. In this thesis, the micro-Raman setup has served as an investigative tool

to analyse changes in protein conformation between sub-strain controls and MPTP-treated groups.

5.2. Methods

5.2.1. Animals

All of the experiments described complied with ARRIVE guidelines and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU directive 2010/63/EU for animal experiments. Prior to commencing experiments, all procedures and protocols were approved the University of Hertfordshire Animal Welfare and Ethical Review Board (AWERB), and carried out under the UK Home Office approved Project Licence PD7255B4. Male mice, aged 8-10 weeks (19-21g) were purchased from Charles River (6J) and Envigo Laboratories (C57b/Ola-HSD). Mice were housed in 3's, and mice from different labs were not housed together. Access to food and water was ad-libitum, room temperature was maintained at 22°C - 24°C and light cycle was automated at 12h(light): 12h(dark). All experiments were carried out under an ambient light intensity of 100 Lux between 09:00h and 14:00h.

5.2.2. Drug

The two C57b sub-strains, 6J and Ola-HSD were split into two groups for treatment with either saline or MPTP, totalling four groups: 6J saline, 6J MPTP, Ola-HSD saline, and Ola-HSD MPTP (n= 6-24). MPTP's administration acutely induces tachycardia but also has an acute effect on adrenergic receptors (AR) in cardiac and vascular tissue, resulting in vasoconstriction and hypothermia which can lead to heart failure and death of the animal (Fuller et al., 1984; Liu et al., 2020). These deleterious acute effects can be avoided if dosing of MPTP is incremental, thereby allowing for de-sensitisation of vascular and peripheral AR's. The procedural technique employed ensured that all MPTP-treated mice survived the sub-chronic MPTP dosing regimen (s.c.), with a single dose administered per day, calculated as MPTP freebase mg/kg (Figure 30).

Day	1	2	3	6	7	8	9	10	15	16	17	27	28	29
Trial	OFT1	EPM1	RR1						OFT2	EPM2	RR2	OFT3	EPM3	RR3
MPTP dose (mg/kg)				11.11	21.37	27.35	29.91	29.91						

Figure 30. Timeline of MPTP treatment and trial series of open field (OFT), elevated plus maze (EPM), and RotaRod (RR). The study spans 29 days, with specific tests administered at various intervals. On Day 1, mice undergo OFT1, followed by the EPM1 on Day 2 and RR1 on Day 3. MPTP is administered in increasing doses from Days 6 to 10, starting at 11.11mg/kg on Day 6 and gradually rising to 29.91mg/kg on Days 9 and 10. Following MPTP administration, the same set of tests is repeated. The Open Field Test is conducted on Days 15 (OFT2) and 27 (OFT3), the Elevated Plus Maze on Days 16 (EPM2) and 28 (EPM3), and the Rotarod Test on Days 17 (RR2) and 29 (RR3). This design allows for the evaluation of MPTP's impact on behaviour and motor performance over time. Mice were culled on day 30 (not shown on timeline).

5.2.3. RotaRod

Mice were trained on the RotaRod (Ugo-Basile 47650) to become familiarised with the apparatus before any motor activity was reordered. Five mice were randomly picked from each group to trial the RotaRod. The test consisted of three trials. One trial in the morning (which was considered as the training trial), and two experimental trials in the afternoon, with each trial separated by one hour. Mice were placed on the RotaRod one by one at a speed of 4 RPM. Mice traversed the RotaRod with the speed increasing incrementally every 2 seconds by 1 RPM, culminating to 35 RPM and continued to maintain at that speed until all mice had fallen off. The duration that mice stayed on the RotaRod was recorded, and a mean value of the two afternoon trials was used as the final result.

5.2.4. Immunohistochemistry

Mice were culled via exposure to CO_2, trans-cardially perfused with ice cold PBS, decapitated, brains fixed in formalin (10% buffered) and prepared accordingly for immunohistochemical (IHC) analyses. Brains were sectioned coronally in 30µm thick sections and analysed for IHC and immunofluorescence (IF) using the following antibodies: tyrosine-hydroxylase (TH) anti-sheep, PA1-4679, 1:500 Invitrogen®, glial fibrillary associated protein (GFAP) anti-chicken, PA1-10004, 1:1000 Invitrogen® and ionised calcium-binding adapter molecule 1 (Iba1) anti-rabbit, ab108539, 1:500 abcam®. Free floating sections were washed in PBS (1X), triton X-100 (0.1%), non-specific binding was inhibited with non-animal protein

blocker (Vector®) and incubated overnight in primary antibody at 4°C. The following day sections were washed and incubated in either fluorescent secondary antibody for one hour at room temp or treated with DAB Substrate Kit, Peroxidase-HRP, SK-4100, 2bscientific® followed by treatment with Vectastain ABC-HRP Kit, Peroxidase, SK-4000, 2bscientific®. Tissue was washed, mounted on slides and images were taken on a Zeiss light microscope.

5.2.5. Stereology

Cell counting was performed using stereological quantification (Ip et al., 2017). From within the boundaries of the nigrostriatal bundle (~Bregma -2.50) and the caudal portion of the SNpc (~Bregma -3.90), five TH+ve sections were observed and cells were digitally identified and counted using ImageJ. The following calculation was used to achieve the final estimate of TH+ve neurons.

$$N = \Sigma^{5}i = 1(A_i \times ssf) \times mcf$$

The estimated number of cells (N) has been derived where Σ is the sum of sections *(i)* ranging from 1 to 5 ($\Sigma^{5}i$). *ssf* (=10) is the sampling fraction which is one section analysed every 10 sections. *mcf* is the missing cell fraction of uncounted cells, cells lay on their side, or layered on top of each other through the Z axis = 1.33.

5.2.6. Raman spectroscopy data collection and analysis

Micro Raman measurements were performed of custom build near infrared micro Raman setup which utilises Ti:Sapphire laser (Newport Spectra Physics) tuned at 725nm. After filtering the laser is fed into an inverted microscope (Nikon, Japan) delivering 135mW irradiance after the water immersion microscope objective (Zeiss). The inelastic scattered Raman photons are collected by the same microscope objective in backscattering configuration and directed via focusing lens and optical fiber towards a spectrograph coupled to a deep cooled Near Ir detector Idus BR-DD 420 (Andor, Belfast, UK). Tissue section for each sub-strain of 40 microns were attached to microscope slide of CaF2 (Crystran, UK). On each section an area of 100 microns × 100 microns was covered by raster scanning with dwell of 1 second per pixel. After acquisition each individual spectrum was subject

to pre-processing and removal of background signal. The peak deconvolution uses curve fitting tool in Matlab (Matworks, USA).

5.3. Results

Two different C57b mouse sub-strains were examined, C57b/6J and C57b/Ola-HSD, examining their body weight, behavioural, molecular spectral, and neuro-histochemical properties

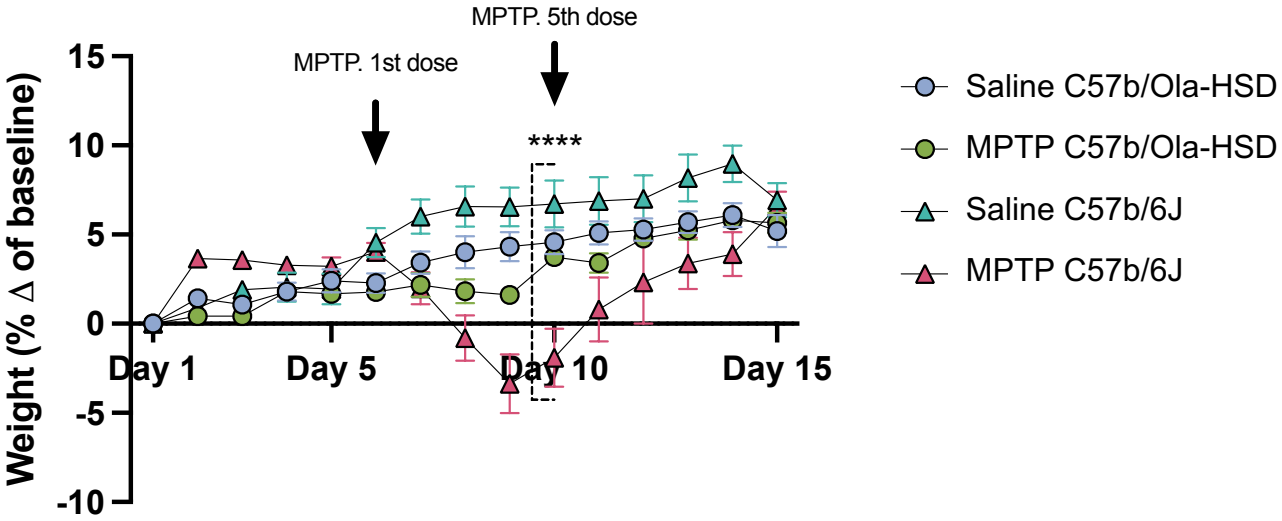


Figure 31. The body weight changes of four groups of mice over time, focusing on the differential impact of MPTP treatment. Light blue circles represent saline-treated C57b/Ola-HSD mice, which maintain relatively stable body weight. Green circles denote MPTP-treated C57b/Ola-HSD mice, which also show stable weight, suggesting resilience to MPTP's acute effects. Blue triangles correspond to saline-treated C57b/6J mice, which display consistent body weight, similar to the C57b/Ola-HSD groups. In contrast, red triangles indicate MPTP-treated C57b/6J mice, which experience a marked decline in body weight over time. The pronounced weight loss in MPTP-treated C57b/6J mice, compared to all other groups, underscores their heightened susceptibility to MPTP's deleterious effects. Two-way ANOVA followed by Fisher's LSD for differences. All group differences marked on figure compared to MPTP C57b/6J. Error bars denote SEM. n= 6-12.

5.3.1. Behavioural assays

In OFT 1, over five minutes C57b/6J had significantly greater locomotor activity than C57b/Ola-HSD [F(1.182 (23, 23) = 0.6925 p = 0.0379] (Figure 32 A), but C57b/Ola-HSD mice spent significantly longer in the centre of the arena compared to C57b/6J [F(1.459 (23, 23) = 0.3712 p = 0.0009] Figure 32 D). In OFT 2, MPTP treated C57b/6J mice had significantly greater locomotor activity compared to all other groups, [F(8.906, (3, 42) = 0.9195 p = 0.0001] (Figure 32 E) whereas saline

treated C57b/Ola-HSD mice spent significantly longer in the centre of the arena compared to all other groups [F(2.590 (3, 42) = 0.7787 p = 0.0654] (Figure 32 G).

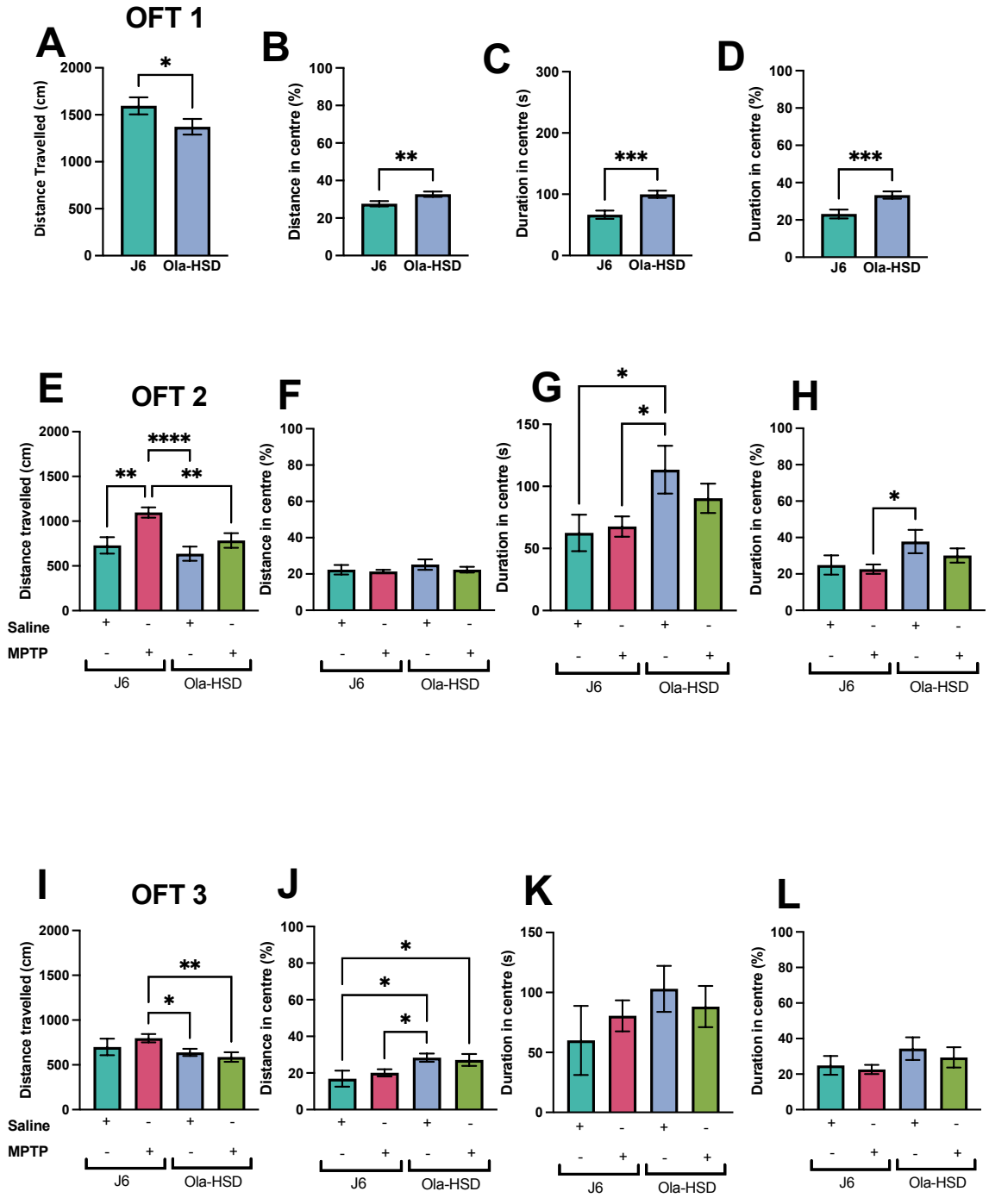


Figure 32. Three Open Field Tests (OFT 1–3), assessing locomotor activity and anxiety-like behaviour in different mouse strains C57b/6J (6J) and C57b/Ola-HSD (Ola-HSD) under various treatment conditions (Saline and MPTP). In OFT1, 6J mice displayed significantly greater locomotor activity than Ola-HSD mice, as indicated by the longer distance travelled (*p < 0.05). Additionally, 6J mice spent significantly more time and a greater percentage of time in the centre of the arena

(**p < 0.01, ***p < 0.001), suggesting reduced anxiety-like behaviour compared to Ola-HSD mice. OFT2 included both saline- and MPTP-treated groups within each strain. 6J mice treated with saline showed the highest distance travelled, which was significantly reduced by MPTP treatment (****p < 0.0001). In contrast, the Ola-HSD strain showed generally lower locomotor activity, regardless of treatment. While the percentage of distance travelled in the centre did not differ significantly among groups, both the time spent in the centre and the percentage of time in the centre were significantly higher in saline-treated 6J mice compared to their MPTP-treated counterparts and Ola-HSD mice (*p < 0.05), indicating a treatment-dependent increase in anxiety-like behaviour. OFT3 again showed that saline-treated 6J mice travelled significantly more than MPTP-treated 6J and both Ola-HSD groups (**p < 0.01, *p < 0.05). 6J mice also spent a significantly higher percentage of distance in the centre of the arena (*p < 0.05), reinforcing findings of reduced anxiety in this strain under saline conditions. However, no significant differences were observed between groups in the duration of time spent in the centre (in seconds or percentage), suggesting that total time-based measures of centre exploration were less sensitive in this test. One-way ANOVA followed by Fishers LSD. n= 6 - 12, error bars denote SEM.

In EPM 2 MPTP induced locomotor activity was reflected through both MPTP treated C57b/6J and C57b/Ola-HSD mice travelling further in the EPM compared to both saline treatment groups [F(2.738 (3, 19) = 0.0720 p = 0.0135] (Figure 33 E), but only MPTP C57b/Ola-HSD mice spent significantly longer in the OA of the EPM compared to all other groups [F(4.111 (3, 19) = 0.0001 p = 0.0209] (Figure 33 F). In OFT 3 the behavioural trends were similar to OFT 2 but not as pronounced. MPTP treated C57b/6J mice still had greater locomotor activity compared to saline C57b/Ola-HSD and MPTP C57b/Ola-HSD mice, but not saline C57b/6J mice [F(3.529 (3, 43) = 0.5822 p = 0.0226] (Figure 32 I), and saline C57b/Ola-HSD mice still spent longer in the centre of the arena compared to all other groups but this difference was no longer significant (Figure 32 L). EPM 3 followed a similar trend to EPM 2, whereby MPTP C57b/6J and MPTP C57b/Ola-HSD had greater locomotor activity throughout the EPM arena compared to both saline groups [F(3.479 (3, 19) = 0.0872 p = 0.0363] (Figure 32 I), and distance in the OA was still markedly greatest from MPTP C57b/Ola-HSD mice compared to both saline groups, but not MPTP C57b/6J mice (Figure 32 J).

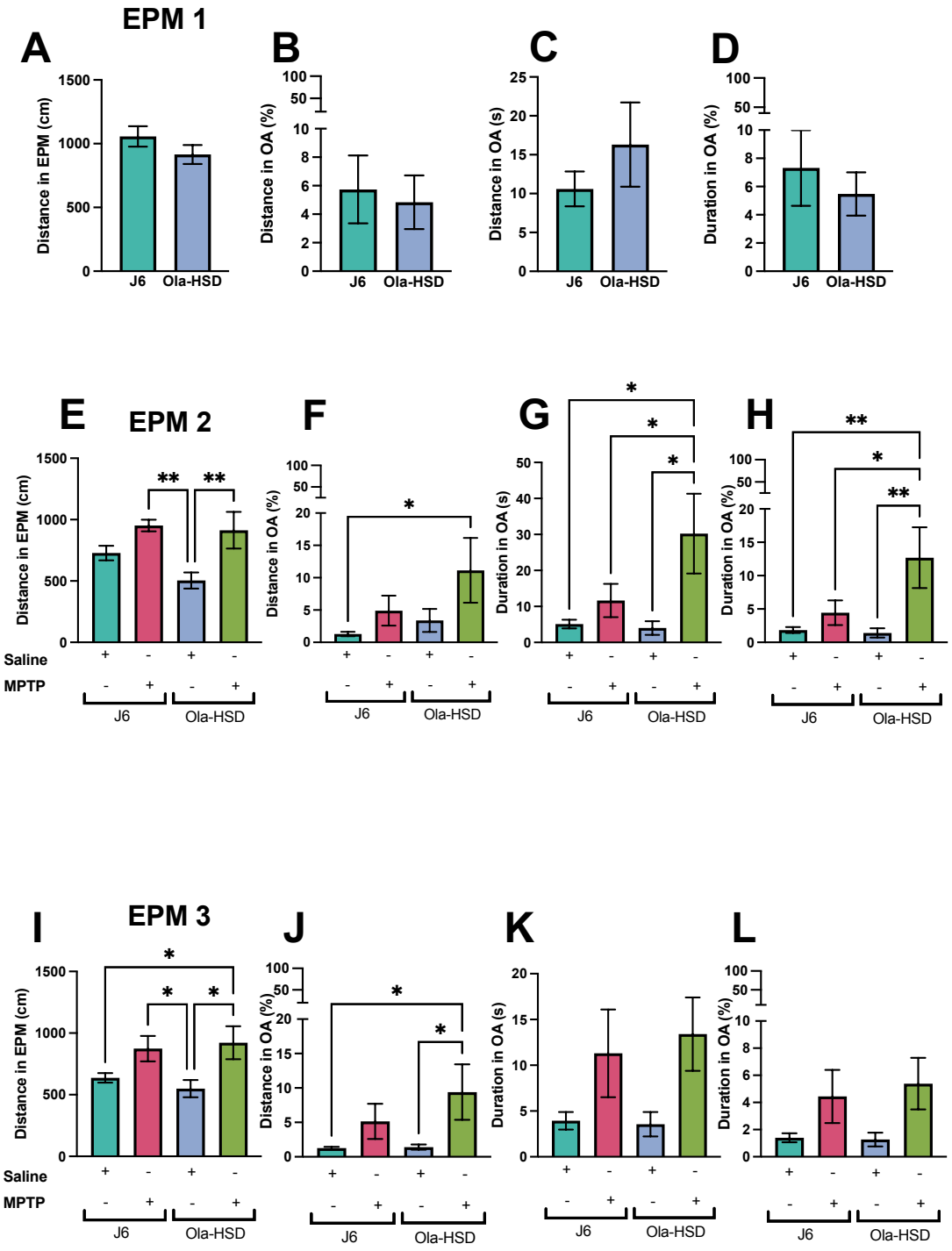


Figure 33. Elevated Plus Maze (EPM) performance across three sessions (EPM 1–3) in C57b/6J (6J) and C57b/Ola-HSD (Ola-HSD) mice following saline or MPTP treatment. There were no statistically significant differences between 6J and Ola-HSD strains in any behavioural measures, including total distance travelled in the maze or exploration of the open arms (distance or duration), indicating similar baseline levels of activity and anxiety-like behaviour. In EPM 2, MPTP treatment significantly increased total distance travelled in both 6J and Ola-HSD mice compared to their saline-treated counterparts (**p < 0.01). In terms of anxiety-like behaviour, Ola-HSD mice treated with MPTP spent significantly more time in the open arms, both in seconds and as a percentage of total time (*p < 0.05, **p < 0.01), and also showed greater distance in the open arms compared to all other

groups (*p < 0.05). In EPM 3, MPTP-treated mice (especially Ola-HSD) showed increased locomotor activity (*p < 0.05) and greater exploration of the open arms compared to saline-treated mice (*p < 0.05). This includes higher percentage of distance and duration in open arms in MPTP groups. One-way ANOVA followed by Fishers LSD. p<0.05, n= 6 - 12, error bars denote SEM.

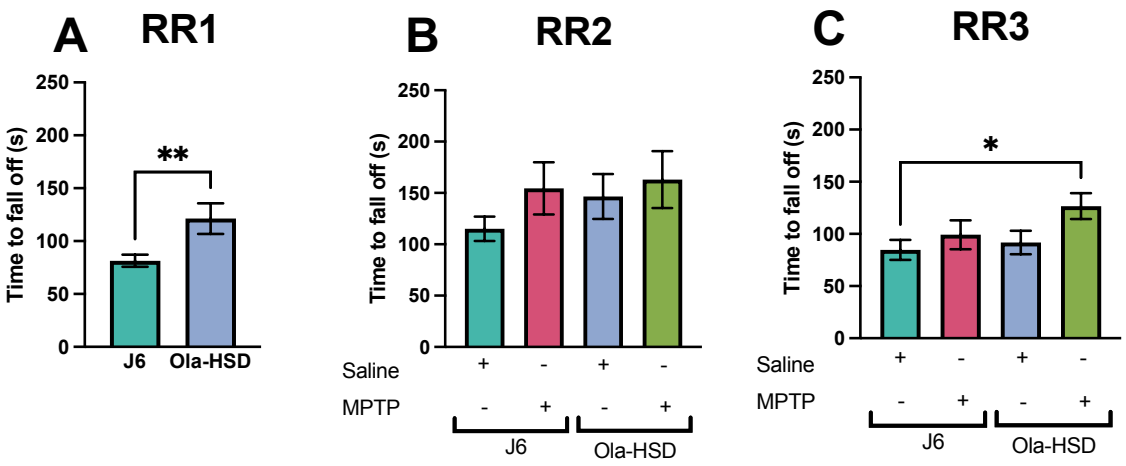


Figure 34. RotaRod endurance across three sessions (RotaRod 1–3) in C57b/6J (6J) and C57/Ola-HSD (Ola-HSD) mice following saline or MPTP treatment. Endurance was assessed as the latency to fall from the RotaRod. In session 1, Ola-HSD mice exhibited greater motor endurance than 6J mice. Across sessions 2 and 3, no significant differences were observed between strains or treatment groups, indicating that MPTP did not impair motor coordination under these conditions. One-way ANOVA followed by Fishers LSD. p<0.05, n= 6, error bars denote SEM.

Baseline time spent on the RotaRod 1 (RR1) by C57b/Ola-HSD was significantly longer, compared to C57b/6J [F(5.217 (9, 11) = 0.0127 p = 0.0065] (Figure 34 A); in RR2 no differences were observed (Figure 33 B) but in RR3 MPTP C57b/Ola-HSD mice spent markedly longer on the RotaRod compared to all other groups [F(2.467 (3, 17) = 0.9485 p = 0.0972] (Figure 34 C).

5.3.2. MPTP lesion

MPTP caused a loss of TH+ve neurons in the SN of both sub-strains of mouse [F(8.605 (3, 17) = 0.7632 p = 0.0011] (Figure 35 A, C). Compared to saline treatment however, MPTP caused a greater level of loss of TH+ve neurons in the SNpc of C57b/Ola-HSD mice (-40%) than it did in C57b/6J mice (-34%), this is highlighted by the fact that there is no significant difference between the two saline groups but there is a significant difference between the two MPTP groups with C57b/Ola-HSD having markedly fewer number of TH+ve neurons than C57b/6J.

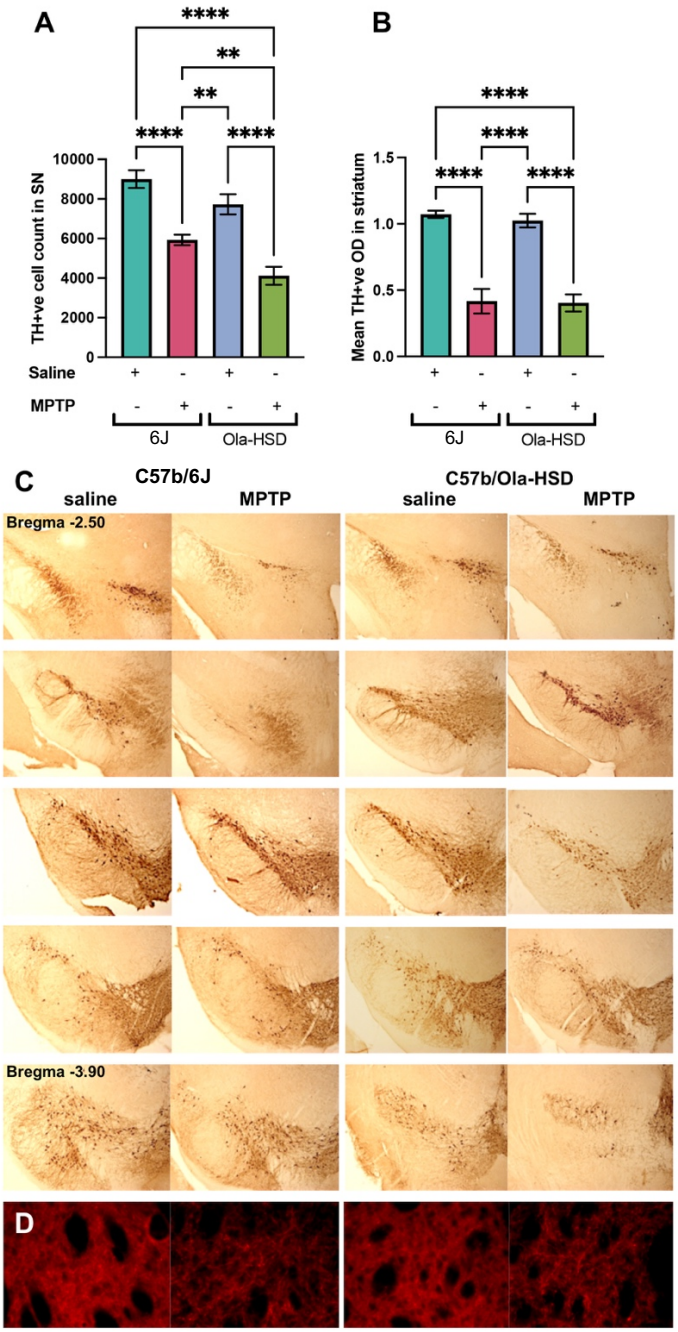


Figure 35. A) The number of TH+ve cells counted in SN from naïve and MPTP treated C57b/6J and C57b/Ola-HSD mice and B) the mean optical density of TH+ve fibres in the CPu. One-way ANOVA followed by Fishers LSD. $p<0.05$, n= 6, error bars denote SEM. C) Representative images of TH+ve stained SN in saline and MPTP treated C57b/6J and C57b/Ola-HSD mice. Sections were taken from Bregma -2.50 at the nigrostriatal bundle through to Bregma -3.90 at the caudal portion of the SN. D) Tyrosine hydroxylase reactivity in caudate putamen of saline and MPTP treated mice.

Loss of Th+ve fibres in the striatum was not dissimilar for both sub-strains administered with MPTP [F(31.16 (3, 15) = 0.4160 p = 0.0001] (Figure 35 B, D) (-63% compared to saline treated mice).

5.3.3. Reactive gliosis

Reactive micro- and astrogliosis was analysed in the striatum and substantia-nigra from all four groups. Reactive astrogliosis in was assessed using GFAP antibody and activated greatest in the striatum of both MPTP treated groups compared to saline treated mice [F(35.55 (3, 14) = 0.6071 p = 0.0001] (Figure 36 B). This trend was not replicated when assessing Iba1+ve binding in the striatum. Interestingly, reactive microgliosis was un-changed in the C57b/6J strain after MPTP treatment, whereas in the C57b/Ola-HSD strain MPTP treatment resulted in the greatest's levels of microglia activation compared to saline treatment; a response likely enhanced due to C57b/Ola-HSD appearing to have a greater basal level activated microglia compared to C57b/6J basal levels (Figure 36 D).

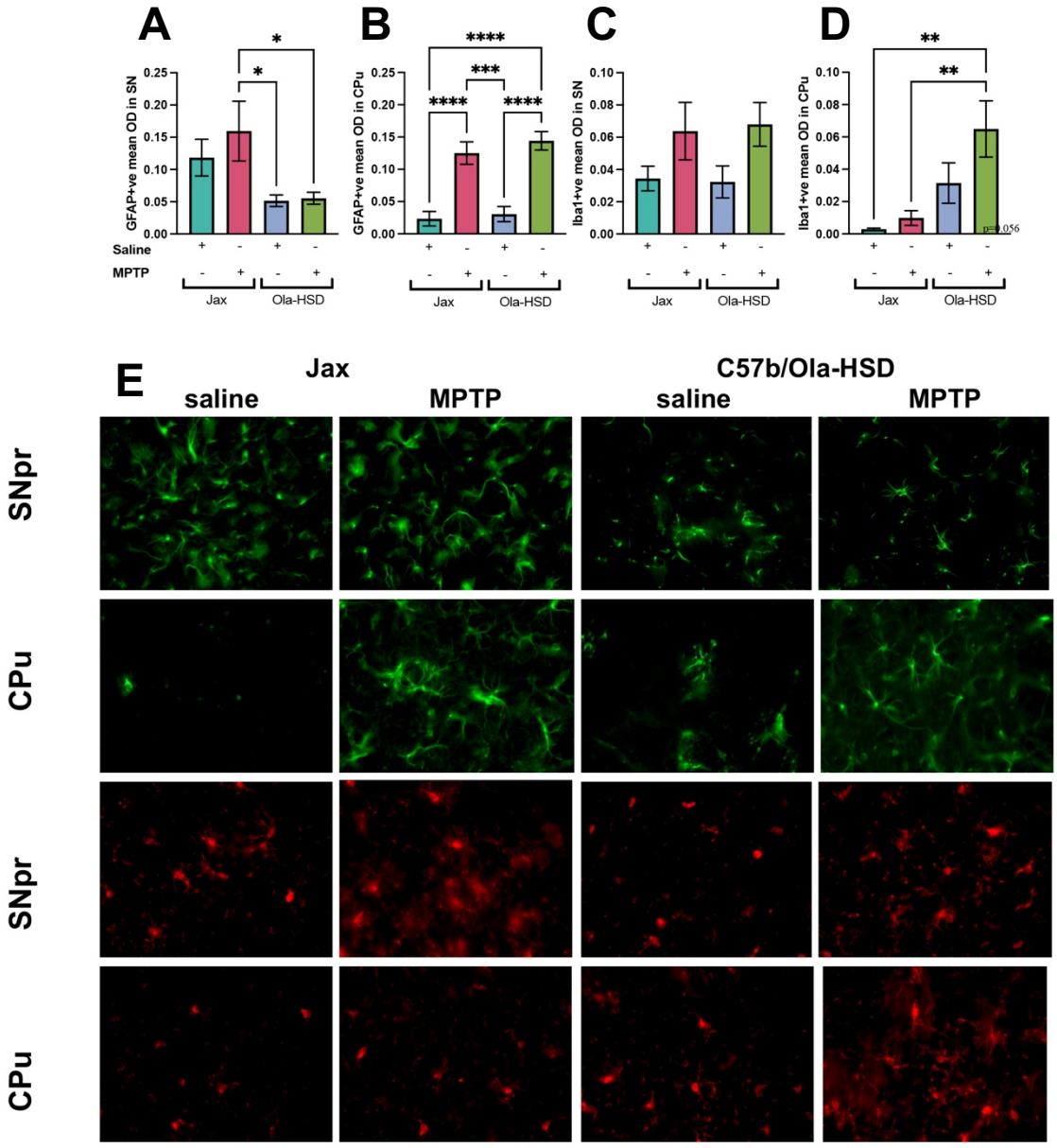


Figure 36. A-D) Mean optical density of GFAP+ve and Iba1+ve immunoreactivity in the SN and CPu. One-way ANOVA followed by Fishers LSD post hoc test. Error bars denote SEM. $p<0.05$, n=5-6. E) Representative images of GFAP+ve reactivity as shown via optical density in the SN. GFAP+ve reactivity as shown via optical density in CPu. Iba1+ve reactivity as shown via optical density, in the SN. Iba1+ve reactivity as shown via optical density in CPu.

Raman spectra presented were obtained from SN. The area of scanning was chosen based by previous designated maps for the excised tissue sections. Raman spectra in the fingerprint region 600-1800 cm-1 are dominated by the massive presence of protein peaks, carbohydrates and lipids. The spectra use in analysis are average over 500 spectra collected in raster scanning mode over 100 microns by 100 microns area on the tissue. The concern of analysis is the amide I

peak around 1663 cm-1. The large full width half maximum and peak asymmetry may indicate that a combination of individual peaks may contribute to the overall profile of amide I. The overall contour of the amide I can be deconvolved in individual peaks using gaussian peak fitting. Changes observed in individual peaks as a result of deconvolution analysis reflect protein conformational change. The convolution of the peaks covers range from 1560 cm^{-1} to 1720 cm^{-1} (Figure 37).

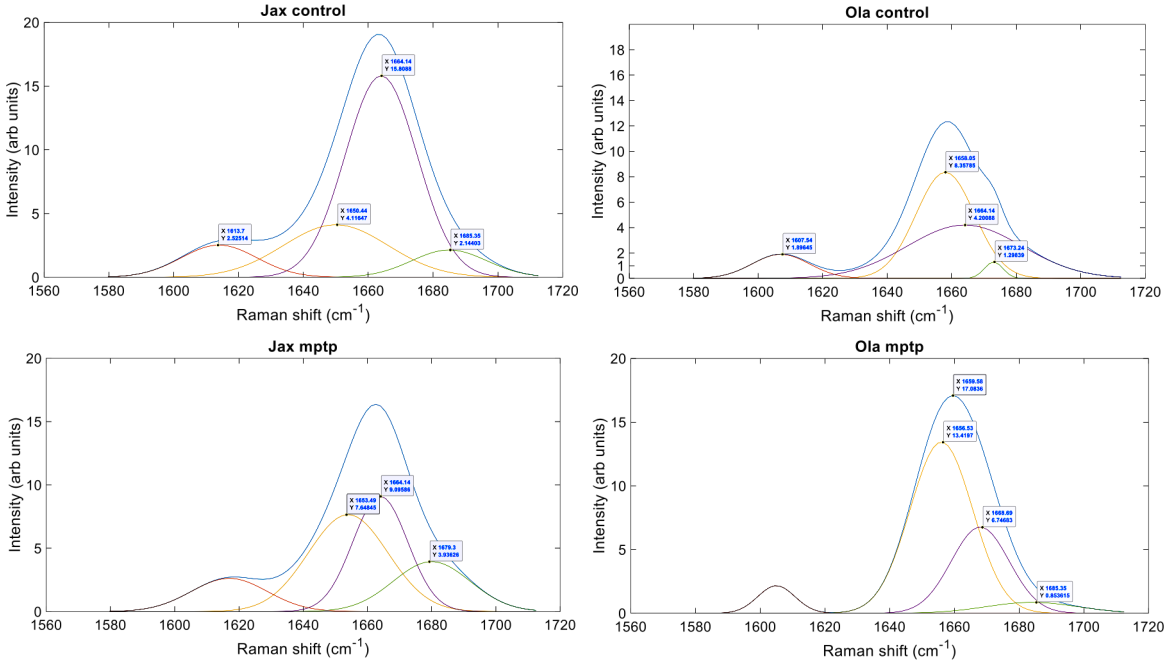


Figure 37. C57b/6J vs C57b/Ola-HSD measurements, Amide -I peak deconvolutions. 4 gaussians are used for analysis. Depicts peak analysis of Amide -I for the average Raman spectrum of the entire scan of each area associated to sub-strains. The average spectrum displays a reasonable signal to noise ratio and Amide -I peak deconvolution represents a good basis for secondary structure analysis. Constraints in peak deconvolution are introduced relative to peak position according to peak analysis of (Maiti et al., 2004)

5.4. Discussion

5.4.1. Behaviour and motor activity

C57b/6J mice exhibited higher locomotor activity, covering more distance and displaying greater velocity than C57b/Ola-HSD mice in the open field, yet they spent more time along the arena's perimeter, indicating increased thigmotaxis. This trend persisted in subsequent tests, suggesting stable behavioural patterns. MPTP

treatment increased locomotor activity in both open field tests (OFT 2 and OFT 3) and elevated plus maze tests (EPM 2 and EPM 3) compared to saline groups. The dosing regimen played a crucial role, as acute MPTP treatment reduced locomotor activity, while chronic and sub-chronic treatments consistently increased it. Notably, MPTP-induced locomotion changes were observed in MPTP-treated C57b/6J mice in the open field but affected both strains equally in the elevated plus maze. Interestingly, RR1 showed C57b/Ola-HSD mice outperformed C57b/6J mice in terms of endurance. This result may appear to contradict the locomotor response findings but aligns with the idea that the RotaRod test measures not just motor function but also perseverance, similar to the forced swim test (Guariglia et al., 2013). Notably, RotaRod-trained mice have larger frontal cortex volumes compared to untrained controls (Scholz et al., 2015).

5.4.2. MPTP lesion and reactive gliosis

This study revealed a more significant loss of tyrosine hydroxylase-positive (TH+ve) DA neurons in the SN of C57b/Ola-HSD mice (-40%), compared to C57b/6J mice (-34%) suggesting that C57b/6J mice may have better protection against MPTP toxicity. This finding is similar to the results of Schluter et al., (2003), who also found that the spontaneous deletion of alpha-synuclein in C57b/Ola-HSD mice does not confer any greater protection toward DA neurons in the SN upon MPTP treatment, than in wildtype C57b mice. The acute toxicity of MPTP appears to have had a distinctive effect upon each strain of mouse. Compared to control, MPTP treatment led to C57b/6J mice losing significantly more weight compared to their respective saline-treated counterparts, whereas C57b/Ola-HSD mice were unaffected in terms of weight loss. Qualitative behavioural observations noted that C57b/Ola-HSD mice recovered more rapidly from the acute adverse effects post-MPTP dosing. There were also distinct differences in basal and reactive gliosis between the two sub-strains. MPTP treatment did not alter the number of reactive astroglia in the SN, but C57b/6J mice had twice as many GFAP+ve cells compared to C57b/Ola-HSD. In the caudate putamen (CPu), MPTP treatment led to a significant increase in the number of GFAP+ve cells in both sub-strains compared to saline treatment. Additionally, Iba1+ve reactivity in the Cpu of C57b/6J mice remained largely unaffected by MPTP treatment, while it significantly increased in MPTP-treated C57b/Ola-HSD mice compared to all other groups. Previous reports

highlight immunological differences between C57b/6J wildtype and C57b/Ola-HSD mice. When C57b/Ola-HSD mice are fed a high-fat diet (HFD) for 21 days, their plasma insulin levels increase four-fold compared to C57b/6J mice, with insulin levels remaining stable compared to day one of an HFD. Additionally, C57b/Ola-HSD mice have a significantly higher whole-body mass and fat mass on day 7, 14, and 21 of an HFD. Liver biopsies from HFD-fed C57b/Ola-HSD mice reveal activated transcription factors, including zinc finger protein (GFI-1) and GATA binding protein 3 (GATA3), both known to influence immune system function, inflammatory processes, and stress response (Li et al., 2012; Zeng, 2013; Kahle et al., 2013), suggesting systemic effects on macrophage lineages, rather than local liver cell-type alterations which indicate significant changes in inflammation-related processes. Inflammatory abnormalities which may also extend to the CNS.

5.4.3. Raman spectra analysis

A hallmark of PD is the formation of Lewy bodies, which are composed of alpha-synuclein protein fibrils. It is believed that the spatial conformation of the peptide backbone of the alpha-synuclein protein adopts a beta-sheet structure (Vidović and Rikalovic, 2022). To analyse protein conformation, we focus on the amide I region in the fingerprint region. This broad peak contains multiple individual peaks. Previous literature has discussed the analysis of the amide I and conformation in various proteins using Gaussian peak analysis. The deconvolution of the amide I region involves fitting a total envelope with four peaks.

Based on previous research, the following peak assignments are considered: around 1650 cm-1 for alpha helix, around 1660 cm-1 for beta sheet, and around 1670 cm-1 for polyproline II. These assignments account for changes in the amide I peak profile and subsequent changes in conformation. After deconvolving the amide I peak for the average Raman spectra obtained from tissue sections excised from C57b/6J and C57b/Ola-HSD mice, we observe a different ratio for the peaks around 1650 cm-1 and 1660 cm-1. It appears that the contribution of alpha helix versus beta sheet is reversed between C57b/6J and C57b/Ola-HSD mice. The peak around 1660 cm-1 displays the highest intensity in C57b/6J mice, both in the control and MPTP-treated groups, suggesting a greater presence of beta-sheet protein formation (Figure 37).

The consistency of these results can be linked to C57b/6J mice to being able to normally express α-synuclein. The heightened intensity of the peak around 1660 cm-1 indicates a pronounced presence of beta-sheet formation, suggesting a change in conformation from a monomer to a filament-like structure. Another hypothesis is the emergence of a completely new protein structure with a beta-sheet as the new dominant secondary structure in C57b/6J mice. As these results are in the preliminary stage, we cannot pinpoint the exact cause. However, we can assert with great certainty that we observe a consistent shift in protein secondary structure.

5.5. Summary and conclusion

The results presented in this chapter provide important insights into the phenotypic differences between two commonly used C57b/6 mouse sub-strains: C57b/6J (6J) and C57b/Ola-HSD (Ola-HSD). The overarching goal of this thesis is to determine whether the selective 5-HT_{1A} receptor agonist NLX-112 confers neuroprotection in a mouse model of PD induced by MPTP. While this central question is addressed in later chapters, the current data establish a necessary behavioural and neuroanatomical baseline upon which neuroprotective effects can be interpreted. Notably, the data indicate that MPTP lesioning results in a comparable reduction of tyrosine hydroxylase-positive (TH+) neurons in the substantia nigra (SN) and TH+ fiber density in the striatum in both sub-strains. This suggests that both 6J and Ola-HSD mice exhibit similar DAergic neurodegeneration in response to MPTP at the anatomical level. However, significant differences emerged in the behavioural consequences of MPTP administration between the two strains. In the open field, MPTP paradoxically increased locomotor activity in both sub-strains, with Ola-HSD mice showing greater centre exploration and reduced anxiety-like behaviour compared to 6J mice. These behavioural profiles suggest a strain-dependent difference in anxiety and exploratory drive, potentially mediated by differences in serotonergic or DAergic tone. In the EPM, similar patterns emerged: MPTP treatment led to increased open arm exploration in Ola-HSD mice, reinforcing the idea that this strain exhibits reduced anxiety-like behaviour, particularly after neurotoxic insult. These findings are intriguing given the typically anxiogenic effects of DAergic lesions, and they underscore the importance of strain-specific

behavioural baselines in interpreting the effects of neurodegenerative processes and potential therapeutics. Importantly, RotaRod testing showed no significant motor deficits in MPTP-treated mice of either strain, suggesting that the degree of DAergic damage induced here does not overtly impair gross motor coordination or endurance. This is relevant for interpreting future treatment effects, as it confirms that MPTP-treated animals retain sufficient motor function for behavioural testing. Taken together, these findings suggest that C57b/6Ola-HSD mice are more resilient to the acute behavioural effects of MPTP, showing enhanced locomotion and reduced anxiety-like responses following treatment. This may position Ola-HSD mice as a more stable platform for testing protective efficacy of compounds like NLX-112, particularly in studies where minimising baseline behavioural variability is critical. Conversely, if the aim is to dissect mechanisms underlying neuroprotection—such as reactive gliosis, neurotrophic signaling, or neuroimmune modulation—then the 6J strain may offer more dynamic or sensitive readouts, as it displays more pronounced behavioural responses to MPTP lesioning.

Ultimately, the choice of sub-strain should be guided by the specific mechanistic or translational focus of the study. The behavioural data from this chapter underscore that strain selection is not a trivial variable, but one that can influence outcomes and interpretations in neurodegeneration and neuroprotection research.

Chapter 6

The Neuroprotective and Behavioural Properties of NLX-112 in C57b/Ola-HSD and C57b/6J Mice

6.1. Introduction

This chapter will address the primary aim of the thesis, which is to investigate the neuroprotective properties of the novel 5-HT_{1A} agonist in a mouse model of PD. This will be accomplished by administering MPTP, a neurotoxic compound, to the mice. As extensively explained in Chapter 1, MPTP selectively destroys DA expressing neurons in the SN by inhibiting mitochondrial respiration, thereby causing a reduction in ATP production and ultimately leading to neuronal apoptosis. Additionally, Chapter 1 provides in depth information on NLX-112, a drug that has successfully completed a Phase 2a clinical trial for alleviating LID in individuals living with PD. On a pre-clinical level, NLX-112 is also likely to be an effective analgesic and psychiatric modulating drug. To test the hypothesis regarding NLX-112's potential neuroprotective properties in vivo, mice were treated with the neurotoxin, MPTP and 5-HT_{1A} agonist, NLX-112.

The experiments in chapter 5 revealed that MPTP is an effective neurotoxic agent for destroying DA neurons in the SN and DA fibres in the striatum of both C57b/6J and C57b/Ola-HSD mice. The experiments also revealed that there are several behavioural differences at baseline between the two sub-strains, differences that appear to be sustained following sub-chronic MPTP treatment.

Upon MPTP treatment, the reduction in the nigral DA cell count is 6% greater in C57b/6J mice compared with C57b/Ola-HSD mice. The fact that C57b/Ola-HSD mice have complete chromosomal absence of α-synuclein but still succumb to MPTP neurotoxicity suggest a more complex role of α-synuclein for exacerbating DA cell loss in PD pathology. It has been reported that un-aggregated α-synuclein, as well as being a major component of PD development, is also found to confer neuroprotection (Chandra et al., 2005). The detrimental effects of α-synuclein on DA neuron function only seem to manifest once it has become aggregated (Villar-Pique et al., 2016). It is plausible that C57b/6J mice enjoy a higher degree of protection against MPTP neurotoxicity due to their normal α-synuclein expression levels, whilst the DA neurons in C57b/Ola-HSD mice might be more vulnerable due to its absence.

With the primary objective of uncovering the potential neuroprotective properties of NLX-112 in an MPTP mouse model of PD, the rationale for selecting the C57b/Ola-HSD strain, over the C57b/6J strain becomes apparent. This choice stems from the fact that both C57b sub-strains experience similar levels of DAergic cell loss in the substantia nigra (SN) and reduced levels of tyrosine hydroxylase-positive (TH+ve) fiber density in the striatum following MPTP treatment. But, it is imperative to prioritise the ethical treatment and welfare of the mice. C57b/Ola-HSD mice exhibit resistance to the acute peripheral toxicity of MPTP, thereby reducing the risk of cardiac arrest and any untimely losses. To investigate the potential neuroprotective properties of NLX-112 in the MPTP mouse model, the C57b/Ola-HSD strain was employed. This choice facilitated the execution of high-throughput experiments crucial for this type of investigation while minimising the risk associated with the acute toxic effects of MPTP.

6.2. Method

6.2.1. Animals

Male C57b/Ola-HSD mice aged 8-10 weeks old were purchased as litter mates from Envigo laboratories and housed in 3's in steel framed Perspex based cages. Food and water were ad-libitum and the amount of materials for nest building, including carboard, tissue, and shredded paper were ample. Exercise equipment such as a running wheel was purposefully omitted due to the fact that exercise can stimulate synaptogenesis in the striatum and basal ganglia of mice that have exercised more than their non-exercising counterparts (Fisher et al., 2004; Smith et al., 2011). A phenomenon which must be avoided when investigating an animal model of neuroprotection.

Ethical awareness must be maintained throughout the entirety of the experimental investigations. From designing the experiments to harvesting the tissue. The three R's: Refine, Reduce, and Replace are key concepts which help researchers carry out animal research with the greatest level of animal care possible whilst also maintaining the appropriate level of scientific rigour.

Before conducting the NLX-112 – MPTP neuroprotection experiments, it is imperative to first establish the pharmacological profile of NLX-112. This initial step

is crucial to identify any physical, behavioural, or motor changes induced by NLX-112 treatment. This is particularly important because mice will receive a dose of NLX-112 and then MPTP, with only a one-hour interval between each. Both drugs target the monoaminergic pathways, as demonstrated in Chapter 2, where we highlighted MPTP's remarkable impact not only on the DAergic pathway, but also on the serotonergic fibre composition of the mouse brain. This includes the reduction in RN 5-HT neuron soma size and hypertrophy of serotonergic fibers in the mPFC and striatum.

Consequently, it is important to consider the potential for acute synergistic interactions between NLX-112 and MPTP that may occur at the same serotonergic sites. To gain a deeper understanding of how this potential interaction might affect the mice, it is necessary to administer NLX-112 to mice on its own, without any MPTP treatment. This approach will in addition allow for the collection of relevant qualitative observations drawn from the initial open-field tests. By doing so, a clearer understanding of the implications of NLX-112 treatment and its potential effects on the treated mice can be achieved.

6.2.2. Drugs

6.2.2.1. MPTP

Preparation, and administration of MPTP is described in detail in Chapter 5. Due to the acute toxicity of MPTP, mice received MPTP following NLX-112 administration (approximately 30 mins).

6.2.2.2. NLX-112

PET radiotracer analysis using the agonist PET tracer [^{18}F]-F13640, reveals NLX-112 is widely distributed throughout the midbrain and cortex of rat, cat, primate, and human brain (Figure 38). The widespread binding distribution of NLX-112 in the brain, coupled with its strong affinity for the 5-HT$_{1A}$ receptor, raises concern about the potential for excessive physiological 5-HT, especially if too great a dose of NLX-112 is administered to the mammalian nervous system (including mice) (Haberzettel et al., 2013: 2015). Conversely, administering too low a dose of NLX-

112 may fail to elicit the required neuroprotective effect on the MPTP-lesioned DAergic nigro-striatal tract.

6.1.1.1. NLX-112

A

Species	Rat	Cat	Primate	Human
Agonist PET tracer: [^{18}F]-F13640 (i.e., ^{18}F-NLX-112)				
Raphe nuclei	++	+++	+++	+++
Striatum	++	++	+++	+++
Amygdala	NV	NV	+++	+++
Hippocampus	++	++	++	++
Thalamus	++	++	+++	++
Frontal Cortex	++	++	++	++
Occipital Cortex	++	++	++	++
Cerebellum	+	+	+	+
Brain stem	–	–	–	–

B

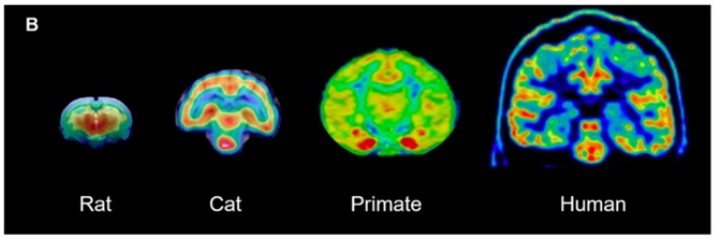


Figure 38. PET imaging with the 5-HT$_{1A}$ agonist tracer [^{18}F]-F13640 shows species-dependent differences in brain labeling. Raphe nuclei and striatum exhibit consistent binding across species, while cortical and limbic regions, particularly the amygdala, show stronger and more widespread binding in primates and humans compared to rodents. Cerebellum and brainstem show no specific labeling in any species. These findings highlight the enhanced cortical distribution of 5-HT$_{1A}$ receptors in higher species. Adapted from Newman-Tancredi et al., (2022).

From our previous research, we established that single doses of NLX-112 are well tolerated within the range of 0.1 mg/kg to 1 mg/kg in older C57b/6J mice. Detailed discussions of this can be found in Chapter 4, where the locomotor-inducing effects of NLX-112 in older C57b/6J mice were reported at these specified doses. It became evident from the data that the most effective dose for inducing locomotor activity was 0.3 mg/kg, and this trend remained consistent for at least 20 minutes after dosing, as confirmed through observations in the Open Field Test (OFT). However, when the dose was increased to 1.0 mg/kg, the magnitude of locomotor activity began to decline (Figure 39). While this dose did result in a significant increase in locomotor activity compared to the saline treatment, it appeared that there might be an emerging dose-toxicity effect. It is important to note that these experiments were conducted on older mice, specifically those at 6 months of age,

and they were of the C57b/6J sub-strain, not the C57b/Ola-HSD sub-strain. Nonetheless, the data strongly suggests that doses at or above 1.0 mg/kg could potentially have adverse effects on the overall health of the mice, introducing a potential source of error to the rest of the experiment. Thus, a daily dose of 1.0 mg/kg has been selected to investigate the potential neuroprotective properties of NLX-112 in vivo. A well-considered decision regarding whether to proceed with this dose was made after the initial series of NLX-112 doses were administered to mice on day 1 of the experiment.

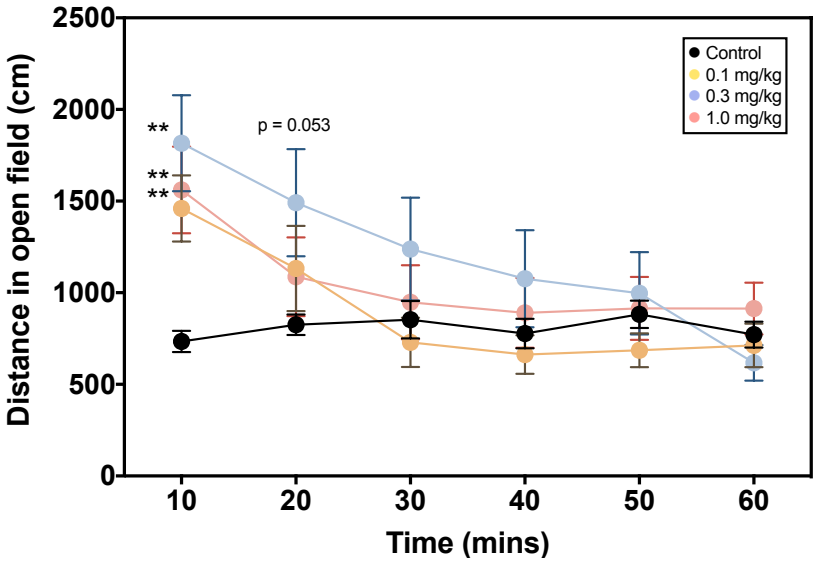


Figure 39. Effect of NLX-112 on locomotor activity in middle aged C57b/6J mice over 60 minutes in the open field test (OFT). Mice were treated with saline (control) or NLX-112 at doses of 0.1, 0.3, or 1.0mg/kg (i.p.) and the distance traveled was recorded in 10-minute intervals. NLX-112 induced a dose-dependent increase in locomotor activity compared to controls, particularly during the initial 20 minutes, with the effect diminishing over time. Data are presented as mean ± SEM (n = 10 per group). Statistical analysis was performed using two-way ANOVA with significance indicated (**p < 0.005) versus control.

6.2.2.3. WAY100635

NLX-112 is an exceptionally selective biased agonist for the 5-HT_{1A} receptor which has been confirmed via signal transduction profile studies performed on various cell lines in vitro (Newman-Tancredi et al., 2017). Further still it has also been confirmed in several animal studies that have investigated the various therapeutic

benefits of NLX-112 by blocking 5-HT_{1A} with the 5-HT_{1A} receptor antagonist, WAY100635. These studies include reversing the benefits of NLX-112 in a PD rat model of LID (Ideberg et al., 2015); inhibiting NLX-112's alleviating properties in the formalin pain test in mice (Salat et al., 2017); and inhibiting NLX-112's effect for alleviating urinary tract dysfunction in rats following spinal cord injury (Lin et al., 2020).

WAY100635 was purchased from Tocris® and used in this study to identify whether any of the potential neuroprotective and/or locomotor effects of NLX-112 could be inhibited by blocking the 5-HT_{1A} receptor with WAY100635. This was achieved by administering WAY10065 at 2.0mg/kg to mice 30 minutes before they were to receive NLX-112.

6.2.3. Dosing protocol

In Chapter 3, it was unveiled that the neuroprotective properties of NLX-112 *in vitro* expressed their protective effects on SH-SY5Y cells once the process of cell death and the apoptotic cascade had already been initiated. Notably, an approximate 10% increase in cell survival was observed in cells treated with MG132 and MPP^+ when NLX-112 was administered subsequently, as opposed to when it was administered beforehand. However, this time point at which NLX-112 confers its protective properties may not translate to an in vivo setting. The *in vitro* findings will serve as a valuable reference for establishing the optimal drug dosing protocol. This is crucial to prevent any false negatives and the potential oversight of NLX-112's neuroprotective effects in vivo. For accurate identification of any potential neuroprotection, it is imperative that all possible scenarios are explored. To achieve this, the dosing regimen must be meticulously designed to include the administration of NLX-112 to animals before, during, and after MPTP treatment. This comprehensive approach helps ensure that chances of capturing NLX-112's neuroprotective properties under various conditions are maximised.

From days 6 – 10, mice from groups 1-4 received 2 x doses (s.c) of either saline + saline, NLX-112 + saline, MPTP + saline, or MPTP + NLX-112, and mice from group 5 received WAY100635 + NLX-112 + MPTP, with all dose treatments separated by 30 minutes. Volumes of these multiple doses were given so as not to

exceed the maximum dose. For example, for a 23 g mouse in the MPTP + NLX-112 group, the cumulative volume of multiple doses must not exceed 20 mL/kg (for a 23 g mouse this would equate to 460 µL); the maximum number of bolus s.c injections must not exceed 60. The maximum for this experiment was 25.

NLX-112 was provided by Neurolixis and prepared freshly in vehicle (0.9% NaCl) at a working solution of 1mg/8mL (0.25% DMSO). Mice from NLX-112 treatment groups received a single daily dose of NLX-112 at 1mg/kg/day for 15 consecutive days (day 1 to day 15). MPTP was purchased from Sigma® and diluted with vehicle (normal saline) on the day of experiment. Mice from MPTP treatment groups were administered with an incremental daily dose of MPTP•freebase from days 6 to 10. The study involved five groups of mice: (i) vehicle-treated (saline + 0.25% DMSO) control mice, (ii) NLX-112-treated mice which received daily doses (1 mg/kg i.p.), (iii) mice treated with MPTP, (iv) mice treated with both NLX-112 and MPTP, and mice treated with WAY100635, NLX-112 and MPTP (Table 8).

As already stated, mice from the NLX-112 treatment group and mice from the NLX-112 + MPTP treatment group received a daily dose of NLX-112 at a concentration of 1mg/kg using the same equation for calculating the dose of MPTP (for drug dose calculation, see Chapter 2). So, for a 20g mouse being administered a dose of NLX-112 at 1mg/kg they will receive a volume of 160µL taken from the 1mg/8mL stock solution.

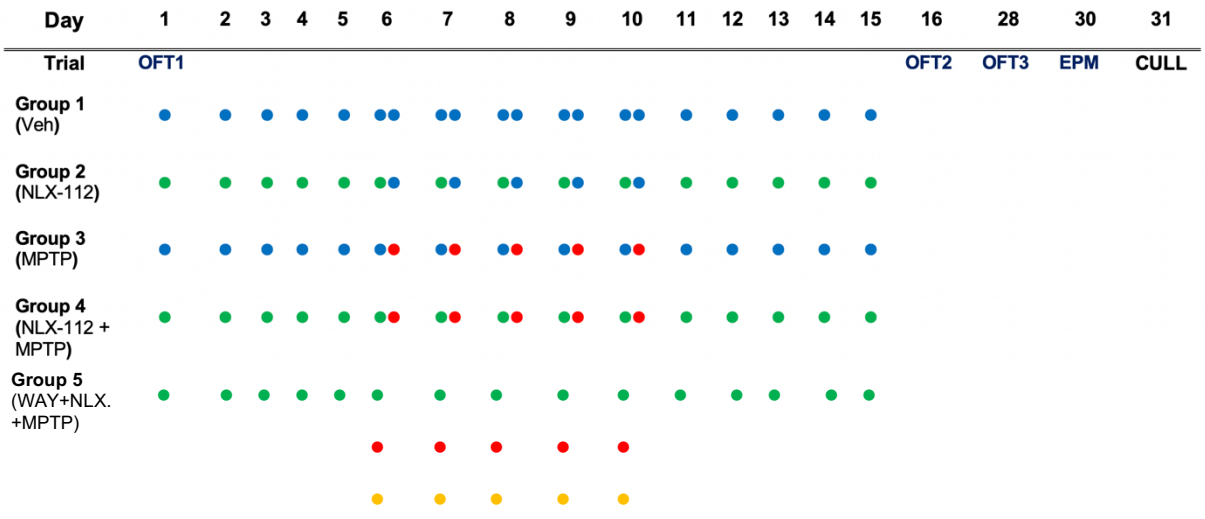
Over the course of 15-day treatment mice being administered with NLX-112 will have received a cumulative dose of 15mg/kg. Over the course of 5-day treatment mice being administered with WAY100635 will have received a cumulative dose of 10mg/kg. Over the course of 5-day treatment mice being administered with incremental single daily doses of MPTP (11.11 /day1; 21.37/day2; 27.35/day3; 29.91/day4; 29.91/day5) will have received a cumulative dose of 119.65 mg/kg (Table 8).

6.2.4. Apparatus

All mice underwent three trials in the open field. The first open field test (OFT) was conducted five minutes immediately after the initial NLX-112 dose. This time delay was also applied to mice receiving saline as their first daily dose. Acute behavioural

changes were assessed for all four groups during the second OFT, which occurred six days after the last MPTP dose. The timing of the second OFT could not be carried out sooner due to the necessary washout period required for MPTP-treated mice, allowing for the elimination of MPTP metabolites through urine and feces (Przedborski et al., 2004). The third OFT was conducted 18 days after the final MPTP dose to evaluate any delayed behavioural changes resulting from vehicle

Table 8. Experimental design and dosing regimen for saline, NLX-112, MPTP, and combinatorial treatments. The timeline depicts the subcutaneous injection schedule and behavioural testing across five experimental groups from Day 1 to Day 31. Each dot represents a single injection: blue = saline (vehicle), green = NLX-112, red = MPTP, and yellow = WAY-100635. Group 1 (Veh) received saline injections daily as a control. Group 2 received NLX-112 alone from Days 1–15. Group 3 received MPTP alone, administered twice daily from Days 6–10 to induce DAergic neurotoxicity. Group 4 received NLX-112 (Days 1–15) in combination with MPTP (Days 6–10), to assess the neuroprotective potential of NLX-112. Group 5 received NLX-112 and MPTP as in Group 4, with the addition of WAY-100635 (Days 6–10) to block 5-HT_{1A} receptors and evaluate receptor-specific mediation of NLX-112's effects. All mice underwent behavioural assessments including Open Field Tests (OFT1, OFT2, OFT3 on Days 1, 16, and 28, respectively) to evaluate locomotor activity, and the EPM, Day 30) to assess anxiety-like behaviour. Animals were sacrificed on Day 31 (CULL) for post-mortem neurochemical and histological analyses. The design enables assessment of acute and long-term effects of NLX-112 treatment, MPTP toxicity, and the role of the 5-HT_{1A} receptor in potential neuroprotection.



Saline 0.9% NaCL (max: 20 ml/kg) ●

NLX-112 (1.0mg/kg) ●

MPTP (11.11 – 29.91 mg/kg freebase) ●

WAY100635 (2.0mg/kg) ●

(saline) NLX-112, MPTP, MPTP+NLX-112 or WAY+MPTP+NLX-112 treatments. Additionally, the elevated plus maze (EPM) was utilised only once on day 30, twenty days after the last MPTP dose. By subjecting the mice to the EPM, two key objectives were achieved: firstly, the EPM was employed to extrapolate anxiety-like behaviour, which may vary upon MPTP or NLX-112 treatment; and secondly, the EPM served as a behavioural control assay, especially for locomotor activity, to contrast with the OFT maze exposure, which could lead to maze habituation (File et al., 1998). Any potential changes in motor activity observed in mice during their trial in the EPM could therefore not be attributed to maze habituation.

6.1.1. Behaviour and locomotor activity

Each trial was carried out with a single cage of littermates, each housed in their box. Each trial took approximately 15 minutes to perform, that is: dosing (5 mins); the trial itself (5mins) and preparation for next group (5 mins), which included cleaning OFT boxes with water, disinfectant, ethanol, and water (all in that order). Each trial was performed with littermates that were assigned to their respective trial. This approach was chosen as opposed to total randomisation due to the potential interference that mice from another cage could have each mouse's scent and thus, awareness (Lacey et al., 2007; Arakawa et al., 2008) - leading to possible error in the assays.

6.1.2. Immunohistochemistry

For a detailed description of brain preparation for IHC analysis, please refer to Chapter 2. In brief, all brains were initially stored in 10% buffered formalin for 48 hours. Subsequently, they were transferred to Eppendorf tubes containing PBS with 30% sucrose. After an additional approximately 48 hours, the brains were removed from the Eppendorf tubes and coronally sectioned into 30µM slices using a cryostat and microtome. For stereological analysis, sections were systematically stored by adding every 10th section to each individual well of a 24-well plate. The caudate putamen (CPu) and substantia nigra (SN) are the two brain regions of primary interest. These regions were examined for DA neurons in the SN and DA nerve fibers in the CPu using a Tyrosine Hydroxylase antibody. Inflammation, specifically reactive gliosis, was assessed in the same brain regions utilising GFAP antibody, which is immunoreactive for astrocytes, and Iba1, which is

immunoreactive for microglia. To explore potential neuroprotective mechanisms through the upregulation of neurotrophic factors, IHC analysis was also conducted using GDNF antibody.

6.1.3. Statistical analysis

Apart from the body weight analysis which was carried out using a two-way ANOVA. All additional data sets were compared for differences using a one-way ANOVA followed by Fishers LSD post hoc. Statistical analysis was performed using the Prism Graph-Pad V9.

6.2. Results (C57b/Ola-HSD)

In these experiments mice were subjected to one of four conditions: vehicle (saline), MPTP, NLX-112 and NLX-112+MPTP. Each of these distinct conditions garnered various results when behaviour locomotor activity was analysed in the OFT-1, -2, and -3, and in the EPM, also. OFT1 and OFT2 were both conducted 5 minutes after dosing with NLX-112 or saline.

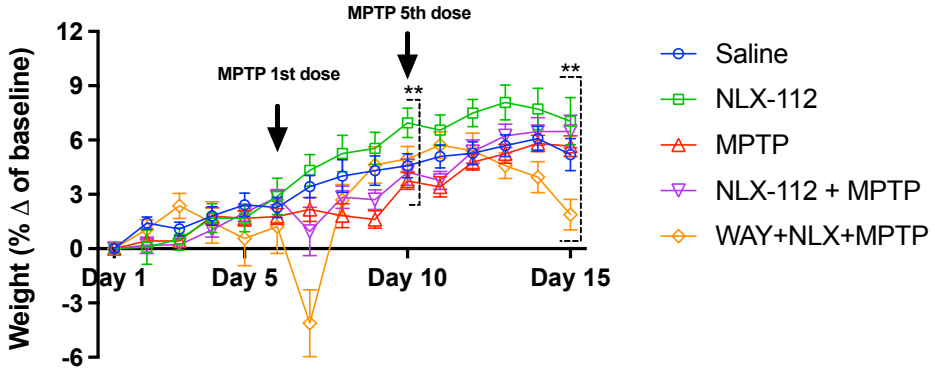


Figure 40. Change in body weight over time across treatment groups**.** Body weight change from baseline (Day 1) is plotted for each treatment group across the 15-day treatment period. Groups include Vehicle (blue), NLX-112 (green), MPTP (red), NLX-112 + MPTP (purple), and WAY-100635 + NLX-112 + MPTP (orange). On Day 10, all groups differed significantly from the NLX-112 alone group (green) **(**$p < 0.005$**)**, indicating the impact of MPTP toxicity and co-treatment on weight gain trajectories. By Day 15**,** all groups differed significantly from the WAY + NLX-112 + MPTP group (orange) **(**$p < 0.005$**)**, suggesting a potential exacerbating or blocking effect of 5-HT_{1A} receptor antagonism on recovery or weight maintenance. This figure highlights distinct physiological trajectories in response to neurotoxic insult and serotonergic modulation. Data represent means ± SEM (n = 7–12 per group). A two-way ANOVA followed by Fisher's Least Significant Difference (LSD) post hoc test.

6.2.1. Pharmacodynamic response to NLX-112

Mice in the neuroprotection group and the NLX-112 control group all received NLX-112 at 1mg/kg. Five minutes after mice had received their first dose, dose effects were assessed in the open field for five minutes duration (Table 9).

Table 9. Acute behavioural effects of NLX-112 (1mg/kg) and reversal by 5-HT_{1A} antagonism. Five minutes post-dose, NLX-112-treated mice displayed atypical open-field behaviour, failing to explore the perimeter and exhibiting subdued activity, backward stepping, rapid rotations, mild piloerection, tail elevation, and slight tremors. Rearing was reduced, and mice showed increased social clustering and appetite in the home cage. All effects resolved within 30 minutes and were completely blocked by pre-treatment with the 5-HT_{1A} antagonist WAY-100635, confirming a 5-HT_{1A} receptor-mediated mechanism.

Pharmacodynamic response to NLX-112 (1mg/kg) (Five mins post-dose)
1. When placed in the open-field mice do not re-locate to the perimeter as per the behaviour of an untreated or saline treated mouse
2. Mice are subdued and seem unsure of their surroundings
3. They perform backwards steps with quick side to side rotations
4. Mild piloerection
5. Mild tail erection
6. Mild shaking
7. Once OFT trial was completed mice were placed back in their cage. Mice housed in 3s would bunch together outside of their nest. An increased appetite in NLX-112 treated mice was seemingly greater than their saline treated counterparts
8. Decreased rearings
9. After 30 minutes from dosing mice resumed normal behaviour comparable to that which was observed in the saline treated group.
10. The above physiological responses to an acute dose of NLX-112 were all blocked following prior treatment with the 5-HT_{1A} antagonist WAY100635

The observations mentioned above were qualitative but consistently exhibited by all mice treated with NLX-112. These results suggest that mice treated with NLX-112 may be displaying mild symptoms associated with excess physiological 5-HT, as noted in previous studies by Haberzettel et al. (2013, 2015). Additionally, 6-month-old mice were treated with acute doses of NLX-112, ranging from 0.1 mg to 1 mg/kg. These doses were well tolerated and, interestingly, resulted in an increase in locomotor activity, contrasting with locomotor suppression observed in younger

mice (see Chapter 4). Given these results and the fact that mice fully recover after 30 mins it was deemed appropriate that administering mice with single daily doses of 1mg/kg is safe and tolerable, and thus the most appropriate dose to keep using for the full duration of the experiment.

6.2.2. The open field and elevated plus maze

The OFT and EPM assays have unearthed a vast array of NLX-112' s properties in mice, that were previously unknown. Over the course of the OFT trials (1 through to 3) saline and MPTP treated mice exhibit a similar response (Figure 41 A and C), in that locomotor activity steadily decreases due to maze habituation – a phenomenon already discussed previously. At 1mg/kg, NLX-112 treatment, as already mentioned likely causes excess availability of physiological 5-HT which has a temporary effect in subduing other fine motor skills, but for no longer than 30 mins, after which mice resumed their normal characteristics and behaviour. In OFT2 there is a slight non-significant increase in locomotor activity in NLX-112 treated mice (Figure 41 G), with that in mind, as with OFT1, the OFT2 trial is carried out 5 minutes after NLX-112 had been administered to mice, therefore the response and behaviour exhibited in OFT1 and OFT2 are reflective of NLX-112's acute effects and not its chronic properties.

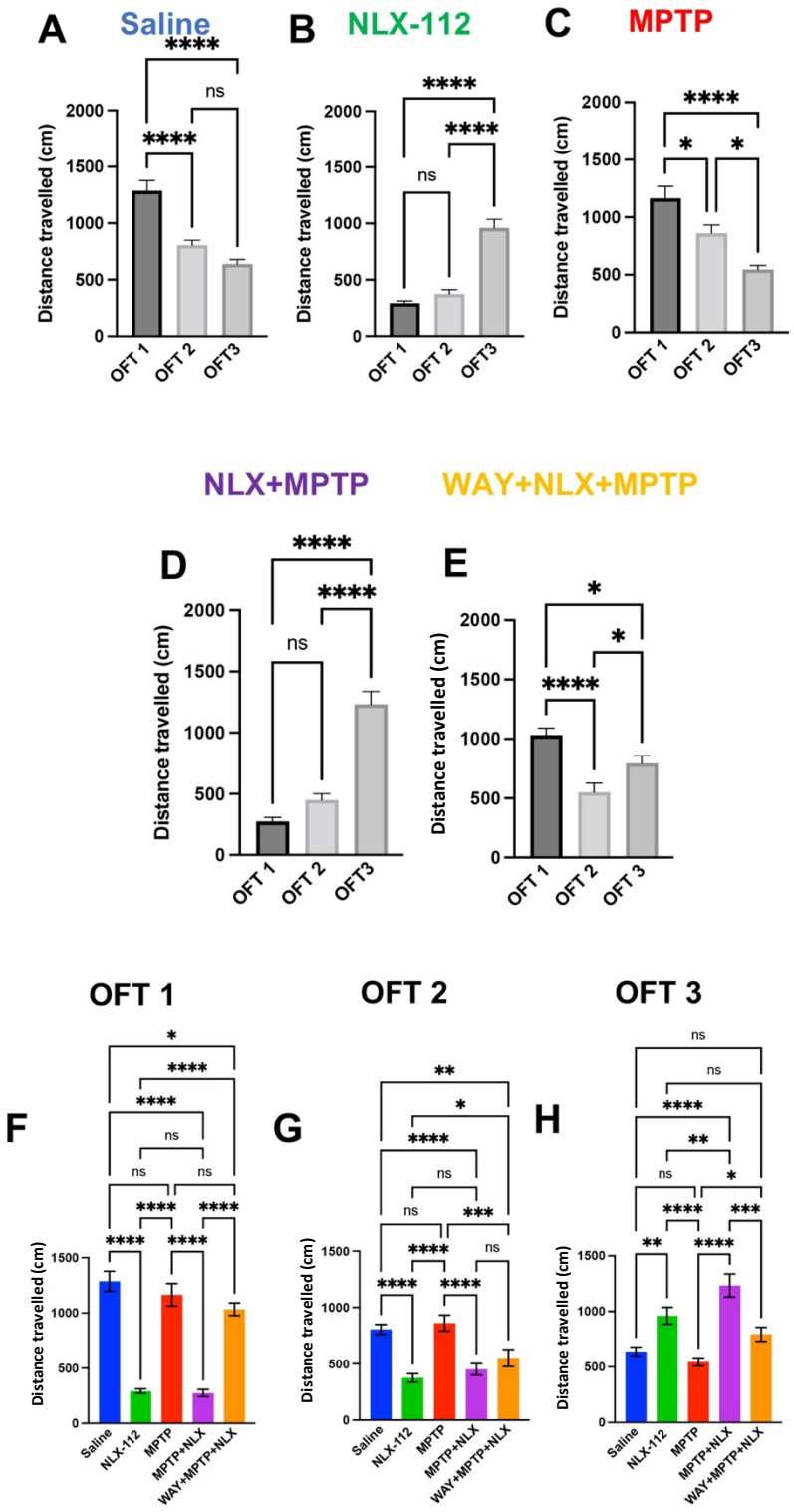


Figure 41. Locomotor activity measured by distance travelled across three open field tests (OFT 1–3) in C57b/6J mice under different treatment conditions. Panels A–E show within-group comparisons across time. Saline-treated mice (Panel A) displayed significantly greater activity in OFT 1 compared to OFT 2 and OFT 3 ($p < 0.0001$). NLX-112-treated mice (Panel B) had low activity in OFT 1, which significantly increased in OFT 2 and OFT 3 ($p < 0.0001$). MPTP-treated mice (Panel C) showed high activity in OFT 1 that progressively declined in subsequent

sessions ($p < 0.05–0.0001$). Mice treated with NLX-112 + MPTP (Panel D) showed low activity in OFT 1 and OFT 2, followed by a significant increase in OFT 3 ($p < 0.0001$). In the WAY-100635 + NLX-112 + MPTP group (Panel E), activity declined from OFT 1 to OFT 2 and OFT 3 ($p < 0.0001–0.05$). Panels F–H compare treatment groups within each session. In OFT 1 (Panel F), MPTP-treated groups travelled significantly more than saline and NLX-112-alone groups ($p < 0.05–0.0001$). In OFT 2 (Panel G), distance travelled was significantly greater in the NLX-112 + MPTP group than in the MPTP-only group ($p < 0.01$), while the addition of WAY-100635 significantly reduced this effect. In OFT 3 (Panel H), the NLX-112 + MPTP group maintained significantly higher activity compared to both MPTP and NLX-112-alone groups ($p < 0.01–0.0001$), while the WAY-treated group remained low. One-way ANOVA n= 7-12. Error bars denote SEM.

Following OFT2, mice were housed back in their normal cages and were not subjected to any additional experimental treatments or maze assays. They were handled daily but only to be weighed and then placed immediately back into their cage. OFT3 was performed two weeks after any saline, NLX-112 or MPTP treatment, and the results were particularly astonishing. NLX-112 appeared to have had a residual effect on locomotor activity whereby the distance travelled by NLX-112+MPTP treated mice was over 100% greater than saline treated mice [$F(19.53 (3, 43) = 4.071$ $p < 0.0001$] (Figure 41 H), an effect that was significantly blocked by WAY100635.

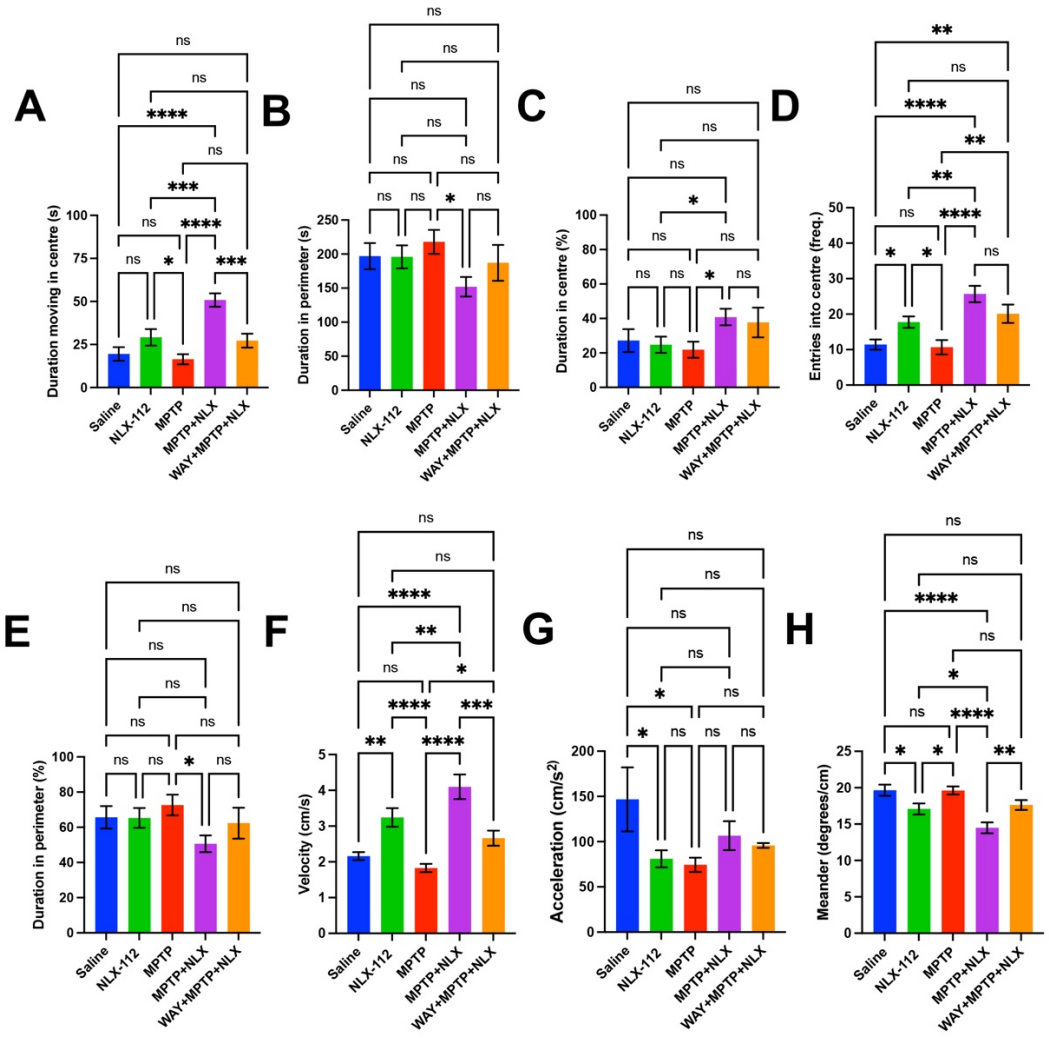


Figure 42. Specific behavioural and kinetic parameters exhibited by C57b/Ola-HSD mice in OFT 3. Open field behavioural metrics for C57b/6J mice treated with saline, NLX-112, MPTP, NLX-112 + MPTP, or WAY-100635 + NLX-112 + MPTP. Panel A shows a significant reduction in time spent moving in the centre for MPTP-treated mice compared to saline, NLX-112, and NLX-112 + MPTP groups ($p < 0.0001–0.05$). The NLX-112 + MPTP group exhibited significantly more centre movement than MPTP alone ($p < 0.001$). Panel B indicates no significant differences in perimeter distance among most groups, except for a small reduction in the MPTP + NLX-112 group compared to MPTP alone ($p < 0.05$). Panel C shows a modest increase in percentage of time spent in the centre for NLX-112 + MPTP compared to MPTP alone ($p < 0.05$). Panel D shows that MPTP-treated mice entered the centre significantly less frequently than all other groups ($p < 0.0001–0.05$), while NLX-112 + MPTP animals had significantly more centre entries than MPTP alone ($p < 0.0001$). Panel E shows no significant differences in perimeter duration between groups, except for a slight reduction in the MPTP group ($p < 0.05$ vs NLX-112 + MPTP). Panel F shows significantly reduced velocity in MPTP mice compared to saline, NLX-112, and NLX-112 + MPTP ($p < 0.0001–0.01$). NLX-112 + MPTP showed greater velocity than NLX-112 alone ($p < 0.05$). Panel G indicates that MPTP significantly reduced acceleration compared to saline and NLX-112 + MPTP ($p < 0.05$), while other comparisons were non-significant. Panel H shows

that MPTP increased path meander ($p < 0.0001$ vs saline), while NLX-112 + MPTP significantly reduced meander compared to MPTP alone ($p < 0.0001$). The addition of WAY-100635 attenuated these effects in some metrics. One-way ANOVA n= 7-12. Error bars denote SEM.

This pattern is continued when other specific behavioural parameters examined in the OFT3. For example, NLX-112+MPTP treated mice have reduced thigmotaxis, spending less time in the perimeter of the open field, an effect which again was blocked by WAY100635 suggesting that NLX-112 has residual anxiolytic property in the MPTP lesioned brain [$F(2.635\ (3, 43) = 0.3657\ p < 0.0619$] (Figure 42 E). Further still, the number of entries made into the centre of the arena were significantly greater in NLX-112 and NLX-112+MPTP treated mice compared to both saline and MPTP treated mice [$F(17.3\ (3, 43) = 0.9372\ p < 0.0001$] (Figure 42 D), however this effect was not blocked by WAY100635. Acceleration was significantly reduced in NLX-112 and MPTP treated mice, but not NLX-112+MPTP treatment, compared to saline treatment (Figure 42 G). Scientifically, a relatively unexplored behaviour, but one which is relevant to address in this instance is the 'meander' behaviour. This behaviour is determined by calculating the amount of times a mouse moves from side to side whilst travelling in a forward direction. In NLX-112+MPTP treated mice, meander is significantly reduced compared to saline and MPTP treated mice but not NLX-112 only treated mice (Figure 42 H), this effect is significantly inhibited by prior treatment with WAY100635. This possibly suggests that NLX-112 induces a more purposeful behavioural state in mice. This is of course speculative, but reflects a consistent pattern repeatedly exhibited by mice that have been treated with NLX-112 but predominately, treatment with NLX-112+MPTP.

It can be argued that these behaviours exhibited by NLX-112 and NLX-112+MPTP mice are simply due to the acute doses of NLX-112 inhibiting the mouse's normal conscious exposure to the maze paradigm. In that, the acute excess levels of physiological 5-HT mute the maze habituation phenomenon. The greatest counter against this argument however, is that in the EPM, in which mice were only exposed to once, NLX-112+MPTP treatment still elicited the same significant increase in locomotor activity compared to all other groups [$F(5.771\ (3, 40) = 0.5968\ p = 0.0022$] (Figure 43 A). But unlike OFT3, this locomotor effect was not

blocked by WAY100635 and restricted to NLX-112+MPTP treated mice only and not NLX-112 treated mice, (Figure 43 A).

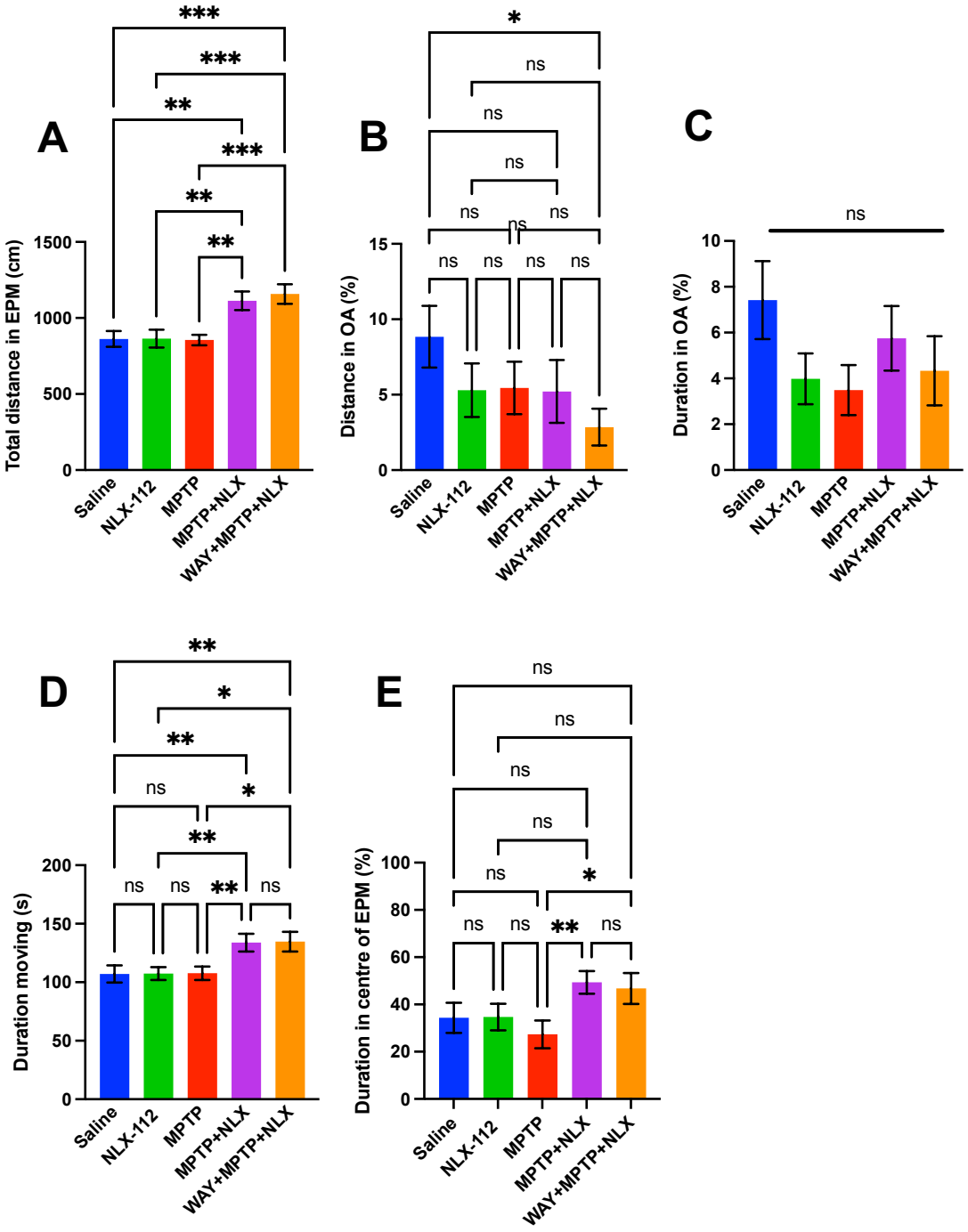


Figure 43. Behavioural effects of various treatments on mice assessed in the EPM. A) Total distance travelled was significantly greater in the MPTP + NLX-112 and WAY + MPTP + NLX-112 groups compared to MPTP alone ($p < 0.001$). B) The WAY + MPTP + NLX-112 group travelled significantly less distance in the open arms compared to the saline group ($p < 0.05$). C) No significant differences were observed in the percentage of time spent in the open arms. D) Duration of movement was significantly greater in the MPTP + NLX-112 ($p < 0.01$) and WAY

+ MPTP + NLX-112 ($p < 0.05$) groups compared to MPTP alone. E) Both MPTP + NLX-112 and WAY + MPTP + NLX-112 groups spent significantly more time in the centre of the EPM than the MPTP group ($p < 0.05$). One-way ANOVA followed by Fishers LSD post hoc. n= 10-12. Error bars denote SEM.

The residual anxiolytic properties are also likely an unknown feature of sub-chronic dosing with NLX-112, as already shown with reduced thigmotaxis in OFT3; this theory is further reinforced as NLX-112+MPTP treated mice spent markedly longer in the centre of the EPM compared to all other treatment groups [$F(2.613\ (3, 43) = 0.8037\ p = 0.0634$] (Figure 43 E). This effect was not blocked by WAY100635, which may be due to WAY'100635 having a known anxiolytic property of its own (Nunes-de-Souza et al., 2011).

6.2.3. TH+ve cells and fibres (stereologiocal count)

Stereological counting of TH+ve neurons in the SNpc has revealed that a sub-chronic MPTP dosing regimen causes loss of these neurons by approximately 40%, compared to saline treatment.

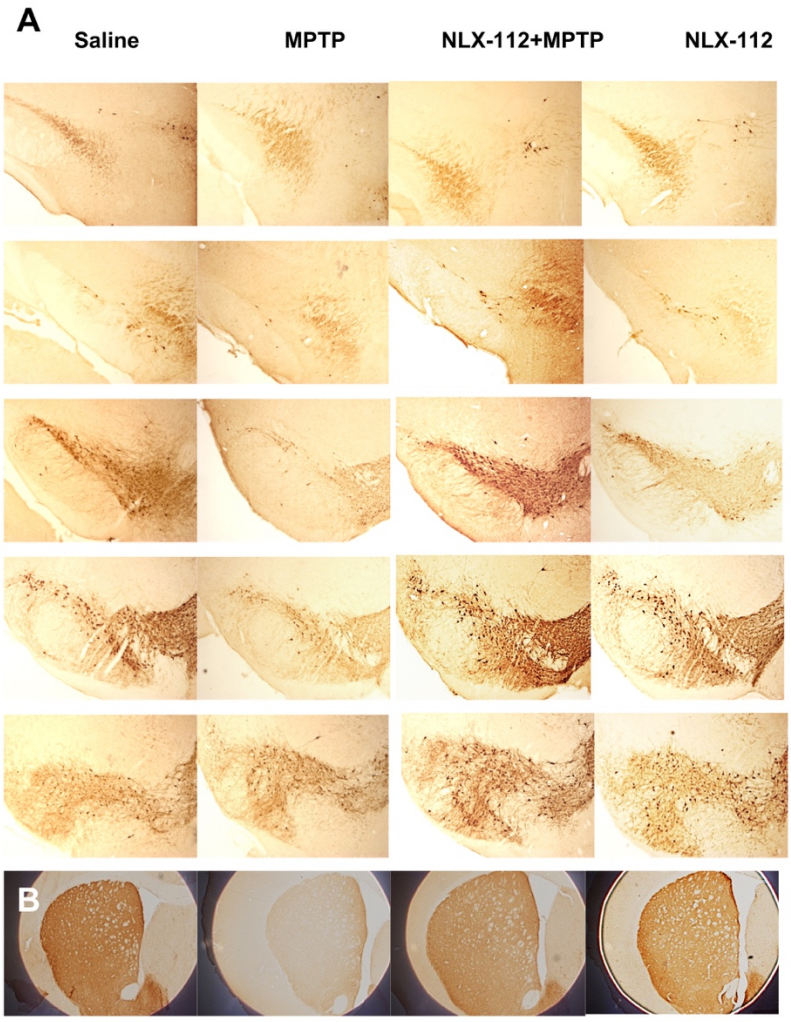


Figure 44. A) Representative images of TH+ve stained SN in saline, MPTP, NLX-112 and NLX-112+MPTP C57b/Ola-HSD mice. Sections were taken from Bregma -2.50 at the nigrostriatal bundle through to Bregma -3.90 at the caudal portion of the SNpc. B) Mouse striatum treated with TH. Somatosensory cortex indicated as this was used to subtract image background for calculating optical density. Bregma 1.09 – 0.85. X 2.5 magnification**.** One-way ANOVA followed by Fishers-LSD. Error bars denote SEM. n = 10-11.

Moreover, the NLX-112 treatment regimen rescues this loss almost completely [$F(15.03\ (3, 29) = 0.2503\ p < 0.0001$] (Figure 44 A and 45 A). These protective effects are further reflected in the striatum where MPTP causes a similar loss in TH+ve fibre density and NLX-112 recovers against this loss [$F(7.804\ (3, 39) = 0.6921\ p < 0.001$] (Figure 44 B and 45 B). Treatment with NLX-112 alone had no effect of TH+ve cell number in the SNpc or TH+ve fibre density in the striatum.

Analysis of TH+ve staining in WAY100635 treated mice was carried out only using a single section from the SN. A comparison to corresponding single sections taken from all four other groups was made to estimate any changes in TH+ve cell number

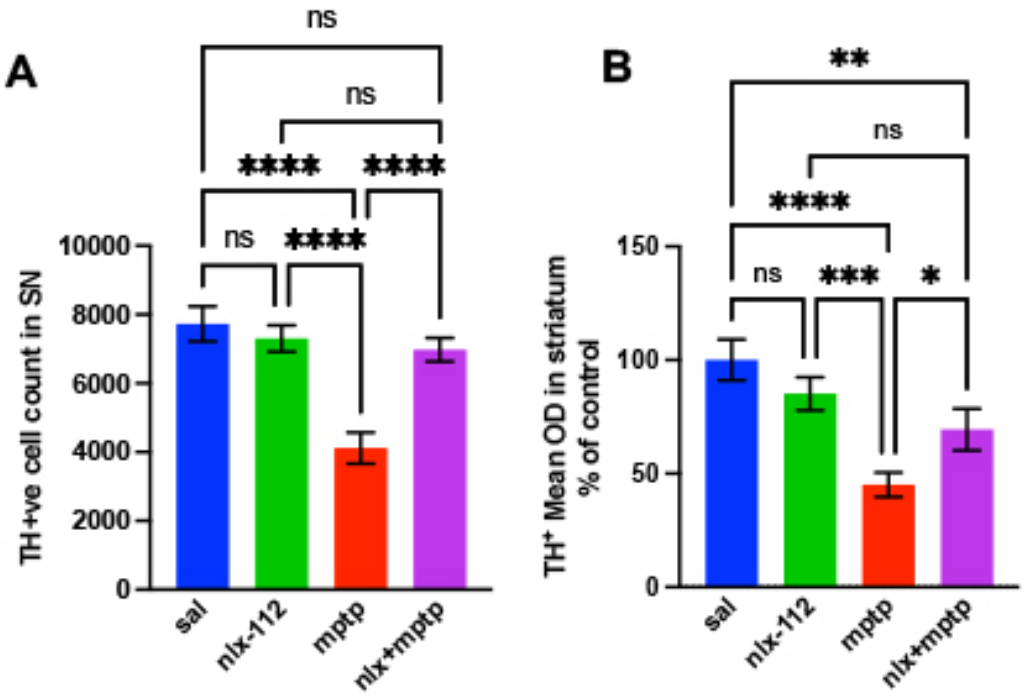


Figure 45. Tyrosine hydroxylase (TH)-positive cell counts and fiber density were assessed in the substantia nigra (SN) and striatum (CPu), respectively. In the SN (Panel A), MPTP treatment significantly reduced the number of TH-positive neurons compared to saline and NLX-112-treated controls (****p < 0.0001), confirming DAergic neurodegeneration. Co-treatment with NLX-112 partially preserved TH+ cell counts compared to MPTP alone (****p < 0.0001), though not fully restoring them to control levels. In the striatum (Panel B), MPTP also significantly decreased TH+ fiber density (****p < 0.0001), while NLX-112 co-treatment provided a modest but significant rescue effect (*p < 0.05). NLX-112 alone did not significantly alter TH+ counts or optical density compared to saline. These results indicate that NLX-112 offers partial neuroprotection against MPTP-induced DAergic damage. One-way ANOVA followed by Fishers LSD. n= 6, error bars denote SEM.

in the presence of WAY+NLX-112+MPTP (see Chapter 2 for description of TH+ve cell counting and comparison analysis). The technique used for estimating TH+ve cell number from a single section is not as valid as comparing multiple tissue sections using stereological quantification, however it does give an indication to any DAergic changes in the SN which can then be compared to other treatment groups.

6.3.4. TH+ve cells (single section count)

As with the stereological TH+ve DAergic cell count in the SN, the analysis comparing single sections for cell count quantification yielded similar results: MPTP

reduces the number of cells in the SN, an effect that is offset in animals supplemented with NLX-112. However, WAY100635 does not block NLX-112's therapeutic effect (Figure 46 A and B).

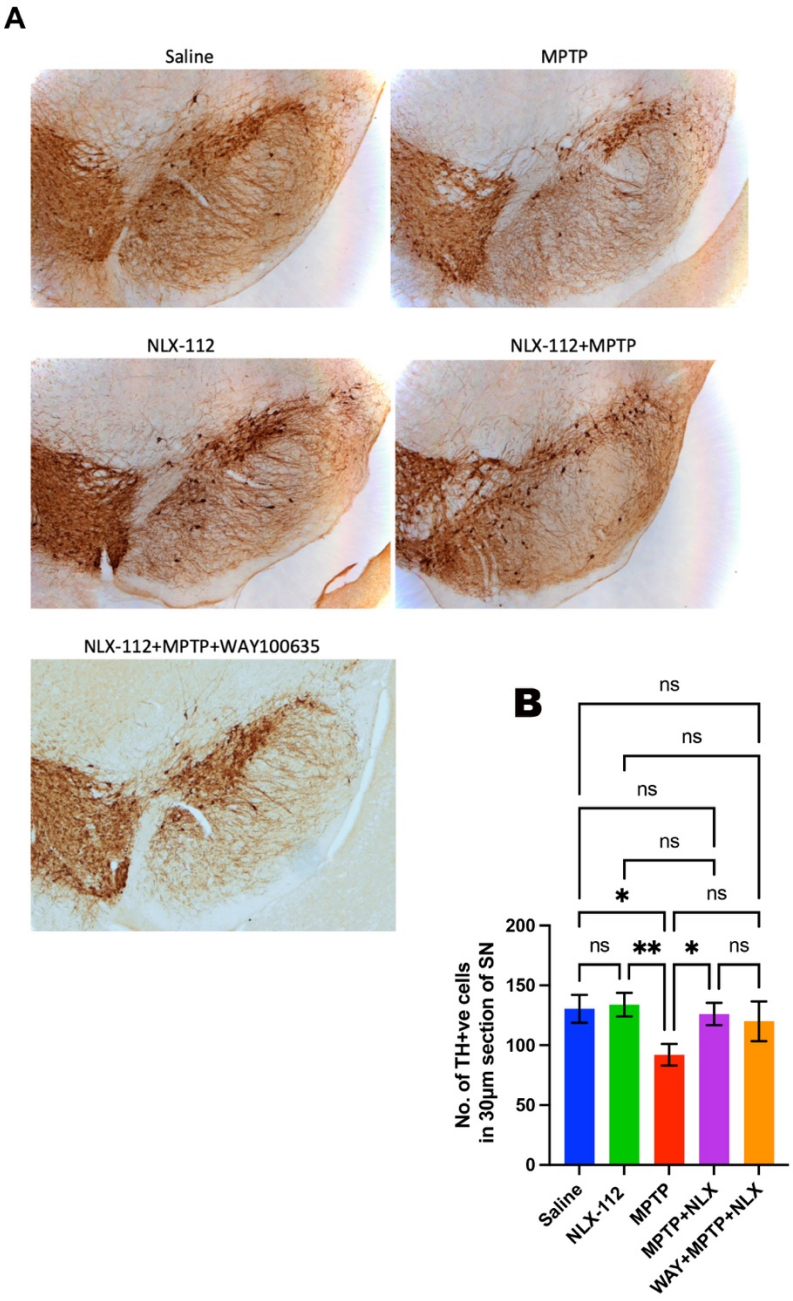


Figure 46. A) Representative TH+ positive (TH+ve) stained images taken from a single section of the substantia nigra (SN), comparing five groups of mice: saline, NLX-112, MPTP, MPTP+NLX-112, and WAY100635+MPTP+NLX-112. B) The number of counted TH+ve neurons in the SN. MPTP significantly reduces the number of neurons compared to saline, which is significantly offset by supplementation with NLX-112. This therapeutic effect is not blocked by prior administration of WAY100635. Statistical analysis was performed using a one-way ANOVA followed by Fisher's LSD test. $p < 0.05$, $n = 12$, error bars denote SEM.

6.3.5. Reactive astrocytosis and microgliosis

Depending on the dosing regimen given, MPTP has the capacity to cause extensive astrocytosis and microgliosis in the lesioned brain. Two key areas that have already shown deterioration of DAergic connectivity, including neurons in the SNpc, and fibres in the striatum and Cpu, were examined for reactive gliosis.

Identification of astrocytosis and microgliosis can be achieved through IHC or IF techniques using antibodies immunoreactive for their respective antigen (Figure 50). Glial Fibrillary Acidic Protein (GFAP) is highly expressed in astrocytes (Figure 50) and Iba1 which is specifically expressed in activated microglia, have each been used to determine the levels of astrocytosis and microgliosis upon MPTP lesioning, respectively.

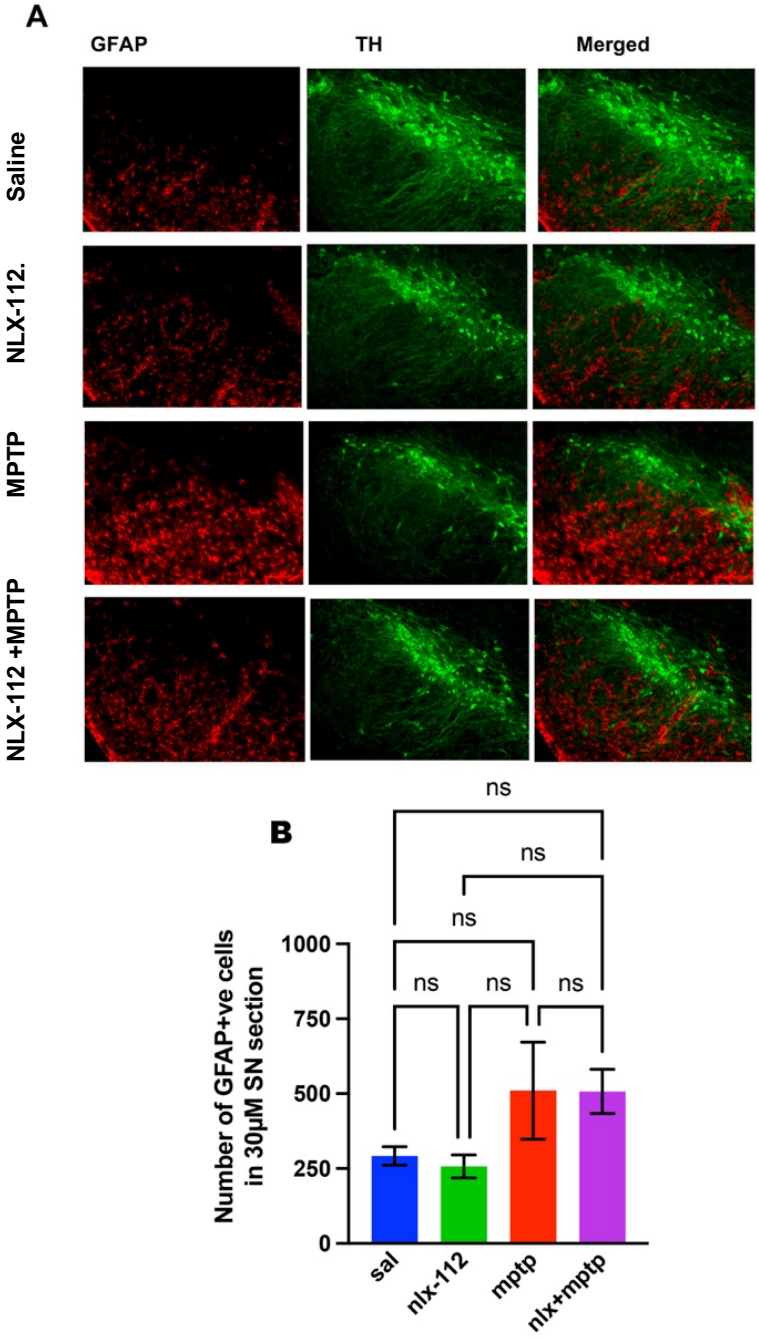


Figure 47. A) Reactive astrocytosis in the SN, stained with TH and GFAP. X 20 magnification. B) There is a clear but non-significant increase in GFAP+ve cells in the in the SN of MPTP treated mice, regardless of NLX-112 supplementation. One-way ANOVA followed by Fishers LSD. Error bars denote SEM. n= 5-6.

Compared to saline treated mice, MPTP caused a loss of TH+ve neurons in the SN (-40%) and TH+ve fibre density in the striatum (-55%), with both effects attenuated by NLX-112 (cell loss only -3% in the SN and fibre loss only -30% in the striatum). The fact that, upon MPTP lesioning, NLX-112 also significantly reduced

Iba1-ir in the SNpr [F(18.60 (3, 18) = 0.4554 p < 0.0001] (Figure 48 A, B) and significantly attenuated GFAP-ir in the mid-CPu [F(10.36 (3, 35) = 0.0006 p < 0.0001] (Figure 51 A, C) suggests that NLX-112 is exerting its protective property via chemical interaction with two major glial pathways.

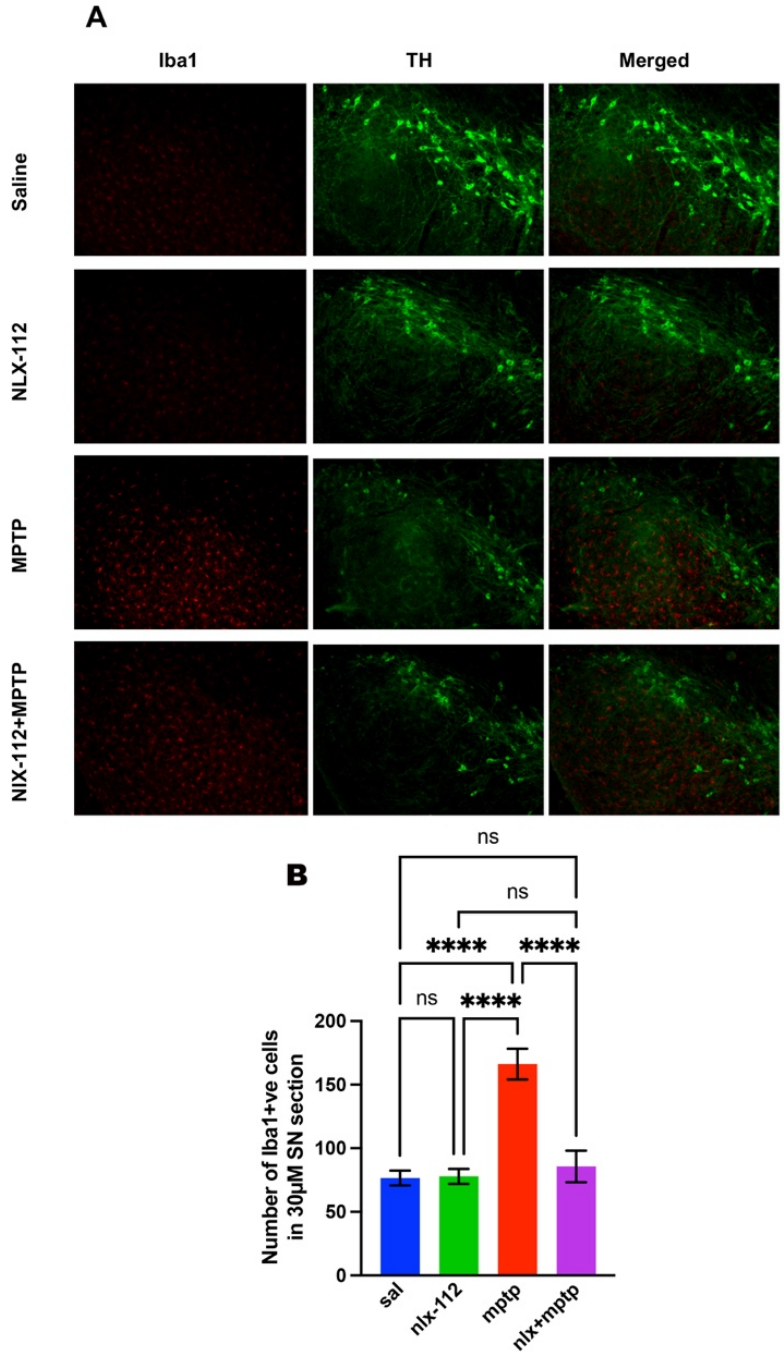


Figure 48. A) Reactive micro-gliosis in the SN stained with TH and ionised calcium-binding adapter molecule 1 (Iba1) X 20 magnification. B) MPTP treatment induces an upregulation in Iba1+ve cells in the SN compared to control, by 56%. This upregulation is attenuated by NLX-112 which brings the number of Iba1+ve cells back down to almost control level. One-way ANOVA followed by Fishers LSD. Error bars denote SEM. n = 5-6.

As previously discussed, these two neuronal subtypes not only modulate the brain's innate and adaptive immune responses, but also play a key role in the release and regulation of cytokines and growth factors. The observed preservation of the nigrostriatal pathway, along with a concurrent reduction in reactive gliosis, prompted the hypothesis that resident astrocytes in the brain may be contributing to this neuroprotection through the release of the glial cell line-derived neurotrophic factor (GDNF).

6.3.6. GFAP-GDNF co-localisation

Thus, what state of activation may GDNF be under in the mid-CPu and SNpr of mice under all experimental treatments? Co-localisation of GFAP-GDNF in the mid-CPu was increased by MPTP (110%), and was increased even further by NLX-112+MPTP (333%) [$F(13.44\ (3, 34) = 0.2363\ p < 0.0001$] (Figure 51D).

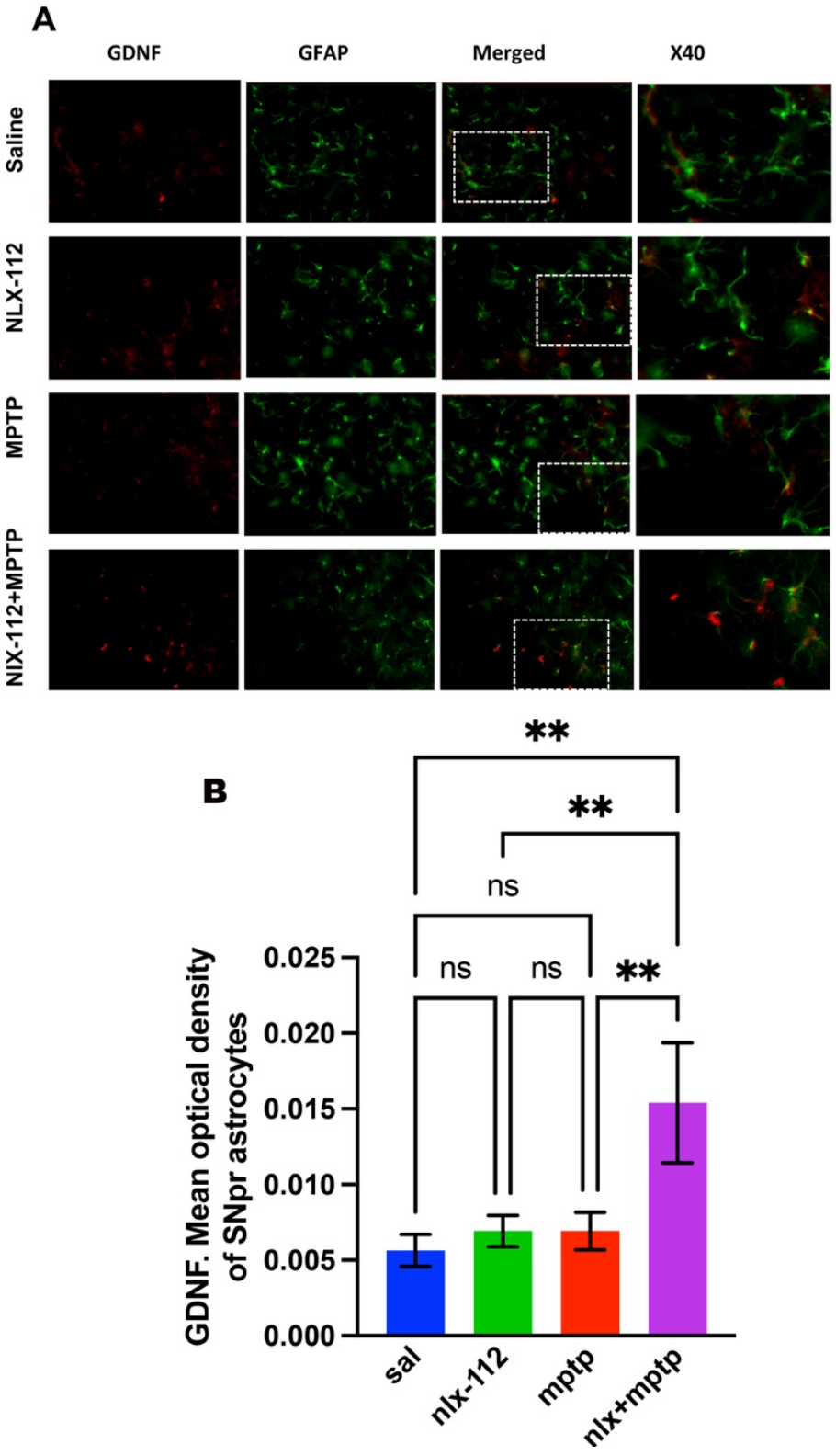


Figure 49. A) GFAP and GDNF co-localisation in SNpr. 40 X magnification. **B)** Compared to saline and nlx-112 the co-localisation of GDNF-GFAP in the SNpr does not change upon treatment with mptp, however, co-localisation becomes markedly greater when mice have been treated with nlx-112. One-way ANOVA followed by Fishers LSD. Error bars denote SEM. n = 7-12.

In the SNpc, GFAP-GDNF co-localisation was unchanged by MPTP, but was increased by NLX-112+MPTP (173%) [F(4.534 (3, 41) = 0.0210 p = 0.0077] (Figure 49 A, B).

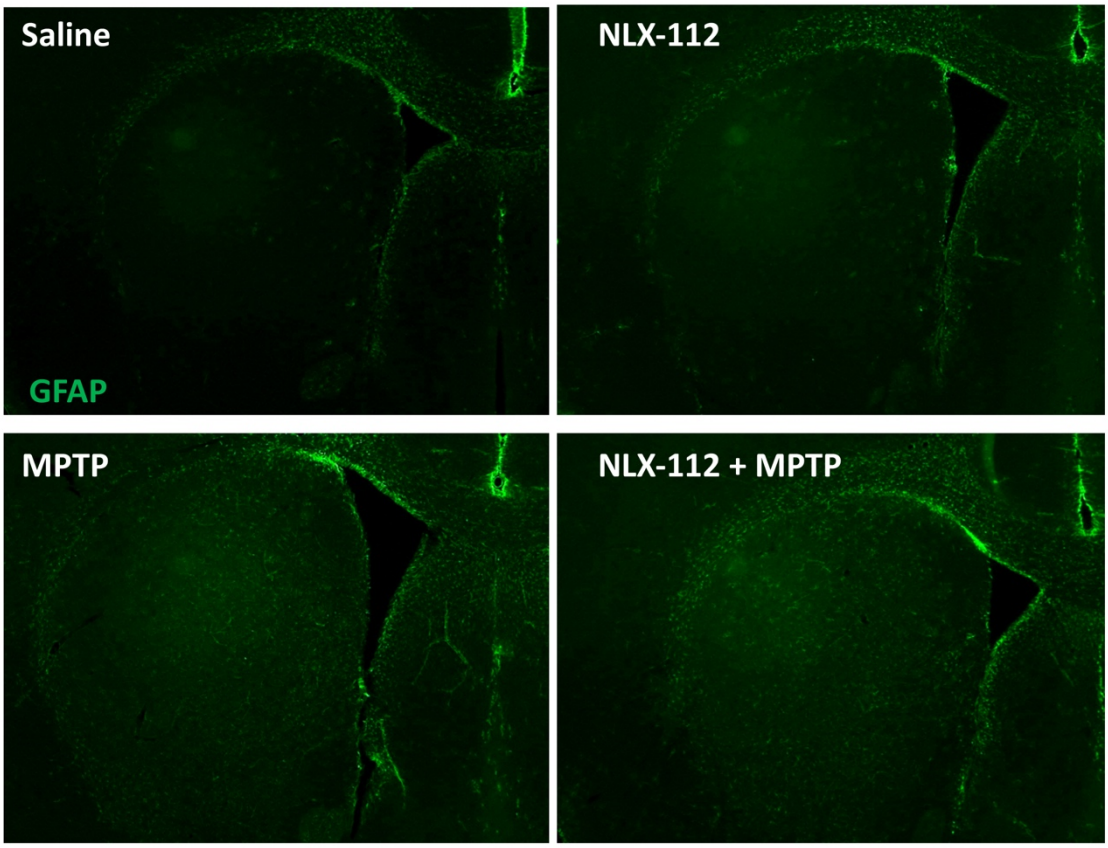


Figure 50. Representative GFAP immunoreactivity in the striatum following treatment with saline, NLX-112, MPTP, or NLX-112 + MPTP. Immunofluorescence images show glial fibrillary acidic protein (GFAP) labeling in the striatum of mice from each treatment group. Sections were imaged at 2.5× magnification. A visibly increased GFAP signal is apparent in the MPTP-treated group, consistent with reactive gliosis. Apparent reduction in GFAP intensity in the NLX-112 + MPTP group suggests a potential gliomodulatory effect of NLX-112; however, quantitative analysis is required to confirm this observation.

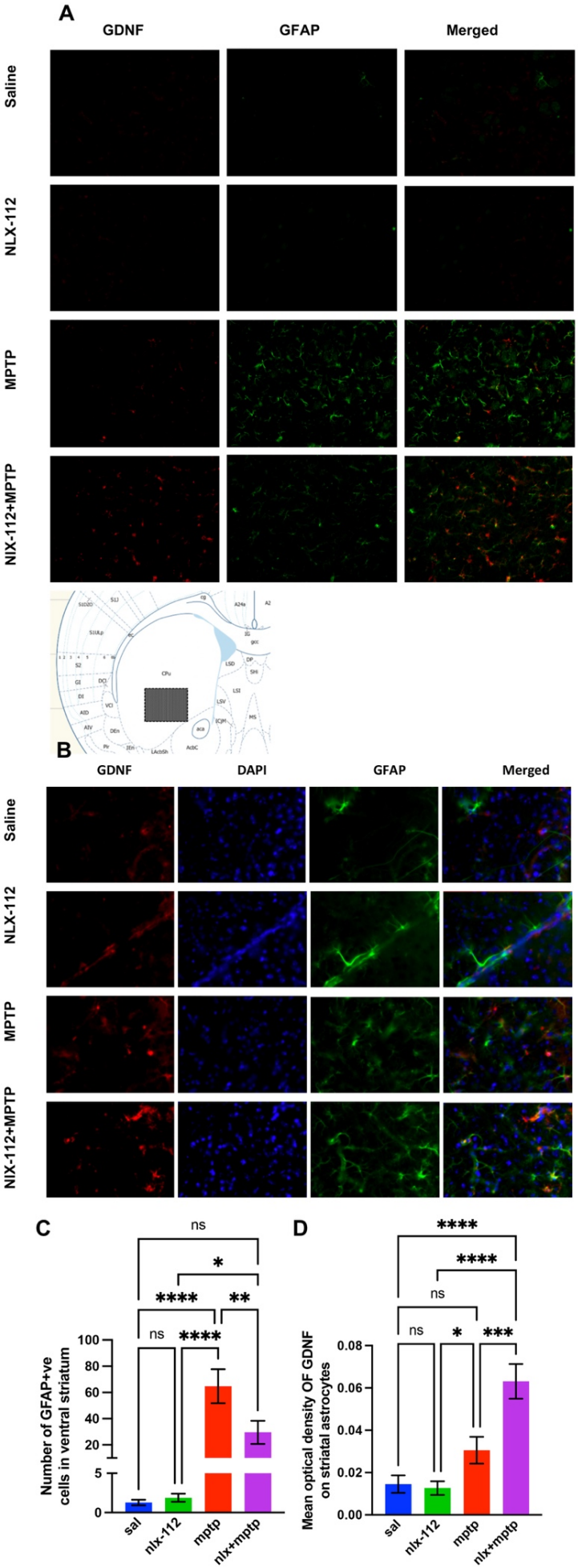

A
GDNF
GFAP
Merged
Saline
NLX-112
MPTP
NIX-112+MPTP
B
GDNF
DAPI
GFAP
Merged
Saline
NLX-112
MPTP
NIX-112+MPTP
C
Number of GFAP+ve cells in ventral striatum
ns
*
****
**
ns
****
100
80
60
40
20
5
0
sal
nlx-112
mptp
nlx+mptp
D
Mean optical density OF GDNF on striatal astrocytes
****
****
ns
ns
*
***
0.08
0.06
0.04
0.02
0.00
sal
nlx-112
mptp
nlx+mptp

Figure 51. A) Representative images of GDNF and GFAP co-localisation in the mid CPu of saline, MPTP, NLX-112 and NLX-112+MPTP treated mice, 20X magnification. B) Representative images of GDNF and GFAP co-localisation at 40X magnification, highlighting the extent of co-localisation of astrocytes and GDNF in MPTP and NLX-112+MPTP conditions. C) When assessed at 20X magnification, there is almost no GFAP immunoreactivity in saline and NLX-112 conditions inferring that resident striatal astrocytes are in a resting state; GFAP immunoreactivity is significantly upregulated in mice administered with MPTP which is significantly attenuated upon treatment with NLX-112. D) GDNF-GFAP co-localisation is minimal in saline and NLX-112 conditions. As indicated by the optical density, there is a modest increase in GDNF-GFAP co-localisation in the mptp group; this becomes markedly greater upon NLX-112 treatment, compared to all other groups. One-way ANOVA followed by Fishers LSD. Error bars denote SEM. n = 7-12.

6.3.7. The neuroprotective properties of NLX-112 in C57b/6J mice

The aforementioned data addresses the neuroprotective properties of NLX-112 in C57b/6J mice of the C57b/Ola-HSD strain. Already discussed as to the rationale of using the C57b/Ola-HSD strain rather than C57b/6J was due primarily to the robustness of C57b/Ola-HSD toward the acute toxicity of MPTP. The primary concern with this approach however is that the C57b/Ola-HSD strain are absent of SNCA1 gene amongst harbouring many other genetic aberrations in comparison to their C57b/6J counterparts.

NLX-112 is protective toward the preservation of the nigrostriatal tract in C57b/Ola-HSD mice. These findings raise several questions. It is known that an MPTP lesion of the SNpc is similar between both strains of mice. However, it remains uncertain whether NLX-112 is also neuroprotective in the C57b/6J strain. Chapter 5 revealed differences between resting and activated astro- and microglia in the two sub-strains. These differences could significantly impact the neuroprotective properties of NLX-112, particularly given previous data indicating that astroglia and microglia play crucial roles in modulating these protective properties.

As an extension of this study, an assessment of the neuroprotective properties of NLX-112 in C57b/6J mice has been undertaken.

The method for this experiment follows exactly the method described previously. Apart from the sub-strain of C57b mouse being of the C57b/6J sub-strain instead

of C57b/Ola-HSD, all other aspects and controls of the experiment remained the same. The dosing regimen, the time of day, housing, food and water, age, the amount of handling, and maze exposure all conferred to the conditions already defined earlier in the methods section of this Chapter.

6.4. Results (C57b/6J)

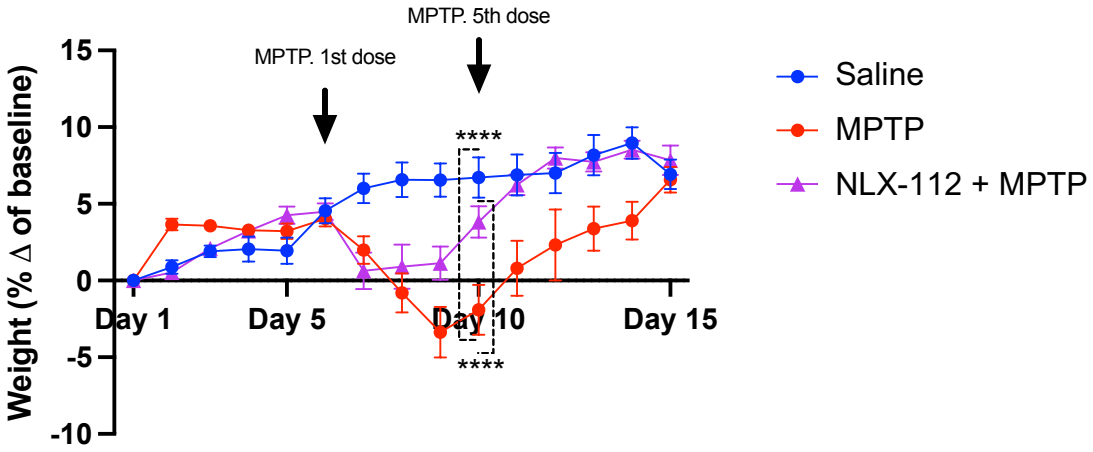


Figure 52. Change in body weight from baseline in C57b/6J mice (Day 1). Body weight was monitored daily from baseline (Day 1) across three experimental groups: saline, MPTP, and NLX-112 + MPTP. MPTP-treated mice exhibited a significant and sustained reduction in body weight beginning around Day 6, coinciding with the start of neurotoxin administration. In contrast, mice co-treated with NLX-112 and MPTP showed a transient drop in weight, but recovered more rapidly and approached saline control levels by Day 10. Two-way ANOVA followed by Fisher's LSD post hoc test revealed significant group differences over time (n = 6–11), data expressed as mean ± SEM).

6.4.1. TH+ve immunoreactivity in SNpc of C57b/6J mouse

The effects of NLX-112 were studied in the C57b/6J mouse sub-strain. Results have revealed many similarities to that which was investigated in the C57b/Ola-HSD sub-strain, in terms of behaviour and nigrostriatal lesion. Already thoroughly analysed in Chapter 5, the differences between each mouse's response to MPTP, compared to saline treated mice will not be repeated in the following paragraphs. The analysis will solely focus on how mice have responded to NLX-112 after receiving a sub-chronic MPTP challenge.

6.4.2. Behavioural differences between C57b/6J and C57b/Ola-HSD

The stand out similarities to the previous experiments are that saline and MPTP treated mice both succumb to maze habituation as seen from OFT1 to OFT3. In OFT 1 and OFT 2 NLX-112+MPTP treated mice undergo slowed movement in response to an acute dose of NLX-112 (1mg/kg), and also have the same delayed excitatory motor activity response as C57b/Ola-HSD mice did, when assessed in OFT 3. After 2 weeks from any drug treatment, NLX-112+MPTP treated mice have over a 100% increase in locomotor activity in OFT 3 compared to saline and MPTP only treated mice [F(41.86 (2, 31) = 0.5292 p < 0.0001] (Figure 53 F).

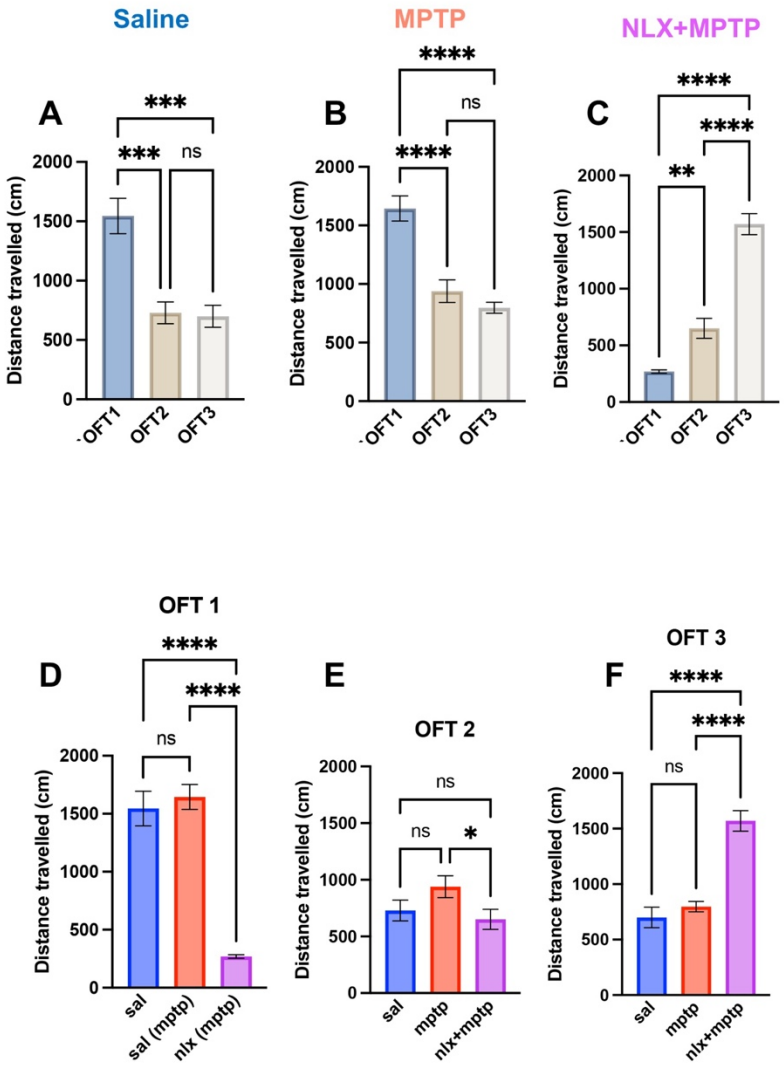


Figure 53. Open Field Test (OFT) performance was used to assess locomotor activity across three trials (OFT1, OFT2, OFT3) in saline-, MPTP-, and NLX-112 + MPTP-treated mice. In saline-treated controls (A), distance travelled decreased significantly from OFT1 to OFT2 and OFT3 (***p < 0.001), indicating normal

habituation. MPTP-treated mice (B) showed a significant reduction in distance from OFT1 to OFT2 (****p < 0.0001), but no further decrease from OFT2 to OFT3, suggesting a plateau of motor impairment. Notably, in NLX-112 + MPTP-treated mice (C), there was a significant and progressive increase in locomotor activity across all trials (**p < 0.01 to ****p < 0.0001). At OFT1 (D), NLX-112 + MPTP mice exhibited significantly less locomotion than both saline and MPTP-only groups (****p < 0.0001), indicating an initial suppression. By OFT2 (E), this difference was no longer significant between NLX-112 + MPTP and controls, though the increase relative to MPTP alone was significant (*p < 0.05). By OFT3 (F), NLX-112 + MPTP mice travelled significantly farther than both other groups (****p < 0.0001). Data are expressed as mean ± SEM (n = 6–11/group), analysed by one-way ANOVA with post hoc testing.

This suggests that these residual locomotor effects are independent of any genetic differences between the two mice sub-strains and is likely an effect largely due to NLX-112's pharmacological activity. There is no difference in the amount of time that NLX-112+MPTP mice spend in the centre of the arena compared to saline or MPTP only treated mice [F(0.513 (2, 25) = 0.3355 p = 6012] (Figure 54 E).

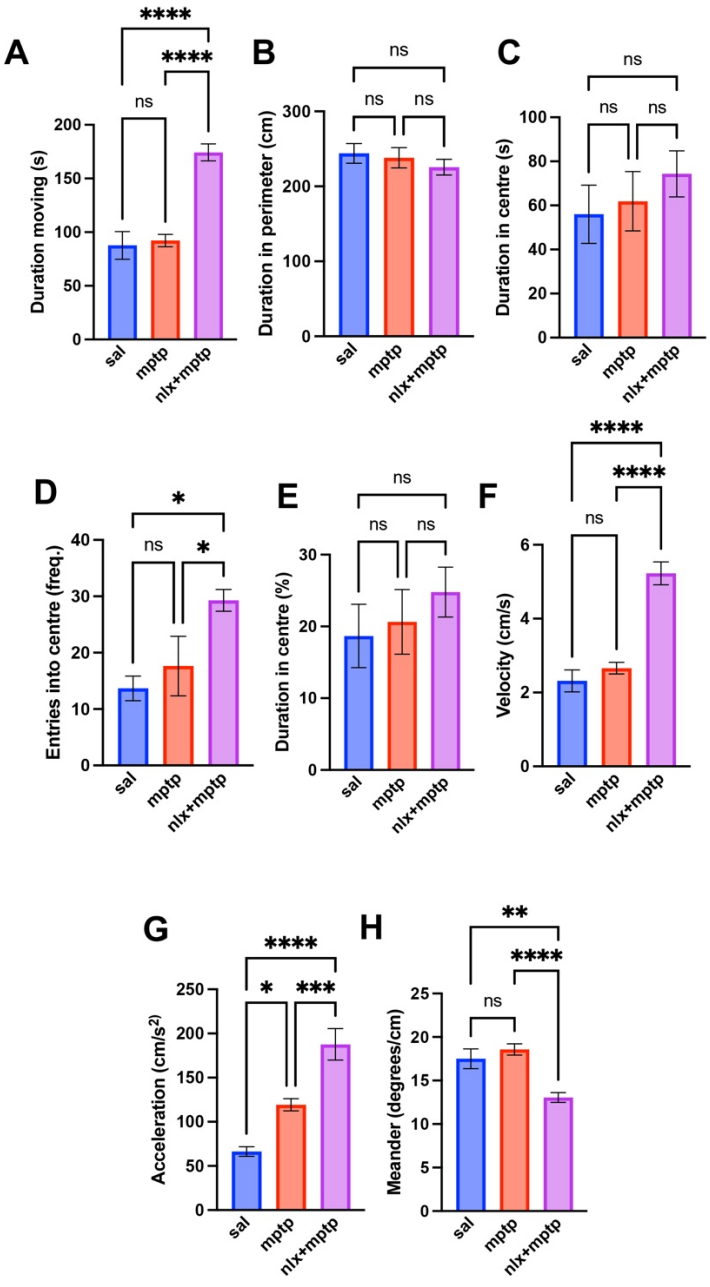


Figure 54. A-H) Specific behavioural and kinetic parameters exhibited by C57b/6J mice in OFT 3. Panel A shows that mice treated with NLX-112 + MPTP spent significantly more time moving than both saline- and MPTP-treated mice ($p < 0.0001$), indicating enhanced locomotor activity. In contrast, no significant differences were observed between groups in time spent along the perimeter of the arena (Panel B) or in the total duration spent in the centre (Panel C), suggesting that anxiety-like behaviour was not notably altered by treatment. However, NLX-112 + MPTP mice entered the centre of the arena significantly more frequently than both saline- and MPTP-treated mice (Panel D, $p < 0.05$), reflecting increased exploratory behaviour. Despite this, the overall percentage of time spent in the centre (Panel E) did not differ significantly between groups. Velocity (Panel F) was significantly elevated in the NLX-112 + MPTP group compared to both controls ($p < 0.0001$), aligning with increased movement duration and suggesting a robust

motor-enhancing effect. Similarly, mice receiving NLX-112 + MPTP showed significantly greater acceleration (Panel G) than both saline ($p < 0.05$) and MPTP-only mice ($p < 0.0001$), indicating increased motor drive or responsiveness. Finally, analysis of meander (Panel H)—a measure of path curvature—revealed that NLX-112 + MPTP-treated mice exhibited significantly more directed movement compared to MPTP-treated mice ($p < 0.01$), while saline and MPTP groups did not differ significantly. One-way ANOVA n= 6-11. Error bars denote SEM.

The lack of effect of NLX-112 being able to induce an anxiolytic like behaviour upon MPTP treatment contrasts with what was observed in the C57b/Ola-HSD experiment. This suggests that in these experiments, NLX-112's action on alleviating anxious behaviour is dependent to some extent on the trait (genetic) behavioural differences expressed by each of the two different mouse strains.

By repeating the neuroprotection experiments in C57b/6J mice, the goal was to determine whether the genetic divergence between the two sub-strains influences NLX-112's ability to provide neuroprotection for the MPTP-lesioned nigrostriatal pathway

6.4.3. MPTP lesion and NLX-112 treatment

In C57b/6J mice, stereological analysis revealed that NLX-112 supplementation warded off the deleterious effects of MPTP on TH+ve neurons in the SNpc. There is a 34% reduction upon MPTP treatment which is significantly attenuated with NLX-112 [$F(19.59\ (2, 24) = 0.8742\ p < 0.0001$] (Figure 55 and 56).

Figure 55. Representative coronal sections showing tyrosine hydroxylase-positive (TH^+) immunostaining in the substantia nigra of C57b/6J mice from three experimental groups: saline-treated, MPTP-treated, and NLX-112 + MPTP-treated. Sections span from Bregma −2.50, capturing the entry of the nigrostriatal bundle, to Bregma −3.90, encompassing the caudal extent of the SN. These images illustrate the extent of DAergic neuron loss induced by MPTP and the relative preservation observed with NLX-112 co-treatment. Scale bar = 100 µm.

Unlike the levels of neuroprotection in the C57b/Ola-HSD experiment where NLX-112 nearly reversed completely the amount of cell loss caused by MPTP, NLX-112 treatment in C57b/6J mice only wards off MPTP induced cell loss by approximately 50% of the possible maximum.

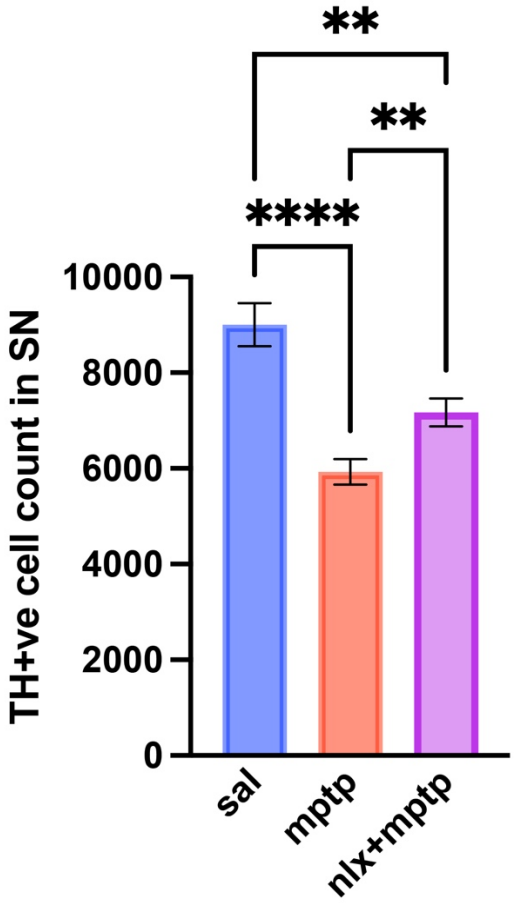


Figure 56. Stereological quantification of TH-positive neurons in the substantia nigra of C57b/6J mice following saline, MPTP, or NLX-112 + MPTP treatment. Mice treated with MPTP showed a significant reduction in TH-positive cell counts compared to saline-treated controls (****$p < 0.0001$). Co-treatment with NLX-112 significantly attenuated this loss (**$p < 0.01$ vs. MPTP), indicating a partial neuroprotective effect. Data were analysed by one-way ANOVA followed by Fisher's LSD post hoc test. Bars represent mean ± SEM (n = 5–14).

6.5. Discussion

This chapter aimed to address the primary hypothesis: whether NLX-112 is neuroprotective in an MPTP mouse model of Parkinson's disease. Additionally, by assessing mouse behaviour at various time points throughout the experiment, a pharmacologically unique property of NLX-112 was uncovered; namely, its residual effect on increasing motor activity in the absence of the drug, as demonstrated in the OFT 3 maze assays conducted two weeks after the last NLX-112 treatment. A phenomenon likely acting via activation of the 5-HT_{1A} receptor considering that this effect was blocked by the 5-HT_{1A} antagonist WAY100635, as well as were several other residual behavioural alterations.

Moreover, it was found that NLX-112 exerts many, but not all, of its effects on two different sub-strains of C57b mice treated with NLX-112 and MPTP. This suggests that the efficacy and response to the drug's effects are possibly dependent on the genetic background of the subjects being treated.

By assessing the immunoreactivity of cells reactive of GFAP and Iba1 it was also discovered that NLX-112 is having an effect on the brains innate immune responses against MPTP toxicity. MPTP significantly increased Iba1-ir by 117% in the SN, an effect almost abolished by NLX-112. This has given a clue as to one of the possible mechanisms that NLX-112 may be exerting its neuroprotective properties via a reduction in inflammation. To explore this mechanism further, an examination of GDNF-GFAP co-localisation in the substantia nigra pars reticulata (SNpr) and the caudate-putamen (CPu) was conducted. In MPTP-treated animals, striatal GFAP-GDNF co-localisation increased by 110%, and this increase was further amplified by 333% in the presence of NLX-112. In the substantia nigra, GFAP-GDNF co-localisation remained unchanged by MPTP compared to control, but NLX-112 treatment significantly increased co-localisation by 173%.

This is an important finding and one which implies that NLX-112's neuroprotective effects are likely mediated through the reversal of MPTP induced inflammation as shown by attenuation of astrocytosis (in ventral striatum but SNpc/pr) and inhibition of microgliosis in the SNpc, but also by upregulation of the neurotrophic factor, GDNF in astrocytes in the SNpr and ventral striatum.

6.5.1. NLX-112 offsets MPTP induced weight loss

Unlike their C57b/Ola-HSD counterparts, C57b/6J mice lesioned with MPTP experience significantly greater weight loss compared to control mice, a condition that is notably mitigated by treatment with NLX-112 (Figure 40 and 52). The exact reason why MPTP induces weight loss in C57b/6J mice but not in C57b/Ola-HSD mice is challenging to determine. It could be an indirect response to MPTP's acute effects on the cardiovascular and respiratory systems. Previous findings, and observations made throughout these experiments, have indicated that C57b/Ola-HSD mice cope with these effects better and recover more quickly post-MPTP dosing than C57b/6J mice.

6.5.2. NLX-112 on mouse behaviour and locomotor activity

In both C57b/Ola-HSD mice and C57b/6J mice, NLX-112 at 1mg/kg elicited similar acute physiological and behavioural responses, notably reduced motor activity, tremors, and piloerection, all indicative of excess 5-HT in the CNS. These responses ceased well before 30 minutes post-dosing. The chronic effects of NLX-112 are particularly fascinating and innovative. The drug demonstrated the capacity to increase locomotor activity and reduce anxious behaviour (specifically observed in C57b/Ola-HSD mice), persisting even in the absence of the drug, as evidenced in OFT 3, two weeks after the last dose of NLX-112. As previously mentioned, NLX-112 is a highly potent and selective agonist for the 5-HT_{1A} receptor. Moreover, NLX-112's binding to the 5-HT_{1A} receptor is widespread across the brain, as illustrated in Figure 38, which shows the radiotracer for NLX-112 being imaged throughout the cortical regions and striatum, areas heavily involved in motor function and control of CNS and PNS motor units. This, paired with the fact that mice were treated with NLX-112 (1mg/kg) for 15 days could have led to changes in synaptic plasticity and long-term-potentiation (LTP) or long-term depression (LDP) of 5-HT_{1A} receptors throughout the serotonergic pathways of the cortical and midbrain regions. And even though mice were treated with the 5-HT_{1A} antagonist for 5 days only, it was enough to significantly attenuate this residual effect, suggesting that 5-HT_{1A} activation for a sub-chronic period of time (15 days), using with a potent and selective 5-HT_{1A} agonist drug, is able to induce synaptic changes long after drug treatment has ceased. This supposition is supported by a large body of evidence, one that has been shown previously whereby horses

injected with Tph into the blood, and rats injected with Tph into the brain undergo an accelerated induction of physical exhaustion (Farris et al., 1998; Soares et al., 2003). In rats that have undergone prolonged exercise, the concertation of 5-HT in the dorsal horn was shown to be 41% greater compared to controls (Gerin et al., 2008). Additionally, the acute effects of 5-HT regulating drugs have been reported in humans performing strenuous exercise, they recounted feeling exhausted after taking an SSRI (Wilson and Maughn, 1992), and similar results were also reported by those performing a comparable exercise task after ingestion of the partial 5-HT_{1A} agonist, buspirone (Marvin et al., 1997). Furthermore, it has been shown that prolonged stimulation of the raphe spinal pathway in turtles decreases the excitability of spinal motoneurons as well as reducing motor activity (Cotel et al., 2013), which is mediated by the activation of axonal 5-HT_{1A} receptors on motoneurons (Cotel et al., 2013). The SSRI, citalopram increases LTP when injected into the motor cortex of healthy human subjects (Batsikadze et al., 2013), and when exposed daily to a novel environment over a prolonged period, rats undergo LTP in the denate gyrus which is blocked by the 5-HT_{1A} antagonist WAY100635, suggesting a key role for the 5-HT_{1A} receptor in facilitating LTP. And when 8-OH-DPAT was injected into the rat denate gyrus it reduces GABAergic inhibition (Sanberg et al., 2005). The fact that an abundance of serotonergic neurons terminate on denate inhibitory interneurons suggests that 5-HT_{1A} activation contributes to LTP via the inhibition of GABAergic interneurons (Orban et al., 2013) The key role that 5-HT_{1A} receptors seemingly play in modulating motor function is substantiated even further after rats receiving a daily dose of 8-OH-DPAT (0.25mg/kg) that had undergone a partial spinal ligation recovered their motor function capability at a far better rate than their saline treated counterparts (Antri et al., 2003), but the same dose of 8-OH-DPAT administered daily directly into the RN worsens immobility and increases struggling in the forced swim test (Cervo and Samanin, 1991). Thus, the targeting of the serotonergic pathway with various serotonin drugs clearly has a marked effect on modulating motor function and physical activity.

Another possible mechanism that could explain why NLX-112+MPTP treated mice experienced astonishingly high levels of motor activity in OFT 3 is due to the role that 5-HT inhibition plays in reducing maze habituation. This was shown clearly in

rats that were treated with the tryptophan enzyme inhibitor, *p*-Chlorophenylalanine which caused a marked reduction in maze habituation compared to their saline treated counterparts (Bidzinkski et al., 1998). However, this theory would not explain the significantly greater levels of locomotor activity seen by NLX-112+MPTP mice in the EPM, to which all treatment groups were only ever exposed to once.

6.5.3. NLX-112 residual locomotor property (in absence of NLX-112)

What is particularly noteworthy is that in C57b/Ola-HSD mice the residual locomotor effects appear to be greatest in NLX-112 treated mice that have undergone MPTP lesioning as opposed to mice that have received NLX-112 only. In NLX-112 treated mice the residual locomotor effect is still present, and in OFT 3 it is still significantly greater than MPTP only or saline treated mice, but the effect size is significantly muted in comparison to NLX-112+MPTP treated mice. Already described in Chapter 2, it is now known that MPTP has a major effect on the serotonergic pathway, as shown by the induction of hypertrophic nerve fibres in the mPFC, striatum, and RN. If MPTP is altering the physiology of the serotonergic pathway to such an extent then it is possible that the locomotor inducing properties of prolonged 5-HT_{1A} receptor activation through 15 daily doses of NLX-112 at 1mg/kg could have been greatly enhanced, than if mice were treated with NLX-112 only.

6.5.4. NLX-112 attenuates microglial activity in SN

A key set of data from this thesis highlight the role that NLX-112 may have on attenuating the brains resident macrophage, microglial cells upon MPTP treatment. NLX-112 treatment led to an almost total reduction of MPTP induced reactive microglial, returning to control and NLX-112 only levels. Previous research has shown that eltoprazine (a 5-$HT_{1A/1B}$ agonist) reduced Iba1 immunoreactive cells in the Cpu and SNpc, as well as reducing IL1β and TNF co-localisation in Iba1+ve microglial cells (Pinna et al., 2021). Further still, AI-8309, a potent 5-HT_{1A} agonist, prevents microglial activation in a model of macular degeneration (Collier et al., 2011). IHC and IF examination of mouse brain tissue was performed 15 days after the last dose of NLX-112 or MPTP, and the fact that NLX-112 was administered to

mice before, during and after treatment, therefore makes it impossible to determine whether NLX-112 prevented the activation of microglial cells in the SN, or whether it was tempered after their activation following MPTP treatment. Regardless, the demonstration that NLX-112 treatment likely plays an active role in suppressing microglia in the substantia nigra further enhances the value of NLX-112's known theoretical properties. This finding underscores the potential of NLX-112 not only in neuroprotection but also in modulating neuroinflammatory responses.

6.5.5. NLX-112 is neuroprotective in an MPTP mouse model

In both C57b/6J and C57b/Ola-HSD strains, the 5-HT_{1A} agonist NLX-112 has been shown to be pharmacologically effective in mitigating the neurotoxic effects of MPTP by attenuating damage to the DAergic nigrostriatal pathway. This finding is consistent with the research of Bezard et al. (2006), which demonstrated that the 5-HT_{1A} agonist BAY639044 is neuroprotective in both mice and macaques lesioned with MPTP. Additionally, this study is the first to report that NLX-112 likely exerts its protective effects through the upregulation of endogenous GDNF on astrocytes, specifically in the SNpc and striatum of MPTP-treated mice (notably in the C57b/Ola-HSD strain). This finding aligns with a substantial body of pre-clinical and clinical research exploring GDNF as a neuroprotective agent to counteract the progressive loss of DAergic cells in Parkinson's disease.

6.5.6. NLX-112’s neuroprotectve properties are not blocked by WAY100635

Although WAY100635 was able to block several NLX-112 induced behavioural changes, whether it was those observed after acute administration with NLX-112 or those observed following two weeks from NLX-112 treatment cessation, it was not able to block NLX-112’s neuroprotective property in the MPTP lesioned SN. This could be due one of several reasons. While WAY100635 is primarily classified as a 5-HT_{1A} antagonist, in some circumstances it can act as a partial agonist at presynaptic 5-HT_{1A} receptors (Fletcher et al., 1996; Rabiner et al., 2002). This means that it might not fully block the neuroprotective effects of NLX-112, particularly if NLX-112 is working predominantly through presynaptic 5-HT_{1A} receptors (Blier et al., 2003; Vidal et al., 2020), which modulate serotonin release and have different dynamics than postsynaptic receptors. The observed neuroprotective effects might not be solely mediated by 5-HT_{1A} receptor activity. In

an MPTP-induced neurotoxicity model, long-term or repeated administration of NLX-112 may induce compensatory changes, such as receptor desensitisation (Riad et al., 2001), adaptation of downstream signaling pathways (Albert et al., 2019), or recruitment of alternative neuroprotective pathways. This could result in neuroprotection that is not entirely dependent on 5-HT_{1A} receptor activation, and thus not fully blocked by WAY100635. It is also possible that WAY100635 might not cross the BBB as efficiently as NLX-112, particularly in the regions where neuroprotection is occurring (e.g., substantia nigra) (Pardridge bet al., 1975). As a result, the antagonist might not reach adequate concentrations to fully block the effects of NLX-112, allowing some neuroprotection to persist. Considering that the motor cortex is more proximal to the BBB, than the SN is, this may explain why NLX-112's inducing motor effects were blocked by WAY100635, but not so its neuroprotective effects. Lastly, the MPTP model causes considerable neuroinflammation and DAergic neurodegeneration, which can alter the expression and sensitivity of 5-HT and DA receptors (Ballanger et al., 2016). The neurotoxic environment caused by MPTP might change the way 5-HT_{1A} receptors respond to both agonists and antagonists. For instance, receptor upregulation or changes in receptor coupling to intracellular signaling pathways could result in reduced effectiveness of WAY100635 in blocking 5-HT_{1A}-mediated effects.

6.5.7. GDNF

GDNF is a protein encoded by the GDNF gene and is a member of the transforming growth factor beta superfamily. It serves as a potent neurotrophic factor, playing a crucial role in the survival, development, and maintenance of various neuronal populations in both the CNS and PNS (Airaksinen and Saarma, 2002). GDNF primarily exerts its effects through interactions with a specific receptor complex comprising GDNF family receptor alpha (GFRα) and the tyrosine kinase receptor, RET. Drinkut et al., (2016) uncovered that presence and function of the Ret receptor was paramount in order to facilitate the neuroprotective properties of GDNF in the nigrostriatal pathway of Ret knockout mice that have undergone MPTP challenge, compared to wildtype controls. Upon binding to this receptor complex, GDNF initiates signaling pathways that promote neuronal survival, regulate neuronal function, and support the growth and differentiation of specific neuron types, particularly DAergic neurons in the SN (Pascual et al., 2008)

Regarding its relevance to PD, GDNF has garnered meaningful attention due to its potential therapeutic implications. Research from preclinical animal models suggest that GDNF exhibits neuroprotective effects by fostering the survival of DA-producing neurons within the SN (Tomac et al., 1995; Kordower et al., 2000; Sun et al., 2005). Additionally, GDNF likely modulates neuronal function and connectivity, potentially aiding in the restoration of impaired motor function in PD (Kirik et al., 2001; Boger et al., 2006). Furthermore, decreased levels of GDNF in PFC of MPTP treated mice causes synaptic degeneration and is associated with cognitive impairment (Tang et al., 2023). Despite promising preclinical studies demonstrating its neuroprotective and restorative properties in animal models, clinical trials exploring GDNF as a direct treatment for PD have yielded mixed results (Gash et al., 2020; Barker et al., 2020; Manfredsson et al., 2020).

6.5.8. GDNF and neuroprotection in PD animal models

Many pre-clinical animal studies, as well as in vitro PD models, have successfully shown that GDNF harbours and exerts neuroprotective properties. For example, GDNF injections into the rat SN shields DA neurons in the SN and VTA from 6-OHDA induced cell death (Kearns and Gash, 1995). This protective effect mirrors GDNF's capacity demonstrated in mice (Tomac et al., 1995) and nonhuman primates (Gash et al., 1996), where it was also shown to foster protection and regeneration in the DAergic nigrostriatal system, upon MPTP lesioning (Iravani et al., 2001), as well as improve motor activity (Costa et al., 2001). But, and importantly, these and other studies have all been carried out through the administration of exogenous GDNF (Winkler et al., 1996; Georgievska et al., 2002; Sun et al., 2005), GDNF-secreting mesenchymal stem cells (Hoban et al., 2015) or GDNF transfected macrophages (Biju et al., 2010; Zhao et al., 2014). A fundamental difference that distinguishes this study from those previously mentioned is the ability to confer protection upon the nigrostriatal pathway by upregulating the brain's own physiological endogenous GDNF. This approach emphasises the use of the body's natural mechanisms for neuroprotection, rather than relying solely on external administration of neuroprotective agents.

This study also demonstrated that GDNF is upregulated solely upon MPTP treatment, a finding similarly shown in rats lesioned with LPS (Iravani et al., 2014).

This is expected due to the activation of GDNF, a crucial protective protein of the CNS, which is triggered by any form of damage or insult to the brain (Cintrón-Colón et al., 2020).

6.5.9. Limits to GDNF delivery

GDNF cannot cross the blood brain barrier (BBB) (Airaksinen et al., 2002). Therefore, any proposed treatment with exogenous GDNF would have to be administered intracranially or possibly intranasally ((which has been successively shown in a 6-OHDA lesion model (Migliore et al., 2014)), that would allow it to bypass the BBB. However, adeno-associated viral vector (AAV) meditated delivery of GDNF into the nigrostriatal tract failed to elicit any neuroprotection in rats lesioned with AAV_2 delivered human α-synuclein (Decressac et al., 2011), suggesting that exogenous GDNF's inability to cross the BBB is not its only impediment to promoting neuroprotection. Although counter to this, AAV mediated delivery of GDNF to the striatum of unilaterally 6OHDA lesioned marmosets protected DA neurons and behaviour (Eslamboli et al., (2003)

6.5.10. GDNF clinal trials in patients with PD

Due to the repeated preclinical success of GDNF being able to exert neuroprotection in the various PD and inflammation models, it was subsequently recognised by researchers and clinicians as a potential therapeutic target for the treatment of PD in humans. Between 2003 and 2019 there have been 7 clinical trials that have each sought to determine if GDNF is neuroprotective in the brain of patients living with PD. Patient UPDRS score is calculated before GDNF or placebo treatment and also at the end of the study. By assessing any changes to motor function clinicians were able to determine whether GDNF had been able to provoke any beneficial effects upon the motor regulating regions of the brain (Table 10).

Table 10. Clinical trials that have assessed the potential of glial cell line-derived neurotrophic factor (GDNF) as a treatment for PD, with outcomes varying based on delivery method, dosage, and study design. In Amgen 1 (Nutt et al., 2003), GDNF was delivered intraventricularly (into the CSF) in 38 patients, but no significant clinical benefit was observed, and serious side effects occurred—likely due to poor targeting of the putamen. By contrast, Gill et al. (2003) and Slevin et al. (2007) delivered GDNF directly into the putamen, reporting robust motor improvements (~39–42% reduction in UPDRS III scores) in small open-label cohorts. However, in the larger Amgen 2 trial (Lang et al., 2006), using a double-blind design, GDNF showed only modest, non-significant effects compared to placebo, despite direct putaminal infusion. Subsequent Bristol studies (Whone et al., 2019a, 2019b) used higher monthly doses (120 µg/side) over 80 weeks. These showed moderate improvements (~17–27% reduction in "off" UPDRS III), particularly with extended treatment durations. Lastly, a gene therapy trial by Heiss et al. (2019) tested escalating GDNF expression in the putamen but found no significant motor improvements after 18 months. Together, these studies suggest that while direct putaminal delivery of GDNF shows promise, consistent therapeutic effects remain elusive—potentially limited by delivery efficiency, treatment duration, and variability in patient response. Adapted from Gash et al., (2020).

Study	Patients	Delivery	Dose	Decrease in UPDRS III
Amgen 1 Nutt et al., (2003)	GDNF n=38 *Placebo n=12*	Ventricle (CSF)	25-4000 µg/month *Vehicle*	No significant improvements, serious side effects
Bristol 1 Gill et al., (2003)	GDNF n=5	Putamen infusion (brain)	14.4-43 µg/day one side	-39% Off at 1 year -37% On at 1 year
Kentucky Slevin et al., (2007)	GDNF n=10	Putamen infusion (brain)	3-30 µg/day per side	-42% Off at 1 year -39% On at 1 year
Amgen 2 Lang et al., (2006)	GDNF n=17 *Placebo n=17*	Putamen infusion (brain)	15 µg/day per side *Vehicle*	11.9% Off at 6 months *-6% Off at 6 months*
Bristol 2 Whone et al., (2019a)	GDNF n=17 *Placebo n=18*	Putamen infusion (brain)	120 µg/month per side for 80 weeks *Vehicle*	17.3% Off at 40 weeks *11.8% Off at 40 weeks*
Bristol 3 Whone et al., (2019b)	GDNF/GDNF n=21 *Placebo/GDNF n=20*	Putamen infusion (brain)	120 µg/month per side for 80 weeks *Vehicle for 40 weeks, 120µg GDNF/moth per side for 40 weeks*	26.7% Off at 80 weeks *27.6% Off at 40 weeks GDNF*
NIH Heiss et al., (2019)	GDNF Gene therapy n=13	Putamen (brain)	3 escalating dose levels tested	No significant change at 18 months

Only the Amgen 1 trial failed to register any improved scores in either drug or placebo groups. This is likely due to the method of GDNF and vehicle delivery which was carried out via monthly pumping into CSF of the frontal horn of the cerebroventricular system. Moreover, patients reported severe adverse side effects including anorexia, nausea and hyponatremia (Nutt et al., 2003), possibly due to the off-target actions of GDNF. All the other trials delivered GDNF and vehicle via perfusion into the putamen. Excluding the Amgen 1 trial, a major confounding outcome of the trials was that placebo treatment was shown to be as effective in reducing UPDRS score as it was in the GDNF treated patients. A

phenomenon in any drug trial and in those particularly concerning patients with neurodegenerative disorders is that by merely contributing to the trial and taking part in something that requires focus and attention, the positive effects that has on patients is so great that the patients' health, in so many different respects, can be majorly improved. Thus, unless sophisticated PET imaging during life or post mortem examination is carried out on the brains of all of those who took part in the clinical trials, it is therefore very difficult to determine whether GDNF did in fact elicit any improvements to the nigrostriatal or mesocortical pathways.

6.5.11.NLX-112 upregulates astrocytic GDNF

Analysis of the present study revealed that the upregulation of GDNF is localised on GFAP+ve cells (astrocytes). Already described in greater detail in Chapter 1, astrocytes are a fundamental homeostatic regulating component of the brain. Astrocytes are extremely multifaceted, facilitating the transport of macromolecules from the BBB to resident neurons, they are also one of the chief cells that secrete GDNF. Knockout of astrocytic SARM1 (member of the Toll/interleukin receptor family and largely found in cell bodies and axons) in mice with experimental autoimmune encephalomyelitis (EAE) (a model of MS) inhibits neuroinflammation through upregulation of GDNF (Jin et al., 2022). Furthermore, upon ischemic damage, neurons are rescued by high frequency magnetic stimulation through the release of GDNF on astrocytes (Gava- Junior et al., 2023).

NLX-112 is highly selective for 5-HT_{1A} and astrocytes are known to express this serotonergic receptor (Whitaker-Azmitia et al., 1993; Miyazaki et al., 2013; 2016; 2017; Lee et al., 2015; Isooka et al., 2020; Narváez et al., 2020; Kikuoka et al., 2020). Though, the majority of these studies have not directly observed the expression of 5-HT_{1A} on nigrostriatal resident astrocytes, be it through autoradiography or IHC, but have determined their presence through the use of 5-HT_{1A} agonist compounds such as 8-OH-DPAT and ipsaperone which have been used to elicit the neuroprotective mechanisms of astrocytes through release of antioxidants such as metallothionine or cell growth regulating proteins such as S-100 (Whitaker-Azmitia et al., 1993; Miyazaki et al., 2013; 2016; 2017). However, the 5-HT_{1A} receptor has been observed directly on astrocytes in the gerbil hippocampus, via IHC, but interestingly the 5-HT_{1A} receptor was only shown to be

colocalised with astrocytes upon ischemic neuronal damage, and not in normal non-hypoxic astrocytes (Lee et al., 2015).

A deeper understanding of 5-HT_{1A} receptor expression in astrocytes was provided by a study that detected mRNA for several serotonin receptor subtypes—including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{5B}, 5-HT_{6}, and 5-HT_{7}—in cultured astrocytes. Notably, 5-HT_{4} and 5-HT_{5A} mRNA were absent, which is surprising given that 5-HT_{4} receptors have previously been reported in abundance on hippocampal astrocytes (Muller et al., 2020). To further investigate 5-HT_{1A} receptor functionality, the study examined cAMP production in cultured astrocytes stimulated with forskolin and isoproterenol, followed by treatment with the 5-HT_{1A} agonist 8-OH-DPAT. The lack of inhibition in cAMP production suggested that while 5-HT_{1A} mRNA is present, the functional G protein–coupled 5-HT_{1A} receptor is not expressed on the cell surface (Hirst et al., 1998).

Therefore, the fact that NLX-112 enhances GDNF to be upregulated by astrocytes to an even greater extent than what is found upon MPTP only treatment indicate that NLX-112 interacts with astrocytes almost certainly through the 5-HT_{1A} GPCR. NLX-112 does not bind to any other receptor other than 5-HT_{1A} as shown by the receptor binding assays performed by Colpaert et al., (2002). Gerbil hippocampal astrocytes highly express the 5-HT_{1A} receptor but only upon ischemic damage. This suggest that the astrocyte does not facilitate the use of 5-HT_{1A} as a basic resting function, but that only upon a toxic insult or damage to resident neurons does it form into a functional active GPCR. This theory is certainly supported by the previously mentioned mechanisms, that resting astrocytes do indeed contain 5-HT_{1A} mRNA and 5-HT_{1A} agonism using 8-OH-DPAT is unable to inhibit cAMP production, signifying the absence of functional 5-HT_{1A} receptors. A more discrete utility of 5-HT_{1A} may be how astrocytes express this serotonergic ligand. Astrocytes certainly harbour 5-HT_{1A} mRNA and the fact that our study shows NLX-112 as being able to elicit a major upregulation of GDNF in mouse nigrostriatal neurons as well as causing a reduction in GFAP+ve astrocytic activation. This all points toward evidence that NLX-112 is exerting its neuroprotective property through 5-HT_{1A}, but perhaps an insult to a specific brain region using a toxin such as MPTP

is required for 5-HT_{1A} receptors to shift from a dormant cytosolic conformation to a functional cell surface bound conformation.

6.5.12.NLX-112 neuroprotection: mechanism of action

NLX-112 crosses the BBB where it is taken up by astrocytes via 5-HT_{1A}, already recruited via apoptotic chemokine and cytokine signalling, thus recruiting one of the primary functions of astrocytes which is to secrete the neurotrophic factor GDNF. NLX-112's potent agonism via the 5-HT_{1A} receptor leads to the sustained activation of astrocytes, leading to LTP and greater secretion of GDNF. Astrocytic secreted GNDF is then taken up via Ret receptors on DA neurons resulting in greater protection of the neuron and its survival against MPP^+ toxicity. Additionally, the signaling pathway of GDNF/Ret helps regulate optimal mitochondrial function and activity through stimulation of complex-I whilst functionally interacting with the NfκB pathway thereby enhancing mitochondrial biogenesis and restoring ATP production (Kramer and Liss et al., 2015)

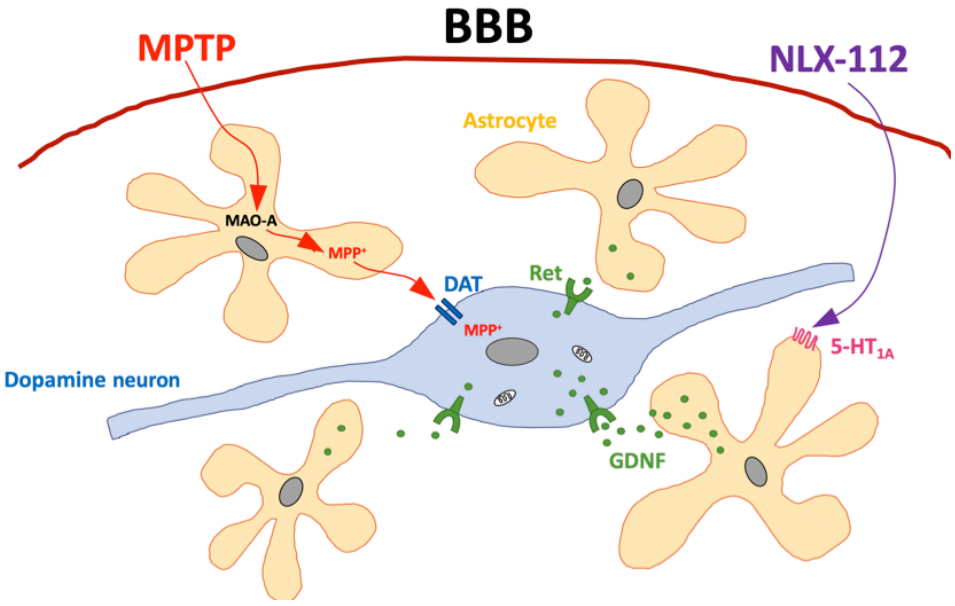


Figure 57. Mechanism of MPTP-induced DAergic toxicity and proposed neuroprotective action of NLX-112. After crossing the blood-brain barrier (BBB), the neurotoxin MPTP is taken up by astrocytes, where it is converted by monoamine oxidase-A (MAO-A) into its active metabolite MPP^+. MPP^+ is then released into the extracellular space and taken up selectively by DAergic neurons via the DA transporter (DAT). Once inside the neuron, MPP^+ accumulates in mitochondria, where it disrupts function and initiates cell death pathways. In response to this stress, surrounding astrocytes can be recruited to release glial cell

line-derived neurotrophic factor (GDNF), which acts via the Ret receptor on DA neurons to promote survival. The serotonin 5-HT_{1A} receptor agonist NLX-112 crosses the BBB and activates astrocytic 5-HT_{1A} receptors, leading to an enhanced release of GDNF. This augments the neuron-supportive response, improving the resilience of DA neurons to MPP^+-induced toxicity. Together, these effects suggest that NLX-112 facilitates an astrocyte-mediated neuroprotective mechanism, helping preserve the integrity of the nigrostriatal DA pathway in models of PD.

6.6. Summary and conclusion

This Chapter has unearthed several unknown quantities regarding NLX-112's property. Firstly, its effects upon locomotor activity and anxiety-like behaviour even two weeks after drug cessation; and secondly, that NLX-112 exhibits neuroprotective properties in MPTP treated mice. In this model, NLX-112's protective effects are likely mediated through reversal of MPTP induced inflammation as shown by attenuation of astrogliosis and inhibition of microgliosis but also by upregulation of the neurotrophic factor, GDNF in astrocytes. Further still, this study demonstrates that C57b/Ola-HSD and C57b/6J mice are equally susceptible to MPTP's deleterious effects on destroying the DAergic nigrostriatal pathway, but equally there are respective behavioural differences between the two sub-strains at basal level but also in response to MPTP and NLX-112+MPTP treatment.

Chapter 7

Summary and Conclusion

7.1. Thesis hypothesis and aims

While not yet realised in clinical settings, 5-HT_{1A} agonists have demonstrated substantial pre-clinical potential as pharmacological agents for inducing neuroprotection in models of PD (Oosterink et al., 1998; Ahlemeyer et al., 1999; Adayev et al., 2003; Madhaven et al., 2003; Druse et al., 2005; Fricker et al., 2005; Bezard et al., 2006; Garrett et al., 2013; Miyazaki et al., 2013; Isooka et al., 2020; Miyazaki et al., 2020). Building upon this evidence, it was hypothesised that NLX-112, a fully selective biased 5-HT_{1A} receptor agonist with nanomolar affinity, may also possess neuroprotective properties. In recent years, NLX-112 has made noteworthy strides in clinical development. It exhibits a unique signal transduction profile, demonstrating second messenger signaling properties at 5-HT_{1A} receptors akin to those of 5-HT itself. It has also shown efficacy in mitigating LID/AIMS in non-human primate models of PD, and most notably, it has achieved success in its Phase 2A clinical trial by significantly countering the deleterious effects of Levodopa treatment. NLX-112 is evidently a drug demonstrating promising therapeutic properties whether in an animal or human experimental setting.

Thus, the aims stipulated in Chapter 1 were those that focussed on carrying out a series of experiments which had the capacity to shed light on whether NLX-112 is neuroprotective.

7.1.1. Summary of findings

- Apoptotic SH-SY5Y cells pre-treated with the mitochondrial inhibitor MPP^+ are modestly protected upon treatment with NLX-112 at ranges from 10nm-1μM. Protection of cells was inferred via measurement of total mitochondria and mitochondrial polarisation.

- Apoptotic SH-SY5Y cells pre-treated with the proteasome inhibitor MG132 are modestly protected upon treatment with NLX-112 at ranges from 100nM-1μM. Protection of cells was inferred via measurement of total mitochondria and total ATP content.

- NLX-112 protected DA neurons against MPTP lesioning in two different sub-strains of C57b mice. In this model NLX-112 also attenuated MPTP induced reactive gliosis, as well as enhancing astrocytic secretion of GDNF (C57b/Ola-HSD strain only).

- NLX-112 had a residual effect on enhancing locomotor activity in C57b mice. More specifically, two weeks after cessation of drug administration, mice from the NLX-112 treatment groups had over a 2-fold increase in the distance travelled in the open field, compared to mice that had received only MPTP or vehicle .

- Middle aged C57b/6J mice treated with acute doses of NLX-112 exhibited reduced anxiety-like behaviour. This was not observed in younger mice (C57b/6J or C57b/Ola-HSD).

The experiments and subsequent findings described in this thesis outline the various mechanisms of action responsible for NLX-112's capacity to confer neuroprotection. These descriptions are not exhaustive and are limited to the models with which they adhere to. This study used a neurotoxin which inhibits the mitochondrial respiratory chain of DA neurons in the SN, and have sought to identify if NLX-112 can counter that neurotoxicity. PD aetiology is not due to a single cause, such as mitochondrial inhibition. As already described, it is comprised of many known, and very likely many unknown causes. Thus, any experiment seeking to model PD will be limited to the variables it is employed to measure. There is no perfect fit, but there is a best fit, at least within the confines of the stated experimental aims, and MPTP is by no means a perfect neurotoxin. The benefits of the MPTP animal model is that it predominantly targets DA producing neurons in the SN but, as identified in this thesis, and others prior (Zeng et al., 2006), it also impedes the normal function of the serotonergic pathway. Loss of DA neurons in the SN is the key pathological feature of PD, therefore the fact that MPTP possess the property to also cause severe injury to nigro-striatal pathway makes utilising the MPTP neurotoxin a useful model for identifying any potential neuroprotective drug.

In Chapter 1 the systematic literature review identified, from the last 10 years, a collection of the most promising drugs shown to elicit neuroprotection in an MPTP mouse model. What immediately stood out from the review is the wide variety of drugs that each target very specific pathways. Therein lies the suggestion that offsetting the loss of DA neurons in the SN can be achieved through the targeting of different pathways. For example, the hormone melatonin acts as a full agonist and binds to the melatonin GPCR, and upon MPTP lesioning it protects DA neurons and reduces inflammation (Jingwen et al., 2022). Necrosulfonamide prevents necroptosis by supressing the membrane expiry mediator MLKL, thus leading to protection of SN DA neurons following MPTP lesioning (Leem et al., 2023). Unsurprisingly, several studies have sought to target the transcription factor Nurr1, due to its key role in modulating inflammatory and DAergic neurochemical systems. Mutations in the Nurr1 gene (NR4A2) are linked to many clinical disorders including PD and schizophrenia (Yi et al., 2014). The Nurr1 agonist, 4A7C-301 protects midbrains DA neurons from MPTP neurotoxicity (Kim et al., 2023). And the PGE1 prostaglandin and its metabolite PGA1 each are found to stimulate Nurr1 activity leading to neuroprotection of nigral DA neurons (Rajan et al., 2020). P110 inhibits the mitochondrial fission regulator peptide, Drp1 demonstrating neuroprotective effects of the DAergic pathway (Filichia et al., 2016), and the microtubule stabiliser epothilone-D mitigates the degeneration of DA neurons in the SNpc (Cartelli et al., 2013. These are but a few of the studies outlined in the systematic review which have successfully conferred neuroprotection in mice lesioned with MPTP. Although each of the aforementioned drugs target distinct neurological molecules, what is apparent is that they all indirectly act upon negating one or several of the key mechanisms responsible for DA cell death in PD pathology; these primarily being reduction of inflammation or restoration of mitochondrial integrity.

This thesis shows that NLX-112 increases the count of mitochondria, boosts ATP content, and restores mitochondrial polarisation in an in vitro cell-line neuroprotection model of PD. Moreover, we have also shown that NLX-112 tempers reactive gliosis and increases astrocytic secretion of GDNF of the nigrostriatal pathway in an in vivo neuroprotection model of PD. Our results, as well as those described in the paragraph above, reveal that there are multiple ways to

confer neuroprotection against MPTP toxicity, be that through improvements to Nurr1 transcription, microtubule stabilisation, or the increased secretion of astrocytic GDNF– all of which are molecular targets shown to yield similar in-direct downstream effects, for either the reduction of inflammation and/or renewal of mitochondrial function.

7.1.2. Is the SH-SY5Y cell line a suitable model for assesing a neuoroprotective agent?

The SH-SY5Y cell line is an immortalised oncogenic neuroblastoma and due to a Ras mutation, it exhibits a profound ability to differentiate at a rapid rate. This quality allows for high throughput experiments, but it also counters the depth of investigation that can be carried out. For example, the results of any neuroprotective assays would be able to tell you what the ratio of viable cells to dead cells is, but it would not be able to tell you whether the viable cells have been recovered by the neuroprotective agent or if they are the remnants of surviving dividing cells (Datky et al., 2003). Nevertheless, the SH-SY5Y cell line has a strong pedigree for modelling neuroprotection in vitro. Extensively used by researchers, SH-SY5Y cells are often differentiated with retinoic acid to create a DAergic phenotype. This differentiation step was omitted from the experiments in Chapter 3 due to NLX-112 not having any binding properties to receptors other than 5-HT_{1A}. Furthermore, conflicting results have been reported that treatment with retinoic acid can increase or decrease susceptibility to DAergic neurotoxins, including MPP^+ (Cheung et al., 2009; Lopes et al., 2010). In its native state SH-SY5Y cells express SERT, MAO-A and 5-HT_{1A}, all very important components of 5-HT signal transduction. To shift the cell-line away from a serotonergic state to a DAergic state would have likely impeded the prospect of NLX-112 exerting any potential protective properties. The validity of the choice to not differentiate the SH-SY cells with retinoic acid to a DA phenotype is strengthened by the fact that the 5-HT_{1A} antagonist WAY100635 was able to block NLX-112's significant, albeit modest, protective effects. However, SH-SY5Y cells are very limited, at least in terms of attempting to model what is occurring to the expiring DA cells in the parkinsonian brain. Therefore, overreaching inferences must not be made, and any data which is uncovered must be carefully considered. It should be noted, that the MPP^+ concentration used to reach EC50 was 1mM, whereas the MG132 concentration

used was 2.5μM. This vast difference in both drug concentrations to achieve the same EC50, over the same time period, is likely due to MPP^+ having only limited toxicity toward SH-SY5Y cells due to its absence of DAT expression, whereas fully functioning proteolysis is integral to the health and viability of all cells.

In summary, we used the mitochondrial inhibitor MPP^+ and the proteasome inhibitor MG132 to model cell death in the SH-SY5Y cell line. Both of these compounds target specific pathways that, when dysfunctional, are responsible for the demise of DA neurons in PD aetiology. And the fact that we assessed cell viability through determination of total mitochondria, mitochondrial re-polarisation, and ATP content provides sound face validity supporting the conclusion that NLX-112's protective effects are mediated via improvements to mitochondrial health and integrity.

7.1.3. Is the MPTP mouse model a suitable experiment for assesing a neuoroprotective agent?

As discussed in Chapter 1, the experimental outcome of using MPTP to mimic PD pathology is varied and depends very much on the species of animal used, the dosing regimen employed, and the type of investigation required. We sought to determine if the novel 5-HT_{1A} agonist, NLX-112 is neuroprotective in mice. In anticipation that any putative neuroprotective properties may be only subtle, it was therefore deemed appropriate to utilise mice as the number of animals required for each group condition could be sizeable, something that is much more difficult to manage when using higher species such as the 6-OHDA rat model or the MPTP non-human primate model. Once the dosing regimen had been refined, a considerable number of animals can be examined in each experiment, a necessary requirement when carrying out exploratory 'blue sky' research on a novel drug. Furthermore, the face validity of modelling PD with MPTP is enhanced all the more, due to it having already shown to cause PD like symptoms in humans (Langston et al., 1984). In our experiments we treated mice with an incremental sub-chronic dose of MPTP, over 5 days culminating in a total of 117mg/kg. This dosing regimen was adequate at causing cell death of approximately -40% and -34% TH+ve cells in the SN of C57b/Ola-HSD and C57b/6J mice, respectively. The extent of this lesion was shown to be sufficient for NLX-112 treatment to robustly counter TH+ve

cell loss and reveal NLX-112's neuroprotective properties against MPTP lesioning. Efforts were made to assess whether MPTP had reduced locomotor activity but we discovered that the opposite had actually occurred, locomotor activity was in fact markedly increased compared to the control group, furthermore NLX-112 treatment enhanced this effect. The reporting of MPTPs effects on mouse behaviour and locomotor activity is extensive but often conflicting. This is likely due to several factors; a key one being the specific time point from last MPTP dose, at which researchers choose to perform the analysis, and another being the type of dosing regimen used. For example, acute MPTP treatment causes reductions in locomotor activity, whereas chronic and sub-chronic MPTP treatments almost always lead to a greater level of locomotor activity (Fredriksson et al., 1990; Colotla et al., 1990; Chia et al., 1996; 1999; Sedelis et a., 2000).

In summary, the fact that locomotor activity increases, should not be entirely surprising, given that: one, this phenomenon has been widely reported by others previously who have utilised the MPTP neurotoxin in mice, and: two, NLX-112 has elicited its own locomotor exerting effects due the chronic dosing regimen mice had received with this very potent 5-HT_{1A} agonist at 1mg/kg/day for 15 days. Whilst these findings are extremely interesting and may provide valuable information to those researching NLX-112 motor function properties in the future, they are in this thesis, a tertiary observation. We wanted to know if NLX-112 was neuroprotective toward the demise of DA neurons that have perished due to MPTP induced mitochondrial inhibition. For all of the MPTP models' limitations, and the surprising supplementary results gained throughout the course of these experiments, this thesis has achieved its primary aim.

7.1.4. Is 5-HT_{1A} a suitable target for the protection of DA neurons in PD

Targeting of the 5-HT_{1A} receptor for the alleviation of affective disorders such as depression and anxiety is well known and 5-HT_{1A} drugs used to attenuate these disorders have been widely prescribed over the last several decades. Enquiry into 5-HT_{1A}'s neuroprotective properties in PD is relatively more recent with research beginning to take off at the turn of the century. In this thesis we have focused our efforts on whether NLX-112 is neuroprotective in vitro and in an MPTP neurotoxin model that targets DAergic neurons. As previously mentioned the majority of

successful research to have unearthed the protective properties of 5-HT_{1A} agonists has been limited to cell culture models, few animal models, and of those, many being carried out using partial 5-HT_{1A} agonists.

The evidence supporting 5-HT_{1A} receptor targeted therapy for inducing neuroprotection is abundant, primarily demonstrated in models not aiming to replicate PD aetiology. Much of this research stems from modeling cerebral ischemia, a condition often proceding stroke or cardiac arrest (de Aguira et al., 2021). In vitro and in vivo models of cerebral ischemia show efficacious prevention by partial (buspirone, gepirone, and ipsapirone) and full (8-OH-DPAT, BayR1531, and NLX-101) 5-HT_{1A} agonists (Bielenberg and Burkhardt 1990; Prehn et al., 1993; Piera et al., 1995; Kamei et al., 2001; Johansen et al., 2014; Aguiar et al., 2020). In contrast to animal PD models seeking protective agents against specific cell types, such as DA neurons, the reversal of neuronal damage in modelling cerebral ischemia is widespread and affects entire brain regions comprising multiple neuronal cell types. This broad impact results from countering the primary cause of ischemic damage, namely the lack of cerebral blood flow. Interestingly, cerebral ischemia is reversed with treatment using the CB1 receptor-binding endocannabinoid cannabidiol, with its therapeutic effects being nullified by the 5-HT_{1A} antagonist WAY100635 (Mishima et al., 2005), further highlighting the multifaceted role of 5-HT_{1A} targeted therapies as well our own limited understanding through which 5-HT_{1A} receptors exert their signaling properties throughout the brain.

Extensive research has gone into understanding the pharmacological profile of NLX-112. Each experiment and piece of research that goes into comprehending a particular property of NLX-112 is buttressed by the preceding body of work. From over a decade of in depth investigation we know many things about NLX-112 including but not limited to: its signal transduction profile at 5-HT_{1A} receptors is exceptionally similar to the endogenous neurotransmitter, serotonin, it has nanomolar affinity at the 5-HT_{1A} receptor, it does not bind to any other receptors even at micromolar concentrations, it preferentially activates Gαo versus other G-protein subtypes, it alleviates non-motor ailments including: reduced aggressive, improved analgesia, depressive- and anxiety-like states, and drug misuse

potential, all of which have been reported in a variety of different rodent models; it alleviates motor dysfunction in rodents and humans mainly those dysfunctions involving LID but also parkinsonian symptoms in humans also (Svenningsson et al., 2023).

7.1.5. Study limitations

The time point at which animals are sacrificed and the subsequent brain sections analysed via IHC allows only for snap shot at a specific point in time. We analysed tissue sections using antibodies for TH, Iba1, GFAP, and GDNF in order to infer the effects of MPTP and NLX-112 on DAergic integrity and inflammation state of the nigrostriatal pathway. This was carried out on mice culled 16 days after their last dose of drug or saline. Therefore, we are analysing the DAergic and gliotic state of the SN and striatum when apoptotic lesioning or reactive gliosis may be in its ascendancy or descendance. It is known that mice can recover considerably from the damage of the nigrostriatal pathway following MPTP treatment (Mitsumoto et al., 1998); thus, any MPTP study, including the ones described in this thesis will always be limited by the fact that metabolic recovery of damaged neurons and nerve fibres in mice occurs at a rapid pace. Regardless, the present treatment conditions elicited a substantial lesion which was negated by NLX-112 treatment whilst also corresponding with reductions in micro- and astrogliosis and enhanced secretion of astrocytic of GDNF.

7.2. Conclusion

The findings presented in this thesis demonstrate that NLX-112 exerts neuroprotective effects in both in vitro and in vivo models of toxin-induced DAergic cell death. In SH-SY5Y cells, which express 5-HT_{1A} receptors (Levitt et al., 2011), NLX-112's protective effects were blocked by the selective 5-HT_{1A} antagonist WAY-100635, confirming receptor involvement. NLX-112 may also provide limited protection against mitochondrial and proteasomal insults in SH-SY5Y cells, potentially via mechanisms such as enhanced calcium buffering or free radical scavenging. However, the absence of glial cells, particularly astrocytes, likely limits these effects, as protection did not exceed 10%.

In contrast, NLX-112 produced robust neuroprotective effects in the MPTP-lesioned C57b mouse model, likely through activation of astrocytic 5-HT_{1A} receptors. This activation appears to stimulate astrocytic GDNF secretion, promoting DAergic neuron survival. While astrocyte-derived GDNF likely plays a key role in this effect, it is unlikely to be the sole mechanism. Given the widespread expression of 5-HT_{1A} receptors across neuronal and non-neuronal populations (Newman-Tancredi et al., 2022; Depoortere et al., 2025), direct modulation of neuronal circuits, reduction of excitotoxicity, modulation of DAergic tone, and potential interactions with microglia may also contribute to NLX-112's neuroprotective actions. Given this, these findings suggest that NLX-112 may offer as a promising therapeutic approach for neurodegenerative disorders such as Parkinson's disease, likely trhough stimulation of endogenous GDNF production through astrocytic 5-HT_{1A} receptor activation.

To conclusively establish the role of astrocyte-derived GDNF in NLX-112-mediated neuroprotection, further targeted studies are warranted. These should include the use of astrocyte-specific GDNF knockout mice (e.g., GDNF floxed crossed with GFAP-Cre lines (Kim et al., 2025)) to assess whether the absence of astrocytic GDNF abolishes NLX-112's neuroprotective effects. Additionally, pharmacological blockade of GDNF signaling using neutralising antibodies or Ret receptor inhibitors would clarify pathway dependency (Lu et al., 2024). Astrocyte-specific 5-HT_{1A} receptor knockout models would further determine if the neuroprotective effects are mediated via these receptors. Finally, direct measurement of GDNF secretion from NLX-112-treated primary astrocytes, with or without 5-HT_{1A} receptor antagonism, would confirm receptor-specific induction of GDNF release (Sandhu et al., 2009). Together, these experiments will clarify the astrocyte-specific and GDNF-dependent contributions to NLX-112's neuroprotective profile.

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