

Tactile Learning within the Early Phase of Experimental Autoimmune Encephalomyelitis in Mice

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The purpose of this study was to assess tactile learning in the early phase of experimental autoimmune encephalomyelitis (EAE), which was induced in C57BL/6 mice by subcutaneous injections on flank of myelin oligodendrocyte glycoprotein, MOG35-55 (250 µg per mouse). Tactile learning was assessed one week after EAE induction using the novel object recognition test (NORT) in a dark room. The procedure consisted of two phases. During the training phase (T1), the animals explored two similar objects; within the test phase (T2, occurring 4 h later) the mice explored one novel and one familiar object. On average, mice developed significant behavioral disabilities related to EAE 13.2 ± 1.9 days following immunization. In the EAE group, the locomotor activity level (assessed by measuring the distance travelled) in the T1 and T2 phases did not differ significantly, as compared to the related phases in the control group ($P > 0.05$). Within phase T1, no reliable differences between experimental groups were found for the frequency (number) of visits to the sample objects and for total exploration time. For phase T2, no difference was also found in the discrimination ratio when comparing the control group with the EAE group. Our study demonstrates that tactile learning in male mice may not be affected 7 days after immunization with MOG35-55 (i.e., within the early EAE phase).

Keywords: multiple sclerosis, experimental autoimmune encephalomyelitis, behavior, tactile learning.

INTRODUCTION

Multiple sclerosis (MS) is a progressive neurodegenerative disease of the CNS characterized by widespread lesions in the myelin sheaths of the fibers and damage to the gray matter [1]. There is a great diversity of motor, somatosensory, visual, coordination, and cognitive symptoms and signs in MS patients [2]. The attention of researches has only recently been focused on cognitive consequences in this disease. A loss of learning and memory abilities is the most common cognitive symptom evident in 40-65% of patients suffering from MS [3]. Certain types of memory are more intensely affected in this disease. For example, it was reported that deficits

in executive functions in MS patients occurs less frequently than memory or processing speed disabilities [4].

Experimental autoimmune encephalomyelitis (EAE) is one of the most used models for studying the etiopathogenesis of MS [5]. Despite being traditionally concentrated on motor dysfunction, some papers have recently reported that there are behavioral changes in EAE. According to Pollak et al. [6, 7], low social interaction and low sucrose consumption were induced in EAE mice when compared to the controls. On the other hand, there are reports demonstrating that spatial learning and recall (e.g., in the Morris water maze spatial memory test) were not impaired following induction of EAE in C57BL/6 mice [8].

In our study, we investigated tactile learning in a model of EAE induced in C57BL/6 mice.

METHODS

Animals. The animals were purchased from the Rafsanjan University of Medical Sciences. Mice were housed in groups (two to four per cage) and

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maintained at a 12 h light/dark cycle (lights on 07:00 to 19:00) with free access to food and water. The animal housing temperature was maintained at $23 \pm 2.0^\circ\text{C}$. During the experiments, all animals were weighed every day.

Reagents. Myelin oligodendrocyte glycoprotein (MOG, Alexis Company, USA) along with complete Ferund's adjuvant (CFA), Cresyl Fast Blue, pertussis toxin, Luxol Fast Blue, and lithium carbonate (Sigma-Aldrich, USA) were used in our experiments.

Experimental Groups. Mice were randomly divided into three experimental groups with seven mice in each group as follows: (i) control, with no interventions, (ii) sham group, in which animals received CFA and pertussis toxin without MOG, and (iii) EAE group, where EAE was induced using MOG35-55, CFA, and pertussis toxin.

Induction of EAE in Mice. EAE was induced using a peptide, MOG35-55 (Alexis, USA), corresponding to the sequence of rodent MOG. Experimental mice received a subcutaneous injection on flank of 250 μg MOG35-55 per mouse emulsified in CFA containing 0.4 mg *Mycobacterium tuberculosis*. Animals also received pertussis toxin (500 ng per mouse) immediately after immunization and 48 h later [10]. In the sham-treated group, animals received CFA and pertussis toxin but did not receive MOG. Mice were monitored daily for weight loss and neurological signs of EAE. The severity of the disease among EAE mice was scored based on the method reported by Onuki et al. [9, 10]: grade 0, no signs of disease; grade 1, partial loss of tail tonicity; grade 2, loss of tail tonicity along with tail righting disabilities; grade 3, unsteady gait and mild paralysis of one hindlimb; grade 4, hindlimb paralysis and incontinence; grade 5, quadriplegia, and grade 6, the animal was died.

Object Recognition Task. The object recognition task assesses recognition memory and is based on a natural tendency of animals to preferentially explore novel objects, as opposed to familiar objects [11]. The experimental apparatus was a Plexiglas box (35 \times 35 \times 35 cm) with a black plastic floor placed in a dimly illuminated room [12]. The objects to be discriminated were square and triangular iron blocks. The behavior of the mice was recorded by a camera positioned directly above the box and subsequently analyzed using Ethovision software (Noldus, Netherlands).

The object recognition task was done in three phases (habituation, training, and test phases) with

a 24-h-long interval between the habituation and training phases and 4-h-long interval between the training and test phases. During the habituation phase, the mice were allowed to freely explore the box in the absence of objects for 30 min. In the training phase (T1), each mouse was placed in the box with one object and was allowed to explore for 10 min. To prevent side preference affecting the results, the position and shape of the object were changed after each animal was tested. All mice were placed in the box at the same point, and they were facing the same direction. Within the test phase (T2), each mouse was returned to the box where it was presented with a familiar object from the training trial (the position of this object was consistent between both training and test phases) and a novel object. Exploration time in phase T2 was 10 min (similar to that in T1). Care was taken to avoid olfactory stimuli by cleaning the box and objects with 70% ethanol between tests [13]. The time (sec) spent for exploring the objects was recorded. Exploration was defined as pointing the nose to the object at a distance ≤ 2 cm. Climbing and/or sitting on an object were not considered as exploration. Within phase T2, the discrimination ratio was calculated as (time spent for exploring novel objects only divided by the total time spent in exploring both objects) $\cdot 100$ (%). Mice showing a total exploration time < 10 sec on either training or testing phases were excluded [13].

Statistical Analysis. Statistical analysis was performed using Excel and SPSS softwares. All data are expressed as means \pm s.e.m. Differences between the groups were determined using ANOVA followed by the Tukey *post-hoc* test. The paired *t*-test was also used to compare activity levels between the trial and test phases. For comparison of behavioral scores in the EAE group, we used repeated measurement ANOVA (RMA); *P* values smaller than 0.05 were considered statistically significant.

RESULTS

Behavioral Scores. In the EAE group, the first statistically significant behavioral scores of EAE became apparent, on average, 13.2 ± 1.9 days after immunization. In this group, the behavioral scores increased to a peak level of 3.4 ± 0.8 (17 days following immunization; RMA, $P = 0.049$). In the sham-treated group, we observed no behavioral scores of EAE throughout the period of study (Fig. 1).

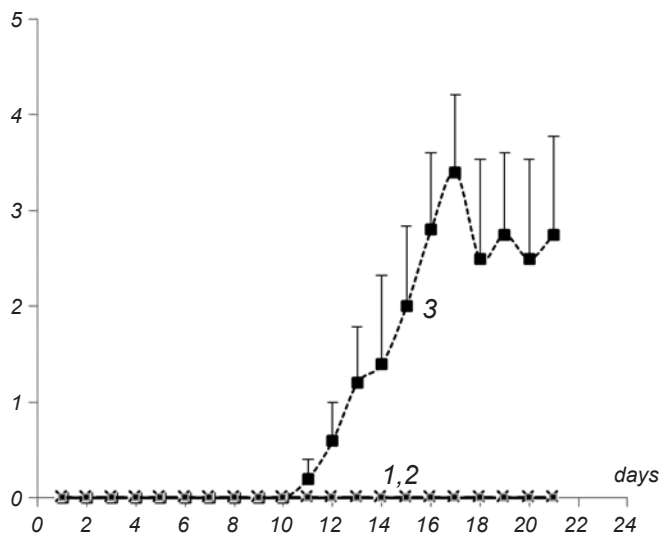


Fig. 1. Means of behavioral scores of mice in different groups. Abscissa) Days after immunization; ordinate) points. 1 and 2) Scores for the control and sham groups; 3) those for the EAE group.

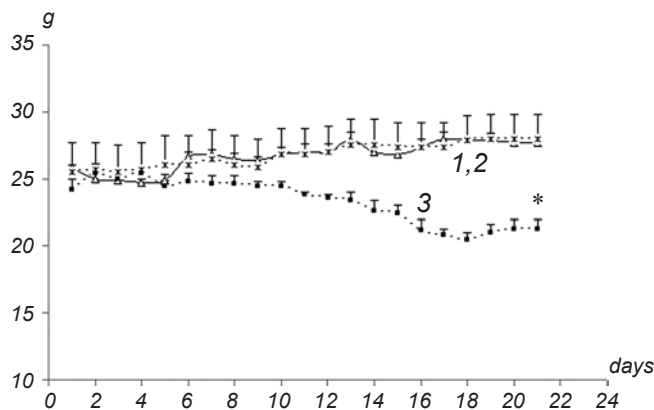


Fig. 2. Mean body mass (g) of mice in different groups within the observation period. Designation of the groups is the same as in Fig. 1. Significant difference between day 1 and day 21 in the EAE group ($P = 0.003$) is shown by asterisk.

TABLE 1. Frequencies and Times of Visits with Respect to Novel or Familiar Objects within Phases T1 and T2 in Three Experimental Groups

Measured index	Groups		
	control	sham	EAE
Trial phase (T1)			
total exploration time, sec	31.83 ± 7.1	38.24 ± 9.2	50.74 ± 2.06
number of visits to both objects within the observation period	39.28 ± 9.3	36.83 ± 6.7	48.25 ± 3.3
time to visit the familiar object, sec	21.24 ± 5.2	25.5 ± 5.6	22.32 ± 6.5
time to visit the novel object, sec	20.07 ± 5.06	29.19 ± 5.4	32.87 ± 5.2
Test phase (T2)			
total exploration time, sec	41.32 ± 10.1	54.69 ± 10.0	48.62 ± 12.9
number of visits to the familiar object	27.85 ± 6.7	31.6 ± 8.2	29.6 ± 8.0
number of visits to the novel object	29.0 ± 7.9	30.6 ± 4.8	31.0 ± 10.6

Footnotes. Data are expressed as means ± s.e.m. The T2 phase was done 24 h after T1.

Body Mass Changes. The mean body mass of mice in the EAE group on the 21st day was significantly lower than their mean mass on the 1st day ($P = 0.003$). For the sham and control groups, we did not observe any body mass loss throughout the study (Fig. 2).

Novel Object Recognition Test: Activity Level. The activity level was assessed by measuring the distance travelled during the trial (T1) and test (T2) phases (Fig. 3). In the control group, the mean

travelled distances in T1 and T2 did not differ significantly from each other ($P = 0.6$). In the sham group, the travelled distances in T1 and T2 were not significantly dissimilar ($P > 0.05$). In the EAE group, the travelled distances in T1 and T2 also did not differ significantly from the respective values in the control group (all $P > 0.05$). Thus, the levels of locomotor activity in all three groups demonstrated no significant differences despite the fact that some dissimilarities between the mean estimates were

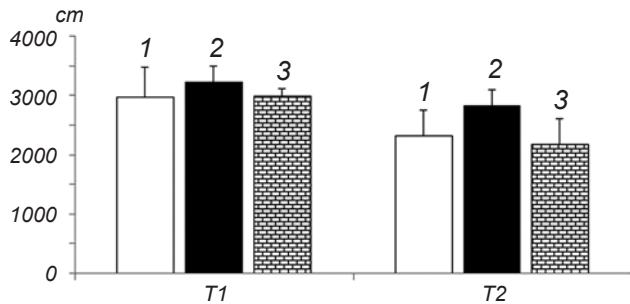


Fig. 3. Locomotor activity level in the control, sham-treated, and EAE groups (1-3, respectively). The activity levels were measured according to the distance travelled within 10 min during both training and test phases (T1 and T2, respectively).

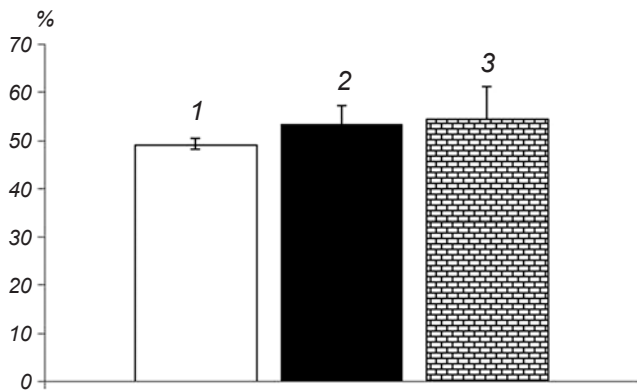


Fig. 4. Mean values of the discrimination ratio in different experimental groups. Designations are the same as in Fig. 3.

noticeable (Fig. 3).

Novel Object Recognition Test: Trial Phase (T1). The total time spent exploring one object in T1 (Table 1) was not statistically different in the control, sham, and EAE groups ($P > 0.5$). Similarly, no reliable differences were found between experimental groups for the frequency (number) of visits to sample objects ($P > 0.09$; Table 1). Differences between the indices measured varied somewhat but demonstrated no systematic trends.

Novel Object Recognition Test: Test Phase (T2). Object exploration times during the test phase (T2) in experimental groups are shown in Table 1. No reliable differences were found for the time spent to explore novel and familiar objects between the experimental groups (all $P > 0.05$). The mean of the total exploration time of both objects (familiar + novel) did not differ statistically from each other in the control and EAE groups ($P > 0.05$). Moreover, no reliable differences were found for the frequency of visits to the novel and familiar objects between

experimental groups (all $P > 0.05$; Table 1).

A comparison of the discrimination ratio (Fig. 4) between the experimental groups also revealed no difference ($P = 0.3$).

DISCUSSION

In our study, we were unable to find clear impairment of tactile learning (assessed by the novel object recognition test) in C57BL/6 male mice within the acute phase of EAE.

Most neurodegenerative diseases, like Alzheimer's, Parkinson's, lateral sclerosis, and multiple sclerosis, exert deleterious effects on learning and memory, and these diseases mainly destroy integrative and cognitive abilities [14]. Experimental autoimmune encephalomyelitis (EAE) is the most frequently studied animal model used for elucidation of the underlying etiopathology of MS; this model has proved to be effective in the development of therapeutic strategy. EAE shares many clinical, histopathological, and immunological features of MS [15]. Nevertheless, there are some differences between EAE and MS. For example, the CNS structure primarily affected in EAE is the spinal cord. However, some recent studies have detected evidence of inflammation and neuronal changes in the brain of mice with EAE [16-19]. Because of brain dysfunction, behavioral disturbances in animals with EAE are to be expected. Thus, recent studies have been focused on cognitive deficits in EAE to find the possible mechanisms underlying cognitive defects.

Recently, Rodrigues et al. [20] checked the indices of memory and anxiety 9 and 60 days after induction of EAE with MOG35-55 in C57Bl/6 mice. They reported no differences in memory and anxiety when comparing controls and animals with induced EAE. In addition, Tu et al. [8], reported that spatial learning and recall (in the Morris water maze spatial memory test) were not affected following induction of EAE in C57Bl/6 mice. Our results also demonstrated that tactile learning is not considerably impaired 7 days after induction of EAE in C57Bl/6 mice. Based on these results, one may conclude that learning and memory are not affected within the early EAE phase. In EAE, it is well established that T-cell infiltration and inflammation are the main cause of CNS demyelinating lesions and neurodegeneration [16]. Therefore, it appears possible that brain inflammation should be

associated with behavioral changes in EAE. There are reports demonstrating little signs of inflammation, T-cell infiltration, or neurodegeneration in the brain of EAE mice within the early period (7-10 days after immunization with MOG35-55) [16, 21]. Conversely, there are some reports showing noticeable behavioral changes in EAE. Pollak et al. [6, 7] reported some behavioral sickness in the acute phase of EAE, including anorexia, decreased preference for sucrose solution, and reduced social exploration. It is worth mentioning that the cited authors used a dissimilar EAE model (induced by a proteolipid protein in SJL/J mice).

Although most studies on EAE reported that clinical signs of EAE would start 9-14 days post immunization [22], there are, however, some communications reporting that clinical signs of EAE start sooner than 7-14 days post immunization [23, 24]. Hence, we tested animals for tactile learning (by the novel object recognition test, NORT) before the appearance of EAE clinical signs (day 7 after immunization). As all behavioral tests depend on motor function, and as it seems that in EAE the behavioral changes coincide with the severe phase of the disease (within this phase, motor function is impaired), this imposes a limit for evaluating behavioral changes within the acute phase of EAE. For example, Jones et al. [21] reported that numbers of crossings in the open field test are dissimilar in EAE and control animals. However, it is not possible to conclude whether this outcome is a result of motor impairment or it is a behavioral change. Some investigators used a mild model of EAE to solve this problem based on reduction of the amount of pertussis toxin [25]. While this seems a good strategy to decrease the motor impairment, the mechanisms that lead to the behavioral changes may be different.

Thus, the results of our study agree with observations reported in a few other studies and demonstrate little behavioral changes within the acute phase of EAE.

Procedures involving animals and their care were conducted in accordance with the Guide to the Care and Use of Experimental Animals (Olfert et al., Canada, 1993 [26]). Approval from the local Ethics Committee was also obtained.

The authors, F. Ayoobi, I. Fatemi, A. Roohbakhsh, and A. Shamsizadeh, have no conflict of interests.

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