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Amino acid and biogenic amine concentrations during experimental autoimmune encephalomyelitis and the disease-modifying effects of phenelzine treatment

by

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ABSTRACT

The project described in this thesis began with a broad analysis of the changes to amino acid and biogenic amine concentrations in the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE) in mice, an animal model of Multiple Sclerosis (MS). That study identified deficits in specific neurotransmitters during EAE that I targeted pharmacologically using the antidepressant drug phenelzine. Phenelzine administration substantially influenced the concentrations of amino acids and biogenic amines in EAE mice in a manner likely to be therapeutic. In the final experiment, I treated EAE mice chronically with phenelzine; This treatment was associated with significant improvements in motor abilities compared to vehicle treated animals. In an open field, improvements were also observed in behavioural indices of depression, physical sickness and anxiety. The results of this thesis may offer new insights into the pathogenesis of EAE and MS and indicate the disease-modifying potential of phenelzine treatment in MS.

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LIST OF ABBREVIATIONS

5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine
ALA	Alanine
ALA_T	Alanine transaminase
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	One-way analysis of variance
APC	Antigen presenting cell
ARG	Arginine
ASP	Aspartate
CFA	Complete Freund's Adjuvant
cm	Centimetre
CNS	Central nervous system
CSF	Cerebrospinal fluid
DSER	D-Serine
EAE	Experimental autoimmune encephalomyelitis
GABA	Gamma-aminobutyric acid
$GABA_T$	GABA transaminase
GAT-1	GABA Transporter-1
GLN	Glutamine
GLU	Glutamate
GLY	Glycine
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
IBC	N-isobutyryl-L-cysteine
IDO	Indoleamine 2,3-dioxygenase
IFNγ	Interferon gamma
IL-17	Interleukin-17
IL-1β	Interleukin-1 beat
kg	Kilogram
LPS	Lipopolysaccharide
LSER	L-Serine

MAO	Monoamine oxidase
MeOH	Methanol
mg	Milligram
MHPG	3-Methoxy-4-hydroxyphenylglycol
MOG_{35-55}	myelin oligodendrocyte glycoprotein 35-55
MS	Multiple Sclerosis
NA	Noradrenaline
NMDA	*N*-Methyl-D-aspartic acid
NO	Nitric oxide
OPA	*Ortho*-pthaldialdehyde
PEH	Phenylethylidenehydrazine
PLP	Proteolipid protein
PLZ	Phenelzine
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
TAUR	Taurine
THF	Tetrahydrofuran
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TRYPT	Tryptophan
α	Alpha
β	Beta
γ	Gamma

CHAPTER 1 GENERAL INTRODUCTION

1.1 Multiple Sclerosis (MS)

Multiple Sclerosis (MS) is the most common neurological disease leading to disability in adults in the developed world, with a prevalence in the Canadian population of 240 cases per 100 000 people and affecting over 2.5 million people in the general population [1-3]. MS typically afflicts young adults and affects women at a threefold higher rate than men [4-5]. Disease frequency generally increases with distance north or south of the equator [6]. With a median survival time of 30 years, consequent losses to quality of life and employability are of concern to not only afflicted individuals and immediate family but furthermore to society at large [5].

MS presents clinically in a variety of basic forms. Most patients are initially diagnosed with a Relapsing-Remitting (RRMS) form of MS, with intermittent neurological attacks and full recoveries. Over time, however, disability accumulates as relapses are followed by only partial recovery. Most RRMS patients eventually enter a phase of progressive deterioration known as secondary progressive MS [5]. Approximately 20% of MS patients are initially diagnosed with a progressive disease [7].

Using histological and imaging methods, MS can be characterized by the hallmark presence of sclerotic plaques that act as lesions in the CNS. Active MS plaques consist of demyelination, oligodendrocyte loss, gliosis and an active immune response generated by accumulating immune cells from the periphery. Inflammatory lesions are allowed for by local breakdown of the blood-brain barrier and consist largely of CD8+ T cells [5, 8-9]. While they distribute randomly throughout the CNS, plaques and active sites of inflammatory activity are found most commonly in perivascular regions. Inactive "shadow" plaques on imaging scans represent areas where remyelination has occurred to some extent [5]. Although MS is historically thought of as a white matter disease, plaques are also consistently found in gray matter such as the cerebral cortex. Gray matter issues of neuronal death, axon transaction, and losses in synaptic density are critical aspects of the disease process and underlie the progression of disability [2, 5].

While losses in motor function are perhaps the most recognizable features of MS, patients also present with a wide variety of physiological, sensory and psychiatric disturbances. The diversity of symptoms reflects the seemingly random appearance and

distribution of lesions in the CNS. These symptoms can profoundly affect quality of life, especially when existing in concert [10-11]. Pain syndromes are common in MS, affecting up to 86% of MS patients, and include issues with chronic pain, neuropathic pain, headache, as well as pain associated with spasticity [10]. Depression and fatigue are also major complaints, affecting over 50% and up to 88% of patients respectively [12-13]. The distinction between depression and fatigue in MS patients, however, is difficult to delineate, albeit it has been reported recently that measures of depression are more strongly linked to fatigue and sleep disturbances than to sickness-associated mechanisms [14]. Pain, depression, and fatigue in MS also frequently present in clusters that synergistically affect patient quality of life [15-17]. Additionally, excessive psychological stress is common in MS patients [18]. While not thought to directly exacerbate the disease, stress can make coping with symptoms far more difficult [1].

1.2 Experimental Autoimmune Encephalomyelitis (EAE)

Many important insights into the mechanisms of MS and the development of new therapies come from research into experimental autoimmune encephalomyelitis (EAE), an animal model of neurological autoimmune disease. In most EAE models, animals are given one or more injections containing an emulsion of one or more CNS antigens along with an adjuvant. The antigen targeted is most often a myelin protein. The autoimmune disease produced can have a variety of symptoms but is most recognized for an ascending paralysis that displays variable degrees of remittances [19]. The major measurement of EAE is a clinical score based on the rate and severity of motor disability accumulation. Importantly, the choice of animal species, animal strain, antigen, and adjuvant can dramatically affect the resulting EAE phenotype, producing a range of diseases from mild, with a clear series of relapses and remittances, to quick, progressive and severe [19-20]. The choice of EAE model also affects the relative amounts of inflammation and demyelination [20]. Observations from our lab (unpublished observations) and others further show that varying the strength of adjuvant or antigen also profoundly affects disease course and EAE-associated symptoms [21]. While not all EAE treatments have translated well to humans, research using the model has resulted in three of the major MS treatments used clinically, ie.

glatiramer acetate, natilizumab, and mitoxantrone, in addition to having a major role in increasing the understanding of immune surveillance and neuroinflammation [19].

EAE has been intensely studied as a model of MS because of shared pathological features. The presence of inflammatory lesions and regions of demyelination reminiscent of MS plaques are perhaps the most important commonality. As in MS, these lesions have a greater propensity for peri-vascular regions and are composed chiefly of peripherally derived macrophages and T lymphocytes. In EAE, however, infiltrating CD4+ T cells are far more common than CD8+ cells. While it is difficult to identify resident macrophages and microglia from those invading from the periphery, these cells hypertrophy during EAE and show large increases in number. Similarly, astrocytes show a substantial increase in activation and proliferation. Lesions in EAE also distribute randomly and can show variable degrees of remyelination [19]. Reflecting the ascending pattern of motor deficits, however, lesions are much more common in the spinal cord and brainstem than in more rostral structures [19-20].

As mentioned previously, the most common experimental outcome in EAE is a clinical score based on the animal's ability to maintain normal locomotion and reflexes. Locomotor ability is also commonly evaluated with the rotorod, a rotating, narrow beam that the animal must walk to stay on. Like MS, however, EAE is additionally accompanied by changes in various non-motor neurological systems. Work in our laboratory, for example, has shown that signs of neuropathic pain are present in EAE, as evidenced by mechanical allodynia and thermal hyperalgesia [22]. Large behavioural changes indicative of sickness behaviour and depression are present, as is evidence of cognitive impairment [23-25]. Signs of rarer neurological dysfunctions in EAE mice such as spasticity and ataxia have also been observed (unpublished observations).

1.3 Immune mechanisms in the pathology of MS and EAE

Although the precise causes of MS are uncertain, both environmental factors and genetic susceptibility have a role in the risk of developing MS. Observations that MS frequency increases with north or south distance from the equator and that disease risk increases substantially if a direct relative is afflicted exemplify this. Despite this uncertainty and the inherent heterogeneity of MS patients, it is widely believed that

pathogenesis begins with autoreactive T cells crossing the blood-brain barrier and initiating a local immune response against host myelin or neuronal components [5]. Along with inflammatory processes, neurodegenerative mechanisms are also crucial and may act independently of inflammation, especially early in disease [5, 25-26]. Inflammation is often more associated with RRMS whereas neurodegenerative processes better reflect primary and secondary progressive phases of MS [5]. This is supported by observations that anti-inflammatory treatments are beneficial if given early in the disease. In patients with progressive MS, these treatments effectively decrease inflammation and plaque load but do not alter the progression of neurological deficits [27]. As disease course proceeds, inflammatory and neurodegenerative mechanisms interact as damaged neurons increase the immune responses that, in turn, further damage neuronal components [20, 26].

Initiation of MS and EAE pathogenesis depends on activated, autoreactive T cells entering into the CNS, coupled with a failure of suppressive regulatory mechanisms that allows for a self-directed immune response [5]. Naive, autoreactive T-cells are first activated in the periphery by specialized antigen presenting cells (APC's) such as dendritic cells. Peripheral activation of T cells is bypassed in some EAE models, where disease can be induced by transplanting autoreactive T cells that are activated in-vitro. After entering the CNS, other APC's such as macrophages, microglia, and astrocytes, which upregulate immune molecules in response to inflammatory stimuli, reactivate T cells which then begin to clonally expand and secrete cytokines and chemokines. The specific cytokine environment during activation determines the phenotype of activated T cells [20]. In MS and EAE, the predominant cytokine environment favours T cell activation into the pro-inflammatory Th17 subclass [28]. The presence of T cells that are reactive to CNS antigens, however, is not enough to initiate MS, since they can be found in both MS patients and healthy subjects. It is consequently hypothesized that a failure of normal regulatory mechanisms to suppress effector T cell activity, namely by regulatory T cells, is a critical component of pathogenesis. This is supported by studies showing that the effectiveness of regulatory T lymphocytes in suppressing cytotoxic T cell activity is deficient in cells taken from RRMS patients [5, 29].

Activated T lymphocytes are capable of damaging neurons directly and through the cytokines they secrete. Pro-inflammatory cytokines and the initial damage to neurons also have the critical role of propagating the immune response by recruiting and activating macrophages/microglia and activating astrocytes [20]. The absolute number of microglia/macrophages in the CNS is a major factor correlating with acute disease severity [30]. Activated macrophages/microglia then release more pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) that amplify the immune response, promote neurodegenerative processes, and alter synaptic transmission [31-33]. Recently, microglial TNFα was linked to enhanced glutamatergic synaptic activity in the striatum during the EAE disease process, even at pre-clinical disease stages. This study linked increased activity of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors to these synaptic changes and also suggested that cytokines have roles in EAE associated cognitive impairments and potential excitotoxic neurodegeneration [34]. Hippocampal atrophy and a loss of GABAergic interneurons are also observed during EAE and underlie deficits in learning and spatial memory abilities. This occurs with relatively few infiltrating immune cells but on a background of demyelination and microglial activation [25]. Interestingly, T lymphocyte infiltration into the spinal cord and the number of activated microglia is also strongly associated with the development of neuropathic pain in other animal models [35-36]. A variety of other soluble factors that damage neurons and oligodendrocytes such as antibodies, reactive oxygen species (ROS), nitric oxide (NO), and complement are also released from lymphocytes, macrophages/microglia, and astrocytes [26]. It is noteworthy though, that neuroprotective activities can also be displayed by these cells [20].

1.4 Neuropathological role of demyelination in MS and EAE

Extensive loss of oligodendrocytes via apoptic and necrotic cell death mechanisms and subsequent demyelination is associated with MS [37-38]. Oligodendrocyte injury can be mediated directly by T cells, B cells and activated glia through Fas ligand interactions and indirectly through the release of cytokines, complement, ROS, NO, and glutamate [26, 39-41].

Demyelination contributes both to neurological deficits and the neurodegenerative process. Axonal demyelination impairs saltatory conduction, resulting in a diminished ability to conduct fast trains of impulses, in slower speeds of action potential conduction or in conduction failure [5]. Impaired motor abilities, decreased sensory function, cognitive dysfunction, and fatigue are foreseeable consequences of this. Additionally, demyelinated axons are capable of spontaneously discharging and cross talk which may explain abnormal sensations in some patients such as the presence of allodynia [5]. Remyelination of axons is associated with disease remittances. However, over time the capacity for remyelination decreases because injured oligodendrocytes die and are not effectively replaced [2, 5].

While axonal injury can occur without myelin loss, chronic demyelination directly influences neurodegenerative processes. In healthy myelinated axons, voltage gated Na^+ channels are expressed mostly in the nodes of Ranvier while voltage gated K^+ channels are found mainly under the myelin sheath. This distribution enables effective saltatory conduction by minimizing current leak through K^+ channels and by placing the lowest membrane capacitance in the nodes [42]. In response to chronic demyelination, however, Na^+ channels upregulate and their expression re-distributes along the axon, allowing for a greater entry of ions upon activation. The greater ion flux across the membrane can disturb the intracellular ionic homeostasis and increase energy demand for ionic regulation [27, 42]. This can alter resting membrane potential, triggering a lethal increase in Ca^{2+} concentration from extracellular and releasable intracellular sources. Mitochondrial dysfunction, which occurs early in EAE, is also a potent source ROS and apoptotic stimuli like Ca^{2+} and cytochrome c [27, 42].

1.5 Neuropathological role of excess excitatory signalling in MS and EAE

Increased glutamatergic activity and excitoxicity is a potent neurodegenerative stimulus in MS, EAE and other neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), and Parkinson's disease [26, 40]. In the excitoxic process, over-activation of AMPA, kainate, and *N*-methyl-D-aspartic acid (NMDA) glutamate receptors causes prolonged and excessive Ca^{2+} influxes that can initiate apoptosis. In addition to neuronal expression, glutamate receptors are also found on

microglia, astrocytes and oligodendrocytes in the gray and white matter [27, 42]. Glutamate concentrations are known to increase in the brain and CSF during MS, and increases in glutamatergic synaptic activity occur during EAE [40, 43-46]. During MS and EAE, in addition to the neuronal glutamate released into synapses, a substantial amount of glutamate is also released from activated astrocytes and macrophages/microglia [40, 47-48]. Activated astrocytes further exacerbate glutamatergic activity in MS and EAE through the downregulation of glutamate transporters. Astrocytic glutamate transporters are largely responsible for the tight regulation of extracellular glutamate concentrations and their downregulation allows glutamate levels to rise inappropriately in synaptic and extra-synaptic spaces [26, 40-41, 49]. The downregulation of glutamate transporters correlates with microglial activation and synaptic protein loss in MS lesions [50]. Simultaneously, AMPA, kainate and NMDA glutamate receptors are upregulated [51]. The selective blocking of different glutamate receptors and limiting of neurotransmission with compounds like memantine, MK-801 and riluzole is neuroprotective in MS and EAE. These compounds can improve disease without significantly altering inflammatory measures like T lymphocyte infiltration [26, 52-55]. Other notable compounds such as D-serine and glycine are also potentially excitotoxic because they are NMDA receptor coagonists that modulate the receptor's sensitivity to glutamate. Excess glutamate receptor activity is also linked to the development of neuropathic pain in EAE, and this can be reversed by antagonizing glutamate receptors or maintaining glutamate transporter activity [56-57].

1.6 Neuropathological role of decreased inhibition in MS and EAE

Decreased inhibitory activity is an additional contributing factor to both neurodegenerative and immune processes in MS and EAE. GABA concentrations decrease during MS and EAE and there is a further loss of GABAergic interneurons and inhibitory synaptic activity [58-60]. Downregulation of transcripts associated with GABAergic transmission is also found in normal appearing cortex of MS patients [61]. Furthermore, the brains of MS patients are hyperexcitable compared to control brains, indicating decreased GABAergic inhibitory activity [62]. The imbalance in excitatory and inhibitory neuronal activity suggested by EAE-induced changes in glutamate and GABA

potentiate excitotoxic processes and contribute to clinical symptoms like pain and spasticity.

GABAergic activity is also critical because it directly suppresses immunological activity. In human tissue, strong expression of GABA receptors and enzymes for GABA synthesis and degradation was identified in astrocytes. Microglia were not found to be GABA synthetic but possessed the receptors and intracellular enzymes necessary to be considered GABA receptive. Furthermore, microglial inflammatory processes were inhibited by GABAergic activity [63]. Similar findings showing that GABAergic activity inhibits the production of pro-inflammatory cytokines by APCs were found by Bhat et. al (2010). This group additionally found that treatment with GABAergic agents suppressed EAE disease progression. Notably, an inhibition of cytokine production by GABAergic agents was not observed on purified T cells but their immunological activity could be suppressed indirectly through GABAergic effects on APCs [64]. An earlier study by Wang et. al. also found that knockout of GABA transporter-1 (GAT-1) exacerbated EAE and increased inflammation [65]. The expression of GAT-1 in their study, however, was restricted to activated T cells and was not found in macrophages, which conflicts with the finding of GAT-1 in macrophages by Bhat et. al. (2010).

1.7 Objectives

This thesis consists of the results of three experiments. The major objective of the first experiment was to characterize changes in the concentrations of amino acids and biogenic amines in the CNS during the disease progression of the MOG_{35-55} EAE model. Amino acid and biogenic amines previously examined in the scientific literature were evaluated as well as some substances never examined in the context of MS and EAE. Based on the observed changes, I further aimed to categorize these substances into groups whose concentrations change together. These distinct groups may reflect similar biological processes. Changes in neurotransmitters and metabolites as they pertain to disease progression, inflammation, and neurodegeneration are the main topics of this experiment. Heterogeneity of MS and EAE is also relevant.

The changes observed in amino acid and biogenic amine concentrations in chapter 2 are the basis for the experiment in Chapter 3. This chapter's main objective

was to target specific deficiencies in neurotransmitter concentrations during EAE with the antidepressant and anxiolytic drug phenelzine (PLZ) and evaluate how an acute administration affects amino acid and biogenic amine concentrations in the CNS. The biological rationale for selecting PLZ will be discussed.

In the final experimental chapter, I show the effects of daily PLZ administration on EAE disease progression and disease-associated symptoms. The effects of PLZ during EAE are evaluated on gross motor coordination, locomotor ability and, in an open field assay that I developed for this experiment, on behavioural measures indicative of sickness, depression and anxiety. A more detailed discussion of depression and sickness behaviour in MS and EAE is included. Additionally, this section will include the rationale behind the open field assay and a discussion of some of its limitations.

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CHAPTER 2 TISSUE CONCENTRATION CHANGES OF AMINO ACIDS AND BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEM DURING EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

2. 1 Introduction

The homeostasis of various neurotransmitters in the central nervous system (CNS) is disturbed during the pathogenesis of Multiple Sclerosis (MS) and this disruption may underlie a variety of the neurological symptoms that the disease can present with. Recent evidence further shows that many of these neurotransmitters can directly regulate activities of the immune system [1-5]. The major excitatory neurotransmitter glutamate, for example, is well known to be released by and act on neurons in addition to non-neuronal cells such as astrocytes, macrophages, microglia and T cells [1, 6-9]. γ-Aminobutyric acid (GABA), which can be released from neuronal and astrocytic sources, is a critical component of inhibitory neurotransmission that has additional anti-inflammatory effects on astrocytes, macrophages/microglia, and T cells [2-3, 10-11]. In a neurodegenerative disorder such as MS that is largely believed to be mediated by the immune system, an understanding of how neurotransmitter concentrations are altered in the CNS is consequently important for understanding disease mechanisms and identifying areas that may warrant further research for therapeutic targeting.

Previous studies examining neurotransmitter concentration changes in MS have been conducted in the cerebrospinal fluid (CSF) and plasma of MS patients. Increased concentrations of the excitatory neurotransmitters glutamate and aspartate in MS patients were strong indicators of excitotoxic processes in this disease. The relationship of these increased concentrations to MS patients with active disease was further suggestive of pathological mechanisms associated with clinical deficits [12-14]. Decreases in CSF concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the metabolite of 5-hydroxytryptamine (5-HT; serotonin), that relate to the rate of disability accumulation indicate that deficits in central serotonergic activity appear to be another important feature of MS [15].

Concentration changes of neurotransmitters in the CNS have also been directly evaluated in experimental autoimmune encephalomyelitis (EAE), an animal model frequently used to study MS. These studies show EAE-associated concentration changes in neurotransmitters such as glycine and GABA that normalize during remissions [16-18].

In contrast to this, spinal cord concentrations of glutamate, 5-HT and noradrenaline are reduced during EAE and do not recover during remissions [16, 19-21]. The relationships of neurotransmitter concentration changes to disability in EAE therefore suggest that different neurotransmitter systems may have different roles in the inflammatory and neurodegenerative aspects of the disease.

MS patients are recognized as clinically heterogeneous according to features such as their different disease progressions and responsiveness to therapies [22-24]. Differences in etiology and genetics that influence the inflammatory and neurodegenerative mechanisms may underlie this variability [22]. Heterogeneity is also inherent in EAE models as the choice of animal species, strain, antigen, and adjuvant can drastically affect disease progression [25-27]. The neurotransmitter concentration studies by Krenger et al. (1986) and Honnegger et al. (1989), for instance, were conducted in a rat model with non-specific antigenic targets. This produced a disease course with several EAE attacks intermittent with full neurological recoveries. In comparison, most modern EAE models specifically target myelin proteins such as the myelin oligodendrocyte glycoprotein 35-55 peptide (MOG_{35-55}), which can produce a relapsing-remitting or chronic progressive disease [26-27]. Full recoveries from clinical deficits are rarely seen. The MOG_{35-55} model further differentiates itself from other modern EAE models because MOG_{35-55} autoreactive T cells also recognize the neurofilament-M protein as an autoantigen [28-29].

The following experiment was undertaken to characterize the changes in concentrations of amino acid and biogenic amine neurotransmitters in five anatomical regions of the CNS (spinal cord, brainstem, cerebellum, hypothalamus, and cerebrum) at disease onset, peak, and the chronic phase of the MOG_{35-55} EAE model using high performance liquid chromatography (HPLC). Concentration changes in a number of other non-neurotransmitters, precursors and metabolites were also evaluated. This included the evaluation of amino acids not examined in previous studies, including D-serine. Relationships between the concentration changes of amino acids and biogenic amines were then established that distinguished most of the analysed substances into two major groups that changed similarly during the disease course of the MOG_{35-55} EAE model. The characterization of neurotransmitter and non-neurotransmitter

concentrations in this model offers a broad picture of the gross changes in the CNS associated with MOG_{35-55} EAE disease progression. It may also provide a valuable comparison with data from previously studied EAE models and from data obtained from MS patients. The relationships between different amino acids and biogenic amines may provide additional insight into disease processes and potential therapeutic targets.

2.2 Materials and Methods

2.2.1 EAE induction

All animal procedures and experiments were performed according to protocols approved by the University of Alberta Animal Care and Use Committee. EAE was induced in female C57/BL mice (Charles River) with MOG_{35-55} according to the methods of Olechowski et. al., 2009 [30]. In two experiments, mice aged 10-12 weeks were divided into a control group (n=5) and an EAE "Peak" group (n=5) or a control group (n=5), an EAE Onset group (n=5) and an EAE "Chronic" group. EAE was induced by subcutaneous injection of 50 µg MOG_{35-55} (obtained from the Peptide Synthesis Facility, University of Calgary) emulsified in Complete Freund's Adjuvant (CFA) at a final concentration of 1mg/mL. Control mice received subcutaneous injections of CFA. An intraperitoneal injection of 300 ng pertussis toxin (Sigma-Aldrich, Oakville, ON) was administered on the day of induction and 48 hours later.

2.2.2 EAE assessment

Mice were monitored daily for clinical signs of EAE and were graded according to the following scale: Grade 0, normal mouse; Grade 1,flaccid tail; Grade 2, mild hind limb weakness with quick righting reflex; Grade 3, severe hind limb weakness with slow righting reflex; Grade 4, hind limb paralysis in one hind limb or both [31].

2.2.3 High Performance Liquid Chromatography (HPLC)

2.2.3.1 HPLC Materials

Standards and samples for the detection of amino acids were run with a Waters Alliance 2690XE pump and sample management system equipped with an autosampler and thermally controlled sample and column compartments. Separation of amino acids was achieved with a Waters Symmetry C18 guard column and column (3.5 µm, 4.6x150 mm). Fluorescence detectors used for amino acid detection were Waters 474 or Shimadzu RF10A (12 µL quartz flow cell) units.

Standards and samples for biogenic amine detection were run through a Waters Alliance 2695 pump and sample management system equipped with an autosampler and thermally controlled sample and column compartments. Separation of biogenic amines was achieved using a dC18 guard column and column (3µm, 3x100 mm) from Waters Atlantis. Detection of biogenic amines was achieved using a Waters 2465 electrochemical detector. Samples for HPLC analysis of both amino acids and biogenic amines were kept at 4˚C and column temperatures were maintained at 30˚C.

All water used was distilled and purified by reverse osmosis using a Milli-Q filtration system from Millipore. Solvent components for amino acids and biogenic amines were all obtained from Fischer Scientific with the exception of ascorbic acid (Sigma). Methanol (MeOH), tetrahydrofuran (THF), and acetonitrile were HPLC grade. Solvents for amino acids and biogenic amines were filtered using Millipore nylon membranes (0.2 µm pore size). *Ortho*-pthaldialdehyde (OPA) was obtained from Sigma Aldrich, N-isobutyryl-L-cysteine (IBC) from Novachem and sodium borate from Fischer Scientific.

2.2.3.2 Dissection and sample preparation

Mice were euthanized with sodium pentobarbital (Euthanyl, 240 mg/mL) for HPLC analysis and the lumbar spinal cord, brainstem, cerebellum, hypothalamus, and cerebrum were rapidly dissected and quickly frozen in liquid nitrogen. Mice were euthanized for HPLC when they reached EAE onset (clinical Grade 1), EAE Peak (clinical Grade 3 or 4), or the chronic phase. The chronic phase was defined as 14 days after mice

achieved EAE Peak, regardless of the clinical score at that time. Tissue samples were stored at -80˚C for later use. Samples were homogenized in a volume, in uL, of ice cold water equivalent to five times sample weight, in mg, after which they were divided into aliquots for HPLC analysis of amino acids or biogenic amines.

2.2.3.3 HPLC separation of amino acids

HPLC separation and detection of amino acids was performed using a chiral derivatizating reagent according to the protocol of Grant et. al. (2006)[32] with the following revisions. Solvent A was composed of 8.40g NaH_2PO_4, 1.42g Na_2HPO_4, 300 mL MeOH, 1700 mL water and adjusted to pH 6.2. Solvent B was composed of 6.43g NaH_2PO_4, 1110 mL MeOH, 1340 mL water, 60 mL THF, and adjusted to pH 6.2. Both Solvent A and Solvent B were filtered and degassed under vacuum pressure. Derivatizing reagent was prepared by combining 2 mg OPA, 3 mg IBC, 150 µL MeOH, and 1350 µL of 0.1M sodium borate. Amino acid stock solutions were prepared at a concentration of 1mg/mL in water. High standards were made to the following concentrations in 40% MeOH: 30 µg/mL glutamate, 20 µg/mL glutamine, 10 µg/mL taurine, alanine, and GABA, and 5 µg/mL for L-serine, aspartate, glycine, and arginine. D-Serine high standards were made to 5 µg/mL of D-serine for brain samples, and either 1 or 2 µg/mL for cerebellum samples, spinal cord and brainstem samples. Standard curves were generated by diluting the high standards to 0.75, 0.5, 0.25, 0.1, 0.025, 0.01, and 0.005 times their original strength. New standard curves were generated for each individual run of samples.

Aliquots of tissue homogenate for amino acids were diluted in 5X volume of MeOH, let sit for at least 10 minutes, and then centrifuged at 10 000xg for 4 minutes. Sample preparation was completed by diluting supernatants in a volume of water needed to make a 30X or a 60X dilution. Automated, precolumn derivatization was achieved by drawing up a 5 µL aliquot of standard, sample or blank along with 5µL derivatizing reagent and holding this in the injection loop for 5 minutes prior to injection. Separation of amino acids was achieved using a concave gradient with initial conditions of 83% Solvent A and 17% Solvent B at a constant flow rate of 0.5 mL/min. Final conditions of 10% Solvent A and 90% Solvent B were achieved within 45 minutes

using convex curve #8 from Waters Empower Pro software. At 50 minutes, conditions were changed to 100% Solvent B and flow rate was increased to 0.8 mL/min using curve #9 from Waters Empower Pro software. After 5 minutes at these conditions, solvent flow and concentrations were returned to initial conditions and let run for a minimum of 10 minutes before the next injection to wash out any late eluting peaks from compounds within the sample. Fluorescence detection was used with an excitation wavelength of 344 nm and an emission wavelength of 433 nm.

2.2.3.4 HPLC separation of biogenic amines

HPLC conditions for the separation and analysis of biogenic amine concentrations was adapted from Parent et. al. (2001)[33] with the following revisions. Mobile phase for the separation of biogenic amines consisted of 920 mL water, 6.6 mg NaH_2PO_4, 197.2 mg sodium octyl sulfate, 137.7 mg EDTA, 80 mL acetonitrile and 117 mg NaCl, and pH was adjusted to 2.9 with *o*-phosphoric acid. Mobile phase was recycled for two weeks before being replaced. Amino acid and biogenic amine stock solutions were prepared at a concentration of 1mg/mL in water. Standard solutions for biogenic amines were created by first adding 10 µL of noradrenaline, dopamine, 3,4 dihydroxyphenylacetic acid (Dopac), 5-hydroxyindoleacetec acid (5-HIAA), Homovanillic acid (HVA), 5-hydroxytryptophan (5-HT) to 940 µL of 0.1N perchloric acid ($HClO_4$). Of this, 100 µL was then diluted with 20 uL tryptophan and 880 µL of 0.1N $HClO_4$. Standard solutions for running were generated from this second solution by diluting it to 0.1, 0.05, 0.025, 0.01, 0.005, and 0.002 times its original strength. New standard curves were generated for each individual run of samples.

Aliquots of tissue homogenate for biogenic amines were diluted 0.9X with 1N $HClO_4$ (composed of 21.2 mL $HClO_4$ (60%) brought up to 250 mL with water and the addition of 22 mg of ascorbic acid and 250 mg EDTA). Sample solutions were vortexed, centrifuged at 10,000xg for 4 minutes and the supernatant removed for separation by HPLC. Separation was conducted by injecting a 10 µL sample, standard or blank into the mobile phase (flow rate of 0.4 mL/min). The applied potential for electrochemical detection was 0.65 V.

Analysis of biogenic amines was only completed on the tissue samples in the second experiment (CFA, onset and chronic). Due to the small size of the samples, aliquots of the hypothalamus samples were also not made for this assay. However, the time courses of concentration changes of biogenic amines during EAE were well described by Krenger et al. (1986) and the majority of equivalent samples in our model appear the same [19].

2.2.3.5 HPLC determination of amino acid and biogenic amine concentrations

All analysis of HPLC standards and samples was completed on Empower Pro software package from Waters. Calibration curves were generated for each amino acid, biogenic amine and acid metabolite using the serial dilution of standard solutions for each run of samples. Of the cerebellum samples, 3 from the first experiment (1 CFA and 2 EAE Peak) were excluded because of contamination during extraction. D-Serine, while clearly detected in the majority of samples, was unquantifiable because of peak interference in 1 chronic phase spinal cord and 2 EAE peak brainstems.

2.2.4 Statistical Analysis

Statistical analysis and the creation of graphics were performed using SigmaPlot software version 11.0. Concentration values for amino acid and biogenic amine concentrations were expressed as percent control. Turnover of 5-HT for each sample was calculated by dividing the percent of control of 5-HT by the percent of control of 5-HIAA. The L-/D-serine ratio was conducted by dividing a sample's percent control value of L-serine by its percent control of D-serine. Tissue concentrations of amino acids and biogenic amines during EAE were evaluated by one-way analysis of variance (ANOVA). Dunnett's post hoc test, using CFA as the reference standard was used to determine statistical differences. Concentrations of dopamine, dopac and HVA were excluded from the results because detection in most of the analysed CNS regions was inconsistent. Because of its close biological association to 5-HT, and thus 5-HIAA and 5-HT turnover, tryptophan, for the purpose of this study, is grouped in the results with the biogenic amines. Pearson product-moment correlation coefficients were used to correlate amino

acid and biogenic amine concentration changes in the spinal cord during EAE. In all instances, results were considered as statistically significant if $P \leq 0.05$.

2.3 Results

2.3.1 Concentration changes of amino acids and biogenic amines in the spinal cord during EAE

Previous studies have shown that concentrations of amino acids and biogenic amines are altered during MS and EAE [12-20]. To determine how the concentrations of amino acids and monoamines are affected in the CNS during MOG_{35-55}-induced EAE, we carried out HPLC analysis on tissue samples from the spinal cords, brainstems, cerebella, hypothalami, and cerebra from mice at different stages of the disease.

In the spinal cord, the vast majority of substances analysed had concentrations that were significantly different from control animals during the disease course of EAE. Eight of 10 amino acids had tissue concentrations that were significantly different during EAE than in CFA mice (Figure 2-1). Significant decreases in concentration were observed for the amino acid neurotransmitters glutamate in the chronic phase (74.89 ± 3.45 %CFA, Fig. 2-1A), GABA at peak and in the chronic phase (Peak: 82.73 ± 2.66 %CFA; Chr: 74.15 ± 3.34 %CFA, Fig. 2-1B) and aspartate at all EAE time points (Onset: 78.78 ± 5.79 %CFA; Peak: 64.13 ± 4.02 %CFA; Chr: 73.54 ± 2.07 %CFA, Fig. 2-1C). Spinal cord concentrations of D-serine were increased at peak and in the chronic phase (Peak: 134.63 ± 5.16 %CFA; Chr: 181.89 ± 21.11 %CFA, Fig. 2-1E) while glycine concentrations were increased at onset and at peak (Onset: 136.37 ± 4.86 %CFA; Peak: 151.38 ± 2.26 %CFA, Fig. 2-1I). In pattern similar to that of glycine concentrations changes during EAE, concentrations of L-serine, alanine, and taurine were elevated at onset and at peak (L-serine, Onset: 155.27 ± 12.07 %CFA; Peak: 175.49 ± 11.65 %CFA, Fig. 2-1D; alanine, Onset: 179.69 ± 11.13 %CFA; Peak: 182.19 ± 3.38 %CFA, Fig. 2-1G; taurine, Onset: 171.37 ± 23.14 %CFA; Peak: 198.38 ± 9.30 %CFA, Fig. 2-1H). The ratio of L- to D-serine in spinal cord was significantly altered at EAE onset only (237.5 ± 42.5 %CFA, Fig. 2-1F).

The spinal cord concentrations of the biogenic amines, 5-HIAA and tryptophan all showed significant changes from CFA levels during EAE (Figure 2-2). Concentrations of 5-HT and noradrenaline decreased during EAE and were substantially below control

concentrations in the chronic phase (5-HT, Onset: 79.93 ± 4.50 %CFA; Chr: 21.92 ± 2.64 %CFA, Fig. 2-2A; Noradrenaline, Chr: 31.42 ± 0.79 %CFA, Fig. 2-2E). Compared to control levels, concentrations of the 5-HT metabolite, 5-HIAA, were increased at onset and decreased in the chronic phase (Onset: 139.51 ± 12.74 %CFA; Peak: 48.19 ± 7.17, Fig. 2-2B). Turnover of 5-HT was increased both at EAE onset and in the chronic phase (Onset: 0.592 ± 0.062 %CFA; Peak: 0.485 ± 0.081 %CFA, Fig. 2-2C). Tryptophan concentrations were greater at onset than those of controls but returned to CFA levels by the chronic phase (Onset: 276.62 ± 45.24 %CFA, Fig. 2-2E). Of the substances examined in this study, only spinal cord concentrations of glutamine and arginine were not altered significantly during EAE (Figure 2-3).

2.3.2 Concentration changes of amino acids and biogenic amines in the brainstem during EAE

In the brainstem, concentrations of 7 out of 10 amino acids in EAE animals were significantly different than CFA concentrations (Figure 2-4). Concentrations of glutamate, glycine, and L-serine were decreased at EAE onset (glutamate, 77.79 ± 9.34 %CFA, Fig. 2-4A; glycine, 83.08 ± 3.88 %CFA, Fig. 2-4C; L-serine, 74.37 ± 9.97 %CFA, Fig. 2-4G) , those of aspartate and arginine were decreased at onset and in the chronic phase (aspartate, Onset: 78.61 ± 5.32 %CFA; Chr: 84.94 ± 2.66 %CFA, Fig. 2-4B; arginine, Onset: 76.14 ± 8.81 %CFA; Chr: 76.94 ± 4.91 %CFA, Fig. 2-4D) and concentrations of alanine and taurine were increased compared to controls at EAE peak (alanine, 130.59 ± 8.73 %CFA, Fig. 2-4E; taurine, 120.68 ± 11.01 %CFA, Fig. 2-4F). Of the biogenic amines (Figure 2-5), 5-HT and noradrenaline concentrations were greater than controls in the chronic phase (5-HT, 154.47 ± 3.79 %CFA, Fig. 2-5A; noradrenaline, 117.48 ± 2.89 %CFA, Fig. 2-5E). Brainstem concentrations of GABA, D-serine , glutamine, tryptophan and 5-HIAA were unaffected during EAE, as were 5-HT turnover and the L-/D-serine ratio (Figures 2-5 and 2-6).

2.3.3 Concentration changes of amino acids and biogenic amines in the cerebellum during EAE

Fewer amino acids and biogenic amines had concentrations different from CFA levels in the cerebellum of mice with EAE: Glutamate concentrations were decreased in the chronic phase (79.92 ± 4.09 %CFA, Fig. 2-7A), glycine and L-serine concentrations were increased at peak and in the chronic phase (glycine, Peak: 123.66 ± 4.91 %CFA; Chr: 122.81 ± 6.83 %CFA, Fig. 2-7B; L-serine, Peak: 125.51 ± 11.83 %CFA; Chr: 122.66 ± 4.61 %CFA, Fig. 2-7C), taurine concentrations were decreased at peak but increased in the chronic phase (Peak: 77.73 ± 6.03 %CFA; Chr: 117.77 ± 2.89 %CFA, Fig. 2-7D) and arginine concentrations were increased at peak (128.29 ± 4.92 %CFA, Fig. 2-7E). No differences from control levels were observed in the cerebellar content of the biogenic amines, 5-HIAA, or tryptophan or the measure of 5-HT turnover (Figure 2-8). Concentrations of the other amino acids and the L-/D-serine ratio were also unaffected (Figure 2-9).

2.3.4 Concentration changes of amino acids and biogenic amines in the hypothalamus and cerebrum during EAE

In contrast with the spinal cord, brainstem, and the cerebellum, concentrations of most amino acids and biogenic amines were not different from those of control animals in the hypothalamus (Figure 2-10) or the cerebrum (Figures 2-11 and 2-12). While concentrations in the cerebrum of 5-HT and its metabolite, 5-HIAA were no different than controls at the measured time points during EAE, a significant increase in 5-HT turnover was observed at onset (0.732 ± 0.037 %CFA, Fig 2-12C).

2.3.5 Correlations of amino acid and biogenic amine concentrations in the spinal cord of CFA and EAE mice

Because the spinal cord concentrations of some amino acids and biogenic amines evaluated in this study appeared to change similarly during the disease course of MOG_{35-55} EAE, the relationships of individual concentration changes to each other were assessed by correlating the concentrations of each substance within each spinal cord. A

number of strong correlations were found between the concentrations of different amino acids and biogenic amines, as exemplified by those of 5-HT and GABA ($r=0.774$, $p<0.001$, Fig. 2-13A), GABA and glutamate ($r=0.794$, $p<0.001$, Fig. 2-13B), L-serine and glycine ($r=0.839$, $p<0.001$, Fig. 2-13C) and L-serine and taurine ($r=0.727$, $p<0.001$, Fig. 2-13D)(Figure 2-13A and Table 1). Interestingly, no correlation existed between amino acids where relationships may be expected such as between glutamate and its precursor, glutamine ($r=0.139$, $p=0.560$); D-serine and its precursor, L-serine ($r=-0.166$, $p=0.497$); or tryptophan and 5-HT ($r=0.299$, $p=279$). Based on these correlations, it appears that two major groups of analytes in this study can be distinguished (Figure 2-13B). These groups consist of amino acids and biogenic amines possessing, with few exceptions, statistically significant correlation coefficients set at $r\geq0.65$ with all other members of that group of analytes. Group I analytes, with the exception of glycine, are non-neurotransmitter amino acids whose concentrations are increased at EAE onset and peak but then decrease in the chronic phase with a large degree of variability. Group II is composed largely of neurotransmitters that change in a progressive manner during EAE. 5-HIAA, correlating strongly with 3 of 6 Group II analytes, nearly fits within Group II. Other than 5-HIAA, arginine and tryptophan are the only analytes not represented within these general groups.

2.4 Discussion

2.4.1 General context of the findings of the present experiment

Previous reports have demonstrated that concentration changes occur for a number of amino acids and biogenic amines in the CSF of MS patients and in the CNS during EAE [12-21, 34-36]. In this study, we evaluated tissue concentrations in the CNS of the MOG_{35-55} EAE model for most of the substances examined in the previous reports in addition to several newly examined amino acids. Many of the concentration changes observed in this study confirm those in the previous reports. However, notable differences were found between the current study in the MOG_{35-55} EAE model, the previously studied EAE models, and the CSF data from MS patients. Furthermore, our study expands the picture of amino acid and biogenic amine changes throughout the CNS. While Krenger et. al. (1986) evaluated the biogenic amines at different levels of the

CNS during successive EAE attacks and remissions, amino acid changes in their later study were not examined in structures rostral to the brainstem or at time points after the initial EAE attack. In addition to analysing the previously evaluated amino acids, new features of this study include the novel evaluation of L-serine, D-serine, alanine, arginine, and aspartate concentrations in the spinal cord and more rostral CNS regions and at later time points during the disease process. Our analysis includes the examination of concentrations in the cerebellum, a region not examined in earlier EAE studies that is now recognized to be affected during EAE [37-40]. The finding that the concentration changes of most amino acids and biogenic amines in the spinal cord occur in two major patterns during EAE may also help provide new insight into disease processes.

2.4.2 The MOG_{35-55} model in the context of previous EAE models

While the progression of symptoms and neuropathological changes in MS is fairly unpredictable, the clinical and pathological disease course of EAE typically begins in the spinal cord and progresses to more rostral structures [25-26]. This general pattern of ascending pathology is reflected by the concentration changes observed in this study and in those of Krenger et al. (1986) and Honegger et al. (1989). In all cases, concentration changes are the most common and of the greatest magnitude in the spinal cord and decrease in both respects as more rostral CNS regions are examined. Significant differences between EAE animals and controls are less common in the brainstem and are mostly restricted to 5-HIAA concentrations and 5-HT turnover in the cerebrum. In the cerebrum, EAE has a variety of effects on neurotransmitter activities [39, 41-42]. However, these changes may not be well-reflected by total tissue concentrations and/or are too subtle to be measured using our HPLC methods.

Beyond the general pattern of clinical and pathological progression in the CNS, our findings show a number of similarities with the previously examined EAE models, especially with regard to the biogenic amines. In the spinal cord, we confirm concentration increases during EAE in glycine and taurine and progressive decreases in the concentrations of the neurotransmitters glutamate, 5-HT, noradrenaline as well as 5-HT turnover [16-21]. The increases in tryptophan concentrations associated with

disease onset observed in this study were also seen by Krenger et al. (1986). They also saw a similar, transient surge in 5-HIAA concentrations during the first attack that decreased below control levels afterwards, regardless of later clinical status [19, 21]. In the brainstem, we see similar concentration increases during the late phases of EAE disease in noradrenaline concentrations and decreases in those of glutamate. Increased brain concentrations of 5-HIAA are also a common feature. Krenger et al. (1986) did not calculate 5-HT turnover in the brain, but a similar increase would likely be observed because 5-HT concentrations are unaltered during EAE.

Concentration changes in the spinal cord during EAE progression differ from the previously studied EAE model in several ways and these differences are suggestive of dissimilar biological processes. Most noticeable are the large changes observed by Honegger et al. (1989) in spinal cord glutamine levels associated with EAE exacerbations that are absent in the MOG_{35-55} model and the prominent increases we observe in brainstem 5-HT concentrations that are absent in theirs. The relationship of the changes in amino acid concentrations to disease course also differs; Honegger et al. (1989) observed changes in spinal concentrations of glutamine, glycine and GABA that generally return to CFA levels during recovery while the only persistent changes were elevated spinal concentrations of taurine. In comparison, our results show a very strong relationship between taurine and glycine concentrations in EAE that do not relate with changes in GABA nor are elevated persistently. The different groups of substances changing in relation to EAE progression we observe may suggest different biological processes. Alternatively, this may also indicate relative differences between EAE models in the magnitudes of processes held in common.

In addition to the increased brainstem 5-HT concentrations in the MOG_{35-55} model, changes in more rostral structures also differ between the EAE models. In the brainstem, concentration changes of glutamine and GABA are absent in our study and changes in glycine, taurine, and 5-HT were observed. In the previously studied model, brainstem concentrations of glutamine and GABA are altered while glycine, taurine, and 5-HT levels are unaffected [16, 19]. Furthermore, in a subsequent report on monoamine concentrations during EAE in the spinal cord and brainstem in 1989, 5-HT concentrations in the brainstem were decreased transiently during EAE attack [21]. In the cerebrum,

Krenger et al. (1986) report 5-HIAA concentration increases at most time points after disease onset and increases in noradrenaline levels in the late stages of EAE. In our model, 5-HIAA and noradrenaline levels in the brain are not statistically different from CFA levels. The reduction at onset in the 5-HT/5-HIAA ratios suggests, though, that changes may have occurred but were not sufficient to pass threshold for statistical significance.

A variety of factors may underlie the differences in amino acid and biogenic amine concentrations during EAE between the MOG_{35-55} model and the one used in earlier reports. Firstly, amino acid concentrations were only evaluated after the first EAE attack in the report by Honegger et al. (1989). Evaluations of those concentrations at later time points may have elucidated similar relationships to the ones we observe. Genetic differences between the species we used in our models may also produce variance in the underlying inflammatory and neurodegenerative mechanisms. Perhaps the most important factor, however, is the difference in clinical progressions. The model used in the reports by Krenger et al. (1986) and Honegger et al. (1989) is a clear relapsing-remitting model that has a series of EAE attacks with remissions during which there are no neurological symptoms; In the MOG_{35-55} model, there are often variations in clinical score after the initial attack but only rarely are full recoveries from neurological deficits observed. Relative differences in inflammation, demyelination, and axonal loss have been observed between EAE models described as relapsing-remitting and chronic progressive [27, 43-46]. Berard et al. (2010) recently showed that these differences are also present in EAE models of the same genetic background that were differentially induced to relapsing-remitting or chronic EAE by varying the concentration of MOG_{35-55}. Inflammation and demyelination correlate best with clinical deficits during acute EAE attacks whereas, at later time points, disability correlates with axonal loss [47-48]. Presumably then, a greater rate and extent of axonal damage in our model, with a relatively smaller inflammatory response, could underlie our different observations.

The different antigens used for inducing EAE may also initiate different pathogenic mechanisms. The previously studied EAE model used a non-specific antigen created by injecting emulsified guinea pig spinal cords. By their methods, it is possible that a range of autoreactive T cells are activated, to various degrees, which could then

attack a diversity of components in the CNS. This is in stark contrast to the MOG_{35-55} antigen that specifically activates autoreactive T cells to target the MOG protein in myelin and the neurofilament-M protein, which is found in neurons and schwann cells [28-29]. These antigenic differences could be expected to translate biologically into differences in the specific mechanisms of pathogenesis and the relative extents of inflammatory and neurodegenerative processes.

2.4.3 The MOG_{35-55} model in the context of MS

Comparing the concentration changes of amino acids and biogenic amines during EAE with those observed in the CSF and plasma of MS patients may help further elucidate common pathogenic processes. Changes in the concentration of amino acid neurotransmitters have been well documented [12-14, 34]. An analysis similar to our own, however, where the same range of amino acids and biogenic amines are analysed in parallel, has not been conducted to our knowledge. Concentration increases of taurine and glycine in MS patients with active relapsing-remitting MS or progressive MS that normalize in quiet disease periods are similar to those observed during EAE attacks [12-13, 16]. Decreases in GABA concentrations and glutamate decarboxylase activity in the plasma are also observed [34]. Interestingly, however, whereas tissue concentrations of glutamate and aspartate decrease during EAE, concentration increases are observed for these excitatory amino acids in the CSF of MS patients with active disease [12-14]. Elevated glutamate concentrations in the CSF correlate with the amount of gadolinium-enhanced lesions, although not with breakdown of the blood-brain barrier [13-14]. The discrepancies in glutamate and aspartate concentrations between MS and EAE are not irreconcilable and a possible biological explanation is presented in discussion section 2.4.3.2.

The concentrations of non-neurotransmitter amino acids are also important to compare between MS and EAE. These amino acids may have crucial roles as precursors for neurotransmitter synthesis and changes in their tissue concentrations may reflect gross changes in cellular metabolism and/or indicate different aspects of inflammatory or neurodegenerative processes. In a manner reminiscent of the conflicting differences between EAE models, CSF concentrations of the neurotransmitter precursors glutamine

(for glutamate) and asparagine (for aspartate) were increased in MS patients in one study but unaffected in another [13-14]. Altered CSF concentrations of tryptophan have not been observed in MS patients [49-50]. Some non-neurotransmitter amino acids may be important but their potential roles are not yet fully understood. The NMDA receptor agonist activity of D-serine, only discovered in recent years, is one such example. While the different enantiomers were not distinguished, Stover et al. (1997) found no change in serine concentrations during MS. This is in contrast to what we observe in the CNS of MOG_{35-55} EAE, where concentrations of both D- and L- serine enantiomers are elevated above control levels. The greater CSF concentrations of taurine during MS correlate well with those of glutamate and support the notion that taurine is released in response to glutamatergic excitotoxicity [13].

The biogenic amine neurotransmitter systems are also affected during MS. Studies have generally made inferences on central changes in biogenic amine activities, though based on changes in the CSF concentrations of their respective metabolites ie. 5-HIAA for 5-HT and 3-methoxy-4-hydroxyphenylglycol (MHPG) for noradrenaline. Decreases in central serotonergic activity during MS are highly suggested by lower CSF concentrations of 5-HIAA in both relapsing-remitting MS and progressive MS patients [15, 35-36]. Changes in 5-HIAA strongly correlate with the rate of disability accumulation in relapsing-remitting MS. In progressive MS, this relationship to disability no longer holds, as the lower concentrations of 5-HIAA may have stabilized by this time [15]. With the exception of the onset-associated increase in spinal 5-HIAA and increases in brainstem 5-HT, decreases in 5-HIAA and 5-HT concentrations and a lower 5-HT turnover are observed in both the MOG_{35-55} EAE model and the previous EAE model[19]. Elevations in 5-HIAA concentrations in MS could potentially be observed if samples could be obtained from patients at a time sufficiently close to disease onset. These results strongly imply that decreased central serotonergic activity is common to MS and EAE.

Increased noradrenaline concentrations in the CSF of MS patients have been reported [12]. Concentrations of MHPG, however, are unaltered during MS, although their concentrations negatively correlate with disease duration and the number of relapses [15]. The past and present EAE studies show noradrenaline increases in

supraspinal CNS regions that are comparable with what is observed by Barkhatova et al. (1998). However, this generalization must be balanced by the absence of concentration changes in CSF MHPG and the negative correlations of MHPG with disease duration and the number of relapses observed by Markianos et al. (2009). In EAE models, this may compare with the progressive losses of noradrenaline, and thus noradrenergic activity, in the spinal cord [19].

2.4.4 Biological implications of the concentration changes in MOG_{35-55} EAE

2.4.4.1 Patterns of concentration changes in the spinal cord during EAE

The spinal cord is the CNS region most affected by EAE and is where inflammatory and neurodegenerative processes are the most severe. The concentration changes in the spinal cord in our study appear to occur in two major groups that likely reflect different biological processes. The first group of changes (Group I) are, with the exception of glycine, non-neurotransmitter amino acids that are altered to the greatest extent at disease onset and peak. Because concentrations in this group decrease in the chronic phase when clinical recovery has occurred to some extent, the changes in this group likely relate closest to the active inflammatory processes associated with clinical deficits during acute EAE attacks [47]. Neurotransmitters are almost exclusively represented within the second group of changes (Group II) and, with the majority showing concentration decreases, probably occur due neurodegenerative processes such as the loss of axons and synapses. Less efficient axonal transport and neurotranmission along intact fibers may also contribute. Why the concentrations of 5-HIAA, arginine, and tryptophan do not change in relation to the major groups distinguished here may be particularly interesting to consider.

Inflammatory and neurodegenerative processes may also have distinct, additive or subtractive influences on substance concentrations relative to each other and to control levels. Consequently, at any given time point during EAE, the tissue concentrations we measure likely reflect the net dominance of one process over another. Glutamate produced and released from microglia and astrocytes at EAE onset and peak, for example, may not change its total spinal cord concentrations because this occurs on a background of decreasing glutamate concentrations due to damage to

and/or loss of neurons, axons and synapses. The loss of sources for glutamate storage and production may be further compelling, given that the enzymes responsible for its degradation are known to decrease [51]. In the chronic phase, when inflammatory processes are relatively less severe, the lower tissue concentrations reflect the now dominant effect of neurodegeneration [27, 47-48, 52].

2.4.4.2 Excitatory amino acid concentration changes in EAE

Substantial evidence exists that supports increases in glutamatergic excitotoxicity in the CNS of MS patients and in EAE [6, 9, 51, 53-54]. These findings are not necessarily challenged by the observation of decreased total tissue concentrations of glutamate in the present study and the previously examined EAE model [16]. This discrepancy may be explained by the decreased expression of astrocytic excitatory amino acid transporters in MS and EAE [54]. Despite decreases in total glutamate concentrations, reduced re-uptake allows extracellular concentrations to rise to inappropriate concentrations at which these neurotransmitters cross into the CSF and plasma [13]. Like in the CSF of MS patients, the concentration changes of glutamate are dissociated from changes in those of its precursor, glutamine [13].

Other molecules associated with glutamatergic neurotransmission and excitotoxicity are also altered in this study. The large, novel increase we observe in spinal concentrations of D-serine, a potent coagonist of the NMDA glutamate receptor at the glycine binding site, has excitotoxic potential that has been demonstrated *in-vitro* in the hippocampus and in the spinal cord *in-vivo* in an animal model of ALS [55-56]. While spinal cord concentrations of D-serine are usually well below 1 µg/g tissue, the changes in D-serine concentrations, to around 175% of control levels by the chronic phase, may be biologically significant. D-Serine concentrations during EAE correlate with the progressive changes in other neurotransmitters identified in group II and are unrelated to the changes of its precursor, L-serine. As the only Group II analyte increasing in concentration during EAE, D-serine changes are curious. Its expression increases in glia in the spinal cord during ALS [55]. Investigations in our lab, however, as to the spinal expression during EAE of D-serine and its generating enzyme, serine racemase, indicate only the presence of neuronal expression (unpublished results).

Reductions in tissue concentrations of aspartate, whose levels are elevated in the CSF of MS patients, are also interesting because it is an agonist of the NMDA receptor and can be co-released synaptically with glutamate [12-14, 57]. Because aspartate can be removed from the synapse using a transporter in common with glutamate, there is the possibility that, like glutamate, a lack of extracellular clearance allows for its concentrations to elevate to levels sufficient to allow it to cross into CSF.

2.4.4.3 Glycine concentration changes in EAE

Concentration increases in glycine during EAE are well known [16-18]. While glycine receptor agonists have been found to improve EAE-associated bladder hyperactivity in rats, glycine, as a topic, has been relatively unexplored in EAE and the implications of glycine changes are unclear [58]. Glycine is a co-agonist at the NMDA glutamate receptor, the presence of which is critical for both normal function and NMDA-receptor mediated excitotoxicity. Through binding to its own glycine receptors, however, it is also the most important inhibitory neurotransmitter in the spinal cord. The presence of glycine in Group I with the non-neurotransmitter amino acids suggests that the large increases in glycine concentrations we observe may relate more to inflammatory events in EAE than to its role as a neurotransmitter. Its correlation with the other non-neurotransmitter amino acids may derive from their metabolic relations in astrocytic metabolism [59]. Whether or not these changes in glycine have coincident effects on NMDA or glycine receptors is unknown. Glycine channels, furthermore, are present on cells of the immune system and could, thereby, also influence EAE [5].

2.4.4.4 GABA concentration changes in EAE

Decreased concentrations of GABA, the major inhibitory neurotransmitter of the CNS, have a number of implications for EAE progression and symptomology. As an inhibitory neurotransmitter, its actions help avert excitotoxic mechanisms and alleviate symptoms caused by excessive neuronal excitability such as pain and spasticity. Drugs that increase GABA concentrations or activate GABA receptors also suppress the inflammatory activities of astrocytes, macrophages/microglia and T cells and the clinical

signs of EAE [2-3, 10-11, 60]. The progressive decrease in GABA concentrations in EAE thus indicates a possible disinhibition of both neurodegenerative and pro-inflammatory mechanisms. Extracellular and intracellular concentrations of GABA may be further dysregulated because GABA transporter expression in the spinal cord is decreased during acute EAE [10]. The close relationship of GABA changes with those of glutamate also suggests that a loss of GABAergic synapses and axons may be occurring in EAE.

2.4.4.5 Biogenic amine concentration changes in EAE

5-HT and noradrenaline influence the function of neurons through a variety of receptors. In the spinal cord, their concentrations depend on transport from brainstem sources and help regulate the excitability of motor neurons and the activity of central neurons of the nociceptive pathways [61-63]. Both serotonergic and noradrenergic neurons appear to be particularly affected in MS and in EAE. This is no less evident than in EAE spinal cords, where 5-HT, 5-HIAA, and noradrenaline concentrations are reduced to around 20%, %50, and 30% of control levels respectively by the chronic phase. Permanent damage to bulbospinal axons that transport 5-HT from the brainstem occurs early in EAE but without the loss of cell bodies in the medulla [19, 64-65]. This likely happens to neurons of the descending noradrenergic system as well [66]. Remaining 5-HT and noradrenaline may come from spared axons or possibly from diffusion across the leaky blood-brain barrier, as is known to occur with noradrenaline in a model of spinal cord injury [67]. Interestingly, even in the rat EAE model with full neurological recoveries, spinal cord concentrations of 5-HT and noradrenaline do not recover; a fact implying that functional recovery is not dependent on the complete restoration of these systems [19]. The constitutive activation of 5-HT and noradrenergic receptors observed after spinal cord injury may play a role in the recovery of motor function as well as the development of spasticity [68-69]. Signals to increase 5-HT production in the brainstem from intact spinobulbar afferents could also explain the progressive increase in 5-HT concentrations we observe in this region. The increase in 5-HIAA concentrations at EAE onset and the persistent decrease in 5-HT turnover may also indicate possible changes in the expression of monoamine oxidases during EAE.

5-HT and noradrenaline both also affect the functioning of the immune system, facts which have been investigated in several EAE studies. Both neurotransmitters can suppress pro-inflammatory responses through receptors and transporters that are present on non-neuronal cells such as astrocytes, macrophages/microglia and T cells [1, 70-72]. Increasing serotonergic activity by decreasing 5-HT reuptake reduces clinical deficits in EAE [4, 73]. Similarly, depleting noradrenaline concentrations exacerbates EAE while elevating its concentrations attenuates its severity [74]. Reducing the reuptake of both with an anti-depressant such as venlafaxine also helps overcome EAE disease processes [75]. Antidepressants targeting 5-HT or noradrenaline specifically additionally help in MS [76-77].

2.4.4.6 Non-neurotransmitter concentration changes in EAE

The non-neurotransmitter amino acids are important as they may possess neuroactive properties, act as neurotransmitter precursors, or indicate gross changes in cellular metabolism. Through metabolic processes in astrocytes and neurons, glutamine is intimately linked to the synthesis of glutamate, via the glutamate-glutamine cycle, to GABA through the GABA shunt as well as to glycine [59, 78]. Tissue concentrations of glutamine are not changed significantly in the MOG_{35-55} EAE model and do not correlate with tissue concentrations of either glutamate or GABA. A strong correlation is found with glycine though, which indicates a metabolic link in acute EAE between glutamine and this important neurotransmitter. Concentrations of arginine, the substrate for nitric oxide synthesis, are also unaltered in the spinal cord during our study, although decreases are observed in the brainstem. Nitric oxide regulates inflammation and EAE through a variety of mechanism [79]. Interestingly, on its own, administration of L-arginine has been shown to have a suppressive effect on EAE [80]. Taurine, an abundant intracellular amino acid, is recognized as having neuroprotective functions in a variety of models and its increased concentrations during EAE may be beneficial [81-83]. It is released in response to glutamatergic excitotoxicity, can act on glycine and GABA receptors and possesses a role in the regulation of cell volume [83-86]. The implications of concentration changes in L-serine and alanine are more ambiguous. Alanine is metabolically related to numerous substances such as glutamine,glutamate, pyruvate

and lactate and possesses weak agonist activity at NMDA receptors [78]. L-Serine is also metabolically related to other amino acids like glycine and taurine and has a central role in cellular proliferation [87].

Concentration changes of tryptophan may be of particular interest. Tryptophan, an essential amino acid, is the precursor for 5-HT and its availability is the synthesis reaction's rate limiting step [88-89]. In the presence of proinflammatory cytokines like IFNγ,TNFα, and IL-1, however, macrophages/microglia upregulate the enzyme indoleamine 2,3-dioxygenase (IDO), which diverts available tryptophan into the kynurenic acid pathway. Tryptophan depletion, which slows the proliferation of T cells, occurs in various autoimmune disorders. Downstream catabolites of IDO can further regulate adaptive immunity [88, 90-91]. Two products of this pathway are kynurenine and quinolinic acid, an antagonist and an agonist at NMDA receptors respectively. Quinolinic acid has further excitotoxic potential because it stimulates glutamate release [89]. Increased activity of IDO has been shown both pre-clinically and at onset during EAE[92]. Inhibition of IDO, though, exacerbates both immune responses and EAE clinical scores through mechanisms that include the suppression of T regulatory cells [92-93]. In the current study, tryptophan concentrations are increased at onset in the spinal cord, a time in which 5-HT concentrations have begun to decrease, and they correlate poorly with 5-HT concentrations. The discrepancy between tryptophan and 5-HT concentrations may indicate a diversion of tryptophan into the kynurenic acid pathway that may be beneficial in EAE. The reason tryptophan concentrations increase at onset is unclear. Tryptophan concentrations in the brain are increased, however, after various forms of stress in animals [91]. Nevertheless, the possible benefits of IDO activity are possibly counter balanced. By diverting available tryptophan from 5-HT synthesis, IDO upregulation may also have a role in the decreased serotonergic activity that is characteristic of sickness and depressive behaviours [89, 94]

2.4.4.7 Supraspinal concentration changes in EAE

In the more rostral CNS structures examined, concentration changes are less frequent and generally of lesser magnitude. In the brainstem, most amino acid concentrations that are changed show decreases, particularly at onset and peak. This

may suggest a transient, general depression of brainstem activities associated with acute EAE. 5-HT concentration increases in the brainstem were previously mentioned, although elevated noradrenaline concentrations are also observed. Greater noradrenaline concentrations could be linked to increased stress and anxiety [95]. Cerebellar changes include lower glutamate concentrations and greater levels of glycine, L-serine, and taurine in the later phases of EAE. These changes may indicate similar processes as to what occurs in the spinal cord, albeit at dissociated time points and of lesser severity. The decreases in cerebellar glutamate concentrations may reflect the gray matter damage seen in other studies [37-38]. With the exception of 5-HT turnover in the brain, EAE has little effect on amino acids and biogenic amine concentrations in the rest of brain and hypothalamus. The increased turnover of 5-HT, however, likely due to increased 5-HIAA levels such as observed in the rats with EAE studied by Krenger et al. (1986), could relate to sickness and/or depressive-like behaviours observed in EAE [96].

Table 2-1: Correlations of amino acid and biogenic amine concentration changes from CFA levels in the spinal cord during EAE. Values are r value (top) and probability (bottom). ASP: Aspartate, GLU: Glutamate, LSER: L-Serine, DSER: D-Serine, GLN: Glutamine, GLY: Glycine, ARG: Arginine, TAUR: Taurine, ALA: Alanine, GABA: γ-aminobutyric acid, 5-HT: 5-Hydroxytryptamine, 5-HIAA: 5-Hydroxyindoleacetic acid, TRYPT: Tryptophan, NA: Noradrenaline.

	GLU	LSER	DSER	GLN	GLY	ARG	TAUR	ALA	GABA	5-HT	5-HIAA	TRYPT	NA
ASP	0.682 <0.001	-0.453 0.045	-0.275 0.255	-0.283 0.226	-0.429 0.059	0.281 0.230	-0.548 0.012	-0.491 0.028	0.720 <0.001	0.691 0.004	0.067 0.813	0.067 0.813	0.709 0.003
GLU	-	-0.101 0.670	-0.576 0.010	0.139 0.560	0.156 0.510	0.327 0.160	-0.144 0.546	-0.041 0.862	0.794 <0.001	0.797 <0.001	0.562 0.029	0.124 0.661	0.693 0.004
LSER		-	-0.166 0.497	0.693 <0.001	0.839 <0.001	0.248 0.291	0.727 <0.001	0.891 <0.001	0.098 0.652	-0.127 0.214	0.340 0.452	0.523 0.045	-0.029 0.681
DSER			-	-0.206 0.398	-0.265 0.273	-0.357 0.134	-0.079 0.747	-0.248 0.307	-0.634 0.004	-0.731 0.003	-0.847 <0.001	-0.543 0.045	-0.673 0.008
GLN				-	0.688 <0.001	0.111 0.642	0.602 <0.001	0.650 0.002	0.172 0.468	-0.069 0.808	0.248 0.374	0.292 0.291	-0.037 0.895
GLY					-	0.068 0.776	0.828 <0.001	0.921 <0.001	0.150 0.753	0.089 0.029	0.564 0.713	0.452 0.091	0.019 0.527
ARG						-	-0.126 0.598	0.126 0.597	0.528 0.017	0.399 .0141	0.224 0.423	0.130 0.644	0.693 0.004
TAUR							-	0.840 <0.001	-0.100 0.297	-0.289 0.429	0.221 0.858	0.309 0.644	-0.393 0.675
ALA								-	0.132 0.579	-0.048 0.864	0.459 0.085	0.520 0.047	0.052 0.854
GABA									-	0.774 <0.001	0.501 0.057	0.281 0.310	0.805 <0.001
5-HT										-	0.698 0.004	0.299 0.279	0.914 <0.001
5-HIAA											-	0.724 0.002	0.580 0.024
TRYPT												-	0.242 0.385

Figure 2-1: Changes in amino acid concentrations in the spinal cord during MOG_{35-55} EAE. (A-I) Normalized data relative to CFA controls (n=10) showing significant changes in the concentrations of (A) glutamate, (B) GABA, (C) aspartate, (D) L-serine, (E) D-serine, (G) alanine, (H) taurine and (I) glycine and (F) the ratio of L-/D-serine concentration changes in the spinal cord at EAE onset (n=5), EAE Peak (n=5) and in the chronic phase of EAE (n=5). (F) Represents the change in concentrations of L-serine, relative to CFA control levels, divided by the change in concentrations of D-serine, relative to CFA controls. Values are mean ± SD. (*P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (µg/g wet tissue): Glutamate 815.9; GABA 107.7; Aspartate 352.9; L-Serine 27.3; D-Serine 0.33; Alanine 28.2; Taurine 484.3; Glycine 250.6.

Figure 2-1

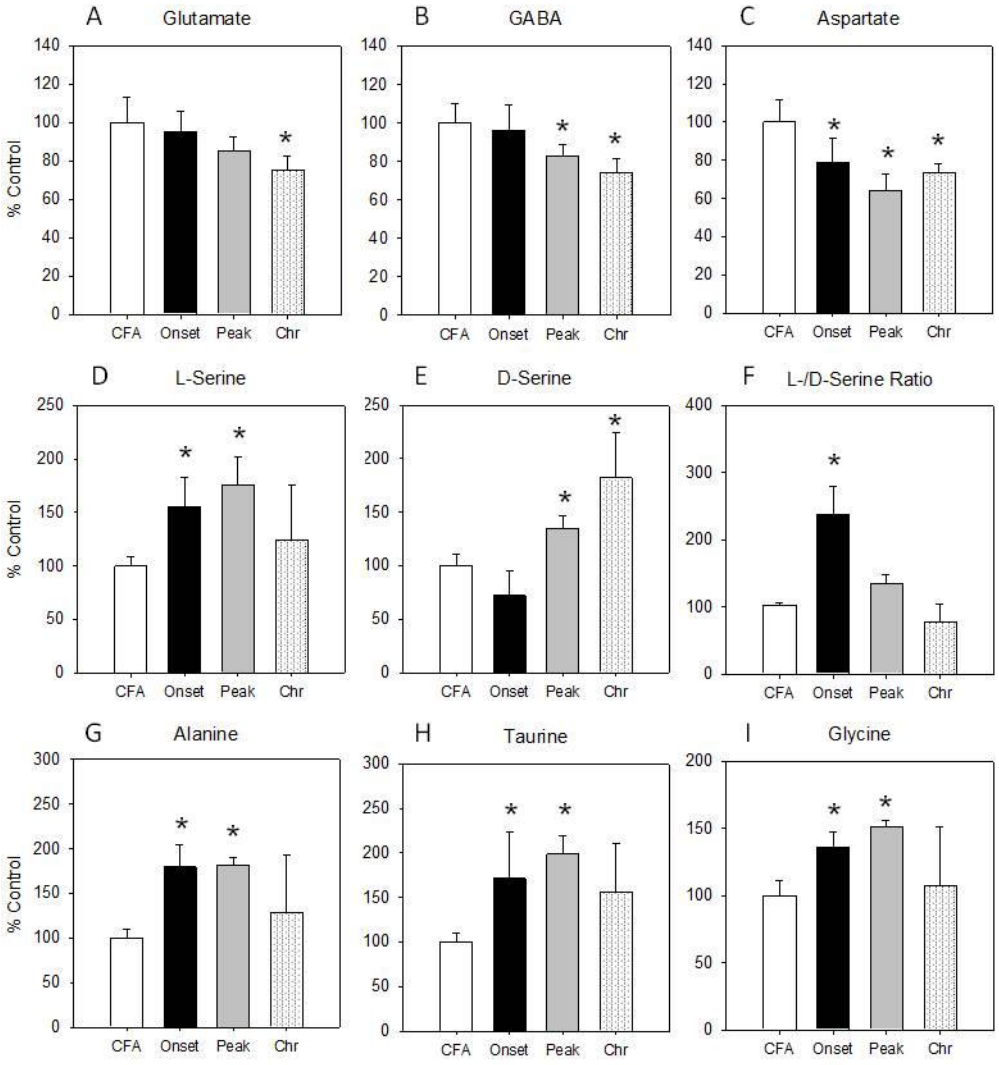

A
Glutamate
B
GABA
C
Aspartate
D
L-Serine
E
D-Serine
F
L-/D-Serine Ratio
G
Alanine
H
Taurine
I
Glycine
% Control
CFA
Onset
Peak
Chr

Figure 2-2: Changes in the concentrations of biogenic amines, 5-HIAA and tryptophan in the spinal cord during MOG_{35-55} EAE. (A-E) Normalized data relative to CFA controls (n=5) showing significant changes in the concentrations of (A) 5-HT, (B) 5-HIAA, (C) 5-HT Turnover, (D) noradrenaline and (E) tryptophan at EAE onset (n=5) and in the chronic phase of EAE (n=5). (C) Represents the ratio of 5-HT concentration changes, relative to CFA controls, divided by the concentration changes of 5-HIAA relative to CFA controls. Values are mean ± SD. (*P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls: 5-HT 778.6 ng/g; 5-HIAA 300.5 ng/g; noradrenaline 242.4 ng/g; tryptophan 1.22 µg/g.

Figure 2-2

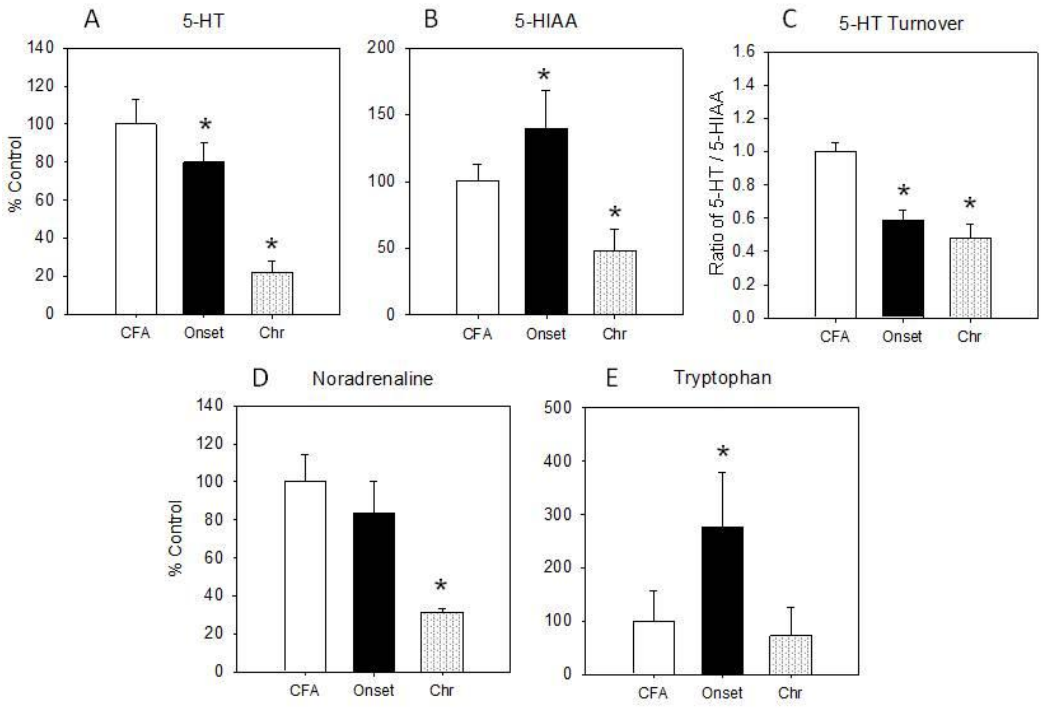
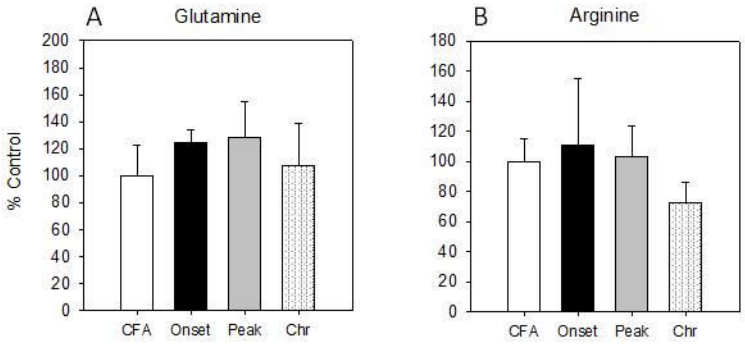

A
5-HT
% Control
140
120
100
80
60
40
20
0
CFA
Onset
Chr
B
5-HIAA
200
150
100
50
0
C
5-HT Turnover
Ratio of 5-HT / 5-HIAA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
D
Noradrenaline
E
Tryptophan
500
400
300
200
100

Figure 2-3: Concentrations of amino acids in the spinal cord during MOG_{35-55} EAE. (A-B) Normalized data relative to CFA controls (n=10) showing non-significant changes in the concentrations of (A) glutamine and (B) arginine at EAE onset (n=5) and in the chronic phase of EAE (n=5). Values are mean ± SD. (* P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's Post Hoc test). Mean concentration values of CFA controls (μg/g wet tissue): glutamine 678.0; arginine 17.7.

Figure 2-3


A
Glutamine
% Control
200
180
160
140
120
100
80
60
40
20
0
CFA
Onset
Peak
Chr
B
Arginine
180
160
140
120
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80
60
40
20
0
CFA
Onset
Peak
Chr

Figure 2-4: Changes in amino acid concentrations in the brainstem during MOG_{35-55} EAE. (A-G) Normalized data relative to CFA controls (n=10) showing significant changes in the concentrations of (A) glutamate, (B) aspartate, (C) glycine, (D) alanine, (E)arginine, (F) taurine and (G) L-serine in the brainstem at EAE onset (n=5), EAE Peak (n=5) and in the chronic phase of EAE (n=5). Values are mean ± SD. (*P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (in µg/g wet tissue): Glutamate 955.4; Aspartate 475.1; Glycine. 213.8; Arginine 22.2; Alanine 38.4; Taurine 465.2; L-serine 29.0.

Figure 2-4

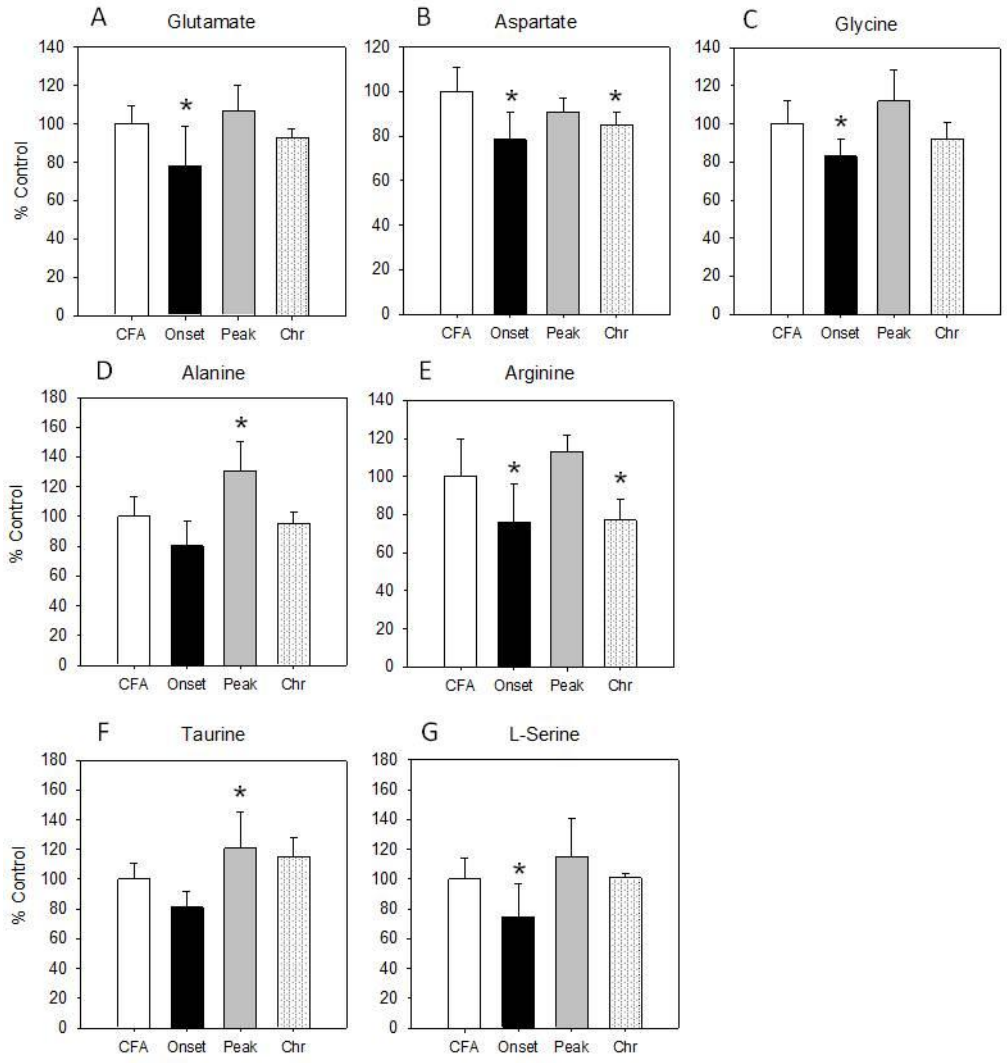

A
Glutamate
B
Aspartate
C
Glycine
D
Alanine
E
Arginine
F
Taurine
G
L-Serine
% Control
CFA
Onset
Peak
Chr

Figure 2-5: Changes in biogenic amine concentrations in the brainstem during MOG_{35-55} EAE. (A-E) Normalized data relative to CFA controls (n=5) showing significant changes in the concentrations of (A) 5-HT and (E) noradrenaline in the chronic phase of EAE (n=5). There were no significant differences in brainstem levels of (B) 5-HIAA, (D) tryptophan or (C) the turnover of 5-HT in EAE mice compared to CFA controls. (C) Represents the ratio of 5-HT concentration changes, relative to CFA controls, divided by the concentration changes of 5-HIAA, relative to CFA controls. Values are mean ± SD. (*P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls: 5-HT 488.7 ng/g; noradrenaline 458.0 ng/g; 5-HIAA 442.6 ng/g; tryptophan 2.9 µg/g.

Figure 2-5

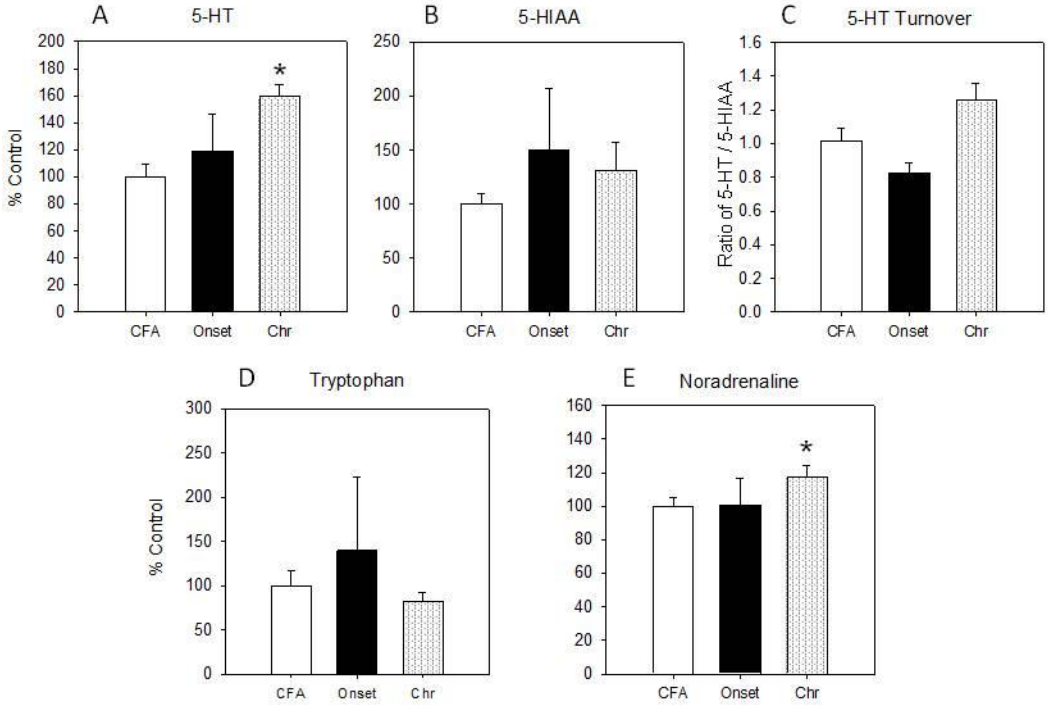

A
5-HT
% Control
200
180
160
140
120
100
80
60
40
20
0
*
CFA
Onset
Chr
B
5-HIAA
250
200
150
100
50
0
CFA
Onset
Chr
C
5-HT Turnover
Ratio of 5-HT / 5-HIAA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CFA
Onset
Chr
D
Tryptophan
% Control
300
250
200
150
100
50
0
CFA
Onset
Chr
E
Noradrenaline
160
140
120
100
80
60
40
20
0
*
CFA
Onset
Chr

Figure 2-6: Concentrations of amino acids in the brainstem during MOG_{35-55} EAE. (A-D) Normalized data relative to CFA controls (n=10) showing non-significant changes in the concentrations of (A) GABA, (B) glutamine, (C) D-serine and (D) the L-/D-serine ratio at EAE onset (n=5), EAE Peak (n=5) and in the chronic phase of EAE (n=5). (D) Represents the change in concentrations of L-serine, relative to CFA control levels, divided by the change in concentrations of D-serine, relative to CFA controls. Values are mean ± SD. (* P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's Post Hoc test). Mean concentration values of CFA controls (µg/g wet tissue): GABA 205.1; glutamine 641.4; D-serine 1.87.

Figure 2-6

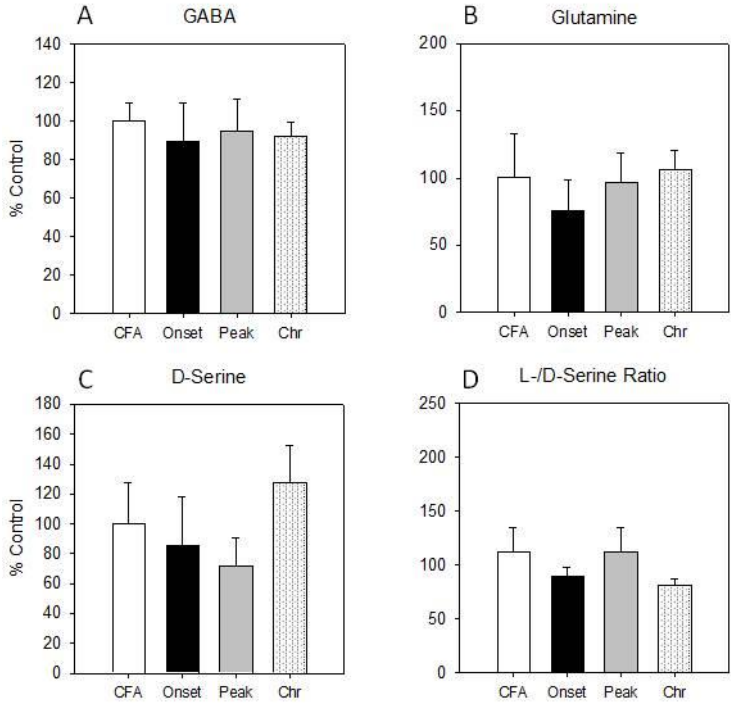

A
GABA
% Control
140
120
100
80
60
40
20
0
CFA
Onset
Peak
Chr
B
Glutamine
200
150
100
50
0
CFA
Onset
Peak
Chr
C
D-Serine
% Control
180
160
140
120
100
80
60
40
20
0
CFA
Onset
Peak
Chr
D
L-/D-Serine Ratio
250
200
150
100
50
0
CFA
Onset
Peak
Chr

Figure 2-7: Changes in the concentrations of amino acids in the cerebellum during MOG_{35-55} EAE. (A-E) Normalized data relative to CFA controls (n=9) showing significant changes in the cerebellum concentrations of (A) glutamate, (B) glycine, (C) L-serine, (D) taurine and (E) arginine EAE onset (n=5), EAE Peak (n=3) and in the chronic phase of EAE (n=5). Values are mean ± SD. (* P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (in µg/g wet tissue): glutamate 1379.1; glycine 46.1; L-serine 31.8; taurine 955.7; arginine 14.0.

Figure 2-7

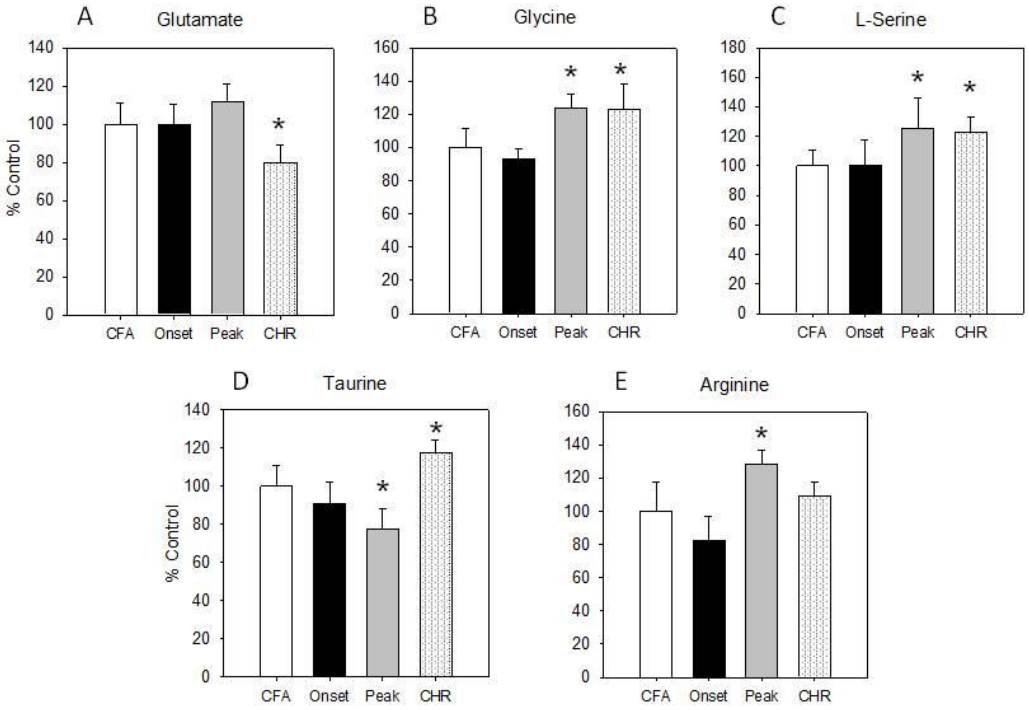

A
Glutamate
% Control
CFA
Onset
Peak
CHR
B
Glycine
C
L-Serine
D
Taurine
E
Arginine

Figure 2-8: Concentrations of biogenic amines, 5-HIAA, tryptophan and 5-HT turnover in the cerebellum during EAE. (A-E) Normalized data relative to CFA controls (n=5) showing non-significant changes in the concentrations of (A) 5-HT, (B) 5-HIAA, (D) tryptophan and (E) noradrenaline and (C) 5-HT turnover at EAE onset (n=5) and in the chronic phase of EAE (n=5). (C) Represents the ratio of 5-HT concentration changes, relative to CFA controls, divided by the concentration changes of 5-HIAA relative to CFA controls. Values are mean ± SD. (* P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's Post Hoc test). Mean concentration values of CFA controls: 5-HT 173.0 ng/g; 5-HIAA 135.1 ng/g; tryptophan 2.4 μg/g; noradrenaline 252.8 ng/g.

Figure 2-8

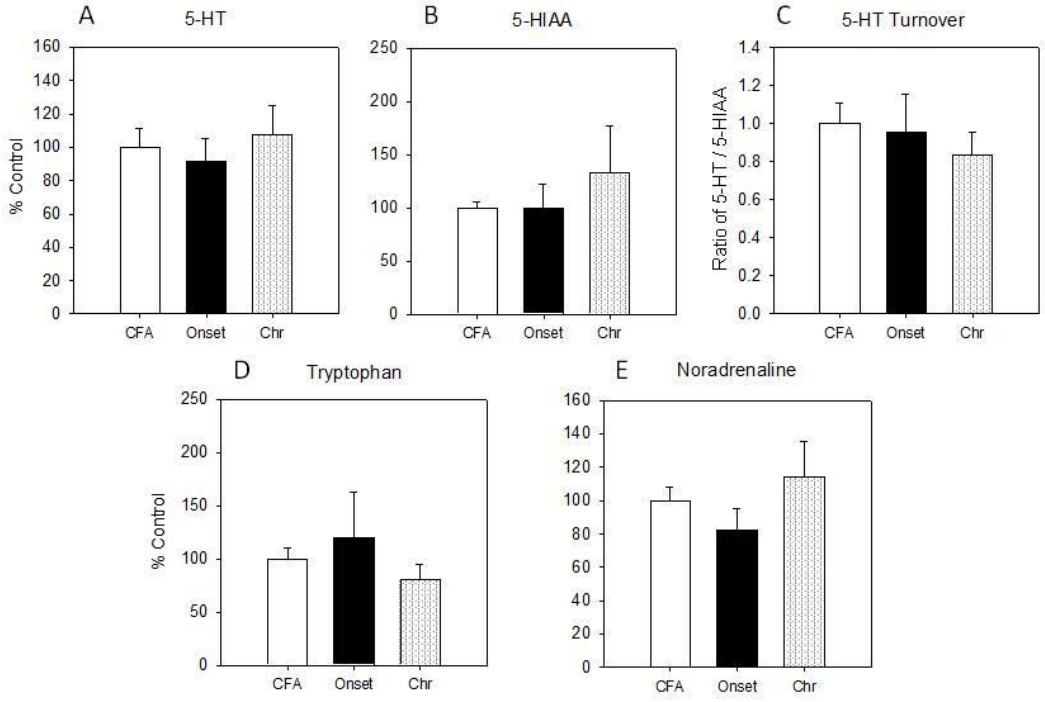

A
5-HT
% Control
160
140
120
100
80
60
40
20
0
CFA
Onset
Chr
B
5-HIAA
250
200
150
100
50
0
CFA
Onset
Chr
C
5-HT Turnover
Ratio of 5-HT / 5-HIAA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CFA
Onset
Chr
D
Tryptophan
% Control
250
200
150
100
50
0
CFA
Onset
Chr
E
Noradrenaline
160
140
120
100
80
60
40
20
0
CFA
Onset
Chr

Figure 2-9: Concentrations of amino acids in the cerebellum during EAE. (A-F) Normalized data relative to CFA controls (n=9) showing non-significant changes in the concentrations of (A) GABA, (B) aspartate, (C) D-serine, (D) glutamine, (E) alanine and (F) the ratio of L-/D-serine concentration changes at EAE onset (n=5), EAE Peak (n=3) and in the chronic phase of EAE (n=5). (F) Represents the change in concentrations of L-serine, relative to CFA control levels, divided by the change in concentrations of D-serine, relative to CFA controls. Values are mean ± SD. (* $P \leq 0.05$ compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (in µg/g wet tissue): GABA 197.6; aspartate 350.9; D-serine 0.55; glutamine 941.8; alanine 32.3.

Figure 2-9

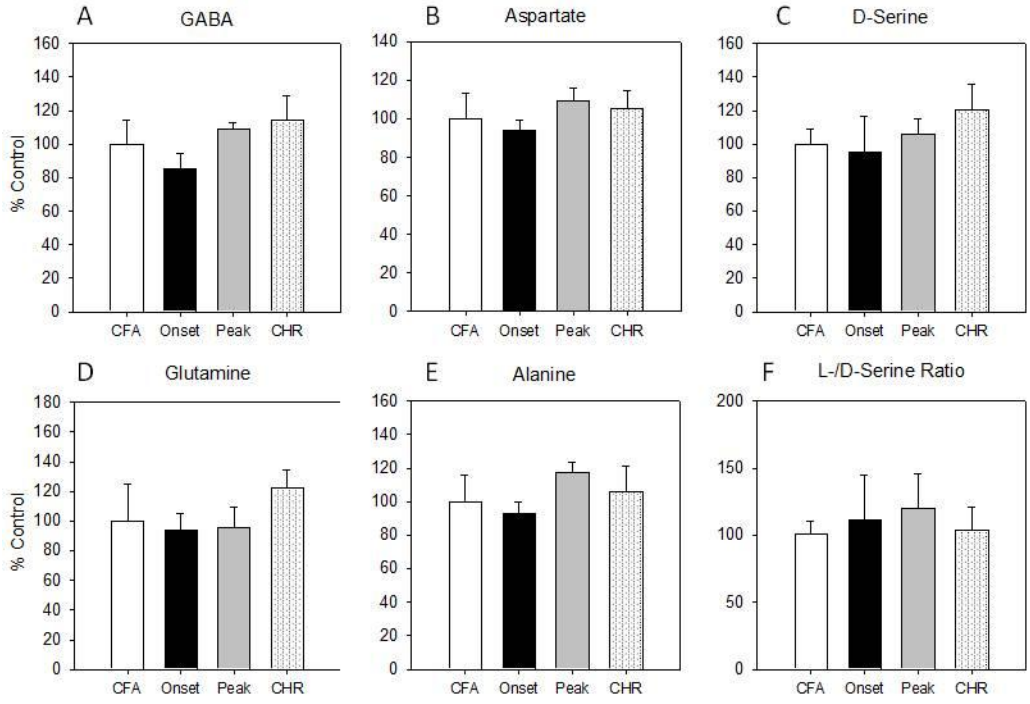

A
GABA
B
Aspartate
C
D-Serine
D
Glutamine
E
Alanine
F
L-/D-Serine Ratio
% Control
CFA
Onset
Peak
CHR

Figure 2-10: Concentrations of amino acids in the hypothalamus during MOG_{35-55} EAE. (A-K) Normalized data relative to CFA controls (n=10) showing non-significant changes in the hypothalamus concentrations of (A) glutamate, (B) GABA, (C) glycine, (D) aspartate, (E) glutamine, (F) L-serine, (G) D-serine, (I) alanine, (J) arginine, (K) taurine and (H) the ratio of L-/D-serine changes at EAE onset (n=5), EAE Peak (n=5) and in the chronic phase of EAE (n=5). (H) Represents the change in concentrations of L-serine, relative to CFA control levels, divided by the change in concentrations of D-serine, relative to CFA controls. Values are mean ± SD. (* P≤0.05 compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (in µg/g wet tissue): glutamate 927.6; GABA 260.5; glycine 42.2; aspartate 271.0; glutamine 634.6; L-serine 20.4; D-serine 7.31; alanine 23.2; arginine 7.9; taurine 539.3.

Figure 2-10

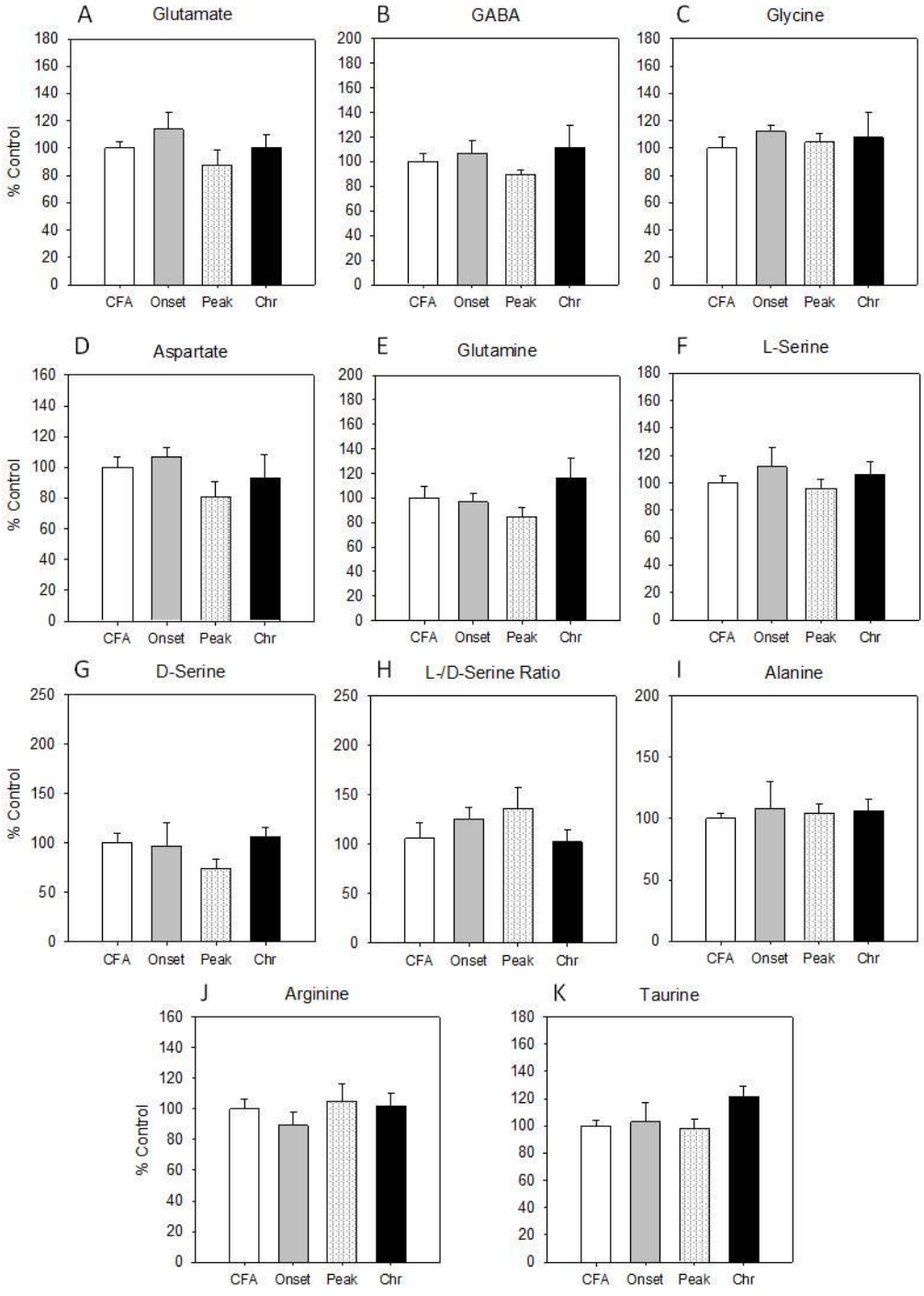

A
Glutamate
B
GABA
C
Glycine
D
Aspartate
E
Glutamine
F
L-Serine
G
D-Serine
H
L-/D-Serine Ratio
I
Alanine
J
Arginine
K
Taurine
% Control
CFA
Onset
Peak
Chr

Figure 2-11: Concentrations of amino acids in the cerebrum during MOG_{35-55} EAE. (A-K) Normalized data relative to CFA controls (n=10) showing non-significant changes in the concentrations of (A) glutamate, (B) GABA, (C) glycine, (D) aspartate, (E) glutamine, (F) L-serine, (G) D-serine, (I) alanine, (J) arginine, (K) taurine and (H) the ratio of L-/D-serine concentration changes at EAE onset (n=5), EAE Peak (n=5) and in the chronic phase of EAE (n=5). (H) Represents the change in concentrations of L-serine, relative to CFA control levels, divided by the change in concentrations of D-serine, relative to CFA controls. Values are mean ± SD. (* $P \leq 0.05$ compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls (in µg/g wet tissue): glutamate 1074.7; GABA 362.4; glycine 73.5; aspartate 353.0; glutamine 846.1; L-serine 42.3; D-serine 21.5; alanine 58.1; arginine 24.7; taurine 1018.4.

Figure 2-11

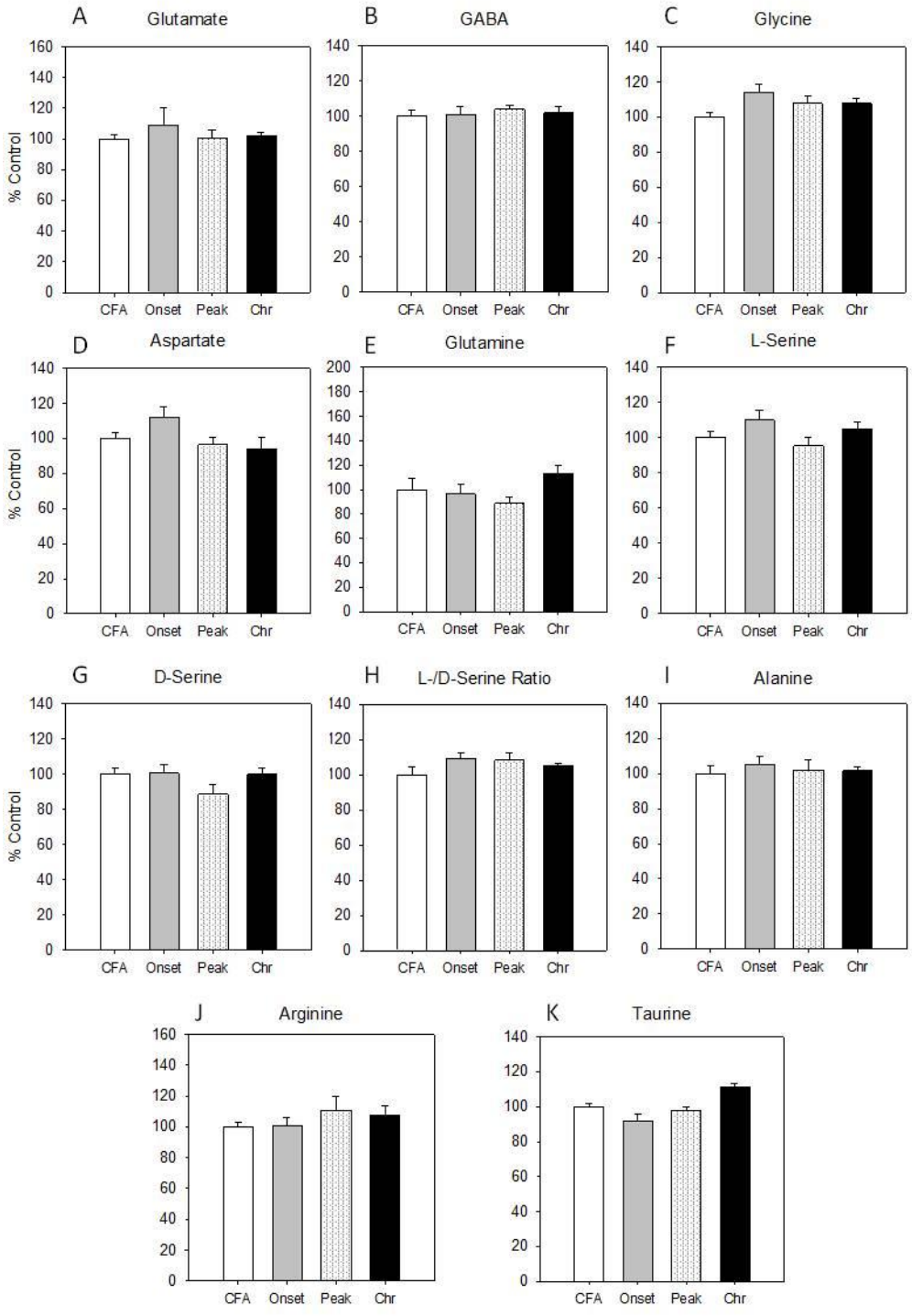

A
Glutamate
B
GABA
C
Glycine
D
Aspartate
E
Glutamine
F
L-Serine
G
D-Serine
H
L-/D-Serine Ratio
I
Alanine
J
Arginine
K
Taurine
% Control
CFA
Onset
Peak
Chr

Figure 2-12: Changes in concentrations of biogenic amines, 5-HIAA and tryptophan in the cerebrum during MOG_{35-55} EAE. (A-E) Normalized data relative to CFA controls (n=5) showing the changes in the concentrations of (A) 5-HT, (B) 5-HIAA, (D) noradrenaline and (E) tryptophan at EAE onset (n=5) and in the chronic phase of EAE (n=5). (C) 5-HT turnover was significantly increased compared to CFA levels at disease onset. (C) Represents the ratio of 5-HT concentration changes, relative to CFA controls, divided by the concentration changes of 5-HIAA relative to CFA controls. Values are mean ± SD. (* $P \leq 0.05$ compared to CFA levels, one-way ANOVA, Dunnett's post hoc test). Mean concentration values of CFA controls: 5-HT 709.6 ng/g; 5-HIAA 273.5 ng/g; noradrenaline 342.9 ng/g; tryptophan 3.1 µg/g.

Figure 2-12

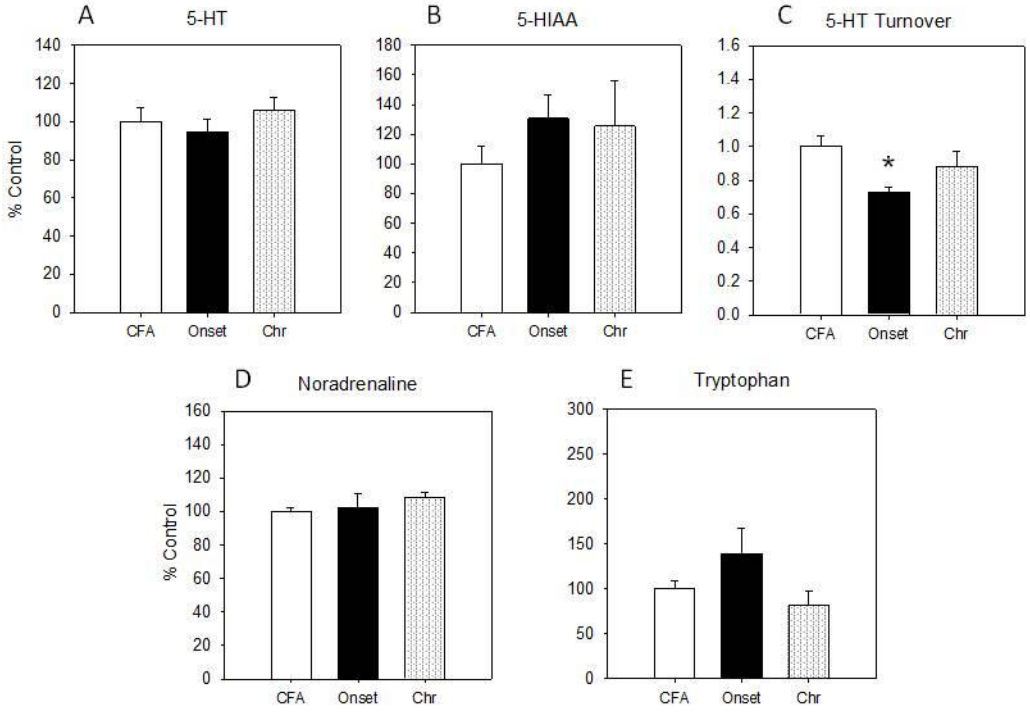
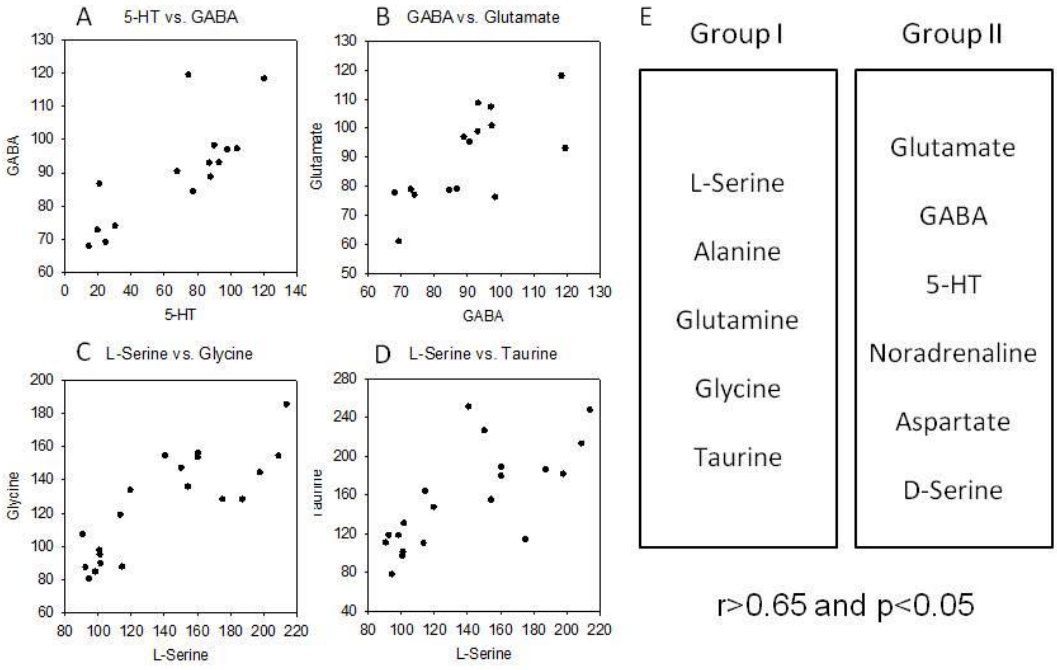

A
5-HT
% Control
140
120
100
80
60
40
20
0
CFA
Onset
Chr
B
5-HIAA
180
160
140
120
100
80
60
40
20
0
CFA
Onset
Chr
C
5-HT Turnover
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
CFA
Onset
Chr
D
Noradrenaline
% Control
160
140
120
100
80
60
40
20
0
CFA
Onset
Chr
E
Tryptophan
300
250
200
150
100
50
0
CFA
Onset
Chr

Figure 2-13: Correlations of amino acid and biogenic amine concentration changes in the spinal cord during MOG_{35-55} EAE. (A-D) Examples of significant correlations between the changes of (A) 5-HT and GABA ($r=0.774$, $P<0.05$), (B) GABA and glutamate ($r=0.794$, $P<0.05$), (C) L-serine and glycine ($r=0.839$, $P<0.05$) and (D) L-serine and taurine ($r=0.727$, $P<0.05$). (E) Concentration changes in the spinal cord occur in two major groups of analytes.

Figure 2-13


A 5-HT vs. GABA
GABA
5-HT
B GABA vs. Glutamate
Glutamate
GABA
C L-Serine vs. Glycine
Glycine
L-Serine
D L-Serine vs. Taurine
Taurine
L-Serine
E
Group I
L-Serine
Alanine
Glutamine
Glycine
Taurine
Group II
Glutamate
GABA
5-HT
Noradrenaline
Aspartate
D-Serine
r>0.65 and p<0.05

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CHAPTER 3 THE EFFECTS OF ACUTE PHENELZINE TREATMENT ON THE CONCENTRATIONS OF AMINO ACIDS AND BIOGENIC AMINES IN THE CENTRAL NERVOUS SYSTEMS OF MICE WITH EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

3.1 Introduction

In the previous chapter, concentration changes of amino acids and biogenic amines were evaluated in the CNS during the disease progression of the MOG_{35-55} model of EAE, an animal model used for the study of MS. Permanent deficits in spinal cord concentrations of noradrenaline, 5-HT and GABA appear to be prominent features of this model. As previously discussed, these neurotransmitters possess functions that are both neuromodulatory and immunomodulatory. Consequently, their reduced signalling and activities may particularly contribute to EAE pathogenesis by exacerbating neuroinflammation and altering neuronal excitability. Recent research has additionally shown that therapeutic agents that selectively increase GABAergic and monoaminergic signalling can lessen the severity of EAE [1-6].

Because of its potent ability to increase the CNS concentrations of biogenic amines and GABA, the antidepressant drug phenelzine (PLZ) is a promising candidate therapy to alleviate the clinical symptoms and neuropathological features of EAE [7-13]. Clinically, PLZ is effective in the treatment of psychiatric disorders like atypical depression, social anxiety disorder and panic disorder [14]. PLZ is also active in animal tests for antidepressants and anxiolytics such as the forced swim test, the elevated-plus maze, fear-conditioned freezing behaviour, and the mouse defence test battery [15-18]. Depression-like symptoms can also be observed in various animal models of CNS disease, including EAE [19]. PLZ's suitability for treating sickness associated behaviours in animal models that are reflective of depression and anxiety is unknown.

PLZ irreversibly and non-selectively inhibits the monoamine oxidase (MAO) enzymes responsible for the degradation of biogenic amines [14]. PLZ is also metabolized by MAO , however, and bioactive metabolites such as phenylethylidenehydrazine (PEH) inhibit the breakdown of GABA by GABA transaminase (GABA-T), resulting in an increase in GABA concentrations in the CNS [13, 20]. PLZ, through PEH, induces similar increases in alanine concentrations by inhibiting alanine

transaminase (ALA-T)[21-22]. PLZ's effects on biogenic amines and amino acids are temporally dissociated, however. While a single, acute dose of PLZ can cause elevations in biogenic amine concentrations detectable up to seven days after administration, changes to amino acid concentrations typically disappear around 24 hours [23]. Concentrations of other amino acids are also affected by PLZ and typical effects include concentration decreases in glutamine, glycine, and serine and concentration increases in tryptophan, asparagine and ornithine [13, 23-24]. Treatment additionally increases the activity of the hypothalamic-pituitary-adrenal (HPA) axis, which normally loses sensitivity in chronic EAE but has been shown to be disease-suppressive [25-26].

Apart from presumably attenuating glutamatergic excitotoxicity and inflammatory injury by increasing the concentrations of GABA, various lines of evidence further suggest that PLZ treatment will be neuroprotective during EAE. *In-vitro* experiments show that PEH reduces seizure activity in brain slices without affecting normal electrophysiology [27]. The hydrazine function of PLZ/PEH confers further protective benefits by reacting with and sequestering reactive aldehydes in a model of ischemia [28]. In conditions of formaldehyde-induced toxicity, PLZ also increases the expression of glutamate transporters and reverses losses in glutamate uptake [29]. Additional effects include reducing the synaptic release of glutamate and aspartate and enhancing GABA extracellular concentrations, synaptic release, and receptor activity [30-33]. Furthermore, PLZ treatment appears to be able to decrease the expression of pro-inflammatory transcripts in the hippocampus [34]. While an evaluation of an MAO inhibitor in EAE has not been conducted, the selective MAO-A inhibitor moclobremide has been shown to decrease pro-inflammatory cytokine production and be neuroprotective [35-38]. By increasing the concentrations of 5-HT and noradrenaline in the spinal cord, locomotor deficits that may be attributed to decreased neuronal excitability could also be prevented.

In this experiment, the potential neuroprotective and clinical benefits of PLZ treatment in EAE are given a preliminary exploration through the assessment of PLZ's effects on the concentrations of amino acids and biogenic amines in mice with EAE in five regions of the CNS (spinal cord, brainstem, cerebellum, hypothalamus and cerebrum) using HPLC. To our knowledge, this constitutes the first evaluation of an MAO

inhibitor in the treatment of EAE. We also examine the relative efficacy of PLZ treatment on 5-HT, noradrenaline and GABA concentrations in CNS regions of CFA and EAE mice. Our results show that PLZ affects the concentrations of various amino acids and biogenic amines in the CNS during EAE, although the specific substances altered and the relative magnitudes of such changes may differ between regions. Notably, however, prominent concentration increases in 5-HT, noradrenaline and GABA are always observed. These results support the concept that PLZ should be capable of modifying the clinical and neuropathological aspects of EAE and may consequently warrant consideration as a therapeutic option in MS.

3.2 Methods

3.2.1 EAE and PLZ administration

This experiment was conducted in conjunction with the second experiment described in the previous chapter. PLZ was administered to mice at EAE "Peak" (clinical score of grade 3) or controls (CFA) via a single, intra-peritoneally (IP) injection at a dose of 30 mg/kg in bacteriostatic water according to PLZ's free base weight. From previous reports showing the clinical and neurochemical effects of PLZ treatment in animals, it was determined that this dose should cause clinically relevant changes in amino acid and biogenic amine concentrations [12, 17-18, 21, 23, 28, 31, 33, 39]. 3 hours after injection, a time at which target concentration changes in amino acids and biogenic amines have neared their peak elevation, mice were euthanized and regions of the CNS were extracted and frozen according to the methods in the previous chapter [21, 28, 39].

3.2.2 High Performance Liquid Chromatography (HPLC)

HPLC materials and methods for the detection and quantification of amino acid and biogenic amine concentrations in the CNS were the same as used in the previous chapter. Concentrations of dopamine and its metabolites were not evaluated in this study because there was no comparative data from the previous experiment. The effects of PLZ treatment on D-serine concentrations in the spinal cord, cerebellum and

brainstem were also not analysed because treatment produced interfering peaks in the chromatograms of these regions that obstructed reliable quantification.

3.2.3 Statistical Analysis

Statistical analysis of the effects of PLZ treatment in CFA and EAE mice was conducted using Student's t-tests and statistical significance was set at $P \leq 0.05$. The relative effects of PLZ treatment in CFA and EAE mice were compared by calculating % change ([concentration-CFA or EAE average concentration]/CFA or EAE average concentration multiplied by 100).

In the previous chapter, biogenic amines were only analysed in samples of the second experiment. No data from mice at EAE Peak, therefore, was obtained that could be compared with the PLZ treated EAE mice in this experiment. To make this comparison, concentrations of biogenic amines from EAE chronic phase mice were selected to represent "EAE." Chronic phase concentrations, rather than those at EAE onset, were selected for the major reason that these mice had previously achieved disease "Peak". In the reports by Krenger et al. (1986), spinal concentrations of 5-HT and noradrenaline decreased progressively after each EAE attack [40]. The previous chapter's results for the MOG_{35-55} EAE model also suggest a pattern of progressive change for 5-HT, noradrenaline and GABA. For the target neurotransmitters in this experiment, therefore, the concentrations obtained from mice at EAE onset would be less representative of EAE Peak because the initial EAE exacerbation was incomplete.

3.3 Results

3.3.1 The effect of PLZ treatment on amino acids and biogenic amines in the spinal cords of CFA and EAE mice

PLZ treatment is shown in this experiment to effectively elevate the CNS concentrations of our target neurotransmitters 5-HT, noradrenaline, and GABA throughout the CNS as well as modulate the concentrations of numerous other substances. In the spinal cord of CFA and EAE mice (Figure 3-1), PLZ significantly elevated the concentrations of 5-HT (Fig. 3-1A), noradrenaline (Fig. 3-1B) and GABA (Fig.

3-1C)(*5-HT*, CFA: 100.00 ± 5.82 %CFA vs. CFA PLZ: 199.27 ± 3.80 %CFA, P<0.001; EAE: 21.92 ± 2.64 %CFA vs. EAE PLZ: 235.09 ± 33.56 %CFA, P<0.001; *noradrenaline*, CFA: 100.00 ± 6.45 %CFA vs. CFA PLZ: 204.35 ± 12.60 %CFA, P<0.001; EAE: 31.42 ± 0.79 %CFA vs. EAE PLZ: 128.58 ± 14.01 %CFA, P<0.001; *GABA*, CFA: 100.00 ± %CFA vs. CFA PLZ: 222.79 ±8.35, P<0.001; EAE: 82.73 ± 2.66 vs. EAE PLZ: 246.56 ± 14.96, P<0.001). PLZ treatment also significantly increased the concentration of alanine (Fig. 3-1H) and tryptophan (Fig. 3-G) in all mice and aspartate in CFA mice (Fig. 3-1E)(*alanine*, CFA: 100.00 ± 3.01 %CFA vs. CFA PLZ: 242.89 ± 7.44 %CFA, P≤0.001; EAE: 182.19 ± 3.38 %CFA vs. EAE PLZ: 516.11 ± 51.33 %CFA, P≤0.001; *tryptophan*, CFA: 100.00 ± 25.22 %CFA vs. CFA PLZ: 197.77 ± 7.55 %CFA, P=0.006; *aspartate*, CFA: 100.00 ± 3.78 %CFA vs. CFA PLZ: 120.50 ± 5.27 %CFA, P=0.008). 5-HIAA concentrations were decreased in CFA mice that received PLZ but increased in EAE mice treated with PLZ (CFA: 100.00 ± 5.63 %CFA vs. CFA PLZ: 72.09 ± 3.81 %CFA, P=0.003; EAE: 48.19 ± 7.17 %CFA vs. EAE PLZ: 97.85 ± 9.15 %CFA, P=0.003, 3-1D). Decreased concentrations of glutamine were also observed in CFA animals (CFA: 100.00 ± 7.08 %CFA vs. CFA PLZ: 77.56 ± 1.33 %CFA, P=0.047, Fig. 3-1F). Spinal concentrations of glutamate, glycine, arginine, taurine and L-serine were not significantly altered by PLZ treatment (Fig. 3-2).

3.3.2 The effect of PLZ treatment on amino acids and biogenic amines in the brainstems of CFA and EAE mice

In the brainstem (Figure 3-3), PLZ treatment increased the concentrations of 5-HT (Fig. 3-3A), noradrenaline (Fig. 3-3B), GABA (Fig. 3-3C), and alanine (Fig.16I) in CFA and EAE mice (*5-HT*, CFA: 100.00 ± 4.31 %CFA vs. CFA PLZ: 411.22 ± 40.14 %CFA, P≤0.001; EAE: 159.47 ± 3.79 %CFA vs. EAE PLZ: 527.47 ± 30.37 %CFA, P≤0.001; *noradrenaline*, CFA: 100.00 ± 2.24 %CFA vs. CFA PLZ: 151.02 ± 9.62 %CFA, P≤0.001; EAE: 117.48 ± 2.89 %CFA vs. EAE PLZ: 168.25 ± 5.63 %CFA, P≤0.001; *GABA*, CFA: 100.00 ± 3.00 %CFA vs. CFA PLZ: 231.72 ± 26.47 %CFA, P≤0.001; EAE: 94.68 ± 7.63 %CFA vs. EAE PLZ: 249.02 ± 30.42 %CFA, P=0.001; *alanine*, CFA: 100.00 ± 4.31 %CFA vs. CFA PLZ: 213.11 ± 14.29 %CFA, P≤0.001; EAE: 130.59 ± 8.73 %CFA vs. EAE PLZ: 260.56 ± 13.86, P≤0.001). EAE mice treated with PLZ had significantly reduced concentrations of glutamate (Fig. 3-

3D), glycine (Fig. 3-3E), and glutamine (Fig. 3-3F) but increased concentrations of tryptophan (Fig. 3-3G)(*glutamate*, EAE: 106.58 ± 6.15 vs. EAE PLZ: 71.66 ± 4.54, P=0.002; *glycine*, EAE: 112.03 ± 7.20 %CFA vs. EAE PLZ: 86.06 ± 3.88 %CFA, P=0.013; *glutamine*, EAE: 96.69 ± 9.64 %CFA vs. EAE PLZ: 67.96 ± 5.23, P=0.031; *tryptophan*, EAE: 81.80 ± 4.76 ± EAE PLZ: 136.48 ± 11.19 %CFA, P=0.002). Arginine (Fig. 3-3H) was significantly increased in the brainstems of CFA mice only (CFA: 100.00 ± 6.13 %CFA vs. CFA PLZ: 123.01 ± 5.45 %CFA, P=0.032). Changes in the brainstem concentrations of 5-HIAA, aspartate, taurine, and L-serine were not significant in CFA and EAE mice after PLZ treatment (Figure 3-4).

3.3.3 The effect of PLZ treatment on amino acids and biogenic amines in the cerebella of CFA and EAE mice

In the cerebella of CFA and EAE mice, PLZ administration increased the concentrations of 5-HT (Fig. 3-5A), noradrenaline (Fig. 3-5B), GABA (Fig. 3-5C), alanine (Fig. 3-5I) and tryptophan (Fig. 3-5F) but decreased the concentrations of glycine (Fig. 3-5E)(*5-HT*, CFA: 100.00 ± 5.17 %CFA vs. CFA PLZ: 247.95 ± 59.29 %CFA, P=0.038; EAE: 107.20 ± 7.98 %CFA vs. EAE PLZ: 309.90 ± 64.11 %CFA, P=0.014; *noradrenaline*, CFA: 100.00 ± 3.70 %CFA vs. CFA PLZ: 159.16 ± 14.90 %CFA, P=0.005; EAE: 114.37 ± 9.32 %CFA vs. EAE PLZ: 156.01 ± 9.10, P=0.013; *GABA*, CFA: 100.00 ± 4.67 %CFA vs. CFA PLZ: 7.64 ± 13.48 %CFA, P≤0.001; EAE: 108.68 ± 2.44 %CFA vs. EAE PLZ: 214.31 ± 6.28 %CFA, P≤0.001; *alanine*, CFA: 100.00 ± 5.32 %CFA vs. CFA PLZ: 209.76 ± 12.83 %CFA, P≤0.001; EAE: 117.01 ± 3.76 %CFA vs. EAE PLZ: 213.67 ± 8.31, P≤0.001; *tryptophan*, CFA: 100.00 ± 4.56 %CFA vs. CFA PLZ: 153.37 ± 2.43 %CFA, P≤0.001; EAE: 80.94 ± 6.06 %CFA vs. EAE PLZ: 107.70 ± 7.60 %CFA, P=0.025; *glycine*, CFA: 100.00 ± 3.84 vs. CFA PLZ: 84.63 ± 4.35, P=0.027; EAE: 123.62 ± 4.91 vs. EAE PLZ: 80.14 ± 4.82, P=0.001). Treatment with PLZ significantly decreased the concentrations of 5-HIAA in CFA mice (Fig. 3-5D) and the concentrations of L-serine in mice with EAE (Fig. 3-5H)(*5-HIAA*, CFA: 100.00 ± 2.24 %CFA vs. CFA PLZ: 71.00 ± 10.81 %CFA, P=0.030; *L-serine*, EAE: 125.51 ± 11.84 %CFA vs. EAE PLZ: 82.38 ± 3.75 %CFA, P=0.005). Significant concentration changes in the cerebellum were not observed for glutamate, aspartate, glutamine or taurine (Figure 3-6).

3.3.4 The effect of PLZ treatment on amino acids in the hypothalami of CFA and EAE mice

In the hypothalamus, PLZ increased the concentrations of GABA (Fig. 3-7A) and alanine (Fig. 3-7E) in CFA and EAE mice (*GABA*, CFA: 100.00 ± 6.24 %CFA vs. CFA PLZ: 256.81 ± 12.18 %CFA, P≤0.001; EAE: 89.28 ± 3.82 %CFA vs. EAE PLZ: 213.99 ± 9.39 %CFA, P≤0.001; *alanine*, CFA: 100.00 ± 4.29 %CFA vs. CFA PLZ: 303.38 ± 18.70 %CFA, P≤0.001; EAE: 104.46 ± 7.29 %CFA vs. EAE PLZ: 320.17 ± 38.58 %CFA, P≤0.001). Observed changes also included significantly increased concentrations of aspartate and arginine in CFA animals (Fig. 3-7B and 3-7D) and decreased concentrations of glutamine in EAE animals (Fig. 3-7C)(*aspartate*, CFA: 100.00 ± 7.04 %CFA vs. CFA PLZ: 127.92 ± 3.73 %CFA, P=0.019; *arginine*, CFA: 100.00 ± 6.13 %CFA vs. CFA PLZ: 140.27 ± 4.33 %CFA, P≤0.001; *glutamine*, EAE: 84.52 ± 8.17 %CFA vs. EAE PLZ: 61.41 ± 4.02 %CFA, P=0.035). Hypothalamic concentrations of glutamate, D-serine, glycine, taurine, and L-serine were unaffected by PLZ administration in CFA or EAE animals (Figure 3-8).

3.3.5 The effect of PLZ treatment on amino acids and biogenic amines in the cerebra of CFA and EAE mice

In the cerebrum, PLZ treatment increased the concentrations, in all mice, of 5-HT (Fig. 3-9A). noradrenaline (Fig. 3-9B), GABA (Fig. 3-9C) and alanine (Fig. 3-9I) and decreased the concentrations of 5-HIAA (Fig. 3-9D) and glycine (Fig. 3-9F)(*5-HT*, CFA: 100.00 ± 3.18 %CFA vs. CFA PLZ: 154.25 ± 1.82 %CFA, P≤0.001; EAE: 106.25 ± 2.93 %CFA vs. EAE PLZ: 200.71 ± 10.98 %CFA, P≤0.001; *noradrenaline*, CFA: 100.00 ± 2.19 %CFA vs. CFA PLZ: 216.87 ± 2.75 %CFA, P≤0.001; EAE: 108.44 ± 2.62 %CFA vs. EAE PLZ: 160.34 ± 12.55, P=0.004; *GABA*, CFA: 100.00 ± 3.62 %CFA vs. CFA PLZ: 209.53 ± 3.45, P≤0.001; EAE: 103.93 ± 1.88 %CFA vs. EAE PLZ: 182.73 ± 10.39 %CFA, P≤0.001; *alanine*, CFA: 100.00 ± 4.19 %CFA vs. 229.86 ± 5.74 %CFA, P≤0.001; EAE: 101.91 ± 5.63 %CFA vs. EAE PLZ: 198.27 ± 9.33 %CFA, P≤0.001; *glycine*, CFA: 100.00 ± 2.81 %CFA vs. CFA PLZ: 81.91 ± 3.83 %CFA, P=0.002; EAE: 107.70 ± 4.48 %CFA vs. EAE PLZ: 91.46 ± 4.96 %CFA,P=0.041). Significant decreases in cerebral glutamine concentrations were observed in EAE mice (Fig. 3-9G) while increases in glutamate concentrations were observed in CFA animals

(Fig. 3-9E)(*glutamine*, EAE: 88.53 ± 4.81 %CFA vs. EAE PLZ: 67.06 ± 4.55 %CFA, P=0.012; *glutamate*, CFA: 100.00 ± 3.04 %CFA vs. CFA PLZ: 138.81 ± 2.99 %CFA, P≤0.001). PLZ did not induce significant changes in the cerebral concentrations of aspartate, D-serine, tryptophan, L-serine, arginine, or taurine (Figure 3-10).

3.3.6 The relative effectiveness of PLZ treatment at altering the concentrations of 5-HT, noradrenaline and GABA in CNS regions of CFA and EAE mice

The relative ability of PLZ to augment target neurotransmitters was also examined by calculating the percent change in concentrations after treatment from CFA or EAE average concentrations. In CFA animals, we find that the effect of PLZ treatment appears to vary between the CNS regions examined. Concentration increases in 5-HT range between 54% in the cerebrum to nearly 300% in the brainstem (Figure 3-11). Differences in noradrenaline alterations are milder, ranging from approximately 50% in the brainstem and cerebellum to 117% in the cerebrum (Figure 3-12). The effects of PLZ on GABA concentrations, though, are relatively consistent, ranging from only 110% in the cerebrum to 132% in the brainstem (Figure 3-13).

The effect of PLZ treatment on concentrations of 5-HT, noradrenaline and GABA were further altered by EAE. Concentration increases in 5-HT after PLZ administration were significantly more robust in the spinal cords and cerebra of EAE mice compared to treated controls but did not differ in the brainstems or cerebella (Figure 3-11) (*Spinal cords*, CFA: 99.27 ± 3.80 %CFA vs. EAE: 972.63 ± 153.10 %EAE, P≤0.001; *Cerebra*, CFA: 54.25 ± 1.82 %CFA vs. EAE: 88.90 ± 10.33 %EAE, P=0.011). Noradrenaline concentration changes after PLZ treatment were more potent in the spinal cord during EAE than in controls, were of lesser magnitudes in the cerebrum during EAE than in CFA but were no different in the brainstem or cerebellum (Figure 3-12)(*Spinal cords*, CFA: 104.35 ± 12.60 %CFA vs. EAE: 309.21 ± 44.56 %EAE, P=0.002; *Cerebra*, CFA: 116.87 ± 2.75 %CFA vs. EAE: 47.86 ± 11.57 %EAE, P≤0.001). The effect of PLZ on concentrations of GABA was significantly greater in the spinal cords, cerebella, and cerebra of EAE mice than in controls but differences did not reach significance in the brainstem (Figure 3-13)(*Spinal cords*, CFA: 122.79 ± 8.53 %CFA vs. EAE: 282.10 ± 23.19 %EAE, P≤0.001; *Cerebella*, CFA:

117.64 ± 13.48 %CFA vs. EAE: 220.37 ± 9.38 %EAE, P≤0.001; *Cerebra*, CFA: 109.53 ± 3.45 %CFA vs. EAE: 250.31 ± 19.92 %EAE, P≤0.001). To see if the relative differences in 5-HT changes translated to differences in 5-HT metabolism, the changes in 5-HIAA were also calculated (Figure 3-14). Treatment with PLZ reduced the concentrations of 5-HIAA in all CNS regions with no relative differences observed between CFA and EAE mice. The increased concentrations of 5-HIAA in the spinal cord after PLZ treatment during EAE (Fig. 3-14A) is the only exception.

3.4 Discussion

3.4.1 The effects of PLZ on concentrations of 5-HT, noradrenaline and GABA

The results of this study provide strong theoretical support for evaluating the effects of PLZ, a MAO inhibitor with effects on GABA concentrations, on the disease progression and symptomology of EAE in subsequent experiments. Critically, concentrations of 5-HT, noradrenaline, and GABA, the specific neurotransmitters we wished to modulate, were strongly enhanced by drug treatment in all regions of the CNS. Strong evidence suggests that increasing the concentrations of these three neurotransmitters will attenuate EAE severity because acting on these systems individually can reduce neurological symptoms [1-2, 4-6, 41]. Neuroprotective benefits of PLZ have been further shown in other models [27-29].

The ability of PLZ treatment to increase the concentrations of 5-HT, noradrenaline and GABA is well known and our results are consistent with previous research [7, 10-14, 23, 33, 42]. The large increase in their concentrations throughout the CNS indicates the widespread, direct inhibition of MAOs by PLZ and the suppression of GABA-T by the PLZ metabolite, PEH [9, 12-13, 20-21, 23]. Interestingly, the anxiolytic effect of PLZ in animals may depend on this effect on GABA concentrations [18]. The trend for PLZ to induce larger concentration increases in 5-HT than noradrenaline is also consistent with results showing that 5-HT concentrations increase at a lower percent inhibition of MAOs than concentrations of noradrenaline [43-44]. Our analysis contributes to the current knowledge by showing that systemic administration of PLZ affects the target neurotransmitters throughout all CNS regions, although the relative

effectiveness of treatment appears to depend on the CNS region being examined. The different regional responses to PLZ likely derive from factors such as the local presence and activity level of specific neuroanatomical networks and the relative abundances of MAO expression.

During EAE, the response of 5-HT, noradrenaline and GABA concentrations to PLZ is further altered. Spinal cord concentration changes of these neurotransmitters with PLZ treatment are consistently greater in EAE animals than in CFA controls, with an especially robust change in 5-HT. The significantly larger responses to PLZ in the spinal cord during EAE suggests that disease-specific factors probably affect regional drug sensitivity, such as the local changes to the integrity of the blood-brain barrier, the state of gliosis, and possibly inflammation-induced changes to MAO expression [45]. Different cerebral responses to PLZ may also be explained by the occurrence of similar inflammation-associated changes or even possibly by altered afferent signalling from caudal CNS structures affected by EAE [46-47]. Interestingly though, the absence of relative differences to PLZ treatment between brainstems of CFA and EAE animals indicate that this area may be particularly resilient to disease state. The disease dependence of PLZ effectivenes may have important implications for future studies because it demonstrates that disease severity could possibly modify required PLZ dosages.

3.4.2 The effects of PLZ on the concentrations of other analytes

The analysis of the other amino acids confirms that PLZ treatment elicits a CNS response more complex than simply the modififcation of 5-HT, noradrenaline and GABA concentrations. While the effects of PLZ inconsistently produced statistically significant results between regions, the general trends associated with its treatment are consistent with the results of previous studies. This includes trends for PLZ to increase CNS concentrations of alanine and tryptophan and decrease the concentrations of glutamine, glycine and 5-HIAA [12, 21, 23-24]. Like GABA and alanine concentrations, PLZ's effect on glutamine concentrations appears to depend on its metabolism to products like PEH [12]. We also see a decrease in L-serine concentrations in the

cerebellum and an increase in spinal cord and cerebral concentrations of aspartate that may relate to changes in serine and asparigine reported by Parent et al. (2000)[23]. Mixed data are obtained with regards to the concentrations of arginine, which are increased in spinal cord and hypothalamus but decreased in the cerebellum. The effects of PLZ on glutamate concentrations are also confounding. The decreased concentrations of glutamate in the spinal cord during EAE are reflective of findings showing PLZ causes decreased glutamatergic function [30-31]. However, we also see increased glutamate concentrations in the cerebrum of CFA animals treated with PLZ. This result is puzzling and probably represents an experimental artefact, possibly caused by seasonal variations in the amino acid concentrations in the CNS of mice.

3.4.3 Limitations of the present study

Several limitations of this study, however, should be considered. Perhaps most notably, the absence of samples at EAE Peak in which to analyse biogenic amine concentrations led us to select samples from chronic phase EAE mice for the generation of EAE average concentrations. As previously discussed in methods section 3.2.3, the samples from EAE chronic phase mice should contain more representative concentrations of 5-HT, noradrenaline and GABA than samples at onset because the initial EAE attack was completed. This selection may confound the results for 5-HIAA, however, because spinal 5-HIAA concentrations increase transiently at EAE onset before falling permanently [40, 48]. At EAE peak, the disease point at which EAE mice treated with PLZ are compared, 5-HIAA levels are probably much higher than what we see in samples from the chronic phase. Consequently, this may explain why PLZ treatment, which inhibits the degradation of 5-HT to 5-HIAA, appears to increase 5-HIAA concentrations in the spinal cord of EAE animals. The low statistical power in this study or the relatively short time of 3 hours after injection that we waited before extracting samples from PLZ treated mice may also explain some of the inconsistent effects of PLZ treatment on the concentrations of other amino acids.

3.4.4 General conclusions

Despite these limitations, the concentration changes we observe in the CNS after an acute administration of PLZ have promising implications during the MOG_{35-55} model of EAE. Our results show that a single dose of PLZ is potentially capable of reversing many EAE-associated concentration changes of amino acids and biogenic amines. The effect of PLZ on 5-HT, noradrenaline and GABA, this study's target neurotransmitters, was particularly strong in the spinal cord, the CNS region most damaged during EAE. Increasing the concentrations of these specific neurotransmitters, in particular, may help reduce disease burden by controlling inflammation, attenuating glutamatergic excitoxicity and maintaining neuronal membrane excitability. Furthermore, while the effects of acute treatment are weaker on the other amino acids, PLZ may potentially act to reverse changes in glutamate, glycine, aspartate, as well as L- and/or D-serine. Increasing tryptophan availability may also promote its degradation to anti-inflammatory catabolites of the kynurenic acid pathway, which can reduce the disease severity of EAE [49-51]. An additional benefit may be the ability of PLZ to reverse decreased activity of the HPA axis during EAE [25-26, 52]. The potential for PLZ to treat EAE is tested in the following chapter.

Figure 3-1: The effects of acute PLZ treatment on the concentrations of amino acids and biogenic amines in the spinal cords of CFA controls and mice with EAE. (A-H) Normalized data relative to CFA controls showing significant changes from untreated CFA (amino acids n=10, biogenic amines n=5) or EAE (n=5) levels in the spinal cord concentrations of (A) 5-HT, (B) noradrenaline, (C) GABA, (D) 5-HIAA, (E) aspartate, (F) glutamine, (G) tryptophan and (H) alanine in CFA (n=5) and/or EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-1

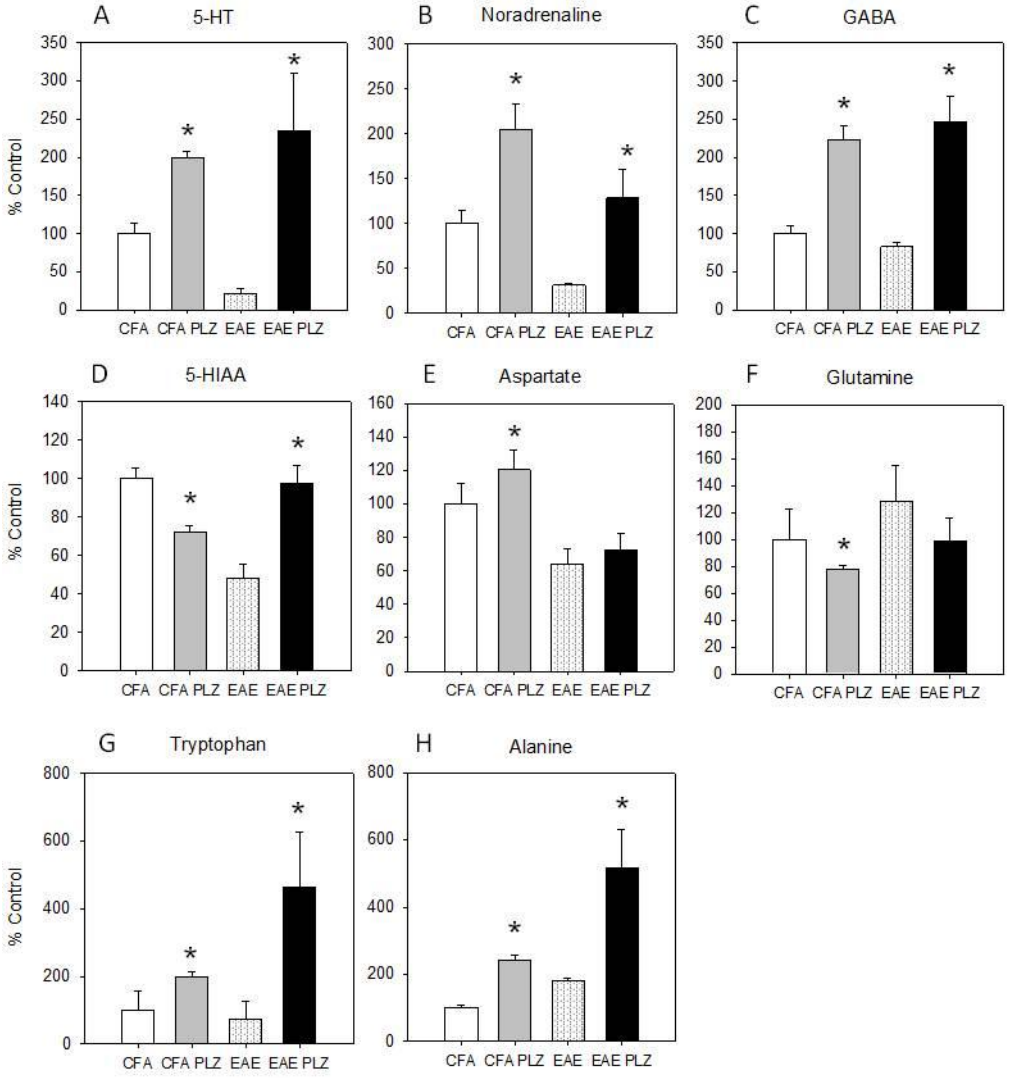

A
5-HT
B
Noradrenaline
C
GABA
D
5-HIAA
E
Aspartate
F
Glutamine
G
Tryptophan
H
Alanine
% Control
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-2: Amino acid concentrations in the spinal cords of CFA controls and mice with EAE that are unaffected by acute PLZ treatment. (A-E) Normalized data relative to CFA controls showing no significant changes from untreated CFA (amino acids n=10) or EAE (n=5) levels in the spinal cord concentrations of (A) glutamate, (B) glycine, (C) arginine, (D) taurine and (E) L-serine in CFA (n=5) and EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-2

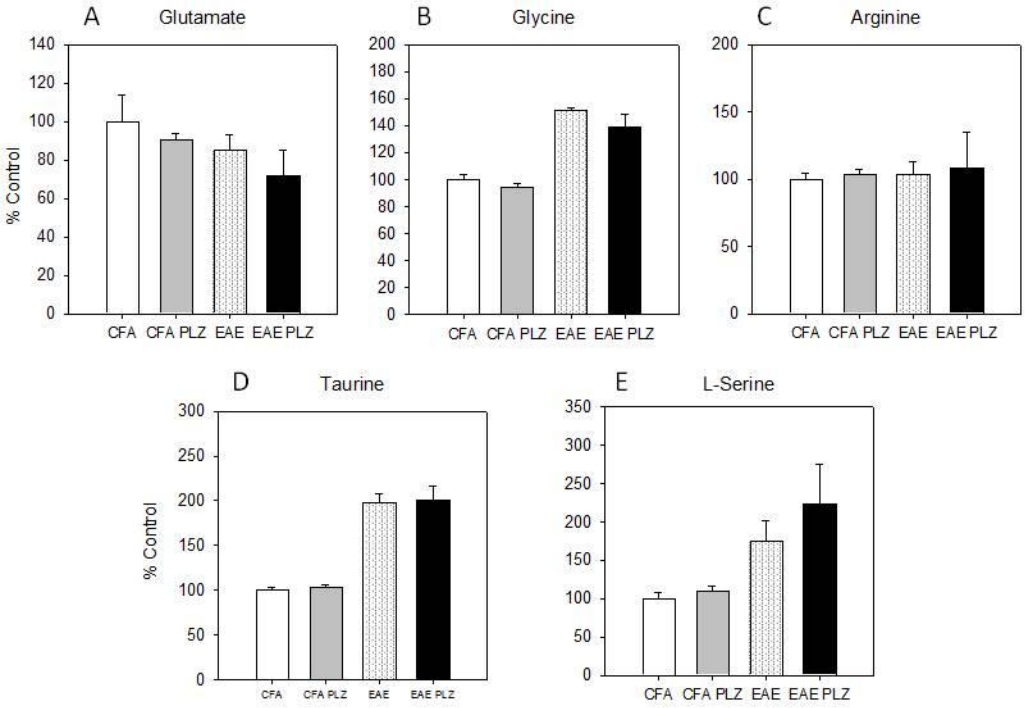

A
Glutamate
% Control
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ
B
Glycine
200
180
160
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ
C
Arginine
200
150
100
50
0
CFA
CFA PLZ
EAE
EAE PLZ
D
Taurine
% Control
300
250
200
150
100
50
0
CFA
CFA PLZ
EAE
EAE PLZ
E
L-Serine
350
300
250
200
150
100
50
0
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-3: The effects of acute PLZ treatment on the concentrations of amino acids and biogenic amines in the brainstems of CFA controls and mice with EAE. (A-I) Normalized data relative to CFA controls showing significant changes from untreated CFA (amino acids n=10, biogenic amines n=5) or EAE (n=5) levels in the brainstem concentrations of (A) 5-HT, (B) noradrenaline, (C) GABA, (D) glutamate, (E) glycine, (F) glutamine, (G) tryptophan, (H) arginine and (I) alanine in CFA (n=5) and/or EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-3

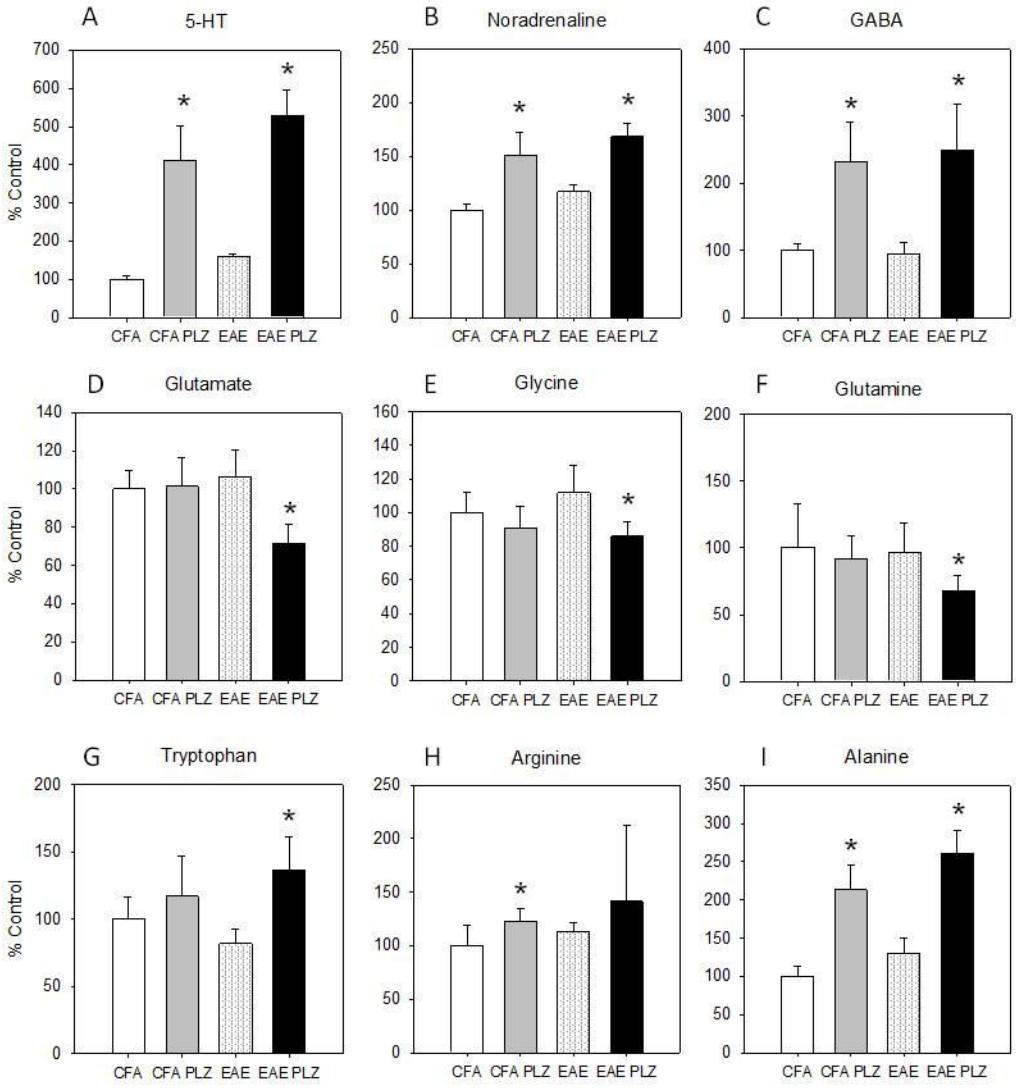

A
5-HT
B
Noradrenaline
C
GABA
D
Glutamate
E
Glycine
F
Glutamine
G
Tryptophan
H
Arginine
I
Alanine
% Control
CFA
CFA PLZ
EAE
EAE PLZ
*

Figure 3-4: Concentrations of amino acids and 5-HIAA in the brainstems of CFA controls and mice with EAE that are unaffected by acute PLZ treatment. (A-D) Normalized data relative to CFA controls showing no significant changes from untreated CFA (amino acids n=10, 5-HIAA n=5) or EAE (n=5) levels in the spinal cord concentrations of (A) 5-HIAA, (B) aspartate, (C) taurine, and (D) L-serine in CFA (n=5) and EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-4

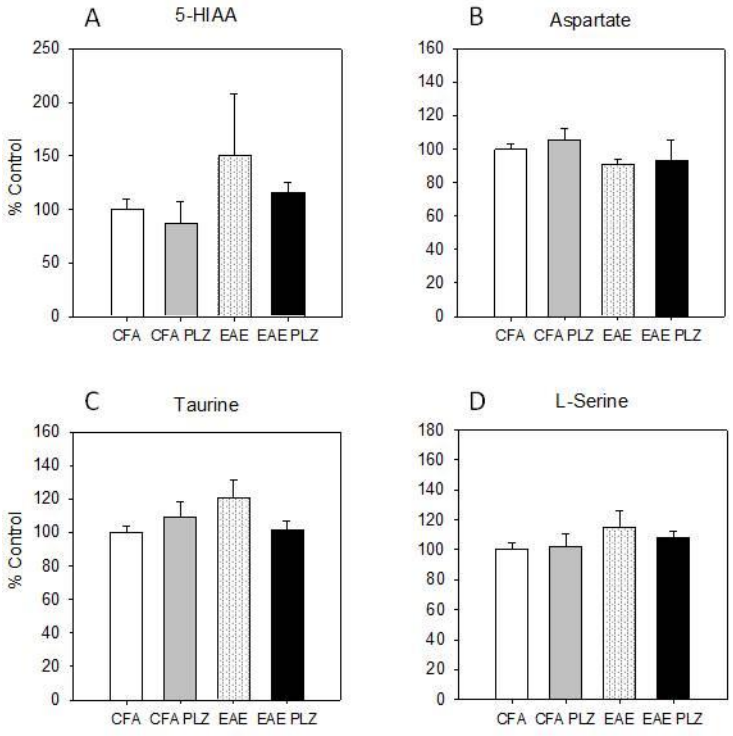

A
5-HIAA
% Control
250
200
150
100
50
0
CFA
CFA PLZ
EAE
EAE PLZ
B
Aspartate
160
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ
C
Taurine
% Control
160
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ
D
L-Serine
180
160
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-5: The effects of acute PLZ treatment on the concentrations of amino acids and biogenic amines in the cerebella of CFA controls and mice with EAE. (A-I) Normalized data relative to CFA controls showing significant changes from untreated CFA (amino acids n=9, biogenic amines n=5) or EAE (n=3) levels in the cerebellum concentrations of (A) 5-HT, (B) noradrenaline, (C) GABA, (D) 5-HIAA, (E) glycine, (F) tryptophan, (G) arginine, (H) L-serine and (I) alanine in CFA (n=5) and/or EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-5

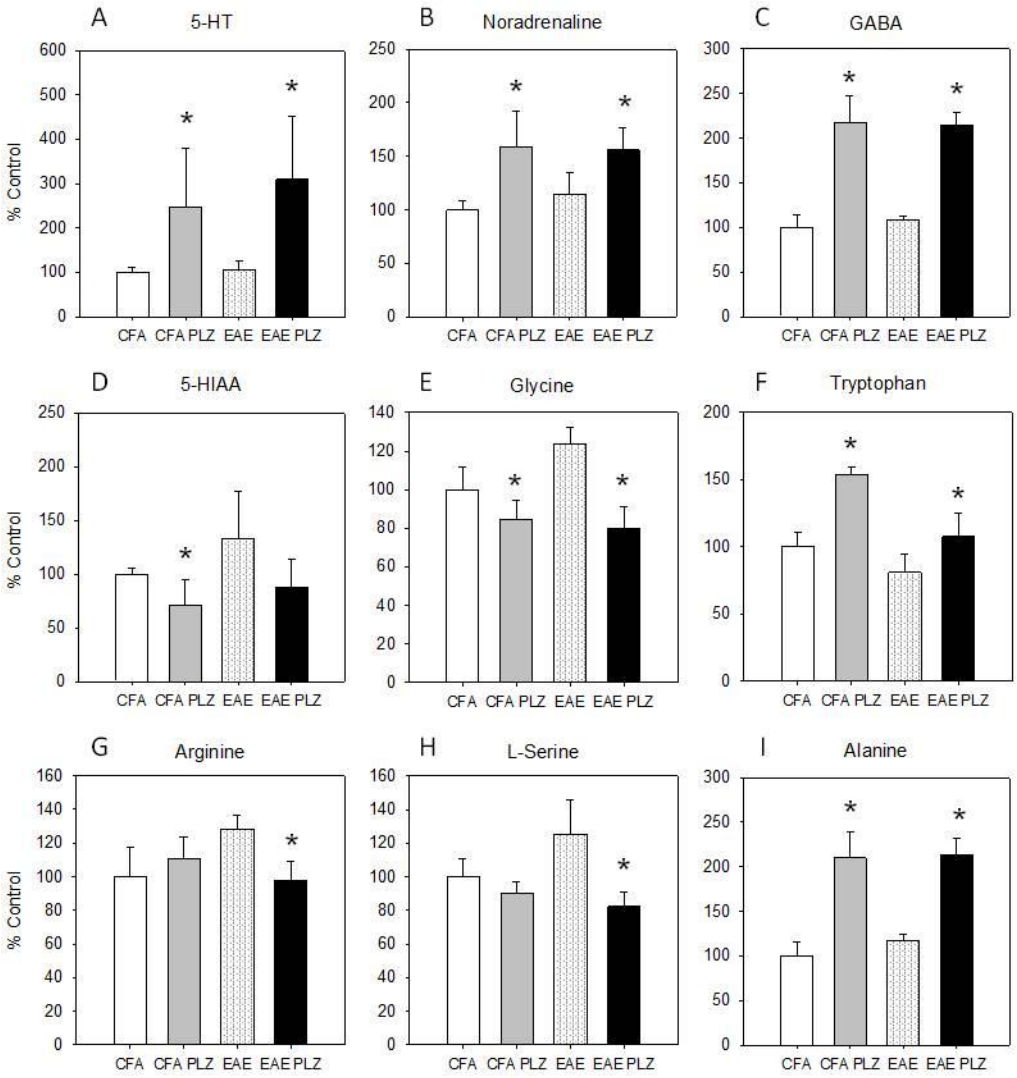

A
5-HT
B
Noradrenaline
C
GABA
D
5-HIAA
E
Glycine
F
Tryptophan
G
Arginine
H
L-Serine
I
Alanine
% Control
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-6: Concentrations of amino acids in the cerebella of CFA controls and mice with EAE that are unaffected by acute PLZ treatment. (A-D) Normalized data relative to CFA controls showing no significant changes from untreated CFA (n=9) or EAE (n=3) levels in the cerebellum concentrations of (A) glutamate, (B) aspartate, (C) glutamine, and (D) taurine in CFA (n=5) and EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-6

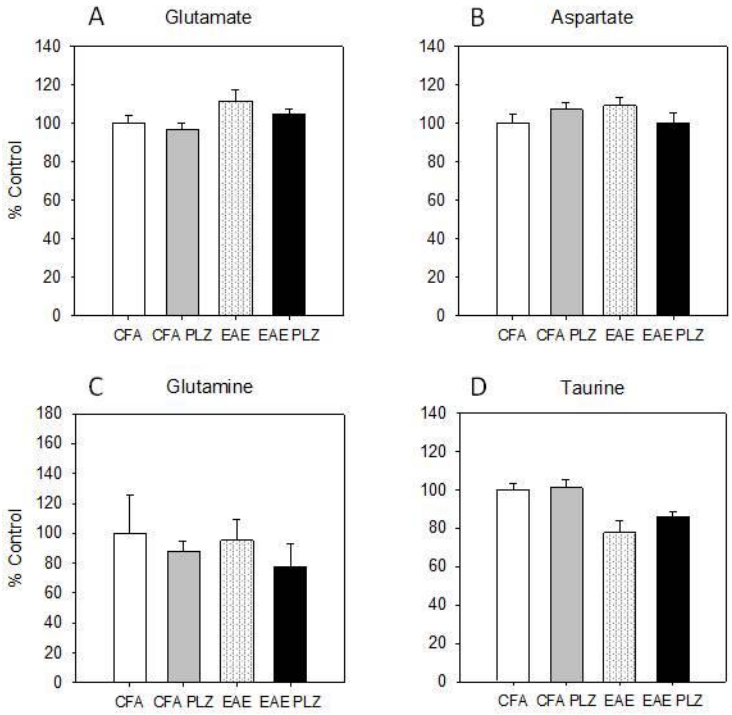

A
Glutamate
% Control
140
120
100
80
60
40
20
0
CFA
CFA PLZ
EAE
EAE PLZ
B
Aspartate
C
Glutamine
180
160
D
Taurine

Figure 3-7: The effects of acute PLZ treatment on the concentrations of amino acids in the hypothalami of CFA controls and mice with EAE. (A-E) Normalized data relative to CFA controls showing significant changes from untreated CFA (n=10) or EAE (n=5) levels in the hypothalamus concentrations of (A) GABA, (B) aspartate, (C) glutamine, (D) arginine and (E) alanine in CFA (n=5) and/or EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-7

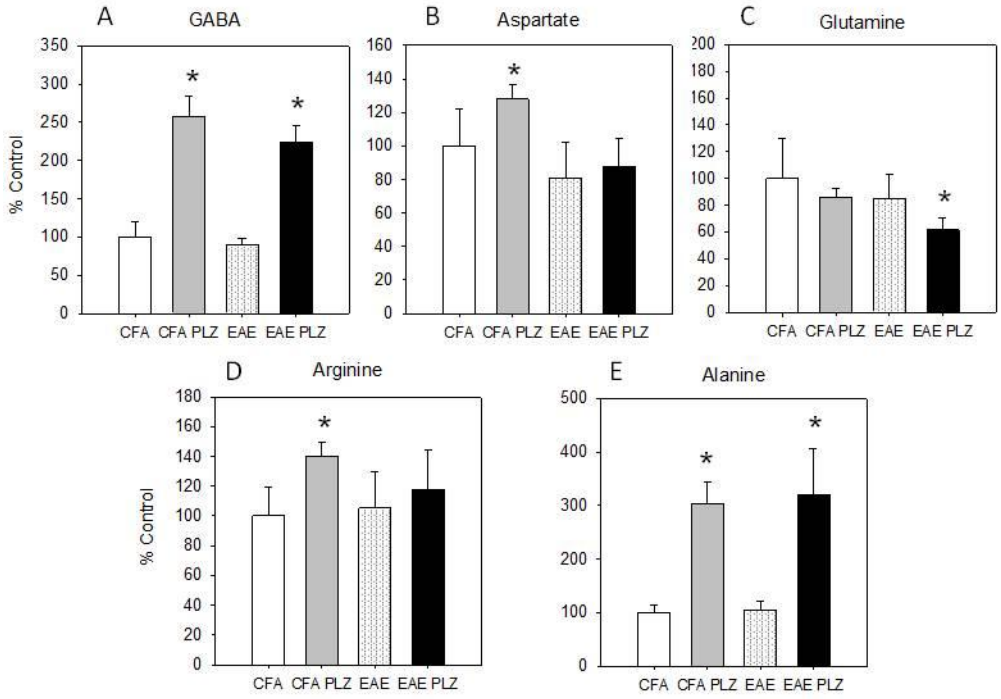

A
GABA
% Control
CFA CFA PLZ EAE EAE PLZ
B
Aspartate
CFA CFA PLZ EAE EAE PLZ
C
Glutamine
CFA CFA PLZ EAE EAE PLZ
D
Arginine
% Control
CFA CFA PLZ EAE EAE PLZ
E
Alanine
CFA CFA PLZ EAE EAE PLZ

Figure 3-8: Concentrations of amino acids in the hypothalami of CFA controls and mice with EAE that are unaffected by acute PLZ treatment. (A-E) Normalized data relative to CFA controls showing no significant changes from untreated CFA (n=10) or EAE (n=5) levels in the hypothalamus concentrations of (A) glutamate, (B) D-serine, (C) glycine, (D) taurine and (E) L-serine in CFA (n=5) and EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-8

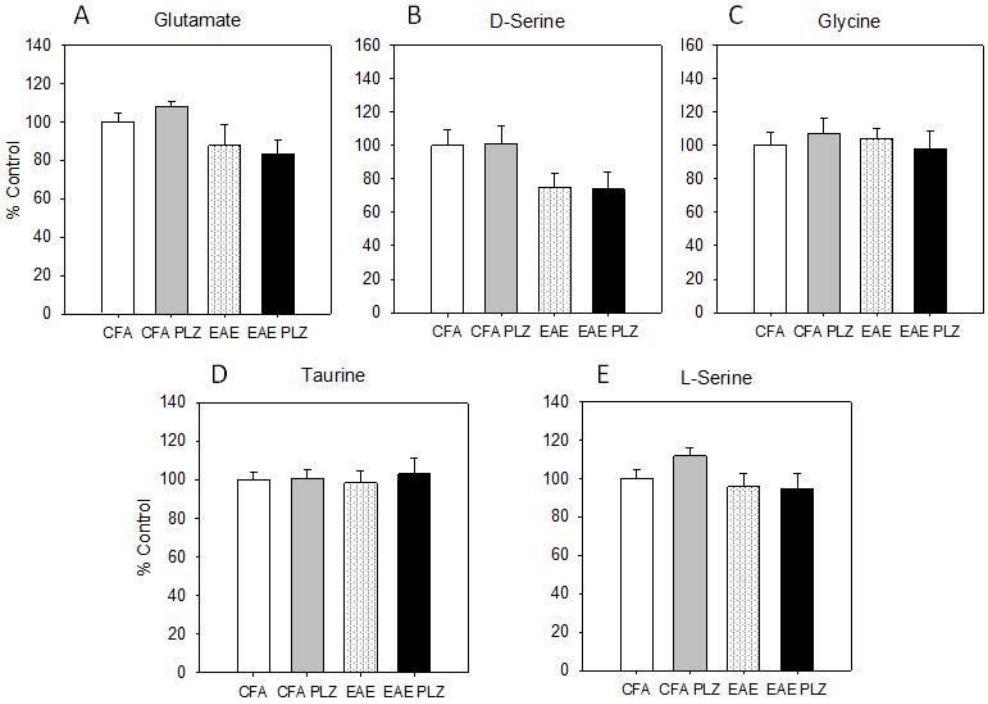

A
Glutamate
% Control
CFA CFA PLZ EAE EAE PLZ
B
D-Serine
CFA CFA PLZ EAE EAE PLZ
C
Glycine
CFA CFA PLZ EAE EAE PLZ
D
Taurine
% Control
CFA CFA PLZ EAE EAE PLZ
E
L-Serine
CFA CFA PLZ EAE EAE PLZ

Figure 3-9: The effects of acute PLZ treatment on the concentrations of amino acids in the cerebra of CFA controls and mice with EAE. (A-I) Normalized data relative to CFA controls showing significant changes from untreated CFA (n=10) or EAE (n=5) levels in the cerebrum concentrations of (A) 5-HT, (B) noradrenaline, (C) GABA, (D) 5-HIAA, (E) glutamate, (F) glycine, (G) glutamine and (I) alanine in CFA (n=5) and/or EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-9

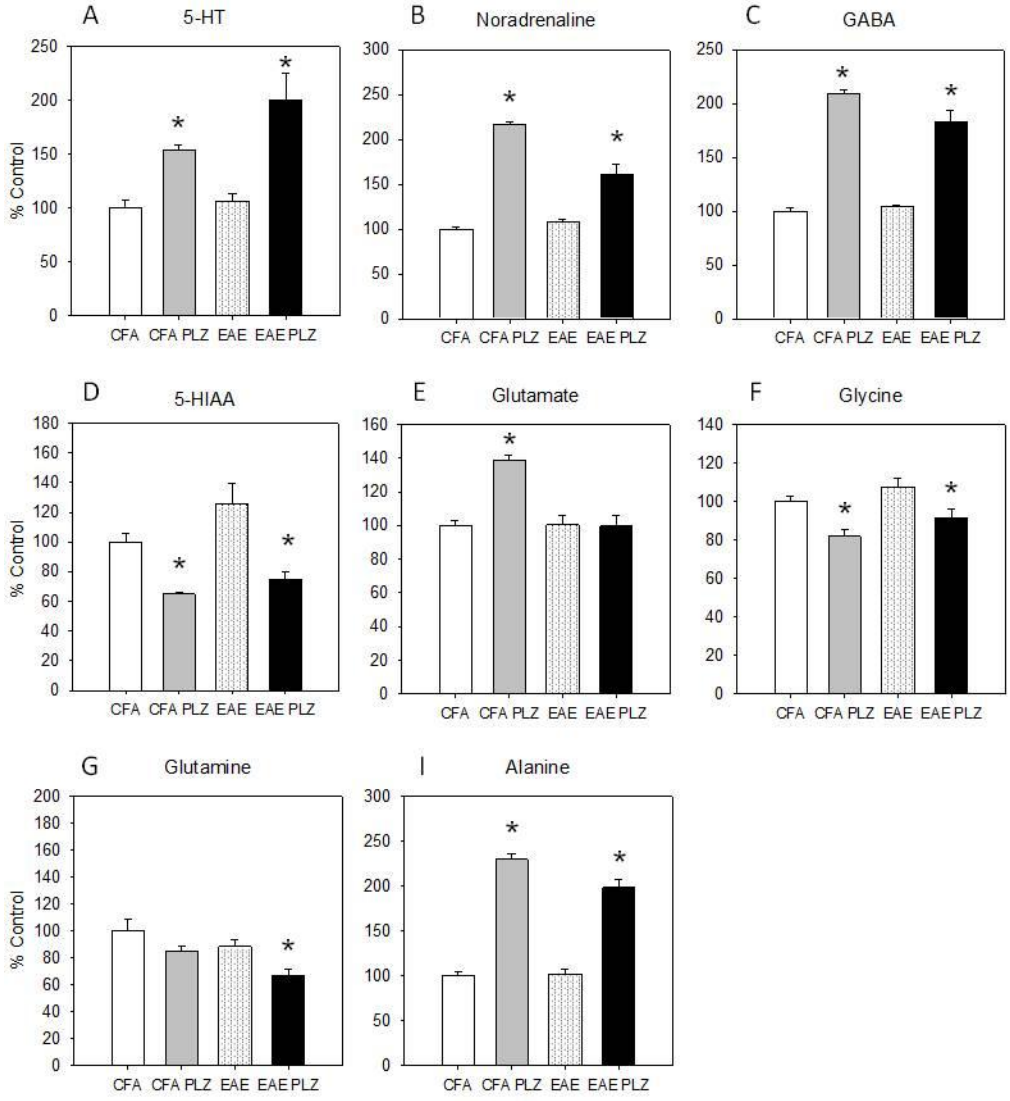

A
5-HT
B
Noradrenaline
C
GABA
D
5-HIAA
E
Glutamate
F
Glycine
G
Glutamine
I
Alanine
% Control
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-10: Concentrations of amino acids in the cerebra of CFA controls and mice with EAE that are unaffected by acute PLZ treatment. (A-F) Normalized data relative to CFA controls showing no significant changes from untreated CFA (n=10) or EAE (n=5) levels in the cerebrum concentrations of (A) aspartate, (B) D-serine, (C) tryptophan, (D) L-serine, (E) arginine and (F) taurine in CFA (n=5) and EAE (n=5) mice treated with PLZ (30 mg/kg). Values are mean ± SD. (*P≤0.05 Student's t-test).

Figure 3-10

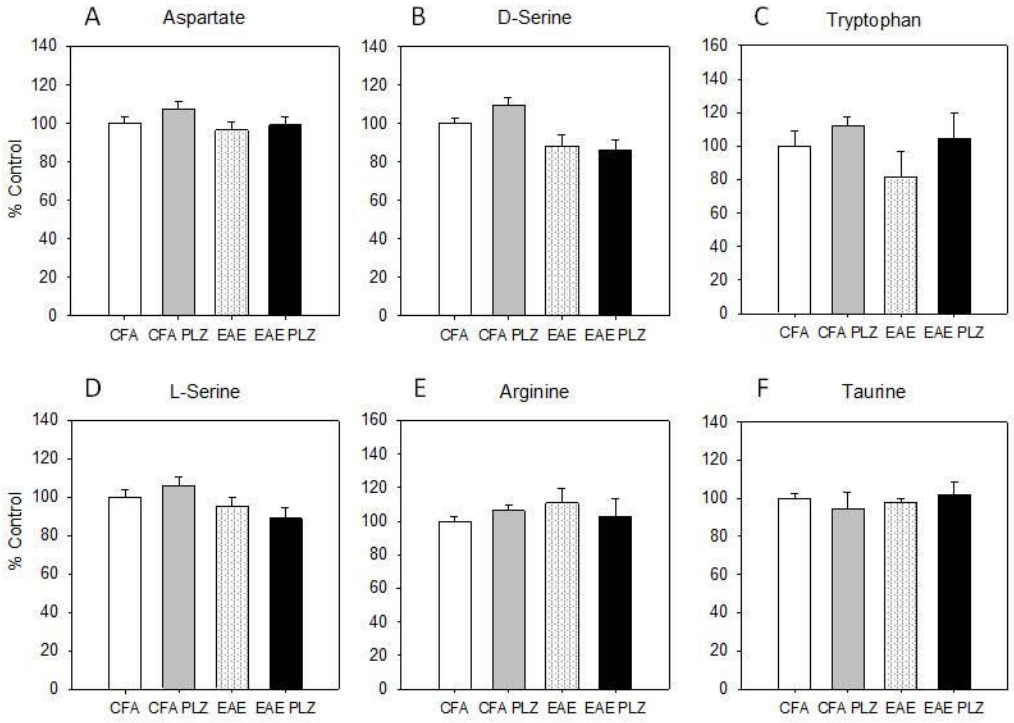

A
Aspartate
B
D-Serine
C
Tryptophan
D
L-Serine
E
Arginine
F
Taurine
% Control
CFA
CFA PLZ
EAE
EAE PLZ

Figure 3-11: The relative effectiveness of PLZ treatment in elevating the concentrations of 5-HT in the CNS of CFA controls and mice with EAE. (A-D) Calculation of percent change from CFA or EAE levels after PLZ treatment (30 mg/kg) shows that 5-HT concentrations are more greatly affected during EAE in the (A) spinal cord and (D) cerebrum but are no different in the (B) brainstems or (C) cerebella. Values are mean ± SEM. (*P≤0.05 Student's t-test).

Figure 3-11

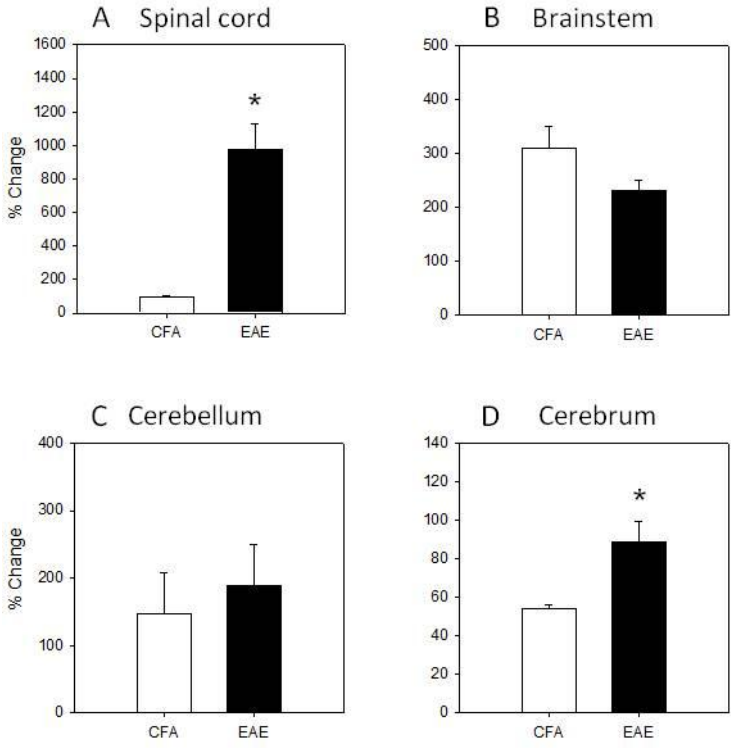

A Spinal cord
% Change
1600
1400
1200
1000
800
600
400
200
0
*
CFA
EAE
B Brainstem
500
400
300
200
100
0
CFA
EAE
C Cerebellum
% Change
400
300
200
100
0
CFA
EAE
D Cerebrum
140
120
100
80
60
40
20
0
*
CFA
EAE

Figure 3-12: The relative effectiveness of PLZ treatment in elevating the concentrations of noradrenaline in the CNS of CFA controls and mice with EAE. (A-D) Calculation of percent change from CFA or EAE levels after PLZ treatment (30 mg/kg) shows that noradrenaline concentrations are (A) more greatly affected during EAE in the spinal cord, (D) affected to a lesser degree in the cerebrum, and (B-C) are no different in the (B) brainstem or (C) cerebellum. Values are mean ± SEM. (*P≤0.05 Student's t-test).

Figure 3-12

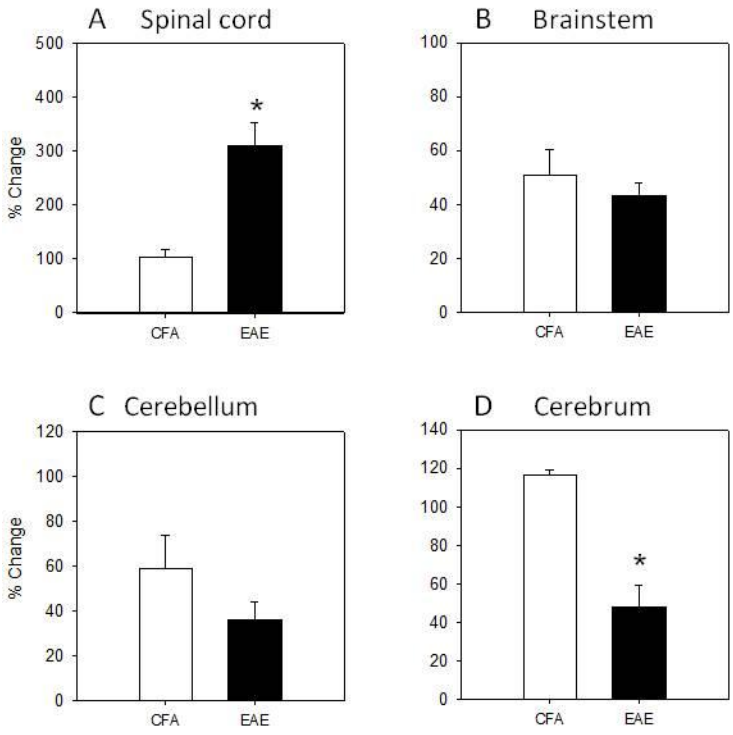

A Spinal cord
% Change
500
400
300
200
100
0
*
CFA
EAE
B Brainstem
100
80
60
40
20
0
CFA
EAE
C Cerebellum
% Change
120
100
80
60
40
20
0
CFA
EAE
D Cerebrum
140
120
100
80
60
40
20
0
*
CFA
EAE

Figure 3-13: The relative effectiveness of PLZ treatment in elevating the concentrations of GABA in the CNS of CFA controls and mice with EAE. (A-D) Calculation of percent change from CFA or EAE levels after PLZ treatment (30 mg/kg) shows that GABA concentrations are more greatly affected during EAE in the (A) spinal cord, (C) cerebellum and (D) cerebrum but are no different in the (B) brainstem. Values are mean ± SEM. (*P≤0.05 Student's t-test).

Figure 3-13

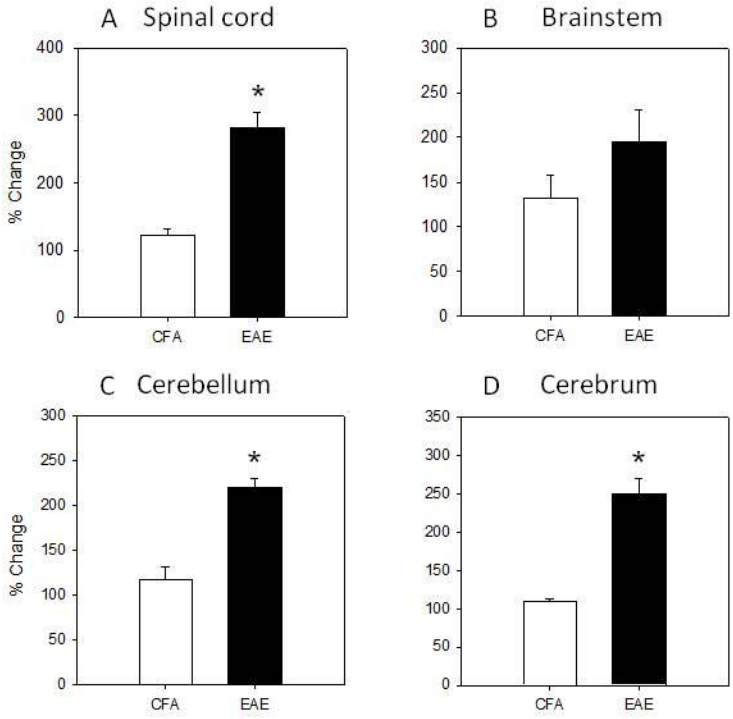
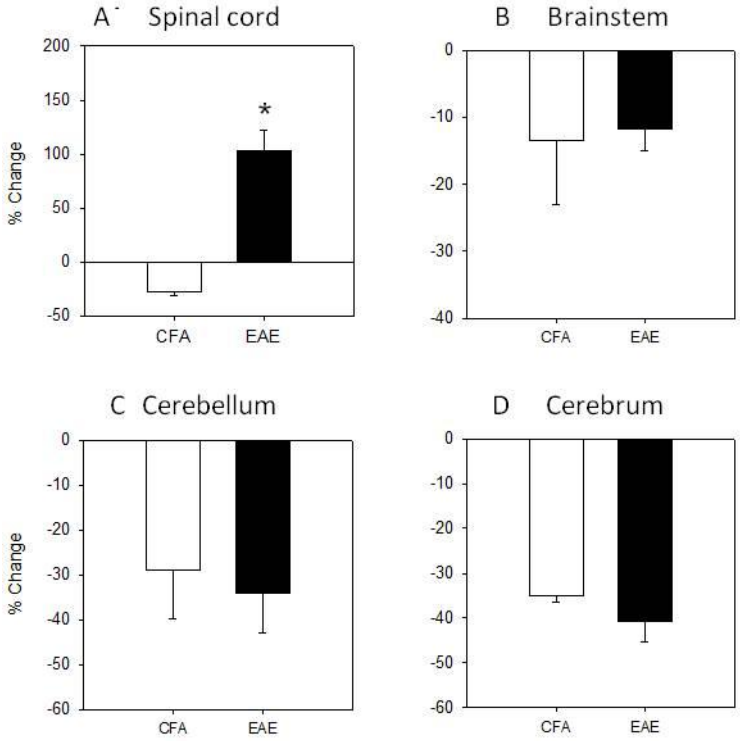

A Spinal cord
B Brainstem
C Cerebellum
D Cerebrum
% Change
CFA
EAE
*

Figure 3-14: The relative effectiveness of PLZ treatment in altering the concentrations of 5-HIAA in the CNS of CFA controls and mice with EAE. (A-D) Calculation of percent change from CFA or EAE levels after PLZ treatment (30 mg/kg) shows that 5-HIAA concentrations are (A) increased during EAE in the spinal cord but are no different in the (B) brainstem, (C) cerebellum or (D) cerebrum. Values are mean ± SEM. (*P≤0.05 Student's t-test).

Figure 3-14


A Spinal cord
B Brainstem
C Cerebellum
D Cerebrum
% Change
% Change
200
150
100
50
0
-50
0
-10
-20
-30
-40
0
-10
-20
-30
-40
-50
-60
0
-10
-20
-30
-40
-50
-60
*
CFA
EAE
CFA
EAE
CFA
EAE
CFA
EAE

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CHAPTER 4 THE EFFECTS OF CHRONIC PHENELZINE TREATMENT ON MOTOR AND BEHAVIOURAL FEATURES OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

4.1 Introduction

In the previous chapters, concentration changes in the CNS of mice during disease progression of MOG_{35-55} EAE, an animal model of MS, were evaluated and the potential therapeutic effect of PLZ was shown. The benefits of PLZ, putatively, would derive from its demonstrated ability to strongly enhance the CNS concentrations of 5-HT, noradrenaline and GABA during EAE. Modulating the activity of these neurotransmitters individually has been shown to alleviate EAE severity [1-4].

Most studies of EAE, however, only evaluate disease severity and potential therapies according to their ability to reduce clinical scores, which are largely a measure of motor disability only. If, however, EAE is to be considered in the context of the human disease it models, it may be valuable to include evaluations of non-motor aspects of disease. A myriad of non-motor symptoms are associated with MS, including pain, fatigue and depression [5-7]. Previous work in our lab demonstrated that signs of neuropathic pain can be found in mice with EAE [8]. EAE is also characterized by depression-like behaviours, in what was described as "EAE-associated behavioural syndrome" by Pollak et. al. (2002)[9]. This syndrome was characterized by weight loss, decreased intake of food and water, decreased social exploration and anhedonia (measured by a decreased preference for a palatable sucrose solution) during acute disease exacerbations [10]. These symptoms are typical of both sickness behaviour and depression [11-12]. Various similarities between affective changes in EAE animals and MS patients should warrant its consideration as a model for evaluating the affective changes associated with MS pathogenesis [9]

Sickness is known to induce behavioural changes and depression in people and animals through a coordinated response in the CNS to pro-inflammatory cytokines [11]. While an infection is a common cause, it is not necessary. Sickness behaviours can also be observed in patients and animals with autoimmune diseases and after the central or peripheral administration of pro-inflammatory cytokines, TNFα and IL-1β in particular. The behavioural changes associated with inflammation are theorized to represent a novel, central motivation state in which the organism's perceptions and actions are re-

organized so as to better combat a possible infection [11]. Immune system dysregulation may also trigger the development of major depressive disorders in otherwise non-clinical patients [13].

Importantly, the depression-like symptoms of sickness behaviour in animals, including during EAE, are responsive to treatments with antidepressants and may therefore positively respond to PLZ [9, 11-12]. The efficacy of PLZ treatment in tests of panic, anxiety and depression in animals is well established but has not been examined in the context of disease-associated signs of depression [14-18]. The potential benefits of PLZ for mice with EAE were demonstrated in the previous chapter after an acute treatment. While PLZ's anxiolytic effects have been reported after a single dose, the benefits of treatment often require chronic administration [15-17]. Physiologically, chronic treatment may confer additional neurological benefits through physiological modifications of excitatory neurotransmitter release, GABA receptor composition, expression of brain-derived neurotrophic factor, and the hypothalamic-pituitary-adrenal axis [19-23]. Long term inhibition of MAO activity also suppresses the production of pro-inflammatory cytokines, which may reduce the intensity of motor and affective changes in EAE [24-25].

The purpose of the present experiment was to evaluate the potential effects of PLZ treatment on the traditional, motor measures of EAE and to assess non-motor aspects of EAE disease progression. The behaviours of EAE and CFA controls in an open field were monitored to assess affective changes. This open field assay includes a trial of the first iteration of the activity score, a novel measure of sickness and depressive-like behaviours developed for this experiment. This experiment's results support the hypothesis that PLZ treatment can successfully attenuate clinical disease severity and locomotor deficits. Additionally, CFA and EAE mice had reduced exploratory behaviours and activity scores in the open field that were substantially improved by PLZ treatment. Interestingly, however, the beneficial effects of PLZ in EAE were not permanent, as measures of motor and affective changes in EAE mice treated with PLZ converge at experiment's end with those of EAE mice receiving vehicle injections. These results suggest the therapeutic potential of PLZ for treating autoimmune neuroinflammation

although they raise interesting questions about the possible effectiveness of PLZ's different pharmacological properties in disease conditions.

4.2 Materials and Methods

4.2.1 EAE and PLZ administration

EAE induction and assessment were conducted according to the methods described in Chapter 2. Starting on the seventh day after induction, CFA and EAE mice were given a daily IP injection of PLZ in bacteriostatic water or vehicle. Mice designated as "Naive" did not undergo the protocol to become CFA or EAE, did not receive injections of PLZ or vehicle but were tested on the rotorod and in the open field. To avoid any possible acute effects of drug or vehicle injection on behaviour, treatments were given in the afternoon after testing was completed. For this experiment, PLZ dose was reduced to 15 mg/kg in order to avoid the signs of lethargy and anorexia that developed in some mice receiving repeated treatments of 30 mg/kg. The reduced dosage of PLZ we used is still capable of effectively modulating the concentrations of amino acids and biogenic amines in the CNS in a manner consistent with our results in Chapter 3 [26]. The dose of 15 mg/kg is also still above the PLZ dose that are effective in animals for improving behavioural performance in tests of depression and anxiety [16-18, 27]. Given that GABA concentrations are elevated at least up to 24 hours after acute PLZ administration, it was determined that our daily treatment protocol should be capable of keeping CNS concentrations of GABA elevated throughout our experiment. After 36 days of treatment, mice were euthanized according the methods described in Chapter 2 and tissue was extracted for future evaluation by high performance liquid chromatography or was fixed for analysis by immunocytochemistry.

4.2.2 Rotorod

Locomotor abilities were assessed using a protocol previously employed by our lab [8]. Mice were given three days of training on the rotorod before induction. Trials were conducted, starting the second day after EAE induction, every second day until day 22, after which rotorod abilities were tested only on days 29 and 36. Naive, CFA and EAE

mice were placed for 3 minutes on the rotating beam of a rotorod (MED Associates Inc., ENV-576M) that was rotating at a fixed rate of 16 revolutions per minute. Each mouse was given three trials, after which the average time a mouse remained on the rotating beam was calculated.

4.2.3 Open field behavioural assay

4.2.3.1 Open field conditions

Open field analysis was conducted in the morning on the same days as the rotorod using a clear plastic container (width: 29 cm, length: 44 cm, height: 17 cm) placed on a grid that divided the box into four quadrants of equal size. Mice were observed in the open field for four minutes, during which the behaviours being evaluated, which are described below, were recorded. The open field was cleaned and wiped with ethanol after each cage of mice completed their trials. A standard electronic timer was used to keep time. No pre-induction habituation was conducted in the open field. In another study, 3 days of habituation in the open field produced similar patterns of behavioural changes after EAE induction (unpublished observations).

4.2.3.2 Open field scoring system

Open field behaviours were recorded during each 4 minute trial on the score sheet shown in Figure 4-1. During each minute, the number of crossings, rearings and groomings were counted and a score corresponding to the amount of time the mouse spend in a sedentary, slouched posture (activity score) was given. The “corner gaze” category was not used and was, instead, replaced with a count of the number of fecal boli left in the open field after each mouse’s trial. These scores were totalled for each mouse at the end of the 4 minute open field trial. The inclusion of naive mice in this experiment allowed us to make comparisons, not only between CFA and EAE, but also between naive (normal) mice and those that received Complete Freund’s Adjuvant (CFA), an immune-active compound [28].

For analysis, open field behaviours were divided as either positive indicators or negative indicators of health. Because withdrawal from environmental and social

environments are symptoms of sickness behaviour and depression, crossings, rearings and groomings were considered positive scores because they demonstrate an absence of fear/freezing behaviour and are objective indicators of the mouse actively attending to cues within its environment or itself. Positive scores also demonstrate the actions that a mouse performs voluntarily. Deviations of positive scores from control levels, therefore, may be broadly interpreted as an abnormal affective status or motivational state. Decreases in exploratory behaviours from control levels have been observed in animal models of autoimmunity or after cytokine administration and were considered as signs of sickness- or inflammation-induced depression [11-12, 29]. Other factors that cause reduced positive scores during EAE may also include fatigue, which is common in MS, and pain, which is commonly present in both MS and EAE [8, 30-31].

"Negative" scores, in comparison, indicate either high amounts of anxiety or the abnormal withdrawal of interest from normal activities. Increases in the number of fecal boli are thought to reflect the presence of anxiety and stress in mice [32]. The activity score is a novel measure that distinguishes an abnormal behaviour (described in section 4.3.2.6) from other behaviours in periods during which no "positive" behaviours are exhibited. This posture in animals is similar to that described as being characteristic of sickness behaviour [12]. Decreased activity scores from control levels reflect an abnormal withdrawal of attention from internal and external cues that normal mice would attend to. Reasons for reduced activity scores could include EAE-induced sickness behaviour and depression, pain and ataxia or unreported phenomena in EAE such as fatigue and anxiety [8, 10].

4.2.3.3 Crossings

"Crossings" were defined as the number of times a mouse steps into an adjacent quadrant. After being placed in the center of the box, mice "select" a quadrant to start when they place all four limbs in that quadrant. After the starting quadrant is noted, movements are marked as crossings if the mouse places all four limbs in an adjacent quadrant. If a mouse moves along the lines demarcating two quadrants, a crossing cannot be counted until all four limbs cross into the adjacent quadrant from where the mouse began.

4.2.3.4 Rearings

"Rearings" were recorded with each discrete instance that a mouse extended itself onto its hind limbs in the vertical direction. This included exploratory behaviours where the mouse extended upright on its hind limbs and placed the forepaws on a wall of the box. Once extended vertically, another rearing was not counted unless the mouse lowered itself so as to place a forepaw on the ground or unless the mouse sat back completely on its hind limbs. For this experiment, the rare act of a mouse jumping was counted as a rearing.

4.2.3.5 Groomings

"Groomings" were counted every time a mouse was observed to initiate a discrete grooming action. Once grooming had begun, another grooming was not counted unless the previous grooming activity stops. For logistical reasons, the type of grooming displayed and duration of grooming were not factors considered in this analysis.

4.2.3.6 Activity score

The activity score is a categorical score given each minute to a mouse based on the duration it spends in a specific sedentary, slouched posture. This posture was defined as having both forepaws on the ground and with the head being slouched, relatively still and with a steady gaze directed below the horizontal. Timing was stopped if the mouse interrupted this posture for any reason, including extensions of the head/neck forward or upward (usually to sniff at the air); large, lateral investigatory head movements; or if grooming was initiated. Small head movements and minute shifts in weight were tolerated, however, so long as the direction of gaze did not change.

The total duration spent in this posture was measured using a standard sports stopwatch. After each minute, if timing had been started, the watch was quickly reset in order to start the next minute's timing from 0 seconds. The time spent in the specified posture during each minute was then converted into a score from 0 to 3 according to the following criteria: 0, mouse is slouched, still and with a low directed gaze for 45 or

more seconds in one minute; 1, mouse is slouched, still and with a low directed gaze for 30 to 45 seconds in one minute; 2, mouse is slouched, still and with a low directed gaze for 15 to 30 seconds in one minute; 3, mouse is slouched, still and with a low directed gaze for 0 to 15 seconds in one minute.

4.2.4 Statistical Analysis

Statistical analyses and the creation of graphics were conducted using SigmaPlot software version 11.0. Two-way repeated measures ANOVA was used to compare clinical scores, rotorod, and open field scores between treatment groups and Tukey's post-hoc test was utilized to reveal specific points of difference between groups. For the analysis of activity scores, the scores of CFA mice treated with PLZ and vehicle were pooled because deviations from scores of 12 were rare in both groups. Results were considered as statistically significant if $P<0.05$.

4.3 Results

4.3.1 The effect of chronic PLZ treatment on the motor symptoms of mice with EAE

Treatment using a number of antidepressants has been shown to alleviate the clinical and neuropathological features of EAE [1, 3-4, 33]. In this experiment, we show that daily treatment with PLZ, an MAO inhibitor with GABA enhancing properties, can reduce both the clinical severity of EAE as well as the behavioural signs indicative of sickness and depression.

Daily PLZ treatment starting at day 7 after EAE induction had a significant effect on the motor impairments that are characteristic of EAE disease progression (Figure 4-2). While peak scores appear to be no different between EAE mice treated with PLZ or vehicle, EAE onset was substantially delayed and the motor impairments in the chronic phase were reduced in EAE animals receiving PLZ treatments. EAE and EAE-PLZ groups showed a significant effect of group ($P=0.005$) and group x time interaction ($P\leq0.001$)(Fig. 4-2A). Locomotor abilities on the rotorod were also better maintained by EAE mice receiving PLZ treatments (Group effect: $P=0.001$, group x time interaction: $P\leq0.001$)(Fig. 4-2B).

4.3.2 The effect of chronic PLZ treatment on positive behaviours in the open field

In the open field, positive scores were significantly altered in EAE and CFA animals but were significantly improved by PLZ treatment (Figure 4-3). In EAE animals, the amount of crossings and rearings (Fig. 4-3A and Fig. 4-3C) decreased dramatically in the initial days after disease induction and well before disease onset. In EAE mice receiving daily vehicle injections, these exploratory behaviours continued to decline during the course of the experiment. While the decline in rearing is expected because of EAE-induced hindlimb weakness/paralysis, all mice were capable of general locomotion, as all mice were observed to move to the periphery upon being placed in the center of the open field. PLZ treatment had an immediate and pronounced effect on crossings in EAE mice when compared to EAE animals receiving vehicle (Group effect: $P \leq 0.001$; group x time interaction: $P=0.006$)(Fig. 4-3A). PLZ appears to exert a smaller effect on rearings (Group effect: $P=0.007$, group x time interaction: $P \leq 0.001$)(Fig. 4-3C). PLZ treatment, however, did not produce a lasting effect in EAE mice, as the amounts of both crossings and rearings saw a sudden decline after about 10 days of treatment.

Surprisingly, when viewed alongside naive mice, the exploratory behaviours observed in CFA animals were also reduced (Fig. 4-3B and 4-3D). In CFA mice receiving vehicle injections, crossings and rearings decreased below the levels of naive mice after the day of induction but appeared to be greater than those of untreated EAE. These reductions appear similar to those observed in asymptomatic EAE mice. Treatments with PLZ, however, significantly increased the number of crossings in the open field by CFA mice (Group effect: $P=0.043$; group x time interaction: $P \leq 0.001$). In contrast to the eventual decline in exploratory behaviours in EAE mice treated with PLZ, the increased number of crossings observed in CFA mice was stable throughout the duration of the experiment. No significant effect of PLZ treatments on rearing behaviour in CFA animals was observed.

The amount of discrete grooming actions observed in this experiment did not appear to be altered EAE or treatment with PLZ (Fig. 4-3E). However, the number of groomings performed by untreated CFA mice may be greater than PLZ-treated CFA mice and EAE mice with or without PLZ treatment.

4.3.3 The effect of chronic PLZ treatment on negative behaviours in the open field

The effects of PLZ treatment are also apparent on negative scores in the open field (fecal boli, activity score)(Figure 4-4). In EAE animals, a group difference was found between those treated with PLZ and those with vehicle (Group effect: P=0.028)(Fig. 4-4A). This likely reflects the general trend for fewer fecal boli to be left by EAE mice treated with PLZ. However, further analysis revealed no distinct time points of statistical difference. No difference in the number of fecal boli left by treated and untreated CFA mice was detected (Fig. 4-4B).

The activity scores of EAE mice responded positively to PLZ treatment (Group effect: P=0.002, group x time effect: P≤0.001)(Figure 4-4C). In vehicle treated EAE mice, the first deviations from a healthy score of 12 appear around disease onset (days 8 -12) before dropping permanently at day 14 (naive mice consistently score an activity score of 12, data not shown). The activity scores of untreated EAE mice were significantly worse than those of CFA mice (group effect P≤0.001, group x time effect: P≤0.001). Post-hoc analysis revealed significant differences between CFA mice and untreated EAE mice beginning at day 14 and persisting throughout the rest of the experiment. In contrast to this, the activity scores of EAE mice treated with PLZ, which also first deviate from a score of 12 at around 8 days post-induction, were far better maintained. EAE mice treated with PLZ were also significantly worse off than CFA controls (group effect: P=0.008, group x time effect: P≤0.001). Post hoc analysis, however, showed that the activity scores of EAE mice treated with PLZ were no different than CFA mice until day 22, after which the activity scores of treated and untreated EAE mice began to converge.

4.4 Discussion

4.4.1 General implications of results

The results of this experiment demonstrate the ability of PLZ treatment to attenuate EAE disease severity. These results further add to the growing knowledge that antidepressants possess the potential to modify EAE disease course and strongly suggest that MAO inhibitors such as PLZ possess therapeutic potential in conditions of

neurological inflammation. While no difference was apparent in the severity of peak clinical scores, mice treated with PLZ had substantially delayed disease onset and reduced disability at the experiment's end. The benefits of PLZ treatment in EAE also extended beyond the traditional measures of motor abilities to the behavioural signs of sickness and depression exhibited in the open field. Additionally, the response to PLZ by EAE mice in activity scores suggests the potential utility for this easy and novel measure of sickness and depressive behaviours. Notably, however, the inability of PLZ treatment to maintain the improvements in the open field behaviours of EAE mice, despite improved motor abilities, raises interesting questions about its pharmacological mechanisms.

4.4.2 The potential mechanisms through which PLZ modulates EAE

Because PLZ exerts strong effects on the CNS concentrations of 5-HT, noradrenaline and GABA (amongst a diversity of mostly smaller effects on neurochemistry), it is difficult to point to the individual mechanisms responsible for improving aspects of EAE observed in this experiment. However, previous research evaluating the effects of 5-HT, noradrenaline and GABA neurotransmitter systems on EAE and inflammation may offer clues. Bhat et. al. (2010) recently demonstrated that GABAergic agents powerfully reduce EAE severity by inhibiting pro-inflammatory mechanisms, effects that were mediated by the GABA receptors present on APCs [1]. The delayed disease onset in our experiment possibly reflects a similar GABAergic suppression of EAE initiating processes. Using mice lacking the expression of 5-HT transporters, Hofstetter et al. (2005) showed that increased 5-HT activity reduces EAE and is associated with fewer inflammatory infiltrates and decreased production of IFNγ. Increasing noradrenaline concentrations after EAE onset, using a combined treatment of the synthetic noradrenaline precursor L-threo-3,4-dihydroxyphenylserine and atomoxetine (a noradrenaline uptake inhibitor), also improved EAE clinical scores. This treatment did not modify T cell production of IFNγ or IL-17 but its influence on APCs was not evaluated [4]. Treatment with venlafaxine, which inhibits the synaptic re-uptake of both 5-HT and noradrenaline, was also shown to successfully suppress the progression of EAE clinical scores [33]. The overall results of the present experiment therefore

suggest that an additive anti-inflammatory response to these neurotransmitters probably occurs, resulting in a smaller magnitude of the neurodegenerative mechanisms that induce permanent disability. An alternative interpretation, possibly, is that elevated concentrations of monoamines masked the normal accumulation of disability by maintaining neuronal excitability in the face of declining spinal concentrations of 5-HT and noradrenaline.

The delayed disease onset, and yet unaffected peak severity, in EAE mice treated with PLZ in this study may implicate the GABAergic system's influence during EAE. Our results, therefore, may specifically suggest a dysinhibition of pathological immune mechanisms. Closer examination of the results of Bhat et. al (2010), where they employ a GABA receptor agonist or an irreversible GABA-T inhibitor in EAE, shows that the suppression of clinical scores by GABAergic agents may lose its absolute potency with time. One possibility is that the enhanced GABAergic activity causes a desensitization of GABA receptors and/or an induced change in their cell surface expression [34]. If GABA concentrations had remained elevated throughout our experiment, GABAergic desensitization may have occurred.

Our results, however, are probably better explained in the context of PLZ pharmacology. PLZ-induced increases in GABA levels require a MAO-dependent metabolism of PLZ to PEH, the active product that inhibits GABA-T [27, 35]. PLZ's effects on monoamine and GABA concentrations are also temporally dissociated, with elevated concentrations of monoamines lasting up to a week after a single injection while those of GABA disappear before 48 hours [26]. As a consequence, successive daily treatments with PLZ may cause a progressively greater inhibition of MAO, which reduces the conversion of PLZ to PEH and causes progressively weaker inhibition of GABA degradation. Eventually, PLZ's increase in GABAergic activity is no longer sufficient to suppress inflammatory disease mechanisms. The effect of successive administrations of PLZ on GABA concentrations has not been examined. Recent analysis in our lab of the CNS tissue generated in this experiment's by HPLC, however, shows that this idea is likely the case, as the GABA levels in PLZ-treated CFA and EAE mice are completely normalized at day 36 (unpublished results). The disease onset and worsening of open field scores in EAE mice treated with PLZ at around 17 days post-induction suggests that

the threshold for GABAergic inhibition of disease mechanisms is no longer passed at about 10 days of treatment.

4.4.3 Open field behaviours of CFA and EAE mice

In this experiment, EAE mice in the open field showed behavioural changes that included weight loss (data not shown), decreased voluntary activity and the adoption of a posture suggestive of environmental and personal withdrawal. Amongst a constellation of other symptoms that were not monitored in this study, which include fatigue, nausea, fever, mild cognitive impairments, social withdrawal, and increased sensitivity to pain, the behaviours we observed are suggestive of both sickness behaviour and/or depression [11-12]. These behavioural changes in rodents are a consequence of the activity of pro-inflammatory cytokines, in particular TNFα and IL-1β, on their corresponding receptors located on neuronal and non-neuronal cells of the CNS [12]. The infusion of TNFα and IL-1β, either centrally or peripherally, can induce the full symptomology of sickness. Factors that induce the endogenous production of pro-inflammatory cytokines such as lipopolysacharride (LPS) also successfully induce sickness behaviour [11]. Peripheral proinflammatory cytokines exert their influence centrally through various mechanisms that stimulate the local production and release of proinflammatory cytokines in the CNS by macrophages and microglia. These mechanisms include the activation of toll-like receptors (TLR) on macrophage-like cells of the circumventricular organs, of IL-1 receptors of perivascular macrophages and brain endothelial cells and by volume diffusion into the CNS. In patients, immunotherapy treatment also induces sickness behaviours and often depression disorders [12, 36-38].

Consistent with idea that endogenous production of proinflammatory cytokines can induce sickness and depressive-like signs in animals, Pollak et al. (2003) found in their previous studies of EAE-associated behavioural syndrome that behavioural changes were best explained by the temporal correlation between behaviour, immune cell infiltration into the brain and the production of IL-1β, TNFα and prostaglandin E_2. During the recovery phase of EAE though, only observed reductions in IL-1β and TNFα were correlated with improvements [39]. Behavioural changes have also been observed in the mouse model used to study systemic lupus erythematosus (SLE), and include anhedonia,

reduced locomotion and exploration in an open field, decreased novel object exploration and cognitive dysfunction. Sickness behaviour is evident in these mice early, when signs of immune system activation become evident in the serum [29, 40-41].

When Pollak et al. first examined sickness and depressive behaviours in EAE, they found that reductions in body weight, food intake, sucrose intake and social exploration preceded motor symptoms [9-10, 39, 42]. Improvements in behavioural signs associated with neither the onset nor the remission of motor impairments [9-10]. This suggested that behavioural and motor alterations in EAE are dissociated [9]. Our results are generally consistent with this observation. The number of crossings and rearings by EAE mice decreased steadily throughout the experiment and were lower than the numbers by corresponding, untreated controls. Interestingly, our results show that exploratory behaviours of CFA mice also declined after the EAE induction procedure, a fact which suggests that the procedure or CFA itself, a known activator of TLRs, may possess anxiogenic and/or depressive effects [28]. Unfortunately, because PLZ treatments were begun before disease onset, no comparison could be made between the open field behaviours and motor impairments of untreated and treated EAE mice at the onset of motor symptoms. Although statistically significant effects of EAE on activity score were not observed before disease onset, EAE mice began showing deviations from the healthy score of 12 before treatments began or the onset of motor symptoms occurred. The observation that CFA mice also do not have consistent scores of 12 like naive animals also suggests an effect of EAE induction procedure or CFA administration on sickness and/or depressive behaviours. While some recovery of clinical scores is observed after peak disease severity in this study's EAE mice, the lack of a coincident improvement in behavioural signs in EAE mice adds support to the idea that motor and behavioural dysfunction in EAE are dissociated phenomena.

4.4.4 The effects of PLZ on behaviours in the open field

The open field results are important because they show that the affective state during EAE is sensitive to antidepressant treatment with PLZ. In EAE mice, PLZ treatments produced immediate, yet unfortunately transient, increases in the amount of exploratory behaviours along with maintenance of activity scores. The loss of

treatment effects on exploratory behaviours and activity scores in this experiment appears to occur at around day 17, which coincides with the onset of motor deficits in these mice. The transiency, therefore, could derive from the temporary suppression of EAE mechanisms by PLZ. Presumably, this effect may be mediated by the temporary GABAergic inhibition of the immune system. Selective inhibition of MAO-A by moclobemide has been shown to reduce cytokine expression *in-vitro*, though, which suggests that beneficial effects of MAO inhibition on both EAE mechanisms and sickness behaviour are possible [24-25]. While GABAergic mechanisms may lose their potency with time, monoamine concentrations continue to be elevated. The failure of PLZ to elicit statistically significant improvements in the late exploratory behaviours of EAE mice suggests that the benefits of increased monoamine concentrations on this measure are relatively mild in the later time points of EAE. In contrast to this, PLZ's anti-depressant and anxiolytic properties are fully effective in otherwise healthy mice, as judged by the persistently greater number of crossings by treated controls in comparison with those of untreated controls. Beneficial effects of elevated monoamine concentrations on sickness behaviour, though, may be evident from the activity scores of EAE mice treated with PLZ because reductions in activity scores occurred much slower than the accumulation of motor disability after disease onset. This may also be interpreted from the statistical improvement on the number of fecal boli left by treated EAE mice, as the differences from untreated EAE levels are mostly observed later in the experiment.

The improved open field behaviours after PLZ treatment in EAE mice in this study are fairly consistent with the studies of Pollak et al., where treating EAE animals with the antidepressant imipramine, a tricyclic antidepressant, or anti-inflammatory therapies of various mechanisms produced improvements in the sickness behaviours of EAE mice [9, 42]. However, in the short time after disease onset that treatment was administered, not all therapies they administered were equally effective; some anti-inflammatory treatments were ineffective unless combined, they often improved only some measures of sickness and depression and only dexamethasone, a general inhibitor of cytokine production, had a mild influence on clinical scores. These therapies were, however, far more successful at attenuating sickness behaviour after LPS injection. Their

results indicate that not all anti-inflammatory therapies will be equally effective and that, further, individual models of sickness behaviour may operate according to different cytokine mechanisms [42]. In our experiment, PLZ's relative successes at improving different behavioural alterations in mice with EAE depended on the measure being observed and the experimental time point. This indicates that PLZ's underlying pharmacological mechanisms may have relatively different potencies according to the behaviour being examined and the cytokine mechanisms that produce it.

4.4.5 Evaluation of the open field assay

Our open field results show promise for this assay as an easy method for evaluating affective changes during EAE. However, despite its successes, the present experiment and open field methods have several limitations worth considering. Firstly, the concept of sickness as a unique motivation state has important implications because motivational states compete for behavioural outputs [11]. Fear, for example, is a strong motivation characterized by freezing behaviour that will compete with and possibly confound the observed behaviours in the open field [40]. Environmental factors that affect fear may consequently affect the behaviours of all mice. A possible indication of this in the present study may be the spikes in the number of fecal boli left by naive mice. Further, the space of the open field itself can be anxiogenic. Consequently, events like the changes in schedule from every two days to every seven days could have altered the anxiogenic properties of the open field, thereby affecting the results of the last two trials where activity scores of treated and untreated EAE mice were seen to converge. The effect of EAE on anxiety is presently unknown, although increased general anxiety was observed in the mouse model of SLE [41].

The specific methods used in the open field could also possibly be improved upon. The groomings measure, for one, was thought to be unsuccessful largely because the total amount of time a mouse spends grooming is probably a better indicator of attention to self than the number of discrete instances. Observations during the experiment support this because during some trials, the total duration of grooming was probably short but the number of starts and stops was very high. Logistically, however, with the other behaviours requiring monitoring and recording, measuring the duration

of grooming is not presently realistic. The number of fecal boli was also deemed largely unsuccessful, as the effects of experimental (CFA, EAE and naive) or treatment group were mild and/or inconsistent. This suggests the influence of uncontrolled factors on the occurrence of fecal boli. Possibly, this may include environmental factors that affect stress levels such as cage transport and cage conditions (ie. dirty, clean, broken water bottles).

Possible improvements in the activity score method may potentially translate into greater sensitivity as well. One example where improvement may be possible is the criterion that both forepaws be on the ground for timing to occur. On several occasions it was observed that otherwise healthy mice displayed a posture reminiscent of the one timed but with the forepaws held off the ground. Removing this requirement may consequently affect the scores of both CFA and EAE mice. Changes in the categorical scores may also be considered. Original iterations of the measure had it as a subjective score modelled after scales of similar styles such as the Basso, Beatie and Bresnahan scale used for evaluating rats after spinal cord injury. However, when the measurement of activity scores evolved to include timing, the categorical score remained. Possibly, expanding the number of categorical scores beyond 4 will improve measurement sensitivity. Alternatively, using time in seconds as the dependent variable may also be worth considering. Greater sensitivity, theoretically, may enable the detection of statistically significant changes that precede EAE disease onset [9-10].

Figure 4-1: The score sheet developed for this experiment to assess behaviors in the open field.

Figure 4-1

Date Experiment

Mouse		min 1	min 2	min 3	min 4	Total
	Crossings					
	Rearing					
	Grooming					
	Corner Gaze (s)					
	Activity Score	R(0) O(1) F(2) C(3)	R(0) O(1) F(2) C(3)	R(0) O(1) F(2) C(3)	R(0) O(1) F(2) C(3)	AVG

Figure 4-2: The effect of daily PLZ treatment starting 7 days after EAE induction on motor symptoms associated with EAE. (A) Clinical scores are significantly improved in EAE mice receiving daily treatment with PLZ (15mg/kg). (B) Locomotor abilities on the rotorod are better maintained in EAE mice receiving daily treatment with PLZ. Values are mean ± SEM. (* $P \leq 0.05$ two-way repeated measures ANOVA, Tukey's post hoc test).

Figure 4-2

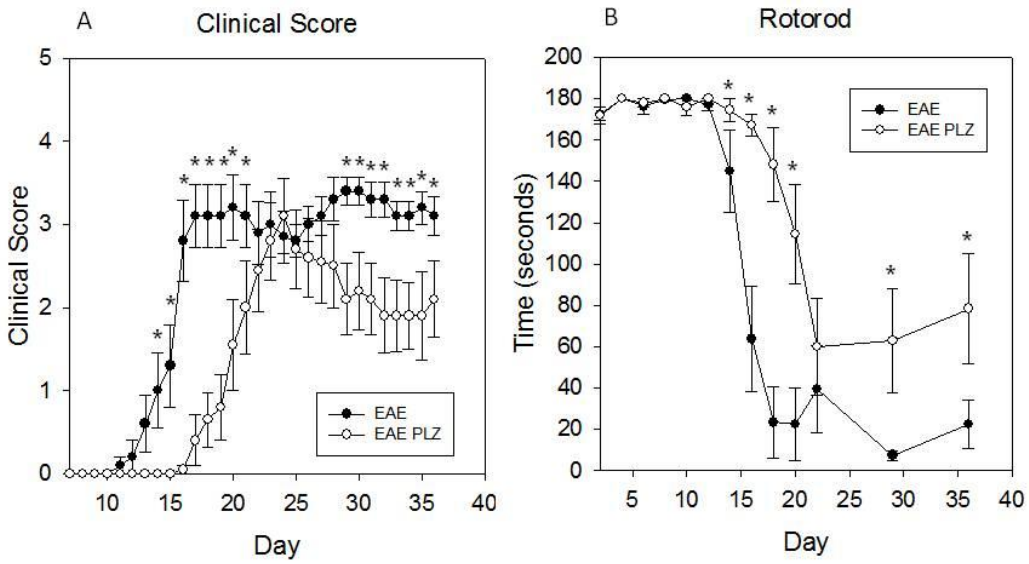

A
Clinical Score
Clinical Score
Day
EAE
EAE PLZ
B
Rotorod
Time (seconds)
Day
EAE
EAE PLZ

Figure 4-3: The effect of daily PLZ treatment starting 7 days after EAE induction on the positive scores of CFA and EAE mice in the open field. (A) EAE mice given daily PLZ treatment (15 mg/kg) have a greater number of crossings temporarily in the open field. (B) CFA controls given daily PLZ treatment have a consistently greater number of crossings in the open field. (C) EAE mice treated with PLZ show a greater amount of rearing behaviors temporarily in the open field. (D) The amount of rearing behavior in CFA controls is unaffected by PLZ treatment. (E) The amounts of discrete grooming events appear to be unaffected by EAE or by treatment with PLZ. Values are mean ± SEM. (* $P \leq 0.05$ two-way repeated measures ANOVA, Tukey's post hoc test).

Figure 4-3

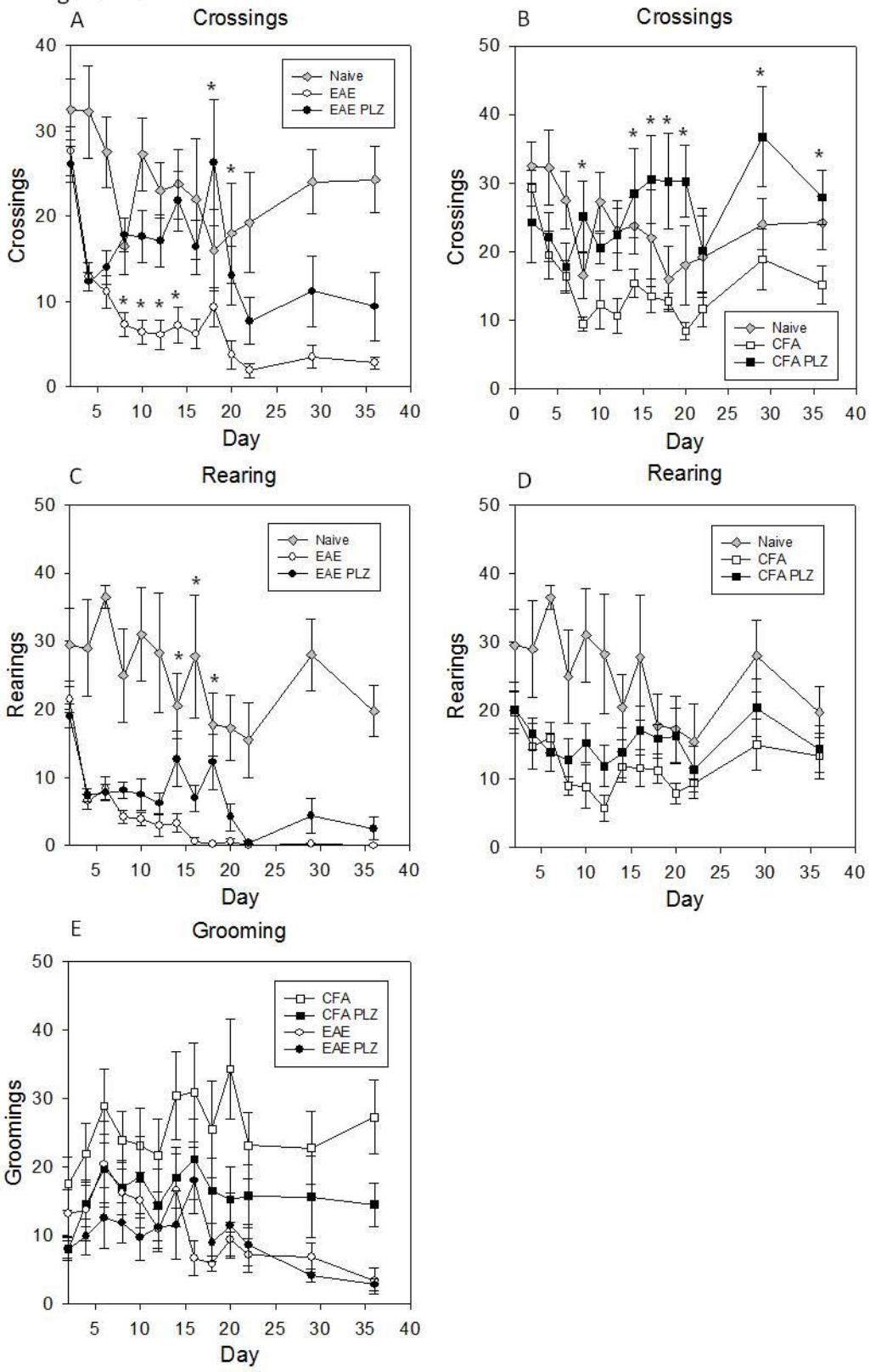
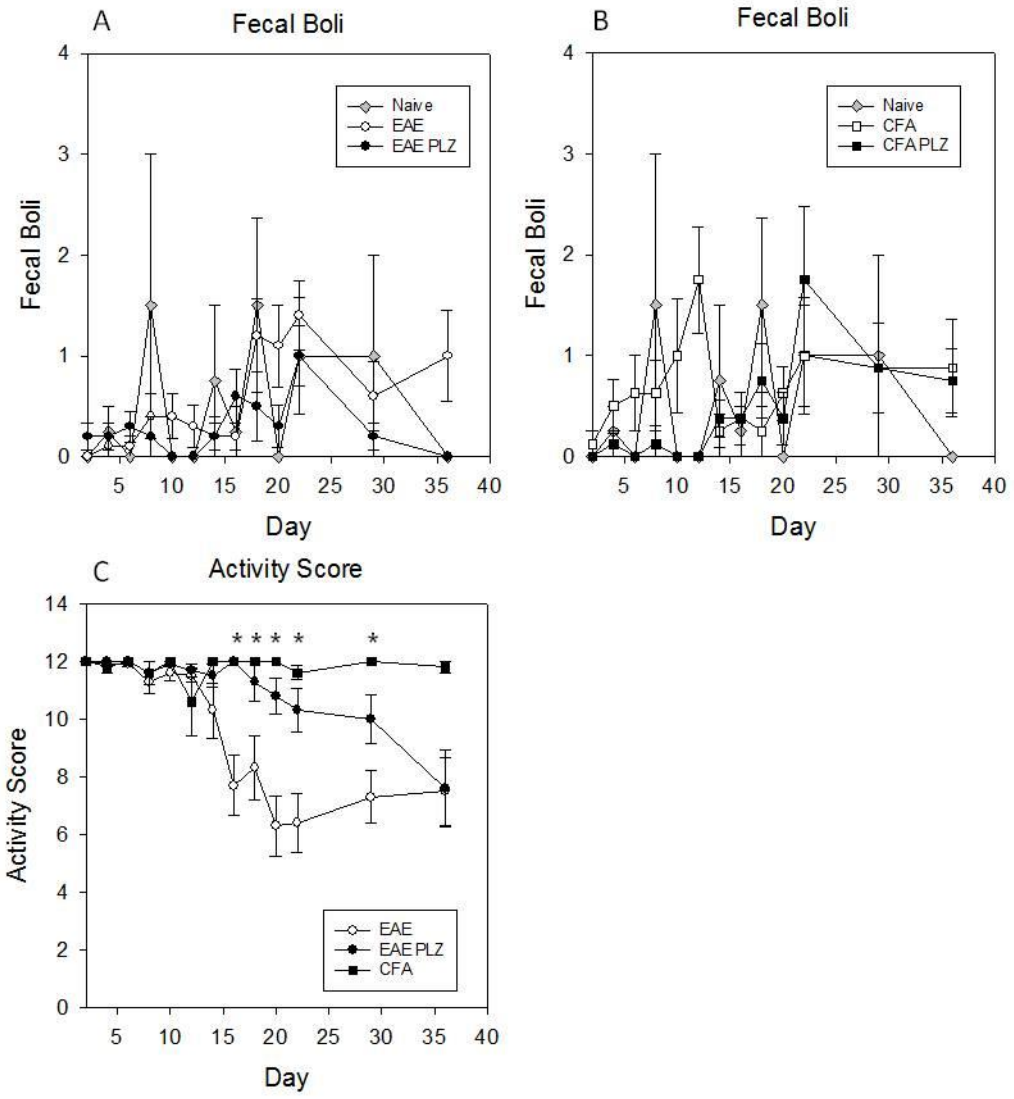

A
Crossings
Naive
EAE
EAE PLZ
Crossings
Day
B
Crossings
Naive
CFA
CFA PLZ
Crossings
Day
C
Rearing
Naive
EAE
EAE PLZ
Rearings
Day
D
Rearing
Naive
CFA
CFA PLZ
Rearings
Day
E
Grooming
CFA
CFA PLZ
EAE
EAE PLZ
Groomings
Day

Figure 4-4: The effect of daily PLZ treatment starting 7 days after EAE induction on the negative scores of CFA and EAE mice in the open field. (A) EAE mice treated with PLZ leave a reduced number of fecal boli in the open field than vehicle treated EAE mice. Post hoc analysis, however, does not distinguish specific days where differences occur. (B) CFA mice treated with PLZ show no difference in the number of fecal boli left in the open field. (C) EAE mice treated with vehicle have reduced activity scores in the open field compared to CFA controls. The activity scores of EAE mice treated with PLZ are maintained at CFA levels and are significantly greater than those of vehicle treated EAE throughout the majority of the experiment. Values are mean ± SEM. (* $P \leq 0.05$ two-way repeated measures ANOVA, Tukey's post hoc test).

Figure 4-4


A
Fecal Boli
Naive
EAE
EAE PLZ
Fecal Boli
Day
B
Fecal Boli
Naive
CFA
CFA PLZ
Fecal Boli
Day
C
Activity Score
EAE
EAE PLZ
CFA
Activity Score
Day

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CHAPTER 5 CONCLUSIONS

5.1 Conclusions

The motor and psychological symptoms associated with MS are considerable burdens for patients that weigh additionally upon the communities that support them. Research efforts directed at understanding disease pathogenesis and treating symptomatology are therefore worthwhile. Further, these investigations establish a foundation upon which new treatments can be developed and evaluated. Ultimately, new discoveries may benefit those afflicted by reversing disease pathogenesis and reducing the debilitating neurological symptoms. While our understanding of MS is incomplete, it is continually progressing, largely aided by research using its animal model, EAE. The commonalities and differences between MS and EAE, therefore, are important to distinguish. Additionally, just as there is a clinical diversity of MS patients, a variety of EAE models with different clinical and pathological profiles are used in research. Greater knowledge of how different EAE models compare may therefore provide helpful insight into the observed heterogeneity of MS.

The experiments described in this thesis were begun with a characterization of the levels of amino acids and biogenic amines in CNS of mice with a chronic model of EAE induced using MOG_{35-55}. This characterization identified a number of substances whose concentrations changed during the course of EAE, but the disease-induced reductions in the concentrations of 5-HT, noradrenaline and GABA were particularly interesting because of their potent dual functions in the nervous and immune systems. 5-HT, noradrenaline and GABA deficiencies were specifically targeted with the selection of the antidepressant drug PLZ. PLZ successfully elevated the CNS concentrations of these neurotransmitters during EAE and the subsequent experiment tested its therapeutic potential as a disease modifying agent. In that latter experiment, chronic treatment with PLZ substantially altered EAE by delaying and reducing motor impairments and decreasing the open field behaviours indicative of sickness behaviour and depression.

The characterization of concentration changes in Chapter 2 was initiated because similar analyses of EAE tissue had not been conducted for several decades and they had only made use of one EAE model that, to my knowledge, is not in use today. Our investigation into the neurochemical changes in a modern EAE model therefore seemed worthwhile because it could be compared with the older model as well as with similar data from MS patients. The refinement of HPLC methods for separating and quantifying analyte concentrations over the years also meant we could evaluate the changes to the concentrations of a number of newly examined amino acids. Our results confirm previous findings that

show EAE has an influence on a great variety of physiological processes. Analysing these changes identified a number of alterations common to previously studied EAE models and to MS patients although major differences were also apparent that suggest pathogenic processes of the MOG_{35-55} EAE model and the previously examined model are not alike. A similar, broad analysis of amino acids and biogenic amines in other EAE models and in samples from MS patients may further help ascertain which changes are common, which of them are model-specific and what individual biological processes drive them. An examination of these substances in the CSF and plasma of MS patients may also find novel biomarkers of disease. Associating these changes with disease-associated factors like fatigue, depression and pain may help elucidate their relative importance to these symptoms. Future experiments may also wish to explore the specific roles of different substances. Concentrations of D-serine and glycine, for example, are greatly affected by EAE but their roles in disease processes and symptomology are largely a mystery.

PLZ was selected as a potential therapeutic agent in EAE because its potent ability to elevate the CNS concentrations of 5-HT, noradrenaline and GABA meant it possessed strong disease-modifying potential. In Chapter 3, this potential was tested with HPLC analyses of amino acid and biogenic amine concentrations in the tissue of treated CFA and EAE mice. The results of PLZ treatment on concentrations of amino acids and biogenic amines are mostly unsurprising since the changes that PLZ treatment elicited were consistent with the scientific literature on PLZ neurochemistry. Nevertheless, this experiment provided critical proof of concept that PLZ is capable of elevating the concentrations of 5-HT, noradrenaline and GABA throughout the CNS. The observations that neurotransmitter concentrations are affected to the greatest degree in the spinal cord are interesting, in particular because disease processes are greatest in this region during EAE (although this is not necessarily the case in MS). The comparison of drug effectiveness on analyte concentrations in control and EAE animals may be important for future work using PLZ because drug effectiveness was clearly influenced by disease status. This may be important for future experiments and therapies as it would have foreseeable implications on effective dosages and side effects. Determining why PLZ effectiveness changes with EAE may also be interesting.

With the potential of PLZ treatment to affect EAE pathology confirmed, the experiment in chapter 4 provided *in vivo* evidence that PLZ treatment can attenuate the severity of neurinflammatory autoimmune disease. Chronic treatment with PLZ, started only a few days before the expected onset of disease, slowed the onset and reduced the severity of motor impairments in EAE. In the open field assay

that I developed for this experiment, PLZ treatment provided further benefits to CFA and EAE mice by reducing signs of anxiety and behavioural indicators of sickness and depression. This assay is simple, flexible and can easily be altered to adjust sensitivity and/or accommodate new ideas. Recent work in our lab starting PLZ administration at disease onset further indicates PLZ's therapeutic potential beyond its use in psychiatric disorders because similar, beneficial findings were seen. However, the improvements associated with PLZ treatment were only transient in EAE mice using this protocol, an observation that raises intriguing questions about which of PLZ's pharmacological mechanisms were effective during disease. Future experiments looking at PLZ's effects *in vitro* or using analogues of PLZ or its bioactive metabolites to individually modulate neurotransmitter systems may help answer those questions.

The larger purpose of this work, however, was to add new insights to the scientific knowledge of MS pathogenesis, symptomatology and treatment through neurochemical and behavioural evaluations of the animal model frequently used for research into MS. A greater understanding of the commonalities and differences between MS and EAE will be important in the search for effective new treatments and therapies. Hopefully, this work furthers that process. In addition to physical disabilities, the psychological aspects of MS are clearly important but are rarely examined in EAE studies. Perhaps this work can also renew interest in the study of EAE-associated psychological changes. The potential value of these studies, however, comes from the fact that drugs such as PLZ are already in use for psychiatric disorders and their impacts on patients' lives are well understood. Consequently, if the effects of PLZ on EAE observed in this thesis can be extended to MS patients, the benefits of its use, or the use of similar therapeutic agents, could quickly be passed on to patients because PLZ may bypass some of the considerable hurdles required for new drugs to enter clinical practice. While there appear to be limitations as to the effectiveness of PLZ treatment in EAE, these may possibly be overcome using different treatment protocols or the addition of complementary therapies. If that is the case, then PLZ treatment could offer considerable relief to patients and their communities by improving their motor, sensory and/or psychiatric symptoms. The experiments of this thesis, therefore, offer new insights into EAE and possibly a new therapeutic agent for treating MS.