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Circulating brain-reactive autoantibodies and behavioral deficits in the MRL model of CNS lupus

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ABSTRACT

Brain-reactive autoantibodies (BRAA) are hypothesized to play a role in the neuropsychiatric manifestations that accompany systemic lupus erythematosus (SLE). The present study tests the proposed relation between circulating BRAA and behavioral deficits in lupus-prone MRL/lpr mice. Two age-matched cohorts born at different times were used to test the relationship in the context of altered disease severity. Significant correlations between autoimmunity and behavior were detected in both cohorts. These results are the first to report correlations between behavior and autoantibodies to integral membrane proteins of brain, supporting the hypothesis that BRAA contribute to the behavioral dysfunction seen in lupus.

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1. Introduction

Almost seventy years ago, the German physician Lechman-Facius proposed the link between autoimmunity and mental illness. He observed that immunoglobulin/protein in sera and cerebrospinal fluid from psychiatric patients may react with neuronal antigens (Lehmann-Facius, 1939). The above notion did not attract significant attention until the 60s, when Fessel and Solomon published a series of reports on "macroglobulins" or "anti-brain factors" in psychotic patients (Fessel, 1962a,b,c; Fessel and Hirata-Hibi, 1963; Solomon et al., 1966, 1969). Although recent work supports the hypothesis that brain-reactive autoantibodies (BRAA) play a role in the pathogenesis of some forms of mental illness, further evidence is required to establish the cause–effect relationship (Ganguli et al., 1993; Tanaka et al., 2003; Schott et al., 2003; Margutti et al., 2006).

One of the conditions with well-documented BRAA involvement is neuropsychiatric lupus (NP-SLE or CNS-SLE), an autoimmune disorder which affects both the central and peripheral nervous systems (Carr et al., 1978; Hoffman and Sakic, 2009). It was in this context that the autoantibody hypothesis received strong impetus from the findings that sera from lupus patients and autoimmune mice contain auto-antibodies reactive with brain tissue (Martin and Martin, 1975; Bluestein and Zvaifler, 1976) and isolated neurons (Hoffman et al., 1978a,b; Harbeck et al., 1978). This hypothesis was refined by distinguishing non-pathogenic from pathogenic BRAA, or a subset that could induce neuropsychiatric manifestations (Narendran and Hoffman, 1989; Hoffman and Madsen, 1990). Subsequently, NP-SLE has been found to be frequently accompanied by increased levels of serum autoantibodies [reviewed in (Hanly, 2005)], which cross-react with diverse brain-specific and systemic antigens (Zandman-Goddard et al., 2007).

Given that the blood-brain barrier (BBB) is compromised in SLE, it is still unclear whether autoantibodies passively diffuse from peripheral blood and/or become synthesized intrathecally [reviewed in (Hoffman and Harbeck, 1989; Abbott et al., 2003)]. The importance of a breached BBB in BRAA pathogenicity has been recently confirmed in animal models. In particular, active immunization with the NR2 antigens of the NMDA receptor (Kowal et al., 2004), or passive infusion of serum with reactivity to the NMDA receptor and DNA (Kowal et al., 2006) led to learning deficits when barrier permeability was increased by systemic administration of lipopolysaccharide. Compared to this antigen-induced, acute model of CNS-SLE, the inbred strain of MRL/MpJ-Fas^{lpr}/J (MRL/lpr) mice develops systemic autoimmune disease spontaneously. Although they show ~50% mortality between 5 and 6 months of age [reviewed in (Theofilopoulos, 1992)], serological changes, such as increased levels of IL-6, can be detected even at 3 weeks (Tang et al., 1991). Similar to SLE, the murine form of SLE has a progressive and chronic time-course, which is accompanied by a constellation of behavioral deficits, operationally labeled "autoimmunity-associated behavioral syndrome", or AABS (Sakic et al., 1997a,b). At the onset of autoimmunity the deficits are most consistently noted in tasks reflective of emotional reactivity and affective behavior (Szechtman et al., 1997), while at advanced stages of lupus-like disease learning/memory deficits may emerge (Sakic et al., 1992; Hess et al., 1993). Autoimmunity-

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induced compromise of the BBB is evidenced in autoimmune mice by immunoglobulin binding in brain (Zameer and Hoffman, 2001), expression of cell adhesion molecules (Zameer and Hoffman, 2003), and infiltration of lymphoid cells into the choroid plexus (Zameer and Hoffman, 2004; Ma et al., 2006; James et al., 2006). Moreover, behavioral deficits and infiltration of immunocytes into the brain tissue coincide with markers of neuronal degeneration and brain atrophy (Sakic et al., 1998, 2000; Ballok et al., 2003a,b, 2004).

An earlier study, in which serum antibodies to Neuro-2A cells were measured, revealed that MRL-lpr mice with serum BRAA differ in behavioral performance from BRAA-negative cagemates (Sakic et al., 1993a). In particular, they moved slowly in a novel environment, groomed less, and showed increased thigmotaxis in comparison to mice that had no detectable levels of BRAA in their serum. The relevance for CNS involvement was tentative, however, because the Neuro-2A cell line is derived from peripheral nerves and surface antigens do not fully match the antigen profile on CNS neurons (Hoffman et al., 1988). In this study, we use a preparation of transmembrane proteins from normal mouse brain. We correlate indices of systemic autoimmunity, brain atrophy, and behavioral deficits in two cohorts of MRL-lpr mice that differ in disease severity. The selection of the transmembrane fraction is based on the evidence that a large set of serum BRAA from several autoimmune strains (including the MRL-lpr strain) are directed against integral membrane proteins extracted from brain homogenates (Narendran and Hoffman, 1989; Hoffman and Madsen, 1990). In addition, using Western blotting to brain homogenates and immunohistochemistry to brain sections we further test the reactivity of BRAA to CNS tissue. Behavioral measures known to reliably detect aberrant performance in diseased MRL/lpr mice were selected (Sakic et al., 1994a, 1996).

Over the past several years, however, the immunological phenotype of the MRL/lpr substrain changed to the point that autoimmune manifestations became mild and their life span significantly extended. This unexpected phenomenon (http://jaxmice.jax.org/strain/006825.html) led to the re-coding of stock #485 to stock #6825, and re-development of the original MRL-lpr population (available from The Jackson Laboratory from fall 2007). This loss of phenotype was also noted at the behavioral level, prompting a comprehensive re-analysis of the MRL/lpr model using a large cohort of diseased animals (Sakic et al., 2005a,b). Multivariate analysis of immunological, neuropathological, and behavioral data revealed that approximately 30% of MRL/lpr mice (born between 1998 and 2000) shows severe brain damage and behavioral dysfunction, however serum BRAA levels were not measured in this study and the current experiment is addressing the correlation between BRAA and brain atrophy/behavior. Therefore, as a secondary focus we also examined the relationship between BRAA and aberrant behavior in the context of declining immunological phenotype. We presently employ MRL/lpr cohorts that were produced over two subsequent years, and tested at these two points in time. The overall expectation was that regardless of diminishing autoimmune profile, increased production of serum BRAA would be associated with impaired behavioral performance in measures of overall activity, motivated behavior, and/or emotional reactivity. There have been no previous reports of a decline in behavioral dysfunction in an autoimmune substrain in parallel with a decline in autoimmune phenotype. The comparisons between these two cohorts provided us with a rare opportunity to test the causal relationship between peripheral autoimmunity and CNS involvement.

2. Materials and methods

2.1. Animals

Compared to the congenic MRL/MpJ substrain, MRL/lpr mice (both male and female) develop an accelerated form of lupus-like disease, with ~50% mortality occurring between 5 and 6 months of age [reviewed in (Theofilopoulos, 1992)]. Serological changes (e.g. increased production of IL-6) can be detected as early as 3 weeks of age (Tang et al., 1991), followed shortly by an excessive production of autoantibodies and infiltration of leukocytes into the choroid plexus (Vogelweid et al., 1991; Ma et al., 2006).

To examine whether the autoimmune phenotype indeed diminished over time, the immune markers were compared at equivalent ages, between fourteen 3–5 month-old, male MRL/lpr mice (first cohort, $N=14$, born in 2004) and forty 3–5 month-old, male MRL/lpr mice (second cohort, $N=40$, born in 2005). For correlations between variables (primarily behavior and immunologic) all animals, from 5 to 34 weeks, were used in the first cohort ($N=30$). Differences in numbers (N) between the figures was due to not being able to use some animals in the correlations or comparisons, e.g., because not all the data were available for specific animals in a group. The cohorts were maintained under comparable housing conditions and tested with the same apparatus one year apart. The 3–5 month time frame was chosen because CNS involvement and behavioral deficits can be readily detected, but potentially confounding clinical manifestations (e.g. dermatitis, lymphadenopathy, and arthritis) are not present yet.

Mice were housed 4–5 per cage, and kept under standard laboratory conditions (light period from 8 A.M. to 8 P.M., room temperature ~22 °C, humidity ~62%, regular rodent chaw, and tap water *ad libitum*, bedding changed every 3–4 days). After acclimation and habituation, they underwent behavioral testing over 3 weeks. During the behavioral testing the animals were housed individually. The sequence of behavioral tests matched the order they are described below. One test was given daily, with no overlapping.

Brains from a 2 and a 4 month-old female C3H/HeJ mice were used for Immunohistochemistry and Western blotting, respectively. Sera from three 4 month-old female C3H/HeJ mice were also used as negative controls in the immunohistochemistry. The C3H strain has no autoimmunity and is a 12% background strain for the MRL mice. We could not use MRL brain, since we have previously reported that both MRL strains have in situ bound Ig (Zameer and Hoffman, 2001), while C3H had none, and this could interfere with testing for serum BRAA binding to brain. The CNS antigens from C3H were expected to be comparable to the antigens in the MRL strains, but free of pre-bound BRAA.

2.2. Behavioral testing

The specific behavioral tests selected were based on our previous research (Sakic et al., 1993a,b, 1996, 1997a,b, 2005a,b), which has reliably shown differences between MRL/lpr mice and controls. Most of the behavioral tests are related to affective disorders, which have been reported in CNS-SLE patients.

2.3. Spontaneous nocturnal activity

Spontaneous ambulation was assessed by computerized activity monitors (AccuScan Instruments, Columbus, Ohio) from 6 P.M. to 8 A.M. During the testing period mice were taken out of home cages (4–5 mice housed per cage) and returned 14 h later. The testing room was equipped with ten activity chambers ($40\times40\times35$ cm) with ventilated lids. The chambers were interfaced with a PC computer running VersaMax software from the same manufacturer. Total distance traveled, moving time, and ambulatory speed were assessed in 30-min intervals. These assessments were designed as a basic test of motor activity, which can be used to assess the effects of disease on motor functioning, as well as to supplement information on the tests of affective behavior. “Lights on” reflected cumulative measures from 6 P.M. to 8 P.M., while “lights off” reflected cumulative score from 8 P.M. to 8 A.M. During the 14-h testing mice did not have access to food and water.

2.4. Sucrose preference test

Reduced sucrose intake in a preference paradigm is proposed to measure sensitivity to reward and model anhedonia (Willner et al., 1992). More extensive analysis of the dose-dependent performance, post-ingestive factors, and taste responsiveness has been reported in our previous studies (Sakic et al., 1996, 1997a,b). Based on an established methodology (Ballok et al., 2003a,b), mice were trained to drink 3 ml of a 4% sucrose solution from a graduated syringe fastened to the cage lid with a 2.5″ paper clip. They had 24-h access to sucrose over three days (the training period), and free access to food and water. Training and testing were performed after the activity monitoring, but before the forced swim test. The criterion for training was that the mouse empties the syringe at least once before testing. All of the mice met this criterion. The solution was then removed for 24-h to allow sugars to clear from their circulation. A 1-h sucrose preference test (between the times 20:30 and 21:30 h) was given over four subsequent nights. Each night syringes were filled with one of four sucrose solutions, presented in ascending order (i.e., 1, 2, 4, or 8%). The volume ingested over three trials (a trial being the 1-h period) was used as an index of responsiveness to palatable stimulation.

2.5. Forced swim test

Increased immobility of rodents in a no-escape situation was proposed to reflect a state of lower "mood", which can be reduced by antidepressants and electroconvulsive shock (Porsolt et al., 1977). It is a good complement, in testing affective behavior, to the sucrose preference test. Presently, a mouse was gently lowered into a circular pool filled with 25 °C water and allowed to swim for 10 min (Sakic et al., 1994a). The time spent in floating was measured by EthoVision XT video tracking software (Noldus, NL). Floating was defined when swimming speed was less than 5 cm/s.

2.6. Tissue collection and indices of autoimmune disease

Mice were anesthetized with an intraperitoneal injection of Somnotol (65 mg/kg) and body weight was measured on a digital scale (Sartorius 2024 MP, VWR, Scientific Canada Ltd.). Blood samples were collected by cutting the vena cava and exsanguinating (approximately 1 ml) within 10–15 s using a needle-free syringe, then left to coagulate for 1 h at 4 °C, and centrifuged for 3 min at 7000 rpm. Serum was separated from blood clots, aliquoted and plastic vials (containing 100 µl of serum/mouse) shipped on dry ice by overnight courier service for further analysis. Blood vessels were flushed with intracardial phosphate buffered saline (~40 ml), brain was extracted within 2 min, and weighed on an analytical scale (AB54-S, Mettler Toledo, Switzerland). Reliable signs of systemic autoimmune disease in MRL-lpr mice are splenomegaly and high serum levels of autoantibodies. Therefore, wet spleen weight was measured on an analytical scale immediately upon extraction. Serum levels of anti-DNA antibodies were measured by ELISA, as described below.

2.7. Integral membrane protein preparation

The protocol used for the extraction of the integral membrane proteins used in the ELISA and Western blotting techniques was previously described by (Narendran and Hoffman, 1988). Briefly, the integral membrane proteins were suspended in phosphate buffer saline (PBS), if being used for ELISA, or 1.0 M Tris (pH 6.8) if being used for Western blotting. The concentration of the integral membrane proteins used for the ELISA was determined using the BCA Assay Kit (Pierce, USA) and the Bradford Test (Sigma-Aldrich, USA) when doing Western blots.

2.8. ELISA for anti-dsDNA and BRAA

The procedure for determining anti-dsDNA and BRAA levels were performed using previously described protocols (Aotsuka et al., 1979; Crimando and Hoffman, 1995; Zameer and Hoffman, 2003) and only brief descriptions, plus differences from the norm, are given here. To test anti-dsDNA antibody levels poly-L-lysine (Sigma, USA) was used to coat the 96-well plates. The odd wells of the plates were incubated with 10 µg/ml of calf-thymus DNA (Sigma, USA; purified for dsDNA), while the even wells received only PBS (paired control wells). One hundred µg/ml of poly-L-glutamate (Sigma, USA) dissolved in PBS was added to the plates and thereafter PBS containing 5% bovine serum albumin (BSA) (Sigma, USA). The plates were incubated with serial dilutions of mouse sera and then incubated with the secondary antibody goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) diluted at 1/1000 in PBS. Lastly citrate buffer containing 2,2′-azino-bis(3ethylbenzthiazoline) sulfonic acid and hydrogen peroxide was added for 30 min and incubated at 37 °C. The plates were then read at 405 nm on a microplate reader. The data was corrected for background binding by subtracting the optical density (OD) values for the paired control wells from the OD values for the wells containing the calf-thymus DNA, which is known as the S-value. The S-value was used for the statistical analysis. Due to correcting for background binding, S-values were allowed to be negative because occasionally the binding in the control well was greater, likely due to high levels of immunoglobulins in the mouse serum. This helps deal with a lot of the variability that occurs in autoimmune mice. Since this calculation was performed on all samples, the binding intensity in individual samples was relative to each other.

S-values were also calculated in the same way for the serum BRAA. We used 10 µg/ml of integral membrane proteins from a brain homogenate of healthy C3H/HeJ mice. The plates were treated with 5% BSA to reduce background reactivity and serial dilutions of the mouse sera were added to the wells. The secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA) was diluted at 1/1000 in PBS. The plates were read at a wavelength of 405 nm on a microplate reader.

2.9. Immunoblot analysis

We further tested the serum samples from the mice using immunoblotting techniques we have previously described (Narendran and Hoffman, 1989; Hoffman and Madsen, 1990) to determine the apparent

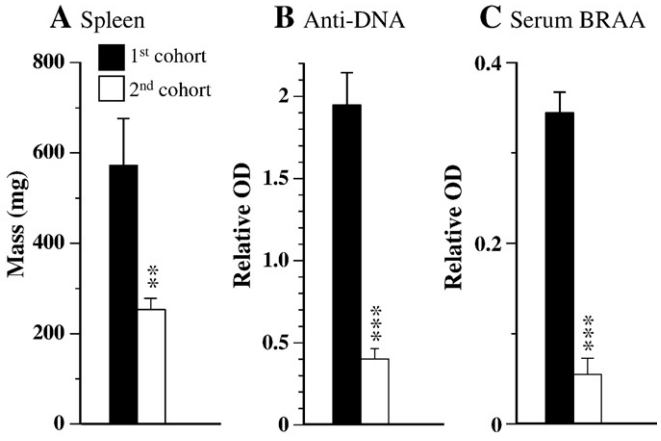


Fig. 1. Differences in spleen weight, anti-DNA autoantibody and BRAA levels between the first ($N=14$) and second cohorts ($N=40$) confirmed a production year-dependent decline in autoimmune phenotype. The mean ± 2 standard errors of the mean are shown. The serum dilutions shown were at 1:400 for the anti-DNA autoantibodies, and 1:25 for the first cohort and 1:20 for the second cohort BRAA. There were statistically significant differences between the groups ($^{**}p=0.01$, $^{***}p=0.001$).

Table 1
Significant partial correlations (controlling for age and/or body weight) for behavioral and immunological variables measured in the first cohort.

Behavioral / Immune Variables (N = 30)	Immune/Organ Weight Variables			
	Serum BRAA	Serum anti-DNA	Spleen Weight	Brain Weight
4% Sucrose consumption	−0.348*	−0.209	−0.352*	0.430*
Average speed (lights off)	−0.399*	−0.203	0.108	0.083
Distance traversed (lights off)	−0.140	−0.366*	−0.299	0.469**
Movement time (lights off)	−0.081	−0.352*	−0.318	0.461**
Brain weight	−0.123	−0.148	−0.699***	
Serum anti-DNA	0.550**			
Spleen Weight	0.231	0.015		

One tailed test; 5–34 weeks.
Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

molecular weight of antigens to which BRAA are binding. Briefly, a polyacrylamide gel was prepared using a 12% resolving gel layer and a 4% stacking gel layer. The stacking gel was then loaded with 0.668 μg/μl of integral membrane proteins from a 4 month C3H/HeJ mouse. The gel was then transferred to nitrocellulose paper (BioRad Laboratories, CA) and then cut into strips and incubated with blocking solution containing TBS, 0.1% Tween-20, 5% albumen, and 1% casein (Sigma). Serum samples from the mice were diluted and added to the strip of nitrocellulose paper overnight. The secondary antibody, goat anti-mouse IgG antibody conjugated with peroxidase (Caltag, USA), was diluted at 1/5000 and the blots were then incubated with a detection solution from a Chemiluminescence Kit (Roche, USA). The blots were exposed to the X-ray film (Kodak, New York) for 1, 5, and 10 min and then processed. The molecular weight (MW) of the bands was determined from the graph with known MW markers and the distance that a given band traveled. Some strips were incubated with goat anti-mouse NK-1R antibody (Zymed, USA) as a positive control.

2.10. Immunohistochemistry

Based on previous techniques of ours (Hoffman et al., 1978a,b; Zameer and Hoffman, 2001), brains from 2 month-old C3H/HeJ mice were blocked by cutting the entire brain into 3 blocks at an angle of 68° from the table top and freezing in Tissue Tek OCT Compound (Sakura, USA) using dry ice and 2-methyl butane. These were then cut on a cryostat into 8 μm sections and heat-fixed onto a microscope slide and acetone-fixed. Subsequently, the slides were immersed in 50 °C citrate buffer containing 1.92 g/l of citric acid at a pH = 6.0 to better expose the epitopes for antibody binding on the brain sections. A 1.5% blocking solution containing PBS and BSA was added to the sections and mouse sera at a 1/10 dilution was then added overnight at 4 °C. After washing, the secondary antibody, FITC-conjugated goat anti-mouse IgG (Caltag, USA), which had been treated with rabbit liver powder, was added to the brain section at a 1/10 dilution. A 1/10 dilution of propidium iodide (to stain the DNA in the cells) to Fluoroguard (to prevent fading of the fluorescence being detected) was added to the sections. Negative controls included slides without primary (i.e., sera) or secondary antibody (controlling for auto-fluorescence), as well as slides without

Table 2
Significant bivariate correlations for behavioral and immunological variables measured in the second cohort.

Behavioral / Immune Variables (N = 39)	Immune/Organ Weight Variables			
	Serum BRAA	Serum anti-DNA	Spleen weight	Brain weight
Floating time	0.463**	0.240	0.308*	0.015
4% Sucrose consumption	0.031	−0.324*	−0.330*	0.274*
Serum anti-DNA	0.358*			0.061
Spleen weight	0.504**	0.633***		0.006

Significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

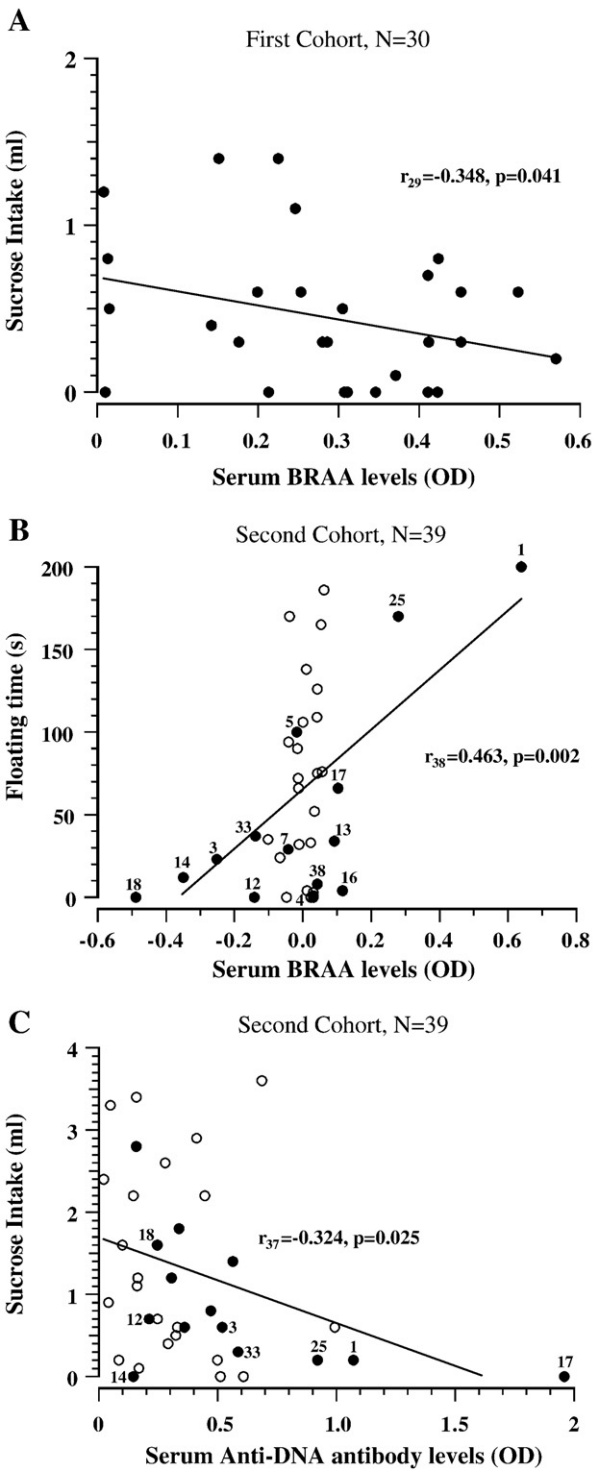


Fig. 2. A. Negative correlation between performance in the sucrose test and serum BRAA levels (1:25 dilution), suggesting that decreased 4% sucrose consumption is associated with high levels of serum BRAA ($r_{29} = -0.348$, $p = 0.041$). This figure shows data from the mice in the first cohort. B. Positive correlation between performance in the forced swim test and serum BRAA levels (1:80 dilution), suggesting that increased immobility is associated with high levels of serum BRAA ($r_{38} = 0.463$, $p = 0.002$). This figure shows data from the mice in the second cohort. Solid symbols denote mice whose sera were used for Western blotting and immunohistochemistry. C. Negative correlation between performance in the sucrose test and serum anti-DNA antibody levels (1:400 dilution), suggesting that decreased 4% sucrose consumption is associated with high levels of serum anti-DNA antibodies ($r_{37} = -0.324$, $p = 0.025$). This figure shows data from the mice in the second cohort. Solid symbols (B and C) denote mice whose sera were used for Western blotting and immunohistochemistry.

primary antibody but having the secondary antibody (as a control for non-specific, secondary antibody binding). Pictures of the cortex and the hippocampus were taken at 100× objective magnification. The brain sections were analyzed using confocal microscopy, using the Keck Lab facilities at Arizona State University.

2.11. Statistical analysis

The Student's *T*-test was used for all simple comparisons between cohorts (Fig. 1). Correlations (Tables 1 and 2, and Fig. 2A, B and C) between the immunological (BRAA, anti-DNA and spleen weight) and neurobehavioral variables (forced swim test, 4% sucrose consumption, activity measures with lights on and off, for distance travelled, movement time and speed, plus the brain weight) were also done for both cohorts. More specifically, linear relationships between scale variables were analyzed by partial correlation, with age and/or body weight as controlling factors, for the first cohort and bivariate correlations for the second cohort. Given that the direction of relationships was known *a priori*, one-tailed significance was used in the overall analysis. Fig. 1 shows mean values ± SEM. Significant differences of $p \leq 0.05$, $p < 0.01$ and $p < 0.001$ are indicated by *, **, and ***, respectively, in the figures. All computations were performed using the SPSS 15 statistical package.

3. Results

3.1. Loss of autoimmune phenotype

As expected, males from the first cohort had bigger spleens ($t_{52} = 4.238$, $p < 0.01$, Fig. 1A) and higher levels of (a 1:400 dilution for both cohorts) anti-DNA antibodies ($t_{49} = 10.012$, $p < 0.001$, Fig. 1B) than animals from the second cohort. Similarly, serum BRAA levels (done at closely comparable dilutions of 1:25 in the first cohort and 1:20 in the second cohort) were higher in mice from the first cohort ($t_{50} = 8.881$, $p < 0.001$, Fig. 1C). These results are consistent with the expectation that the autoimmune phenotype of the MRL/lpr substrain diminished over two years.

3.2. The relationship between immune measures, brain size, and behavior

3.2.1. First cohort

The same tests and correlations were performed on both cohorts, only those correlations which were significant are shown in Tables 1 and 2. It should be noted that there are 8 significant correlations to behavior in the first cohort and only 5 in the second cohort, consistent with the reduced autoimmune phenotype. Significant relationships obtained by partial correlations for the first cohort are shown in Table 1. Consistent with the hypothesis that BRAA play a role in the etiology of behavioral dysfunction, increased serum BRAA levels (1:20 dilution) were associated with impaired 4% sucrose consumption, ($r_{29} = -0.348$, $p = 0.041$; Fig. 2A) and reduced speed during spontaneous nocturnal ambulation ($r_{29} = -0.399$, $p = 0.022$). The latter result is consistent with our earlier report, in which serum levels of antibodies to the Neuro-2A cell line correlated with low ambulatory speed and increased anxiety-related behaviors (Sakic et al., 1993a).

As one may expect, increasing levels of anti-DNA autoantibodies (a serological marker of disease activity) were associated with increasing BRAA levels ($r_{29} = 0.550$, $p = 0.002$). More importantly, increased anti-DNA autoantibody levels correlated with shorter distances traveled at night ($r_{24} = -0.388$, $p = 0.025$), as well as with shorter movement times ($r_{30} = -0.366$, $p = 0.033$). As observed in our previous studies (Sakic et al., 2005a,b; Ma et al., 2006;Ballok et al., 2006), mice with bigger spleens had lower brain weights ($r_{30} = -0.699$, $p < 0.001$). Conversely, increased brain weight positively correlated with distance traveled ($r_{30} = 0.469$, $p = 0.008$), movement time at night ($r_{30} = 0.461$,

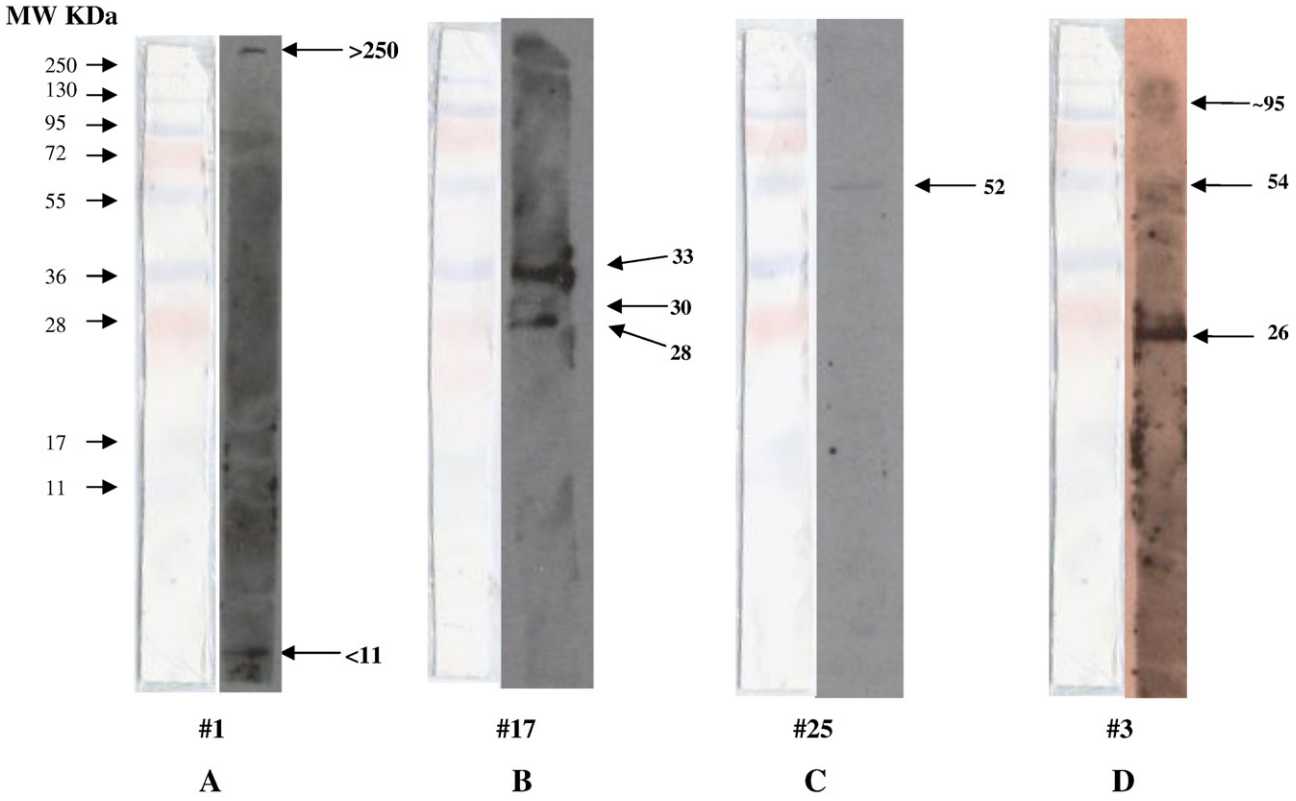


Fig. 3. Representative Western blots of MRL-lpr sera (A–D), corresponding to high or moderate float time animals 1, 17, 25 and low float time animal 3 (see Fig. 2B, from the second cohort), reactive with integral membrane proteins from brain of a non-autoimmune mouse. Different brain antigens were detected and their number or specificity did not match serum BRAA levels measured by ELISA. (Also see Table 4.)

Table 3
Molecular weights of Western blot bands (second cohort).

Mouse ID	Band MW (kDa)
1	>250, 12
3	54, 26
4	48
5	65
7	68, 59, 43
13	47
14	42
16	48
17	33.3, 30, 28
25	52
37	38

$p = 0.009$), and volume of 4% sucrose solution consumed ($r_{30} = 0.430$, $p = 0.014$). Splenomegaly was associated with reduced consumption of 4% sucrose ($r_{30} = -0.352$, $p = 0.039$).

3.2.2. Second cohort

Since the direction of relationships has been established previously, a one-tailed test of significance was accepted in the bivariate correlation analysis (selected data summarized in Table 2). Increased floating time in the forced swim test was associated with higher BRAA levels, tested at a dilution of 1:80 ($r_{38} = 0.463$, $p = 0.002$; Table 2 and Fig. 2B). Similar to the first data set, BRAA (1:80) levels correlated with splenomegaly ($r_{38} = 0.504$, $p = 0.001$) and anti-DNA autoantibody levels ($r_{37} = 0.358$, $p = 0.015$), indicating a correlation to disease activity.

Anti-DNA autoantibody levels and splenomegaly correlated positively ($r_{37} = 0.633$, $p < 0.001$), further supporting the relationship between serological markers and organ pathology in lupus-like disease. Increased consumption of 4% sucrose ($r_{37} = -0.324$, $p = 0.025$; Fig. 2C) correlated negatively with increased levels of anti-DNA autoantibodies, suggesting an impaired motivated response in mice with severe systemic autoimmunity. Further support for this notion came from the negative correlations between spleen weight and 4% sucrose consumption ($r_{38} = -0.330$, $p = 0.022$), and the positive correlation between splenomegaly and floating time ($r_{38} = 0.308$, $p = 0.03$). Lastly, brain weight correlated positively with increased 4% sucrose consumption ($r_{38} = 0.274$ $p = 0.048$), suggesting that mice with brain atrophy due to autoimmunity were also poor responders to a palatable stimulation.

3.3. Western blot analysis

To characterize BRAA on the basis of molecular weight of the antigen bound, we used the remaining sera for the Western blot analysis. Those mice showing the greatest immobility in the forced swim test and the highest BRAA levels was used in the initial analysis. Fig. 3 illustrates BRAA banding patterns (and their approximate molecular weights) in these mice (samples 1, 17 and 25). For the mice that showed low BRAA levels and low float times (far left, Fig. 2B), three out of the five samples (i.e. #12, #18, and #33) had no bands. Bands were, however, detected in sample #3 (Fig. 3D) and sample #14, suggesting that sensitivity and specificity of Western blotting and ELISA were different.

Bands were detected as listed in Table 3. No bands were detected in sample numbers 2, 6, 8–10, 12, 18–24, 26, 29, 30, 32–35, 38, and 39. Samples 11, 15, 27, 28, 31, 36, and 40 were not tested due to an insufficient amount of sera. As previously reported (Narendran and Hoffman, 1989), there was a diversity of antigens bound by the BRAA.

3.5. Immunohistochemistry

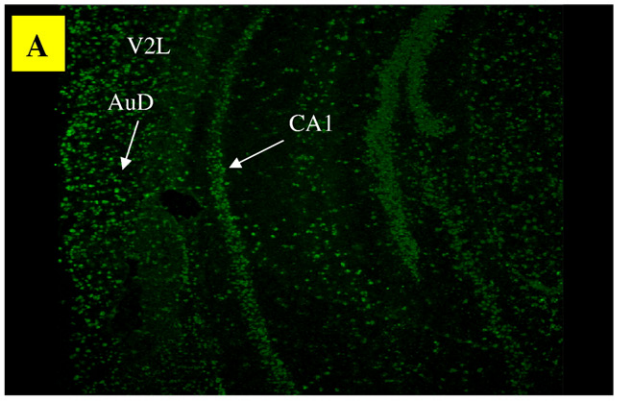
Immunohistochemistry was also performed using sera from eight MRL/lpr (the three samples with high float times and five samples with low float times in Fig. 2B) and three non-autoimmune C3H/HeJ mice, our normal age-matched control strain. Red propidium iodide (PI) staining (of DNA) was used to show cells, while the antibodies were detected using a green fluorescein isothiocyanate (FITC) stain, as described in the Materials and methods. In Fig. 4, all the figures on the right correspond to dual staining using both PI and FITC and all the figures on the left are showing staining with FITC only. No binding was seen in any of the controls where no primary (sera) or secondary (anti-Ig) was added, or where secondary antibody was added only, or in the C3H serum samples tested (Fig. 4C and 4D). Binding to brain sections was observed in all of the eight MRL/lpr samples, not distinguishing between mice with high or low BRAA levels (as determined by ELISA). Moderate binding to the hippocampus and the cortex (Fig. 4A,B) was seen in serum from mouse #1, who has shown excessive floating, high ELISA BRAA levels, and BRAA positivity on Western blots. Fig. 4E and F shows moderate binding to the cortex for sample #33, however binding was also seen in the hippocampus for this animal (data not shown). Fig. 4G and H shows moderate binding in the hippocampus for sample #18. These mice (sample #33 and #18) showed low floating time, low BRAA levels and were negative for brain antigens on Western blotting. These results clearly show that there are autoantibody to brain in the sera of the MRL/lpr mice, but also suggest that the levels of BRAA detected in the sera by ELISA, or immunoblotting and the intensity of their binding to the brain (as determined by immunofluorescence) do not necessarily correspond with one another. The sample from mouse #12 (data not shown) further illustrated this lack of correspondence because in comparison to sample #1 (Fig. 4A and B), a stronger binding to the hippocampal region and throughout the brain was observed, even though this mouse had low BRAA levels, low float time and was negative by Western blotting.

What is interesting, however, is that mice #1, #12 and #33, even though they vary in float times, show low sucrose consumption, often considered to reflect a dysfunctional reward circuitry. This suggests that the immunohistochemistry is more sensitive in the detection of functionally important BRAA, likely because targeted brain antigens are not denatured as much as they are in ELISA and Western blotting. It is important to keep in mind that any of these methods for detecting BRAA can be valid and detect different autoantibodies, but will not necessarily correspond to one another.

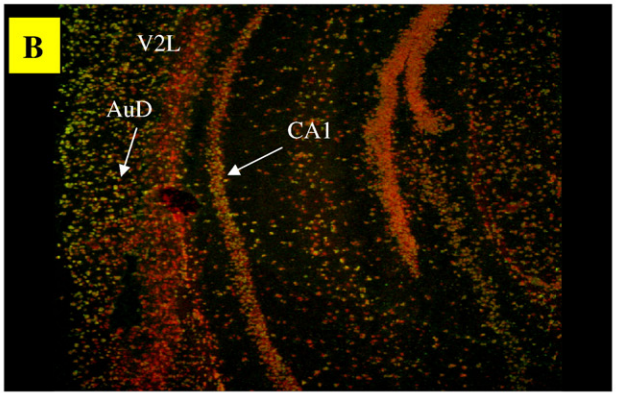
Table 4 shows a comparison of data from the 8 MRL/lpr mice (from the second cohort) used for immunohistochemistry. The first 3 animals have high BRAA levels as determined by ELISA and also showed banding by the Western blot. These mice all showed abnormally high float times, indicative of depressive-like behavior. The other 5 mice had low ELISA BRAA levels, but were positive by immunohistochemistry. Some showed

Fig. 4. Representative images showing binding between serum BRAA and brain sections from healthy C3H/HeJ mice by immunohistochemistry. The green fluorescence (FITC) shows antibody–antigen binding, while the red color shows propidium iodide (PI) staining of the cell nucleus which allows for easier identification of brain structures. Dual staining with PI and FITC is shown in B, D, F and H, i.e., all the figures on the right. A, C, E and G shows the BRAA green fluorescence only, using FITC, i.e., all the figures on the left. (A) Hippocampus and cortex showing only green fluorescence after exposure to serum from MRL-lpr mouse #1. (B) Hippocampus and cortex showing both green and red fluorescence after exposure to serum from MRL-lpr mouse #1. The combination staining appears as yellowish/green. (C) Lack of binding in the hippocampus and cortex using a serum from a non-autoimmune C3H/HeJ mouse with green fluorescence only. No staining indicates no BRAA binding. (D) Red and green fluorescence showing binding in the hippocampus and cortex using a serum from a non-autoimmune C3H/HeJ mouse. Only the red PI staining appears, indicating lack of BRAA binding. (E) Green fluorescence in the cortex only, obtained with serum from MRL-lpr mouse #33. (F) Green and red fluorescence in the cortex only, obtained with serum from MRL-lpr mouse #33. (G) Green fluorescence in the hippocampus only, obtained with the serum sample from MRL-lpr mouse #18. (H) Green and red fluorescence in the hippocampus only, obtained with the serum sample from MRL-lpr mouse #18. Abbreviations: CA, hippocampal regions; AuD, secondary auditory cortex, dorsal area; V2L, secondary visual cortex, lateral part. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3-4 Months MRL/lpr #1 serum, FITC staining

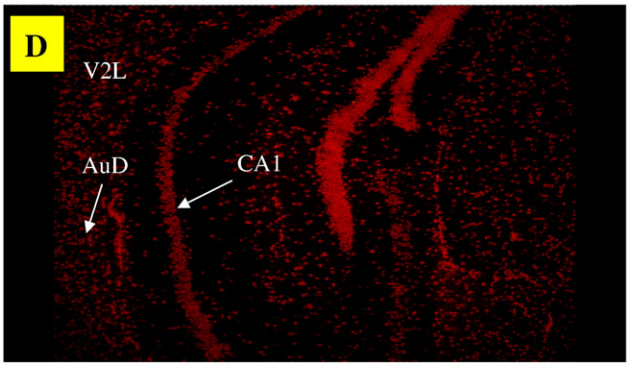


3-4 Months MRL/lpr #1 serum, FITC and PI staining

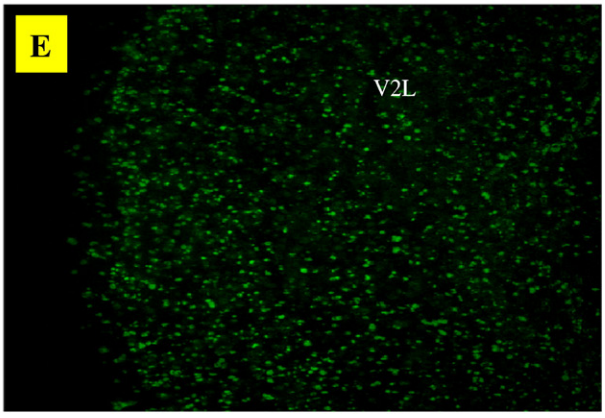


4 Months C3H/HeJ serum, FITC staining

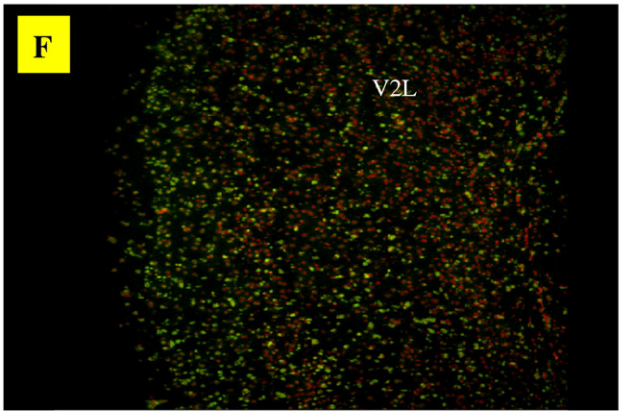
4 Months C3H/HeJ serum, FITC and PI staining



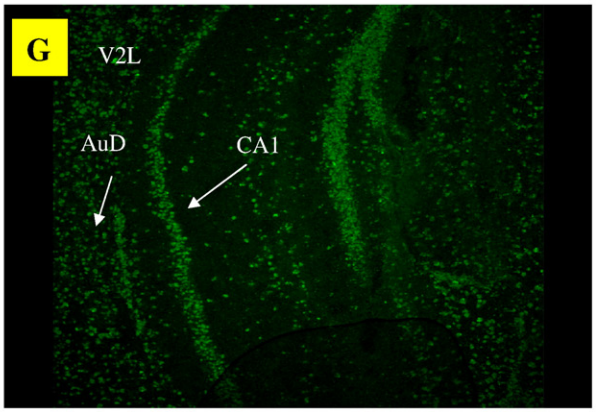
3-4 Months MRL/lpr#33 serum, FITC staining



3-4 Months MRL/lpr#33 serum, FITC and PI staining



3-4 Months MRL/lpr#18 serum, FITC staining



3-4 Months MRL/lpr#18 serum, FITC and PI staining

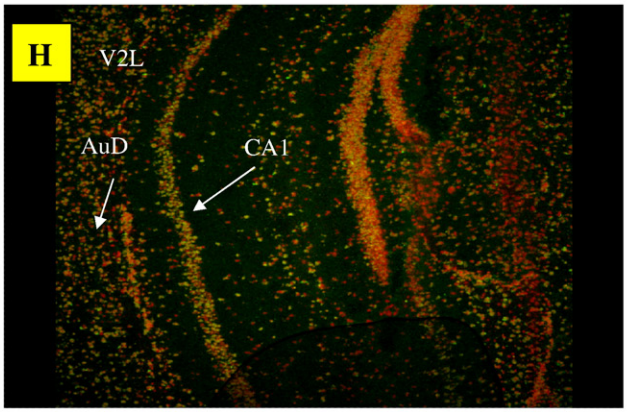


Table 4
Comparisons from second cohort.

Mouse number	Forced swim test	Sucrose test	Western blot number of bands (molecular weight)	Immunohistochemistry	BRAA levels
1	High float time	Low consumption	2 (>250, 12)	+	High
17	High float time	Low consumption	3 (33.3, 30, 28)	+	High
25	High float time	Low consumption	1 (52)	+	High
3	Low float time	Low consumption	2 (54, 26)	+	Low
14	Low float time	Low consumption	1 (42)	+	Low
12	Low float time	Low consumption	0	+	Low
33	Low float time	Low consumption	0	+	Low
18	Low float time	Moderate consumption	0	+	Low

Western blot bands, while others did not. All had anhedonia as determined by the relatively low sucrose consumption.

4. Discussion

Our previous studies have shown that behavioral changes and disease-dependent overproduction of BRAA occur in parallel in the MRL/lpr substrain (Hoffman and Madsen, 1990; Zameer and Hoffman, 2001; Sakic et al., 1994b, 2005a,b). The levels of circulating BRAA peak around 3 months of age (Hoffman et al., 1987) with parallel deficits in spatial learning and memory (Sakic et al., 1993b). In addition, circulating autoantibodies to neuroblastoma cells were associated with changes in locomotor activity in a novel environment, short grooming episodes, and enhanced thigmotaxis (Sakic et al., 1993a). A neuroblastoma cell line does not, however, reflect the antigenic spectrum of a normal mouse brain. In addition, paradigms reflective of motivated behavior were not employed in these early behavioral studies. For the first time, the present study shows a consistent set of correlations between circulating BRAA to native brain antigens (as confirmed by Western blot and immunofluorescence) in the context of autoimmune phenotype (both declining and natural). As such, it provides further evidence of the importance of naturally occurring, peripheral BRAA in the etiology of structural and functional CNS damage during lupus-like disease.

It was found, over a ten year period, that there was a declining autoimmune phenotype in MRL/lpr mice (http://jaxmice.jax.org/strain/006825.html) produced in The Jackson Laboratories. This phenomenon of unknown etiology allowed us to test the hypothesis that behavioral abnormalities could occur even in weak autoimmune disease and correlate with BRAA. The present study confirms the declining phenotype and extends this by showing a cohort-dependent decrease in splenomegaly, serum autoantibody levels, and their declining association with behavioral deficits. Nevertheless, associations were found in both cohorts (with mild and more severe autoimmune disease), supporting the hypothesis that circulating BRAA alter behavior, even with fading autoimmunity. The fact that there were fewer correlations between behavior and immunological parameters in the second cohort is consistent with our expectation, due to declining autoimmune phenotype in this cohort. We would also expect differences in correlations between the two cohorts, as were seen, due to the differences in autoimmune phenotype. Although these correlations suggest a link between BRAA and behavioral alterations, they do not prove a causal connection. Other researchers have supplemented this by using passive or active BRAA transfer to specific antigens (Kowal et al., 2004; Lawrence et al., 2007; Mondal et al., 2008). Given the diversity of behavioral manifestations in NP-SLE and AABS, as well as the results of our current study, it is expected that a variety of BRAA account for deficits in different domains of behavior.

In the first cohort high BRAA levels were associated with low sucrose consumption and low ambulatory speed. In addition, high anti-DNA autoantibody levels were a predictor of attenuated locomotor activity. Finally, splenomegaly was a predictor of reduced sucrose consumption and increased immobility. It should also be noted that speed, distance traveled, and movement time reflect the same aspect of behavior, viz., reduced locomotor activity. Correlations between BRAA and ambulatory speed, and anti-DNA and ambulation time and distance, may reflect an overlapping of autoantibody action. Taken together, these correlations suggest that more severe lupus-like disease is associated with the emergence of behavioral dysfunction, largely in the domain of locomotion and “affect”. They also suggest that autoimmune factors different from BRAA (e.g., cytokines, immune complexes, complement components, and vascular pathology) affect behavior. The relationship between brain weight and sucrose consumption/movement suggests that the reduction in brain mass affects behavioral performance of autoimmune animals. The negative correlation between brain and spleen mass further supports the possibility that brain growth is retarded during progression of lupus-like disease (Sakic et al., 2005a,b; Ma et al., 2006; Ballok et al., 2006). The second cohort confirmed the relationship between BRAA and behavior, but likely due to the declining autoimmune phenotype, fewer correlations were observed. Although the relationships were not identical to the pattern seen in the first cohort, they were consistent with the notion that circulating BRAA alter emotional reactivity by inducing functional deficits in the limbic system (Sakic et al., 1994a). Taken together, the above results suggest that the emergence of lupus-like manifestations is associated with brain atrophy and impaired exploration/motivated behavior in MRL/lpr mice. Importantly, although circulating BRAA may account for specific changes in behavior, they seem not to be related to the structural brain damage.

To confirm the presence of BRAA in these mice, we used Western blotting and immunohistochemistry, in addition to ELISA. When Western blot data are considered, antigen-specific bands were expected in three mice with high serum BRAA levels (as determined by ELISA) and high float times. Although bands were detected in these animals, no bands were common, precluding inference on a unique, antigen-specific pathogenic BRAA. As would be expected, in three mice showing an absence of BRAA by ELISA and low float times, no bands were seen. There were, however, bands seen in two other such mice, suggesting, as we have previously predicted, that non-pathogenic BRAA can exist. With respect to immunohistochemistry of brain, all MRL/lpr sera showed different levels of fluorescence in the hippocampus and the cortex. The binding to the hippocampus is consistent with the notion that areas involved in emotional reactivity are affected, as evidenced by deficits in the forced swim and the sucrose preference tests.

In order for serum BRAA to mediate their effects, they must gain access to the brain. Increased BBB permeability was first shown in experimental models of immune complex disease (Harbeck et al., 1979; Hoffman et al., 1983), a pathogenic mechanism of lupus. Several lines of evidence suggest a breached blood-brain barrier in diseased MRL/lpr mice. Perivascular leakage of IgG (Vogelweid et al., 1991), immunoglobulins bound to the MRL/lpr brain (Zameer and Hoffman, 2001) and increased levels of IgG in the CSF (Sidor et al., 2005) point to IgG diffusion via a more permeable blood-brain barrier. These observations are consistent with recent studies showing increased local expression of cell adhesion molecules and entry of immunocytes

(Zameer and Hoffman, 2003, 2004;Ma et al., 2006;James et al., 2006). Therefore it is reasonable to assume that the principal pathogenic mechanisms include entrapment of circulating leukocytes by cell adhesion molecules on endothelial cells, increased permeability of the BBB, and BRAA diffusion and/or intrathecal synthesis before they bind to brain tissue.

Except for a few monoclonal antibodies we have produced (Khin and Hoffman, 1993; Crimando and Hoffman, 1995) there have been limited attempts to systematically characterize the BRAA in autoimmune mice. The specificity of these antibodies is for the most part not known, nor where they bind in brain (e.g., what cell types bear the reactive antigens). Nonetheless, there are new attempts to characterize BRAA in SLE patients (Zandman-Goddard et al., 2007). One study (Gitlits et al., 2001) has gone in this direction, identifying synapsin I as a reasonable candidate autoantigen for mediating CNS manifestations. In addition, Kowal et al. (2004) have focused on the cross-reactivity between anti-DNA autoantibodies and the NMDA receptor, providing evidence for their role in neurobehavioral changes. Similarly, using the autoimmune NZM strain, Lawrence and colleagues produced a monoclonal autoantibody that was directed against mouse dynamin-1 (Lawrence et al., 2007; Mondal et al., 2008). More importantly, when the antibody was injected intravenously into non-autoimmune Balb/C mice, they developed behavioral manifestations similar to those seen in the NZM mice. Nonetheless, there are likely many more BRAA involved in neuropsychiatric manifestations, which have not been identified.

One of the weaknesses of our present study is in not knowing the identity of the bands in the Western blots. We focused on the relationship between overall BRAA and behavior. If we measured levels of specific BRAA (such as anti-NR2, anti-ribosomal P, anti-cardiolipin, or others not identified), then we would be in a better position to find more significant correlations. In the future, we plan to develop studies that will do this, by identifying the molecules to which the BRAA bind and correlating them to behavior.

Our study also points to the possibility that BRAA binding is not the sole mechanism by which CNS dysfunction is induced. For example, much of the neurologic involvement in SLE patients has been attributed to vascular lesions (Bluestein et al., 1981; West, 1996). It is also well known that cytokines can affect brain function (Dunn, 1988; Bindoni et al., 1988; Bartholomew and Hoffman, 1993; Zalcman et al., 1994; Kim et al., 1998), as can immune complex disease (Hoffman et al., 1978a,b, 1998), which may be mediated by other soluble components, such as complement (Hoffman et al., 1982; Schupf and Williams, 1987). Along this line, it has been shown that complement can mediate apoptosis in the brains of MRL/lpr mice (Alexander et al., 2007). Therefore, it is likely that multiple mechanisms, including BRAA, mediate behavioral deficits in SLE-like disease.

In conclusion, dissimilar immune status between two cohorts produced in different years confirms fading autoimmune phenotype (which has since been corrected) in the MRL/lpr substrain. Despite this phenomenon of unknown origin, the relationship between serum BRAA to surface neuronal antigens and behavioral dysfunction is detectable. This study is the first to identify BRAA to naturally occurring integral membrane proteins of murine brain and correlate them to behaviors in autoimmune mice. Although the presence of BRAA did not account for the entire constellation of behavioral deficits, the obtained results are consistent with the notion that a subset (rather than the entire class) of circulating BRAA is pathogenic. As such, they further support the hypothesis that peripheral BRAA enter the brain, bind to integral membrane proteins, and contribute to emergence of behavioral dysfunction in lupus-like disease.

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