

**OLFACTORY DYSFUNCTION IN CNS LUPUS-LIKE DISEASE**

**OLFACTORY DYSFUNCTION IN THE MRL MOUSE MODEL OF CNS SLE**

**By**

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## **Abstract**

Systemic lupus erythematosus (SLE) is a chronic autoimmune / inflammatory disease that is frequently accompanied by brain atrophy and neuropsychiatric (NP) manifestations. CNS involvement ranges from focal abnormalities to diffuse disorders, and according to more recent clinical data, may also include olfactory dysfunction of unknown etiology. Similar to CNS SLE, spontaneous development of lupus-like disease in MRL/lpr mice coincides with neurodegeneration in periventricular regions and behavioural impairments in paradigms dependent on olfactory function. However, previous studies could not resolve whether exploration-dependant deficits reflect changes in emotional reactivity / exploratory drive, or altered olfactory capacity. The latter possibility was also supported by evidence of disturbed projection of neuroblasts to the olfactory bulb. The aim of this thesis was then to examine whether lupus-like autoimmunity alters olfactory function, thereby affecting performance in other behavioural tasks. Indeed, behavioural testing in a battery of paradigms revealed that lupus-prone MRL/lpr males spend less time exploring unfamiliar conspecifics and demonstrate age-dependant changes in responsiveness to attractant and repellant scents. Sustained treatment with the immunosuppressant cyclophosphamide abolished signs of autoimmunity and improved responsiveness to an attractant and a novel object, thus indirectly supporting a cause-effect relationship. In order to probe for pathogenic autoimmune factors, we administered CSF from CNS SLE patients and purified autoantibodies into the brains of healthy mice. Sustained infusion of autoimmune CSF induced olfactory dysfunction, excessive immobility in the forced swim test, enhanced

perseveration in a learning task, and altered several home-cage behaviours. Direct exposure to purified antibodies produced broad, but relatively mild, functional changes in olfactory function, spatial learning / memory, and home-cage behaviour. These findings provide an initial step toward understanding the nature and immunopathogenic mechanisms underlying early neurofunctional deficits in human and murine forms of CNS lupus.

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## List of Abbreviations

<b>AABS</b>	Autoimmune associated behavioural syndrome
<b>aCL</b>	Anti-cardiolipin antibodies
<b>ACR</b>	American College of Rheumatology
<b>aCSF</b>	Artificial cerebrospinal fluid
<b>ANA</b>	Anti-nuclear antibodies
<b>ANOVA</b>	Analysis of variance
<b>AQP4</b>	Aquaporin 4
<b>ARPA</b>	Anti-ribosomal P protein antibodies
<b>BBB</b>	Blood-brain barrier
<b>BCSFB</b>	Blood-cerebrospinal fluid barrier
<b>BRA</b>	Brain-reactive autoantibodies
<b>CP</b>	Choroid plexus
<b>CNS</b>	Central nervous system
<b>CSF</b>	Cerebrospinal fluid
<b>CY</b>	Cyclophosphamide
<b>DCX</b>	Doublecortin
<b>dsDNA</b>	Double-stranded deoxyribonucleic acid
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>FasR</b>	Fas receptor
<b>FITC</b>	Fluorescein isothiocyanate
<b>HPA</b>	Hypothalamus–pituitary–adrenal
<b>HRP</b>	Horseradish peroxidase
<b>IFA</b>	Immunofluorescence assay

<b>IFN</b>	Interferon
<b>IgG</b>	Immunoglobulin G
<b>IL</b>	Interleukin
<b>icv</b>	Intracerebroventricular
<b>mg</b>	Milligram
<b>min</b>	Minute
<b>ml</b>	Milliliter
<b>mm</b>	Millimeter
<b>MRI</b>	Magnetic resonance imaging
<b>MRL/lpr</b>	Murphy Roth's Large/MpJ-Tnfrsf6 <sup>lpr</sup>
<b>MRL +/+</b>	Murphy Roth's Large/MpJ +/+
<b>MWM</b>	Morris water maze
<b>NAA</b>	<i>N</i> -acetylaspartate
<b>NMO</b>	Neuromyelitis optica (Devic's disease or Devic's syndrome)
<b>NMDAR</b>	<i>N</i> -methyl-D-aspartate receptor
<b>NP</b>	Neuropsychiatric
<b>NP-SLE</b>	Neuropsychiatric systemic lupus erythematosus
<b>NZBWF1/J</b>	New Zealand black/white F1 hybrid
<b>OB</b>	Olfactory bulb
<b>PBS</b>	Phosphate buffered saline
<b>PFA</b>	Paraformaldehyde
<b>PNS</b>	Peripheral nervous system
<b>PR3</b>	Proteinase 3
<b>RH</b>	Room humidity

<b>RT</b>	Room temperature
<b>RNA</b>	Ribonucleic acid
<b>RMS</b>	Rostral migratory stream
<b>SAB</b>	Spontaneous alternation behaviour
<b>SEM</b>	Standard error of the mean
<b>SGZ</b>	Subgranular zone
<b>SVZ</b>	Subventricular zone
<b>TNF</b>	Tumor necrosis factor
<b>TNFRSF6</b>	Tumor necrosis factor receptor superfamily member 6
<b>μl</b>	Microliter
<b>μm</b>	Micrometer
<b>veh</b>	Vehicle

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## **Chapter 1: Introduction**

### **Systemic Lupus Erythematosus**

Systemic lupus erythematosus (SLE) is a chronic immunological disorder with a clinical course that can vary from mild symptoms to life-threatening multi-organ disease [1]. Colloquially referred to as “the disease with a thousand faces” [2], lupus presents with a broad spectrum of symptoms that are easily mistaken for other illnesses [3]. Typical manifestations include inflammation of the skin, joints, kidneys and heart that vary greatly with respect to onset, distribution, and severity. This clinical heterogeneity poses several problems for clinicians, who must rely on a shifting set of criteria to confer a diagnosis of SLE (reviewed in [4]). Several classification standards have been proposed over the years (e.g. [5-7]), but the most widely used are those developed by the American College of Rheumatology (ACR). The first version of the criteria was proposed in 1971 [8], updated in 1982 [9] and subsequently revised by a committee in 1997 [10]. According to the most recent guidelines, the patient must satisfy at least four diagnostic criteria from a list of 11 which includes skin lesions, arthritis, renal disorder, neurologic disorder, hematologic changes among others (Table 1.1).

Despite the ongoing diagnostic problems posed by its clinical diversity, the prevalence of SLE appears to be increasing as the disease is recognized more readily and survival increases [11]. There are marked disparities in SLE rates worldwide, ranging from 20 to 150 cases per 100,000 population [12]. This variability may reflect genuine differences across populations and regions, or be a consequence of methodological

differences in studies. In North America alone, SLE is estimated to affect 1:1000 Canadians (Lupus Canada, 2015) and more than 1.5 million Americans (Lupus Foundation of America, 2015). In comparison, there is a trend towards higher rates in Europe, with the highest prevalence reported in Sweden, Iceland and Spain [13]. The burden of the disease is likely to fall disproportionately on certain ethnic groups, notably individuals with African, Caribbean or Asian ancestry, who tend to have a higher risk of developing SLE and greater involvement of vital organs than patients of European descent [14]. The majority of these patients will be women aged between the late teens and early 40s, with an effective female-to-male ratio of about 9:1 commonly reported in literature [15]. In spite of improvements in life expectancy [16], SLE patients continue to have a higher mortality risk than that of the general population [17]. A 20-year-old patient diagnosed with lupus has a 1 in 6 chance of dying by 35 years of age, most often succumbing to active lupus or infection involving the lungs, kidneys and nervous system [18]. In later stages of the disease, complications arising from accelerated atherosclerosis represent a significant cause of death [19].

The clinical and epidemiological heterogeneity of SLE points to a complex etiology reflecting the combined effects of various predisposing genetic, environmental, and hormonal factors [20]. A strong genetic link is supported by high heritability, elevated sibling recurrence-risk ratio, and higher concordance rates among monozygotic twins in comparison to dizygotic twins [21-23]. Although in rare cases SLE may be associated with the deficiency of a single gene (e.g., the complement components C1q and C4 [24]), the genetic risk seems to be more commonly derived from variations in at

least 28 confirmed disease susceptibility loci, each of modest effect size [25]. Several of these variants fall into key immunopathogenic pathways implicated in SLE, but together account for only about 10% of the genetic heritability [26]. Disease activity and severity among genetically-predisposed individuals is likely to be influenced by several environmental factors [27]. Exposure to sunlight (and ultraviolet radiation) is the most thoroughly described of such factors and has been linked to exacerbation of cutaneous manifestations in patients [28]. The possibility that viral infections trigger SLE is also supported by several lines of evidence, but confirming causative roles has proved challenging [29-34]. Other potential environmental risk factors include cigarette smoke [35], certain drugs [36], and occupational exposure to harmful compounds [37;38]. Considering women of childbearing age are disproportionately affected and the severity of disease varies with pregnancy / menstrual cycle, it seems likely that female hormones contribute to SLE pathogenesis [39]. Factors such as early menarche, oral contraceptive use, menopause, and postmenopausal hormone replacement therapy are reported to be associated with increased risk of SLE [40]. However, treating women with active disease with hormonal supplements has yielded only moderate improvements in symptomatology [41;42]. The results support the argument that increased prevalence of SLE among women reflects a protective role for male hormones [43] and/or hormone-independent effects of genes on the X chromosome [44]. Notwithstanding these insights into the multi-factorial nature of SLE, the precise cellular and molecular mechanisms leading to phenotypic manifestation remain poorly understood. However, it is worth noting that the factors mentioned above merely represent potential triggering events that drive

disease process forward and are unlikely to function as direct mechanisms of pathology themselves [45].

A substantial body of evidence supports the notion that SLE-associated pathology is mediated by a failure or breakdown of mechanisms responsible for maintenance of self-tolerance [46;47]. The breaching of tolerance checkpoints gives rise to a microenvironment in which immune cells routinely mount an aberrant response to self-antigens that would normally be bypassed. The result is a self-sustaining and detrimental positive feed forward loop characterized by immune-cell differentiation, secretion of proinflammatory cytokines, activation of autoreactive T- and B- cell populations, hyperproduction of autoantibodies by plasma cells and deposition of antigen-antibody complexes into the walls of blood vessels in several tissues. The accumulation of immune complexes in turn initiates systemic inflammation culminating in organ damage and clinical manifestations. The overall goal of disease management is to suppress inflammation and prevent organ damage. Therapy revolves around five drug classes: non-steroidal anti-inflammatory drugs, anti-malarials, steroids (prednisone), immunosuppressives (methotrexate, azathioprine, mycophenolate mofetil and cyclophosphamide) and biologics [i.e., rituximab (anti-CD20) and belimumab (anti-BAFF)][11].

### **Neuropsychiatric Systemic Lupus Erythematosus**

In addition to diverse symptoms related to systemic autoimmunity and inflammation, SLE patients may experience a wide array of neurologic and psychiatric

events [48]. The phenomena was first documented over a century ago in lupus patients displaying altered states of consciousness [49]. Since then, a multitude of neuropsychiatric (NP) syndromes affecting the central, peripheral and autonomic nervous systems have been described [50-53]. In that time, the ACR case definition for neuropsychiatric systemic lupus erythematosus (NP-SLE) has changed substantially – from seizures and psychosis to 19 distinct and classifiable syndromes (Table 1.2) [54]. Central nervous system (CNS) involvement may take the form of diffuse disorders (acute confusional state, anxiety, cognitive deficits, depression, psychosis) or focal abnormalities (seizures, cerebrovascular disease, chorea, myelopathy, transverse myelitis, demyelinating syndrome, aseptic meningitis, headaches) depending upon the anatomic location of pathology. Deficits of the peripheral nervous system (PNS) include autonomic disorders, plexopathy, myasthenia gravis, polyneuropathies and mononeuropathies.

The standardized ACR case definitions provide systematic diagnostic / exclusion criteria for each NP syndrome, but there are limitations regarding their use in clinical practice. For one, none of the individual NP manifestations are unique to SLE, and some (e.g. headache, mild forms of anxiety, depression, cognitive dysfunction, and polyneuropathy) occur with considerable frequency in the general population [55]. Secondly, the list itself is not an exhaustive one and does not preclude SLE patients from presenting with other NP manifestations. For example, more recent observations suggest that a large proportion of SLE patients show signs of hyposmia (reduced smell capacity), and many suffer from a complete loss of smell, or anosmia [56]. Lastly, there is no

formal system for grading the severity of most NP syndromes and the diagnostic criteria for others, such as demyelinating syndromes and headache, are either poorly defined or inferior to existing criteria. In the absence of diagnostic gold standards, the assessment of NP-SLE in individual patients is ultimately dependent upon the best available clinical, serological, and neuroimaging data.

The clinical heterogeneity of manifestations, coupled with a lack of established diagnostic criteria, has meant that the reported frequency of NP-SLE among different populations varies between 12 to 95% [55;57-60]. Based on more recent data obtained from large cohorts [61-63], cumulative incidence of NP events is probably closer to 30–40% [64]. Events can manifest at any time during the course of the disease and present as single or multiple episodes in the same individual. A large proportion of NP-SLE events occur at disease onset or within the first two years after SLE diagnosis [65;66]. They may also manifest during quiescent periods without serologic activity [67;68], often preceding the onset of lupus [69]. Despite the substantial variability in presentation, diffuse CNS involvement is consistently reported to predominate over PNS disease (Table 1.3) [70]. The most common manifestations of NP-SLE, include headache [71], mild to moderate cognitive dysfunction [72], mood disorders [73], seizures [74], cerebrovascular disease [75] and anxiety disorders [76]. The cumulative occurrence of NP events increases over time, though cognitive dysfunction and atherosclerotic cerebrovascular disease correlate with advancing age [70].

Several SLE-related risk factors have been associated with the development of NP manifestations. The most commonly reported include generalized (non-CNS) lupus activity, cumulative organ damage, treatment with high doses of corticosteroids / cytotoxic agents, clinical history of major NP-SLE events (previous or concurrent) and cardiovascular disease [64]. Clinical predictors of NP events are particularly relevant considering NP-SLE is proposed to represent a more severe form of lupus [70;77-82]. However, individual NP manifestations can differ widely in their prognostic implications [83-87] and this variance may help explain why some studies have reported increased mortality in patients with NP events [88-91], whereas others have not [92-94]. Variability in the prognosis aside, early treatment is likely to result in more favourable outcomes [95]. Evidence from randomized controlled trials is limited [96], but general consensus supports the use of corticosteroids, alone or in combination with immunosuppressive therapies (such as azathioprine or cyclophosphamide, CY) [64].

While a histologically normal brain is a possible finding in NP-SLE, histopathological and neuroimaging studies commonly reveal a wide range of brain abnormalities in both white [97;98] and grey matter [99]. Global and regional cerebral atrophy are frequently observed [100-102] and proposed to reflect functionally relevant brain damage in NP-SLE patients [103]. The frontal, parietal and occipital lobes seem particularly vulnerable to cortical thinning [104], while subcortical tissue loss is common in periventricular regions [101;104-107]. Brain atrophy progresses over a relatively short period of time [108] and can be detected very early in the course of SLE [109], but does not always correlate with disease duration [103]. Biochemical analysis indicates that

neurodegeneration and inflammation are key contributors [110;111]. Levels of *N*-acetylaspartate (NAA), an amino acid marker of axonal integrity, are significantly reduced in lesions, either permanently [112] or reversibly in a disease-dependent manner [113]. Additional markers of brain damage, including neurofilament triplet protein and glial fibrillary acidic protein, are significantly increased in the cerebrospinal fluid (CSF) of NP-SLE patients [114]. In addition to neuronal and glial damage, cerebrovascular abnormalities involving small intracranial vessels are prominent [52;53] and considered to be a major, if not the predominant, component of CNS damage [115-117]. Cerebrovascular events such as haemorrhages and ischemic infarcts are common [118;119] and associated with stroke and brain lesions [75]. Various degrees of cerebral hypoperfusion have also been characterized, ranging from focal abnormalities [120] to moderate defects in the frontal and parietal lobes [121], and even global perfusion deficits [122]. Taken together, data from neuroimaging studies overwhelmingly point to a complex brain disorder characterized by profound metabolic alterations including impaired blood flow, ischemia, decreased aerobic metabolism, patchy multiple sclerosis-like demyelination and progressive neuronal loss [123;124].

### **Proposed Pathogenic Factors**

It is unlikely that there is a single pathogenic mechanism to explain the broad NP manifestations and histopathological abnormalities reported in SLE patients. While neuroimaging, neuropsychological testing, and soluble markers are helpful in contemporary diagnosis [125], the attribution of individual NP events to SLE or to an alternative etiology remains a challenge. This concern has led to the development of at

least two different attribution models, both of which attempt to broadly classify NP events as primary manifestations, secondary complications of the disease or a concurrent, non-SLE NP disorder [77;126]. Based on these criteria, less than 60% of all NP events are estimated to be directly attributable to active disease or so called ‘primary NP-SLE’ [127]. The remaining ~40% of cases are hypothesized to arise from complications related to SLE or its therapy (‘secondary NP-SLE’), a concomitant disease process unrelated to lupus, or some combination thereof. Common confounding factors include the accumulation of toxic metabolites due to glomerulonephritis, opportunistic infections as a result of immunosuppression, adverse effects of treatment with high-dose corticosteroids, and hypertension [95]. As SLE progresses, secondary and non-SLE-specific factors are hypothesized to contribute to a substantial proportion of NP events [70]. However, these factors are insufficient to account for early CNS involvement (i.e., before lupus affects peripheral organs), when no infections can be detected, and/or when steroids are not used in therapy. Accordingly, NP events in these cases are regarded to be attributable to SLE – that is, as primary manifestations of the disease.

There are a variety of mechanisms by which the immune system can affect the nervous system and produce NP events. Immunological responses such as inflammation and autoimmunity are known to compromise the cytoarchitecture of the CNS [128]. On the other hand, it is conceivable that alterations in the immune system lead to transient, non-destructive changes in brain functioning in a relapsing / remitting fashion. Yet, the anatomy of the CNS poses unique challenges to both these processes because the brain is protected from harmful formed blood elements by a system of barriers containing tight

junctions. These include the blood-brain barrier (BBB) formed by cerebrovascular endothelial cells around parenchymal venules, the blood–cerebrospinal fluid barrier (BCSFB) that surrounds the choroid plexus (CP) epithelium, and arachnoid epithelial cells encapsulating meningeal vessels [129]. Dysregulation of the barrier system seems to be among the earliest pre-conditions permitting the establishment of autoimmune responses in the CNS [130]. Barrier damage, as evidenced by an increased albumin quotient, is common in SLE patients [131;132] and correlates with clinical relapses [133], diffuse CNS injury [134], and intrathecal oligoclonal immunoglobulin G (IgG) synthesis [132-134]. Further evidence for barrier dysfunction in NP-SLE patients stems from the increased expression of soluble adhesion molecules [135;136], which could provide a potential mechanism by which immune cells and soluble factors gain access to the CNS.

The current etiological theory proposes that the local secretion of various proinflammatory mediators and autoantibodies drive brain damage via immune complex deposition, inflammation, vasculopathy, and ultimately, neurodegeneration [137]. While the role of proinflammatory cytokines in promoting barrier dysfunction and activating endothelial cells is not to be overlooked in NP-SLE [138-148], considerable attention has been given to antibodies reactive to diverse brain antigens (brain-reactive autoantibodies, BRA) [149]. The data from clinical studies is largely correlational in nature and based on the temporal relationship between clinical events and serologic autoimmunity [72;150-155], binding of serum autoantibodies to neuronal cells [156-161], the presence of autoantibodies in CSF [157;162-166], and, to a very limited extent, the identification of autoantibodies in post-mortem neuronal tissues [167;168]. The mechanism by which

BRAs gain access to the CNS in lupus patients is not well understood. One possibility involves the perivascular leakage of antibodies through a permeable or damaged BBB [132]. Another possibility is that they are synthesized intrathecally [169] by activated leukocytes that extravasate between barrier cells of the BBB and BCSFB [170;171]. The latter is supported by an elevated IgG index and CSF oligoclonal banding in lupus patients with CNS dysfunction [133;134;169]. Several authors have argued that BRA from CSF are not only more pathogenic than BRA originating in serum [163;164;172], but also better predictors of CNS involvement [173]. However, the extent to which specific BRA mediate NP manifestations remains unknown given that diverse binding mechanisms may affect neurons [174]. This is particularly relevant for antibodies that may not be cytotoxic, but affect neuronal functioning in transient fashion, or are epiphenomena associated with CNS damage [175]. Therefore, it is hypothesized that only certain subsets induce NP manifestations [70;176;177], but the correlational nature of clinical data makes it difficult to study cause-effect relationships between specific BRA, regional changes in brain morphology, and distinct NP manifestations. As such, the pathogenic mechanisms underlying primary NP-SLE remain mostly speculative at this point [95].

### **Mouse Models of NP-SLE**

Given the complexity of autoimmune / inflammatory conditions, animal models are indispensable tools for understanding principal pathogenic factors and mechanisms not feasible in clinical studies. In experimental studies, interactions between autoimmune / inflammatory phenomena and brain function can be examined in a more systematic and

direct way using well-controlled animal models that have significant face, construct, and predictive validity [178]. In this regard, both induced and spontaneous murine models of NP-SLE have proven to be extremely valuable in investigating cause-effect relationships between specific immune factors and changes in behaviour. The most commonly studied spontaneous models include the New Zealand black/white F1 hybrid (NZBWF1/J), BXSB and MRL (Murphy Roth's Large) strains [179]. These inbred mice develop a wide spectrum of autoimmune manifestations [180] and share common serological hallmarks of SLE, such as hypergammaglobulinemia and elevated anti-nuclear antibodies (ANA).

Considering that no animal model provides a complete representation of a disease, each strain has distinct features which make it more or less suitable for examining specific aspects of SLE [179]. With respect to the examination of neurobehavioural manifestations, the MRL model (developed in The Jackson Laboratory in the late 70s) has several advantages over other strains. In contrast to NZBWF1/J and BXSB mice which lack an adequate (genetically-similar) control group, the MRL model consists of the MRL/MpJ-Tnfrsf6<sup>lpr</sup> (MRL/lpr) substrain and the MRL/MpJ +/+ (MRL +/+) substrain. The existence of two congenic substrains which differ less than 0.1% in their genome diminishes the influence of dissimilar genetic backgrounds on behavioural patterns and brain morphology [181]. Furthermore, MRL mice do not display a high incidence of inherited neuroanatomical abnormalities [182] that may confound assessment of autoimmunity-induced changes in brain function in NZB and BXSB mice [183;184]. Finally, unlike other autoimmune strains, MRL mice produce anti-Ro, anti-La

and anti-Sm autoantibodies which have been associated with NP manifestations in SLE [177;181]. These traits render the MRL substrains an appropriate model to exam the interrelationships between systemic autoimmunity, neuropathology, and aberrant behaviour [185].

MRL/lpr and MRL +/+ mice are comparable in many respects (e.g., appearance, size, reproductive age) and primarily distinguished by the onset of systemic autoimmune disease. While MRL +/+ mice manifest signs of autoimmunity relatively late in life, the MRL/lpr substrain develops an accelerated form of SLE-like disease that is attributable to a spontaneous lymphoproliferation mutation ( $Fas^{lpr}$ ) and a deficit in the expression of the apoptotic Fas receptor (FasR, encoded by TNFRSF6 gene) [186]. The resultant defect in Fas-mediated apoptosis, coupled with the unchecked proliferation of autoreactive T and B cells [187;188] causes circulating levels of cytokines, autoantibodies, and immune complexes to rise within the first two months of life [181]. These serological changes manifest as characteristic skin lesions, alopecia, and arthritis in older MRL/lpr mice, which typically succumb to complications relating to massive lymphadenopathy, splenomegaly and glomerulonephrosis around five to six months of age. Conversely, the congenic MRL +/+ substrain develops a milder form of lupus-like disease much later and can have a lifespan of up to two years in comparison.

Accelerated disease progression in MRL/lpr mice is accompanied by widespread brain damage and a constellation of behavioural deficits, operationally termed “autoimmunity-associated behavioural syndrome” or AABS [189] [190]. In comparison

to age- and sex-matched MRL  $+/+$  controls, MRL/lpr mice display increased thigmotaxic behaviour [191-193], impaired exploration of novel objects and spaces [193;194], excessive immobility when forced to swim [194], reduced responsiveness to palatable stimulation [195;196], and diminished isolation-induced inter-male fighting [197]. The brains of MRL/lpr mice exhibit widespread morphologic alterations characterized by increased BBB / BCSFB permeability [198;199], lymphoid cell infiltration [198;200-203], as well as, age- and disease-dependent damage to cortical and periventricular neurons [204-210]. Further support for autoimmune-induced neuropathology comes from studies demonstrating that IgG-rich CSF from behaviourally-impaired MRL/lpr mice is toxic to mature neurons [211] and neuronal stem cells [212]. The notion that neural progenitors are targeted by neurotoxic CSF is strengthened by morphological abnormalities in neurogenic niches surrounding the ventricles [206;210;213-215]. In support of a cause-effect relationship, sustained treatment with the immunosuppressant CY abolishes several behavioural deficits [195;207;216], prevents neuronal damage [208;217] and arrests the entry of immunocytes into the CP [218]. Taken together, the above evidence points to a complex pathogenic cascade involving toxic CSF IgG, neuronal / progenitor cell death and progressive behavioural deficits [199].

As discussed above, several lines of evidence support a causative role of autoimmunity and particularly intrathecal autoantibodies in the induction of behavioural changes in the MRL/lpr substrain. However, the precise nature of AABS and terminal pathologic mechanisms remains unclear. Apart from subtle learning / memory deficits [193;203;207] and mild deficiencies in neurological [203;219] and psychomotor tests

[220] at more advanced stages of lupus-like disease, performance deficits in MRL/lpr mice are most consistently noted in tasks reflective of emotional reactivity and affective behaviour [190]. Yet previous studies could not address whether exploratory deficits in approach-avoidance paradigms reflect a progressive anxious / depressive-like state [189] or as of yet unknown alterations in a separate, unrelated behavioural domain. Indeed, the recent findings of smell dysfunction in humans with SLE [56], and morphological changes in MRL/lpr neurogenic niches that normally replenish the olfactory bulb (OB) with neuroblasts [213], raised the possibility that MRL/lpr mice concomitantly develop olfactory dysfunction that impairs performance in other behavioural tasks. In other words, the unique behavioural, immunological and neuropathological profiles of MRL/lpr mice provide a useful preparation to study the interrelationships between olfaction and autoimmunity that have thus far eluded clinicians. The first two studies presented in this thesis describe original work examining this association in the MRL model.

As appropriate as the MRL model has been in uncovering complex neuro-immuno interactions in SLE, it is not without limitations. The MRL/lpr substrain develops a progressive and unrelenting disease course, in contrast to the fluctuating course of flares and remissions typical in human SLE [181;221]. SLE is also overwhelmingly more common in women (i.e. 9:1 female-to-male ratio), but MRL/lpr mice do not exhibit such a strong gender bias in favour of females. However, the disease does seem to manifest a few weeks earlier in female mice [193;222;223], possibly due to a different hormonal milieu in human and murine forms of lupus. Lastly, MRL/lpr mice

develop a complex SLE-like disease that includes, hyperproduction of various autoantibodies [173;223-231], secretion of neuroactive cytokines [232-236], disturbances in the endocrine network [197;237-242] and severe organ damage [243]. While these characteristics are useful to examine the multifaceted interplay between genetic, immune, and hormonal factors, they are less suitable for identifying cause-effect relationships between specific pathogenic factor(s) and behavioural changes. This difficulty to attribute brain damage to a particular mechanism in MRL/lpr mice (and other genetically-susceptible autoimmune strains for that matter) necessitates inducible SLE models in asymptomatic animals, in which potential confounds outside experimental interest can be better controlled for [185].

Several inducible models of CNS SLE have been developed over the years, and most have corroborated the link between BRA and behavioural dysfunction in human and murine forms of lupus. Preliminary evidence came from independent studies demonstrating that BRA-rich CSF from SLE-affected patients is neurotoxic to murine hippocampal and retinal neurons *in vivo* [159;212]. These effects have been partially linked to anti-DNA antibodies in the CSF that cross-react with neuronal glutamate receptors [159;244]. They specifically bind the NR2 subunit of the *N*-methyl-D-aspartate receptor (NMDAR) and have been shown to induce neuronal damage [159;245] and aberrant behaviour in healthy mice [168;246] when the BBB is damaged or bypassed. More relevant to the following discussion were the findings from Katzav and colleagues, who demonstrated that a single injection of human anti-ribosomal P antibodies (ARPA) into the brain of healthy mice induces “autoimmune depression” characterized by

olfactory dysfunction and excessive immobility in the forced swim test [247-249]. In another study, mice injected intravenously with anti-dynamin-1 antibodies were shown to develop behavioural disturbances, despite the absence of overt neuropathology or a mechanism to explain how they had infiltrated the brain [250]. Lastly, emerging evidence from experimental studies point to a pathogenic role for antibodies directed towards highly-conserved cytoskeletal proteins [251;252]. In summary, the above evidence suggests that diverse BRA bind multiple antigens that are preserved across species, induce neuronal damage and ultimately aberrant behaviour. Based on previous work from other inducible models, the last study in this thesis uses a similar, but refined approach to examine potential pathogenic factors that may mediate olfactory dysfunction in MRL/lpr mice. In this final experiment, we specifically administer CSF containing autoantibodies directly into the brains of asymptomatic, healthy animals and assess performance in tests probing olfactory function and other domains of behaviour. In summary, each study described in this report logically progresses from the one before it. The first study is designed to longitudinally explore the association between systemic autoimmunity and performance in olfactory tests. The second study attempts to dissociate the relationship using sustained immunosuppression. The final study addresses the pathogenic factors that induce functional changes in olfaction and other domains of behaviour.

**Table 1.1 - ACR classification criteria for SLE**

<b>CRITERION</b>	<b>DEFINITION</b>
Malar rash	A red or purplish rash on the cheeks and nose, often in the shape of a butterfly
Discoid rash	A scaly rash that appears as red, raised, disk-shaped patches on the face, scalp, ears, chest or arms
Photosensitivity	An excessive skin reaction to sunlight that causes a rash to appear or get worse
Oral ulcers	Small, usually painless, sores that occur on the mucous lining of the mouth
Arthritis	Pain involving two or more peripheral joints characterized by tenderness or swelling
Serositis	Inflammation of the lining around the lungs (pleuritis) or the heart (pericarditis)
Renal disorder	Persistent urinary protein or cellular casts
Neurologic disorder	Seizures or psychosis
Hematologic disorder	Anemia (low red-cell count), leukopenia (low white-cell count), lymphopenia (low level of specific white cells) or thrombocytopenia (low platelet count)
Immunologic disorders	Positive test for anti-double stranded DNA, anti-Sm, or anti-phospholipid autoantibodies
Anti-nuclear antibodies	Positive anti-nuclear antibody (ANA) test

*Note: Adapted from Tan et al (1999).*

**Table 1.2 - ACR case definitions for NP-SLE**

<b>CENTRAL NERVOUS SYSTEM</b>	<b>PERIPHERAL NERVOUS SYSTEM</b>
Aseptic meningitis	Acute inflammatory demyelinating polyradiculoneuropathy (Guillain-Barré syndrome)
Cerebrovascular disease	Autonomic disorder
Demyelinating syndrome	Mononeuropathy, single/multiplex
Headache (migraine and benign intracranial hypertension)	Myasthenia gravis
Movement disorder (chorea)	Cranial neuropathy
Myelopathy	Plexopathy
Seizure disorders	Polyneuropathy, unspecified
Acute confusional state	
Anxiety disorder	
Cognitive dysfunction	
Mood disorder	
Psychosis	

*Note:* Adapted from the American College of Rheumatology Case Definitions (1999).

**Table 1.3 - Cumulative incidences of NP-SLE syndromes**

<b>NP-SLE SYNDROMES</b>	<b>ESTIMATED CUMULATIVE INCIDENCE (%)</b>
<b>COMMON (&gt;5%)</b>	
Headache	20-40
Cognitive dysfunction	10-20
Mood disorder	10-20
Seizure disorder; Epilepsy	7-10; 12-22
Cerebrovascular diseases	7-10
Anxiety disorder	4-8
<b>UNCOMMON (1-5%)</b>	
Acute confusional state	1-5
Psychosis	3.0-4.5
Polyneuropathy	2.0-3.0
Myelopathy	1.0-1.5
<b>RARE (&lt;1%)</b>	
Cranial neuropathy	0.5-1.0
Mononeuropathy, single/multiplex	0.5-1.0
Aseptic meningitis	0.5-1.0
Movement disorders	0.4
Demyelinating syndrome	0.3
Acute inflammatory demyelinating polyradiculoneuropathy (Guillain-Barré syndrome)	0.1
Autonomic disorder	0.1
Myasthenia gravis	0.1
Plexopathy	0.1

*Note:* Adapted from Bertias and Boumpas (2010).

## **Chapter 2: Central Hypothesis and Specific Aims**

The overall objective of this work is to elucidate the nature and pathogenic mechanisms underlying functional deficits in SLE. In particular, this thesis examines whether autoimmunity in murine forms of lupus alters olfactory function and changes performance in other behavioural tasks.

### **Central Hypothesis**

*Autoimmunity in general, and autoantibodies in particular, alter olfactory function in lupus-prone MRL/lpr mice.*

### **Specific Aims**

- 1) To elucidate the nature of behavioural changes in MRL/lpr mice using a battery of paradigms dependent on smell capacity alongside spontaneous progression of autoimmune disease.
- 2) To examine odor-guided behaviours and immunological outcomes of chronic immunosuppression in MRL/lpr mice.
- 3) To investigate the behavioural effects of sustained autoantibody-rich CSF administration into the healthy mouse brain.

### **Chapter 3: Altered Olfactory Function in the MRL Model of CNS Lupus**

#### **Chapter Link**

The following chapter has been published in its entirety in *Behavioural Brain Research* [Kapadia, M., Stanojic, M., Earls, A. M., Pulapaka, S., Lee, J., & Sakic, B. (2012). Altered olfactory function in the MRL model of CNS lupus. *Behavioural brain research*, 234(2), 303-311. doi: 10.1016/j.bbr.2012.07.005].

Previous studies in the MRL model pointed to a complex behavioural phenotype most consistent with an anxiety-like profile in lupus-prone MRL/lpr mice [189;190]. However, more recent findings of CSF neurotoxicity [211;212] and morphological alterations in periventricular regions linked to olfactory function [213] raised the possibility that MRL/lpr mice develop impairments in olfaction that contribute to deficits in exploratory and other tasks. The chapter below addresses this question in a longitudinal set of experiments utilizing several odor-guided behavioural paradigms.

## **Altered olfactory function in the MRL model of CNS lupus**

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## **Abstract**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder that damages several bodily systems, including the CNS. Brain atrophy and diverse neuropsychiatric manifestations are common and serious complications of SLE. Recently, it has been reported that many patients with CNS involvement also present with olfactory deficits of unknown etiology. Similar to CNS SLE, spontaneous development of lupus-like disease in MRL/lpr mice is accompanied by neurodegeneration in periventricular regions and a constellation of behavioral deficits dependent on olfaction. To test the possibility that olfactory dysfunction also occurs in autoimmune mice, we presently examine odor-guided behaviors using a battery of paradigms. Indeed, lupus-prone males spent less time exploring unfamiliar conspecifics and demonstrated age-dependant performance deficits when exposed to low concentrations of attractant and repellent odors. The emergence of olfactory changes was associated with a skewed distribution of DCX<sup>+</sup> cells in the proximal portion of the rostral migratory stream (RMS). The present results are consistent with the hypothesis that the onset of a SLE-like condition affects periventricular regions, including the RMS, as evidenced by disrupted migration of neuronal precursor cells toward the olfactory bulb. If so, ensuing hyposmia and/or olfactory memory deficit may contribute to altered performance in other behavioral tasks and reflect a prodrome of brain damage induced by chronic autoimmune disease.

## 1. Introduction

Systemic lupus erythematosus (SLE) is a relapsing/remitting autoimmune disorder which can be life threatening when major organs are affected. Inflammation of the skin, joints, kidneys, and heart are typical manifestations, which differ largely with respect to onset, distribution, and severity among individual cases. In addition to diverse peripheral signs, many patients present with a broad spectrum of neurologic and psychiatric (NP) disorders of unknown etiology [1;2]. These complications range from focal abnormalities (i.e., seizures, cerebrovascular disease, aseptic meningitis, demyelinating syndrome, myelopathy) to diffuse symptoms, including anxiety, depression, cognitive deficits, and psychosis [3]. According to more recent observations, reduced smell capacity (hyposmia) may be yet another presentation of CNS involvement accompanying SLE [4]. In the particular study, patients with a history of NP manifestations were found to have deficits in both discrimination and identification of odorants. Although these olfactory impairments were proposed to reflect a consequence of systemic autoimmunity, the origin and pathologic mechanisms remain unexplained.

Animal models are indispensable tools for examining causal relationships between autoimmune/inflammatory phenomena and brain dysfunction, not feasible in clinical studies [5]. The MRL model consists of two congenic substrains which share more than 99.9% of their genomes [6], yet differ in disease onset. While MRL/MpJ (MRL<sup>+/+</sup>) mice manifest signs of autoimmunity relatively late in life, the MRL/MpJ-Fas<sup>lpr</sup>/J (MRL/lpr) substrain develops an accelerated form of lupus-like disease attributed

to deficiencies in Fas receptor (FasR) expression [7]. Changes in the immune status coincide with the emergence of behavioral deficits within the first three months of life [8]. These manifestations (operationally termed “autoimmunity-associated behavioral syndrome”) are largely characterized by blunted responsiveness to unfamiliar environments, objects, and conspecifics. In particular, when introduced to an open field, MRL/lpr mice exhibit increased thigmotaxis (ambulation along the perimeter) and confined exploration in the vicinity of their “home-base” [8,9]. In the presence of a novel object, lupus-prone mice are slower to approach and investigate it by sniffing, touching or biting [9]. Consistent with the notion of increased anxiety-like and/or impaired motivated behavior, they also show reduced intermale aggression, as evidenced by fewer fights, longer attack latency, and shorter fighting bouts following prolonged social deprivation [10].

The constellation of deficits in exploratory tasks develops in the wake of widespread neurodegeneration and brain atrophy [11–13]. Periventricular regions seem to be particularly vulnerable to cellular demise, as suggested by reduced neuronal density, aberrant morphology of pyramidal neuron dendrites [14], enhanced Fluoro Jade B staining [15,16], increased ubiquitination [16] and DNA fragmentation [17]. Consistent with these results, analysis of major neurogenic regions (the subgranular zone and the subventricular zone, SVZ) reveals distinct morphological abnormalities. In particular, we observed marked alterations in the proximal portion of the rostral migratory stream (RMS) [18], a well-defined pathway that facilitates tangential migration of neuroblasts from the SVZ to the olfactory bulb (OB). Discontinuous projection of neuroblasts and

decreased numbers of proliferating Ki67<sup>+</sup> and BrdU<sup>+</sup> cells pointed to aberrant migration in RMS. Such alterations in the number and distribution of progenitor cells raised the possibility that aberrant turnover of olfactory neurons in the OB contributes to changes in social and exploratory behaviors.

This assumption is supported by evidence that olfactory information from chemosensory cues influences a wide range of behavioral patterns (particularly in nocturnal animals), such as navigation, foraging, avoidance of predators, kin recognition, aggression, bond formation, and mate selection [19–21]. Although mild impairments in sensorimotor function [22] and learning/memory have been observed in diseased MRL/lpr mice [16,23], previous behavioral studies could not resolve whether deficits in approach-avoidance paradigms reflect changes in emotional reactivity/motivation, or altered olfactory capacity. The latter interpretation is supported by the link between certain behavioral responses (e.g., short-term olfactory memory) and continuous replacement of OB interneurons by the influx of neuroblasts from the RMS [24]. Based on this relationship, as well as exploration-dependant deficits and altered distribution of neural progenitor cells in the SVZ–RMS junction, we presently examine whether performance in olfactory tasks is altered in the MRL/lpr substrain. Using a battery of paradigms largely dependent on smell capacity, we assess sensitivity to different olfactory cues longitudinally at ages characterized by mild, modest, and severe lupus-like disease. To further enhance our understanding of neuropathogenic mechanisms, we examine the distribution of neuroblasts along the length of the RMS in separate cohorts of young, mid-aged, and old mice.

## 2. Methods

### 2.1. Experiment 1

Social relationships in rodents depend on the ability to acknowledge unfamiliar conspecifics through sniffing, licking, biting, fighting, and vocalizing [25]. The formation of social memories is important for establishing social bonds (both sexual and affiliative) and hierarchy, which allows group living with minimal aggressive tendencies [26]. In rodents, social recognition and memory are primarily based on chemosensory cues originating from the olfactory system [20]. Here we explore social interactions amongst unfamiliar conspecifics from two MRL substrains in parallel with the progression of lupus-like disease. To avoid the confounding effects of estrus-cycling on behavioral performance, male mice were used exclusively in the current and subsequent experiments.

#### 2.1.1. Animals

Four-week-old MRL/lpr and MRL+/+ males were purchased from the Jackson Laboratories (Bar Harbor, ME). Upon arrival, mice (n = 12/substrain) were left to acclimate for 7 days in groups of 3/cage to standard laboratory conditions: light cycle 7:00 a.m.–7:00 p.m., t = ~22 °C, relative humidity 35–37%, and *ad lib* access to food and water. At 5 weeks of age, the groups were matched for body weight, and each mouse was tail-tattooed (AIMS Inc., Hornell, NY) and shaved from the base of the neck to the hind legs. The exposed area was then marked with a blue, red, or green non-toxic marker

(Sharpie, Oakville, ON) for color-based video tracking. Mice were handled by the experimenter one week prior to behavioral testing. Social interactions were assessed at 7, 11 and 17 weeks of age, corresponding to “Young”, “Middle”, and “Old” groups, respectively. To confirm autoimmune disease and CNS involvement in MRL/lpr mice, spleen and brain weight were recorded at sacrifice (~18 weeks of age). All protocols and procedures in the present and following experiments were approved by a local committee and performed in accordance with the rules and regulations of the Canadian Council of Animal Care.

### *2.1.2. Interactions between Unfamiliar Conspecifics*

The presently used paradigm is a modification of dyad interaction tests previously employed in studies with socially-deprived males [26–28]. Interactions between two conspecifics from different home cages were assessed in a closed arena (45.5 cm × 45.5 cm × 12 cm) made of black plexiglass (Warehoused Plastic Sales, Toronto, ON). Dark brown top soil (Fafard Gardening Soil, Agawam, MA) was autoclaved and spread on the arena floor to provide contrast and a urine-absorbent, non-reflective surface. Three days prior to testing, mice were permitted to explore the empty arena for 5 min. Subsequently, each mouse was exposed to an unfamiliar conspecific for 10 min, to two mice on the same day, and at three ages (this resulted in six exposures in total). Interactions between two mice were filmed with an overhead camcorder and analyzed with EthoVision Color Pro 2.3 software (Noldus Information Technology, Leesburg, VA). A dyadic “encounter” was defined as an event when the distance between body midpoints was less than 3 cm

apart, characterized by sniffing (including ano-genital), biting, grooming, licking, and chasing of a conspecific. Inter-male aggression was not observed as mice were housed in groups throughout the study. Parameters measured included latency to the first encounter, as well as encounter frequency and duration. Individual measures of activity, including distance traveled in the arena and mean velocity of ambulation, were also recorded and analyzed. Arena walls were cleaned with Mikro Quat Germicide (1:125 dilution, Ecolab, Mississauga, ON) for sterilization and odor elimination between trials. Top soil in the arenas was replaced after each individual session.

## ***2.2. Experiment 2***

The utilization of a dyadic interaction paradigm in Experiment 1 did not allow the assessment of individual performance in odor-guided behaviors. This lack of information required additional scent-dependent tasks to obtain individual measures in a separate cohort of mice at identical time points. Such an experimental design also excluded the potential confounding effects of stress induced by social interactions on performance in spontaneous olfactory exploration tests.

### ***2.2.1. Animals***

Four week-old MRL/lpr and MRL+/+ males (n=20/group) were purchased from the Jackson Laboratories. Upon arrival, mice were matched for body weight, housed 4/cage, and kept under the laboratory conditions described above. One MRL/lpr mouse died prematurely, thus reducing the sample size to n=19 in the group.

### *2.2.2. Buried Food Pellet Test*

The response to a salient odor was used to examine whether lupus-prone mice develop a complete loss of smell (anosmia). Therefore, we employed the buried food pellet test, a paradigm proposed to rely on a rodent's intrinsic tendency to follow olfactory cues when foraging [29,30]. Prior to testing at 7 weeks of age, a 3-day shaping procedure was used to familiarize animals to a novel, palatable food (~0.3 g pellets, Cap'n Crunch cereal, The Quaker Oats Company, Chicago, IL). More specifically, individually-housed mice were given over-night access to three pieces of cereal in a white, plastic weigh boat placed in their home cage. Subsequently, mice were food-deprived 12 h prior to testing. One 5 min trial was conducted daily over three consecutive testing days at 7, 11 and 17 weeks of age, corresponding to Young, Middle and Old time-points. In each trial, a mouse was placed in a cage (45 cm × 24 cm × 20 cm) to recover one piece of cereal buried ~1 cm beneath the surface of 3 cm-deep bedding. Mice were lowered into the corner opposite to the buried pellet. To prevent entry of other odors, the cage was covered with a large piece of plexiglass. Each day, the pellet was placed in a different location to preclude place preference learning. Performance was digitally recorded and then scored with Observer XT software (Noldus Information Technology, Leesburg, VA) by an experimenter blind to group origin. Latency to locate the food pellet was defined as the time between mouse placement into the cage and the instant when it grasped the pellet with either forepaws, or teeth. Rearing behavior was also counted. Mice were allowed to consume the pellet at the end of the trial and were then returned to their home cage. If a mouse did not locate the pellet within 5mins, it was removed and

placed back into his home cage. The test chamber was cleaned with Quatricide Disinfectant (1:256 dilution, PRL Pharmacal, Naugatuck, CT) between the trials and filled with fresh bedding.

### *2.2.3. Olfactory Sensitivity Tests*

To identify more subtle alterations in olfactory function, a battery of sensitivity tests employing different concentrations of attractant and repellent scents was carried out at 7, 11 and 17 weeks of age [31,32]. Habituation to the experimental environment consisted of placing a mouse into the test chamber (45 cm × 24 cm × 20 cm) 6 min prior to the trial. Subsequently, a 5 cm × 5 cm piece of filter paper (Whatman Inc., Piscataway, NJ) scented with an odorant (~.25 ml) was placed into the corner opposite from the mouse. A repellent (Peppermint Essential Oil, Aura Cacia, Urbana, IA), an attractant (peanut butter), and a neutral-like scent (Pure Vanilla Extract, Club House, London, ON) were used in 1 min trials. Each odorant was diluted to .01, 1, 10, and 50%. The utilization of four different concentrations allowed us to produce a dose–response curve with which we could compare olfactory responsiveness between MRL substrains to the same olfactory stimulus. The aim of the study was not to assess the absolute detection threshold for each odor, but to compare olfactory responsiveness between the groups. Mice were exposed to a single concentration on each testing day. Both peppermint and vanilla were dissolved in distilled water, while smooth peanut butter (Kraft Canada Inc., North York, ON) was dissolved in mineral oil (Exact Mineral Oil, Loblaws Inc., Brampton, ON). To control for responsiveness to a wet filter paper, a drop of water was

applied in a separate test and introduced in a manner identical to odorants. Testing cages were covered with a clear piece of plexiglass to limit evaporation and entry of external odors. Behavioral performance was digitally recorded and analyzed with Observer XT software. Active investigation was defined as directed sniffing within .5 cm of the odor source. After each trial, mice were returned to their home cage, while the experimental cage was cleaned with Quatricide.

### ***2.3. Experiment 3***

We previously reported that disease onset in MRL/lpr mice is associated with reduced expression of Ki67 and BrdU proliferation markers in the SVZ [18]. However, this analysis was limited to the junction between SVZ and the proximal portion of the RMS. Here we expand these observations by assessing the distribution of neuroblasts along the length of the RMS, including the distal segment of the RMS in the vicinity of the OB. The focus of the current study was not the cytoarchitecture of the RMS *per se*, but rather to investigate whether substrain differences in immature neurons coincide with the development of autoimmunity in lupus-prone mice.

#### ***2.3.1. Animals***

Twenty-eight 4-week-old MRL/lpr and 27 MRL<sup>+/+</sup> male mice were purchased from the Jackson Laboratories. They were housed in groups of 4 mice/cage under the laboratory conditions described above. Distribution of neuronal precursor cells along the RMS was examined at 7, 11, and 17 weeks of age. At each time point, mice were

anaesthetized with an intraperitoneal injection of a ketamine/xylazine mixture, exsanguinated by severing the inferior vena cava, and intracardially perfused over 5 min with cold phosphate buffered saline (PBS). Following extraction, brains were fixed in 4% paraformaldehyde (PFA) overnight and then transferred to increasing concentrations of PFA/sucrose solution (10–30%).

### 2.3.2. RMS Immunohistochemistry

Brains were cut sagittally into 30  $\mu\text{m}$ -thick uni-hemispheric sections (save the OB, which was hard to keep intact during sagittal sectioning and slide mounting). Starting at the mid-sagittal plane, the first 20 cuts from medial to lateral were discarded and the full length of the RMS was defined as the region encompassing lateral planes .6–1.32 mm (~5 sections). The distribution of DCX<sup>+</sup> cells was predominantly localized to two regions, operationally defined as proximal and distal RMS. They included cell clusters in the SVZ–RMS junction and the rostral-inferior extent prior to the OB. Primary goat anti-DCX antibodies (1:100, Santa Cruz, CA) and secondary donkey anti-goat Cy3 antibodies (1:250, Jackson ImmunoResearch, PA) were diluted in PBS containing .25% Triton X-100 and 3% donkey serum. Digital images of the RMS were taken from the right hemisphere using Axio Imager.Z1 (Carl Zeiss Canada Ltd., Toronto, ON) at 10 $\times$  magnification. Area of DCX<sup>+</sup> cells was assessed using Axiovision 4.8 and Interactive Measurement plug-in. Neuroblast distribution ratio was estimated by dividing distal and proximal DCX<sup>+</sup> cell areas for each RMS region.

## **2.4. Statistical Analysis**

Raw data were analyzed using SPSS 16 software package (SPSS Inc., Chicago, IL). Analysis of variance (ANOVA) was used in the overall analysis, with Substrain and Age as between-group factors. When measures were taken repeatedly (e.g., trials or sections), they were considered within-group factors in ANOVA with repeated measures. If significant interactions were detected, Student's t-test was used in post-hoc comparison. The significance level was set at  $p \leq .05$  in all two-way comparisons. Graphs indicate mean values  $\pm$  SEM, with significant differences of  $p \leq .05$ ,  $p < .01$  and  $p < .001$ , shown as \*, \*\*, and \*\*\*, respectively.

## **3. Results**

### **3.1. Interactions between Unfamiliar Conspecifics**

The latency to approach an unfamiliar conspecific increased over time (Age:  $F_{1,22}=5.699$ ,  $p < .05$ ; Figure 1A), but group differences did not reach statistical significance at any time point. While frequency of contacts declined in both substrains (Age:  $F_{2,44}=17.307$ ,  $p < .001$ ; Figure 1B), the exploration time between unfamiliar mice increased with aging (Age:  $F_{2,44}=5.722$ ,  $p < .006$ ). More importantly, total exploration time was lower between unfamiliar MRL/lpr conspecifics, than in age-matched controls (Substrain:  $F_{1,22}=15.515$ ,  $p < .001$ ), particularly at middle and old ages (Figure 1C). While distance traversed and mean velocity of ambulation ( $6.0 \pm 0.3$  vs.  $5.5 \pm 0.3$  vs.  $3.9 \pm 0.2$  cm/s) declined during the study (for Distance: Age:  $F_{2,44}=25.525$ ,  $p < .001$ ; for Velocity: Age:

$F_{2,44}=24.157, p<.001$ ), significant group differences were not detected. Taken together, the results obtained suggest disease progression in MRL/lpr mice is paralleled by activity-independent alterations in social interactions.

Confirming previous findings of severe autoimmune manifestations and brain atrophy [13], aged MRL/lpr mice had enlarged spleens ( $347\pm 35\text{mg}$  vs.  $90.8\pm 5\text{mg}$ ;  $t_{22}=7.151, p<.001$ ) and lower brain weights ( $498.9\pm 5\text{mg}$  vs.  $521.9\pm 8\text{mg}$ ;  $t_{22}=-2.348, p=.028$ ) at sacrifice.

### **3.2. Buried Food Pellet Test**

Throughout the 3-day training period, mice readily consumed the food pellets provided, thus confirming Cap'n Crunch cereal is highly palatable. Since overall analysis did not reveal a significant Group x Age x Trial interaction ( $p=.59, \text{n.s.}$ ), group data are shown as a function of Age (Figure 2). Accordingly, when the mean of 3 trials was taken, both groups of younger mice spent comparable time to locate the buried pellet. However, at 17 weeks, MRL/lpr males showed a shorter latency to uncover the pellet in comparison to age-matched MRL+/+ controls (Age x Substrain:  $F_{2,76}=3.762, p<.05$ ; Figure 2A). This difference was associated with increased rearing behavior in the control group (Age:  $F_{2,76}=57.547, p<.001$ ; Figure 2B). Collectively, the data obtained suggest diseased MRL/lpr mice have no difficulties in detecting salient odors (i.e., they do not show signs of anosmia).

### **3.3. Olfactory Sensitivity Tests**

The overall shape of the dose–response curves confirmed that: (1) vanilla has neutral-like properties, (2) peppermint has repellent properties, as indicated by decreased exploration time as the odor becomes more concentrated and (3) peanut butter is indeed an attractant, given the positive relationship between exploration time and concentration (Figure 3). Across scents and ages, group differences were consistently noted at the lowest concentration (0.01%), but not at higher concentrations. The latter result is consistent with the performance in the buried food pellet test, suggesting MRL/lpr mice do not have difficulties detecting salient odors. Due to the aforementioned discriminatory capacity of 0.01% tests, further analysis focused on performance at this concentration. Although, MRL substrains showed comparable exploration of wet filter paper at a young age, MRL/lpr mice unexpectedly spent more time investigating it at the Middle Age (Age x Substrain:  $F_{2,76}=5.250$ ,  $p<.01$ ;  $t_{38}=2.760$ ,  $p<.01$ ; Figure 4A). Both groups of mice increased investigation of 0.01% vanilla over time (Age:  $F_{2,74}=24.425$ ,  $p<.001$ ; Figure 4B), but no significant substrain differences were noted at any age. Similar to the test with wet filter paper, increased exploration time with an aversive scent (peppermint) emerged in diseased MRL/lpr mice at 11 and 17 weeks of age (Age x Substrain:  $F_{2,76}=5.257$ ,  $p<.01$ ; Figure 4C). Although exploration of paper soaked with 0.01% peanut butter increased with age (Age:  $F_{2,74}=40.116$ ,  $p<.001$ ), MRL/lpr mice consistently spent less time in contact with it than age-matched MRL+/+ males (Substrain:  $F_{1,37}=14.194$ ,  $p<.01$ ; Figure 4D).

At the time of sacrifice, MRL/lpr mice expectedly manifested severe splenomegaly ( $542\pm 48\text{mg}$  vs.  $100\pm 11\text{mg}$ ;  $t_{37}=9.162$ ,  $p<.001$ ). Reduced brain weight in

the MRL/lpr substrain ( $481 \pm 3$  mg vs.  $513 \pm 3$  mg,  $t_{36} = -6.554$ ,  $p < .001$ ) was consistent with previously-reported attenuation in brain growth [13].

### ***3.4. RMS Immunohistochemistry***

Assessment of spleen weight confirmed that cohorts of MRL +/+ mice were asymptomatic across the three ages, while MRL/lpr groups exhibited an accelerated disease process (Figure 5A). Splenomegaly was not evident in young MRL/lpr mice, but substrain differences were identifiable by the third month of life. Although significant group difference in body weight could not be detected, lupus-like disease in MRL/lpr mice was associated with reduced brain weight at all three age points (Figure 5B). The area of DCX<sup>+</sup> cells in the RMS was quantified in a series of sagittal sections (representative composite images from two aged mice are shown in Figure 6). Planimetric assessment of the proximal and distal RMS regions revealed no significant differences at 7 weeks of age. However, 11 week-old MRL/lpr mice had a larger cell area in the proximal RMS region in comparison to age-matched controls ( $t_{18} = 2.25$ ,  $p < .05$ ; Figure 7A). In contrast, DCX<sup>+</sup> distal cell area did not differ between the groups across the ages (Figure 7B). Along the same line, the distal/proximal area ratio revealed significant increases in both MRL strains from 11 to 17 weeks of age ( $t_{19} = 2.554$ ,  $p = .032$ , Figure 7C). However, MRL/lpr mice did not show equal ratio increase in comparison to the control MRL+/+ group (Age:  $F_{1,41} = 11.78$ ,  $p < .001$ ; Substrain:  $F_{1,41} = 5.78$ ,  $p = 0.022$ ). The progressive decrease in proximal area and parallel increases in the distal portion of the RMS at older age suggests defects in tangential neuroblast migration. However, this

process seems to be attenuated in MRL/lpr mice at an age that coincides with more severe manifestations of lupus-like disease.

#### **4. Discussion**

Shoenfeld et al. reported that smell deficit correlates with male sex and older age, both in SLE patients and controls [4]. More importantly, hyposmia was observed in 46% of SLE patients (vs. 25% controls), while 10% of patients suffered from a complete loss of smell, or anosmia. Moreover, this clinical impairment was associated with disease severity and CNS manifestations. The present longitudinal study supports these findings in a spontaneous model of CNS lupus. In particular, we found that aging is a contributing factor to alterations in olfaction. Furthermore, when compared to asymptomatic MRL<sup>+/+</sup> controls, MRL/lpr mice spent less time in contact with unfamiliar conspecifics. Although they did not have difficulties in detecting salient odor cues, changes in performance were evident in tasks employing low-concentrated scents. As they were becoming sicker, MRL/lpr mice showed prolonged response to a repellent and attenuated response to an attractant. This altered performance was also detected when sick MRL/lpr mice were exposed to filter paper soaked with water at Middle Age. Taken together, the results obtained suggest hyposmia and/or olfactory memory deficit are exacerbated by the progression of systemic autoimmune disease. Such alterations in sensory capacity are likely to affect performance in paradigms probing different domains of behavior, including exploratory drive and/or emotional reactivity. Coinciding with the onset of severe disease manifestations and aberrant odor-guided behavior, MRL/lpr mice had

altered distribution of DCX immunoreactivity within the RMS, suggesting impaired migration of neuronal precursor cells and immature neurons toward the OB. If so, this deficiency may ultimately compromise the replacement of mature neurons and contribute to olfactory deficits at advanced stages of lupus-like disease.

Reduced contact duration between unfamiliar males is consistent with previous findings of aberrant social behavior, exemplified by impaired isolation-induced aggression between MRL/lpr males [10]. Although the analysis of dyadic encounters was useful in the assessment of exploration, two additional tasks were employed to provide more sensitive measures of individual olfactory performance. In particular, in the buried food pellet test, food-deprived MRL males found the pellet within the first minute, similar to non-autoimmune strains of mice [30]. However, the shorter latency to locate the odor source at an older age suggests that control MRL+/+ mice employ an exploration strategy characterized by increased rearing, as shown earlier [8]. Another viable explanation for a faster response in diseased MRL/lpr mice may involve their known preference for carbohydrate-rich food [33]. The lack of a dose-effect response in the MRL subgroups to increasing concentrations of vanilla is consistent with its neutral like-properties [31]. Along the same line, negative and positive associations between concentration and exploration time confirm the aversive and appetitive properties of peppermint and peanut butter, respectively. More importantly, prolonged investigation of peppermint-scented paper by diseased MRL/lpr mice is in accordance with impaired avoidance to menthol (the primary constituent of peppermint oil) in an inducible model of CNS SLE [34,35]. Given peanut butter's potency as a palatable food source for mice

(e.g., as bait in mouse traps), reduced investigation of a peanut butter-scented paper supports the notion of olfactory dysfunction in MRL/lpr mice. Similar impairments in response to peanut butter have been shown in mice with OB lesions [31,36].

Planimetric analysis of DCX immunoreactivity along the RMS reveals alterations in the distribution of immature neurons migrating toward the OB. Particularly, while insignificant variations in proliferative cell area are noted between groups of young MRL mice, the onset of systemic autoimmunity at 11 weeks of age in the MRL/lpr substrain is associated with aberrant precursor cell projections along the RMS. These findings extend previously-reported disruptions in the number and distribution of BrdU<sup>+</sup> cells in major neurogenic regions, including the SVZ–RMS region [18]. Similarly, delayed migration of neuronal precursors from the SVZ to the RMS occurs in lupus-prone MRL<sup>+/+</sup> mice when compared to healthy, ancestral strains [37]. In a broader context, the present results are consistent with the hypothesis that immature neurons around the SVZ are susceptible to systemic autoimmune disease [18,38,39]. Despite growing evidence that migrating neuroblasts differentiate into bulbar interneurons incorporated into pre-existing networks of the OB [40], the functional importance and contribution of a neurogenic cascade remains unclear. Nevertheless, newborn neurons in the adult OB have been linked to roles in odor discrimination [41], parental-offspring recognition [42], predator avoidance and sex-specific responses [43], perceptual learning [44], as well as short-term [24] and long-lasting olfactory memory [45,46].

The etiology of observed substrain differences in odor-guided behaviors and aberrant neuroblast migration remains unknown. Although our results imply that changes in olfactory function develop alongside aging and may be exacerbated by the progression of systemic autoimmunity in MRL/lpr mice, they do not prove a cause-effect relationship. Indeed, one must take into consideration the possibility that other factors can account for substrain differences in response to .01% peanut butter before overt disease manifestations are present at a young age. They may include deficient FasR expression in the MRL/lpr brain [47] or in utero exposure to maternal autoimmunity [48]. Therefore, the performance in olfactory tasks is likely under the influence of multiple factors, including genetic make-up, aging and systemic autoimmune disease.

Without further analysis, it is difficult to fully understand the abundance of DCX<sup>+</sup> cells in the proximal RMS at the onset of SLE-like disease in mid-aged MRL/lpr mice. However, one may hypothesize that enhanced neurogenesis in the SVZ region reflects a compensatory response to widespread neuroinflammation and neurodegeneration in the autoimmune brain [13,14,49]. In addition, it is possible that soluble autoimmune factors originating from the cerebrospinal fluid, CSF (e.g., autoantibodies) diffuse into the periventricular SVZ region and interfere with normal migration of neuroblasts along the RMS. The end result of these two processes would be that a smaller proportion of cells from the initial progenitor pool reach the distal portion of the RMS.

Antibodies reactive to brain antigens (brain-reactive autoantibodies, BRA) have attracted considerable attention as pathogenic factors in the etiology of CNS

manifestations induced by SLE [50]. Disturbed migration of neuroblasts along the RMS is consistent with antibody-induced demise of immature and adult neurons in MRL/lpr brains. In particular, elevated immunoglobulin G (IgG) levels in autoimmune CSF [51], CSF neurotoxicity [38,52,53], and intensive binding of CSF IgG to the SVZ [54] collectively point to a complex pathogenic cascade. This process may involve increased blood–brain barrier permeability, drainage and/or intrathecal production of neurotoxic BRA, and deposition of CSF antibodies, followed by damage to neurogenic regions. Supporting the role of intrathecal BRA neurotoxicity in CNS SLE, other groups have demonstrated that autoantibodies from lupus patients induce neuronal death [55,56] and behavioral deficits when introduced into the mouse brain [57–59]. Of particular relevance, administration of anti-ribosomal P protein antibodies to healthy rodents induces neuronal apoptosis [60], depressive-like behavior, olfactory dysfunction [34] and alterations in the olfactory pathway [35]. Along the same line, our recent work revealed enhanced reactivity of MRL/lpr BRA to evolutionary conserved proteins which form the cytoskeleton [61]. These findings parallel clinical evidence pointing to several cytoskeletal proteins as autoantigens in CNS SLE [62–65]. If these BRA are indeed pathogenic, they may interfere with the normal assembly of microtubules and other cytoskeleton-related proteins (e.g., DCX) instrumental in the migration of immature neurons [66]. In addition, it is plausible that altered morphology of the RMS-OB pathway is not an exclusive contributor to olfactory changes. This is based on evidence that anti-ribosomal P antibodies bind to neurons in the hippocampus, cingulate cortex, and the primary olfactory piriform cortex [67]. Along the same line, other autoantibodies (e.g.,

anti-*N*-methyl- D-aspartate receptor), may also be involved in the pathogenesis that leads to altered activity in limbic structures [58]. Considering odor information from the OB ultimately projects to the amygdala and hippocampus [68,69], autoantibody-mediated damage in these limbic regions may also contribute to changes in olfactory function.

The current findings further corroborate the link between autoimmune / inflammatory phenomena and olfactory dysfunction [70]. In addition, the longitudinal assessment of odor-guided behaviors suggests that hyposmia (rather than anosmia) develops alongside the progression of spontaneous lupus-like disease. Lastly, our study points to the role of neurogenic pathways in an animal model with documented blood–brain barrier dysfunction and numerous neuropathogenic autoantibodies [49,51,71].

Despite the wealth of behavioral and neuropathological data collected over the life-span of MRL mice, the current study lacks information about damage in primary and accessory olfactory systems. More specifically, to determine whether differences in cell distribution have morphological and/or behavioral sequelae, the survival, differentiation, and integration of neuroblasts in the OB needs to be determined. Along the same line, characterization of the molecular mechanisms involved in altered migration of immature neurons would be an important step in revealing the etiology of autoimmunity-induced neurodegeneration and psychiatric manifestations in SLE.

## **5. Conclusions**

Olfactory capacity changes alongside the aging process in the murine MRL strain. Similar to CNS SLE patients, accelerated development of lupus-like disease in MRL/lpr

mice further alters their responsiveness to low-concentrated repellents and attractants. It is hypothesized that systemic autoimmunity affects distribution of neuroblasts, thus compromising olfactory function and performance in other odor-guided behaviors.

### **Author Contributions**

M.K., M.S., and B.S. were involved in manuscript drafting and contributing to the intellectual content. B.S. takes responsibility for the integrity and accuracy of results.

**Study conception and design:** B.S.

**Data Acquisition:** M.K., M.S., A.E., S.P. and J.L.

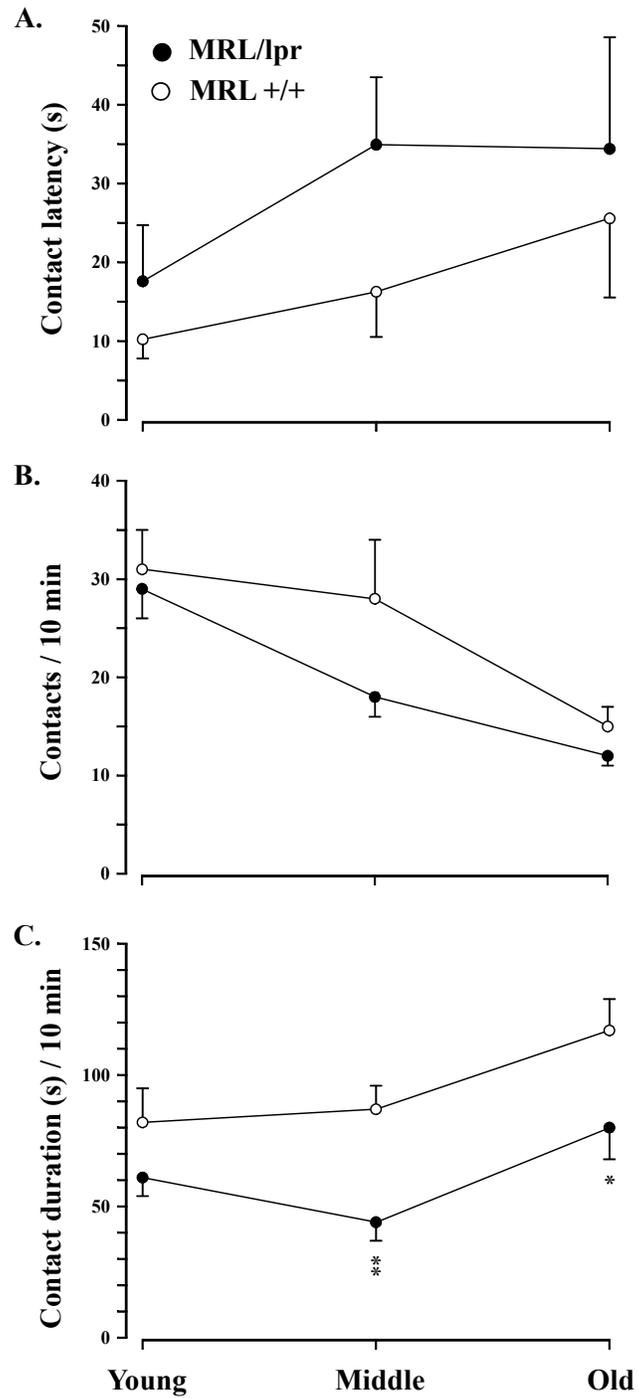
**Data Analysis and Interpretation:** B.S., M.K., and M.S.

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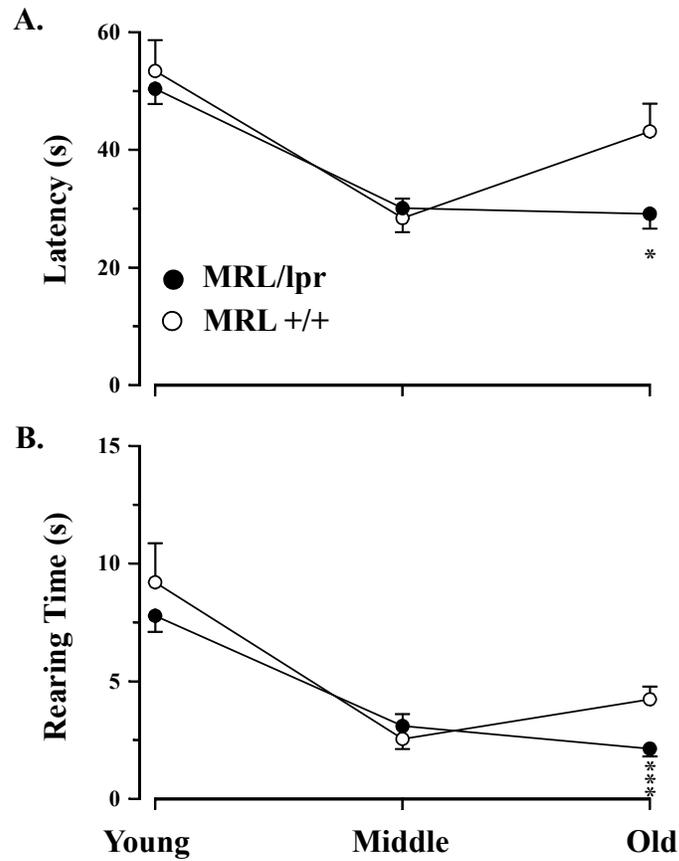
## Figures

Figure 3.1 - Dyadic encounters between unfamiliar conspecifics



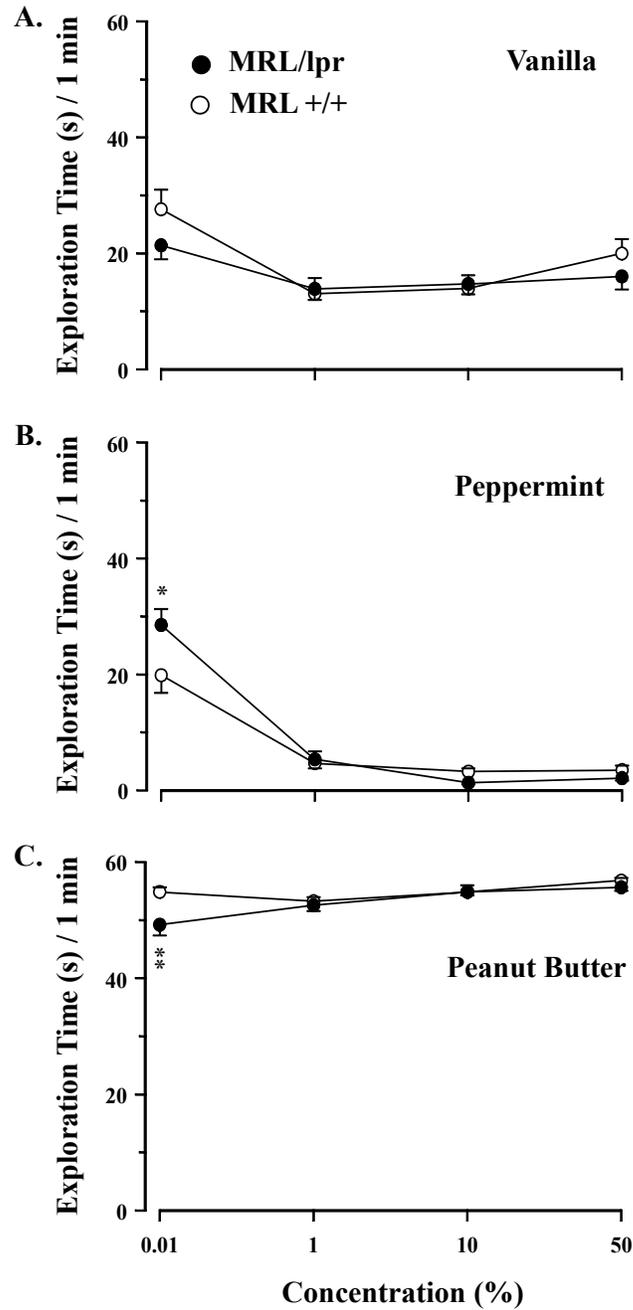
**Figure 1. Dyadic encounters between unfamiliar conspecifics.** (A) Although latency to approach unfamiliar conspecifics increased throughout the lifespan, no significant differences between the MRL substrains were noted during the study. (B) Conversely, contact frequency declined across ages in both groups. (C) While contact duration increased with age, it was significantly lower in diseased MRL/lpr mice. These results suggest that MRL/lpr mice spend less time exploring unfamiliar conspecifics when lupus-like disease manifests.

**Figure 3.2 - Buried food pellet test**



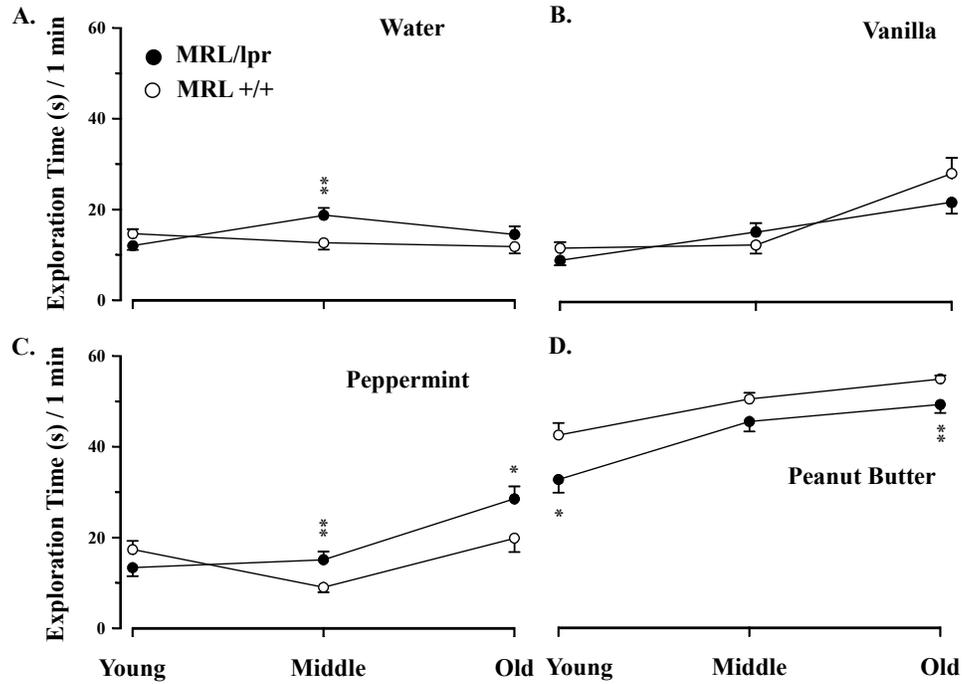
**Figure 2. Buried food pellet test.** (A) At younger ages, both groups of MRL mice readily located the buried pellet of Cap'n Crunch cereal. However, 17 week-old MRL+/+ mice took more time to discover the pellet in comparison to MRL/lpr mice. (B) The longer latency was accompanied by increased rearing in comparison to MRL/lpr mice. These results suggest that diseased MRL/lpr mice have no difficulties in locating a salient odor.

**Figure 3.3 - Concentration-dependent response to neutral-like, repellent, and attractant scents at 17 weeks of age (Old Age)**



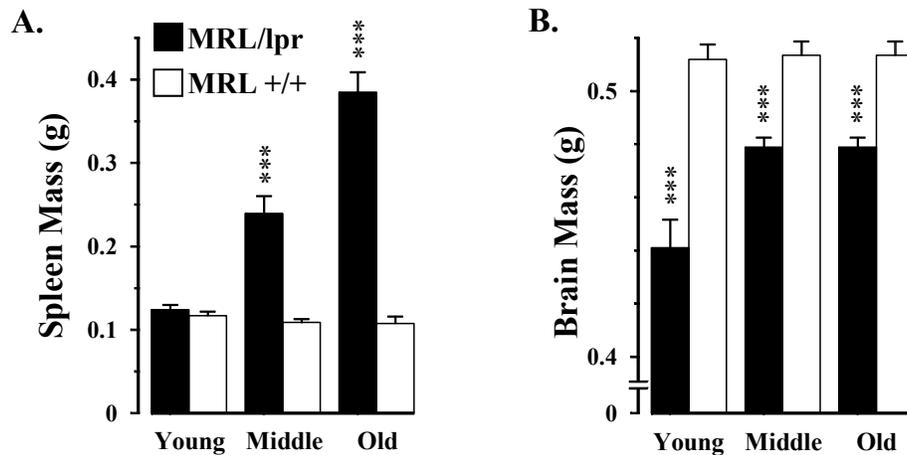
**Figure 3. Concentration-dependent response to neutral-like, repellent, and attractant scents at 17 weeks of age (Old Age).** Both groups of MRL mice showed comparable responses to highly-concentrated scents. However, the 0.01% concentration discriminated the groups when exposed to either (A) vanilla, (B) peppermint, or (C) peanut butter. Given this, the 0.01% concentration was selected in the subsequent analysis.

**Figure 3.4 - Olfactory sensitivity tests**



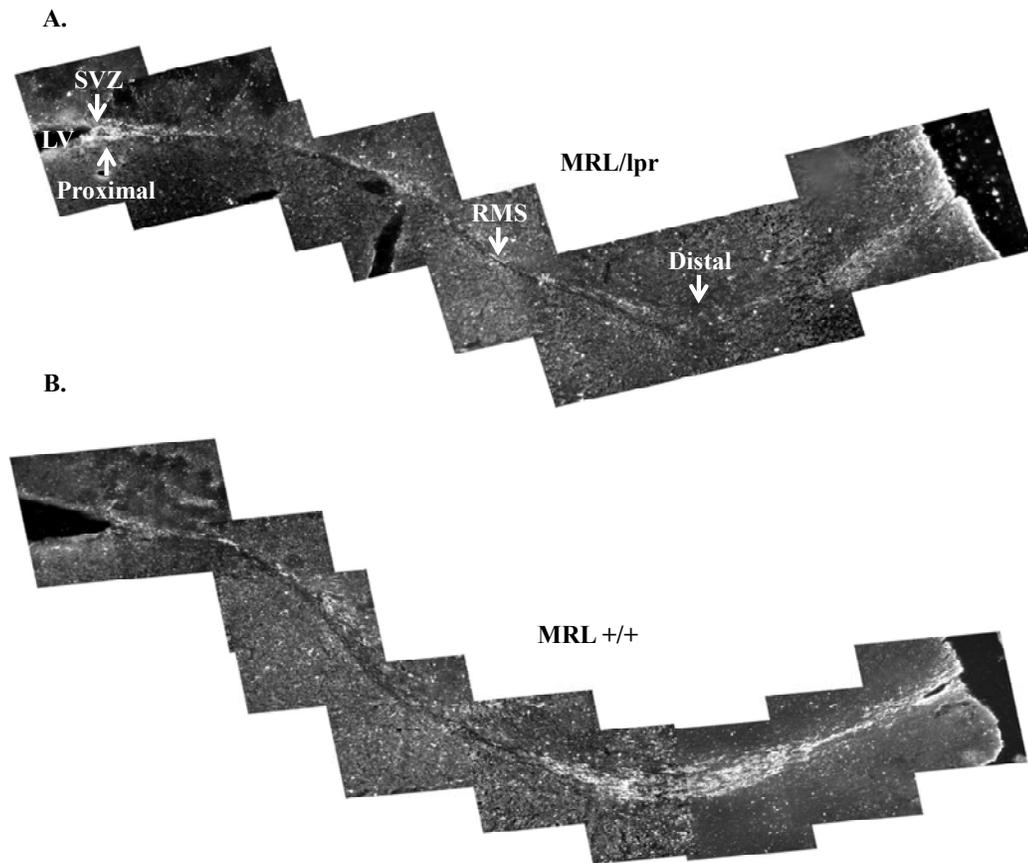
**Figure 4. Olfactory sensitivity tests.** (A) MRL/lpr mice spent more time exploring wet filter paper at the onset of disease (Middle Age). (B) As expected, MRL substrains did not differ in their exploration of 0.01% vanilla across all three time points. (C) At young age, MRL groups did not differ in responding to the aversive scent. However, at older age, MRL/lpr mice spent more time exploring peppermint-scented paper than age-matched controls. (D) Conversely, their responsiveness to the lowest concentration of peanut butter was significantly reduced at all ages. Taken together, the results pointed to altered olfactory sensitivity in the MRL/lpr group, in particular at the onset of severe autoimmunity and inflammation.

**Figure 3.5 - Spleen and brain mass in separate age cohorts**



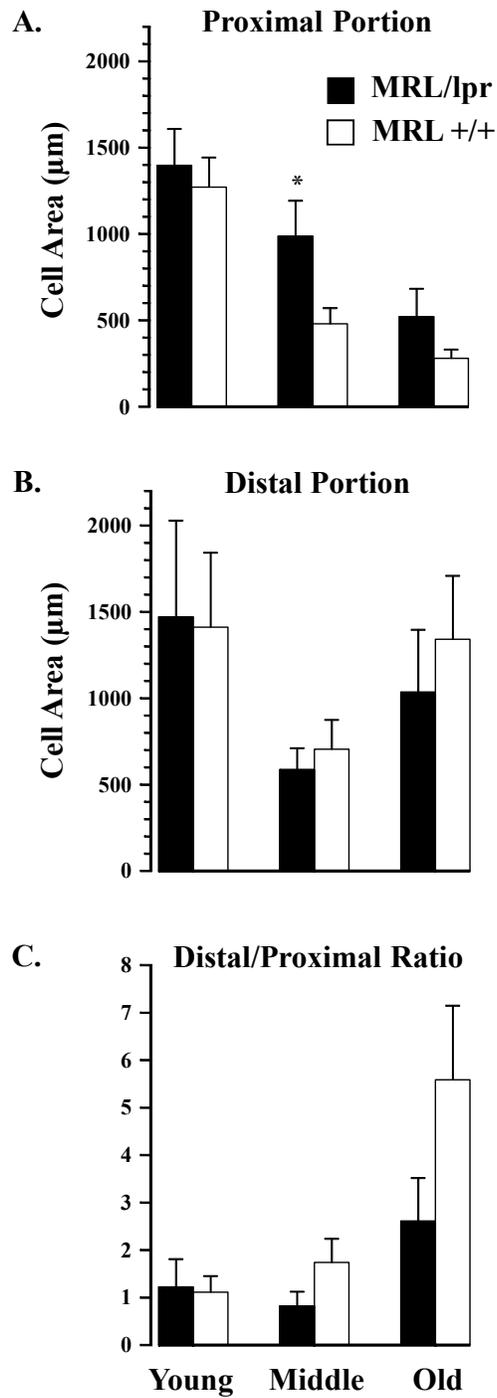
**Figure 5. Spleen and brain mass in separate age cohorts.** (A) The assessment of wet-spleen weight confirmed that splenomegaly (a reliable marker of autoimmunity) is detected in MRL/lpr mice from Middle Age, while the MRL +/+ groups remain asymptomatic at each time point. (B) Conversely, reduced brain weight was observed in the MRL/lpr group as early as 7 weeks of age, confirming results from a recent longitudinal study [13].

**Figure 3.6 - Composite images of sagittal brain sections illustrating DCX staining along the RMS**



**Figure 6. Composite images of sagittal brain sections illustrating DCX staining along the RMS.** (A) An example of increased proximal and reduced distal DCX<sup>+</sup> cell area in an old MRL/lpr mouse. (B) An example of decreased proximal and increased distal DCX<sup>+</sup> area in the RMS of an age-matched MRL+/+ male. Abbreviations: LV, lateral ventricle; RMS, rostral migratory stream; Proximal, proximal portion of RMS; Distal, distal portion of RMS.

**Figure 3.7 - Planimetric assessment of DCX<sup>+</sup> cell area in the RMS**



**Figure 7. Planimetric assessment of DCX<sup>+</sup> cell area in the RMS.** (A, B) The density of DCX<sup>+</sup> cells in both proximal and distal regions is comparable in the MRL substrains at a young age. However, the onset of overt SLE-like disease manifestations at Middle Age was accompanied by increased DCX<sup>+</sup> area in the proximal region. (C) This discrepancy resulted in an imbalanced increase in the distal/proximal ratio at older ages. Taken together, the results obtained suggest an altered migration of immature neurons along the RMS at the time when lupus-like disease becomes severe.

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## **Chapter 4: Sustained Immunosuppression Alters Olfactory Function in a Model of CNS SLE**

### **Chapter Link**

Portions of the following chapter have been published in an original *PLoS ONE* article addressing a separate question related to “self-healing” process in the MRL model [Kapadia M, Zhao H, Ma D, Hatkar R, Marchese M, et al. (2014) Zoopharmacognosy in Diseased Laboratory Mice: Conflicting Evidence. *PLoS ONE* 9(6): e100684. doi: 10.1371/journal.pone.0100684].

The experimental findings detailed in Chapter 3 corroborated the clinical assessment of olfaction in SLE patients, and strengthened the face validity of the MRL/lpr substrain as a model of CNS SLE. Although alterations in odour-guided behaviours coincided with the development of systemic autoimmunity, they did not prove a cause–effect relationship between the two. Several alternative pathological mechanisms, none of which are mutually exclusive, need to be taken into consideration. They include deficient FasR expression in the MRL/lpr brain [186;253], *in utero* exposure to maternal autoimmunity [254], and/or asymmetric activation of the hypothalamus–pituitary–adrenal (HPA) axis [242]. One method to clarify the relative contribution of autoimmunity versus genetic, endocrine and other factors in the MRL/lpr substrain involves generalized suppression of immune hyperactivity. If olfactory dysfunction is indeed related to autoimmunity, then immunosuppression should abolish behavioural deficits in tests probing olfactory function.

## **Sustained Immunosuppression Alters Olfactory Function in a Model of CNS SLE**

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## **Abstract**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder that adversely affects many organs, including the brain. Recently, it has been reported that CNS involvement in SLE patients includes olfactory deficits of unknown origin. Similar to SLE patients, spontaneous development of lupus-like disease in MRL/lpr mice is accompanied by changes in olfactory function and brain regions important in neurogenesis and smell capacity. To further assess the construct validity of this model, the current study examines the possibility that systemic autoimmune disease alters olfaction in MRL/lpr mice. Over 12 weeks, young MRL/lpr and control MRL +/+ males had *ad lib* access to a solution containing the immunosuppressant cyclophosphamide (CY). Concurrently, responsiveness to several concentrations of different scents was longitudinally assessed at mild, modest and severe stages of disease development. Odor-guided exploratory behaviour was further assessed in the novel object test at 21 weeks of age, shortly before terminal assessment of tissue pathology. In comparison to the vehicle-treated group, sustained immunosuppression prevented autoimmunity and improved blunted responsiveness to an attractant and a novel object. However, CY treatment worsened aberrant response in MRL/lpr mice to a repellent scent, suggesting a dual mode of action on olfactory circuits. Taken together, the present study reveals that prolonged, generalized immunosuppression modulates odor-guided behaviours in autoimmune MRL/lpr mice. Although key pathogenic mechanisms remain to be elucidated, it supports the hypothesis that lupus-like disease alters olfaction and related domains of behaviour.

## 1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disorder that typically manifests in inflammation of the skin, joints, kidneys, and heart, albeit to varying degrees of severity. Diverse signs of peripheral inflammation are often accompanied by a broad spectrum of neurologic and psychiatric (NP) disorders of unknown etiology [1;2]. These complications range from focal abnormalities (i.e., seizures, cerebrovascular disease, demyelinating syndrome, myelopathy) to diffuse symptoms such as anxiety, depression, cognitive deficits, and psychosis [3]. Recent observations suggest that SLE patients with a history of NP symptoms also exhibit reduced smell capacity or hyposmia [4]. Impairments in both discrimination and identification of odorants were noted at different stages of disease development. Although these olfactory deficits are proposed to reflect a consequence of autoimmunity, the origin and pathologic mechanisms remain largely unknown.

The MRL murine model of SLE has proven valuable in examining causal relationships between autoimmune/inflammatory phenomena and brain dysfunction not feasible in clinical studies [5]. Due to a *lpr* mutation on chromosome 19 and dysfunctional FasR expression during negative selection of autoreactive T cells [6;7], MRL/*lpr* mice develop florid disease by 3 months of age [8] with the clinical and serological manifestations reminiscent of human SLE [9]. Conversely, the congenic MRL/MpJ (MRL +/+) substrain displays lupus-like disease later in life, thus representing an adequate control. In comparison to sex- and age-matched MRL+/+ controls, the

behavioural profile of MRL/lpr mice is largely characterized by increased anxiety-like, impaired motivation and “cognitive” inflexibility, jointly termed “autoimmunity-associated behavioural syndrome” [10]. In particular, autoimmune MRL/lpr mice confine their exploration to the perimeter of an open field [11;12], are slower to approach and investigate novel objects [11], and display impaired inter-male aggression [13]. These alterations in odor-guided behaviors coincide with widespread neurodegeneration [14-16], with the periventricular region seeming particularly vulnerable to cellular demise [17-20]. Accordingly, major neurogenic regions display altered distribution and decreased numbers of proliferating cells in the dorsal portion of the rostral migratory stream (RMS) [21], a well-defined pathway that facilitates tangential migration of neuroblasts from the subventricular zone (SVZ) to the olfactory bulb (OB). Such morphological abnormalities raised the possibility that aberrant turnover of olfactory neurons in the OB contributes to changes in social and exploratory behaviours.

Based on clinical evidence of olfactory dysfunction in NP-SLE, olfaction-dependent deficits in autoimmune mice, altered distribution of neural progenitor cells in the SVZ-RMS junction, and the functional importance of SVZ-derived neuroblasts in the OB [22], we recently examined odor-guided behaviours longitudinally in the MRL model [23]. In comparison to asymptomatic MRL+/+ congenic controls, MRL/lpr males spent less time exploring unfamiliar conspecifics during dyadic encounters. Although they did not have difficulties detecting salient odor cues in the buried food pellet test (i.e., they are not anosmic), changes in performance were evident in olfactory preference tests employing low-concentrated scents. As they aged and became sicker, MRL/lpr mice

show prolonged response to a repellent (peppermint) and attenuated response to an attractant (peanut butter). This altered performance was detected even when sick MRL/lpr mice were exposed to filter paper soaked with water. Collectively, the results obtained suggested that hyposmia develops alongside aging and is exacerbated by the progression of systemic autoimmune disease.

Generalized suppression of immune hyperactivity is an effective approach to clarify the contribution of autoimmunity in the MRL model. If behavioural changes are indeed induced by autoimmune disease, then reducing disease severity in MRL/lpr mice should eliminate, or at least attenuate differences between behavioural profiles of diseased and treated groups. Chronic administration of the immunosuppressant cyclophosphamide (CY) has previously been shown to abolish substrain differences in behaviour [24;25], indices of autoimmunity [26;27], and neuropathology [28]. Therefore, sustained, non-stressful treatment with CY was currently used to test cause-effect relationship by exploring whether attenuation of the autoimmune process abolishes abnormalities in olfactory function in the MRL/lpr group

## **2. Methods**

### ***2.1. Animals***

Thirty 8-week-old MRL/MpJ-Fas<sup>lpr</sup>/2J (MRL/lpr, stock 485) and 30 age-matched MRL/MpJ (MRL +/+, stock 486) males were purchased from the Jackson Laboratories (Bar Harbour, ME). Upon arrival, mice were tail-tattooed for identification purposes (AIMS, Inc., Hornell, NY) and housed in groups of 5 mice/cage under standard

laboratory conditions (light period from 7:00 A.M. – 7:00 P.M., *ad lib* access to rodent chow and tap water). Two mice (two MRL/lpr) fulfilled exclusion criteria for early euthanasia based on loss >20% of body mass, necrotic ear tips, and/or severe alopecia/dermatitis. An additional two MRL +/+ mice were excluded from the study due to excessive inter-male aggression, thus reducing the sample size to N=56. All procedures were approved by the local Animal Care Committee (McMaster University AUP # 11-03-11) and performed in accordance to guidelines set out by the Canadian Council of Animal Care.

## ***2.2. Immunosuppressive Treatment***

In order to minimize distress brought on by weekly intraperitoneal injections, treated groups were given access to drink CY-laced sucrose voluntarily. A similar paradigm utilizing chocolate milk as the vehicle was previously shown to be effective in reducing lymphadenopathy [26] and hypergammaglobulinemia in MRL/lpr mice [27]. Although a more appropriate design may have employed an aqueous solution of CY, dissolving it in water results in an unpalatable taste and negligible intake by mice [27]. To avoid the protein content in chocolate milk, increase palatability and achieve the therapeutic dose range of CY (~70-100 mg/b.w.), we mixed CY with 16% sucrose solution. The use of 16% sucrose as the vehicle (Veh) was based on previous findings that MRL substrains do not differ in sucrose intake at this concentration [25;29]. Mice were assigned to one of 4 groups (n = 15 mice/group), according to substrain (MRL/lpr vs. MRL +/+), and treatment (CY vs. Veh). For a 3 month period (beginning at 11 weeks

of age), mice were individually housed and provided *ad lib* access to a top-mounted 10 ml plastic syringe containing CY ("Procytox", Baxter, Mississauga, ON) diluted to 0.4 mg/ml in 16% sucrose solution, or Veh (16% aqueous solution of sucrose). Intake of sucrose and CY-laced sucrose solutions was recorded daily, when syringes were re-filled with fresh solution. Both solutions were available over 3 days each week, after which mice were returned to their home cage.

### ***2.3. Olfactory Preference Test***

The presently used paradigm to assess olfactory function was described in detail earlier [23]. Testing was carried out with mice at 9 ("Young"), 15 ("Middle") and 20 ("Old") weeks of age corresponding to mild, modest and severe disease manifestations. Following habituation to a new test chamber (45 × 24 × 20 cm), each mouse was introduced to a 5×5 cm piece of filter paper scented with ~0.25 ml of an odorant. The scented filter paper (Whatman Inc., Piscataway, NJ) was always placed in the opposite corner and for a duration of 1 min. Mice were presented with one repellent (Peppermint Essential Oil, Aura Cacia, Urbana, IA) and one attractant (Smooth Peanut Butter, Kraft Canada Inc., North York, ON) diluted to a particular concentration each day. Four different concentrations (0.01, 0.1, 1, and 10%) were used to produce a dose–response curve to compare olfactory responsiveness between MRL substrains to the same olfactory stimulus. The aim of the study was not to assess the absolute detection threshold for each odor, but to compare olfactory responsiveness between groups. To control for responsiveness to a wet filter paper, water was applied in a separate test and introduced in

a manner identical to odorants. Testing cages were covered with a clear piece of Plexiglas to limit evaporation and entry of external odours. Behavioural performance was digitally recorded and analyzed with Observer XT 7.0 software (Noldus Information Technology, Leesburg, VA) by an individual blind to group origin. Active investigation was defined as directed sniffing within 0.5 cm of the odour source. After each trial, mice were returned to their home cage, while the experimental cage was cleaned with Quatricide disinfectant (1:256 dilution, PRL Pharmacal, Naugatuck, CT).

#### ***2.4. Novel Object Test***

The novel object test was carried out at 21 weeks of age, just prior to sacrifice. Performance in the novel object test is proposed to reflect explorative and emotional responses in an approach-avoidance situation. Mice were allowed to explore an arena (45 × 45 × 20 cm) for 3 min before a small pyramid was positioned in the center of the field for an additional 3 min. Measures of activity including distance traversed, velocity and the ratio of time spent in the center versus periphery were analyzed in both “empty” and “full” phases. Latency to approach the object, contact frequency and duration of snout contact with the object were assessed using EthoVision XT 7.0 software (Noldus Information Technology, Leesburg, VA). Following each session, the field was cleaned with Quatricide Disinfectant.

#### ***2.5. Immunopathology***

At 22 weeks of age, mice were exsanguinated by severing the inferior vena cava and perfused intracardially with phosphate buffered saline under ketamine/xylazine

anaesthesia. Serum was separated from the blood clot and stored at -20 °C until further analysis. Extracted organs were weighed immediately using an analytical balance (Mettler Toledo AB54-S; VWR Scientific of Canada, Mississauga, ON). Wet spleen mass was recorded as a measure of splenomegaly, a reliable indicator of autoimmune disease in lupus-prone mice [30]. Assessment of brain weight was used to confirm attenuation of brain growth and/or brain atrophy in the MRL/lpr substrain [31]. Along with spleen enlargement, hyperproduction of serum autoantibodies is a typical manifestation of lupus-like disease in MRL/lpr mice [30]. Anti-ribosomal P antibodies (ARPA) in particular have garnered attention due to their high specificity for CNS SLE [33-34] and ability to induce olfactory dysfunction in an induced murine model of NP-SLE [35]. We quantified serum levels of ARPA using microplate ELISA kits (Euroimmun AG, Mississauga, ON). Briefly, 100 µl of each sample (serum 1:50 dilution in sample buffer) was transferred into the corresponding microtiterplate well coated with ribosomal P proteins purified by affinity chromatography from bovine and rabbit thymus (EUROIMMUN pre-coated microtiterplate). Each sample was incubated for 30 min at RT and then washed three times with 450 µl of working strength wash buffer. One hundred microliters of 1:2000 diluted rabbit anti-mouse IgG-HRP conjugate (Promega) was pipetted into each of the microtiterplate wells, left to incubate, and washed to remove unbound HRP enzyme conjugate. Subsequently, 100 µl of 3,3',5,5'-tetramethylbenzidine enzyme/substrate solution was pipetted into each well of the microtiterplate and incubated for another 20 min at RT. One hundred microliters of stop solution was added to each well in the same order and at the same speed as when the chromogen/substrate

solution was introduced. The microtiterplate was shaken at a speed of 20 Hz for 5 s to ensure a homogeneous distribution of the solution. Optical density was determined at a wavelength of 450 nm and a reference wavelength of 620 nm within 10 min of adding the stop solution. Observed results are expressed as relative optical densities.

## ***2.6. Statistical Analysis***

Normal distribution of the data was tested using the Shapiro-Wilk test. Assuming normal data distribution, equal samples sizes or comparable population variances, dependent measures were analyzed using analysis of variance (ANOVA), with Substrain (MRL/lpr vs. MRL +/+) and Treatment (CY vs. Veh) as between-group factors. When measures were taken repeatedly (e.g., age, concentration or phase), they were considered within-group factors in ANOVA with repeated measures. If significant interactions were found, Student's t-test was used in post-hoc comparisons. In instances involving non-homogeneity of variance or departure from normal distribution, nonparametric tests (e.g., Kruskal-Wallis, Mann-Whitney) were used. Criterion for statistical significance was set at  $p \leq .05$  for all group comparisons. Graphs indicate mean values and  $\pm$  SEM with significant differences of  $p \leq .05$ ,  $p < .01$  and  $p < .001$ , shown as \*, \*\*, and \*\*\*, respectively. All calculations were performed using SPSS 20 software package (SPSS Inc., Chicago, IL).

### 3. Results

#### 3.1. *CY Dose*

Both MRL substrains drank CY-laced sucrose solution, but autoimmune MRL/lpr mice consumed significantly less CY solution in comparison to MRL +/+ mice, particularly after the first week of treatment (Substrain  $\times$  Treatment  $\times$  Age:  $F_{1,51} = 3.253$ ,  $p < 0.05$ ; data not shown). Consequently, the weekly CY dose consumed via voluntary drinking was higher in less symptomatic MRL +/+ than in MRL/lpr mice with early disease manifestations (100–150 mg/kg in +/+ mice vs. 60–100 mg/kg b.w. in lpr mice; Substrain:  $F_{1,27} = 28.322$ ,  $p < 0.001$ ; Figure 1). Significant correlations between CY dose and performance measures in the olfactory preference and novel object tests were not detected (data not shown).

#### 3.2. *Olfactory Preference Test*

At all ages, MRL/lpr mice spent more time exploring unscented filter paper than MRL +/+ controls (Strain:  $F_{1,51} = 21.532$ ,  $p < 0.001$ ; Figure 2), but exposure to CY did not affect this group discrepancy. Baseline assessment of olfactory function at 9 weeks of age revealed reduced exploration of peanut butter-scented filter paper in MRL/lpr mice (Strain:  $F_{1,51} = 20.465$ ,  $p < 0.001$ , Figure 3A). However, CY exposure increased exploration of 0.1% peanut butter by treated MRL/lpr mice in comparison to the Veh MRL/lpr group at 15 weeks of age (Concentration  $\times$  Strain  $\times$  Treatment:  $F_{3,153} = 3.310$ ,  $p < 0.05$ , Figure 3B). Along the same lines, CY also restored investigation of 1 and 10% peanut butter in 20-week-old MRL/lpr mice (Concentration  $\times$  Strain  $\times$  Treatment:  $F_{3,156} =$

2.816,  $p < 0.05$ , Figure 3C), and normalized overall substrain differences (Strain:  $F_{1,52} = 1.363$ , n.s.). Similar to the test with wet filter paper, baseline assessment revealed that autoimmune MRL/lpr mice spent more time investigating a repellent than MRL +/+ controls (Strain:  $F_{1,52} = 7.712$ ,  $p < 0.01$ , Figure 4A). In contrast to the findings with peanut butter, CY therapy failed to attenuate substrain differences in peppermint-scent exploration at 15 weeks of age (Strain:  $F_{1,52} = 10.089$ ,  $p < 0.01$ , Figure 4B). Unexpectedly, immunosuppressed MRL/lpr mice showed increased responsiveness to 0.01% and 0.1% peppermint at 20 weeks of age (Strain  $\times$  Treatment:  $F_{1,51} = 4.576$ ,  $p < 0.01$ , Figure 4C). Taken together, these results suggest chronic CY therapy restores MRL/lpr responsiveness to an attractant, but has an opposite effect on the investigation of a repellent.

### ***3.3. Novel Object Test***

When placed in an arena devoid of an object at 21 weeks of age, MRL/lpr mice traversed longer distances than MRL +/+ controls (Strain:  $F_{1,51} = 7.213$ ,  $p < 0.05$ , Figure 5A). When the novel object was introduced, CY treatment significantly reduced the ratio of time spent in the centre versus periphery (Treatment:  $F_{1,51} = 5.236$ ,  $p < 0.05$ , Figure 5B). Despite similar contact frequencies and latencies (data not shown), CY treatment significantly increased contact duration in MRL/lpr mice, but reduced time spent in contact with the novel object in MRL +/+ mice (Strain  $\times$  Treatment:  $F_{1,51} = 4.245$ ,  $p < 0.05$ , Figure 5C). The results suggest CY therapy has a positive effect on the

performance of MRL/lpr mice, but adversely alters behaviour of less-sick MRL +/+ controls.

### **3.4. Immunopathology**

Chronic immunosuppressive treatment prevented splenomegaly in MRL/lpr mice (Substrain  $\times$  Treatment:  $F_{1,52}=11.350$ ,  $p < 0.001$ , Figure 6A). Normalized spleen mass was accompanied by significantly lower serum ARPA levels in this group ( $K_3=38.860$ ,  $p < 0.001$ ,  $U = 0$ ,  $p < 0.001$ , Figure 6B). In summary, unrestricted exposure to sweetened CY solution abolished severe signs of systemic autoimmunity. However, it did not prevent well-documented low brain weight in the MRL/lpr substrain (data not shown) [17;36].

## **4. Discussion**

Apart from complications induced by kidney damage, infections, and steroid therapy, autoimmunity has been hypothesized to be the primary mechanism in the etiology of CNS lupus. Shoenfeld and colleagues found that olfactory dysfunction was common in SLE patients and associated with disease severity and CNS manifestations [4]. Yet, much like our previous findings in a spontaneous model of CNS lupus [23], they could not demonstrate a cause-effect relationship between autoimmunity and changes in olfaction. The present longitudinal study employing chronic administration of an immunosuppressant supports such a relationship in MRL/lpr mice. Sustained CY exposure abolished signs of systemic autoimmunity including splenomegaly and elevated ARPA in lupus-prone mice. Amelioration of autoimmune symptoms prevented blunted

responsiveness to an attractant (peanut butter), evident in vehicle-treated MRL/lpr mice. Sustained CY therapy also improved performance of treated MRL/lpr mice, but adversely affected performance of the MRL +/+ group in the novel object test. Along the same lines, CY treatment unexpectedly worsened aberrant response of MRL/lpr mice to a repellent scent (peppermint). Taken together, the present results suggest that a treatment which ameliorates autoimmune symptoms can concurrently abolish substrain difference in certain odor-guided behaviours. They support the hypothesis that autoimmunity and/or inflammation are key pathogenic factors in altering olfactory function in lupus-prone mice.

The results of the current study are largely consistent with our previous report detailing alterations in odor-guided behaviours at the onset of lupus-like disease. In particular, we confirmed that autoimmune MRL/lpr mice display aberrant responses to low concentrations of attractant and repellent odors consistent with hyposmia at an early age. Prolonged sniffing of peppermint-scented filter paper by MRL/lpr mice is in accordance with impaired avoidance to menthol (the primary constituent of peppermint oil) in an inducible model of CNS SLE [35;37]. In the same manner, reduced investigation of diluted peanut butter, a palatable food source for rodents, is consistent with the findings of others who found comparable impairments in mice with olfactory dysfunction [38]. Consistent with the effects of aging previously described, age-dependent performance deficits were evident in both vehicle groups as they grew older. Conversely, CY-treated MRL/lpr mice displayed increased responsiveness to peanut butter and improved performance in the novel object test at time points associated with

florid disease and olfactory dysfunction. Similar behavioural effects of chronic CY exposure have previously been reported in MRL/lpr mice. More specifically, our lab has shown that pre-treatment with CY prevents deficits in novel object exploration and normalizes sensitivity to palatable stimulation in the MRL/lpr substrain [24;25]. Behavioural changes coincided with the amelioration of systemic immunopathology, as shown by reduced spleen weight and autoantibody levels in the MRL/lpr substrain. The potent immunosuppressive properties of CY are consistent with previous observations that brief access to CY-laced chocolate milk results in significant reductions in palpable lymphadenopathy and serum anti-DNA antibody titers [26;27]. Generalized immunosuppression after repeated 100 mg/kg/week intraperitoneal CY injections similarly decreases anti-nuclear antibody levels [24;25] and prolongs the life-span of MRL/lpr mice [39].

The behavioural effects of CY in the MRL/lpr group are believed to reflect the suppression of pathogenic immune factor(s) that damage neuronal structures involved in olfactory function during the onset of autoimmune disease. This hypothesis is based on previous findings demonstrating that sustained CY immunosuppression significantly reduces the infiltration of mononuclear CD45<sup>+</sup> leukocytes, but not of CD45R<sup>+</sup> B cells into the choroid plexus of the MRL/lpr substrain [40]. Despite its differential effect on leukocytic infiltration, CY preserves neuronal morphology *in vivo* [28] and attenuates cerebrospinal (CSF) neurotoxicity *in vitro* [41]. Analysis of autoimmune CSF reveals an abundance of immunoglobulin G (IgG) antibodies [42] that seem to largely account for its cytotoxicity to mature and immature neurons [43]. Intensive binding of CSF IgG to

different regions of brain parenchyma [44] lends further support to a complex pathogenic cascade initially involving increased infiltration of activated leukocytes and soluble immune factors (such as brain-reactive autoantibodies, BRA) into the CSF through a permeable, damaged blood-brain barrier [42;45]. Within the CNS, intrathecal production of neurotoxic BRA, and deposition of CSF antibodies, would then culminate in damage to neurons in surrounding limbic regions and aberrant behaviour in lupus-prone mice. Supporting the role of intrathecal BRA neurotoxicity in CNS SLE, autoantibodies from lupus patients induce neuronal death [46;47] and behavioural deficits when introduced into the mouse brain [48-50]. ARPA, which we found to be decreased by CY treatment, have been shown to induce neuronal apoptosis [51], depressive-like behaviour, olfactory dysfunction [35] and alterations in the olfactory pathway [37] when administered to healthy rodents.

The interpretation of the present findings is complicated by the observation that CY treatment seemingly has opposite effects on MRL/lpr responsiveness to the two scents. In particular, CY-treated MRL/lpr mice increased investigation of an attractant odour, suggestive of improved olfactory function, but also spent significantly more time with low-concentrations of a repellent, consistent with hyposmia. One may surmise that the heightened response to scented-filter paper in either case merely reflects CY-induced changes in exploratory drive. However, this seems unlikely given that CY exposure did not alter investigation of unscented filter paper in control trials with water. A more likely explanation may involve the differential properties of the two scents themselves. Like many other odorous volatiles [52], peppermint causes considerable trigeminal nerve (CN

V) stimulation in addition to the primary olfactory nerve, CN I [53]. Given that trigeminal chemoreception contributes significantly to olfaction in many ways [54], it is intriguing to hypothesize that CY itself may modulate trigeminal function either directly or indirectly. This would be in line with studies suggesting its effects are exerted in the periphery and do not cross the blood-brain barrier to enter the CNS [55;56]. However, recent experimental findings demonstrate that intraperitoneal administration of CY (40-50 mg/kg) is associated with impairments in hippocampal neurogenesis [57;58]. Considering odor information ultimately projects to the hippocampus among other structures [59;60], CY-related alterations in hippocampal function may underlie the apparent worsening of responsiveness to peppermint by lupus-prone mice. Nevertheless, further study of this seemingly selective CY effect on odor-guided behaviours is necessary.

Despite the longitudinal assessment of olfactory function, as well as the analysis of immunopathological measures following chronic immunosuppression, the current study lacks information regarding the effects of CY at the neuronal and molecular levels. For example, details relating to the morphology of the primary and accessory olfactory systems would go a long way in determining the mechanisms underlying the apparent conflicting effect of CY in olfactory tests with an attractant or repellent. Along the same line, further characterization of the specific inflammatory / autoimmune factors is needed to reveal the etiology of olfactory dysfunction and neurodegeneration in MRL/lpr mice. To address this issue of causality, the logical step forward would include investigating the behavioural effects of specific factors in healthy animals.

The current study support the hypothesis that systemic autoimmune disease in MRL/lpr mice alters responsiveness to an attractant odor and affects behaviors controlled by the limbic system. Although considerable research is needed to elucidate the mechanisms underlying this phenomenon, these findings represent an important step forward in characterizing the behaviour of lupus-prone mice as it relates to autoimmune manifestations. They also strengthen the hypothesis that NP manifestations, autoimmunity and olfaction are tightly interconnected and may represent an underlying pathology in many CNS disorders involving the immune system.

#### **Author Contributions**

M.K. and B.S. were involved in manuscript drafting and contributing to the intellectual content. B.S. takes responsibility for the integrity and accuracy of results.

**Conceived and designed the experiments:** M.K. and B.S.

**Performed the experiments:** M.K., H.Z., and B.T.

**Analyzed the data:** M.K. and B.S.

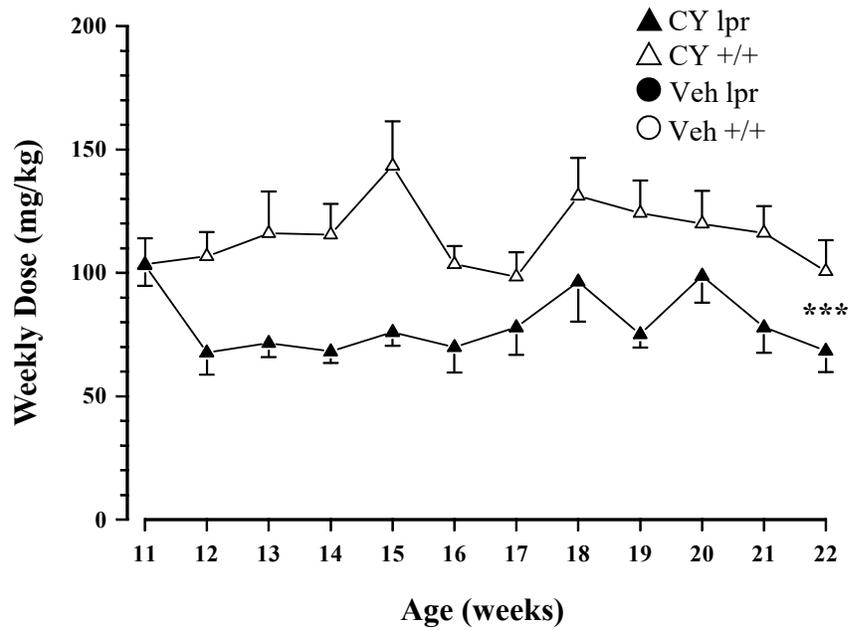
**Contributed reagents/materials/analysis tools:** D.M. and B.S.

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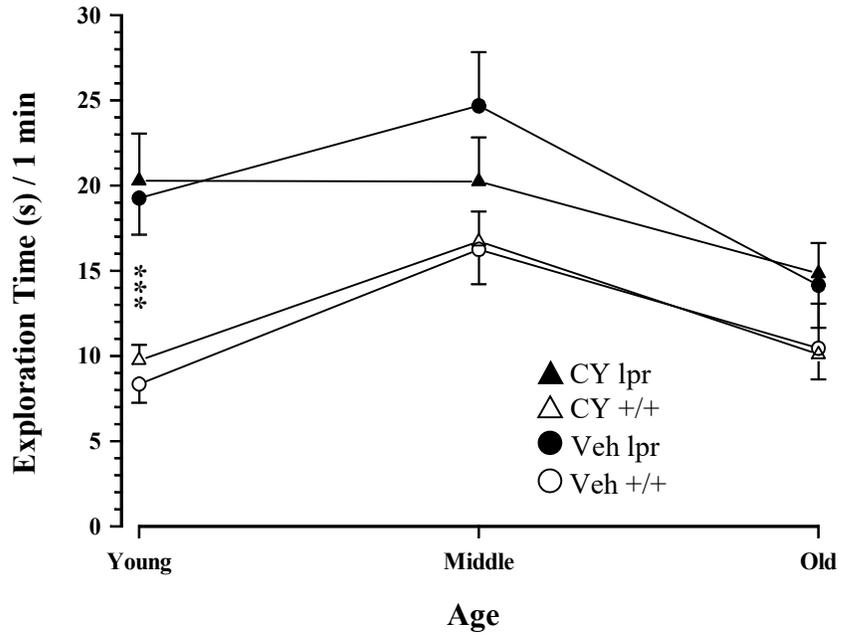
## Figures

Figure 4.1 - Weekly CY dose ingested



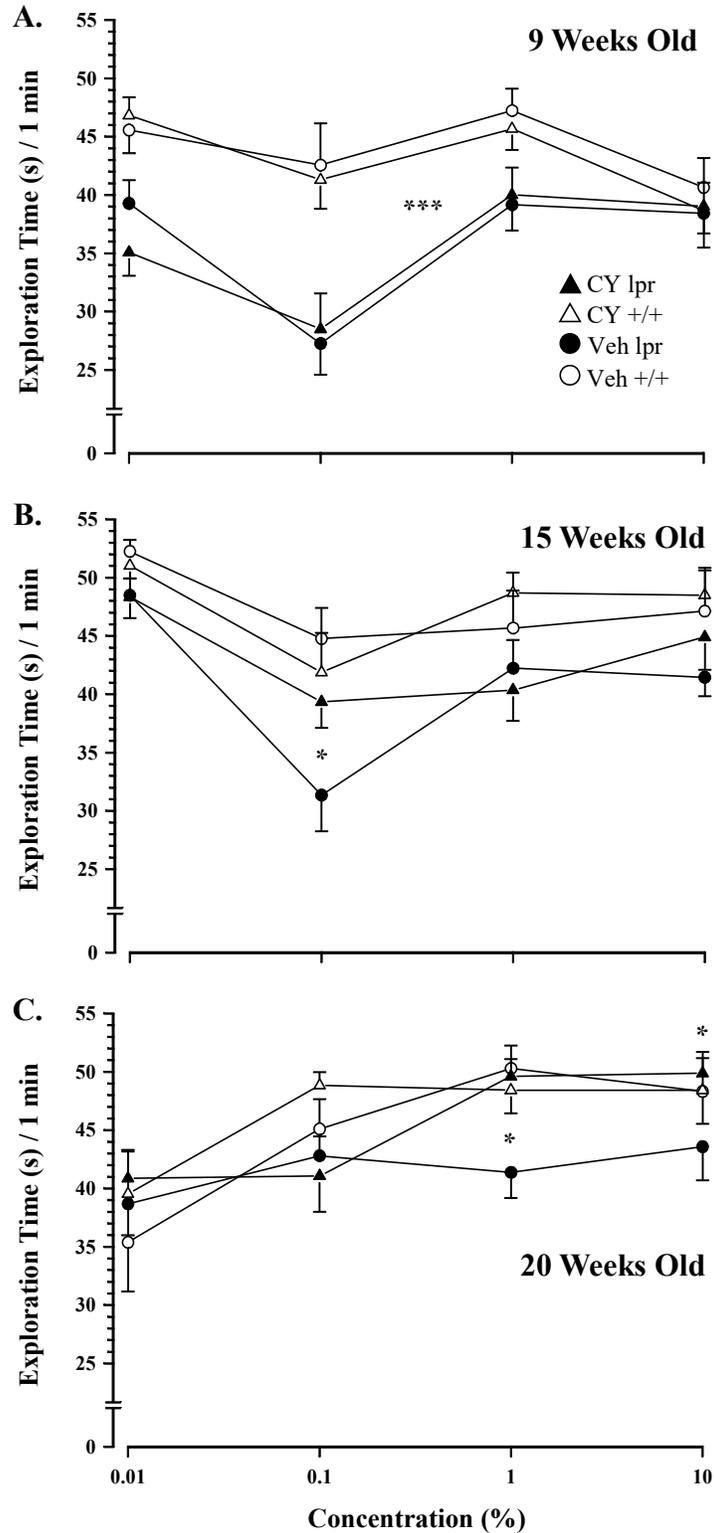
**Figure 1. Weekly CY dose ingested.** Although both substrains received similar CY dose at the starting of the treatment period, voluntary drinking in subsequent weeks was higher in less symptomatic MRL +/+ than in MRL/lpr mice.

Figure 4.2 - Olfactory sensitivity test – control



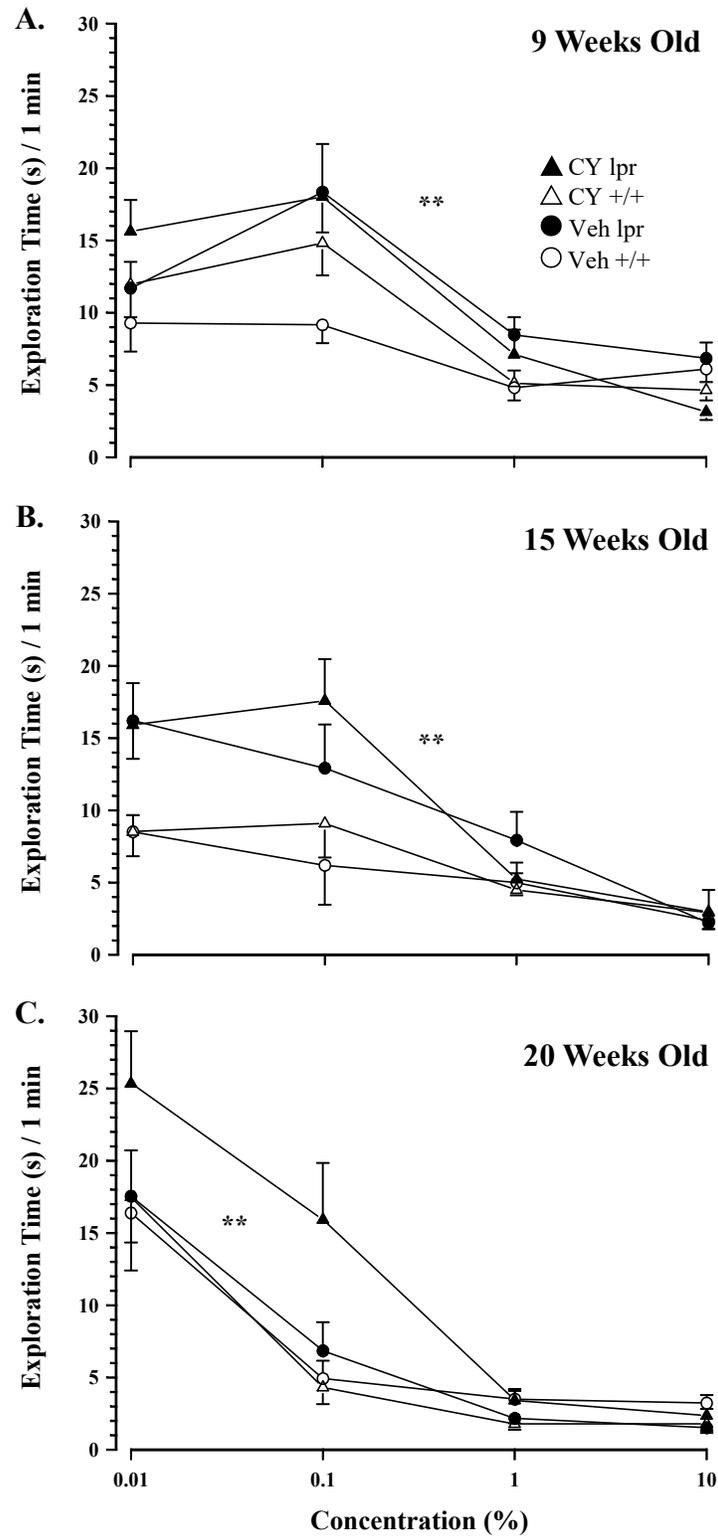
**Figure 2. Olfactory sensitivity test – control.** MRL/lpr mice spend significantly more time exploring wet filter paper when compared to MRL +/+ controls at all ages. These results confirm previous findings of increased responsiveness in MRL/lpr mice and suggest subsequent tests may be confounded by altered reactivity to the paper itself.

Figure 4.3 - Olfactory sensitivity test – peanut butter



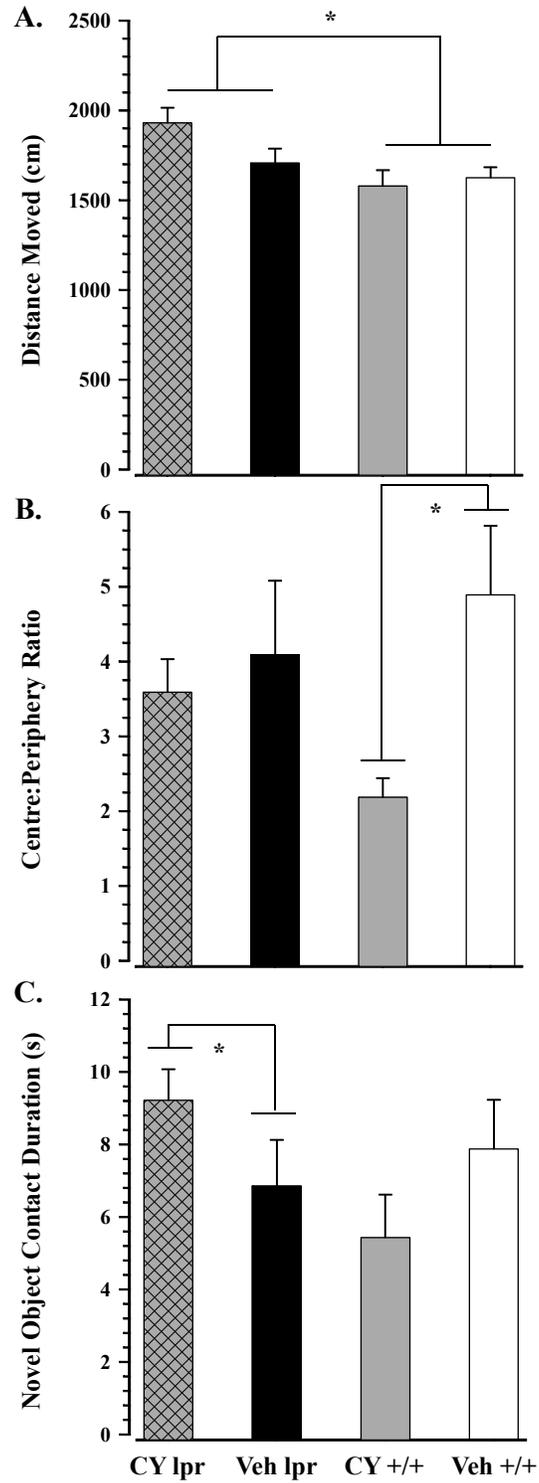
**Figure 3. Olfactory sensitivity test – peanut butter.** (A) Prior to CY treatment, the MRL/lpr substrain spends more time investigating diluted peanut butter scented filter paper when compared to age-matched MRL +/+ controls. (B) Exposure to CY, but not vehicle, significantly increased exploration of 0.1% peanut butter by MRL/lpr mice at 15 weeks of age. (C) Similar restoration in the investigation of 1 and 10% peanut butter was noted in 20-week-old MRL/lpr. Collectively, these findings suggest chronic immunosuppression with CY normalizes differences in olfactory function in MRL/lpr mice when exposed to an attractant such as peanut butter.

Figure 4.4 - Olfactory sensitivity test – peppermint



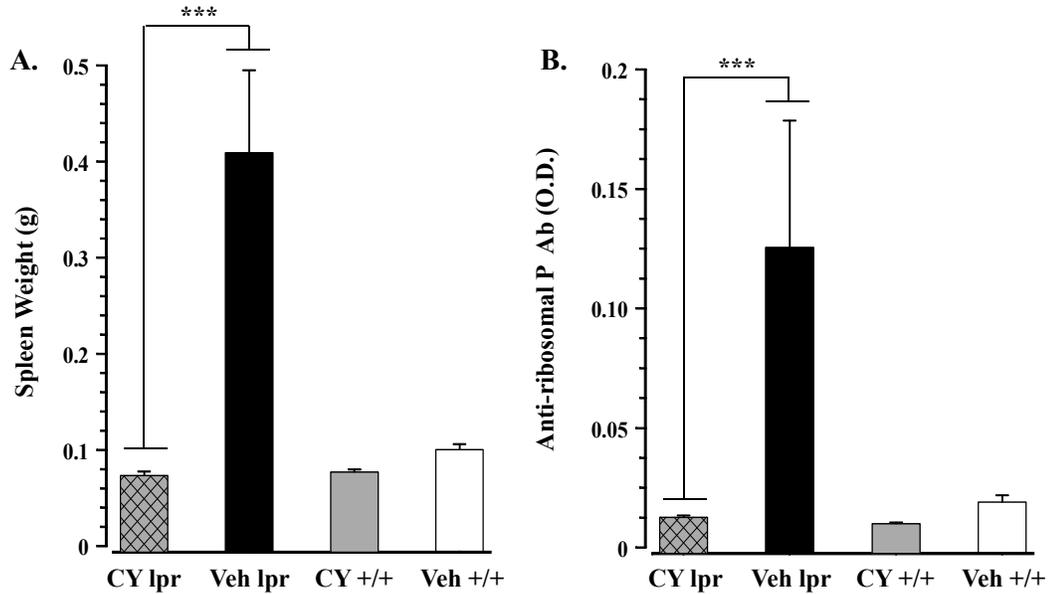
**Figure 4. Olfactory sensitivity test – peppermint.** (A) The MRL/lpr substrain demonstrated increased exploration of the repellent scent as early as 9 weeks of age. (B) In contrast to the findings with an attractant at 15 weeks of age, CY therapy did not attenuate substrain differences in the exploration of peppermint-scented filter paper. (C) At an Old age, immunosuppressed MRL/lpr increased exploration of 0.01 and 0.1% peppermint. Taken together, these results suggest chronic CY therapy is associated with a detrimental effect on MRL/lpr behaviour to a repellent.

Figure 4.5 - Novel object test



**Figure 5. Novel object test.** (A) MRL/lpr mice traversed longer distances when placed in an empty arena. (B) Introduction of the novel object coincided with a significant reduction in the ratio of time spent in the centre versus periphery for CY-treated animals. (C) CY treatment significantly increased contact duration in MRL/lpr mice, but reduced time spent in contact with the novel object in MRL +/+ mice. These findings are consistent with our previous studies [27] and suggest CY therapy improves the performance of MRL/lpr mice, but has adverse effects on the behaviour of MRL +/+ controls.

**Figure 4.6 - Immunosuppressive effects of CY**



**Figure 6. Immunosuppressive effects of CY.** (A) Assessment of wet-spleen weight confirmed that chronic CY exposure prevented splenomegaly (a reliable marker of autoimmunity) in lupus-prone mice. (B) Normalized spleen mass in the CY-treated MRL/lpr group was accompanied by lower serum ARPA levels. Overall, these findings suggest CY therapy abolished substrain signs of systemic autoimmunity.

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## **Chapter 5: Role of intrathecal antibodies on olfactory dysfunction and other behavioural deficits**

### **Chapter Link**

Several lines of evidence support a causative role of autoimmunity and intrathecal antibodies in the development of olfactory dysfunction and other behavioural deficits in MRL/lpr mice. Like alterations in olfaction detailed above, the behavioural profile of MRL/lpr mice changes contemporaneously with the emergence of serologic autoimmunity [193] and the magnitude of many functional impairments correlate with disease severity [191;194;218]. Furthermore, the brains of MRL/lpr exhibit increased BBB / BCSFB permeability [198;199], lymphoid cell infiltration [198;200-203], and disease-dependent damage to cortical and periventricular neurons [204-210]. Likewise, neurogenic niches surrounding the ventricles show morphological abnormalities [206;210;213-215] and altered distribution of neuronal DCX<sup>+</sup> cells [255] consistent with CSF neurotoxicity in the MRL model [211;212]. Lastly, sustained treatment with the immunosuppressant CY not only improves responsiveness to an attractant and a novel object, but also abolishes other behavioural deficits [195;207] and prevents neuronal damage [208;217]. Taken together, the above evidence points to a complex pathogenic cascade involving toxic CSF IgG, neuronal / progenitor cell death and progressive behavioural deficits in the MRL/lpr model [199]. We address this possibility below using an extended behavioural battery consisting of several tests of olfactory function.

**Role of intrathecal antibodies on olfactory dysfunction and other behavioural deficits**

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## **Abstract**

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that is accompanied by damage to the CNS, diverse neuropsychiatric manifestations and olfactory deficits of unknown etiology. Similar to CNS SLE patients, lupus-prone MRL/lpr mice manifest alterations in odor-guided behaviours that coincide with morphological changes in the brain, some of which have been implicated in olfactory function. Sustained immunosuppression improves behavioural performance in many measures of exploration, including responsiveness to an attractant scent and a novel object. While a cause-effect relationship is hypothesized in MRL mice, the origin and terminal pathologic mechanisms remain unknown. Based on the proposed role of intrathecal brain-reactive autoantibodies in human and murine forms of SLE, the current study addresses whether sustained intracerebroventricular administration of autoantibody-rich CSF compromises olfactory function and produces functional changes in asymptomatic mice. Infusion of CSF from CNS SLE patients induces olfactory dysfunction in an odor discrimination task, increases immobility in the forced swim test, enhances perseveration in the water maze, and alters several home-cage behaviours. A purified antibody to ribosomal P protein produces a deficit in spatial reference memory, without affecting other behaviours. Infusion of an anti-NMDAR antibody reduces responsiveness to scents in the olfactory discrimination task, but also improves the acquisition of a novel escape location in the water maze. Mice treated with an antibody to  $\alpha$ -tubulin show superior water maze reversal learning and are more active in a home-cage environment. Taken together, sustained exposure to antibodies in CSF recapitulates

several, but not all facets of the complex behavioural manifestations noted in lupus-prone MRL/lpr mice and humans. The broad behavioural changes with purified antibodies, oftentimes in opposite directions, suggest some BRA have a stimulatory effect, while other subsets act as antagonists.

## 1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune / inflammatory disorder accompanied by damage to multiple organs, including the brain [1]. Neurologic and psychiatric (NP) manifestations of varying severity are common, often conferring a graver prognosis and increased mortality rates [2]. Central nervous system (CNS) symptoms occur most frequently and can range from focal abnormalities (i.e., seizures, cerebrovascular disease etc.) to diffuse disorders such as anxiety, depression, cognitive impairment, and psychosis [3]. Recent observations suggest that a significant portion of SLE patients with a history of NP symptoms also develop hyposmia at different stages of disease development [4]. The interpretation that olfactory impairments are related to autoimmunity is intriguing, but difficult to confirm, partly because assessment of CNS function in patients can be confounded by peripheral organ damage, opportunistic infections and treatment with high doses of corticosteroids / cytotoxic agents [5;6].

The MRL murine model of SLE has proven versatile in studying the interrelationships between systemic autoimmunity, neuropathology, and aberrant behaviour not feasible in clinical studies [7]. Due to a spontaneous lymphoproliferation (*lpr*) mutation [8] and a subsequent defect in Fas-induced apoptosis [9;10], MRL/*lpr* mice develop an accelerated form of lupus-like disease characterized high levels of circulating cytokines, autoantibodies, and immune complexes within the first two months of life [11]. Conversely, the congenic MRL/MpJ (MRL +/+) substrain, which shares 99.9% of its genome with *lpr* mice, is asymptomatic at this age, thus representing an adequate, genetically-similar control. In comparison to sex- and age-matched MRL+/+ controls,

MRL/lpr mice confine their movement to the perimeter of open field [12;13], spend less time investigating novel objects [12], and display impaired inter-male aggression [14]. Our recent findings suggest that some of the aforementioned deficits in approach-avoidance paradigms are confounded by the development of hyposmia alongside disease progression [15]. The emergence of olfactory changes in MRL/lpr mice coincides with a skewed distribution of neuronal precursor cells in the rostral migratory stream, a well-defined pathway involved in the migration of neuroblasts from the subventricular zone to the olfactory bulb. In support of a cause-effect relationship between lupus-like disease and olfactory dysfunction in this strain, sustained treatment with the immunosuppressant cyclophosphamide abolishes autoimmunity and improves responsiveness to an attractant and a novel object. Although alterations in odour-guided behaviours emerge contemporaneously with the development and arrest of systemic autoimmunity, their origin and terminal pathologic mechanisms remain largely unknown.

Antibodies reactive to diverse brain antigens (brain-reactive autoantibodies, BRA) have attracted considerable attention as terminal factors mediating behavioural manifestations [16]. The data from clinical studies is largely correlational in nature and based on the identification of BRA in serum [17-23], cerebrospinal fluid, CSF [24-29], and post-mortem neuronal tissues [30;31]. It is still unclear whether circulating antibodies passively diffuse through a permeabilized blood-brain barrier, BBB [32] or are synthesized intrathecally by activated leukocytes [33-37]. However, antibodies in CSF have been proposed to be more pathogenic than BRA originating in the serum of patients [25;26;38;39]. Indeed, immunoglobulin G (IgG)-rich fractions of CSF from CNS SLE

patients and behaviourally-impaired MRL/lpr mice induce long-term toxicity of adult and immature neurons in vitro [40;41] and murine neurons in vivo [41;42]. Corroborating this evidence of CSF neurotoxicity, the brains of MRL/lpr mice exhibit increased BBB / BCSFB permeability [43;44], lymphoid cell infiltration [43;45-48], disease-dependent damage to cortical and periventricular neurons [49-55] and morphological abnormalities in neurogenic niches surrounding the ventricles [51;55-58]. Taken together, the data suggest that antibodies present in the CSF bind highly-conserved antigen(s) that are shared amongst different populations of immature and differentiated neurons.

Several candidate autoantigens, expressed in the brain and systemically, have been proposed as targets of more than 20 potentially pathogenic BRA (Table 1) [59-62]. For example, a subset of anti-DNA antibodies that cross-react with the NR2 subunit of the N-methyl-D-aspartate receptor (NMDAR) [42;63] have been shown to induce deficits in emotionality and learning / memory in healthy mice following chemical disruption of the BBB [31;64;65]. When the BBB is bypassed altogether, acute intracerebroventricular (icv) infusion of anti-ribosomal P antibodies (ARPA) from NP-SLE patients produces an “autoimmune depression” profile characterized by olfactory dysfunction [66;67] and excessive immobility in the forced swim test [68;69]. More recently, several antibodies against highly-conserved cytoskeletal proteins including microtubule-associated protein 2 [23;70],  $\alpha$ -tubulin [71], and  $\alpha$ -internexin [72] have also been linked to behavioural manifestations. Although their pathogenic relevance remains to be explored in further detail, a recent study in MRL/lpr mice revealed prominent CSF IgG reactivity to several cytoskeletal proteins [73].

Based on the current state of evidence, we hypothesize that BRA in CSF compromises olfactory function and produces functional changes in other domains of behaviour as noted in MRL/lpr mice. In order to test this hypothesis, the current study examines the behavioural outcomes of sustained icv administration of human NP-SLE CSF and purified autoantibodies in asymptomatic, healthy mice.

## **2. Methods**

### **2.1. Experiment 1: Effects of NP-SLE CSF**

Despite the aforementioned findings in inducible and spontaneous models of SLE, there remain several gaps in the present knowledge that make it difficult to distinguish pathogenic BRA from epiphenomenal classes. Previous studies involving direct CNS administration of CSF [42] or BRA [66-69] only examined the effects of acute exposure, despite NP-SLE being chronic in nature. When antibodies were not delivered directly into the CNS, infiltration of circulating BRA was dependant on BBB disruption using potent toxins and neuropeptides [31;64;65;72;74], which per se have profound effects on neuronal plasticity and behaviour. Issues related to repeated immunization, unbalanced study design, inadequate sample sizes, lack of baseline behavioural assessment, reliance on isolated, standardized cross-sectional behavioural tests and absence of home-cage monitoring represent additional factors that may confound behavioural assessment and increase the risk of data misinterpretation. Experiment 1 addresses these concerns by examining the behavioural effects of sustained and direct CNS exposure to NP-SLE CSF in a more comprehensive, integrative, and standardized manner.

### ***2.1.1. NP-SLE Samples***

Matching serum and CSF samples from patients who fulfilled classification criteria for NP-SLE were kindly provided by Dr. Ljudmila Stojanovic (Dept. of Internal Medicine, Bezhanijska Kosa University Medical Center, Belgrade, Serbia). Samples were obtained from 26 patients (22 females, 5 males; mean age  $54.3 \pm 2.1$  years; mean SLE duration  $9.69 \pm 1.0$  years; average SLE Disease Activity Index score  $10.1 \pm 0.8$ ). Neuropsychiatric symptoms in order of frequency included cognitive dysfunction ( $n = 10$ ), cerebrovascular incident ( $n = 6$ ), seizures ( $n = 5$ ), psychosis ( $n = 5$ ), anxiety ( $n = 5$ ), depression ( $n = 4$ ), headache / migraine ( $n = 3$ ), chorea ( $n = 1$ ) and demyelinating syndrome ( $n = 1$ ). Immunosuppressive therapy included prednisone ( $n = 25$ ) and cyclophosphamide ( $n = 12$ ). Serum and CSF samples from an age-matched, female patient presenting with neuromyelitis optica (NMO) were used as a non-SLE control. All samples were aliquoted into sterile 100  $\mu$ l vials and stored at 4 °C until further analysis.

### ***2.1.2. Autoantibody Profile***

Qualitative / quantitative screening of samples involved assessment of several antibody subclasses including anti-nuclear antibodies (ANA), ARPA, anti-cardiolipin antibodies (aCL), anti-proteinase 3 antibodies (PR3), anti-dsDNA antibodies and anti- $\alpha$ -tubulin antibodies.

#### ***2.1.2.1. Immunofluorescence Assay***

ANA levels were assessed using a fully-automated IFA analyzer (EUROIMMUN IF Sprinter). Sera were diluted to 1:80 using PBS buffer (pH 7.2) and 30  $\mu$ l of the diluted

serum was pipetted into the corresponding well of the HEp-2 cell slides (EUROIMMUN). The slides were washed 4 times using PBS-Tween20 after 30 min of incubation at RT. Thirty  $\mu$ l of 1:100 diluted rabbit anti-mouse IgG-FITC conjugate (Sigma-Aldrich) was pipetted onto each well. The slides were washed again as above after 30-min incubation with the conjugate. Using 10  $\mu$ l of the mounting medium, the slides were sealed with a cover glass. The results were obtained by viewing slides under a LED-fluorescence microscope (EUROIMMUN EUROStar III).

#### *2.1.2.2. Enzyme-linked Immunosorbent Assay*

Levels of anti-ribosomal P, anti-cardiolipin (aCL), anti-proteinase 3 (PR3) and anti-dsDNA autoantibodies were quantified using a fully-automated ELISA analyzer (EUROIMMUN Analyzer I). Briefly, 100  $\mu$ l of each sample (serum: 1:50 dilution; CSF: 1:1 dilution in sample buffer) was transferred into the corresponding microtiterplate well (EUROIMMUN pre-coated microtiterplate). Analysis of ARPA involved transferring samples to wells coated with ribosomal P proteins purified by affinity chromatography from bovine and rabbit thymus. Quantification of aCL antibodies was performed using wells lined with purified cardiolipin isolated from bovine heart. For assessment anti-PR3 antibodies, microplate wells were coated with a mixture of recombinant and native PR3. Lastly, for anti-dsDNA analysis, each well contained antigen substrate of dsDNA complexed with nucleosomes and coupled to the solid phase. Irrespective of the antibody tested, each sample was incubated for 30 min at RT and then washed three times with 450  $\mu$ l of working strength wash buffer. One hundred microliters of 1:2000 diluted rabbit anti-mouse IgG-HRP conjugate (Promega) was pipetted into each of the microtiterplate

wells, left to incubate, and washed to remove unbound HRP enzyme conjugate. Subsequently, 100  $\mu$ l of 3,3',5,5'-tetramethylbenzidine enzyme/substrate solution was pipetted into each well of the microtiterplate and incubated for another 20 min at RT. One hundred microliters of stop solution was added to each well in the same order and at the same speed as when the chromogen/substrate solution was introduced. The microtiterplate was shaken at a speed of 20 Hz for 5s to ensure a homogeneous distribution of the solution. Optical density was determined at a wavelength of 450 nm and a reference wavelength of 620 nm within 10 min of adding the stop solution. Observed results are expressed as relative optical densities.

#### *2.1.2.3. Electrophoresis and Western Blotting*

10  $\mu$ g of recombinant human GST-tagged  $\alpha$ -tubulin (Abnova, Taipei, Taiwan) was separated on a 10% sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a 4.5% stacking gel and electroblotted to nitrocellulose membranes (Hybond-C super, Amersham Biosciences, Freiburg, Germany) as described by [71]. The blot was blocked with 3% bovine serum albumin in phosphate buffered saline (PBS) for 1 h at RT and individual lanes were separated into strips and incubated with patients' sera at a dilution of 1:200 and CSF at a dilution of 1:1 (1 h at RT). Membrane strips were washed in PBS and incubated with HRP-conjugated secondary antibody for 1 h at RT. The reaction was visualized with 3-amino-9-ethyl-carbazole as substrate. Results are expressed as absorbance multiplied by 1000.

#### *2.1.3. Blood-brain Barrier Permeability and Intrathecal IgG Synthesis*

To assess BBB permeability and intrathecal synthesis of IgG, paired serum and CSF samples were run in parallel and analyzed together in one analytical run using a fully-automated Dade Behring Nephelometer II System (BN II, Siemens AG, Germany). Briefly, samples were diluted for albumin (1:400 for serum and 1:5 for CSF) and IgG (1:400 for serum and 1:1 for CSF) and pipetted into cuvettes accordingly. Following the addition of anti-albumin and anti-IgG reagents, antigen-antibody complexes were detected using incident light at 840 nm. Light scatter was measured by a photo-detector, compared with the corresponding measured values on the Heidelberger-Kendall reference curve and converted to albumin or IgG concentrations. Integrity of the BBB was assessed by calculating the albumin quotient as follows (CSF albumin  $\times 10^2$  / serum albumin). Similarly, intrathecal IgG synthesis was estimated by calculating the CSF IgG index, based on a general formula [(CSF IgG / serum IgG)  $\times$  (serum albumin / CSF albumin)].

#### **2.1.4. Animals**

Sixteen 6-week old MRL +/- male mice were purchased from the Jackson Laboratories (Bar Harbour, ME) and tail-tattooed for identification purposes (AIMS, Inc., Hornell, NY). Animals were housed four per cage under standard laboratory conditions: light cycle from 19:00 – 9:00, *ad lib* access to rodent chow and tap water. Baseline assessment of behaviour commenced at 10 weeks of age, and was followed by survival surgery ~2 weeks thereafter. Post-surgery monitoring of behavioural performance lasted for an additional ~2.5 weeks. Male mice were used exclusively to avoid the confounding effects of estrus cycling on behavioural performance. Pre- and post-operative body

weight was measured each day to monitor malnutrition. At 20 weeks of age, mice were sacrificed, the position of the cannula was verified, and brain and spleen weight were recorded. Serum was collected and analyzed for anti-dsDNA as described above. All procedures were approved by the local Animal Care Committee (McMaster University AUP # 11-03-11) and performed in accordance to guidelines set out by the Canadian Council of Animal Care.

### ***2.1.5. Survival Surgery***

Aseptic survival surgery involved ketamine/xylazine anesthesia, icv implantation of low-cap cannula (model C315GS-4; Plastics One Inc., Roanoke, VA) into the right ventricle (-0.5 mm from Bregma, lateral 1.0 mm), and subcutaneous implantation of primed mini-pumps with a release rate of 0.25  $\mu\text{l/hr}$  (model 1002; Alzet, Cupertino, CA). Figure 1 exemplifies technical aspects of cannula and Alzet 1002 mini-pump implantation in an adult MRL<sup>+/+</sup> male mouse. Briefly, each mini-pump was filled with ~100  $\mu\text{l}$  of undiluted CSF from one of four NP-SLE patients. The vinyl tube (model C312VT; Plastics One Inc.) connecting the mini-pump to the cannula was pre-loaded with artificial CSF (aCSF) to allow for 4-day post-operative recovery. aCSF was prepared using instructions from Alzet's Osmotic Pumps website ([www.alzet.com/products/guide\\_to\\_use/cfs\\_preparation.html](http://www.alzet.com/products/guide_to_use/cfs_preparation.html)) and separated from patient CSF by a 2 mm air "spacer". Control animals were treated in an identical manner, with the exception that the mini-pump was filled instead with CSF from a patient with NMO. Each mini-pump was designed to ensure continuous solution delivery for ~2 weeks.

### ***2.1.6. Behavioural Battery***

As alluded to above, the protocol sequence included baseline performance, post-surgical performance, and “experimental” performance (i.e., during the infusion of patient CSF). In each phase, mice were exposed to a diverse battery of behavioural paradigms reflective of olfactory function, emotional reactivity, locomotor activity, sensorimotor function, and learning capacity that have confirmed discriminatory power in previous studies of lupus-prone MRL/lpr mice [12;13;15;75-78]. Such a design offered an opportunity to perform multiple within- and between-group comparisons and control for carry-over effects expected from repeated exposures to the same testing apparatus. In other words, behavioural changes induced by the administration of BRA-rich CSF could be identified by comparing pre-operative vs. post-operative performance, as well as to the performance of a control group.

#### ***2.1.6.1. Olfactory Function***

Olfactory tests were used to examine whether mice are able to detect (sensitivity test), differentiate (discrimination test), and remember (memory test) scents. In all three tests, animals were habituated in an empty, clean cage (45×24×20 cm) for 8 min and subsequently exposed to a 3×3 cm piece of filter paper (Whatman Inc., Piscataway, NJ) scented with an odorant (60 µl) for 2 min. In olfactory sensitivity tests, varying dilutions of peanut butter were tested (diluted to 0.0001, 0.001, 0.01 and 0.1% in mineral oil) to estimate the detection threshold. The olfactory discrimination test examined the ability to distinguish different scents using a habituation-dishabituation paradigm [79] with an inter-trial interval of 4 minutes. Each mouse had four successive exposures to the first

odorant (cinnamon, 0.1% concentration) before being presented with a dissimilar odorant (paprika, 0.1% concentration). An increase in sniffing duration with the novel scent is indicative of intact discriminatory abilities. The olfactory memory test provides information regarding the ability to remember a previously exposed scent. Mice were exposed to an odorant twice, with a short interval (30, 60, 90, and 120 min) between the two trials. Odors were randomized, comprising of several commercially-available extracts including vanilla, banana, almond, and coconut (0.1% concentration; Club House, London, ON). A significant decrease in exploration time upon re-exposure was considered an indication of “olfactory memory”. Individuals blind to treatment code manually scored duration of sniffing using Observer XT 7.0 (Noldus Information Technology).

#### *2.1.6.2. Novel Object Test*

The novel object test, which relies on the approach-avoidance conflict, was used to investigate exploratory drive and anxiety-like response to a novel object [12]. Mice were allowed to explore an arena (45×45×20 cm) for 5 min before a small pyramid was positioned in the center for an additional 5 min. Measures of activity including distance traversed, velocity and time spent in the center and periphery were analyzed in both “empty” and “full” phases. Latency to approach the object, contact frequency and duration of snout contact with the object were assessed using a 3-point detection module in EthoVision XT 8.0 software (Noldus Information Technology, Leesburg, VA).

#### *2.1.6.3. Open Field*

The open field is a classical test of exploratory locomotor behaviour and emotionality in a spacious environment [78]. In the current study, each mouse was gently lowered along the wall of an enclosed circular arena (diameter = 113 cm) and permitted to explore, uninterrupted for 30 min. Topography of locomotion (center vs. transition vs. thigmotaxic zones), total distance moved, and velocity were automatically analyzed using EthoVision XT 8.0 software.

#### *2.1.6.4. Step-down Test*

The step-down test is designed to measure anxiety-related behaviour through assessing the readiness of a mouse to descend from an elevated platform (15×9×9 cm) onto a firm, dark surface in a brightly-lit room [12;80]. Latency to step down with all 4 paws was recorded using a stop-watch with a maximum trial duration set at 20 min.

#### *2.1.6.5. Porsolt (forced) Swim Test*

Increased immobility (floating) of rodents in a situation with no escape has been proposed to reflect a state of "behavioural despair" [81]. In the present study, mice were lowered into a large pool (diameter = 120 cm) filled with water (temperature ~25°C) and left to swim for 10 min. An overhead digital camera was used for video-tracking with EthoVision XT 8.0 software. Floating was scored using the built-in mobility module, with "immobility" defined as <5% change in surface area between samples.

#### *2.1.6.6. Spontaneous T-maze Alternation*

Spontaneous alternation behaviour (SAB) refers to the intrinsic tendency of mice to alternate their choice of maze arms on successive opportunities. Alternation has been

commonly used to assess hippocampal-dependent spatial learning and memory [82]. The discrete-trial procedure was employed as described previously [52].

#### *2.1.6.7. Morris Water Maze*

Spatial learning and memory were assessed in the Morris water maze (MWM) over the course of 8 days as recently described in detail [83]. Mice were initially trained in four, 2 min cue trials with a visible platform (Day 1) as a control procedure. On the following four days, the platform was hidden and 4 daily acquisition trials from different starting locations were performed. On Day 6, mice were permitted to swim in an empty pool to examine whether they employed spatial learning (2 min probe trial) and whether they could shift their learning strategy (3 x 2 min extinction trials). Subsequently, a cued platform was placed in the opposite quadrant and mice were permitted 2 min to locate it. “Cognitive flexibility” was measured in 4 reversal trials with a hidden platform on Day 8. Measures including platform latency, swim path, speed and time spent in the quadrant(s) of interest were obtained with EthoVision XT 8.0.

#### *2.1.6.8. Beam-walking Test*

The beam walking test exploits an animal’s innate aversion to height and bright lights as a means to assess fine motor coordination/balance [84-86]. The animal is tasked to traverse a narrow beam connecting a brightly-lit starting platform to an elevated escape platform supporting a dark shelter. Following a brief shaping procedure, a single test was recorded and latency to traverse and foot slips were manually scored via video playback (reviewed in [75]).

#### *2.1.6.9. Rotarod Test*

Balance, muscle strength and acquisition of sensorimotor coordination were assessed using the rotarod test as previously reported [83;87]. Each mouse was positioned on a rotating drum (ENV-575M, Med Associates Inc., St. Albans, VT) which gradually accelerated from 4 to 40 RPM over 5 min. The latency and speed at fall was recorded automatically and the trial was stopped manually for mice which reached the maximum speed in the allotted time.

#### *2.1.6.10. Integrated Behavioural Station ('INBEST')*

Mice were placed in customized activity boxes for 10 hours every other day [88]. Each 'INBEST' box comprised of a computer-controlled light stimulus, photocell-controlled lickometers, automated food dispenser, computerized running wheel and shelter (Med Associates Inc.). Latencies, frequencies, and durations of several behaviours were collected by customized MedPC IV software, in parallel with live-tracking of mice ambulation by EthoVision XT 8.0.

## **2.2. Experiment 2: Effects of Purified BRA**

Although Experiment 1 is a necessary first step in validating our hypothesis and paradigm, it cannot resolve the identity of pathogenic factors. Using a design similar to Experiment 1 with autoimmune CSF, we sought to determine whether sustained exposure to three proposed pathogenic BRA subclasses (anti-NMDAR, ARPA or anti- $\alpha$ -tubulin) induces olfactory dysfunction and functional deficits in other behavioural tests.

### ***2.2.1. Animals***

A total of 72, 8-9 week old male CD1 mice were purchased from Charles River Laboratories (Quebec, Canada) and tail-tattooed. Mice were housed 4 per cage under standard laboratory conditions as described above. Pre-operative behavioural assessment commenced at 11 weeks of age in batches of 8. Subsequently, mice were assigned to one of 6 groups (n=12 mice/group), according to antibody (anti-NMDAR vs. anti-Ribosomal P vs. anti-cytoskeletal) and treatment (experimental vs. control). Consistent with Experiment 1, post-surgery monitoring of behavioural performance was started immediately and lasted for ~2.5 weeks.

### ***2.2.2. Antibodies***

Commercially-available rabbit polyclonal IgG antibodies to NMDAR (anti-NR2A, cat.#07-632; Millipore, Billerica, MA), Ribosomal P (anti-RPLP0, cat.#NBP1-49979; Novus Biologicals, Oakville, ON), and cytoskeletal protein (anti- $\alpha$ -tubulin, cat.#600-401-880; Rockland Immunochemicals Inc., Limerick, PA) were used for experimental groups. Prior to dilution in aCSF, antibody solutions were dialyzed as per instructions from Rockland Immunochemicals (<http://www.rockland-inc.com/AzideRemovalbyDialysis.aspx>) using Slide-A-Lyzer Dialysis Device (10K MWCO, cat.# PI69574, Fisher Scientific, Ottawa, ON).

### ***2.2.3. Survival Surgery***

Sterile implantation of low-cap cannulae and primed mini-pumps took place under ketamine/xylazine anesthesia as described above. Each mini-pump was filled with

~20 µg of the experimental antibody and diluted with aCSF to achieve an antibody delivery rate of 1.2µg/per day. The cumulative dose over ~2.5 weeks of treatment was calculated to be higher than the dose shown to induce apoptosis of hippocampal neurons in vivo [42]. Control mice were treated in the same way, with the exception that the pump was filled with only aCSF. Although a more appropriate design may have employed control IgG, even normal IgG in the CSF can cause behavioural changes including hyperactivity and depressive-like behaviour [89].

#### **2.2.4. Behavioural Battery**

Animals underwent an identical sequence of protocols 15 days prior to and after surgery. Performance was assessed using the same tests as described above (see Figure 2). However, the behavioural battery was expanded to include an additional set of sensorimotor tests to identify neurological deficits undetectable by gross home-cage observation. They included the hind limb clasping reflex [90], visual placing reflex [91], geotaxis test [92] and the basket test [93].

#### **2.3. Statistical Analysis**

All statistical calculations were performed using SPSS 20 software package (SPSS Inc.; Chicago, IL) with the criterion for statistical significance set at  $p \leq .05$  for all group comparisons. Dependent measures were analyzed using Student's t-test or, with Treatment (Experimental vs. Control) as a between-group factor. When measures were taken repeatedly (e.g., Trial, Concentration, Days and/or Phase), they were considered within-group factors in Analysis of variance (ANOVA) with repeated measures. If

significant interactions were detected, Student's t-test was used in post-hoc comparisons. When appropriate, rates of post-surgery data were taken as a percentage of pre-surgery performance. Rates were calculated using the 1) last mean data point, when pre-operative performance was increasing or decreasing, 2) average, when pre-surgery behaviour was stable or variable and, 3) the last, three mean data points when pre-operative performance was initially increasing or decreasing. Graphs indicate mean values and  $\pm$  SEM with significant differences of  $p \leq .05$ ,  $p < .01$  and  $p < .001$ , shown as \*, \*\*, and \*\*\*, respectively.

### **3. Results**

#### **3.1. NP-SLE Samples**

From the cohort of 26 NP-SLE patients, 4 CSF samples were chosen on the basis of significant elevations in specific autoantibody levels as confirmed by IFA, ELISA and WB techniques. Demographic, clinical and immunological profile of NP-SLE patients is detailed in Table 2. Undiluted CSF from these 4 patients were then administered directly into the brains of healthy animals.

#### **3.2. Behavioural Effects of CSF Infusion**

##### ***3.2.1. Olfactory Function***

Considering mice were matched for baseline behavioural performance and assigned to treatment groups accordingly, pre-surgery differences were undetected unless specified otherwise. The olfactory discrimination test was one such exception. In particular, assessment of baseline performance revealed that mice assigned to receive NP-

SLE CSF spent significantly more time investigating cinnamon in their first trial (Trial x Treatment:  $F_{1,14} = 8.771$ ,  $p < 0.01$ , Figure 3A). However, differences subsided thereafter, as both groups showed comparable habituation to repeated exposures of cinnamon (Treatment:  $F_{1,14} = 0.6$ , n.s.) and similar exploration of a paprika-laced filter paper in the dishabituation trial (Treatment:  $F_{1,14} = 0.02$ , n.s.). When exposed to the same paradigm following mini-pump implantation and CSF infusion, no group differences were noted during cinnamon habituation, although an overall drop in mean sniffing duration was evident. Importantly, mice treated with lupus CSF spent significantly less time investigating the dishabituation odour in comparison to control animals ( $t_{14} = -2.184$ ,  $p < 0.05$ , Figure 3B). Other tests of olfactory function failed to distinguish the groups. In particular, mice spent comparable time investigating several different concentrations of peanut butter in the olfactory sensitivity test and various scents in the short-term olfactory memory test (data not shown). The results suggest that sustained transfer of NP-SLE CSF impairs the ability to discriminate scents, without altering the detection and memory for scents.

### ***3.2.2. Tests of Emotional Reactivity and Motivated Behaviour***

When exposed to the novel object test, groups did not differ in overall locomotion and topography in both empty and full phases (data not shown). Infusion of NP-SLE CSF also failed to alter responsiveness to the novel object as mice in both groups had similar durations of sniffing, frequency of contacts and latencies to approach the object (data not shown). The groups also performed comparably in the open field in terms of distance moved, velocity and time spent in the center and thigmotaxic zones (data not

shown). Taken together, CSF infusion did not seem to alter exploratory behaviour in approach-avoid scenarios with novel objects or brightly-lit open spaces. Between-group comparisons in the step-down task revealed comparable latencies to step-down from the elevated platform ( $t_{14} = -0.262$ , n.s.). When considered in the context of the findings in the novel object and open field tests, the data is consistent with the notion that chronic administration of NP-SLE does not induce anxiety-like behaviour. In contrast to dry-land paradigms discussed above, administration of NP-SLE CSF led to a significant reduction in swimming distance (Treatment:  $F_{1,14} = 4.870$ ,  $p < 0.05$ , Figure 4A) in the forced swim test. The overall decrease in swimming coincided with increased floating in NP-SLE CSF-treated animals (2-4 min interval:  $t_{14} = 2.213$ ,  $p < 0.05$ , Figure 4B).

### ***3.2.3. Tests of Learning and Memory***

Assessment of SAB revealed that both groups showed comparable alternation rates before surgery ( $t_{14} = 0.640$ , n.s.). However, infusion of control CSF from an NMO patient led to a significant reduction in spontaneous alternation post-surgery (Treatment:  $t_{13} = 2.213$ ,  $p < 0.05$ , Figure 4C). When tested in the MWM, there were no group differences in the latency to find the cued platform (Treatment:  $F_{1,14} = 0.784$ , n.s.) or the hidden platform over the next 4 days (Treatment:  $F_{1,14} = 1.968$ , n.s.). However, examination of swimming distance revealed that NMO CSF-treated mice displayed longer swim paths to locate the platform over the same acquisition trials (Treatment:  $F_{1,14} = 4.932$ ,  $p < 0.05$ , Figure 4D). Both groups exhibited similar performance when the platform was removed or moved to the opposite quadrant and made visible. When the same platform was submerged in reversal trials, mice receiving NP-SLE CSF displayed a

significantly stronger perseveration response for the quadrant where the platform used to be (Treatment:  $F_{1,14} = 4.676$ ,  $p < 0.05$ , Figure 4E). Taken together, NP-SLE CSF did not affect initial learning, but impaired performance of mice when the task parameters were made more difficult. In the context of the SAB data, our findings further suggest that NMO CSF may have detrimental effects during the acquisition phases in tests of spatial learning and memory.

### ***3.2.4. Tests of Sensorimotor Functioning***

No significant differences in the latency to traverse a narrow beam (Treatment:  $F_{1,14} = 0.79$ , n.s.) or number of beam slips (Treatment:  $F_{1,14} = 0.27$ , n.s.) were noted throughout the study. Consistent with the results from the beam-walking task, latency to fall from the rotarod was comparable among mice receiving NP-SLE CSF and those treated with NMO CSF (Treatment:  $F_{1,14} = 2.489$ , n.s.). Taken together, these results suggest post-surgical behavioural performance was not confounded by deficits in balance, motor coordination or muscle strength.

### ***3.2.5. Body Weight and Home-cage Monitoring***

Post-surgery monitoring revealed a significant drop in body weight (taken as a percentage of body weight at surgery) in both groups. However, mice receiving CSF from lupus patients exhibited a more pronounced reduction in body weight (Day 4:  $t_{14} = -2.529$ ,  $p < 0.05$ ; Day 5:  $t_{14} = -2.841$ ,  $p < 0.05$ ; Day 6:  $t_{14} = -2.724$ ,  $p < 0.05$ ; Figure 4F). Group differences were transient in nature, only being detected in the initial days of BRA-rich CSF infusion, but subsiding thereafter. Body weight reductions were

associated with a profound and chronic decrease in water consumption (Treatment:  $F_{1,14} = 9.909$ ,  $p < 0.01$ , Figure 5A) and food intake (Treatment:  $F_{1,13} = 28.044$ ,  $p < 0.001$ , Figure 5B). Changes in ingestive behaviour were accompanied by a reduction in running wheel activity, as measured by the rate of wheel rotations (Treatment:  $F_{1,14} = 28.722$ ,  $p < 0.001$ , Figure 5C) and time spent in the running wheel (Treatment:  $F_{1,14} = 12.672$ ,  $p < 0.01$ , Figure 5D). Coinciding with lower running wheel duration, mice treated with NP-SLE CSF spent more time in the shelter (Treatment:  $F_{1,14} = 22.657$ ,  $p < 0.001$ , Figure 5E). Infusion of CSF from NP SLE patients resulted in less overall ambulation (Treatment:  $F_{1,14} = 12.637$ ,  $p < 0.01$ , Figure 5F) without affecting velocity of movement (Treatment:  $F_{1,14} = 0.347$ , n.s., data not shown).

### ***3.2.6. Tissue Sampling***

At sacrifice, there were no significant group differences in body, brain or spleen weight (data not shown). Moreover, analysis of serum autoantibodies revealed comparable levels of anti-dsDNA antibodies ( $t_{14} = -1.519$ , n.s.), suggesting behavioural differences were related to CSF treatment, and not due to the spontaneous development of autoimmune disease in MRL +/+ mice.

## **3.3. Behavioural Effects of Purified BRA**

### ***3.3.1. Anti-NMDAR***

Behavioural performance between mice receiving anti-NR2A antibodies and control animals treated with only aCSF was largely comparable, but task-specific differences were noted. In particular, before surgery, both groups displayed similar

responses to cinnamon and paprika in the olfactory discrimination task (Cinnamon:  $F_{1,22} = 0.092$ , n.s., Paprika:  $t_{22} = 1.0$ , n.s., Figure 6A). Conversely, post-surgery assessment revealed that mice treated with anti-NR2A antibodies spent significantly less time exploring both scents across all trials (Treatment:  $F_{1,22} = 5.755$ ,  $p < 0.05$ ; Figure 6B). These results suggest anti-NMDAR treatment may alter responsiveness to previously-exposed scents, without necessarily affecting habituation or discriminatory capacity. Other tests of olfactory function revealed similar performance in terms of odor sensitivity and short-term memory for different scents (data not shown).

Analysis of the MWM revealed two outliers that consistently failed to find the platform. As a result, both mice (one from each treatment group) were excluded from MWM analyses. Performance in the cued and acquisition phases of the test was comparable between groups (path length data illustrated in Figure 6C). When the task was made more difficult in reversal trials, mice that received anti-NR2A antibodies exhibited shorter path lengths to locate the newly-positioned hidden platform (Treatment:  $F_{1,20} = 5.161$ ,  $p < 0.05$ ; Figure 6C). They also swam shorter distances in an empty pool devoid of a platform during probe and extinction trials (Treatment:  $F_{1,20} = 4.611$ ,  $p < 0.05$ ; Figure 6D). Taken together, sustained anti-NMDAR administration seemingly improved the ability to reach a relocated, hidden platform, suggestive of enhanced “cognitive” flexibility. Performance measures in all other tests of sensorimotor function, locomotion, motivated behaviour, learning / memory capacity and behaviour in a home-cage setting were comparable between the groups (data not shown).

### ***3.3.2. Anti-Ribosomal P***

Similar to results seen with anti-NR2A, infusion of anti-RPLP0 antibodies did not have an effect on overall performance measures in the majority of behavioural paradigms. In particular, mice in both groups had no trouble detecting increasingly lower concentrations of peanut butter, discriminating cinnamon from paprika and remembering different scents (data not shown). The results suggest that experimental administration of ARPA does not alter olfactory function. Similar results were noted in other odor-guided behaviours related to the exploration of a novel object and an open field (data not shown). The only paradigm which differentiated treatment groups was the MWM test of spatial learning and memory. While there were no differences in the latency to locate a cued or hidden platform during acquisition and reversal trials (Figure 7A), ARPA-treated mice displayed poorer spatial bias for the platform location when it was removed from the pool altogether ( $t_{14} = -2.224$ ,  $p < 0.05$ , Figure 7B). Eight animals had to be excluded from the analysis of the probe and extinction trials due to a technical error. The results suggest that ARPA infusion into the brains of otherwise healthy animals alters the spatial search strategy employed in the MWM. Both treatment groups performed comparably in all other behavioural paradigms (data not shown).

### ***3.3.3. Anti- $\alpha$ -tubulin***

Assessment of olfactory function in anti- $\alpha$ -tubulin-treated and control animals revealed comparable exploration of scents in discrimination, sensitivity and short-term

memory paradigms (data not shown). The results demonstrate that odor-guided behaviours in otherwise healthy animals are not altered by the sustained administration of anti-cytoskeletal antibodies. Although olfactory tests failed to discriminate groups, post-surgery monitoring of home-cage behaviour revealed that infusion of anti- $\alpha$ -tubulin antibodies increased running wheel activity, as measured by wheel rotations (Treatment:  $F_{1,19} = 5.163$ ,  $p < 0.05$ , Figure 8A) and time spent in the running wheel (Treatment:  $F_{1,19} = 5.518$ ,  $p < 0.05$ , Figure 8B). Coinciding with higher running wheel duration, mice treated with anti- $\alpha$ -tubulin antibodies also had a tendency to spend less time in the shelter (Treatment:  $F_{1,19} = 9.431$ ,  $p < 0.05$ , Figure 8C). Other INBEST measures related to ingestive behaviours, ambulation and velocity were similar between groups (data not shown).

In the MWM, mice had similar latencies to locate cued and hidden platforms (Figure 8D) and performed comparably in probe / extinction trials (data not shown). When the task difficulty was increased by placing the hidden platform in the opposite quadrant, anti-cytoskeletal antibody administration led to significantly shorter escape latencies (Treatment:  $F_{1,19} = 4.405$ ,  $p < 0.05$ , Figure 8D). Coupled with the data from INBEST, these results are consistent with the notion that sustained administration of anti- $\alpha$ -tubulin antibodies has a beneficial, stimulatory effect on certain behaviours. Mice treated with anti- $\alpha$ -tubulin performed comparably to animals receiving only aCSF in all other respects (data not shown).

#### **4. Discussion**

The current study sought to investigate the direct, functional effects of sustained icv administration of autoantibody-rich NP-SLE CSF, as well as three potentially-pathogenic BRA on olfactory function and other behaviours. Diverse alterations in behaviour were noted during the infusion of undiluted autoimmune NP-SLE CSF. In the olfactory discrimination task, administration of lupus CSF reduced exploration of a dissimilar scent, indicative of impaired discriminatory abilities. Although olfactory capacity was not altered in other odor-guided tasks, these mice exhibited increased immobility in the forced swim test and enhanced perseveration in MWM reversal trials. A transient reduction in body weight coincided with decreased intake of food and water in home-cages. Mice receiving autoimmune CSF also showed a persistent reduction in running wheel activity, failing to restore their wheel preference even after CSF infusion had ceased. Prolonged assessment of home-cage behaviour also revealed a significant increase in time spent in the shelter, and a concurrent decrease in overall ambulation. In comparison to the robust effects elicited by undiluted NP-SLE CSF, icv administration of purified, commercially-available antibodies resulted in relatively mild, task-specific functional changes. Infusion of anti-NR2A antibodies, for example, resulted in a diminished response to previously-exposed scents in the olfactory discrimination task, but improved performance in acquiring the novel escape location in the MWM. Interestingly, mice treated with anti- $\alpha$ -tubulin antibodies demonstrated a similar beneficial response in MWM reversal trials. Moreover, assessment of home-cage behaviour revealed that these mice also spend more time on the running wheel and less

time in the shelter in comparison to control animals. In contrast to these seemingly positive improvements in behaviour, ARPA treatment was associated with a deficit in spatial reference memory during MWM probe trials. Taken together, our findings suggest that sustained exposure of the healthy brain to BRA in CSF recapitulates several, but not all facets of the complex behavioural deficits noted in lupus-prone MRL/lpr mice and humans. The broad behavioural changes, oftentimes in opposite directions, suggest some BRA have a stimulatory effect, while other subsets have deleterious, inhibitory effects.

The results obtained following administration of NP-SLE CSF are largely consistent with previous reports detailing CSF neurotoxicity in human and murine forms of lupus [40-42]. Whereas these earlier studies focused on effects at a cellular level, our results suggest that BRA-rich CSF promotes an assortment of impairments in olfactory function, metabolic demands, motivated behaviours and spatial learning / memory. In this regard, the functional profile of animals receiving autoimmune CSF mimics several aspects of so-called autoimmunity-associated behavioural syndrome that coincides with the onset of systemic autoimmunity in lupus-prone MRL/lpr mice [94;95]. These include confined exploration in the vicinity of a “home-base” [12;13], reduced responsiveness to palatable stimulation [76;96], response perseveration in spatial learning tasks [13;48;52], excessive immobility when forced to swim [12], and altered olfactory function [15]. The latter impairments, operationally termed “autoimmune depression” by Katzav and colleagues, have also been noted in another murine model of CNS SLE induced through the acute icv infusion of ARPA [66-69]. Yet, the results from the current study do not

support such a relationship under sustained administration. Instead, the finding of a spatial reference memory deficit in ARPA-treated mice is in line with more recent evidence of learning impairments following passive transfer of ARPA in otherwise healthy animals [69;74]. The results are intriguing given that we could not reproduce similar deficits using anti-NMDAR antibodies, as others have noted in paradigms of learning and memory [31;64;97]. On the other hand, several reports have also been unable to replicate the association between anti-NR2 antibodies and behavioural impairments in SLE patients [98-104] and lupus-prone mice [105]. We did, however, document diminished exploration of scents in the olfactory discrimination task in anti-NR2A-treated animals. It is interesting to speculate that reduced responsiveness in these mice reflects improved memory function post-surgery. If so, they would be in line with relative improvements in acquiring and remembering a novel escape location following anti-NR2A exposure. Together, these results suggest that anti-NMDAR antibodies may actually have a beneficial and/or stimulatory role under certain experimental conditions. Such results would be in line with neuroprotective effects elicited by anti-NR1/2 antibodies in experimental models of stroke, epilepsy and neuropathic pain [106;107]. By the same account, anti- $\alpha$ -tubulin antibodies also seemed to have a stimulatory effect, one that was more robust and detectable in multiple performance measures. These findings, like in anti-NMDAR-treated animals, seem to run contrary to clinical associations in NP-SLE patients [71] and require further studies to clarify the role of anti-cytoskeletal antibodies.

The heterogeneity of behavioural changes induced by NP-SLE CSF and the potency (or lack thereof) of purified commercially-available antibodies to do the same requires further consideration. Firstly, CSF samples from NP-SLE patients are likely to contain a plethora of antibodies with differing reactivities to neuronal tissues. Indeed, our own analysis of CSF revealed elevations in several antibodies that were not tested in the current study, but have been linked to NP-SLE manifestations including anti-dsDNA [31;42;63;108], aCL [20;22;109;110] and anti-PR3 antibodies [111]. Therefore, it is plausible that the relatively pronounced effects elicited by undiluted NP-SLE CSF are mediated by these BRA classes. Given the abundance of autoantibodies in lupus [112], an alternative, but equally-likely possibility is that behavioural manifestations are induced by as of yet undetectable BRA in the CSF. While some of antibodies in CSF may ultimately represent epiphenomena, other classes may have potent effects across a wide range of concentrations, even in negligible amounts. In the current study, we used a cumulative dose of 20  $\mu\text{g}$  over  $\sim 2$  weeks, with a daily delivery rate of 1.2 $\mu\text{g}$ /per day. The final dose was significantly larger than the amount used by Degiorgio and colleagues to induce local neuronal loss with an anti-NMDAR antibody [42], but considerably smaller than the dose used by Katzav and colleagues to induce behavioural changes using ARPA [66;68;69]. Although beyond the scope of this report, one may further assume that different concentrations of a particular antibody, much like the binding of different antigen targets, have variable effects. Recent findings show that low concentrations of anti-NMDAR antibodies selectively amplify NMDAR-mediated synaptic signaling, but promote excitotoxic cell death via mitochondrial dysfunction at high concentrations [113].

Relatively low concentrations may help explain why we noted stimulatory roles for both anti-NR2A and anti- $\alpha$ -tubulin antibodies in certain paradigms. However, concentration effects alone cannot resolve why we also documented seemingly antagonistic effects in other tasks under the same dose or when using ARPA. Another factor to consider is the source of the antibodies. Whereas the current study used polyclonal antibodies against singular antigens in isolation, previous studies involving direct CNS administration have employed purified human antibodies against multiple antigens and epitopes [42;66;68;69]. Therefore, a possible combined effect of these antibodies against multiple epitopes might explain the pathological effects seen in previous studies. The data may further serve as a potential model to explain discrete CNS symptoms along a wide spectrum, some that are caused by a transient effect on neuronal functioning and others caused by permanent neuronal damage [114]. Taken together, significant differences were found in several behavioural domains, but sustained icv administration of autoantibodies did not account for the broad spectrum of behavioural alterations noted in SLE patients, spontaneous murine models of lupus or following administration of NP-SLE CSF. The potential complex modes of action detailed above, underlie the necessity for further studies aimed at systematically addressing these relationships in the future.

The interpretation of the present findings is complicated by the observation that CSF from an NMO patient seemingly impaired performance in spatial learning and memory tasks. NMO is an inflammatory demyelinating disorder of the CNS that is primarily characterized by the presence of autoantibodies to aquaporin-4 (AQP4) in serum [115] and CSF [116]. The target antigen is an integral membrane protein that

forms the most abundant water channel in the CNS. Anti-AQP4 antibodies have been specifically implicated in BBB dysfunction, altered glutamate homeostasis, and induction of necrotic cell death in the optic nerve and the spinal cord [117]. Passive transfer of purified patient NMO IgG fractions, as well as recombinant human anti-AQP4 IgG, produces key aspects of NMO-like CNS lesion pathology including loss of AQP4 expression, myelin breakdown, axonal injury, extensive inflammatory cell infiltration, astrocyte depletion, and neuronal cytotoxicity in a complement-dependent manner [118-120]. More recently, intrathecal administration of anti-AQP4 was found to elicit similar, but reversible histopathological changes independently of complement activation and immune cell infiltration [121]. One may surmise that impaired performance in both the T-maze and water maze noted in the current study reflects anti-AQP4-induced vision deficits. However, this possibility seems unlikely given that mice performed comparably to NP-SLE CSF-treated animals in cued platform trials. Given the high expression of AQP4 messenger RNA by neurons in periventricular structures of the rodent brain [122], a more likely explanation may involve preferential binding of anti-AQP4 IgG to regions like the hippocampus that are implicated in the acquisition, consolidation, storage and retrieval of spatial information [123]. Nevertheless, further study of this selective effect of NMO CSF on behavioural performance in spatial learning tasks is necessary.

While the current study had several research strengths (e.g., sustained administration of both NP-SLE CSF and 3 candidate BRA, comprehensive behavioural battery, within- and between-group comparisons), it also had a number limitations that can be resolved in the future. For one, it lacks information regarding the effects of NP-

SLE CSF and BRA at the neuronal and molecular levels. For example, details relating to the morphology of the periventricular regions would go a long way in determining the structures and mechanisms underlying the apparent stimulatory and inhibitory effects of specific BRA. Secondly, a dose-response study for each individual BRA would corroborate the hypothesis that the potency and/or direction of effects elicited by specific autoantibodies are concentration-dependent. Lastly, based on the post-hoc analysis of results obtained following NMO CSF administration, a more appropriate design in hindsight may have included a third group of animals that received only aCSF and would thus serve as true negative controls.

Despite the aforementioned drawbacks of the current study, it nevertheless provides strong evidence suggesting a pathogenic role for BRA in the CSF of NP-SLE patients. In addition to supporting a long-hypothesized mechanism of NP manifestations in lupus, the data may one day provide the conceptual basis for the identification of diagnostic markers [124] and novel approaches to the treatment of NP-SLE. Identification of other pathogenic BRA in the same manner may help to shorten the list of candidate BRA in SLE, advance our understanding of immunopathogenic mechanisms in other CNS disorders, and open new vistas in pharmacotherapy.

### **Author Contributions**

M.K. and B.S. were involved in manuscript drafting and contributing to the intellectual content. B.S. takes responsibility for the integrity and accuracy of results.

**Conceived and designed the experiments:** M.K. and B.S.

**Performed the experiments:** M.K., H.Z., M.C., S.E.T., M.S.,B.Z.

**Analyzed the data:** M.K. and B.S.

**Contributed reagents/materials/analysis tools:** D.M., R.K. and B.S.

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**Table 5.1 - Proposed BRA involved in NP-SLE manifestations**

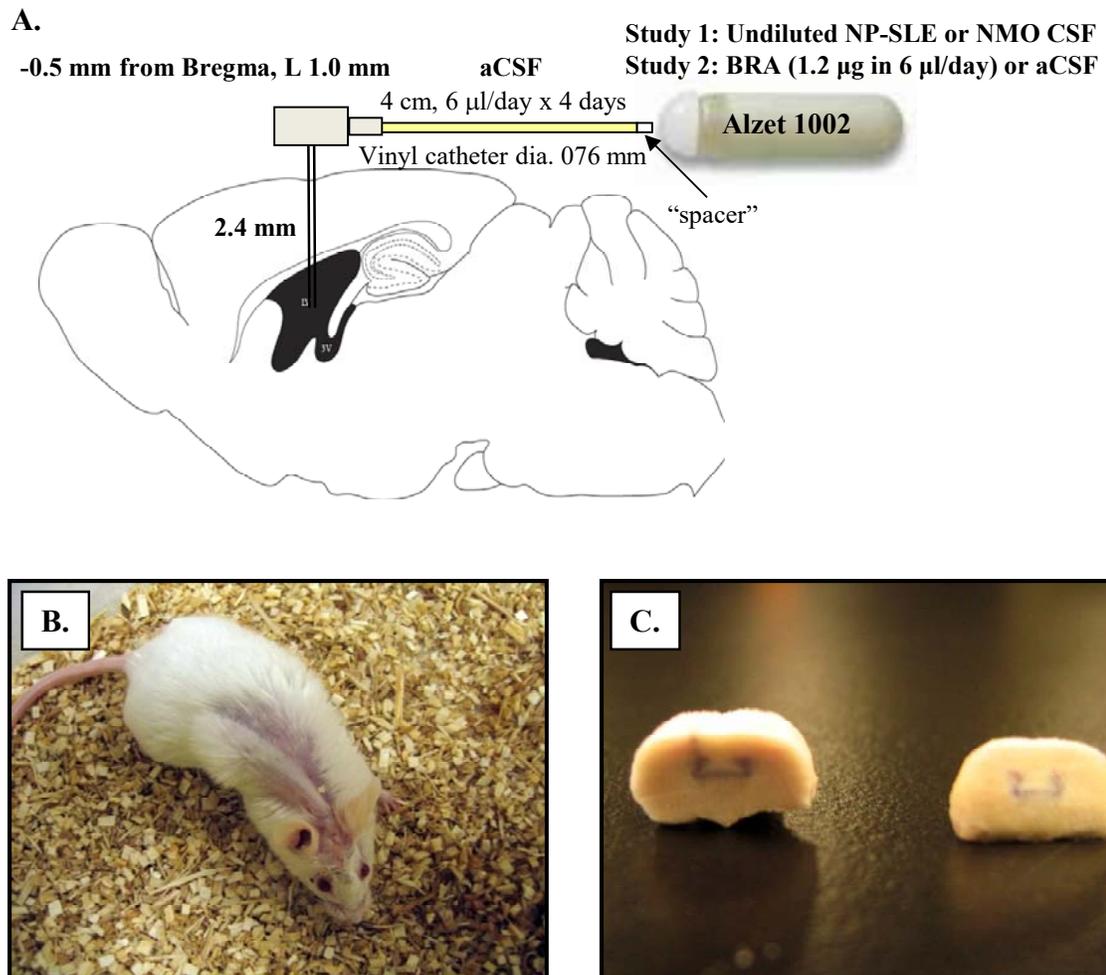
<b>GROUP</b>	<b>NP-SLE MANIFESTATIONS</b>
<b>BRAIN-SPECIFIC</b>	
Anti-neuronal	Cognitive dysfunction, psychosis
<b>Anti-N-methyl-D-aspartate receptor</b>	Cognitive dysfunction, depression
Anti-microtubule-associated protein 2	Depression
Anti-ganglioside	Psychosis
Anti-neurofilament	
Anti- $\alpha$ -internexin	Non-specific NP manifestations
Anti-glia fibrillary acidic protein	Non-specific NP manifestations
Anti-synapsin I	Undetermined
<b>SYSTEMIC</b>	
Anti-phospholipid	
Anti-cardiolipin	Epilepsy, seizures, dementia, psychosis, depression, cognitive dysfunction, migraine
Anti- $\beta_2$ -glycoprotein I	Undetermined
Lupus anti-coagulant	Cognitive dysfunction, chorea
Anti-triosephosphate isomerase	Non-specific NP manifestations
<b>Anti-ribosomal P</b>	Psychosis, depression
Anti-Ro/SSA	Chorea, cognitive dysfunction, psychosis
Anti-smith	Seizures, psychosis
Anti-endothelial cell	
Anti-Nedd5	Non-specific NP manifestations
Serum lymphocytotoxic	Cognitive dysfunction
Anti-neutrophil cytoplasmic	
Anti-proteinase 3	Non-specific NP manifestations
Anti-myeloperoxidase	Non-specific NP manifestations
<b>Anti-<math>\alpha</math>-tubulin</b>	Non-specific NP manifestations

**Table 5.2 - Demographic, clinical and immunological profile of NP-SLE patients**

	<b>NP-SLE Sample #1</b>	<b>NP-SLE Sample #2</b>	<b>NP-SLE Sample #3</b>	<b>NP-SLE Sample #4</b>
Age	59	60	65	49
Sex	Female	Female	Female	Female
SLE Duration (years)	6	19	9	8
SLEDAI	12	12	9	12
NP Manifestations	Psychosis Cognitive Dysfunction Vasculitis	Headaches Vasculitis	Depression Cognitive Dysfunction Vasculitis	Anxiety Cerebrovascular Diseases
<b>Serological Antibody Profile</b>				
ANA staining pattern	Homogenous <sup>+</sup>	Homogenous <sup>+++</sup>	Homogenous <sup>++</sup>	Speckle <sup>+++</sup>
Anti-ribosomal P IgG	3.33	0	191.34	2.09
Anti-cardiolipin IgG	120	2.36	4.08	0
Anti-proteinase 3 IgG	5.21	3.48	9.64	2
Anti-dsDNA IgG	163.72	514.44	297.88	25.7
Anti- $\alpha$ -tubulin IgG	557	494	742	629
<b>Cerebrospinal fluid Antibody Profile</b>				
Anti-ribosomal P IgG	7.83	2	<b>89.24</b>	3.12
Anti-cardiolipin IgG	<b>52.68</b>	2.75	3.45	2.1
Anti-proteinase 3 IgG	2.16	2.66	4.3	2.9
Anti-dsDNA IgG	49.46	<b>253.61</b>	77.85	13.46
Anti- $\alpha$ -tubulin IgG	572	577	777	<b>1229</b>
<b>BBB permeability</b>				
Serum IgG (g/l)	9.04	11.8	11.7	9.96
CSF IgG (g/l)	0.026	0.042	0.031	0.095
Serum albumin (g/l)	37.8	35.4	30.6	34.8
CSF albumin (g/l)	0.233	0.269	0.183	0.506
Albumin quotient	0.616	0.760	0.598	1.454
CSF IgG index	0.467	0.468	0.443	0.656

## Figures

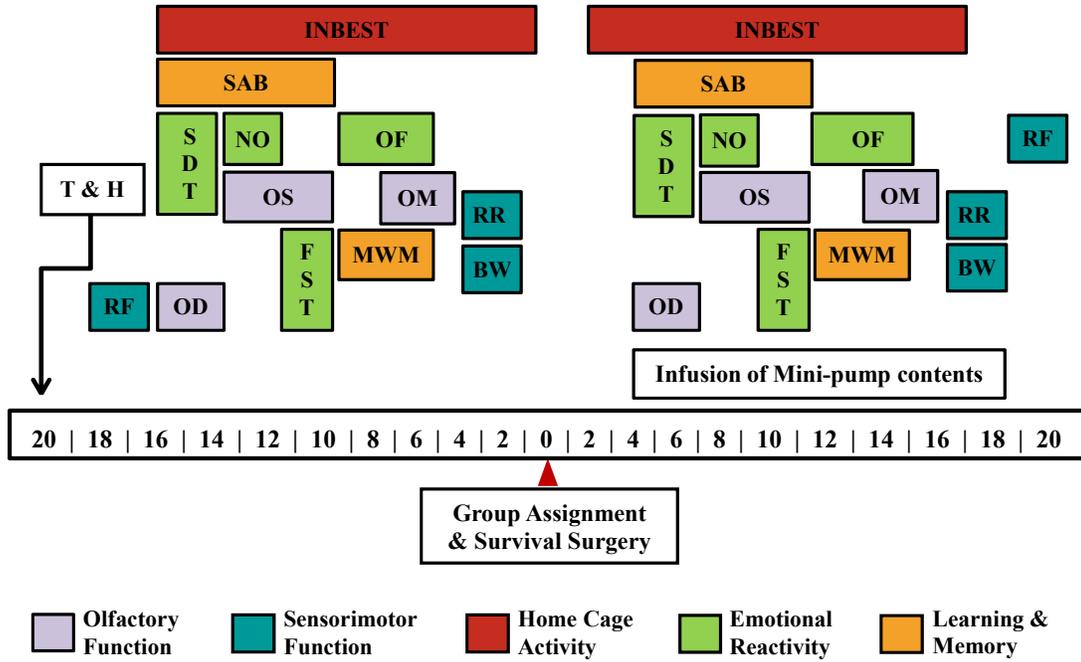
**Figure 5.1 - Technical details of survival surgery**



**Figure 1. Technical details of survival surgery.** (A) Assessment of pre-operative behavioural performance was followed by assignment into either experimental or control group. All mice underwent unilateral implantation of a sterilized cannula into the right lateral ventricle and subcutaneous insertion of a primed Alzet mini-pump connected to a cannula via vinyl catheter tubing. Animals in both experimental and control groups

received aCSF for four days to facilitate post-operative recovery. Hereafter, infusion of the solution of interest was initiated (NP-SLE or NMO CSF in study 1, purified BRA or aCSF in study 2) and continued for two weeks. An oil drop “spacer” was used to prevent mixing of aCSF in the tubing and the experimental solution in the primed pump. **(B)** An MRL +/- animal moving freely following survival surgery. **(C)** Histological verification of co-ordinates obtained by post-mortem injection of Toluidine blue into the vinyl tubing cut at the neck level.

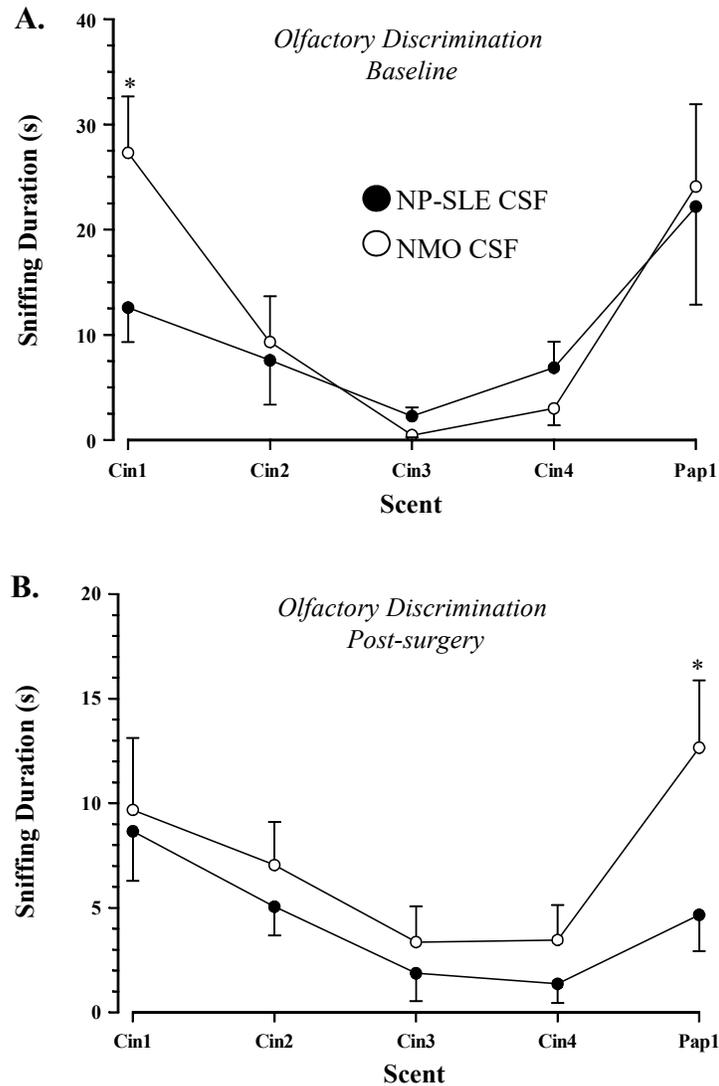
**Figure 5.2 - Schematic representation of experimental design**



**Figure 2. Schematic representation of experimental design.** Prior to testing, all animals were tail-tattooed and habituated to the experimenters. Mice from both groups underwent an identical sequence of behavioural protocols 15 days prior to and after survival surgery. The behavioural battery was designed to evolve from less towards more strenuous tasks in an attempt to mitigate residual stress effects on subsequent tests. **Abbreviations:** T – Tattooing; H – Habituation; RF – Reflexes; INBEST – Integrated Behavioural Station; SAB – Spontaneous Alternation Behaviour; SDT – Step Down Test; NO – Novel Object Test; FST – Forced Swim Test; OF – Open Field Test; MWM – Morris Water Maze; OS – Olfactory Sensitivity; OM – Olfactory Memory; OD – Olfactory Discrimination; BW – Beam-Walking test; RR – Rotarod. *Note: Tests are categorized into their respective behavioural domains. However, this classification is not*

*mutually exclusive, as some tasks may be used to assess behaviour in domains other than what the legend suggests.*

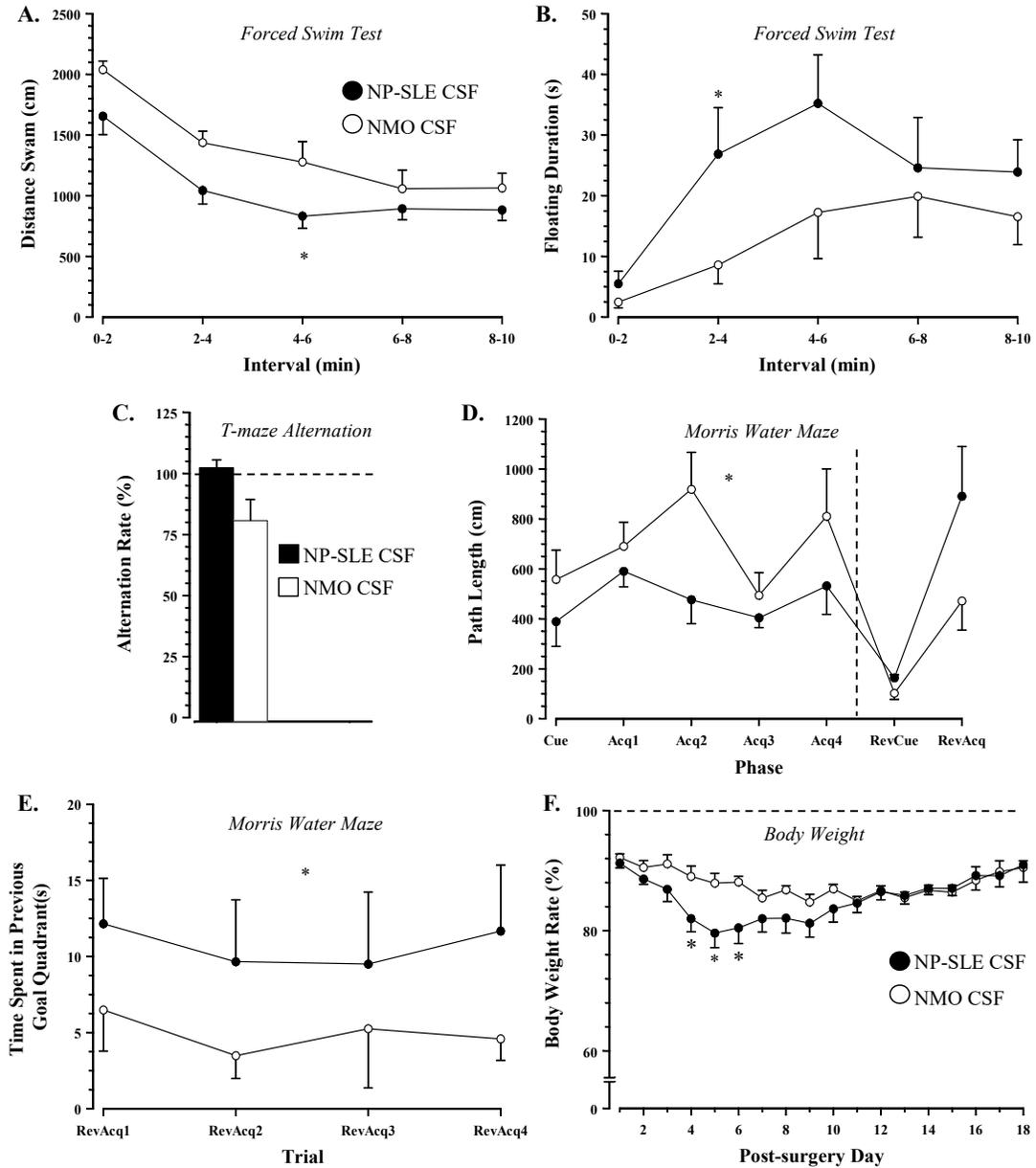
**Figure 5.3 - Olfactory discrimination test pre- and post-infusion of CSF**



**Figure 3. Olfactory discrimination test pre- and post-infusion of CSF. (A)** When initially exposed to the paradigm during baseline assessment, mice assigned to receive NP-SLE CSF spent significantly more time investigating cinnamon in their first trial, but performed comparably to control animals in subsequent exposures at the time point. **(B)** Following surgery, both groups seemed to habituate to repeated exposures to cinnamon-

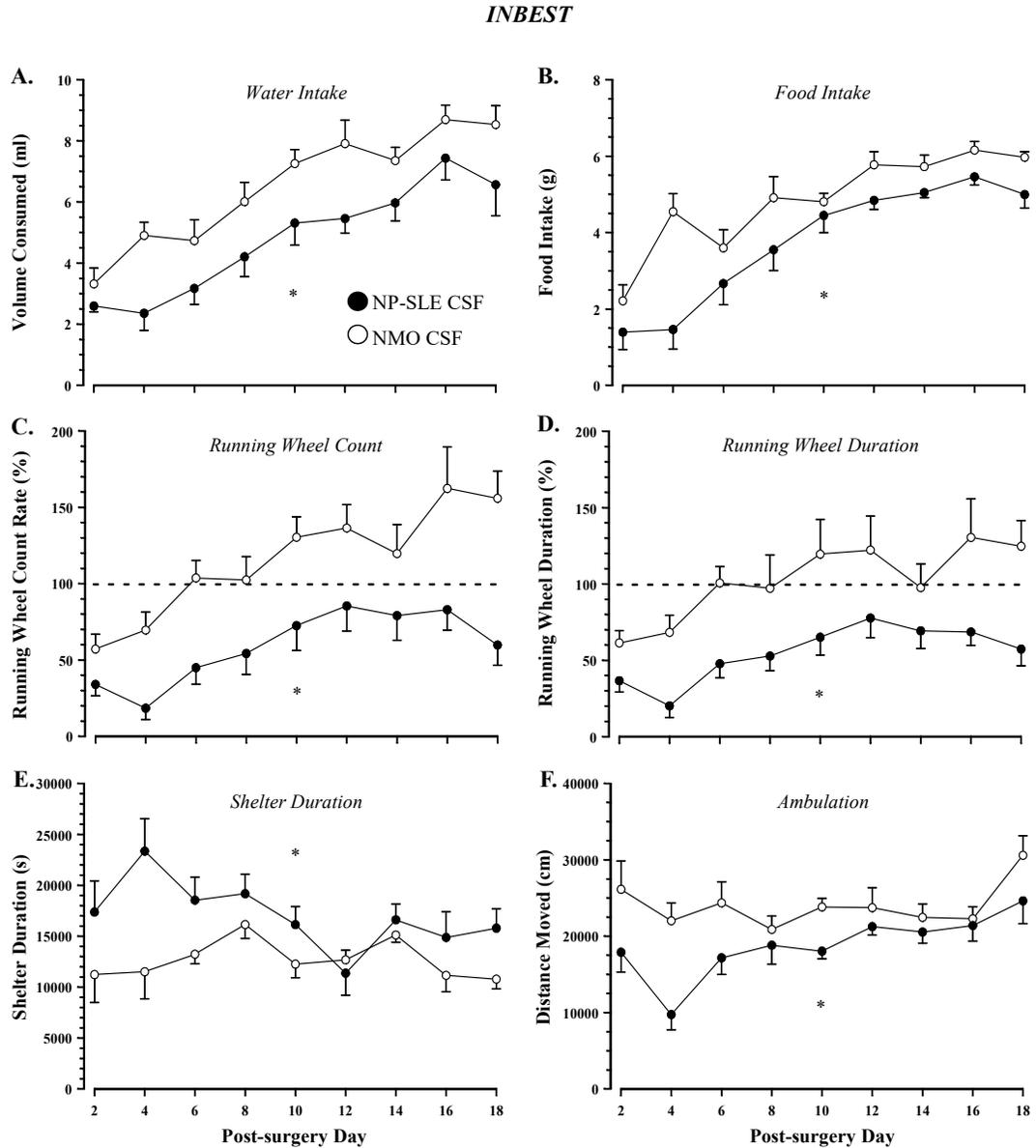
scented filter paper. However, NP-SLE CSF-treated mice spent significantly less time investigation paprika-laced filter paper in the final (dishabituation) trial. The results suggest that post-surgery administration of NP-SLE CSF induces olfactory dysfunction, particularly in the discriminatory capacity of mice

**Figure 5.4 - Post-surgical effects of NP-SLE and NMO CSF**



**Figure 4. Post-surgical effects of NP-SLE and NMO CSF. (A)** Sustained administration of CSF from NP-SLE patients reduces overall swimming distance and **(B)** increases floating in the forced swim test. **(C)** Control animals receiving NMO CSF exhibit a significant post-surgery drop in spontaneous alternation rates that was not noted in animals treated with NP-SLE CSF. **(D)** Despite similar path lengths to locate the cued platform on Day 1, control animals swam longer distances to find a submerged platform on subsequent acquisition trials. **(E)** Even though NMO CSF-treated animals showed relative deficits in acquiring the location of a hidden platform, administration of NP-SLE CSF induced increased perseveration when the platform was re-located in reversal trials. **(F)** Behavioural changes in mice treated with lupus CSF is accompanied by a significant, but transient, reduction in body weight when CSF infusion begins. Taken together, NP-SLE CSF produced increased immobility in the forced swim, but did not impair learning in the MWM, until the task parameters were made more difficult. Conversely, mice treated with NMO CSF swim longer distances to locate the hidden platform and do not alternate as frequently, suggesting that NMO CSF may have detrimental effects in tests of spatial learning and memory.

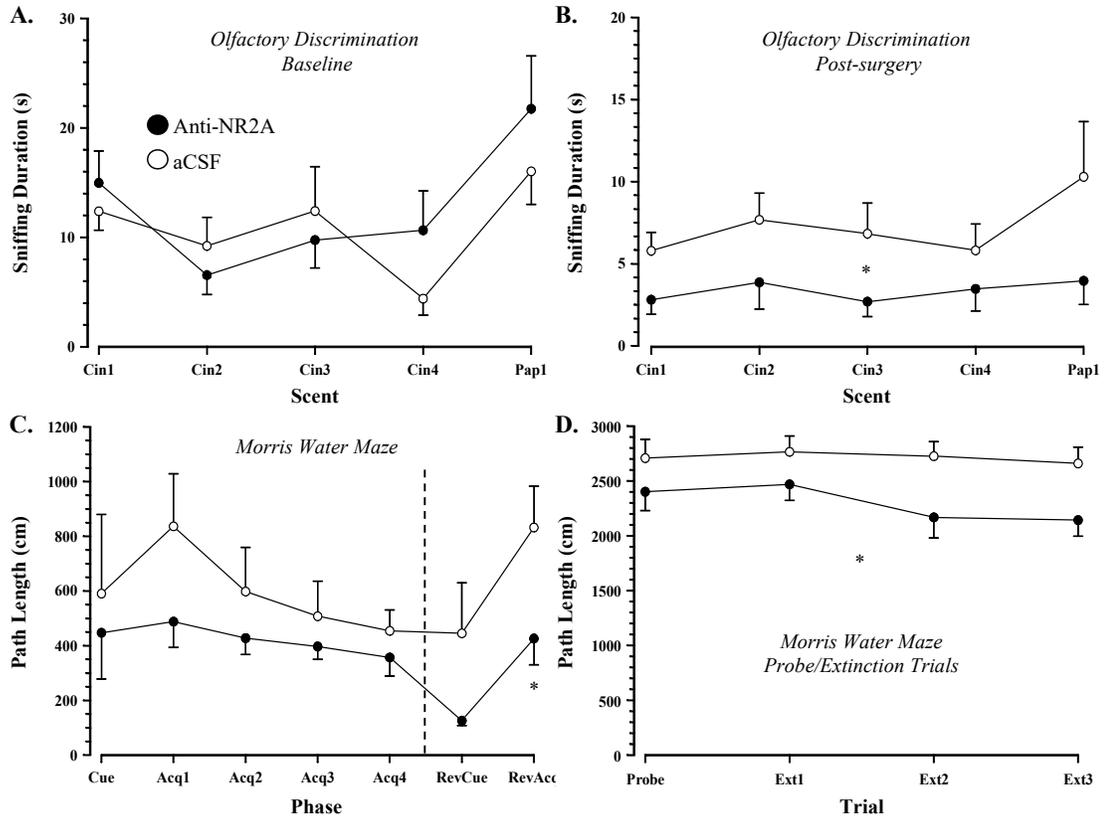
**Figure 5.5 - Home-cage behaviour in INBEST post-surgery**



**Figure 5. Home-cage behaviour in INBEST post-surgery.** (A) In comparison to control mice, experimental animals exposed to NP-SLE CSF (A) drank less water and (B) consumed less food during the testing period (Days 4 – 16). Coinciding with these changes, they also show impaired activity, as exemplified by (C) diminished running

wheel rotations, **(D)** reduced time spent in the running wheel, **(E)** prolonged stay in the shelter, and **(F)** decreased ambulation in the home-cage environment. The results are consistent with notion that NP-SLE CSF induces diverse changes in several motivated behaviours.

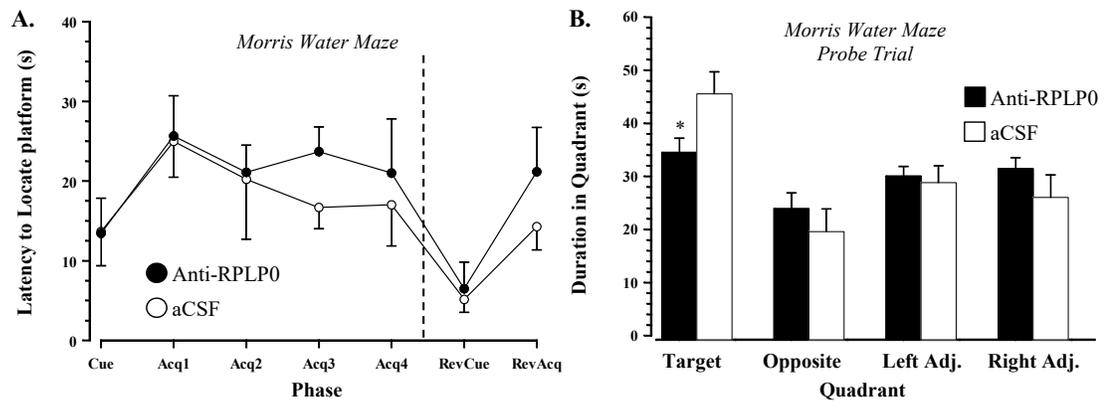
**Figure 5.6 - Behavioural effects of anti-NR2A administration**



**Figure 6. Behavioural effects of anti-NR2A administration.** (A) During baseline assessment, both treatment groups performed comparably when exposed to cinnamon and paprika in the olfactory discrimination task. (B) Post-surgical infusion of anti-NR2A antibodies resulted in a significant reduction in time spent exploring both scents across all trials, compared to mice that were administered only aCSF. These results suggest anti-NMDAR treatment attenuates responsiveness to previously-exposed scents. (C) Spatial learning / memory assessment in MWM revealed similar performance outcomes in cued and hidden versions of the test. However, when the platform was relocated to the opposite quadrant, mice administered anti-NR2A antibodies exhibited significantly

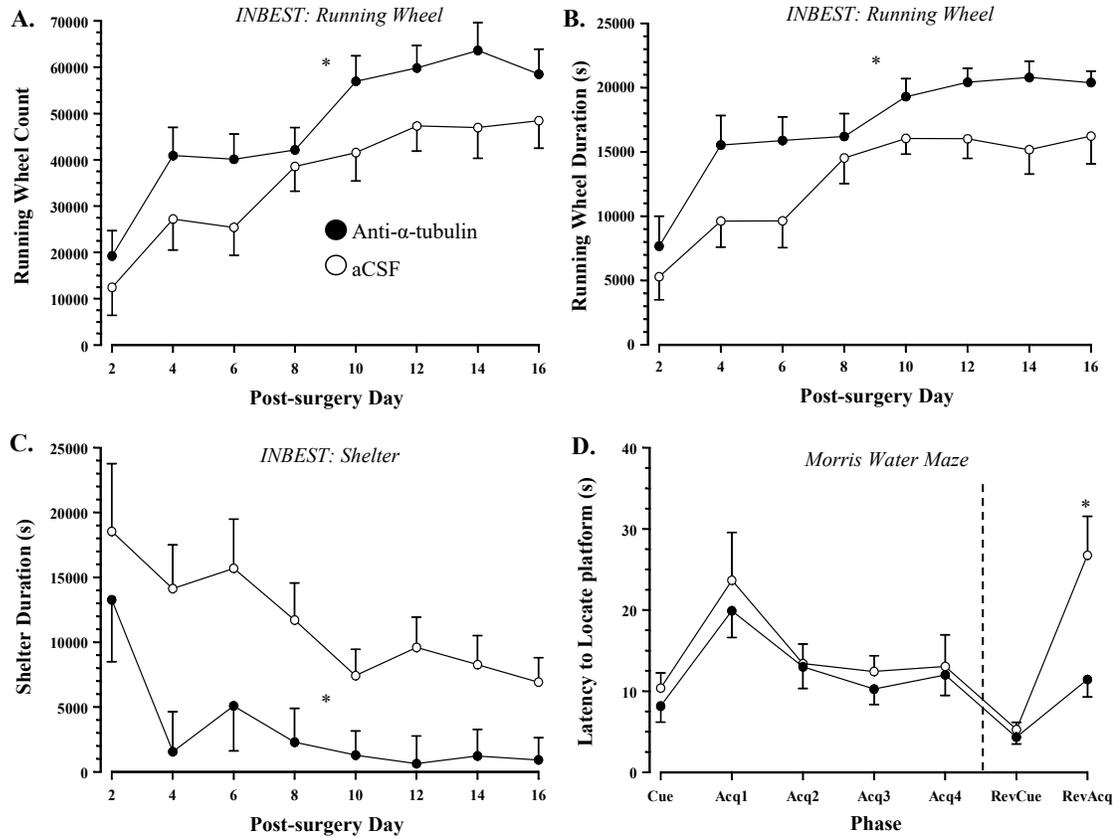
shorter path lengths to locate it. **(D)** They also swam shorter distances in an empty pool devoid of a platform during probe and extinction trials. Taken together, anti-NMDAR administration did not seem to impair performance in the MWM as previously described [64]. Rather, our results point to improved performance in these mice, particularly when tasked to reach a relocated, hidden platform in spatial reversal trials. *Note: Graphs exclude data from two mice (one from each treatment group) that consistently failed to find the platform.*

**Figure 5.7 - Behavioural effects of anti-RPLP0 administration**



**Figure 7. Behavioural effects of anti-RPLP0 administration.** (A) When exposed to the MWM, no group differences were detected in the latency to locate a cued or hidden platform in acquisition and reversal trials. (B) However, ARPA-treated mice displayed poorer memory for the platform location when it was removed from the pool during probe trials. The results suggest that ARPA infusion into the brains of healthy animals alters spatial search strategy in the MWM. *Note: Eight animals had to be excluded from the analysis of the probe and extinction trials due to a technical error.*

**Figure 5.8 - Behavioural effects of anti- $\alpha$ -tubulin administration**



**Figure 8. Behavioural effects of anti- $\alpha$ -tubulin administration.** (A) Infusion of anti- $\alpha$ -tubulin antibodies increased home-cage activity, as measured by elevated running wheel counts, (B) more time spent in the running wheel, and (C) less time spent in the shelter. (D) In the MWM, mice had similar latencies to locate cued and hidden platforms. However, relocation of the platform resulted in significantly shorter escape latencies in mice treated with anti-cytoskeletal antibodies. Coupled with home-cage behaviour, MWM results suggest that sustained administration of anti- $\alpha$ -tubulin antibodies has a beneficial, stimulatory effect on certain behaviours.

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## **Chapter 6: Summary of findings and general discussion**

This dissertation set out to further our understanding of the nature and pathogenic mechanisms underlying behavioural dysfunction in murine forms of lupus-like disease. This was accomplished in a series of experiments which specifically examined (1) olfactory function in the MRL model alongside disease progression, (2) the behavioural and immunological outcomes of chronic immunosuppression in lupus-prone MRL/lpr mice as it pertains to olfactory function, and (3) the effects of sustained intracerebroventricular autoantibody-rich CSF administration on olfaction and other behaviours in asymptomatic, healthy mice. The central hypothesis tested in all three studies was that autoimmunity in general, and autoantibodies in particular, alter olfactory function in lupus-prone mice, thus accounting for performance deficits in other domains of behaviour. Taken together, the results described in the thesis largely support this hypothesis.

In the first study, we investigated whether spontaneous development of autoimmunity in MRL/lpr mice is associated with impairments in odor-guided behaviours. We noted that lupus-prone males spent less time exploring unfamiliar conspecifics and exhibited age-dependant alterations in responsiveness to low concentrations of attractant and repellent odors. Aberrant behaviour in tests of olfactory function coincided with a skewed distribution of DCX<sup>+</sup> cells within the RMS of MRL/lpr brains. The findings suggest that the onset of systemic autoimmunity in MRL mice produces morphological abnormalities in periventricular regions, including in the migration of neuronal precursor cells from the RMS toward the olfactory bulb. If so,

ensuing impairments in olfactory function may contribute to altered performance in other behavioural tasks.

In the second study, the cause-effect relationship between accelerated autoimmune processes and olfactory function was examined by comparing the performance of diseased MRL/lpr mice to animals treated with CY. The overall expectation was that prolonged immunosuppression would abolish severe signs of systemic autoimmunity and attenuate group differences in odor-guided behaviours. Indeed, we found that sustained administration of CY prevented splenomegaly and reduced serum autoantibodies in MRL/lpr males. Amelioration of autoimmune indices in treated mice was associated with improved responsiveness to an attractant scent, as well as to a novel object at ages typically associated with florid disease. Although CY treatment seemingly worsened MRL/lpr performance in olfactory tests using a repellent scent, the results nevertheless strengthened the hypothesis that systemic autoimmune disease is a causal factor in the etiology of alterations in olfactory function, as well as, other behavioural deficits in the MRL/lpr substrain.

Subsequently, the final study attempted to identify the specific autoimmune factor(s) that mediate functional changes in olfaction and other behaviours. In the first experiment, serum and CSF from NP-SLE patients was obtained and screened for autoantibodies. CSF samples containing relatively high levels of autoantibodies were then delivered directly into the brains of young, asymptomatic animals over a two-week period. Diverse alterations in behaviour were detected during the infusion of

autoimmune CSF. Robust changes were noted in the home cage-like environment and included reduced food / water intake, impaired running wheel activity, increased shelter time and decreased overall ambulation. Consistent with previous studies in lupus-prone animals, infusion of CSF from CNS lupus patients also resulted in increased immobility in the forced swim test and olfactory dysfunction in an odour discrimination task. In the second experiment, we sought to determine which antibodies present in the CSF may have accounted for the behavioural changes. Using an experimental design identical to that used to investigate the behavioural effects of autoimmune CSF, purified commercially-available antibodies were administered in a sustained manner directly into the brains of healthy mice. Comprehensive behavioural assessment revealed varied, but relatively mild functional changes following purified autoantibody infusion. We could not replicate previous findings of “autoimmune depression”, but ARPA administration produced a spatial reference memory deficit in the water-maze. Administration of an anti-NMDAR antibody reduced responsiveness to scents in the olfactory discrimination task and unexpectedly improved reversal learning in the water-maze. Mice treated with an antibody to  $\alpha$ -tubulin also showed superior water-maze learning in reversal trials and were more active in a home-cage environment. Taken together, sustained administration of NP-SLE CSF and purified autoantibodies reproduces many, but not all, manifestations of lupus-like disease in MRL/lpr mice and SLE in humans. NP-SLE CSF proved to be much more potent in eliciting behavioural changes when compared to purified antibodies, which we hypothesize may be related to concentration effects or the combined effects of

multiple BRA. Our results also suggest that certain BRA have a stimulatory effect, while other subsets act as antagonists under specific conditions.

The findings contribute to a better understanding of the nature and origin of behavioural dysfunction in the MRL model. They strengthen the validity of the MRL/lpr substrain as a useful model of lupus-like disease that recapitulates several aspects of human SLE [256], including hyposmia [56]. More specifically, the results demonstrate that the behavioural profile of autoimmune MRL/lpr mice is confounded by alterations in olfactory function that may contribute to performance deficits noted in other behavioural tasks. The findings underscore the need to refine previous interpretations of anxiety-like and/or impaired motivated behaviour in the MRL/lpr substrain, constructs that were primarily based on their increased thigmotaxis in large arenas, impaired exploration of novel objects and spaces, performance deficits in the plus-maze and step-down tests [191;193;194], and impaired inter-male aggression [197]. Indeed, the current results may help explain why assessment of anxiety-like behaviours in MRL/lpr mice have thus far yielded conflicting results [194;222;257;258]. At the same time, alterations in olfactory function are unlikely to fully account for the broad constellation of behavioural changes that also include excessive immobility in the forced swim test from an early age [194;222;257;259;260], persistent reduction in the preference for palatable solutions [195;196;208;261;262], as well as, mild impairments in tests of sensorimotor function [203;219;220] and learning / memory [192;207;263]. Given the complexity of the behavioural phenotype, one may surmise that multiple neuroimmunological pathways are involved in the etiology of aberrant behaviours in MRL/lpr mice [264]. That CY

significantly increased responsiveness to peanut butter and a novel object, in the same way it has been shown to improve other behaviours [195;207;216;218;260] points to some form of shared or converging pathogenic cascade.

Our results suggest that this pathogenic cascade involves humoral autoimmunity and the binding of BRA to highly-conserved CNS antigens across species. Indeed, a plethora of antigen-specific BRA have been implicated to varying extents in promoting aberrant behavioural and emotional manifestations in human and murine forms of lupus [70;159;177;191;222;246;257;265-268]. Sustained administration of purified antibodies against three candidate autoantigens yields diverse behavioural changes ranging from reduced responsiveness in the olfactory discrimination task and spatial reference memory deficits in the water-maze to improved performance in reversal learning and increased activity in the home-cage. The broad effects are not surprising given that auto-reactive antibodies to neuronal epitopes may alter function and viability of central neurons in a variety of ways [174;176]. More specifically, BRA can have both agonistic and antagonistic effects on neurotransmitter and ion channel receptors on the cell surface, as well as on different intracellular targets located in nuclei and organelles [128]. Whether the autoantibody-antigen interaction elicits a functional response is dependent on several factors. For example, even though 25-50% of SLE patients are positive for anti-DNA antibodies, their association with behavioural manifestations remains questionable [269]. Yet, a subset of these antibodies cross-react with neuronal NMDAR in the brain and their presence seems to strongly correlate with NP manifestations [270]. Moreover, anti-NMDAR antibodies act as positive modulators of NMDAR function *in vitro*, capable of

increasing the magnitude of excitatory postsynaptic potentials [245]. At a higher concentration, the same antibody promotes excitotoxicity by altering mitochondrial metabolism, suggesting that anti-NMDAR elicit graded cellular alterations dependent on the amount of BRA present. In contrast to anti-NMDAR, ARPA are documented to penetrate into cells and induce cellular dysfunction through inhibition of protein synthesis [271;272]. However, ARPA can also recognize a neuronal integral membrane protein, the binding of which triggers a rapid and sustained increase in calcium influx and apoptosis in cultured neurons [273]. Thus, the same auto-reactive antibody can have markedly different effects on neuronal function, based on concentration and cross-reactivity. These complex intricacies may help to explain the diversity of CNS SLE manifestations mediated by common autoimmune / inflammatory pathways. Addressing these questions, as they pertain to anti-NMDAR, ARPA, anti- $\alpha$ -tubulin and other candidate BRA, is a logical step forward in supporting a role for autoantibodies in the induction of CNS SLE.

The inability to reproduce the robust effects of NP-SLE CSF using purified BRA suggest that early dysregulation of inflammatory mediators play a crucial role in driving CNS SLE pathogenesis, well before other soluble factors like autoantibodies reach the CNS [274]. Early elevations in IL-1 $\beta$ , TNF- $\alpha$ , IL-6, and IFN- $\gamma$ , for example, facilitate the persistent hyperproduction of autoantibodies in the MRL/lpr substrain [196;234;235;275]. Moreover, serum IL-6 correlates with reduced responsiveness to palatable stimulation [276], while sustained exposure to circulating IL-6 [261] and IFN- $\gamma$  [277] impairs ingestive behaviour. Although evidence that cytokines directly induce

neurodegeneration *in vivo* remains tentative [278], they may promote the upregulation of adhesion molecules [279;280], breakdown of the BBB [199], differentiation of infiltrating leukocytes [200], initiation of the complement cascade [209;281-283] and activation of microglia [214;284] in the MRL/lpr brain. Cytokines can also have profound effects on behaviour through the activation of the HPA axis [285-289]. In particular, sustained activation of the HPA axis in MRL/lpr mice is evidenced by increased central expression of arginine vasopressin mRNA [241;290] and high levels of corticosterone [238], the latter of which may make neurons more vulnerable [291] to the effects of BRA, thus providing the basis for *bona fide* neurodegeneration and behavioural dysfunction [205-207]. However, future studies examining neuropathogenic mechanisms in the MRL/lpr substrain are required to characterize the contribution of inflammatory mediators and the neuroendocrine axes alike.

The results of this thesis support the role of autoimmunity in the etiology of olfactory dysfunction. In the first study, the onset of lupus-like disease was shown to coincide with the development of age-associated olfactory dysfunction in MRL/lpr mice. In the second study, generalized immunosuppression of lupus-prone animals abolished signs of autoimmunity, and resulted in significant improvements in responsiveness to an attractant and a novel object. In the final study, sustained administration of NP-SLE CSF and a purified anti-NMDAR antibody induced an attenuated response to scents in the olfactory discrimination task. Although, we could not replicate previous findings of olfactory dysfunction following ARPA treatment under the current experimental conditions [247-249], our results do strengthen the notion that pathogenic BRA in the

CSF have the capacity to induce changes in olfactory function and other behaviours when introduced directly into the ventricles of healthy mice. The results are intriguing in light of accumulating evidence confirming the presence of olfactory dysfunction not just in SLE, but in several neurodegenerative and developmental conditions including multiple sclerosis, Alzheimer's disease, Parkinson's disease, schizophrenia, autism and depression [292]. Some researchers have argued that varying forms of immunodysregulation in each of these conditions impairs olfactory function at an early stage, reflecting a prodrome for future NP manifestations and more severe brain damage [293]. If so, the results presented in this thesis may have indirect clinical applications and eventually assist in the development of novel diagnostic approaches to detect early CNS involvement in patients with SLE. The implications go beyond lupus, and may apply to other CNS disorders that are accompanied by olfactory dysfunction, resulting in earlier treatment options and better prognostic outcomes.

In summary, the correlational nature of clinical data has led to the necessity for animal models in which interactions between specific autoimmune phenomena and brain function can be examined in a more systematic and direct manner. This thesis has detailed the development of olfactory dysfunction alongside the disease and aging process in lupus-prone MRL/lpr mice. Certain deficits in odor-guided behaviours could be reversed by generalized immunosuppression with CY, supporting a cause-effect relationship. Direct administration of autoantibody-rich CNS SLE CSF produced robust behavioural effects including olfactory dysfunction in a discrimination task, as well performance deficits across multiple measures in a diverse behavioural battery.

Sustained infusion of purified antibodies into the brains of healthy animals led to several task-specific alterations, but failed to reproduce the behavioural profile of MRL/lpr mice or the impairments detected in healthy animals receiving undiluted CSF. As such, the studies described in this thesis support the central hypothesis that autoimmunity in general, and autoantibodies specifically, alter olfactory function in lupus-prone mice. These results suggest that the characteristics of AABS in MRL/lpr mice need refinement. A more complete understanding of the molecular mechanisms underlying olfactory dysfunction and behavioural deficits, as well as the terminal pathologic factor(s) may provide a basis for the advancement of novel diagnostic criteria and development of therapeutic interventions for hyposmic SLE patients.

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