PIOGLITAZONE IS SUPERIOR TO QUETIAPINE, CLOZAPINE AND TAMOXIFEN AT ALLEVIATING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

by

Matthew Assad Joseph Chedrawe

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Sid, Sally, Joseph, Mark, Laura, and Amira

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ABSTRACT

Recent evidence suggests that clozapine, quetiapine, tamoxifen and pioglitazone may improve functional recovery in multiple sclerosis (MS). We have compared the effectiveness of oral administration of these drugs, beginning at peak disease, at reducing disease severity and demyelination in mice subjected to experimental autoimmune encephalomyelitis (EAE). Unlike clozapine, quetiapine and tamoxifen, administration of pioglitazone beginning at peak disease decreased clinical scores and lumbar white matter loss in EAE mice. Using kinematic gait analysis, we found that pioglitazone also maintained normal movement of the hip, knee and ankle joints for 44 days after EAE induction. This long-lasting preservation of hindleg joint movements was accompanied by reduced white matter loss, microglial and macrophage activation and the expression of pro-inflammatory genes in the lumbar spinal cord of EAE mice. These results support clinical findings that suggest pioglitazone may reduce the loss of motor function in MS by decreasing inflammation and myelin damage.

LIST OF ABBREVIATIONS AND SYMBOLS USED

4-HT	4-Hydroxytamoxifen
AD	Alzheimer's Disease
ALS	Amyotrophic lateral sclerosis
CFA	Complete Freund's adjuvant
CLO	Clozapine
CNS	Central nervous system
DPI	Days post immunization
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disability status scale
ER	Estrogen receptor
FBS	Fetal bovine serum
FDA	Food and Drug Administration
ip	Intraperitoneal
KGA	Kinematic gait analysis
L2	Lumbar spinal cord vertebrae 2
L3-4	Lumbar spinal cord vertebrae 3-4
L5	Lumbar spinal cord vertebrae 5
LPC	Lysophosphatidylcholine
MCU	Mitochondrial calcium uniporter
MOG_{35-55}	Myelin oligodendrocyte glycoprotein peptide 35-55
MS	Multiple sclerosis
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OPC	Oligodendrocyte progenitor cell
PBS	Phosphate buffered saline
PBS-TX	Phosphate buffered saline with Triton-X100
PD	Parkinson's Disease
PFA	Paraformaldehyde
PIO	Pioglitazone
$PLP_{139-151}$	Proteolipid protein peptide 139-151
PMCA-2	Plasma membrane calcium ATPase-2
po	oral administration
PPARγ	Peroxisome proliferator-activated receptor gamma
PPMS	Primary progressive multiple sclerosis
PRMS	Primary relapsing multiple sclerosis
PTX	Pertussis toxin
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
QUE	Quetiapine
RMS	Root mean squared
ROM	Range of motion
RRMS	Relapse remitting multiple sclerosis
sc	subcutaneous
SERM	Selective estrogen receptor modulator
SPMS	Secondary progressive multiple sclerosis
TAM	Tamoxifen
TZD	Thiazolidinedione

VEH	Vehicle
%	Percent
°C	Degrees Celsius

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CHAPTER 1: INTRODUCTION

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1.1 OVERVIEW

Multiple sclerosis (MS) is primarily an inflammatory disease characterized by autoimmune-mediated demyelination in the central nervous system (CNS) (Loma and Heyman, 2011). This results in a variety of neurological symptoms such as limb weakness and sensory loss. In most cases, the disease progressively worsens over time, leading to fatigue, cognitive decline and motor deficits as well as visual impairments (Compston and Coles, 2008; Loma and Heyman, 2011). Experimental autoimmune encephalomyelitis (EAE) is one of the most commonly used experimental methods to model MS in rodents that recapitulates autoimmune mechanisms, demyelination, axonal damage, sensory impairments and motor deficits in MS. To date, EAE has assisted in the development of four Food and Drug Administration (FDA)-approved drugs used to treat MS (Brinkmann et al., 2002; Constantinescu et al., 2011; Steinman and Zamvil, 2006). Current therapies suppress disease relapses by targeting various aspects of immune dysfunction in MS. However, these treatments have minimal-to-no capacity to stimulate remyelination. Although preclinical studies have identified a variety of novel remyelinating therapeutics, the human trials required to establish

their clinical effectiveness are lengthy, expensive, complex and risky (Collier, 2009; Martin et al., 2017). One way to mitigate this problem is to identify drugs already approved by the FDA that could be repositioned for the treatment of MS. This repositioning strategy may thus overcome the long developmental timelines and high clinical failure rates associated with *de novo* CNS drug discovery. The aims of the current study are the following: 1) to identify which of four FDA-approved drugs, known to stimulate remyelination - quetiapine, clozapine, tamoxifen and pioglitazone - is most effective at alleviating EAE disease severity in mice; 2) to determine the mechanism of action for the most effective drug candidate and 3) to measure the beneficial effects of the drug candidate on gait deficits in EAE mice that resemble those seen in MS.

1.2 MS: PATHOPHYSIOLOGY, EPIDEMIOLOGY, CLINICAL FEATURES, AND TREATMENTS

1.2.1 PATHOPHYSIOLOGY AND EPIDEMIOLOGY OF MS

MS is an autoimmune disease caused by myelin-reactive immune cells that destroy the myelin sheath which surrounds axons in the CNS (Loma and Heyman, 2011). Although the precise disease triggers are unclear, MS is thought to arise from a complex interplay between environmental and genetic factors (Milo and Kahana, 2010) that give rise to the activation, expansion and infiltration of myelin-autoreactive T-cells and macrophages (Stys et al., 2012). Remyelination occurs in MS, but is typically inefficient and eventually fails after repeated cycles of demyelination (Franklin and Ffrench-Constant, 2008). The expansion of sodium channels along a denuded axon slows conduction velocity

and places a considerable metabolic burden on mitochondria to buffer calcium and provide the necessary energy for increased ion pumping (Loma and Heyman, 2011; Waxman et al., 2004). This renders axons highly vulnerable to damage that is responsible for the irreversible loss of sensory, motor and cognitive function in MS (Compston and Coles, 2008; Trapp et al., 1998; Trapp and Stys, 2009). Immune dysfunction in MS may also contribute to fatigue and general weakness accompanied by mood changes and attention deficits (Compston and Coles, 2008; Loma and Heyman, 2011). MS affects a wide variety of people, however, the ratio of females to males with MS is approximately 3:1 with onset typically occurring between 20-40 years of age (Constantinescu et al., 2011). The 3:1 ratio of females to males is not well understood, but is thought to arise from environmental, hormonal and genetic factors. Smoking, which is linked to increased risk for MS, is one potential factor underlying this ratio because of its higher prevalence among females (Alonso and Hernan, 2008; Ascherio and Munger, 2007). The hormonal difference between females and males likely contributes to MS susceptibility. This is substantiated by evidence showing altered disease severity during pregnancy due to increased levels of progesterone, estrogen and oestriol (Harbo et al., 2013) and an increase in relapse rate following *in vitro* fertilization (Laplaud et al., 2006). The X chromosome is thought to have a role in autoimmune disorders (Selmi, 2008); consequently, the presence of two X chromosomes would likely increase susceptibility (Harbo et al., 2013). As of 2013, there were 2.3 million individuals worldwide living with MS (Evans et al., 2013). Canada represents one

of the highest prevalence rates of MS in the world at approximately 240:100,000 (Evans et al., 2013).

1.2.2 CLINICAL DISEASE COURSES OF MS

There are four main clinical subtypes of MS (Figure 1). Approximately 85% of individuals are diagnosed with a form of MS called relapse remitting MS (RRMS; Figure 1A) (Compston and Coles, 2008). In RRMS, patients experience episodes of increased disability and symptom severity, clinically referred to as relapses, resulting from inflammation and demyelination. These occurrences are sporadic and can happen several times in a year (Compston and Coles, 2008). Between relapses, symptoms and disability diminish, or remit, as the inflammatory processes subside; this allows the patient to recover, yet seldom completely (Waxman et al., 2004). Disability gradually increases throughout the course of RRMS due to the accumulation of demyelination and axonal damage (Franklin and Ffrench-Constant, 2008). Roughly 80% of individuals with RRMS will eventually develop secondary progressive MS (SPMS; Figure 1B) (Dendrou et al., 2015). Individuals with SPMS generally have a continuous and gradual increase in neurological disability without relapses or remissions (Lublin and Reingold, 1996). Approximately 10% of patients are diagnosed with primary progressive MS (PPMS; Figure 1C) which is characterized by an unrelenting deterioration of neurological function (Dendrou et al., 2015). Unlike RRMS, PPMS is thought reflect a chronic neurodegeneration condition that appears to be driven by distinct but poorly understood disease mechanisms (Baecher-Allan

et al., 2018; Loma and Heyman, 2011). The last, and least common, form of MS is progressive relapsing MS (PRMS) characterized by a steady increase in functional disability with sporadic relapses (Figure 1D). Around 5% of MS patients are diagnosed with PRMS (ECTRIMS, 2012). Like PPMS, PRMS does not respond well to current therapies (Koch et al., 2013).

1.2.3 MS THERAPEUTICS

Since the majority of people with MS have RRMS, it has served as the central focus for research and therapeutic development (Stys et al., 2012). Disease-modifying treatments for MS, such as interferon beta-1α (Jacobs et al., 1996) and glatiramer acetate (Johnson et al., 1995) are formulated for intramuscular or subcutaneous injection, respectively. Newer oral medications, like teriflunomide (O'Connor et al., 2011) and fingolimod (Cohen et al., 2010), have shown similar efficacy with higher compliance rates. However, current disease modifying therapies for MS are only successful at reducing the frequency and/or severity of disease relapses and have limited value in slowing disease progression (Cohen et al., 2010; Jacobs et al., 1996; Johnson et al., 1995; O'Connor et al., 2011). Present drugs for RRMS are most beneficial when given to an individual early in their disease course since the drugs reduce autoimmune mechanisms responsible for neurodegeneration (Loma and Heyman, 2011). However, current therapies for MS only suppress the autoimmune and inflammatory aspect of RRMS and fail to directly target the neurodegenerative process

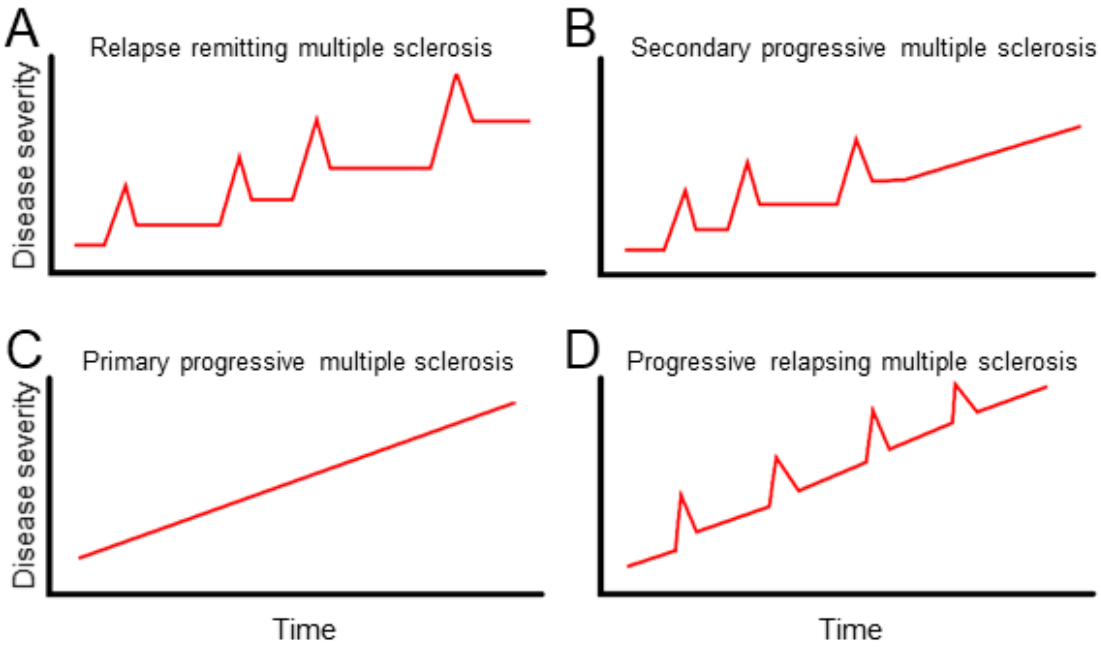

Figure 1. The four main subtypes of multiple sclerosis (MS). (A) Relapsing remitting MS (RRMS), (B) secondary progressive MS (SPMS), (C) primary progressive MS (PPMS) and (D) progressive relapsing MS (PRMS).

(Arun et al., 2013; Sormani and Bruzzi, 2015). Hence, there are no effective treatments that slow neurodegeneration and/or enhance remyelination for MS (Ransohoff, 2012).

1.3 EAE: PATHOPHYSIOLOGY AND LIMITATIONS

EAE is an animal model of MS that was first described 85 years ago (Rivers et al., 1933). Today, it remains the most extensively used animal model for MS because it recapitulates several pathophysiological processes observed in MS patients. EAE is characterized by autoimmune-mediated demyelination that results in paralysis and locomotor deficits, resembling MS (Warford et al., 2014). Moreover, preclinical studies using the EAE model have assisted in the development of four approved therapies for MS: glatiramer acetate, mitoxantrone, natalizumab, and fingolimod (Brinkmann et al., 2002; Constantinescu et al., 2011; Steinman and Zamvil, 2006). These findings support the clinical relevance of EAE for MS.

1.3.1 PATHOPHYSIOLOGY FEATURES OF EAE IN MICE

EAE has proven to be a versatile model of MS. Using different antigens for immunization and mice with distinct genetic backgrounds, it is possible to model both RRMS and SPMS (Constantinescu et al., 2011). For instance, SJL mice immunized with the proteolipid protein peptide 139-151 ($PLP_{139-151}$) produce a relapsing/remitting disease course resembling RRMS (Bittner et al., 2014). By contrast, immunization of C57BL/6 mice with myelin oligodendrocyte glycoprotein

peptide 35-55 (MOG_{35-55}) emulsified in complete Freund's adjuvant (CFA) produces a monophasic and chronic disease with limited recovery that mimics progressive MS (Bittner et al., 2014).

Since the MOG_{35-55} model of EAE was employed in the current study, the focus will be on the features of this model. Subcutaneous (sc) injections of MOG_{35-55} emulsified in CFA followed by intraperitoneal (ip) injections of pertussis toxin (PTX) trigger the production of myelin-reactive $CD4^+$ T cells in the lymph nodes and spleen that migrate to the CNS, resulting in demyelination (Bannerman et al., 2005). Macrophage activation also contributes to EAE. M1 macrophages release pro-inflammatory mediators that damage the CNS while macrophages of an anti-inflammatory M2 phenotype promote the resolution of EAE (Huitinga et al., 1990; Kotter et al., 2001). $CD4^+$ T cells and M1 macrophages have been shown to degrade myelin and trigger the death of myelin-producing oligodendrocytes (Kieseier et al., 1999; Smith, 1999). Demyelination and the release of reactive oxygen species by activated immune cells causes axonal damage resulting in permanent sensory and motor deficits (Kornek et al., 2000; Nikic et al., 2011). PTX enhances MOG_{35-55}-induced EAE disease severity. The mechanism of action of PTX is not well understood, however, it is thought to increase blood-brain barrier permeability which enables immune infiltration (Hofstetter et al., 2002). Motor deficits in EAE have been linked to axonal damage and motor neuron loss in the spinal cord and inflammation in the cerebellum (Bannerman et al., 2005). The CNS pathology in EAE causes ascending paralysis in mice

(beginning at the tail) that manifests approximately 9- to 12-days post-immunization (Constantinescu et al., 2011).

1.3.2 LIMITATIONS OF THE EAE MODEL

Although EAE models many features of MS, it is not without its limitations. The main difference between MS and EAE is that the etiology of MS remains unknown but appears to involve a complex combination of genetic and environmental factors. By contrast, specific antigens, that may not be the initial triggers of MS, are used to induce EAE in animals such as mice, rats and monkeys (Constantinescu et al., 2011). EAE is also unable to fully recapitulate the pathological features of MS because of differences in the immune system and CNS between animals and humans. EAE therefore fails to precisely mimic the immunological and neuropathological mechanisms implicated in MS (Emerson et al., 2009). For example, EAE pathology is driven primarily by infiltrating $CD4^+$ T cells whereas MS lesions are comprised chiefly of $CD8^+$ T cells (Gay et al., 1997).

1.4 KINEMATIC GAIT ANALYSIS (KGA)

1.4.1 CURRENT METHODS USED TO ASSESS EAE DISEASE SEVERITY

EAE disease severity in mice is traditionally measured using a clinical rating scale due to its simplicity and convenience (Emerson et al., 2009). This rating scale is similar to the expanded disability status scale (EDSS), which is a 10-point scale used to assess neurological disability in people with MS (Kurtzke,

1983). Higher values on the EAE rating scale and EDSS reflect increased disease severity in EAE and MS, respectively (Emerson et al., 2009; Kurtzke, 1983). Although all neurologists use the same EDSS to assess disabilities in MS, EAE clinical rating scales can vary between laboratories (Croxford et al., 2006; Kalyvas and David, 2004; Mendel et al., 1995; Takeuchi et al., 2013). This lack of standardization is problematic because clinical scores may not always reflect similar EAE disease severity between studies. A further disadvantage of EAE rating scales is that they yield only ordinal data that cannot detect subtle changes in disease progression and provide limited information about the precise nature of the deficits. Lastly, clinical scores for EAE reflect a subjective impression of the rater concerning animal health that may vary between laboratories. To improve upon these drawbacks of clinical scoring, KGA was employed in the current study.

1.4.2 KGA: ADVANTAGES IN THE ASSESSMENT OF LOCOMOTOR DEFICITS FOR EAE MICE

KGA employs sophisticated behavioural techniques to assess specific aspects of leg movements during walking for humans and laboratory animals. We have previously shown that KGA is superior to clinical scoring in EAE mice because it can detect movement deficits prior to the onset of clinical scores and identify precise joint angle impairments (Fiander et al., 2017a; Fiander et al., 2017b). This resultant interval data thus provides more precise information that can be used to inform clinical studies that use similar behavioural techniques. KGA has frequently been used to study the abnormal gait of patients with

neurodegenerative/neurological disorders (Cameron and Wagner, 2011; Motl, 2013) and the animal models for these conditions (Akay, 2014; Balkaya et al., 2013; Menalled et al., 2009; Olivan et al., 2015; Pajoohesh-Ganji et al., 2010; Taylor et al., 2010; Wirths and Bayer, 2008). KGA can be performed in the ventral plane (underneath a subject) and in the sagittal plane (to the side of a subject); each technique yields different information about gait. In the ventral plane, KGA can detect gait parameters such as stride length and paw placement of mice (Mitra et al., 2015; Silva et al., 2014), however, the way in which limbs move to achieve the paw placements cannot be viewed in the ventral plane. Sagittal plane KGA has the distinct advantage of being able to measure the precise mechanics of leg movements while mice walk on a treadmill (Akay, 2014; Fiander et al., 2017a).

Although KGA has been used to analyze locomotor deficits in mouse models of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson's Disease (PD), Huntington's Disease, Alzheimer's Disease (AD), stroke and spinal cord injury (Akay, 2014; Balkaya et al., 2013; Menalled et al., 2009; Olivan et al., 2015; Pajoohesh-Ganji et al., 2010; Taylor et al., 2010; Wirths and Bayer, 2008), sagittal plane KGA has only recently been employed in EAE studies (de Bruin et al., 2016; Fiander et al., 2017a; Fiander et al., 2017b). Incorporating gait-related measures into preclinical studies for EAE are important because ambulation is a commonly faced challenge in people with MS and locomotor deficits are frequently assessed using KGA in MS studies (Cameron

and Wagner, 2011; Coghe et al., 2015; Goodman et al., 2010; Larocca, 2011; Zorner et al., 2016).

1.5 DRUG REPOSITIONING

Drug repositioning, also known as drug repurposing, is the application of existing drugs for the treatment of new indications (Oprea and Mestres, 2012). This strategy simplifies approval for human testing and reduces the risk of clinical failure from adverse side effects. Typically, a drug is targeted for repositioning when it has an expired patent or has failed in a Phase III clinical trial due to poor efficacy, not safety-related reasons (Sleigh and Barton, 2010).

1.5.1 BENEFITS TO DRUG REPOSITIONING

This strategy of drug development has many advantages over *de novo* drug discovery. First, the developmental timeline for a drug can be substantially reduced since a wide body of relevant preclinical data is available (Ashburn and Thor, 2004). Phase I and IIa clinical trials are likely to be bypassed if the drug is repositioned at a similar or lower dose (Oprea et al., 2011). This will ultimately reduce the costs involved in drug development and provide a quicker time-to-market (Sleigh and Barton, 2010). Second, there is a reduced risk associated with drug administration and consequently, a potentially higher rate of success (Ashburn and Thor, 2004; Oprea and Mestres, 2012). This decreases the chances that a drug will fail due to poor toxicology or safety profiles in the preliminary phases of a clinical trial. Third, the economics underlying mass

production and synthesis of a drug have already been established (Oprea and Mestres, 2012). Lastly, repurposing a drug can increase the patent life of a molecule so market exclusivity for certain formulations or indications can be achieved.

1.5.2 CHALLENGES IN DRUG REPOSITIONING

Although drug repositioning can be advantageous to *de novo* drug discovery, repositioning drugs comes with a different set of limitations and challenges. New safety concerns associated with a drug may arise in a different patient population; this may require safety assessment by phase I clinical testing (Sleigh and Barton, 2010). Also, the drug candidate still has the potential to fail at effectively treating the new indication (Sleigh and Barton, 2010). Legal considerations, such as implementing strategies to adequately address intellectual property and regulation must also be taken into account (Sleigh and Barton, 2010). If a drug is not generic (still under patent protection), licensing or acquiring the intellectual property from a company to reposition a drug can be difficult (Ashburn and Thor, 2004). The process is simpler if a drug is generic (not under patent protection) since a company can create a new patent for method of use, formulation, dosing or drug combinations (Ashburn and Thor, 2004).

1.6 DRUG CANDIDATES TO REPOSITION FOR THE TREATMENT OF MS

Among the classes of FDA-approved drugs that have been shown to reduce inflammation, demyelination and clinical disease signs in EAE mice are the

atypical antipsychotics (Mei et al., 2012; O'Sullivan et al., 2014), selective estrogen receptor modulators (SERMs) (Bebo, Jr. et al., 2009) and thiazolidinediones (TZDs) (Feinstein et al., 2002; Niino et al., 2001).

1.6.1 QUETIAPINE AND CLOZAPINE

Quetiapine and clozapine are atypical antipsychotic drugs used primarily in the treatment of schizophrenia (Gugger and Cassagnol, 2008). They are typically administered orally (Citrome, 2012) and exert their beneficial functions by acting as antagonist at dopamine D_2 and serotonin 5-HT_{2A} receptors (Schotte et al., 1996). It is thought that the positive and negative symptoms of schizophrenia are alleviated by the binding of atypical antipsychotics to D_2 and 5-HT_{2A} receptors, respectively (Schotte et al., 1996). The side effects associated with quetiapine or clozapine administration include sedation and weight gain (Leucht et al., 2013). A severe side effect brought by clozapine is agranulocytosis, a condition in which white blood cell levels fall to dangerously low levels (Alvir et al., 1993).

Oral administration of the atypical antipsychotic clozapine to mice via the drinking water, beginning one day before immunization with MOG_{35-55}, has been reported to produce dose-dependant reductions in EAE disease severity (O'Sullivan et al., 2014). Clinical signs of EAE were also reduced by the oral administration of quetiapine (10 mg/kg/day, po) at the onset of clinical signs (Mei et al., 2012). Unlike quetiapine (Mei et al., 2012), clozapine did not appear to reduce clinical disease signs in the EAE model by suppressing effector T cell function or

enhancing T regulatory cell activity (Zareie et al., 2017). Alternatively, the ability of clozapine to improve myelin integrity by increasing oligodendrocyte energy production and lipid synthesis (Ferno et al., 2005; Steiner et al., 2014) may contribute to the beneficial effects of this antipsychotic in models of autoimmune- and cuprizone-induced demyelination (O'Sullivan et al., 2014; Xu et al., 2010; Xu et al., 2014; Zhang et al., 2008).

1.6.2 TAMOXIFEN

Tamoxifen, an orally administered SERM (Greco, 1998), is used in the treatment of estrogen receptor (ER) positive breast malignant carcinoma (Jordan and Brodie, 2007). Due to its modulatory properties, tamoxifen acts as an antagonist at the ER in breast tissue (Wang et al., 2004) and, for example, an agonist for the ER in bone (Nakamura et al., 2007). By blocking ER-mediated growth in breast tissue, tamoxifen assists in the prevention of ER positive breast cancer tumor growth (Jordan, 1993). Tamoxifen should only be taken for 10 years since it increases the risk of endometrial cancer (Davies et al., 2013). Other adverse symptoms associated with tamoxifen administration include feminizing effects, flushing and cognitive decline (Moore et al., 2014; Paganini-Hill and Clark, 2000).

Tamoxifen has been shown to suppress relapsing EAE in $PLP_{139-151}$ immunized SJL mice (Bebo, Jr. et al., 2009). In this study, tamoxifen was delivered subcutaneously by a slow-release implant beginning 7 days before immunization. T cell proliferation assays and cytokine measurements suggested that tamoxifen

suppressed the development of EAE by inducing a Th2 immune bias that reduced autoimmune-mediated demyelination (Bebo, Jr. et al., 2009).

1.6.3 PIOGLITAZONE

Pioglitazone is classified as a TZD, indicating it is an agonist for the nuclear peroxisome proliferator-activated receptor gamma (PPARγ) (Shah and Mudaliar, 2010). It is currently used to treat type II diabetes by increasing insulin sensitivity, mainly in skeletal tissue, to reduce blood glucose levels (Ferre, 2004; Shah and Mudaliar, 2010). This effect occurs primarily by increasing the expression of adiponectin and decreasing TNFα expression from adipocytes (Maeda et al., 2001; Walczak and Tontonoz, 2002; Yu et al., 2002). There are some adverse effects brought by pioglitazone administration, such as peripheral edema and weight gain (Shah and Mudaliar, 2010). Pioglitazone also was initially thought to increase the risk of bladder cancer; however, a recently published meta analysis found no link between pioglitazone and bladder cancer (Filipova et al., 2017). TZDs such as pioglitazone, rosiglitazone and troglitazone have been shown to reduce EAE disease severity in association with decreased inflammation and demyelination (Feinstein et al., 2002; Niino et al., 2001). Pioglitazone, however, appears to be more effective at reducing EAE disease severity than rosiglitazone and troglitazone (Feinstein et al., 2002; Niino et al., 2001).

1.7 RATIONALE AND RESEARCH OBJECTIVES

A major obstacle in the repurposing of clozapine, quetiapine, tamoxifen and pioglitazone for MS is the considerable resources required to test their clinical effectiveness. Preclinical studies are therefore required to identify the most promising of these therapeutic candidates for clinical testing. It is important in these studies that drug administration to EAE mice be delayed until peak disease to gauge their therapeutic potential at reducing demyelination, inflammation and motor deficits that are characteristic of MS.

The present work has three major research aims: 1) to determine which of four FDA-approved drugs (clozapine, quetiapine, tamoxifen or pioglitazone) is most effective at alleviating MOG_{35-55}-induced EAE; 2) to determine the mechanism of action for the most effective drug candidate and 3) to measure the beneficial effects of the drug candidate on gait deficits in EAE mice that resemble those seen in MS.

1.7.1 AIM 1

The first objective of this study was to determine if quetiapine, clozapine, tamoxifen or pioglitazone was most effective at reducing EAE disease severity in mice. Although these four drugs have previously been shown to reduce EAE disease severity when administered before peak disease (Bebo, Jr. et al., 2009; Feinstein et al., 2002; Mei et al., 2012; O'Sullivan et al., 2014), mice were treated at peak disease in the current study to identify whether these drugs could reverse

disease progression instead of prevent disease onset. To elucidate this, MOG_{35-55}-immunized EAE mice were treated with either quetiapine, clozapine, tamoxifen or pioglitazone beginning at peak clinical scores. EAE disease severity was measured behaviourally using a clinical rating scale and histologically via white matter loss quantification of lumbar spinal cords.

1.7.2 AIM 2

The second objective of this study was to investigate the effects of the most effective drug candidate on immune cell activation in the spinal cord and protection of cortical neuron cultures against exposure to a lethal concentration of glutamate. Immunohistochemistry was performed to detect $Iba1^+$ macrophages and microglia in the spinal cord that were counted by computer-assisted image analysis. Quantitative reverse transcriptase-polymerase chain reaction was employed next to measure pro- and anti-inflammatory cytokine mRNA levels in the spinal cord. Lastly, the protective effects of the most effective drug candidate on the viability of primary mouse cortical neurons exposed to a lethal concentration of glutamate was assessed.

1.7.3 AIM 3

The last aim of this study was to employ KGA (Akay, 2014) to determine if the most effective of the four FDA-approved drugs prevented or reversed impaired joint movements in EAE mice. This was done by examining whether the top clinical candidate alleviated the disruption of hip, knee and ankle joint

movements in the hindlimb of EAE mice that resemble those seen in MS patients (Fiander et al., 2017a; Fiander et al., 2017b).

CHAPTER 2: MATERIALS AND METHODS

2.1 ANIMAL CARE

The current project was done in accordance with the Canadian Council on Animal Care guidelines and was approved by the Dalhousie University Committee on Laboratory Animals. All mice were housed in the Life Sciences Research Institute Animal Care Facility on a 12-hour light/dark cycle (7:00am/7:00pm); food and water were provided *ad libitum*. Behavioural experiments were performed at the same time of day using 10-week old female C57BL/6 mice obtained from Charles River Canada (St. Constant, QC, CAN). Mice were given one week to habituate to the facility prior to experimentation.

2.2 EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

2.2.1 EAE INDUCTION

A peptide corresponding to amino acids 35-55 (MEVGWYRSPFSRWHLYRNGK; Gen Script, Piscataway, NJ, USA) of myelin oligodendrocyte glycoprotein (MOG_{35-55}) was dissolved in phosphate buffered saline (PBS; pH = 7.4) and emulsified in complete Freund's adjuvant (CFA) at a 1:1 ratio. The CFA solution (10 mg/ml) included incomplete Freund's adjuvant mixed with heat-killed *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). All mice were anesthetized using isoflurane (2%; 1.5 liters/min O_2) and kept under anesthesia during EAE induction. Two bilateral subcutaneous (sc) injections (100µl) of the MOG_{35-55}/CFA mixture (300µg/mouse) were administered near the base of the tail to mice on day 0 (EAE group). Pertussis toxin (PTX, 300 ng;

Sigma Aldrich, St. Louis, MO, USA) was administered by intraperitoneal (ip) injection in a volume of 200 µl on day 0 as well as 2 days post-immunization (DPI) to each mouse. Immunization controls underwent the same procedure, but received injections of CFA emulsified in PBS without MOG_{35-55} (CFA group).

2.2.2 BEHAVIOURAL ASSESSMENT OF CLINICAL SCORES

Starting on DPI 7, mice were weighed and assessed for disease severity each day using a clinical rating scale. The following 11-point ordinal scale was used to assess motor deficits: 0, no motor deficits; 0.5, hooked tail; 1.0, fully flaccid tail; 1.5, bilateral hindlimb splay; 2.0, minor walking deficits; 2.5, major walking deficits; 3.0, dropped pelvis; 3.5, unilateral hindlimb paralysis; 4.0, bilateral hindlimb paralysis; 4.5, forelimb paralysis; and 5.0, moribund (Table 1). Minor walking deficits can include general weakness of the lower body and slight loss of coordination; they may not be visible to an untrained experimenter. Major walking deficits are clearly visible to an untrained experimenter and can include paraparesis (partial paralysis of hindlimbs), a major sway of the lower body and difficulty righting themselves. For each experiment, one experienced individual, blind to the experimental conditions, performed the clinical scoring.

Table 1. EAE clinical scoring scale used to assess disease severity in EAE mice.

Clinical Score	Description
0.0	No motor deficits
0.5	Hooked tail
1.0	Fully flaccid tail
1.5	Bilateral hindlimb splay
2.0	Minor walking deficits (weakness in lower body or slight loss of coordination)
2.5	Major walking deficits (partial paralysis of hindlimbs and major sway of lower body)
3.0	Dropped pelvis
3.5	Unilateral hindlimb paralysis
4.0	Bilateral hindlimb paralysis
4.5	Forelimb paralysis
5.0	Moribund

2.2.3 EAE ANIMAL HUSBANDRY

All mice were given access to mashed kibble and Dietgel Recovery (ClearH2O, Westbrook, ME, USA) at disease onset. If body weight loss exceeded 10% of pre-immunization values, mice were handfed with DietGel Boost (Clear H2O, Westbrook, ME, USA) and given 0.9% sodium chloride solution (25 ml/kg/day, sc). Humane end-points were one of the following: 1) weight loss exceeded 20% of the pre-immunization values; 2) clinical score of 5; 3) loss of right reflex or 4) inability to access food or water for 24 hours. A humane end-point was reached in less than 5% of EAE mice.

2.3 DRUG DOSING FOR EAE STUDIES

Mice were orally (po) gavaged once daily with vehicle (Neobee 1053; Stepan, Northfield, IL, USA; 5 ml/kg), clozapine (Sigma Aldrich, St. Louis, MO, USA; 10 mg/kg), quetiapine (Sigma Aldrich, St. Louis, MO, USA; 10 mg/kg), tamoxifen (Cayman Chemical, Ann Arbor, MI, USA; 5 mg/kg) or pioglitazone (Santa Cruz Biotechnology, Dallas, TX, USA; 15 mg/kg) beginning at peak disease (DPI 15) and until the experimental endpoint. In a subsequent set of experiments, vehicle (Neobee 1053; 5 ml/kg/day, po) or pioglitazone (15 mg/kg/day, po) was administered to mice 24 hr after disease onset until the end of the experiment. The rationale for the dosages used for each drug is explained in Section 4.2.

2.4 HISTOLOGY

2.4.1 TISSUE PREPARATION

Mice were humanely euthanized with an overdose injection of sodium pentobarbital (150 mg/kg, ip) and perfused with PBS and 4% paraformaldehyde (PFA). Spinal cords were dissected and post-fixed in 4% PFA for 48 hours before being cryoprotected in 30% sucrose. Three segments of the spinal cord (L2, L3-4 and L5) were embedded in tissue-tek (Sakura Finetek, Torrance, CA, USA), frozen and sectioned using a cryostat (Leica, model CM1950) set to -17°C. Serial coronal sections (30 µm) were mounted on Superfrost glass slides (Fisher Scientific, Nepean, ON, CAN).

2.4.2 ERIOCHROME CYANINE AND NEUTRAL RED STAIN

All sections underwent serial rehydration by being passed through xylenes and then through descending concentrations of ethanol (100% to 75%) before being incubated in a water bath for 1 min at room temperature. The tissue was stained with eriochrome cyanine (Sigma Aldrich, St. Louis, MO, USA) for 15 minutes, differentiated with ammonium hydroxide (5-7 dips), and counter stained with neutral red (Acros Organics, New Jersey, NJ, USA) for 2 minutes. The tissue was dehydrated in a series of ethanol concentrations (75% to 100%) and cleared with xylene.

2.4.3 WHITE MATTER LOSS ANALYSIS

Spinal cord sections were imaged using an Axioplan II with an Axiocam HRC Colour Camera and analyzed using ImageJ. An area of white matter loss was defined as a region without eriochrome cyanine staining. White matter loss was traced by a trained experimenter, unaware of the treatment conditions. The area of white matter loss was measured for two sections within L2, L3-4 and L5, yielding measurements for a total of six spinal cord sections per mouse. Percent white matter loss was calculated by dividing the white matter lesion area by total white matter and multiplying by 100. Mean percent white matter loss across L2, L3-4, and L5 was used for data analysis.

2.5 IBA1 IMMUNOHISTOCHEMISTRY AND IMAGE ANALYSIS

Spinal cord sections on Superfrost glass slides were washed 3 times with PBS containing 1% triton X100 (PBS-TX) for 10 min and incubated in 20% normal goat serum diluted in PBS-TX for 60 min. Next, sections were incubated in a polyclonal rabbit anti-Iba1 antibody (Wako, Dallas, TX, USA; 1:1000 dilution) for 22 hrs at 4ºC. After the primary antibody incubation, the slides were washed 3 times with PBS and incubated in a goat anti-rabbit 488 secondary antibody (Invitrogen, Carlsbad, CA, USA; 1:250) for 2 hrs at room temperature. The sections were then washed 3 times in PBS and cover-slipped using Fluoromount (Sigma Aldrich, St. Louis, MO, USA). For all immunohistochemistry, a control slide underwent the same procedure without receiving the primary antibody. For

each mouse, the white matter on an L3-4 spinal cord section was traced, and the mean gray value was calculated using ImageJ.

2.6 RNA EXTRACTION AND QUALITY CONTROL

Mice were humanely euthanized with an overdose injection of sodium pentobarbital (150 mg/kg, ip) and perfused with PBS. The spinal cord was dissected, homogenized in pureZOL RNA isolation reagent (Bio-Rad, Hercules, CA, USA) using a bead homogenizer (Benchmark Scientific, Sayreville, NJ, USA) and stored at -80ºC. Total RNA was extracted using the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturers protocol. An Experion bioanalyzer with an RNA StdSns analysis kit (Bio-Rad, Hercules, CA, USA) was used to assess the RNA quality and purity. Only RNA values of 7.5 or above were used for further processing to ensure degraded RNA was not analyzed. RNA was then quantified using the SPECTROstar Nano (Mandel, Guelph, ON, CAN) in duplicates.

2.7 QUANTITATIVE RT-PCR (qRT-PCR)

The iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) was used to generate cDNAs using 1000 ng of template RNA from each sample following manufacturers protocol. Each qRT-PCR experiment was done using a Bio-Rad CFX 96 real time system C1000 Touch thermal cycler in accordance with the MIQE guidelines (Bustin et al., 2009). For each experiment, the SsoFast EvaGreen Supermix kit (Bio-Rad, Hercules, CA, USA) was used with GAPDH

and HPRT1 as the reference genes. Triplicate measurements were performed for each sample. The qRT-PCR program used was: 95ºC x 30s + (95ºC x 5s + 58ºC x 5s + plate reading) x 40 cycles. Analysis was completed by the Bio-Rad CFX Manager 3.1 software using the △△Cq method. The primer sequences used for qRT-PCR are displayed in Table 2.

2.8 PRIMARY CORTICAL NEURON CULTURES

Embryonic brains were aseptically removed from a pregnant mouse at E16 and put into Neurobasal medium (Thermo Fisher, Waltham, MA, USA) with 10% fetal bovine serum (FBS) on ice. The cortices were extracted from each embryonic brain and placed in a petri dish with Hanks Balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA). All cortices were then pooled and mixed with 2 ml of Stem-Pro Accutase cell dissociation reagent to detach cells. After, the cortices were triturated 3 - 4 times and incubated at 37ºC for 15 min; this process was completed twice. FBS (1 ml) was added to the cortices to stop protease activity, and then the cortices were transferred to a 15 ml conical with 3 ml of neurobasal mix (500 ml Neurobasal + B27 + 500 mM glutamine + 20 mg/ml gentamicin) at 4ºC. This cortex/neurobasal solution was triturated 3 more times and allowed to settle so the supernatant could be collected and filtered using a 40 µm filter. The neurons were centrifuged at 300 g for 7 min and re-suspended in neurobasal mix, counted and plated for experiments. To maintain cellular viability, 60% of the total media was changed four days after seeding the cells, and every three days afterwards.

Table 2. Forward and reverse primer sequences of target genes in qRT-PCR.

Gene target	Forward primer sequence	Reverse primer sequence
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA
HPRT	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
IFNγ	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IL1β	GAAATGCCACCTTTTGACAGTG	CTGGATGCTCTCATCAGGACA
IL6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
TNFα	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
TGFβ	AGCTGGTGAAACGGAAGCG	GCGAGCCTTAGTTTGGACAGG
IL4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG

2.9 MTT [3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE] CELL VIABILITY ASSAY

After 10 days *in vitro*, pioglitazone (0.1μM) was added to primary cortical neurons for 24 hrs (37ºC, 5% CO_2). Following pioglitazone treatment, cells were incubated with 50 μM glutamate for 30 mins (37ºC, 5% CO_2). Glutamate exposure was terminated by replacing the media with new neurobasal medium, and cellular viability was measured 24 hrs later. An MTT (Sigma Aldrich, St. Louis, MO, USA) assay was used to assess viability of primary cortical neuron cultures in 48-well plates seeded at a density of 150,000 neurons/well. MTT (55 ml; 5 mg/ml, dissolved in PBS) was added to each well and incubated (37ºC, 5% CO_2) for 1 hr. Following incubation, the supernatant was removed and 400 ml of dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO, USA) was added to each well to dissolve the formazan crystals. The absorbance of each well was then measured at 592 nm with a microplate reader (ELx 800; BioTek, Winooski, VT, USA). The net absorbance of control wells not treated with glutamate was defined as 100% cell viability.

2.10 TREADMILL GAIT RECORDINGS

Movements of the right hindleg were recorded during walking on a treadmill as previously described (Fiander et al., 2017a; Fiander et al., 2017b). A baseline recording was done prior to EAE inoculation to establish typical walking in C57BL/6 mice. Subsequent recordings were performed starting DPI 9, and weekly thereafter.

2.10.1 PREPARATION OF MICE FOR TREADMILL RECORDINGS

Reflective markers were placed on the right hindlimb to follow the movement of the leg during locomotion. First, mice were anesthetized using isoflurane (2%, 1.5 litre/min O_2) to facilitate shaving and preparation of the right hindlimb. Next, five reflective markers (2 mm diameter) were placed on the shaven right hindlimb of a mouse on the following locations: the iliac crest, the femoral head, the ankle joint, the metatarsophalangeal joint, and the fourth digit (Figure 2A). Reflective markers were not placed on the knee joint since this method of locating the knee is inaccurate due to skin slippage. Instead, the lengths of the femur and tibia were measured to triangulate the position of the knee with motion analysis software.

2.10.2 TREADMILL RECORDINGS

Mice were transported to a designated room for treadmill recordings and given time to recover from anesthesia before walking on the treadmill. Once awake, each mouse walked on a treadmill at a specified speed while a high-speed camera (250 frames/sec; Fastec IL3-100, San Diego, CA, USA) recorded gait in the sagittal plane. The resultant video recording for one mouse would include 8 – 12 step cycles of steady walking. The treadmill was sanitized with peroxigard (Virox, Oakville, ON, CAN) between each recording session to reduce any anxiety-driven behaviour from olfactory cues during locomotion for following mice.

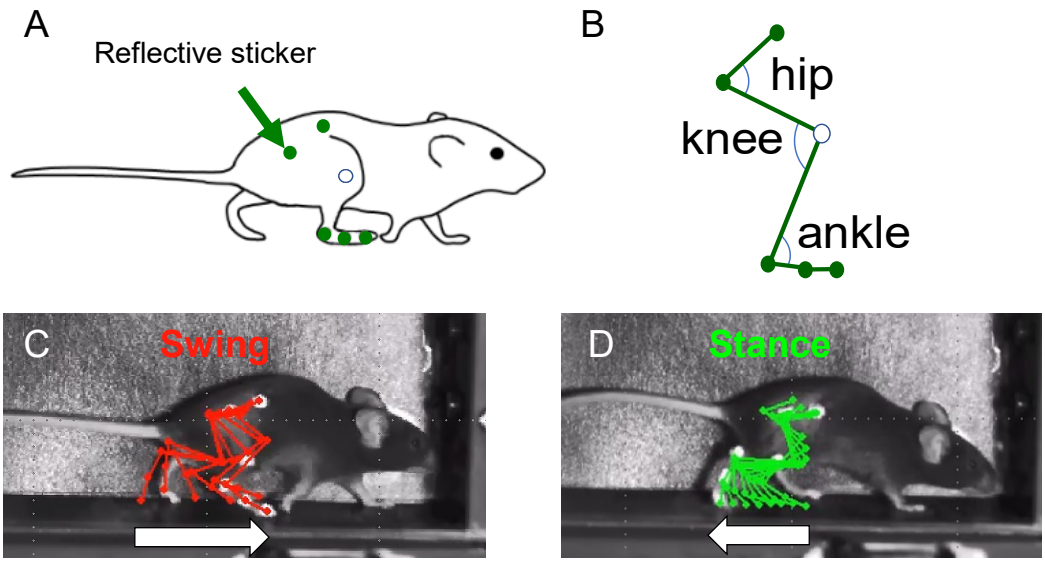

Figure 2. Illustration of kinematic treadmill recording for mice. (A) Reflective markers are attached to five regions on each mouse: the iliac crest, hip joint, ankle joint, metatarsophalangeal joint and tip of the fourth digit. X and Y coordinates of each marker are determined for each frame throughout a video recording. The knee joint is located by triangulation using the lengths of the femur and tibia. (B) The hip, knee and ankle joint angles are extracted from a constructed stick model made from the X and Y coordinates. Video frames showing stick representations for movements of the right hindlimb during swing (C) and stance (D) phases of a step cycle for a mouse walking on a treadmill. Adapted from (Chedrawe et al., 2018).

2.10.3 QUALITY OF RECORDINGS

Treadmill recordings for mice that walked steadily were used for data analysis to ensure representative walking was assessed. Five certain behaviours were avoided in treadmill recordings since they have been shown to skew data: 1) attempting to jump out of the treadmill; 2) swaying from side-to-side; 3) walking too close to the back of the treadmill belt; 4) rearing on hindlimbs and 5) sprinting to the front and lagging to the back of the treadmill. All mice ran at a maximum of 20 cm/sec on the treadmill. In some instances, EAE mice would not be able to run at 20 cm/sec due to motor disability from the EAE disease. When this occurred, the treadmill speed was lowered until steady walking was achieved. Certain kinematic parameters such as walking speed, cadence (step cycles/min), and stride length require the same treadmill speed between recordings for accurate comparisons. The kinematic parameters used in this study are extracted from the sagittal plane and are not highly sensitive to the speed at which a mouse is walking (Baker et al., 2009; Batka et al., 2014). Therefore, different treadmill speeds between mouse recordings did not negatively impact comparisons of kinematic parameters.

2.11 KINEMATIC GAIT ANALYSIS

2.11.1 DIGITIZING MARKER MOVEMENTS

Video recordings of mice walking on a treadmill were analyzed using custom scripts created for ImageJ and R. The threshold intensity of each video was adjusted, using ImageJ, such that only the 5 reflective markers placed on the

mouse would be detected throughout the recording. Next, the X and Y coordinates of each reflective marker were determined, in pixels, for every frame throughout each video recording.

2.11.2 EXTRACTION OF KINEMATIC DATA

A custom script made for R was used to extract kinematic data from the X and Y coordinates of each marker determined using ImageJ thresholding. First, the pixel measurements were transformed into centimeters (cm) to account for variability between recordings on different days. This was accomplished by recording a short video (2 sec) of a block with known dimensions on the treadmill before each recording session. These short videos were used by R to calculate a conversion coefficient so pixels could be transformed into cm. Next, the location the knee joint was determined, via triangulation, using measurements of the tibia and femur for each mouse. Lastly, the angular excursions of the hip, knee and ankle joints were calculated for each frame of a video, and then extracted (Figure 2B).

2.11.3 DETECTION OF SWING AND STANCE PHASE

The swing (Figure 2C) and stance phases (Figure 2D) of the step cycle were detected, using R, throughout each video recording. A step cycle consists of a swing and stance phase. The swing phase occurs when the foot is moving forward and, in a healthy mouse, lifted off the ground. This phase was detected by R when there was positive movement of the foot in the X coordinate. The

stance phase happens after the swing phase when the foot is in contact with the ground and is moving backwards to propel the mouse. This phase was detected when there was negative movement of the foot in the X coordinate. In the final step, the duration of the swing and stance phases in a recording were determined by calculating the number of frames devoted to each phase.

2.11.4 NORMALIZING SWING AND STANCE PHASE

Custom R scripts were used to normalize the duration of the swing and stance phases. Each of the swing and stance phases within a video recording were normalized to 100 frames, resulting in normalized step cycles consisting of 200 frames. The step cycles were normalized to 200 frames to account for the different walking speeds of mice and were used for all further data analysis.

2.11.5 AVERAGING OF STEP CYCLE WAVEFORMS

All normalized step cycles for a video recording were averaged into one normalized step cycle. An averaged normalized step cycle is therefore comprised of 8 – 12 step cycles and divides the swing and stance phases evenly (100 frames devoted to each phase). This was done by averaging the coordinate values at the same frame between step cycles. Coordinates within the averaged normalized step cycles of the hip, knee and ankle joints were used to calculate range of motion (ROM) and root mean squared (RMS) difference. ROM is defined as the largest joint angle achieved (during joint extension) subtracted by the smallest joint angle achieved (during joint flexion) throughout an average

normalized step cycle. RMS difference is a commonly used measure in human KGA studies of MS (Baker et al., 2009). It is a metric used to assess the deviation of joint movements (in EAE mice) from a baseline recording (performed in healthy mice). Extracting these kinematic parameters from an averaged normalized step cycle is optimal because they account for outliers and best represent how a mouse walked during a recording session.

2.12 STATISTICAL ANALYSES

EAE clinical score curves with 3 groups were analyzed using area under the curve followed by a one-way ANOVA with Tukey's multiple comparisons tests (Fleming et al., 2005). A Kruskal Wallis test with Dunn's multiple comparisons was used to analyze non-Gaussian data sets with three or more groups. A one-way ANOVA test with Tukey's multiple comparisons tests was used to analyze data sets with three or more groups. Multiple t-tests with Bonferroni corrections were used to analyze the qRT-PCR data. The area under the curve followed by a student's t-test was used to analyze EAE clinical score curves involving 2 groups (Fleming et al., 2005), and the Mann-Whitney U test was used to analyze white matter loss between 2 groups. The kinematic parameters were analyzed using a two-way repeated measures ANOVA in which the repeated factor is time (DPI) and the second factor is treatment (vehicle or pioglitazone). Custom scripts made for ImageJ and R were used to analyze and process the kinematic treadmill data. All statistical tests were performed using GraphPad Prism (Version 7.00; GraphPad Software, San Diego, CA) with an alpha level set to 0.05.

CHAPTER 3: RESULTS

3.1 ASSESSMENT OF THE EFFECTS OF QUETIAPINE, CLOZAPINE, TAMOXIFEN AND PIOGLITAZONE ON EAE DISEASE SEVERITY AND WHITE MATTER LOSS

Four FDA-approved drugs – quetiapine, clozapine, tamoxifen and pioglitazone – were tested to see which was most effective in a mouse model of MS. To determine this, 10-week-old female C57Bl/6 mice were subjected to MOG_{35-55}-induced EAE. Starting on DPI 7, and daily afterwards, mice were weighed and given a score based on their EAE disease severity. Mice were treated orally (po) with either quetiapine, clozapine, tamoxifen, or pioglitazone beginning DPI 15. At the end of the experiment, mice were humanely euthanized, and the lumbar spinal cords were dissected to determine the percentage of white matter loss.

3.1.1 ORAL ADMINISTRATION OF QUETIAPINE OR CLOZAPINE, BEGINNING AT PEAK DISEASE, FAIL TO REDUCE EAE DISEASE SEVERITY

Clinical scores were assigned to each mouse to measure EAE disease severity. EAE mice that received either vehicle (5 ml/kg/day, po) or quetiapine (10 mg/kg/day, po) or clozapine (10 mg/kg/day, po), beginning at peak disease (DPI 15), displayed similar clinical scores from DPI 16 - 34 (Figure 3A). The number of days each mouse had a clinical score of ≥ 2.0 was determined to measure the frequency of paraparesis in these groups. Relative to EAE mice that received vehicle, neither quetiapine nor clozapine reduced the number of days each mouse had a clinical score of 2.0 or greater (Figure 3B).

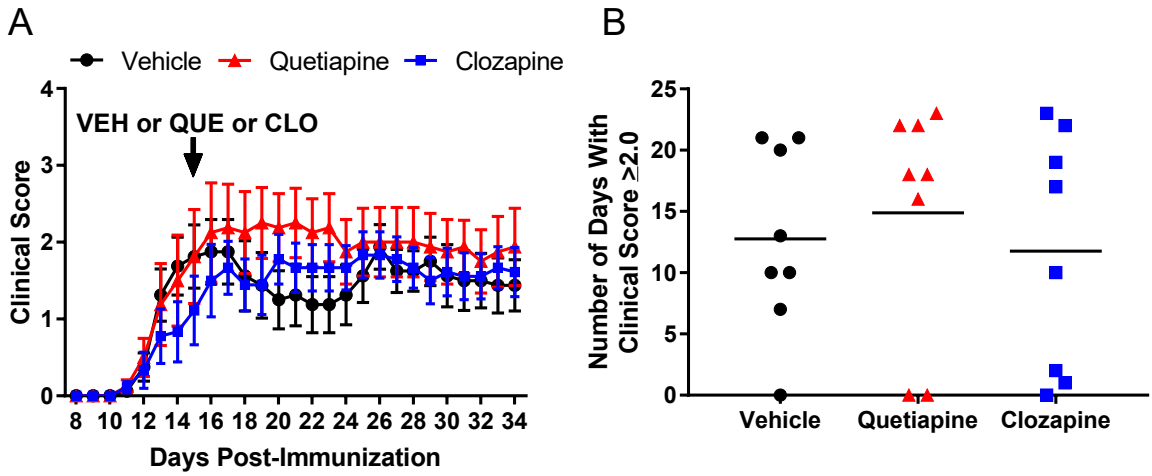

Figure 3. Effects of oral administration of quetiapine (QUE; 10 mg/kg/day, po; n = 8) or clozapine (CLO; 10 mg/kg/day, po; n = 8) beginning at peak disease (DPI 15) on clinical scores relative to vehicle (VEH; 5 ml/kg/day, po; n = 8). (A) No differences in clinical scores were found between vehicle-, quetiapine- and clozapine-treated mice from DPI 16 – 34 ($p > 0.05$). (B) The number of days each mouse spent at a clinical score of 2.0 or greater was similar between all groups ($p > 0.05$). Data are expressed as the mean ± SEM and were analyzed using the area under the curve followed by a one-way ANOVA test with Tukey's post-hoc test (A) and a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons (B). Adapted from (Chedrawe et al., 2018).

3.1.2 CLOZAPINE, BUT NOT QUETIAPINE, PRODUCES A DECREASE IN WHITE MATTER LOSS IN THE LUMBAR SPINAL CORD FROM EAE MICE

To determine whether quetiapine or clozapine reduced white matter loss, spinal cord sections were stained with eriochrome cyanine and neutral red. Relative to EAE mice treated with vehicle (5 ml/kg/day, po), quetiapine (10 mg/kg/day, po) failed to reduce white matter loss in the lumbar spinal cord (Figure 4B and D). Although clozapine (10 mg/kg/day, po) did not attenuate EAE clinical severity, this antipsychotic drug produced a decrease of spinal cord white matter loss (Figure 4C-D).

3.1.3 ORAL ADMINISTRATION OF PIOGLITAZONE, BUT NOT TAMOXIFEN, BEGINNING AT PEAK DISEASE, REDUCES EAE SEVERITY

Vehicle (5 ml/kg/day, po), tamoxifen (5 mg/kg/day, po) or pioglitazone (15 mg/kg/day, po) were administered to EAE mice beginning at peak disease (DPI 15) until the end of the experiment (DPI 27). Unlike the previous experiment, it was necessary to terminate this study at DPI 27 for ethical reasons because of marked EAE severity for the vehicle and tamoxifen groups. Relative to EAE mice that received vehicle, tamoxifen did not lower clinical scores (Figure 5A). Tamoxifen also failed to decrease the number of days EAE mice had a clinical score of 2.0 or greater when compared to vehicle-treated mice (Figure 5B). By contrast, pioglitazone reduced clinical scores and the number of days EAE mice had a clinical score of 2.0 or greater (Figure 5A and B).

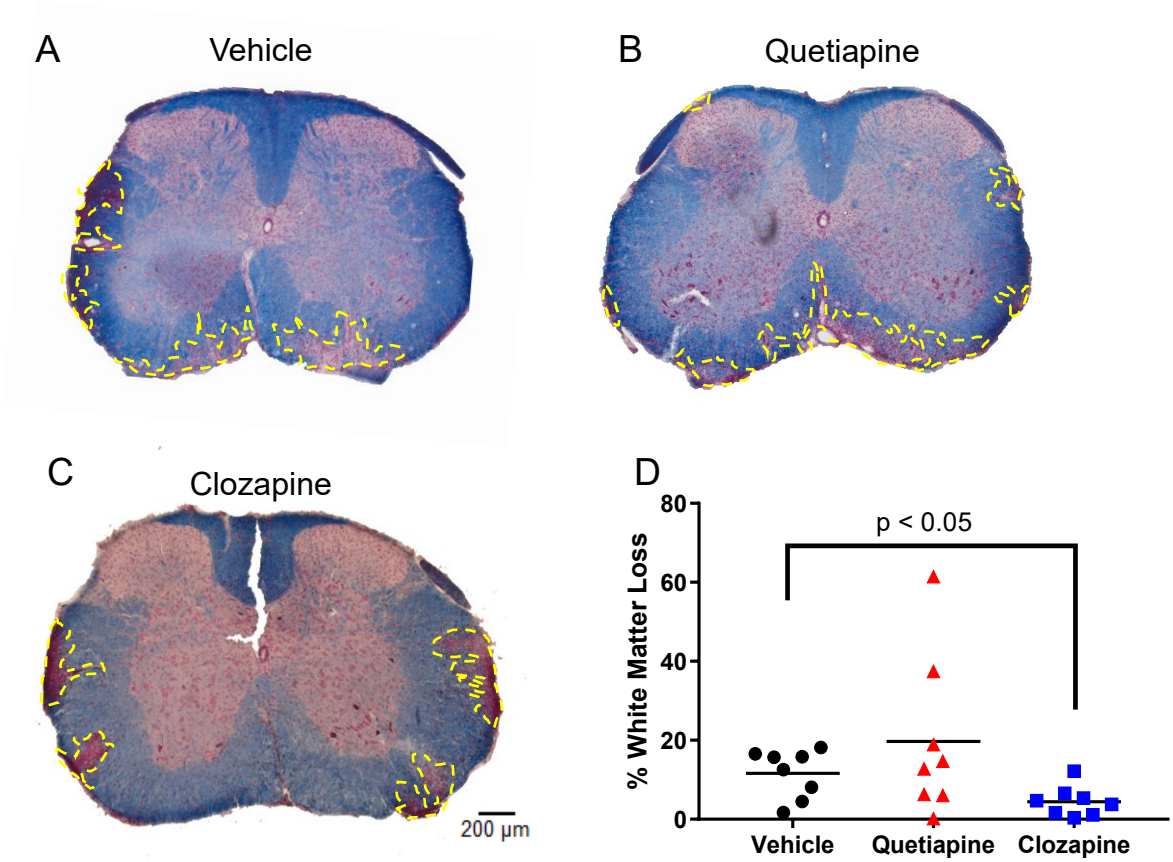

Figure 4. Effects of oral administration of quetiapine (10 mg/kg/day, po; n = 8) or clozapine (10 mg/kg/day, po; n = 8) beginning at peak disease (DPI 15) on spinal cord white matter loss relative to vehicle (5 ml/kg/day, po; n = 8). (A-C) Representative photomicrographs of the lumbar spinal cord of vehicle-, quetiapine- and clozapine-treated EAE mice (scale bar represents 200 µm). The percentage of white matter loss was comparable between vehicle- and quetiapine-treated EAE mice ($p > 0.05$). Clozapine produced a reduction of white matter loss in EAE mice ($p < 0.05$). The yellow outline depicts regions of white matter loss in the spinal cord. (D) Data were analyzed using the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons. Adapted from (Chedrawe et al., 2018).

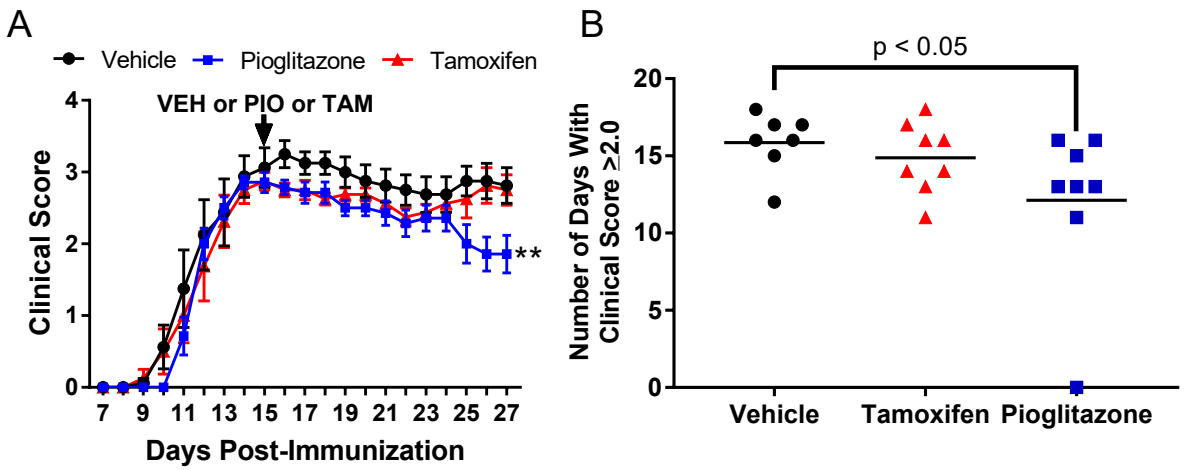

Figure 5. Effects of oral administration of tamoxifen (TAM; 5 mg/kg/day, po; n = 8) or pioglitazone (PIO; 15 mg/kg/day, po; n = 8), beginning at peak disease (DPI 15), relative to vehicle (VEH; 5 mg/kg/day, po; n = 7) on clinical scores. (A) Pioglitazone reduced clinical scores relative to EAE mice treated with vehicle from DPI 16 – 27 (**p < 0.01). (B) Pioglitazone treated EAE mice spent fewer days at a clinical score of 2.0 or greater than animals treated with vehicle (p < 0.05). (A-B) Data are expressed as the mean ± SEM and were analyzed using the area under the curve followed by a one-way ANOVA test with Tukey's post-hoc test (A) and a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons (B). Adapted from (Chedrawe et al., 2018).

3.1.4 PIOGLITAZONE, BUT NOT TAMOXIFEN, REDUCES LUMBAR SPINAL CORD WHITE MATTER LOSS IN EAE MICE

Tamoxifen (5 mg/kg/day, po) did not reduce white matter loss in the spinal cords of EAE mice, which is consistent with its lack of benefit against EAE disease severity (Figure 6B and D). By contrast, reduced EAE disease severity following administration of pioglitazone (15 mg/kg/day, po) was associated with a decrease in lumbar white matter loss (Figure 6C and D).

3.2 CHARACTERIZATION OF THE IMMUNE MODULATORY AND NEUROPROTECTIVE EFFECTS OF PIOGLITAZONE

To further investigate the mechanism of pioglitazone, histological and biochemical tests were performed on EAE mice treated with vehicle and pioglitazone. $Iba1^{+}$ cell density in the white matter of the lumbar spinal cord in EAE mice was determined to assess macrophage and microglial activation. A separate set of experiments were performed to assess gene expression of pro- and anti-inflammatory cytokines within the spinal cord of EAE mice treated with vehicle or pioglitazone. In this experiment, two additional groups of MOG_{35-55} immunization controls (CFA mice) were treated in the same manner with either vehicle or pioglitazone. The inclusion of CFA control groups permitted the study of the relative mRNA expression attributed directly to EAE pathogenesis in addition to any non-specific effects of vehicle or pioglitazone. Lastly, an *in vitro* viability assay was performed in primary cortical neurons to assess the effects of pioglitazone against glutamate-induced cell death.

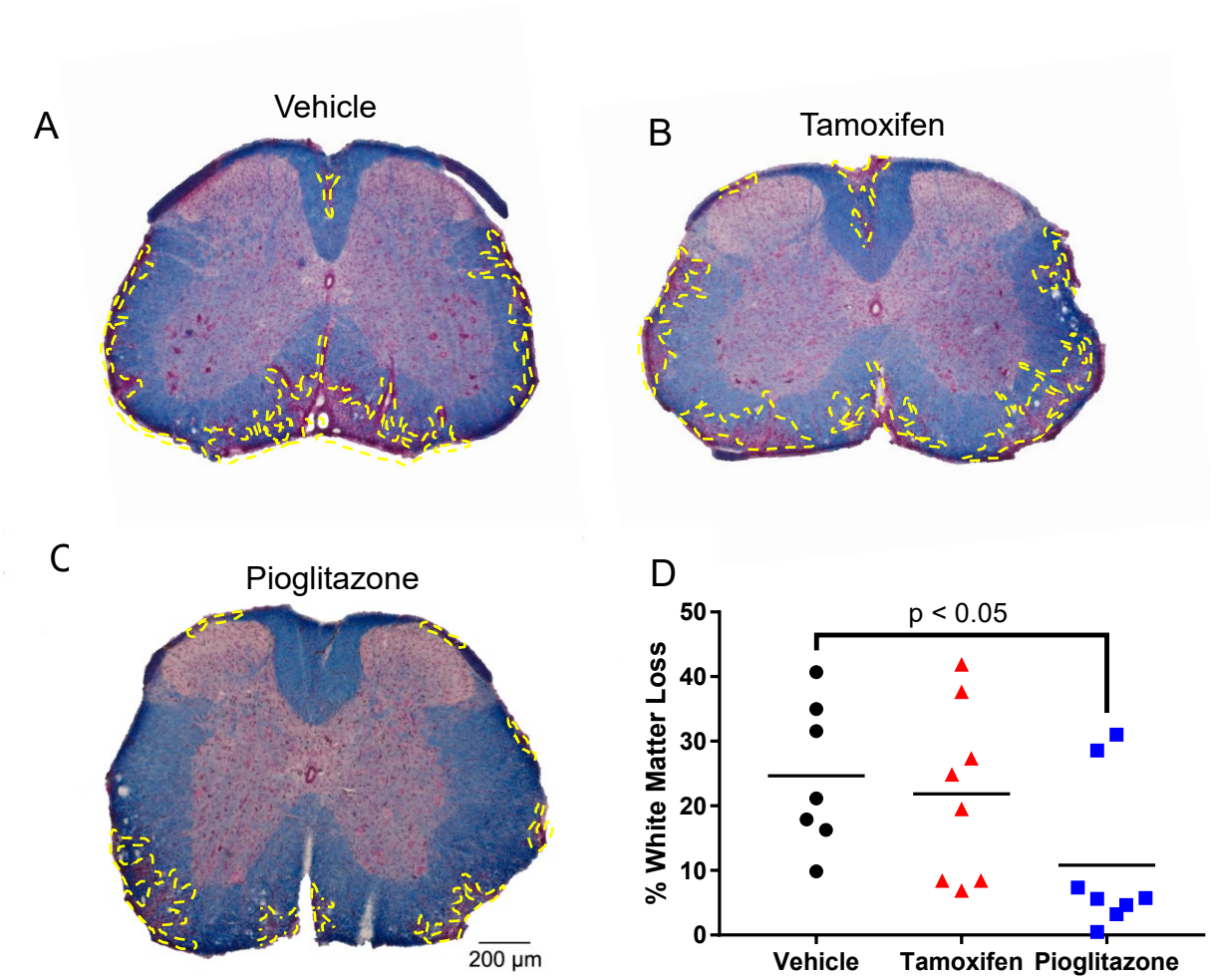

Figure 6. Effects of oral administration of tamoxifen (5 mg/kg/day, po; n = 8) or pioglitazone (15 mg/kg/day, po; n = 8), beginning at peak disease (DPI 15), relative to vehicle (5 mg/kg/day, po; n = 7) on spinal cord white matter loss. (A-C) Representative photomicrographs of the lumbar spinal cord from vehicle-, tamoxifen- and pioglitazone-treated EAE mice stained with eriochrome cyanine and neutral red (scale bar represents 200 µm). The yellow outline depicts regions of white matter loss in the spinal cord. (D) Spinal cord white matter loss was substantially reduced by pioglitazone ($p < 0.05$) but not tamoxifen ($p > 0.05$). Data were analyzed using the Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons (D). Adapted from (Chedrawe et al., 2018).

3.2.1 PIOGLITAZONE REDUCES THE DENSITY OF IBA1⁺ MACROPHAGES AND MICROGLIA WITHIN WHITE MATTER OF THE LUMBAR SPINAL CORD IN EAE MICE

Sections cut at the level of the lumbar spinal cord were processed for the immunohistochemical detection of Iba1 to quantify macrophage and microglia activation in EAE mice treated with either vehicle (5 ml/kg/day, po) or tamoxifen (5 mg/kg/day, po) or pioglitazone (15 mg/kg/day, po; Figure 7A-C) beginning at peak disease (DPI 15) until DPI 27. Mice treated with either vehicle or tamoxifen appeared to display similar levels of Iba1 immunoreactivity in the lumbar spinal cord (Figure 7A-B). By comparison, pioglitazone reduced Iba1 immunoreactivity (Figure 7C). These qualitative observations were confirmed by the quantification of Iba1 immunoreactivity within the spinal cord white matter (Figure 7D). Relative to EAE mice that received vehicle, pioglitazone reduced the number of Iba1⁺ cells, or Iba1 expression, in the spinal cord white matter of EAE mice (Figure 7D).

3.2.2 ORAL ADMINISTRATION OF PIOGLITAZONE, BEGINNING AFTER DISEASE ONSET, REDUCES mRNA LEVELS FOR PRO-INFLAMMATORY GENES IN THE SPINAL CORDS OF EAE MICE

To assess neuroinflammation, mRNA levels of pro-inflammatory cytokines were measured at peak disease (DPI 16) in EAE mice treated with vehicle (5 ml/kg/day, po) or pioglitazone (15 mg/kg/day, po), beginning 24 hr after disease onset (DPI 12).

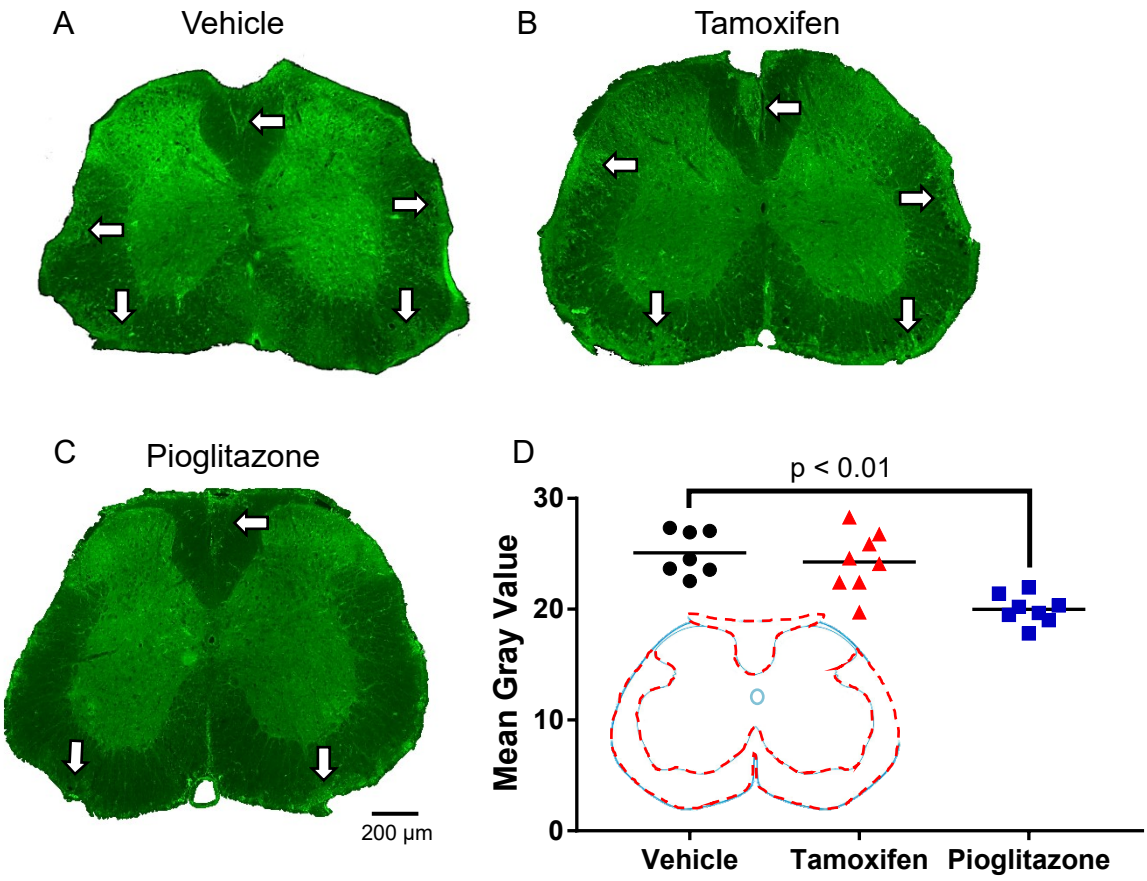

Figure 7. Representative photomicrographs and quantification of Iba1 immunoreactivity in the lumbar spinal cord white matter of EAE mice at DPI 27 treated with either vehicle (5 ml/kg/day, po; n = 7) or tamoxifen (5 mg/kg/day, po; n = 8) or pioglitazone (15 mg/kg/day, po; n = 8) from DPI 15 - 27. (A) Areas of increased Iba1 staining (arrows) are visible in the white matter of EAE mice that received vehicle. (B) Similar amounts of Iba1 immunoreactivity (arrows) appear visible in the white matter of EAE mice treated with vehicle or tamoxifen. (C) Iba1 immunoreactivity appears to be reduced in the white matter of an EAE mouse treated with pioglitazone. (D) Quantification of Iba1 staining in EAE mice that received either vehicle or tamoxifen or pioglitazone. Relative to EAE mice that received vehicle, Iba1 immunoreactivity was reduced by pioglitazone ($p < 0.01$) but not tamoxifen ($p > 0.05$). The regions quantified in the spinal cord are depicted by red outlined areas for the insert in panel D. The scale bar (C) represents 200 µm. Data are analyzed using a Kruskal-Wallis ANOVA followed by Dunn's multiple comparisons (D). Adapted from (Chedrawe et al., 2018).

Relative to EAE mice treated with vehicle (5 ml/kg/day, po), pioglitazone (15 mg/kg/day, po) reduced clinical scores from DPI 13 - 16 (Figure 8A). Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) revealed that mRNA levels of IFNγ, IL1β, IL6 and TNFα were markedly enhanced in EAE mice, relative to CFA mice, at DPI 16 (Figure 8B-E). By contrast, TGFβ mRNA levels were decreased in EAE, compared to CFA, mice (Figure 8F). In CFA mice, pioglitazone produced reductions of IFNγ, IL1β, IL6, TNFα and TGFβ mRNA levels (Figure 8B-F). Pioglitazone also reduced IFNγ, IL1β, IL6, TNFα, and TGFβ mRNA levels in EAE mice (Figure 8B-F).

3.2.2 ORAL ADMINISTRATION OF PIOGLITAZONE, BEGINNING AFTER DISEASE ONSET, DOES NOT ALTER mRNA LEVELS FOR ANTI-INFLAMMATORY GENES IN THE SPINAL CORDS OF EAE MICE

We also compared the effects of vehicle (5 ml/kg/day, po) or pioglitazone (15 mg/kg/day, po) administration, beginning 24 hr after disease onset (DPI 12), on mRNA levels of genes encoding anti-inflammatory cytokines in the spinal cords of EAE mice at peak disease (DPI 16).

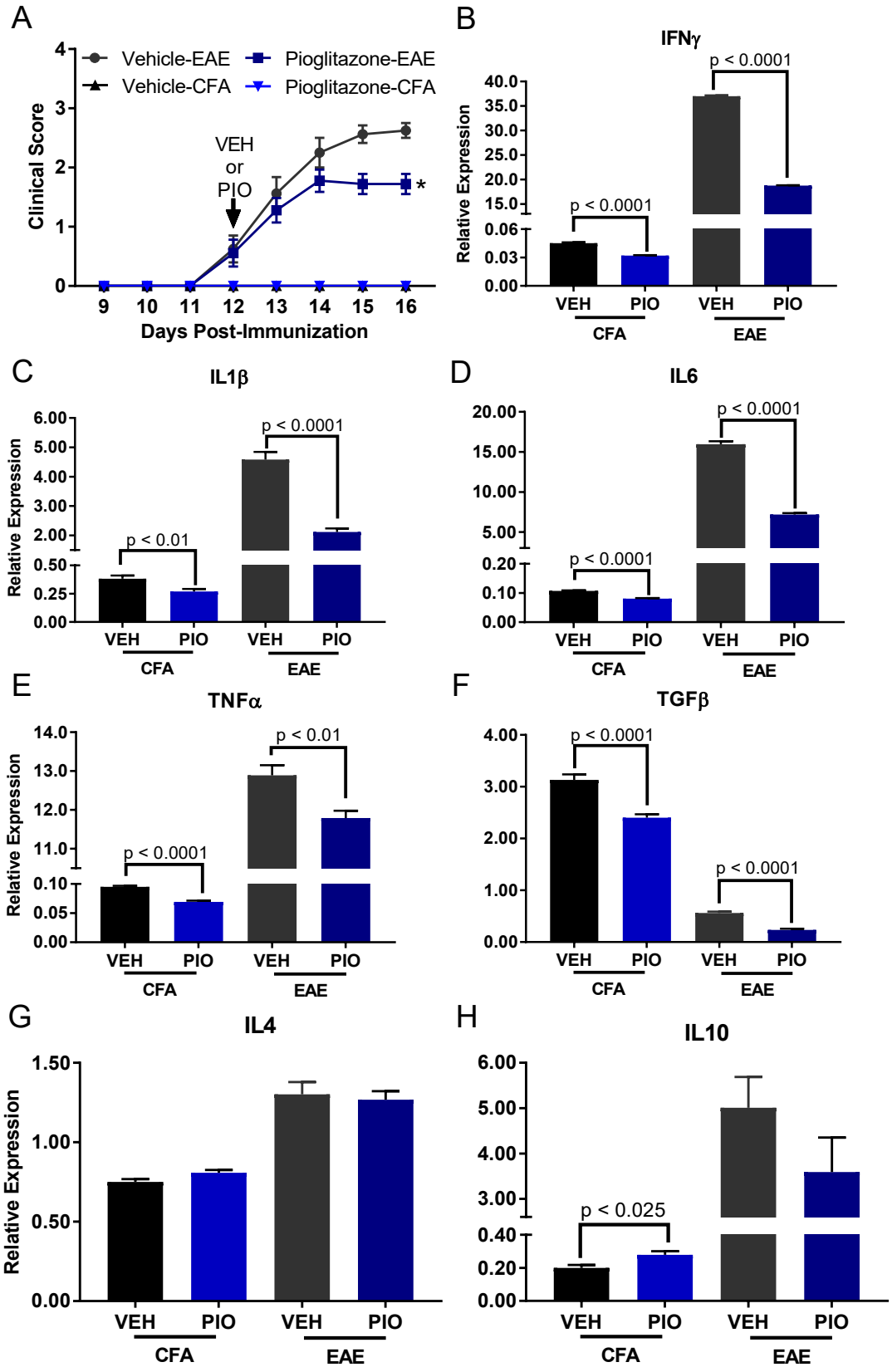
A
Vehicle-EAE
Pioglitazone-EAE
Vehicle-CFA
Pioglitazone-CFA
Clinical Score
VEH
or
PIO
*
Days Post-Immunization
B
IFNγ
p < 0.0001
p < 0.0001
Relative Expression
VEH
PIO
VEH
PIO
CFA
EAE
C
IL1β
p < 0.0001
p < 0.01
Relative Expression
D
IL6
p < 0.0001
p < 0.0001
E
TNFα
p < 0.01
p < 0.0001
F
TGFβ
p < 0.0001
p < 0.0001
G
IL4
H
IL10
p < 0.025

Figure 8. Pioglitazone (PIO; 15 mg/kg/day, po; n = 8) reduces the expression of pro-inflammatory cytokines in the spinal cord of EAE mice at peak disease (DPI 16). (A) Pioglitazone reduced clinical scores relative to vehicle-treated EAE mice (*$p < 0.05$; VEH; 5 ml/kg/day, po; n = 8). (B-H) The mRNA levels for all genes, except TGFβ, were elevated in EAE, compared to CFA, mice. (B-H) Relative to vehicle-treated CFA mice, pioglitazone reduced mRNA levels of IFNγ ($p < 0.0001$), IL1β ($p < 0.01$), IL6 ($p < 0.0001$), TNFα ($p < 0.0001$) and TGFβ ($p < 0.0001$) and elevated mRNA levels of IL10 ($p < 0.025$) in CFA mice. (B-F) Pioglitazone also reduced mRNA levels of IFNγ ($p < 0.0001$), IL1β ($p < 0.0001$), IL6 ($p < 0.0001$), TNFα ($p < 0.01$) and TGFβ ($p < 0.0001$) compared to vehicle-treated EAE mice. Similar mRNA levels of IL4 and IL10 were detected in vehicle- and pioglitazone-treated EAE mice ($p > 0.05$; G and H). Data are expressed as the mean ± SEM. The area under the curve followed by a student's t-test (A) and multiple t-tests with Bonferroni corrections (B-H) were used to analyze these data. Adapted from (Chedrawe et al., 2018).

Transcripts of the anti-inflammatory cytokines IL4 and IL10 were elevated in EAE, relative to CFA, mice at DPI 16 (Figure 8G-H). In CFA mice, pioglitazone (15 mg/kg/day, po) produced an elevation of IL10 mRNA levels (Figure 8H). Pioglitazone, however, did not alter mRNA levels for the anti-inflammatory cytokines IL4 and IL10 in EAE mice (Figure 8G-H).

3.2.3 PIOGLITAZONE REDUCES GLUTAMATE-INDUCED CELL VIABILITY IN PRIMARY CORTICAL NEURONS

Primary cortical neurons were pretreated with or without pioglitazone and subjected to a lethal concentration of glutamate to assess the neuroprotective properties of pioglitazone. Cellular viability was decreased in neurons exposed to 30 mins of glutamate (50 μM; Figure 9). Pioglitazone (0.1 μM) treatment for 24 hrs did not alter levels of neuronal viability in the absence of glutamate (Figure 9). Pioglitazone treatment (0.1 μM, 24 hrs) followed by a 30 min glutamate incubation (50 μM) resulted in elevated cellular viability relative to glutamate-exposed neurons not pretreated with pioglitazone (Figure 9).

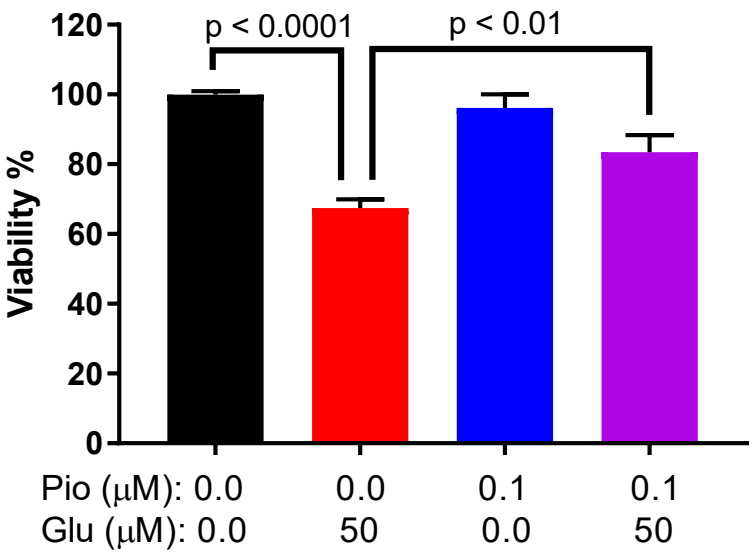

Figure 9. Protective effects of pioglitazone against glutamate-induced cell death in mouse primary cortical neurons. Neurons were treated with pioglitazone (0.1 μM) for 24 hrs prior to a 30 min incubation with glutamate (50 μM). MTT assay results show that glutamate caused a marked decrease in cellular viability ($p < 0.0001$). Pioglitazone reduced the decrease in viability caused by glutamate exposure ($p < 0.01$). Data are from 3 separate experiments performed in duplicate and are expressed as mean ± SEM. A one-way ANOVA followed by Tukey's multiple comparisons tests was used to analyze the data.

3.3 APPLICATION OF KINEMATIC GAIT ANALYSIS TO DETERMINE THE EFFECT OF PIOGLITAZONE ON WALKING DEFICITS IN MICE WITH EAE

Kinematic gait analysis (KGA) was employed to overcome the limited information yielded by clinical scoring in the assessment of disease progression and benefits of pioglitazone on motor deficits. This sophisticated behavioural technique provided more detailed information as to how pioglitazone improved specific motor deficits in EAE mice. Baseline recordings of treadmill walking were performed on female C57Bl/6 mice 2 days prior to immunization (DPI -2). Treadmill recordings were performed next on DPI 9 and weekly thereafter. Twenty-four hrs after disease onset (DPI 12), mice were randomly assigned to the vehicle-treated and pioglitazone-treated groups. All mice were humanely euthanized on DPI 44 and their spinal cords were collected for white matter loss analysis.

3.3.1 ORAL ADMINISTRATION OF PIOGLITAZONE, BEGINNING AFTER DISEASE ONSET, LOWERS EAE DISEASE SEVERITY AND REDUCES WHITE MATTER LOSS

Vehicle (5 ml/kg/day, po) or pioglitazone (15 mg/kg/day, po) were administered to mice with EAE beginning 24 hr after disease onset and until the end of the experiment. Consistent with the findings from the previous experiment, pioglitazone reduced clinical scores (Figure 10A) and spinal cord white matter loss relative to vehicle-treated EAE mice (Figure 10B-D).

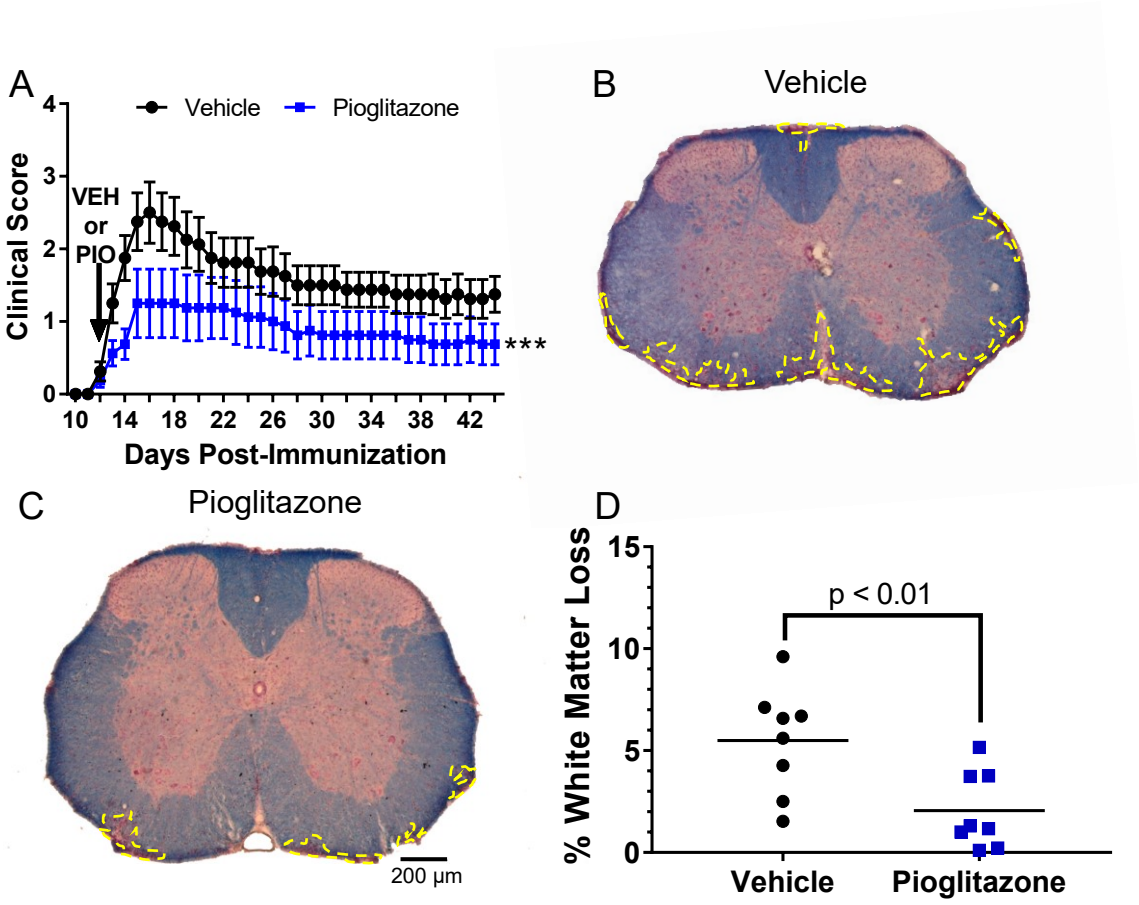

Figure 10. Oral administration of pioglitazone (PIO; 15 mg/kg/day; n = 8), beginning 24 hr after disease onset, reduces EAE disease severity and spinal cord white matter loss. (A) Relative to vehicle-treated EAE mice (VEH; 5 ml/kg/day, po; n = 8), pioglitazone-treated EAE mice had lower clinical scores from DPI 13 – 44 (***p < 0.001). (B-C) Representative photomicrographs of the lumbar spinal cord from vehicle- (B) and pioglitazone-treated (C) EAE mice. (D) Pioglitazone-treated EAE mice had less white matter loss in the lumbar spinal cord compared to control mice (p < 0.01). The yellow outline depicts regions of white matter loss in the spinal cord. The scale bar (C) represents 200 µm. Data are expressed as mean ± SEM and were analyzed using the area under the curve followed by a one-tailed student's t-test (A) and a Mann Whitney-U test (D). Adapted from (Chedrawe et al., 2018).

3.3.2 ORAL ADMINISTRATION OF PIOGLITAZONE PREVENTS REDUCTIONS IN HIP RANGE OF MOTION AND LOWERS RMS DIFFERENCE IN EAE MICE

Pioglitazone treated mice (15 mg/kg/day, po) had greater hip extension throughout a step cycle when compared to vehicle-treated mice (5 ml/kg/day, po). Pioglitazone prevented further reductions in hip range of motion (ROM) observed in vehicle-treated EAE mice (Figure 11A). Moreover, mice treated with pioglitazone experienced a reduction in the elevation of hip root mean squared (RMS) difference at DPI 16, with values remaining lower than vehicle-treated mice until DPI 44 (Figure 11B).

3.3.3 ORAL ADMINISTRATION OF PIOGLITAZONE PREVENTS INCREASED KNEE RMS DIFFERENCE IN EAE MICE

The ROM between vehicle- (5 ml/kg/day, po) and pioglitazone-treated EAE mice (15 mg/kg/day, po) did not differ throughout the experiment (Figure 11C). A general impairment in knee joint movements observed in vehicle-treated EAE mice, characterized by elevated RMS differences, was prevented by oral administration of pioglitazone (Figure 11D).

3.3.4 ORAL ADMINISTRATION OF PIOGLITAZONE RESTORES ANKLE ROM IN MICE SUBJECTED TO EAE

Pioglitazone treatment (15 mg/kg/day, po) restored ankle ROM (Figure 11E), whereas vehicle-treated EAE mice (5 ml/kg/day, po) exhibited a decline in ankle ROM. No observable difference in ankle RMS difference was detected between vehicle- and pioglitazone-treated EAE mice (Figure 11F).

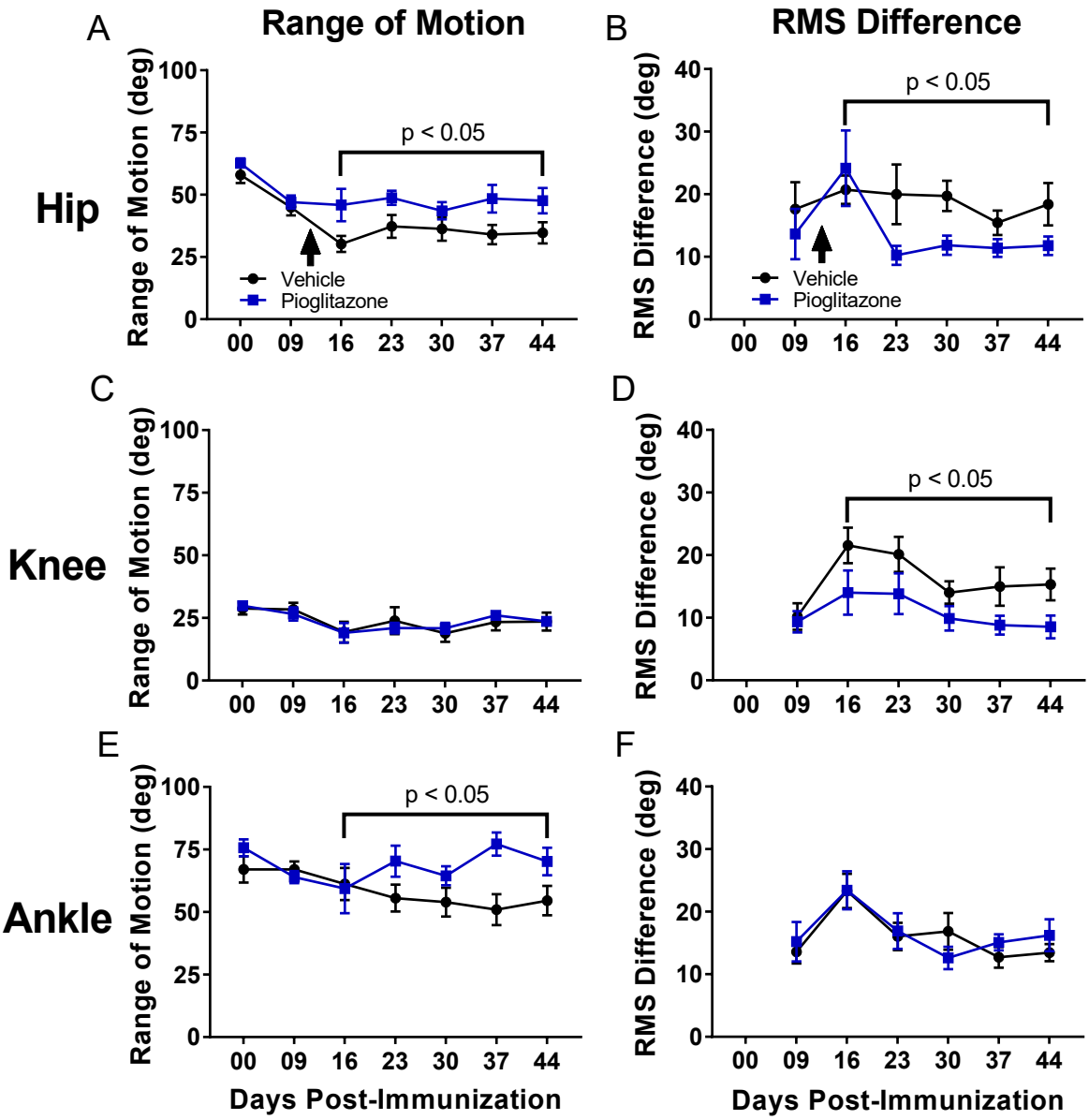

Figure 11. Pioglitazone treatment (15 mg/kg/day, po; n = 8) preserves normal movement of the hip, knee and ankle during walking in EAE mice. (A) The reduction in hip range of motion observed in vehicle-treated EAE mice (5 ml/kg/day, po; n = 8) was prevented in EAE mice treated with pioglitazone ($p < 0.05$). (B) Hip RMS difference was lower for pioglitazone-treated EAE mice than vehicle-treated EAE mice ($p < 0.05$). (D) The pioglitazone-treated EAE mice also had lower knee RMS difference across the study, relative to vehicle-treated EAE mice ($p < 0.05$). (E) Pioglitazone restored ankle range of motion ($p < 0.05$). Data are expressed as the mean ± SEM and were analyzed using a two-way repeated measures ANOVA. Adapted from (Chedrawe et al., 2018).

CHAPTER 4: DISCUSSION

4.1 RESEARCH OBJECTIVES AND OUTCOMES

The main objectives of the current study were to: 1) identify which of the four selected FDA-approved drugs (clozapine, quetiapine, tamoxifen or pioglitazone) was most effective at alleviating EAE in mice, 2) determine the mechanism of action of the best drug candidate and 3) investigate the extent to which the drug candidate benefitted certain aspects of gait. Overall, pioglitazone was superior to clozapine, quetiapine, and tamoxifen at alleviating EAE in mice. Pioglitazone attenuated EAE disease severity by reducing CNS demyelination and inflammation as well as maintaining normal movement of the hip, knee and ankle joint during gait of EAE mice.

4.2 RATIONALE FOR THE ORAL ROUTE OF ADMINISTRATION AT PEAK DISEASE AND THE DOSAGES USED FOR EACH DRUG

Clozapine, quetiapine, tamoxifen and pioglitazone are typically administered orally for their primary indications (Citrome, 2012; Greco, 1998; Pfutzner and Forst, 2006). Each of these drugs was therefore delivered by oral gavage in the current study. To further mimic the typical presentation of MS patients, clozapine, quetiapine, tamoxifen or pioglitazone were given at the peak of clinical scores to determine if these drugs could reduce disease severity in mice with established EAE.

4.2.1 RATIONALE FOR QUETIAPINE AND CLOZAPINE DOSE SELECTION

Quetiapine (10 mg/kg/day, po) and clozapine (10 mg/kg/day, po) have previously been shown to reduce EAE disease severity in MOG_{35-55} immunized mice (Mei et al., 2012; O'Sullivan et al., 2014). Therefore, the dose of 10 mg/kg/day (po) for quetiapine and clozapine was used in the current study.

4.2.2 RATIONALE FOR TAMOXIFEN DOSE SELECTION

Tamoxifen is a prodrug that is metabolized into the active metabolite 4-hydroxytamoxifen (4-HT) (Lim et al., 2005). Tamoxifen was given orally to EAE mice because this route of administration generates higher levels of 4-HT relative to subcutaneous injections (Kisanga et al., 2004). *In vitro* evidence suggests that 4-HT may improve EAE by enhancing the differentiation of oligodendrocyte progenitor cells (OPCs) into myelin-producing oligodendrocytes responsible for remyelination (Barratt et al., 2016). Oral administration of tamoxifen at 5 mg/kg (po) produces greater serum concentrations of both tamoxifen and 4-HT than subcutaneous injections of the same dose in rats (Kisanga et al., 2004). Brain levels of tamoxifen and 4-HT were also higher after 21 days of oral delivery compared to subcutaneous administration (Kisanga et al., 2004). Since subcutaneous delivery of tamoxifen (25 mg) by a slow release pellet over 60 days reduced disease severity in the $PLP_{139-151}$ mouse model of relapsing-remitting EAE (Bebo, Jr. et al., 2009), we selected an oral dose of 5 mg/kg/day (po) that was estimated on the basis of previous pharmacokinetic studies to

produce equivalent drug exposure (DeGregorio et al., 1987; Kisanga et al., 2004; Reid et al., 2014).

4.2.3 RATIONALE FOR PIOGLITAZONE DOSE SELECTION

Feinstein et al. (2002) showed that oral consumption of pioglitazone, milled into mouse chow to deliver an estimated dose of 10 mg/kg/day, reduced EAE severity in MOG_{35-55}-immunized mice (Feinstein et al., 2002). To approximate similar drug exposure in this study, a dose of 15 mg/kg/day (po) of pioglitazone was used.

4.3 ORAL ADMINISTRATION OF QUETIAPINE, CLOZAPINE OR TAMOXIFEN, BEGINNING AT PEAK DISEASE, DOES NOT REDUCE EAE SEVERITY WITH ONLY CLOZAPINE PRODUCING A MILD DECREASE IN SPINAL CORD WHITE MATTER LOSS

Oral administration of either quetiapine (10 mg/kg/day, po) or clozapine (10 mg/kg/day, po) or tamoxifen (5 mg/kg/day, po), beginning at peak disease (DPI 15), failed to reduce EAE disease severity. Furthermore, neither quetiapine nor tamoxifen lowered spinal cord white matter loss in EAE mice. However, a mild, but significant, reduction in white matter loss with clozapine was detected at DPI 34. This is consistent with evidence demonstrating that clozapine may stimulate myelin lipid synthesis (Steiner et al., 2014). The degree to which clozapine lowered spinal cord white matter loss, however, may not have been sufficient to reduce EAE disease severity. These three drugs have been previously shown to reduce EAE severity at these doses when administered before peak disease (Bebo, Jr. et al., 2009; Mei et al., 2012; O'Sullivan et al., 2014). Clinical signs of EAE were reduced by treatment with tamoxifen 7 days before EAE immunization

(Bebo, Jr. et al., 2009), administration of clozapine immediately after immunization (O'Sullivan et al., 2014), or dosing with quetiapine beginning 24 hr after disease onset (Mei et al., 2012).

Measurements of immune function in these studies suggested that efficacy was mediated, in part, by reprogramming the immune system to suppress disease development as opposed to reversing disease progression. For instance, tamoxifen lowered myelin-specific T cell proliferation which would attenuate the development of EAE (Bebo, Jr. et al., 2009). Although this mechanism would oppose early T cell-mediated events responsible for disease development, this mode of action is less likely to be effective at reducing subsequent pathological processes responsible for demyelination and axonal damage in mice with established EAE. Furthermore, tamoxifen has only been tested by Bebo Jr. et al. (2009) in the $PLP_{139-151}$ model of relapsing EAE in which there is near-complete disease remission and little demyelination between relapses. By contrast, the MOG_{35-55} model of chronic EAE is characterized by permanent motor deficits with consistent demyelination (Jones et al., 2008; Najm et al., 2015). Drugs that are effective in the $PLP_{139-151}$ model may therefore be less effective in the MOG_{35-55} model, particularly when administered after EAE onset, because of different disease mechanisms in the $PLP_{139-151}$ and MOG_{35-55} models (Najm et al., 2015). This renders the MOG_{35-55} model more suitable to study the potential of drugs to reduce myelin loss than the $PLP_{139-151}$ model of EAE.

4.4 ADMINISTRATION OF PIOGLITAZONE AFTER THE FIRST ONSET OF EAE OR AT PEAK DISEASE LOWERS CLINICAL SCORES AND WHITE MATTER LOSS IN THE SPINAL CORD

Unlike clozapine, quetiapine and tamoxifen, administration of pioglitazone, beginning at peak clinical scores, reduced EAE disease severity. These findings follow Feinstein et al. (2002) for which oral consumption of pioglitazone in the lab chow, beginning at peak disease, reduced EAE severity (Feinstein et al., 2002). In the case of EAE mice treated prophylactically with pioglitazone, demyelination in the spinal cord at DPI 11 was also found to be suppressed (Feinstein et al., 2002). We have extended these findings by showing that oral administration of pioglitazone, beginning 24 hr after the first onset of clinical signs or at peak disease, reduced white matter loss in the spinal cord at DPI 44 and 27, respectively. Given that a there is significant demyelination at peak disease with limited subsequent remyelination in the MOG_{35-55} model of EAE (Jones et al., 2008), pioglitazone may have reduced demyelination as well as promote remyelination. For instance, when administered at peak disease, pioglitazone likely reduced disease severity by promoting remyelination. The ability of pioglitazone to suppress disease progression when administered after the first onset of clinical signs suggests that this TZD may also suppress the events responsible for demyelination. These findings are consistent with the results of previous studies which have demonstrated that pioglitazone not only reduces white matter loss (Park et al., 2007), but also enhances remyelination following nerve injury (Eto et al., 2008). The suppression of white matter loss in

pioglitazone-treated EAE mice likely stems from the protective effects of pioglitazone on OPCs *in vitro* (Bernardo et al., 2009; Peymani et al., 2018).

4.5 PIOGLITAZONE LOWERS PRO-INFLAMMATORY GENE EXPRESSION IN THE SPINAL CORDS OF EAE MICE

Relative to vehicle-treated EAE mice, pioglitazone (15 mg/kg/day, po; DPI 12-16) lowered mRNA levels for pro-inflammatory genes in the spinal cords of EAE mice at DPI 16. A similar suppressive effect of pioglitazone on functionally related pro-inflammatory cytokines has been previously demonstrated in MOG_{35-55}-induced EAE (Feinstein et al., 2002) and spinal cord injury models as well (Park et al., 2007). Another TZD, trogliazone, reduced TNFα, but not IFNγ, gene expression in EAE mice spinal cords at a high dose of 100 mg/kg/day (Niino et al., 2001). Thus, the ability of pioglitazone (15 mg/kg/day, po) to reduce both TNFα and IFNγ mRNA levels demonstrates that this TZD is more effective than troglitazone at reducing pro-inflammatory gene expression in the spinal cords of EAE mice.

Macrophages and microglia express PPARγ receptors (Bernardo et al., 2000; Pascual et al., 2005; Tontonoz et al., 1998; Xu and Drew, 2007) and are known to produce IFNγ, IL1 β, IL6, TNFα, and TGFβ (Arango and Descoteaux, 2014; Darwich et al., 2009; Kallfass et al., 2012; Kiefer et al., 1998; Lue et al., 2001; Luo et al., 2007). The reductions of pro-inflammatory cytokines (IFNγ, IL1β, IL6, TNFα and TGFβ) produced by pioglitazone therefore likely stem from the activation of PPARγ receptors within macrophages and microglia. This proposed mechanism is supported by reductions in the number of activated microglia and

macrophages in the spinal cords of EAE mice treated with pioglitazone. The cytokine TGFβ can exert both pro- and anti-inflammatory properties; this action largely depends on the spatiotemporal microenvironment of the affected tissues (Sanjabi et al., 2009). In the context of EAE, TGFβ has been shown to be involved in the initiation of the disease process (Luo et al., 2007). Pharmacological inhibition of TGFβ signalling delayed EAE onset and lowered peak disease severity (Luo et al., 2007). Suppression of TGFβ gene expression by pioglitazone during the acute phase of the disease may therefore contribute to reduced EAE disease severity.

Pioglitazone treatment also produced a minor, yet significant, reduction in the pro-inflammatory response that occurred in the spinal cords of CFA mice. This finding suggest that CFA causes mild inflammation in the spinal cord that is opposed by pioglitazone. To the best of our knowledge, this finding has not been reported previously and underscores the importance of using CFA mice, rather than healthy mice, as controls for the effects of the immunization procedure. In contrast to the suppression of pro-inflammatory gene expression, pioglitazone did not alter the levels of mRNAs encoding the anti-inflammatory genes IL4 and IL10 in EAE mice. Taken together, pioglitazone reduced pro-inflammatory gene expression without hindering the potentially beneficial effects of elevated IL4 and IL10 levels on the resolution of inflammation, remyelination and neuronal cell survival (Klose et al., 2013; Pluchino and Peruzzotti-Jametti, 2016; Spittau, 2017).

4.6 SUPPRESSION AND REVERSAL OF EAE-INDUCED GAIT DEFICTS BY PIOGLITAZONE

4.6.1 PIOGLITAZONE MAINTAINS NORMAL MOVEMENT OF THE HIP, KNEE AND ANKLE JOINTS IN EAE MICE

We have previously shown that kinematic gait analysis (KGA) yields highly sensitive measures of motor deficits for EAE mice which are not detected by clinical scoring or fall latencies from a rotarod (Fiander et al., 2017b). KGA was therefore employed to assess the beneficial effects of pioglitazone on the locomotion of EAE mice. In keeping with previous findings from our lab, mild-moderate EAE (clinical scores ≤2.5) for vehicle-treated mice was characterized by impaired movement of the hip, resulting in decreased range of motion (ROM) (Fiander et al., 2017a; Fiander et al., 2017b). Pioglitazone not only maintained normal hip movements in EAE mice by preventing reductions of hip ROM, but also reversed the elevation of root mean squared (RMS) difference observed at DPI 16. Therefore, this normalization of RMS difference indicates that pioglitazone reversed variations in movements of the hip joint caused by EAE (Fiander et al., 2017a; Fiander et al., 2017b). Deficits in knee movements displayed by vehicle-treated EAE mice were also typical of those we have observed previously for EAE mice with comparable clinical scores (Fiander et al., 2017a; Fiander et al., 2017b). Pioglitazone prevented a substantial elevation of RMS difference for the knee joint observed at DPI 16 and kept the RMS difference at lower levels relative to vehicle-treated EAE mice at all subsequent time points. Since vehicle-treated EAE mice in the present study suffered only mild paralysis, marked impairments of ankle movements (sustained elevation of

RMS difference) typical of severe EAE were not observed (Fiander et al., 2017b). However, the progressive loss of ankle ROM was reversed by pioglitazone. Pioglitazone administration, beginning 24 hr after disease onset, therefore suppressed, and in some case even reversed, gait deficits typical of EAE mice by maintaining normal movements of the hip, knee and ankle.

4.6.2 THE PRESERVATION OF GAIT SUGGESTS THAT PIOGLITAZONE PROTECTS THE INTRINCATE NEURAL CIRCUITRY INVOLVED IN LOCOMOTION

Pioglitazone has previously been shown to be neuroprotective in models of ALS (Schutz et al., 2005), PD (Breidert et al., 2002), traumatic brain injury (Semple and Noble-Haeusslein, 2011; Zhu et al., 2013), AD (Searcy et al., 2012) and stroke (Kaundal et al., 2009). The neuroprotective properties of pioglitazone treatment in stroke were associated with improved locomotor activity (Kaundal et al., 2009). The present findings have shown that pioglitazone partially prevented the reduction of glutamate-induced neuronal viability *in vitro,* suggesting that pioglitazone may also have preserved gait by suppressing excitotoxicity known to promote spinal cord damage in EAE (Centonze et al., 2010; Kanwar et al., 2004). Furthermore, pioglitazone is neuroprotective under NO- and H_2O_2-mediated injury (Gray et al., 2012). Taken together, these findings further suggest that pioglitazone maintained the complex leg movements of mice subjected to MOG_{35-55}-induced EAE by protecting the intricate neural circuitry responsible for gait from damage.

4.6.3 EFFECTS OF FINGOLIMOD ON GAIT DEFICITS FOR EAE MICE

To the best of our knowledge, only one other study has performed sagittal plane KGA on EAE mice to test the efficacy of a drug on locomotion. De Bruin et al. (2016) tested the effect of fingolimod, an approved MS therapeutic, on gait in the $PLP_{139-151}$ model of relapsing EAE. The single timepoint of locomotor testing and minimal gait parameters investigated were two limitations of this study. De Bruin et al. (2016) assessed the effect of fingolimod on gait only once at the end of the experiment. Furthermore, these investigators only detected a deficit in peak tail height off the ground during locomotion. This experimental design provides little information as to how fingolimod may influence walking for two reasons: 1) there is a lack of understanding as to when, and for how long, fingolimod benefits gait in EAE mice, and 2) it is still unknown whether fingolimod improved clinically relevant aspects of locomotion, such as how hindlimb joints move during a step cycle. The current study improved upon these limitations by assessing gait in EAE mice on a weekly basis to identify when pioglitazone benefitted certain aspects of gait. We also investigated two kinematic parameters (ROM and RMS difference) for the hip, knee and ankle joints which are more relevant to human studies.

4.7 THERAPEUTIC POTENTIAL OF PIOGLITAZONE FOR THE TREATMENT OF MS

EAE and MS are characterized by similar gait deficits such as decreased hip, knee and ankle joint range of motion (Fiander et al., 2017a; Fiander et al., 2017b; Kelleher et al., 2010; van der Linden et al., 2014). Pioglitazone maintained

normal movement of the hip, knee and ankle joint throughout an average step cycle for EAE mice. Reducing locomotor deficits in people with MS is important because falls can lead to a worsened quality of life (Gijbels et al., 2010; Peterson et al., 2007) and an economic burden on the health care system (Rizzo et al., 1998). Pioglitazone may thus reduce the development of deficits in hip, knee and ankle joint movements that impair mobility and increase the risk of debilitating falls for people with MS (Motl, 2013; van der Linden et al., 2014).

Findings from three small clinical trials and one case-study suggest that pioglitazone may be an effective treatment for MS when administered alone (Negrotto et al., 2016; Pershadsingh et al., 2004) or as an add-on therapy (Kaiser et al., 2009; Shukla et al., 2010). These studies have shown that pioglitazone reduces relapses and lesion burden as measured by fluid-attenuated inversion recovery and fractional anisotrophy changes on diffusion weighted magnetic resonance tensor images (Shukla et al., 2010), gray matter atrophy (Kaiser et al., 2009) and the number of new or enlarging T2 and gadolinium-enhancing lesions in MS patients (Negrotto et al., 2016). Our findings further suggest that pioglitazone may improve these MRI measures in MS by suppressing neuroinflammation and white matter loss.

4.8 FEASIBILITY OF REPOSITIONING PIOGLITAZONE FOR THE TREATMENT OF MS

4.8.1 PIOGLITAZONE HAS LESS SEVERE ADVERSE EFFECTS THAN QUETIAPINE, CLOZAPINE AND TAMOXIFEN

The adverse effects associated with the therapeutic use of pioglitazone are less severe relative to quetiapine, clozapine and tamoxifen. For example, quetiapine and clozapine are sedating at therapeutic doses (Leucht et al., 2013). This side effect is particularly problematic for MS patients who frequently suffer from physical and cognitive fatigue (Compston and Coles, 2008; Loma and Heyman, 2011). Tamoxifen is rarely used for more than 10 years (Davies et al., 2013) due to its adverse effects, some of which include: feminizing effects (Moore et al., 2014), endometrial cancer (Grilli, 2006; Moore et al., 2014) and a decline in cognition (Paganini-Hill and Clark, 2000). The most common side effects associated with pioglitazone are mild weight gain (0.9 - 2.6 kg) and edema, which occur to a lesser extent than other TZDs (Shah and Mudaliar, 2010). Long-term administration of pioglitazone is also well-tolerated and associated with a reduced risk of myocardial infarction and stroke (Lincoff et al., 2007). Early concerns about the increased risk of bladder cancer with prolonged pioglitazone use have not been supported by a detailed meta-analysis of drug exposure and cancer incidence (Filipova et al., 2017).

4.8.2 REPOSITIONING PIOGLITAZONE

Drug repositioning is beneficial for two main reasons: 1) reduced timeline for therapeutic development and 2) decreased risk of late-stage clinical failure

because of unacceptable adverse side-effects (Ashburn and Thor, 2004). Following approval by the FDA as a generic drug, brand and generic formulations of pioglitazone have been shown to produce equivalent drug exposure and efficacy in patients with type II diabetes (Sugita et al., 2014; Temboonkiat et al., 2012). Since the composition-of-matter patent for pioglitazone has ended, obtaining a method-of-use, dosing and/or formulation patent for the treatment of MS is now possible (Ashburn and Thor, 2004). Such patents would incentivize biotechnology or pharmaceutical companies to reposition pioglitazone for the treatment of MS (Ashburn and Thor, 2004).

Drug repositioning is not a foreign concept within the MS therapeutic landscape. Dimethyl fumarate, previously approved for the treatment of psoriasis, has recently been repurposed by Biogen Idec. as an MS therapeutic named Tecfidera® (Spencer, 2014). Biogen Idec. accomplished this by filing method of use, dosing, and formulation patents to protect the use of dimethyl fumarate for MS until 2028 (Spencer, 2014). By repositioning dimethyl fumarate, Biogen Idec. earned $478.5 million in revenue from Tecfidera® sales in the first nine months of 2013 (Spencer, 2014). A similar approach could therefore be adopted to reposition pioglitazone for the treatment of MS.

4.9 LIMITATIONS OF THE PRESENT STUDY

One limitation of this study is the degree to which the mechanism of pioglitazone was established in EAE. Pioglitazone was found to reduce EAE disease severity

in association with decreased white matter loss, macrophage and microglial activation and the expression of pro-inflammatory cytokine genes in the spinal cord. However, axonal damage is thought to be responsible for the permanent loss of neurological function in MS (Trapp and Nave, 2008). Determining if pioglitazone reduces axonal damage in EAE mice would further encourage testing of this drug in people with MS.

A second limitation of this study was not examining the effects of an approved MS therapeutic on disease severity, white matter loss and kinematic gait parameters for EAE mice. The results of such studies would provide a benchmark by which to gauge the clinical potential of pioglitazone for MS.

A third, but lesser, limitation of this study was not including a cohort of MOG_{35-55} immunization controls (CFA mice that do not develop EAE symptoms) in the KGA experiments. This would have enabled further assessment of the degree to which pioglitazone improved the gait of EAE mice relative to CFA controls. However, this was estimated in the present study by comparing gait performance in EAE mice relative to their pre-immunization recordings. Furthermore, our previous work has demonstrated that CFA mice do not develop gait deficits as detected by KGA (Fiander et al., 2017b). Gait performance before MOG_{35-55} immunization should therefore be the same as that for CFA mice. However, inclusion of CFA mice treated with vehicle or pioglitazone would have made it

possible to further evaluate the ability of pioglitazone to completely normalize gait in EAE mice.

4.10 FUTURE RESEARCH DIRECTIONS

4.10.1 FURTHER CHARACTERIZATION OF THE MECHANISM OF ACTION OF PIOGLITAZONE

Based on the findings of the present work, further investigation into the mechanism through which pioglitazone is functioning to alleviate EAE is warranted. Immunohistochemical detection of markers for astrocytes (GFAP), OPCs (PDGFRα), oligodendrocytes (OSP), neurons (NeuN) and axons (SMI 312) should be performed on spinal cord sections from vehicle- and drug-treated CFA and EAE mice. This would enable determination of the effects of pioglitazone on astrocytic activation, OPC loss, oligodendrocyte destruction, neuronal cell death and axonal damage in the spinal cords of EAE mice. It would also be useful to measure the effects of pioglitazone on levels of mRNAs encoding calcium handling genes such as the plasma membrane calcium ATPase-2 (PMCA-2) and mitochondrial calcium uniporter (MCU). This would allow the assessment of whether pioglitazone prevented the loss of PMCA-2 expression, which contributes to toxic calcium overloading and neuronal cell death in the spinal cords of EAE mice (Kurnellas et al., 2005; Kurnellas et al., 2010; Nicot et al., 2003). Induction of MCU expression could contribute to the preservation of denuded axons in pioglitazone-treated EAE mice by enhancing calcium buffering and energy production. This line of reasoning is supported by evidence that pioglitazone increases neuronal mitochondrial biogenesis that

would elevate MCU-mediated mitochondrial calcium uptake and energy production (Agarwal et al., 2017; Ghosh et al., 2007). Employing a second model to assess the effects of pioglitazone on remyelination, such as the lysophosphatidylcholine (LPC) model of focal demyelination (Keough et al., 2015), would be useful. LPC mainly functions by targeting and destroying myelin, oligodendrocytes and OPCs (Blakemore and Franklin, 2008). Therefore, the LPC model may help further substantiate the beneficial effects of pioglitazone on remyelination and establish a potential mechanism linked to oligodendrocytes and OPCs. Lastly, additional *in vitro* assays could be employed to identify mechanisms that occur in single-cell types. This would overcome problems associated with determining the relative roles of various cell types *in vivo*. One particular *in vitro* experiment that would be useful to perform is gene ontology and proteomic analyses (Subramanian et al., 2005) in neurons or macrophages treated with vehicle or pioglitazone. This would allow the biological or immunological processes influenced by pioglitazone in neurons or macrophages to be identified.

4.10.2 IDENTIFICATION OF OTHER FDA-APPROVED DRUGS THAT MAY BE REPOSITIONED FOR THE TREATMENT OF MS

Several research groups have developed high-throughput screening techniques that identified several FDA-approved drugs which promote remyelination and thus may potentially be repositioned for the treatment of MS (Deshmukh et al., 2013; Mei et al., 2014; Najm et al., 2015). Given the considerable resources required to complete phase III clinical trials, the development of improved

preclinical methods to prioritize these compounds for human trials is crucial. The present findings suggest that KGA of EAE mice may be a highly useful method to identify the most promising therapeutic candidates for MS. Since KGA is frequently used to assess the effectiveness of new MS therapies (Cameron and Wagner, 2011; Coghe et al., 2015; Goodman et al., 2009; Zorner et al., 2016), findings from such preclinical studies could inform the selection of gait measures and definition of clinical endpoints for human trials.

4.10.3 ASSSSMENT OF PIOGLITAZONE EFFICACY IN THE CLINIC

Preclinical findings from this thesis suggest that pioglitazone should be given priority for repositioning in the treatment of MS. This conclusion is supported by the results of four small human studies for MS. These studies have shown that pioglitazone caused no adverse effects in people with MS and provided extra benefit when administered either alone (Negrotto et al., 2016; Pershadsingh et al., 2004) or as an add-on therapy (Kaiser et al., 2009; Shukla et al., 2010). These findings, combined with the low cost and well-demonstrated safety of pioglitazone, are strong incentives to further characterize the potential benefits of this FDA-approved drug in MS.

4.11 CONCLUSIONS

We have shown that oral administration of pioglitazone, but not clozapine, quetiapine or tamoxifen, beginning at peak clinical scores, reduced disease progression and suppressed white matter loss in the spinal cords of mice with

MOG_{35-55}-induced EAE. Pioglitazone also protected neurons from glutamate-induced injury. These findings are consistent with evidence that pioglitazone protects neural cells from injury by pro-inflammatory mediators, oxidative stress and hypoxia (Bernardo et al., 2009; Gray et al., 2012; Kaundal et al., 2009; Peymani et al., 2018). Furthermore, pioglitazone decreased the number of $Iba1^+$ macrophages and microglial cells in the spinal cords of EAE mice. This was accompanied by reductions in mRNA levels for pro-inflammatory cytokines produced by these immune cells (Arango and Descoteaux, 2014; Darwich et al., 2009; Kallfass et al., 2012; Kiefer et al., 1998; Lue et al., 2001; Luo et al., 2007). Interestingly, pioglitazone did not dampen the expression of anti-inflammatory cytokines (IL4 and IL10) known to promote EAE recovery. The ability of pioglitazone to promote OPC differentiation necessary for remyelination (Bernardo et al., 2009; Peymani et al., 2018) may have further contributed to the therapeutic effects of this TZD in the MOG_{35-55} model of EAE. We therefore propose that these diverse mechanisms allowed pioglitazone to the preserve the intricate neural circuitry responsible for complex movements of the hip, knee and ankle joints in EAE mice. These results support the use of KGA in the EAE model to identify the most promising therapeutic candidates for clinical testing in MS. Since gait analyses have been employed to test the efficacy of MS therapeutics (Cameron and Wagner, 2011; Coghe et al., 2015; Goodman et al., 2009; Zorner et al., 2016), our findings encourage further use of these behavioural techniques to assess motor recovery in future clinical trials.

REFERENCES

Agarwal, S., Yadav, A., Chaturvedi, R.K., 2017. Peroxisome proliferator-activated receptors (PPARs) as therapeutic target in neurodegenerative disorders. Biochem. Biophys. Res. Commun. 483, 1166-1177.

Akay, T., 2014. Long-term measurement of muscle denervation and locomotor behavior in individual wild-type and ALS model mice. J. Neurophysiol. 111, 694-703.

Alonso, A., Hernan, M.A., 2008. Temporal trends in the incidence of multiple sclerosis: a systematic review. Neurology 71, 129-135.

Alvir, J.M., Lieberman, J.A., Safferman, A.Z., Schwimmer, J.L., Schaaf, J.A., 1993. Clozapine-induced agranulocytosis. Incidence and risk factors in the United States. N. Engl. J. Med. 329, 162-167.

Arango, D.G., Descoteaux, A., 2014. Macrophage cytokines: involvement in immunity and infectious diseases. Front Immunol. 5, 491.

Arun, T., Tomassini, V., Sbardella, E., de Ruiter, M.B., Matthews, L., Leite, M.I. et al., 2013. Targeting ASIC1 in primary progressive multiple sclerosis: evidence of neuroprotection with amiloride. Brain 136, 106-115.

Ascherio, A., Munger, K.L., 2007. Environmental risk factors for multiple sclerosis. Part II: Noninfectious factors. Ann. Neurol. 61, 504-513.

Ashburn, T.T., Thor, K.B., 2004. Drug repositioning: identifying and developing new uses for existing drugs. Nat. Rev. Drug Discov. 3, 673-683.

Baecher-Allan, C., Kaskow, B.J., Weiner, H.L., 2018. Multiple Sclerosis: Mechanisms and Immunotherapy. Neuron 97, 742-768.

Baker, R., McGinley, J.L., Schwartz, M.H., Beynon, S., Rozumalski, A., Graham, H.K., Tirosh, O., 2009. The gait profile score and movement analysis profile. Gait. Posture. 30, 265-269.

Balkaya, M., Krober, J.M., Rex, A., Endres, M., 2013. Assessing post-stroke behavior in mouse models of focal ischemia. J. Cereb. Blood Flow Metab 33, 330-338.

Bannerman, P.G., Hahn, A., Ramirez, S., Morley, M., Bonnemann, C., Yu, S., Zhang, G.X., Rostami, A., Pleasure, D., 2005. Motor neuron pathology in experimental autoimmune encephalomyelitis: studies in THY1-YFP transgenic mice. Brain 128, 1877-1886.

Barratt, H.E., Budnick, H.C., Parra, R., Lolley, R.J., Perry, C.N., Nesic, O., 2016. Tamoxifen promotes differentiation of oligodendrocyte progenitors in vitro. Neuroscience 319, 146-154.

Batka, R.J., Brown, T.J., Mcmillan, K.P., Meadows, R.M., Jones, K.J., Haulcomb, M.M., 2014. The need for speed in rodent locomotion analyses. Anat. Rec. (Hoboken.) 297, 1839-1864.

Bebo, B.F., Jr., Dehghani, B., Foster, S., Kurniawan, A., Lopez, F.J., Sherman, L.S., 2009. Treatment with selective estrogen receptor modulators regulates myelin specific T-cells and suppresses experimental autoimmune encephalomyelitis. Glia 57, 777-790.

Bernardo, A., Bianchi, D., Magnaghi, V., Minghetti, L., 2009. Peroxisome proliferator-activated receptor-gamma agonists promote differentiation and antioxidant defenses of oligodendrocyte progenitor cells. J. Neuropathol. Exp. Neurol. 68, 797-808.

Bernardo, A., Levi, G., Minghetti, L., 2000. Role of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and its natural ligand 15-deoxy-Delta12, 14-prostaglandin J2 in the regulation of microglial functions. Eur. J. Neurosci. 12, 2215-2223.

Bittner, S., Afzali, A.M., Wiendl, H., Meuth, S.G., 2014. Myelin oligodendrocyte glycoprotein (MOG35-55) induced experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice. J. Vis. Exp.

Blakemore, W.F., Franklin, R.J., 2008. Remyelination in experimental models of toxin-induced demyelination. Curr. Top. Microbiol. Immunol. 318, 193-212.

Breidert, T., Callebert, J., Heneka, M.T., Landreth, G., Launay, J.M., Hirsch, E.C., 2002. Protective action of the peroxisome proliferator-activated receptor-gamma agonist pioglitazone in a mouse model of Parkinson's disease. J. Neurochem. 82, 615-624.

Brinkmann, V., Davis, M.D., Heise, C.E., Albert, R., Cottens, S., Hof, R. et al., 2002. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J. Biol. Chem. 277, 21453-21457.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M. et al., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611-622.

Cameron, M.H., Wagner, J.M., 2011. Gait abnormalities in multiple sclerosis: pathogenesis, evaluation, and advances in treatment. Curr. Neurol. Neurosci. Rep. 11, 507-515.

Centonze, D., Muzio, L., Rossi, S., Furlan, R., Bernardi, G., Martino, G., 2010. The link between inflammation, synaptic transmission and neurodegeneration in multiple sclerosis. Cell Death. Differ. 17, 1083-1091.

Chedrawe, M.A.J., Holman, S.P., Lamport, A.C., Akay, T., Robertson, G.S., 2018. Pioglitazone is superior to quetiapine, clozapine and tamoxifen at alleviating experimental autoimmune encephalomyelitis. J. Neuroimmunol. 321, 72-82.

Citrome, L., 2012. Oral antipsychotic update: a brief review of new and investigational agents for the treatment of schizophrenia. CNS. Spectr. 17 Suppl 1, 1-9.

Coghe, G., Pau, M., Corona, F., Frau, J., Lorefice, L., Fenu, G. et al., 2015. Walking improvements with nabiximols in patients with multiple sclerosis. J. Neurol. 262, 2472-2477.

Cohen, J.A., Barkhof, F., Comi, G., Hartung, H.P., Khatri, B.O., Montalban, X. et al., 2010. Oral fingolimod or intramuscular interferon for relapsing multiple sclerosis. N. Engl. J. Med. 362, 402-415.

Collier, R., 2009. Rapidly rising clinical trial costs worry researchers. CMAJ. 180, 277-278.

Compston, A., Coles, A., 2008. Multiple sclerosis. Lancet 372, 1502-1517.

Constantinescu, C.S., Farooqi, N., O'Brien, K., Gran, B., 2011. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). Br. J. Pharmacol. 164, 1079-1106.

Croxford, J.L., Miyake, S., Huang, Y.Y., Shimamura, M., Yamamura, T., 2006. Invariant V(alpha)19i T cells regulate autoimmune inflammation. Nat. Immunol. 7, 987-994.

Darwich, L., Coma, G., Pena, R., Bellido, R., Blanco, E.J., Este, J.A. et al., 2009. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. Immunology 126, 386-393.

Davies, C., Pan, H., Godwin, J., Gray, R., Arriagada, R., Raina, V. et al., 2013. Long-term effects of continuing adjuvant tamoxifen to 10 years versus stopping at 5 years after diagnosis of oestrogen receptor-positive breast cancer: ATLAS, a randomised trial. Lancet 381, 805-816.

de Bruin, N.M., Schmitz, K., Schiffmann, S., Tafferner, N., Schmidt, M., Jordan, H., Haussler, A., Tegeder, I., Geisslinger, G., Parnham, M.J., 2016. Multiple rodent models and behavioral measures reveal unexpected responses to

FTY720 and DMF in experimental autoimmune encephalomyelitis. Behav. Brain Res. 300, 160-174.

DeGregorio, M.W., Wilbur, B.J., Coronado, E., Osborne, C.K., 1987. Serum tamoxifen concentrations in the athymic nude mouse after three methods of administration. Cancer Chemother. Pharmacol. 20, 316-318.

Dendrou, C.A., Fugger, L., Friese, M.A., 2015. Immunopathology of multiple sclerosis. Nat. Rev. Immunol. 15, 545-558.

Deshmukh, V.A., Tardif, V., Lyssiotis, C.A., Green, C.C., Kerman, B., Kim, H.J. et al., 2013. A regenerative approach to the treatment of multiple sclerosis. Nature 502, 327-332.

ECTRIMS., 2012. Setting new standards in multiple sclerosis care and research. Lancet Neurol. 11, 835.

Emerson, M.R., Gallagher, R.J., Marquis, J.G., LeVine, S.M., 2009. Enhancing the ability of experimental autoimmune encephalomyelitis to serve as a more rigorous model of multiple sclerosis through refinement of the experimental design. Comp Med. 59, 112-128.

Eto, M., Sumi, H., Fujimura, H., Yoshikawa, H., Sakoda, S., 2008. Pioglitazone promotes peripheral nerve remyelination after crush injury through CD36 upregulation. J. Peripher. Nerv. Syst. 13, 242-248.

Evans, C., Beland, S.G., Kulaga, S., Wolfson, C., Kingwell, E., Marriott, J. et al., 2013. Incidence and prevalence of multiple sclerosis in the Americas: a systematic review. Neuroepidemiology 40, 195-210.

Feinstein, D.L., Galea, E., Gavrilyuk, V., Brosnan, C.F., Whitacre, C.C., Dumitrescu-Ozimek, L., Landreth, G.E., Pershadsingh, H.A., Weinberg, G., Heneka, M.T., 2002. Peroxisome proliferator-activated receptor-gamma agonists prevent experimental autoimmune encephalomyelitis. Ann. Neurol. 51, 694-702.

Ferno, J., Raeder, M.B., Vik-Mo, A.O., Skrede, S., Glambek, M., Tronstad, K.J., Breilid, H., Lovlie, R., Berge, R.K., Stansberg, C., Steen, V.M., 2005. Antipsychotic drugs activate SREBP-regulated expression of lipid biosynthetic genes in cultured human glioma cells: a novel mechanism of action? Pharmacogenomics. J. 5, 298-304.

Ferre, P., 2004. The biology of peroxisome proliferator-activated receptors: relationship with lipid metabolism and insulin sensitivity. Diabetes 53 Suppl 1, S43-S50.

Fiander, M.D., Chedrawe, M.A., Lamport, A.C., Akay, T., Robertson, G.S., 2017a. Sagittal Plane Kinematic Gait Analysis in C57BL/6 Mice Subjected to

MOG35-55 Induced Experimental Autoimmune Encephalomyelitis. J. Vis. Exp.

Fiander, M.D., Stifani, N., Nichols, M., Akay, T., Robertson, G.S., 2017b. Kinematic gait parameters are highly sensitive measures of motor deficits and spinal cord injury in mice subjected to experimental autoimmune encephalomyelitis. Behav. Brain Res. 317, 95-108.

Filipova, E., Uzunova, K., Kalinov, K., Vekov, T., 2017. Pioglitazone and the Risk of Bladder Cancer: A Meta-Analysis. Diabetes Ther. 8, 705-726.

Fleming, K.K., Bovaird, J.A., Mosier, M.C., Emerson, M.R., LeVine, S.M., Marquis, J.G., 2005. Statistical analysis of data from studies on experimental autoimmune encephalomyelitis. J. Neuroimmunol. 170, 71-84.

Franklin, R.J., Ffrench-Constant, C., 2008. Remyelination in the CNS: from biology to therapy. Nat. Rev. Neurosci. 9, 839-855.

Gay, F.W., Drye, T.J., Dick, G.W., Esiri, M.M., 1997. The application of multifactorial cluster analysis in the staging of plaques in early multiple sclerosis. Identification and characterization of the primary demyelinating lesion. Brain 120 (Pt 8), 1461-1483.

Ghosh, S., Patel, N., Rahn, D., McAllister, J., Sadeghi, S., Horwitz, G., Berry, D., Wang, K.X., Swerdlow, R.H., 2007. The thiazolidinedione pioglitazone alters mitochondrial function in human neuron-like cells. Mol. Pharmacol. 71, 1695-1702.

Gijbels, D., Alders, G., Van, H.E., Charlier, C., Roelants, M., Broekmans, T., Eijnde, B.O., Feys, P., 2010. Predicting habitual walking performance in multiple sclerosis: relevance of capacity and self-report measures. Mult. Scler. 16, 618-626.

Goodman, A.D., Brown, T.R., Edwards, K.R., Krupp, L.B., Schapiro, R.T., Cohen, R., Marinucci, L.N., Blight, A.R., 2010. A phase 3 trial of extended release oral dalfampridine in multiple sclerosis. Ann. Neurol. 68, 494-502.

Goodman, A.D., Brown, T.R., Krupp, L.B., Schapiro, R.T., Schwid, S.R., Cohen, R., Marinucci, L.N., Blight, A.R., 2009. Sustained-release oral fampridine in multiple sclerosis: a randomised, double-blind, controlled trial. Lancet 373, 732-738.

Gray, E., Ginty, M., Kemp, K., Scolding, N., Wilkins, A., 2012. The PPAR-gamma agonist pioglitazone protects cortical neurons from inflammatory mediators via improvement in peroxisomal function. J. Neuroinflammation. 9, 63.

Greco, F.A., 1998. Evolving role of oral chemotherapy for the treatment of patients with neoplasms. Oncology (Williston. Park) 12, 43-50.

Grilli, S., 2006. Tamoxifen (TAM): the dispute goes on. Ann. Ist. Super. Sanita 42, 170-173.

Gugger, J.J., Cassagnol, M., 2008. Low-dose quetiapine is not a benign sedative-hypnotic agent. Am. J. Addict. 17, 454-455.

Harbo, H.F., Gold, R., Tintore, M., 2013. Sex and gender issues in multiple sclerosis. Ther. Adv. Neurol. Disord. 6, 237-248.

Hofstetter, H.H., Shive, C.L., Forsthuber, T.G., 2002. Pertussis toxin modulates the immune response to neuroantigens injected in incomplete Freund's adjuvant: induction of Th1 cells and experimental autoimmune encephalomyelitis in the presence of high frequencies of Th2 cells. J. Immunol. 169, 117-125.

Huitinga, I., van, R.N., de Groot, C.J., Uitdehaag, B.M., Dijkstra, C.D., 1990. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. J. Exp. Med. 172, 1025-1033.

Jacobs, L.D., Cookfair, D.L., Rudick, R.A., Herndon, R.M., Richert, J.R., Salazar, A.M. et al., 1996. Intramuscular interferon beta-1a for disease progression in relapsing multiple sclerosis. The Multiple Sclerosis Collaborative Research Group (MSCRG). Ann. Neurol. 39, 285-294.

Johnson, K.P., Brooks, B.R., Cohen, J.A., Ford, C.C., Goldstein, J., Lisak, R.P., Myers, L.W., Panitch, H.S., Rose, J.W., Schiffer, R.B., 1995. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase III multicenter, double-blind placebo-controlled trial. The Copolymer 1 Multiple Sclerosis Study Group. Neurology 45, 1268-1276.

Jones, M.V., Nguyen, T.T., Deboy, C.A., Griffin, J.W., Whartenby, K.A., Kerr, D.A., Calabresi, P.A., 2008. Behavioral and pathological outcomes in MOG 35-55 experimental autoimmune encephalomyelitis. J. Neuroimmunol. 199, 83-93.

Jordan, V.C., 1993. Fourteenth Gaddum Memorial Lecture. A current view of tamoxifen for the treatment and prevention of breast cancer. Br. J. Pharmacol. 110, 507-517.

Jordan, V.C., Brodie, A.M., 2007. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids 72, 7-25.

Kaiser, C.C., Shukla, D.K., Stebbins, G.T., Skias, D.D., Jeffery, D.R., Stefoski, D., Katsamakis, G., Feinstein, D.L., 2009. A pilot test of pioglitazone as an add-on in patients with relapsing remitting multiple sclerosis. J. Neuroimmunol. 211, 124-130.

Kallfass, C., Ackerman, A., Lienenklaus, S., Weiss, S., Heimrich, B., Staeheli, P., 2012. Visualizing production of beta interferon by astrocytes and microglia in brain of La Crosse virus-infected mice. J. Virol. 86, 11223-11230.

Kalyvas, A., David, S., 2004. Cytosolic phospholipase A2 plays a key role in the pathogenesis of multiple sclerosis-like disease. Neuron 41, 323-335.

Kanwar, J.R., Kanwar, R.K., Krissansen, G.W., 2004. Simultaneous neuroprotection and blockade of inflammation reverses autoimmune encephalomyelitis. Brain 127, 1313-1331.

Kaundal, R.K., Iyer, S., Kumar, A., Sharma, S.S., 2009. Protective effects of pioglitazone against global cerebral ischemic-reperfusion injury in gerbils. J. Pharmacol. Sci. 109, 361-367.

Kelleher, K.J., Spence, W., Solomonidis, S., Apatsidis, D., 2010. The characterisation of gait patterns of people with multiple sclerosis. Disabil. Rehabil. 32, 1242-1250.

Keough, M.B., Jensen, S.K., Yong, V.W., 2015. Experimental demyelination and remyelination of murine spinal cord by focal injection of lysolecithin. J. Vis. Exp.

Kiefer, R., Schweitzer, T., Jung, S., Toyka, K.V., Hartung, H.P., 1998. Sequential expression of transforming growth factor-beta1 by T-cells, macrophages, and microglia in rat spinal cord during autoimmune inflammation. J. Neuropathol. Exp. Neurol. 57, 385-395.

Kieseier, B.C., Storch, M.K., Archelos, J.J., Martino, G., Hartung, H.P., 1999. Effector pathways in immune mediated central nervous system demyelination. Curr. Opin. Neurol. 12, 323-336.

Kisanga, E.R., Gjerde, J., Guerrieri-Gonzaga, A., Pigatto, F., Pesci-Feltri, A., Robertson, C., Serrano, D., Pelosi, G., Decensi, A., Lien, E.A., 2004. Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. Clin. Cancer Res. 10, 2336-2343.

Klose, J., Schmidt, N.O., Melms, A., Dohi, M., Miyazaki, J., Bischof, F., Greve, B., 2013. Suppression of experimental autoimmune encephalomyelitis by interleukin-10 transduced neural stem/progenitor cells. J. Neuroinflammation. 10, 117.

Koch, M.W., Cutter, G., Stys, P.K., Yong, V.W., Metz, L.M., 2013. Treatment trials in progressive MS--current challenges and future directions. Nat. Rev. Neurol. 9, 496-503.

Kornek, B., Storch, M.K., Weissert, R., Wallstroem, E., Stefferl, A., Olsson, T., Linington, C., Schmidbauer, M., Lassmann, H., 2000. Multiple sclerosis and chronic autoimmune encephalomyelitis: a comparative quantitative study of axonal injury in active, inactive, and remyelinated lesions. Am. J. Pathol. 157, 267-276.

Kotter, M.R., Setzu, A., Sim, F.J., van, R.N., Franklin, R.J., 2001. Macrophage depletion impairs oligodendrocyte remyelination following lysolecithin-induced demyelination. Glia 35, 204-212.

Kurnellas, M.P., Li, H., Jain, M.R., Giraud, S.N., Nicot, A.B., Ratnayake, A., Heary, R.F., Elkabes, S., 2010. Reduced expression of plasma membrane calcium ATPase 2 and collapsin response mediator protein 1 promotes death of spinal cord neurons. Cell Death. Differ. 17, 1501-1510.

Kurnellas, M.P., Nicot, A., Shull, G.E., Elkabes, S., 2005. Plasma membrane calcium ATPase deficiency causes neuronal pathology in the spinal cord: a potential mechanism for neurodegeneration in multiple sclerosis and spinal cord injury. FASEB J. 19, 298-300.

Kurtzke, J.F., 1983. Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS). Neurology 33, 1444-1452.

Laplaud, D.A., Leray, E., Barriere, P., Wiertlewski, S., Moreau, T., 2006. Increase in multiple sclerosis relapse rate following in vitro fertilization. Neurology 66, 1280-1281.

Larocca, N.G., 2011. Impact of walking impairment in multiple sclerosis: perspectives of patients and care partners. Patient. 4, 189-201.

Leucht, S., Cipriani, A., Spineli, L., Mavridis, D., Orey, D., Richter, F. et al., 2013. Comparative efficacy and tolerability of 15 antipsychotic drugs in schizophrenia: a multiple-treatments meta-analysis. Lancet 382, 951-962.

Lim, Y.C., Desta, Z., Flockhart, D.A., Skaar, T.C., 2005. Endoxifen (4-hydroxy-N-desmethyl-tamoxifen) has anti-estrogenic effects in breast cancer cells with potency similar to 4-hydroxy-tamoxifen. Cancer Chemother. Pharmacol. 55, 471-478.

Lincoff, A.M., Wolski, K., Nicholls, S.J., Nissen, S.E., 2007. Pioglitazone and risk of cardiovascular events in patients with type 2 diabetes mellitus: a meta-analysis of randomized trials. JAMA 298, 1180-1188.

Loma, I., Heyman, R., 2011. Multiple sclerosis: pathogenesis and treatment. Curr. Neuropharmacol. 9, 409-416.

Lublin, F.D., Reingold, S.C., 1996. Defining the clinical course of multiple sclerosis: results of an international survey. National Multiple Sclerosis

Society (USA) Advisory Committee on Clinical Trials of New Agents in Multiple Sclerosis. Neurology 46, 907-911.

Lue, L.F., Rydel, R., Brigham, E.F., Yang, L.B., Hampel, H., Murphy, G.M., Jr., Brachova, L., Yan, S.D., Walker, D.G., Shen, Y., Rogers, J., 2001. Inflammatory repertoire of Alzheimer's disease and nondemented elderly microglia in vitro. Glia 35, 72-79.

Luo, J., Ho, P.P., Buckwalter, M.S., Hsu, T., Lee, L.Y., Zhang, H., Kim, D.K., Kim, S.J., Gambhir, S.S., Steinman, L., Wyss-Coray, T., 2007. Glia-dependent TGF-beta signaling, acting independently of the TH17 pathway, is critical for initiation of murine autoimmune encephalomyelitis. J. Clin. Invest 117, 3306-3315.

Maeda, N., Takahashi, M., Funahashi, T., Kihara, S., Nishizawa, H., Kishida, K. et al., 2001. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. Diabetes 50, 2094-2099.

Martin, L., Hutchens, M., Hawkins, C., Radnov, A., 2017. How much do clinical trials cost? Nature Reviews Drug Discovery 16, 381-382.

Mei, F., Fancy, S.P.J., Shen, Y.A., Niu, J., Zhao, C., Presley, B. et al., 2014. Micropillar arrays as a high-throughput screening platform for therapeutics in multiple sclerosis. Nat. Med. 20, 954-960.

Mei, F., Guo, S., He, Y., Wang, L., Wang, H., Niu, J., Kong, J., Li, X., Wu, Y., Xiao, L., 2012. Quetiapine, an atypical antipsychotic, is protective against autoimmune-mediated demyelination by inhibiting effector T cell proliferation. PLoS. One. 7, e42746.

Menalled, L., El-Khodor, B.F., Patry, M., Suarez-Farinas, M., Orenstein, S.J., Zahasky, B. et al., 2009. Systematic behavioral evaluation of Huntington's disease transgenic and knock-in mouse models. Neurobiol. Dis. 35, 319-336.

Mendel, I., Kerlero de, R.N., Ben-Nun, A., 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. Eur. J. Immunol. 25, 1951-1959.

Milo, R., Kahana, E., 2010. Multiple sclerosis: geoepidemiology, genetics and the environment. Autoimmun. Rev. 9, A387-A394.

Mitra, N.K., Bindal, U., Eng, H.W., Chua, C.L., Tan, C.Y., 2015. Evaluation of locomotor function and microscopic structure of the spinal cord in a mouse model of experimental autoimmune encephalomyelitis following treatment with syngeneic mesenchymal stem cells. Int. J. Clin. Exp. Pathol. 8, 12041-12052.

Moore, S.M., Khalaj, A.J., Kumar, S., Winchester, Z., Yoon, J., Yoo, T., Martinez-Torres, L., Yasui, N., Katzenellenbogen, J.A., Tiwari-Woodruff, S.K., 2014. Multiple functional therapeutic effects of the estrogen receptor beta agonist indazole-Cl in a mouse model of multiple sclerosis. Proc. Natl. Acad. Sci. U. S. A 111, 18061-18066.

Motl, R.W., 2013. Ambulation and multiple sclerosis. Phys. Med. Rehabil. Clin. N. Am. 24, 325-336.

Najm, F.J., Madhavan, M., Zaremba, A., Shick, E., Karl, R.T., Factor, D.C. et al., 2015. Drug-based modulation of endogenous stem cells promotes functional remyelination in vivo. Nature 522, 216-220.

Nakamura, T., Imai, Y., Matsumoto, T., Sato, S., Takeuchi, K., Igarashi, K. et al., 2007. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. Cell 130, 811-823.

Negrotto, L., Farez, M.F., Correale, J., 2016. Immunologic Effects of Metformin and Pioglitazone Treatment on Metabolic Syndrome and Multiple Sclerosis. JAMA Neurol. 73, 520-528.

Nicot, A., Ratnakar, P.V., Ron, Y., Chen, C.C., Elkabes, S., 2003. Regulation of gene expression in experimental autoimmune encephalomyelitis indicates early neuronal dysfunction. Brain 126, 398-412.

Niino, M., Iwabuchi, K., Kikuchi, S., Ato, M., Morohashi, T., Ogata, A., Tashiro, K., Onoe, K., 2001. Amelioration of experimental autoimmune encephalomyelitis in C57BL/6 mice by an agonist of peroxisome proliferator-activated receptor-gamma. J. Neuroimmunol. 116, 40-48.

Nikic, I., Merkler, D., Sorbara, C., Brinkoetter, M., Kreutzfeldt, M., Bareyre, F.M., Bruck, W., Bishop, D., Misgeld, T., Kerschensteiner, M., 2011. A reversible form of axon damage in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat. Med. 17, 495-499.

O'Connor, P., Wolinsky, J.S., Confavreux, C., Comi, G., Kappos, L., Olsson, T.P., Benzerdjeb, H., Truffinet, P., Wang, L., Miller, A., Freedman, M.S., 2011. Randomized trial of oral teriflunomide for relapsing multiple sclerosis. N. Engl. J. Med. 365, 1293-1303.

O'Sullivan, D., Green, L., Stone, S., Zareie, P., Kharkrang, M., Fong, D., Connor, B., La Flamme, A.C., 2014. Treatment with the antipsychotic agent, risperidone, reduces disease severity in experimental autoimmune encephalomyelitis. PLoS. One. 9, e104430.

Olivan, S., Calvo, A.C., Rando, A., Munoz, M.J., Zaragoza, P., Osta, R., 2015. Comparative study of behavioural tests in the SOD1G93A mouse model of amyotrophic lateral sclerosis. Exp. Anim 64, 147-153.

Oprea, T.I., Bauman, J.E., Bologa, C.G., Buranda, T., Chigaev, A., Edwards, B.S. et al., 2011. Drug Repurposing from an Academic Perspective. Drug Discov. Today Ther. Strateg. 8, 61-69.

Oprea, T.I., Mestres, J., 2012. Drug repurposing: far beyond new targets for old drugs. AAPS. J. 14, 759-763.

Paganini-Hill, A., Clark, L.J., 2000. Preliminary assessment of cognitive function in breast cancer patients treated with tamoxifen. Breast Cancer Res. Treat. 64, 165-176.

Pajoohesh-Ganji, A., Byrnes, K.R., Fatemi, G., Faden, A.I., 2010. A combined scoring method to assess behavioral recovery after mouse spinal cord injury. Neurosci. Res. 67, 117-125.

Park, S.W., Yi, J.H., Miranpuri, G., Satriotomo, I., Bowen, K., Resnick, D.K., Vemuganti, R., 2007. Thiazolidinedione class of peroxisome proliferator-activated receptor gamma agonists prevents neuronal damage, motor dysfunction, myelin loss, neuropathic pain, and inflammation after spinal cord injury in adult rats. J. Pharmacol. Exp. Ther. 320, 1002-1012.

Pascual, G., Fong, A.L., Ogawa, S., Gamliel, A., Li, A.C., Perissi, V., Rose, D.W., Willson, T.M., Rosenfeld, M.G., Glass, C.K., 2005. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. Nature 437, 759-763.

Pershadsingh, H.A., Heneka, M.T., Saini, R., Amin, N.M., Broeske, D.J., Feinstein, D.L., 2004. Effect of pioglitazone treatment in a patient with secondary multiple sclerosis. J. Neuroinflammation. 1, 3.

Peterson, E.W., Cho, C.C., Finlayson, M.L., 2007. Fear of falling and associated activity curtailment among middle aged and older adults with multiple sclerosis. Mult. Scler. 13, 1168-1175.

Peymani, M., Ghaedi, K., Hashemi, M.S., Ghoochani, A., Kiani-Esfahani, A., Nasr-Esfahani, M.H., Baharvand, H., 2018. Ameliorating the Effect of Pioglitazone on LPS-Induced Inflammation of Human Oligodendrocyte Progenitor Cells. Cell Mol. Neurobiol. 38, 517-527.

Pfutzner, A., Forst, T., 2006. Pioglitazone: an antidiabetic drug with the potency to reduce cardiovascular mortality. Expert. Opin. Pharmacother. 7, 463-476.

Pluchino, S., Peruzzotti-Jametti, L., 2016. Interleukin-4 induced 1 (IL4I1) promotes central nervous system remyelination. Brain 139, 3052-3054.

Ransohoff, R.M., 2012. Animal models of multiple sclerosis: the good, the bad and the bottom line. Nat. Neurosci. 15, 1074-1077.

Reid, J.M., Goetz, M.P., Buhrow, S.A., Walden, C., Safgren, S.L., Kuffel, M.J., Reinicke, K.E., Suman, V., Haluska, P., Hou, X., Ames, M.M., 2014. Pharmacokinetics of endoxifen and tamoxifen in female mice: implications for comparative in vivo activity studies. Cancer Chemother. Pharmacol. 74, 1271-1278.

Rivers, T.M., Sprunt, D.H., Berry, G.P., 1933. OBSERVATIONS ON ATTEMPTS TO PRODUCE ACUTE DISSEMINATED ENCEPHALOMYELITIS IN MONKEYS. J. Exp. Med. 58, 39-53.

Rizzo, J.A., Friedkin, R., Williams, C.S., Nabors, J., Acampora, D., Tinetti, M.E., 1998. Health care utilization and costs in a Medicare population by fall status. Med. Care 36, 1174-1188.

Sanjabi, S., Zenewicz, L.A., Kamanaka, M., Flavell, R.A., 2009. Anti-inflammatory and pro-inflammatory roles of TGF-beta, IL-10, and IL-22 in immunity and autoimmunity. Curr. Opin. Pharmacol. 9, 447-453.

Schotte, A., Janssen, P.F., Gommeren, W., Luyten, W.H., Van, G.P., Lesage, A.S., De, L.K., Leysen, J.E., 1996. Risperidone compared with new and reference antipsychotic drugs: in vitro and in vivo receptor binding. Psychopharmacology (Berl) 124, 57-73.

Schutz, B., Reimann, J., Dumitrescu-Ozimek, L., Kappes-Horn, K., Landreth, G.E., Schurmann, B., Zimmer, A., Heneka, M.T., 2005. The oral antidiabetic pioglitazone protects from neurodegeneration and amyotrophic lateral sclerosis-like symptoms in superoxide dismutase-G93A transgenic mice. J. Neurosci. 25, 7805-7812.

Searcy, J.L., Phelps, J.T., Pancani, T., Kadish, I., Popovic, J., Anderson, K.L. et al., 2012. Long-term pioglitazone treatment improves learning and attenuates pathological markers in a mouse model of Alzheimer's disease. J. Alzheimers. Dis. 30, 943-961.

Selmi, C., 2008. The X in sex: how autoimmune diseases revolve around sex chromosomes. Best. Pract. Res. Clin. Rheumatol. 22, 913-922.

Semple, B.D., Noble-Haeusslein, L.J., 2011. Broad-spectrum neuroprotection against traumatic brain injury by agonism of peroxisome proliferator-activated receptors. Exp. Neurol. 229, 195-197.

Shah, P., Mudaliar, S., 2010. Pioglitazone: side effect and safety profile. Expert. Opin. Drug Saf 9, 347-354.

Shukla, D.K., Kaiser, C.C., Stebbins, G.T., Feinstein, D.L., 2010. Effects of pioglitazone on diffusion tensor imaging indices in multiple sclerosis patients. Neurosci. Lett. 472, 153-156.

Silva, G.A., Pradella, F., Moraes, A., Farias, A., dos Santos, L.M., de Oliveira, A.L., 2014. Impact of pregabalin treatment on synaptic plasticity and glial reactivity during the course of experimental autoimmune encephalomyelitis. Brain Behav. 4, 925-935.

Sleigh S, Barton C. Repurposing Strategies for Therapeutics. 24[3], 151-159. 2010. Pharmaceutical Medicine.
Ref Type: Generic

Smith, M.E., 1999. Phagocytosis of myelin in demyelinative disease: a review. Neurochem. Res. 24, 261-268.

Sormani, M.P., Bruzzi, P., 2015. Can we measure long-term treatment effects in multiple sclerosis? Nat. Rev. Neurol. 11, 176-182.

Spencer, R.P., 2014. Tecfidera((R)): an approach for repurposing. Pharm. Pat Anal. 3, 183-198.

Spittau, B., 2017. Interleukin 4-induced neuroprotection and regulation of microglia activation as a therapeutic approach in the MPTP model of Parkinson's disease. Neural Regen. Res. 12, 1433-1434.

Steiner, J., Martins-de-Souza, D., Schiltz, K., Sarnyai, Z., Westphal, S., Isermann, B. et al., 2014. Clozapine promotes glycolysis and myelin lipid synthesis in cultured oligodendrocytes. Front Cell Neurosci. 8, 384.

Steinman, L., Zamvil, S.S., 2006. How to successfully apply animal studies in experimental allergic encephalomyelitis to research on multiple sclerosis. Ann. Neurol. 60, 12-21.

Stys, P.K., Zamponi, G.W., van, M.J., Geurts, J.J., 2012. Will the real multiple sclerosis please stand up? Nat. Rev. Neurosci. 13, 507-514.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S., Mesirov, J.P., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U. S. A 102, 15545-15550.

Sugita, M., Kataoka, M., Sugihara, M., Takeuchi, S., Yamashita, S., 2014. Effect of excipients on the particle size of precipitated pioglitazone in the gastrointestinal tract: impact on bioequivalence. AAPS. J. 16, 1119-1127.

Takeuchi, C., Matsumoto, Y., Kohyama, K., Uematsu, S., Akira, S., Yamagata, K., Takemiya, T., 2013. Microsomal prostaglandin E synthase-1 aggravates inflammation and demyelination in a mouse model of multiple sclerosis. Neurochem. Int. 62, 271-280.

Taylor, T.N., Greene, J.G., Miller, G.W., 2010. Behavioral phenotyping of mouse models of Parkinson's disease. Behav. Brain Res. 211, 1-10.

Temboonkiat, S., Satyapan, N., Benjasuratwong, Y., Patarakitvanit, S., Tunkanitlert, J., Vudhironarit, T., Klangkalya, B., 2012. Clinical efficacy of pioglitazone: generic vs. original product. J. Med. Assoc. Thai. 95 Suppl 5, S58-S62.

Tontonoz, P., Nagy, L., Alvarez, J.G., Thomazy, V.A., Evans, R.M., 1998. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93, 241-252.

Trapp, B.D., Nave, K.A., 2008. Multiple sclerosis: an immune or neurodegenerative disorder? Annu. Rev. Neurosci. 31, 247-269.

Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mork, S., Bo, L., 1998. Axonal transection in the lesions of multiple sclerosis. N. Engl. J. Med. 338, 278-285.

Trapp, B.D., Stys, P.K., 2009. Virtual hypoxia and chronic necrosis of demyelinated axons in multiple sclerosis. Lancet Neurol. 8, 280-291.

van der Linden, M.L., Scott, S.M., Hooper, J.E., Cowan, P., Mercer, T.H., 2014. Gait kinematics of people with multiple sclerosis and the acute application of functional electrical stimulation. Gait. Posture. 39, 1092-1096.

Walczak, R., Tontonoz, P., 2002. PPARadigms and PPARadoxes: expanding roles for PPARgamma in the control of lipid metabolism. J. Lipid Res. 43, 177-186.

Wang, D.Y., Fulthorpe, R., Liss, S.N., Edwards, E.A., 2004. Identification of estrogen-responsive genes by complementary deoxyribonucleic acid microarray and characterization of a novel early estrogen-induced gene: EEIG1. Mol. Endocrinol. 18, 402-411.

Warford, J., Jones, Q.R., Nichols, M., Sullivan, V., Rupasinghe, H.P., Robertson, G.S., 2014. The flavonoid-enriched fraction AF4 suppresses neuroinflammation and promotes restorative gene expression in a mouse model of experimental autoimmune encephalomyelitis. J. Neuroimmunol. 268, 71-83.

Waxman, S.G., Craner, M.J., Black, J.A., 2004. Na+ channel expression along axons in multiple sclerosis and its models. Trends Pharmacol. Sci. 25, 584-591.

Wirths, O., Bayer, T.A., 2008. Motor impairment in Alzheimer's disease and transgenic Alzheimer's disease mouse models. Genes Brain Behav. 7 Suppl 1, 1-5.

Xu, H., Yang, H.J., Li, X.M., 2014. Differential effects of antipsychotics on the development of rat oligodendrocyte precursor cells exposed to cuprizone. Eur. Arch. Psychiatry Clin. Neurosci. 264, 121-129.

Xu, H., Yang, H.J., McConomy, B., Browning, R., Li, X.M., 2010. Behavioral and neurobiological changes in C57BL/6 mouse exposed to cuprizone: effects of antipsychotics. Front Behav. Neurosci. 4, 8.

Xu, J., Drew, P.D., 2007. Peroxisome proliferator-activated receptor-gamma agonists suppress the production of IL-12 family cytokines by activated glia. J. Immunol. 178, 1904-1913.

Yu, J.G., Javorschi, S., Hevener, A.L., Kruszynska, Y.T., Norman, R.A., Sinha, M., Olefsky, J.M., 2002. The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. Diabetes 51, 2968-2974.

Zareie, P., Connor, B., La Flamme, A.C., 2017. Amelioration of experimental autoimmune encephalomyelitis by clozapine is not associated with defective CD4 T cell responses. J. Neuroinflammation. 14, 68.

Zhang, Y., Xu, H., Jiang, W., Xiao, L., Yan, B., He, J., Wang, Y., Bi, X., Li, X., Kong, J., Li, X.M., 2008. Quetiapine alleviates the cuprizone-induced white matter pathology in the brain of C57BL/6 mouse. Schizophr. Res. 106, 182-191.

Zhu, J., Zhang, J., Ji, M., Gu, H., Xu, Y., Chen, C., Hu, N., 2013. The role of peroxisome proliferator-activated receptor and effects of its agonist, pioglitazone, on a rat model of optic nerve crush: PPARgamma in retinal neuroprotection. PLoS. One. 8, e68935.

Zorner, B., Filli, L., Reuter, K., Kapitza, S., Lorincz, L., Sutter, T. et al., 2016. Prolonged-release fampridine in multiple sclerosis: Improved ambulation effected by changes in walking pattern. Mult. Scler. 22, 1463-1475.

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Title: Pioglitazone is superior to quetiapine, clozapine and tamoxifen at alleviating experimental autoimmune encephalomyelitis in mice

Author: Matthew A.J. Chedrawe,Scott P. Holman,Anna-Claire Lamport,Turgay Akay,George S. Robertson

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