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Short Communication

Strain and sex differences in somatosensation and sociability during experimental autoimmune encephalomyelitis

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ABSTRACT

Multiple Sclerosis (MS) is an immune-mediated disease that results in major locomotor deficits. However, recent studies have revealed that fatigue, slow processing speed, and memory impairment are the top variables impacting employment status for MS patients. These suggest that cognitive effects may have a greater impact on productivity, lifestyle, and quality of life than do disease-related motor deficits. However, these debilitating non-locomotive effects have been largely overlooked in rodent models of the disease, such as experimental autoimmune encephalomyelitis (EAE). We hypothesized that murine EAE can also be used to assess non-locomotive dysfunctions (mood, sociability, muscle strength, and balance), as well as potential biases in these dysfunctions due to sex and/or strain. We actively immunized male and female C57BL/6 (B6) and SJL mice for EAE and evaluated their performance on the Deacon's weight grip test, Kondziela's inverted screen test, Hall's rope grip test, manual von Frey test for somatic nociception, and a three-chamber social preference paradigm. We hypothesized that EAE progression is associated with changes in muscle strength, balance, pain, and sociability and that these variations are linked to sex and/or strain. Our results indicate that strain but not sex influenced differences in muscle strength and balance during EAE, and both sex and strain have an impact on mechanical nociception, regardless of EAE disease status. Furthermore, both sex and strain had complex effects on differences in sociability. In conclusion, testing these additional modalities during EAE helps to unveil other signs and symptoms that could be used to determine the efficacy of a drug or treatment in the modulation of a MS-like behavior.

1. Introduction

Multiple Sclerosis (MS) is an autoimmune disorder of the central nervous system that affects about 1 million Americans (Nelson et al., 2019; Wallin et al., 2019; Reyes et al., 2020). As one of the most common causes of neurological disability in young adults, MS has significant health, psychological, and social consequences. In addition to the commonly observed symptoms of the disease, such as physical weakness, numbness, and fatigue, behavioral and pain symptoms can dramatically affect the quality of life of the patients (Osterberg and Boivie, 2010; Arewasikporn et al., 2018; Ferraro et al., 2018; Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019), who routinely report social withdrawal and isolation, as well as feeling a lack of purpose and meaning in life (Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Amtmann et al., 2015; Benson and Kerr, 2014; Pinkston et al., 2007; Pinkston and Alekseeva, 2006). Among these patients,

Abbreviations: Multiple Sclerosis, MS; Experimental Autoimmune Encephalitis, EAE; Relapsing-Remitting Multiple Sclerosis, RR-MS; Days post-immunization, dpi; C57BL/6, B6.

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approximately 50–80% experience pain, and 47%–66% of those with pain have reported that their symptoms interfered with their work, with their household, or with their enjoyment of life (Osterberg and Boivie, 2010; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Amtmann et al., 2015; Shahrbanian et al., 2013; Kratz et al., 2017, 2017). MS shows considerable sexual bias, with a female to male ratio of 3:1, more likely to experience a relapsing-remitting (RR) form, and reporting more pain symptoms than do males with MS (Ferraro et al., 2018; Kratz et al., 2017; Kratz et al., 2017, 2017; Newland et al., 2012, 2016; Vitkova et al., 2016). There are also substantial associations between pain in MS patients and psychosocial factors such as depression and anxiety (Marck et al., 2017; Amtmann et al., 2015). Moreover, the current COVID-19 pandemic is affecting people all over the world. Although vaccines are emerging, facial covering, sanitation and social distancing and/or quarantining to break the chain of transmission are still the norm. Additionally, research groups have been reporting COVID-19 neurotropism (Koralnik and Tyler, 2020; Buzhdygan et al., 2020; Armocida et al., 2020; De Felice et al., 2020; Paniz-Mondolfi et al., 2020; Conde Cardona et al., 2020; Baig et al., 2020; Keller et al., 2020; Natoli et al., 2020; Murta et al., 2020; Alam et al., 2020; Mohammadi et al., 2020), and in some instances MS patients had a more severe disease course (Mohammadi et al., 2020; Chaudhry et al., 2020; Demir et al., 2020; Alnajashi and Jabbad, 2020; Reguera-Garcia et al., 2020; Naser Moghadasi, 2020; Peeters et al., 2020). The notion of MS as a potential risk factor to COVID-19 severity, and the necessary social distancing, exacerbates the psychological impact of MS.

Addressing the psychological aspects of MS can have a positive impact on the physical symptoms of the disease, as having a high level of personal relationships has been associated with lower self-rated physical and psychological symptoms in MS patients (Reyes et al., 2020).

Experimental autoimmune encephalitis (EAE) is a widely used model for some aspects of MS pathology (Terry et al., 2014; Robinson et al., 2014; Miller et al., 2010). Neurologic or clinical evaluations have been well-characterized in C57BL/6 (B6) mice, which exhibit a monophasic or chronic progression, using a standardized scale for locomotive dysfunction (Terry et al., 2014; Robinson et al., 2014; Miller et al., 2010). EAE can also be induced in SJL mice, which model the sexual bias of RR-MS (Terry et al., 2014; Robinson et al., 2014; Miller et al., 2010; Rahn et al., 2014; Voskuhl, 2011). This strain has gained popularity for studying non-locomotive effects of EAE, such as behavioral changes and nociception (Rahn et al., 2014; Lu et al., 2012; Pollak et al., 2000; Rodrigues et al., 2011; de Bruin et al., 2016). A recent study on behavioral changes during the course of EAE found that SJL females with RR-EAE exhibited decreased social interaction and decreased sucrose consumption when compared to controls (Pollak et al., 2000). Additional studies have observed that EAE mice developed thermal hyperalgesia during the chronic phase of the disease, and they show a significant decrease in tail withdrawal latencies compared to the control group (Lu et al., 2012; Aicher et al., 2004).

While different behavioral and physical aspects of EAE have been studied in isolation, a comprehensive assessment of the relationship between motor deficits, nociception, and sociability among different sexes and strains of mice has been absent. In this article, we address this gap in knowledge with a longitudinal analysis of motor deficits, mechanical nociceptive responses, and social behavior in both sexes of B6 and SJL mice. We hypothesized that non-locomotive signs would precede the onset of motor deficits and have a stronger influence in social behavior. This will elucidate the mechanism behind the variation seen in non-locomotive signs among different mouse strains and provide valuable information about putative sexual dimorphisms in these symptoms. Ultimately, we seek to enhance the understanding of sex-specific psychosocial and physical effects of MS in order to improve quality of life for these patients.

2. Material and methods

2.1. Animals and experimental groups

C57BL/6 (B6) and SJL mice of each sex (Jackson Laboratories, Sacramento, CA) were kept in pathogen-free conditions, according to the guidelines of the University of California, Davis (UCD) Institutional Animal Care and Use Committee (IACUC). All procedures throughout this study were in conformity with the UCD IACUC. Animals underwent pretraining for muscle strength and balance tests, and those that failed to meet training criterion were excluded from the study (Supplemental Figure 1, n = 12 F and M SJL mice). Remaining mice of each sex and strain were randomly assigned to sham control (n = 4) or EAE (n = 8) groups. EAE is associated with an anticipated mortality rate of ~20% (Wolfensohn et al., 2013). Animals that lost ≥20% initial body weight and/or became moribund (spontaneous movement was absent) received a clinical score of 5 and were humanely euthanized by CO_2 inhalation (n = 2 for F B6, n = 2 for F SJL, n = 2 for M B6, n = 2 M SJL). Final statistical analyses were done with group sizes of n = 4 for sham and n = 6 for EAE. At the end of the study, all mice were humanely euthanized by CO_2 inhalation.

2.2. Immunization and clinical scoring

Active immunization of 10-week-old mice was performed following standard protocols for each strain (Cruz-Orengo et al., 2011, 2014) (Fig. 1). Briefly, EAE was induced by subcutaneous injection of myelin emulsion containing Complete Freund's Adjuvant (CFA). B6 mice received 50 μg msMOGp35-55 (GenScript, Piscataway, NJ) with 500 μg *Mycobacterium tuberculosis* (Mtb) H37Ra peptide (Difco Laboratories, Detroit, MI), and SJL mice were immunized with 150 μg msPLPp139-151 (GenScript, Piscataway, NJ) with 200 μg Mtb. Total emulsion volume per mouse was 100 μl, equally distributed at four injection sites at the shoulder blades and flanks, ~25 μl per site. On the day of immunization, and again 2 days post-immunization (dpi), mice received intraperitoneal (i.p.) injections of pertussis toxin (PTX, List Biological Laboratories, Inc, Campbell CA) in 0.9% NaCl injectable saline; 300 ng and 200 ng for B6 and SJL, respectively. Sham mice for both B6 and SJL were immunized with CFA with strain-equivalent amounts of Mtb and received 0.9% NaCl injectable saline i. p. Starting at 7 days post-immunization (dpi), clinical score and body weight of all mice were assessed daily. Clinical scores were assigned based on the EAE standard scoring system (Terry et al., 2014; Robinson et al., 2014; Miller et al., 2010).

- Score 0 = no disease
- Score 1 = limp tail
- Score 2 = hindlimb paresis
- Score 3 = hindlimb paralysis
- Score 4 = forelimb paralysis
- Score 5 = moribund or death

In addition to *ad libitum* chow and water, mice were supplemented with Diet Gel®Boost (Clear H_2O, Portland, ME) once they reached a clinical score of 2.

2.3. Somatosensory assessments

1) **Muscle strength and balance.** Prior to immunization and data collection, all mice were trained on all motor function assessments: Deacon's weights, Hall's rope grip, and Kondziela's inverted grid (Fig. 1 and Supplemental Figure 1) (Deacon, 2013; Peled-Kamar et al., 1997), using either Kellogg's Froot Loops or peanut butter as rewards, depending on individual preference. B6 mice received 3 training

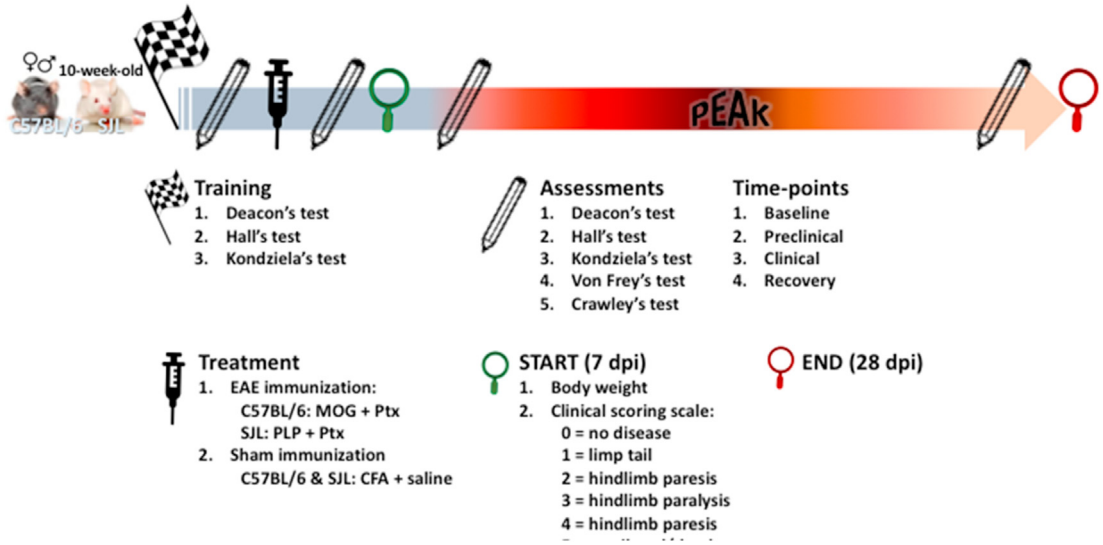

Fig. 1. Experimental approach. Layout to describe the interventions performed on C57BL/6 (B6) and SJL mice. Prior to group assignment, young adult mice were trained on the Deacon's, Hall's and Kondziela's, tests, starting at 9 and 7 weeks for B6 and SJL mice, respectively. Mice that failed to meet training criteria were excluded from the study (n = 12 M SJL and F SJL). Baseline measurements for these, as well as for the von Frey's and three-chambered sociability tests, were recorded one or two days prior to immunization. Subsequent assessments were acquired at the preclinical, clinical, and recovery stages of EAE disease. Gradient arrow = EAE progression on color scale; checkered flag = training; syringe = immunization for either EAE or sham; green loupe = initiation of daily clinical evaluation; red loupe = end of study; pencil = time-points of somatosensory (Deacon's, Hall's, Kondziela's and von Frey's tests) and social assessments (three-chambered sociability test) clinical evaluation, 7 dpi; red loupe = end of study, final clinical evaluation, 28 dpi. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sessions within a week when they were ~9 weeks old. SJL mice required earlier onset of training and received up to 6 sessions, starting ~7 weeks (Figure S1). The somatosensory test and data collection were performed at 4 points during the course of the disease:

- Baseline = 2 days prior to immunization
- Pre-clinical = 7 dpi, score 0
- Clinical = ~10 dpi, score 2
- Recovery = 21 dpi, score 1-2

The evaluator was blind to immunization status of the mice for baseline and pre-clinical assessments. Assessments were not performed during the peak clinical phase (score >2), since motor deficits (hindlimb and/or forelimb paralysis) at these scores constrained the ability to grasp the weights, rope, and grid. Each test included 3 trials, with 30 min inter-trial intervals. White noise was used during the tests to block any outside noises and prevent distraction. The devices used in these tests were custom made (UC Davis Biological & Agricultural Engineering Shop).

a) **Deacon's weight-lift test** (Deacon, 2013)**.** A series of 5 wt constructed from balls of tangled fine gauge stainless steel wire (domestic kettle scale collector) attached to varying numbers of steel chain links (Supplemental Figure 1B). The wire balls were attached to 1, 2, 3, 4, or 5 chain links, resulting in final masses of 20 g, 33 g, 46 g, 59 g, and 72 g, respectively. Each mouse was suspended by the base of the tail and lowered over the smallest weight, such that it could grasp the wire ball with its forepaws. Then the mouse was lifted until the weight was suspended off the bench. The criterion for a successful trial was the ability to grasp the weight for 3 s; if the mouse met this criterion, it was tested on the next heaviest weight was tested. The score for each mouse was calculated:

$$(3 * h) + (s) = score,$$

where $\boldsymbol{h}$ = links on heaviest weight held for 3 s; $\boldsymbol{s}$ = number of sec holding the next-heaviest weight. For instance, a mouse holding a 4-link weight for 3 s, but unable to lift a 5-link weight, is assigned a score of (3 × 4) = 12. If it holds the 5-link weight for 1 s, it scores (3 × 4) + (1) = 13.

b) **Hall's rope grip test** (Peled-Kamar et al., 1997)**.** A 2 mm wire rope was suspended on a stand 80 cm above the laboratory bench (Supplemental Fig. 1D–F). Mice were hung from the rope by their forepaws, and performance was assessed as the latency to grip the rope with the hind paws as well and maintain this grip for a minimum of 10 s. Each of the three trials were 60 s, and mice that failed to grip the rope with their hind paws during this time were scored as 60 s.

c) **Kondziela's inverted grid test** (Deacon, 2013)**.** Each mouse was placed in the center of a 40 cm^2 metal grid with 12 mm^2 openings (Supplemental Figure 1C). In less than 2 s the grid was inverted ~30 cm above a padded surface. Trials lasted 60 s, and mice were scored based on the time to fall from the grid:

- score 1 = 1–10 s
- score 2 = 11–25 s
- score 3 = 26–60 s
- score 4 = 60 s

2) **Mechanosensitivity** (Barrot, 2012)**.** A von Frey filament kit (Semmes-Weinstein monofilaments, Stoelting Co., Wood Dale, IL) was used to evaluate sensitivity to a noxious mechanical stimulus. Mice were placed in a small plexiglass cylinder on a custom-designed, elevated mesh platform (UC Davis BAE Shop, Supplemental Figure 2). After a 40-min habituation, during which a white noise generator was used to block any outside noise, a von Frey monofilament with a pre-determined force was inserted through the mesh perpendicular to the surface of the mice's paws and used to probe the hind paws. The assessment started with the monofilament of lowest force, and the force was gradually increased. The lowest force that elicited a startle response was recorded as the "awareness threshold," and the lowest force that induced any nociceptive behaviors, such as quick paw withdrawal, licking the paw, or vocalization, was recorded as "nociceptive threshold". Each mouse received three trials for each pelvic limb. The evaluator was blind to immunization status of the mice, sham or EAE, for baseline and pre-clinical assessments. Assessments were not performed during the peak clinical phase (score >2), since motor deficits (hindlimb and/or forelimb paralysis) at these scores would prevent an escape response to mechanical stimuli.

2.4. Sociability assessments

A three-chambered apparatus was custom-designed (UC Davis Biological & Agricultural Engineering Shop, Supplemental Figure 3) to assess sociability and social novelty seeking (Moy et al., 2004). The apparatus was a transparent, rectangular, plexiglass box (60 cm × 30 cm x 15.5 cm) over a metal base (62 cm × 31 cm x 2 cm). The plexiglass box was divided into 3 chambers (20 cm × 30 cm x 15.5 cm), with circular openings between chambers so the mice could access each freely. White noise was used to block any outside noise. Each test consisted of three phases:

(1) **Habituation Phase.** The test mouse was placed in the middle chamber for 5 min to habituate to the apparatus.
(2) **Social Novelty Phase.** A novel conspecific of the same sex and strain was placed in a plexiglass cylinder (h = 20 cm, d = 10 cm) in one of the side chambers. The cylinder had circular apertures (d = 1 cm) that allowed for visual and olfactory social contact between the test mouse and novel mouse while obviating physical contact or aggression. An empty cylinder was placed in the other side-chamber. The test mouse was allowed to explore the apparatus for 10 min.
(3) **Social Preference Phase.** Without removing the novel conspecific, a novel object (rubber duck, toy car, etc.) was placed in the previously empty cylinder, and the test mouse was allowed to explore the apparatus for an additional 10 min.

The tests were video recorded, and each phase was scored by a blind observer for the following events:

- time in each chamber (sec)
- entries into each chamber, defined as all four paws inside that chamber
- interactions with the cylinders, including rearing to place the forepaws on the cylinder, inserting the nose through the apertures, or otherwise physically contacting the cylinder
- grooming for ≥30 s

Analyses of social preference were calculated as the difference between encounters/time with the novel conspecific in the social novelty (no distractor) and social preference (novel object distractor) phases. Additional analyses are reported in Supplemental Figures 7–8. No sociability assessments were performed during the peak of disease since severe motor deficits would compromise free locomotion.

2.5. Statistical analysis

All analyses were performed using SAS software, version 9.4 (SAS Institute, Cary, NC). Descriptive statistics were computed as means and standard deviations for continuous variables and frequencies and percentages for categorical variables. We provided boxplots of the outcomes summarized (mean, median, quartiles) over time by treatment, sex, and strain. We corrected for between group (average) effects across time by adjusting outcomes for baseline scores. Models also adjusted for clinical stages of disease. Time was considered as a covariate in the models, two-way and three-way interactions were investigated for treatment (EAE vs. Sham), sex (Female vs. Male), and strain (B6 vs. SJL). Models Clinical outcomes of somatosensory (muscle strength & balance), mecanosensitivity, and social scores were averaged over time and studied with linear mixed models of three main effects (treatment, sex, strain), two-way, and three-way interactions. We tested the statistical significance of the main effects and interaction terms using the F test. Statistical significance was determined as $p < 0.05$ and all tests were 2-sided. A factorial design study with three factors (treatment, sex, strain) requires 40 mice to achieve 87% power when an F test is used to test main effects at a 5% significance level (effect size of 0.50) and achieves 100% power when an F test is used to test interactions at a 5% significance level (effect size of 1).

3. Results

3.1. Clinical assessment

A total of 40 mice (16 sham and 24 EAE, including F B6, M B6, F SJL and M SJL), were monitored daily from 7 dpi to 28 dpi for EAE disease progression, including body weight and standardized EAE clinical scoring of neurologic deficits (Fig. 2 and Supplemental Figure 4). As anticipated, we observed the typical disease progression for EAE mice and lack thereof for sham mice (Supplemental Figure 4A), and statistical evaluation showed these variations in clinical score over time to be significant ($p < 0.001$, Fig. 2A and Table 1).

Likewise, weight loss during EAE was as anticipated for EAE mice (Supplemental Figure 4B). Statistical analysis of weight loss progression over the course of disease showed that active immunization for EAE was a primary effector ($p < 0.0001$) and also that variations were sexually-bias ($p < 0.0001$, Fig. 2B and Table 1). Notably, interactions of some factors also contributed to these variations. The most influential interaction was between treatment group and strain ($p < 0.0001$, Fig. 2B and Table 1). The interactions between active immunization for EAE and sex ($p = 0.0002$) and between sex and strain ($p = 0.0385$) were also significant (Fig. 2B and Table 1). Moreover, these three factors together (treatment*sex*strain) contributed significantly to the variations observed ($p = 0.0005$, Fig. 2B and Table 1).

3.2. Locomotive training and assessment

We evaluated muscle strength and balance using the Deacon's weight lift, Hall's rope grip, and Kondziela's inverted grid tests (Fig. 3 and Supplemental Figure 5). All animals were trained on these tests prior to testing and immunization. Initial attempts to train SJL mice for these tests started at ~9-weeks old were unsuccessful (Supplemental Figure 1A and 1D, n = 12, data not shown). Instead, SJL mice had to begin training

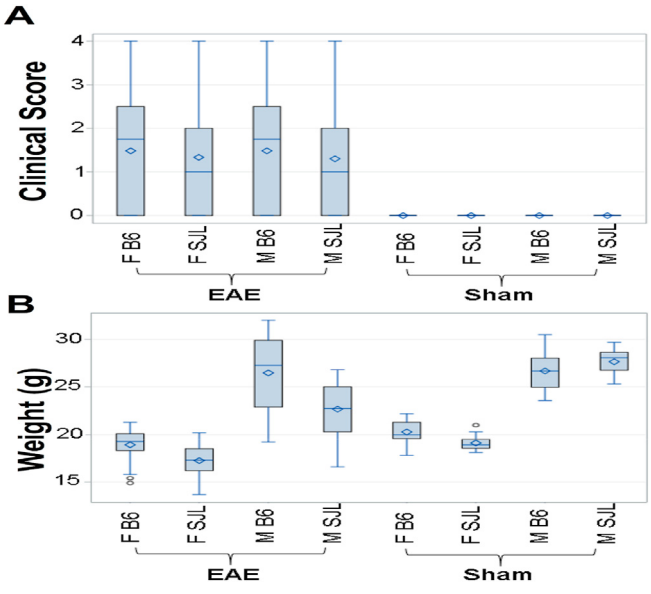

Fig. 2. Clinical scoring and body weight loss. Clinical progression of EAE for mouse cohorts of female C57BL/6 (F B6), female SJL (F SJL), male C57BL/6 (M B6) and male SJL (M SJL); n = 6 for EAE and n = 4 for sham. Results are shown as Mean ± Std Dev, analyzed by ANOVA Type I/II/III SS for disease stage: preclinical, clinical, peak, and recovery; treatment: EAE or sham; and sex, and adjusted for baseline measurements. **A)** Distributions of clinical score revealed a primary effect of treatment ($p < 0.001$). **B)** Distributions of weight loss revealed significant effects of treatment ($p < 0.0001$) and sex ($p < 0.0001$), as well as significant interactions between treatment and sex ($p < 0.001$), treatment and strain ($p < 0.001$), sex and strain ($p < 0.05$), and between all three factors, treatment*sex*strain ($p < 0.001$).

Table 1
Effect of treatment, sex and strain on EAE score, muscle strength and balance, mechanosensitivity, and social responses.

Outcome	Treatment	Sex	Strain	Treatment*Sex	Treatment*Strain	Sex*Strain	Treatment*Sex*Strain
	EAE vs. Sham	Female vs. Male	B6 vs. SJL				
EAE Score							
Clinical Score (0–5)	**1.635; <0.001**	0; 1.00	0; 1.00	0.042; 0.9303	0.229; 0.6305	0; 1.00	−0.042; 0.9507
Weight (g)	**−5.917; <0.0001**	**−8.675; <0.0001**	−0.863; 0.2105	**3.858; 0.0002**	**4.563; <0.0001**	**2.300; 0.0385**	**−4.429; 0.0005**
Somatosensory Integration							
Muscle strength & balance							
Deacon's Score	−0.389; 0.7499	0.750; 0.5749	−0.500; 0.7083	−0.917; 0.5954	**−5.389; 0.0022**	0.333; 0.8600	2.000; 0.4130
Hall's Score	3.417; 0.2761	−3.817; 0.2668	−2.525; 0.4618	3.022; 0.4950	−5.847; 0.1881	3.275; 0.4997	−2.258; 0.7183
Kondziela's Score	−0.277; 0.2935	0; 1.00	−0.250; 0.3877	−0.056; 0.8816	**−1.528; <0.0001**	0.167; 0.6835	0.3889; 0.4616
Mechanosensitivity							
Awareness Threshold (N)	0.019; 0.2291	**0.041; 0.0223**	**0.047; 0.0093**	0.037; 0.1124	**−0.078; 0.0010**	**−0.093; 0.0003**	0.055; 0.0916
Nociceptive Threshold (N)	0.063; 0.6767	0.099; 0.5529	**−0.967; <0.0001**	0.220; 0.3046	−0.364; 0.0906	**0.670; 0.0050**	0.246; 0.4002
Social Interaction							
Time (sec)	−26.667; 0.6630	−44.813; 0.5040	87.25; 0.1942	48.583; 0.5746	−1.646; 0.9848	−14.813; 0.8758	−18.833; 0.8777
Entries (number)	0.959; 0.6712	1.000; 0.6859	1.313; 0.4691	−4.375; 0.1718	−2.313; 0.4691	**−10.688; 0.0026**	**14.438; 0.0033**
Encounters (number)	2.375; 0.5863	−7.563; 0.1150	−8.188; 0.0882	−1.313; 0.8315	7.896; 0.2018	8.32; 0.2199	2.354; 0.7873

Estimates of mean differences were adjusted for clinical stages of disease, and each outcome was adjusted for baseline response; p values. Significant differences are in bold.

at ~7-weeks of age and required 3 additional training sessions, compared to C57BL/6 mice, in order to meet training criteria.

After training, assessment of baseline performance on the Deacon's, Hall's, and Kondziela's tests was done on all mice (Fig. 3 and Supplemental Figure 5). Following immunization, cohorts of n = 6 for all EAE groups and n = 4 for all sham groups were evaluated for Deacon's (Fig. 3A and Supplemental Figure 5A), Hall's (3 B and Supp. 5 B), and Kondziela's (3C and Supp. 5C) tests at pre-clinical, clinical, and recovery stages of EAE disease progression. Type III SS statistical analyses run on baseline-normalized values showed that score variations for the Deacon's weight lift test score variations were significantly influenced by the interaction of treatment and strain ($p = 0.0022$, Fig. 3A and Table 1). Similarly, the Kondziela's inverted grid test responses were significantly affected by treatment and strain ($p < 0.0001$, Fig. 3C and Table 1). No statistically significant differences were obtained for the responses on the Hall's rope grip test (Fig. 3B and Table 1).

3.3. Mechanosensitivity

Mechanical sensitivity and nociception were assessed with the von Frey test throughout the course of EAE at the aforementioned time points (Fig. 4 and Supplemental Figure 6). After normalizing awareness and nociceptive thresholds to baseline values, Type III SS statistical analysis showed primary effects of sex ($p = 0.0223$) and strain ($p = 0.0093$) on the awareness threshold variation (Fig. 4A and Table 1), as well as significant interactions between treatment and strain ($p = 0.0010$) and between sex and strain ($p = 0.0003$, Fig. 4A and Table 1). Although it did not reach significance, the interaction of the three factors (treatment, sex, and strain) did show a relevant trend ($p = 0.0916$, Table 1).

Interestingly, variations in nociceptive threshold (Fig. 4B and Table 1) did not follow the same trends as those in the awareness threshold. There was a significant primary effect of strain ($p < 0.0001$) and a significant interaction of sex and strain ($p = 0.0050$, Fig. 4B and Table 1). The interaction of treatment and strain showed a trend towards significance ($p = 0.0906$, Table 1).

3.4. Sociability tests

Mice were evaluated using the three-chamber sociability test (Fig. 5 and Supplemental Figures 7–8). We assessed the time spent in the chamber with a novel conspecific in the absence (social novelty phase) and presence (social preference phase) of a novel object distractor (Fig. 5A and Supplemental Fig. 7A–D). We also quantitated the number of entries into the chamber containing the novel conspecific for both phases (Fig. 5B and Supplemental Fig. 7E–H), as well as the number of physical interactions with the novel conspecific's cylinder (Fig. 5C and Supplemental Fig. 8A–B).

Interestingly, we did not observe significant effects on the variations in time with the novel mouse between social novelty and preference phases for any of the factors (treatment, sex, and strain), nor for any of their interactions (Fig. 5A and Table 1). For the number of entries into the mouse-occupied chamber, we observed significant interactions between sex and strain ($p = 0.0026$) and between treatment, sex, and strain ($p = 0.0033$, Table 1). There was a trend towards a significant effect of strain on the number of interactions ($p = 0.0882$, Table 1).

However, not all time spent in the mouse-occupied chamber represented a social interaction with the novel mouse. We also analyzed periods of grooming for 30 s or more, a self-focused behavior (Supplemental Fig. 8C–D), but there were no significant differences between groups in these data (data not shown).

4. Discussion

"That which does not kill us, makes us stronger ". This aphorism, attributed to Nietzsche, is far from accurate when we consider quality of life for patients with MS. The physical and psycho-emotional sequelae result not in endurance and resilience but rather in pain, financial burden, and other significant impacts on lifestyle (Osterberg and Boivie, 2010; Arewasikporn et al., 2018; Ferraro et al., 2018; Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Amtmann et al., 2015; Benson and Kerr, 2014; Pinkston et al., 2007; Pinkston and Alekseeva, 2006; Shahrbanian et al., 2013; Kratz et al., 2017, 2017; Harding et al., 2019). Typically, standard of care therapies for neurological disorders focus only on primary (physical/motor) symptoms, when it is actually the non-physical effects that have the greatest impact on quality of life (Zivadinov et al., 2016; Tornatore et al., 2016; Kappos et al., 2015; Harel et al., 2018; Bovis et al., 2019; Sormani et al., 2019; Mattioli et al., 2014; Maruszczak et al., 2015). As such, there has recently been a concerted effort to better characterize the neuropsychological aspects of MS (Benedict et al., 2017; Isernia et al., 2019; Migliore et al.,

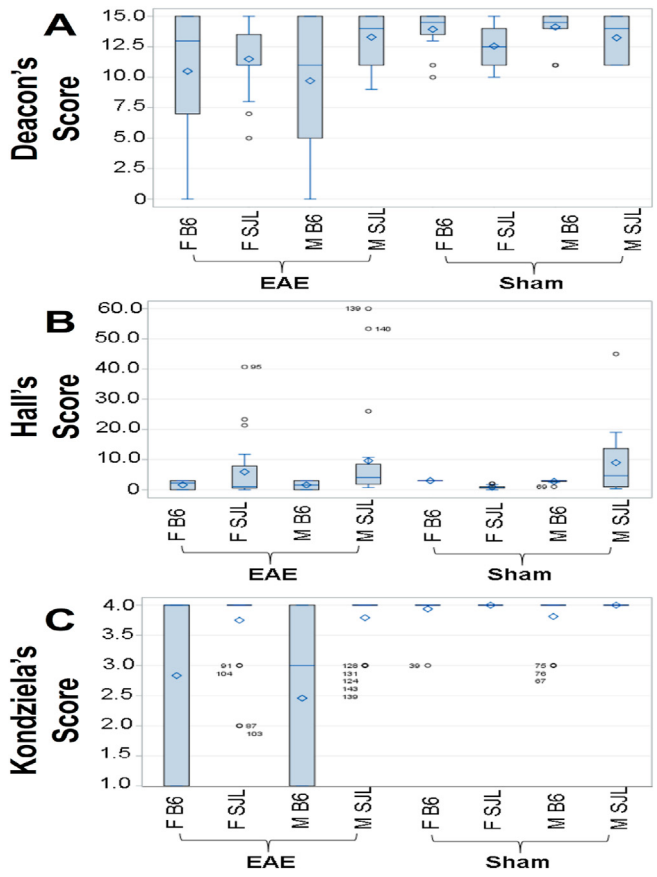

Fig. 3. Somatosensory: muscle strength and balance during EAE. Performance on muscle strength and balance tests for F B6, F SJL, M B6, and M SJL mice; n = 6 for EAE and n = 4 for sham. Results are shown as Mean ± Std Dev, analyzed by ANOVA Type I/II/III SS for disease stage: preclinical, clinical, and recovery; treatment: EAE or sham; and sex, and adjusted for baseline measurements. **A)** Distributions in Deacon's weight lift scores also showed a significant interaction between treatment and strain ($p < 0.01$). **B)** There were no significant differences in the distributions of scores for the Hall's rope grip test. **C)** Variations in Kondziela's inverted grid over the course of disease were significantly affected by the interaction of treatment and strain ($p < 0.0001$).

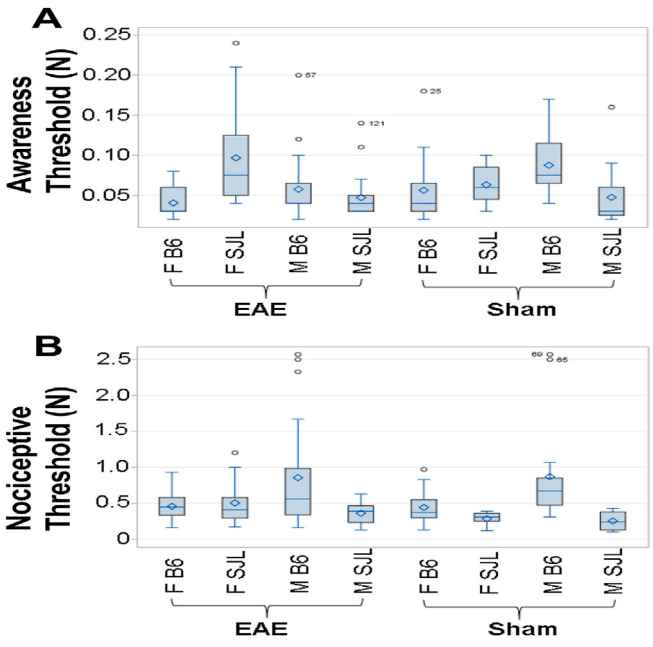

Fig. 4. Somatosensory: mechanical nociception during EAE. Performance on the von Frey test for F B6, F SJL, M B6, and M SJL, n = 6 for EAE and n = 4 for sham. Results are shown as Mean ± Std Dev, analyzed by ANOVA Type I/II/III SS for disease stage: preclinical, clinical, and recovery; treatment: EAE or sham; and sex, and adjusted for baseline measurements. **A)** "Awareness" threshold was defined as the lowest force needed to elicit a startle response. There were significant main effects of sex ($p < 0.05$) and strain ($p < 0.01$), as well as interactions between treatment and strain ($p = 0.001$) and between sex and strain ($p < 0.001$) **B)** Nociceptive threshold on the von Frey's test was defined as the lowest force needed to induce nociceptive responses. There was a significant main effect of strain ($p < 0.0001$) and a significant interaction between sex and strain ($p < 0.01$). There was a trend towards a significant interaction between treatment and strain ($p = 0.0906$).

2019; Macias Islas and Ciampi, 2019; Di Stefano et al., 2019; Scherder et al., 2017, 2018; Khan et al., 2018), including pain and cognitive, psychological, and social deficits, and to redefine them as essential aspects of MS pathology (Arewasikporn et al., 2018; Ferraro et al., 2018; Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Kratz et al., 2017, 2017; Newland et al., 2016; Benedict et al., 2017; Isernia et al., 2019; Chalah and Ayache, 2017; Lex et al., 2018). Anxiety, depression, and pain significantly contribute to social alienation, decreased productivity, and diminished sense of purpose and delight in life; therefore, they should be considered when therapies for MS are being developed (Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Kratz et al., 2017,2017; Newland et al., 2016; Benedict et al., 2017; Isernia et al., 2019; Chalah and Ayache, 2017). Additionally, MS's sexual bias contributes to the frequency and severity of these signs and symptoms, enhancing the need for more appropriate interventions to solve health disparities (Osterberg and Boivie, 2010; Young et al., 2017; Marck et al., 2017; Hakansson et al., 2019; Kratz et al., 2017; Amtmann et al., 2015; Benson and Kerr, 2014; Pinkston et al., 2007; Pinkston and Alekseeva, 2006; Shahrbanian et al., 2013; Kratz et al., 2017, 2017).

In uncertain times like the current COVID-19 pandemic, patients with MS are among the first groups that are impacted by restriction of access to health resources and social interactions (Chaudhry et al., 2020; Demir et al., 2020; Alnajashi and Jabbad, 2020; Reguera-Garcia et al., 2020; Peeters et al., 2020). This puts them at a higher risk of developing mental health conditions in addition to COVID-19 reported neurological involvement (Koralnik and Tyler, 2020; Buzhdygan et al., 2020; Armocida et al., 2020; De Felice et al., 2020; Paniz-Mondolfi et al., 2020; Conde Cardona et al., 2020; Baig et al., 2020; Keller et al., 2020; Natoli et al., 2020; Murta et al., 2020; Alam et al., 2020; Mohammadi et al., 2020; Chaudhry et al., 2020; Demir et al., 2020; Alnajashi and Jabbad, 2020; Reguera-Garcia et al., 2020; Naser Moghadasi, 2020; Peeters et al., 2020; Chen et al., 2020). Patients with MS have expressed increased anxiety about their disease and treatment, increased levels of depression and mental fatigue, and decreased quality of life during the COVID-19 pandemic (Demir et al., 2020; Alnajashi and Jabbad, 2020). Developing a comprehensive and multifactorial assessment of the social and behavior symptoms of patients with MS is helpful in addressing the neuropsychiatric challenges faced by this vulnerable patient population at critical periods.

We used EAE, a common murine model of MS, to investigate non-motor sequelae, aspects of MS pathology not commonly assessed with this animal model. We included both B6, in which the nociceptive pathways and social behaviors have been well-characterized, and SJL, which models MS sexual bias, to display relapse-remission cycles in EAE (Terry et al., 2014; Robinson et al., 2014; Miller et al., 2010; Voskuhl, 2011; Sexton et al., 2018; Pham et al., 2019). Past research has assessed changes in behavior, including pain, during EAE and RR-EAE (Rahn et al., 2014; Voskuhl, 2011; Lu et al., 2012; Pollak et al., 2000; Rodrigues et al., 2011; de Bruin et al., 2016; Aicher et al., 2004), but none have examined the effects of strain, sex, and disease stage on muscle strength and/or balance, pain, and sociability. We compared EAE mice to

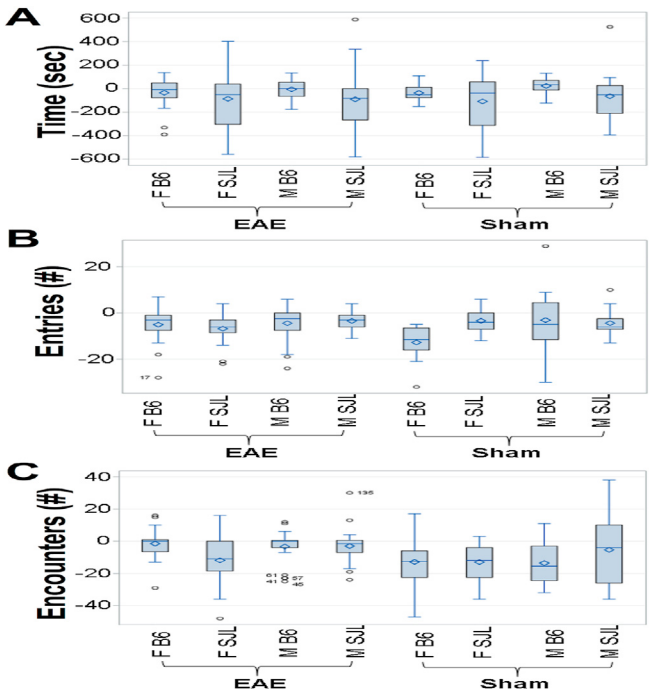

Fig. 5. Social behavior during EAE. Distributions of differences between the social novelty phase (novel conspecific vs empty cylinder) and social preference phase (novel conspecific vs novel object) for F B6, F SJL, M B6, and M SJL mice), n = 6 for EAE and n = 4 for sham. Results are shown as Mean ± Std Dev, analyzed by ANOVA Type I/II/III SS for disease stage: preclinical, clinical, and recovery; treatment: EAE or sham and sex, and adjusted for baseline measurements. **A)** There were no significant differences in time (in seconds) spent in the chamber with the novel conspecific versus the time spent in the opposite chamber, **B)** Distributions in the number of entries into the chambers. There were significant interactions between sex and strain ($p < 0.01$) and between treatment, sex, and strain ($p < 0.01$). **C)** Distributions of the number of physical interactions with the novel conspecific's cylinder. Variation between phases for number of interaction events showed a trend for strain, $p = 0.0882$.

CFA-immunized sham mice to dissociate if an observation was the result of myelin-reactive neuroinflammation or CFA-induced and centrally-induced peripheral neuropathy (Martinov et al., 2013; Lees et al., 2015; Ransohoff et al., 2015; Frezel et al., 2016).

4.1. Motor effects of EAE

We experienced an unforeseen difference between strains during the motor test training phase. Initially, all mice received 3 training sessions a week prior to active immunization for EAE, when they were ~9 weeks old. However, SJL mice were resistant to training (Supplemental Figure 1), exhibiting freezing behavior (Supplemental Figure 1D) when placed on the rope for the Hall's test. Likewise, they refused to grip the Kondziela's inverted grid and the Deacon weights, despite receiving a palatable reward (Kellogg's Froot Loops and/or peanut butter, Supplemental Figure 1A). The mechanisms behind this strain difference are beyond the scope of the current paper, but we found that beginning training earlier, ~7 weeks old, and extending the number of training sessions from 3 to 6 led to a meaningful improvement in their performance. When mice were tested for their baseline performance prior to active immunization, SJL and B6 mice showed no difference in their execution on Deacon's, Hall's, and Kondziela's tests (Fig. 3 and Supplemental Figure 1).

Once we achieved equal levels of trainability for B6 and SJL mice, we proceeded to compare changes in muscle strength and balance resulting from EAE pathology. Despite equal levels of training, B6 and SJL mice performed differently on the Deacon's weight lift and Kondziela's inverted grid tests during EAE (treatment* strain interactions, $p < 0.01$ and $p < 0.0001$, respectively, Table 1 and Supplemental Figure 5). There were no significant differences in Hall's test performance based on treatment, sex, or strain (Table 1 Supplemental Figure 5). We also performed a pilot with a cohort of B6 mice and SJL male mice at chronic stage, after 28 dpi, and found no difference with recovery stage scores (data not shown). Overall, these data suggest that tests like Deacon's and Kondziela's can be valuable assays to elucidate motor and balance capabilities during EAE neuropathology. To our knowledge, this is the first report of the use of these tests to evaluate EAE and RR-EAE mice.

We initially presumed that SJL mice would show worse execution than B6. This assumption was not only based on their reluctance for training as adults, but because SJL mice are deficient in the dysferlin protein encoded by the gene *DYSF* (Rayavarapu et al., 2010; Weller et al., 1997; Bittner et al., 1999; Vafiadaki et al., 2001). Dysferlin is one of six ferlins involved in calcium-mediated cell dynamics necessary for muscle fiber repair (Bulankina and Thoms, 2020). In humans, mutations to ferlin proteins had been implicated in multiple myopathies (Bulankina and Thoms, 2020; Cardenas et al., 2016; Koutsoulidou and Phylactou, 2020; Patel et al., 2017). More specifically, mutations to *DYSF* can cause an array of muscle diseases collectively known as dysferlinopathies, including limb-girdle muscular dystrophy type 2 B and Miyoshi myopathy (Bulankina and Thoms, 2020; Cardenas et al., 2016; Koutsoulidou and Phylactou, 2020; Patel et al., 2017). At 6 months of age, SJL mice develop spontaneous myopathy as determined by histology, muscle wasting (body weight loss), tail-pinching, open-field and grip tests, and decreased soleus muscle force *ex vivo* (Rayavarapu et al., 2010; Weller et al., 1997; Bittner et al., 1999; Vafiadaki et al., 2001). However, all the aforementioned assays were performed when myopathy was fully established. That said, we were expecting SJL mice dysferlin deficiency to have a negative impact on overall weight-bearing, muscle strength and proprioceptive balance prior to 6 months, and we were surprised with the poorer Deacon's and Kondziela's performances of EAE B6 mice compared to SJL EAE mice (Supplemental Figure 5). A plausible explanation is that early training compensated for the putative role of dysferlin deficiency on younger SJL mice.

We did not investigate the performance of female SJL after a relapse, nor the other groups (M SJL, F B6, M B6) at that chronic stage (~42 dpi). This might be considered as a limitation of our study. However, SJL mice undergoing recovery/chronic or relapse/remission stages are closer in the age to the landmark for dysferlinopathy establishment. Although the Deacon's, Hall's and Kondziela's tests differ from the tail-pinching, open-field and grip tests, all these assays rely on muscle strength and balance. We assumed that once myopathy is fully established it will exert synergism with ongoing EAE neurologic deficits. Prior attempts to ameliorate SJL inflammation against dysferlin-deficient myopathies through pharmacologic treatment, showed no effect on muscle fiber degeneration despite inhibition of macrophage infiltration (Rayavarapu et al., 2010). Moreover, this treatment produced worst scores for muscle strength and balance tests (Rayavarapu et al., 2010). That said, we are convinced that the efficacy of using Deacon's, Hall's and Kondziela's tests to assess strain and sex differences beyond recovery stage is limited because of SJL idiopathic myopathy.

4.2. Mechanosensitivity effects of EAE

Sex differences in mechanical nocioperception during EAE have been assessed in B6 mice using the von Frey's test, but this strain does not exhibit a sexual bias in EAE presentation (Rahn et al., 2014; Lu et al., 2012). Separately, strain differences have been assessed in female SJL and B6 mice but not their male counterparts (Rahn et al., 2014; Lu et al., 2012). Additionally, both studies reported different disease progression than what we commonly obtain with our active immunization protocols (Supplemental Figure 4). Specifically, it is typical for mice to experience paraplegia at the peak of disease progression (clinical score = 3), and deficits are expected to resolve during the recovery phase to a clinical

score of 1 (tail hypotonia) or 2 (hindlimb paresis). The aforementioned studies reported moderate to severe paresis at peak, which persisted at recovery, and in one study the CFA-immunized control mice also exhibited some neural deficits (Rahn et al., 2014; Lu et al., 2012). We interrogated mechanical nociception using an immunization protocol that produces a more severe peak clinical score and a partial resolution of deficits during the recovery phase.

Importantly, we observed dramatic effects of strain on mechanical nociception, particularly the nociceptive threshold at baseline (Supplemental Figure 6). Once statistical analyses were performed, we were able to assess the individual sources of these variations (Fig. 4 and Table 1). Strain had significant effects on both awareness ($p < 0.01$) and nociceptive ($p < 0.0001$) thresholds (Table 1). Interestingly, we also observed that changes in awareness did not necessarily parallel changes in nociception (Fig. 4 and Table 1). As an example, sex alone was a source of variations for awareness threshold ($p < 0.05$, Table 1) but not for nociceptive threshold. However, the interaction of sex and strain was significant for both awareness and nociceptive thresholds ($p < 0.001$ and $p < 0.01$, respectively, Table 1). More notably, treatment – EAE vs sham – was not a statistically significant source of variation (Table 1). One explanation for this effect could be the CFA-induced systemic inflammatory responses (Martinov et al., 2013; Sorge and Totsch, 2017; Sorge et al., 2015; Cruz-Orengo et al., 2008). Most studies using CFA are done by footpad or nerve injection, which induces peripheral immune induction and inflammatory cytokine release. Thus, it is reasonable to consider that it could elicit a central neuropathic pain response when injected subcutaneously at the flanks and shoulder blades (Barrot, 2012; Pham et al., 2019; Martinov et al., 2013; Sorge and Totsch, 2017; Sorge et al., 2015; Cruz-Orengo et al., 2008). Many groups are working to elucidate sexual dimorphisms in neuroimmune interactions and their link to triggering central and peripheral neuropathic pain (Barrot, 2012; Khan et al., 2018; Pham et al., 2019; Lees et al., 2015; Sorge et al., 2015; Tsuda, 2017; Salter and Stevens, 2017). Inducing EAE via adoptive transfer of myelin-reactive T cells, a. k.a. Passive immunization, may be the best approach for distinguishing EAE-specific mechanical pain by avoiding the confounding effect of peripheral immune responses elicited by CFA. This approach was used by others when interrogating thermal hyperalgesia and other behaviors on SJL females during EAE (Pollak et al., 2000; Aicher et al., 2004). One study reported reduction of food and sucrose intake, body weight, and social exploration after passive induction of EAE (Pollak et al., 2000). The other compared tail and forepaw withdrawal latency after active and passive immunization of male and female SJL (Aicher et al., 2004). They found tail latency was prolonged (hypoalgesia) during peak of disease and was decreased (hyperalgesia) during chronic EAE, while forepaw latencies remained the same during the length of the study, regardless of sex. They concluded that both types of immunization are equally useful to assess thermal pain on SJL mice during EAE (Aicher et al., 2004).

4.3. Behavioral effects of EAE

Previous studies on social behavior in EAE have only been conducted in female SJL mice (Pollak et al., 2000; de Bruin et al., 2016), and the use of the three-chambered sociability test has been limited to the recovery time point, 26–28 dpi (de Bruin et al., 2016). We sought to extend these studies to elucidate if SJL and B6 mice behave differently during novel social encounters throughout EAE pathology. Because of this, we evaluated the number of entries, or physical translocations with all four paws, not only solely time spent within a mouse-occupied chamber, with or without an object distractor (Fig. 5B and Supplemental Fig. 7E–H). Notably, our statistical analysis showed that time spent with the novel mouse was not different between social novelty and preference phases (Fig. 5A and Table 1). We interpreted this as the mice being eager to socialize despite their neurologic deficits. We were surprised about this finding at first, because male SJL mice are well known to exhibit extreme levels of aggression (Azkona and Caballero, 2019; Theil et al., 2020; Van Loo et al., 2003). Indeed, male SJL mice aggression even among littermates is so severe that it is consider inhumane not to house individuals separately (Azkona and Caballero, 2019; Theil et al., 2020; Van Loo et al., 2003). We were expecting male SJL mice to dislike the presence of a novel mouse to the extent of showing signs of aggression when entering the mouse occupied chamber or by avoiding that chamber entirely. Although SJL mice showed lesser entries to the mouse-occupied chamber was different between phases (Fig. 5B, Table 1 and Supplemental Fig. 7E–H), they did not show signs of seeking an aggressive interaction with the novel mouse (data not shown).

Second, during EAE, mice tended to ambulate less between chambers and spent approximately the same time with a novel conspecific, whether or not there was a distractor (novel object) present (Fig. 5A, Table 1 and Supplemental Figures 7A and 7C). We had two plausible explanation for this result. One is that mice learned the three-chamber paradigm and, being willing to socialize, they showed a preference for the mouse-occupied chamber. Also, their social enthusiasm made them compensate for their ambulatory insufficiency caused by hind limb paresis by spending more time within the mouse-occupied chamber.

Furthermore, we were able to distinguish self-oriented (grooming) versus mouse-oriented (interacting with the cylinder containing the novel conspecific) behavior (Fig. 5C and Supplemental Figure 8). Consistent with our previous interpretation regarding societal eagerness, grooming behavior was not a major distractor for social interaction between phases (data not shown). Interactions included placing the forepaws on the cylinder, inserting the nose in any of the cylinder's holes, or otherwise physically contacting the cylinder. In any case these interactions didn't show a tendency towards hostility, especially among male SJL mice as it was our concern as aforementioned. Interestingly, our data showed a trend towards significance attributed to strain alone ($p = 0.0882$, Table 1 and Supplemental Figure 8).

We speculate that technical limitations are partly responsible for our inability to identify more significant differences with the social preference approach. As an example, videos were hand-scored instead of using automated video-tracking software, so we were unable to measure the exact time spent executing these behaviors or to set a distance criterion for what constituted an interaction. While the analysis was blinded, it could have been more rigorous and unbiased if automated.

Additionally, hind limb paresis may have confounded our results during the clinical stage of sociability test, as mice could be physically unable to complete some of the behaviors, such as rearing, during that stage. Motor dysfunction can have a significant impact on both ability and desire to socialize and engage in self-care behaviors like grooming. As a token of proof, EAE mice look very disheveled, some even extreme, when body weight loss and neurologic disfunction are at peak. Likewise, pain might be a contributing factor for the lack of robustness of social preference approach. Although SJL mice and female B6 mice showed no significant changes in nociceptive threshold, male B6 mice did (Supplemental Figure 6). Since, sham mice were immunized with CFA which causes peripheral inflammation, this might have triggered a painful response that made sham B6 mice indistinguishable from male EAE B6. That said, we believe the results of this study are still informative, while future studies should consider modifying the behaviors measured to account for decreased motor ability and pain augmentation during the clinical stage.

Furthermore, we used both sham and EAE mice, rather than naïve individuals, as novel conspecifics for the three-chambered sociability test, in an effort to reduce the total number of mice in the study. It is possible that the health status of the novel mouse may have either facilitated or deterred interactions from the test mouse. Future studies should use naïve mice as novel conspecifics to prevent this potential confounder.

5. Conclusions

We conducted longitudinal analyses of the motor, somatosensory, and

behavioral effects of EAE progression on C57/BL6 and SJL mice. Our results indicate that a multifactorial approach provides a better view of EAE neuropathology, with a much broader spectrum than the classical EAE scoring. Although the standardized EAE scoring is the current gold-standard, it focuses solely on ascending paralysis. This approach is insufficient for the understanding of the complexity of MS pathology and neuropsychology. These non-locomotive effects and their impact on quality of life are important areas of focus for future research on MS mechanisms and therapies. Therefore, assessing the feasibility of the EAE murine model to elucidate non-locomotive effects is a dire task.

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Declaration of competing interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2021.100262.

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