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Depression is an Early Disease Manifestation in Lupus-Prone MRL/lpr Mice

Hua-Xin Gao[a], **Sean R. Campbell**[a], **Min-Hui Cui**[b], **Pu Zong**[b], **Jong hee Hwang**[b], **Maria Gulinello**[c], and **Chaim Putterman**[a,d]

a *The Department of Microbiology & Immunology, Albert Einstein College of Medicine, Bronx, NY, USA*

b *Magnetic Resonance Research Center, Albert Einstein College of Medicine, Bronx, NY, USA*

c *Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, USA*

d *Division of Rheumatology, Albert Einstein College of Medicine, Bronx, NY, USA*

Abstract

Many lupus patients develop neuropsychiatric manifestations, including cognitive dysfunction, depression, and anxiety. However, it is not clear if neuropsychiatric lupus is a primary disease manifestation, or is secondary to non-CNS disease. We found that MRL/lpr lupus-prone mice exhibited significant depression-like behavior already at 8 weeks of age, despite normal visual working memory, locomotor coordination and social preference. Moreover, depression was significantly correlated with titers of autoantibodies against DNA, NMDA receptors and cardiolipin. Our results indicate that lupus mice develop depression and CNS dysfunction very early in the course of disease, in the absence of substantial pathology involving other target organs.

Keywords

Systemic Lupus Erythematosus; Neuropsychiatric lupus; Anti-DNA antibodies; Depression

1. Introduction

Systemic Lupus Erythematosus (SLE) is an autoimmune disease affecting multiple organ systems, including the heart, skin, joints, and kidney. In addition, approximately 70% of lupus patients suffer central nervous system (CNS) manifestations including cognitive dysfunction and mood disorders (Bluestein, 1992), with major depression being one of the most common psychiatric presentations (as high as 40%). However, it is unclear if the neuropsychiatric signs and symptoms are secondary manifestations of widespread organ dysfunction, or if the CNS itself is a primary target of autoimmune dysfunction in lupus. Some studies suggest that the neurological manifestations in lupus may be a secondary consequence of lupus nephritis, due to uremia or inflammatory changes and increased permeability of the brain-blood-barrier (BBB). Alternatively, although lupus neuropathology can include neuronal death (Kowal et al., 2004) and loss of brain volume, behavioral abnormalities may also be evident when there

Address Correspondence to: Chaim Putterman, MD, Division of Rheumatology, Forchheimer 701N, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY, 10461, USA, Phone: (718) 430-4266, Fax: (718) 430-8789, Email: E-mail: putterma@aecom.yu.edu.

is no gross CNS pathology (Hermosillo-Romo and Brey, 2002), suggesting that chemokines, autoantibodies and other inflammatory mediators may be instrumental in the pathogenesis of the neuropsychiatric manifestations of lupus (Fragoso-Loyo et al., 2007). Furthermore, it has been reported that the mood and cognitive deficits prevalent in lupus patients may not reliably correlate with measures of active disease and/or disease flares involving other organ systems (Hermosillo-Romo and Brey, 2002), suggesting primary CNS involvement in this disease. Thus, behavioral outcomes, such as depression, may represent a sensitive measure of underlying CNS disease mechanisms that have yet to be elucidated.

The diagnostic criteria for lupus include high titers of anti-nuclear autoantibodies (ANA), especially anti-double stranded (ds) DNA antibodies, which are particularly important in the pathogenesis of lupus nephritis (Deocharan et al., 2002; Putterman, 2004). In addition, autoantibodies to neuronal and/or other CNS antigens are also typically present, including N-methyl-D-aspartate receptors (NMDAR). SLE autoantibodies can damage neurons and cause cognitive deficits when the blood brain barrier is breached (Kowal et al., 2004), as can occur as a consequence of lupus related nephritis. Moreover, high immunoglobulin G (IgG) and albumin levels are present in cerebrospinal fluids in murine models of lupus, and these are cytotoxic to neurons *in vitro* (Sidor et al., 2005). However, it remains unclear whether the CNS lupus syndromes are directly caused by neurocytoxicity induced by high titers of autoantibodies, since immunoglobulins can not cross the intact blood brain barrier in normal individuals.

There are several lupus-prone mouse strains in current use, including MRL/lpr, NZB/W F1, and BxSB (Sakic et al., 1997b). Among the listed strains, MRL/lpr mice have been most extensively used in lupus related neuropsychiatric studies. In MRL/lpr mice, an insertion of a retrotransposon in the gene for Fas (CD95) results in defective apoptosis of lymphocytes and massive lymphoproliferation, resulting in impaired regulation of autoreactive B cells and high autoantibody titers (Chu et al., 1993). Previous studies in MRL/lpr mice have indicated numerous characteristics validating the model, such as a higher prevalence in females, increased ANA titers, and severe kidney pathology (Theofilopoulos, 1992). Moreover, emotional and cognitive deficits present in human lupus such as depression and impaired memory have been demonstrated in older male MRL/lpr mice (12–16 weeks of age) (Sakic et al., 1994), although female mice have not been systematically studied.

Lupus becomes manifest earlier, and is notably more severe, in females as compared to males in human patients as well as in most murine models of the disease, including MRL/lpr mice. Therefore, we hypothesized that neuropsychiatric manifestations would be present even earlier in female than in male mice. Thus, we decided to investigate the age of onset and severity of cognitive and affective outcomes in female MRL/lpr mice, and explore a possible relationship of neuropsychiatric manifestations to several categories of autoantibodies. To this end, we conducted a behavioral battery including tests of depression (forced swim), anxiety (elevated plus maze), sickness behavior (social preference), locomotor activity (open field), motor coordination (balance beam), and cognition (novel object recognition test). Finally, we investigated neuropathology in this lupus-prone strain by magnetic resonance imaging (MRI) and magnetic resonance spectroscopic imaging (MRSI).

2. Materials and methods

2.1. Mice

Ten 3–4 week old MRL/MpJ-Fas^{lpr} (MRL/lpr; stock #006825) female mice and 10 age and background matched MRL/MpJ (MRL/+; stock #000486) female mice were purchased from The Jackson Laboratory (Bar Harbor, Maine), and housed five mice per cage in the animal facility of the Albert Einstein College of Medicine (Bronx, NY). The housing conditions were

controlled, with the temperature at 21–23°C and a 12:12 hours light:dark cycle. All animal studies were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine. All the behavior tests were done in the light phase (7AM–7PM).

2.2. Assessment of lupus

Mice were monitored bi-weekly for the development of proteinuria and autoantibody titers. Urinary protein excretion was measured by dipstick analysis (Uristix; Bayer), where +1 is 30 mg/dl, +2 is 100 mg/dl, +3 is 300 mg/dl, and +4 is ≥2000 mg/dl. Mouse IgG anti-double stranded (ds) DNA antibody titers were determined by ELISA, as previously described in detail (Deocharan et al., 2007). Similar ELISA protocols were also used for anti-chromatin, anti-cardiolipin and anti-N-methyl-D-aspartate receptor antibody ELISAs (Putterman and Diamond, 1998). Briefly, antigens were coated on 96-well plates overnight (chromatin 5 μg/ml; cardiolipin at 75 μg/ml; multimeric DWEYSVWLSN (containing a linear peptide sequence present in NMDA receptors) at 20 μg/ml) and blocked with 3% fetal calf serum in PBS for 1 hour. Sera samples were diluted 1:250 in PBS before being added to the plates for a 2 hour incubation. Alkaline phosphotase-conjugated goat anti-mouse IgG was used as the detection antibody.

2.3. Behavioral testing

For all the behavioral tests, mice were transferred to the test room and equilibrated for 30–40 minutes prior to the tests which were performed under low incandescent light. Except for the balance beam walking test, all tests were digitally recorded by Viewer tracking software (Biobserve, Bonn, Germany).

2.3.1. Open field test—General locomotor activity was assessed in an open field (39 cm × 39 cm) for 15 minutes. Central and peripheral zone entries, track lengths and durations were recorded in 5 minute bins using Viewer Software, with the middle of the body of the animal defined as the criteria point for tracking a zone entry. The central zone was defined as a 15 cm × 15 cm area in the center of the box (Sakic et al., 1994; Ziporen et al., 1997).

2.3.2. Balance beam walking test—The beam used for the test is 100 cm long with a diameter of 1.5 cm. The start end was brightly illuminated whereas the goal box was dimly lit and contained a palatable food (cocoa puffs) to foster reliable beam crossing. The animals were pre-exposed to the palatable food for 3–5 days prior to testing to reduce neophobia. Motor coordination was assessed as the latency to cross the beam (time in seconds) and the number of slips (both recorded manually). A slip was defined as one of the paws passing below the midline of the beam (Sakic et al., 1994; Shoenfeld et al., 2003).

2.3.3. Social preference test—The typical preference for a conspecific over an inanimate object was assessed in a Y maze (each arm 5 cm × 30 cm). Within a 5 minute period, social preference was assessed as time spent exploring a novel, ovariectomized stimulus mouse or a novel object, placed at the end of each of two arms with a transparent plastic barrier (with holes) separating them from the rest of the maze. Social preference was defined as the percent time exploring the ovariectomized mouse/total exploration time. Normal mice generally spend >50% of the time exploring the conspecific living mouse, whereas animals with high levels of anxiety, depression, or sickness behavior demonstrate reduced social exploration (Dantzer et al., 1987).

2.3.4. Object recognition (visual memory) test—The novel object recognition test of memory is based on the robust tendency of rodents to preferentially explore novel objects, and was performed essentially as described (Ennaceur et al., 1997) in an opaque Perspex arena (39 × 39 cm). In Trial 1, mice were given 3 minutes to freely explore 2 identical, non-toxic objects

(such as glass, plastic and ceramic items). Mice were then removed to the home cage for a 45 minute retention interval, and returned to the arena for 3 minutes after one object had been replaced with a novel object (Trial 2). Data from Trial 2 were expressed as a preference score (time exploring novel object/total exploration) × 100. A preference score of 50% indicates chance performance. Exploration of the objects was defined as whisking, sniffing, rearing on or touching the object, and approach and obvious orientation to the object within 3–5 cm. Novelty-induced exploration in Trial 1 was also assessed and expressed as the total time spent exploring both the objects. This ensures that all groups had equal tendency to explore novel objects. One of the 8 week old MRL/+ mice was used for setting up the system and was excluded from the analysis.

2.3.5. Forced swim test—Individual mice were placed into a glass beaker (25 cm height, 15 cm diameter) containing 3000 ml (17 cm height) of water maintained at approximately 27° C. Each 10 minute test session was digitally recorded with the Viewer software and manually scored. Mice placed in this situation with no way to escape begin struggling and swimming but eventually exhibit behavioral despair, assessed as immobility (Porsolt et al., 1977a; Katzav et al., 2007). Depression-like behavior was defined as immobility (floating) and is illustrated as the % time spent immobile (Porsolt et al., 1977a). The latency to begin floating was also assessed and was defined as the first bout of immobility lasting at least 8 seconds. The scorers were blinded to the experimental groups. Two of the 8 week MRL/+ mice were used for setting up the system, and their total floating time were not recorded for the analysis.

2.3.6. Elevated-plus-maze test—Mice were tested on a plus maze, elevated 50 cm above the floor, in a room with low, indirect incandescent lighting and low noise levels. The plus maze consists of 2 enclosed arms (10 cm × 43 cm × 20 cm) and 2 open arms (10 cm × 43 cm × 20 cm), and has been validated in detail elsewhere (Pellow et al., 1985). At the time of testing, each animal was evaluated for 15 minutes after exiting to the center platform of the plus maze. To be considered as an entry into any arm, the mouse must pass the line of the open platform with all four paws. The duration (in seconds) of time spent in the open arm from the time of entry was recorded, in addition to the number of entries into both arms. Decreased % of open arm entries and decreased % time spent in the open arm generally indicates higher levels of anxiety-like behavior (Pellow et al., 1985).

2.3.7. Estrous cycle measurement—Estrous cycle stage was determined by microscopic examination of the vaginal lavage, as described previously (Montes and Luque, 1988) daily (5 pm) for 2 weeks.

2.4. Proton magnetic resonance imaging and magnetic resonance spectroscopic imaging of mouse brain

Proton magnetic resonance spectroscopic imaging (^{1}H MRSI) of mice brains was performed on a 9.4T 21 cm horizontal bore imaging system (Varian INOVA, Palo Alto, CA), equipped with home-made coils. The coils included an actively detunable volume transmitter coil and a 12 mm × 8 mm elliptical surface receiver coil. Animals were anesthetized and maintained on 2% isoflurane administered through a specially designed nose cone. The animals were maintained at a constant temperature throughout the experiment using heated circulating water.

An inversion recovery gradient echo imaging sequence (128 × 128 acquisition matrix, 17 slices, 0.5 mm slice thickness, 24 mm × 24 mm field of view, 900 ms inversion time) was employed to acquire scout images. After performing non-iterative shimming as previously described (Miyasaka et al., 2006), water suppressed MRSI was obtained using an adiabatic refocusing method (LASER) (Garwood and DelaBarre, 2001) with 1 mm slice thickness in combination with a broad-band semiselective excitation sequence and a selective 90 degree water

suppression pulse. Two dimensions of 24 × 24 phase encodes were acquired over a FOV of 24 mm × 24 mm. The data was acquired with a repetition time of 2000 ms, and an echo time of 50 ms using two averages resulting in an acquisition time of approximately 38 minutes. Spatial dimensions of the data were filtered with a Hanning filter. After the acquisition of MRSI data, images with a higher resolution (256 × 256 acquisition matrix) were acquired using the same inversion recovery gradient echo imaging sequence with two averages in order to quantify the hippocampal volume of the mouse brain.

To compare differences in brain metabolites between control and MRL/lpr mice, N-acetyl aspartate/creatine (NAA/Cr), NAA/choline (NAA/Ch) and creatine/choline (Cr/Ch) ratios were measured from the spectra obtained from the hippocampus, thalamus and cortex in each animal. The voxels across each brain region, e.g., hippocampus, thalamus and cortex, were selected using an image-guided single voxel reconstruction algorithm via voxel-shifting methods (Miyasaka et al., 2006; Pan et al., 2005; Twieg et al., 1989). The values over the reconstructed voxels were averaged to give a mean value in each region. All data were processed using MATLAB® (The MathWorks, Natick, MA).

2.5. Statistics

Graphpad/Prism4 software was used for the statistical analysis. An unpaired t test (two tailed) or non-parametric Mann-Whitney test was used to compare the differences between two groups. Two way repeated measures ANOVA (age by genotype) with Bonferroni post-hoc tests were used to analyze the data from repeated measurements. Correlation and linear regression analysis were also performed. For all the statistics, significance was defined as $p<0.05$.

3. Results

3.1. Lupus-prone MRL/lpr mice develop higher autoantibody titers by 8 weeks of age

The onset and severity of murine lupus is usually measured by autoantibody levels, especially titers of anti-dsDNA and other autoantibodies, and the amount of urinary protein excretion (proteinuria), which reflects kidney dysfunction. We monitored these parameters in lupus-prone MRL/lpr female mice and the congenic wild type control MRL/+ mice over the time-course of the study, starting from 6 weeks to the mid-disease point at 18 weeks of age.

We found that typical serologic indicators of lupus were evident by 8 weeks in MRL/lpr female mice, and continued to increase over time. At 6 weeks of age, anti-dsDNA Ab titers were similar in MRL/lpr and the congenic MRL/+ mice. Starting at week 8 and throughout week 18, MRL/lpr mice displayed significantly higher anti-dsDNA autoantibody titers than MRL/+ controls (Fig. 1A). A two-way repeated measures (RM) ANOVA analyzing anti-dsDNA Ab titers revealed a main effect of genotype ($F_{(1,18)}=32.80$; $p<0.0001$) and a main effect of age ($F_{(4,18)}=26.80$; $p<0.0001$). The Bonferroni post-hoc tests indicated that the anti-dsDNA Ab titers were significantly different between strains at week 8 ($p<0.05$) and weeks 10–18 ($p<0.001$), but not week 6 ($p>0.05$). This result is consistent with previous reports that no disease is yet present in MRL/lpr mice at 6 weeks of age, and that the disease-onset time is around 8 weeks of age (Sakic et al., 1992).

We also measured titers of anti-chromatin antibodies, another anti-nuclear autoantibody characteristic of SLE. At 6 weeks of age, anti-chromatin antibody titers were similar in MRL/lpr and MRL/+ mice. Starting at week 8, MRL/lpr mice developed significantly higher anti-chromatin autoantibody titers than the congenic MRL/+ controls, which continued to increase throughout week 18 (Fig. 1B). A two-way RM ANOVA analyzing anti-chromatin Ab titers revealed a main effect of genotype ($F_{(1,18)}=117.30$; $p<0.0001$) and a main effect of age

($F_{(4,18)}$ =27.03; p<0.0001). The Bonferroni post-hoc tests indicated that the anti-chromatin antibody titers were significantly different between strains from week 8 to week 18 (p<0.01 or 0.001 at each time point), but not at week 6 (p>0.05).

It has been reported that the anti-dsDNA antibodies from SLE patients can cross-react with NMDA receptors and mediate neuronal cell death or damage (DeGiorgio et al., 2001). The resulting damage to relevant brain regions, such as the hippocampus, cortex (DeGiorgio et al., 2001), and amygdala (Emmer et al., 2006) may manifest in several possible behavioral outcomes, including cognitive and emotional dysfunction. Thus, we also examined anti-NMDAR autoantibody titers over time. At 6 weeks of age, anti-NMDAR autoantibody titers were similar between MRL/lpr and MRL/+ controls. From week 6 to week 10, a rapid increase in anti-NMDAR Ab titers was found in the MRL/lpr group but not in the MRL/+ group (Fig. 1C). After week 10, the anti-NMDAR Ab titers reached a plateau in MRL/lpr mice but still remained significantly higher than in the MRL/+ group. A two-way RM ANOVA (age by genotype) analyzing anti-NMDAR Ab titers revealed a main effect of genotype ($F_{(1,18)}$ =132.3; p<0.0001) and a main effect of age ($F_{(4,18)}$ =8.129; p<0.0001). Bonferroni post tests showed significantly higher levels of anti-NMDAR autoantibodies in MRL/lpr mice than the control group from week 8 to week 18 (p<0.001 at each time point). No significant difference between the two groups was present at the week 6 time point (p>0.05).

Anti-cardiolipin (aCL) antibodies, which react against phospholipid antigens, are associated with manifestations of neuropsychiatric SLE (NPSLE) including stroke and transient ischemic attack (Brey et al., 2001; Gorelick, 2002; Petri, 2000). At 6 weeks of age, higher aCL Ab titers were already detected in MRL/lpr mice, compared to MRL/+ controls. From week 6 to week 18, aCL Ab titers progressively increased in the MRL/lpr group, but rose much more slowly in MRL/+ mice (Fig 1D). From week 6 to week 18, the aCL Ab titers were consistently higher in MRL/lpr mice than the MRL/+ group. A two way RM ANOVA (age by genotype) analyzing aCL Ab titers revealed a main effect of genotype ($F_{(1,18)}$ =282.4; p<0.0001) and a main effect of age ($F_{(4,18)}$ =32.51; p<0.0001). Bonferroni post tests showed significantly higher levels of aCL autoantibodies in MRL/lpr mice than in the control group at each time point tested between week 6 and week 18 (p<0.001 at each time point).

In contrast to the early onset of autoantibody formation, proteinuria increased much more gradually. Proteinuria began to increase in MRL/lpr mice at week 14, and was significantly different in MRL/lpr as compared to MRL/+ mice at 18 weeks of age (Fig. 1E, p<0.001). A two way RM ANOVA (age by genotype) analyzing proteinuria revealed a main effect of genotype ($F_{(1,18)}$ =4.28; p<0.05) and a main effect of age ($F_{(4,18)}$ =3.57; p<0.01). Bonferroni post tests indicated that the proteinuria levels were significantly higher in 18w old MRL/lpr mice than in control group (p<0.001). No significant difference were found from week 6 to week 14 (p>0.05). Severe proteinuria (>300 mg/dL urine protein) was not observed in the period of study (≤18 weeks of age). These results indicate that despite displaying increased autoantibody titers, kidney function was not yet severely affected by 18 weeks of age in the studied mice.

3.2. MRL/lpr mice had normal general activity, motor coordination, cognition activity and social preference

To examine the time course of potential behavioral outcomes of lupus, we tested the mice at 8 weeks (approximate time of lupus onset) and at 18 weeks of age. A battery of behavioral tests was selected to investigate general activity, motor coordination, cognition, anxiety-like behavior, and depression at these time points.

To test the general activity of the mice, open field tests were performed. We found that MRL/lpr mice had similar total track lengths and center track lengths as MRL/+ controls at both age

8w (Fig. 2A) and 18w (Fig. 2B), suggesting that normal general locomotor activity was present in MRL/lpr mice. Two way RM ANOVA (age by genotype) analyzing total track lengths and center track lengths revealed no main effect of genotype and age in the performance of mice in this open field test.

There were no evident deficits in motor coordination in MRL/lpr mice, as assessed in the balance beam test (number of slips and latency to cross a round beam). We found that MRL/lpr mice made a similar number of slips as controls while crossing the beam (Fig. 3A, 3.4±0.3 vs. 4.2±1.2, $p>0.05$). Interestingly, MRL/lpr mice actually crossed the beam more quickly than MRL mice (Fig. 3B, 9.98±0.96 vs. 13.60±1.25 seconds, $p=0.034$). The open field and balance beam experiments also indicate that impaired locomotor function (caused by inflammatory joint disease, general disability, or other causes) is not likely to be a confounding factor in the subsequent behavioral tests.

An object recognition (visual memory) test was performed to evaluate cognition and memory performance. In this behavioral test, both 8w old and 18w old MRL/lpr mice showed similar visual memory preference as MRL/+ controls (Fig. 4A & B, $p>0.05$), indicating normal cognition in these lupus-prone mice. Two-way RM ANOVA (age by genotype) analyzing visual memory preference revealed no main effect of genotype or age in the performance of mice in this test.

3.3. Lupus mice did not exhibit typical anxiety-like behavior in the elevated plus maze test

Anxiety is a mood disorder often found in NPSLE patients (Hermosillo-Romo and Brey, 2002). To investigate anxiety levels in lupus-prone mice and the disease-free controls, we utilized the elevated plus maze (EPM). Anxiety-like behavior in this test was defined as decreased exploration of the open arms during the first exposure to the EPM test (Pellow et al., 1985). Since responses in subsequent exposures to the EPM are regulated by a complicated interplay between learning, anxiety, habituation and sensitization (Bessa et al., 2005), we only assessed 18 week old mice. Within 5 minutes in the EPM, MRL/lpr mice exhibited significantly lower levels of anxiety-like behavior than MRL/+ mice, as evident in the higher percentage of open arm exploration (9.27±1.96% vs. 1.93±0.73% of the total time, respectively, Fig. 5A; $p=0.005$) and a similar number of open arm entries as MRL/+ mice (Fig. 5B; $p>0.05$). Interestingly, MRL/lpr mice showed significantly more stretched attend posture (Fig. 5C, $p=0.021$) than MRL/+ mice. Together, these data indicate a moderate but significantly lower level of anxiety-like behavior in female MRL/lpr mice.

One hallmark of sickness behavior and anxiety-like behavior is social withdrawal (Dantzer et al., 1987), as assessed in a social preference test. We found that both 8w old and 18w old MRL/lpr mice had normal preference for a conspecific living mouse compared to an inanimate object (Fig. 6A & B, $p>0.05$). Thus, the normal social preference test, together with results in the EPM, did not indicate any increase in anxiety-like behavior or general debility.

3.4. Lupus mice develop depression very early in the forced swim test

As one of the most prevalent behavioral manifestations of lupus is a major depressive disorder, we assessed depressive like behavior in the forced swim test in lupus-prone female MRL/lpr mice and in age matched MRL/+ mice at early (8 weeks) and later (18 weeks) stages of the disease. In the forced swim test, depression was assessed as increased time spent floating (as opposed to swimming and/or struggling) and shorter latency to begin floating. Lupus-prone MRL/lpr exhibited significantly higher levels of behavioral despair at both time points (8 weeks and 18 weeks old; Fig. 7) than age-matched MRL/+ mice. Lupus-prone MRL/lpr had more than a 100% increase in their % floating time compared to MRL/+ mice, even at the earliest age tested (Fig. 7A; 7C). In Figure 7E, a two-way RM ANOVA (age by genotype) analyzing

total floating time in a 9 minute test revealed a main effect of genotype ($F_{(1,16)}$ =25.70; p<0.0001) and no main effect of age ($F_{(1,16)}$ =0.056; p>0.05). In addition to increased total time spent immobile, the MRL/lpr mice also had a shorter latency to begin floating. As shown in Figure 7B, the 8w MRL/lpr mice started floating after 160.5±18 seconds, significantly earlier than the MRL/+ controls (255.0±32 seconds, p<0.03). The 18w MRL/lpr mice started floating at 118.6±20 seconds (Fig. 7D), earlier than in their previous exposure 10 weeks earlier, but not significantly earlier than the MRL/+ controls (158.1±32 seconds, p> 0.05). These results indicate that the 8w and 18w old MRL/lpr mice had distinct depression-like behavior, which was not found in the control strain. In addition, the data are consistent with the suggestion that repeated exposure to the forced swim test tends to increase some indices of behavioral despair (i.e. latency to float) in both control MRL/+ and MRL/lpr female mice. In Figure 7F, a two-way RM ANOVA (age by genotype) analyzing floating latency revealed a main effect of age ($F_{(1,18)}$ =7.855; p<0.05) and no main effect of genotype ($F_{(1,18)}$ =2.596; p>0.05).

3.5. Depression scores correlate with anti-dsDNA, anti-NMDAR and anti-cardiolipin autoantibody titers

To address a possible pathogenic link of autoantibodies to the development of NPSLE, we correlated the results on the depression test with autoantibody titers at each test age (8w or 18w) in a correlation study. The result of the correlation analysis showed that anti-dsDNA Ab levels in 18w mice significantly correlated with the % floating time in the forced swim test (Fig. 8D, p=0.005, r=0.598), while the correlation was not significant in 8w mice (Fig. 8A, p>0.05).

At both age 8w and 18w, serum anti-NMDAR antibody levels significantly correlated with % floating time in the forced swim test. As shown in Figure 8B, anti-NMDAR Ab titers of 8 week old mice significantly correlated with the % floating time (p=0.015, r=0.596). A similar correlation was also found at 18 weeks of age (Figure 8E, p=0.007, r=0.583). Significant correlations were also found between the % floating time and aCL Ab titers (Fig. 8C, 8 weeks, p=0.001, r=0.708; Fig. 8F, 18 weeks, p=0.004, r=0.621), and anti-chromatin Ab titers (data not shown, p<0.05) at both ages tested.

3.6. Estrous cycle stage was not likely to confound the behavior tests

To exclude the possibility that hormonal influences may have confounded our study, we examined the estrous cycle stages of the mice for two weeks. Table 1 shows the respective stages on the day we tested 8w old mice in the forced swim test. The stages of estrous cycle found in the tested mice were randomly distributed across all the cycle phases, and there was no synchronized or systematic trend in either group of mice. This observation indicated that at least in our study, sex hormones were not likely to have had any systematic effect on the results of the behavior tests.

3.7. MRSI confirms regional brain dysfunction

In addition to the behavioral tests, a MRI examination of the brain was performed on those MRL/lpr and MRL/+ mice which presented behavior differences in the previous depression tests. Mice at 18 weeks of age were tested in pairs, with one lupus mouse and one control mouse in each experimental set (total lupus n=6, control n=6). Both groups appear to display grossly normal brain structure by MRI (data not shown). However, subtle changes in brain anatomy may not have been detectable by this method.

To analyze major brain metabolites which reflect brain cell activity, including total creatine (Cr), choline-containing compounds (Ch), and neuron-specific product N-acetyl aspartate (NAA), magnetic resonance spectroscopic imaging (MRSI) was performed (Fig. 9). These metabolites are traditionally standardized by creatine, a stable marker, and are expressed as

ratios of each metabolite to the creatine standard (e.g. NAA/Cr). Furthermore, choline levels reflect membrane turnover or genesis, and are expressed as Ch/Cr. These metabolic ratios have been extensively used to identify regional brain dysfunction or damage in human brain studies (Brooks et al., 1997;Sibbitt et al., 1994;Sibbitt et al., 1997;Sibbitt and Sibbitt, 1993) as well as in studies of the neuronal pathogenesis of anti-dsDNA autoantibodies (Kowal et al., 2004). As shown in Table 2, MRL/lpr mice had significantly lower Ch/Cr ratios in the hippocampus (0.61 ± 0.010 vs. 0.66± 0.014, p=0.044) and cortical regions (0.67± 0.020 vs 0.74± 0.016, p=0.039) than the control group. As compared to non-autoimmune mice with induced lupus (Kowal et al., 2004), MRL/lpr mice with spontaneous disease did not however display decreased hippocampal NAA/Cr ratios (Table 2). In contrast, a higher NAA/Ch ratio was found in the cortex of MRL/lpr mice than in the MRL/+ controls (1.39± 0.056 vs. 1.24± 0.055, p=0.027), indicating that cortical brain cells (neurons and/or glia) in MRL/lpr mice were more active than those in MRL/+ mice. These results suggest that brain cell activity changes in the hippocampal and cortical regions may be important in regulation of emotion in brains of MRL/lpr lupus mice.

4. Discussion

Major depression is one of the most common neuropsychiatric manifestations in SLE patients (Wekking, 1993). In our study, female MRL/lpr mice showed depressive behavior as early as 8 weeks of age, the onset of the lupus-like disease. This significant increase in behavioral despair in the forced swim test was not accompanied by abnormal behavior in tests of general activity, motor activity, anxiety, social activity and visual memory. Excessive floating and early cessation of attempts to escape have been used to characterize the depression in the forced swim test model (Porsolt et al., 1977a; Porsolt et al., 1977b). Both 8w old and 18w old MRL/lpr mice were immobile for a significantly longer period of time, and the 8w old MRL/lpr mice stopped struggling much earlier than the MRL/+ congenic controls. Surprisingly, MRL/lpr mice actually displayed significantly less anxiety behaviors as compared to the background matched MRL/+ mice, despite more severe depression. Finally, the fact that the mice did not have severe nephritis while displaying depressive behavior indicates that neuropsychiatric outcomes of SLE are dissociable from kidney pathology. Since in this study we compared MRL/lpr lupus mice to background matched MRL/+ mice, any relative differences observed between these mice strains are solely attributable to disease acceleration mediated by the lpr trait. However, it is important to point out that the MRL background, which itself promotes autoimmune manifestations late in life, may also have contributed to the absolute severity of neurobehavioral abnormalities described above.

SLE is much more highly prevalent in women, with a 9:1 female:male ratio in human patients. It is thought that sex hormones (such as estrogens and prolactin) have a potent role in disease development (Cohen-Solal et al., 2006; Grimaldi, 2006; Lahita, 1999). This sex difference is also evident in several rodent models of lupus including MRL/lpr; however, female mice have rarely been systematically studied (Sakic et al., 1997a; Sakic et al., 1993; Sakic et al., 1992; Sakic et al., 1996). As one of the very few studies using female MRL/lpr mice for behavioral tests, our results demonstrate that female MRL/lpr mice develop depression at a very young age, much earlier than the 12–16 weeks of age time point previously reported in studies of MRL/lpr males (Sakic et al., 1994). Nevertheless, the fact that female MRL/lpr mice were in different stages of the estrous cycle at the time of testing in the forced swim test, indicates that there is not a simple relationship between circulating sex hormones and manifestations of depression-like behavior.

There is debate in the literature whether neuropsychiatric symptoms of lupus are a secondary complication of peripheral organ pathology (such as severe nephritis) or reflect a primary CNS etiology. Though renal dysfunction in lupus may indeed affect neuropsychiatric manifestations

through metabolic disturbances, our results suggested that CNS lupus can occur long before kidney insufficiency in female lupus-prone mice, since severe nephritis (≥ 300mg/dL proteinuria) was not found at the age that depression was demonstrated. This finding is consistent with previous studies in another lupus-prone strain, NZM88 mice, which developed behavioral changes before the onset of nephritis (Rudofsky et al., 1993; Rudofsky and Lawrence, 1999). In our study of MRL/lpr mice, we found that development of depression does not require end-organ injury such as renal failure. Moreover, other target organ involvement observed in MRL/lpr mice including rash, arthritis, and lymphadenopathy were also not present when marked depression was already manifest (data not shown). Taken together, our data suggest that the brain is a primary target organ in lupus, and that depression-like behavior may be an early indicator of lupus target organ involvement.

It is interesting to note that we did not find increased anxiety in the MRL/lpr mice, in either the EPM or the social preference tests. Anxiety is often co-morbid with depression in lupus patients (Nery et al., 2008). Previous studies have demonstrated increased anxiety in lupus models: 12–16 week old MRL/lpr male mice that floated extensively in the forced swim test had higher levels of anxiety in the EPM test (Sakic et al., 1994), while increased anxiety was found in lupus-prone NZB/W F1 mice as well (Schrott and Crnic, 1996). In contrast, we found in the current study that MRL/lpr female mice were depressed but ***not*** anxious, raising the possibility that at least in female MRL/lpr mice there are separate pathways contributing to the development of depression and anxiety.

Since behavioral changes can occur before other disease manifestations in SLE patients, and lupus-prone NZM88 (Rudofsky and Lawrence, 1999) and MRL/lpr mice (current study) as well develop behavioral deficits before the onset of nephritis, there has been a great deal of interest in possible underlying mechanisms for the lupus associated behavioral syndrome. It has been suggested that certain brain antigen reactive antibodies can independently cause or accelerate the development of NPSLE-like manifestations in mice (Lawrence et al., 2007). To be sure, several previous studies have shown that lupus associated autoantibodies can promote behavioral changes. Inducing high titers of anti-phospholipid antibodies in non-autoimmune mice led to a loss of reflexes and gripping potential (Ziporen et al., 1997), while mice injected with anti-ribosomal P autoantibodies from human patients exhibited depression that was reversible with anti-depressant treatment (Katzav et al., 2007). In the current study we found that the depression-like behavior of lupus-prone MRL/lpr mice correlated with autoantibodies against dsDNA, chromatin, NMDA receptors, and cardiolipin, further supporting a mechanistic link between the depression syndrome and circulating autoantibodies. While some authors have reported that behavioral deficits such as cognitive decline do not parallel lupus activity (Leritz et al., 2000) and that autoantibody titers do not linearly correlate with CNS pathology (Kowal et al., 2004), there is also convincing evidence that cognitive deficits (reduced novel object exploration) in MRL/lpr mice do correlate with ANA titers. Moreover, this behavioral abnormality in lupus mice was significantly improved by immunosuppressive drug therapy which dramatically reduced ANA titers (Sakic et al., 1994). In our study, we found a significant correlation between depression and anti-NMDAR autoantibody titers in MRL/lpr female mice, consistent with the previous finding that high serum anti-NMDAR antibody titers were associated with depression, but not cognitive deficits, in SLE patients (Lapteva et al., 2006). It is possible that different types of pathogenic autoantibodies affect certain brain regions separately, according to their antigen specificity. Interestingly, female MRL/lpr mice already showed definite depression-like behavior even at the age when only subtle or insignificant increases of autoantibodies were detected. This raises the question whether lowering the currently accepted threshold for detection of anti-nuclear antibodies (i.e. making the test more sensitive) would result in an earlier clinical diagnosis of NPSLE.

Human SLE and murine models of the disease are characterized serologically by a broad array of autoantibodies against nuclear antigens and other cellular components. Of these, several autoantibody specificities have been associated with focal and diffuse neuropsychiatric syndromes affecting both adult and pediatric lupus populations (recently reviewed in Muscal and Myones, 2007; Stojanovich et al., 2007; Zandman-Goddard et al., 2007; Toubi and Shoenfeld, 2007). Autoantibodies linked to neuropsychiatric lupus include autoantibodies to brain components (e.g. anti-neuronal antibodies, brain reactive antibodies, anti-microtubule-associated protein 2 (MAP-2) antibodies), and systemic antigens (e.g. anti-cardiolipin/anti-phospholipid antibodies, anti-ribosomal P antibodies, anti-endothelial cell antibodies). However, no autoantibody specificity for any brain specific or systemic antigen has been linked with a single neuropsychiatric manifestation (Zandman-Goddard et al., 2007).

Of the systemic autoantibodies related to neuropsychiatric lupus, antibodies to ribosomal P have been particularly well studied and linked in several studies in lupus patients to psychosis and depression (Eber et al., 2005; Schneebaum et al., 1991; Zandman-Goddard et al., 2007; Toubi and Shoenfeld, 2007). Anti-ribosomal P antibodies have the ability to penetrate into live cells, induce apoptosis and modulate cytokine secretion (Shoenfeld, 2007). In addition, recent studies by Shoenfeld and colleagues (Katzav et al., 2007) have implicated a novel mechanism by which anti-ribosomal P antibodies may contribute to depression. It was previously shown that defects of the smell apparatus can lead to depression in mice (Zueger et al., 2005) and rats (Pause et al., 2001). Interestingly, human anti-ribosomal P antibodies were shown to bind to olfactory-related brain structures including the piriform and cingulate cortex, while injection of anti-ribosomal P antibodies intracerebroventricularly in non-autoimmune C3H mice directly caused depression-like behavior (Katzav et al., 2007). While any possible effect on smell capabilities was not directly evaluated in injected mice, these studies raise an interesting possible connection between anti-ribosomal P antibodies, disturbed olfaction, and depressive behavior. However, MRL/lpr mice display a relatively low incidence (<15%) of this particular autoantibody specificity (Bonfa et al., 1988; Elkon et al., 1989), and therefore we did not measure anti-ribosomal P antibodies in our studies. The severe depression we found in young MRL/lpr mice, despite low titers of anti-ribosomal P antibodies described in this strain, indicates that several distinct pathways may contribute to depression in SLE.

The mechanism by which cross-reactive autoantibodies pass through the blood brain barrier to affect brain cell function is a matter of great interest. It was previously reported that 33% of 8 week old MRL/lpr mice had inflammation and perivascular leakage of the BBB, which becomes more prevalent as the mice age (Vogelweid et al., 1991). The early disruption of the BBB presumably can provide an entry for circulating factors such as inflammatory cytokines and pathogenic autoantibodies to the inner brain regions in lupus-prone mice even early on in the disease. Thus, one would expect a positive correlation between brain-reactive pathogenic antibodies and brain dysfunction as observed in behavior tests, as we did find in our study. There is indeed convincing experimental evidence that a breach of the blood brain barrier can result in circulating autoantibodies gaining access to the brain, leading to manifestations of NPSLE. In a series of elegant studies, Diamond and colleagues have shown that in normal BALB/c mice, NMDAR-reactive anti-DNA antibodies can penetrate into the mouse brain following an induced breach of the BBB and cause apoptotic death of hippocampal neurons, leading to significant cognitive deficits in hippocampal-dependent functions of learning and memory (Huerta et al., 2006; Kowal et al., 2006; Kowal et al., 2004). Moreover, treatment with a NMDA receptor antagonist prevents neuronal cell death. Interestingly, the agent used to open the BBB determines which brain region is made vulnerable to these neurotoxic autoantibodies. Using lipopolysaccharide to breach the BBB, induction of high titer anti-NMDAR Abs resulted in neuronal death in the hippocampus, cognitive dysfunction and altered hippocampal metabolism (Kowal et al., 2004), while a combination of epinephrine plus autoantibodies resulted in neuron loss in the lateral amygdala and emotional dysfunction characterized by

defective fear conditioning (Huerta et al., 2006). The observation that floating time and latency were not increased in older MRL/lpr mice as compared to younger mice of this strain, together with these other studies, suggests that BBB breach and the early exposure to autoantibodies could indeed be the key to trigger NPSLE. However, what factors increase BBB permeability in young lupus mice is not currently known.

In human brain studies, MRSI is used to detect inflammation, gross pathology, and for localization of abnormalities in regional brain function. We applied this method to mouse studies and found significant metabolic differences between the normal control mice and lupus mice in both the hippocampus and cortex regions, which are involved in cognition and regulation of emotion. These metabolic abnormalities reflected abnormal neuronal (and/or glial) activity, but had no structural correlate detectable by MRI. The results in our studies suggest that these brain regions may be particularly susceptible to the effects of SLE autoimmunity. Moreover, no gross structural abnormalities (such as cerebral infarction, increased ventricular volume or hippocampal or cortical atrophy) were found in MRL/lpr mice despite high aCL autoantibody titers, similar to results reported in a previous study (Hess et al., 1993).

In conclusion, we found that female lupus mice developed depression-like behavior as early as 8 weeks of age, the onset of the disease, and that the behavior deficits correlated with autoantibody levels. Moreover, at least in female MRL/lpr mice, anxiety and depression were dissociated. Our results indicate that NPSLE can develop at a very early stage of the disease when only subtle changes of autoimmunity are present in MRL/lpr mice, and encourage additional study into further understanding the important role of autoantibodies in the pathogenesis of neuropsychiatric SLE.

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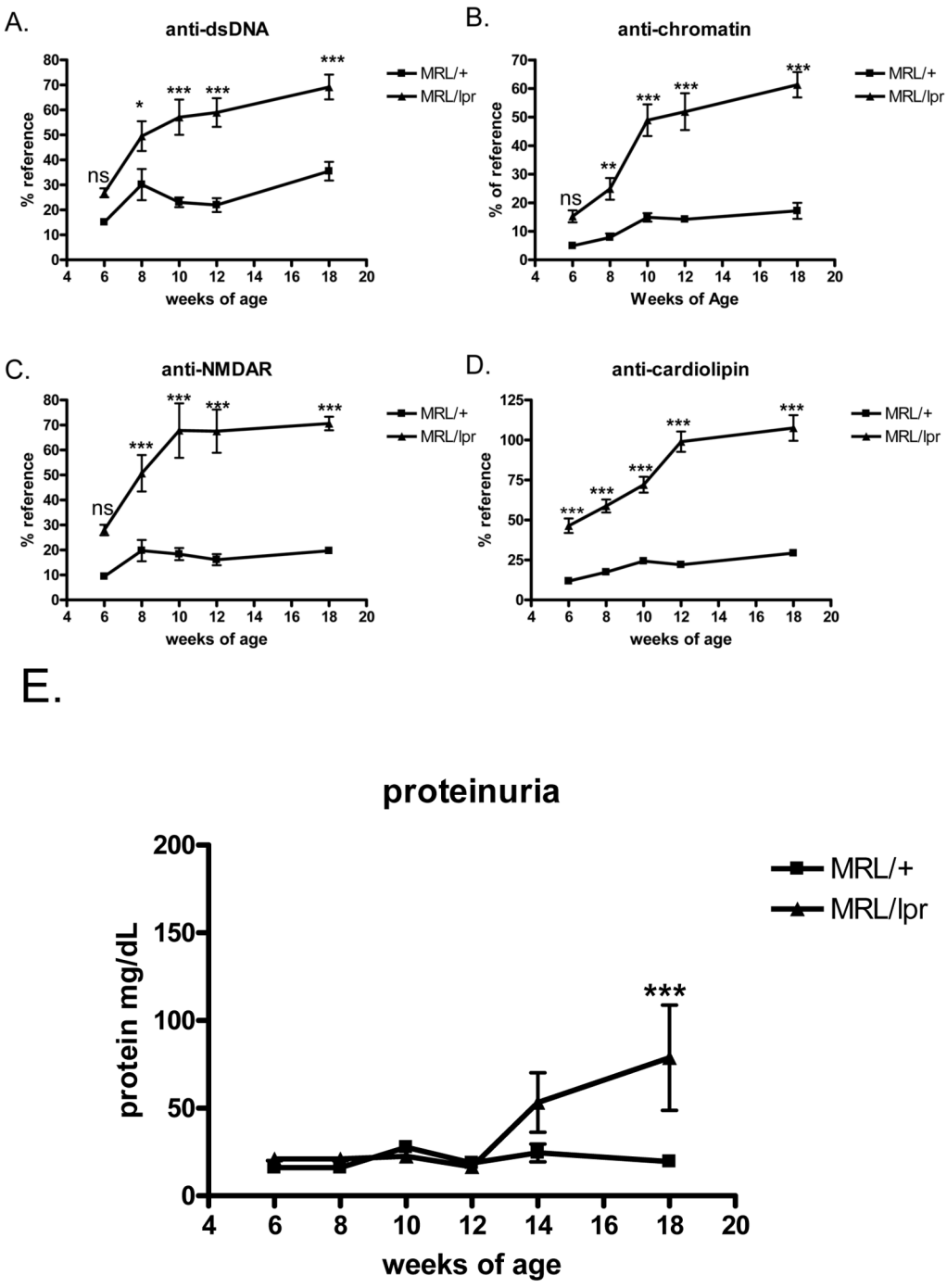

Fig. 1.

Proteinuria and autoantibody levels in MRL/lpr and MRL/+ mice. A–D, Mouse serum autoantibody titers examined by ELISA, from age 6w to 18w. A. IgG anti-dsDNA titers. B. IgG anti-chromatin titers. C. IgG anti-NMDAR titers. D. IgG anti-cardiolipin titers. E. Urine protein concentrations in MRL/+ and MRL/lpr mice at 6–18 weeks. N=10 in each of the mice groups in this Figure. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$

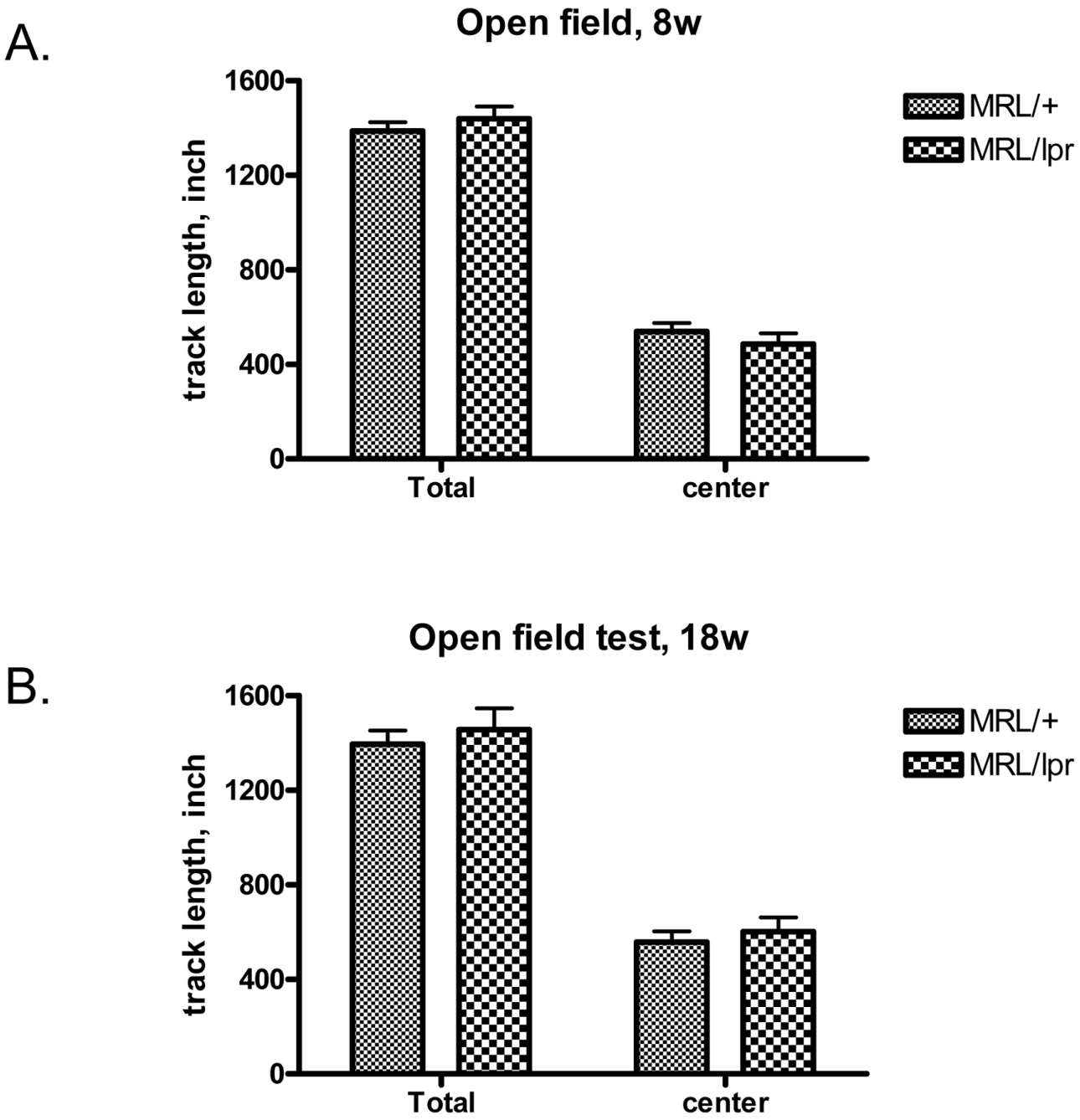

Fig. 2.

Lupus-prone MRL/lpr mice behave normally in the open field test. The total track lengths and center track lengths in the open field of 8w (A) and 18w (B) MRL/+ and MRL/lpr mice (n=10) were measured. Total time in the field: 15 minutes.

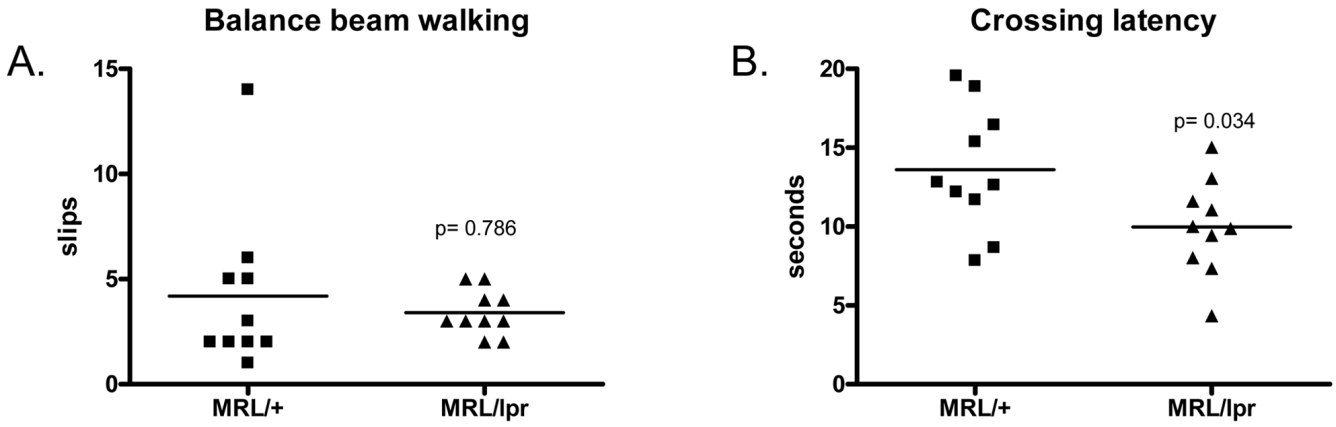

Fig. 3.

Lupus-prone MRL/lpr mice display normal behavior in the balance beam walking test. 8w old MRL/+ and MRL/lpr mice were tested in beam walking for locomotor coordination. A. Number of slips recorded during beam crossing. B. Total time each group spent crossing the beam.

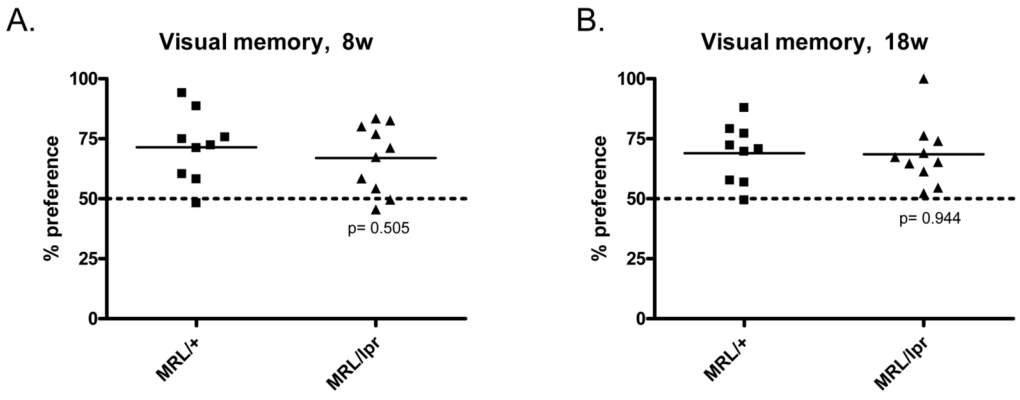

Fig. 4.

Cognition is preserved in 8w and 18w MRL/lpr mice. Object recognition (visual memory) tests on 8w (A) and 18w (B) mice were performed. A 45 minute delay time was used between the first and second object exposures. Mice having normal visual memory are expected to have >50% preference for a novel object (above the dotted line).

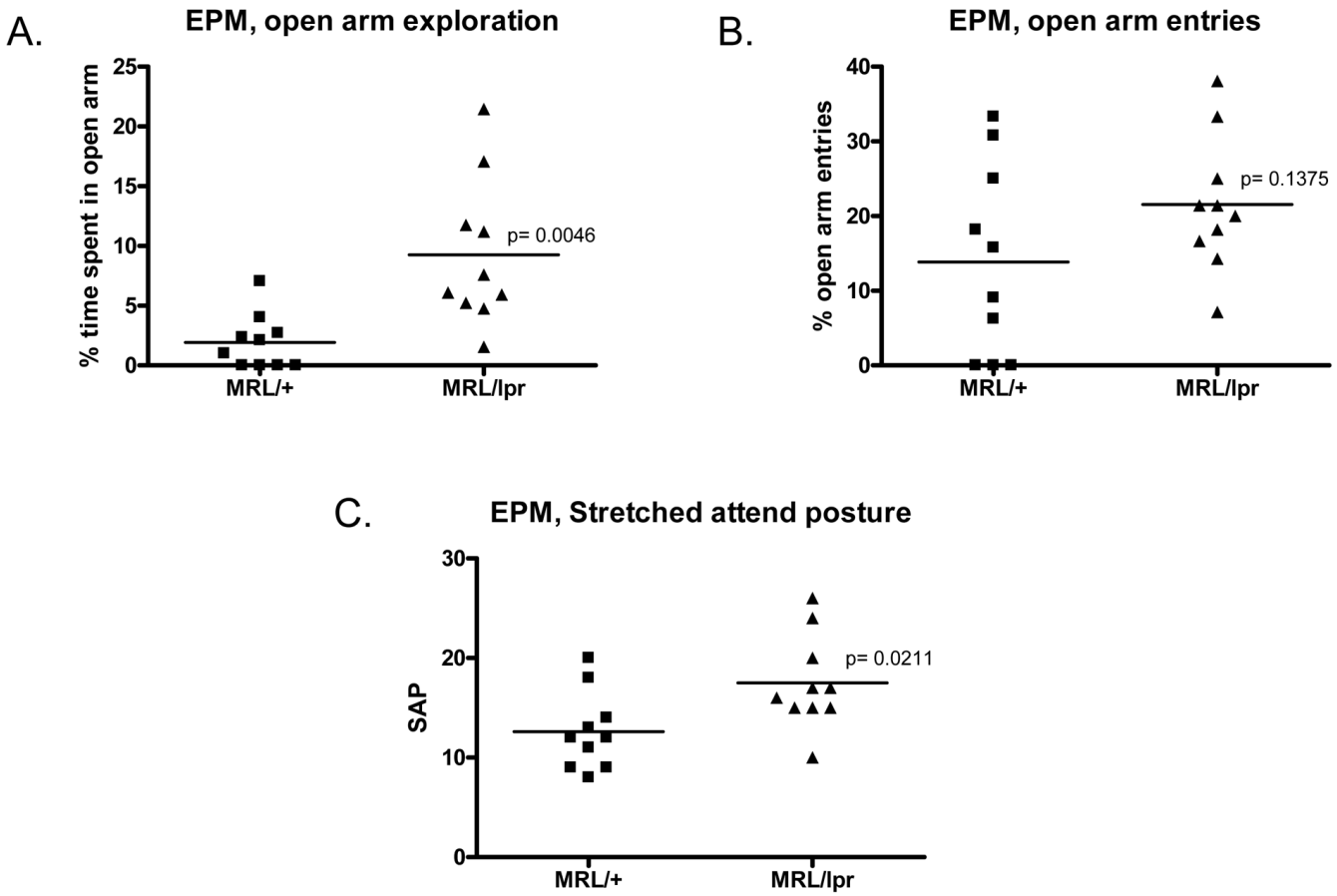

Fig. 5.

Elevated plus maze test detects less anxiety in lupus mice than control mice. Anxiety levels were tested in an EPM test. A. Time spent in the open arms was recorded and converted to a percentage of the total time in the EPM (5 minutes). The percent time spent on exploration is shown in the graph. B. Open arm entries were recorded. C. stretched attend postures counted during EPM tests.

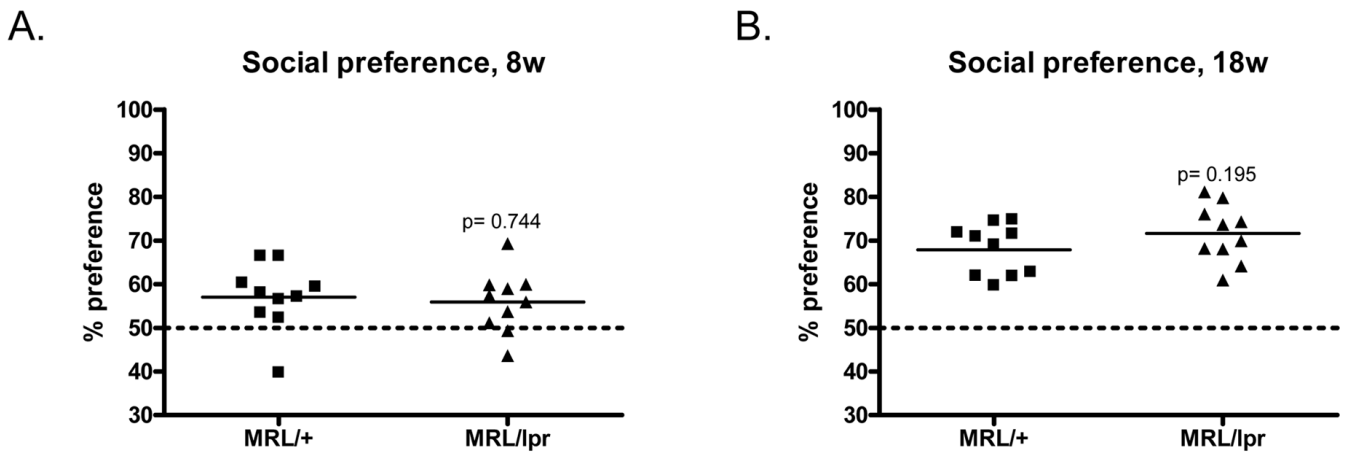

Fig. 6.

MRL/lpr mice had similar social preference as MRL/+ controls. 8w (A) and 18w (B) MRL/lpr mice were tested for social activity in a Y maze. Preference was calculated based on the social target contacting time over total exploring time. Mice having normal social activities are expected to have >50% preference to a social target (above the dotted line).

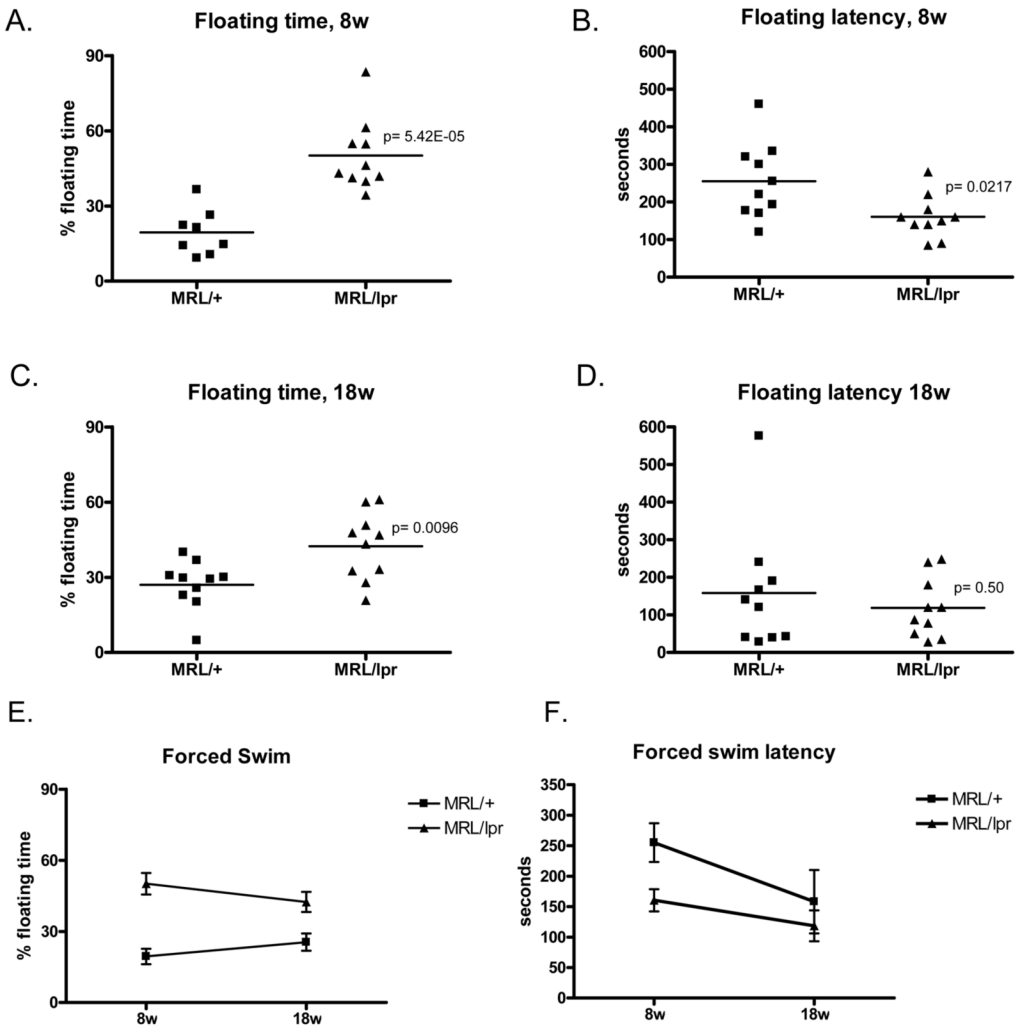

Fig. 7.

MRL/lpr mice display depression-like behavior at 8 weeks of age. The forced swim test was used to assess depression-like behavior in mice. % floating time: total floating time converted to percentage of total test time (A, test on 8 weeks of age. C, test on 18 weeks of age). B and D, floating latency is the delay time until mice give up struggling in the water and start to show the 'still' floating behavior. Time was recorded in seconds. B, testing 8 week old mice. D, testing 18 week old mice. E, F. Two way ANOVA analysis of the effects of age and genotype on total floating time (E) and floating latency (F) in the tested mice.

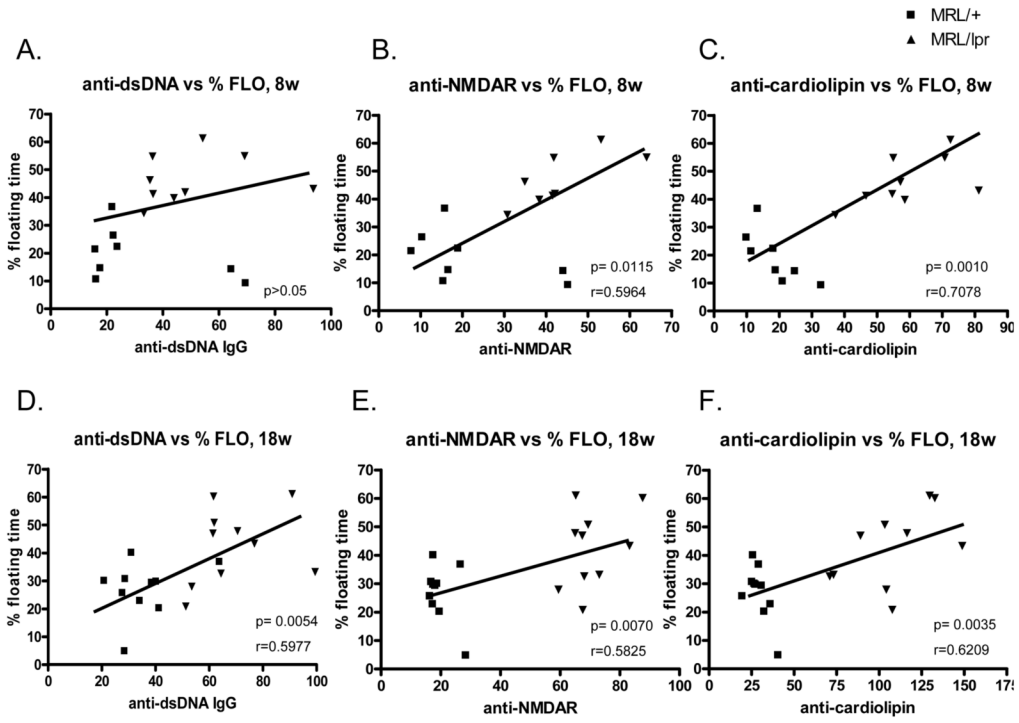

Fig. 8.

Lupus autoantibody levels correlate with depression behavior. Linear regression correlation of % floating time in forced swim test with serum anti-dsDNA titers (A, 8w; D, 18w), anti-NMDAR antibody titers (B, 8w; E, 18w), or anti-cardiolipin antibody titers (C, 8w; F, 18w) were shown. MRL/+: ■, MRL/lpr: ▼. P values and Pearson's R values for each correlation are also shown in the graphs.

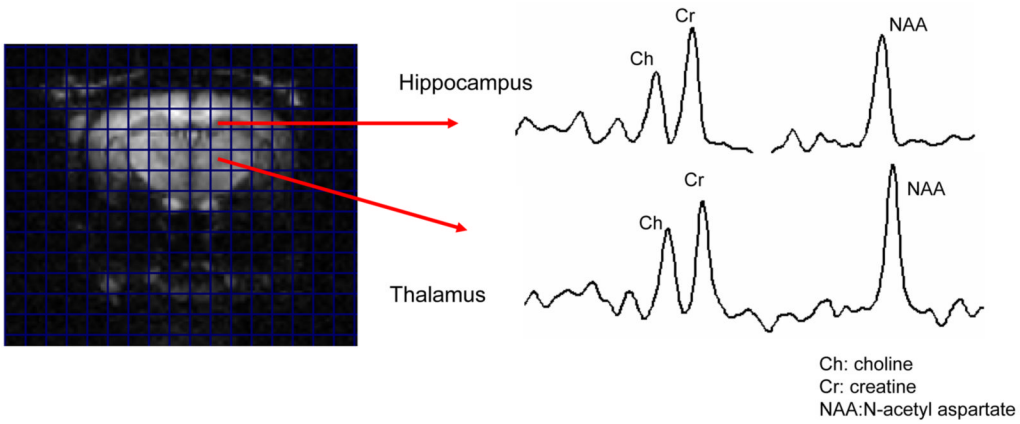

Fig. 9.

MRI/MRSI analyses show differences in regional brain metabolism between control and lupus mice. The figure illustrates the type of spectra obtained from the hippocampus (top) and thalamus (bottom), with a corresponding anatomical image of a lupus mouse brain. Data from the comparative analysis of lupus and control mouse brains is provided in Table 2.

Table 1

Estrous cycle stages of tested mice were randomly distributed. Estrous cycle (EC) stages were monitored along the test. The Table shows the phase on the day of forced swim test in 8w mice. P, proestrus; E, Estrous; M, Metestrus; D2, Diestrus II.

EC phase	P	E	M	D2
MRL/+	3	4	0	3
MRL/lpr	3	2	2	3

Table 2

Mouse brain regional NAA/Ch, NAA/creatine and Ch/Cr ratios. N=6 for each group.

	Hippocampus			Thalamus			Cortex		
	NAA/Cr	**NAA/Ch**	**Ch/Cr**	**NAA/Cr**	**NAA/Ch**	**Ch/Cr**	**NAA/Cr**	**NAA/Ch**	**Ch/Cr**
MRL/+	0.92±0.03	1.40±0.05	0.66±0.01	1.25±0.02	1.73±0.04	0.73±0.02	0.90±0.03	1.24±0.06	0.74±0.02
MRL/lpr	0.91±0.03	1.50±0.05	0.61±0.01	1.24±0.03	1.76±0.05	0.71±0.02	0.92±0.04	1.39±0.06	0.67±0.02
t test	ns	ns	0.0438*	ns	ns	ns	ns	0.0273*	0.0393*