Fall 12-18-2015

Compensatory Mechanisms and T Cell Migration In Mouse Models of Dopaminergic Loss

Kristi M. Anderson
University of Nebraska Medical Center

Follow this and additional works at: https://digitalcommons.unmc.edu/etd

Part of the Behavioral Neurobiology Commons, Developmental Neuroscience Commons, Immunology and Infectious Disease Commons, Laboratory and Basic Science Research Commons, Other Neuroscience and Neurobiology Commons, and the Pharmacology Commons

Recommended Citation
https://digitalcommons.unmc.edu/etd/39

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.
Compensatory Mechanisms and T Cell Migration

In Mouse Models of Dopaminergic Loss

By

Kristi M. Anderson

A Dissertation

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

Department of Pharmacology and Experimental Neuroscience

Under the Supervision of Professor R. Lee Mosley

University of Nebraska Medical Center

Omaha, Nebraska

September, 2015
Acknowledgements

Completing the requirements for my Ph.D. was not always an easy task and it certainly wasn’t one that I could have done without the support of those who are closest to me.

My parents, Tim and Julie Anderson, were always supportive of my decision to work towards a Ph.D. degree. Your encouragement and pride mean more to me than you probably realize. Mom, thank you for always listening to me as I talked about everything that had happened over the week – good, bad, or indifferent. Dad, thank you for your eagerness to hear about the finer details of my research. I truly enjoyed each and every time we discussed my new research, whether in person or over the phone.

Brett, we’ve been together for 8 years and I’ve been a student for every single one of them. You were never very good at helping me with my studies or research, but you made me look forward to coming home every day. You were such a huge source of support during my Ph.D. work and I can’t image having done this without you.

Dana, Erin, and Cortney – you guys made that first year of BRTP exponentially more enjoyable. Because of you three, I always knew I would have a group of people that not only knew the frustrations of grad school, but who would also be willing to listen when things got especially stressful. I’m very grateful that I came in with a group of such smart and outgoing people. I
especially owe Dana a heartfelt thank you for your critical evaluation of my dissertation.

There are too many students throughout the department to thank each one individually. So many of you have been such a large part of my life these past years at UNMC and I know it would have much more difficult to complete my degree without your help and moral support. The students within our department have a real feeling of comradery and friendship with each other and I was fortunate to be a part of it. From intramurals, to happy hours, to rounds of golf, we always enjoyed being around one another and I can honestly say I will miss all of you.

My committee members – Drs. Jyothi Arikkath, Howard Gendelman, Tammy Kielian, Charles Murrin, and Larisa Poluektova have been immensely helpful and supportive during my time at the University of Nebraska Medical Center. Thank you all for your guidance in selecting appropriate coursework, furthering my research and dissertation project, and taking an interest in discussing career opportunities after a Ph.D.

My lab mates Adam, Rebecca, Kate, and Charles – being in a lab with people you hate would be miserable. I’m very lucky in that I ended up in the exact opposite situation. Of course you were all available to help with experiments or troubleshoot protocols, but you also helped to make the lab fun and enjoyable on a daily basis. I don’t think I could have picked a better group of people to work with.
Dr. R. Lee Mosley – over the past 5 years you have helped to make the lab a fun environment to work in. Your frequent presence in the lab was something that really drew me in 5 years ago because it showed your high level of commitment to your students. Due in large part to your critical evaluation of my papers and presentations, I know how to tell a story with my research and to always ask “what is the question?”. I know I’ve become a better writer and public speaker because of your thorough mentoring. I appreciate all the opportunities you have afforded for me from sending me to conferences, applying for travel grants and award, and authoring book chapters. It’s a rare mentor that takes such an interest in their students and I will be forever grateful to you.
Parkinson’s disease (PD) is the most common neurodegenerative movement disorder and second most common neurodegenerative disorder. PD is characterized by the selective loss of dopaminergic neurons and dopamine neurotransmitter within the substantia nigra and termini in the striatum. Progressive loss of dopaminergic neurons occurs over many years in PD, and by the time movement disorder symptoms manifest, up to 50-70% of dopaminergic neurons have been lost. Several aspects of PD pathology have been described in detail, but a better understanding of PD progression is needed to develop more efficient treatments.

Motor symptoms associated with PD do not manifest until significant numbers of dopaminergic neurons are lost, suggesting compensatory mechanisms play a role in maintaining normal motor function. However, little is known about these mechanisms and the role they play in delaying PD symptom onset.

Only palliative treatment is now available for PD. This consists of principally of dopamine replacement therapy and L-DOPA considered the gold
treatment standard. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD, chronic administration of dopamine replacement drugs, L-DOPA or BL-1023 in the absence of further degeneration, resulted in improved motor function and consistent increases in the number of TH+ neurons in the substantia nigra. The increase in TH+ neurons was not associated with dopaminergic neurogenic activity, but rather a phenotypic shift of GAD67+ GABAergic neurons to express TH. These data represent a novel effect of dopamine replacement therapy as triggering putative compensatory mechanisms, presumably to restore dopamine levels in a dopamine depleted environment.

An interleukin-23 (IL-23) knock-out mouse strain proven to have significantly reduced dopaminergic neuron population was used to test motor control and behavior. No significant differences were observed between knock-out and wild-type in any of the forced or unforced motor tests. These data suggest either insufficient dopaminergic loss to afford functional alterations or that compensation to the dopaminergic signaling pathway allowed for normal functioning.

Taken together, compensatory mechanisms represent a novel pathway for PD treatment that include symptomatic benefits as well as potential regenerative strategies. Targeting such pathways may provide more effective therapeutics by avoiding the secondary toxicities of current pharmaceuticals.
# Table of Contents

1. Introduction 1-24
   1.1 Parkinson’s disease overview 1-3
   1.2 Immunity and neurodegeneration 3-8
   1.3 Dopaminergic neurons and tyrosine hydroxylase in the developing midbrain 8-12
   1.4 GABAergic neurons 12-14
   1.5 Dopaminergic/GABAergic interactions 14-21
   1.6 Compensatory mechanisms in Parkinson’s disease 21-23
   1.7 Summary and conclusions 23-24

2. Phenotypic shift of GABAergic neurons in MPTP intoxicated mice 25-87
   Introduction 26-31
   Materials and methods 31-42
   Results 42-51
   Discussion 51-58
   Tables and figures 59-87

3. Neuronal and behavioral analysis of p19 deficient mice 88-147
   Introduction 89-91
   Materials and methods 91-99
   Results 99-107
   Discussion 107-113
   Tables and figures 114-147
4. Targeting cellular migration as a novel treatment for PD 148-167
   Introduction 148-152
   Materials and methods 152-155
   Results 156-158
   Discussion 158-161
   Figures 162-167
5. Conclusions 168-178
6. Bibliography 179-212
List of Abbreviations

6-OHDA – 6 hydroxy-dopamine

AD – Alzheimer’s disease

ALS – amyotrophic lateral sclerosis

APC – antigen presenting cell

BBB – blood brain barrier

CAM – cell adhesion molecule

CFA – complete Freund’s adjuvant

CNS – central nervous system

DAB - diaminobenzidine

DAT – dopamine transporter

EAE – experimental autoimmune encephalomyelitis

GABA – gamma-aminobutyric acid

GAD – glutamic acid decarboxylase

GFP – green fluorescent protein

HCM – home cage monitoring

IFA – incomplete Freund’s adjuvant

IFNγ – interferon gamma
IL – interleukin

L-DOPA – L-3,4-dihydroxyphenylalanine

LB – Lewy body

MAP2 – microtubule associated protein 2

MCAM – melanoma cell adhesion molecule

MHC II – major histocompatibility complex II

MPTP – 1-methyl-2-phenyl-1,2,3,6-tetrahydropyridine

MS – multiple sclerosis

N-α-syn – nitrated α-syn

OFAT – open field activity testing

PD – Parkinson’s disease

PFA - paraformaldehyde

RFP – red fluorescent protein

ROI – region of interest

SEM – standard error of mean

SRS – simple random sampling

TH – tyrosine hydroxylase

TNFα – tumor necrosis factor alpha
TR – Texas red

VTA – ventral tegmental area

α-syn – alpha synuclein
CHAPTER ONE
INTRODUCTION

Parkinson’s disease overview

Parkinson’s disease (PD) is a chronic progressive disease, the most common neurodegenerative movement disorder and second most common neurodegenerative disorder. Currently, there is no cure for PD, and therapies are purely palliative (Mosley et al., 2012). Pathologically, PD is characterized by the progressive loss of tyrosine hydroxylase (TH) expressing dopaminergic neurons in the substantia nigra pars compacta (SNpc) and striatal dopaminergic termini (Dauer and Przedborski, 2003). The loss of the neurotransmitter dopamine accounts for characteristic motor dysfunction presented in PD (Dauer and Przedborski, 2003). Arvid Carlsson played an instrumental role in arguing for dopamine’s role in controlling motor function and playing a part in PD (Rubin, 2007; Murrin, 2012): first, large amounts of dopamine are present in the corpus striatum. Second, depletion of dopamine from the striatum results in hypokinesis. Lastly, L-DOPA administration can counteract reserpine-induced hypokinesis (Carlsson et al., 1957; Bertler and Rosengren, 1959a, b; Carlsson, 1959; Murrin, 2012). Another, hallmark of PD neuropathology is the presence of Lewy bodies (LB); neuronal inclusions composed primarily of ubiquitin and misfolded and modified α-synuclein (Zhang et al., 2005). The slow, insidious nature of disease progression in PD determines that by the time motor disturbances first appear, more than 50-70% of dopaminergic neurons in the substantia nigra and 60-80% of striatal termini have been lost (Dauer and Przedborski, 2003). The selective
vulnerability of dopaminergic neurons in the substantia nigra is believed to be due to their sensitivity to combinations of oxidative stress, low levels of antioxidants, and high iron content (Chinta and Andersen, 2005). While the initiating factor in PD development is still unknown, several risk factors are proposed to play a role – age, rural residence, herbicide/pesticide exposure, high intake of dietary fats, heavy metal exposure, head trauma, and a history of neuroinflammation (Siderowf and Stern, 2003; Singh et al., 2007; Thomas, 2009; Mosley et al., 2012; Blesa et al., 2015). Physical symptoms manifest in the form of bradykinesia, resting tremor, muscular rigidity, and gait disturbances (Bernheimer et al., 1973; Hornykiewicz and Kish, 1987; Dauer and Przedborski, 2003; Olanow et al., 2009).

In 1982, Dr. William Langston described the “The Case of the Frozen Addicts” (Langston et al., 1983) - young men and women who after self-administering what was thought to be heroin, were found conscious, but unable to move or speak (Langston, 2014). It was later discovered that the patients had unknowingly administered 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a contaminant of illicit meperidine synthesis (Langston et al., 1983). The ability of MPTP to cause PD-like motor symptoms is due to its ability to cross the blood brain barrier (BBB), where it is taken up by resident glial cells of the CNS, and converted into the active metabolite MPP+ (Schneider, 2008). MPP+ has a strikingly similar molecular structure to dopamine and can therefore be selectively transported by the dopamine transporter. Once inside the neuron, MPP+ is transported by the organic cation transporter-1 (OCT1) across the
mitochondria membrane and reversibly binds complex I of the electron transport chain, interfering with ATP synthesis, depleting the neuron of energy, and resulting in injury and cell death. Since the discovery of MPTP and its ability to selectively destroy nigrostriatal dopaminergic neurons and induce PD-like neurodegeneration, it has become one of the most widely used animal models of PD (Schneider, 2008). While MPTP is able to recapitulate the loss of dopaminergic neurons and striatal termini and microglial activation seen in PD, it does not result in the formation of Lewy bodies as seen in PD patients (Przedborski et al., 2001; Dauer and Przedborski, 2003; Schneider, 2008). However, the model provides an important system in which to test new therapeutic strategies for dopaminergic neurodegeneration and replacement therapies for PD (Schneider, 2008).

**Immunity and neurodegeneration**

The barrier between the brain and peripheral immune system presents a unique scenario as to the mechanisms by which pathogenic agents are cleared from the brain. It is believed that during early PD, misfolded and modified proteins are not adequately cleared from the neurons and accumulate intracellularly (Thomas, 2009; Blesa et al., 2015). Upon release from the neurons, extraneuronal modified self-proteins lead to breakage of or deviation from immunological tolerance resulting in an immune response (Mosley et al., 2012). Although the initiating event of PD is not known, our laboratory has shown that modification or aggregation of alpha synuclein (α-syn) breaks immunological tolerance and triggers the initial immune response. (Benner et al., 2004; Olanow et al., 2009).
Nitration of the self-protein, α-syn (N-α-syn), is believed to initiate an immune response following its release from injured or dying neurons and drainage into the cervical lymph nodes where it is taken up by antigen presenting cells (APCs) and presented to naïve T cells through major histocompatibility complex II (MHC II) (Benner et al., 2008; Reynolds et al., 2008; Mosley et al., 2012). The T cells now specific for N-α-syn circulate throughout the body and cross the BBB at sites of inflammatory foci where they encounter cognate antigen and induce pro-inflammatory and neurotoxic effector functions. These perpetuate dopaminergic cell death either directly or through indirect mechanisms involving microglia and alternative states of activation (Guadagno et al., 2013).

The roles of the peripheral immune system in patients are supported by initial studies of peripheral lymphocyte populations from PD patients showing decreased frequencies and total numbers of CD4+ T lymphocytes compared to controls (Shalit et al., 1995; Richartz-Salzburger et al., 2007; Bonotis et al., 2008; Larbi et al., 2009). More detailed studies reporting changes in T cell populations in PD patients are sparse and do not reach a consensus on the specific CD4+ T cell populations altered in patients with PD. For instance, in one study the diminution of CD4+ T cell numbers in PD patients was found chiefly from decreased numbers of CD4+CD45RA+ naïve T cells and to a lesser extent from CD4+CD29+ memory subsets (Shalit et al., 1995); whereas, Stevens and colleagues reported decreased numbers of CD4+CD45RO+ memory T cells (Bonotis et al., 2008). A recent study by Saunders and colleagues showed slight, but significant increases in frequencies of CD4+CD45RO+ memory/effector T
cells with concomitant diminution of CD4+CD45RA+ resting/naïve T cell levels (Richartz-Salzburger et al., 2007; Saunders et al., 2012). Additionally, frequencies of peripheral CD4+ T cells with effector-associated phenotypes such as FAS+ were increased in patients, whereas those expressing α4β7 integrins and CD31 (PECAM1) were diminished. Notably, these changes in CD4+ T cell phenotypes were correlated with severity of motor function as scored by Unified Parkinson’s Disease Rating Scale, part III (UPDRS III). Differences in these immunological profiles among the few reports may range from the heterogeneity of disease to individual laboratory methodologies, but clearly will require further investigation to attain consensus profiles.

Post-mortem studies of PD patient brain tissues showed both CD4+ T cells and CD8+ T cells in close proximity to dopaminergic neurons within the substantia nigra at levels exceeding 10-fold those found in control brains (Speciale et al., 2007; Brochard et al., 2009). Moreover, these increased levels of T cells were specific to lesioned brain areas. Microarray analysis of peripheral blood leukocytes and substantia nigra brain tissue showed many genes expressed were in common with those expressed by TH17-mediated immune reactions and suggested that idiopathic Parkinsonism is a TH17 dominant autoimmune disease (Saresella et al., 2011). However, whether T cell infiltration is primary or secondary to PD progression is still unclear.

Our laboratories have substantial experience with the MPTP model, indicating that neuroinflammation is enhanced by the adoptive transfer of effector T cells, particularly those specific for N-α-syn, (Benner et al., 2004; Reynolds et
Adoptive transfer studies demonstrated that following MPTP-intoxication, T cells exacerbate both the level of neurodegeneration and neuroinflammation as well as prolong cell death and microglial activation with the lesion (Benner et al., 2004; Benner et al., 2008; Reynolds et al., 2008; Reynolds et al., 2009; Reynolds et al., 2010; Mosley et al., 2012; Kosloski et al., 2013). Recent evidence from clinical studies indicated that T cells with an activated or memory/effector phenotype are present in greater frequencies in PD patients compared to age- and environment-matched caregiver controls (Saunders et al., 2012). Increased proportions of those T cell subsets were directly correlated with diminished motor function and associated with diminished Treg function in PD patients (Saunders et al., 2012). Taken together, the detection of CD4+ and CD8+ T cells within the substantia nigra of mice treated with MPTP and in PD patients, the proximity of infiltrating T cells to MHC expressing microglia/macrophages, and CD4/CD8 ratios of infiltrating T cells that are reversed from those expected in peripheral circulation (Kurkowska-Jastrzebska et al., 1999; Ozaki et al., 2000; Benner et al., 2004; Benner et al., 2008; Brochard et al., 2009) provide strong evidence for the directed extravasation and migration of activated T cells to sites of inflammation and for the association of increased disease or lesion progression with increased T cell infiltration (Brochard et al., 2009). However, whether extravasation and migration of T cells is necessary and the mechanism(s) associated with T cell migration in Parkinsonism have not been adequately evaluated.
A newly described T cell lineage, TH17, has shed new light on PD pathology. TH17 cells are characterized by their proinflammatory functions and the secretion of interleukin-17 (IL-17) and are thought important in defense of extracellular pathogens and parasites. Differentiation of TH17 cells from a naïve T cell is dependent on multiple cytokines including IL-6, TGF-β, and IL-23 (Benwell and Lee, 2010). IL-23 plays a dual role in both driving the TH17 cell phenotype and also stabilizing TH17 cells whilst inhibiting the formation of TH1 cells. TH17 cells were first described in the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) (Rostami and Ciric, 2013; Robinson et al., 2014; Sie et al., 2014). They have also been shown to play an important role in disease progression in rat models of Alzheimer's disease (AD), and human amyotrophic lateral sclerosis (ALS) (Chen and Shannon, 2013; Rostami and Ciric, 2013; Luchtman et al., 2014; Robinson et al., 2014; Sie et al., 2014).

When specifically evaluating the role the adaptive immune system plays in PD, our laboratory and others have shown T cells specific for N-α-syn of the TH17 cell phenotype have an increased ability to exacerbate neurodegeneration compared to TH1 cells (Benner et al., 2004; Benner et al., 2008; Brochard et al., 2009; Reynolds et al., 2010; Mosley et al., 2012) even though both cell types are classically defined as pro-inflammatory T cells. Unlike TH1 cells, TH17 cells rely on IL-23 for survival and maintenance of their phenotype (Damsker et al., 2010). In the absence of IL-23, TH17 cells are not long lived and are unable to carry out
their effector function (Langrish et al., 2005; Chen et al., 2006; Gaffen et al., 2014).

The discovery of TH17 cells, their detrimental effects in neurodegenerative diseases and their respective models, has led to the development of multiple therapies targeting IL-23 expressing/responsive T cells that show significant efficacy (Chen et al., 2006; Gaffen et al., 2014). These include phase I and II clinical trials in immune-mediated inflammatory diseases (Gaffen et al., 2014) such as psoriasis, rheumatoid arthritis, Crohn’s disease, and ankylosing spondylitis (Gaffen et al., 2014). In the murine model of MS, mice that are unable to develop antigen specific TH17 cells are resistant to disease development (Sutton et al., 2006). Additionally, experiments using mice that are deficient in expression of IL-17 or p19 (an IL-23 component), or IL-23 receptor demonstrated a requirement for these cytokines and receptors in various inflammatory TH17-mediated diseases, such as EAE, psoriasis, inflammatory bowel disease, and collagen antibody-induced arthritis (Murphy et al., 2003; Langrish et al., 2005; Chan et al., 2006; Chen et al., 2006; Sherlock et al., 2012). Taken together, these results suggest that TH17 targeted therapy has the potential to improve therapy and quality of life for patients with inflammatory diseases. They also provide the basis for targeting TH17 cells in PD and animal models of PD.

**Dopaminergic neurons and tyrosine hydroxylase in the developing midbrain**

Dopaminergic neurons within the ventral midbrain make up almost 75% of all dopaminergic neurons in the CNS (German et al., 1983; Pakkenberg et al., 1991;
Blum, 1998; Wallen and Perlmann, 2003). Dopaminergic neurons in the ventral midbrain ultimately give rise to three clusters of neurons, termed A8, A9, and A10, that will develop into anatomically and functionally distinct populations. A8 and A10 populations will ultimately develop into the retrorubral field and ventral tegmental area, respectively (Hegarty et al., 2013). The A9 group of neurons, which give rise to the those in the substantia nigra, are particularly vulnerable to cell death compared to the other two groups and loss of A9 dopaminergic neurons is a driving force behind motor deficiencies in PD (Hornykiewicz and Kish, 1987; Hegarty et al., 2013).

Innervation of dopaminergic neurons into the striatum is critical during the establishment of the nigrostriatal pathway due to its role in motor function and control (Bourdy et al., 2014; Tremblay et al., 2015). Axons from midbrain dopaminergic neurons, which begin to appear at embryonic day 11 in mice, are guided by extrinsic cues in the dorsal midbrain and repulsive cues in the caudal brain stem (Gates et al., 2004). Dopaminergic axons from the substantia nigra run via the medial forebrain bundle where they terminate in the striatum, guided by additional chemoattractant factors released from the striatum and chemorepulsion factors released from the cortex (Specht et al., 1981b, a; Gates et al., 2004; Zhao et al., 2004). Loss of dopaminergic neurons and their axons during PD progression leads to significant reductions in the levels of striatal dopamine, resulting in the hallmark symptoms of PD – resting tremor, muscle rigidity, gait abnormalities, and slowness of movement (Antony et al., 2013).
Therefore, re-establishment of dopaminergic axons into the striatum would provide a novel approach in the treatment of PD.

Tyrosine hydroxylase is used to identify dopaminergic neurons (Chinta and Andersen, 2005). TH, the rate-limiting enzyme in catecholamine synthesis, is responsible for the conversion of L-tyrosine into L-DOPA, the precursor to dopamine (Haavik and Toska, 1998). The discovery of TH, and the description in its role in synthesis and metabolic fate of catecholamines, is due to the work of Julius Axelrod (Axelrod, 1971{Rubin, 2007 #418}) and Ulf von Euler (Von Euler, 1946b, a; Rubin, 2007) which ultimately led to the 1970 Nobel Prize in Physiology or Medicine with Bernard Katz (Shafir, 1994{Rubin, 2007 #418}). The physical symptoms associated with PD are largely a consequence of the death of TH expressing dopaminergic neurons and the ensuing decline in dopamine production. Early studies demonstrating the efficacious outcome of L-DOPA administration in combating akinesias in PD (Birkmayer and Hornykiewicz, 1961, 1962) proved restoration of dopamine levels in PD patients could alleviate symptoms of disease and initiated today’s gold standard of PD treatment (Murrin, 2012). The study of dopaminergic neurons was aided and advanced upon generation of a transgenic mouse line that expresses green fluorescent protein (GFP) under control of the rat TH promoter. Use of the TH-GFP mouse, has led to extensive characterization of the expression pattern of TH within the developing midbrain of mice. Such studies have shown that expression of TH begins in the early embryonic stages of development with sharp decreases in expression in the later stages of embryonic development. Researchers have
hypothesized that the decrease in TH expression coincides with the timeframe of lateral and rostral migration of dopaminergic neurons. The expression of TH is then increased in the very late stages of embryonic development and continues to increase into the period of postnatal development until approximately postnatal day 14 (p14) when the levels are similar to those seen in the adult mouse (Matsushita et al., 2002).

In the TH-GFP mouse model, in addition to the expression of TH as indicated by expression of GFP, the intensity of GFP expression varies during development in the midbrain. As development progresses the number of neurons that express both TH and GFP increase, as does the intensity of GFP by TH immunoreactive neurons. This leads to the possibility that the expression pattern of GFP could be an indicator of not only a dopaminergic neuronal phenotype, but also the developmental stage of the neuron relative to the intensity of the GFP signal. Furthermore, the intensity of GFP expression could indicate how active and functional the neuron may be. In other words, a neuron expressing high GFP intensity may indicate a more active neuron compared to lowly expressing GFP+ neurons, capable of producing more dopamine than a neuron with low GFP expression in the same region.

Several theories have been proposed to explain the sharp rise in GFP expression between embryonic days 12 and 14, including an overlap in transcription factor activation. Alternatively, others have postulated that an increase in the stability of TH mRNA helps to regulate the level of TH expression (Tank et al., 2008). Still others have stated that during development, a silencer
gene is switched on and off and directs the increase and decrease in TH expression. It is thought that the level of dopamine metabolism may play a role in the expression of TH, and that as dopamine metabolism increases, the gene silencer involved in TH expression is itself silenced, leading to increased TH expression. This increase in dopamine metabolism occurs as dopaminergic neurons are closer to a more mature state and more synapses are made as a result (Schimmel et al., 1999; Kim et al., 2006; Calcagno et al., 2013). A better understanding of the regulation and expression pattern of TH during development will help develop therapeutics that target increased TH expression or activity by surviving neurons.

**GABAergic Neurons**

GABAergic neurons are an inhibitory class of neurons that utilize gamma-aminobutyric acid (GABA) as their primary neurotransmitter. GABAergic neurons also referred to as medium spiny neurons and, as their name suggests, are medium sized compared to other neuron populations and have extensive dendritic projections (Nair-Roberts et al., 2008; Tepper, 2010; Brown et al., 2012). Expression of glutamic-acid decarboxylase (GAD) is the most commonly used marker for GABAergic neurons. In mammals, GAD exists in two isoforms with molecular weights of 65 kDa (GAD65) or 67 kDa (GAD67), with neurons of the CNS expressing GAD67 most commonly (Soghomonian and Chesselet, 1992; Pinal et al., 1997).

As a result of the developmental patterning in the embryonic midbrain, the midbrain is divided into dorsoventral subdivisions termed m1 – m7 which are
characterized by specific homeodomain transcription codes and the specific
neuronal subtypes to which these regions give rise (Nakatani et al., 2007; Kala et
al., 2009). GAD67 expressing GABAergic neurons originate from the m1, m2,
m3, and m5 regions. GABAergic neurons of the midbrain show unique
characteristics compared to GABAergic neurons in other brain regions. First,
GABAergic neurons originate from multiple regions (m1, m2, m3, and m5) and
show little tangential migration. This is in stark contrast to GABAergic neurons of
the forebrain, for example, which have a propensity to migrate from their region
of origin to their target location (Anderson et al., 1997; Tamamaki et al., 1997).
Additionally, midbrain GABAergic neurons tend to function as projection neurons,
whereas GABAergic neurons of the forebrain are local interneurons, controlling
the action of other neurons in the immediate vicinity (Lahti et al., 2013).

Putative therapies for the treatment of PD have focused, in large part, on
restoring the levels of dopamine within the midbrain (Haavik and Toska, 1998;
Siderowf and Stern, 2003; Hutter-Saunders et al., 2011). Studies showing
reduced levels of GABA and GABA_A receptor density in PD patients suggest that
GABA deficiency may be associated with dyskinesia, and therefore may a
secondary deficit in PD patients. Within the basal ganglia of PD patients,
GABAergic neuronal dysfunction has been described and this reduction in
signaling may contribute to the altered motor function associated with PD. Using
this knowledge, studies have shown that GABA therapy reduce motor asymmetry
in rats, and that GABA receptor agonists suppress limb tremor in PD patients
(Bartholini et al., 1987; Levy et al., 2001). Taken together, this provides strong
evidence for GABA therapy as a promising target to relieve motor symptoms in PD patients.

**Dopaminergic-GABAergic interactions**

Within the substantia nigra of mice, GABAergic neurons intermingle with dopaminergic neurons, making these two neuronal populations highly interactive and connected. Proper migration and connectivity into the substantia nigra depends on intact dopaminergic and GABAergic systems. Mice birth-dating studies showed at embryonic day 13, the ventral mesencephalon containing the anlages for the substantia nigra, ventral tegmental area, and retrorubral field, is completely devoid of GABAergic neurons, but rich in dopaminergic neurons. Shortly after embryonic day 17, GABAergic neurons enter to reside along with the dopaminergic neurons (Vasudevan et al., 2012). Furthermore, in the absence of complete dopaminergic architecture, GABAergic neurons fail to migrate and form the correct neuronal networks within the ventral mesencephalon (Vasudevan et al., 2012). These essential interactions within the ventral mesencephalon will be useful in furthering our understanding of neurodegenerative diseases and may provide novel insight into treatments for brain disorders.

GABAergic neurons are widely co-distributed with other types of neurons in the midbrain, but are enriched in specific regions of the mature midbrain. Within the substantia nigra, populations of GABAergic neurons are intimately associated with dopaminergic neurons (Lahti et al., 2013). Within the ventral tegmental area (VTA), 20-35% of neurons are of the GABAergic lineage and are
thought to regulate the activity of dopaminergic neurons (Fields et al., 2007; Nair-Roberts et al., 2008; Omelchenko and Sesack, 2009; Brown et al., 2012). The GABAergic neurons of the substantia nigra project to the thalamus, superior colliculus, and basal ganglia where they play a part in regulating voluntary movements (Zhou and Lee, 2011). Because of the unique and intimate association that GABAergic neurons in the midbrain (substantia nigra and VTA) share with dopaminergic neurons in this region, they are frequently referred to as D-GABAergic neurons (Lahti et al., 2013). Fate mapping experiments in mice showed the majority of D-GABAergic neurons originate outside of the midbrain, specifically in rhombomere 1, at approximately embryonic day 12.5 (Achim et al., 2012) and migrate to the midbrain mid-gestation (Lahti et al., 2013). The exact migratory pattern of midbrain GABAergic neurons and timing of their appearance has yet to be described.

In addition to identifying the origin of D-GABAergic neurons, experiments showed that within the substantia nigra are two developmentally different subgroups of D-GABAergic neurons: anterior and posterior rhombomere 1-derived neurons, aD-GABAergic and pD-GABAergic, respectively (Lahti et al., 2013); however their phenotypic expression as to neuronal subsets have not been defined. This distinction between populations of GABAergic neurons in adjacent regions may prove to be invaluable in future studies that attempt to target a specific region of GABAergic neurons for therapeutic approaches. Furthermore, additional studies will help to build the knowledge as to how
GABAergic neurons control behaviors and the behaviors in which they play a particularly important role.

Dopaminergic neurons in the nigrostriatal pathway interact with GABAergic neurons both at their cell body and termini regions, and loss of dopaminergic neurons in PD leads to dysregulation of GABAergic neurons, resulting in symptoms associated with PD (Chesselet and Delfs, 1996; Chesselet, 2002). Additional evidence on the effects of dopamine on GABAergic neurons comes from studies of lesioned rodents. Lesions to the nigrostriatal dopaminergic pathway leads to an increase in GAD immunoreactivity in the striatum, elevated levels of GAD67 mRNA, and increased GAD activity (Segovia et al., 1990; Soghomonian and Chesselet, 1992; Soghomonian et al., 1992). Furthermore, transplants of dopamine producing cells in the striatum reverse the increase in GAD activity induced by dopaminergic loss (Segovia et al., 1989). The presence of D1 receptors on GABAergic afferent from the striatum points to a facilitory effect of dopamine on GABA release in the substantia nigra through a D1-mediated action (Floran et al., 1990; Cameron and Williams, 1993). Taken together, these data suggest dopaminergic neurons exert complex modulatory effects along the nigrostriatal pathway and changes on GABAergic transmission due to effects of dopaminergic lesion play a critical role in PD symptoms (Sesak, 2002).

Within the striatum, dopamine concentrations appear to regulate the numerical density of TH+ neurons in a dose-dependent manner. Separate studies examining the number of TH+ neurons within the striatum showed clear
discrepancies between the effects of dopamine replacement therapy in the form of L-DOPA, one demonstrating decreases in the number of TH+ striatal neurons (Lloyd et al., 1975; Huot and Parent, 2007) and the other demonstrated an overall increase in striatal TH+ neurons (Jollivet et al., 2004; Tande et al., 2006). Ultimately these contradictory findings may be explained through a dual effect hypothesis wherein at higher levels, dopamine is proposed to act in conjunction with growth factors to up-regulate TH expressing neurons within the striatum (Huot and Parent, 2007). At lower levels, dopamine may control TH expression by means of a more conventional and straightforward feedback mechanism. Regardless, it is apparent that local dopamine concentration appears to regulate the numerical density of TH expressing neurons within the striatum. In the absence of dopamine, due to PD progression or neurotoxin-induced neuronal death, the increase in TH+ neurons is likely the result of a phenotypic shift of striatal interneurons so that they may act as a local source of dopamine, and as such be part of a compensatory mechanism for dopaminergic neuronal death (Jollivet et al., 2004; Abe et al., 2010; Busceti et al., 2012).

The most common marker for dopaminergic neurons is TH; however TH expression also occurs transiently in some neurons derived from the neural crest (Jaeger and Joh, 1983). While cells that retain TH expression may signify the establishment of these cells developing into a mature dopaminergic neuron (Black, 1982), the following findings suggest the mere presence of TH may not be sufficient to determine if a neuron is dopaminergic. Several reports provide significant evidence that TH and GAD67 co-localize (TH+GAD67+) naturally in
the brain of mammals, birds, and reptiles (Kosaka et al., 1987a; Kosaka et al., 1987b; Kosaka et al., 1988; Wulle and Wagner, 1990). While the exact number or percentage of neurons in mouse or primate brains that are positive for both TH and GAD67 expression has not yet been reported, Abe and colleagues successfully used double immunofluorescent labeling of neurons to show co-expression of these two markers in neurons at postnatal day 8 (Abe et al., 2010). While the presence of TH+GAD67+ neurons have been demonstrated by multiple investigations, this neuronal population does not appear to be stable or long lived. However, the exact timeline for when these neurons begin to decline in number is an area of debate as later studies reported their existence as late as postnatal day 18 (p18) (Masuda et al., 2011), in stark contrast to p8 reported by Abe and colleagues. Regardless of the temporal appearance and disappearance of the neurons, there is a consensus that these neurons begin to wane during development and their presence in the brain of adult mice could signify a previously unreported phenomenon.

Two independent studies reported seeing an increase in TH+ neurons in the substantia nigra following MPTP assault (Mao et al., 2001; Tande et al., 2006), contradictory to the previously described actions of MPTP. Initially this increase was explained by neurogenesis from cells originating in the subventricular zone or progenitors within the striatal parenchyma (Bjorklund and Dunnett, 2007). This theory was later disproved by the studies due to a lack of bromodeoxyuridine (BrdU) incorporation to label newly formed cells (Mao et al., 2001; Tande et al., 2006; Bjorklund and Dunnett, 2007). This led to the theory
described and tested herein that pre-existing neurons in the substantia nigra begin expressing TH, possibly as a compensatory mechanism for MPTP-induced neuronal loss and reduction in dopamine.

GABAergic neurons immunoreactive for TH represent a unique and novel type of neuron that has yet to be further characterized and explained. Several lines of evidence suggest that these neurons exist naturally, but are normally eliminated by the action of extracellular dopamine between postnatal day 4 and postnatal day 8 (Masuda et al., 2011). The olfactory bulb is an area where this unique population of TH+GAD67+ neurons is especially plentiful; at least for a short period of time. In reptiles, it has been reported that in the glomerular layer and external plexiform layer/mitral cell layer approximately 91% of TH immunoreactive cells were also GAD67 immunoreactive (Kosaka et al., 1991), but this number has not been validated in rodents or primates.

GABAergic neurons immunoreactive for TH were originally described in the striatum of adult monkeys (Dubach et al., 1987) and have since been reported in other species including rat (Tepper, 2010). The description of TH+ neurons within the striatum are shockingly variable, ranging from tens of thousands as described by Dubach et al. to only 1 neuron per section in humans (Huot and Parent, 2007). TH+ neurons present in the striatum are often reported to also be immunoreactive for the dopamine transporter (DAT), GABA, or GAD67 suggesting that not all TH+ neurons are purely dopaminergic in nature, but rather could be of the GABAergic lineage (Betarbet et al., 1997; Cossette et al., 2005b; Cossette et al., 2005a; Mazloom and Smith, 2006; Tande et al., 2006; Huot and
Parent, 2007; Tepper et al., 2010). Still, some investigators find these cases unconvincing and argue that TH+ neurons do not naturally occur in the striatum of animals, but are only seen after MPTP or 6-OHDA lesion (Tepper, 2010). While others argue that TH+ GABAergic neurons are not present in control or lesioned rodent models, but only are detected in the striatum of primates (Dubach et al., 1987; Betarbet et al., 1997; Tepper, 2010).

GABAergic neurons of the midbrain are a highly heterogeneous group of neurons in terms of their location, morphology, connections, functions, developmental origins, and molecular regulatory mechanism. Of specific importance, are the GABAergic neurons that are closely associated with dopaminergic neurons in the substantia nigra such as the previously mentioned D-GABAergic neurons. These neurons are distinct from other midbrain GABAergic neurons as they are derived from adjacent brain regions and under control of a distinct set of transcription factors (Lahti et al., 2013). Because of these unique properties, GABAergic neurons of the substantia nigra may be especially sensitive to changes in the environmental milieu, and as such, have the capacity to act as a source of dopamine in a dopamine-depleted environment.

What remain to be described are the cellular mechanisms by which TH expression is being triggered in a neuronal population not known to express this marker under normal physiological conditions. In order to further understand the expression and function of TH in GABAergic neurons, we would need to elucidate the regulating mechanism(s). As discussed above TH can be regulated
by multiple mechanisms, phosphorylation playing the most prominent role. For example, direct phosphorylation of serine 31 by ERK1 and ERK2 will increase TH activity. Whereas phosphorylation of serine 19 by either mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) or calcium-calmodulin-dependent protein kinase II (CaMKII) will increase the phosphorylation of serine 40 ultimately resulting in an increase in TH activity. The chemical compound nicotine has been proposed to have an interesting role in the regulation of TH by phosphorylation for as long as 48 hour in vitro (Haavik and Toska, 1998; Bobrovskaya et al., 2007). Furthermore, there is an inverse correlation between smoking and developing PD, suggesting a neuroprotective action of nicotine (Quik, 2004).

Another avenue by which TH expression may be suddenly triggered in GABAergic neurons is due to removal of inhibitory signals. Since TH is regulated via feedback inhibition, following dopaminergic neuronal death the feedback inhibition may be lifted due to the diminished dopamine levels. More research is needed to evaluate the phosphorylation pattern of TH in GABAergic neurons as well as the level of protein kinases, second messengers, TH mRNA and protein levels.

**Compensatory mechanisms in Parkinson’s disease**

Pathological hallmarks of PD include a significant loss of the dopaminergic neurons in the substantia nigra and striatal termini. However, by the time symptoms appear greater than 50% of dopaminergic neuronal bodies and up to 80% of striatal termini have been lost. This suggests a physiological
compensation for the slow loss of dopaminergic neurons (Lloyd et al., 1975). It is unlikely that only one compensatory mechanism ensues during PD progression, but rather several mechanisms come into play during different stages of PD development. These compensatory mechanisms include, but are not limited to, increased sensitivity to dopamine, increased expression of dopamine receptors, and increased enzymatic activity of TH. While these compensatory mechanisms may delay symptom onset, they do not stop disease progression, and as dopaminergic neurons continue to die the compensatory mechanisms ultimately fail and physical symptoms appear. There is strong evidence for a striatal dopamine:acetylcholine imbalance in PD (Greenblatt and Shader, 1973) and it has been proposed that in early PD, a homeostatic dampening of striatal cholinergic activity maintains a normal striatal dopamine:acetylcholine balance (Lloyd et al., 1975). This mechanism is proposed to be active during early PD, but upon continued dopaminergic neuron loss, can no longer maintain the homoeostatic balance.

Dopaminergic:GABAergic interactions within the nigrostriatal pathway play a large role in maintaining proper signaling pathways. Normally, GABAergic neurons inhibit dopaminergic cell bodies in the substantia nigra, controlling the firing rate of the cells (Lloyd et al., 1975). During the early stages of PD, one of the proposed compensatory mechanisms exploits the dopaminergic:GABAergic interaction. Decreasing the activity of GABAergic neurons allows the surviving dopaminergic neurons to fire at an increased rate and maintains normal motor control. However, as previously stated, this compensatory mechanism cannot
keep up with the continuous dopaminergic cell death and will eventually be insufficient to overcome the severe dopamine deficit with presentation of PD symptoms and signs.

Additional compensatory mechanisms have been proposed, all with the endpoint of enhancing the effects of remaining dopamine in the striatum. These mechanisms include increased synthesis and release of dopamine by surviving dopaminergic neurons (Zigmond et al., 1990), increasing the number or sensitivity of dopamine receptors in the striatum, decreasing the turnover of striatal dopamine, using striatal interneurons as an alternative source for dopamine production, upregulation of the enzymes responsible for dopamine synthesis, and increased sprouting of neuron terminals by surviving dopaminergic neurons (Turjanski et al., 1997; Brotchie and Fitz-Attas, 2009). While evidence exists that support a role for these mechanisms in the delaying of PD symptom onset (Brotchie and Fitz-Attas, 2009), symptoms do ultimately appear indicating the mechanisms do not afford a definitive solution, but are merely delaying the inevitable. Gaining insight as to which compensatory mechanisms come into play at which stages of disease, may help to develop treatments that target prolonging the effectiveness of these mechanisms and help to delay intervention by L-DOPA and dopamine replacement therapy.

Summary and conclusions

Neurodevelopment of the midbrain that can adequately support proper brain function requires very complex and multifaceted processes that interact both at the temporal and spatial levels. Loss of dopaminergic neurons within the
substantia nigra and their striatal termini, as in PD and MPTP models of PD, results in deficits in motor function and control. While these physical symptoms can be lessened through the use of dopamine replacement therapy, it affords no cure for PD, and patients will most likely become refractory after long-term use. Interestingly, classical physical symptoms associated with PD do not become apparent until greater than 50% of neurons are lost within the substantia suggesting either that a threshold concentration of dopamine must be maintained or intrinsic compensatory mechanisms are in place to maintain proper function and control within the CNS. However, these mechanisms untimely fail as the levels of dopamine continue to drop with the progression of PD. With an extensive understanding of the homeostatic balance in the CNS, new therapies aimed at stopping, slowing, or even reversing disease progression will lead to restoration of neuron numbers and dopamine concentrations, ultimately improving neurologic and motor deficits seen in disorders of the CNS. We further posit that under pathological conditions such as neuroinflammation and dopaminergic neuronal loss, that mature neurons in the midbrain shift phenotype and as such, could serve as a source of neurons capable of producing dopamine and restoring proper motor control. *In toto*, increased dopaminergic neuron numbers, termini densities, and/or endogenous dopamine levels achieved either by neuron or dopamine replacement therapies or by targeting upregulation of compensatory mechanisms may provide improved therapeutic outcomes for PD patients.
CHAPTER TWO

PHENOTYPIC SHIFT OF GABAERGIC NEURONS IN MPTP INTOXICATED MICE

ABSTRACT

We previously showed that chronic administration for 35 days of L-3,4-dihydroxyphenylalanine (L-DOPA) or BL-1023 [an L-DOPA-gamma-aminobutyric acid (GABA) conjoined compound] improved locomotor function and increased numbers of tyrosine hydroxylase (TH) immunoreactive (TH+) neurons in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice compared to those given no drug. We therefore sought to identify whether the increase in TH+ neurons was a consequence of neurogenesis or rather from a phenotypic shift of surviving neurons in the substantia nigra. Using the thymidine analogue bromodeoxyuridine (BrdU), we sought to identify newly formed neurons after 21 days of L-DOPA or BL-1023 treatment, but were unable to do so. Dual immunofluorescent analysis of tissues for TH and glutamic acid decarboxylase-67 (GAD67), a marker for GABAergic neurons revealed that mice treated with either L-DOPA or BL-1023 showed significant increases in the numbers of TH+GAD67+ neurons in the substantia nigra compared to controls. These findings support the notion that numbers of GAD67+ neurons upregulate TH expression and effectively increase the numbers of TH+ neurons in response to MPTP-intoxication, loss of dopaminergic neurons, and/or chronic administration of dopamine-replacement drugs.
INTRODUCTION

Parkinson’s disease (PD) is the most common neurodegenerative movement disorder and the second most common neurodegenerative disorder overall next to Alzheimer’s disease (AD). PD is characterized by the progressive loss of TH+ dopaminergic neurons in the substantia nigra and their termini in the striatum. The symptoms and clinical signs of PD originate from the loss of dopamine characteristically produced by the neurons that are progressively lost. Current therapeutics are primary palliative and include dopamine replacement and dopamine receptor agonists, which decrease in efficacy over time and can cause significant side effects that may become intolerable (Bjorklund and Cenci, 2010). Thus, therapies that slow or reverse neuronal death and disease progression, rather than treating symptoms, are greatly needed.

TH is the rate-limiting enzyme in catecholamine synthesis and loss of TH+ neurons in PD results in a significant reduction in striatal dopamine levels. L-DOPA is the gold standard for the treatment of PD (Agid, 1998; Agid et al., 1998) and works to effectively bypass TH in the dopamine synthesis pathway to restore striatal dopamine levels. While therapies aimed at restoration of dopamine levels are beneficial to patients, targeting gamma-aminobutyric acid (GABA) may also prove to be a valuable treatment option. In rats, GABA therapy was shown to reduce motor asymmetry (Mehta and Chesselet, 2005) and GABA receptor agonists suppress limb tremor in PD patients (Pahapill et al., 1999). However, the therapeutic potential of GABA is hindered by its chemical structure. The hydrophilic functional groups on GABA greatly limit its ability to cross the
blood brain barrier (BBB), thus covalently linking L-DOPA and GABA in the form of the drug BL-1023 effectively increases the transport of GABA across the BBB (Stein, 2012).

Recent studies demonstrated that TH+ neurons in the substantia nigra and striatum of MPTP-intoxicated animals are increased several weeks post-intoxication (Mao et al., 2001; Tande et al., 2006; Bjorklund and Dunnett, 2007; Hutter-Saunders et al., 2011). Initially the increase in TH was explained by neurogenesis of dopaminergic neurons. However, this theory lacks critical support in studies where dopaminergic neurogenesis has been examined after MPTP treatment (Mao et al., 2001; Tande et al., 2006; Bjorklund and Dunnett, 2007), but has never been tested in the MPTP murine model. In light of the lack of substantial neurogenic activity, subsequent theories herein suggested the increase in TH is due to phenotypic shift or up-regulation of TH from surviving cells within the substantia nigra. However, the cell type responsible for increased TH production had not been identified. Furthermore, whether purported neuroprotective pharmaceuticals function via induction of TH has not been assessed.

Dopaminergic neurons represent a heterogeneous group of cells localized to specific regions in the brain including the diencephalon, mesencephalon [including the substantia nigra and ventral tegmental area (VTA)], olfactory bulb, and retina with approximately 75-90% of the total number of brain dopaminergic neurons residing in the mesencephalon (Dahlstroem and Fuxe, 1964; Wallen and Perlmann, 2003; Chinta and Andersen, 2005; Bjorklund and Dunnett, 2007). TH
expression occurs only transiently in neurons derived from the neural crest (Jaeger and Joh, 1983) and cells that retain TH expression may signify the establishment of these cells developing into a mature dopaminergic neuron (Black, 1982). Appearance of dopaminergic neurons within the ventral neural tube begins between embryonic day 11 and 13 in mice. These neurons then migrate to locations within the substantia nigra compacta and VTA (Kawano et al., 1995; Matsushita et al., 2002), become post-mitotic and begin to innervate other brain regions (Wallen and Perlmann, 2003; Chinta and Andersen, 2005). Axonal processes from the dopaminergic neurons that remain within the substantia nigra and VTA target the striatum, with the former comprising the nigrostriatal dopaminergic pathway and the latter comprising the mesocorticolumbic dopaminergic pathways. The importance of nigrostriatal development is underscored as the loss of neuronal bodies and axons along the nigrostriatal pathway due to PD or MPTP-intoxication is thought to play a critical role in development of motor deficits associated with PD (Chesselet, 2002).

Dopaminergic systems (nigrostriatal, mesolimbic, and mesocortical) interact with GABAergic neurons, both at their cell body and termini. Dopamine within the nigrostriatal pathway plays a crucial role in the regulation of GABAergic neurons, and a reduction in dopamine levels in PD can lead to dysregulation of GABAergic neurons which in turn, can contribute to PD symptomology (Chesselet, 2002). In the context of dopaminergic-GABAergic interactions, several reports provided significant evidence that the neuronal markers TH (dopaminergic) and GAD67 (GABAergic) co-localize naturally in the brain of
mammals, birds, and reptiles (Kosaka et al., 1987b; Kosaka et al., 1988; Wulle and Wagner, 1990; Kosaka et al., 1991), but whether a direct relationship between dopaminergic and GABAergic neurons exists in the substantia nigra is unknown (Chesselet, 2002). Previous reports showed that within the glomerular layer and external plexiform layer/mitral cell layer approximately 91% of TH immunoreactive cells were also GAD67 immunoreactive for a period of time (Kosaka et al., 1991). Using a TH-GFP transgenic mouse, wherein green fluorescent protein (GFP) transcription and expression is under control of the TH promoter, studies revealed that not all GFP+ neurons within the substantia nigra and VTA were dopaminergic, and were postulated to be of a GABA lineage (Matsushita et al., 2002). While evidence suggest that during development, co-expression of both TH and GAD67 is normal, these neurons are typically eliminated by the action of extracellular dopamine between postnatal day 4 and postnatal day 8 (Busceti et al., 2012) with some studies reporting their existence as late as postnatal day 18 (Masuda et al., 2011). However, to date, no reports have been provided with definitive evidence for the co-expression of TH and GAD67 in mature neurons within the substantia nigra of adult animals, or that this phenotype is inducible.

Expression and activity of TH within the CNS is controlled by multiple short- and long-term regulatory mechanisms including, but not limited to, dopamine concentrations (Ames et al., 1978; Busceti et al., 2012) and post-translational modifications (Haycock and Haycock, 1991). Phosphorylation of TH at various serine residues within the N-terminal regulatory domain has been
shown to have an effect on both the activity and stability of the enzyme (Lazar et al., 1981; Vrana et al., 1981; Vrana and Roskoski, 1983; Kumer and Vrana, 1996; Nakashima et al., 2013b). Findings suggest that phosphorylation of TH converts the enzyme to a more active state that is simultaneously less stable, as demonstrated by a shorter half-life compared to its non-phosphorylated counterpart (Gahn and Roskoski, 1995; Kumer and Vrana, 1996). In models of neurodegeneration, reports indicate that phosphorylation state of serine 31 (Ser31) and serine 40 (Ser40) within the regulatory domain of TH are altered, presumably in an attempt to increase the activity of remaining TH and restore levels of endogenous dopamine (Ames et al., 1978; Vrana et al., 1981; Kumer and Vrana, 1996; Kumar et al., 2003; Bobrovskaya et al., 2007; Ong et al., 2012; Nakashima et al., 2013a; Salvatore, 2014). Whether neuroprotective pharmaceuticals function via induction of TH transcription or through post-translational phosphorylation of TH to increase activity has not been thoroughly assessed. The latter is supported by long-term studies that showed an inverse correlation between cigarette smoking and the development of PD (Fratiglioni and Wang, 2000; Galanaud et al., 2005) whereby nicotine, a major component of cigarette smoke, has been shown to induce phosphorylation of TH in vitro for up to 48 hours (Haavik and Toska, 1998; Bobrovskaya et al., 2007). Therefore the potential neuroprotective role of nicotine, and possibly other drugs, could be through the induction of TH phosphorylation (Quik, 2004).

Herein we hypothesize that a novel compensatory mechanism exists in which surviving GABAergic neurons within the substantia nigra undergo a
phenotypic shift and begin expressing TH as a putative mechanism to increase dopamine production in a dopamine-depleted environment.

MATERIALS and METHODS

Animals

C57BL/6J (wild-type) mice were purchased from Jackson Laboratory. For direct visualization of dopaminergic neurons, mice on the C57BL/6 background expressing GFP driven by the TH promoter (C57BL/6.TH-GFP, or TH-GFP mice) were obtained from Osaka, Japan (Sawamoto et al., 2001) and bred in our facility. All mice were housed and maintained on a 12:12 hr light/dark cycle with ad libitum access to food and water and were randomly assigned to treatment groups. Studies were conducted in accordance with the animal care guidelines issued by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. TH-GFP mice were crossed with C57BL/6J mice and pups genotyped to identify mice positive for the GFP gene. Primers for each marker were custom designed (Table 1) using Invitrogen Primer Design, reconstituted in water to a concentration of 20 nM, aliquotted, and stored at -20° C. PCR cycle was as follows: 94° C for 3 min, 31 cycles (94° C for 30 sec, 56° C for 1 min, and 72° C for 1 min), 72° C for 2 min, and held at 4° C. DNA samples were loaded onto 1.5% agarose gels and ran at 110 V for 35 minutes to obtain sufficient band separation. The fluorescent DNA stain GelRed was added to the agarose mixture to identify DNA bands at the correct molecular weight.

Acute MPTP intoxication
To address the sensitivity of dopaminergic neurons in TH-GFP mice to MPTP induced death we directly compared the level of neuronal death as a function of neuronal survival observed in wild-type and TH-GFP mice when intoxicated with increasing levels of MPTP. Male mice, 8 - 10 weeks of age, were randomized into 4 treatment groups, on day 0 four subcutaneous injections (s.c.) were administered at 2 hour intervals per injection of either PBS (10 ml/kg) or MPTP (calculated for free base dose of 10, 14 or 18 mg/kg). For drug treatment studies, male TH-GFP mice, 8-10 weeks old, were randomized into 8 treatment groups and immunized with 4 s.c. injections of MPTP (18 mg/kg in a 10 ml/kg volume) or PBS (10 ml/kg) on day 0; each injection given at 2 hour intervals. MPTP handling and safety measures were in accordance with the National Institutes of Health, the University of Nebraska Medical Center, and prior published guidelines (Przedborski et al., 2001).

**Drug treatment**

MPTP-treated mice were administered daily injections for 21 days of either L-DOPA or BL-1023. L-DOPA was reconstituted in water, pH 2.7, to a concentration of 28.4 mg/mL which is equimolar to the amount of L-DOPA in the BL-1023 compound. BL-1023 (BioLineRx, Ltd., Jerusalem, Israel) was reconstituted in water, pH 7, to a concentration of 40 mg/mL. Drugs were made fresh every 2 days. Daily intraperitoneal (i.p.) drug treatments began on day 7 post-MPTP and continued until day 28. To detect possible neurogenic activity occurring as a consequence of neuronal assault and/or drug treatment, the thymidine analogue bromodeoxyuridine (BrdU) was added to the drinking water
of mice at a concentration of 1 mg/mL starting on day 7 post-MPTP. To ensure animals did not decrease water intake due to taste aversion, glucose was added to BrdU-containing water for the first 7 days. BrdU-containing water was protected from light and changed every 7 days until day 21 post-MPTP when animals returned to normal water for the remainder of the study.

*Immunohistochemistry*

Upon completion of study, 7 or 28 days post-MPTP, animals were terminally anesthetized with pentobarbital and perfused first with PBS at a flow rate of 10.5 – 11.5 mL/minute for 3 minutes. Animals were then perfused with 4% paraformaldehyde (PFA) dissolve in PBS (pH 7.4) for 8 minutes. After perfusion, brains were carefully harvested and post-fixed in 4% PFA overnight, and cryopreserved in 30% sucrose/PBS for 48 hours. Brains were then snap-frozen in 2-methylbutane that had been cooled on dry ice and were embedded in optimum cutting temperature (OCT) compound (TissueTek, Sakura Finetek, Torrance, CA). Thirty µm sections collected through the midbrain. Tissue sections were processed free-floating in 48-well plates and neuron numbers quantified using stereological software (StereoInvestigator, MBF Bioscience, Williston, VT).

Stereological analysis of neurons in the substantia nigra was performed using optical fractionator module stereology software (StereoInvestigator, MBF Bioscience, Williston, VT) interfaced with a Nikon Eclipse 90i fluorescence microscope equipped with a monochrome camera and three filter blocks. Using simple random sampling (SRS) image series workflow, the region of interest
(ROI) containing the substantia nigra was outlined for one hemisphere of each tissue section at a 4x magnification. Fluorescent images of each ROI taken using a 40x objective with FITC, Texas red (TR), and DAPI fluorescent cubes, and merged Workflow parameters were defined to count approximately 20% of the total neuron population in the substantia nigra (counting frame size 120 x 100 um, 245 x 240 um grid size) and Gunderson coefficient of error (C.E. m=1) was ≤ 0.10. Estimated population size was generated by the software and multiplied by 2 to obtain the overall population estimation for both hemispheres of the brain.

*Verification of TH-GFP animals.*

Following MPTP-intoxication, mice were terminally-anesthetized and brains were harvested and sectioned as previously described (Hutter-Saunders et al., 2011). Following sectioning, 30 μm tissue sections were processed free-floating in 48-well plates. Tissues were incubated with one of the following primary antibodies: rabbit anti-TH (1:2000, Calbiochem/EMD Chemicals, Billerica, MA); rabbit anti-glutamic acid decarboxylase 67 (GAD67) (1:500, Spring Bioscience, Pleasanton, CA); rabbit anti-microtubule-associated protein 2 (MAP2) (1:1000, EMD Millipore, Billerica, MA). Detection of TH+ neurons was carried out using both HRP and immunofluorescent conjugated secondary antibodies. Anti-TH stained tissues were incubated with either biotinylated goat anti-rabbit IgG (1:400, Vector Laboratories, Inc., Burlingame, CA) or Texas red (TR) conjugated donkey anti-rabbit IgG (DyLite 594 1:1000, Jackson ImmunoReserach, West Grove, PA). Color development was carried out in a streptavidin-horseradish peroxidase (HRP) solution (ABC Elite vector kit, Vector Laboratories, Burlingame, CA) and
Nissl substance counter stained with thionin. Tissues stained in anti-GAD67 or anti-MAP2 antibodies were incubated with TR DyLight-594 donkey anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA). Nuclei were visualized with 4',6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector Laboratories, Inc., Burlingame, CA). Expected outcome of wild-type and TH-GFP mice stained with anti-TH, anti-GAD67, and anti-MAP2 antibodies outlined in Table 2.

**Dual immunofluorescent staining.**

For simultaneous visualization of dopaminergic and GABAergic neurons in a single tissue, a free-floating method of immunofluorescence was used. Thirty μm tissue sections from drug-treated mice were incubated with sheep anti-TH (1:2000 EMD Millipore, Billerica, MA) followed by fluorescein isothiocyanate (FITC) conjugated donkey anti-sheep IgG [1:2000, DyLight 488 (FITC), Jackson ImmunoResearch, West Grove, PA)]. Sections were then incubated with rabbit anti-GAD67 (1:500, Spring Bioscience) followed by TR-conjugated donkey anti-rabbit IgG [1:2000, DyLight 594 (Jackson ImmunoResearch, West Grove, PA). All antibodies were absorbed by the vendor to remove inter-species cross-reactive antibodies. Nuclei were visualized with DAPI-containing mounting medium (Vector Laboratories, Inc., Burlingame, CA).

**Staining of drug-treated TH-GFP mice.**

Upon completion of drug injections, L-DOPA or BL-1023 treated mice were terminally anesthetized, perfused, and brains removed. Thirty μm sections were
stained with rabbit anti-GAD67 (1:500, Spring Bioscience, Pleasanton, CA) followed by TR-conjugated donkey anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA). Tissues were mounted onto slides and cover-slipped with DAPI containing mounting medium (Vector Laboratories, Inc., Burlingame, CA). Use of TH-GFP mice allowed for the detection of TH+ dopaminergic neurons without the need for immunofluorescent staining.

**BrdU detection.**

To detect incorporation of BrdU, tissue sections were incubated in 2 M HCl for 15 minutes at 37° C to denature DNA (Matsuura and Suzuki, 1997) prior to incubation in rat anti-BrdU primary antibody (1:1000, Santa Cruz Biotechnology, Dallas, TX) followed by incubation in TR-conjugated donkey anti-rat IgG secondary antibody (1:2000, Molecular Probes, Carlsbad, CA). To serve as a positive control, tissue sections from the olfactory bulb, an area of known neurogenesis, were collected and stained in the same manner. Tissues were mounted onto slides and cover-slipped with Vectashield Hardset mounting medium (Vector Laboratories, Inc., Burlingame, CA).

**Western blot analysis**

Western blot analyses were performed to evaluate the expression level of TH and phosphorylated TH within the substantia nigra and striatum of treated mice. Using the same MPTP and drug treatment regimen described above, mice were sacrificed on day 28 and brains removed. The midbrain containing the substantia nigra and striatum was isolated separately, placed in tubes containing RNAlater
(Applied Biosystems, Carlsbad, CA) and held on ice. Tissue was homogenized and protein purified using protein and RNA isolation system (PARIS) (Life Technologies, Carlsbad, CA) per the manufactures instructions. Protein concentration in the samples was quantified using Pierce BCA Protein Assay Kit (Life Technologies, Carlsbad, CA) and RNA samples quantified using NanoDrop 1000 (Fisher Scientific, Waltham, MA).

Forty µg of total protein was resuspended in a reducing sample buffer and boiled for 5 minutes at 95° C, electrophoresed onto 4 – 20% PAGE gel (GenScript, Piscataway, NJ) and transferred to a PVDF membrane. Membranes were blocked with 5% BSA/TBST or 5% dry milk/TBST and subsequently probed with primary antibodies specific for TH (1:500, Santa Cruz Biotechnology), phospho-tyrosine hydroxylase serine 31 (Ser31) (1:500, Cell Signaling Technology, Danvers, MA), or phospho-tyrosine hydroxylase serine 40 (Ser40) (1:500, Cell Signaling Technology, Danvers, MA). Membranes were incubated with HRP-conjugated goat anti-donkey IgG (1:20,000, Santa Cruz Biotechnology, Dallas, TX) prior to being developed with SuperSignal West Femto substrate (Life Technologies, Carlsbad, CA) for 1 minute and imaged using enhanced chemiluminescence (FluorChem HD2, Protein Simple, San Jose, CA). HRP-conjugated β-actin (1:5000, Sigma Aldrich, St. Louis, MO) was used as a protein loading control. Protein band density was determined using Image J software (National Institutes of Health).

Initial experimental design included the analysis of RNA isolated from the substantia nigra and striatum for expression of TH and GAD67 using quantitative
PCR (qPCR) to further verify increases in TH with MPTP and/or drug treatment. However, the highly lipid concentration of the brain and small tissue volume collected made RNA quality and yield sub-optimal. Therefore we were not able to proceed with qPCR protocols.

In vitro neuron culture

Currently, few useful GABAergic cell lines are suitable for in vitro studies. Cell lines exist that express or over-express GAD65 or GAD67 or that produce GABA in vitro (Giordano et al., 1993; Giordano et al., 1996; Eaton et al., 1999; Behrstock et al., 2000; Conejero-Goldberg et al., 2000); however to date none have been studied in the context of increasing TH expression. The P19 embryonic carcinoma cell line (Pinal et al., 1997), human fibroblasts, and neuroectodermal and insulinoma cell lines (Kono et al., 2001; Salazar et al., 2001; Varju et al., 2002) have all been evaluated for use in GABAergic neuronal studies, but none have been proven to be of long-term use that will further the field of GABAergic studies. The majority of published studies on GABAergic neurons have used cell lines that require a high degree of manipulation and sub-culturing techniques to yield consistent results (Trojanowski et al., 1997; Sanchez et al., 2006). Therefore a GABAergic cell line relevant to studies of neurodegeneration would be a very useful tool.

AF5 cells, an immortalized rat CNS progenitor cell line, were obtained as a generous gift from Dr. William J. Freed (National Institute of Health, New Jersey). AF5 cells are a CNS-derived line immortalized with the N-terminal fragment of the large SV40 T antigen (Truckenmiller et al., 1998; Sanchez et al., 2006).
Differentiated AF5 cells significantly upregulate their expression of Pitx2 mRNA, the GABAergic neuron specification transcription factor. Additionally, expression of GAD65 and GAD67 are upregulated in differentiated AF5 cells, suggesting that AF5 cells have adopted a GABAergic lineage (Sanchez et al., 2006). AF5 cells were cultured in T75 tissue culture flasks at a density of $5 \times 10^6$ in maintenance media [high glucose media (DMEM/F12 Glutamax) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin] and incubated at 37°C/5% CO$_2$. To differentiate AF5 cells into a GABAergic lineage, after 5 days maintenance media was replaced with serum-free differentiating media [neural basal media (Invitrogen, Waltham, MA) supplemented with B27 serum free supplement (Invitrogen, Waltham, MA) and 50 µM of the anti-proliferative agent N6,2’O-dibutyryladenosine 3’:5’-cyclic monophosphate sodium (dbcAMP, Sigma, St. Louis, MA) to prevent growth of any non-neuronal cells and shown to aid in the differentiation of AF5 cells to a GABAergic lineage. As an additional study, I addressed whether AF5 cells could retain their GABAergic markers for a longer period of time. AF5 cells were maintained in differentiating media for 21 days with half-media exchanges twice a week.

Limited availability of reliable GABAergic cell lines necessitated an alternative approach to study the induction of TH, and underlying mechanisms, *in vitro*. Therefore, primary neurons were isolated from the midbrain of postnatal mice. When isolated from mice between postnatal days 1 and 7, the neuronal populations isolated retain the ability to survive in culture and respond to conditions chosen, in this case the presence or absence of dopamine
replacement therapy. Brains were isolated from wild-type mice pups and midbrain containing the substantia nigra carefully isolated. Care was taken to remove any meninges surrounding brain tissue. Neural tissue was dissociated using a kit specifically designed for the isolation of neurons from postnatal mice (neural tissue dissociation kit – postnatal neurons, Miltenyi Biotec, San Diego, CA). Neurons were made into single cell suspensions and plated on poly-D-lysine coated flasks at a concentration of 1 x 10^6 cells/tissue flask in plating media: MEM, 10% filtered, head inactivated FBS, 0.45% glucose (20% w.v.), 100 mM (1x) sodium pyruvate, 200 mM (1x) glutamine, 1x penicillin/streptomycin (Beaudoin et al., 2012). Cells were incubated at 37°C for 2 - 6 hours until cells were attached to flask at which time media was replaced with maintenance media: Neuralbasal media supplemented with B-27 serum-free supplement, 200 mM (1x) glutamine, 1x penicillin/streptomycin. Cells were maintained at 37°C with half-media exchanges twice a week. Due to the heterogeneous population of neurons in the midbrain, steps were taken to deplete the number of non-GABAergic cells in primary culture. MPP+, the active metabolite of the neurotoxin MPTP, is routinely added to culture resulting in the selective killing of dopaminergic cells (Notter et al., 1988; Radad et al., 2015). Five µM MPP+ (Sigma, St. Louis, MO) was added to primary neuron cultures 24 hours after cells were switched to maintenance media. MPP+ was maintained in media for 48 hours.

Various concentrations of nicotine (positive control to induce TH expression), L-DOPA, and BL-1023, and pro-inflammatory cocktails were added
to AF5 or primary neuronal cultures for predefined periods of time prior to RNA isolation and quantitative PCR analysis of DNA expression for genes of interest (Table 4). The pro-inflammatory cytokines IFN-γ (2 ng/mL), IL-23 (10 ng/mL), and IL-1β (5 ng/mL) were added to cultures to address the hypothesis that the inflammatory response following MPTP intoxication plays a role in upregulation of TH expression *in vivo*. Preliminary tests showed an unusual effect of L-DOPA and BL-1023 on the culture media in that both compounds caused a significant color change to the culture media after 24 hours, even at very low concentrations. Therefore, experiments using L-DOPA or BL-1023 in culture were kept to time points less than 24 hours. At end-points stated in Table 3, cells were removed from flasks and RNA isolated to be used in quantitative PCR reactions as described in the following section.

*Quantitative PCR*

Cells were kept in differentiating media for 4 or 21 hours following which the cells were detached and lysed with 0.05% trypsin and RNA extracted using RNeasy mini kit (Qiagen) per manufacture’s protocol. RNA concentration was approximated using NanoDrop 1000 (Thermo Scientific) and reverse transcribed into cDNA using RT First Strand kit (Qiagen). cDNA was used in quantitative PCR reactions to look for the expression of astrocytic markers [glial fibrillary acidic protein (GFAP)], GABAergic markers (GAD67, Pitx2), dopaminergic markers (TH, Pitx3), and β-actin as a housekeeping gene. Primers for each marker were custom designed (Table 4) using Invitrogen Primer Design and
reconstituted in water to a concentration of 20 nM, aliquoted, and stored at -20°C.

SYBR GreenSyrbr green (Qiagen, Valencia, CA) was used as a detection method to quantify the amount of cDNA present in AF5 cells and primary neurons cultured under various conditions. SYBR Green is used as a nucleic acid stain that binds in the minor groove of double-stranded DNA and emits a signal at 520 nm. The resulting signal intensity is measured with a real-time thermocycler (Eppendorf Mastercycler ep realplex) and an increase in signal correlates with an increase in mRNA concentration in the sample. cDNA samples were mixed with RT² SYBR Green qPCR master mix and the appropriate primer pair (Table 4). Eppendorf Mastercycler ep realplex with ROX filter set was used to run samples in triplicate with the following cycle conditions: 95°C, 10 minutes; 40 cycles of (95°C, 15 seconds; 60°C, 60 seconds); melting curve cycle. The number of cycles required to produce a Sybr green signal above threshold (delta-CT values) of sample triplicates were averaged and samples that did not produce a signal during melting curve analysis excluded.

Statistical analysis

Tests were performed using Statistica (StatSoft, Inc. Tulsa, OK) or Prism GraphPad and data expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way ANOVA followed by post-hoc comparisons using Fisher’s LSD or Tukey’s correction.

RESULTS
**TH-GFP mice are not more susceptible to MPTP-intoxication**

Preliminary studies were performed to determine the practicality of using TH-GFP mice in further MPTP studies. Because homozygosity of the GFP gene is lethal when under control of the chicken β-actin promoter, I verified overlapping expression of TH and GAD67 by comparing unstained TH-GFP mice (Figure 1A) to wild-type (Figure 1B) and TH-GFP (Figure 1C) mice with anti-TH antibodies conjugated with Texas red. A summary of the expected immunohistochemical and immunofluorescent stained results of wild-type and TH-GFP mice is in Table 2. Next, I explored the possibility that TH+GFP+ neurons may be more susceptible to MPTP-induced death. To address that possibility, we compared the number of surviving dopaminergic neurons in wild-type and TH-GFP mice treated with MPTP or PBS. To ensure that larger doses of neurotoxin would not over-reach a GFP-toxic threshold, we administered small, medium, and large doses of MPTP (10, 14, or 18 mg/kg) or PBS (10 mL/kg). Mice were treated with 4 s.c. injections of either PBS or MPTP at 10, 14, or 18 mg/kg in 10 mL/kg PBS. Seven days following MPTP intoxication, mice were sacrificed, brains removed, embedded in OCT, and cut into 30 μm serial sections through the substantia nigra. Serial sections were immunostained with primary antibodies (anti-TH, anti-GAD67, or anti-MAP2) and probed with the appropriate biotinylated or fluorochrome-conjugated anti-IgG.

No significant differences were detected in numbers of dopaminergic neurons between wild-type and TH-GFP mice in the PBS control group using immunohistochemical (Figure 2A) or immunofluorescent (Figure 2B) analysis,
suggesting that GFP expression is an accurate method to quantify surviving dopaminergic neurons. Moreover, linear regression showed that both strains responded similarly to MPTP dose in both the immunohistochemical and immunofluorescent stained tissue groups (immunohistochemical: wild-type $r^2 = 0.90$, $p = 0.014$ and TH-GFP $r^2 = 0.939$, $p = 0.007$; immunofluorescent: wild-type $r^2 = 0.959$, $p = 0.021$ and TH-GFP, $r^2 = 0.996$, $p = 0.002$). Loss of dopaminergic neurons due to MPTP dose between wild-type or TH-GFP strains were not different (immunohistochemical $p = 0.89$; immunofluorescent $p = 0.83$), indicating that TH+GFP+ neurons are no more sensitive to MPTP than those of wild-type animals. No significant differences were seen in the number of MAP2+ (Figure 2C) or GAD67+ (Figure 2D) neurons between strains of mice within treatment groups.

To verify all GFP positive neurons were also positive for TH expression, and that all TH expressing neurons expressed GFP, we utilized stereological analysis to first assess the numbers of TH+, GFP+, and TH+GFP+ neurons in TH-GFP mice. No significant differences in the total number of TH+ or GFP+ neurons were detected at any MPTP dose, suggesting all GFP+ neurons are also TH expressing and all TH+ neurons express GFP (Figure 3A). To further verify that GFP expression is restricted to dopaminergic neurons, the total number of GAD67+ neurons were quantified and compared to the number of neurons that were positive for GAD67 expression and negative for GFP expression (GAD67+GFP-). Numbers of neurons expressing both GAD67 and GFP (GAD67+GFP+) averaged less than 1% of the total number of GAD67+ and
GFP+ neuron population (data not shown). No significant differences were seen between the number of GAD67+GFP- neurons and total GFP+ neurons (Figure 3B). If GFP expression was not specific to dopaminergic neurons, we would expect to see differences between the numbers of total GAD67+ neurons and those that are GAD67+GFP-. These results show that GFP expression is restricted to dopaminergic neurons at day 7 post-MPTP.

**Number of MAP2+ neurons compared to TH+ plus GAD67+**.

MAP2 is a non-specific neuronal marker expressed by all neurons within the region being evaluated, in this case the substantia nigra. MAP2 is commonly used to ensure the reduction in dopaminergic neurons following MPTP intoxication is due to neuronal death and not merely a temporary loss of TH expression. Since MAP2 is expressed by all neurons, the number of MAP2+ neurons determined by stereological analysis should very closely match those numbers of TH+ neurons added to the number of GAD67+ neurons. Consistent differences in the number of MAP2+ neurons compared to population estimates of TH+ and GAD67+ neurons were summed. (Figure 4A, B). This was hypothesized to be due to some neurons expressing both TH and GAD67, and thus being included in both populations of neuron counts. This is in contrast to MAP2 expression when TH+ and GAD67+ neurons would be included only once in population estimates. The differences in neuron counts were especially different with higher doses of MPTP intoxication indicating neuronal assault causes changes to neuronal profiles not seen in PBS animals.
Dual immunofluorescent staining reveals co-localization of TH and GAD67 in neurons of the substantia nigra

To look for induction of TH expression by GABAergic neurons following MPTP assault, we first assessed the extent of changes in TH+GAD67+ neuron numbers in the substantia nigra through two-color immunofluorescent staining. Tissues were isolated from MPTP-intoxicated mice sacrificed after 21 – 28 days post-MPTP (14 or 21 days of dopamine replacement therapy injections). Stereological analysis showed a 7.8-fold increase in the number of TH+GAD67+ neurons following MPTP intoxication and L-DOPA administration compared to animals given only PBS. A 5.6-fold increase was seen in animals following MPTP assault and BL-1023 administration (Figure 5). No statistically significant change in the number of TH+GAD67+ neurons was seen in animals treated with MPTP only. Moreover, the increase in TH+GAD67+ neurons were directly correlated with the loss of neurons that express only GAD67 (TH-GAD67+) (r = 0.896, p = 0.0018) (data not shown). This suggests that after MPTP intoxication, TH expression is induced in surviving GABAergic neurons as a compensatory mechanism for dopaminergic neuronal loss resulting in a phenotypic shift of GABAergic neurons. This led to question whether the increase in TH by GABAergic neurons was a consequence of drug treatment alone or a combination of MPTP-induced lesion and drug treatment. Additionally, this led us to further characterize the phenotypic profile of substantia nigra neurons from drug-treated animals to identify the source of increased TH+ neurons.
Increase of TH+ neurons is due to a phenotypic shift of GABAergic neurons and not neurogenesis

Having demonstrated TH+ neurons in the substantia nigra of TH-GFP mice are no more susceptible to MPTP-induced death, we next sought to evaluate the number of TH+GAD67+ neurons 28 days post-MPTP using TH-GFP animals as well as address the alternative hypothesis that the increase in TH+ neurons are a result of neurogenesis using BrdU. The experimental timeline is provided in Figure 6. Tissues from TH-GFP animals were stained with anti-GAD67 followed by TR-conjugated secondary antibody. Expression of GFP was used to identify TH+ neurons. Representative images showing TH+GAD67- (Figure 7A), TH-GAD67+ (Figure 7B), and TH+GAD67+ (figure 7C) neurons demonstrate the co-localization of TH and GAD67 results in yellow staining. Co-expression of TH-driven GFP and GAD67 was detectable in neuronal populations within the substantia nigra following MPTP intoxication alone, drug treatment alone, or MPTP intoxication and drug treatment (Figure 8A, B). In contrast to the low level of neurons expressing TH and GAD67 in PBS-treated, treatment with L-DOPA, or BL-1023 alone, or MPTP + PBS increased TH+GAD67+ neurons by 3.2-, 3.8-, and 2.6-fold compared to PBS controls. Interestingly, MPTP + L-DOPA or MPTP + BL-1023 treatment increased TH+GAD67+ neuron numbers 5.0- and 5.4-fold above PBS control levels and 1.9- and 2.1-fold above MPTP controls indicating an additive effect of neuronal insult and dopamine replacement therapy.

Incorporation of the thymidine analog, BrdU, is considered the gold standard for detection of neurogenesis in the adult mammalian brain (Arias-
Carrion et al., 2009) which is normally limited to specific areas of the adult mammalian brain, specifically the hippocampus, olfactory bulb, and epithelium (Altman and Das, 1965; Altman, 1969). Newly synthesized DNA will incorporate BrdU which can be detected with an anti-BrdU antibody. Past studies of neurogenesis within the substantia nigra were limited to short term administration of BrdU, thus due to the short in vivo half-life of BrdU in circulation, approximately 2 hours (Deng et al., 2010), the possibility existed that using those regimens, nigral-associated neurogenesis could have been underevaluated; particularly should neurogenic differentiation be slower than BrdU is cleared. To preclude that possibility, BrdU was administered in the drinking water of animals to detect any neurogenic activity occurring over the course of the study.

To serve as a positive control that both BrdU administration and the staining protocol were appropriate for the experiment, sections containing the olfactory bulb (Figure 9A) and substantia nigra (Figure 9B) were stained and analyzed. Nuclear BrdU+ cells were found the olfactory bulb indicating the methodology was capable of detecting neurons derived from neurogenesis and thus validating the results obtained from the substantia nigra. MPTP alone or MPTP followed by treatment with L-DOPA or BL-1023 for 21 days did not significantly increase the total number of nuclear BrdU+ neurons or TH+ nuclear BrdU+ neurons within the substantia nigra (Figure 10). Interestingly, mice that exhibited the greater number of and increase in TH+GAD67+ neurons (Figure 7) had fewer nuclear BrdU+ neurons (Figure 10), although not at significant levels. Initial analysis of tissue sections stained with BrdU revealed cells that appeared
to be positive for BrdU staining. However, upon closer evaluation of these cells, BrdU incorporation was only in the cytoplasmic area (Figure 11) and is therefore indicative of a reparative mechanism instead of a neurogenic one (Cooper-Kuhn and Kuhn, 2002). Therefore, data presented here represents stereological results only from cells that had BrdU expression in the nuclear region. This suggests that the phenotypic shift of GABAergic neurons to express TH in response to L-DOPA or BL-1023, suppresses the normally low levels of BrdU incorporation within the substantia nigra.

*In vitro changes of TH mRNA and protein in response to drug*

In an attempt to explain the mechanism responsible for TH upregulation by GABAergic neurons two *in vitro* approaches were used; a transformed cell line (AF5) and primary neuronal cultures. Quantitative PCR analysis of mRNA from cells was measured for expression of GFAP, GAD67, Pitx2, Pitx3, TH, and β-actin. Primers were designed using InVitrogen Primer Design and listed in Table 4. Despite using multiple culture conditions (Table 3), neither *in vitro* method utilized resulted increased measurable TH expression. The lack of positive data points to the likelihood that upregulation of TH by GABAergic neurons is a highly regulated process that only occurs under a set of very specific and extraordinary conditions. If GABAergic neurons easily express TH, the effects could be detrimental to the homeostatic environment of the CNS and therefore upregulation of TH may be seen as a “last resort” to restore the environmental milieu to normal.
Post-translations phosphorylation is increased following MPTP and drug treatment

To evaluate the changes in TH phosphorylation following dopaminergic insult, PBS and MPTP-intoxicated mice were administered PBS, L-DOPA or BL-1023, for 21 days post-MPTP and mice were sacrificed on day 28 post MPTP. Ventral midbrain containing the substantia nigra and striatum was isolated, homogenized, and protein extracted. Western blot analysis was performed for quantification of TH, phospho-TH (Ser31), phospho-TH (Ser40), and β-actin. Protein levels were normalized to β-actin and fold changes relative to PBS/PBS controls calculated (fold change ± SEM for n=4 mice/group).

While no significant increases were seen in Ser40 phosphorylation with MPTP and drug treatment (data not shown), phosphorylation of Ser31 was increased compared to PBS controls in MPTP intoxicated animals who received L-DOPA or BL-1023 daily for 21 day by 2.47- and 7.89-fold respectively (Figure 12A) within the substantia nigra. Drug treatment in the absence of dopaminergic insult did not yield any significant increases in phosphorylation of TH, suggesting initial insult to neurons, or decreased levels of endogenous dopamine, is necessary to induce the phosphorylation of TH Ser31 within the substantia nigra. Analysis of protein levels within the striatum showed no significant increase in the level of phosphorylated Ser31 (Figure 12B). Unlike the stereological results, Western blot analysis for TH did not show any significant increases between the drug or vehicle treated groups within the substantia nigra (Figure 13A) or striatum
(Figure 13B), presumably is due to analysis of whole brain lysate versus evaluation of TH on a per-cell basis.

DISCUSSION

Herein using dual immunofluorescent staining and TH-GFP transgenic mice, we showed that surviving GABAergic neurons within the substantia nigra undergo a phenotypic shift and begin expressing TH following neuronal assault and/or dopamine replacement therapy. To address the possibility that the increase in TH+ cells was due to an increase in neurogenic activity, the thymidine analogue BrdU was given over the course of 14 days. Results demonstrated a lack of BrdU+ neurons in the substantia nigra of animals and thus ruled out the possibility of newly formed TH+ neurons. Furthermore, the majority of BrdU incorporation was detected within the cytoplasm of cells rather than in nuclear DNA suggesting either a DNA reparative mechanism rather than neurogenesis or merely uptake and retention of BrdU in the cytoplasm (Arias-Carrion et al., 2009). Interestingly, the combination of MPTP followed by dopamine replacement therapy in the form of either L-DOPA or BL-1023 after lesion development yields an additive increase in numbers of GABAergic neurons that upregulate TH when compared to both PBS and MPTP controls. These findings are the first of their nature to successfully demonstrate that dopamine replacement therapy can have an effect on surviving GABAergic neurons within the substantia nigra of adult mice and provide evidence as to a putative compensatory or reparative mechanism to increase dopamine production in a dopamine-depleted environment.
Within the striatum, dopamine concentrations appear to regulate the numerical density of TH+ neurons in a dose-dependent manner. α-Methyl-p-tyrosine, a TH inhibitor, given to animals at post-natal day 4 significantly reduces the concentration of dopamine within the striatum, but results in an overall increase in the number of TH+ neurons (Busceti et al., 2012). It was shown that the TH+ neurons within the striatum did not incorporate BrdU and were therefore not a result of neurogenesis. While not specifically addressed by Busceti and colleagues, the increase in TH+ neurons following α-methyl-p-tyrosine administration, and subsequent dopamine decrease, was proposed to be a result of unopposed trophic factors that have yet to be elucidated. However, the finding that TH+ neurons in these studies were also GAD+ implicate GABAergic neurons as a potential source for the increased number of TH+ neurons in these studies. Data provided herein supports the hypothesis that dopamine negatively regulates the number of TH+ neurons and a reduction in endogenous dopamine results in an increased survival of TH+GAD67+ neurons normally eliminated by extracellular dopamine (Busceti et al., 2012). Separate studies examining the number of TH+ neurons within the striatum showed clear discrepancies between the effects of dopamine replacement therapy in the form of L-DOPA; one set demonstrating decreases in the number of TH+ striatal neurons (Lloyd et al., 1975; Huot et al., 2008) and another set showing an overall increase in striatal TH+ neurons (Lopez-Real et al., 2003; Jollivet et al., 2004; Tande et al., 2006). Ultimately these contradictory findings may be explained by the fact that the studies utilized very different doses of L-DOPA and the effects of L-DOPA effects
may be highly dose-dependent. Above a certain level, as with the studies performed by Lopez-Real and Jollivet, dopamine is proposed to act in conjunction with growth factors to up-regulate TH expressing neurons within the striatum (Huot and Parent, 2007). At lower levels, dopamine is suggested to control TH expression by means of conventional feedback mechanisms. These findings allude to the hypothesis that local dopamine concentrations regulate the numerical density of dopaminergic neurons in the substantia nigra and/or the relative density of TH termini within the striatum.

In the absence of dopamine, due to PD progression or neurotoxin induced neuronal death, the increase in TH+ neurons is feasibly due to a de-repression of TH expression and a phenotypic shift of surviving GABAergic neurons so that they may act as a local source of dopamine-secreting cells. As such they would be part of a compensatory mechanism in response to dopaminergic neuronal death (Kosaka et al., 1991; Jollivet et al., 2004; Abe et al., 2010; Busceti et al., 2012). Our studies suggest that dopamine replacement therapy, given daily for 21 days, elevates dopamine levels beyond the inhibitory threshold for TH expression. It is interesting however, that dopamine replacement therapy can increase the number of TH+GAD67+ neurons with and without initial dopaminergic neuronal assault, but an increase in Ser31 phosphorylation only occurs in the presence of dopaminergic insult. This finding suggests that either a threshold level of dopamine depletion must be reached before changes in phosphorylation are detectable or, alternatively, increased levels of pro-inflammatory cytokines in the MPTP-intoxicated animals (Koprich et al., 2008)
further increase phosphorylation of TH (Nakashima et al., 2013b). This also suggests that additional mechanisms, as yet unknown, are playing a role in the induction of TH expression by GABAergic neurons. These data also support the hypothesis that TH expression is regulated by multiple mechanisms which are highly dependent on the surrounding environmental milieu. Our results indicate that at the dosage given in our study, dopamine was present at concentrations that affected multiple TH regulatory mechanisms; one mechanism that ultimately led to increase expression of TH by GABAergic neurons and one resulting in increased phosphorylation of Ser31 within the substantia nigra. Whether these mechanisms represent separate pathways or are intricately associated has yet to be determined.

Another regulatory mechanism is concerned with the continuous presence of transcription factors that are needed to maintain cellular phenotype. The loss or expression of one or more specific transcription factor may influence the loss or activation of a critical cell type necessary for the production of dopamine or dopaminergic function. It may be worth exploring the many different TF’s needed to maintain/trigger a dopaminergic phenotype and evaluate whether they are upregulated in GABAergic neurons. Another method of regulation may center around the different neuronal firing patterns associated with dopaminergic and GABAergic neurons and how the induction of TH expression and production of dopamine by GABAergic neurons may influence not only the firing patterns of those neurons, but also on the surrounding surviving dopaminergic neurons. Further characterization of the TH+GAD67+ neurons would be informative as to
the possibility of this phenotype aligning with any of the subtype(s) of GABAergic interneurons and whether those shift phenotypes in response to MPTP and/or dopamine replacement therapy. Because the interneuron subtypes are so distinct in their transcription factors and topographical organization, and are rather numerous, it is likely that not all subtypes may be capable of expressing TH; however the elucidation of specific TH-producing phenotypic subsets have yet to be initiated.

Several reports have alluded to the fact that GABAergic research is leagues behind research into other neuronal populations within the midbrain. This underscores the minimum level of effort afforded to the development of a stable GABAergic cell line. One of the few GABAergic cell lines available, AF5 cells, is an immortalized rat CNS progenitor cell line that is able to assume differentiated states with neuronal properties (Sanchez et al., 2006). While AF5 cells can be induced into a GABAergic lineage, they did not prove to be beneficial in regards to evaluating the upregulation of TH mRNA by GABAergic neuron in vitro. Because of the heterogenetic nature of GABAergic neurons in the CNS, it is plausible that AF5 cells may lack essential factors that GABAergic neurons in the substantia nigra possess. However, still unknown are the factor(s) that induce GABAergic neurons to undergo this phenotypic shift in vivo in response to dopamine depletion and/or dopamine replacement therapy. Data suggesting that even subtle differences between GABAergic neurons in distinct brain regions have profound effects on neuron function (Tepper, 2010; Lahti et
al., 2013; Achim et al., 2014; Morales and Root, 2014), warrants further research into GABAergic neuronal phenotype, function, and plasticity.

Extensive data suggests that post-translation phosphorylation of serine residues has a large effect on TH expression and activity (Vrana et al., 1981; Vrana and Roskoski, 1983; Gahn and Roskoski, 1995; Kumer and Vrana, 1996; Wallen and Perlmann, 2003; Bobrovskaya et al., 2007); however the kinase(s) responsible in our model remain unknown. In vitro, more than 7 protein kinase systems have been identified that can modulate TH phosphorylation (Kumer and Vrana, 1996). Not only does phosphorylation of serine residues within TH occur by multiple mechanisms, but reports show these mechanisms are activated under a number of different conditions (Lazar et al., 1981; Kumar et al., 2003; Bobrovskaya et al., 2007; Ong et al., 2012). This helps to explain our findings that suggest phosphorylation of TH is differently regulated within the substantia nigra and striatum in the MPTP model of PD. Specifically, our data indicates a compensatory mechanism to increase phosphorylation of Ser31 is present in the substantia nigra, but not the striatum; results that are consistent with other reports (Salvatore, 2014). Alternatively, prior to detectable changes to the phosphorylation status of serine residues in TH, a threshold level of TH loss might be necessary and our MPTP regimen may not have been sufficient to attain that level of loss. A clearer picture as to the level of kinase expression and activity within the midbrain and striatum following MPTP or dopamine replacement therapy, and whether it corresponds to an increase in dopamine concentrations, would provide a better understanding as to the role of
phosphorylated TH behind the mechanism of TH expression by GABAergic neurons.

Due to multiple reports indicating that midbrain GABAergic neurons are an exceptionally heterogeneous subset of neurons (Floran et al., 1990; Soghomonian et al., 1992; Nakatani et al., 2007; Tepper, 2010; Achim et al., 2012; Vasudevan et al., 2012; Lahti et al., 2013; Achim et al., 2014; Li et al., 2014), I focused my efforts on isolating neurons from only this specific region to have the greatest chance of replicating in vivo results in vitro. Neurons were isolated from the midbrain from postnatal tissues and depleted of dopaminergic neurons with MPP+. However, this approach yielded similar results to those obtained with AF5 cells in that upregulation of TH mRNA was not seen ex vivo. Multiple concentrations of nicotine were used as positive controls due to the known ability of nicotine to modulate TH in vitro (Radcliffe et al., 2009; Ferrari et al., 2011; Suen et al., 2013). This likely may be due to a very unique phenotype or idiosyncratic characteristic that midbrain GABAergic neurons possess as well specific cues derived from the surrounding environmental milieu. The possibility exists that to induce TH expression by GABAergic neurons requires either one or more factors that include 1) a pro-inflammatory environment either due to MPTP-induced cell death or T cell infiltration; 2) the presence of actively dying or dead dopaminergic neurons; or 3) a reduction in the concentration of endogenous dopamine in the area. Without reproducing one or more of these factors, induction of TH by GABAergic neurons in vitro may be difficult.
Taken together, we believe these findings serve to demonstrate the ability of dopamine replacement therapy to induce a phenotypic shift of surviving neurons in the MPTP model of PD. Additionally, we provide evidence that dopamine replacement therapy acts through a protein kinase pathway to increase the phosphorylation of Ser31 that corresponds to a significant increase in TH+ neurons within the substantia nigra. Ultimately, these findings will help improve PD drug development and advance drug design for the treatment of PD by providing novel targets to which new lines therapeutic strategies can focus.
Table 1. TH-GFP genotyping primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’ – 3’</th>
<th>Product size (b.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type (forward)</td>
<td>CAAATGTTGCTTGTCTGGTG</td>
<td>200 b.p.</td>
</tr>
<tr>
<td>Wild-type (reverse)</td>
<td>GTCAGTCGAGTGCACAGTTT</td>
<td></td>
</tr>
<tr>
<td>GFP (forward)</td>
<td>AAGTTTCATCTGCACCACCG</td>
<td>475 b.p.</td>
</tr>
<tr>
<td>GFP (reverse)</td>
<td>TGCTCAGGTAGTGGTTGTCG</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Expected immunohistochemical and immunofluorescent staining results of wild-type and TH-GFP mice.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th>TH-GFP</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dopaminergic</td>
<td>GABAergic</td>
<td>Dopaminergic</td>
<td>GABAergic</td>
</tr>
<tr>
<td>TH IHC</td>
<td>Brown</td>
<td>Blue</td>
<td>Brown</td>
<td>Blue</td>
</tr>
<tr>
<td>TH TR</td>
<td>Red</td>
<td>None</td>
<td>Yellow</td>
<td>None</td>
</tr>
<tr>
<td>GAD67 TR</td>
<td>None</td>
<td>Red</td>
<td>Green</td>
<td>Red</td>
</tr>
<tr>
<td>MAP2 TR</td>
<td>Red</td>
<td>Red</td>
<td>Yellow</td>
<td>Red</td>
</tr>
</tbody>
</table>
Table 3. Culture conditions to study induction of TH expression by AF5 cells in vitro.

<table>
<thead>
<tr>
<th>Media additive</th>
<th>Concentration</th>
<th>Duration of treatment (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotine</td>
<td>1, 10, 100 uM</td>
<td>24 hrs</td>
</tr>
<tr>
<td></td>
<td>10, 100 uM</td>
<td>48 hrs</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>10, 100 uM</td>
<td>4 hrs</td>
</tr>
<tr>
<td></td>
<td>10, 100 uM</td>
<td>12 hrs</td>
</tr>
<tr>
<td></td>
<td>10, 50, 100 uM</td>
<td>24 hrs</td>
</tr>
<tr>
<td>BL-1023</td>
<td>10 uM</td>
<td>4 hrs</td>
</tr>
<tr>
<td></td>
<td>10 uM</td>
<td>12 hrs</td>
</tr>
<tr>
<td></td>
<td>10 uM</td>
<td>24 hrs</td>
</tr>
<tr>
<td>Pro-inflammatory cytokines (IFNγ, IL-23, IL-1β)</td>
<td>2 ng/mL, 10 ng/mL, 5 ng/mL</td>
<td>4 hrs</td>
</tr>
<tr>
<td></td>
<td>2 ng/mL, 10 ng/mL, 5 ng/mL</td>
<td>12 hrs</td>
</tr>
<tr>
<td></td>
<td>2 ng/mL, 10 ng/mL, 5 ng/mL</td>
<td>24 hrs</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Primer Sequence (5’ – 3’)</td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>GFAP (forward)</td>
<td>TCAACGTTAAGCTAGCCCTGGACA</td>
<td></td>
</tr>
<tr>
<td>GFAP (reverse)</td>
<td>TCTGTACAGGAATGGTGATGCGGT</td>
<td></td>
</tr>
<tr>
<td>GAD67 (forward)</td>
<td>GCCGGGGGCTGCCTTGGGCTTTG</td>
<td></td>
</tr>
<tr>
<td>GAD67 (reverse)</td>
<td>TGGGCGGTGCTTCCGGGACATGAGC</td>
<td></td>
</tr>
<tr>
<td>Pitx2 (forward)</td>
<td>CAAATGGGAGAAAGCGGGAGC</td>
<td></td>
</tr>
<tr>
<td>Pitx2 (reverse)</td>
<td>ATGGATGAGATGGATGGTTGCG</td>
<td></td>
</tr>
<tr>
<td>TH (forward)</td>
<td>TTGAAAAACTCTCCACGGGTACT</td>
<td></td>
</tr>
<tr>
<td>TH (reverse)</td>
<td>TACGGGTCAAACTTCACAGAGAAT</td>
<td></td>
</tr>
<tr>
<td>Pitx3 (forward)</td>
<td>CTCTCTGAAGAGAAGCAGCG</td>
<td></td>
</tr>
<tr>
<td>Pitx3 (reverse)</td>
<td>CCGAGGGCACCATGGAGGCAGC</td>
<td></td>
</tr>
<tr>
<td>β-actin (forward)</td>
<td>TGAGAGGGAAATCGTGCGTGACAT</td>
<td></td>
</tr>
<tr>
<td>β-actin (reverse)</td>
<td>ACCGCTCGTTGCCAATAGTGATGA</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Representative image of wild-type and TH-GFP tissues stained with anti-TH and Texas red-conjugated IgG.
Figure 1. Staining outcome of the substantia nigra in wild-type versus TH-GFP mice. (A) In the absence of staining, identification of dopaminergic neurons is still plausible when using the TH-GFP mouse. Because GFP is under control of the TH promoter in these mice, expression of GFP is an indicator of a dopaminergic neuron. (B) In order to identify dopaminergic neurons in wild-type mice the tissues must first be incubated in a primary antibody specific for TH followed by the appropriate secondary antibody conjugated with a fluorescent fluorochrome (TR). To ensure GFP expressing neurons were also immunoreactive for TH, tissues from TH-GFP mice were incubated with anti-TH antibodies followed by TR- conjugated secondary IgG. The resulting dopaminergic neurons in the substantia nigra of TH-GFP mice were yellow due to the co-expression of TR and GFP. All tissue were mounted on slides and coverslipped with DAPI containing mounting medium to identify nuclei. All images acquired using a 4x objective and Nikon 90i microscope equipped with a monochrome camera and 3 filter blocks.
Figure 2. Dopaminergic neurons expressing GFP are not more susceptible to MPTP
Figure 2. Dopaminergic neurons expressing GFP are not more susceptible to MPTP. (A) Quantification of TH+ neurons using immunohistochemical analysis and DAB color generation in wild-type and TH-GFP mice 7 days post PBS or MPTP (10, 14, or 18 mg/kg). (B) Quantification of TH+ neurons using immunofluorescent analysis in wild-type and TH-GFP mice 7 days post PBS or MPTP (10, 14, or 18 mg/kg). (C) Quantification of MAP2+ neurons in wild-type and TH-GFP mice 7 days post PBS or MPTP (10, 14, or 18 mg/kg). (D) Quantification of GAD67+ neurons in wild-type and TH-GFP mice 7 days post PBS or MPTP (10, 14, or 18 mg/kg). Linear regression showed that both strains responded similarly to MPTP dose in both the immunohistochemical and immunofluorescent stained tissue groups: (A) wild-type $r^2 = 0.90$, $p = 0.014$ and TH-GFP $r^2 = 0.939$, $p = 0.007$; (B) wild-type $r^2 = 0.959$, $p = 0.021$ and TH-GFP, $r^2 = 0.996$, $p = 0.002$. No significant differences were seen in the number of (C) MAP2+ or (D) GAD67+ neurons between strains of mice within treatment groups. Data represented as mean ± SEM for n = 5 – 7 animals.
Figure 3. GFP expression is restricted to TH expressing dopaminergic neurons.
Figure 3. GFP expression is restricted to TH expressing dopaminergic neurons. 30 μm sections from the midbrain of TH-GFP mice intoxicated with MPTP (10, 14, or 18 mg/kg) or PBS controls were stained with anti-GAD67 and TR-conjugated IgG. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken at 40x and neurons classified as (A) TH+ or GFP+ (green); (B) GAD67+/GFP- (red) and GAD67+ (red). (A) No significant differences in the total number of TH+ or GFP+ neurons were detected at any MPTP dose. (B) No significant differences were seen between the number of GAD67+GFP- neurons and total GFP+ neurons. Data presented as mean ± SEM for n = 5 – 9 animals.
Figure 4. MAP2+ neuron populations are consistently less than the populations of TH+ + GAD67+ neurons.
Figure 4. MAP2+ neuron populations are consistently less than the populations of TH+ + GAD67+ neurons. Tissue sections from mice intoxicated with increasing doses of MPTP (10, 14, 18 mg/kg) or PBS were stained with anti-TH, anti-GAD67, and anti-MAP2 antibodies followed by TR-conjugated IgG. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 40x objective. (A) Neurons were quantified as TH+, GAD67+, MAP2+ based upon the expression criteria summarized in Table 2. Mean number of neurons were determined for 10-16 mice per group. Significant differences (p ≤ 0.05) in neuron numbers from mice treated with graded doses of MPTP compared to non-intoxicated controls (0 mg/kg) were denoted for each neuronal phenotype expressing compared to: aMAP2; bTH; or cTH + GAD67. Notably, no significant differences were noted in neurons expressing only GAD67. (B) In addition to the number of TH+, GAD67+, and MAP2+ neurons, the numbers of TH+ and GAD67+ neurons were summed and the means ± SEM determined for 10 – 16 animals per group. The numbers of MAP2+ neurons and the sum of the TH+ and GAD67+ neurons (TH + GAD67) were compared at each dose level without discernible significant differences. Significant differences were detected in the mean neuron number in mice treated with each dose of MPTP: p ≤ 0.05 compared to non-intoxicated controls (0 mg/kg MPTP) within aMAP2/PBS or bTH + GAD67 neuronal phenotypes./PBS.
Figure 5. Dopamine replacement therapy following MPTP causes significant fold change in the number of TH by GABAergic neurons.
Figure 5. Dopamine replacement therapy following MPTP causes significant fold change in the number of TH by GABAergic neurons. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 40x objective. Fold changes of TH+GAD67- or TH+GAD67+ neurons within the substantia nigra of drug treated animals. Mean ± SEM determined for 5-7 mice per group and $P \leq 0.05$ compared to neuron numbers from controls treated with $^a$PBS at day 0 and days 7-34 (PBS) or $^b$MPTP on day 0 and PBS on days 7-34 (MPTP/PBS).
Figure 6. Experimental timeline to assess upregulation of TH expression by GABAergic neurons. The ability of MPTP-intoxication and/or dopamine replacement therapy was assessed using the MPTP mouse model of PD. On day 0 mice were intoxicated with 4 doses of MPTP and 7 days later daily i.p. injections of dopamine replacement therapy began (L-DOPA or BL-1023) or PBS. Concurrently, BrdU was administered via the drinking water for the first 14 days of treatment. Dopamine replacement therapy continued until day 28 post-MPTP (21 days of injections) at which time mice were sacrificed and numbers of TH+ and GAD67+ neurons quantified using stereological analysis.
Figure 7. TH and GAD67 co-localize in neurons.
Figure 7. TH and GAD67 co-localize in neurons of the midbrain of mice. Representative image of (A) TH+ dopaminergic neurons (green) (B) GAD67+ (red) and (C) TH+GAD67+ neurons. 30 µm sections were collected through the midbrain of TH-GFP mice and stained with anti-GAD67 primary antibodies followed by a Texas red-conjugated secondary IgG. Nuclei were stained using DAPI containing mounting medium. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 40x objective.
Figure 8. Dopamine replacement therapy increases number of TH+ GABAergic neurons with and without neuronal lesion.
Figure 8. Dopamine replacement therapy increases number of TH+ GABAergic neurons with and without neuronal lesion. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 40x objective. (A) Quantification of TH+GAD67− and TH+GAD67+ neurons from TH-GFP animals following 21 days of PBS, L-DOPA, or BL-1023 injection. Numbers of neurons were determined by stereological analysis by sampling approximately 20% of the neuronal population and achieving a coefficient of error (C.E.) of less than 0.10 (10%). Means ± SEM were determined for 7-8 mice per group, and P ≤ 0.05 compared to mice treated with aPBS on day 0 and days 7-27 (PBS/PBS) or bMPTP on day 0 and PBS on days 7-27 (MPTP/PBS) (B) Fold changes in TH+GAD67− and TH+GAD67+ neuron populations were calculated relative to PBS/PBS control animals. Means of fold changes ± SEM were calculated for 7-8 mice per group, and significant differences determined wherein P ≤ 0.05 from mice treated with aPBS on days 0 and 7-27 (PBS/PBS); bPBS on day 0 and L-DOPA on days 7-27 (PBS/L-DOPA); cPBS on day 0 and BL-1023 on days 7-27 (PBS/BL-1023); or dMPTP on day 0 and PBS on days 7-27(MPTP/PBS).
Figure 9. BrdU incorporation (red) was detected in the olfactory bulbs (A), but not the SN (B) of mice following 14 days of continuous BrdU administration.
Figure 9. BrdU incorporation was detected in the olfactory bulbs, but not the SN of mice following 14 days of continuous BrdU administration. To assess neurogenic activity of TH-GFP mice, BrdU was administered for 14 days in the drinking water at 1 mg/mL starting at day 7 post-MPTP (time of drug initiation). To avoid taste aversion, glucose was added to the water and mice were observed to ensure normal drinking. Tissues known to contain neurogenic cells, such as (A) olfactory bulbs served as positive controls for incorporation of BrdU and were compared to tissues sections containing the (B) substantia nigra. Sections, 30 μm thick, were stained with rat anti-BrdU primary antibodies following denaturation of DNA with 2M HCl for 15 minutes and TR-conjugated secondary IgG. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 4x objective.
Figure 10. Quantification of BrdU+ cells in numbers of the substantia nigra. The increase in TH+GAD67+ neurons are not associated with increased neurogenesis.
Figure 10. Increase in TH+GAD67+ neurons does not appear to be a consequence of neurogenesis. Quantification of BrdU+ neurons as determined by stereological analysis. Sections, 30 μm thick, were stained with rat anti-BrdU primary antibodies following denaturation of DNA with 2M HCl for 15 minutes and TR-conjugated secondary IgG. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one image with a 40x objective. Number of TH+, Nuclear BrdU+, and TH+Nuclear BrdU+ neurons were determined by stereological analysis by sampling approximately 20% of the neuronal population and achieving a coefficient of error (C.E.) of less than 0.10 (10%). Mean neuron numbers ± SEM were determined for 7 - 8 mice per group, and P ≤ 0.05 compared to control mice treated with \(^a\)PBS on days 0 and 7-27 (PBS/PBS).
Figure 11. BrdU incorporation in the cytoplasmic region of neuron in the substantia nigra.
Figure 11. BrdU incorporation in the cytoplasmic region of neuron in the substantia nigra. Representative image showing cytoplasmic incorporation of BrdU (white box). Tissue sections, 30 μm thick, were stained with rat anti-BrdU primary antibodies following denaturation of DNA with 2M HCl for 15 minutes and TR-conjugated secondary IgG. Sections were imaged using a Nikon 90i microscope equipped with a monochrome camera and 3 fluorescent filter cubes. Images were taken with separate fluorescent filter cubes and merged into one Image with a image with a 40x objective.
Figure 12. Phosphorylation of TH at serine 31 is increased by dopamine replacement therapy following MPTP intoxication.
Figure 12. Phosphorylation of TH at serine 31 is increased by dopamine replacement therapy following MPTP intoxication. Western blot analysis of protein levels within the ventral midbrain containing the substantia nigra or striatum was isolated, homogenized, and protein extracted following 21 days of PBS, BL-1023, or L-DOPA administration and 28 days post-MPTP to assess changes to TH phosphorylation. (A) Fold changes of phosphorylation of ser31 within the substantia nigra and (B) striatum. Protein levels were normalized to β-actin and fold changes relative to PBS/PBS controls calculated. Mean fold change ± SEM were calculated for 4 mice per group.
Figure 13. TH protein concentration is not significantly altered with MPTP and/or drug treatment
**Figure 13. Phosphorylation of TH at serine 31 is increased by dopamine replacement therapy following MPTP intoxication.** Western blot analysis of protein levels within the ventral midbrain containing the substantia nigra or striatum was isolated, homogenized, and protein extracted following 21 days of PBS, BL-1023, or L-DOPA administration and 28 days post-MPTP to assess changes to TH phosphorylation. (A) Fold changes of TH within the substantia nigra and (B) striatum. Protein levels were normalized to β-actin and fold changes relative to PBS/PBS controls calculated. Mean fold change ± SEM were determined for 4 mice per group.)
CHAPTER THREE

Neuronal and behavioral analysis of p19 deficient mice

ABSTRACT

Behavioral and motor characteristics were evaluated in mice deficient in one or both copies of the IL-23p19 chain, the unique subunit of the proinflammatory cytokine IL-23. This genotype leads to an absence of CD4+ TH17 cells in circulation. Such mice were shown to have a significantly reduced number of dopaminergic neurons in the substantia nigra compared to wild-type controls. The dopaminergic termini in the striatum were also aberrant, however only in the homozygote p19 knockout mice. Furthermore, p19 knockout mice showed no difference in the sensitivity to MPTP-induced lesion. Behavioral and motor characterization of p19 knockout mice using multiple forced and unforced tests ultimately showed that knockout mice, despite their significantly reduced neuron numbers exhibited few behaviors or abilities in motor functions compared to wild type control mice.
INTRODUCTION

Multiple lines of evidence have pointed to the detrimental role of TH17 cell in PD and the MPTP mouse model of PD (Benner et al., 2008; Reynolds et al., 2010; Gaffen et al., 2014; Sie et al., 2014). The findings that TH17 rely on IL-23 for survival suggested IL-23 as a promising therapeutic target in immunological diseases driven by aberrant TH17 cell responses. IL-23 itself is a heterodimer composed of a p40 and p19 subunit. While p40 is a common subunit with IL-12, p19 is unique to IL-23. This led to the development of a mouse model wherein the 4 exon coding region of p19 has been replaced with GFP, effectively preventing transcription of p19. These animals display no overt phenotype and the unaffected p40 subunit is available for normal generation of IL-12 and humoral responses (Ghilardi et al., 2004). Based on findings that adoptive transfer of TH17 cells exacerbate MPTP lesion, the loss of TH17 cells was hypothesized to result in the lack of neurodegenerative exacerbation and possible neuroprotection in MPTP-intoxicated mice (Reynolds et al., 2010).

Pro-inflammatory cytokines such as those secreted by TH1, TH17, and activated mononuclear phagocytes typically serve as major players in inflammatory responses and clearance of pathogens, but they also play an important role in neurogenesis and proper neuronal patterning. Neurogenesis refers to the process of generating new neurons from neural stem cells (Emsley et al., 2005) and consists of multiple steps including proliferation, differentiation into the correct neuronal lineage, and integration into the proper brain region to form functional neuronal circuitry (Ming and Song, 2005; Kim et al., 2015).
Recently, a collective of evidence suggests that neurogenesis is affected by the dysregulation of cytokines, chemokines, and neurotransmitters (Whitney et al., 2009). For example, during the early period of development pro-inflammatory cytokines may exert detrimental effects on the brain (Kim et al., 2015), but during mild acute inflammation, inflammatory cytokines can stimulate neurogenesis (Whitney et al., 2009). Tumor necrosis factor-α (TNF-α) is one of the major pro-inflammatory cytokines that plays a role in neurogenesis; either supporting or suppressing it depending on the type of TNF receptor it binds (Arnett et al., 2001; Dybedal et al., 2001; Ben-Hur et al., 2003; Monje et al., 2003; Chen et al., 2004; Iosif et al., 2006; Keohane et al., 2010). A broader picture of the interplay between PD, neuroinflammation, cytokines, and neurogenesis would vastly improve our understanding of the chronic nature of PD and aid the development of therapeutic modalities aimed at restoring neuronal numbers. In PD, clinical symptoms do not present until a significant number of dopaminergic neurons have been lost, therefore knowing the cytokines and levels needed to support neurogenesis would benefit the design of neuroprotective or neuroregenerative efforts before disease progresses to later stages.

To restore normal motor function and control in PD patients, appropriate animal models will be essential to test behavior and demonstrate improvements in behavior following treatment. Several forced and unforced motor tests have been extensively used and described in mice models of PD. Many of these tests have been found to be of little utility due in part to the lack of sensitivity, but also due to the relative small lesions induced in most animal models. Our laboratory
has previously implemented several of these tests and successfully demonstrated differences between drug-treated and controls following MPTP-intoxication (Hutter-Saunders et al., 2011). Due to the results that successfully demonstrated motor impairments following MPTP-intoxication, any improvements seen in this test can be confidently reported to be a consequence of disease intervention and possible restoration of striatal dopamine levels. These animal-based behavioral tests will help create the foundation on which human therapies will be built that target restoring proper motor control in PD. Herein, we evaluate the involvement of IL-23, a cytokine known to be critical in TH17 development, in dopaminergic neurodevelopment and the extent that Mendelian genotypes affect behavioral phenotypes.

MATERIALS and METHODS

Animals

IL-23p19 deficient (p19-/-) mice were obtained from Genetech (San Francisco, CA) and wild-type (WT) C57BL/6J mice were obtained from Jackson Laboratories (Bar Harber, ME). Animals were housed and bred in our animal facilities, and maintained on a 12:12 hr light/dark cycle with ad libitum access to food and water. The study was conducted in accordance with the animal care guidelines issued by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Generation of p19+/- mice were generated by crossing female p19-/- homozygous knockout mice and C57BL/6J males. F1 offspring were then genotyped to ensure all were heterozygous p19 knockout. Male and female F1
heterozygous animals were then crossed, the resulting litters genotyped, and assigned to p19+/+ WT, p19+/− heterozygotes, and p19−/− homozygotes.

Genotyping

Genotyping was carried out to correctly identify wild-type, heterozygous, and homozygous knockout animals. DNA was isolated from ear tissue using DNeasy Blood and Tissue kit (Qiagen, Valencia, CA). For routine genotyping, a PCR-based method with a common antisense primer, wild-type specific and knockout specific sense primers. Primers for each marker were custom designed (Table 1) using Invitrogen Primer Design and re-constituted in water to a concentration of 20 nM, aliquotted, and stored at -20°C. The primer triplet amplifies a 210-bp fragment for the wild-type allele and a 289-bp fragment for the knockout allele. PCR was conducted in a thermocycler using the following conditions: 1 cycle of 94°C, 60 s; 35 cycles of 94°C, 30 s; 58°C, 30 s; 72°C, 60 s; 1 cycle of 72°C, 7 min; and held at 4°C. DNA samples were loaded onto 3% agarose gels and ran at 110 V for 35 minutes to obtain sufficient band separation. The fluorescent DNA stain GelRed (Biotium, Inc., Hayward, CA) was added to the agarose mixture to identify DNA bands at the correct molecular weight.

Acute MPTP intoxication

To address sensitivity of dopaminergic neurons among the 3 different genotypes under neurodegenerative conditions, we compared the level of neuronal survival to MPTP-intoxication in p19 WT, heterozygous, and homozygous mice. Mice received 4 subcutaneous (s.c.) injections of MPTP-HCL (18 mg/kg free base in a
volume of 10 ml/kg) in PBS or PBS (10 mL/kg); each injection administered every 2 hours. MPTP handling and safety measures were in accordance with the National Institutes of Health, the University of Nebraska Medical Center, and prior published guidelines (Przedborski et al., 2001).

Immunohistochemistry

Seven days after MPTP or PBS administration, mice were terminally anesthetized, transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were harvested and post-fixed in 4% PFA overnight, cryoprotected in 30% sucrose/PBS for 48 hours, snap frozen in 2-methylbutane, embedded in OCT compound, and 30 µm sections collected through the midbrain. Tissue sections were processed free-floating in 48-well plates. Sections containing the substantia nigra were immunostained with sheep anti-TH primary antibody (1:2000, EMD Millipore, Billerica, MA) and FITC conjugated donkey anti-sheep IgG (1:1000, Vector laboratories, Burlingame, CA). Tissue sections were mounted onto slides, cover-slipped with DAPI containing mounting medium (Vector laboratories, Burlingame, CA), and neuronal numbers quantified using stereological software (StereoInvestigator, MBF Bioscience, Williston, VT). Tissue sections containing the striatum were immunostained with rabbit anti-TH (1:1000, Cal-Biochem/EMD Millipore, Billerica MA) primary antibody and HRP-conjugated goat anti-rabbit IgG (1:400 Vector laboratories, Burlingame, CA), and visualized with diaminobenzidine (DAB). Relative striatal density was quantified using ImageJ software (National Institute of Health).
Stereological analysis of neurons in the substantia nigra was performed using optical fractionator module stereology software (Stereoinvestigator, MBF Bioscience, Williston, VT) interfaced with a Nikon Eclipse 90i fluorescence microscope equipped with a monochrome camera and three fluorescence filter blocks. Using simple random sampling (SRS) image series workflow for every 4th serial section, the region of interest (ROI) containing the substantia nigra was outlined for one hemisphere of each tissue section at a 4x magnification. Fluorescent images of each ROI were taken using a 40x objective with FITC and DAPI fluorescent cubes, and merged. Workflow parameters were defined to count approximately 20% of the total neuron population in the substantia nigra (counting frame size 120 x 100 um, 245 x 240 um grid size) and Gunderson coefficient of error (C.E. m=1) was ≤ 0.10. Estimated population size was generated by the software and multiplied by 2 to achieve an overall population estimation for both hemispheres of the brain.

Behavioral testing

Natural behavior and movement were measured using the home cage monitoring system, open field test, and analysis of syntactic grooming. Forced motor function was measured using both constant speed rotarod and accelerating rotarod methods and adhesive removal test.

Home cage monitoring (HCM)

Existing behavioral assays typically examine behavior over a limited time and focus on a single behavioral domain (Tecott and Nestler, 2004; de Visser et al.,
Furthermore, these behaviors tested under forced conditions may differ from the behavior of animals acting freely in their home cage leading to results that may not accurately reflect behavior. Using a unique home cage monitoring (HCM) system allow for simultaneous measure of patterns of feeding, drinking, activity, and movement in individually housed animals so as to elucidate complex organization of diverse behaviors exhibited by mice (Goulding et al., 2008). Mouse position was determined by the force and torques acting on the system strain gauges; feeding events were detected by a photobeam across the food hopper, and drinking events were detected by a capacitive lick sensor. Validation studies show agreement between mouse position and drinking events measured by the system compared with video-based observation (Parkison et al., 2012).

Baseline mouse day-to-day behavior was measured using a custom-designed HCM that measures behaviors with 1 ms temporal and 0.5 cm spatial resolution. Thirty-two mice (10 p19+/+ wild-type, 12 p19+/- heterozygous, and 10 p19-/- homozygous) 8-10 weeks of age, were acclimated for 5 days and observed for 16 days. All data was sampled at 1 kHz, and written to disk using a real-time computer (to prevent potential skipped data points). All mouse data underwent rigorous quality control to eliminate known spurious points (arising from blocked photobeams or sipper tubes, sudden changes in cage center of mass, etc.), followed by a data classification workflow to determine mouse active and inactive states, mouse bouts of feeding, drinking, and movement, and differences in circadian variation (Goulding et al., 2008).

Constant speed and accelerating rotarod
A pilot study was conducted to first determine the appropriate speed at which to test the motor function of wild-type and knockout mice. The apparatus was fitted with a 7-cm diameter rod and was interfaced with automatic timing instrumentation (Rotamex, Columbus Instruments, Inc., Columbus, OH). A preliminary study was performed to determine the appropriate rotational speed(s) at which to test mice so that differences in motor control between wild-type and knockout mice could be detected, if present. To do this, mice were acclimated and trained to perform on the rotarod using an accelerating method (0 – 16 rpm) for 5 minutes x4 daily sessions on 3 consecutive days. On the 4th and 5th days animals were tested at constant speeds of 6, 8, 10, 12, 14, and 16 rpm for runs with a maximum run time of 90 seconds. Latency to fall was recorded for each animal and averaged for the 3 runs calculated.

Preliminary experiments indicated two speeds at which rotarod testing had the potential to show differences between strains of mice, 14 and 16 rpm. Following 3 days of acclimation to the apparatus using an accelerating method (0 – 16 rpm) for 5 minutes x4 daily sessions, experimental animals (p19+/+ wild-type, p19+/- heterozygous, and p19-/- homozygous knockout animals) were tested at constant speeds of 14 and 16 rpm for 3 runs with a maximum run time of 90 seconds. Latency to fall was recorded for each animal. Due to previous reports suggesting the accelerating rotarod may be more sensitive to motor function differences than traditional constant speed rotarod (Keshet et al., 2007), on the 5th day, the animals were evaluated for their ability to perform on an accelerating rotarod model. The accelerating rotarod differs from the constant
speed in that the apparatus increases in speed at a constant rate over a defined period of time. Mice were placed on the stationary rod and tested for their ability to stay on the apparatus as it increased in speed from 0 rpm to 18 rpm over the course of 5 minutes. Animals were tested for 3 runs with a maximum time of 5 minutes. Latency to fall was recorded for each animal and averages calculated.

Open field activity testing

Evaluation of normal activity and behaviors were evaluated using an automated open field activity system (Tru Scan 2.0, Coulbourn Instruments, Whitehall, PA, USA) to measure different types of unforced movements over the course of 20 minutes. Activities evaluated included total movements within the floor and vertical (i.e. rearing) planes, move and rest time, total distance covered, stereotypic behaviors (i.e. grooming), zone time (center vs. margin). Movements of each mouse were automatically recorded and measured by recording breaks in photo beams spaced 2.4 cm apart and span floor and vertical planes. The area measured 40.64 cm x 40.64 cm x 40.64 cm and was kept in a quiet and undisturbed area whilst recording mouse behaviors. Per the manufacturer, stereotypic movements are repetitive movements that start and return to the original position in less than 2 seconds with at least 3 such movements occurring prior to the event being considered a stereotypic episode. OFAT distinguishes between two types of stereotypic behaviors, movements that changes less than ±0.999 bean spaces are denoted type 1, and movements that change less than ±1.499 bean spaces are denoted type 2.

Syntactic grooming evaluation
Syntactic grooming consists of serially-ordered movements of four phases (elliptical strokes, unilateral strokes, bilateral strokes, and flank licking) and together is considered a form of stereotypic behavior. The chain of grooming events occurs in a frequency that is over 13,000x greater than could be expected by chance (Berridge et al., 1987). Due to the nature of the grooming chains, the OFAT arena and software cannot distinguish syntactic versus non-syntactic grooming. The chain of movements that distinguish syntactic grooming are controlled by neurons in the striatum and a depletion of dopamine should interrupt syntactic grooming. Each of the movements in the syntactic grooming chain can occur alone, or out of order, but the individual movements are similarly dependent on dopamine (Aldridge and Berridge, 1998; Aldridge et al., 2004).

Male wild-type, heterozygous, and homozygous knockout mice (n = 7 – 8) were videotaped in their home cage and visually scored for complete syntactic grooming sequences over the course of 10 minutes in a blinded fashion (Hutter-Saunders et al., 2011). Numbers of incomplete grooming events, either an incomplete grooming cycle or grooming movement that occurred alone, were recorded.

Adhesive removal test

Sensitive and reliable behavioral test are critical in testing the therapeutic efficacy and potential disease-modifying therapies in preclinical trials for PD (Peghini et al., 1990). The ability of an animal to remove an adhesive dot from the snout requires fine control of the forepaws. Published data supports the use of the adhesive removal test as a method to detect sensorimotor function differences in
newer strains of mice. In Pitx3-aphakia mice, wherein nigrostriatal dopamine neurons are significantly reduced, the adhesive removal test successfully showed a significantly increased time to remove the adhesive dot compared to wild-type controls (Peghini et al., 1990). To evaluate differences in the ability of knockout and wild-type animals to perform this task, we used a small adhesive dot (8-mm) and placed it on the snout of the animal using forceps. The animal was then placed in a clean cage and the time to remove the sticker recorded. Each trial run was conducted 3 times per animal.

Statistical analysis

All tests were performed using Statistica (StatSoft, Inc. Tulsa, OK) and data expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way ANOVA followed by post-hoc comparisons using Tukey’s comparison or Fisher’s LSD.

RESULTS

Stereological analysis of naïve mice

To assess the effects that p19 deficiencies on nigrostriatal dopaminergic neuron populations, we assessed the number of TH+ neurons in the substantia nigra and terminal densities in the striatum for p19+/+ wild-type, p19+/- heterozygous, and p19-/- homozygous knockout mice at 7 – 8 weeks of age. Stereological evaluation of dopaminergic neurons of naïve mice revealed significant differences in the number of TH+ neurons between wild-type, heterozygous knockout, and homozygous knockout mice, (8905 ± 692, 3724 ± 291 and 4003 ±
437 respectively) (Figure 1A). Unlike neuronal bodies in the substantia nigra, wild-type mice did not show a significant difference in the striatal dopaminergic density compared to heterozygous knockout mice (29.5 ± 3.1 and 36.5 ± 2.3 respectively, Figure 1B). However, striatal density in p19-/- homozygous knockout mice (16.9 ± 3.5) was significantly reduced compared to both wild-type and heterozygous mice.

MPTP intoxication

To assess whether dopaminergic neurons were more sensitive to neurodegenerative processes, we treated the 3 strains of mice with the neurotoxin MPTP which induces death of dopaminergic neurons along the nigrostriatal pathway. Previous studies showed that T cell-mediated immunity plays a pivotal role in exacerbating and attenuating neuronal death (Kurkowska-Jastrzebska et al., 1999; Benner et al., 2004; Reynolds et al., 2007; Benner et al., 2008; Reynolds et al., 2008; Schneider, 2008; Reynolds et al., 2009; Reynolds et al., 2010; Mosley et al., 2012). Particularly noteworthy was that TH17 and to a lesser degree TH1 cells exacerbate neuroinflammation and subsequent neurodegeneration. Therefore, we sought to determine if animals lacking the p19 subunit of IL-23 would be protected from MPTP-induced cell death. At 7 days post-MPTP or PBS treatment, the numbers of surviving TH+ neurons in the substantia nigra were quantified using stereological software. The number of surviving TH+ neurons in wild-type mice injected with PBS (7765 ± 538) was significantly higher compared to PBS injected p19+/− heterozygous knockout (4204 ± 197) and p19−/− homozygous knockout (2544 ± 223) mice.
Among all three genotypes, MPTP-intoxication yielded significant reduction in the numbers of surviving TH+ neurons compared to the respective genotype treated PBS control animals. At 7 days post-MPTP intoxication all animals exhibited similar reduction in the percentage of TH+ neurons lost within the substantia nigra (56% for wild-type, 53% for heterozygous, mice, and 58% for homozygous knockout mice; p = 0.7804) (Figure 2B). Similar to numbers of nigral neurons, the relative striatal densities of control mice were correlated with the three genotypes of p19 (p19+/+ wild-type, 77 ± 1.4; p19+/- heterozygous, 69 ± 2.5; and p19-/- homozygous, 46 ± 2.4) (Figure 2C). However, the percentages of lost striatal densities in MPTP-intoxicated mice among all genotypes were not significantly different compared to PBS controls (wild-type 41%; heterozygous 51%, and homozygous knockout 45%; p = 0.214) (Figure 2D).

Home cage monitoring

Home cage monitoring of mice for 14 days allows for constant monitoring of animals and provides information about their habitual behaviors. Using sensitive and cost-effective home cage monitoring systems that utilize force and torque measurements to determine behavior bouts such as feeding and drinking as well as temporal inactive versus active behaviors. Behaviors of mice were categorized as inactive (black), active but not in forward locomotion (red), forward locomotion (green), drinking (blue), and food (orange) (Figure 3 A-C). Percentage of a 24-hour cycle spent in each category was calculated for p19+/+ wild-type (Figure 3A), p19+/- heterozygous (Figure 3B) and p19-/- homozygous mice (Figure 3C). Time percentages were calculated for each 24-hour period and
averaged over the course of the observational period (16 days). While no significant differences were detected in any of these behavioral categories between strains of mice, wild-type mice consistently spent lesser time eating and more time in forward locomotion compared to heterozygous and homozygous knockout strains of mice.

Activities of mice greatly differ between light and dark cycles with the most active periods being during dark cycles. Therefore, I sought to evaluate whether or not differences in behaviors would become apparent when directly comparing light and dark cycle time budgets (Figure 4). In a similar manner to the 24-hour time budget analysis, mice were observed and behaviors categorized for p19+/+ wild-type (Figure 4A and B), p19+/- heterozygous (Figure 4C and D), and p19-/- homozygous knockout (Figure 3E and F) mice with time being broken down into the dark cycle (Figure 4A, C, and E) and light cycle (Figure 4B, D, and F). Similar to the 24-hour time budget results (Figure 3), no significant differences were detected in any of these behavioral categories between strains of mice. However, a proclivity of wild-type mice to feed less and exhibit forward locomotion more compared to knockout mice was demonstrated.

Because of the consistent finding that wild-type mice show differences in percentage of the time spent in locomotion, despite reaching a significance of $p \leq 0.05$, this behavior was further analyzed (Figure 5). Upon completion of the 16-day study, averages were calculated for the percentage of each hour spent in locomotion over the course of a 24-hour period for each animal. The hourly averages for each strain of mice were calculated and plotted against averages of
other strains. Figure 5A wild-type (green) versus heterozygous knockout (red); Figure 5B wild-type (green) versus homozygous knockout (red); Figure 5C heterozygous knockout (green) versus homozygous knockout (red). The plots were each divided (dashed bars) into light and dark cycles with X-axis values of 6 – 12 and 24 – 30 representing light cycle and 12 – 24 representing dark cycles. Expectedly, all mice were more active during the dark cycle compared to light. While significant differences were detected in the amount of time heterozygous mice spend in locomotion during the dark cycle compared to wild-type (Figure 5A), other behaviors involving locomotion were not significantly different, and therefore, locomotor behaviors could not be confidently reported as being different in heterozygous mice. No significant differences were detected between wild-type and homozygous knockout (Figure 5B) or homozygous and heterozygous knockout (Figure 5C).

**OFAT**

Automated open field activity testing (OFAT) allows for measurement of multiple unforced movements such as total distance travelled over the course of the observation period, average speed of movements, time spent in areas of the arena, and rearing and stereotypic behaviors. Animals with neurological impairments may not move normally or explore the arena in a similar manner to wild-type animals. Therefore, we chose to evaluate wild-type and p19 deficient animals over the course of 20 minutes and identify differences in these measurements.
Comparisons of movement are shown in Figure 6. The following type of movements were measured for wild-type, heterozygous, and homozygous knockout mice and evaluated for statistical significance: total floor plane move time (seconds) (Figure 6A); total floor plane rest time (seconds) (Figure 6B); total floor plane movements (Figure 6C); total floor plane distance travelled (cm) (Figure 6D); mean floor plane velocity (Figure 6E); average distance travelled (cm) per movement (Figure 6F); total number of jumps (Figure 6G); mean number counter-clockwise turns (Figure 6H); mean number clockwise turns (Figure 6I). We did not find any significant differences in these types of movement in wild-type mice or mice deficient in one or two p19 genes.

Comparisons of stereotypic movements are shown in Figure 7. Stereotypic-1 (Figure 7A, B, and C) and stereotypic-2 (Figure 7D, E, and F) movements were observed over the course of 20 minutes for wild-type, heterozygous, and homozygous knockout mice. Stereotypic events included total number stereotypic-1 moves (Figure 7A); total number stereotypic-1 episodes (Figure 7B); total stereotypic-1 time (seconds) (Figure 7C); total number stereotypic-2 moves (Figure 7D); total number stereotypic-2 episodes (Figure 7E); total stereotypic-2 time (seconds) (Figure 7F). No significant differences were observed between p19+/+, p19+-/-, or p19-/- mice.

Comparisons of zone time are shown in Figure 8. The OFAT arena is sectioned into a center and margin portion. Time budgeted in these two areas were calculated as total margin distance covered (cm) (Figure 8A); total time spend in arena margin (seconds) (Figure 8B); total arena center distance
covered (cm) (Figure 8C); total time spend in arena center (seconds) (Figure 8D); total number of arena center entries (Figure 8E); percentage of time spent in area center (Figure 8F); and percentage of time spent in arena margin (Figure 8G). No significant differences were observed between wild-type mice or either strain deficient in one or two p19 genes.

Comparisons of vertical plane movements (rearings) are shown in Figure 9. Beam breaks were used to detect movements in the vertical plane and categorized as: total number vertical plane entries (Figure 9A); total number vertical plane moves (Figure 9B); total time spent in vertical plane (seconds) (Figure 9C); and total vertical plane distance travelled (cm) (Figure 9D). No significant differences were observed between wild-type and p19 knockout mice.

Comparisons between p19+/+ wild-type, p19+- heterozygous, and p19-/- homozygous knockout mice in a plethora different activities (total movements within the floor and vertical, planes, move and rest time, total distance covered, stereotypic behaviors, zone time) showed no significant differences between strains of mice.

Rotarod

The rotarod is a widely used motor function test in mice and can be used in the traditional constant speed mode or an accelerating mode. Initial studies aimed at determining a constant speed at which differences between strains of mice become apparent, demonstrated 14 and 16 rpms were best suited to testing wild-type and p19 knockout mice (Figure 10). No significant differences in the latency
to fall between strains of mice were found when tested at 14 rpm (p = 0.274) (Figure 11A), at 16 rpm (p = 0.525) (Figure 11B), or at 0-18 rpm accelerating rotarod (p = 0.573) (Figure 11C). Average latency to fall for 3 trials were 64 ± 10.6 sec, 44 ± 8.4 sec, and 230 ± 12.7 sec for wild-type mice; 48 ± 10.5 sec, 36 ± 6.9 sec, and 211 ± 16.9 sec for heterozygous mice; and 69 ± 6.9 sec, 48 ± 7.1 sec, and 229 ± 12.8 sec for homozygous knockout mice at 14 rpm, 16 rpm, and accelerating respectively.

Adhesive removal test

The ability of an animal to remove an adhesive dot from the tip of their nose requires fine motor control of the forepaws. Altered neuronal numbers or pathways involved in motor control could therefore lead to differences in the ability of animals to complete the task. To evaluate this, a small adhesive dot was carefully placed on the nose of the mouse and the mouse placed in an empty cage. The latency to successfully remove the sticker was recorded and repeated for a total of 3 trials (Figure 12A). Interestingly, homozygous knockout animals exhibited the smallest mean latency to remove the adhesive dot (4.1 ± 1.1 sec) which was significantly faster than mean time for wild-type mice (7.5 ± 0.61 sec) (p < 0.01), but not the mean for heterozygous mice (5.4 ± 0.85 sec). No significant difference was detected in the mean latency to remove the adhesive dot between wild-type and heterozygous animals, though the trends were relatively strong (p = 0.068). Evaluation of each of the 3 individual runs demonstrated that all animals became faster at removing the adhesive dot with
each subsequent trial, but latency to remove the sticker was not different between strains at the same trial run (Figure 12B).

Syntactic grooming

Grooming patterns of mice have been well-studied and shown to occur in a predictable pattern. Dopamine levels in the basal ganglia play a role in controlling this grooming pattern, and therefore, altered dopaminergic neurons and reduced dopamine production in knockout animals could lead to differences in the number of completed grooming cycles (Berridge et al., 1987; Taylor et al., 2010; Hutter-Saunders et al., 2011). Animals were recorded for 10 minutes in their home cage and the number of complete and incomplete grooming cycles recorded in a blinded fashion. No significant differences were seen between genotypes in the mean number of complete grooming cycles (wild-type: 2.25 ± 0.90; heterozygous: 0.29 ± 0.29; homozygous knockout: 0.89 ± 0.35) or incomplete grooming cycles (wild-type: 1.5 ± 0.33; heterozygous: 2.9 ± 0.67; homozygous knockout: 2.9 ± 0.68) (Figure 13). While no significant differences were detected between any of the genotypes, clear trends were evident that the loss of p19 gene and dopaminergic neurons were associated with fewer complete and greater interrupted grooming cycles.

DISCUSSION

Extensive data exists that support the hypothesis implicating the immune system in PD progression as well as in the MPTP-mouse model of PD. Studies from our own laboratory have shown that antigen specific TH17 cells are especially potent
in their ability to exacerbate MPTP-induced dopaminergic neuronal lesions within the nigrostriatal axis (Benner et al., 2004; Benner et al., 2008; Reynolds et al., 2010). This effect is not seen to such an extent with TH1 cells even though both are classically considered pro-inflammatory immune cells. However, other cells in the T cell lineage, regulatory T cells (Tregs), have the ability to mitigate MPTP-induced lesions and significantly reduce dopaminergic cell loss (Reynolds et al., 2010).

Evidence suggests that inflammatory processes and the CD4+ T cells responsible for these responses show a degree of plasticity. Therefore in mice lacking fully functional TH17 cells, other CD4+ T cells may take over the usual TH17 cell role and carry exacerbation of the inflammatory process. This would provide an explanation as to why p19 knockout mice afford no obvious neuroprotection against MPTP-intoxication, even though they lack the ability to induce TH17 cells. The other explanation is that TH17 cells may not be induced until the lesion or disease develops into a chronic state. These findings will help in the development of therapeutic approaches to treating neuroinflammatory diseases by shedding light on the complex and multifaceted aspect of the disease. The balance between pro- and anti-inflammatory cells must be altered in a way that keeps neuroinflammation to a minimum, but does not deplete the immune system completely and leave the CNS vulnerable to other pathogenic entities. Data from p19 knockout mice indicates that monotherapy targeting only TH17 cells may not reduce neuroinflammation to a level whereby disease progression and neurodegeneration are slowed or stopped. However,
immunomodulatory agents that target multiple immune cells may prove to be beneficial and should be further investigated.

The physical symptoms associated with PD generally do not appear until greater than 50% of dopaminergic neurons in the substantia nigra have been lost. The number of existing dopaminergic neurons in the substantia nigra compared to the number of neurons needed helps to explain why PD symptoms do not appear until late in the disease process, and provides an example of “safeguards” set up in the CNS to maintain normal processes. However below a certain level, the surviving dopaminergic neurons are thought no longer able to produce enough dopamine to fully support normal functions. Therefore, we hypothesized that p19 knockout mice, newly found to have significantly reduced numbers of dopaminergic neurons in the substantia nigra, would have similar motor and behavioral deficiencies.

Using multiple motor and behavior tests proven to demonstrate motor dysfunction in other models of nigrostriatal damage, including the MPTP model, motor behavior deficits were found to be few in p19 knockout mice. These tests included forced motor tests (rotarod), normal behavior observations (home cage monitoring, open field activity testing, syntactic grooming), and fine motor skills (adhesive dot removal). Previous studies in the MPTP model have been able to successfully demonstrate motor or behavior deficits with far less severe substantia nigra neuron and striatal termini loss (Hutter-Saunders et al., 2011; Bove and Perier, 2012); therefore we are confident that the appropriate tests and conditions were utilized in this study. Home cage monitoring (HCM), which
observed animals over the course of 16 days in an undisturbed environment, was the most wide-ranging and sensitive test carried out, and would have therefore detected any abnormal behaviors in knockout mice compared to controls. Thus, unexpected results of finding no significant differences using the HCM system in any behaviors (movement, locomotion, feeding, drinking) proves that knockout mice are fully functional and are indistinguishable from wild-type mice in terms of unforced behavior and movement.

While not specifically validated by this study, we posit that the number of dopaminergic neurons in p19 knockout mice is significantly reduced from the time of birth, rather than a gradual decline in neuron numbers as the animal ages. This points to a role of p19 or IL-23 in the early stages of neuronal patterning. Taking cues from findings in Pitx3 knockout mice (ak/ak), which also display significant reductions in substantia nigra dopaminergic neurons number (Vasudevan et al., 2012), it is possible that dopaminergic neurons in p19 knockout mice are unable to reach their final destination and fully differentiate into a dopaminergic phenotype. The hypothesis that neuron numbers are reduced from before birth, would help to explain why p19 knockout mice showed no significant differences in the behavioral tests performed. As in the ak/ak mouse model, which show no behavior abnormalities despite significantly reduced dopaminergic neuron numbers (Vasudevan et al., 2012), p19 knockout mice are hypothesized to utilize the same compensatory mechanisms to function in a manner that is indistinguishable from wild-type mice. The data presented herein is consistent with other reports, showing that preserved motor function is
associated with residual dopamine and that the behavioral and motor consequences of decreased dopaminergic neuron number are dependent on whether neurons are lost pre- or post-natally (Golden et al., 2013). Whereas substantial lesions to the substantia nigra in adult mice result in significant and measurable deficits in motor function, mice born with fewer dopaminergic neurons show no measurable deficits.

The time of lesion development may also play a crucial role on the behavioral outcome can possibly be explained through multiple mechanisms. First, additional mechanisms of plasticity may be induced when damage occurs in developing animals (Golden et al., 2013). Reduced excitation of the indirect dopamine pathway, through a reduction in glutamatergic excitation or reduction of GABA release, can help to alleviate PD symptoms (Stephens et al., 2005; Day et al., 2006; Brotchie and Fitzer-Attas, 2009). Secondly, the decreased level of dopamine in knockout mice from birth could result in a hypersensitivity of the neurons in the striatum to dopamine (Hussong, 2014). Alternatively, the number of dopamine receptors in the striatum could be increased compared to wild-type mice. As such, even small amounts of dopamine in the area would be able to sufficiently control motor functions. Third, the intrinsic regulatory property of TH, i.e., that it can be regulated by dopamine itself, suggests that in the absence of normal levels of dopamine, the activity of TH can be upregulated resulting in the increased production of dopamine by remaining neurons (Zigmond et al., 1984).

The use of animal models has led to multiple advances in our understanding of diseases and how to best treat them. However, it is important to
understand that all animal models have limitations and may not be a perfect model to study human diseases. Two common rodent models to study PD use the neurotoxins MPTP or 6-hydroxy dopamine (6-OHDA); each having their own advantages and disadvantages. Therefore, new animal models for studying PD are necessary. Data from these studies could lead to the use of p19 knockout mice in future studies of neurodegeneration. Due to the inherent characteristics of p19 knockout mice to present a significant reduction in the number of dopaminergic neurons and termini in the nigrostriatal pathway, these mice could be useful for potential methods to increase the levels of striatal dopamine. This may prove to be advantageous over traditional MPTP or 6-OHDA models wherein the initial reduction along the nigrostriatal pathway is achieved with chemical compounds or neurotoxins. The novel finding regarding the neuron numbers in p19 knockout mice suggests multiple uses for p19 knockout mice reaching beyond our laboratory.

The results from this study will help to further our understanding of interactions between the brain and immune system and how these two work in synergy to establish proper neuronal patterning and connections. Results showing that mice lacking the distinct p19 subunit of IL-23 have significantly reduced numbers of dopaminergic neurons in the substantia nigra and striatal density provides insight into a previously undescribed relationship between IL-23 and dopaminergic neurodevelopment. Even more importantly however, is the finding that mice are able to function normally despite their significantly reduced nigrostriatal pathway. This points to possible compensatory mechanism(s) that
allow proper neuronal signaling with minimal amounts of dopamine. Using these findings we can better develop therapeutic strategies for neurodegenerative diseases during the early stages of disease and help maintain or regain normal motor function (Hussong, 2014).
Table 1. p19 knockout and wild-type genotyping primers.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence 5’ – 3’</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common reverse</td>
<td>GCCTGGGCTCACTTTTTCTG</td>
<td>N/A</td>
</tr>
<tr>
<td>Wild-type specific (forward)</td>
<td>GCGTGAAGGGAAGGACACC</td>
<td>210 b.p.</td>
</tr>
<tr>
<td>Knock-out specific (forward)</td>
<td>AGGGGGAGGATTGGGAAGAC</td>
<td>289 b.p.</td>
</tr>
</tbody>
</table>
Figure 1. Quantification of TH+ cells in substantia nigra and density of striatal termini.
Figure 1. Quantification of TH+ cells and striatal termini. Number of TH+ neurons in the substantia nigra of naïve wild-type and p19 deficient mice. Naive mice were terminally anesthetized, transcardially perfused with PBS, fixed with 4% paraformaldehyde, and brains removed and processed for immunohistochemistry. Brains were sectioned through the midbrain containing the substantia nigra and striatum. Substantia nigra sections were immunostained with sheep anti-tyrosine hydroxylase and FITC-conjugated donkey anti-sheep IgG. Neuron numbers were quantified by stereological analysis (Stereo Investigator, MBF Bioscience). Striatal sections were immunostained with sheep anti-tyrosine hydroxylase and biotinylated goat anti-sheep IgG. Relative striatal densitometry was quantified using ImageJ. Means ± SEM of total numbers of surviving neurons or striatal density was determined for 6-8 mice per group and analyzed by one way ANOVA and Fisher’s LSD post-hoc test with. Each group is compared to aB6.IL-23 p19 (+/+) and bB6.IL-23 p19 (+/-).
Figure 2. Neuronal quantification and striatal density in PBS and MPTP-intoxicated mice.
Figure 2. Neuronal quantification and striatal density in PBS and MPTP-intoxicated mice. Animals were intoxicated with four injections of PBS or MPTP-HCL (18 mg/kg free base). Seven days post-MPTP, mice were terminally anesthetized, transcardially perfused with PBS, fixed with 4% paraformaldehyde, and brains removed and processed for immunohistochemistry. (A) Quantification of surviving TH+ dopaminergic numbers within the midbrain of PBS and MPTP-intoxicated B6.IL-23 p19 (+/+) and B6.IL-23 p19 (-/-); (B) Calculation in the percentage of neuronal loss following MPTP-intoxication in B6.IL-23 p19 (+/+) and B6.IL-23 p19 (-/-) mice. Neuron numbers were quantified by stereological analysis (Stereo Investigator, MBF Bioscience) and data presented as means ± SEM of total numbers of surviving neurons or striatal density was determined for 7-8 mice per group and analyzed by one way ANOVA and Tukey’s post-hoc test. Each group is compared to aPBS-B6.IL-23 p19 (+/+); bMPTP-B6.IL-23 p19 (+/+); cPBS-B6.IL-23 p19 (+/); dPBS-B6.IL-23 p19 (-/-) with p < 0.05 indicating statistical significance.

(C) Relative TH striatal density of PBS and MPTP-intoxicated mice (B6.IL-23 p19 (+/+); B6.IL-23 p19 (+/); and B6.IL-23 p19 (-/-). (D) Calculation of percent reduction in striatal density following MPTP compared to PBS control. Striatal densitometry was quantified using ImageJ. Means ± SEM of total numbers of surviving neurons or striatal density was determined for 7-8 mice per group and analyzed by one way ANOVA and Fisher’s LSD post-hoc test. Each group is compared to aPBS-B6.IL-23 p19 (+/+) and bMPTP-B6.IL-23 p19 (+/+).
B6.IL-23 p19 (+/-); \textsuperscript{d}MPTP-B6.IL-23 p19 (+/-); \textsuperscript{e}PBS-B6.IL-23 p19 (-/-) with p < 0.05 indicating statistical significance.
Figure 3. Categorization of behaviors in a 24-hour time period.
Figure 3 Categorization of behaviors in a 24-hour period. Following 5 days of acclimation, mice were placed singly in cages to monitor precise temporal and spatial measurement of mouse home cage behavior (including movement, feeding, and drinking). Behaviors were monitored continuously for 16 days followed by robust statistical evaluation. (A) Wild-type p19 +/+; (B) heterozygous p19 knockout; and (C) homozygous knockout p19 mice underwent 24-hour monitoring. Behaviors were analyzed and categorized as inactive (black); active (not forward locomotion, red); forward locomotion (green); time spent at water (blue); time spent at food (orange). N = 10 – 12 animals per group.
Figure 4. Time budgets in 12-hour light and dark cycles.
Figure 4. Categorization of behaviors in a 12-hour light and dark cycles. Behaviors during (A, C, D) 12-hour dark cycle and (B, D, F) light cycles were monitored and analyzed for (A, B) wild-type p19 (+/+); (C, D) heterozygous p19 knockout mice; and (E, F) homozygous knockout mice. Behaviors were categorized as the following: inactive (black); active (not forward locomotion, red); forward locomotion (green); time spent at water (blue); time spent at food (orange) and percentage of each 12 hours cycle spend in that behavior calculated. N = 10 – 12 animals per group.
Figure 5. Differences in locomotion time between strains of mice during 12-hour periods.
Figure 5. Differences in locomotion time between strains of mice between 12-hour light and dark cycles. Comparisons between (A) B6.IL-23 p19 (+/+) (green) and B6.IL-23 p19 (+/-) (red); (B) B6.IL-23 p19 (+/+) (green) and B6.IL-23 p19 (-/-) (red); and (C) B6.IL-23 p19 (-/-) (green) and B6.IL-23 p19 (+/-) (red). Data expressed as means ± SEM for n = 10 – 12 animals per group with percent locomotion (Y-axis) plotted against hour (X-axis) with hours 6 – 12 and 24 – 30 representing the light cycle and 12 – 24 representing dark cycle. *p ≤ 0.05
Figure 6 (continued on pages 45 and 46)
Figure 6 (continued on page 46)…
Figure 6 Open field activity testing (movements).
Figure 6. Analysis of movements observed during open field activity testing. Analysis of general activity and natural behaviors were assessed using an automated open field activity system (Tru Scan 2.0, Coulbourn Instruments, Whitehall, PA, USA) to measure different types of movement recorded over a 20-min observation period for each animal. Types of movement, classified as X-Y coordinate changes in the floor plane, were measured and broken down into the following categories: (A) total floor plane move time; (B) total floor plane rest time; (C) total floor plane movements; (D) total floor plane distance travelled; (E) mean floor plane velocity; (F) average distance traveled per movement; (G) total number of jumps; (H) mean number counter-clockwise turns; and (I) mean number clockwise turns. Means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
Figure 7 (continued on page 49)…
Figure 7. Analysis of stereotypic movements observed during open field activity testing.
Analysis of stereotypic movements observed during open field activity testing. Stereotypic movement is described as repetitive behaviors that do not contribute to large location changes progressively further from the starting point. Stereotypic movements are further defined as type-1 (plus or minus 0.999 bean spaces) and type-2 (plus or minus 1.499 beam spaces). Movement is considered stereotypic when movement is made in the X-Y dimensions and back to the original starting point that do not exceed 2 seconds apart. Three such movements must be made before a stereotypy episode starts. When the subject moves outside of the region of qualified coordinates, or fails to move within them for 2 seconds, the stereotypy episode breaks. OFAT data was categorized as (A) total number stereotypic-1 movements; (B) total number stereotypic-1 episodes; (C) total stereotypic-1 time; (D) total number stereotypic-2 moves; (E) total number stereotypic-2 episodes; (F) total stereotypic-2 time. Means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
Figure 8 (continued on page 52)…
Figure 8 (continued on page 53)…
Figure 8. Analysis of zone time during open field activity testing.
Figure 8. Analysis of zone time observed during open field activity testing. Time spent in distinct areas of the open field arena was observed and recorded, specifically the time that was spent in the margin of the arena (within a 2.5-beam margin of the walls) and center of the arena (region that is more than 2.5-beam spaces away from the arena walls) and the distance that was covered in these two areas of the arena. (A) Total distance travelled in the area margin; (B) total time spent in arena margins; (C) total center distance travelled; (D) total time spent in area center); (E) total number of area center entries; (F) percentage of time spent in arena center; (G) percentage of time spent in arena margins. Data expressed as means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
A

![Bar chart for Total Number of Vertical Plane Entries ± SEM.

- B6.IL-23 p19 (+/+)
- B6.IL-23 p19 (+/-)
- B6.IL-23 p19 (-/-)

p = 0.4719

B

![Bar chart for Total Number of Vertical Plane Moves ± SEM.

- B6.IL-23 p19 (+/+)
- B6.IL-23 p19 (+/-)
- B6.IL-23 p19 (-/-)

p = 0.5269

Figure 9 (continued on page 56)…
Figure 9. Analysis of vertical plane movements observed during open field activity testing.
Figure 9. Analysis of vertical plane movements observed during open field activity testing. In addition to movements along the floor plane, movements in the vertical plane are also observed and measured. These include (A) the total number of vertical plane entries; (B) total number vertical plan movements; (C) total time spent in the vertical plane; and (D) the total distance traveled in the vertical plane. Means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
Figure 10. Pilot study of latency to fall in wild-type and p19 knockout mice.
Figure 10. Pilot study of latency to fall in wild-type and p19 knockout mice. Preliminary rotarod speed testing. Animals were habituated to the rotarod using the accelerating setting (6 - 16 rpm) over the course of 5 minutes. Mice were habituated 4 times a day for 3 consecutive days. On day 4, mice were tested for their ability to remain on the rotarod at a constant speed. Animals were 6, 8, 10, 12, 14, and 16 rpm. Three trials were performed at each of the speed and latency to fall recorded for each animal with a maximum run time of 90 seconds. Averages for the 3 runs at each speed were calculated. Results expressed as average latency to fall ± SEM for n = 7 – 8 animals.
Figure 11. Evaluation of forced behavior using two rotarod methods.
Figure 11. Evaluation of forced behavior using two rotarod methods. Animals were habituated on an accelerating rotarod (6 – 16 rpm) for 5 minutes x 3 trials on 4 consecutive days. Following habituation, animals were placed on the rotarod at a constant speed of (A) 14 or (B) 16 rpm for a maximum of 90 seconds x 3 trials. Latency to fall was recorded and trials were averaged. (C) For the accelerating rotarod test, animals were tested for a maximum of 5 minutes on a rotarod accelerating from 0 – 18 rpm for 3 trials. Latency to fall was recorded and trials averaged. Means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
**Figure 12. Evaluation of fine motor skill using adhesive removal test**
Figure 12. Evaluation of fine motor skills using adhesive removal test. Analysis of fine motor skills was performed by evaluating latency for animals to remove a small adhesive dot from the tip of their nose. Adhesive dots were carefully placed on the nose of the animals and the animal was placed in a clean cage. Latency to successfully remove the dot was timed for three separate trials. Means ± SEM for 7 animals per group were calculated and analyzed by one way ANOVA and Tukey’s post-hoc test. (A) 3-trial average latency to remove sticker. (B) Latency to remove sticker for each of the 3 trials. Means ± SEM were determined for 10 – 12 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
Figure 13. Syntactic grooming in wild-type and p19 knockout mice
Figure 13. Syntactic grooming in wild-type and p19 knockout mice. Each mouse was videotaped by an observer for 10 minutes in their home cage. Numbers of complete and incomplete grooming cycles were counted in a blinded fashion. Data presented as means ± SEM were determined for n = 7 - 8 animals per group and analyzed by one way ANOVA and Tukey’s post-hoc test.
CHAPTER FOUR

TARGETING CELLULAR MIGRATION AS A NOVEL TREATMENT FOR PARKINSON’S DISEASE

ABSTRACT

Infiltration of CD4+ T cells in the substantia nigra has been described in post mortem tissues from Parkinson’s disease (PD) patients and these findings were later replicated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. While halting infiltration of CD4+ T cells has proven beneficial in other neurodegenerative disease, it has yet to be adequately tested or utilized in PD and PD models. Using therapies targeting CD4+ T cell adhesion molecules we aimed to block the infiltration of antigen-specific T cells into the brain of MPTP-intoxicated mice and elicit dopaminergic neuroprotection. We conclude that monotherapy did not elicit significant neuroprotection given at the dose and times chosen.

INTRODUCTION

The brain was once thought to be an immune privileged site and not subject to the same immune surveillance as in other areas of the body. This theory was first disproven using the experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis (MS) wherein injections of radioactively labeled encephaliotgenic T cell blasts were found within the brain parenchyma after 6 hours (Engelhardt, 2010). Since then studies have worked to understand how the immune system can play a role in several
neurodegenerative diseases including MS, amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD) and PD. Data from post-mortem studies provided the first evidence for neuroinflammatory processes in PD. In these studies both CD4+ and CD8+ T cells were found in close proximity to dopaminergic neurons in PD brains as well as MPTP-intoxicated mice at levels exceeding 10-fold those found in brains of healthy controls (Speciale et al., 2007; Brochard et al., 2009; Saunders et al., 2012).

In order for peripheral immune cells to remain in the brain they must be antigen specific and encounter their antigen of interest. Within the substantia nigra of PD patients, nitration of α-syn (N-α-syn) leads to accumulation within Lewy bodies (McCormack et al., 2012). Upon examination of the microglial phenotype induced by nitrated and aggregated α-syn, it was shown that the modified protein induces a neurotoxic inflammatory phenotype that accelerates the death of dopaminergic neurons (Bassotti et al., 2000; Zhang et al., 2005; Zhou et al., 2005; Thomas et al., 2007; Reynolds et al., 2008). This suggests aggregated α-syn or N-α-syn, when released from dying neurons, may stimulate resident brain microglia resulting in their sustained activation (Benner et al., 2008). This theory was confirmed in vitro showing N-α-syn can activate microglia causing them to secrete inflammatory proteins (Reynolds et al., 2009). Compounding evidence specifically examining effects of N-α-syn in mouse models of PD have shown the presence of N-α-syn in draining cervical lymph nodes that in turn “educate” peripheral leukocytes. Furthering the interest in N-α-syn and its effects on neurodegeneration came from findings that showed
adoptive transfer of N-α-syn specific T cells exacerbated MPTP-induced dopaminergic cell death (Benner et al., 2008). These studies show that N-α-syn specific T cells accelerate neuronal death by activating microglia and promoting the release of neurotoxic cytokines. Taken together, these studies make a strong case implicating N-α-syn as a contributing factor to neuroinflammation in PD.

While the brain is not completely devoid of immune cells, entry of peripheral immune cells into the brain is tightly controlled and regulated. Expression of correct cell adhesion molecules (CAMs) on peripheral immune cells and brain endothelial cells is absolutely essential for extravasation from circulation into the brain (Figure 1). An additional level of regulation is provided by the unique profile of CAMs upregulated in response to different antigens and disease states. For example, the ability to transfer EAE using myelin basic protein (MBP) T cells was dependent on glycosylation of PSGL-1, the binding ligand for the CAM P-selectin (Deshpande et al., 2006). In human MS patients, the number of circulating CD4+ T cells expressing high levels of PSGL-1 was shown to be increased, indicating these cells have an enhanced ability to interact with brain endothelial cells (Bahbouhi et al., 2009; Engelhardt, 2010). Therefore, therapies that are aimed at modifying cellular migration and targeting specific CAMs may prove to be very beneficial in neurodegenerative diseases including PD.

Research looking at infiltration of circulating lymphocytes across ECs has proven that the expression of CAMs and cell adhesion ligands changes following antigen activation and/or cytokine release. These changes typically precede
disease initiation and work to either increase or decrease the binding affinity of circulating lymphocytes to target endothelium. Therefore, release of N-α-syn could be the initiating factor that leads to changes in CAM expression on peripheral immune cells and leads to their enhanced ability to cross the BBB and exacerbate dopaminergic neurodegeneration.

Unlike MS, the CAM(s) and CAM ligands involved in T cell infiltration in PD and PD models have not yet been identified. Studies have shown that the T cell subset of major interest in the MPTP-model are CD4+ TH17 cells (Benner et al., 2004; Reynolds et al., 2007; Reynolds et al., 2009; Reynolds et al., 2010; Mosley et al., 2012). The more prominent ability of TH17 cells to exacerbate neurodegeneration in MPTP-models may, in part, be explained by the CAM expression on these cells. A recent study identified melanoma cell adhesion molecule [MCAM (CD146)] expressed on TH17 cells and its ligand, laminin 411, expressed within the vascular endothelial basement membrane (Flanagan et al., 2012), which does not appear to be expressed by TH1 cells. Anti-MCAM antibodies inhibited the in vitro interaction of MCAM and laminin 411, and administration of anti-MCAM antibodies to recipient mice prior to adoptive transfer of encephalitogenic TH17 cells reduced TH17 cell infiltration and ameliorated disease in EAE. These data suggested that MCAM expression, if highly restricted to TH17 cells and this may provide insight into the role of the adaptive immune system in PD progression as well as other TH17-driven immune reactions. Thus, the goal of the current study was to evaluate whether
blocking MCAM could provide a level of protection against MPTP and adoptive transfer of N-α-syn specific T cells.

MATERIALS AND METHODS

*Animals*

Adult male C57BL/6J and B6.Cg-Tg (CAG-DsRed.MST) (DsRed) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice 6 – 10 weeks of age were housed and maintained on a 12:12 hr light/dark cycle with *ad libitum* access to food and water and were randomly assigned to treatment groups. The study was conducted in accordance with the animal care guidelines issued by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

*N-4ySyn immunization*

DsRed mice that possess red fluorescent protein (RFP) expression under control of the chicken β-actin promoter were used as donor mice. DsRed mice were immunized with N-4ySyn (10 µg) emulsified Freund’s complete adjuvant (CFA) containing 1mg/mL *Mycobacterium tuberculosis*. Immunized animals were boosted 2 weeks later with N-4ySyn emulsified in Freund’s incomplete adjuvant (IFA). 5 days after boost, splenocytes were isolated and resuspended as a single cell suspension in Hank’s balanced salt solution (HBSS) to be used in adoptive transfer.

*Acute MPTP intoxication and adoptive transfer*
Mice received 4 subcutaneous (s.c.) injections of MPTP-HCL (18 mg/kg free base in a volume of 10 ml/kg) or PBS (10 mL/kg) every 2 hours for 4 doses. Twelve hours after the last MPTP dose, $30 \times 10^6$ donor cells from N-4ySyn immunized animals were adoptively transferred via tail vein injection. MPTP handling and safety measures were in accordance with the National Institutes of Health, the University of Nebraska Medical Center, and prior published guidelines (Przedborski et al., 2001).

**Blocking T cell infiltration**

Studies done by our lab have previously shown that adoptive transfer of TH1 or TH17-polarized T cells can exacerbate MPTP-induced neuronal lesion. Therefore, blockage of T cell infiltration into the CNS could lead to a level of neuroprotection or reduction in MPTP-induced neuroinflammation. Using the sphingosine-1-phosphage inhibitor, fingolimod, prevents peripheral immune cells from exiting secondary lymph organs, effectively reducing the number of circulating immune cells. Therefore, we chose to evaluate the efficacy of fingolimod to prevent or reduce T cell infiltration into the midbrain of MPTP-intoxicated mice and quantify surviving dopaminergic neurons. Twenty-four hours prior to the first MPTP injection and adoptive transfer, groups of animals were treated with either 1 mg/kg fingolimod (MPTP/fing/AT) or 10 mg/kg anti-MCAM antibody (MPTP/anti-MCAM/AT). Daily injections with anti-MCAM or fingolimod continued until the end of the study. To serve as controls, one group of MPTP-intoxicated mice received 10 mg/kg rat isotype control antibody (MPTP/Isotype/AT) and one group received no additional treatment (MPTP/AT).
**Immunohistochemistry and stereological analysis**

7 days post-MPTP or PBS administration, mice were terminally anesthetized, transcardially perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Brains were harvested and post fixed in PFA overnight, cryoprotected in 30% sucrose in PBS for 48 hours, snap frozen in 2-methylbutane, embedded in OCT compound, and 30 µm sections collected through the midbrain. Tissue sections were processed free-floating in 48-well plates. Tissues were immunostained with rabbit anti-TH (1:2000 CalBiochem/EMD Millipore, Billerica, MA) and HRP-conjugated goat anti-rabbit IgG (1:400, Vector laboratories, Burlingame, CA), and visualized with diaminobenzidine (DAB). Tissues were mounted on slides and counterstained for Nissl substance using thionin. Neurons expressing TH and nissl (TH+Nissl+) were considered dopaminergic and quantified by stereological analysis (StereoInvestigator, MBF Bioscience, Williston, VT). Additional sets of tissues were mounted on slides and coverslipped with Vectashield mounting medium for fluorescent tissues (Vector laboratories, Burlingame, CA) to be evaluated for the expression of infiltrating cells from DsRed donor mice.

Stereological analysis of neurons in the substantia nigra was performed using optical fractionator module stereology software (StereoInvestigator, MBF Bioscience, Williston, VT) interfaced with a Nikon Eclipse 90i fluorescence microscope equipped with a bright camera. Using simple random sampling (SRS) image series workflow, the region of interest (ROI) containing the substantia nigra was outlined for one hemisphere of each tissue section at a 4x
magnification. Images of each ROI taken using a 40x were taken at random, equally spaced intervals. Workflow parameters were defined to count approximately 20% of the total neuron population in the substantia nigra (counting frame size 120 x 100 um, 245 x 240 um grid size) and Gunderson coefficient of error (C.E. m=1) was ≤ 0.01. Estimated population size was generate by the software and multiplied by 2 to get an overall population estimation for both hemispheres of the brain.

Flow cytometric analysis

Prior to perfusion, a small volume of whole blood was collected from mice (PBS, MPTP/AT, and MPTP/Fing/AT) and held in EDTA coated tubes. Red blood cells (RBCs) were lysed and cells incubated with FITC-conjugated anti-CD4+ antibodies. Cells were then fixed and submitted to the University of Nebraska Center flow cytometric core facility. Using forward scatter (FSC) and side scatter (SSC) lymphocyte populations were first gated from the total cell population. Using the quadrant gate CD4+ and DsRed+ cells were isolated to identify single and double positive cells. Percentages of CD4+, DsRed+, and CD4+/DsRed+ cells was calculated based on the parental population.

Statistical analysis

All tests were performed using Statistica (StatSoft, Inc. Tulsa, OK) and data expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated by one-way ANOVA followed by post-hoc comparisons using Fisher’s LSD.
RESULTS

Blocking MCAM provides partial neuroprotection against N-4ySyn-specific splenocytes following MPTP

TH17 cells have been shown to play a role in exacerbating dopaminergic neurodegeneration. Therefore specific targeting of TH17 using an antibody specific to MCAM was utilized in the MPTP/adoptive transfer model of PD. Splenocytes isolated from donor mice immunized with the antigen N-4ySyn were adoptively transferred into recipient mice 12 hours after the last MPTP dose. To determine the effects blocking TH17 cell infiltration would have we administered an antibody specific for MCAM to beginning 24-hours pre-MPTP and continuing until day 7 post-MPTP, wherein the number of surviving neurons were quantified. Additionally, a group of animals were administered fingolimod, a sphingosine-1 phosphate inhibitor known to sequester T cells within secondary lymphoid organs (Mehling et al., 2008; Mehling et al., 2010; Slavin and Zamvil, 2010; Sheridan and Dev, 2014). Mice that received anti-MCAM therapy showed a slight increase in the number of surviving dopaminergic neurons compared to those mice treated with MPTP/AT or MPTP/Isotype/AT (Figure 2).

Fingolimod did not decrease number of CD4+ cells in circulation

Fingolimod is a sphingosine-1 phosphate inhibitor that is an approved drug for the treatment of MS. By sequestering lymphocytes in lymph nodes, the cells are unable to circulate and infiltrate the brain where they would elicit a response. By administering fingolimod to MPTP-intoxicated animals that received donor cells
from N-4ySyn immunized animals, we hypothesized that the MPTP-intoxicated animals would be protected from MPTP-induced neurodegeneration in a similar manner to mice receiving anti-MCAM therapy. However, we did not show any neuroprotection in the fingolimod group, but rather we saw increased neurodegeneration compared to mice who received anti-MCAM (Figure 2).

Whole blood was collected from PBS and MPTP-intoxicated animals who received adoptively transferred cells and lymphocytes stained with anti-CD4 antibodies. Adoptively transferred cells were isolated from N-4ySyn-immunized DsRed donor mice so that donor antigen-specific cells could be easily detected in the circulation and CNS of MPTP-intoxicated mice. Using forward and side scatter flow cytometric data, CD4+ lymphocytes populations were gated based upon parent lymphocyte populations (Figure 3A) and DsRed expression (Figure 3B). CD4+DsRed+ cells were calculated based upon the parental lymphocyte population (Figure 3C). Unexpectedly, an increased percent of CD4+ cells were found in circulation in fingolimod treated animals compared to PBS controls, however it did not reach statistical significance. The percentage of DsRed+ (donor cells) in circulation was marginally decreased in fingolimod treated mice compared to controls, but not to the level of statistical significance (Figure 3B). Similarly, slight differences were seen in the percentage CD4+DsRed+ in animals that received fingolimod compared to those who did not (Figure 3C).

Donor cells were not detectable in the substantia nigra of MPTP-intoxicated recipient mice
In this study we chose to use DsRed mice as the source of adoptively transferred N-4ySyn splenocytes. DsRed mice expressed RFP under control of the β-actin promoter so all their cells express RFP. By using these mice we would be able to identify and quantitate infiltrating splenocytes in the substantia nigra of MPTP-intoxicated mice. Following processing of tissues and mounting on slides, tissues were analyzed under fluorescent microscopy. In this study we were unable to identify any cells within the substantia nigra that expressed RFP.

DISCUSSION

The lack of significant neuroprotection afforded with anti-MCAM therapy in this study could be due to a number of factors. First, administration of the MCAM antibody may have been needed at times other than 1 hour prior to adoptive transfer or the antibody may have needed to be given at a greater concentration to effectively inhibit MCAM binding. Secondly, a relatively low percentage of adoptively transferred cells expressed MCAM prior to adoptive transfer and this would have reduced, even further, the chance of anti-MCAM antibodies finding its target within the entire circulation. Third, because we did not activate splenocytes from donor mice prior to AT, the cells may not have upregulated MCAM expression as they would have if stimulated with N-4ySyn or anti-CD3. Lastly, T cells extravasation from the circulation is not dependent on only one CAM but rather a multiple CAMs that act in redundant and synergistic mechanisms. This leads to the possibility that MCAM may be playing a minor role in the infiltration of TH17 cells following MPTP lesion or, alternatively, other subsets of CAMs may be compensating for the blockage of MCAM and allowing
for CD4+ T cells to enter the. Interestingly, animals that received fingolimod treatment following MPTP and AT had significantly fewer surviving dopaminergic neurons suggesting the sequestration of all T cells in lymphoid organs is particularly detrimental. This explanation is in agreement with other findings that show CD4+ regulatory T cells provide a level of protection against MPTP-lesion and blocking all CD4+ T cells is a therapeutic approach that should be avoided but rather a variety of CAMs should be administered to block only the CAMs that play a large role in cell extravasation.

Fingolimod is an approved drug for the treatment of relapsing/remitting MS and works by preventing lymphocytes from leaving the lymph nodes and returning to circulation. We hypothesized that administering fingolimod in the MPTP-mouse model of PD would work in much the same way and sequester lymphocytes in the lymph nodes, thus reducing the number of lymphocytes in circulation. Administration of fingolimod at 1 mg/mL staring 1 day prior to MPTP-intoxication and continuing until day 7 did not result in reduced number of circulating lymph nodes as measured by flow cytometric analysis. Fingolimod has been proven to sequester T cells in secondary lymph organs, effectively reducing the number of T cells in circulation. In the study discussed here, we were unable to validate these results in the MPTP mouse model of PD. We expected that in MPTP-intoxicated mice that received cells from donor mice, there were be a reduced number of T cells in the blood of mice that also received fingolimod to those that did not. These unexpected results could be a result of inappropriate
fingolimod dosage or time of administration or a result of the collection process used when obtaining a sample of blood from the animals.

The lack of red donor cells within the substantia nigra of MPTP/AT control mice does not in itself prove cells never crossed the BBB and infiltrated into the substantia nigra but rather can be explained by several mechanisms. First, MPTP induces peak neuroinflammation and rate of neuronal death by 48 hours, both of which are typically resolved by 4 days post-treatment (Jackson-Lewis et al., 1995; Kurkowska-Jastrzebska et al., 1999; Jackson-Lewis and Przedborski, 2007). Therefore at 7 days post-MPTP, when the tissues were harvested, donor CD4+ T cells that infiltrated into the substantia nigra could have migrated back out of the substantia nigra or undergone cell death. Secondly, the number of cells adoptively transferred, 30 x 10^6, could have been too low for a large enough number of cells to successfully cross the BBB and be visible with the microscopy method utilized in our study. Lastly, the stability and intensity of RFP on CD4+ T cells was not specifically addressed in this study and it is plausible that RFP expression was lost or significantly reduced so as these cells were no longer distinguishable by the presence of RFP.

In conclusion, we demonstrated that a dose of 10 mg/kg of anti-MCAM therapy alone is not sufficient to elicit neuroprotection in the MPTP-mouse model. While our results were not as expected, it does not rule out that a combination therapy that includes anti-MCAM as well as other CAM blocking agents would not be protective. The tests described herein should provide a foundation for
which additional studies could be performed aimed at blocking T cell entry to the substantia nigra and provide a level of neuroprotection to dopaminergic neurons.
Figure 1. Migration of activated T cells into brain. Peripherally, naïve T cells (N) encounter APC that present peptides from aberrant, misfolded, or aggregated proteins associated with neuroinflammatory processes. Upon presentation of antigen and delivery of appropriate co-stimulatory signals by APC, naïve T cells recognizing the antigen/MHC complex via the TCR become activated (A) leading to upregulation of CAMs on the T cell surface. These receptors and ligands include, but are not limited to, integrins, MCAM, and PSGL-1. Similarly, at sites of neuronal injury and neuroinflammation, danger signals, pro-inflammatory cytokines, and chemokines induce upregulation of endothelial associated CAMs on the basolateral side of the blood brain barrier. Following upregulation of CAMs, activated T cells (such as pro-inflammatory, anti-inflammatory or regulatory T cells) enter the vasculature and begin the process of extravasation via either a trans- or para-cellular route. This migratory process occurs in a step-
wise manner beginning with T cells loosely tethering to endothelial cells via the binding of T cell ligands to selectins, such as E-selectin and other CAMs, such as VCAM, ICAM, and laminin 411 on the luminal side of the endothelial cells. Loose tethering allows the cell to roll along the luminal side of the endothelium and interact with CAMs, pulling it closer to the endothelial cell layer to eventual capture. Upon clustering of receptors and ligands on T cell and endothelial cell surfaces, the T cell begins “crawling” across the endothelial surface until reaching an endothelial cell junction, which signals the initiation of extravasation. Transmigration proceeds, via a chemotactic gradient allowing antigen-specific T cells entrance to the brain. Once in the parenchyma, activated T cells recognize antigen presented by MHC, initiating the efferent response program of the T cells to deliver either effector or regulatory function that supports the respective neurodegenerative or neuroprotective outcome.
Figure 2. Blocking MCAM following N-4YSyn-specific splenocyte transfers elicits partial neuroprotection.
Figure 2. Blocking MCAM following N-4YSyn-specific splenocyte transfers elicits partial neuroprotection. N-4YSyn-specific Teffs were adoptively transferred into MPTP-intoxicated mice. Appropriate controls included MPTP-only mice, MPTP + adoptive transfer, MPTP + adoptive transfer + isotype control. Antibody and fingolimod treatments began the day before adoptive transfer and continued until the end of study. One group was treated with only PBS (PBS), and served as total neuron control. Brains were sectioned through the midbrain, immunostained with rabbit anti-TH and HRP-conjugated goat anti rabbit IgG, and visualized with DAB. Total numbers of surviving dopaminergic neurons (TH+) in the SN were quantified by stereological analysis (Stereo Investigator, MBF Bioscience). Means ± SEMs of total numbers of surviving dopaminergic neurons were determined from 5–8 mice per treatment group and were compared by one way ANOVA and Fisher’s LSD post-hoc test.
Figure 3. Flow cytometric analysis of blood from animals with and without fingolimod treatment.
Figure 3 Flow cytometric analysis of blood from animals with and without fingolimod treatment. Seven days post-MPTP, blood samples were collected from mice who received adoptive transfer of DsRed N-4ySyn specific splenocytes with and without fingolimod or anti-MCAM antibodies as well as no-treatment controls. Red blood cells were lysed with ACK lysis buffer and remaining cells were stained with FITC-conjugated anti-CD4+ antibodies. Single cell suspensions were submitted to the University of Nebraska Medical Center flow cytometric core facility for expression of DsRed and FITC. Flow cytometric data was first gated on the parental lymphocyte population based on forward and side scatter. The parental population was then gated on (A) FITC (CD4+) and (B) DsRed (N-4ySyn adoptively transferred) and percentages of CD4+DsRed+ calculated (C). Values represented as mean ± SEM for n = 4 mice/group.
CONCLUSIONS

From the data presented above, it is obvious that many safeguards are in place to protect and maintain normal neuronal signaling. This includes the possession of an excess number of dopaminergic neurons which allow maintenance of normal motor and behavior functions in the event that a significant number of dopaminergic neurons are lost. Secondly, a short time after ablation of dopaminergic neurons, surviving non-dopaminergic neurons undergo a phenotypic shift which is hypothesized to compensate for the loss of neurons and allow normal levels of dopamine signaling. Third, from a decreased number of dopaminergic neurons, surviving neurons can possibly express double the number of termini as a normal neuron, presumably to preserve adequate signaling in the striatum.

Unlike GABAergic neurons in other areas of the CNS (Achim et al., 2012; Achim et al., 2014) molecular cues that midbrain GABAergic neurons rely on are poorly understood and described. This extends to their migration patterns and temporal appearance, as well as transcriptional regulation of these processes (Achim et al., 2012). Throughout the CNS, dopaminergic neurons are known to have a direct effect on GABAergic neurons; negatively regulating TH expression by GABAergic neurons and positively regulating GABAergic neuron migration and establishment. As shown in chapter 2, these neurons, previously believed to represent distinct and non-overlapping populations, exhibit some plasticity upon injury with MPTP. This opens new possibilities for PD therapy. For example, as an alternative to restoring dopamine levels using dopamine replacement therapy
with drugs such as L-DOPA, other strategies could instead target GABAergic neurons in the vicinity to induce TH expression and synthesize dopamine. These neurons would then comprise a novel population in the adult midbrain; namely functional TH+GAD67+ neurons. Determining the conditions and factors necessary to trigger phenotypic and/or functional changes in GABAergic neurons would greatly aid in the development of novel therapies for PD.

An increase in phosphorylated serine residues within the regulatory domain of TH was demonstrated in this study and has been well described by others. The lingering question is whether the increased phosphorylated Ser31 is on TH expressed on the surviving dopaminergic neurons or if it is a consequence of TH expression in the newly described TH+GAD67+ neurons. If the latter, then that suggests an additional mechanism by which dopamine synthesis could be increased in an otherwise dopamine-depleted environment. Because phosphorylation controls both the expression and activity of TH, it is plausible that TH produced by both the dopaminergic and GABAergic neurons is being phosphorylated to provide the best opportunity to increase dopamine synthesis.

In addition to the TH expression in neurons, cytokines involved in neuroinflammation have been proven to play a role in the migration, differentiation, and survival of neurons (Whitney et al., 2009; Kim et al., 2015). The severity and duration of neuroinflammation, as well as the profile of inflammatory molecules involved in neuroinflammation has a profound effect on the neurogenic or neurodestructive outcome (Whitney et al., 2009). Extensive studies have shown that inflammation and cytokines affect neurogenesis in
neurodegenerative diseases in a positive and negative manner (Arnett et al., 2001; Dybedal et al., 2001; Ben-Hur et al., 2003; Ming and Song, 2005; Iosif et al., 2006; Whitney et al., 2009; Keohane et al., 2010; Kim et al., 2015). However, current literature does not adequately address the role that IL-23 or TH17-associated cytokines play in dopaminergic neurogenesis, survival, or migration. Data presented herein in p19 knockout mice, suggests that IL-23 plays a role in the establishment of normal numbers of dopaminergic neurons in the substantia nigra, but whether that role lies in neurogenic, migration, integration, or survival processes remains enigmatic. One possible mechanism explaining the significantly reduced number of dopaminergic neurons in p19 knockout mice draws on findings that show cytokines play a role in the expression of CAMs on cellular surfaces. CAMs play an important role in neuronal development, aiding in migration from sites of neurogenesis to their final target location as well as ensuring permanent residence in the area. In mice lacking p19 and IL-23, the expression of adhesion molecules on dopaminergic neurons is plausibly insufficient to allow integration into the midbrain due to inadequate cytokine levels. Neurons without the proper expression pattern of CAMs on their surface fail to take permanent residence in the substantia nigra and innervate into the striatum and signaling along the IL-23/IL-17 axis has been shown to regulate migration of cells (Kawano et al., 1995; Blum, 1998; Mehler and Kessler, 1998).

In contrast to homozygous p19-/- knockout mice, heterozygous p19+/- mice have normal densities of dopaminergic termini within the striatum. The normal striatal density, despite the significant reduction in neuronal bodies within
the substantia nigra, suggests that each dopaminergic neuron in the substantia nigra possesses roughly double the number of termini that innervate into the striatum. I posit that alterations in cytokine profiles between heterozygous and homozygous knockout mice are, in part, responsible for this finding. Heterozygous mice presumably have some level of functional TH17 cells and therefore are capable of secreting TH17 associated cytokines. If cytokine levels are responsible for the development or migration of neurons versus sprouting of neurons, higher amounts of cytokines present in heterozygous knockout mice may result in the establishment of proper striatal innervation regardless of the number of cell bodies present (Mehler and Kessler, 1998). Homozygous knockout mice lacking all TH17 cells also lack the cytokine signals needed for neuronal establishment of the substantia nigra and innervation into the striatum.

Cell replacement therapy is an area of research that is gaining popularity as a method to restore both the numbers of dopaminergic neurons in the substantia nigra as well as the amount of dopamine available in the striatum without the use of pharmaceuticals. The first steps toward clinical utilization of cell replacement therapy have been taken using transplantation of human fetal midbrain tissue. These studies have provided proof of concept for cell replacement therapy being well-tolerated in PD (Lindvall and Bjorklund, 2004; Drouin-Ouellet and Barker, 2012; Thomsen et al., 2014), and human midbrain dopaminergic neurons have been successfully generated from multiple cell types in vitro including by lineage reprogramming (Caiazzo et al., 2011; Kim et al., 2011; Pfisterer et al., 2011; Arenas et al., 2015). However, major impediments
with neuronal replacement strategies are the non-autonomous development of Lewy bodies in transplanted neurons and the susceptibility and eventual loss of the Lewy body-containing neurons. Another interesting strategy could achieve successful reprogramming of surviving cells in the substantia nigra which would lead to monumental advances in the treatment of PD. In order for this to occur, a full picture of the essential mechanisms that control dopaminergic neuron development is needed to enable the application to generation of functional dopaminergic neurons from other neuronal lineages, such as those along the TH-inducible GABAergic lineage. In order for neuron reprogramming to be efficacious in PD treatment, newly formed dopaminergic neurons must meet a number of criteria (Arenas et al., 2015). First, the neurons must acquire a stable mature dopaminergic phenotype. Second, neurons must re-innervate into only the striatum and no other off-target locations. Third, they must synthesize and release dopamine and exhibit appropriate firing patterns associated with dopaminergic neurons. Fourth, reprogramming of cells into a dopaminergic lineage must result in improvements in physical symptoms associated with PD. Lastly, the newly transplanted or reprogrammed neurons must remain resistant to Lewy body development and induced susceptibility to injury and degenerative processes. Along those same strategies, if utilized together an interesting question arises as to whether too much dopamine production by transplanted neurons and reprogrammed neurons would produce untoward outcomes such as dyskinesias in the PD patient.
The data showing significantly decreased numbers of dopaminergic neurons in p19 knockout leads to the question as to whether this is a consequence of abnormal neurogenesis, neuronal migration, neuronal differentiation, or survival of terminally differentiated neurons. To date, over 50 genetic mutations and mutant mice have been described that lead to effects on dopaminergic neuron development and survival (Hegarty et al., 2013). Using findings from these modes in combination with my data will help to pinpoint factors required for normal midbrain development. This would have direct clinical applications by targeting newly identified factors with the goal of restoring neuron numbers by mobilizing dopaminergic neurons from neurogenic areas to areas of neurodegeneration.

If we can delineate the mechanism(s) to increase neuronal migration, multiple benefits over traditional cell replacement therapy would be gained. Clinical trials evaluating the efficacy of fetal stem cell transplantation into the striatum of PD patients failed to show significant improvements to patients compared to those who underwent sham surgery. In addition to the technical difficulty of this therapy, fetal stem cell transplantation carries multiple ethical issues (Chinta and Andersen, 2005). As an alternative to fetal stem cell transplantation and the associated issues, information collected from my studies regarding how neuronal phenotypes are controlled could help develop techniques to enable stem cells or non-dopaminergic neurons from PD patient’s own bodies to be induced and become dopaminergic neurons. These neurons can then be triggered to migrate to areas of neurodegeneration and effectively
restore the number of dopaminergic neurons in the substantia nigra. This will treat not only the physical symptoms of the disease, but possibly reverse the disease itself. If dopaminergic neurons can be replaced by one's own cells at a rate equal to the rate of neuronal loss, the neuronal population can be maintained resulting in alleviation or disappearance of physical symptoms.

Based upon original findings from our laboratory showing a detrimental effect of TH17 cells in the MPTP mouse model of PD (Benner et al., 2004; Reynolds et al., 2010), we hypothesized knockout of TH17 cells in a transgenic mouse would result in neuroprotection from MPTP induced cell death. However, this hypothesis was disproven due to the finding that p19 knockout mice show the same sensitivity to MPTP within the substantia nigra and striatum compared to wild-type mice (chapter 4). Expanding on data described above (chapter 3) wherein pro-inflammatory cytokines were implicated in controlling neuronal migration (Arnett et al., 2001; Dybedal et al., 2001; Ben-Hur et al., 2003; Monje et al., 2003; Chen et al., 2004; Iosif et al., 2006; Whitney et al., 2009; Keohane et al., 2010; Kim et al., 2015), I posit that by blocking TH17 cells with the use of anti-MCAM or fingolimod, the previously undescribed beneficial effects of pro-inflammatory cytokines were unknowingly blocked. This conclusion is supported by data in chapter 3 (figures 5 and 8). These figures demonstrate that in MPTP intoxicated mice, even in the absence of dopamine replacement therapy, the numbers of TH+GAD67+ neurons are significantly increased. This suggests that in the absence of continued neurodegeneration, pro-inflammatory cytokines elicit
a novel compensatory mechanism to upregulate the expression of TH by GABAergic neurons.

These results propose three compensatory mechanisms in the MPTP mouse model of PD in response to a reduction in striatal dopamine levels. First, I demonstrated a phenotypic shift of GABAergic neurons in the substantia nigra following MPTP-intoxication. The number of GABAergic neurons that undergo this phenotypic shift and begin expressing TH increases with the addition of dopamine replacement therapy in MPTP-lessoned mice. This leads to a potential increase in the production of dopamine by increasing expression of the rate-limiting enzyme involved in dopamine synthesis. Secondly, using p19 deficient mice, I showed that a significant reduction of dopaminergic neurons does not result in measurable behavior or motor deficits compared to wild-type mice. This could be for a number of reasons: slower removal of dopamine, increased hypersensitivity of dopamine receptors, or increases in the number of dopamine producing striatal interneurons (Brotchie and Fitzter-Attas, 2009). The finding that heterozygous p19+/- mice show no reduction in striatal density compared to wild-type mice, but the number of dopaminergic neurons in the substantia nigra is roughly half of that found in a wild-type, points to a compensatory mechanism that results in increased synaptogenesis or sprouting of new terminals by surviving dopaminergic neurons (Rosenblad et al., 1998; Backman et al., 2006; Brotchie and Fitzter-Attas, 2009). Lastly, I presented data showing a lack of dopaminergic neuroprotection with the TH17 blocking agent, MCAM or sphingosine-1 phosphate inhibitor fingolimod – contradictory to the original
hypothesis. This finding points to a novel effect of pro-inflammatory in which TH expression by surviving neurons is dependent on resolution of neurodegeneration and presence of pro-inflammatory cytokines.

Herein, I have presented data showing novel avenues by which to approach the treatment of PD and other neurodegenerative diseases. First, I presented the novel finding that GABAergic neurons in the substantia nigra undergo a phenotypic shift in response to dopaminergic neurodegeneration or dopamine replacement therapy, in the form of L-DOPA or BL-1023, and begin expressing TH. This phenotypic shift was seen with and without initial MPTP-induced lesion, but the greatest increase in TH+ GABAergic neurons was in lesioned animals treated with drug. Showing the initial increase in TH+ neurons was not due to neurogenesis, proved GABAergic neurons in the substantia nigra show a degree of plasticity in their neuronal phenotype. This finding can be applied to future PD treatments aimed at increasing dopamine synthesis and restoration of dopamine levels in the striatum without the need for dopamine replacement therapy. Secondly, I presented data that showed significantly fewer dopaminergic cells are required to control motor function and behavior in p19 knockout animals compared to wild-type. It is hypothesized that the reduced number of dopaminergic neurons and dopamine levels in knockout animals results in increased sensitivity of dopamine receptors or an upregulation in the number of dopamine receptors themselves. This is especially relevant to PD, as the same reduction in dopaminergic neurons is seen in patients. Understanding how knockout animals are able to function normally, despite their significantly
affected neuronal profile, could lead to more efficacious therapies for the treatment of neurodegenerative disorders. Using the p19 knockout mouse will help us to better understand how the CNS can function with lower levels of dopaminergic neurons and dopamine with any consequent neuronal signaling modifications.

The fact that PD symptom onset does not begin until the disease has substantially progressed and a majority of dopaminergic neurons in the substantia nigra have been lost, leads to the hypothesis that multiple compensatory mechanisms are involved to maintain normal CNS function. It is likely the numerous compensatory mechanisms are activated at different stages of disease, and understanding the sequence of action will vastly improve clinical treatment of disease. The potential for significant improvement in the lives of PD patients is the rationale for continuing research into these areas. However, as previously stated, PD symptoms do not begin until well into disease progression, so a first step would be to identify a biomarker that could identify the very early stages of PD, and treat accordingly. One such proposed biomarker is the balanced neurochemical index of the ratio of nigrostriatal pathway traffic versus dopamine stores (Goldstein, 2013). As disease progresses, it is hypothesized that different compensatory mechanisms are initiated as a method to maintain normal dopaminergic function. Each compensatory mechanism results in different neurochemical profiles in the cerebrospinal fluid that theoretically can be detected in clinical tests. By tracking compensatory activation in presymptomatic individuals, the best treatment plan could be established to attenuate or prevent
further dopaminergic cell death (Goldstein, 2013). Using data from findings presented in this report will help to reveal some of these compensatory mechanisms and aid in developing the most efficacious treatment plans for patients at different stages of PD progression.
BIBLIOGRAPHY


Birkmayer W, Hornykiewicz O (1961) [The L-3,4-dioxyphenylalanine (DOPA)-

Birkmayer W, Hornykiewicz O (1962) [The L-dihydroxyphenylalanine (L-DOPA)
effect in Parkinson's syndrome in man: On the pathogenesis and
treatment of Parkinson akinesis]. Arch Psychiatr Nervenkr Z Gesamte
Neurol Psychiatr 203:560-574.


Bjorklund A, Cenci MA (2010) Recent Advances in Parkinson's disease -

Science 215:1198-1204.


Blum M (1998) A null mutation in TGF-alpha leads to a reduction in midbrain

Bobrovskaya L, Gilligan C, Bolster EK, Flaherty JJ, Dickson PW, Dunkley PR
(2007) Sustained phosphorylation of tyrosine hydroxylase at serine 40: a
novel mechanism for maintenance of catecholamine synthesis. J
Neurochem 100:479-489.

Systemic immune aberrations in Alzheimer's disease patients. J


hematopoietic cells modulates proliferation via MAP kinase--ERK-1/2 and does not require tyrosine docking sites in the EPO receptor. Exp Cell Res 298:155-166.


Flanagan K et al. (2012) Laminin-411 is a vascular ligand for MCAM and facilitates TH17 cell entry into the CNS. PloS one 7:e40443.


Kosaka K, Hama K, Nagatsu I, Wu JY, Kosaka T (1988) Possible coexistence of amino acid (gamma-aminobutyric acid), amine (dopamine) and peptide (substance P); neurons containing immunoreactivities for glutamic acid decarboxylase, tyrosine hydroxylase and substance P in the hamster main olfactory bulb. Exp Brain Res 71:633-642.


system for rodent behavioral phenotyping in a home cage environment.


dopaminergic cell death in primary mesencephalic cell culture. Neurosciences (Riyadh) 20:10-16.


Schneider JS, Anderson, D.W., Decamp, E. (2008) 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced mammalian models of Parkinson's disease: potential uses and misuses of acute, sub-acute, and chronic models. In:
Parkinson’s disease: Molecular and therapeutic insights form model systems (Richard Nass SP, ed), pp 87-103: Elsevier.


spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells. Nat Med 18:1069-1076.


dysfunction of basal ganglia subterritories: From movement to behavioral
disorders. Mov Disord.

Trojanowski JQ, Kleppner SR, Hartley RS, Miyazono M, Fraser NW, Kesari S,
Lee VM (1997) Transfectable and transplantable postmitotic human
neurons: a potential "platform" for gene therapy of nervous system

Truckenmiller ME, Tornatore C, Wright RD, Dillon-Carter O, Meiners S, Geller
HM, Freed WJ (1998) A truncated SV40 large T antigen lacking the p53
binding domain overcomes p53-induced growth arrest and immortalizes

Turjanski N, Lees AJ, Brooks DJ (1997) In vivo studies on striatal dopamine D1
and D2 site binding in L-dopa-treated Parkinson's disease patients with

embryonic and adult forms of glutamic acid decarboxylase during in vitro-
induced neurogenesis in cloned neuroectodermal cell-line, NE-7C2. J
Neurochem 80:605-615.

neurons modulate GABA neuron migration in the embryonic midbrain.
Development 139:3136-3141.

Von Euler US (1946a) The presence of a sympathomimetic substance in extracts


