NEURODEGENERATION IN AN ANIMAL MODEL OF NEUROPSYCHIATRIC LUPUS

By

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NEURODEGENERATION IN LUPUS

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ABSTRACT

Systemic lupus erythamatosus (SLE) affects not only peripheral organs, but also the most vital central organ, the brain. When the brain is involved, this often denotes the graver prognosis of Neuropsychiatric (NP)-SLE. In a special breed of lupus-prone mice, the onset of systemic autoimmunity and inflammation is accompanied by various deficits in brain structure and function, providing a useful model to study NP-SLE. While previous studies suggested brain cell death in MRL/MpJ-Fas^{lpr} (MRL-lpr) mice, direct evidence that neurons were dying was lacking. The aim of this thesis was to examine connections between neurodegeneration and autoimmune / inflammatory disease, behavioural dysfunction, and cytotoxic cerebrospinal fluid (CSF) in these animals. In addition, this work attempted to elucidate the mode of cell death and investigate the importance of microglial cell activation and dopaminergic pathways in the manifestation of disease. Behavioural data, brain, spleen, blood and CSF samples were obtained from mice at various ages. In addition to FACS analysis, numerous enzyme-linked immunosorbant assays (ELISAs), immunohistochemical and cytochemical stains, and microscopic techniques were employed. Subsequently, it has been demonstrated that neuronal loss and degeneration are associated with 1) the progression of systemic autoimmune manifestations, 2) emergence of behavioural deficits, 3) increased microglia activation, 4) damage of dopaminergic pathways, and 5) toxicity of CSF. Moreover, destruction of mesonigral and mesolimbic circuits appear to contribute to the etiology of some aberrant behaviours, and lesions in germinal layers suggest a compromised reparative capacity of MRL-lpr brains. These findings provide an initial step toward understanding mechanisms, modes, and targets of neuronal death in the MRL model, and may lead to therapeutic interventions for NP-SLE patients.

EXTENDED ABSTRACT

Systemic lupus erythamatosus (SLE) does not only affect peripheral organs such as skin and kidneys, but frequently the most vital central organ, the brain. When the brain is involved, mental problems such as anxiety, depression, memory loss, or psychosis often denote a more severe form of lupus, and the graver prognosis of Neuropsychiatric (NP)-SLE. In a special breed of lupus-prone (MRL) mice, the onset of systemic autoimmunity and inflammation is accompanied by various deficits in brain structure and function, providing a useful model to study NP-SLE.

The aim of this thesis was to examine neurodegeneration (brain cell demise) and possible connections with autoimmune / inflammatory disease, behavioural dysfunction, and cytotoxic cerebrospinal fluid (CSF) in lupus-prone MRL/MpJ-Fas^{lpr} (MRL-lpr) mice. In addition, this thesis attempted to elucidate the mode of cell death and investigate the importance of microglial cells and dopaminergic pathways in the manifestation of disease. Behavioural data, brain, spleen, blood and CSF samples were obtained from mice at various ages. In addition to FACS analysis, numerous enzyme-linked immunosorbant assays (ELISAs), immunohistochemical and cytochemical stains, and microscopic techniques were performed.

While experimental studies suggested brain cell death in MRL-lpr mice (eg. reduced dendritic spine densities), direct evidence that cells were indeed dying and that these cells were neurons was lacking. Consequently, Fluoro Jade B (FJB), a novel fluorescent dye which has a high affinity for dying neurons (both apoptotic and necrotic) was employed. In comparison to allogenic and congenic control groups, the brains of diseased MRL-lpr mice showed increased numbers of FJB-positive (+) cells. To examine the mode of cell death, in particular apoptosis, Terminal

deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) was also employed. TUNEL+ cells co-localized with FJB+ cells suggesting that apoptotic-like cell death mechanisms are associated with neuronal degeneration. While immunostaining for CD4 and CD8 T-lymphocyte markers did not correlate with the number of FJB+ cells, their presence in brain tissue appeared concomitantly with neuronal demise. Similarly, indices of systemic autoimmunity (splenomegaly and high serum anti-nuclear antibody levels) were associated with increased FJB+ cell numbers in brains of autoimmune MRL-lpr mice, supporting the causal link between autoimmunity and neurodegeneration.

To assess the time course and mechanism of neuronal damage in young and aged mice, FJB staining and anti-ubiquitin (anti-Ub) immunocytochemistry were used. The causal relationship between autoimmunity and neuropathology was tested by prolonged administration with the immunosuppressive drug cyclophosphamide (CY). To examine the relationship between specific structural damage and behavioural impairment, spontaneous alternation behaviour (SAB) was used to estimate the functional severity of hippocampal dysfunction. Young, asymptomatic MRL-lpr mice and congenic age-matched mice performed equally well in the SAB test. When autoimmune manifestations were full-blown, deficits in memory and learning emerged in MRL-lpr animals, concomitant with increased FJB+ neurons and anti-Ub particles in the hippocampal CA3 region. The increased anti-Ub particles suggested that mechanistically, the Ub pathway is important in neuronal death. Additionally, increased FJB and Ub positivity coincided with decreases in the density of hippocampal H&E stained neurons. The hippocampus from a deceased NP-SLE patient also showed reduced neuronal density and increased FJB positivity in the CA3 region, strengthening

the face validity of the MRL model of lupus. The SAB deficit and overall FJB staining was attenuated by CY treatment. While this suggested a link between autoimmunity, structural damage and functional impairment, inference of a causal relationship between CNS damage and functional loss remains difficult due to the systemic nature of disease.

The mode of neuronal death within the apoptosis-necrosis continuum also remained unclear, given that merely 7% of FJB+ neurons co-localized with TUNEL+ cells in the periventricular nucleus immediately surrounding the third ventricle of diseased MRL-lpr brains. Treatment with CY attenuated neurodegeneration and behavioural dysfunction in MRL-lpr mice. CY, however, is a drug which affects broad populations of cells. To better understand the contribution of inflammation in the etiology of brain damage, the non-steroid anti-inflammatory drug (NSAID) ibuprofen (IBU) was used. Following chronic administration, IBU failed to normalize behavioural performance and immune status. It also did not normalize brain morphology in MRL-lpr mice. More specifically, IBU did not reduce the density of CD3+ lymphocytes in the choroid plexus, or FJB+ neurons in the hypothalamus. While microglia activation increased with age in MRL-lpr brains (shown by FACS analysis), IBU treatment was not effective in reducing F4/80+ cell numbers. Consequently, despite relative co-localization of FJB+ and F4/80+ cells, whether age-dependant microglia activation causes neuronal demise remains unknown. Toluidine blue staining revealed numerous dark cells in functionally critical brain regions such as the subgranular zone of the hippocampus and substantia nigra (i.e. areas populated with progenitor cells), but defining characteristics of typical apoptotic or necrotic cell death were not seen with transmission electronic microscopy (EM). Profound differences between MRL-lpr and control brains suggest that dark cells

may be due to profound metabolic perturbations (both in neurons and accessory cells) during the development of systemic autoimmune disease.

Considering that the hippocampus is part of the mesolimbic dopamine (DA) system (involved in the control of behavioural reward), the functional status of central dopaminergic circuits was pharmacologically probed. Based on the stimulatory effects of *d*-amphetamine sulfate (Amph) on sucrose intake, the response rates were compared between diseased MRL-lpr mice and congenic MRL +/+ controls using the sucrose preference paradigm. While control mice significantly increased intake of sucrose solutions after administration of Amph, the intake of drugged MRL-lpr mice was comparable to those given SAL injections. Increased FJB staining was detected in mesolimbic areas (i.e. the nucleus accumbens and hippocampus) of diseased mice, and Amph treatment neither altered this nor other measures of organ pathology. Despite the systemic nature of disease, the results obtained through Amph administration point to neurotransmitter-specific regional brain damage which may account for changes in reward behaviour of MRL-lpr mice.

To further explore the possibility that autoimmunity affects other parts of the central dopaminergic system in diseased MRL-lpr animals, functional damage of the nigrostriatal pathway was assessed from rotational behaviour after a single injection of the D1/D2-receptor agonist apomorphine (Apo). Apomorphine increased circling in the diseased MRL-lpr group. The contribution of autoimmunity to dopamine system damage was further assessed by comparing asymptomatic and diseased MRL-lpr mice, and by employing CY. Neurodegeneration in the midbrain was estimated by FJB staining, and damage / loss of dopaminergic neurons was specifically assessed by tyrosine hydroxylase (TH) staining. Increased FJB staining and reduced TH positivity

in the substantia nigra (SN) pars compacta and ventral tegmental area (VTA) were detected in only behaviourally impaired MRL-lpr mice. A three-fold increase in serum brain-reactive antibodies (BRA) accompanied the loss of TH neurons in diseased animals. In addition, CSF from the behaviourally impaired autoimmune mice was neurotoxic to a DA progenitor cell line. CSF from young mice was not toxic, and immunosuppression attenuated both CSF cytotoxicity and midbrain neurodegeneration. The source of the neurotoxic factor(s), however, were not elucidated.

This thesis has demonstrated that neuronal loss and neurodegeneration is associated with 1) the progression of systemic autoimmune manifestations, 2) emergence of behavioural deficits, 3) increased microglia activation, 4) damage of dopaminergic pathways, 5) and toxicity of CSF. More specifically, destruction of mesonigral and mesolimbic dopaminergic circuits appear to contribute to the etiology of aberrant behaviour in this animal model of neuropsychiatric lupus. Evidence of TUNEL and Ub positivity suggests a mode and mechanism of neuronal cell death which may predominate in MRL-lpr brains. F4/80 immunohistochemistry and FACS analysis also support the possibility of a microglia-induced neuronal excitotoxicity event, resulting in a non-apoptotic cell death process. CSF toxicity (but not serum) to DA progenitor cells suggests that neurotoxic metabolites are synthesized within the brain (intrathecally) of diseased MRL-lpr mice and underlie cell death at advanced stages of lupus-like disease. The effects of CY on abolishing neurodegeneration and CSF cytotoxicity, along with the behavioural effects of the DA agonists Amph and Apo supports the notion that disease-induced neurotransmitter-specific brain damage may underlie the autoimmune associated behavioural syndrome (AABS) in lupus-prone animals. A similar process leading to cognitive and affective deficits, as well as CNS damage may occur in NP-

SLE patients. While this thesis provided an initial step, a better understanding of neuronal death (i.e. mechanisms, modes, and targets) in the MRL model of NP-SLE may provide a basis for therapeutic interventions in this poorly understood and complex neuroimmunological condition.

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First and foremost, I would like to thank Dr. Boris Sakic for accepting me as a graduate student in his laboratory. From the beginning I knew that Dr. Sakic would not lead me astray in the hectic world of research and he has not. Boris is a truly inspirational mentor, especially when you begin to feel uncertain or discouraged. He has a remarkable ability to create enthusiasm for a project and I know this fueled much of the creativity within his lab. I also thank him for his help with my computer woes and his expertise and undying patience in 'doing things right'. He also instructed me on the 'ways of the world', and for these reasons and many more, thank you Boris.

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Many, many thanks to the McMaster crew: Jason Millward who introduced me to the laboratory lifestyle, Mary Jo Smith and Xiaoxing Ma who always found time to assist me with immunohistochemistry, and Marnie Timleck and Ernie Spitzer for sharing their vast technical knowledge. Last but not least, I thank my mother and father for always being there to encourage me

in my academic endeavors throughout the years, for listening, and for keeping my spirits high. For these reasons and many more, I would like to dedicate this thesis to all of you.

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LIST OF ABBREVIATIONS

AABS	Autoimmune associated behavioural syndrome
Amph	*d*-amphetamine sulphate
ANA	Anti-nuclear antibodies
ANOVA	Analysis of variance
Anti-Ub	Ubiquitin antibodies
Apo	Apomorphine
BBB	Blood brain barrier
BRA	Brain reactive antibodies
C	Celsius
C17.2	Immortalized progenitor cell line
CD	T lymphocyte cell surface marker
CD1	Healthy mouse strain
CD69	Cell activation marker
CIC	Circulating immune complex
CNS	Central nervous system
CO_2	Carbon dioxide
COX	Cyclooxygenase
CRF	Corticotropin-releasing factor
CSF	Cerebrospinal fluid
CY	Cyclophosphamide

DA	Dopamine
ddH_2O	Distilled deionized water
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dpi	Dot pixel per inch
dsDNA	double strained DNA
ELISA	Enzyme-linked immunosorbant assay
EM	Electronic microscopy
F4/80	Microglia / macrophage cell surface marker
FACS	Fluorescent activated cell sorter
FasL	Fas Ligand
FST	Forced swim test
FJB	Fluoro Jade B
g	Gram
h	Hour
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin and eosin
IBU	Ibuprofen
Ig	Immunoglobulins
IL	Interleukin
i.p.	Intraperitoneal

KA	Kainic acid
kg	Kilogram
kV	Kilovolt
ME	Median eminence
MHC	Major histocompatibility complex
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
MRL-lpr	Murphy Roth's Large/MpJ-Tnfrsf6^{lpr}
MRL +/+	Murphy Roth's Large/MpJ +/+
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NE	Norepinephrine
NIH	National Institutes of Health
nm	Nanometer
NP-SLE	Neuropsychiatric systemic lupus erythematosus
NSAID	Non-steroidal anti-inflammatory drug
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGE_2	Prostaglandin E2

PNS	Peripheral nervous system
ppm	Parts per million
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
SAB	Spontaneous alternation behaviour
SAL	Saline
s.c.	Subcutaneous
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SN	Substantia nigra
S/W	Swiss / Webster mouse strain
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
Ub	Ubiquitin
µl	Microliter
µm	Micrometer
VTA	Ventral tegmental area

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PREFACE

The following studies have been published or accepted for publication and form the basis of chapters two, three, four, five and six of this thesis:

NEURODEGENERATION IN AUTOIMMUNE MRL-LPR MICE AS REVEALED BY FLUORO JADE B STAINING. David A. Ballok, Jason M. Millward, and Boris Sakic. Published: Brain Research. 2003; 964(2): 200-10.

HIPPOCAMPAL DAMAGE IN MOUSE AND HUMAN FORMS OF SYSTEMIC AUTOIMMUNE DISEASE. David A. Ballok, John Woulfe, Monalisa Sur, Michael Cyr, and Boris Sakic. Published: Hippocampus. 2004; 14(5): 649-61.

IBUPROFEN FAILS TO PREVENT BRAIN PATHOLOGY IN AN ANIMAL MODEL OF NEUROPSYCHIATRIC LUPUS. David A. Ballok, Xiaoxing Ma, Judah A.Denburg, Larry Arsenault and Boris Sakic. *In Press*: Journal of Rheumatology. 2006.

IMPAIRED RESPONSE TO AMPHETAMINE AND NEURONAL DEGENERATION IN THE NUCLEUS ACCUMBENS OF AUTOIMMUNE MRL-LPR MICE. Kelly K. Anderson, David A. Ballok, Nadia Prasad, Henry Szechtman, and Boris Sakic. Published: Behavioural Brain Research. 2006; 166(1): 32-38.

AUTOIMMUNE-INDUCED DAMAGE OF THE MIDBRAIN DOPAMINERGIC SYSTEM IN LUPUS-PRONE MICE. David A. Ballok, Aoife M. Earls, Catherine Krasnik, Steve A. Hoffman, and Boris Sakic. Published: Journal of Neuroimmunology. 2004; 152(1-2): 83-97.

These studies are basic research studies which required input from many people. Dr. Boris Sakic provided and organized laboratory space and equipment, and some internal financial support combined with my Douglas C. Russell Memorial Scholarship and Father Sean O'Sullivan Research Centre Studentship. Sources of external financial support included my Ontario Graduate Scholarships and Canadian Institute of Health Research Doctoral Award. Jason Millward, Mary Jo Smith, Xiaoxing Ma, Marnie Timleck and Ernie Spitzer provided technical support on numerous occasions. Dr. Henry Szechtman, Dr. Judah Denburg and Dr. Laurie Doering offered considerable expertise and laboratory space to analyze collected samples. Dr. Monalisa Sur and Dr. Larry Arsenault provided additional knowledge and support.

I am the first author on four of the five papers which form the basis of this thesis. I was involved with the planning and design of most of the experiments (submitting the proposals outlining the purpose, methods and analysis of the experiments). I performed the animal behavioural testing procedures and was involved in animal surgeries which included perfusions, blood and CSF collection, and organ extractions. I was responsible for many of the subsequent assays on the samples collected including brain and spleen processing, ELISAs, and cytochemical and immunochemical staining of the tissues. Pilot studies were required to modify the dose of drug given in some of the experiments. I collected most of the data and completed the required statistical analyses for each study. The majority of the figures and tables included in this thesis are my own work (or that of a co-author) and although feedback was provided by other authors, it was my responsibility to write four first author articles (of the five papers comprising the thesis).

I am second author on one of the five papers comprising the thesis. In this study I was

involved in the technical procedures of animal surgery, sample collection, processing brain tissue, performing cytochemical staining, quantifying data, and provided input into the preparation of the manuscript.

CHAPTER 1

INTRODUCTION

Systemic Lupus Erythematosus (SLE) and Neuropsychiatric SLE

The worldwide prevalence of Systemic lupus erythematosus (SLE) ranges from between 1:245 to 1:1000 and in North America is up to 1.5 million (Lahita 1995). According to recent estimates, individuals suffering from SLE has more than tripled over the past 40 years (Scolding & Joseph 2002). As with many related disorders, such as rheumatoid arthritis, scleroderma, and Sjogren's syndrome, SLE affects nine to ten times more women than men (Scolding & Joseph 2002; Brey et al. 2002). The predilection of this disease for females after puberty cannot be adequately explained, however, suspected pathogenic factors such as sex steroid hormones or gonadotrophins (e.g. estrogen or prolactin), may play a role in the severity of the disease and the different clinical presentations (Elgarf et al. 1996; Walker & Jacobson 2000). The diagonosis of SLE should be made principally on clinical grounds with the support of laboratory tests (van Dam 1991). While diagnostic criteria have been proposed for the classification of SLE, they are not universally applied in practice. Eleven criteria have been designated by the American College of Rheumatology (Tan et al. 1982) for classification. The presence of 4 or more criteria is mandatory for the appropriate classification of SLE.

SLE is a chronic autoimmune / inflammatory disease with a broad spectrum of clinical and immunological manifestations (Isenberg et al. 1989) which affect the peripheral nervous system (PNS) as well as the central nervous system (CNS). Multiple organs can be targeted by SLE including the muscle and joints, lungs, heart, kidneys, skin, serous membranes, and components of the blood. While other organ and organ systems can be involved, they are affected with lesser

frequency (Omdal et al. 1991; Manger et al. 2002; van Dam 1991). Patients with SLE usually have autoantibodies to various self antigens. For example, antibodies reacting with ubiquitin (Ub) have been reported in almost 80% of lupus patients (Muller & Schwartz 1995). The most common autoantibody is antinuclear antibodies (ANA), the serological hallmark of SLE; they are present in more than 95% of all SLE patients at sometime during the course of the disease. Less frequently, other antibodies against DNA, small ribonucleoproteins, blood cells (platelets and red blood cells), and phospholipid complexes (antiphospholipid antibodies) are also observed (Greenwood et al. 2002; Swaak et al. 1990; West et al. 1995).

Besides some of the physical symptoms and signs such as malar rash, discoid rash, and arthritis (Hochberg 1992), many patients with SLE also present with neuropsychiatric problems (Bombardier et al. 1992; Denburg et al. 1993a; Wekking 1993). Neurologic and psychiatric (NP) manifestations of unknown etiology are common in SLE and have been proposed to represent a more severe form of the disease, often denoting a graver prognosis (Scolding & Joseph 2002; Navarrete & Brey 2000). For many years, the manifestations of NP-SLE (e.g. psychosis, depression, anxiety, and cognitive deficits) were considered to be consequences of systemic complications, such as kidney damage, infections, or steroid therapy. More recent evidence obtained from contemporary imaging studies have helped clarify that autoimmune disease is the primary factor which induces NP-SLE, simulating functional disorders such as schizophrenia and depression (Perry & Miller 1992). These findings also have lead to the inclusion of psychiatric manifestations as one of the diagnostic criteria of disease (Hochberg 1992). It has since been reported that NP-SLE occurs during the course of illness in 15-75% of patients (Hanly 2001; Bluestein 1992; Adelman et al. 1986; Mcnicholl et al. 1994; Komatsu et al. 1999). NP-SLE syndromes range from diffuse CNS disorders (i.e. acute

confusional state, psychosis, anxiety and depressive disorders, clinical to subclinical cognitive disorder of variable functional significance) to focal CNS syndromes (i.e. seizures, cerebrovascular disease, chorea and myelopathy, transverse myelitis, demyelinating syndrome and aseptic meningitis, headaches) and PNS disorders (i.e. polyneuropathies and mononeuropathies, autonomic disorders, plexopathy, myasthenia gravis) (Tincani et al. 1996).

Systemic lupus erythematosus is a disease with a fluctuating course and NP-SLE manifestations can occur as a single or multiple events at any time during the course of the disease, including periods in which no nervous system SLE disease activity is detected (Hanly 1998). Approximately 40% of the NP-SLE manifestations develop before the onset of SLE or at the time of diagnosis and about 60% within the first year after diagnosis (van Dam 1991). The pathogenic etiology of NP-SLE is likely to be multifactorial (Iverson & Anderson 1994) and may involve autoantibody production, microangiopathy, and intrathecal production of pro-inflammatory cytokines (Nakamura 1997; Svenungsson et al. 2001). While a histologically normal brain with no specific pathognomonic brain lesions is a possible finding in NP-SLE, histopathologic and neuroimaging studies commonly reveal a wide range of brain abnormalities, all of which are diagnostic criteria for NP-SLE. These abnormalities include multifocal microinfarcts, cortical atrophy, gross infarcts, hemorrhage, ischemic demyelination, and patchy multiple-sclerosis-like demyelination (Baum et al. 1993; Bosma et al. 2000), as well as neuronal and astrocytic damage (Trysberg et al. 2003). The various neuroimaging techniques such as magnetic resonance imaging, positron emission tomography, and single-photon emitted computerized tomography have become important in the evaluation of SLE patients with neurologic symptoms and have helped to sort through the diagnostic possibilities (Carbotte et al. 1992; Carbotte et al. 1986; Hanly et al. 1992b; Brooks et al. 1997;

Sibbitt et al. 1994).

Etiology of SLE

Systemic lupus erythematosus results from the interplay among multiple factors at different levels (Fessel 1988). There are at least three etiologies of SLE including genetic, hormonal, and environmental factors (Hochberg 1990). Although these factors do not function as direct mechanisms of pathology, they may be important triggers of the disease (Shoenfeld & Mozes 1990). Genetic factors may determine the way an individual copes with various infections and how patients respond or react to certain drugs. Hormonal factors may be modified by genes, leading to abnormal immune functioning. Environmental factors may or may not trigger the disease in highly susceptible individuals (Cooper & Parks 2004).

From the genetic point of view, there are a number of alleles that influence the expression of SLE and a number of genes that function together in additive fashion (Tsao 2003). Since SLE has low penetrance, the fact that a person has a combination of alleles which are presumably required to develop SLE does not mean that SLE will present in that person. Also, SLE highly depends on an individuals ethnicity (Mccarty et al. 1995). For example, African American women comprise the highest subpopulation of patients (Hochberg 1990). Lastly, the third level of complication arises from allelic heterogeneity. There is no single genetic variant of a gene that's more important than others, but there are many combinations of alleles of the same gene that might contribute to development of SLE (Tsao 2003). Despite the complications in genetic studies of SLE, current literature suggests that SLE is in fact a genetic disease. Some genes found to be defective in human family studies also are involved in animal models of SLE, such as C4A (C4AQ0) (Alexander et al.

2003) and the fas gene (Nagata 1994).

Since the majority of SLE patients are females, there appears to be a link between hormonal factors and disease. As seen in patients with SLE (Lahita et al. 1982; Jara et al. 2001), murine models of SLE have revealed that there are three main hormones which play a role in disease: estrogen, testosterone and prolactin (Wilder 1995). A study using MRL-lpr animals showed a disease-accelerating effect of estrogens (Carlsten et al. 1990), while the administration of testosterone was observed to have a protective effect on disease development in autoimmune-prone mice (Carlsten et al. 1989). Hyperprolactinemia has been found to be associated with a higher production of autoantibodies in lupus patients and autoimmune mice (Neidhart 1996; McMurray et al. 1991).

Environmental factors such as UV light, a number of drugs, and foodstuffs may also play a significant role in the etiology of SLE. People with a genetic predisposition may or may not develop SLE depending on the presence of environmental triggers (Hochberg 1990). Environmental factors may also change the intensity of the disease state and interact with the other two factors (i.e. genetics and hormones). As all of these basic factors interplay together, they appear to have various effects on the immunological function of SLE patients.

Although the etiology of SLE remains elusive, there is a large number of studies supporting the genetic and hormonal hypotheses. Environmental factors, including chemical exposures, may also be important triggers of disease. Regardless of the causative factors, immune dysfunction or dysregulation underlies a diagnosis of SLE. Ultimately, autoimmunity and aberrant immune processes appear to lead to long-term morbidity and an unfavorable impact on a patient's health status. The etiopathogenic factors include T-cell and B-cell dysfunctions as well as abnormal

cytokine production which in turn can influence B cells to produce pathogenic autoantibodies (Kyttaris et al. 2005). These dysfunctions of T-cells and B-cells lead to the production of an array of inflammatory cytokines, diverse autoantibodies, and immune complexes that in turn activate effector cells and the complement system leading to a viscous self-destructive cycle, and the clinical manifestations of disease.

Pathogenesis of SLE

It is believed that T-cell, B-cell and cytokine abnormalities and dysfunction ultimately produce pathogenic autoantibodies which have a direct effect on the manifestation of clinicopathologic symptoms of SLE (Denburg et al. 1994; Hanly 1998). SLE may be categorized as an immune complex-mediated disease in which autoantibodies participate in the pathogenesis of disease by depositing preformed circulating immune complexes (CIC) at the site of damage (Hoffman et al. 1988). Although this is possible, there is a more direct way in which autoantibodies can damage tissue; they locate antigens that are deposited in organs (e.g. DNA attached to basement membranes of the skin or glomeruli). Both ways may participate in SLE, more particularly in lupus nephritis, vasculitis, interstitial cystitis, pulmonary hemorrhage, neuronitis, and through immune complex deposition in the choroid plexis in CNS lupus. Autoantibodies may also directly damage target tissues causing a lytic effect and facilitating the removal of damaged cells by the phagocytic system (Casiano & Tan 1996). Specific examples include hemolytic anemia, thrombocytopenia, neutropenia and perhaps some aspects of the lymphopenia seen in SLE. Two general types of autoantibody systems in lupus lead to this effect: autoantibodies directed to specific antigens on the cell surface and autoantibodies directed to more ubiquitous antigens such as phospholipids and / or

their protein cofactors (van Dam 1991).

Psychiatric Disorders and Cognitive Dysfunction related to NP-SLE

There is a wide presentation of mood disorders in SLE. This includes major depressive episode, mood disorders with depressive manic, or mood disorders with mixed features. These disorders are estimated to affect up to 75% of lupus patients (Kandel 1991). The prevalence of anxiety disorders is seen in up to 70% of patients, and psychosis and depression are two other psychiatric disorders which affect up to 40% of SLE cases, not always in the context of exacerbated disease activity (Lindal et al. 1995; Wekking 1993; Iverson & Anderson 1994). Psychiatric disorders in SLE have been linked to anti-ribosomal P and anti-cardiolipid antibodies (Isshi & Hirohata 1996; Schneebaum et al. 1991; Lai & Lan 2000), and cognitive dysfunctions have been linked to anti-phospholipid antibodies (Denburg & Denburg 2003; Denburg et al. 1997).

Mild to severe cognitive dysfunction in NP-SLE presents with a prevalence of over 65% (Carbotte, Denburg & Denburg 1986) and remains one of the most common type of NP-SLE manifestations. Most prominently there is a compromise in the areas of processing efficiency / speed and attention / concentration, memory function, conceptual reasoning, and cognitive flexibility, which approaches dementia in 20 to 40% of NP-SLE cases (Hanly et al. 1992a). The etiology of cognitive dysfunction in NP-SLE still remains unknown, but it is clear that it cannot be fully accounted for by past or current corticosteroid treatment, disease duration, disease activity or its associated psychological / emotional distress (Hanly et al. 1994; Gladman et al. 2000). Sociodemographic and psychosocial factors, however, need to be explored further (Waterloo et al. 1998; Denburg et al. 1993b). Also, cognitive impairment in SLE is not consistently related to

psychiatric manifestations and can be detected even in the absence of other current or past overt CNS manifestations (Carbotte et al. 1995). Recent imaging studies, however, support the notion of an organic brain etiology to account for the neurological and neuropsychiatric dysfunctions observed in NP-SLE, relative to non-NP-SLE patients (Rocca et al. 2006; Ainiala et al. 2005).

Pathogenesis of NP-SLE

The pathogenic etiology of NP-SLE is likely to be multifactorial and may involve autoantibody production, microangiopathy, and intrathecal production of proinflammatory cytokines. Vasculopathy consisting of proliferative changes of the intima, vascular hyalinization, and perivascular lymphocytosis have also been seen in SLE patients with only psychiatric symptoms, as well as those with focal neuropsychiatric symptoms (Smith et al. 1994; Omdal et al. 1989). In addition, chronic cerebral ischemia has been linked to the cerebral atrophy and cognitive decline often documented in NP-SLE patients (Yamauchi et al. 1994; Chinn et al. 1997). Cytokines also appear to play crucial roles in NP-SLE, such as TNF-alpha and Interleukin (IL)-6. Both have been found in CSF and serum samples of NP-SLE patients (Baraczka et al. 2004; Trysberg et al. 2000). These cytokines can enter the brain through a compromised (leaky) area of the blood-brain-barrier (BBB) by specific transport systems, or by binding to other receptors on endothelial cells of brain vasculature, leading to endothelial release of other mediators into the CSF and brain parenchyma (Tsai et al. 1994; Svenungsson et al. 2001). For example following the onset of systemic autoimmunity, endothelial cells associated with perivascular microglia synthesize prostaglandin E2 (PGE_2) using cyclooxygeneases (COX) and prostaglandin E synthases. The PGE_2 is released into the CSF and brain parenchyma and acts on the corticotropin-releasing factor (CRF)-secreting

neurons of the paraventricular nucleus of the hypothalamus which ultimately regulates glucocorticoids. While it's role in NP-SLE is unknown, glucocorticoids may have a regulatory role in disease. For example, corticosterone is elevated in a model of neuropsychiatric lupus (Shanks et al. 1999; Lechner et al. 2000), and are the most frequently used therapy for patients with NP-SLE (Iverson & Anderson 1994; Tincani et al. 1996).

Neurodegenerative disorders are characterized by a gradual and relentlessly progressive neuronal loss that is often selective in that it occurs in anatomically and physiologically related brain areas. For many years researchers believed that structural damage to the brain may lead to abnormal mental function, but due to limitations in technical methods and imaging technology, this possibility was not extensively explored until recently. Imaging studies of NP-SLE support the notion that brain cell death could indeed account for the emergence of neurologic and psychiatric symptoms (Rocca et al. 2006; Ainiala et al. 2005). More interestingly, in the case of NP-SLE, evidence confirms that it is an autoimmunity-induced brain disorder characterized by profound metabolic alterations and progressive neuronal loss (Brooks et al. 1997; Sibbitt & Sibbitt 1993).

Autoantibodies have been linked to CNS involvement in NP-SLE. For example, anti-phospholipid antibodies are strongly associated with localized NP-SLE brain damage, including transient ischemic attack, stroke, seizure, and cerebral vein thrombosis in lupus (Tincani et al. 1996). These antibodies are directed against plasma proteins bound to negatively-charged phospholipids which lead to hypercoaguability through their effects on platelets, endothelial cells and complement activation. Studies have also illustrated the association between anti-phospholipid antibodies and cognitive dysfunction, such as verbal memory, speed of attention and concentration, cognitive flexibility, and psychomotor speed (Denburg & Denburg 2003; Sanna et al. 2003), but this is not

consistent across studies (Emori et al. 2005). In addition, anti-cardiolipin, a member of anti-phospholipids, have been detected in SLE patients with lupus headache, acute psychosis, cognitive dysfunction, and altered consciousness (Lai & Lan 2000). Neuronal antibodies are also believed to be involved in the pathogenesis of psychiatric diseases, including NP-SLE (Quismorio & Friou 1972; Vincent et al. 2003; Diederichsen & Pyndt 1970; Bluestein & Zvaifler 1983). In many cases however, the correlational nature of clinical data has led to the necessity for animal models. Using animal models, interactions between autoimmune / inflammatory phenomena and brain function can be examined in a more systematic and direct way.

Animal Models of Lupus

The existence of several murine models of SLE has been extremely valuable to researchers in evaluating various behavioural manifestations and autoimmune abnormalities which present in this disorder. The development of a fatal immune complex-mediated glomerulonephritis associated with immunological abnormalities, such as autoantibody production, makes several animal models very similar to the salient features of human SLE. Linkage analysis using simple sequence length polymorphisms performed in backcross, and intercross cohorts involving lupus-prone and immunologically normal mouse stains, revealed approximately 30 lupus susceptibility loci linked to various phenotypic trains. This knowledge lead to the development of several mouse strains with a spontaneous lupus-like disease (Theofilopoulos 1992). The most commonly studied spontaneous models of lupus include the (NZB×NZW)F1(BWF1) hybrid, Murphy Roth's Large (MRL), and BXSB mice, which are characterized by a wide spectrum of autoimmune manifestations (Dixon et al. 1978). These strains all share common characteristics such as hypergammaglobulinemia,

antinuclear antibodies (ANA) and glomerulonephritis, but they also have distinctive characteristics which are beneficial when examining specific aspects of the disease (Andrews et al. 1978).

The MRL Model of NP-SLE

The MRL/MpJ-Tnfrsf6^{lpr} (MRL-lpr) strain has a number of resemblances to human disease, including the neurobehavioural dysfunctions (Sakic et al. 1997). The MRL-lpr and the congenic MRL/MpJ +/+ (MRL +/+) substrains of mice are comparable in many respects (appearance, size and reproductive age), except in the onset of autoimmune disease. MRL-lpr mice were produced after a series of crossings among four inbred strains. More specifically, during the process of inbreeding it was observed that a subpopulation of mice developed a massive lymphoadenopathy, induced by the accumulation of abnormal T-lymphocytes. This subpopulation was separated from offsprings with normal phenotype and after a series of reciprocal backcrosses, it was observed that the lymphoid accumulation was due to the mutation of a single autosomal recessive gene, designated lymphopoliferation, or lpr (Theofilopoulos 1992). Due to the presence of the lymphoproliferative gene on chromosome 19, and a subsequent deficit in apoptotic Fas receptor expression (Singer et al. 1994), MRL-lpr mice develop an accelerated form of chronic autoimmune, lupus-like disease. The onset and progression of disease parallels the emergence of aberrant behaviours in these mice (Szechtman et al. 1997). Since MRL-lpr mice share 99.9% of their genome with MRL +/+ congenic mice, when studied together they are considered to be a natural, well controlled model of NP-SLE.

While MRL-lpr mice have rapid onset of lupus-like autoimmunity and inflammatory lesions beginning around 7 weeks of age, MRL +/+ controls develop similar symptoms later in their lifetime, usually by 12 months (Theofilopoulos 1992). The MRL-lpr strains have spontaneous

loss-of-function mutations in Fas, also known as APO-1 or CD95 (Nagata 1994). In brief, Fas is a 306 amino acid, 45-kDa, cell surface membrane protein related to the TNF receptor superfamily of type I membrane glycoproteins. This receptor is expressed rather ubiquitously in various tissues such as on actively proliferating cells in the thymus, liver, ovary, heart, skin, and gut epithelium, with particularly high levels on CD4+CD8+ thymocytes, activated T and B cells, and some neoplastic cells (Nagata & Golstein 1995). The main function of Fas is to bind to its ligand (FasL), and transduce signals leading to apoptotic cell death (Nagata & Suda 1995). FasL, may contribute to the maintenance of immune privilege by inducing Fas-mediated apoptosis in invading inflammatory cells (Bechmann et al. 1999). The well characterized apoptotic pathway defect in MRL-lpr mice has lead to much speculation as to which cell death (e.g. apoptosis vs. necrosis) and compensatory processes (e.g. the ubiquitin-proteasome system) are most likely to predominate in CNS disease (Elouaai et al. 1994; Alves-Rodrigues et al. 1998).

Autoimmune Associated Behavioural Syndrome (AABS)

Similarly to many patients with SLE, profound deficits in behaviour appear at a high frequency during the onset of spontaneous lupus-like manifestations in MRL-lpr mice (Szechtman, Sakic & Denburg 1997; Sakic, Szechtman & Denburg 1997). The specific patterns of behaviour (i.e. aberrant emotional reactivity and affective behaviour) shown at the onset of systemic autoimmunity and inflammation in MRL-lpr mice (Sakic et al. 1992), in reference to congenic MRL+/+ controls, is operationally labeled "autoimmune associated behavioural syndrome", or AABS (Sakic, Szechtman & Denburg 1997). More specifically, behavioural deficits in MRL-lpr mice which develop along the progression of systemic autoimmune disease is defined as the departure from the

behavioural performance of congenic MRL +/+ mice (Szechtman, Sakic & Denburg 1997).

The nature of AABS suggests a progressive anxious- and depressive-like behavioural state, and differences in emotionality, as indicated by increased thigmotaxic behaviour, impaired exploration of novel objects and spaces, excessive floating in the forced swim test (FST), and performance in the plus-maze and step-down tests (Sakic et al. 1992; Sakic et al. 1993a; Sakic et al. 1994), reduced responsiveness to a palatable stimulus (Sakic et al. 1996a), and reduced isolation-induced inter-male fighting (Sakic et al. 1998a). Moreover, impaired "cognitive" flexibility was suggested by response perseveration and longer escape latencies in the spatial learning task known as the Morris water maze (Sakic et al. 1993b). MRL-lpr mice also show lower nocturnal and open-field activity, and impaired performance in a psychomotor (beam-walking) task, (Sakic et al. 1993b). Interestingly, the presence of BRA within MRL-lpr mice was associated with thigmotactic swimming (i.e. along the wall of a large water pool), slower locomotion, and impaired exploration (Sakic et al. 1993a). The behavioural aspects of the MRL model have been extensively reviewed (Brey et al. 1997; Denburg et al. 1999).

The fact that the deficits in behaviour (e.g. blunted responsiveness to palatable food, immobility in the FST, reduced activity in a novel environment) appear before signs of severe peripheral symptomatology suggests that impaired performance in behavioural tasks is not an epiphenomenon produced by systemic organ involvement (Brey et al. 1995; Sakic et al. 1992; Sakic et al. 1996b). The emergence of behavioural impairments are also not evident before the development of manifestations of autoimmunity (Sakic et al. 1994). In addition to this circumstantial evidence, the causative role of autoimmunity and inflammation in the pathogenesis of AABS has been supported by studies employing the immunosuppressive drug cyclophosphamide

(CY), which prevented some of the behavioural deficits (Sakic et al. 1995; Sakic et al. 1996a; Farrell et al. 1997). More specifically, CY prevented anxiety- and depressive-like behaviour in MRL-lpr mice, indicated by the restoration of novel object exploration, increased responsiveness to a sweet palatable solution, and reduced floating in the forced swim test. In addition to autoimmunity, other factors have been suggested to be involved in the emergence of AABS such as genetics (Fas mutation), endocrine factors (CRF, glucocorticoid, prolactin), and multisystem disease (kidneys, joints, skin, eyes), as similarly seen in patients (Theofilopoulos 1992).

Pathogenesis of MRL-lpr NP-SLE

The MRL strain does not show a high incidence of inherited neuroanatomical abnormalities (Sherman et al. 1987), which minimizes the confounding influence of congenital defects in the study of disease-induced neurodegeneration. Reduced complexity of pyramidal neurons has been interpreted as evidence of neuronal atrophy in these brains (Sakic et al. 1998b) and the onset of autoimmune symptoms in MRL-lpr mice correlates with a variety of changes in behaviour, as seen in human NP-SLE (Sakic et al. 1992; Sakic et al. 1994; Denburg et al. 1995). For example, marked behavioural deficits in the MRL-lpr substrain coincides with an early imbalance in the cytokine network and hyperproduction of autoantibodies, but antedate generalized lymphadenopathy, arthritis, glomerulonephritis, and skin lesions (Andrews et al. 1978). AABS coincides temporally with the infiltration of lymphoid cells into the brain around 8 weeks of age (Farrell et al. 1997; Vogelweid et al. 1991), ventricular enlargement around 14 weeks of age (Denenberg et al. 1992), as well as expression of mRNA for proinflammatory cytokines from 11-30 weeks of age (Tomita et al. 2001a; Tomita et al. 2001b).

The BBB appears to be disrupted early on and thus could play a key role in SLE-related neurological dysfunction in the MRL-lpr model, especially if antibodies or other systemic mediators of inflammation (e.g. cytokines, prostaglandins) are causally related to disease manifestations. It has been documented that there is an age-related increase in the frequency of both CNS inflammation, composed predominantly of CD4+ cells and perivascular leakage of IgG around brain vessels of MRL-lpr mice (Vogelweid et al. 1991), and neurotoxic CSF from old MRL-lpr mice (Maric et al. 2001). Antibody-producing cells are present in the brains of MRL-lpr mice late in the course of disease and could play a role as well. For example, B cells are present in the choroid plexus (CP) of MRL-lpr mice (Zameer & Hoffman 2004) along with circulating immunoglobulins (Ig) in their CSF (Sidor et al. 2005). Together, these data suggest that autoantibodies reactive to brain antigens could gain access or be produced in the CNS, but whether or not they are actually neuropathogenic is unknown.

Pre-treatment with the immunosuppressive drug CY had a differential effect on the infiltration of CD45+ leukocyte subtypes (Farrell et al. 1997). More specifically, a six week treatment of CY reduced the infiltration of CD45 (a marker for T-cells, and cells of the monocyte-macrophage lineage), but not CD45R+ cells (B-cells) into the CP of the MRL-lpr substrain. CY treatments also successfully prevented atrophy of dendritic spines in MRL-lpr brains (Sakic et al. 2000a). Since CY is a cytotoxic drug which affects broad populations of cells, it is unknown which pathways (cytologic or inflammatory) play a predominate role in the etiology of brain damage in MRL-lpr mice.

In some conditions, activated microglia are proposed to contribute to the pathogenesis of neurologic and psychiatric disease (Gwag et al. 1997), and cytokine-producing microglia may also

play a role in CNS damage (Piani et al. 1992; Allan & Rothwell 2001). Previous studies suggest that microglia-induced neuronal excitotoxicity may account for brain damage in MRL-lpr mice. More specifically, class II MHC upregulation (McIntyre et al. 1990), deposition of complement proteins C3 and C9 (Alexander et al. 2005), and increased mRNA expression for pro-inflammatory cytokines has been documented in brains of MRL-lpr mice (Tomita, Holman & Santoro 2001a; Tomita et al. 2001b). Dysregulation of corticosteroids in these lupus-prone mice revealing a dysfunctional hypothalamic-pituitary axis (Shanks et al. 1999; Lechner et al. 1996), may also account for changes observed in their paraventricular nucleus (Sakic et al. 1999). Increased numbers of TUNEL+ cells in the periventricular areas and lymphocytes in the ventricle lumens of MRL-lpr mice was additional evidence of accelerated cell loss (Sakic et al. 2000b).

Depressive-like behaviours are often shown to be influenced by altered central neurotransmitters such as dopamine (DA), serotonin (5-HT) and norepinephrine, or NE (Elhwuegi 2004). MRL-lpr brains have been found to express increased levels of DA in the paraventricular nucleus and median eminence (ME), suggesting impaired catabolism in the turberinfindibular pathway. Decreased concentrations of 5-HT in the paraventricular nucleus and enhanced levels in the hippocampus where also detected, along with decreased levels of NE in the prefrontal cortex of these mice. Of all transmitters examined, the evidence suggested that central dopaminergic system activity was most profoundly altered in MRL-lpr brains. More specifically, acute injection with a selective D2/D3 agonist, quinpirole, increased floating time of MRL-lpr mice in the FST, and chronic injection resulted in self-injurious behaviour (i.e. limb gnawing and chest-skin pulling and biting). While impaired catabolism and / or acquired dopaminergic cell damage appeared to be the most likely cause for the drug induced behavioural manifestations, evidence to confirm selective

neuronal demise was lacking.

The accumulation of unwanted proteins often underlie the pathogenesis of several major human neurodegenerative diseases. For example, as a result of defective ubiquitin-dependent proteolysis, neurodegeneration can occur (Layfield et al. 2001). Evidence of this possibility in MRL-lpr brains, however, was lacking. Despite this, neuron-like cytoplasmic staining of some TUNEL+ cells had previously suggested that neurons were affected in autoimmune mice, which was supported by evidence that CSF from lupus-prone mice was specifically neurotoxic in co-cultures of hippocampal astrocytes / neurons (Maric et al. 2001). Therefore, there was no direct evidence which confirmed and detailed neuronal death within the MRL-lpr CNS.

Limitations of the MRL-lpr Model

Although the MRL-lpr strain displays many characteristics that resemble human NP-SLE, there are a number of limitations we must consider when studying this murine model. First of all, human NP-SLE often has oscillating relapsing-remitting presentations of symptoms, but MRL-lpr mice show a progressive and unrelenting course of disease. This difference appears to be the most fundamental difference between human and murine SLE. Secondly, human SLE shows a strong gender preference (i.e. about nine to ten times more female patients than male). The MRL-lpr strain, however, has no gender bias, possibly suggesting a different hormonal differentiation between two sexes in human and murine SLE. Lastly, affective states of MRL-lpr mice can only be assessed by behavioural tests while more sophisticated and systematic assessments are administered in human SLE. Therefore, care is required when transferring information obtained from an animal model of SLE to human disease.

Despite the parallel between the emergence of behavioural dysfunction with systemic autoimmune disease, so far there has been no direct evidence that brain pathology is associated with aberrant behaviour. Based on clinical reports that neuropsychiatric manifestations are accompanied by cerebral atrophy (Chinn et al. 1997) and progressive neuronal loss (Brooks et al. 1997; Sibbitt & Sibbitt 1993), we sought to further test the validity and limits of the MRL model of NP-SLE. The aim of this thesis is to establish the phenomena and the extent of neurodegeneration in MRL-lpr brains, as well as examine the associations between brain morphology, structural and functional damage, and progression of autoimmune / inflammatory disease using parallel analyses of neuropathology, behaviour, and immunity.

Overall Hypothesis

The spontaneous manifestation of systemic autoimmune / inflammatory disease factors propagates a selective non-apoptotic central neuron demise in lupus-prone brains, which overtly presents as a departure from the normal behavioural performance of healthy MRL animals.

Specific Hypotheses

1) Apoptotic neurodegeneration will be associated with the onset of systemic autoimmune disease in MRL-lpr mice.

2) Neurodegeneration will occur through ubiquitin pathways, concomitantly with cognitive-behavioural deficits in lupus-prone mice.

3) Inflammatory microglia cells contribute to the neurodegenerative process, and a non-steroidal anti-inflammatory drug treatment will attenuate brain damage in lupus-prone mice.

4) Dopaminergic neuron circuit damage can account for some deficits in reward behaviour, commonly seen in MRL-lpr mice.

5) Autoimmune-associated dopaminergic neurodegeneration and cerebrospinal fluid toxicity will be abolished through immunosuppressive treatment in behaviourally impaired MRL-lpr mice.

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CHAPTER 2
NEURODEGENERATION IN AUTOIMMUNE MRL-LPR MICE AS REVEALED BY FLUORO JADE B

Published in Brain Research in 2003

David Ballok's contribution:

I am the first author on this paper. I was involved in the planning and design of the experiment. I injected mice with kainic acid (KA) and was involved in animal surgeries which included perfusions, blood collection and organ extractions. I was responsible for the majority of the subsequent assays on the samples collected including brain and spleen processing / sectioning, performing the ELISA for ANA, and employing FJB and TUNEL staining to the brain tissues. CD4 and CD8 staining was performed by another author. Technicians ignorant to the study design and blind to sample origin quantified cell numbers both manually and with computer imaging software. I completed the required statistical analyses with the assistance of my supervisor. The figures included in the paper are my own work, and I wrote the article.

Dear Natalie David,

I am completing a Ph.D. thesis at McMaster University entitled "Neurodegeneration In An Animal Model Of Neuropsychiatric Lupus". I would like your permission to reprint the following journal article in my thesis:

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Research report

Neurodegeneration in autoimmune MRL-lpr mice as revealed by Fluoro Jade B staining

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Abstract

As in many humans suffering from lupus erythematosus, the development of systemic autoimmunity and inflammation in Fas-deficient MRL-lpr mice is accompanied by CNS dysfunction of unknown etiology. Experimental studies revealed infiltration of lymphoid cells into the choroid plexus, reduced neuronal complexity, retarded brain growth, and enlargement of cerebral ventricles. Moreover, an increased presence of cells with nicked-DNA (TUNEL+ cells) in the periventricular areas suggested accelerated apoptosis in brain cells of MRL-lpr mice. However, direct evidence that the dying cells were neurons was lacking. For this purpose, we presently use Fluoro-Jade B (FJB), a novel fluorescent dye which has high affinity for dying neurons (both apoptotic and necrotic). As expected, in comparison to the control groups, the brains of diseased, 5-month-old MRL-lpr mice showed increased numbers of FJB-positive (+) cells in cortical and periventricular regions. The FJB+ cells were significantly more numerous than TUNEL+ cells, and only ~7% co-localized with TUNEL. Immunostaining for CD4 and CD8 markers did not correlate with the number of FJB+ cells, suggesting that T-lymphocyte infiltration into the brain tissue is not a reliable predictor of neuronal demise. Conversely, indices of systemic autoimmunity (splenomegaly and high serum anti-nuclear antibody levels) were associated with increased FJB+ cell numbers in brains of autoimmune MRL-lpr mice, supporting the causal link between autoimmunity and neurodegeneration. Taken together, the above results suggest that factors other than T-cell infiltration and cell death mechanisms other than Fas-mediated apoptosis dominate neuronal degeneration in lupus-prone MRL-lpr mice.

Keywords: Autoimmunity; Inflammation; Lupus; Neurodegeneration; Fluoro-Jade B method; TUNEL method; T-Lymphocyte; MRL mouse; Autophagic; Autophagocytosis

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune/inflammatory disease with diverse clinical manifestations. The psychiatric aspect of SLE involves depression, anxiety, psychosis, and cognitive deficits of unknown etiology [25,80]. Contemporary imaging techniques, such as magnetic resonance imaging and single-photon emitted computerized tomography have demonstrated morphological abnormalities in brains of SLE patients with central nervous system (CNS) involvement. They include focal edema, infarction, and brain atrophy [4,9,16,17,40,74,76]. In addition to morphological imaging, positron emission tomography scanning and nuclear magnetic resonance spectroscopy provide further evidence that NP-SLE is an autoimmunity-induced brain disorder characterized by profound metabolic alterations and progressive neuronal loss [12,73].

The existence of animal models has been essential to exploring the etiopathogenesis of many neuroimmunologic diseases, including SLE. The murine inbred MRL strain develops a spontaneous disease that has clinical and serologic manifestations reminiscent of human SLE, including hyperproduction of autoantibodies, immune complex formation, inflammation and multiple organ damage [78]. The autoimmune MRL-MpJ-Tnfrsf6^{lpr} (MRL-lpr) substrain has a congenic control, MRL/MpJ (MRL +/+), which develops similar manifestations of lupus-like disease substantially later in life. The behavioural aspects of so-called 'autoimmunity-associated behavioral syndrome' or

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AABS in MRL-lpr mice have been extensively reviewed elsewhere [11,23,65,77]. In brief, an accelerated development of lupus-like disease in the MRL-lpr substrain is accompanied by a constellation of behavioral deficits [32,68,69,83], most consistently noted in tasks reflective of emotional reactivity, affective behaviour [64,77] and spatial learning/memory [32,68]. Behavioral abnormalities appear concurrently with serological manifestations of autoimmunity and neuropathology, including infiltration of lymphoid cells into the choroid plexus and brain parenchyma [28,82], reduced complexity of pyramidal neurons [66], and ventricular enlargement at an older age [26]. Progression of autoimmune disease seems to have a causal role since immunosuppressive treatment with cyclophosphamide prevented neuronal atrophy and severity of autoimmune symptoms was found to correlate with dendritic spine loss [62]. Furthermore, immunofluorescent staining with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) technique has revealed an increased incidence of cells with fragmented DNA (a putative marker of apoptosis) in brains of MRL-lpr lupus prone mice [63]. The fact that 70% of the total number of TUNEL-positive cells did not co-localize with CD4 or CD8-positive cells [63] suggested that a significant proportion of resident brain cells die as a result of an autoimmune/inflammatory insult. However, whether these dying brain cells were indeed neurons remained an open question. Support for this notion comes from an in vitro study in which cerebrospinal fluid (CSF) from lupus-prone mice was found to be neurotoxic in co-cultures of hippocampal neurons and astrocytes [47]. The hypothesis of autoimmunity-induced brain cell demise is rather important in the light of evidence that the MRL strain does not show a high incidence of inherited neuroanatomical abnormalities [72].

However, the evidence obtained in the above studies was not specific for neuronal death, or was obtained in vitro. To provide direct evidence that neurons are dying in vivo we used a relatively novel method with the Fluoro-Jade B (FJB) dye, which is an anionic fluorescein derivative used for visualization of neuronal degeneration in brain tissue sections [37,71]. Given that this method has not been previously used in our laboratory, a group of healthy Swiss Webster (SW) mice were injected with kainic acid, a well-known neurotoxin [56] to assess the effectiveness of the FJB technique. Subsequently, the presence and distribution of dying neurons was compared among diseased MRL-lpr mice, asymptomatic MRL +/+, and non-autoimmune SW mice. In addition, the relationships between FJB positivity and indices of autoimmunity, T-cell infiltration and TUNEL positivity were examined. Based on a significant correlation between reduced neuronal complexity and anti-nuclear antibody titers [62], it was expected that severe disease manifestations would be associated with increased neuronal death. Similarly, in further exploring the role of T-cell infiltration in neuronal demise [63], we expected that severe influx of T-lymphocytes into the brain tissue will enhance neuronal death, which will be predominantly of apoptotic type. Finally, cohorts of non-autoimmune SW mice were used in the experiments to provide a healthy, age-matched negative control for the FJB method.

2. Methods

2.1. Validation of the FJB method

Eight SW mice were injected with either kainic acid (10 mg/kg, i.p.; Sigma, St. Louis, MO, USA) or phosphate-buffered saline (PBS), according to a previously published protocol [37]. Kainic acid is a rigid structural analog of glutamate and powerfully reproduces the excitatory neurotoxic (excitotoxic) action of glutamate on central neurons [56]. Animals were sacrificed 4 days later and tissue was processed by a modified FJB method. Namely, incubation times were increased from 10 to 15 min for potassium permanganate and from 20 to 30 min for staining solution. In addition, the concentration of the fluorochrome was increased (as specified below). These changes were made with an expectation that degenerating neurons would be more readily detected by an inexperienced observer. Indeed, the presence of clearly distinguished FJB-labelled neurons led to subsequent employment of these modifications in the actual experiment.

The Fluoro-Jade B stain has an affinity for the entire degenerating neuron including cell body, dendrites, axon and axon terminals, regardless of the type of cell death [37,71,88]. The degenerating tissue components (biomolecules) to which the dye has an affinity is currently unknown and the exact chemical identity of FJB remains to be confirmed [71]. Despite this incomplete knowledge of the staining mechanisms, this relatively simple method reliably detects dying neurons, with results comparable to the traditional silver method when animals are treated with neurotoxins [88].

2.2. Assessment of FJB positivity in brains from autoimmune mice

2.2.1. Animals

Three-week-old male MRL-lpr and MRL +/+ mice ($n=15$ mice/strain) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), while age-matched, non-autoimmune Swiss Webster (SW, $n=15$) males were purchased from Charles River Canada (St. Constant, Canada). They were habituated to the colony room and housed in groups of five per cage. The mice were maintained under standard laboratory conditions (light phase: 08:00–20:00 h; water and rodent food ad lib) until 19 weeks of age. One MRL-lpr mouse died prematurely due to early disease onset. The experimental protocols

were carried out in accordance with the rules and regulations of the Canadian Council of Animal Care.

2.2.2. Tissue preparation

Mice were anaesthetized with Somnotol (60 mg/kg), transcardially perfused with 0.9% saline, and extracted brains were immersed into 4% paraformaldehyde (PFA) for fixation at 4 °C for 72 h. Post-fixed brains were washed three times in PBS before being immersed into 30% sucrose (in PBS) for 5 days. Before sectioning, the brains were placed into aluminum freezing capsules filled with O.C.T. histological embedding medium (Somagen Diagnostics, Edmonton, Canada) and frozen by immersion in isopentane cooled in liquid nitrogen. Eight-micron coronal sections were cut from the olfactory bulb through the cerebellum using a Jung Frigocut 2800 E cryostat. Sections were placed on APTEX-coated glass microscope slides, and left to dry at room temperature (RT) for 24 h before processing.

2.2.3. Fluoro-Jade B method

The standard histological processing of FJB involves pretreatment with 1% sodium hydroxide in 80% alcohol [71]. This step was omitted due to a methodological error, and instead slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. The slides were then rinsed in distilled water (dH_2O) for 1 min, before being transferred to a 0.06% potassium permanganate solution and gently shaken for 15 min. Subsequently, they were rinsed in dH_2O for 1 min before immersion into a 0.001% FJB staining solution, prepared from a stock solution (Histo-Chem, Jefferson, AR, USA). The stock solution was made by adding 50 mg of the dye powder to 500 ml of dH_2O, and stored in darkness at 4 °C. To make up 200 ml of staining solution, 20 ml of stock solution was added to 180 ml 0.01% acetic acid in dH_2O. The staining solution was prepared immediately before use and was not reused. After 30 min of gentle shaking in the staining solution, slides were rinsed (for 1 min in each of three dH_2O washes) and left to dry overnight in darkness, at RT. They were immersed in three 2-min xylene washes, before coverslipping with DPX (Sigma), a non-aqueous, non-fluorescent plastic mounting media. Results were assessed with an epifluorescent microscope with blue (450–490 nm) excitation light (Diastar Fluoresence Microscope, Reichert Scientific, Buffalo, NY, USA). Photographs of coronal sections were taken at magnifications ×200 and ×400. Numbers of FJB+ cells were quantified with NIH Image analysis software (Scion, Frederick, MD, USA). The validity of counts obtained by software was confirmed by manual quantification of several sections.

2.2.4. Indices of autoimmunity

High levels of serum anti-nuclear antibodies (ANA) and increased spleen weight are typical manifestations of the lupus-like disease in MRL-lpr mice [62,78], and both were presently used to confirm differences in immune status among the groups. Blood collection was performed under Somnotol anaesthesia (i.p. 60 mg/kg body weight). Blood was left to coagulate in 1.5-ml plastic vials, and later centrifuged for 10 min at 3000 rpm. Serum was separated from the clot and stored at −20 °C until further analysis. Relative ANA titres were measured using a sandwich ELISA kit (catalog No. 5200), according to the manufacture's instructions (Alpha Diagnostic International, San Antonio, TX, USA) and the protocol previously described [62]. Spleen weights were determined immediately upon extraction on an analytic scale (Sartorius 2024 MP, VWR Scientific Canada Ltd.).

2.3. Association between FJB positivity, T-lymphocyte infiltration, and TUNEL staining

To examine whether neuronal death is associated with other putative markers of brain inflammation, a larger cohort of MRL-lpr males was employed. In particular, to examine whether influx of T-lymphocytes is associated with increased FJB positivity, the FJB method was combined with immunofluorescent detection of CD4 and CD8 T-cell markers. To examine whether the neurodegenerative processes involves DNA nicking, brain sections were double-labelled with FJB and TUNEL staining, and co-localized cells were revealed by confocal microscopy.

2.3.1. Animals

Twenty 3-week-old MRL-lpr male mice were purchased from the Jackson Laboratory and maintained under the conditions specified above. To control for false positive or background staining, age-matched healthy male SW controls were used ($n=20$). Mice were housed in groups of five per cage until 20 weeks of age. Three MRL-lpr animals died prematurely due to disease complications. Mice were anaesthetized and tissue was prepared as outlined above. Based on results from the first experiment, the locations with the most intense FJB staining were examined. They included regions of the hippocampus and choroid plexus of the third ventricle, at an approximate location between Bregma −0.94 and −1.58 mm [29].

2.3.2. Immunohistochemical detection of T-lymphocytes

Four serial sections per slide were processed for the presence of CD4 and CD8 markers. Sections were incubated overnight at RT with monoclonal rat-anti-mouse CD4 antibody (IgG2b isotype, diluted 1:200), or anti-mouse CD8a antibody (IgG2a isotype, diluted 1:600). Antibodies were purchased from Sigma and diluted in antibody diluent (DAKO®, Carpinteria, CA, USA). The primary antibodies were visualized using an FITC-conjugated goat anti-rat IgG antibody (Jackson Immunoresearch, West Grove, PA, USA), diluted 1:50 in 1% normal goat serum in PBS. Sections were incubated with secondary antibody for 4 h at RT, rinsed three times in PBS and

coverslipped in Fluorescence Mounting Medium (lot No. 011-3, DAKO®). Preliminary studies revealed that immunostaining for CD4 and CD8 markers was not compatible with the FJB staining protocol, thus precluding double-labelling. Therefore, CD4+ and CD8+ cells were first manually quantified by an unbiased observer, and after the removal of mounting medium (10 min washing in PBS) the same sections were reprocessed for FJB. Both stains fluoresced at similar wavelengths (488 nm). The FJB+ cells were then manually counted by a second unbiased observer, focusing on the periventricular regions, where the majority of T-lymphocyte infiltrates were located.

2.3.3. TUNEL staining

In situ detection of DNA nicks was performed using a Neuro TACS II TdT Labelling Kit (Trevigen, Gaithersburg, MD, USA). Fixed-frozen sections from five animals from each group were permeabilized using Neuropore for 30 min at RT. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 3 min. After washing, the sections were exposed to the mixture of TdT, Mn^{2+} and biotinylated-dNTP for 1 h at 37 °C. The reaction was stopped by putting slides into a jar with TdT Stop Buffer for 5 min. After washing three times in distilled–deionized water (ddH_2O), streptavidin-Alexa 594 (diluted 1:200 in normal goat serum diluent) was applied to each section for 1 h at RT, and then washed three times in ddH_2O before proceeding to FJB staining. In the case of the TUNEL method, we were able to combine this technique with FJB on the same sections.

2.3.4. Modified Fluoro-Jade B method

The FJB method was modified for double labelling of neurons and combined with the TUNEL stain for the purpose of co-localization (http://pappone.ucdavis.edu/tom/protocol/FJ). Briefly, slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. They were rinsed in dH_2O for 1 min before being transferred to 0.0001% FJB staining solution (Histo-Chem) prepared immediately before use. In brief, the composition of the stain vehicle was 2 ml of the stock solution added to 198 μl acetic acid in 198 ml of dH_2O. After 1 h of gentle shaking in the staining solution at 4 °C, slides were rinsed for 1 min in each of three dH_2O washes. The slides were left to dry at RT overnight in darkness. Once dried, they were immersed in three xylene washes, each for 2 min, before coverslipping with the non-aqueous, non-fluorescent plastic mounting media, DPX (Sigma).

Three areas, previously shown to have a high incidence of TUNEL nuclei [63], were selected for analysis (Fig. 6). TUNEL-stained nuclei and FJB+ cells were visualized using a Zeiss Laser Scanning Confocal Microscope (LSM-510). Two channels were tracked with excitations of 543 nm (for TUNEL cell nuclei) and 488 nm (for FJB+ neurons) yielding red and green scans, respectively. In brief, a helium–neon laser (wavelength 543 nm, red emission) was used for the visualization of Alexa-594 (Molecular Probes, Eugene, OR, USA) and an argon laser (wavelength 488 nm, green emission) was employed for visualization of FJB. Sections were scanned using a C-Apochromat 63x/1.2 W Corrected objective in combination with a 1024×1024 pixel resolution. For purposes of co-localization of fluorochromes, separate confocal images were pseudo-coloured to yield composite images. Stained nuclei/cells which co-localized appeared yellow. Scans were imported as TIFF files into Photoshop 4.5 (Adobe Systems) and nuclei/cells were counted manually by an unbiased observer.

2.3.5. Indices of autoimmunity

To confirm the disease in MRL-lpr mice, levels of serum ANA and spleen weights were measured in all animals according to the protocol described above.

2.3.6. Statistics

The data were analysed by a one-way analysis of variance (ANOVA) and Student's *t*-test where appropriate. Pearson's correlation was used to measure association between the variables. Computations were performed using the SPSS 11.0 statistical package, and the accepted level of significance was $P \leq 0.05$. Graphs show means±S.E.M., with $***P<0.001$ representing between group differences.

3. Results

In comparison to mice injected with saline (Fig. 1A), numerous FJB+ cells were observed in coronal sections from mice injected with kainic acid (Fig. 1B). They were identifiable by their apparent neuronal-like morphology in the pyriform cortex and hippocampus, as well as other regions previously reported [37]. Similarly to this drug-induced damage, sections from autoimmune MRL-lpr mice showed increased numbers of FJB+ cells in comparison to congenic and allogenic controls (Fig. 2A, C, E). They were most notably detected in the parietal cortex (Fig. 2B), and periventricular regions of the hippocampus and the paraventricular nucleus of the hypothalamus (Fig. 2D, F). Other areas showing variable staining included the arcuate nucleus/median eminence region, central amygdala (Fig. 3A), and cerebellum. Furthermore, in contrast to the cerebellum (which showed scattered patches of FJB+ cells around the fourth ventricle, data not shown), the CA2, CA3 (as shown in Fig. 2D) and dentate gyrus of the hippocampus were distinctly labelled in MRL-lpr mice. The total number of FJB+ stained neurons (per brain) was significantly higher in MRL-lpr mice in comparison to the MRL +/+ and SW groups [$F(2,43)=249.36$, $P<0.001$; Fig. 3B]. As expected, the ANA titers and spleen weight were highly elevated in the MRL-lpr group (ANA: 1.36±0.18; spleen: 515.7±40.4) in comparison to the two

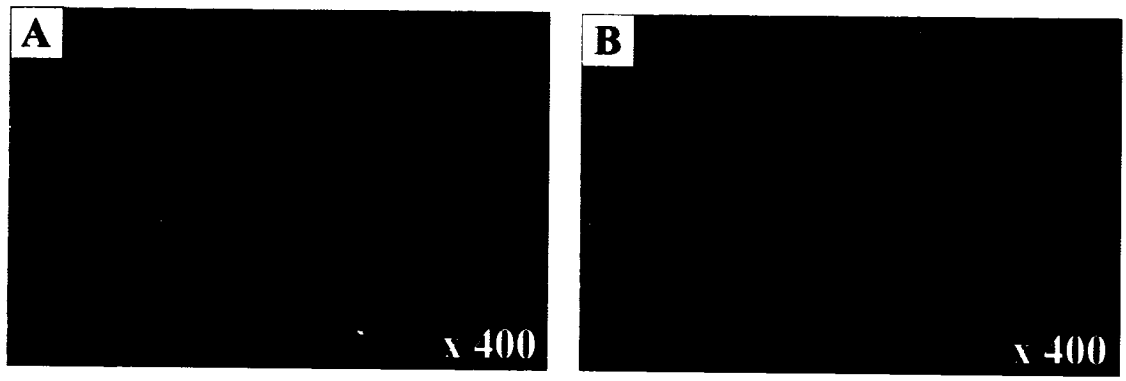

Fig. 1. Verification of Fluoro-Jade B (FJB) positive staining. The effects of a single i.p. injection of (A) neutral phosphate buffered saline or (B) kainic acid (10 mg/kg i.p.) to a group of healthy SW mice. As expected, 4 days after the injection, a yellowish staining of hippocampal neurons in brains of mice injected with kainic acid suggested the neurodegenerative process. Note: changes made to the FJB method may account for a higher degree of background staining; magnification on this and other figures is shown in the lower, right corner.

controls (for MRL +/+ ANA: 0.15±0.03, spleen: 250.3±14.4; for SW ANA: 0.03±0.01, spleen: 160.6±11.7). More importantly, the extent of FJB positivity correlated with immune measures in MRL mice, and in particular within the MRL-lpr group (for ANA: $r_{14}=0.627$, $P=0.016$; for spleen weight: $r_{14}=0.654$, $P=0.011$; Fig. 4A, B). We also observed non-specific Fluoro-Jade B staining of blood cells and vascular elements in the choroid plexus and brain parenchyma (Fig. 5A). This latter finding is consistent with reported FJB-staining of vascular elements in hemispheres and the choroid plexus of damaged rat brains [70] (L. Schmued, personal communication). The former finding is rather interesting and may reflect two possibilities. The first involves poor perfusion of the blood vessels and the second involves reported infiltration of leukocytes into the choroid plexus and brain parenchyma of MRL-lpr mice [28,82]. Whichever is the case, this observation appears important for future studies that are aimed at assessing neuronal demise by FJB in the context of brain inflammation.

Along this line, the second cohort of mice was used to examine the relationship between T-lymphocyte markers and FJB+ staining. As expected [63], CD4+ and CD8+ cells were found in clusters in the choroid plexus and periventricular region (Fig. 5B) of MRL-lpr brains. Consequently, this area was chosen to examine the above relationship. The mean number of CD4+ cells was two-fold higher than CD8+ cells (data not shown), but the infiltration of both cell types did not correlate linearly with the extent of FJB staining. In addition, when the TUNEL method was used in combination with the FJB method, the majority of FJB+ cells (more than 90%) in the MRL-lpr brains did not have TUNEL-stained nuclei (Fig. 6A). Conversely, the number of TUNEL+ cells was substantially lower than the number of FJB+ cells, and TUNEL nuclei almost completely co-localized with FJB+ cells (Fig. 6B). Despite a relatively small sample ($n=5$ mice group), significantly more TUNEL cells were confirmed in the MRL-lpr brains than in controls (for MRL-lpr: 5.6±0.51; for SW: 2.4±0.4; $t_8=4.938$, $P=0.001$) [63]. As seen in the first cohort, the autoimmune status of the second cohort was confirmed by significantly higher ANA levels and spleen weights in MRL-lpr mice then in healthy controls (for ANA: $t_{35}=7.087$, $P=0.001$; for spleen weight: $t_{35}=8.991$, $P=0.001$; data not shown).

4. Discussion

By using a stain for degenerating neurons, the present study supports the hypothesis that central neurons are targeted during the development of systemic autoimmune disease in the Fas-deficient MRL-lpr strain [57,62]. Although a modified FJB method was used, discernable differences between groups were revealed. The fact that the majority of TUNEL+ cells co-localized for FJB strengthens the notion (obtained in vitro) that neurons are more susceptible to autoimmunity-induced toxic mediators than astrocytes [47]. These results are consistent with previously reported neuronal atrophy [62,66], which may represent an initial step in excessive neuronal demise. The lack of correlation between CD4+/CD8+ cells and FJB+ cells suggests that in addition to T-lymphocyte infiltration other factors (e.g., brain-reactive antibodies, upregulated glucocorticoid production, etc.) are involved in induction of neurodegeneration in periventricular regions. Conversely, a positive correlation between the extent of FJB+ staining, serum ANA levels, and spleen weights supports the hypothesis that autoimmunity is a key factor in the pathogenesis of brain damage during lupus-like disease. Finally, since most of the FJB+ neurons were not concurrently TUNEL+, Fas-mediated neuronal apoptosis is unlikely a mode of cell death in this model of neuroimmunologic disease.

The terminal cause of brain damage in many neuroimmunological disorders remains unknown. However, it is

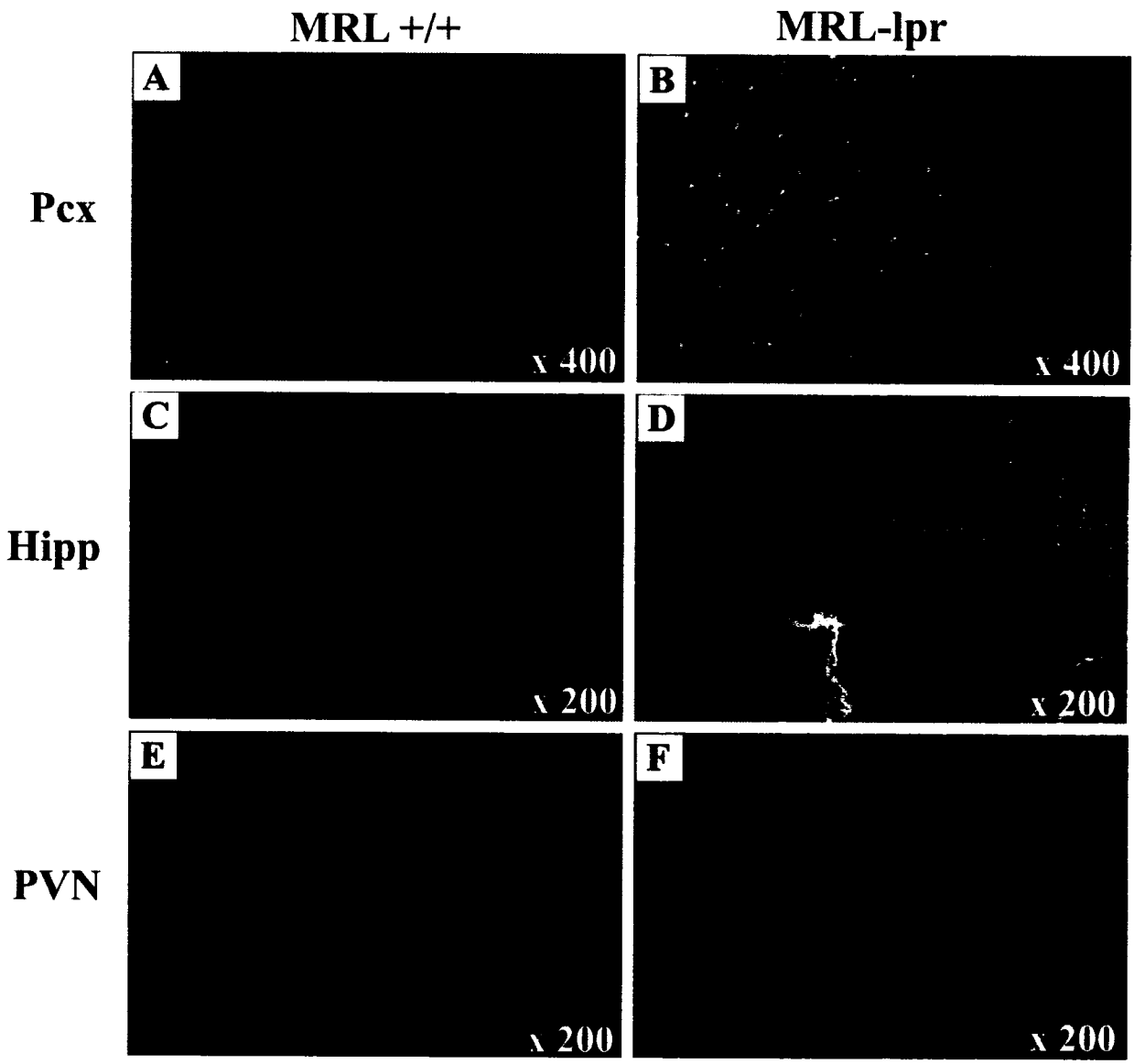

Fig. 2. Regional distribution of dissimilar FJB staining between 19-week-old mice from two MRL substrains. Although variation was seen within the MRL-lpr group as to the number of FJB+ cells in a region, there were clear differences between the substrains. They were most notably seen in the (A) cortex of MRL +/+ vs. (B) cortex of MRL-lpr mice, (C) hippocampus of MRL +/+ vs. (D) hippocampus of MRL-lpr mice, and in the (E) paraventricular nucleus of MRL +/+ vs. (F) paraventricular nucleus of MRL-lpr mice. Age-matched SW mice were comparable to MRL +/+ controls in the number of FJB staining seen within the brain (photos not shown). Note: red arrows and (D) inset show FJB+ cells with neuronal morphology.

well-documented that the integrity of the blood–brain barrier is compromised in human and animal forms of lupus [28,35,42,58,82]. This deficiency facilitates entry of various types of immune cells into the choroid plexus and brain parenchyma [1,28,42]. Since treatment with anti-CD monoclonal antibodies was shown to inhibit the development of CNS lesions in MRL-lpr mice [54], an overall assumption was that severe influx of leukocytes will be associated with intense FJB+ staining, induced largely by infiltrated cytotoxic T-lymphocytes [5,33]. However, the present lack of linear correlation between CD4+/CD8+ and FJB+ cells is consistent with the notion that brain damage in MRL-lpr mice is a consequence of multiple factors, which may include (and are not limited to) cytokines of glial or immunocyte origin [27,61], intrathecally produced brain-reactive antibodies [20,21,47], or sustained binding of adrenal hormones [49], which are dysregulated in this and other strains of autoimmune animals [45].

The role of antibodies in the etiology of SLE with CNS involvement has been proposed for at least two decades [6,7,10,22,24,38,51,84]. The possibility that antibody-producing, CNS infiltrating lymphocytes contribute to disturbed emotionality in MRL-lpr mice has been initially supported by the presence of CD45R+ cells in the choroid plexus and brain parenchyma [28]. Similar to T-lympho-

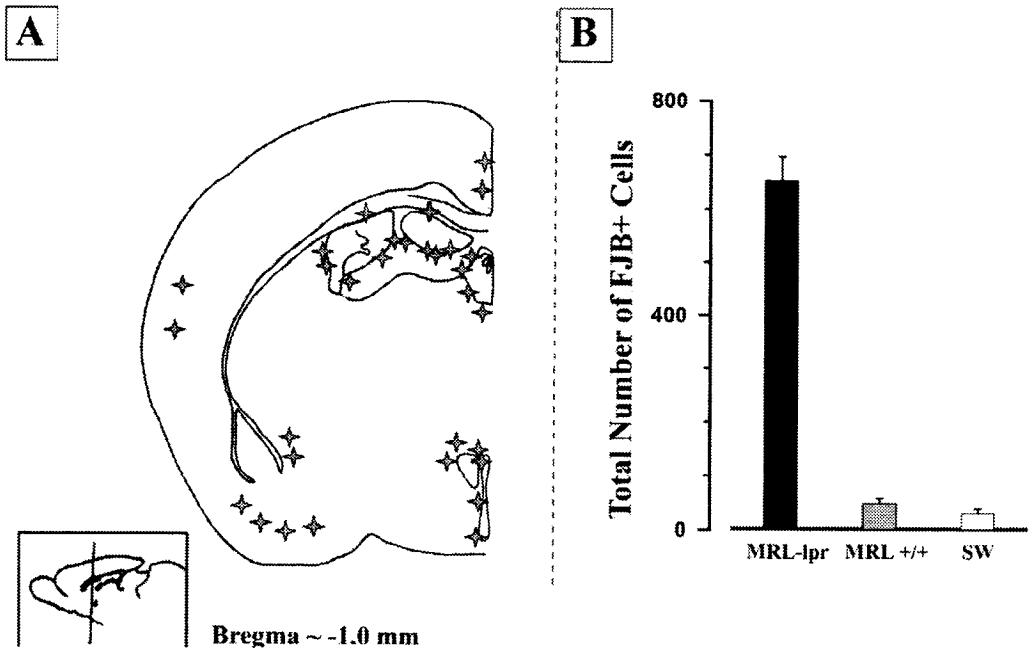

Fig. 3. Schematic distribution and number of FJB+ cells within the MRL-lpr brain. (A) The accumulations of FJB+ cells (shown as stars) were confined to discrete locations, such as periventricular areas of the limbic system and cortex. Inset: the approximate location of a coronal section assessed. (B) The mean number of FJB+ cells was more than 10-fold higher in diseased 19-week-old MRL-lpr mice than in either asymptomatic autoimmune-prone MRL +/+ or healthy SW controls.

cytes, B-cells remain in the brain upon recognizing specific antigens [85] and may turn into plasma cells which produce brain-reactive antibodies in autoimmune mice [89]. Their levels increase substantially with age in the MRL-lpr strain [19,34,36,52,67] and can directly disrupt neuronal functioning [18] and neuronal viability [21]. Zameer and Hoffman have recently shown the periventricular pattern of immunoglobulin binding in brains of autoimmune, 4-month-old MRL-lpr and BXSB mice [89], which matches with increased FJB positivity in the present study and the previously reported TUNEL positivity [63].

While the mechanism by which brain-reactive antibodies

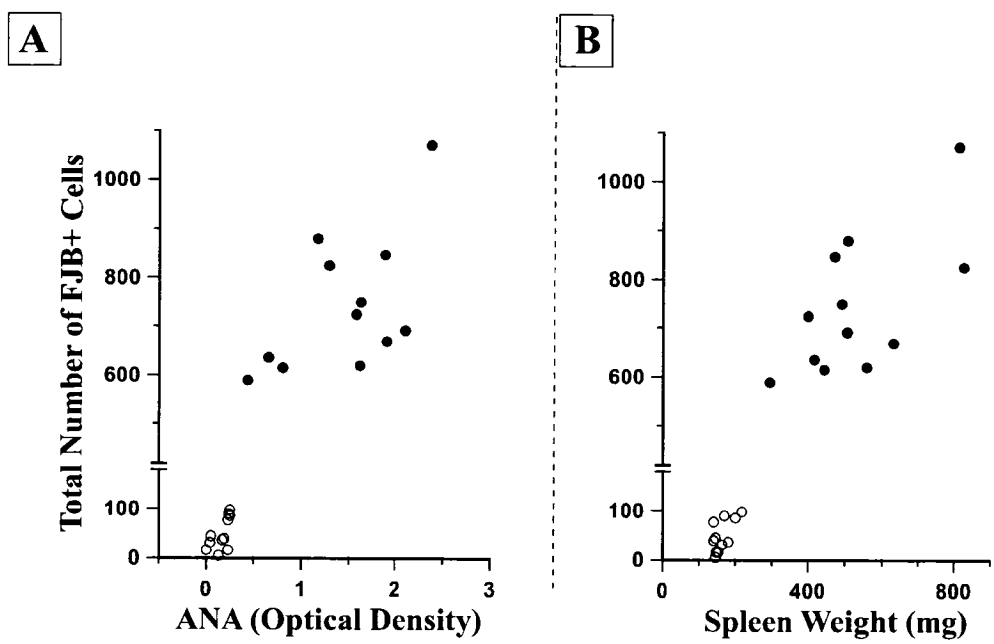

Fig. 4. The relationship between FJB staining and indices of autoimmunity. (A) MRL-lpr mice with high levels of systemic ANA (B) or enlarged spleens appear to show an increased number of FJB+ cells in the brain.

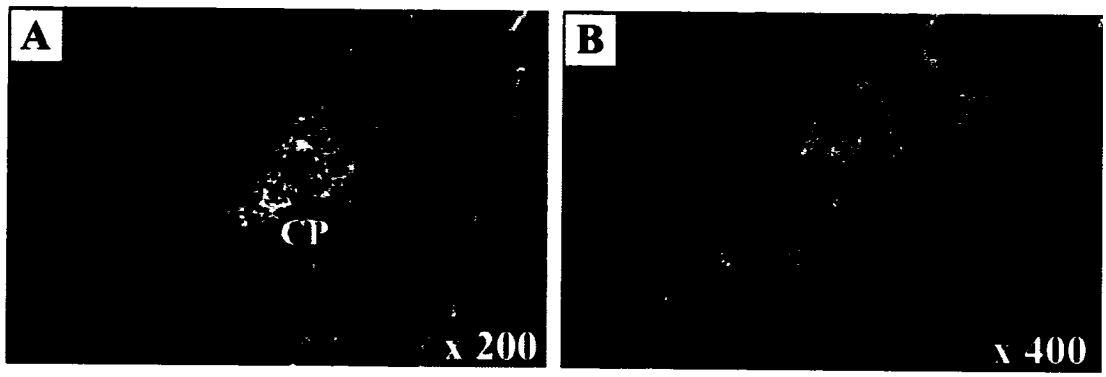

Fig. 5. Non-specific FJB staining and presence of lymphocytes. (A) In several MRL-lpr brains the choroid plexus (CP) was abundant with rounded FJB+ cells (presumably red blood cells or leukocytes). (B) CD4+ and CD8+ cells were often found in the periventricular layer, with the number of CD4 cells being double that of the CD8 cell markers. However, their total number did not correlate with the density of FJB staining in the adjacent brain parenchyma.

may induce neuronal death is not well understood, the recent observation that human anti-DNA antibodies in the CSF cross react with glutamate NMDA receptors in the murine brain and induce neuronal death by apoptosis [21] points to at least one of the important modes of action. This effect is consistent with the evidence that glutamate (one of the principal excitatory transmitters in the CNS) can become neurotoxic through sustained activation of its receptors [48]. From the evidence outlined above, one may speculate that excessive FJB staining in MRL-lpr brains is a consequence of sustained activation of excitatory receptors by IgG in the CSF [47]. In addition to immune mechanisms, glutamate excitotoxicity can be evoked through glucocorticoid-mediated increases in neurotransmission [75] and compromised neuronal survival [2]. Since MRL-lpr mice develop high basal levels of serum corticosterone [39,45], neuronal death could be facilitated through endocrine mechanisms and the accumulation of glutamate [50]. In addition to apoptosis, a considerable body of evidence suggests that an enhancement of glutamate activity can induce necrosis of neurons [13]. Whichever mechanism is dominant, diverse behavioral manifestations

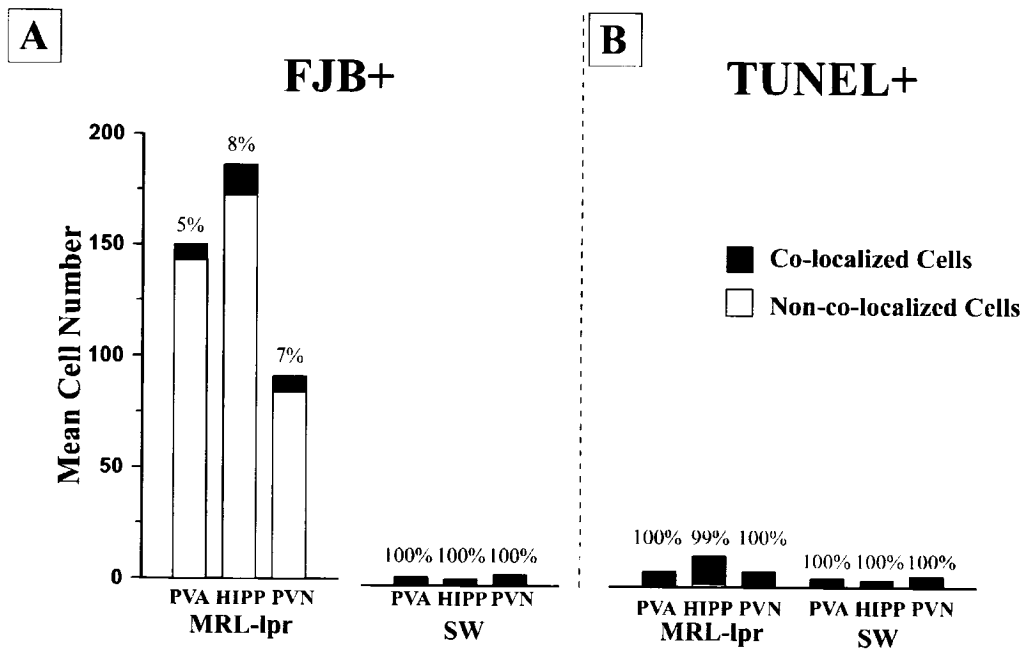

Fig. 6. The relationship between FJB and TUNEL staining. (A) TUNEL+ cells co-localized with FJB+ cells, but accounted for less than 7% of the total FJB stained cells in lupus-prone mice. (B) When present, TUNEL+ cells co-localized with FJB+ cells in both diseased and healthy brains. Abbreviations: periventricular area (PVA), hippocampus (HIPP), periventricular nucleus (PVN).

in human and murine forms of lupus suggest that multiple CNS antigens and sites are targeted during systemic autoimmune disease.

Although the TUNEL assay is a reliable technique for the detection of DNA nicks that can lead to fragmentation, unless combined with other methods it is not regarded as conclusive proof of an apoptotic process [8,15,30]. Additionally, the 'DNA nicking' step occurs relatively late in the apoptosis process, and has also been shown in cells undergoing necrosis [31,81] as well as in 'hybrid' neurons having overlapping characteristics of both necrosis and apoptosis [60]. Since a small percent of all FJB+ neurons were double-labelled by TUNEL, it is presently not clear whether a large proportion of remaining FJB+/TUNEL-negative cells are in an early phase of apoptosis, or are in fact necrotic. Given that MRL-lpr mice are deficient in the expression of Fas receptor in the brain [57], one may at least conclude that neuronal death does not involve the FasL-Fas receptor pathway.

In addition to typical apoptosis, autophagic degeneration is considered as a subtype of eukaryotic death in which cells digest/degrade themselves from within via increased activity of autolysosomes and autophagosomes [14,43]. It is characterized by the lack of caspase activation and limited DNA nicking [44,79,86,87], and has been reported in several neurodegenerative diseases [3,41,46,53], the latter is consistent with the present observation of minimal TUNEL+ nuclei in FJB+ neurons of lupus-prone mice. Autophagocytosis of neurons can also be demonstrated by cytoplasmic ubiquitin inclusions [55,59], which were recently observed in the hippocampus and brain parenchyma of diseased MRL-lpr mice (manuscript in preparation). Therefore, it is possible that neurodegeneration in autoimmune brains involves the autophagic type of cell death.

Further studies assessing the ultrastructure of dying neurons (i.e., chromatin condensation in apoptotic neurons or vacuoles in autophagic neurons) will help to confirm the mode of cell death. In addition, age-related changes in FJB staining, their association to behavioral deficits and the damage of specific neurotransmitter systems may provide instrumental evidence in understanding the nature and mechanisms of brain atrophy and diverse behavioral deficits in humans and animals affected by chronic autoimmune disease.

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CHAPTER 3
HIPPOCAMPAL DAMAGE IN MOUSE AND HUMAN FORMS OF SYSTEM AUTOIMMUNE DISEASE

Published in Hippocampus in 2004

David Ballok's contribution:

I am the first author on this paper. I was involved with the planning and design of the experiment. I performed the animal behavioural testing, administered the immunosuppressive treatment, and conducted the animal surgeries which included perfusions, blood collection and organ extractions. I was responsible for performing the subsequent ELISA (ANA), H&E staining, cytochemical (FJB) and immunochemical (anti-Ub) staining of the tissues. Experimenters blind to sample origin quantified cell numbers both manually and with computer imaging software. A co-author obtained human tissue and worked with me in assessing brain pathology in deceased patients. I completed the required statistical analyses for this study with my supervisor. The figures and tables are my own work, and although feedback was provided by other authors, it was my responsibility to write the article.

Dear Sheik Safdar,

I am completing a Ph.D. thesis at McMaster University entitled "Neurodegeneration In An Animal Model Of Neuropsychiatric Lupus". I would like your permission to reprint the following journal article in my thesis:

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Sincerely,
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Ph.D. Candidate, McMaster University, Faculty of Health Sciences

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Hippocampal Damage in Mouse and Human Forms of Systemic Autoimmune Disease

David A. Ballok,[1] John Woulfe,[5] Monalisa Sur,[4] Michael Cyr,[3] and Boris Sakic[1,2]*

ABSTRACT: Systemic lupus erythematosus (SLE) is frequently accompanied by neuropsychiatric (NP) and cognitive deficits of unknown etiology. By using autoimmune MRL-lpr mice as an animal model of NP-SLE, we examine the relationship between autoimmunity, hippocampal damage, and behavioral dysfunction. Fluoro Jade B (FJB) staining and anti-ubiquitin (anti-Ub) immunocytochemistry were used to assess neuronal damage in young (asymptomatic) and aged (diseased) mice, while spontaneous alternation behavior (SAB) was used to estimate the severity of hippocampal dysfunction. The causal relationship between autoimmunity and neuropathology was tested by prolonged administration of the immunosuppressive drug cyclophosphamide (CY). In comparison to congenic MRL +/+ controls, SAB acquisition rates and performance in the "reversal" trial were impaired in diseased MRL-lpr mice, suggesting limited use of the spatial learning strategy. FJB-positive neurons and anti-Ub particles were frequent in the CA3 region. Conversely, CY treatment attenuated the SAB deficit and overall FJB staining. Similarly to mouse brain, the hippocampus from a patient who died from NP-SLE showed reduced neuronal density in the CA3 region and dentate gyrus, as well as increased FJB positivity in these regions. Gliosis and neuronal loss were observed in the gray matter, and T lymphocytes and stromal calcifications were common in the choroid plexus. Taken together, these results suggest that systemic autoimmunity induces significant hippocampal damage, which may underlie affective and cognitive deficits in NP-SLE.

KEY WORDS: autoimmunity; inflammation; lupus; hippocampus; Fluoro Jade B; ubiquitin; spontaneous alternation behavior; cyclophosphamide; MRL mice

INTRODUCTION

Cognitive and affective disorders are frequent manifestations of the systemic autoimmune/inflammatory disease lupus erythematosus (SLE). Largely due to multi-system involvement and confounding factors (e.g., uremia and treatment with corticosteroids), clinical and experimental studies have thus far not succeeded in elucidating the principal factors and mechanisms involved in the pathogenesis of neuropsychiatric (NP)-SLE (Bruyn, 1995). With an ultimate goal of understanding the central nervous system (CNS) targets and functional consequences of SLE-like disease, we have been examining the neuropathology and behavioral dysfunction in the MRL-MpJ-Tnfrsf6^{lpr} (MRL-lpr) mouse substrain. This substrain is known to develop systemic autoimmune disease due to a *lpr* mutation on chromosome 19 and a dysfunctional Fas (CD95, APO-1) receptor in a negative selection of autoreactive T cells (Watanabe-Fukunaga et al., 1992a,b). Along with early onset of inflammation and autoimmunity, MRL-lpr mice display changes in emotional reactivity (Sakic et al., 1994b), deficits in spatial learning/memory tasks (Sakic et al., 1992, 1993; Hess et al., 1993), accumulation of serotonin in the hippocampus (Sakic et al., 2002), and atrophy of pyramidal neurons (Sakic et al., 1998). Consistent with a case report on isolated hippocampal damage (Schnider et al., 1995) and impaired cognitive function in SLE patients (Carbotte et al., 1995; Denburg et al., 1997; Hanly et al., 1999), these results suggested structural and functional damage of the hippocampus during systemic autoimmunity and inflammation.

Behavioral deficits in MRL-lpr mice are defined as the departure from the behavioral performance of congenic MRL-MpJ +/+ (MRL +/+) controls (Szechtman et al., 1997), which develop similar disease symptoms later in life (Theofilopoulos, 1992). Marked behavioral deficits in the MRL-lpr substrain have been observed in mice as early as 7 weeks of age (Sakic et al., 1994a) and coincide with the emergence of humoral autoimmunity, but antedate generalized lymphadenopathy, arthritis, glomerulonephritis, and skin lesions (Andrews et al., 1978). The initial support for the hypothesis that spatial learning and memory are impaired by lupus-like disease came from studies in which MRL-lpr mice perseverated in their response bias during extinction and reversal learning in the Morris water-maze task (Sakic et al., 1992, 1993). The aim of the present study is to evaluate the time course of spontaneous alternation behavior (SAB), a functional trait proposed to be highly sensitive to hippocampal damage (reviewed in Richman et al., 1986). More specifically, it is well documented that spatial working memory and the reliable alternation of rodents in a T-maze in two consecutive trials largely depend on an intact hippocampus (Lalonde, 2002). If development of autoimmunity induces hippocampal damage, one might expect that in comparison to congenic MRL +/+ controls, diseased

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MRL-lpr mice would show a reduced SAB rate at advanced stages of lupus-like disease, but not before the serological signs of autoimmunity are manifest.

Although neuronal degeneration in the CA2/CA3 regions of MRL-lpr brains was recently demonstrated with a novel Fluoro Jade B (FJB) cytochemical stain (Ballok et al., 2003), it has not been determined whether this damage is functionally important. In addition, it remains to be determined whether neuronal loss is associated with the progress of autoimmune disease or, alternatively, reflects a developmental deficiency related to impaired expression of the apoptotic Fas receptor in the MRL-lpr brain (Park et al., 1998). Although the latter possibility appears less viable in the light of evidence that cortical architectures in young, pre-diseased MRL-lpr and MRL +/+ mice are comparable (Sherman et al., 1990), and that size of hippocampal fields and neuronal density are not reduced in Fas-deficient *lpr* mice (Kovac et al., 2002), we assess age-related changes in densities of hippocampal neurons stained by the hematoxylin-eosin (H&E) method. The present analysis involves the comparison between young (asymptomatic) and old (diseased) MRL-lpr and MRL +/+ mice and employs ubiquitination as an additional marker of cell degeneration (Alves-Rodrigues et al., 1998). Specifically, ubiquitin (Ub) binds to damaged or misfolded proteins, targeting them for degradation by the Ub-proteasome pathway. If ubiquitinated proteins are not eliminated by this pathway, neurodegeneration may occur. In addition to cellular demise, ubiquitination of proteins may be involved in a DNA repair mechanism (Jentsch et al., 1987). Therefore, it was expected that an increased density of FJB-positive cells and alterations in the Ub-proteasome degradation system in the hippocampus would parallel both the emergence of autoimmunity and impaired SAB performance. To test for a cause-effect relationship between neuronal degeneration and autoimmunity, the immunosuppressive drug cyclophosphamide (CY) was used as previously reported (Farrell et al., 1997; Sakic et al., 1995, 2000a).

Despite the well-acknowledged construct validity and theoretical usefulness of animal models, it is equally important to demonstrate their face validity (Henn and McKinney, 1987). Our experimental study coincided with the death of an SLE patient who had CNS involvement. This provided us with a unique opportunity to compare neuropathological changes in animal and human forms of systemic autoimmune disease in which brain function is compromised.

MATERIALS AND METHODS

Experiment I: Age-Related Changes in SAB Response Rate and Neuromorphology

Animals

Three-week-old (±3 days) MRL-lpr and MRL +/+ male mice (n = 20 mice/substrain) were purchased from the Jackson Laboratory (Bar Harbor, ME); each group was left for 7 days to habituate to local laboratory conditions (light phase: 8 AM–8 PM, food, and water ad libitum; 5 mice/cage; level E, cages sanitized during regular housing). Five days prior to behavioral testing, mice were singly caged and habituated to the experimenter (Sakic et al., 1992). In addition, mouse cages were wheeled on three occasions from the colony room to the testing room, to reduce stress induced by transportation and a novel environment. Ten mice from each strain were tested and sacrificed at either 6 or 16 weeks of age (cohort 1). Because of dissimilar fixation protocols for the assessment of dying neurons and ubiquinated particles, a second batch of mice (cohort 2; 20 males/substrain) was obtained from our recently established MRL colony (housing level A, cages sterilized, replaced, and manipulated under laminar flow protection). At 3 weeks of age (±3 days), the mice were transferred to a housing room and left to habituate over 7 days, for assessment of brain morphology at 4 and 14 weeks of age. All experimental protocols were performed in accordance with the rules and regulations of the Canadian Council of Animal Care.

Spontaneous alternation behavior

There is substantial evidence that the hippocampus is one of the structures most intimately involved in SAB (Lalonde, 2002). Indirect but compelling evidence that supports this notion comes from developmental studies in which hippocampal maturation and level of SAB show parallel courses (Deacon et al., 2002). Considering that hippocampal damage reduces the rate of SAB in mice (Deacon et al., 2002), it was expected that young MRL-lpr mice would show a comparable SAB rate to congenic controls before the onset of autoimmunity and, conversely, that their SAB rate would decline with advancing manifestations of systemic autoimmune disease.

The T-maze (made of black Plexiglas) consisted of four perpendicular arms (H = 15 × L = 25 × W = 10 cm) and sliding separators that could modify the maze into an L, T, or + shape. The discrete-trial procedure (Richman et al., 1986) was employed, with a daily session consisting of trial 1 (5 s in a start position and entry into an unblocked arm), 60-s intertrial period (mouse restrained in the arm) and trial 2, in which both arms were open and a mouse was expected to choose an unvisited arm after leaving the start position. An arm was considered to have been chosen if all four limbs were within the selected arm. A guillotine door was lowered behind the mouse, immediately after the entry. Urinary trails were removed, and the maze was cleaned by a cloth moistened with Windex® after each mouse was tested. The sequence of blocked arms (left or right) in trial 1 was randomly generated by Microsoft Excel software. Following 5 days of SAB testing, mice were given a reversal trial on day 6, to examine whether the nonspatial learning strategy was employed (Bertholet and Crusio, 1991), such as taxis or praxis (Sutherland and McDonald, 1990; Whishaw, 1991). Trial 1 consisted of placing a mouse in the usual starting position. However, following a 60-s intertrial period, trial 2 was initiated from the arm located 180 degrees from the trial 1 arm. Assuming that a mouse had used a spatial strategy to memorize external cues, it was expected that after leaving the new start position, an unvisited arm would be chosen by a body turn identical to trial 1.

Indices of autoimmunity

High levels of serum antinuclear antibodies (ANA) and splenomegaly are typical manifestations of systemic autoimmune lupus-like disease (Theofilopoulos, 1992) and were presently examined. Mice were anesthetized with Somnotol (i.p. 60 mg/kg body weight; MTC Pharmaceuticals, Cambridge, ON) and perfused with 40 ml of phosphate-buffered saline (PBS) after terminal bleeding from the vena cava. Blood samples were left to coagulate in 1.5-ml plastic vials and centrifuged for 10 min at 3,000 rpm. Serum was separated from the clot and stored at −20°C until further analysis. ANA concentration was measured using a sandwich Anti-Nuclear Antibody ELISA kit (Cat. no. 5200), according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX) and the protocol previously described (Sakic et al., 2000a). In brief, serum samples were diluted 1:100 in the kit diluent and applied to both experimental and control wells to assess the specificity of binding. Optical density was determined using a microplate ELISA reader set to 450 nm. The wet spleen weight was determined on an analytical scale (Sartorius 2024 MP, VWR Scientific of Canada Ltd.) shortly after extraction.

Fluoro Jade B method

The Fluoro-Jade B stain has an affinity for the entire degenerating neuron, regardless of the type of cell death (Hopkins et al., 2000; Schmued and Hopkins, 2000). Despite incomplete knowledge of the staining mechanisms, the FJB method shows high reliability in the detection of dying neurons (Ye et al., 2001).

Extracted brains were fixed in 4% paraformaldehyde (PFA) for 24 h and were then immersed in 30% sucrose (in PBS) for 4 days before frozen sections were processed according to the previously published protocol (Ballok et al., 2003). In brief, slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. The slides were rinsed in distilled water (dH_2O) for 1 min before being transferred to a 0.06% potassium permanganate solution and gently shaken for 15 min. Slides were rinsed in dH_2O for 1 min before immersion in a 0.001% FJB/0.1% acetic acid staining solution (prepared from a 0.01% stock solution; Histo-Chem, Jefferson, AR). In our experience, the stock solution produced optimal staining results after 2 months of storage at 4°C in darkness. After 30 min of gentle shaking in the staining solution, slides were rinsed for 1 min in each of three dH_2O washes and left to dry for several hours in darkness. Subsequently, they were processed in three 2-min xylene washes before being coverslipped with DPX (Sigma, St. Louis, MO). The reactivity in the CA3 sector and nonhippocampal periventricular areas were examined using an epifluorescent microscope with 450–490-nm excitation light (Diastar Fluorescence Microscope, Reichert Scientific, Buffalo, NY) at ×200 magnification. To quantify the number of FJB-positive neurons, sections were photographed on 400 ASA 35-mm print film (Eastman Kodak, Rochester, NY), using a Nikon N90s camera. The pictures were scanned at 600-dpi resolution and digitized into TIFF files using Adobe Photoshop software (Adobe Systems, San Jose, CA) for assessment by NIH Image analysis software (Scion, Frederick, MD). To produce representative high-quality images, a Zeiss Laser Scanning Confocal Microscope (LSM 510, Carl Zeiss) argon laser (wavelength 488 nm) was employed for visualization of FJB. Confocal micrographs were obtained using a Fluar 20×/0.75 objective in combination with a 1,024 × 1,024-pixel resolution and were saved in the TIFF format.

The overall neuronal density was assessed by H&E staining of coronal sections (bregma approximately −1.0 mm) in the parietal cortex and CA2/CA3 area that were less densely packed with neurons and thus amenable to quantification. Sections were stained with an automated slide stainer (Leica Instruments, Germany), and 1-mm^2 areas were examined with a stage micrometer by an unbiased observer. Sections of the highest quality were included in the analysis (n = 4–8 brains/group) and four counts (2 sections × 2 hemispheres) were performed at ×400 magnification.

Immunohistochemical localization of Ub

Mice were perfused with PBS, and the brains were extracted within 2 min, immersed into 10% neutral buffered formalin, and left in formalin for 4 days at room temperature (RT) until processing. Subsequent to fixation, brains were embedded in paraffin and cut in the coronal plane at 4 μm. Serial adjacent sections were stained with H&E and processed immunohistochemically for Ub, using an anti-Ub polyclonal antiserum (1:400; Dako, Burlington, Canada) and the labeled streptavidin-biotin-peroxidase technique (Vectastain; Shandon, Pittsburgh, PA). Sections were briefly counterstained with hematoxylin, dehydrated in a graded series of ethanol, mounted in xylene, and coverslipped. For each animal, the number of Ub-immunopositive dot-like structures was counted in five nonoverlapping fields within the strata oriens and pyramidale of the CA3 sector of the hippocampus. All counts were performed at the same coronal level (bregma −1.6 to −2.4 mm) under oil immersion (×1,000) by an experimenter blind to the experimental design.

Experiment II: Effects of Immunosuppression on SAB and Neuromorphology

Animals

To examine whether prolonged immunosuppressive treatment modifies SAB performance, 24 MRL-lpr and 20 MRL +/+ male mice (3 weeks old ±3 days) were purchased from the Jackson Laboratory. An additional batch of 12 MRL-lpr and 12 MRL +/+ mice was purchased to confirm behavioral observations and examine the effects of sustained immunosuppression on neuropathology seen in Experiment I. Each group was managed and maintained under conditions as outlined above. Two weeks later (i.e., 5 weeks of age), mice were housed singly to receive treatment.

Immunosuppressive treatment

The therapeutic effect of CY on the development of autoimmune symptoms was demonstrated previously (Shiraki et al., 1984; Grota et al., 1989, 1990; Sakic et al., 1995, 1996). In addition to the reduction of leukocyte numbers (Snippe et al., 1976), CY makes these cells unresponsive to stimuli, leading to generalized immunosuppression (ten Berge et al., 1982). CY was injected

weekly (100 mg/kg i.p.; mouse LD_{50} = 405 mg/kg i.p.; Procytox, Horner, Montreal, Canada) to half of the mice in each group. The treatment started during the 5th week of life and ended during the14th week. The other half of the animals received nine injections of an equivalent volume (~0.2–0.3 ml) of saline (SAL). Mice were assigned into one of four groups, according to substrain (MRL-lpr vs MRL +/+) and treatment (CY vs SAL). In both batches, two mice died prematurely in the CY group before treatment was completed. To avoid acute effects of CY and stress induced by injection, the SAB testing commenced 7 days after the last injection and mice were sacrificed at around 16 weeks of age. FJB and Ub staining was analyzed as described above, and all counts were performed by an observer blind to the experimental design. ANA levels, which correlate highly with the spleen weight (Sakic et al., 2000a), were assessed as in Experiment 1.

Experiment III: Human Specimens

Brain tissue was obtained from a 58-year-old woman who died from NP-SLE and had a history of psychosis and seizures. She presented with status epilepticus that was managed with phenytoin and diazepam. She remained comatose after her seizures. Despite aggressive immunosuppression, including high-dose methylprednisolone, plasmapheresis, and CY, her condition deteriorated; she developed disseminated intravascular coagulation and died. The brain was examined after 14-day fixation in 10% buffered formalin. Sections were taken from the meninges, blood vessels, pons, medulla oblongata, cerebellum, mamillary bodies, right and left hippocampus, basal ganglia, frontal watershed area, temporal, parietal and occipital lobes, periventricular area around lateral and third ventricles, and choroid plexus. Coronal sections of the brain were serially sectioned and representative paraffin wax embedded tissue sections were stained with Luxol fast blue/H&E. Other stains used were Bielschowsky silver and Congo red. Using standard immunohistochemical methods, sections of the periventricular area, hippocampus, and temporal gray matter were stained for Ub; sections of the choroid plexus were stained for leukocyte common antigen (LCA), T-cell markers (CD3, CD4, CD8), B-cell marker (CD20), and macrophage marker (CD68). All sections were examined under light microscopy. Additional sections were frozen, stained with FJB, and examined with confocal microscopy. Control sections were obtained from a female subject of similar age who died of causes unrelated to SLE and epilepsy.

Statistical Analysis

The data were analyzed by analysis of variance (ANOVA) with substrain (MRL-lpr vs MRL +/+), treatment (CY vs SAL), and age as between-group factors, and slide as the repeated measure. Student's *t*-test was used in the post hoc analysis. Fisher's exact test was used to assess the difference in group performance in the SAB tests. Significance level was set at $P < 0.05$, and all computations were performed using the SPSS 11.0 statistical package. Graphs show means ± SEM.

TABLE 1.

SAB Rates (%) of Young (6-wk) and Older (16-wk) MRL-lpr and Control MRL +/+ Mice Over 5-Day Response Acquisition and Single Reversal Trial†

Group	Acquisition at 6 wk	Acquisition at 16 wk	Reversal at 16 wk
MRL-lpr (n = 10)	76 ± 5	64 ± 6*	50** (5/10)
MRL +/+ (n = 10)	76 ± 5	80 ± 4	100 (10/10)

SAB, spontaneous alternation behavior.
†The alternation rate was lower in diseased 16-week-old MRL-lpr mice; a deficit in the spatial learning strategy was detected in the "reversal" task. This task differed from testing in the acquisition phase as Trial 2 was initiated 180 degrees away from the box arm where Trial 1 was given.
*In comparison to MRL+/+, $t_{18} = 2.228$, $P = 0.039$.
**In comparison with Fisher's exact test $P = 0.016$.

RESULTS

Experiment I

The SAB performance in young mice from the two MRL substrains was comparable at 6 weeks of age. However, 16-week-old MRL-lpr mice performed poorly in comparison to age-matched MRL+/+ controls ($t_{18} = 2.228$, $P = 0.039$, Table 1). This deficit was confirmed in the "reversal" trial, where the alternation rate of diseased MRL-lpr mice dropped to chance levels (Fisher's exact test, one-tailed $P = 0.016$, Table 1). Neuropathological assessment revealed higher numbers of FJB-positive neurons (Fig. 1A) in the CA3 region (strain by age: $F(1,36) = 39.944$, $P < 0.001$, MRL-lpr vs age-matched MRL +/+, $t_{18} = 6.583$, $P < 0.001$, Fig. 2A). In a separate cohort of 14-week-old MRL-lpr mice, immunostaining for Ub showed intensely immunoreactive spherical dot-like structures (Fig. 1C), which were numerous in the neuropil of the stratum oriens and superficial stratum pyramidale of the CA3 region (strain $F(1,18) = 8.093$, $P < 0.011$, $t_{18} = 3.000$, $P < 0.008$; Fig. 2A). In addition to the hippocampus, the substantia nigra and brainstem tegmentum also showed more Ub particles than in MRL +/+ controls (strain $F(1,18) = 8.998$, $P < 0.008$, $t_{18} = 2.845$, $P < 0.011$; data not shown). Examination at higher magnification showed that the morphology of the structures was reminiscent of axon terminals. Indeed, occasional linear arrays of these structures indicated the presence of immunoreactive varicose axonal segments.

Reduced density of cortical neurons in diseased MRL-lpr mice has been initially revealed with the cresyl violet method, but this observation was not quantified (Sakic et al., 1998). We presently confirmed this by assessing neuronal densities in the parietal cortex and CA2/CA3 region on sections stained with the standard H&E method. Comparisons across ages and to age-matched controls showed a paucity of cells in older MRL-lpr mice (Table 2). Despite a relatively small sample in some groups (n = 4 brains), the counts obtained suggest that significant group differences at an older age is a combination of an age-related increase in neuronal density in the

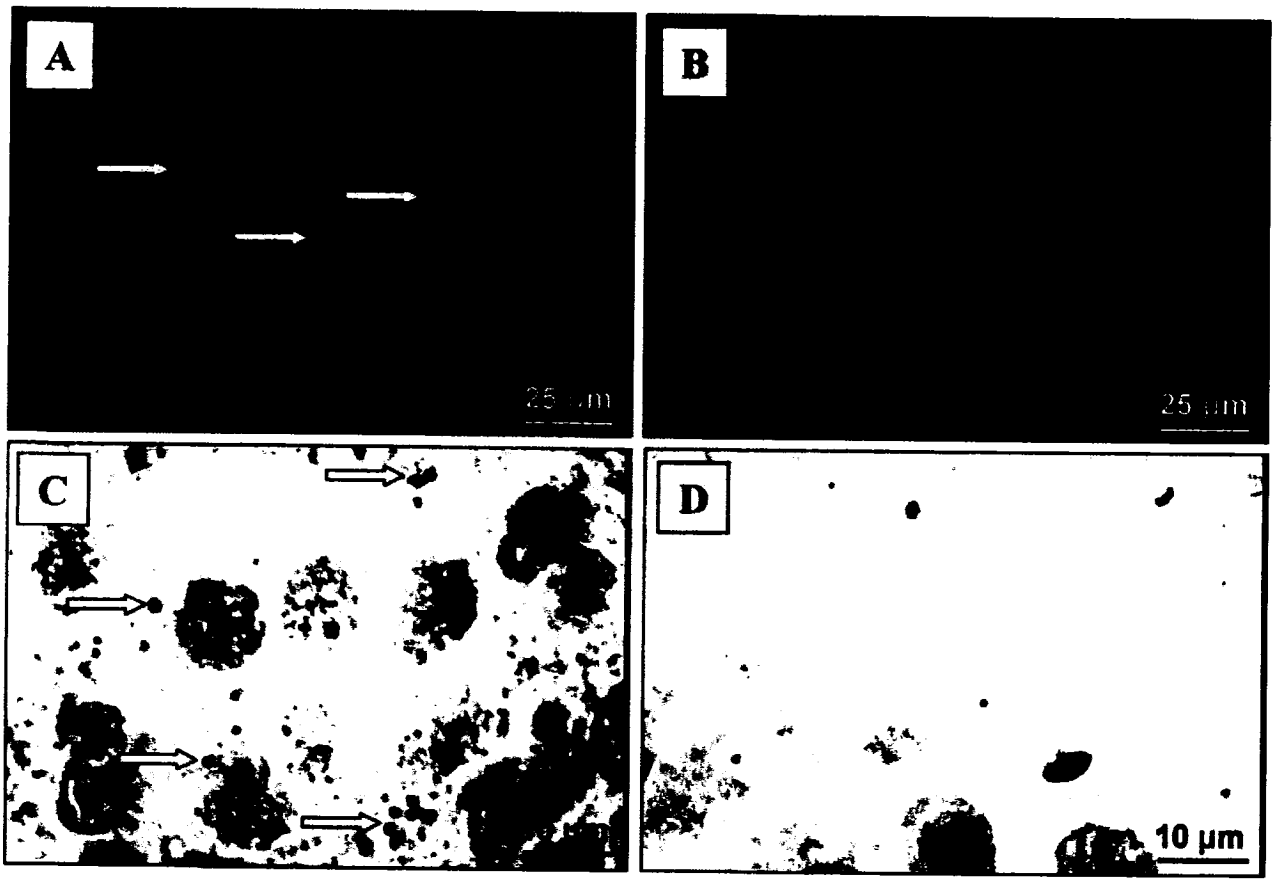

FIGURE 1. Representative micrographs showing Fluoro Jade B (FJB) and ubiquitin (Ub) labeling in the CA3 region. Numerous FJB-positive cells with neuronal morphology (shown by arrows) were common in brains from MRL-lpr mice (A) in comparison to asymptomatic MRL +/+ controls (B). Intensely immunoreactive Ub-positive spherical particles (shown by arrows) were detected in brains from diseased lupus-prone mice (C), likely reflecting degenerating axon terminals. Such particles were not abundant in the control brains (D). ×400 in A,B; ×1,000 in C,D. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

MRL +/+ substrain and genuine loss of neurons during the development of systemic autoimmunity in the MRL-lpr substrain. This is supported by comparable immune statuses at younger ages, and the emergence of the SAB deficit when spleen weight and serum ANA titers increased in aged MRL-lpr mice (at 16 weeks of age, strain by age: for spleen, $F(1,36) = 22.448$, $P < 0.001$; for ANA, $F(1,35) = 28.110$, $P < 0.001$; at 14 weeks of age, strain by age: for ANA, $F(1,34) = 15.006$, $P < 0.001$; for spleen, $F(1,36) = 36.568$, $P < 0.001$; Fig. 2B,C).

Experiment II

The SAB deficit was less severe in CY-treated MRL-lpr mice compared to the SAL-treated MRL-lpr group ($t_{22} = 2.691$, $P = 0.013$; Table 2). CY-treated MRL-lpr mice also did not differ in the "reversal" test when compared to other groups (Table 3). Although the acquisition of SAB response in the CY-MRL +/+ group was comparable to that in the SAL-MRL +/+ group, their performance in the "reversal" task dropped to a chance level. This detrimental effect of CY on performance of control mice is not presently clear, but it is consistent with our previous observations (Sakic et al., 1995, 1996). Our recent study demonstrated that sustained CY treatment normalizes neuronal morphology, as evidenced by Golgi impregnation (Sakic et al., 2000a). In the present study, extensive FJB staining appeared to be attenuated after immunosuppressive treatment. However, likely due to the small sample size (n = 5–6 mice/group), a trend was seen in the CA3 region (strain by treatment: $F(1,17) = 3.956$, $P = 0.063$; Fig. 3A) and a statistically significant difference was detected in other periventricular gray matter regions (strain by treatment: $F(3,17) = 5.549$, $P = 0.008$; between SAL and CY MRL-lpr groups, $t_9 = 2.161$, $P = 0.05$; Fig. 3B). This reduction in cytochemical staining was associated with the profound immunosuppressive effect of CY, as indicated by low ANA levels in the CY groups across two batches (batch: $F(1,56) = 0.022$, n.s.; strain by treatment: $F(1, 56) = 47.376$, $P < 0.001$; CY-lpr mice: 0.28 ± 0.06; SAL-lpr mice:1.58 ± 0.14; CY +/+ mice:0.13 ± 0.02; SAL +/+ mice: 0.24 ± 0.05). However, analysis of the CA3 region with the present sample size could not detect a beneficial effect of CY on the density of Ub-positive particles. Namely, sustained immunosuppression did not appear to change the difference between the MRL substrains (strain: $t_{19} = 3.006$, $P = 0.007$; treatment: $t_{19} = 0.157$, n.s.).

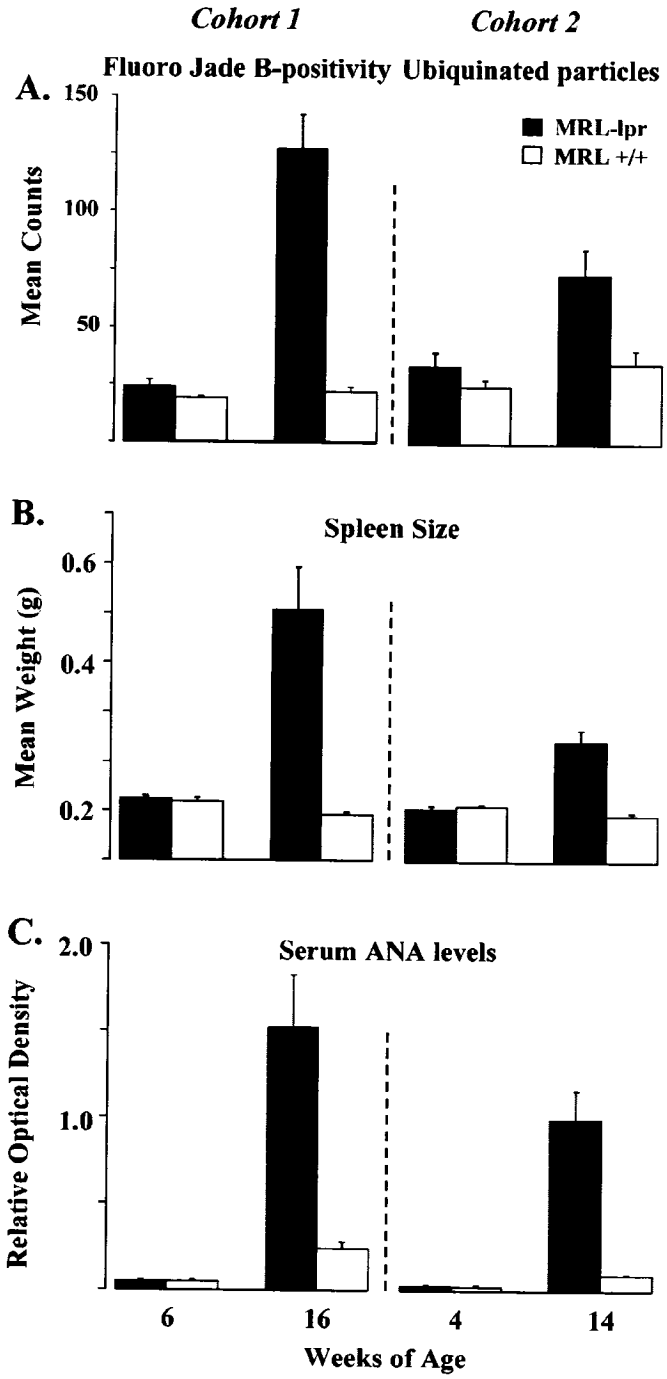

FIGURE 2. Total numbers of Fluoro Jade B (FJB)-positive and ubiquitin (Ub)-positive cells and the immune status in MRL mice at different ages. Cells/particles staining for FJB or Ub significantly increased following the onset of autoimmunity in older MRL-lpr mice (A). Spleen weight (B) and antinuclear antibody (ANA) levels (C) also increased significantly with age, confirming the autoimmune status in the MRL-lpr substrain.

Experiment III

The brain of the lupus patient weighed 1,145 g. There was no evidence of a subdural hematoma, and both hemispheres were symmetrical with no evidence of meningeal exudates or herniations. Blood vessels, dissected out from the circle of Willis, did not show any thromboembolic or atherosclerotic changes, but they did show myointimal proliferation with some hyalinization of smaller blood vessels (features suggestive of a history of benign hypertension). On serial sectioning, there was no evidence of infarction or of intracerebral or intraventricular bleeding. There was dilation of the lateral and third ventricles, left more than right, with thinning of the cortex around the ventricles. Some of the intraparenchymal blood vessels showed perivascular cuffing by chronic inflammatory cells and deposition of hemosiderin, features suggesting previously healed vasculitis. This is further corroborated by the fact that there were foci of demyelination around these vessels, indicating a vasculitic mechanism probably related to immune complex deposi-

TABLE 2.

Neuronal Density (Total Count ± SEM) as Assessed by Hematoxylin and Eosin Staining†

Group	Parietal cortex	CA2/CA3 area
6-wk MRL-lpr	514 ± 32 (n = 4)	210 ± 34 (n = 8)
6-wk MRL +/+	573 ± 32 (n = 7)	237 ± 33 (n = 7)
16-wk MRL-lpr	451 ± 43 (n = 8)*	159 ± 28 (n = 8)**
16-wk MRL +/+	691 ± 83 (n = 4)	250 ± 29 (n = 7)

†The total number of H&E-stained neurons was obtained from four 1-mm^2 areas (2 sections × 2 hemispheres) that were well preserved and amenable to counting (number of brains processed is shown in parentheses). Consistent with previous reports on reduced growth of brain mass and atrophy of pyramidal neurons, reduced neuronal density in diseased 16-week-old MRL-lpr mice supported the hypothesis that progress of systemic autoimmune disease impairs parenchymal growth in the parietal cortex and hippocampus, likely by inducing neuronal loss.

In comparison with age-matched MRL +/+ control, $^*t_{10} = 2.868$, $P = 0.017$, and $^{**}t_{13} = 2.238$, $P = 0.043$.

TABLE 3.

SAB Rates (%) of 14-Week-Old MRL-lpr and Control MRL +/+ Mice Following 8 Weekly i.p. Injections With Immunosuppressive Drug Cyclophosphamide (100 mg/kg body weight) or Saline (SAL)†

Group	Acquisition	Reversal
MRL-lpr SAL (n = 12)	60 ± 3*	50 (6/12)
MRL +/+ SAL (n = 10)	84 ± 7	90 (9/10)
MRL-lpr CY (n = 12)	75 ± 4	67** (8/12)
MRL +/+ CY (n = 8)	83 ± 8	50 (4/8)

SAB, spontaneous alternation behavior; CY, cyclophosphamide; SAL, saline.

†Sustained immunosuppressive treatment reduced the substrain difference in SAB acquisition rate. However, this treatment appeared to have detrimental effects in the MRL +/+ CY group in the "reversal" form of the test.

*In comparison to MRL-lpr CY, $t_{22} = 2.691$, $P = 0.013$.

**Not statistically different from other groups in multiple comparisons using the Fisher test.

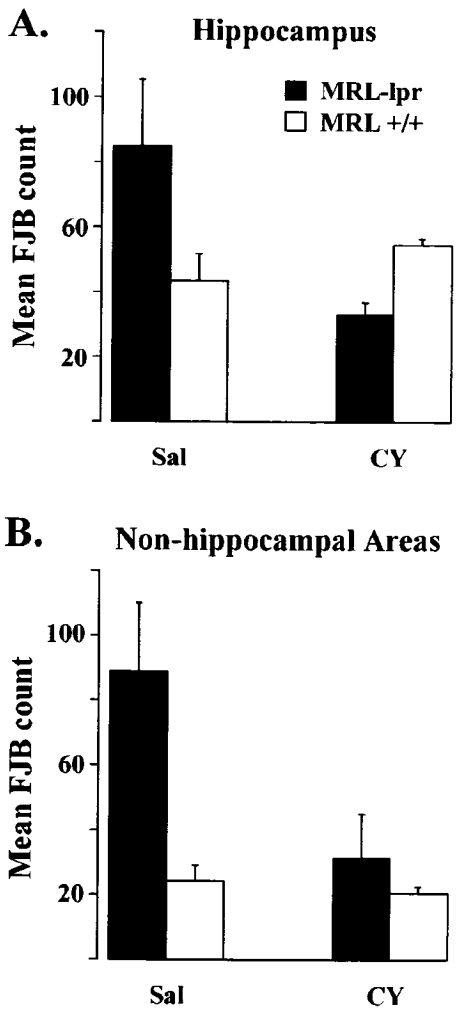

FIGURE 3. Effects of immunosuppressive treatment on brain pathology in MRL mice. Cyclophosphamide (CY) treatment appeared to attenuate the incidence of Fluoro Jade B (FJB)-positive cells in the hippocampus (A) and significantly reduced the staining in other brain regions of MRL-lpr mice (B).

tion. There was no microscopic evidence of abnormal neuronal migration, infarction, intraparenchymal, or intraventricular hemorrhage. Conversely, gross examination of the control brain did not show ventricular dilation of the lateral and third ventricles.

An interesting feature was the presence of increased densities of scattered inflammatory cells within the choroid plexus of the lupus patient (Fig. 4A). Mononuclear lymphocytes displayed positive immunostaining for LCA, CD3, CD4, and CD8, indicating that these were T lymphocytes. More CD8 than CD4 cells were observed, suggesting that most of the T lymphocytes were cytotoxic (Fig. 4C). These were not present in the control brain (Fig. 4D). Furthermore, periventricular tissue around the lateral and third ventricles showed loss of neurons, gliosis, satellitosis (microglial cells surrounding occasional viable neurons), and "brain sands"/ corpora amylacea, not present in the control (Fig. 5A–D). The parietotemporal cortex also showed gliosis and ischemic changes in the neurons (likely secondary to status epilepticus). The neuronal loss in the CA3 region of the hippocampus was accompanied by surviving neurons, gliosis, and focal satellitosis (Fig. 6A). This was also seen above the dentate gyrus in the pyramidal layer, in addition to patchy areas of cell loss (Fig. 6C). In contrast, the hippocampus sections from the control brain did not show neuronal loss, gliosis, or satellitosis in the CA3 region. There were no patchy areas of cell loss in the pyramidal layer above the dentate gyrus. Neuronal demise in the hippocampus of the NP-SLE patient was confirmed by FJB staining. More specifically, FJB-positive neurons (Fig. 6E) and few scattered Ub-stained particles were observed in the CA3 and dentate gyrus. However, with respect to Ub staining, no significant difference between lupus and control brains could be detected.

DISCUSSION

The emergence of FJB-positive neurons and Ub-positive structures in the hippocampus of mice with impaired SAB and the beneficial effects of immunosuppressive treatment point to the causal link between chronic autoimmunity/inflammation, structural damage, and aberrant behavior. These findings complement documented atrophy of basilar and apical dendritic branches in the CA1 region and parietal cortex (Sakic et al., 1998, 2000a) and neurotoxic properties of cerebrospinal fluid (CSF) from diseased MRL-lpr mice (Maric et al., 2001). In comparison to the murine form of the disease (Denenberg et al., 1992; Farrell et al., 1997; Sakic et al., 2000a,b), similar changes are seen in the brain from the NP-SLE patient. They include neuronal loss in the hippocampus and parietal regions, T-lymphocyte infiltration into the choroid plexus, and ventricular enlargement. Overall, the present results further support our hypothesis that systemic autoimmune disease induces brain damage and subsequently, behavioral dysfunction. In addition, the above evidence strengthens the face and construct validity of MRL-lpr mice as a model of NP-SLE.

The SAB paradigm is a procedure proposed to reflect exploratory behavior, which also depends on the formation of working memory (Richman et al., 1986). However, anxiety and stress may negatively affect SAB (Bats et al., 2001) to the extent that it does not reflect exploratory behavior (Gerlai, 2001). In our study, the animals were initially habituated to the experimenter and the testing environment to reduce these confounding effects. In addition to consistently poorer performance over successive trials, a profound deficiency in the "reversal" trial was observed in aged MRL-lpr mice. If the spatial strategy was employed, MRL-lpr mice would be expected to alternate to the novel arm by making the same body turn on the second trial. However, the MRL-lpr mice used the opposite body turn to enter the unvisited arm, thus ending up in the same arm as in trial 1. This observation suggests that during the acquisition phase (across-the-day SAB rate in MRL+/+ mice increased from 70% to 90%, in MRL-lpr mice from 40% to 80%) the diseased mice did not rely on an extramaze spatial map to the same extent as MRL +/+ controls. Detrimental effects of prolonged CY treatment on performance of control MRL +/+ mice in the "reversal" trial is consistent with its negative

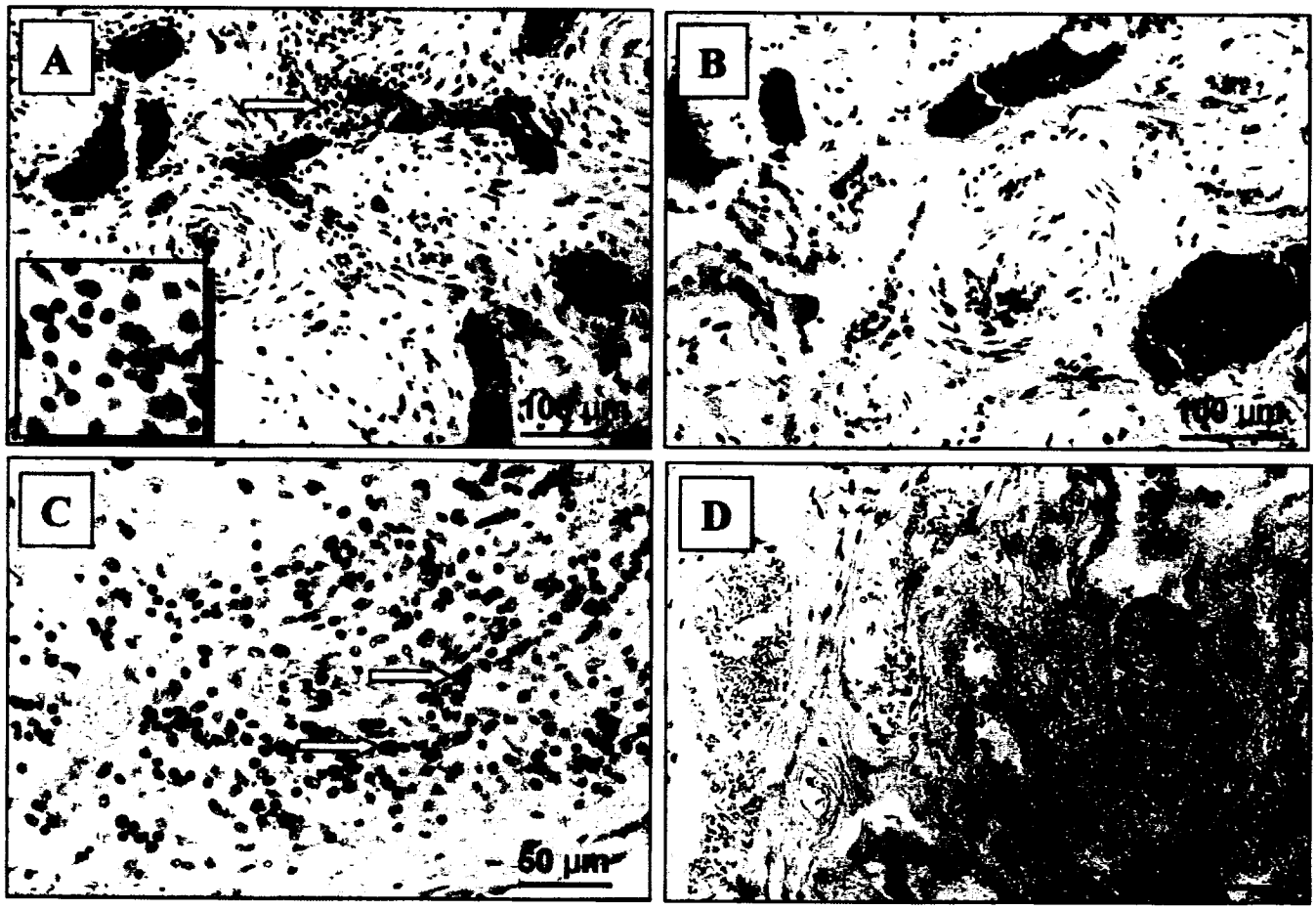

FIGURE 4. Chronic mononuclear inflammatory cells in the human brain. H&E staining showed a cluster of lymphocytes (arrow) in the choroid plexus of the lupus patient (A), but not in the control brain (B). Subsequent immunohistochemical staining for leukocyte common antigen (LCA), CD3, CD4, and CD8 antigens (shown by arrows) confirmed that the cells in the stroma were T lymphocytes (C), while the control brain was negative for the same markers (D). ×100 in A,B; ×200 in C,D; ×600 (inset in A).

effects in paradigms that measure exploration and motivated behavior (Sakic et al., 1995, 1996). Although the principal mechanisms are unclear, one may hypothesize that by cross-linking strands of DNA/RNA and inhibiting protein synthesis, CY treatment impairs molecular mechanisms (e.g., phosphorylation) required for short-term memory formation and consolidation (Ng et al., 1991). Over the past 40 years, lesion studies demonstrating reduced SAB rate in rodents have implicated the role of the hippocampus in attention control and memory formation (Roberts et al., 1962). If so, increased degeneration in the CA3 region of MRL-lpr brains may account for deficits in formation of a spatial map. However, more recent evidence suggests that in addition to hippocampal damage, the SAB deficit may reflect severed connections with other brain regions vital for the formation of working memory (Lalonde, 2002).

The loss of hippocampal neurons may occur via several non-mutually exclusive mechanisms. First, a direct pathway may involve increased permeability of the blood-brain barrier and infiltration of circulating immune factors into the CNS. The choroid plexus appears to be the primary site of immune complex deposition (Lampert and Oldstone, 1973; Vogelweid et al., 1991), which facilitates increased entry of soluble immune factors, monocytes and lymphocytes into the parenchyma (Hess et al., 1993; Farrell et al., 1997) and hippocampal regions of lupus-prone mice (Kier, 1990). Subsequent leukocyte clustering may lead to the dissemination of autoreactive clones into the CSF (Sakic et al., 2000b). Whether systemically or intrathecally, these lymphocytes could produce neuroactive cytokines, chemokines, and/or brain-reactive antibodies (Hoffman and Madsen, 1990; Khin and Hoffman, 1993; Crimando and Hoffman, 1992, 1995; Hickey et al., 1997; Zameer and Hoffman, 2001), which compromise the survival of differentiated neurons (Maric et al., 2001). Indeed, when injected into the mouse hippocampus, the CSF from a demented NP-SLE patient induced apoptotic neuronal death via cross-reactive binding of anti-DNA antibodies to an N-methyl-D-aspartate (NMDA) receptor subtype (DeGiorgio et al., 2001). Given that neuronal survival is dependent on the activity of supporting cells, activation of microglia during the autoimmune disease (Hickey et al., 1997) may also lead to the generation of potentially neurotoxic factors (e.g., reactive oxygen species) which contribute to neuronal demise (Wood, 1998).

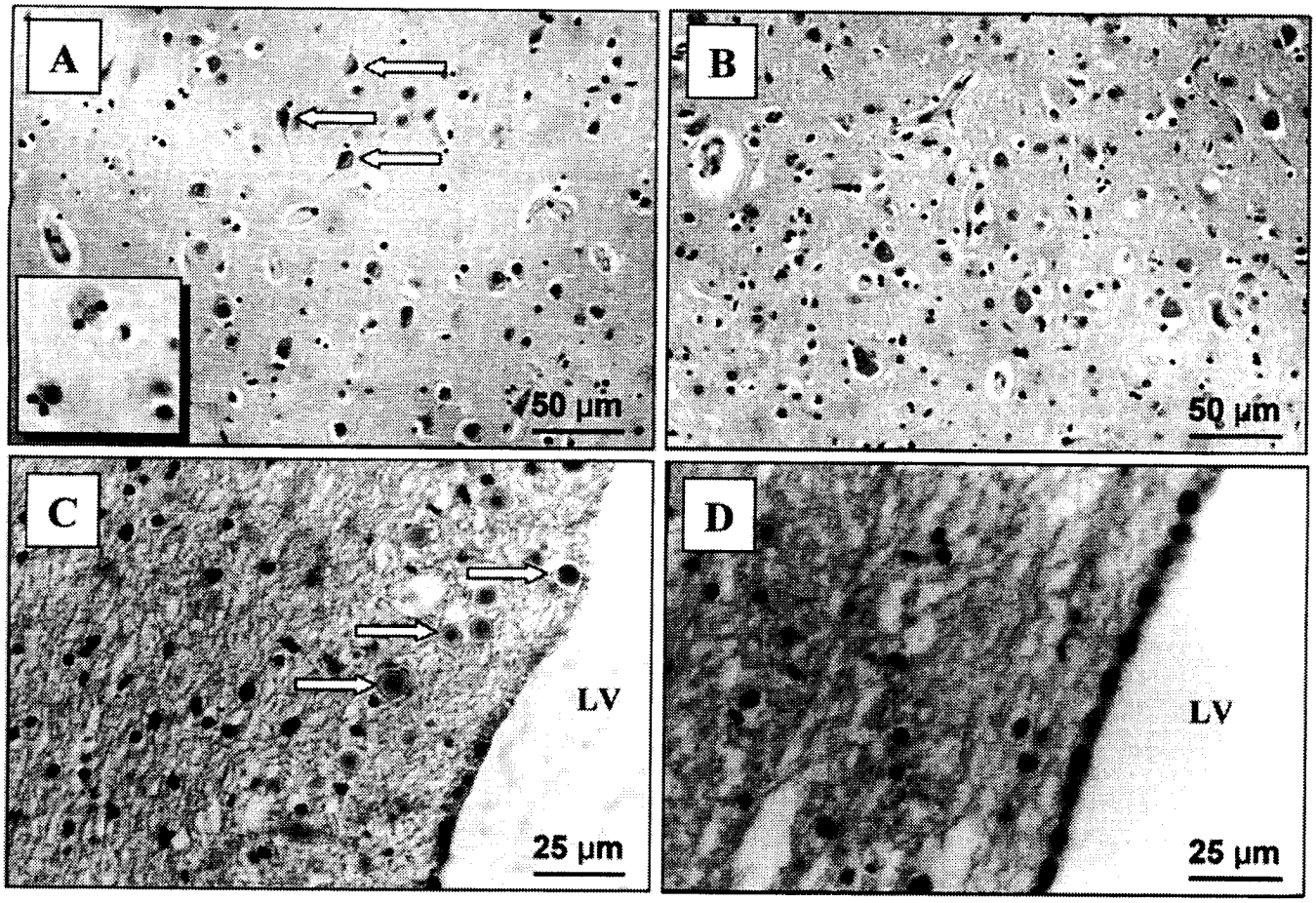

FIGURE 5. H&E staining of the parietotemporal cortex and periventricular regions of the human brain. "Dying neurons" (shown by arrows) and satellitosis (Inset) in the gray matter of the lupus patient (A). Normal neurons and resting glial cells in the control brain (B). Numerous particles identified as "brain sand" were common around the ventricles in the patient's brain (C), and were rarely seen in the control (D). LV, lateral ventricle. ×200 in A,B); ×400 in C,D.

A second possibility is that glucococorticoids contribute to the loss of hippocampal neurons via cytokine-induced sustained activation of the hypothalamo-pituitary-adrenal (HPA) axis (Wick et al., 1993) and intracellular glucocorticoid receptors (McEwen et al., 1992). It is documented that glucocorticoids potentiate the release and postsynaptic actions of glutamate, which likely account for loss of neurons in the CA3 region (Magarinos et al., 1997). In vivo studies confirm that this region is vulnerable to endogenous glucocorticoids (Armanini et al., 1990) and that memory deficits and structural damage are prevented by an inhibitor of corticosterone synthesis (Roozendaal et al., 2001). Since MRL-lpr mice have chronically elevated basal levels of corticosterone (Hu et al., 1993; Lechner et al., 2000), this steroid may act in a similar fashion to induce neuronal loss in the CA3 region.

Pro-inflammatory cytokines are upregulated systemically (Tang et al., 1991; Tsai et al., 1995) and in the hippocampus of MRL-lpr mice (Tomita et al., 2001). Since they appear to alter central neurotransmission (Zalcman et al., 1994), one may hypothesize that an accumulation of serotonergic neurotoxins, such as 5,7-dihydroxytryptamine (Tabatabaie et al., 1993), could compromise the survival of hippocampal neurons. Indeed, we recently identified by HPLC excessive postmortem levels of serotonin (5-HT) in the hippocampus of MRL-lpr mice (Sakic et al., 2002). The observed increase in FJB and Ub staining in the CA3 region may reflect neuronal loss (due to an intracellular accumulation of toxic metabolites), which leads to impaired performance in tasks contingent upon the integrity of hippocampal circuits. However, more detailed assessment of the phenotype of dying neurons (e.g., serotonin transporter) combined with in situ analysis of central cytokines is required to confirm this relationship.

Highly Ub-immunoreactive structures were observed in brains from autoimmune MRL-lpr mice. They likely represent degenerating axon terminals, and their location suggests that they belong to mossy fibers. The damage observed in the hippocampal synapses in human neuropathological diseases (DeKosky and Scheff, 1990; Samuel et al., 1994) and aging (Gray et al., 2003) is proposed to have significant cognitive and behavioral consequences. Interestingly, antibodies reacting with Ub and ubiquitinated histones are present in SLE, with almost 80% of patients having antibodies against Ub-protein conjugates (Muller and Schwartz, 1995). However, the present treatment with CY neither reduced expression of Ub in brains from MRL-lpr mice nor did the analysis of the

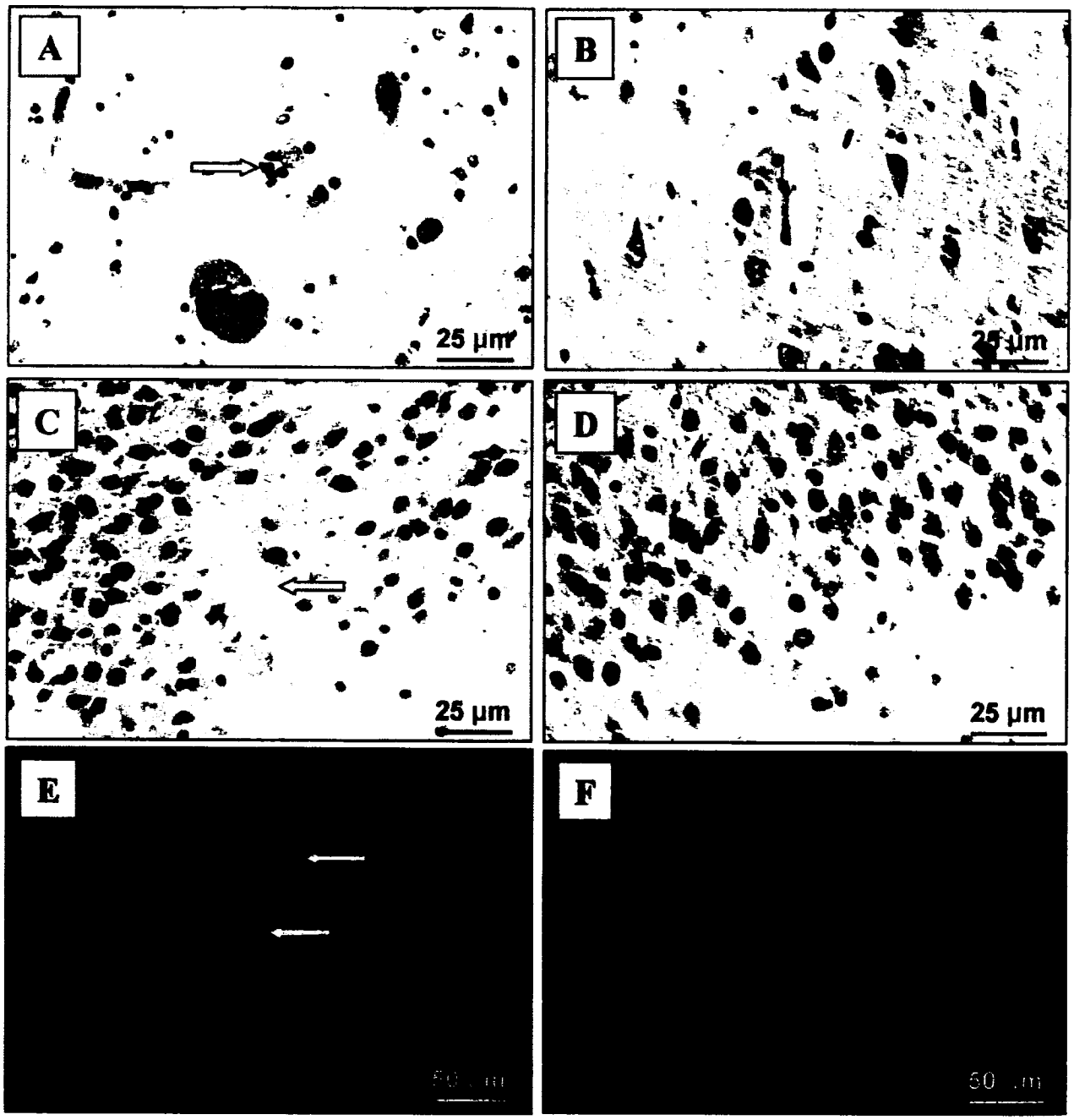

FIGURE 6. Neuronal loss and degeneration in the human hippocampus as revealed by H&E and FJB staining. The brain from the neuropsychiatric-systemic lupus erythematosus (NP-SLE) patient revealed a paucity of neurons and satellitosis (arrow) in the CA3 region (A), while the control brain showed normal neuronal density (B). A patchy area of cell loss was also seen in the dentate gyrus (arrow) of the lupus brain (C), but not in the control (D). Subsequently, bright green Fluoro Jade B (FJB)-positive neurons confirmed a degenerative process in the CA3 region (E), which was not observed in the control brain (F). ×400 in A–D; ×200 in E,F; ×600 (inset in E, under oil immersion). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com].

brain from a NP-SLE patient show increased density of Ub-positive particles. With respect to the animal model, it needs to be determined whether overexpression of Ub is associated with anti-brain reactivity, such as autoantibodies to Ub particles in kidneys (Elouaai et al., 1994), is a consequence of nonimmune pathogenic mechanisms (e.g., hormonal), or is merely an epiphenomenon of a systemic autoimmune disease.

The CNS and choroid plexus pathology in the MRL-lpr strain has been reported extensively (Alexander et al., 1983; Vogelweid et al., 1991; Hess et al., 1993; Farrell et al., 1997; Sakic et al., 2000b).

Similarly, clinical reports point to the possibility that the choroid plexus is a site for immune complex and leukocyte deposition (Atkins et al., 1972; Gershwin et al., 1975; Peress et al., 1981; Duprez et al., 2001). In the present study, we provide a direct comparison of animal and human brain pathology by using the same protocols and reagents for FJB staining. In addition, the cortical thinning, dilation of ventricles, and infiltration of T lymphocytes into the stroma of the choroid plexus are comparable to ventricular enlargement (Denenberg et al., 1992) and the infiltration of cells immunoreactive for CD3, CD4, and CD8 antigens in the choroid plexus of MRL-lpr mice (Sakic et al., 2000b; Ballok et al., 2003). The neuropsychiatric manifestations reported in human SLE have also been shown to accompany cerebral atrophy (Chinn et al., 1997) and progressive neuronal loss (Sibbitt and Sibbitt, 1993; Brooks et al., 1997). In line with this evidence, the parietotemporal cortex of our patient showed gliosis and ischemic changes in neurons. Consistent with the notion of cortical damage, reduced neuronal density was presently observed by H&E in the parietal cortex of diseased MRL-lpr mice.

Schnider et al. (1995) described an SLE patient who presented with severe amnesia due to isolated hippocampal damage. Similar to this case report, the present analysis revealed neuronal loss in the CA3 region and the dentate gyrus, with surviving neurons in the pyramidal layer showing focal satellitosis and gliosis. Although CY treatment may have beneficial effects in preventing NP-SLE manifestations (Boumpas et al., 1991; Ramos et al., 1996), it neither prevented a fatal outcome in our patient nor showed effects comparable to those seen in lupus-prone mice. One explanation is that the dose of CY used in mice (100 mg/kg) is essentially myeloablative in humans and substantially higher then the standard treatment for human autoimmune diseases (1–2 mg/kg i.v. was given daily to our patient). Second, in experimental studies, effective immunosuppressive treatments are started well before overt signs of the disease (O'Sullivan et al., 1995; Sakic et al., 1995; Walker, 2001). This is similar to the clinical protocols in which CY treatment (often associated with side effects) needs to be employed early in order to prevent or minimize irreversible organ damage (Ioannou and Isenberg, 2002). However, the SLE patient in our study received the CY treatment when she became comatose (i.e., 4 days after she presented with status epilepticus), which was probably not sufficient to reduce existing brain damage.

In summary, the present results support the hypothesis that systemic autoimmune/inflammatory disease impairs hippocampal function and compromises neuronal survival in lupus-prone mice. Damage in the CA3 region is detectable after the spontaneous onset of the disease, affects performance in the SAB and "reversal" test, and can be attenuated by immunosuppressive treatment. Further studies are required to elucidate neuropathogenic mechanisms and neuronal phenotypes susceptible to the disease process.

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CHAPTER 4
IBUPROFEN FAILS TO PREVENT BRAIN PATHOLOGY IN A MODEL OF NEUROPSYCHIATRIC LUPUS

Published in Journal of Rheumatology in 2006

David Ballok's contribution:

I am the first author on this paper. I planned and designed the experiment. I oversaw the formulation and preparation of the non-steroidal anti-inflammatory diet, supervised technicians who preformed the animal behavioural testing, and conducted the animal surgeries which included perfusions, blood collection and organ extractions. I was responsible for performing the subsequent ELISAs (CIC, dsDNA, IL-1beta and TNF-alpha), H&E staining, cytochemical (FJB) and immunochemical (F4/80) staining of the tissues. A co-author performed the CD3 immunostaining and FACS analysis. Experimenters blind to sample origin manually quantified cell numbers, as well as employing imaging software. Toluidine blue staining and EM tissues were prepared by technicians following my direction. I completed the required statistical analyses for this study. The figures are my own work and although feedback was provided by other authors, it was my responsibility to write the article.

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Ph.D. Candidate, McMaster University, Faculty of Health Sciences

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Ibuprofen Fails to Prevent Brain Pathology in a Model of Neuropsychiatric Lupus

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Keywords:	Systemic lupus erythematosus, Animal disease models, Nervous system diseases

Abstract Neurologic and psychiatric manifestations are severe complications of systemic lupus erythematosus. As commonly seen in patients, spontaneous development of lupus-like disease in MRL-lpr mice is accompanied by brain atrophy and behavioral dysfunction. We presently examine inflammatory and ultrastructural aspects of the CNS involvement using a non-selective COX-2 inhibitor and measuring effects on behavior, microglial activation, and neuronal morphology. Ibuprofen (IBU) was provided in a rodent chow (375 ppm) from 5-19 weeks of age. Exploration of a novel environment and performance in the forced swim test assessed effects on behavior. Immunohistochemistry, Fluoro-Jade B (FJB) staining, and flow cytometry were employed in neuropathological analysis. Transmission electron microscopy examined ultrastructural morphology of cortical, hippocampal, hypothalamic, nigral and cerebellar cells. Chronic IBU treatment failed to normalize immune status, behavior, and brain mass in lupus-prone MRL-lpr mice. It also did not reduce density of CD3+ lymphocytes in the choroid plexus, or FJB+ neurons in the hypothalamus. Activated F4/80+ microglia increased with age, but IBU treatment was not effective in reducing their numbers. Although numerous dark cells were seen in functionally critical brain regions (e.g. the PVN and the subgranular zone), ultrastructural morphologies of classical apoptosis or necrosis were not detected. The COX-dependent pathway does not seem to be critical in the etiology of CNS disease in this model of neuropsychiatric lupus. Reduced brain mass significantly correlated with increased splenomegaly and serum dsDNA levels suggesting chronic autoimmune disease is associated with neuropathology. Increased microglial activation and condensation of cytoplasm point to a metabolic perturbation (e.g. excitotoxic damage) which compromises function and survival of central neurons during lupus-like disease.

Keywords: autoimmunity; inflammation; ibuprofen; microglia; lupus; excitotoxicity; apoptosis; oncosis; subgranular zone; MRL mice; electron microscopy.

1. Introduction

Although development of systemic autoimmunity and inflammation clearly account for peripheral manifestations (e.g., dermatitis, vasculitis, glomerulonephritis), mechanisms underlying central nervous system (CNS) damage in up to 50% of patients with systemic lupus erythematosus (SLE) largely remain unknown. Brain atrophy (Steens et al., 2004; Bosma et al., 2002) and biochemical signs of neuronal/astrocytic damage are reported in a significant proportion of patients with clinically verified CNS involvement (Trysberg et al., 2003). With an ultimate goal of understanding the relationship between SLE-like disease and aberrant behavior, we study brain pathology and behavioral dysfunction in the MRL/MpJ-Fas^{lpr} (MRL-lpr) murine substrain. MRL-lpr mice lack a functional Fas receptor and develop lupus-like manifestations earlier in life than congenic MRL/MpJ (MRL+/+) controls (Theofilopoulos, 1992). Along with spontaneous onset of inflammation and autoimmunity, MRL-lpr mice show retarded brain growth, infiltration of leukocytes into the choroid plexus, neuronal atrophy, and deterioration in behavioral performance (Farrell et al., 1997; Sakic et al., 1992; Sakic et al., 1998). The constellation of behavioral deficits which distinguish two MRL substrains has been operationally defined as "autoimmunity-associated behavioral syndrome" (Sakic et al., 1997b), and has been proposed to model neuropsychiatric lupus, or NP-SLE (Szechtman et al., 1997).

As reported in NP-SLE patients (Abbott et al., 2003; Duprez et al., 2001), the blood-brain barrier in MRL-lpr mice becomes more permeable with the progress of lupus-like disease (Vogelweid et al., 1991; Sidor et al., 2005). Since T- and B-cells infiltrate brain (Zameer and Hoffman, 2004), their soluble mediators may drain into the CSF, diffuse into neighboring interstitial tissue and have detrimental effects on neuronal cell functioning (Maric et al., 2001). Although recent

evidence emphasizes the role of autoantibody-mediated neuronal damage (DeGiorgio et al., 2001; Kowal et al., 2004; Huerta et al., 2006), inflammatory mechanisms in the etiology of mental dysfunction (e.g. depressive behavior) remain largely unexplored (Raison et al., 2006; Sakic et al., 1996; Sakic et al., 1994). Circulating pro-inflammatory cytokines facilitate infiltration of macrophages and other monocytes into the brains of lupus-prone mice by upregulating expression of cell adhesion molecules (Marshall et al., 2003; Zameer and Hoffman, 2003). Activated microglial cells/macrophages often migrate to sites of injury where they proliferate and undergo morphological and functional changes (Gonzalez-Scarano and Baltuch, 1999). This includes expression of molecules which orchestrate the inflammatory cascade, such as MHC class II, tumor necrosis factor-alpha (TNF-alpha), interleukin (IL)-1, and IL-6. Expressed primarily on activated microglial cells (Nakanishi, 2003), MHC class II molecules interact with T cells, thus contributing to local synthesis of inflammatory factors and recruitment of other inflammatory cells. Indeed, excessive levels of MHC class II (as determined by Ia mRNA) were observed in the diencephalon of MRL-lpr mice (McIntyre et al., 1990). Along the same line, overexpressed IL-6 and interferon-gamma mRNAs in the hippocampus and cerebellum (Tomita et al., 2001b; Tomita et al., 2001a) further supported the notion of a central inflammatory response in these animals.

The periventricular regions and hippocampus of diseased MRL-lpr mice shows increased density of TUNEL+ cells (Sakic et al., 2000b), but merely ~7% of this population is accounted by degenerating neurons (Ballok et al., 2003a). Although this suggests that the majority of dying neurons do not undergo synchronized DNA fragmentation, the prevalent mode of neuronal death within the apoptosis-necrosis continuum remained unclear. The present study focuses on the "inflammatory aspect" of brain involvement by attenuating activity of cyclooxygenase-2 (COX-2),

an enzyme instrumental in the inception of inflammatory processes (Dubois et al., 1998). In addition, given that neuronal necrosis is often accompanied by an inflammatory response (Kagan and Zakeri, 1999) and sustained microglial activation may contribute to chronic neuropathology (Hanisch, 2002), microglial/macrophage population and ultrastructural morphology of cells in several brain regions were examined.

Non-steroidal anti-inflammatory drugs (NSAIDs) are effective inhibitors of COX-1/COX-2 pathways (Garavito and Mulichak, 2003) and the microglia/macrophage-driven neurotoxic cascade (Klegeris and McGeer, 2002; Shibata et al., 2003). In vitro studies had showed that the non-selective COX-2 inhibitor ibuprofen (IBU) can induce apoptosis of activated microglial cells (Elsisi et al., 2005), reduce glutamate neurotoxicity (Iwata et al., 2004) and attenuate drug-induced damage of dopaminergic neurons (Carrasco et al., 2005), which are a proposed target of autoimmune processes in the MRL model (Ballok et al., 2004a; Sakic et al., 2002; Anderson et al., 2006). Moreover, supplementing rodent chow with IBU led to attenuation of amyloid plaque deposition and microglia-mediated brain inflammation in murine models of Alzheimer's disease (Lim et al., 2000; Yan et al., 2003). The above beneficial effects and stress-free chronic administration of the drug led us to select the above treatment for the present behavioral/neuropathological study. The overall expectation was that mice fed with IBU would show normalized behavioral performance, immune status, and brain morphology.

2. Materials and methods

2.1 Animals, drug treatment, and tissue collection

Twenty MRL-lpr and 10 MRL +/+ males (5 mice /cage) were obtained from a local specific-

pathogen free colony and maintained under standard laboratory conditions (light phase: 8 A.M. - 8 P.M., food and water *ad libitum*; t^{o} = 24-26 °C). Ten age-matched CD1 males (Charles River, Canada) were housed in the same colony room and used as non-autoimmune controls. Ibuprofen (IBU) was purchased from Sigma (St. Louis, MO) and subsequently formulated into color-coded, AIN-76A rodent diet by Research Diets (New Brunswick, NJ) at a final concentration of 375 ppm. This dose was selected on the evidence that it prevents CNS pathology and inflammation in a mouse model for Alzheimer's disease (Lim et al., 2000). From 5-19 weeks of age half of each group was fed *ad libitum* with either drug-supplemented chow or control chow. At the end of the study mice were anesthetized with Somnotol (i.p., 60 mg/kg body weight; MTC Pharmaceuticals, Cambridge, ON) and after terminal bleeding from the vena cava, they were intracardially perfused with 20 ml of phosphate buffered saline (PBS) and 20 ml of fresh 4% paraformaldehyde (PFA). Extracted brains and spleens were weighed on an analytical balance (AB54-S, Mettler Toledo, Switzerland). Brains were used to examine CD3+ cell (T-lymphocyte) infiltration into the choroid plexus and brain parenchyma.

Given dissimilar fixation protocols, a second cohort of 4-week old mice (MRL-lpr, MRL +/+, and CD1 males; n = 18/strain; 3 mice/cage) was used to examine effects of the IBU treatment on neuropathological indices (presence of FJB+ neurons, expression of a F4/80 marker on microglia/macrophage cell line) and behavioral performance (MRL-lpr vs. MRL +/+ only). As with the first cohort, half of each group was fed *ad libitum* with either drug-supplemented chow or control chow from 4-18 weeks of age (n = 9 mice/strain/treatment). The body weight and food consumption were monitored weekly. Averaged food consumption was ~5 g/day/animal, resulting in a final daily dose of ~62.5 mg/kg in the IBU-treated groups. Mice were sacrificed at 18 weeks by overdose with

Somnotol. After terminal bleeding from the vena cava, they were perfused as described above.

To assess the time-course of microglial/macrophage activation, PBS-perfused brains from 5, 12, 18-week-old MRL-lpr (n = 8/age group) and MRL+/+ mice (n = 5/age group) were pooled for the purposes of flow cytometry. Mice were maintained under conditions as outlined above. A fourth cohort of males was used for the purpose of electron microscopy. It included three 23-week old MRL-lpr mice, an asymptomatic 8-week old MRL-lpr mouse, a 22-week old MRL +/+ congenic control, and a healthy, aged-matched CD1 mouse. After overdose with Somnotol, they were heparinized and perfused by gravity via the left ventricle with lactated Ringer's solution, followed by Karnofsky's fixative (phosphate buffered 4% gluteraldehyde). The brains were dissected and postfixed in the Karnofsky's fixative at 4°C for 2 days. A stainless steel coronal brain matrix (Stoelting Co., Wood Dale, IL) was used to obtain 1mm-thick sections. Using a scalpel blade, blocks of tissue (~2 mm^2) were dissected from hypothalamus, cerebellar cortex, hippocampus, and substantia nigra (SN). To prevent tissue drying, sections remained submerged in Karnofsky's fixative throughout the micro-dissection. All experimental protocols were approved by a local animal care committee and carried out in accordance with the rules and regulations of the Canadian Council of Animal Care.

2.2 Immunohistochemistry for CD3+ cells

Immunohistochemistry to the CD3 T-lymphocyte marker was used to examine the degree of leukocyte infiltraton into the choroid plexus and brain parenchyma in the first cohort of mice. Twelve-µm coronal sections (Bregma ~ -1.0) were fixed in aceton at -20° C for 3 min and air dried. Following two 5 min washes in PBS, slides were placed into avidin-blocking solution (Vector

Laboratories, CA) for 15 min, washed in PBS and immersed into biotin-blocking solution (Vector Laboratories, CA) for 5 min. After a 5 min wash, sections were incubated with 10% normal goat serum (Vector Laboratories, CA) in PBS for 30 min at room temperature (RT). After incubation with primary antibody (hamster anti-mouse CD3e diluted 1:30 with 5% goat serum/PBS, BD Pharmingen, Cat# 550275) overnight at 4°C, three additional PBS washes (5 min each) were followed by incubation with secondary antibodies (biotinylated anti-hamster IgG diluted 1:250 with 5% goat serum/PBS; Vector Laboratories, Cat# BA-9100) for 1 h at RT. After a 5 min wash in PBS, slides were immersed into 0.03% H_2O_2 in PBS for 10 min at RT, and following an additional wash, incubated with streptavidin/HRP (BD Pharmingen from the Anti-Ig HRP Detection Kits, Cat# 551013) for 30 min at RT. Following three additional PBS washes (5 min each), slides were incubated with DAB for several minutes, and washed in tap water. A brief counterstain with hematoxylin, dehydration through graded alcohol and xylene preceded three 5-min PBS washes before cover slipping. Counting of CD3+ cells from digitized photos (depicting choroid plexus of the third ventricle) was performed in one section / brain under ×400 magnification by an observer blinded to the group origin.

2.3. Behavioral testing

In comparison to congenic MRL +/+ controls, diseased MRL-lpr mice reliably show impaired locomotor activity in a novel environment and excessive floating in the forced swim test (Sakic et al., 1994; Sakic et al., 1992). In the present study, the second cohort of age-matched MRL-lpr and MRL +/+ males was tested between 18 and 19 weeks in computerized activity boxes (Digiscan-16, Omnitech Electronics, Columbus, OH). Distance traveled and time spent in ambulation over two

30-min periods (6:00-7:00 P.M.) were measured by VersaDat software (Accuscan Instruments Inc., Columbus, OH). Floating in the swimming pool (dia. 1.83 m, water $t^{o} = 25^{o}C$) was defined by the absence of any paw and tail movements over a 6-min trial and recorded by an unbiased observer, as described earlier (Sakic et al., 1994).

2.4 Fluoro-Jade B (FJB) staining

FJB is an anionic fluorescein derivative used for the localization of degenerating neurons in brain tissue sections. This dye has an affinity for the entire degenerating neuron including cell body, dendrites, axon and axon terminals. However, the degenerating tissue components (biomolecules) to which the dye has an affinity is currently unknown. Ten-μm coronal sections (Bregma ~ -1.0) were cut with a Jung Frigocut 2800E cryostat and sections were mounted onto Aptex-coated slides according to the previously published protocol (Ballok et al., 2004a). The sections were subsequently examined using a Zeiss Laser Scanning Confocal Microscope (LSM 510, Carl Zeiss Inc.) argon laser (wave-length 488 nm). Two confocal micrographs from the paraventricular hypothalamic region were obtained using a Fluar 20x/0.75 objective in combination with a 1024x1024 pixel resolution and FJB+ cells were manually counted by an observer blinded to group origin.

2.5 Immunohistochemistry for F4/80+ cells

F4/80, a 120-160kD glycoprotein, is highly and constitutively expressed on most resident tissue macrophages (Morris et al., 1991), including microglia (Perry et al., 1985). Resting microglia possess a characteristic ramified morphology, which can be visualized with antibodies towards the

F4/80 antigen. However, immunohistochemical staining to F4/80 is proposed to become more intense after microglial activation (Chen et al., 2005). We employed the rat monoclonal antibody F4/80 (1:100 dilution in 5% goat serum/PBS, Serotec) and the biotinylated anti-rat IgG secondary antibodies (1:200 dilution in 5% goat serum/PBS, Serotec) to morphologically characterize microglia on horizontal sections. Incubation times for the primary and secondary antibodies were overnight at 4°C and 1h at RT, respectively. Following incubation, brain sections were treated with Vectastain ABC reagent (Vector Laboratories, CA) according to the manufacturer's directions and the resulting avidin-biotin-peroxidase complex was visualized with diaminobenzidine (DAB substrate kit, Vector Laboratories, CA). The stained brains were observed by confocal light microscopy under differential interference contrast. In addition to more intense staining, activated microglia were distinguished by a more rounded morphology than when in a resting state (Perry and Gordon, 1997). Three adjacent 0.5 mm^2 areas from the paraventricular region of the diencephalon were selected from each brain, and F4/80+ cells were manually counted from by an observer blinded to group origin at ×200 magnification. The sparse presence of F4/80+ cells in the cerebellum and cortex was also noted.

2.6 Flow cytometry forCD69+F4/80+ cells

A useful approach to assess activated micrcroglia/macrophages in the brain is the combination of immunohistochemistry and flow cytometry (Merrill et al., 1992). Therefore, the third cohort of mice was used to confirm activation of microglial/macrophage cell lineage during the progress of lupus-like disease in MRL-lpr mice. CD69 antigen (also known as an activation inducer molecule or a very early activation marker) is a member of the natural killer (NK) cell gene complex family of signal transducing receptors. CD69 expression can be induced *in vitro* on cells of most

hematopoietic lineages, including T- and B-lymphocytes, NK cells, murine macrophages, neutrophils and eosinophils (Marzio et al., 1999). According to a pilot study (data not presented), 0.5-1.5 x 10^5 of mononuclear cells can be obtained from a single MRL-lpr brain. Since this yield was not optimal for flow cytometry (~ 5 x 10^5), 5-8 brains for each age point and substrain were pooled from 5, 12 or18-week old MRL-lpr and MRL +/+ mice. Freshly extracted brains were minced in a glass homogenizer containing cell culture media, Dulbecco's Modified Eagle Medium (DMEM, GIBCO, Invitrogen Corporation, Grand Island, NY). Tissues were transferred into 50-ml centrifuge tubes and a single-cell suspension was obtained by vigorous pipetting. A Percoll separation was used to isolate the infiltrating mononuclear cells, as previously described (Lindsley and Rodriguez, 1989). Briefly, nine parts of Percoll (Amersham Bioscience, Uppsala, Sweden) to one part of 10x PBS was added to brain tissue suspension at a final concentration of 30% Percoll and centrifuged at 27000G for 30 min at 4°C. This resulted in top myelin/cellular debris fraction, middle glial fraction, and bottom mononuclear cell fraction. The latter fraction was collected and washed with DMEM. The number of collected living cells was counted in a hemacytometer.

Fluorescence-labeled antibodies used for staining were R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD69 monoclonal antibody (0.25μg for 1x10^6 cells, BD Pharmingen) and RPE-Cy5-conjugated rat anti-mouse F4/80 monoclonal antibody (5μl for 1x10^6 cells, Serotex, Oxford). The procedure was followed as described previously (Luzina et al., 1999). In particular, aliquots of mononuclear cells (0.5-1x10^6) were suspended in 0.1 ml of PBS containing 0.1% sodium azide and incubated with predetermined optimal concentration of R-PE-conjugated anti-mouse CD69 and RPE-Cy5-conjugated anti-F4/80 for 30 min in the dark at 4°C. Cells were washed three times with PBS, resuspended in 0.3 ml of PBS (containing 0.1% sodium azide), and subjected to FACScan

analysis. Mononuclear splenocytes without fluorescence-labeled antibodies added were used as a negative control. Background autofluorescence was determined by incubating mononuclear cells (from brains of 18-week-old MRL-lpr mice) with PBS. The analysis was performed using FACScan (Becton Dickinson, Mountain View, CA) and regions of interest were set according to forward scatter (roughly proportional to the diameter of the cell) and side scatter characteristics of splenocytes (proportional to the granularity). A total of 10,000 cells were analyzed and the frequency of each cell surface marker was determined using WinMDI 5.1 software (Joseph Trotter; Scripps Clinic, La Jolla, CA). Data were represented as dot plots, where each cell is represented by a dot, positioned on the X and Y axis according to the fluorescence intensities. These two-color dot plots are divided into four quadrants, including double negative cells, green-only, red-only, and double stained cells. Percentage of different cell populations in each quadrant was calculated for comparative purposes.

2.7 Light microscopy and transmission electron microscopy (EM)

In preparation for the EM analysis, 1mm × 2 mm × 1mm blocks of brain tissue were isolated from the regions of interest (described above) and stored in Karnofsky's fixative until processing. Samples were rinsed in 0.1M sodium-cacodylate buffer twice for 10 min each, and post-fixed in 1% osmium-textroxide (in 0.1M sodium cacodylate buffer) for 1h at RT. Tissue was dehydrated in ascending concentrations of ethanol, followed by two changes with propylene-oxide (10 min each). Subsequently, samples were immersed in 1:1 and 1:3 propylene-oxide:Spurr's resin (Marivac-Canemco) for 1 h before being placed into 100% Spurr's resin overnight. The next day samples were embedded into fresh Spurr's resin and polymerized overnight at 60°C. Blocks were trimmed

and semi-thin sections (0.5 μm) were cut and stained for light microscopy with toluidine blue, a stain which delineates both perikarya and proximal dendrites (Trump et al., 1961). In brief, 5g of toluidine-blue powder (Marivac-Canemco, St. Laurent, Quebec) and 5g sodium-borate (Caledon, Georgetown, Ontario) were combined in deionized water to make a 500 ml staining solution. The solution was heated to ~65°C and sections were transiently immersed for staining. Sections were washed three times in distilled water before mounting and cover slipping for assessment. Finite neuroanatomical areas were identified from each section. From each slide, an area common to a particular region was demarcated for further processing. Ultra-thin sections (90 nm) of each finite area were then cut and placed onto 200 mesh copper palladium grids. The grids were post-stained with saturated uranyl-acetate in 50% ethanol and Reynold's Lead Citrate. Grid sections were examined and photographed using a JEOL 1200 EX (Tokyo, Japan) transmission electron microscope at an accelerating voltage of 80 kV. Some sections were also processed with standard H&E as previously described (Ballok et al., 2004b).

2.7. Indices of systemic disease

Profound changes in the systemic cytokine network are one of the earliest manifestations of autoimmune disease in murine models of lupus (Theofilopoulos, 1992). We presently measured TNF-alpha, which is proposed to contribute to progressive microglia-induced cell damage in neurodegenerative diseases (Ahmad et al., 2000), and is elevated in MRL-lpr mice (Tsai et al., 1995; Ballok et al., 2003b). Similarly, high concentrations of IL-1beta may have detrimental effects on cultured neurons (Strijbos and Rothwell, 1995) and hippocampal tissue (Depino et al., 2005). Therefore, serum levels of TNF-alpha and IL-1beta were analyzed by an ELISA kit (R&D Systems,

Minneapolis, MN) according to the manufacturer's directions and protocol described previously (Ballok et al., 2003b).

Considering that death of MRL-lpr mice is usually attributed to immune complex-mediated glomerulonephritis, and deposits of IgG and DNA immune complexes have been demonstrated in the kidneys (Theofilopoulos and Dixon, 1985), circulating immune complexes (CIC) and antibodies to double-stranded DNA (dsDNA) were presently measured as additional markers of disease severity. Blood samples were left to coagulate in 1.5-ml plastic vials and centrifuged for 10 min at 3000 rpm. Serum was separated from the clot and stored at -20°C until further analysis. CIC and dsDNA concentrations was measured using qualitative sandwich ELISA kits (CIC: Cat. #5900; anti-dsDNA: Cat.#5100), according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX). In brief, serum samples were diluted 1:100 in diluent included in the kit, and applied to both experimental and control wells to assess the specificity of binding. The optical density of each well was determined using a microplate ELISA reader set to 450 nm. Given that splenomegaly is a well-established marker of severe autoimmunity in the MRL model (Theofilopoulos, 1992), wet spleen was weighted immediately upon extraction.

2.8. Statistical analysis

The data were analyzed by analysis of variance (ANOVA) with Substrain (MRL-lpr vs. MRL +/+; CD1) and Treatment (IBU-rich vs. control food) as between-group factors. Student's t-test was used in the post-hoc analysis because of the 2 x 2 design in most comparisons. Pearson's correlation was used to measure association between variables. Significance level was set at $p \leq .05$ and graphs show means ± SEM. All computations were performed using the SPSS 13 statistical package.

3. Results

Dermatitis, alopecia and necrosis of ear tips appeared earlier and were more common among the IBU-treated MRL-lpr mice in comparison to three other groups (~30% vs. ~10%). This suggested that chronic ingestion of IBU-laced food exacerbated lupus-like disease in some animals. However, this observation needs to be repeated in a separate cohort by formulating an *a priori* hypothesis and by systematically measuring peripheral manifestations.

3.1. Infiltration of T-cells and neuropathology

As shown previously (Sakic et al., 2000b), T-cells were dense in the choroid plexus and sparsely scattered throughout the brain parenchyma of MRL-lpr mice. Although the difference in the number of T-lymphocytes in the choroid plexus and the third ventricle was not statistically significant, there was a trend for more T-cells in brains from the IBU-treated MRL-lpr group (83 ± 23 vs. 53 ± 13 in control MRL-lpr mice, $t_{18} = 1.197$, $p > .1$). Regardless of the treatment, T-lymphocytes were not observed in brains of congenic or allogenic controls.

As expected, reduced brain weight ($F(1,32) = 4.704$, $p < .05$) was accompanied by increased FJB and F4/80 positivity in brains of diseased MRL-lpr mice. Hypothalamic, cortical and cerebellar regions were frequently populated with brightly stained FJB+ neurons or Purkinje cells (Figure 1). Contrary to a scattered distribution in the hypothalamus (Figure 2A), clusters of intensely stained F4/80+ cells were observed in the parieto-temporal cortex of MRL-lpr brains (Figure 2C). The size and morphology of some F4/80+ cells was reminiscent of macrophage-like cells (Figure 2C inset). While F4/80+ cells were rarely seen in the cerebellum of MRL-lpr mice, discretely stained cells were

frequently noted in the proximity of the Purkinje layer (Figure 2E). The above observations were more profound in comparison to brains of congenic MRL +/+ controls (Figures 2B, 2D and 2F) and healthy CD1 mice (data not shown). The treatment with IBU did not change established group differences, as evidenced by undiminished counts in the hippocampal region (Figure 3). However, similar to enhanced T-cell infiltration, there was a trend for more FJB+ neurons in the IBU-treated MRL-lpr mice than MRL-lpr mice fed with the control diet (102 ± 8 vs. 81 ± 7, $t_{16} = .081$; Figure 3A).

A novel contribution to the previously described neuropathology (Ballok et al., 2004b; Ballok et al., 2003a; Sakic et al., 2000b; Sakic et al., 1998; Farrell et al., 1997) is the observation that (regardless of treatment) intensely stained "activated microglia" are more common in the hypothalamus of diseased MRL-lpr mice (Substrain: $F(2,25) = 17.667$, $p < .001$, Figure 3B). This notion of enhanced microglial activation was supported by the FACS analysis, which revealed ~20-fold increase in the number of CD69+F4/80+ cells from 5 - 18 weeks of age (Figure 4). Although resident CD69+F4/80+ cells were less abundant than other leukocyte populations (Ma et al., 2006), their percentage increased with age and was higher in brains pooled from 12-week and 18-week old MRL-lpr mice than in age-matched MRL +/+ controls.

3.2. Behavior

MRL-lpr mice consumed less food and water compared to congenic controls, and the IBU-rich diet did not affect these measures (for food, Substrain: $F(1,32) = 6.639$, $p < .02$; for water, Substrain: $F(1,32) = 7.062$, $p < .02$). As expected, MRL-lpr males moved less and traveled shorter distances when exposed to a novel environment (activity box), and this deficit was not ameliorated

by the IBU treatment (for movement time, Substrain: $F(1,32) = 9.153$, $p < .01$; for distance, Substrain: $F(1,32) = 12.909$, $p < 0.001$). Chronic IBU treatment also failed to reduce floating time in MRL-lpr mice (Substrain: $F(1,32) = 13.352$, $p < 0.01$). Since these observations were reported previously (Sakic et al., 1997a; Ballok et al., 2003b; Sakic et al., 1994), they are not presently shown. The CD1 mice showed food/water consumption and behavioral performance similar to MRL +/+ mice (data not shown).

3.3. Indices of autoimmune disease

MRL-lpr mice had significantly larger spleen weights compared to the other two control groups (Substrain: $F(2,46) = 33.778$, $p < .001$), but IBU did not attenuate this difference. Splenomegaly correlated with reduced brain weights within the MRL-lpr mice ($r_{16} = -.486$, $p < .05$) and serum dsDNA levels ($r_{16} = -.573$, $p < .05$), suggesting a relationship between systemic autoimmunity and brain damage in this substrain. Serum levels of TNF-alpha and IL-1beta were elevated in MRL-lpr mice exclusively, and the treatment with IBU did not alter these measures (for TNF-alpha Substrain: $F(2,47) = 8.058$, $p < 0.001$; for IL-1beta Substrain: $F(2,47) = 4.719$, $p < 0.02$). Similarly, increased levels of CIC and anti-dsDNA antibodies in sera of MRL-lpr mice were not attenuated by the anti-inflammatory treatment (for CIC Substrain: $F(2,47) = 30.016$, $p < 0.001$; for anti-dsDNA antibodies Substrain: $F(2,39) = 21.407$, $p < 0.001$; data not shown). The MRL +/+ mice fed control chow, and both groups of CD1 mice were negative for CIC and anti-dsDNA antibodies.

3.4. Microscopic observations

As expected, spleen weight of three MRL-lpr mice employed in the EM analysis were

heaviest in the 23-week old MRL-lpr animals (0.45 ± 0.03) relative to controls (0.11 ± 0.02), thus confirming their autoimmune status (data not shown). Increased presence of "dark" cells in two MRL-lpr brains was already observed at the level of light microscopy by enhanced affinity for staining with toluidine blue (Figure 5). Interestingly, densely packed cells were prominent in the subgranular zone, one of few brain areas which is populated with neuronal and glial precursors (Alvarez-Buylla and Lim, 2004). This observation is consistent with our previous reports in which FJB+ cells were detected in the dentate gyrus of MRL-lpr mice (Ballok et al., 2003a) and an NP-SLE patient (Ballok et al., 2004b). Many scattered "dark" cells were seen in the substantia nigra, but less frequently in the periventricular hypothalamus and in the Purkinje and granular cell layers of the cerebellar vermis (Figure 5), and cerebral cortex (data not shown). Similarly to toluidine blue staining, H&E revealed accumulations of basophilic cells in the subgranular zone of the dentate gyrus (Figure 6). Although a degenerative process is anticipated (Ballok et al., 2004b; Ballok et al., 2003a), it is generally considered that with H&E staining only, a clear distinction between cells in prophase and cells undergoing pycnosis or apoptosis cannot be made (Brenner et al., 2003).

At the level of electron microscopy, an abundance of "electron-dense" cells was confirmed in the hypothalamus, hippocampus, cerebellum (Figure 7) and the substantia nigra. Nuclear and cytoplasmic condensation and clumping/condensation of chromatin were ubiquitous across different brain regions. However, "dark" cells neither contained apoptotic bodies, nor showed evidence of budding, which are classical signs of apoptosis (Schmechel, 1999). Although intracellular organelles showed good ultrastructural preservation and membrane integrity, their swollen shape suggested an initial stage of a metabolic insult. Cells adjacent to the "dark Purkinje layer" were often of normal appearance despite increased amounts of lipofuscin-like particles. Occasionally, the cellular plasma

membrane was ruffled, giving a scalloped appearance to these cells (Figure 7C). Electron-dense cells were confirmed in the subgranular zone and the CA2/CA3 region, with mitochondria preserving double-membrane appearance. While cristae appeared condensed, they were relatively intact within the condensed cytoplasm of dark neurons. Blebbing of cell membranes or profound internal changes in organelles was generally absent. The dysmorphic cells in MRL-lpr brains resembled reversible ultrastructural changes documented by Auer and colleagues, who proposed non-lethal alterations to the neurons after hypoglycemic brain damage (Auer et al., 1985). In contrast to the above observations, cells in control brains had evenly dispersed nuclear chromatin and rounded/oval shaped nuclei with prominent nucleoli (data not shown).

Overall, these dark cells failed to exhibit classical signs of apoptosis, such as formation of apoptotic bodies secondary to nuclear and cytoplasmic fragmentation, or blebbing of the cytoplasmic membrane. Moreover, there was no evidence of large discrete masses (crescentic caps) of chromatin aggregation around the perimeter of nuclear membranes (Kerr et al., 1972). These negative findings would argue against a classical form of apoptosis within brain cells of Fas-deficient autoimmune mice. Similarly, there were no signs of classical oncosis or necrosis, characterized by vacuolization, nuclear and plasma membrane breaks, and spilling of cell contents. Despite such an "intermediate form" of cellular pathology (Martin et al., 1998), a large proportion of cells in 2 out of 3 brains extracted postmortem from aged MRL-lpr mice appear to have undergone profound metabolic perturbations.

4. Discussion

Immunosuppression with cyclophosphamide attenuates infiltration of leukocytes (Farrell et

al., 1997), reduces neuronal atrophy (Sakic et al., 2000a), lowers serum and CSF levels of TNF-alpha, and abolishes *in vitro* CSF cytotoxicity in lupus-prone MRL-lpr mice (Ballok et al., 2004a). Since cyclophosphamide is a cytotoxic drug which affects broad populations of cells, it was of interest to understand whether inflammatory pathways associated with prostaglandin production play a role in the etiology of brain damage. In the present study we used a non-steroid anti-inflammatory drug ibuprofen (IBU), which is commonly used in treatment of SLE. The present dose was comparable to therapeutic doses used in other models of CNS inflammation (Lim et al., 2000; Yan et al., 2003; Teismann et al., 2003). In this model of NP-SLE however, the IBU-rich diet did not attenuate behavioral dysfunction, serologic markers of autoimmune disease, T-lymphocyte infiltration or microglia/macrophage activation. This lack of beneficial effects was also observed in prospective clinical trials with Alzheimer's disease patients (Lucas et al., 2006), and when kidney pathology was examined in lupus-prone mice (Kelley et al., 1986). When infiltrated CD3+ lymphocytes are considered, chronic IBU treatment showed a trend for increased severity of leukocyte infiltration into the third brain ventricle. Consistent with this observation are commonly reported adverse CNS reactions to IBU in SLE patients (Samuelson, Jr. and Williams, 1979; Sonnenblick and Abraham, 1978; Hoppmann et al., 1991; Mou et al., 2006). In addition, our negative finding on the importance of the inflammatory pathway is in line with a classic description of brain pathology in NP-SLE, generally negative for vasculitis (Johnson and Richardson, 1968). Although this reasoning indirectly points to the importance of autoimmune and/or COX-independent mechanisms, one may assume that the present treatment regimen failed due to limited penetration of IBU into the brain (Mannila et al., 2005) or because systemic inflammation in MRL-lpr mice is more aggressive (hence the dose used was inadequate) than in models with Alzheimer's-like disease

brain pathology (Lim et al., 2000; Yan et al., 2003). However, given that the blood-brain barrier is breached in diseased MRL-lpr mice (Vogelweid et al., 1991; Sidor et al., 2005), the former possibility is unlikely. Using light and electron microscopy, numerous dark cells were observed throughout brains from diseased MRL-lpr mice. Condensed nuclear and cytoplasmic material, swollen mitochondria and ruffled cell membranes suggest events which may precede excitotoxic/oncotic cell death. Transient dark neurons, however, are common in ischemic brain (Auer et al., 1985), and also in normal CNS (Cohen and Pappas, 1969) as a consequence of improper fixation or handling of brain tissue (Schmechel, 1999). Although the present sample size was not large enough to estimate the severity of the phenomenon, profound differences between MRL-lpr and control brains suggest that dark cells are not procedural artifacts. They rather point to profound metabolic perturbations (both in neurons and accessory cells) during the development of systemic autoimmune disease.

The Fas antigen (Fas/Apo-1/CD95) is a cell surface receptor which is critical in mediating apoptosis in the CNS (Griffith et al., 1995). This receptor is not expressed in brains of MRL-lpr mice (Park et al., 1998), which is generally consistent with the lack of typical apoptotic morphology in the present study. However, an apoptotic mode of neuronal demise can be mediated by mechanisms such as TNF-alpha or granzyme B receptors. If so, there are at least two lines of supportive evidence. Namely, Alexander and colleagues combined immuohistochemistry to the neurofilament, TUNEL staining, DNA laddering, and caspase-3 activity to infer increased neuronal apoptosis in MRL-lpr brains (Alexander et al., 2005a). Additionally, caspase-3-mediated apoptosis in animal preparations treated with neurotoxic CSF (DeGiorgio et al., 2001) points to a causal relationship between NR2 receptor-reactive autoantibodies and brain damage in NP-SLE patients

(Omdal et al., 2005). However, the inability of the TUNEL method to discriminate apoptosis from necrosis in solid tissues calls into question the specificity of this assay (Yasuda et al., 1995; Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp et al., 1995). Similarly, the evidence that caspase-3 activation also occurs in neuronal necrosis (Niquet et al., 2004) is in accordance with the general consensus that transmission electron microscopy is the most reliable identification method (Otsuki et al., 2003). This dilemma on whether apoptotic or necrotic mechanisms prevail in autoimmune brain is further complicated by markers of excitotoxic and/or pro-oncotic signaling. In particular, significant increases in glutamine, glutamate and lactate concentrations have recently been reported in MRL-lpr brains (Alexander et al., 2005b). In addition to free oxygen species, intracellular glutamine accumulation and release of glutamate are known to result both in apoptotic and necrotic neuronal demise (Brown and Bal-Price, 2003). However, acute excitotoxic damage is considered physiologically closer to the necrotic end of the apoptosis-necrosis continuum (Martin et al., 1998).

There are numerous factors that can induce excitotoxic damage, and in injured or immunological challenged brain, cytokine-producing microglia play an important role (Piani et al., 1992; Allan and Rothwell, 2001). Together with class II MHC upregulation (McIntyre et al., 1990), deposition of complement proteins C3 and C9 (Alexander et al., 2005a), and increased mRNA expression for pro-inflammatory cytokines in brains of MRL-lpr mice (Tomita et al., 2001a; Tomita et al., 2001b), our results are consistent with the notion of microglia-induced neuronal excitotoxicity. Considering that splenomegaly and serum dsDNA correlated with reduced brain weights within the MRL-lpr group, the autoimmune process may also contribute to the neuropathologcal changes. Densely packed dark cells in the subgranual zone is a potentially important observation in the light

of the evidence that CSF from lupus-prone mice is cytotoxic to proliferating brain cells (Sakic et al., 2005).

In addition to immune-mediated insults, altered production of steroid hormones is likely another important factor in the pathogenic circuitry during systemic autoimmune disease. Plasma corticosterone is chronically elevated in MRL-lpr mice (Lechner et al., 2000), and contrary to its suppressive effect on peripheral inflammation, it may exacerbate excitotoxicity by glutamate accumulation and non-apoptotic death of central neurons (Roy and Sapolsky, 2003; Dinkel et al., 2002). Despite relative colocalization of FJB+ and F4/80+ cells, the present study does not answer whether age-dependant microglial activation causes neuronal demise, reflects a "scavenging response" to necrosis or alternatively, a reparative process in the CNS (Lazarov-Spiegler et al., 1996; Prewitt et al., 1997). Along the same line, as commonly seen in neurodegenerative disorders associated with glutamate receptor-mediated excitotoxicity (Martin et al., 1998), we could not provide conclusive evidence which documents typical modes of cell death in brains of autoimmune mice. The presence of clustered and scattered dark cells may not necessarily indicate the same pathogenic mechanism in major divisions of the brain because peripheral inflammation, permeable blood-brain barrier, disturbed ionic transport, and altered glucose metabolism may individually and/or synergistically compromise survival of mature and immature neurons in different brain regions (Sakic et al., 2005; Maric et al., 2001; Sidor et al., 2005; Vogelweid et al., 1991; Alexander et al., 2005b).

Defining characteristics of typical apoptotic or necrotic cell death were not found in MRL-lpr brains, despite relatively broad EM screening. However, condensation of nuclear and cytoplasmic material, swollen mitochondria and ruffled appearance of cell membranes suggest profound

metabolic perturbations, which may precede excitotoxic/oncotic cell death. This possibility needs to be further explored in a larger sample size, throughout the whole brain, and using a combination of methodological approaches. A better understanding of neuronal death in the MRL model of NP-SLE may provide a basis for novel, non-anti-inflammatory drugs in treating this poorly understood neuroimmunological condition.

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Figure legends

Figure 1. Representative fields of FJB staining in MRL brains. More numerous, brightly stained FJB+ cells suggested a neurodegenerative process in the diencephalon of MRL-lpr mice (A) in comparison to age- and sex-matched MRL +/+ controls (B). Similarly, scattered FJB+ cells were commonly seen in the cortical parenchyma (C) in comparison to comparable areas from control brains (D). Some contiguous Purkinje cells distinctly stained in MRL-lpr mice (E), but were not seen in congenic MRL +/+ controls (D).

Figure 2. Representative fields of F4/80 staining in MRL brains. Intensely stained F4/80+ cells were commonly observed in hypothalmic regions from diseased lupus-prone mice (A), suggesting increased microglial activation. While also seen in control brains, F4/80+ cells appeared less dense (B). Patches of cortical microglia cells were frequently observed in lupus brains (C) in comparison to age-matched controls (D). Although sparse F4/80 staining was seen in the Purkinje layer of the MRL-lpr brain (E), this was not observed in age- and sex-matched MRL+/+ controls (F).

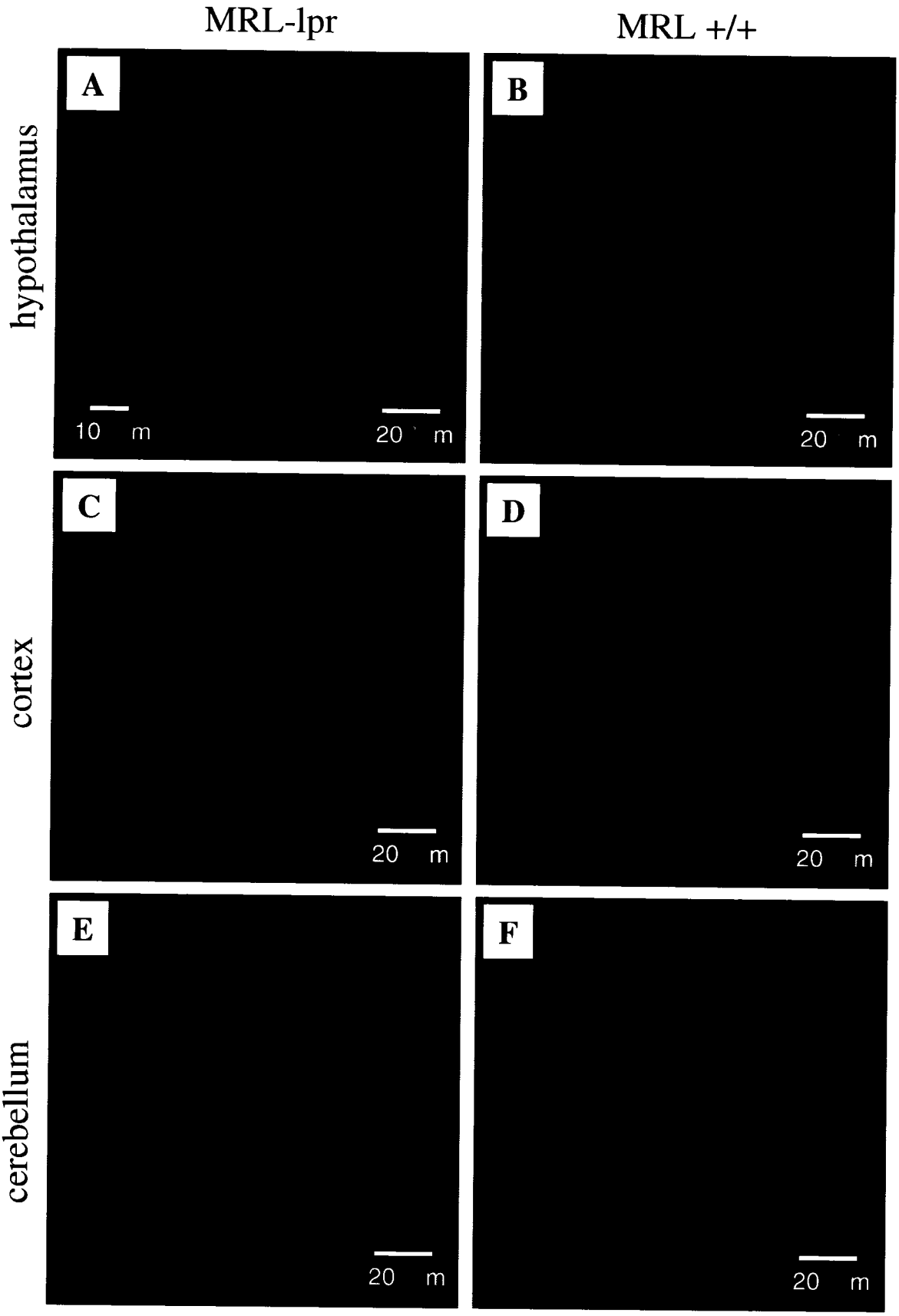
Figure 3. Quantification of degenerating neurons and microglial cells, as revealed by FJB and F4/80 staining. Compared to age-matched controls, the density of FJB+ counts was significantly higher in the hypothalamus of 19-week old MRL-lpr mice than in other groups (A). Similarly, the number of intensely stained F4/80+ cells was increased in MRL-lpr mice in comparison to controls (B). The chronic treatment with IBU did not affect these parameters.

Figure 4. Dot-plot scatter analysis of CD69+F4/80+ cell infiltration in MRL-lpr and MRL+/+ brains pooled at 5, 12 and 18 weeks of age. The percentage of CD69+F4/80+ cells increased from 0.06% to 1.12% in the MRL-lpr groups (A, C, E), while the same population was less consistent (B, F) and less abundant in age-matched MRL+/+ controls (D, F).

Figure 5. Toluidine blue staining of various brain regions inspected by light microscopy. The CA2/CA3 region (not shown) and subgranular zone of the dentate gyrus of two diseased MRL-lpr mice were frequently populated with densely packed, elongated dark cells. Although round, dark cells were clustered in the substanta nigra, they were scattered in the PVN, and Purkinje and granule cell layers of the cerebellum.

Figure 6. H&E staining of dentate gyrus inspected by light microscopy. The subgranular zone of diseased MRL-lpr mice was populated with densely packed basophilic cells in both hemispheres (contralateral hemisphere not shown). Although H&E cannot reliably distinguish degenerating cells from cells in the prophase, the notion of proliferating, immature neurons "at risk" is consistent with our previous reports on hippocampal damage in MRL-lpr mice (Ballok et al., 2004b; Ballok et al., 2003a). *Inset:* the enlarged area showing neighboring cells with dark, basophilic nucleoli.

Figure 7. Electron microscopy (EM) revealing ultrastructural features of dark cells in brain of an MRL-lpr mouse. The increased electron density was unaccompanied by blebbing of cell membranes or internal changes in organelles, other than occasional swelling. **A.** Hypothalamic neuron (N) with densely compacted karyoplasm and cytoplasm, as well as an enlarged Golgi apparatus (GA) and endoplasmatic reticulum (ER); **B.** Densely-packed dark cells between healthy neurons (N) in the subgranular zone; **C.** Cerebellar neuron with condensed cytoplasm, swollen mitochondria (M) and ruffled outer membrane; **D.** Dark neuron (N) surrounded by healthy-looking satellite oligodendrocyte (SO) and shrunken glial cell (G); **E.** Hippocampal oligodendrocyte (O) with electron-dense cytoplasm and karyoplasm. No evidence of apoptotic bodies, nuclear fragmentation, or ruptured membranes could be observed in any preparation.



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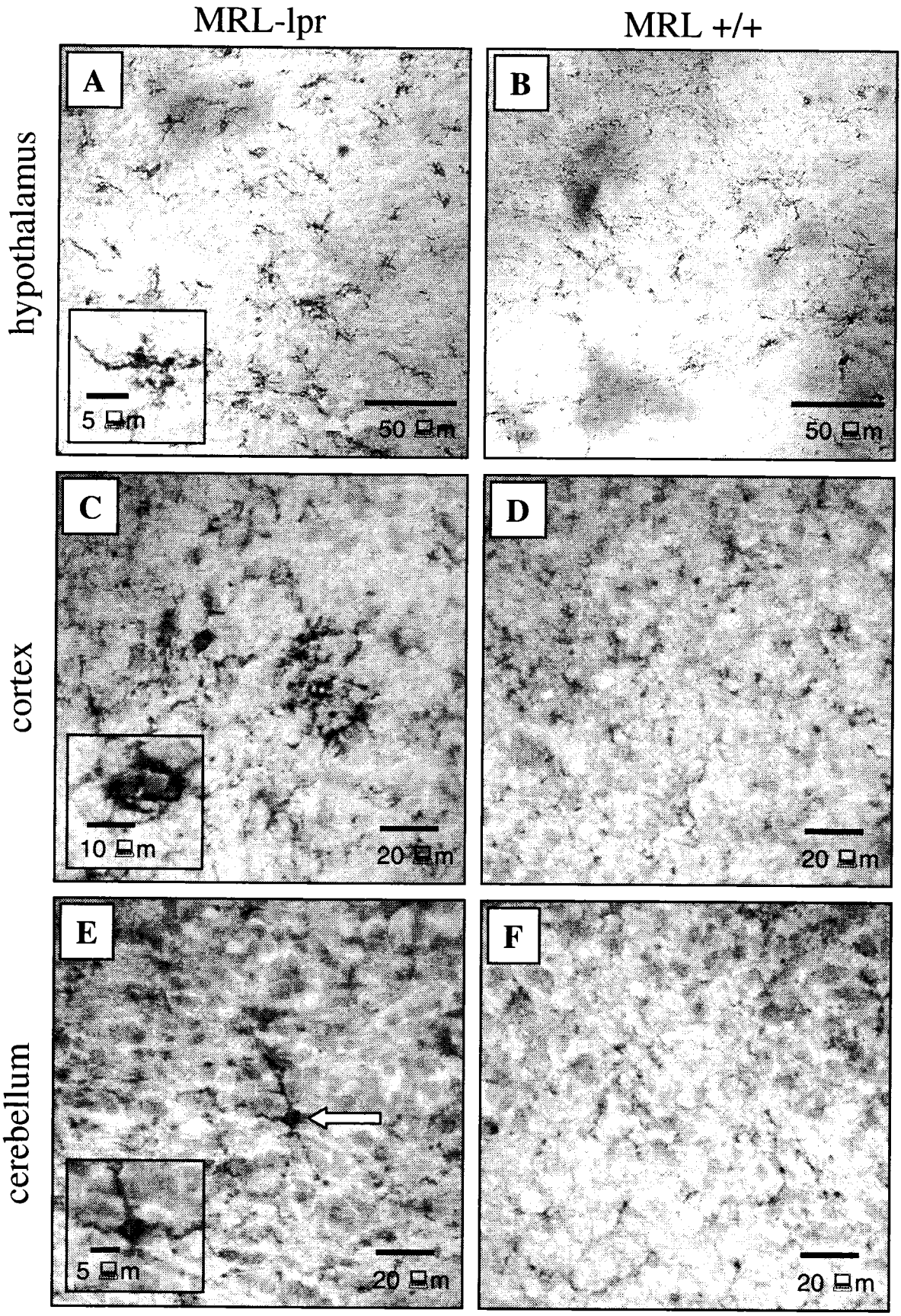

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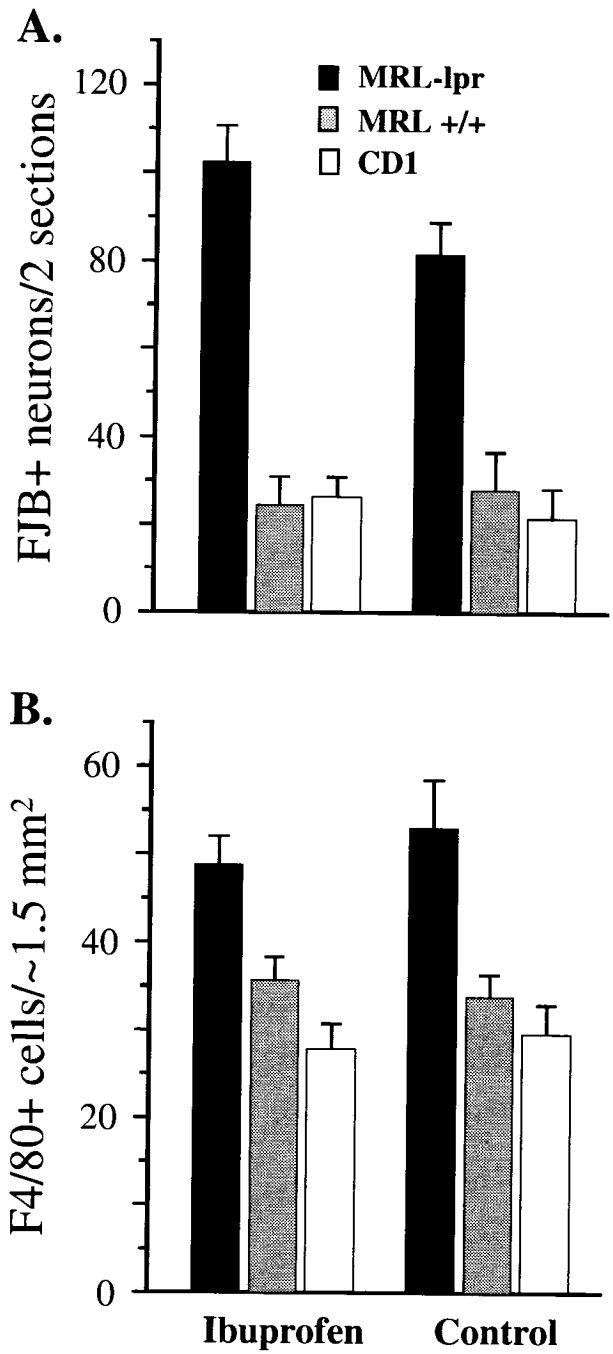
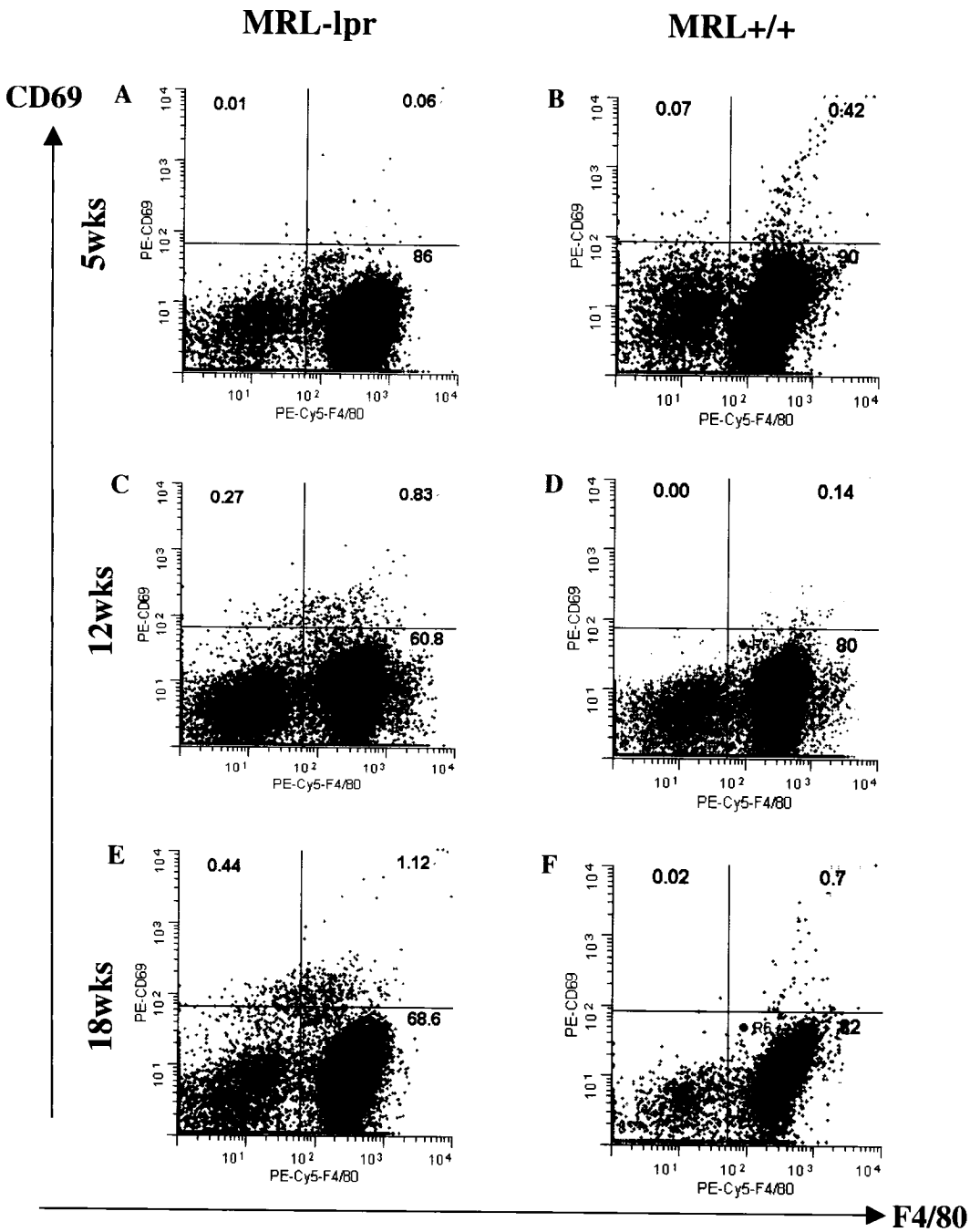


Ballok et al. 2006, **Figure 3.**



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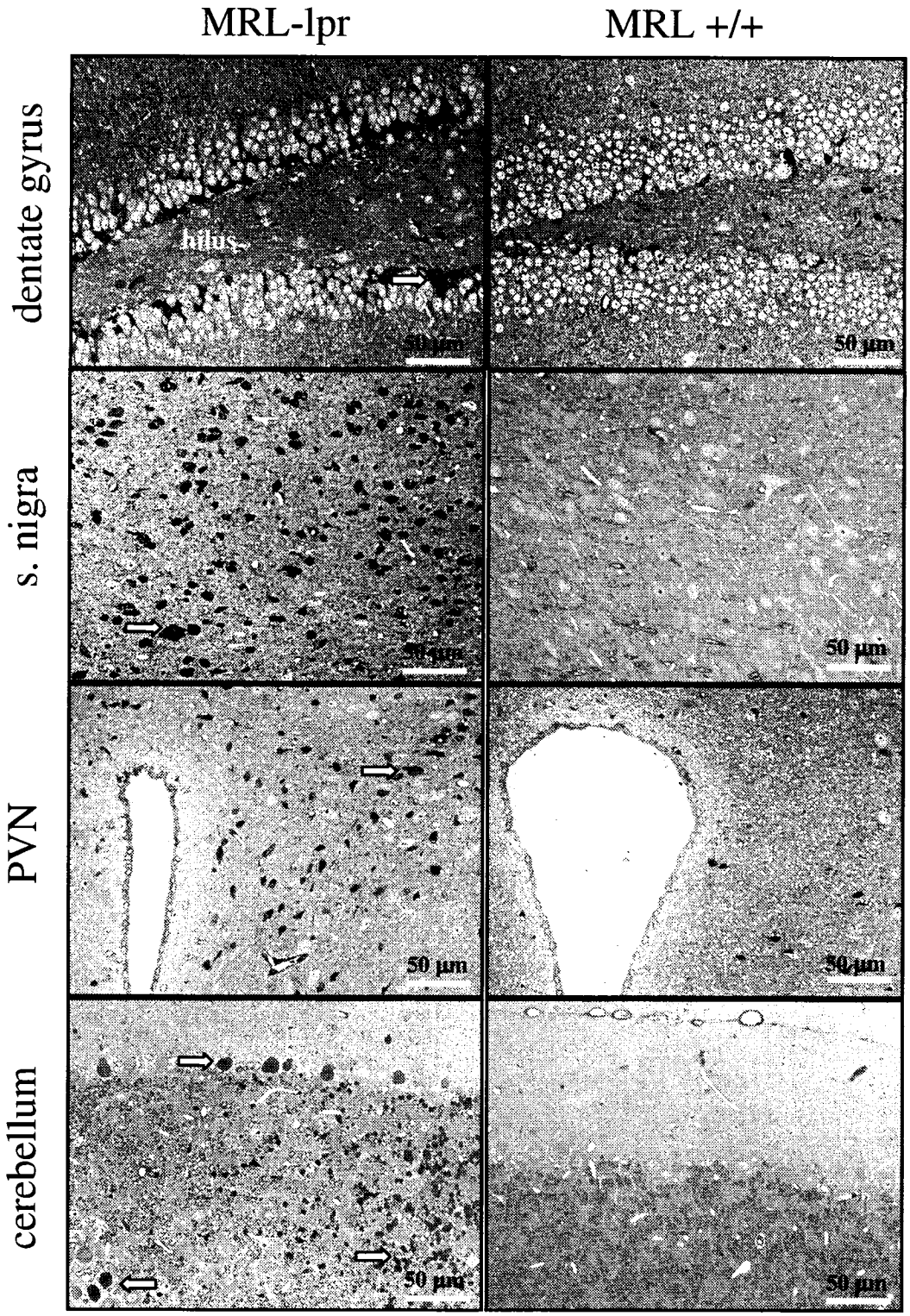

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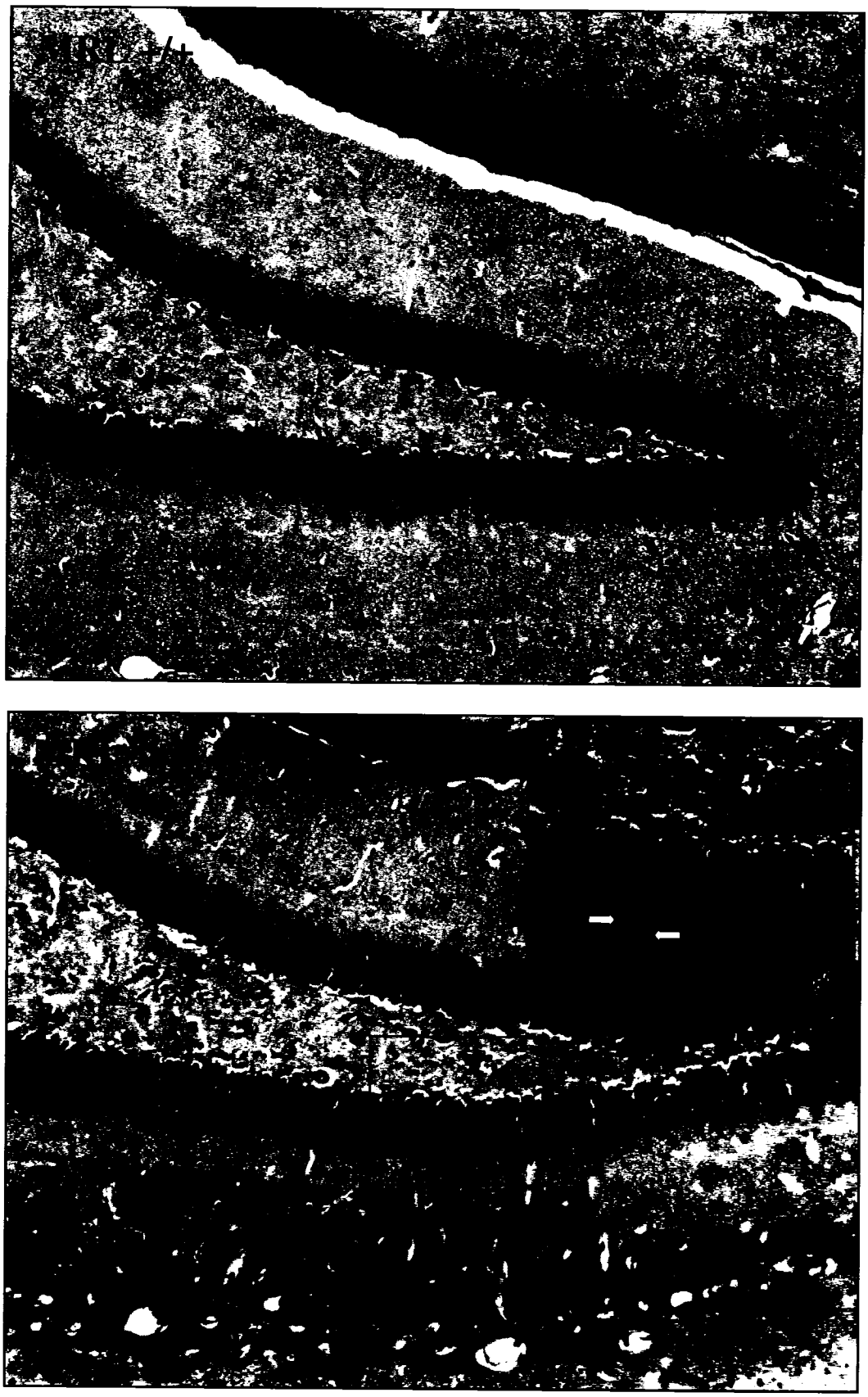

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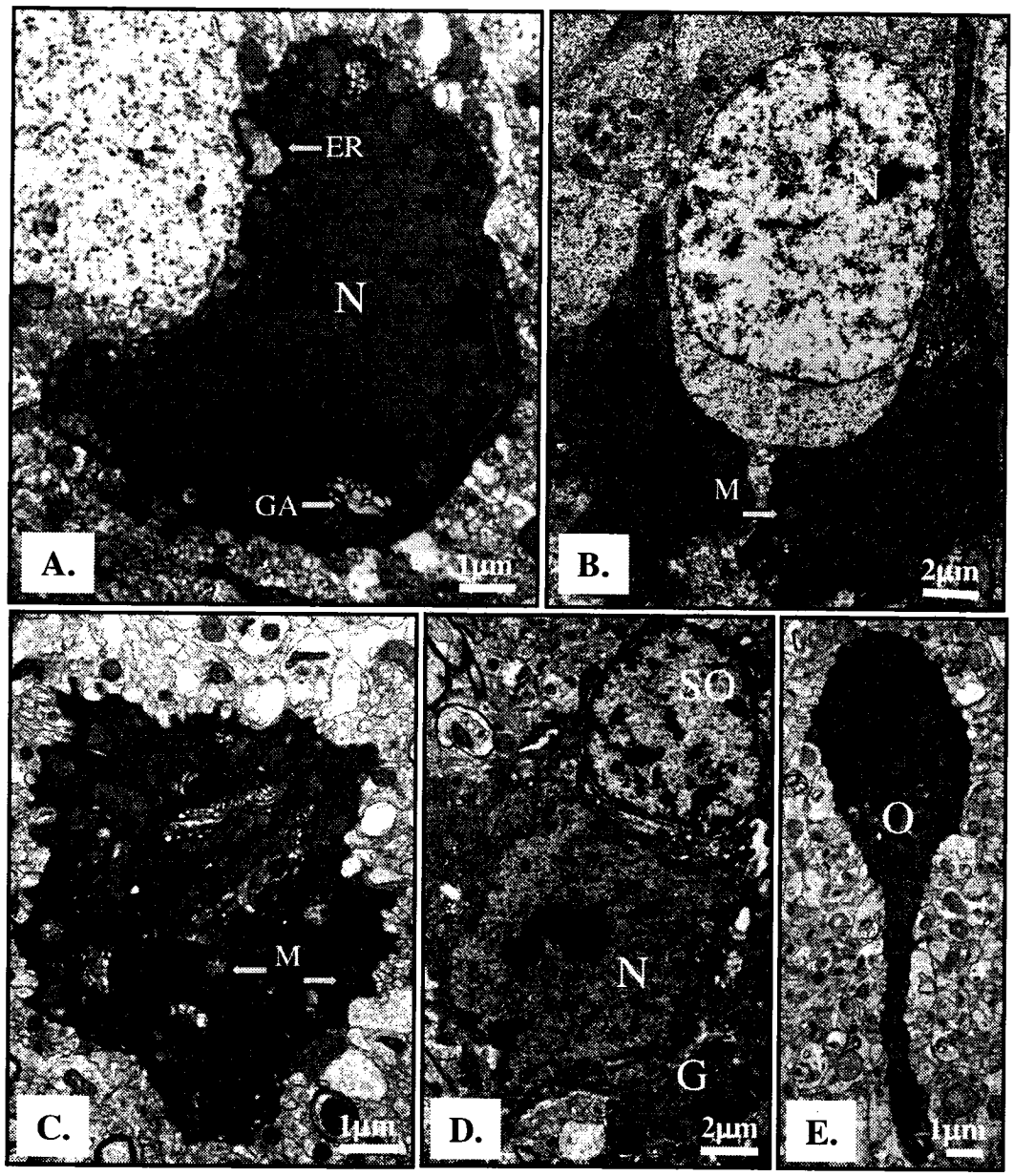

Ballok et al. 2006, **Figure 7.**

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CHAPTER 5
IMPAIRED RESPONSE TO AMPHETAMINE AND NEURONAL DEGENERATION IN THE NUCLEUS ACCUMBENS OF AUTOIMMUNE MRL-LPR MICE

Published in Behavioural Brain Research in 2006

David Ballok's contribution:

I am the second author on this paper. Other experimenters designed the study, administered treatment, and collected the behavioural data. I was involved with the animal surgeries which included perfusions, blood collection and organ extractions. I was also responsible for processing brain tissues and performing the cytochemical (FJB) staining. An experimenter blind to sample origin quantified cell numbers manually. I assisted in preparing figures for the paper and provided ideas and extensive feedback during the preparation of the article.

Dear Steph Smith,

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Research report

Impaired response to amphetamine and neuronal degeneration in the nucleus accumbens of autoimmune MRL-lpr mice

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Abstract

Spontaneous development of lupus-like disease in MRL-lpr mice is accompanied by a constellation of behavioral deficits, including blunted responsiveness to sucrose. Although autoimmunity-induced damage of limbic areas is proposed to underlie this deficit, the systemic nature of the disease precludes inference of a causal relationship between CNS damage and functional loss. Based on the stimulatory effects of *d*-amphetamine sulfate (AMPH) on sucrose intake, the present study pharmacologically probes the functional status of central dopaminergic circuits involved in control of behavioral reward. The response rates were compared between diseased MRL-lpr mice and congenic MRL +/+ controls tested in the sucrose preference paradigm. Neuronal loss was assessed by Fluoro Jade B (FJB) staining of nucleus accumbens and the CA2/CA3 region. While control mice significantly increased intake of sucrose solutions 60 min after administration of AMPH (i.p., 0.5 mg/kg), the intake in drugged MRL-lpr mice was comparable to those given saline injection. Increased FJB staining was detected in the nucleus accumbens and hippocampus of diseased mice, and AMPH treatment neither altered this nor other measures of organ pathology. The results obtained are consistent with previously observed changes in the mesolimbic dopamine system of MRL-lpr mice and suggest that the lesion in the nucleus accumbens and deficits in dopamine release underlie impaired responsiveness to palatable stimulation during the progress of systemic autoimmune disease. As such, they point to a neurotransmitter-specific regional brain damage which may account for depressive behaviors in neuropsychiatric lupus erythematosus.

Keywords: Lupus; Autoimmunity; Limbic system; Nucleus accumbens; Amphetamine; Motivated behavior; Depression; Sucrose intake; MRL model

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic, multisystemic autoimmune disease that is characterized by the damage of many vital organs, including the brain [15]. In addition to serological manifestations (e.g. imbalanced cytokine network and increased production of autoreactive antibodies), significant number of SLE patients develop various neurologic and psychiatric (NP) symptoms, ranging from seizures and strokes to depression, anxiety, and psychosis [16]. The most frequent psychiatric symptoms include depression and anxiety [71], of which lower mood often heralds clinical manifestations of the disease [67].

Similar to humans, the MRL/MpJ-Fas^{lpr}/J (MRL-lpr) mice spontaneously develop an accelerated form of lupus-like disease accompanied by CNS involvement. In comparison to age-matched congenic MRL/MpJ (MRL +/+) controls, a substantial proportion of MRL-lpr mice develop deficits reflective of goal-directed and motivated behavior. They include blunted responsiveness to sucrose and saccharine solutions [6,43], impaired exploration of novel environments [50,52], impaired isolation-induced aggressiveness [44], and increased immobility (floating) in the forced swim test [53]. The constellation of behavioral deficiencies has been operationally termed "autoimmunity-associated behavioral syndrome" (AABS) and it coincides with a profound divergence in the immune statuses of the two MRL substrains around 7–8 weeks of age [52,54]. Increased neurodegeneration (as revealed by Fluoro Jade B, FJB staining), reduced dendritic complexity and density of pyramidal neurons are common observations in brains of diseased MRL-lpr mice [5,46,49]. Brain growth appears retarded [49] and ventricles increase in size along an early and accelerated development

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of autoimmune manifestations [17]. Taken together, there is considerable evidence that systemic inflammation and autoimmunity induce degeneration of central neurons, thus likely forming the structural basis of behavioral deficits in the MRL-lpr substrain.

Although the contribution of peripheral disease manifestations on behavioral performance could not be excluded, blunted responsiveness to palatable stimulation pointed to the mesolimbic dopamine system as one of the targets of the systemic autoimmune/inflammatory disease. It is well-documented that this system plays a significant role in goal-directed and reward-mediated behavior [74], and that the nucleus accumbens (NAc) is one of the primary reward centers, receiving dopaminergic inputs from ventral tegmental area, VTA [66]. Not surprisingly, the VTA of diseased MRL-lpr mice brains shows increased number of FJB-positive neurons and reduced staining for tyrosine-hydroxylase, TH [4]. Post-mortem analysis of brains from MRL-lpr mice by HPLC reveals significant imbalances in dopamine and 5-HT contents in the paraventricular nucleus [47]. These changes coincide with aberrant performance on the sucrose preference test, thus suggesting that lesions in the mesolimbic dopamine system contribute to impaired motivated behavior.

We presently use the appetitive response to sucrose and central psychostimulant to pharmacologically probe the functional status of the NAc dopamine system. In particular, sucrose licking was shown to be associated with release of DA from NAc and pharmacological blockade of DA turnover augmented the licking response [22]. Diseased MRL-lpr mice were challenged with the *d*-amphetamine sulfate (AMPH), known to be effective in activating central dopaminergic circuits within major compartments of the brain reward system [38], including the NAc [26]. The rationale for using *d*-amphetamine sulfate was that it has the capacity to significantly increase sugar intake in rodents [11,19]. In addition, in rats that are high responders, sugar consumption correlates significantly with AMPH-stimulated accumbens-dopamine overflow [60]. These studies also suggested that the responsiveness to sucrose reflects the NAc dopaminergic response to the AMPH treatment. Our overall expectation was that similar stimulation with AMPH will produce an attenuated effect on sucrose consumption in diseased MRL-lpr mice due to dysfunctional and/or damaged mesolimbic dopamine pathways. The results obtained contribute to the nature of the sucrose preference deficit by documenting a centrally-mediated mechanism and neurodegenerative process in the nucleus accumbens of autoimmune MRL-lpr mice. They also suggest that spontaneous progress of SLE-like disease is detrimental to the reward system function, as revealed by the lack of responsiveness to amphetamine challenge. As such, present observations are consistent with the hypothesis that lupus-like disease compromises dopaminergic neurotransmission in the CNS.

2. Methods

2.1. Animals

Four- to five-month-old MRL-lpr and age-matched MRL +/+ mice were obtained from the breeding colony at McMaster University, with the original stock purchased from The Jackson Laboratories (Bar Harbor, ME). Mice were housed singly with 8:00 a.m.–8:00 p.m. light schedule and ad lib access to food and water. The temperature of the colony room was kept at 25 ± 2 °C with a relative humidity of $45 \pm 5\%$. In Experiment 1, 10 age-matched females of each substrain were used, while the groups of males in Experiment 2 were as follows: MRL-lpr AMPH ($n = 10$), MRL-lpr saline ($n = 8$), MRL +/+ AMPH ($n = 10$) and MRL +/+ saline ($n = 10$). The experimental protocols were approved by the McMaster Animal Care Committee and carried out in accordance with rules and regulations of the Canadian Council of Animal Care.

2.2. Drug administration

d-Amphetamine sulfate (AMPH, Sigma–Aldrich Canada, Oakville, Ont.) was dissolved in 0.9% saline and 0.5 mg/kg was injected i.p. using a 26.5 gauge needle (5 ml/kg of body weight; e.g., 0.2 ml/40 g mouse). This dose was chosen based on a previously reported effect [11] and our pilot study where 0.5 and 1.5 mg/kg doses were compared in a small cohort of mice exposed to the sucrose preference test [41].

2.3. Sucrose preference test

Reduced preference for sweet solution (chocolate) was first noted in one of the pioneering studies on behavior of MRL-lpr mice [21]. This phenomenon was further explored using the sucrose preference paradigm (proposed to measure sensitivity to reward), and as such a reduction in sucrose intake was taken to have face validity to anhedonia (loss of interest or pleasure) in human depression [73]. More extensive analysis of the dose-dependent performance, post-ingestive factors, and taste responsiveness has been performed by our group in previous studies [6,43,48]. Based on an established methodology, mice were presently trained to drink 3 ml of a 4% sucrose solution from a graduated syringe fastened to the cage lid with a 2.5 in. paper clip. They had 24 h access to sucrose over 3 days, and free access to food and water. The solution was then removed for 24 h to allow sucrose to clear from circulation. Two cohorts of mice were used to examine whether substrain-specific responsiveness to AMPH is general, or depends on gender, time between AMPH administration and exposure to sucrose, and/or order of sucrose concentrations. In Experiment 1, mice were first injected with 0.9% saline 60 min (~19:30 h) before the 1 h sucrose preference test (20:30–21:30 h) was given over four consecutive nights. Each night syringes were filled with one of four sucrose solutions, presented in ascending order (i.e. 1, 2, 4, or 8%). Upon the assessment of "baseline" performance, mice were given for 3 days tap water only. Following this break, animals were injected with AMPH 60 min prior to testing and the sucrose test was conducted in the same manner as described above. In Experiment 2, separate cohorts of mice (as described above) were used to control for a possible gender-specific difference [14], circulating levels of AMPH, and the "carry-over" effect noted when mice were exposed for prolonged periods of time to sucrose solutions. Namely, as in other healthy mice, the MRL +/+ mice tend to increase sucrose intake over a 10-day period [51]. In summary, this design differed from Experiment 1 such that males were injected with AMPH 12 h before the test, "baseline" performance was not measured, and sucrose solutions were given in random order to minimize "carry-over" and "learning" effects. In addition, a 0.5% dose of sucrose was included to better estimate responsiveness to low concentrations. During all testings, mice had uninterrupted access to bottles filled with fresh tap water.

2.4. Tissue preparation

Upon completion of the sucrose preference test (16–23 weeks of age), mice from Experiment 2 were sacrificed for the purpose of brain and spleen collection and weighing. They were anaesthetized with Somnotol (60 mg/kg) and transcardially perfused with 40 ml of 0.9% saline. Extracted brains were immersed into 4% paraformaldehyde (PFA) for fixation at 4 °C for 2 days, transferred to 30% sucrose (in PBS) for 3 days, and frozen by immersion in isopentane (cooled in liquid nitrogen) before cutting. Horizontal sections (~3.16 mm interaural and −6.84 mm Bregma) were cut with a Jung Frigocut 2800 E cryostat to concurrently obtain workable fields of the NAc and CA2/CA3 region. They were subsequently placed on APTEX-coated glass microscope slides, and left to dry at room temperature for 24 h before processing.

2.5. FJB staining procedure

The Fluoro Jade B (FJB) stain has an affinity for the entire degenerating neuron regardless of the type of cell death [29,56]. Despite incomplete knowledge of the staining mechanisms, the FJB method shows high reliability in the detection of dying neurons [75].

Brain sections were processed according to the previously published protocol [5]. The staining solution was a 0.001% FJB in 0.1% acetic acid (prepared from a 0.01% stock solution, Histo-Chem Inc., Jefferson, AR). Slides were processed in three 2 min xylene washes before being coverslipped with DPX (Sigma Chem. Co., St. Louis, MO). The FJB reactivity in the mesolimbic system was quantified using a Zeiss Laser Scanning Confocal Microscope (LSM 510, Carl Zeiss Inc.) argon laser (wavelength 488 nm). Confocal micrographs were obtained using a Fluar 20×/0.75 objective in combination with a 1024 × 1024 pixel resolution, and saved in the TIFF format. FJB-positive neurons were counted manually from TIFF files by an unbiased observer using standard imaging software (Adobe Photoshop 7).

2.6. Statistical analysis

In Experiment 1, the data were analyzed using an ANOVA with substrain as a main factor and treatment and concentration as repeated measures. In Experiment 2, in addition to substrain, treatment was considered as a main factor in an ANOVA with repeated measures (concentration). Significance level was set at $p < 0.05$ and all computations (including Pearson's correlation) were performed using the SPSS 13 statistical package. One mouse in each AMPH-treated group died prematurely thus reducing the sample size for neuropathological analysis to $N = 36$. Graphs show means ± S.E.M.

3. Results

As expected [43], MRL-lpr females (injected with Sal) showed a lower intake of sucrose in comparison to the MRL +/+ group (substrain, $F(1, 18) = 9.104$, $p < .01$; Fig. 1A). However, this difference was exacerbated when Sal was replaced with AMPH (substrain by treatment, $F(1, 18) = 8.561$, $p < .01$; Fig. 1B). Since substrain by treatment by concentration interaction was not significant ($F(3, 54) = 0.753$), the assumption was that when 0.5 mg/kg of AMPH was injected 60 min before the preference test, MRL +/+ mice increased sucrose intake comparably at all concentrations. In contrast, the MRL-lpr group failed to show enhanced response to AMPH.

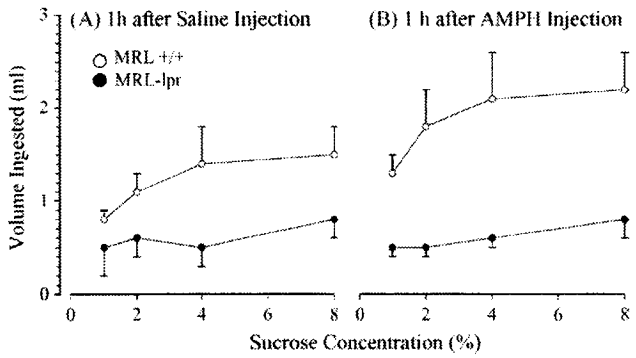

Fig. 1. Consumption of sucrose in the brief sucrose preference test 60 min after i.p. injections. (A) Diseased MRL-lpr females injected with saline (Sal) showed a lower intake of sucrose in comparison to age-matched MRL +/+ controls. (B) This substrain difference was exacerbated when the same group of animals was injected with *d*-amphetamine (AMPH, 0.5 mg/kg b.w.), as MRL +/+ mice significantly increased their performance and MRL-lpr mice showed intake comparable to performance after Sal injections.

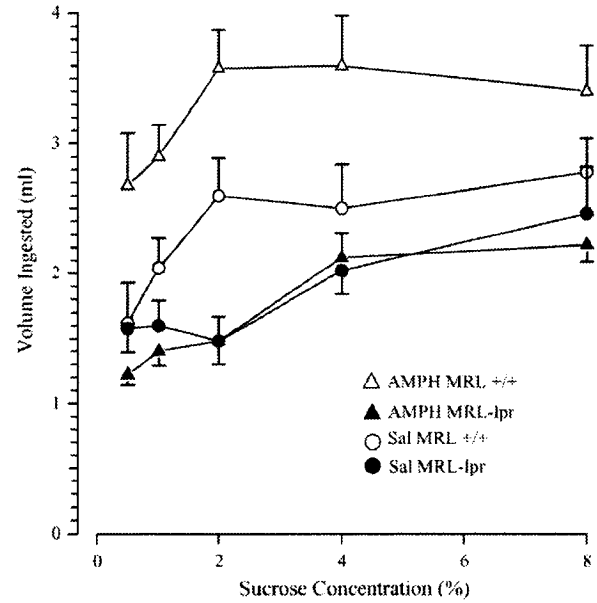

Fig. 2. Sucrose intake in diseased MRL-lpr and age-matched MRL +/+ males. As expected, Sal-treated MRL +/+ controls had higher consumption than diseased, Sal-treated MRL-lpr mice. Although administration of AMPH further increased the intake in the MRL +/+ substrain, it had no effects in the AMPH-treated MRL-lpr group tested at different sucrose concentrations.

In Experiment 2, a similar phenomenon was observed without exposing mice to sucrose beforehand (the "baseline" assessment), despite the fact that AMPH was injected 12 h before the preference test, and that sucrose concentrations were randomized (substrain by treatment, $p < .02$; Fig. 2). The intake of sucrose solutions was generally higher in the second experiment, likely reflecting higher demands for liquids in bigger males [48]. Increased spleen weight (substrain: $F(1, 33) = 18.822$, $p < .001$; Fig. 3A) and lower brain weight in diseased MRL-lpr mice (substrain: $F(1, 33) = 59.855$, $p < .001$; Fig. 3B) confirmed the autoimmune status and brain atrophy in this substrain. As observed earlier [7], the hippocampal CA2/CA3 region showed an increased number of FJB+ neurons in MRL-lpr brains (substrain: $F(1, 31) = 39.034$, $p < .001$) and this was not altered by

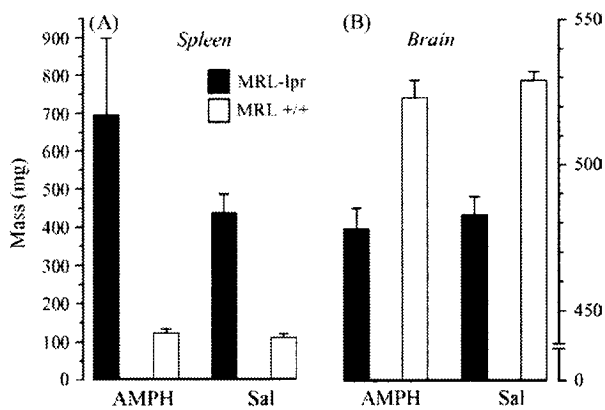

Fig. 3. Substrain-related differences in spleen and brain weights. (A) Increased spleen weight and (B) lower brain weight confirmed autoimmune status and brain atrophy in diseased MRL-lpr mice.

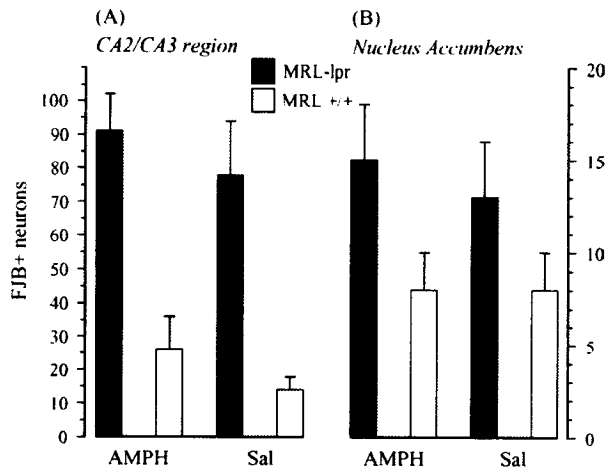

Fig. 4. Significant increase in the density of FJB+ neurons was observed in the hippocampus CA2/CA3 region and nucleus accumbens of the MRL-lpr group, suggesting an enhanced neurodegenerative process in limbic structures during a more severe development of lupus-like disease.

the AMPH treatment (Fig. 4A). A similar drug-independent neurodegenerative process was observed in the NAc (substrain: $F(1, 31) = 5.674$, $p < .05$; Fig. 4B) and correlated with the reduced brain mass in MRL-lpr mice ($r_{15} = -.0488$, $p < .05$). The staining method and horizontal sectioning used in the present study did not allow us to clearly delineate the shell and core regions of the NAc. Consequently, a whole count in the NAc region was taken and a representative image of brightly lit FJB+ neurons is shown in Fig. 5.

4. Discussion

Amphetamine increases release of catecholaminergic neurotransmitters [28] and blocks their reuptake by presynaptic axonal terminals [27]. Although the pharmacological effects of *l*-AMPH and *d*-AMPH involve both norepinephrine and dopamine release, behavioral effects of *d*-AMPH are largely mediated via the central dopamine system [42]. Amphetamine easily crosses the blood–brain barrier [42] and exerts stimulatory effects in the CNS, including limbic structures [37]. In the current experiment we used *d*-AMPH sulfate to probe the central dopaminergic system and further test the hypothesis that dysfunctional mesolimbic dopamine pathways mediate impaired motivational behavior of lupus-prone animals. Indeed, the results obtained reveal that diseased MRL-lpr mice fail to increase sucrose intake in response to systemic injection of *d*-AMPH sulfate repeatedly administered in two different experimental designs. This was consistent with evidence that D1 receptor-deficient mice [18] and mice that cannot synthesize DA demonstrate deficits in goal-directed response to sucrose [12]. Increased FJB staining in the NAc of MRL-lpr animals suggests neuronal degeneration in this region, known to have a significant role in control of the neural reward circuitry [74]. Viewed from a more general perspective, obtained results imply that at least some deficits in behavioral performance of diseased MRL-lpr mice are not an epiphenomenon due to peripheral symptomatology.

The midbrain has a significant role in goal-directed and reward-mediated behavior [57], and dopaminergic inputs from the ventral tegmental area (VTA) are important in activity of the NAc [74]. Consumption of food increases the release of dopamine in the NAc of rodents [8] and this release is mediated by the VTA [62]. Similarly, consumption of sucrose has been shown to increase the release of DA in the NAc [22–24]. In the present study neurodegenerative changes in the NAc may provide a structural basis of impaired response to sucrose in autoimmune MRL-lpr animals. Signs of neurodegeneration of dopaminergic neurons in the VTA has been found in diseased animals in the past [4], potentially impairing activity of the NAc and behavioral performance in MRL-lpr animals. Neural degeneration in the CA2/CA3 region was not affected by repeated administration of *d*-AMP and the severity of damage is consistent with previous findings [7]. The NAc receives glutamatergic inputs from a variety of sources, including the limbic neocortex, the ventral subiculum and hippocampal formation, the amygdala and the dorsal medial thalamus [40]. Hippocampal input appears to play a critical role in modulating resting membrane potential of medium spiny neurons, the output neurons of the NAc [39]. Our previous studies revealed that neuronal complexity and spine density of hippocampal neurons is profoundly reduced by the onset of systemic autoimmunity

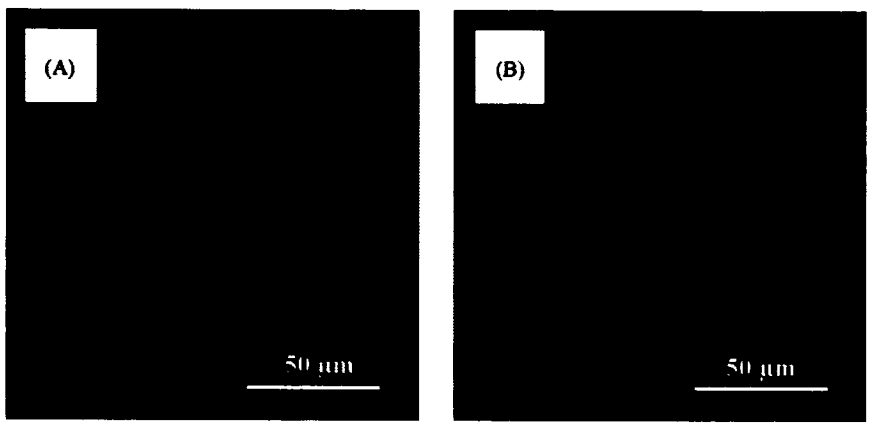

Fig. 5. Representative images (obtained by a confocal microscope) of the horizontal sections of the nucleus accumbens (NAc) in autoimmune mice (A) and control mice (B). Numerous brightly stained FJB-positive neurons confirmed a degenerative process in MRL-lpr brains in comparison to asymptomatic MRL +/+ controls.

and inflammation in MRL-lpr mice [46,49]. Taken together, degeneration in the NAc, VTA, and hippocampus may jointly contribute to the behavioral impairment of MRL-lpr animals in the sucrose preference paradigm. Conversely, increased sucrose intake in AMPH-treated MRL +/+ controls might be associated with increased spine density in the hippocampus, as shown in the CA1 region after AMPH self-administration and sucrose-reward experience [13].

The progress of lupus-like disease in the MRL-lpr substrain is accompanied by elevated serum levels of pro-inflammatory cytokines, such as IL-1, IL-6 and TNF-alpha [63,65]. Given their well-documented capacity to alter motivated behavior and emotional reactivity [2], one can assume that circulating cytokines contribute to impaired performance in the sucrose preference test. Indeed, we show this relationship in our previous studies [6,45,48,51], which did not involve assessments of neurodegeneration. However, the precise mechanisms by which cytokines can impair motivated behavior in the MRL model is far from being understood because of their multiple effects on system organs and the complexity of lupus-like disease. Namely, it is known that exogenous administration or increased endogenous synthesis of interleukin (IL)-1, IL-6, TNF-alpha and interferons can activate major endocrine pathways, alter metabolism of precursor molecules and affects metabolism of central neurotransmitters [3], including the dopamine system [36,58,76]. In addition to hypothalamus and hippocampus, dopamine metabolism in the nucleus accumbens can be significantly affected by systemic administration of IL-6 [61] or lipopolysaccharide, a non-specific activator of pro-inflammatory cytokine release [10]. In additional to "functional damage", several lines of evidence suggest that cytokines promote neurodegenerative process when the brain is injured [1,20,25,34,68]. Given that the blood–brain barrier is breached at an early age [59,69] and that the HPA axis is dysfunctional in diseased MRL-lpr mice [30,32,33], it is presently difficult to dissociate central from peripheral effects, or functional impairment from structural damage produced by neuroactive cytokines.

Secretion of the pituitary hormone prolactin is under the control of dopamine. Given that elevated secretion of prolactin is common in SLE [31], one may wonder whether this endocrine imbalance is a consequence of impaired dopamine regulation in the CNS. Although neuronal damage has been recently reported [64], there is no evidence on whether dopaminergic neurons die excessively in neuropsychiatric SLE (NP-SLE). Consistent with this notion, the dopamine agonist bromocriptine suppresses secretion of prolactin and ameliorates affect in SLE patients [70] and disease activity in autoimmune mice [35]. If the loss of dopaminergic neurons occurs in human and animal forms of SLE, an immediate question would be whether endocrine, immune, or other factors induce neurodegeneration. Our previous studies suggest that dopamine accumulates in the paraventricular nucleus and median eminence/arcuate nucleus areas [47]. Even if increased intracellular levels of dopamine are neurotoxic [9], the dilemma about factors that lead to dopamine accumulation would still remain. One possibility is that the enzymatic system is affected by the autoimmune process (such as autoantibodies binding to and inactivating different kinases, transferases, proteases, etc.). Another mechanism may include sustained binding of corticosteroids [33,72] and increased vulnerability of neurons to various metabolic insults [55]. However, at this stage of knowledge conclusive statements about an etiologically and clinically complex condition such as NP-SLE would be premature.

In summary, the present study reveals profound differences in response to palatable stimulation between MRL-lpr and MRL +/+ mice when their dopamine system is pharmacologically probed. More importantly, significant differences in the number of dying neurons are observed in the NAc, a neural area known to be involved in reward modulation. These results are consistent with the hypothesis that the progression of autoimmune disease impairs motivated behavior by producing a lesion in the dopaminergic reward system. However, there are other neuronal systems that innervate and originate from the NAc and are able to modulate responsiveness to palatable stimulation [11]. Whether they are also affected by the progress of spontaneous systemic autoimmunity and inflammation needs to be examined in future studies.

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CHAPTER 6
AUTOIMMUNE-INDUCED DAMAGE OF THE MIDBRAIN DOPAMINERGIC SYSTEM IN LUPUS-PRONE MICE

Published in Journal of Neuroimmunology in 2004

David Ballok's contribution:

I am the first author on this paper. I designed and planned the experiment. I supervised and assisted in the animal behavioural testing, administered the dopamine agonist and immunosuppressive treatment, and conducted the animal surgeries which included perfusions, blood and CSF collection, and organ extractions. I was responsible for performing the ELISAs for serum (ANA and TNF-alpha) and CSF (TNF-alpha), as well as the cytochemical (FJB) staining of the brains. Technicians ignorant to group origin scored circling behaviour from video recordings, and manually quantified cell numbers. A co-author unaware of the study design performed TH immunostaining and performed the subsequent quantitative and qualitative assessment of TH+ cells. Another co-author unaware of group origin assessed BRA in serum. A technician blind to group origin assessed CSF toxicity to C17.2 stem cells. I completed the required statistical analyses for this study. The figures and tables are my own work and although feedback was provided by other authors, it was my responsibility to write the article.

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Autoimmune-induced damage of the midbrain dopaminergic system in lupus-prone mice

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Abstract

Spontaneous development of lupus-like disease is accompanied by impaired dopamine catabolism and degenerating axon terminals in the mesencephalon of MRL-lpr mice. We presently examine the hypothesis that systemic autoimmunity affects the central dopaminergic system in behaviorally impaired animals. The functional damage of the nigrostriatal pathway was assessed from rotational behavior after a single injection of the D1/D2-receptor agonist apomorphine. Neurodegeneration in the midbrain was estimated by Fluoro Jade B (FJB) staining. The causal role of autoimmunity was tested by comparing asymptomatic and diseased MRL-lpr mice, and by employing the immunosuppressive drug cyclophosphamide. Damage of dopaminergic neurons was assessed by tyrosine-hydroxylase (TH) staining of the midbrain. Apomorphine induced significant asymmetry in limb use, which lead to increased circling in the diseased MRL-lpr group. While FJB-positive somas were not seen in the striatum, increased staining in the substantia nigra (SN) and ventral tegmental area (VTA) were detected in behaviorally impaired MRL-lpr mice, but not in age-matched controls. Reduced brain mass and increased levels of TNF-α in their cerebrospinal fluid (CSF) suggested cerebral atrophy and inflammation. In addition, CSF was neurotoxic to a dopaminergic progenitor cell line. Immunosuppression attenuated CSF cytotoxicity, TNF-α levels, and midbrain neurodegeneration. Supportive of the notion that dying neurons were dopaminergic, the SN of autoimmune mice showed approximately a 35% reduction in the number of TH-positive cells. A three-fold increase in serum brain-reactive antibodies accompanied this loss. Although the source of toxic mediator(s) remains unknown, present results are consistent with the hypothesis that autoimmunity-induced destruction of mesonigral and mesolimbic dopaminergic pathways contributes to the etiology of aberrant behavior in an animal model of neuropsychiatric lupus.

Keywords: Autoimmunity; Inflammation; Lupus; Dopamine; Tyrosine hydroxylase; Substantia nigra; Cerebrospinal fluid; Cyclophosphamide; Neural progenitor cells; Fluoro Jade B; Brain-reactive autoantibodies; MRL mice; Behavior; Ventral tegmental area; TNF-α

1. Introduction

In the systemic autoimmune/inflammatory disease lupus erythematosus (SLE), involvement of the CNS is well recognized and frequently includes deficits in neurologic function, cognition, and affect (Denburg et al., 1993; Wekking, 1993). Although a pathologic neuro-immuno-endocrine circuitry has not yet been elucidated, significant loss of central neurons seems to underlie changes in sensorimotor function and behavior in many SLE patients (Brooks et al., 1997; Sibbitt and Sibbitt, 1993). The MRL-MpJ-Tnfrsf6^{lpr} (MRL-lpr) murine substrain spontaneously develops systemic autoimmune disease with clinical and serological manifestations reminiscent of human SLE (Andrews et al., 1978). Due to a *lpr* mutation on chromosme 19 and dysfunctional Fas receptor in negative selection of autoreactive T cells (Watanabe-Fukunaga et al., 1992a,b), MRL-lpr mice develop florid disease by 3 months, with few surviving beyond 6 months of age (Dixon et al., 1978; Andrews et al., 1978). Age-matched congenic MRL-MpJ+/+(MRL+/+) controls develop a mild form of the disease and have a life span of up to 2 years (Theofilopoulos, 1992).

In addition to the immunological profile, their similar genetic background, appearance, size and reproductive age render these two MRL substrains a useful preparation to examine mechanisms by which chronic autoimmunity and

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inflammation damage brain morphology and function (Alexander et al., 1983). More specifically, the accelerated autoimmune manifestations in MRL-lpr coincide with a deviation in their behavioral performance from congenic controls. The constellation of performance deficits in motivated behavior, emotional reactivity and learning/memory capacity in MRL-lpr mice (Szechtman et al., 1997) has been operationally defined as 'autoimmunity-associated behavioral syndrome' or AABS (Sakic et al., 1992).

Although the MRL strain does not show a high incidence of congenic brain abnormalities (Sherman et al., 1990), significant changes in brain morphology have been observed at the onset of lupus-like disease in the MRL-lpr substrain. They include enlarged cerebral ventricles (Denenberg et al., 1992), lymphocyte infiltration into the choroid plexus and brain parenchyma (Vogelweid et al., 1991; Farrell et al., 1997; Hess et al., 1993; Zameer and Hoffman, 2004), a high incidence of apoptotic terminal deoxynucleotidyl transferase-mediated dUTP nick end labeled-positive (TUNEL+) cells (Sakic et al., 2000b), neuronal atrophy (Sakic et al., 1998b), and neuronal limbic degeneration (Ballok et al., 2003a). Regions of the substantia nigra (SN) and ventral tegmental area (VTA) also show a high incidence of ubiquitinated particles when lupus-like disease is florid (Ballok et al., 2004), suggesting disease-associated axonopathy. It has not been determined, however, whether this midbrain lesion involves a genuine neuronal loss, underlies impaired behavioral performance, and is associated with the progress of systemic autoimmunity and inflammation.

The post-mortem analysis by HPLC revealed impaired catabolism of neurotransmitters in brains of MRL-lpr mice (Sakic et al., 2002). Together with the evidence that CSF (and not serum) of many diseased MRL-lpr mice is toxic to cultured neurons (Maric et al., 2001) and a neuronal progenitor line (manuscript submitted), one may wonder whether specific neuronal phenotypes (dopaminergic, serotonergic, glutamatergic, etc.) are affected during sustained activation of the immune system. Considering that the dopaminergic system of the mesencephalon is important in the control of movements, emotion, and motivated behavior (Majovski et al., 1981), we presently examine whether autoimmunity and inflammation are associated with midbrain degeneration and if CSF from MRL-lpr mice is toxic to neural progenitor cells which express a dopaminergic phenotype (Wagner et al., 1999; Yang et al., 2002).

Following damage of the nigrostriatal pathway, D1 and D2 dopamine receptor agonists can induce excessive turning behavior, which can be accounted for by the supersensitivity of persevering cells (Winkler et al., 1988) and an asymmetry of limb use (Ziegler and Szechtman, 1988). To test for the possibility that the SN is damaged in autoimmune mice, we pharmacologically challenged the dopaminergic system and quantified rotational behavior. For the purpose of assessing neuronal degeneration in the SN and VTA, we employed the Fluoro-Jade B (FJB) cytochemical method (Schmued and Hopkins, 2000; Hopkins et al., 2000; Ye et al., 2001), while loss of dopaminergic neurons was assessed by immunohistochemistry to tyrosine hydroxylase, TH (Kumer and Vrana, 1996; Hattori, 1993). To test the hypothesis that autoimmunity is a condition necessary for neuronal degeneration, young (asymptomatic) and old (diseased) groups of mice were used, as well as a group treated with the immunosuppressive drug cyclophosphamide, CY (Farrell et al., 1997; Sakic et al., 1995, 2000a). Tumor necrosis factor alpha (TNF-α) is associated with changes in motivated behavior (Ballok et al., 2003b) and systemic inflammation in the MRL-lpr substrain (McHale et al., 1999), and is elevated in the CSF of SLE patients (Robak et al., 1996). Based on this evidence, we screened serum and pooled CSF samples for the presence of this pro-inflammatory cytokine. In addition, circulating brain-reactive autoantibodies have been proposed to play a key role in the etiology of aberrant behavior and brain damage in lupus-like disease (Denburg et al., 1987; Bluestein and Zvaifler, 1976; Hoffman et al., 1978; Moore, 1997; Zameer and Hoffman, 2001; Sakic et al., 1993). Therefore, these antibodies were also presently measured in the sera of aged mice.

Our overall expectation was that apomorphine would induce rotational behavior in diseased MRL-lpr mice, thus confirming a dysfunctional mesonigral dopamine pathway. We also anticipated that the emergence of autoimmunity would parallel the manifestation of aberrant behaviors, increased FJB-positive cells in the SN and VTA, and CSF toxicity, all of which would be attenuated by CY treatment. Furthermore, we expected fewer TH-positive cells in the SN and VTA, and elevated BRA levels in sera from diseased mice in comparison to controls.

2. Materials and methods

2.1. Pharmacological probing of the damaged substantia nigra

Apomorphine, a well-known D1/D2-receptor agonist (Di Chiara and Gessa, 1978; Starr and Starr, 1987), was selected for this experiment. The principal mesencephalic projection from the SN is to the striatum, and non-uniform SN cell loss (due to disease or injury) results in an interhemispheric asymmetry of striatal dopamine (Arai et al., 1990). Damage to the nigrostriatal dopamine system is associated with dopamine receptor supersensitivity on the denervated side (Morelli et al., 1989). As a result of this imbalance in striatal receptor sensitivity, dopamine receptor agonists (such as apomorphine) induce rotational behavior in affected mice (Randall, 1984). To determine whether this is the case in our model apomorphine induces rotational behaviour, eight drug-naive, 4-month-old male MRL-lpr males were selected from our colony, and were injected with different doses of apomorphine. While higher doses caused stereotypic mouthing, rearing, and grooming behaviors, a single subcutaneous (s.c.) dose of 0.2 mg/kg induced circling around their body

axis. Based on this observation, the behavioral effect of apomorphine was re-assessed in a new batch of 4-month-old male MRL-lpr, MRL+/+ and CD1 mice (four per group). Video recordings were made using a Sony camcorder (DCR-PC101) mounted on a tripod directly above a 14-cm diameter clear glass bowl containing a thin layer of black soil. On the first day, mice were habituated to a novel environment by being exposed to the bowl for 10 min. On the second day, mice were weighed and injected s.c. with a comparable volume of saline (~ 0.1 ml). After injection, a mouse was immediately placed gently into the bowl and filmed for 10 min. A paper towel moistened with Windex® was used to clean the bowl between filmings, and the soil was replaced to remove urinary trails. The procedure was repeated on day 3, when mice received apomorphine hydrochloride (Sigma-Aldrich, St. Louis), which was dissolved in 2% ascorbic acid and phosphate-buffered saline (PBS). The drug was prepared immediately before use, kept on ice, and protected from light. Subsequently, rotational behavior was scored from videotapes by an experimenter unaware of group treatment or origin. Score criteria for a turn was a complete and fluent 360° rotation. This and other experimental protocols were carried out in accordance with the rules and regulations of the Canadian Council of Animal Care.

2.2. Assessment of age-related behavioral, neuropathological and immunological changes

Three-week-old MRL-lpr and MRL+/+ males ($n=20$/substrain) were purchased from the Jackson Laboratory (Bar Harbor, ME) and left for 7 days to habituate to local laboratory conditions (light phase: 8:00 AM–8:00 PM, food and water ad lib; 5 mice /cage; standard housing level). At 4 weeks of age, the first cohort of MRL mice ($n=10$/substrain) was singly caged, habituated to an experimenter prior to behavioral testing (Sakic et al., 1992) and sacrificed at 5 weeks of age. A second cohort (10 males/substrain) was separated for behavioral testing at 17 weeks, and sacrificed 1 week later.

2.2.1. Behavioral testing

Three tests that reliably detect AABS were selected from our behavioral battery (Sakic et al., 1994, 1992, 1997). The brief sucrose preference test was designed to assess response to a palatable food, and is proposed to model anhedonia, one of two core symptoms of depression (Monleon et al., 1995; Sakic et al., 1996a). In addition to standard tap water, a plastic 10-cc syringe filled with 2 ml of 4% sucrose solution was added onto the cage lid, allowing a mouse to drink from it over a 24-h period. Training started during the 4th or 17th week, lasted 3 days, and was aimed at habituating mice to both a novel taste (sucrose) and a novel object (syringe). All mice emptied a syringe at least once before the actual testing, which consisted of 60-min access to the solution (9:30–10:30 PM) over three consecutive nights. To evaluate hyperactivity induced by a novel environment (Sakic et al., 1992), distance traveled and time spent in ambulation over a 30-min period (6:00–6:30 PM) were assessed in computerized activity monitors (Digiscan-16, Omnitech Electronics, Columbus, OH). The forced swim test (FST) is a procedure often used in screening for novel antidepressants (Thiebot et al., 1992). Based on the evidence that immobility (floating) of rodents in a no-escape situation can be reduced by antidepressant drugs (Porsolt et al., 1977; Porsolt, 1979), it has been proposed that increased floating time reflects a state of lower mood ("behavioral despair"). In the present study, a large pool filled with water (temperature ~ 25 °C) was used as previously described (Sakic et al., 1994). The animal was placed into the pool along the inner side of the wall, and left for 6 min. Floating was defined by the absence of any paw and tail movements. Cumulative time spent floating during 2-min intervals was recorded directly by an unbiased observer sitting at the side of the pool.

2.2.2. Neuropathology

Mice were anesthetized with Somnotol (i.p. 60 mg/kg body weight; MTC Pharmaceuticals, Cambridge, ON) and cardially perfused with 40 ml of PBS after terminal bleeding from the vena cava. The CSF was collected as described previously (Maric et al., 2001). In brief, a custom-made glass micropipette was inserted into the cisterna magna to collect 15–25 μl of CSF. Clear CSF was transferred to small vials and immediately centrifuged. One MRL+/+ sample was discarded due to blood contamination, while the other clean CSF samples were kept at room temperature (RT) and assessed within 24 h of collection. Upon CSF collection, mice were perfused with 40 ml of 4% paraformaldehyde (PFA) and extracted brains were weighed on a digital scale (Sartorius 2024 MO, VWR Scientific of Canada) before immersion into 4% PFA for fixation. Fixed brains were immersed into 30% sucrose (in PBS) before sectioning (Ballok et al., 2003a). Ten-micrometer coronal sections were cut with a Jung Frigocut 2800E cryostat at the level of the SN and VTA (Bregma −3.52 and −3.64 mm), characterized by the presence of the third cranial nerve (Franklin and Paxinos, 1997).

Briefly, sections were mounted onto Aptex-coated slides and immersed into absolute ethanol for 3 min, followed by 70% ethanol for 2 min, and distilled water for an additional 2 min. The slides were transferred to 0.06% potassium permanganate for 15 min. After rinsing with distilled water for 2 min, sections were incubated for 30 min in FJB solution (Histo-Chem, Jefferson, AR) prepared by adding 10 ml of a 0.01% FJB stock solution to 90 ml of 0.1% acetic acid. The slides were then rinsed in distilled water, dried at RT, dehydrated in xylene, and cover slipped using DPX. These sections were examined using a Zeiss Laser Scanning Confocal Microscope (LSM 510, Carl Zeiss) argon laser (wavelength 488 nm). Confocal micrographs were obtained using a Fluar 20 × /0.75 objective in combination with a 1024 × 1024 pixel resolution, and saved as TIFF files.

2.2.3. Assessment of CSF toxicity in vitro

To further test the hypothesis that dopaminergic cells are susceptible to toxic CSF factors, we examined the viability of cultured cells which differentiate into neurons (Ryder et al., 1990; Snyder et al., 1992) expressing the dopamine-synthesizing enzyme tyrosine hydroxylase (Yang et al., 2002). The C17.2 dopamine progenitor cells (Wagner et al., 1999) were grown on uncoated 10-cm tissue culture dishes (Corning, Corning, NY) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 5% horse serum and 2 mM glutamine. Cells were passaged weekly (to a maximum of six passages), removed by trituration and their viability determined by Trypan blue exclusion. Approximately 2×10^4 cells were transferred to each well of Falcon 24-well plates and grown for 48 h on 18-mm glass cover slips coated with 0.1% poly-L-Ornithine (Sigma, St. Louis, MO) in DMEM. All tissue cultures were maintained in a standard, humidified (5% CO_2) air incubator at 37 °C. For the bioassay, the volume of the culturing medium was reduced from 1 to 0.25 ml and 10 μl of a sample supernatant (obtained by brief centrifugation of CSF or serum) was added within 2 min upon extraction (each well containing $\sim 5 \times 10^4$ C17.2 stem cells at the time). The cells were incubated at 37 °C for 24 h before cell viability was assessed by standard Trypan blue exclusion. Toxicity was assessed as the ratio between Trypan-positive cells and the total number of cells (50,000).

2.2.4. Indices of autoimmunity

High levels of serum anti-nuclear antibodies (ANA) and splenomegaly are typical manifestations of systemic autoimmune, lupus-like disease (Theofilopoulos, 1992) and were presently examined. Blood samples were left to coagulate in 1.5-ml plastic vials and centrifuged for 10 min at 3000 rpm. Serum was then separated from the clot and stored at −20 °C until further analysis. ANA concentration was measured using a sandwich Anti-Nuclear Antibody ELISA kit (Cat. #5200), according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX) and the protocol previously described (Sakic et al., 2000a). In brief, serum samples were diluted 1:100 in diluent included in the kit, and applied to both experimental and control wells to assess the specificity of binding. The optical density of each well was determined using a microplate ELISA reader set to 450 nm. The wet spleen weight was determined on an analytical scale (Sartorius 2024 MP, VWR Scientific of Canada) immediately upon extraction.

Profound changes in the cytokine network are one of the earliest manifestations of autoimmune disease in murine models of lupus (Theofilopoulos, 1992). Increased levels of circulating TNF-α are associated with early changes in behavioral performance (Ballok et al., 2003b) and upregulation of cell adhesion molecules on endothelial cells in brains of MRL-lpr mice (McHale et al., 1999). To assess peripheral and central levels of TNF-α, serum and pooled CSF samples were analyzed by an ELISA kit (Cat. #MTA00, R&D Systems, Minneapolis, MN). The procedure was modified due to the small volume of CSF collected from a mouse. In brief, 28 μl of frozen serum (or fresh, pooled CSF samples) and 28 μl of calibrator diluent were mixed. Assay diluent was then added to each well; 50 μl of standard, control, or sample was added per well and gently shaken for 1 min. Each plate was covered with an adhesive strip and incubated for 2 h at RT. After aspiration with wash buffer solution, 100 μl of the cytokine conjugate was added and following a second 2-h incubation at RT, the plate was washed and 100 μl of substrate solution was added to each well (before a 30-min incubation in darkness at RT). Stop solution (100 μl) was added to the plate and the optical density of each well was determined within 10 min using a microtiter plate reader set to 450 nm.

2.3. The effects of immunosuppressive treatment

To evaluate the effects of sustained immunosuppression on disease manifestations, 24 mice were housed singly at 5 weeks of age to receive immunosuppressive treatment ($n = 6$ mice/substrain/treatment group). The therapeutic effect of cyclophosphamide (CY) on the development of autoimmune symptoms has been previously demonstrated (Grota et al., 1989, 1990; Shiraki et al., 1984; Sakic et al., 2000a). In addition to the reduction of leukocyte numbers (Snippe et al., 1976), CY also makes these cells unresponsive, leading to generalized immunosuppression (ten Berge et al., 1982) without increasing serum corticosterone levels (Fast et al., 1982). CY was injected weekly (100 mg/kg i.p.; mouse $LD_{50} = 405$ mg/kg i.p.; "Procytox", Horner, Montreal, Canada) to half of the mice in each group. The treatment started in the 5th week of life and ended in the 17th week. The other half of the animals received 12 injections of an equivalent volume (~ 0.1 ml) of saline (Sal). Mice were assigned into one of four groups, according to substrain (MRL-lpr vs. MRL+/+) and treatment (CY vs. Sal). Two mice died prematurely in the CY groups before the treatment was completed. The beneficial effects of chronic CY treatment on behavioral performance have been reported previously (Farrell et al., 1997; Sakic et al., 1995, 1996a; Ballok et al., 2004) and were not presently assessed. Along the same line, the apomorphine-induced rotation was not examined due to confounding effects of repeated injections and interaction between an immunosuppressive and a psychotropic drug. CSF collection and FJB staining were performed, and ANA levels, spleen weight and TNF-α were assessed as described above.

2.4. In situ assessment of TH-positive neurons

To determine if central dopaminergic neurons are affected in diseased mice, eight MRL-lpr males and seven MRL+/+ controls were used for anti-TH staining. Given that TH immunohistochemistry has not been previously used in our laboratory, eight age-matched, non-autoimmune male CD1

mice (Charles River Canada) were employed as positive controls. Mice were separated at 17 weeks of age for behavioral testing and sacrificed at 18 weeks. Mice were perfused with 30 ml ice-cold PBS followed by 30 ml ice-cold 4% PFA. Behavioral tests, ANA levels, spleen weight and TNF-α were measured as described above.

Brains were stored at +4 °C in PFA for 8 days before transferring them to 30% sucrose solution and refrigerating them before sectioning. Free-floating coronal sections were cut at 40 μm at the level of the SN, between Bregma −3.52 and −3.64 mm (Franklin and Paxinos, 1997). They were kept at +4 °C in 24-well plates containing 30% sucrose (with two drops of PFA) and three sections of the SN pars compact/VTA from each animal were selected for TH-staining. Sections were incubated in 0.3% hydrogen peroxide for 30 min at RT, rinsed three times and incubated with primary antibody and rabbit anti-TH (Vector Labs Canada) for 48 h at +4 °C. After three rinses in PBS, tissues were incubated in biotinylated anti-rabbit IgG secondary antibody (Vectastain Elite, Vector Labs Canada), for 1 hr at RT. Subsequently, the sections were rinsed in PBS and incubated in ABC solution for 1 h to form an Avidin/Biotin complex. All sections were washed again in PBS three times and the TH immunocomplex was visualized with chromogen diaminobenzidine tetrahydrochloride (Sigma). The sections were mounted onto gelatin-increasing concentrations of ethanol (70%, 95%, 100%), placed in xylene and coverslipped using Gurr DePeX mounting medium (BDH Labs, UK). The total number of TH-positive cells in the SN region (obtained from three serial sections) were manually quantified by an observer unaware of the experimental design and confocal light micrographs were obtained using a Fluar 5×/0.25 objective in combination with a 1024 × 1024 pixel resolution, and saved as TIFF files. In comparison to the SN region, the high density of TH staining in the VTA of control brains precluded manual counting and lead to a descriptive assessment.

2.4.1. Serum brain-reactive autoantibody levels

Due to the small volume of CSF collected from mice, we limited our analysis of BRA to serum. An ELISA was used for detecting reactivity against brain antigens as previously described (Crimando and Hoffman, 1992; Zameer and Hoffman, 2001). This procedure is used to measure serum titers of autoantibodies that bind integral membrane proteins, without the cytoplasmic proteins found in common detergent lysates. In brief, odd-numbered wells of 96-well microtiter plates (Corning) were coated with 10 μg/ml of integral brain membrane antigens in 0.5 M carbonate–bicarbonate buffer (pH 9.4). Even-numbered wells received only 0.5 M carbonate–bicarbonate buffer. These integral membrane proteins were prepared according to the method of Narendran and Hoffman (1988). To do this, brain membrane antigens (BMA) were isolated from Balb/c mice by removing the brains and mincing the tissue through a nylon mesh. The dissociated cells were treated with 1.5% Triton X-114 (detergent extraction). Detergent suspended integral membrane proteins were phase separated by incubating at 37 °C and resuspended in the carbonate–bicarbonate buffer. Plates were masked with a 2% bovine serum albumin solution to reduce nonspecific reactivity. Mouse serum was added in a series of dilutions and the plates incubated for 1 h at 37 °C. Following washing, a secondary antibody, goat anti-mouse IgG (light and heavy chain specific), peroxidase conjugated (ICN Pharmaceuticals, CA), was added to the wells and incubated for 1 h at 37 °C. The wells were washed again and color was developed using hydrogen peroxide (100 μl/100 ml) and 2,2′ -azino-bis(3-ethylbenzthiazoline) sulfonic acid (17 mg/100 ml) (Sigma) in 0.1 M citrate buffer pH 4.4. Absorbance was read at 405 nm using a microplate reader (BIORAD laboratories, CA). Optical densities for the even-numbered wells were subtracted from the odd-numbered wells to obtain net reactivity for each dilution. Net absorbance values were compared at an optimal differential reactivity, viz., a serum dilution of 1:320.

2.5. Statistical analysis

The data were analysed by ANOVA with Substrain (MRL-lpr vs. MRL+/+), Treatment (CY vs. Sal) and Age as between-group factors. Student's *t*-test was used in the post-hoc analysis. Significance level was set at $p \leq 0.05$ and all computations were performed using the SPSS 11.0 statistical package. Graphs show means ± S.E.M., with $*p \leq 0.05$; $**p < 0.01$; and $***p < 0.001$ between the two MRL groups.

3. Results

3.1. Apomorphine-induced rotations

Fig. 1 shows the number of rotations induced by acute injections of apomorphine and Sal. While CD1 mice as-

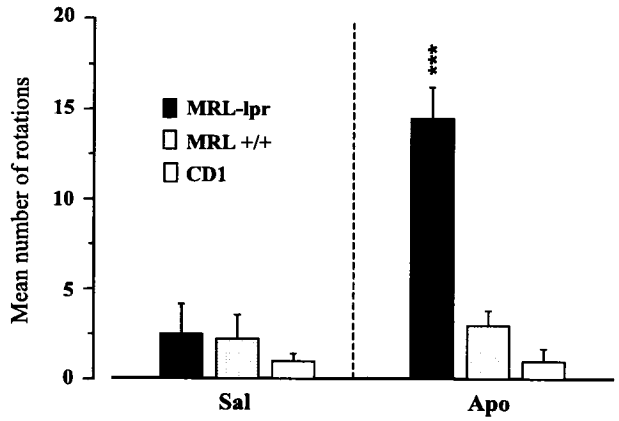

Fig. 1. Rotations induced by apomorphine (Apo, 0.2 mg/kg, s.c.) in the 10-min session. Neither saline (Sal) injection in all mice nor Apo injection in congenic (MRL+/+) and non-autoimmune (CD1) controls increased the number of turns. However, MRL-lpr mice injected with Apo rotated more in comparison to other groups. $*p \leq 0.05$; $**p < 0.01$; $***p < 0.001$ in this and other figures.

sumed a catatonic phase and were immobile approximately 5 min following an apomorphine injection, both MRL substrains continued to ambulate over 10 min. This observation suggests a dissimilar responsiveness to dopaminergic activation between the lupus-prone MRL substrains and non-autoimmune CD1 strain. However, compared to CD1 and MRL+/+ mice, apomorphine induced an asymmetry in limb use in the MRL-lpr substrain, resulting in tight circling around their body axis ($F(2,9)=37.471$, $p<0.001$). This drug-induced behavior suggested that the nigrostriatal dopamine system is out of balance (Tillerson et al., 2001), and justified further study.

3.2. Behavior of MRL mice

Since behavioral profiles and immune statuses did not differ between the batches of 18-week-old mice used in studies 2.1 and 2.4, these data were combined for the statistical analysis. While young MRL mice did not differ in their performance, old MRL-lpr mice showed impaired performance in all tests compared to age-matched controls. Total intake of a 4% sucrose solution across three 60-min tests was lowest in the 17-week-old MRL-lpr group ($t_{33}=6.972$, $p=.001$, Fig. 2A). The discrepancy in older MRL +/+ consumption with a previous study is likely due to its longitudinal design, where sucrose restricted MRL +/+ mice with an intact VTA respond more vigorously following a bingeing period (Ballok et al., 2003b; Bello et al., 2003). Similarly, during the 30-min exposure to a novel environment, diseased MRL-lpr mice locomoted less than age-matched controls. More specifically, distance travelled (age by substrain: $F(1,51)=3.769$, $p=0.05$, Fig. 2B) and the time spent in locomotion (age by substrain: $F(1,51)=12.849$, $p=0.001$; data not shown) were lower in 17-week-old MRL-lpr mice than in age-matched MRL+/+ mice. However, the speed of movements in these groups did not differ (MRL-lpr: 9.2 ± 0.46 vs. MRL+/+: 8.9 ± 0.25 cm/s), suggesting that potentially important impairments in sensorimotor function (resulting from joint inflammation, neuropathy, or lymph node enlargement) did not occur yet (Tanaka et al., 1988; O'Sullivan et al., 1985). Similarly, the immobility time (which was nearly absent in the first 2 min of the forced swim test) was the highest in diseased MRL-lpr mice (age by substrain: $F(1,51)=5.258$, $p=0.026$, Fig. 2C). Gross observation suggested that many of the MRL-lpr mice swam in a circular pattern during this period, in contrast to the controls that explored the whole area of the pool (data not shown). Immobility increased progressively along the session when MRL-lpr mice appeared to "freeze" for prolonged periods (defined as floating time). The appearance of behavioral deficits appeared concurrently with CSF toxicity, elevated BRA levels and neuronal demise. Upon examination of neuropathology, the time spent in locomotion over the 30-min period negatively correlated with the FJB-positivity in the SN of the diseased MRL-lpr group ($n=10$, $r=-0.722$, $p=0.019$), suggesting that increased loss of dopaminergic neurons is associated with impaired activity levels. (Note: lower sample size is a result of 10 animals examined for FJB staining.)

3.3. Neuropathological changes

While no difference was observed between the brains of young animals, most of the 18-week-old MRL-lpr mice had lower brain mass than age-matched controls and young mice (age by substrain: $F(1,36)=9.83$, $p=0.003$, Fig. 3A). The

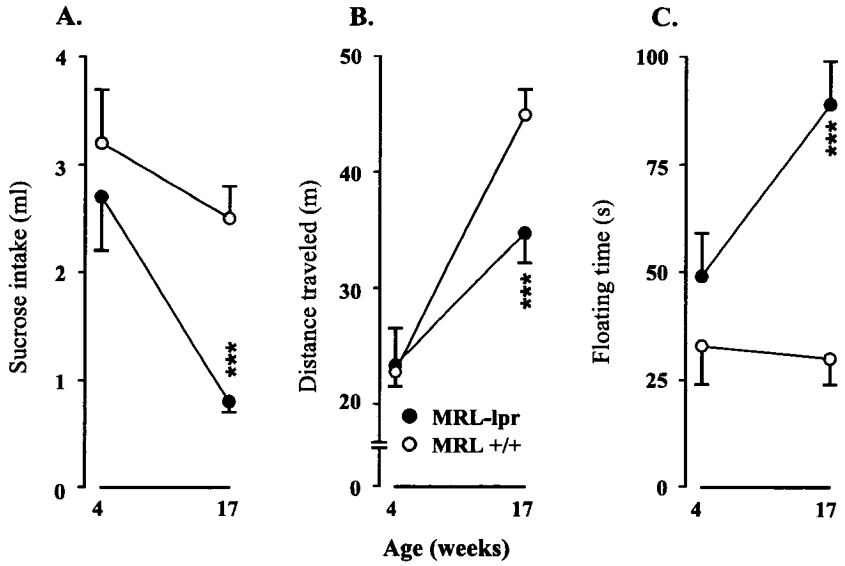

Fig. 2. Age-related differences in behavioral performance between MRL substrains. (A) Three 1-h tests revealed a sharp decline in intake of a 4% sucrose solution (ml) in diseased MRL-lpr mice. (B) Similarly, distanced traversed during 30-min exposure to a novel environment was shorter in aged MRL-lpr mice. (C) When exposed to a no-escape situation of the forced swim test (6-min session), autoimmune mice showed excessive immobility when lupus-like disease was florid. Note: Although independent batches were used for the 4- and 17-week groups, line graphs were selected to improve data legibility.

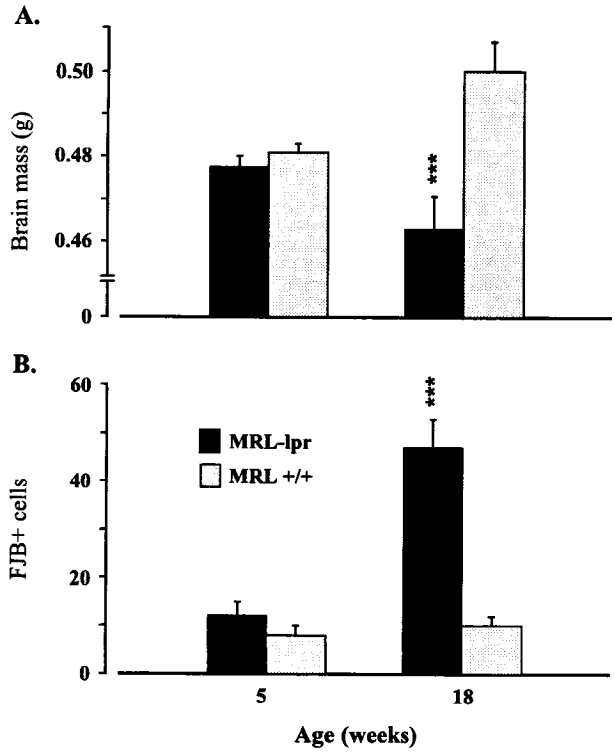

Fig. 3. Age-dependent changes in brain mass and markers of neurodegeneration (as revealed by Fluoro Jade B staining). (A) While young MRL mice had comparable brain mass, diseased MRL-lpr brains were significantly lighter in comparison to age-matched, congenic controls. (B) Similarly, while no differences are observed in Fluoro Jade B (FJB) staining at the younger age, 18-week-old MRL-lpr mice had increased densities of FJB-positive cells in the substantia nigra (SN).

smaller brain mass of old MRL-lpr mice suggests atrophy along the development of lupus-like disease. A significant increase of FJB staining in the SN pars compacta (age by substrain: $F(1,36)=20.428$, $p<0.001$, Fig. 3B) of MRL-lpr mice also implied reduced neuronal survival. Neurodegeneration in the VTA of MRL-lpr brains (age by substrain: $F(1,36)=13.224$, $p=0.001$) also suggested that the mesolimbic system is damaged, an interpretation based on FJB-positivity in the hippocampus, amygdala, and hypothalamus (Ballok et al., 2003a). Consistent with the previous study, we presently did not observe cell bodies or axons stained for FJB in the striatum.

Table 1
In vitro toxicity (%) to C17.2 progenitor cell line after 24-h incubation with CSF from young (5 weeks) and old (18 weeks) MRL-lpr and MRL+/+ mice

Group	5 weeks	18 weeks
MRL-lpr	0	73 ± 6***
MRL+/+	0	8 ± 3

The CSF toxicity was absent in the young MRL animals, but was significantly increased in aged MRL-lpr mice relative to age-matched MRL+/+ controls (***$p<0.001$). The sample size in each age group was $n=10$, except for the older MRL+/+ group in which one CSF sample was contaminated with blood.

3.4. Immune status

The emergence of the behavioral deficits coincided with the appearance of autoimmune manifestations in MRL-lpr mice at an older age. More specifically, spleen weight, circulating ANA, and TNF-α levels in serum and CSF were significantly higher in 18-week-old MRL-lpr males compared to age-matched MRL+/+ controls (for spleen, substrain by treatment: $F(1,51)=19.442$, $p<0.001$; for serum TNF-α, substrain by treatment: $F(1.51)=14.122$, $p<0.001$; for ANA, substrain by treatment: $F(1.51)=35.591$, $p<0.001$). These measures were also employed to confirm the healthy status of CD1 mice (spleen weight: 0.17 ± 0.01, ANA: 0.00 ± 0.00, serum TNF-α: 0.00 ± 0.00). More interestingly, serum TNF-α levels correlated with spleen weights

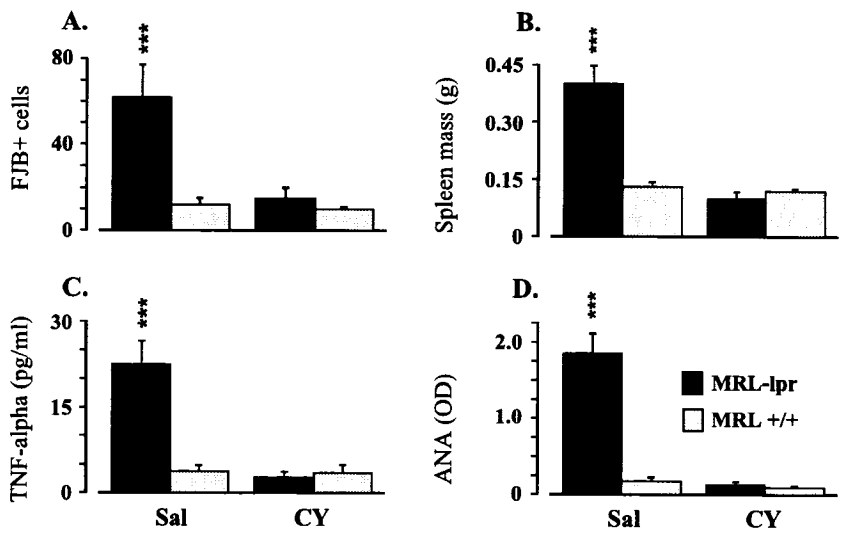

Fig. 4. Effects of immunosuppressive treatment on brain pathology and immune status in MRL mice. (A) Cyclophosphamide (CY) treatment attenuated the incidence of Fluoro Jade B (FJB)-positive cells in the SN, and significantly reduced (B) splenomegaly, (C) serum levels of TNF-α, and (D) ANA.

Table 2
The toxicity (%) of CSF from 17-week-old MRL-lpr and MRL+/+ mice chronically treated with saline (Sal) or cyclophosphamide (CY) in the C17.2 progenitor cell line bioassay

Group	Sal	CY
MRL-lpr	56 ± 3	24 ± 12*
MRL+/+	12 ± 4	8 ± 4

In comparison to the Sal-treated control, chronic CY treatment of MRL-lpr mice significantly attenuated the toxicity of their CSF (*$p \leq 0.05$).

within the diseased MRL-lpr group ($n=18$, $r=0.471$, $p=0.049$). In addition to elevated peripheral measures at an old age, increased levels of TNF-α were also detected in pooled CSF samples ($n=4-5$ samples /group) from diseased MRL-lpr mice (substrain by age: $F(1,13)=4.37$, $p=0.05$).

3.5. Effects of immunosuppressive treatment

In comparison to Sal groups, the rate of body growth slowed in mice chronically treated with CY at 14 weeks of age (treatment: $F(1,22)=5.768$, $p=0.025$). Post-hoc analysis revealed that CY mice were significantly lighter at 15, 16, and 17 weeks of age, while trends toward this end were seen at 12 and 13 weeks. The protective effect of immunosuppression on neuronal survival was confirmed by a marked reduction in FJB staining in the SN (substrain by treatment: $F(1,18)=4.469$, $p=0.049$, Fig. 4A) and VTA (substrain by treatment: $F(1,18)=5.003$, $p=0.038$) of MRL-lpr-CY mice. This reduction in cytochemical staining was associated with the profound immunosuppressive effect of CY, as indicated by low spleen weight, serum TNF-α, and ANA in the MRL-lpr-CY group (for spleen, substrain by treatment: $F(1,18)=27.781$, $p<0.001$; for serum TNF-α, substrain by treatment: $F(1,17)=18.813$, $p<0.001$; for ANA substrain by treatment: $F(1,18)=32.522$, $p<0.001$; Fig. 4B–D). Moreover, pooled TNF-α levels in the CSF were reduced in the MRL-lpr-CY group compared to MRL-lpr-Sal controls (treatment: $t_5=3.366$, $p=0.02$; MRL-lpr-CY, $n=3$, 0.87 ± 0.52; MRL-lpr-Sal, $n=4$, 6.51 ± 1.37).

3.6. In vitro cytotoxicity

The neural progenitor cells incubated with CSF samples from young mice grew without any signs of toxicity or dysmorphology (Table 1). Conversely, the cells incubated with CSF samples from 18-week-old MRL-lpr mice died excessively after 24 h when compared to age-matched controls ($t_{17}=9.134$, $p<0.001$). More importantly, the toxicity of

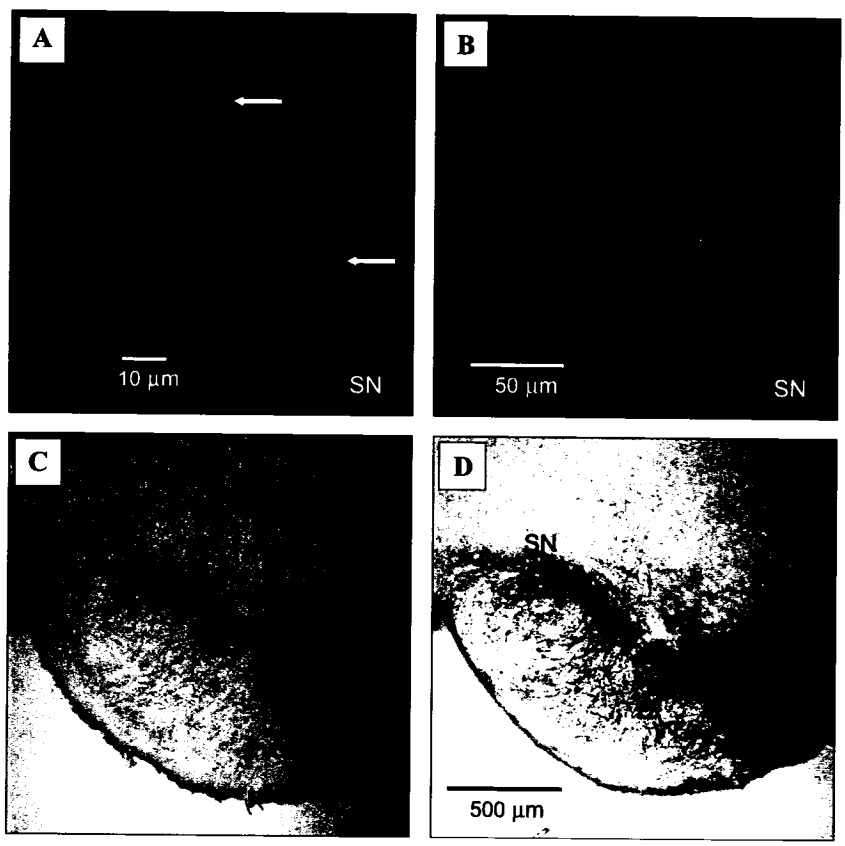

Fig. 5. Representative fields of Fluoro Jade B (FJB) and tyrosine-hydroxylase (TH) stainings in the substantia nigra (SN) pars compacta and ventral tegmental area (VTA). Numerous brightly stained FJB-positive neurons confirmed a degenerative process in the SN of MRL-lpr brains (A) in comparison to asymptomatic MRL+/+ controls (B). A paucity of dark-stained TH-immunoreactive neurons were observed on brain sections from diseased lupus-prone mice (C), supportive of progressive dopaminergic cell loss in the SN and VTA. The control brains showed normal neuronal densities of TH-positive cells (D).

their CSF appeared to be attenuated after prolonged CY treatment (Table 2). However, one CSF sample from the MRL-lpr-Sal group had no effects, which significantly affected normal distribution of the data. This variability is not a surprising observation, considering that it was commonly observed in previous studies that focussed on behavioral performance and infiltration of CD-positive cells (Sakic et al., 1994, 2000b). Due to a small group of MRL-lpr mice presently employed ($N = 5 + 6$), this sample has been considered an outlier and removed from the comparison between the means. Indeed, such analysis revealed attenuated toxicity of CSF in the group of MRL-lpr mice chronically treated with CY ($t_8 = 2.407, p = 0.047$). As previously reported, serum had no toxic effects (Maric et al., 2001). These results further support the hypothesis that the progress of systemic autoimmune disease renders CSF cytotoxic and compromises the survival of midbrain dopaminergic cells.

3.7. Dopaminergic cell loss and brain-reactive autoantibodies (BRA)

In addition to the increased FJB staining in midbrains of MRL-lpr mice (Fig. 5A,B), TH-related hypocellularity was also observed in a separate cohort of brains (Fig. 5C,D). More specifically, cell loss was confirmed by a profound (~ 33%) reduction in the number of TH-positive cells in the SN pars compacta of aged MRL-lpr mice when compared to congenic controls ($t_{13} = 9.857, p < 0.001$), and 37% reduction relative to CD1 mice ($t_{14} = 12.649, p < 0.001$; Fig. 6A). In addition to the SN, the TH-related hypocellularity was clearly apparent (although not quantified) in the VTA of all eight brains from MRL-lpr mice (Fig. 5C,D). Since TH-immunoreactivity did not differ between asymptomatic congenic MRL+/+ and allogenic non-autoimmune CD1 mice, this suggests that autoimmune disease is a predisposing factor of cell loss. Supportive of this notion, levels of brain reactive antibodies were found to be three-fold higher in the diseased MRL-lpr mice at 18 weeks of age ($F(18,21) = 14.839, p < 0.001$, Fig. 6B).

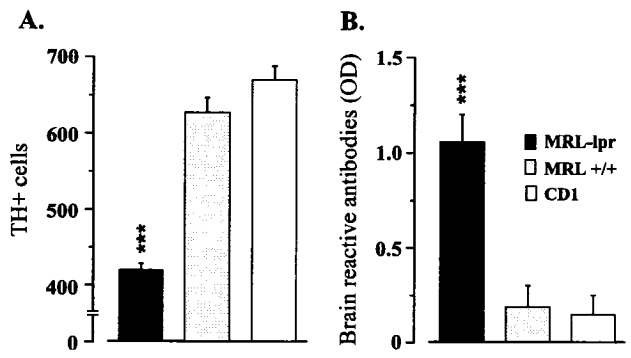

Fig. 6. Assessment of the density of dopaminergic neurons and serum levels of brain-reactive antibodies (BRA) at a dilution of 1:320. Compared to age-matched controls, the sum of TH-positive counts (obtained from three serial sections) was significantly lower in the substantia nigra (SN) pars compacta of 18-week-old MRL-lpr mice. In addition, serum titres of BRA were 3-fold higher in the same mice relative to controls (B).

4. Discussion

The present results are consistent with the hypothesis that dopaminergic neurons of the mesonigral and mesolimbic pathways are targeted during the development of systemic autoimmune disease in behaviorally impaired MRL-lpr mice. This assumption is based on several lines of evidence. First, apomorphine-induced rotational behavior temporally coincides with TH-hypocellularity in the SN, increased FJB staining, CSF cytotoxicity, and spontaneous behavioral deficits. Second, there is a negative correlation between neurodegeneration in the SN and ambulation of diseased mice. Third, their CSF is toxic to dopamine progenitor cells. Finally, evidence that early, prolonged immunosuppressive treatment suppresses the occurrence of both peripheral and central disease manifestations supports the causal relationship between systemic autoimmunity/inflammation, CSF toxicity, and neurodegeneration. Reduced TH-positivity in the VTA, and FJB staining in the hippocampus, amygdala, and hypothalamic areas (Ballok et al., 2003a) is also supportive of the notion of degeneration in the mesolimbic system. Consistent with our present finding, earlier studies on lupus mice revealed that brain mass was smaller in aging MRL-lpr mice (Sakic et al., 1998b). We presently observed that diseased mice with a damaged mesencephalon had high levels of serum antibodies reactive to brain antigens. It should be kept in mind, however, that serum from age-matched animals was not toxic in our in vitro bioassay (Maric et al., 2001). From the present set of data, we could not determine whether brain reactive antibodies are one of the markers of brain damage or an instrumental factor in the pathogenesis of CNS dysfunction and neuropathology. This same reasoning can be applied to TNF-α, which was observed in the CSF of diseased MRL-lpr mice.

SLE patients display a variety of neurologic manifestations, which may include Parkinsonian-like deficits and changes in the basal ganglia (reviewed in Garcia-Moreno and Chacon, 2002). Although it is known that degeneration of dopaminergic systems leads to movement pathology (Bosboom et al., 2003), the causes and mechanisms underlying the selective loss of these neurons are poorly understood. The most profound functional consequences of SN damage are inhibited locomotor activity and dyskinesia (Arai et al., 1990). This has been demonstrated by studies showing that mice with drug-induced cell loss in the SN have decreased movement capacity (Hu et al., 1991), and mice with reduced THr SN staining show reductions in motor coordination and locomotor activity (Thiruchelvam et al., 2003). Similar to these reports, diseased MRL-lpr mice have impaired coordination in a beam-walking task (Sakic et al., 1992, 1996b) and neurological deficits (Hess et al., 1993; Brey et al., 1995). In

the present study, diseased mice showed lower locomotor activity, which correlated with increased FJB neurodegeneration in the SN. One may speculate that this is a direct consequence of SN cell loss, which was functionally exacerbated by acute apomorphine administration. Although not examined in the present study, a dysfunctional serotonergic raphe system (Murai et al., 2001) and deterioration of cholinergic and noradrenergic systems (Bosboom et al., 2003) may also contribute to the etiology of neurologic and psychiatric symptoms (Wolters, 2001).

Following destruction of dopaminergic systems, the remaining dopaminergic cells become hypersensitive to dopamine (Cai et al., 2002), and postsynaptic denervation supersensitivity of striatal neurons appears to be a contributing factor to behavioral abnormalities in rodents and humans (Lau and Fung, 1986; Rinne et al., 1983; Donnan et al., 1991; Schultz et al., 2001). If so, apomorphine-induced rotations in diseased lupus mice suggest asymmetrical dysfunction in the nigrostriatal dopaminergic pathway, which is likely a consequence of receptor supersensitivity following neuronal damage (Randall, 1984). Along the same line, the present findings are comparable to decreased locomotor activity and a 33% SN cell loss in mice, after administration of the selective dopaminergic neurotoxin 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (Arai et al., 1990). Additional support for the notion that behavioral impairments and neuronal demise are a consequence of autoimmunity comes from several case studies in which CY effectively reversed Parkinsonian-manifestations in SLE patients (Kwong et al., 2000; Tan et al., 2001; Osawa et al., 1997). CY treatment has also been shown to attenuate CSF-induced neurotoxicity in rats (Shahani et al., 2001), and we speculate that it similarly exerted a neuroprotective effect by attenuating toxic CSF factor(s) in the present study.

Dopaminergic input from the VTA is connected to a number of limbic structures modulating hedonia (ability to experience pleasure) in humans (Cantello et al., 1989) and reward processes in mice (David et al., 1998). Chronic mild stress and blunted responsiveness to palatable stimulation are proposed as a putative model of stress-induced anhedonia (Dziedzicka-Wasylewska and Papp, 1996; Willner, 1997). The same group of authors revealed that changes in the mesolimbic dopamine system (the VTA in particular) underlie prolonged "anhedonic" and depressive-like behaviors in rodents exposed to chronic stress. Autoimmune MRL-lpr mice exhibit behaviors reminiscent of stressed animals in a number of performance tests (Sakic et al., 1992, 1994). In the present study, the profound reduction of VTA neurons in MRL-lpr brains was observed at a time when diseased mice reduced their sucrose intake in the sucrose preference test and floated excessively in the forced swim test. Therefore, we hypothesize that anhedonic- and depressive-like behaviors may reflect an effect of autoimmunity-induced damage to the mesolimbic dopamine system. As previously shown in areas of the hippocampus, amygdala and hypothalamus of MRL-lpr brains (Ballok et al., 2003a), increased FJB staining in these regions further suggest degeneration of mesolimbic pathways which may have profound consequences on motivated behavior. Similarly, if the remaining cells in the VTA become hypersensitive to dopaminergic stimulation, abnormal social behaviors can be expected in diseased MRL-lpr mice, similar to reports in rats (Glenthoj, 1995). Indeed, autoimmune MRL-lpr mice do exhibit altered affective responsiveness (Sakic et al., 1998a) and aberrant social interactions at the onset of autoimmune disease (manuscript in preparation).

The pleiotropic cytokine TNF-α (Probert and Selmaj, 1997) is elevated in the CSF of SLE and Parkinson's disease patients (Robak et al., 1996; Mogi et al., 1994). Based on reports that TNF-α is selectively toxic to dopaminergic neurons in Parkinson's disease (McGuire et al., 2001; Clarke and Branton, 2002), and mice lacking TNF receptors are protected against dopaminergic neurotoxicity (Sriram et al., 2002), we expected TNF-α to be associated with midbrain neurodegeneration in MRL-lpr mice. Indeed, TNF-α was detected in both serum and CSF samples of diseased mice when neurodegeneration was observed. However, while serum levels of TNF-α were four-fold higher than in CSF, only CSF was toxic to dopamine progenitor cells. This indicates that TNF-α (at least in MRL-lpr mice) is not the cytotoxic factor to dopamine progenitor cells. This finding is consistent with reports that TNF-α is non-toxic to other progenitor (Beran et al., 1988) or differentiated cell lines (Barone et al., 1997; Koch et al., 1990). Despite this, the fact that TNF-α was detected in the CSF of MRL-lpr mice is important when considering that intracerebroventricular TNF-α can exacerbate CSF leukocytosis and perivascular infiltration (Seabrook and Hay, 2001), observed at terminal stages of lupus-like disease (Alexander et al., 1983; Vogelweid et al., 1991; Farrell et al., 1997; Sakic et al., 2000b; Ballok et al., 2003a). Our novel finding that CSF is toxic to progenitor cells is also interesting given that a population of actively dividing progenitor cells reside in the SN of rodents (Lie et al., 2002).

Deficits in neurotransmitter catabolism are often a consequence of aberrant synthesis and/or enzymatic activity (Curzon, 1977; Roth et al., 1982). It is known that excessive levels of dopamine can be neurotoxic (Blum et al., 2001; Asanuma et al., 2003) and one possibility is that accumulated metabolites of dopamine catabolism are some of the factors contributing to the reduced density of dopaminergic cells in the present study. Elevated levels of dopamine in the brains of MRL-lpr mice and their increased sensitivity to the dopamine receptor agonist quinpirole (Sakic et al., 2002) are observations consistent with this notion. Along the same line, abnormal dopamine metabolism may result in harmful CNS toxins (Cohen, 1984), which induce increased activity of the ubiquitin-proteasome system (reviewed in Dauer and Przedborski, 2003) and lead to dopaminergic cell death (McNaught and Olanow, 2003). Indeed, we have recently observed increased numbers of ubiquinated particles in the mesencephalon of MRL-lpr mice (Ballok et al., 2004).

Accumulating evidence suggests that peripheral autoimmune/inflammatory factors may expand to the CNS initiating detrimental intrathecal immune processes, such as cytokine production, complement activation, free-radical formation, lymphocyte infiltration, and binding of brain-reactive autoantibodies (Svenungsson et al., 2001; Jongen et al., 1990; Hafler and Weiner, 1989; Hoffman et al., 1983, 1988). The role of systemic autoantibodies reactive to brain tissue in the etiology of NP-SLE has been proposed over the last few decades (Bluestein et al., 1981; Williams et al., 1981; Bresnihan et al., 1979; Denburg et al., 1994; Hoffman et al., 1988; How et al., 1985; Moore, 1997). There are, however, studies discrepant with this hypothesis (Hanly et al., 1994; Greenwood et al., 2002). While the mechanism by which circulating BRAs access the brain is not well understood (Hoffman and Harbeck, 1989; Harbeck et al., 1978), diverse behavioral manifestations in human and murine forms of lupus suggest that multiple CNS antigens and sites are targeted (Hoffman and Madsen, 1990; Hoffman et al., 1978, 1987; Crimando and Hoffman, 1992). Yet specific autoantibodies may account for certain behavioral impairments. For example, after a single injection of anti-dopamine antibodies motor activity of healthy mice was reduced (Trekova et al., 2001) and autoantibodies targeting dopamine neurons were associated with rapidly progressing Parkinsonian symptomatology in a SLE patient (Kunas et al., 1995). Although we presently found abundant serum BRAs in behaviorally impaired lupus mice with TH-hypocellularity, the fact that CSF (but not serum) was cytotoxic to dopamine progenitor cells suggests that intrathecal autoantibodies are the source of neurotoxicity. Along the same line, circulating BRA can be related to the CNS damage but are not present at a sufficient concentration to induce cytotoxicity in vitro. Alternatively, they might be a marker (i.e. a consequence of damage to neurons) which does not induce brain damage. Considering that CY treatment in the present study abolished both systemic markers of autoimmunity and attenuated CSF toxicity, we are currently unable to elucidate the significance of circulating BRA on CNS manifestations.

There is the possibility that infiltrating lymphocytes produce antibodies intrathecally that interfere with normal brain functioning or neuronal survival (DeGiorgio et al., 2001; Lai and Lan, 2000; Mevorach et al., 1994; Brey et al., 1997; Maric et al., 2001). Indeed, antibodies have been found to bind to the brain of autoimmune mice (MRL/lpr and BXSB) and could mediate functional, or toxic effects to neurons directly (Zameer and Hoffman, 2001). In autoimmune mice, this has been supported by numerous studies pointing to a breached blood–brain barrier and leukocytes infiltrating the choroid plexus and brain parenchyma (Alexander et al., 1983; Kier, 1990; Vogelweid et al., 1991; Hess et al., 1993; Farrell et al., 1997; Zameer and Hoffman, 2004). There is also a possibility that autoantibodies themselves can cross the blood–brain barrier (Hoffman and Harbeck, 1989). Whatever the mechanism of antibody appearance, the potential importance of in situ brain-reactive autoantibodies are further suggested by our recent study showing that the demise of proliferating neuronal precursors can be induced by an IgG-rich fraction from the CSF of MRL-lpr mice, but not by proteins in their serum (manuscript submitted). Unfortunately, due to the limited volume of CSF samples in the present study, we could not explore the relationship between intrathecal BRA, cytotoxicity, neuronal loss, and behavioral deficits. This line of investigation will be pursued in our future studies.

In summary, the present results support the hypothesis that the emergence of lupus-like disease is associated with damage to the mesencephalic dopamine neurons and the cytotoxicity of CSF. Due to the parallel courses of behavioral dysfunction and disease manifestations, as well as the beneficial effects of immunosuppression on neuronal survival, it is likely that sustained activation of the immune system is a necessary condition for CNS damage or dysfunction. Future studies need to examine the status of other neuronal phenotypes and elucidate terminal mechanisms involved in neuronal demise and dysfunction, such as BRA, cytokines and other soluble toxic mediators (Piani et al., 1992). Despite this incomplete knowledge, the present results corroborate the notion that AABS is a valuable paradigm to explore the mechanisms of CNS damage in neuropsychiatric lupus.

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CHAPTER 7

SUMMARY OF FINDINGS AND GENERAL DISCUSSION

SUMMARY OF FINDINGS

The results of studies described in this thesis indicate that the manifestation and progression of lupus-like disease is associated with the etiology of specific organic neuronal damage in MRL-lpr brains. The neurodegenerative changes do not appear to involve inflammatory prostaglandin pathways, but may be propagated by systemic autoimmune factors such as BRA or toxic mediators present in the CSF of diseased animals. In addition, these studies suggest that destruction of dopaminergic pathways and progenitor cells can contribute to the development of behavioural abnormalities which spontaneously arise in MRL-lpr mice.

In the first study, we examined whether neurons were dying in brains of autoimmune MRL-lpr mice, as well as the association between neuronal demise and markers of disease. We demonstrated, for the first time, profound neurodegeneration in diseased lupus animals using the cytochemical stain FJB. Neuronal demise correlated with splenomegaly and anti-DNA autoantibodies. While T-lymphocytes were present in the third ventricle of diseased mice, a linear correlation between numbers of immune cells and periventricular neurodegeneration could not be shown. TUNEL co-localized with FJB+ cells, initially indicating that neuronal death occurred via an apoptotic mechanism.

In the second study, the relationship between structural damage, functional changes, and autoimmune disease was examined. The causal relationship between neuropathology and autoimmunity was tested by comparing young, asymptomatic and old, diseased MRL mice, as well as by prolonged administration of immunosuppressive CY. The relationship between hippocampal

damage and behavioural dysfunction was assessed by using FJB staining, anti-Ub, and the SAB test. In comparison to young MRL mice and old congenic MRL +/+ controls, SAB acquisition rates and performance in the "reversal" trial were impaired in old, diseased MRL-lpr mice. Frequent anti-Ub particles and FJB staining was observed in the CA3 region of the hippocampus in diseased animals. CY treatment attenuated the SAB deficit and overall FJB staining. These findings suggest that systemic autoimmunity induces significant hippocampal damage which may account for the emergence of impairments in spatial learning. It was initially suspected that the numerous anti-Ub particles in the hippocampus evidenced that the Ub pathway was involved in the neuronal degenerative process. Sustained immunosuppression, however, failed to reduce Ub particles in the CA3 region which suggests that overexpression of Ub is a consequence of nonimmune pathogenic mechanisms (e.g. hormonal), or is merely an epiphenomenon of aging in MRL-lpr mice. Strengthening the face validity of the MRL model of NP-SLE, the hippocampus from a patient who died from NP-SLE showed reduced neuronal density and increased FJB positivity; this regional damage may underlie affective and cognitive deficits in neuropsychiatric lupus.

In the third study, we examined inflammatory and ultrastructural aspects of the CNS involvement using a non-selective COX-2 inhibitor, ibuprofen (IBU), and measured effects on behaviour, microglia activation, and neuronal morphology. Exploration of a novel environment and performance in the FST assessed effects on behaviour. Immunohistochemistry for F4/80 and CD3, FJB and Toluidine blue staining, and flow cytometry were employed in neuropathological analysis. Transmission electron microscopy (EM) examined ultrastructural morphology of cortical, hippocampal, hypothalamic, midbrain, and cerebellar cells. Chronic IBU treatment failed to normalize immune status, behaviour, and brain mass in lupus-prone mice. It also did not reduce

density of CD3+ lymphocytes in the choroid plexus, or FJB+ neurons in the hypothalamus. We showed that activated F4/80+ microglia increased with age in MRL-lpr mice, but IBU treatment was not effective in reducing their numbers. These findings suggest that the COX-dependent pathway is not critical in the etiology of neurodegeneration in MRL-lpr brains. At the level of EM, an abundance of dark cells was confirmed in the hypothalamus, hippocampus, cerebellum and the SN pars compacta. Nuclear and cytoplasmic condensation / clumping of chromatin were ubiquitous across different brain regions, but these cells neither contained apoptotic bodies, nor showed evidence of budding, which are classical signs of apoptosis (Schmechel 1999). Reduced brain mass correlated with increased spleen mass within the MRL-lpr group suggesting a link between chronic systemic disease and neuropathology. Increased microglia activation, and condensation of cytoplasm point to a metabolic perturbation (e.g. excitotoxic damage) which compromises function and survival of central neurons during lupus-like disease.

In the fourth and fifth study, we pharmacologically probed the functional status of central dopaminergic circuits by examining behavioural changes in mice following the administration of DA agonists. More specifically, the response rates were compared between diseased MRL-lpr mice and congenic MRL +/+ controls in the sucrose preference paradigm following Amph injection. Rotational behaviour of MRL mice and CD1 controls was examined following Apo injection. In the fourth study, Amph treatment did not increase the intake of sucrose solutions in MRL-lpr mice, suggesting that reward circuits were impaired / damaged. Subsequent FJB staining in the NAc and hippocampus of MRL-lpr brains reveal that a lesion of the mesolimbic DA system may account for deficits in reward motivated behaviours. In the fifth study, rotational behaviour was assessed after a single injection of the DA-receptor agonist, Apo. This drug is thought to have induced significant

asymmetry in limb use, leading to increased circling in the autoimmune animals. This finding provides evidence of functional damage of the nigrostriatal pathway in diseased MRL-lpr mice.

The fifth study found more evidence consistent with the notion that autoimmunity-induced destruction of mesonigral and mesolimbic dopaminergic pathways contributes to the etiology of aberrant behaviour in lupus-prone mice. We confirmed that spontaneous development of lupus-like disease in MRL-lpr mice is accompanied by a constellation of behavioural deficits, including blunted responsiveness to sucrose, hypoactivity in a novel environment, and excessive immobility in the FST. We found a reduction in the number of TH+, DA cells in the SN and VTA of diseased MRL-lpr brains. A three-fold increase in serum brain-reactive antibodies (BRA) accompanied this loss. In addition, we demonstrated for the first time, that CSF from aged, diseased MRL-lpr mice (but not young or asymptomatic MRL mice) was neurotoxic to a DA progenitor cell line. While CY treatment abolished both toxicity *in vitro* and neuronal death *ex vivo*, the source and identity of the toxic mediator(s) remains unknown.

In summary, the studies described in this thesis have demonstrated a neurodegenerative process in brains of MRL-lpr mice, associated with the manifestation of spontaneous systemic autoimmune disease. The studies also showed that specific regional and neuroreceptor damage may be linked to specific deficits in behaviour. Since CSF toxicity developed alongside the emergence of neuronal death, this suggests a connection between progressive systemic disease and central toxic mediators. The ability of immunosuppressive treatment to attenuate neurodegeneration, behavioural dysfunction, systemic autoimmunity, and CSF toxicity strengthens the causal relationships between selective brain damage, functional loss, and pervasive disease in this animal model of neuropsychiatric lupus.

The aim of this thesis was to investigate the mode and mechanism of neurodegeneration, along with the phenotype of the dying neurons. Specifically, we hypothesized that neuronal demise would be associated with the manifestation of autoimmune disease, and that neuronal destruction would involve dopaminergic cells, accounting for behavioural dysfunctions in MRL-lpr mice. These data are consistent with our overall hypothesis and our specific hypotheses 1, 2, 3, 4 and 5. These data suggest that DA neurons died via non-apoptotic mechanisms involving the Ub pathway following the onset of SLE-like disease, giving rise to behavioural dysfunctions in lupus-prone animals. Also, while immunosuppressive treatment may abrogate neuronal death, systemic autoimmunity and CSF toxicity, it does not incontrovertibly prove that autoimmunity *per se* is the cause of neurodegeneration in MRL-lpr mice, as CY also suppressed the inflammatory arm of the immune response. However, the inflammatory prostaglandin pathway is unlikely a factor in cell death since chronic IBU treatment did not prevent neurodegeneration. Therefore, the precise cause of this disease-associated neuronal demise (i.e. the role of excitotoxic BRA and activated microglia) will require further investigation.

GENERAL DISCUSSION

This thesis was aimed at establishing the phenomena and extent of CNS neuropathology, and subsequently try to better understand the mode and mechanisms, and phenotype of dying neurons. Connections between neuronal demise, behavioural dysfunction, and autoimmune / inflammatory disease in lupus-prone mice was also a focus of this work. The results obtained suggest that neurotoxic factors associated with the autoimmune disease process underlie the development of progressive neuron pathology and behavioural dysfunctions in MRL-lpr animals. While the systemic

nature of SLE disease precludes inference of a causal relationship between specific regional brain damage and functional deficits, pharmacological evidence and TH-immunohistochemistry suggests destruction of dopaminergic circuits are important in some AABS behaviours. Progression of autoimmune disease also seems to have a causal role since severity of autoimmune symptoms was found to correlate with neuronal loss, and immunosuppressive treatment prevented neurodegeneration. While not seen in young, asymptomatic mice, the cytotoxic properties of CSF from old, autoimmune MRL-lpr mice and the ability of CY to abolish the toxicity is further support for the notion that disease factors are linked to neuronal demise.

An accelerated development of lupus-like disease in the MRL-lpr substrain is accompanied by a constellation of behavioural deficits (Brey et al. 1997b), most consistently noted in tasks reflective of emotional reactivity, affective behaviour and spatial learning / memory (Sakic et al. 1994; Sakic et al. 1992; Szechtman et al. 1997). Behavioural abnormalities appear concurrently with serological manifestations of autoimmunity and brain pathology, including infiltration of lymphoid cells into the choroid plexus and brain parenchyma (Brey et al. 1997a; Vogelweid et al. 1991; Farrell et al. 1997), reduced complexity of pyramidal neurons (Sakic et al. 1998), and ventricular enlargement at an older age (Denenberg et al. 1992). Furthermore, immunofluorescent staining with the TUNEL method has revealed an increased incidence of cells with fragmented DNA (a putative marker of apoptosis) in brains of MRL- lpr lupus prone mice (Sakic et al. 2000). The fact that 70% of the total number of TUNEL+ cells did not co-localize with CD4+ or CD8+ cells suggested that a significant proportion of TUNEL+ cells were resident brain cells, however, whether these dying brain cells were neurons remained an open question. Support for the notion of neuron cell demise came from an *in vitro* study in which CSF from lupus-prone mice was found to be neurotoxic in co-

cultures of hippocampal neurons and astrocytes (Maric et al. 2001). However, to provide direct evidence that neurons were dying, a relatively novel method with the FJB dye was used in this thesis (Schmued & Hopkins 2000; Hopkins et al. 2000).

By using the FJB stain, the present thesis supports the hypothesis that central neurons are targeted during systemic autoimmune disease in the Fas-deficient MRL-lpr strain. In brief, FJB has an affinity for the entire degenerating neuron including cell body, dendrites, axon and axon terminals, regardless of the type of cell death (Schmued & Hopkins 2000; Hopkins, Wang & Schmued 2000; Ye et al. 2001). The fact that the majority of TUNEL+ cells co-localized with FJB also strengthens the notion, obtained *in vitro*, that neurons are more susceptible to autoimmune-induced toxic mediators (Maric et al. 2001), and are consistent with previously reported neuronal atrophy (Sakic et al. 1998), which may represent an initial step in excessive neuronal demise. The lack of correlation between CD4+ / CD8+ cells and FJB+ cells suggests that in addition to T-lymphoid infiltration, other factors such as chronically elevated systemic glucocorticoids may be instrumental in the neurodegenerative events seen in MRL-lpr mice (Lechner et al. 2000), and in NP-SLE (Ainiala et al. 2005). Conversely, a positive correlation between the extent of FJB+ staining, serum ANA levels, and spleen weights supports the hypothesis that autoimmune factors are a key factor in the pathogenesis of brain damage during lupus-like disease.

It remained to be determined whether neuronal loss is associated with the progress of autoimmune disease or, alternatively, reflects a developmental deficiency related to impaired expression of the apoptotic Fas receptor in the MRL-lpr brain (Park et al. 1998). Although the latter possibility appears less viable in the light of evidence that cortical architectures in young, pre-diseased MRL-lpr and MRL +/+ mice are comparable (Sherman et al. 1990), and that size of

hippocampal fields and neuronal density are not reduced in Fas-deficient lpr mice (Kovac et al. 2002), age-related changes in densities of hippocampal neurons stained by the standard H&E method were assessed in this thesis. Comparisons across ages showed a paucity of cells in older MRL-lpr mice and suggest a genuine loss of neurons during the development of systemic autoimmunity in the MRL-lpr substrain, concomitant with behavioural deficits. This is supported by comparable immune statuses and hippocampal densities at younger ages, and the emergence of the SAB deficit when spleen weight and serum ANA titers increased in aged MRL-lpr mice.

Ubiquitin (Ub) binds to damaged or misfolded proteins, and if ubiquitinated proteins are not eliminated, neurodegeneration may occur. Consequently, Ub can be used as an additional marker of cell degeneration (Alves-Rodrigues et al. 1998). Indeed, increased density of FJB+ cells and alterations in the Ub-proteasome degradation system in the hippocampus appeared to parallel both the emergence of autoimmunity and impaired SAB performance. The beneficial effects of immunosuppressive treatment on the emergence of FJB+ neurons in the hippocampus of mice and improvement in SAB performance point to the causal link between chronic autoimmunity / inflammation, structural damage, and aberrant behaviour. Complementing a study showing progressive atrophy of dendritic spines in the MRL-lpr hippocampus (Sakic et al. 1998), the highly Ub-immunoreactive structures seen in this thesis were identified as degenerating axon terminals. Enhanced Ub staining was also observed in the midbrain of MRL-lpr mice. Interestingly, antibodies reacting with Ub and ubiquitinated histones are present in both MRL-lpr mice (Elouaai et al. 1994) and lupus patients (Muller & Schwartz 1995). However, since the treatment with CY failed to reduce expression of Ub in brains from MRL-lpr mice it appears that overexpression of Ub is a consequence of nonimmune pathogenic mechanisms (e.g. stress hormones), and may constitute an

initial pathologic insult prior to full-blown autoimmune mediated cell destruction.

Inflammatory mechanisms in the etiology of mental dysfunction (e.g. depressive behaviour) remain largely unexplored (Raison et al. 2006; Sakic et al. 1996). To better understand whether inflammatory pathways associated with prostaglandin production play a role in the etiology of brain damage, the non-steroid anti-inflammatory drug IBU, was given at a therapeutic dose in this thesis, comparable to other models of CNS inflammation (Lim et al. 2000; Yan et al. 2003; Teismann et al. 2003). Chronic treatment failed to normalize exploration behaviour and performance in the FST, nor did it reduce microglia activation or reduce the degree of leukocytosis into the choroid plexus and brain parenchyma. For the first time, we showed that microglia / macrophage brain activation increases with age, but IBU treatment was not effective in attenuating this process. These results obtained by immunohistochemistry and FACS analysis, however, are consistent with the notion of microglia-induced neuronal excitotoxicity. The IBU-rich diet also did not prevent neurodegeneration or normalize immune status. In addition to FJB, neuronal death has been determined using Toluidine blue (Ohtaki et al. 2003; Noraberg et al. 1999; Strasser & Fischer 1995), and this stain was employed. Despite relative co-localization of FJB, Toluidine Blue, and F4/80 stained cells, this thesis could not answer whether age-dependant microglia activation causes neuronal demise, reflects a "scavenging response" to necrosis or alternatively, a reparative process in the CNS (Lazarov-Spiegler et al. 1996; Prewitt et al. 1997). In addition, the COX-dependent pathway does not seem to be critical in the etiology of CNS disease in this model of neuropsychiatric lupus.

Transmission electron microscopy (EM) was used to delineate if apoptotic processes were occurring in MRL-lpr brains, through fine structural evaluation to detect morphological events of cell death (Colbourne et al. 1999). This was an important tool in this thesis given merely 7% of

FJB+ neurons co-localized with TUNEL+ cells in periventricular areas of diseased MRL-lpr mice. To elucidate the mode of neuronal death within the apoptosis-necrosis continuum, EM was used to examine ultrastructural morphology of cortical, hippocampal, hypothalamic, nigral, and cerebellar cells. We could not provide conclusive evidence which documents typical modes of cell death in brains of autoimmune mice and condensed Toluidine blue stained neurons were identified as dark, electron-dense cells in our EM analysis. More specifically, defining characteristics of typical apoptotic or necrotic cell death were not found in MRL-lpr brains, despite relatively broad EM screening. Given that these dark cells appeared both in clustered and scattered patterns, cell demise is likely caused by different pathogenic mechanisms in major divisions of the brain. This notion is most plausible when considering that peripheral inflammation, permeable BBB, disturbed ionic transport, and altered glucose metabolism may individually and / or synergistically compromise survival of mature and immature neurons in different brain regions (Vogelweid et al. 1991; Sidor et al. 2005; Maric et al. 2001; Alexander et al. 2005b; Sakic et al. 2005).

A substantial proportion of MRL-lpr mice develop deficits reflective of goal-directed and motivated behaviour which includes blunted responsiveness to sucrose and saccharine solutions (Sakic et al. 1996; Ballok et al. 2003). Blunted responsiveness to palatable stimulation points to a dysfunctional mesolimbic DA system, known to play a significant role in goal-directed and reward-mediated behaviour (Wise 2002). More specifically, the NAc is one of the primary reward centers, receiving dopaminergic inputs from the VTA (Tzschentke & Schmidt 2000) and consumption of sucrose has been shown to increase the release of DA in the NAc (Hajnal & Norgren 2001; Hajnal et al. 2004; Hajnal & Norgren 2002). Using Amph to probe the central dopaminergic system, this thesis provided evidence that mesolimbic DA pathways are indeed dysfunctional in behaviourally-

impaired autoimmune animals. Consistent with evidence of goal-directed response deficits to sucrose in D1 receptor-deficient and DA deficient mice (Cannon & Palmiter 2003; El-Ghundi et al. 2003), our functional results revealed that diseased MRL-lpr mice do not increase sucrose intake in response to systemic injection of Amph. Increased FJB staining in the NAc, and a paucity of TH+ neurons in the VTA of MRL-lpr animals provided structural evidence of neuronal degeneration and cell loss in the mesolimbic circuitry.

In addition to mesolimbic pathways, we found evidence that the mesonigral DA circuitry was targeted during the development of systemic autoimmune disease in behaviourally impaired MRL-lpr mice. More specifically, a single injection of the D1/D2 DA receptor agonist, Apo, induced rotational behaviour in diseased MRL-lpr mice, suggesting asymmetrical dysfunction in the nigrostriatal dopaminergic pathway, which is likely a consequence of receptor supersensitivity following neuronal damage (Randall 1984). The drug-induced rotational behaviour temporally coincided with the emergence of increased FJB staining in the SN, CSF cytotoxicity to dopaminergic progenitor cells, and other spontaneous behavioural deficits in diseased lupus mice. Together, smaller brain masses in aged MRL-lpr mice, TH-hypocellularity in the SN, and a negative correlation between SN neurodegeneration and ambulation in diseased mice supported a link between progressive neuroatrophy, dysfunctional behaviour, and autoimmunity. Evidence that early, prolonged immunosuppressive treatment suppressed the occurrence of both peripheral and central disease manifestations also supported the causal relationship between systemic autoimmunity, CSF toxicity, and neurodegeneration in this thesis.

While we expected to find TNF-alpha in serum, a somewhat surprising result from this thesis was detecting TNF-alpha in the CSF from diseased mice. We suspected that TNF-alpha was the

factor associated with the midbrain neurodegeneration in MRL-lpr mice, given that TNF-alpha is selectively toxic to dopaminergic neurons in neurological disease (McGuire et al. 2001; Clarke & Branton 2002), and mice lacking TNF receptors are protected against dopaminergic neurotoxicity (Sriram et al. 2002). However, while serum levels of TNF-alpha were four-fold higher than in CSF, only CSF was toxic to DA progenitor cells in our *in vitro* cytotoxicty assay. This indicated that TNF-alpha (at least in MRL-lpr mice) was not the cytotoxic factor to C17.2 progenitor cells. The fact that TNF-alpha was detected in the CSF of MRL-lpr mice is important when considering that intracerebroventricular injection of this cytokine can exacerbate CSF leukocytosis and perivascular infiltration (Seabrook & Hay 2001), observed at terminal stages of lupus-like disease (Alexander et al. 1983; Vogelweid et al. 1991). Since TNF-alpha can also mediate an apoptotic mode of neuronal death, it's role in the CNS of lupus mice needs to be explored further. Along the same lines, while abundant serum BRA were found in behaviourally impaired lupus mice with TH-hypocellularity, the fact that CSF (but not serum) was cytotoxic to DA progenitor cells suggests that systemic autoantibodies are not the source of neurotoxicity. Although not measured, it is possible that intrathecal BRA may also contribute to CNS manifestations.

The Fas antigen (Fas/Apo-1/CD95) is a cell surface receptor which is critical in mediating apoptosis in the immune system (Singer et al. 1994) and in the CNS (Park et al. 1998). This receptor is not expressed in brains of MRL-lpr mice (Tucekszabo et al. 1996) which is generally consistent with the lack of typical apoptotic morphology found in this work. The defective regulation of apoptosis is proposed to play a role in the etiology of many conditions, including neurodegenerative diseases (Fadeel & Orrenius 2005). Several apoptosis-inducing factors have been identified: lack of neurotrophic support, neurotransmitters, neurotoxicants, modulators of protein phosphorylation

and calcium homeostasis, DNA-damaging agents, oxidative stress, nitric oxide, and ceramides (Sastry & Rao 2000). Pro-inflammatory cytokines synthesized by immune cells, neurons and activated glial cells appear to have neurotoxic effects (Allan 2000) and apoptotic mechanisms are presumed to play a role in this process (Mattson & Duan 1999). The precise signaling cascade of apoptosis is not well established, and there are gaps in many suggested pathways. Apoptotic biochemical cascades can be activated in synaptic terminals and neurites, thus inducing local functional and morphological alterations which can propagate to the cell body, resulting in neuronal death. The coexistence of reduced neuronal complexity (Sakic et al. 1998) and increased brain cell death in MRL-lpr brains is consistent with the above concept, in particular to the extent that stimulus intensity (e.g. disease chronicity) can be instrumental for the simultaneous presence of neuronal atrophy and brain cell loss.

In this thesis, our findings of condensation of nuclear and cytoplasmic material, swollen mitochondria and ruffled appearance of cell membranes suggest profound metabolic perturbations, which may precede excitotoxic / oncotic cell death. This notion is supported by a recent study which found significant increases in glutamine, glutamate and lactate concentrations in MRL-lpr brains (Alexander, Zwingmann & Quigg 2005b), which are considered to be markers of excitotoxic and / or pro-oncotic signaling. A closer examination of the ultrastructural level revealed what appeared to be early and late stages of neuronal degeneration. We propose that the early phase is characterized by markedly increased electron density, a massive shrinkage of the whole somato-dendritic domain, swelling of the nucleolus and condensation of the nuclear chromatin. In the late stage, subcellular organelles are hardly recognizable due to the extremely high electron density and dramatic shrinkage of the cytoplasm. Also, degenerating neurons frequently occurred adjacent to neurons with normal

morphology. We speculate that these morphological features may indicate a delayed degeneration and cell death process which proceeds through cytoskeletal collapse.

While we found evidence to support previous studies of hippocampal damage and dysfunction (Sakic et al. 1998; Sakic et al. 2000) employing various staining techniques (eg. FJB, Ub, H&E, Toluidine blue) and procedures (eg. light, confocal, EM), an important novel finding described in this thesis is the neurodegeneration in functionally critical brain regions such as the subgranual zone and substantia nigra of MRL-lpr brains. These observations of densely packed dark cells in the two (of very few) regions known to contain proliferative progenitor cells capable of neurogenesis (Suh et al. 2005; McGuire et al. 2001; Zhao et al. 2003), appears even more substantial when considering our novel evidence that CSF from lupus-prone mice is cytotoxic to neuronal progenitor cells. When evaluating the global pattern of neuronal damage in diseased MRL-lpr brains, the pattern of degeneration is similarly seen in cases of hydrocephalus, meningoencephalitis, and hypoglycemic encephalopathy (Weller et al. 1978; Del Bigio 1993; Gerber et al. 2001; Alexander & Alexander 1983; Auer et al. 1984; Fujioka et al. 1997), all resulting in cerebritis. Relevant to this thesis, others have reported manifestations of hydrocephalitis (Denenberg et al. 1992), meningoencephalitis (Alexander et al. 1983) and altered glucose metabolism (Alexander, Zwingmann & Quigg 2005b) in the brains of MRL-lpr mice. Our novel finding of microglia activation in the CNS, as well as the distinct distribution and ultrastructural appearance of neurodegeneration in these animals, is further evidence supporting the notion of murine cerebritis (McIntyre et al. 1990; Alexander et al. 2005a). For example, the SN in mice has more microglia than other regions (Lawson et al. 1990), and considering this neuroanatomical trait, one may assume that the dense population of dark cells in the SN reflects region-specific susceptibility to inflammatory

and excitotoxic metabolites produced by activated microglia in MRL-lpr brains (ultimately leading to neuronal damage and cell loss).

In summary, progressive neuronal damage / death, microglia activation, and spontaneous behavioural deficits are concomitant with the onset and development of systemic autoimmune / inflammatory disease in the MRL-lpr model of NP-SLE. DA neurons and their progenitor cells appear to be one target of the disease process, possibly accounting for some of the aberrant behaviours (such as impaired goal-directed behaviour and hypoactivity) in lupus mice. While it is not clear whether microglia are instrumental in CNS disease or whether autoantibodies play a role in the neurodegenerative process in MRL-lpr brains, our evidence suggests that regional dark cell neuronal damage may account for neuropsychiatric manifestations in the MRL model of NP-SLE. For example, an impaired capacity for hippocampal neurogenesis could account for the cognitive impairments of lupus mice reported in this thesis. The therapeutic implications of this research support the use of immunosuppressive drugs, but not NSAIDS, in treating NP-SLE patients with suspected CNS pathology. Further work demonstrating the precise disease factor(s) as well as other cell targets of disease is needed to confirm the hypothesis that autoimmunity *per se* causes brain cell death, although it is likely an interplay of numerous factors which lead to neurodegeneration. For example, in addition to immune-mediated insults, altered production of steroid hormones is likely another important factor in the pathogenic circuitry during systemic autoimmune disease. Finally, the findings of this research greatly strengthen the face and construct validity of the MRL model of NP-SLE.

LABORATORY TECHNIQUES

The first study established that a modified Fluoro Jade B technique could be used as a reliable cytochemical stain for identifying dying neurons in murine brains. Initially, Swiss Webster (SW) mice were injected with either the neurotoxin kainic acid (KA) or PBS. Breaking away from standard histological processing with FJB (Schmued & Hopkins 2000) which was ineffective, our modification of the technique resulted in clearly distinguished FJB-labeled neurons in injured SW brains. Since introducing this modification into the literature, our staining process has been adopted by many others, confirming that it is reliable and repeatable (Kelly et al. 2003; Yu et al. 2004; Voutsinos-Porche et al. 2004; Hunsberger et al. 2005; Csernansky et al. 2006; Mitruskova et al. 2005; Bonde et al. 2005). To examine whether neuronal death is associated with other putative markers of brain inflammation, the FJB method was combined with staining for CD4 and CD8 T-lymphocytes. Preliminary studies revealed that immunostaining for CD4+ and CD8+ markers was not compatible with the FJB staining protocol, thus precluding double-labeling. Therefore, CD4+ and CD8+ cells were first manually quantified before the same sections were reprocessed for FJB. Double-labeling of FJB with TUNEL staining was successfully performed to determine if the neurodegenerative process involved DNA nicking. The benefit of this design was that markers could be co-localized to produce a more accurate picture of the brain architecture at one point in time. Complementing the spleen weight data and extending this work, we employed an ELISA to quantify levels of serum ANA. This technique has previously been used to confirm the disease status of autoimmune mice. All measures were made by a third, blinded party or computerized software.

In the second study, CY was used to assess the effects of sustained immunosuppression on neuronal death and behaviour. In addition to the reduction of leukocyte numbers (Snippe et al. 1976), CY also makes these cells unresponsive to stimuli, leading to generalized immunosuppression

(ten Berge et al. 1982). While the therapeutic effect of CY on the development of autoimmune symptoms was demonstrated previously (Shiraki et al. 1984; Grota et al. 1989; Grota et al. 1990; Sakic et al. 1995; Sakic et al. 1996), our treatment extended over a much longer period of time (i.e. 12 weeks versus 6 weeks). The benefit of this design was that the causal effect of disease progression on neurodegenerative and cognitive changes could be directly assessed. In a pilot study, it was observed that mice not habituated prior to the SAB test appeared stressed and performed erratically. Therefore, to minimize anxiety and stress, and to obtain robust SAB data, animals were initially habituated to the experimenter and the testing environment. For example, mouse cages were wheeled on three occasions from the colony room to the testing room to reduce stress induced by transportation and a novel environment. In addition to FJB cytochemical staining, standard H&E staining, and anti-Ub immunohistochemistry was employed to assess neuronal death, loss and damage, respectively. Although an attempt was made to adapt the FJB stain for use with paraffin embedded sections, this was not successful. Therefore, due to dissimilar fixation protocols for assessment of dying neurons and ubiquitinated particles, an additional batch of mice were required to complete this study. Both spleen weight data and ANA ELISA confirmed the disease status of autoimmune mice, and all measures were made by a third, blinded party or computerized software.

In the third study, the effects of an NSAID on CNS involvement in MRL-lpr mice was examined. The benefit of this design was the stress-free chronic administration of the drug prior to examining effects on behaviour and neuropathology. Since our laboratory does not have methodology to measure drug levels in blood and tissues, and this information was lacking (as it lacks in reports on beneficial effects of IBU). However, the treatment was limited to a "one-dose design", and the dosage was comparable to therapeutic doses used in other studies (Lim et al. 2000;

Yan et al. 2003). Despite this, the inflammatory disease in MRL-lpr mice was not attenuated, and therefore may be more aggressive than in other models (which implies that the dose of IBU was not adequate). To examine neuroinflammation, the CD3 T-lymphocyte and F4/80 microglia marker were employed. Given that blood-brain and CSF-brain barriers are breached in diseased MRL-lpr mice (Vogelweid et al. 1991; Sidor et al. 2005), the F4/80 stain could not distinguish infiltrating macrophages from resident microglia, which share surface antigens and originate from the monocyte line. Since the combination of immunohistochemistry and flow cytometry is a useful approach to assess activated microglia / macrophages in the brain (Merrill et al. 1992), we also employed the Flow cytometry technique. This procedure is a rapid and reproducible means of quantifying surface marker expression. Specifically, the surface expression of CD69+ and F4/80+ cells using antibodies directed against these molecules was examined. While a drawback of flow cytometry is that often only a small number of cells are present that are positive for the markers of interest and it can be difficult to distinguish a true positive from non-specific or artificial staining, this can be overcome by the use of the multi-parameter sequential-gating strategy to gradually eliminate contaminating cells that stain non-specifically with anti-CD69 and anti-F4/80. This technique also allows for the accurate enumeration of activated microglia cells and was employed in our study. Despite relative co-localization of FJB+ and F4/80+ cells, because IBU was ineffective, this study could not answer whether age-dependent microglia activation caused neuronal demise.

Interestingly and unexpectedly, systemic disease in MRL-lpr mice appeared to have been exacerbated by IBU treatment. However, this observation (i.e an increased incidence of dermatitis, alopecia and necrosis of ear tips) remains anecdotal since peripheral manifestations were not systematically measured. This drug effect needs to be repeated in a separate cohort of mice by

formulating an *a priori* hypothesis and by systematically measuring peripheral manifestations. Similar to peripheral manifestations, trends toward increased numbers of CD3+ cells and dying neurons were observed in brains of drug treated MRL-lpr animals.

Complementing the FJB data and extending our assessment of neurodegeneration, Toluidine blue staining and EM was also employed. As with FJB, both of these techniques have been used to confirm neuronal demise (Noraberg, Kristensen & Zimmer 1999; Strasser & Fischer 1995; Ohtaki et al. 2003; Colbourne, Sutherland & Auer 1999). However, while FJB (an acid dye) stains degenerating / dead neurons, Toluidine blue (a basic stain) demarcates highly condensed cells, which may or may not ultimately die (Auer et al. 1985). Using an alternative dye such as Acid Fuschin would have identified neurons at a similar stage of death, comparable to the FJB stain. Another drawback of our evaluation was that the sample size was not large enough to estimate the severity of the observed phenomenon of dark cell neurodegeneration revealed by Toluidine blue and EM screening. Although dark neurons are also a consequence of improper fixation or handling of brain tissue (Schmechel 1999), profound differences between diseased and control brains discredits the notion of procedural artifacts and supported our previous data. In addition to wet spleen weight, changes in the systemic cytokine network (TNF-alpha and IL-1beta), CIC, and antibodies to dsDNA were measured as additional markers of disease severity. All assays included diluent controls to exclude overestimation of results, and all cell quantifications were performed by an experimenter blinded to sample origin.

In the fourth and fifth study, we pharmacologically probed the functional status of central dopaminergic circuits involved in control of behavioural reward and locomotion in autoimmune animals. To do this, we used Amph to probe the mesolimbic DA system and Apo to probe the

mesonigral DA system. While it is known to be effective in activating central dopaminergic circuits within major compartments of the brain reward system, the drawback of Amph is that it increases release of catecholaminergic neurotransmitters (Hoffman 2001), blocks their reuptake by presynaptic axonal terminals (Heikkila et al. 1975), and has pharmacological effects on both NE and DA release. Fortunately, behavioural effects of Amph are largely mediated via the central DA system (Rang et al. 2003). Although neuronal demise was detected in the NAc, the phenotype of the dying cells were not determined. Given that DA receptors are highly enriched in the NAc, however, it may be assumed that the dying cells were predominantly of a dopaminergic phenotype. While the DA D1/D2 agonist Apo is very specific for DA receptors and acts upon the nigrostriatal DA pathway, it also has been found to induce rotation in unlesioned rodents (Dankova et al. 1978). Also, the sample size of Apo treated animals was relatively small in our study. Directionality and direct assessment of limb use were not taken into consideration when turning behaviour was scored. To optimize the most appropriate dose of the drug, however, eight additional MRL-lpr males were used and the circling phenomena was found to be reproducible. A major benefit of the designs of both pharmacological studies was that the pre-drug SAL baseline behaviours were recorded prior to drug testing, and acted as the subject's own control.

The final (fifth) study used C17.2 DA progenitor cells (Wagner et al. 1999) which can differentiate into neurons (Ryder et al. 1990; Snyder et al. 1992) expressing the DA synthesizing enzyme TH (Yang et al. 2003). Using this new and useful *in vitro* cytotoxicity assay, we screened the toxicity of CSF from autoimmune mice. Since our findings have been subsequently confirmed and expanded upon (Sakic et al. 2005), this technique appears to be a reliable tool to assess toxic CSF. Due to the small volume of CSF collected from a mouse, we modified the TNF-alpha ELISA

procedure and pooled CSF samples. Pooling samples precluded further correlational analyses. The modified ELISA, however, confirmed elevated levels of TNF-alpha in CSF and serum from autoimmune animals. ANA ELISA and wet spleen weights were also used to confirm disease status. Supporting data from our pharmacological studies and the *in vitro* assay, we found that doapminergic neurons were excessively destroyed by co-localizing TH immunohistochemistry with FJB staining in MRL-lpr brains. Twelve week CY treatment abolished both CSF toxicity and neurodegeneration, but the drawback was that extensive behavioural testing could not be performed due to the weakened physical condition of these chronically treated mice. Also, due to a small group of MRL-lpr mice employed in the CSF toxicity screening (N = 6), one CSF sample having no toxic effects significantly affected normal distribution of the data. Consequently, this sample (considered an outlier) was removed from the comparison between the means.

In addition, BRA were three-fold higher in serum from diseased MRL-lpr mice with TH-hypocellularity. Due to the small volume of CSF collected from a mouse, however, this precluded an assessment of BRA levels in CSF. We also did not formulate an *a priori* hypothesis that TH+ cell densities would differ between hemispheres of MRL-lpr brains and consequently this was not systematically recorded for comparison. While cell counts were obtained from the SN, due to the high density of TH+ cells in the VTA of control mice, this region was not amenable to quantification. This limited us to a descriptive assessment of the area. While the co-localized paucity of TH and excess in FJB staining support the notion of selective dopminergic neuron cell death, systemic BRAs and intrathecal TNF-alpha in behaviourally impaired lupus mice are unlikely the cytotoxic agents. Since CSF was toxic to the C17.2 DA progenitor cells, but not serum, these results have to be interpreted with care. Most procedures and all measures were carried out by a

third, blinded party.

STATISTICS

MRL-lpr and MRL +/+ mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), or obtained from a MRL colony in McMaster University's Animal Quarters (housing level A, cages sterilized and manipulated under laminar flow protection). Swiss Webster (SW) mice were purchased from Charles River Canada (St. Constant, Canada), and CD1 mice were obtained from Cedar Lane (Canada). All results in this thesis were graphed for distribution, and all normally distributed data was analyzed by either a one-way analysis of variance (ANOVA), ANOVA with substrain as a main factor and treatment and concentration as repeated measures, or ANOVA with treatment and age or substrain as between-group factors, and slide as the repeated measure. Student's t-test was used in the post-hoc analysis because of the 2 x 2 design in most comparisons. Pearson's correlation was used to measure association between variables, where appropriate. Fisher's exact test was used to assess the difference in group performance in the SAB tests. Computations were performed using the SPSS 11.0, 12.0, and 13.0 statistical packages, and the accepted level of significance was $P \leq 0.05$. Graphs show means ± S.E.M., with $*p \leq 0.05$; $**p < 0.01$; and $***p < 0.001$ representing between group differences.

FUTURE DIRECTIONS

We have demonstrated that neurons are dying in brains of lupus-prone mice and that this process is associated with the spontaneous progression of autoimmune disease and behavioural dysfunction. However, these studies have not answered a number of central questions. More

specifically, future studies need to examine the status of other neuronal phenotypes and elucidate terminal mechanisms involved in neuronal demise and dysfunction, such as BRA, cytokines and other soluble toxic mediators. This thesis has established that the MRL model of NP-SLE holds the potential of explaining mechanisms by which chronic autoimmunity / inflammation damages brain morphology and function in neuroimmunological disease.

Our thesis results indicate that in addition to the periventricular region, degenerating neurons were found in areas remote from ventricles, as revealed by FJB, Toluidine blue, and EM. In addition to these stains and techniques, other methods can be employed to examine dying neurons. For example, silver staining methods selectively delineate dark neurons (Switzer, III 2000; Ye et al. 2001). While we could not provide conclusive evidence which documents typical modes of cell death in brains of autoimmune mice, future work is needed to examine the effects of disturbed ionic transport and alterations in glucose metabolism on the viability of mature and immature neurons in different brain regions, and whether there are definitive region specific modes of cell death. Of major clinical importance is the observation that this brain damage occurs in regions that control emotions, memory, and the release of hormones, and a complete understanding of mechanisms of cell death will likely provide a bases for the development of novel therapies.

For many years autoreactive antibodies (particularly BRA) were the focus of intensive research, which has lately expanded to the recognition of neuroactive cytokines and altered regulation of the neuroendocrine axes (Besedovsky & Del Rey 1992). Although this thesis has supported the hypothesis that the progression of autoimmune disease plays a principal role by showing that immunosuppressive treatment (i.e. inhibiting antibody production) with CY prevents neuronal demise, and that autoimmune symptoms correlate significantly with neurodegeneration, CY

inhibits protein synthesis, DNA replication, and cell division in all cells (Miller 1997). Therefore, knowledge of the precise immune components and biochemical profile of neurotoxic factors would help identify pathogenic circuits in the MRL model. This would likely have implications on designing protocols which could foster both neuroprotection and immunosuppression during relapses of autoimmunity in NP-SLE patients.

Based on the findings of this thesis, COX mediated mechanisms (i.e. prostaglandin pathways) are unlikely involved in the observed progressive brain degeneration. However, another pathway which may play an important role in neurodegeneration involves p53. As a well-characterized transcription factor, p53 is known to respond to DNA damage and other genotoxic stresses by the activation of downstream targets that are involved with repair, differentiation, senescence, growth arrest, and apoptosis (Resnick-Silverman & Manfredi 2006). In a recent pilot study, we examined densities of p53 dot-like particles in the brain parenchyma of MRL mice. Unexpectedly, we observed significantly reduced anti-p53 immunohistochemical staining in MRL-lpr brains relative to congenic controls. Consistent with this finding, a recent study reported that cellular entry of anti-DNA antibodies into glomerular tissue of MRL-lpr mice resulted in an inhibition / reduction of p53 and suppression of apoptosis (Yanase & Madaio 2005). When considering that elevated immunoglobulin levels are seen in the CSF and brain of MRL-lpr animals (Sidor et al. 2005; Zameer & Hoffman 2001) one may assume that a similar autoantibody-mediated process is occurring within neurons and accessory cells of the CNS. Therefore, disrupted p53 functions may account for the morphological features and "intermediate form" of cell demise revealed by our EM analysis, indicative of a delayed degeneration / cell death process which proceeds through cytoskeletal collapse. Future studies should examine the role of antibodies to p53 in MRL-lpr brains more

closely.

Changes in brain water and cerebral volume can lead to brain edema that may be one of the underlying causes of death in many neurological diseases. In SLE, magnetic resonance imaging studies of the brain have demonstrated lesions with the prominent appearance of edema (van Dam 1991) and activation of complement may play a significant role in the pathogenesis of lupus cerebritis by causing inflammation (as described in this thesis) that leads to edema. In MRL-lpr animals, IgG and C1q have been found to co-localize in perivascular deposits, indicating that the BBB is compromised (Alexander et al. 2003). Interestingly, the hormone progesterone and it's precursors and metabolites have been shown to enhance functional and structural recovery in rats and patients with brain edema (Shear et al. 2002; Guo et al. 2006; Sayeed et al. 2006), likely by reconstituting the BBB and reducing lipid peroxidation (Stein 2001). Considering the compromised status of the BBB in this model of NP-SLE, future studies should examine the effects of progesterone treatment on functional and structural recovery of MRL-lpr brains.

This thesis showed that profound neurodegeneration in the limbic system of MRL-lpr mice is associated with cytotoxicity of their CSF to C17.2 cells, and this has subsequently been shown to be mediated largely by IgG (Sidor et al. 2005; Sakic et al. 2005), to mature and immature neurons. Complementing these findings, in this thesis we observed accumulations of basophilic cells and FJB staining in the subgranular zone of the dentate gyrus of MRL-lpr hippocampi. Taken together, a link between toxic CSF IgG and progenitor cell damage would suggest that lesions of germinal layers may reduce development and regenerative capacity of autoimmune brains. The possible effect of autoimmunity on neural stem cells may be extended into pre-natal life, where mulipotent brain cells may be the target of an attack mounted by the immune system of autoimmune mothers. For

example, ova-transfer experiments in mice showed that profound deficiencies in offspring behaviour are produced if embryos were reared in an autoimmune uterine environment and conversely, that the severity of behavioural dysfunction in autoimmunity-prone mice is reduced if they were transferred as embryos into a non-autoimmune uterine environment (Denenberg et al. 1991). Consistent with the possibility that the blood-placental barrier does not provide sufficient protection from mother's autoimmunity is the fact that neonatal lupus is a well-documented phenomenon in humans (Cabanas et al. 1996; Prendiville et al. 2003). Therefore, more work needs to be done to determine if conditions for progressive neurodegenerative events later in life are antedated by early neural precursor cell damage.

More work also needs to be done on the role of hormones in the progression of NP-SLE. For example, secretion of the pituitary hormone prolactin is under the control of DA, and given that elevated secretion of prolactin is common in SLE (Jara et al. 2001), one may wonder whether this endocrine imbalance is a consequence of impaired DA regulation in the CNS. Consistent with this notion, the DA agonist bromocriptine suppresses secretion of prolactin and ameliorates affect in SLE patients (Walker et al. 2000) and disease activity in autoimmune mice (McMurray et al. 1991). Whether dopaminergic neurons die excessively in NP-SLE patients is still an unexplored question, however, based on the finding of basal ganglia damage in this thesis (observed in the lupus patient's brain), this line of investigation needs to be examined in future studies. The role of corticosterone also appears to be important in SLE-like disease given that glucocorticoids are innately elevated in MRL-lpr mice (Lechner et al. 2000), and the iatrogenic effects of sustained corticosteroid therapy on brains of NP-SLE patients (Ainiala et al. 2005).

Similar to the effects of chronic stress, MRL-lpr mice show deficits in cognition, brain

atrophy, and alterations in neural chemistry and morphology at the onset of disease (Szechtman, Sakic & Denburg 1997; Sakic et al. 1998; Sakic et al. 2002), and neuronal loss as autoimmunity progresses. Although an imbalanced neuro-immuno-endocrine network plays a key role in etiology of brain damage, it is still not clear whether central neurons are initially damaged by an autoimmunity-driven upregulation in corticosterone production. In a pilot study, we found that prolonged treatment with corticosterone attenuated signs of autoimmune disease and lead to profound deterioration of neuronal morphology in both MRL substrains. As previously reported (Sakic et al. 1998), while untreated autoimmne MRL-lpr mice showed profound neuronal spine loss relative to asymptomatic MRL +/+ controls, chronic corticosterone treatment induced significant dendritic deterioration in all animals. These changes may be reversible but can also be indicative of neurons in the early stages of degeneration (McEwen 1999; Woolley et al. 1990). Together with the evidence that basal corticosterone levels are chronically elevated in autoimmune MRL-lpr mice (Lechner et al. 2000), our pilot data suggests that sustained endogenous immunosuppresion may be the precursor that predisposes neurons to the degenerative autoimmune / inflammatory cascade seen at later stages of disease (e.g. following a breach in the BBB). More specifically, studies have revealed changes in the morphology of neuronal dendrites, cerebral atrophy, and highly Ub-immunoreactive structures (representing degenerating axon terminals) occur by 14 weeks of age in MRL-lpr brains, and neurodegeneration and microglia activation ensue at terminal stages of disease (e.g. by 5 months). Indeed, few MRL-lpr mice survive beyond 6 months of age (Dixon et al. 1978), which may be attributed to profound CNS damage. Future studies examining the possibility that adrenalectomy could prevent or delay the degeneration and death of cells in MRL-lpr brains should be explored.

In summary, the correlational nature of clinical data has led to the necessity for models in which interactions between autoimmune phenomena and brain function can be examined in a more systematic and direct way. This thesis has detailed the topography of brain lesions and their relationship to some behavioural deficits. As such, the studies described in this thesis corroborate the notion that AABS is a valuable paradigm to explore the mechanisms of CNS damage in the MRL model of neuropsychiatric lupus. A more complete understanding of the fundamental concepts of the mode and mechanisms of brain cell death in MRL-lpr mice will provide a basis for the development of novel therapeutic interventions and potentially explain discrepancies in current interventions used in SLE.

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APPENDIX 1

MATERIALS AND METHODS

Overall Design

The overall research design involves standardized analysis of behaviour, sacrifice, CSF collection, serological assessment of autoimmunity, and tissue perfusion, extraction and fixation. From previous studies the effect size 0.5, power 80% (at alpha2 = 0.05) can be achieved with 10 mice / strain (Sakic et al. 1993b; Sakic et al. 1992; Sakic et al. 1994b). This number was obtained by applying PASS 6.0 (NCSS Statistical Software, Kaysville, UT) before detecting group differences in a univariate design. However, because of unforseen circumstances (e.g. premature death of diseased or drug-treated animals) or shipper / supply demands, the sample size fell below this number in some studies. In most cases, cohorts of n = 10 mice / substrain / treatment or more was used in our studies and mice were matched by age, sex, and body weight. The SPSS 11, 12, and 13 statistical packages (SPSS Inc, Chicago, IL) were used for parametric and non-parametric tests in detecting group differences (ANOVA, student's t-test, fisher's exact t-test, etc.) and relationships among different sets of data (e.g. Pearson's correlation).

Animals

Mice were obtained from either a supplier or from a local breeding colony. MRL-lpr and age-matched MRL +/+ mice were obtained from either the Jackson Laboratories (Bar Harbor, ME) or from the breeding colony at McMaster University, with the original purchased from Jackson Laboratories. SW and CD1 were purchased from Charles River and Cedar Lane Canada, respectively. In all studies, mice were housed singly prior to behavioural testing, with a 8:00 AM

– 8:00 PM light schedule and *ad libitum* access to food and water. The temperature of the colony room was kept at 25 ± 2 °C. All experimental protocols were approved by the McMaster Animal Care Committee and carried out in accordance with rules and regulations of the Canadian Council of Animal Care. To complete this thesis project, 257 MRL-lpr, 204 MRL +/+, 43 SW and 41 CD1 mice were used, for a total of 545 mice. Two human brains were also employed.

Behavioural Tests

Mice were habituated to the experimenters prior to behavioural testing to reduce confounding effects of stress (Sakic et al. 1992). Tests which reliably discriminate behavioural profiles of MRL-lpr mice and MRL +/+ controls were selected from the well-established behavioural battery used for detecting AABS (Sakic et al. 1994a). These tests included computerized measurement of nocturnal activity, the FST, 4% sucrose preference test, and the sucrose response rate test using protocols previously described (Sakic et al. 1994a; Sakic et al. 1996). These tests are proposed to measure anxiety-, depressive-, and anhedonic-like behaviour. The SAB test, novel to the AABS paradigm, was also employed to assess the cause-effect relationship between neuronal degeneration and autoimmunity. Rotational behaviour was also examined following an acute Apo-injection. Sucrose response rates were examined following an acute Amph-injection. Given that all tests were activity-dependant, they were performed between 6 PM and 12 AM, during the period of elevated locomotor activity and optimal (noise-free) testing conditions in the local animal facility.

Neurotoxic and Immunosuppresive Treatment

To assess the effectiveness of the FJB technique, a well-known neurotoxin was injected into

a group of healthy Swiss Webster (SW) mice. Kainic acid (KA) is a rigid structural analog of glutamate and powerfully reproduces the excitatory neurotoxic (excitotoxic) action of glutamate on central neurons. Eight mice received a single injection of KA (10 mg/kg, i.p.; Sigma, St. Louis, MO, USA), while controls were injected with an equivalent volume of PBS. All mice were sacrificed 4 days later. Brains were subsequently processed and assessed for the presence of brightly-lite FJB stained neurons.

Prolonged treatment with CY has successfully been used to restore food / water intake, exploratory and motivated behaviour, and reduce infiltration of leukocytes into the choroid plexus (Farrell et al. 1997; Sakic et al. 1995; Sakic et al. 1996). It also normalizes CRF mRNA expression (Sakic et al. 1999) and prevents neuronal atrophy in MRL-lpr brains (Sakic et al. 2000a). MRL-lpr and MRL +/+ mice were assigned into two groups. The first cohort was injected weekly between 5 - 16 weeks of age with CY (100 mg/kg i.p.; mouse LD50 = 405 mg/kg i.p.; "Procytox", Horner, Montreal), and the second cohort received twelve injections of an equivalent volume (~ 0.2 - 0.3 ml) of SAL. Treatment began at 5 weeks of age because this is an age which antedates hyperproduction of autoantibodies and pro-inflammatory cytokines, such as IL-1 and IL-6, hallmarks of systemic autoimmune disease in lupus-prone mice (Theofilopoulos & Dixon 1985; Ballok et al. 2003). The effectiveness of the CY treatment was assessed by measuring serum levels of ANA and cytokines, and behaviour and neuropathology was examined between 7 and 10 days after the last CY injection.

Non-steroidal Anti-inflammatory Treatment

Ibuprofen (IBU) was purchased from Sigma (St. Louis, MO) and subsequently formulated

into color-coded, AIN-76A rodent diet by Research Diets (New Brunswick, NJ) at a final concentration of 375 ppm. This dose was selected on the evidence that it prevents CNS pathology and inflammation in a mouse model for Alzheimer's disease (Lim et al. 2000; Yan et al. 2003). From 5-19 weeks of age half of each group was fed *ad libitum* with either drug-supplemented chow or control chow. The body weight and food consumption were monitored weekly, and averaged food consumption was ~5 g/day/animal, resulting in a final daily dose of ~62.5 mg/kg in the IBU-treated groups. The effects of IBU treatment on neuropathological indices and behavioural performance was subsequently examined.

Probing Dopaminergic Circuitry

d-amphetamine sulfate (Amph, Sigma-Aldrich Canada, Oakville, Ont.) was dissolved in 0.9% saline and 0.5 mg/kg was injected i.p. using a 26.5 gauge needle (5 ml/kg of body weight; e.g., 0.2 ml/40 g mouse). Since pharmacological blockade of DA turnover augments the licking response (Hajnal & Norgren 2001), diseased MRL-lpr mice were challenged with Amph, known to be effective in modulating the mesolimbic brain reward system (Naranjo et al. 2001). This dose was chosen based on a previously reported effect (Brennan et al. 2001) and a pilot study where 0.5 and 1.5 mg/kg doses were compared in a small cohort of mice exposed to the sucrose preference test (Prasad 1999).

Apomorphine hydrochloride (Apo, Sigma-Aldrich, St. Louis) was dissolved in 2% ascorbic acid and PBS. The drug was prepared immediately before use, kept on ice, and protected from light. As a result of an imbalance in mesonigral receptor sensitivity, DA receptor agonists (such as Apo) induce rotational behaviour in affected mice (Randall 1984). While our pilot study revealed that

higher doses caused stereotypic mouthing, rearing, and grooming behaviours in MRL-lpr mice, a smaller single subcutaneous (s.c.) dose of 0.2 mg/kg induced tight random and intermittent circling behaviour around their body axes. Although this was seen much more in diseased mice, it occurred at a relatively slow rate (Buonamici et al. 1990; Thornburg & Moore 1975) of 15 times on average over a 10 min observation period.

Tissue Preparation

Upon completion of behavioural testing, mice were sacrificed for the purpose of blood and CSF collection, and brain and spleen weighing. Mice were anaesthetized with Somnotol (60 mg/kg) and transcardially perfused with 0.9% SAL before CSF collection. CSF was stored in an incubator (37°C) prior to analysis. Extracted brains were weighed on a digital scale (Sartorius 2024 MO, VWR Scientific of Canada) before immersion into a fixative (either 4% PFA or 10% Formaldehyde) for varying times, depending on procedural staining protocols. Before processing and analysis, brains were cut in coronal, horizontal, or sagittal sections with a Jung Frigocut 2800 E cryostat. Spleen weight was also taken because spenomegaly is one of the most reliable manifestations of severe autoimmunity in the MRL-lpr strain (Theofilopoulos 1992).

Serological and CSF Assessment

At the end of a study mice were anesthetized with Somnotol (i.p., 60 mg/kg body weight; MTC Pharmaceuticals, Cambridge, ON) and after terminal bleeding from the vena cava, they were intracardially perfused with 20 ml of PBS, prior to CSF collection. Blood was left to coagulate in 1.5-ml plastic vials, and later centrifuged for 10 min at 3000 rpm. Serum was separated from the clot

and stored at -20°C until further analysis. The CSF collection is a well-established procedure in our laboratory. In brief, posterior neck muscles were removed with a surgical blade and a glass micropipette (custom-made from 75 ml Natelson blood-collecting tubes) was inserted through the arachnoid membrane into the cisterna magna. 15-25 ml of CSF was aspirated by capillary forces and slight suction through rubber tubing attached to the micropipette. Clear CSF was transferred to small vials and immediately centrifuged. The samples were kept at RT and assessed within 24 h of collection. Following CSF collection, mice were further perfused with 40 ml of fresh 4% PFA.

Significant correlations between circulating immune factors and behavioural deficiency have been documented previously (Sakic et al. 1993a; Ballok, Szechtman & Sakic 2003; Szechtman et al. 1997). To compare the severity of autoimmune manifestations, as well as to assess the efficacy of immunosuppressive treatment, standard serological measures of autoimmunity were performed. In particular, ELISA kits (R&D Systems, MN) were used to measure serum levels of TNF-alpha and IL-1beta (Cat. #MTA00 and ML401). Semi-quantitative ELISA kits (ADI, San Antonio, TX) were used to assess serum levels of ANA (Cat. #5200), and CIC and dsDNA concentrations were measured using qualitative sandwich ELISA kits (CIC: Cat. #5900; anti-dsDNA: Cat.#5100) according to the manufacturer's instructions (Alpha Diagnostic International, San Antonio, TX). A modified TNF-alpha ELISA procedure was developed in our lab to assess cytokine levels in CSF from autoimmune animals. An ELISA was also used for detecting reactivity against brain antigens as previously described (Crimando & Hoffman 1992; Zameer & Hoffman 2001). This procedure is used to measure serum titers of autoantibodies that bind integral membrane proteins of the CNS. Results from the immunological assays were correlated with behavioural and neuromorphological measures.

Antibodies and Stains

FJB stock solution was purchased from Histo-Chem, USA (Jefferson, AR). Monoclonal rat-anti-mouse CD4 antibody (IgG2b isotype), and rat-anti-mouse CD8a antibody (IgG2a isotype) were purchased from Sigma Chemicals, USA (St. Louis, MO). FITC-conjugated goat anti-rat IgG antibody was obtained from Jackson Immunoresearch, USA (West Grove, PA). Trevigen, USA (Gaithersburg, MD) supplied the Neuro TACS II TdT Labeling Kit and Alexa-594 was obtained from Molecular Probes, USA (Eugene, OR). Anti-Ubiquitin polyclonal antiserum was purchased from Dako, Canada (Burlington, ON), and the labeled streptavidin-biotin-peroxidase kit from Vectastain, USA (Pittsburgh, PA). Hamster anti-mouse CD3e antibody and Streptavidin/HRP was used from the Anti-Ig HRP Detection Kits (BD Pharmingen; Mississauga, ON). Biotinylated anti-hamster IgG antibody was obtained from Vector Laboratories, Canada (Burlington, ON). Rat monoclonal antibody F4/80 and the biotinylated anti-rat IgG secondary antibodies were supplied by Serotec, USA (Raleigh, NC). Antibody diluent and Fluorescence Mounting Medium was obtained from DAKO , USA (Carpinteria, CA). Vectastain ABC reagent and DAB substrate kits were purchased from Vector Laboratories, Canada (Burlington, ON). R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD69 monoclonal antibody and RPE-Cy5-conjugated rat anti-mouse F4/80 monoclonal antibody were supplied by Serotec, USA (Raleigh, NC). Toluidine-blue powder was from Marivac-Canemco, Canada (St. Laurent, QB) and sodium-borate from Caledon, Canada (Georgetown, ON). Rabbit anti-TH and biotinylated anti-rabbit IgG secondary antibody were both purchased from Vector Labs, Canada (Burlington, ON), and chromogen diaminobenzidine tetrahydrochloride from Sigma-Aldrich, Canada (Oakville, ON).

Cytochemical and Immunohistochemical Staining

To examine neuronal degeneration and cell loss, the stains used included the cytochemical stains FJB, Toluidine blue, EM and H&E, as well as the DA cell marker TH. FJB, an anionic fluorescein derivative, was used for the localization of degenerating neurons in brain tissue sections (Schmued & Hopkins 2000). While this dye has an affinity for the entire degenerating neuron, the degenerating tissue components (biomolecules) to which the dye has an affinity is currently unknown. Extracted brains were fixed in 4% PFA for 24 - 48 h and were then immersed in 30% sucrose (in PBS) for up to 4 days before frozen sections were processed. Ten-μm coronal, horizontal and sagittal sections were cut with a Jung Frigocut 2800E cryostat and sections were mounted onto Aptex-coated slides before staining. Slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. The slides were rinsed in distilled water (dH_2O) for 1 min before being transferred to a 0.06% potassium permanganate solution and gently shaken for 15 min. Slides were rinsed in dH_2O for 1 min before immersion in a 0.001% FJB / 0.1% acetic acid staining solution (prepared from a 0.01% stock solution). In our experience, the stock solution produced optimal staining results after 2 months of storage at 4°C in darkness. After 30 min of gentle shaking in the staining solution, slides were rinsed for 1 min in each of three dH_2O washes and left to dry for several hours in darkness. Subsequently, they were processed in three 2-min xylene washes before being coverslipped with DPX. The reactivity of the stain to dying / dead neurons was examined using an epi-fluorescent microscope with 450–490-nm excitation light at ×200 magnification. To quantify the number of FJB+ neurons, sections were photographed on 400 ASA 35-mm print film, using a Nikon N90s camera. The pictures were scanned at 600-dpi resolution and digitized into TIFF files using Adobe Photoshop software (Adobe Systems, San Jose, CA) for assessment by NIH

Image analysis software (Scion, Frederick, MD) or manually counted by an observer blinded to group origin. To produce representative high-quality images, a Zeiss Laser Scanning Confocal Microscope argon laser (wavelength 488 nm) was employed for visualization of FJB. Confocal micrographs were obtained using a Fluar 20×/0.75 objective in combination with a 1024 × 1024-pixel resolution and were saved in the TIFF format.

The FJB method was modified for double-labelling of neurons and combined with the TUNEL stain for the purpose of co-localization. Briefly, slides were immersed in 100% ethanol for 3 min, followed by 1 min in 70% ethanol. They were rinsed in dH_2O for 1 min before being transferred to 0.0001% FJB staining solution prepared immediately before use. The composition of the stain vehicle was 2 ml of the stock solution added to 198 µl acetic acid in 198 ml of dH_2O. After 1 h of gentle shaking in the staining solution at 4°C, slides were rinsed for 1 min in each of three dH_2O washes. The slides were left to dry at RT overnight in darkness. Once dried, they were immersed in three xylene washes, each for 2 min, before coverslipping with the non-aqueous, plastic mounting media, DPX. Three areas, previously shown to have a high incidence of TUNEL nuclei (Sakic et al. 2000b), were then selected for analysis. TUNEL-stained nuclei and FJB+ cells were visualized using a Zeiss Laser Scanning Confocal Microscope and two channels were tracked with excitations of 543 nm (for TUNEL cell nuclei) and 488 nm (for FJB+ neurons) yielding red and green scans, respectively. In brief, a helium–neon laser (wavelength 543 nm, red emission) was used for the visualization of Alexa-594 (Molecular Probes, Eugene, OR, USA) and an argon laser (wavelength 488 nm, green emission) was employed for visualization of FJB. Sections were scanned using a C-Apochromat 63x/ 1.2 Water Corrected objective in combination with a 1024 x 1024 pixel resolution. For purposes of co-localization of fluorochromes, separate confocal images were pseudo-

coloured to yield composite images. Stained nuclei / cells which co-localized appeared yellow. Scans were imported as TIFF files into Photoshop 4.5 and nuclei / cells were counted manually by an unbiased observer.

In preparation for the EM analysis, 1mm × 2 mm × 1mm blocks of brain tissue were isolated from different regions and stored in Karnofsky's fixative until processing. Samples were rinsed in 0.1M sodium-cacodylate buffer twice for 10 min each, and post-fixed in 1% osmium-textroxide (in 0.1M sodium cacodylate buffer) for 1h at RT. Tissue was dehydrated in ascending concentrations of ethanol, followed by two changes with propylene-oxide (10 min each). Subsequently, samples were immersed in 1:1 and 1:3 propylene-oxide: Spurr's resin (Marivac-Canemco) for 1 h before being placed into 100% Spurr's resin overnight. The next day samples were embedded into fresh Spurr's resin and polymerized overnight at 60°C. Blocks were trimmed and semi-thin sections (0.5 μm) were cut and stained for light microscopy with Toluidine blue, a stain which detects neurodegeneration (Ohtaki et al. 2003). In brief, 5g of Toluidine blue powder and 5g sodium-borate were combined in dH_2O to make a 500 ml staining solution. The solution was heated to ~65°C and sections were transiently immersed for staining. Sections were washed three times in dH_2O before mounting and cover slipping for assessment.

Finite neuroanatomical areas were identified from each section. From each slide, an area common to a particular region was demarcated for further processing. Ultra-thin sections (90 nm) of each finite area were then cut and placed onto 200 mesh copper palladium grids. The grids were post-stained with saturated uranyl-acetate in 50% ethanol and Reynold's Lead Citrate. Grid sections were examined and photographed using a JEOL 1200 EX transmission EM at an accelerating voltage of 80 kV.

Employing H&E staining, the overall neuronal density of coronal sections was assessed in the parietal cortex and CA2/CA3 area (i.e. areas that were less densely packed with neurons and thus amenable to quantification). Sections were stained with an automated slide stainer (Leica Instruments, Germany), and 1-mm^2 areas were examined with a stage micrometer by an unbiased observer. Sections of the highest quality were quantified at ×400 magnification.

Given that TH immunohistochemistry was not previously used in our laboratory, eight age-matched, non-autoimmune male CD1 mice were employed as positive controls. Mice were perfused with 30 ml ice-cold PBS followed by 30 ml ice-cold 4% PFA. Brains were stored at 4°C in PFA for 8 days before transferring them to 30% sucrose solution and refrigerating them before sectioning. Free-floating coronal sections were cut at 40 μm at the level of the SN, between Bregma -3.52 and -3.64 mm (Franklin & Paxinos 1997). They were kept at 4°C in 24-well plates containing 30% sucrose (with two drops of PFA) and three sections of the SN pars compact / VTA from each animal were selected for TH staining. Sections were incubated in 0.3% hydrogen peroxide (H_2O_2) for 30 min at RT, rinsed three times and incubated with primary antibody and rabbit anti-TH for 48 h at 4°C. After three rinses in PBS, tissues were incubated in biotinylated anti-rabbit IgG secondary antibody, for 1 hr at RT. Subsequently, the sections were rinsed in PBS and incubated in ABC solution for 1 h to form an Avidin / Biotin complex. All sections were washed again in PBS three times and the TH immunocomplex was visualized with chromogen diaminobenzidine tetrahydrochloride. The sections were mounted onto gelatin-increasing concentrations of ethanol (70%, 95%, 100%), placed in xylene and coverslipped using Gurr DePeX mounting medium. The total number of TH+ cells in the SN region (obtained from three serial sections) were manually quantified by an observer unaware of the experimental design and confocal light micrographs were

obtained using a Fluar 5×/0.25 objective in combination with a 1024 × 1024 pixel resolution, and saved as TIFF files. In comparison to the SN region, the high density of TH staining in the VTA of control brains precluded manual counting and lead to a descriptive assessment.

To examine brain inflammation the stains used included T-lymphocyte markers CD4, CD8, CD3, and the microglial marker F4/80. Four serial sections per slide were processed for the presence of CD4 and CD8 markers. Sections were incubated overnight at RT with monoclonal rat-anti-mouse CD4 antibody (IgG2b isotype, diluted 1:200), or anti-mouse CD8a antibody (IgG2a isotype, diluted 1:600). The primary antibodies were visualized using an FITC-conjugated goat anti-rat IgG antibody, diluted 1:50 in 1% normal goat serum in PBS. Sections were incubated with secondary antibody for 4 h at RT, rinsed three times in PBS and coverslipped in Fluorescence Mounting Medium. Preliminary studies revealed that immunostaining for CD4 and CD8 markers were not compatible with the FJB staining protocol, thus precluding double-labelling. Therefore, CD4+ and CD8+ cells were first manually quantified by an unbiased observer, and after the removal of mounting medium (10 min washing in PBS) the same sections were reprocessed for FJB. Both stains fluoresced at similar wavelengths (488 nm). The FJB+ cells were then manually counted by a second unbiased observer, focusing on periventricular tissue (around the third ventricle), where the majority of T-lymphocyte infiltrates were located.

Immunohistochemistry to the CD3 T-lymphocyte marker was also used to examine the degree of leukocytosis into the choroid plexus and brain parenchyma. Twelve-μm coronal sections were fixed in aceton at -20° C for 3 min and air dried. Following two 5 min washes in PBS, slides were placed into avidin-blocking solution for 15 min, washed in PBS and immersed into biotin-blocking solution for 5 min. After a 5 min wash, sections were incubated with 10% normal goat

serum in PBS for 30 min at RT. After incubation with primary antibody (hamster anti-mouse CD3e diluted 1:30 with 5% goat serum/PBS) overnight at 4°C, three additional PBS washes (5 min each) were followed by incubation with secondary antibodies (biotinylated anti-hamster IgG diluted 1:250 with 5% goat serum/PBS) for 1 h at RT. After a 5 min wash in PBS, slides were immersed into 0.03% H_2O_2 in PBS for 10 min at RT, and following an additional wash, incubated with streptavidin/HRP for 30 min at RT. Following three additional PBS washes (5 min each), slides were incubated with DAB for several minutes, and washed in tap water. A brief counterstain with hematoxylin, dehydration through graded alcohol and xylene preceded three 5-min PBS washes before cover slipping. Counting of CD3+ cells from digitized photos (depicting choroid plexus of the third ventricle) was performed in one section / brain under ×400 magnification by an observer blinded to the group origin.

F4/80, a 120-160kD glycoprotein, is highly and constitutively expressed on most resident tissue macrophages (Morris et al. 1991), including microglia (Perry et al. 1985). Resting microglia possess a characteristic ramified morphology, which can be visualized with antibodies towards the F4/80 antigen. However, immunohistochemical staining to F4/80 is proposed to become more intense after microglia activation (Chen et al. 2005). We employed the rat monoclonal antibody F4/80 (1:100 dilution in 5% goat serum/PBS) and the biotinylated anti-rat IgG secondary antibodies (1:200 dilution in 5% goat serum/PBS) to morphologically characterize microglia on horizontal sections. Incubation times for the primary and secondary antibodies were overnight at 4°C and 1 h at RT, respectively. Following incubation, brain sections were treated with Vectastain ABC reagent according to the manufacturer's directions and the resulting avidin-biotin-peroxidase complex was visualized with diaminobenzidine. The stained brains were observed by confocal light microscopy

under differential interference contrast. In addition to more intense staining, activated microglia were distinguished by a more rounded morphology than when in a resting state (Perry & Gordon 1997). Three adjacent 0.5 mm^2 areas from the paraventricular region of the diencephalon were selected from each brain, and F4/80+ cells were manually counted from by an observer blinded to group origin at ×200 magnification. The sparse presence of F4/80+ cells in the cerebellum and cortex was also noted.

To examine mechanisms and modes of cell death the stains used were TUNEL and Ub. In situ detection of DNA nicks was performed using a Neuro TACS II TdT Labelling Kit. Fixed-frozen sections from each group were permeabilized using Neuropore for 30 min at RT. Endogenous peroxidase activity was blocked using 0.3% H_2O_2 in methanol for 3 min. After washing, the sections were exposed to the mixture of TdT, Mn and biotinylated-dNTP for 1 h at RT. The reaction was stopped by putting slides into a jar with TdT Stop Buffer for 5 min. After washing three times in distilled–deionized water (ddH_2O), streptavidin-Alexa-594 (diluted 1:200 in normal goat serum diluent) was applied to each section for 1 h at RT, and then washed three times in ddH_2O before proceeding to combine this technique with FJB on the same sections (detailed above).

For Ub, mice were perfused with PBS, and the brains were extracted within 2 min, immersed into 10% neutral buffered formalin, and left in formalin for 4 days at RT until processing. Subsequent to fixation, brains were embedded in paraffin and cut in the coronal plane at 4 μm. Serial adjacent sections were stained with H&E and processed immunohistochemically for Ub, using an anti-Ub polyclonal antiserum (1:400) and the labeled streptavidin-biotin-peroxidase technique. Sections were briefly counterstained with hematoxylin, dehydrated in a graded series of ethanol, mounted in xylene, and coverslipped. For each animal, the number of Ub-immunopositive dot-like

structures was counted in five non-overlapping fields within the strata oriens and pyramidale of the CA3 sector of the hippocampus. All counts were performed at the same coronal level under oil immersion (×1000) by an experimenter blind to the experimental design.

Light, Confocal and Transmission Electron Microscopy

Light microscopy was employed for the morphometric assessment of sections stained using standard histological methods for H&E, Ub, CD3, F4/80, Toluidine blue and TH with the epifluorescent Diastar Fluoresence Microscope (Reichert Scientific; Buffalo, USA) or LSM-510 Zeiss Confocal microscope (Zeiss, Germany). Fluorescent labeling was used for CD4 and CD8 T-cell markers, and FJB and TUNEL staining were visualized with the Diastar Fluoresence Microscope (blue excitation light, wavelength 450–490 nm), or Zeiss Confocal microscope (LSM-510) equipped with helium–neon (wavelength 543 nm, red emission) and argon (wavelength 488 nm, green emission) lasers. In preparation for the EM analysis, finite neuroanatomical areas were identified from sections placed on 200 mesh copper palladium grids, examined and photographed using a JEOL 1200 EX (Tokyo, Japan) transmission EM at an accelerating voltage of 80 kV.

Flow Cytometry

All samples were run by a third blinded party. Fluorescence-labeled antibodies used for staining were R-phycoerythrin (R-PE)-conjugated rat anti-mouse CD69 monoclonal antibody (0.25μg for $1x10^6$ cells) and RPE-Cy5-conjugated rat anti-mouse F4/80 monoclonal antibody (5 μl for $1x10^6$ cells). Aliquots of mononuclear cells ($0.5\text{-}1x10^6$) were suspended in 0.1 ml of PBS containing 0.1% sodium azide and incubated with predetermined optimal concentration of R-PE-

conjugated anti-mouse CD69 and RPE-Cy5-conjugated anti-F4/80 for 30 min in the dark at 4°C. Cells were washed three times with PBS, resuspended in 0.3 ml of PBS (containing 0.1% sodium azide), and subjected to FACScan analysis. Mononuclear splenocytes without fluorescence-labeled antibodies added were used as a negative control. Background autofluorescence was determined by incubating mononuclear cells from brains of old MRL-lpr mice with PBS. The analysis was performed using FACScan (Becton Dickinson, Mountain View, CA) and regions of interest were set according to forward scatter (roughly proportional to the diameter of the cell) and side scatter characteristics of splenocytes (proportional to the granularity). A total of 10,000 cells were analyzed and the frequency of each cell surface marker was determined using WinMDI 5.1 software (Scripps Clinic, La Jolla, CA).

Cytotoxicty Assay

In this thesis, the possibility that CSF from autoimmune mice with severe behavioural deficits is excessively toxic to progenitor cells was also explored. Since the C17.2 cell line is routinely grown in Dr. Doering's laboratory at McMaster University, we used it as a target to test the toxicity of CSF from mice. This cell line is easily maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 5% horse serum and 2 mM glutamine. The cultures were maintained in a standard humidified 5%CO_2 air incubator at 37°C and split when the cells reached approximately 90% confluency. Approximately 20,000 cells were seeded on coverslips in 24 well plates, followed by the addition of the diluted CSF samples from CY-treated and SAL-treated MRL-lpr and MRL +/+ groups. The morphology and viability of cells were determined after 24 h incubation with 10 μl of CSF or serum.

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