Journal of the Neurological Sciences 399 (2019) 15–21

Contents lists available at ScienceDirect

Journal of the Neurological Sciences

journal homepage: www.elsevier.com/locate/jns

Cognitive dysfunction in mice with passively induced MuSK antibody seropositive myasthenia gravis

Liis Sabre[a,*], Amelia Evoli[b], Anna Rostedt Punga[a]

[a] Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Uppsala, Sweden
[b] Department of Neurology, Catholic University, Rome, Italy

ARTICLE INFO

Keywords:
MuSK
Myasthenia
Mice
Cognitive dysfunction
Behavioral
Hippocampus

ABSTRACT

Recent reports on cognitive dysfunction, in addition to skeletal muscle fatigue, in muscle-specific tyrosine kinase antibody seropositive (MuSK+) myasthenia gravis (MG) patients led us to study cognition in mice with MuSK+ passive transfer MG (PTMG). Twelve 7-week-old female wild-type C57BL/6J mice were passively immunized with IgG from MuSK+ MG patients and 12 control mice received intraperitoneal saline injections. Mice were evaluated with clinical, neurophysiological and behavioral tests (Barnes maze (BM) and novel object recognition (NOR)), and the muscles were immunostained to evaluate the neuromuscular junction in the end of the study. Two-thirds of the immunized mice developed clinically distinct MuSK+ PTMG. MuSK+ PTMG mice spent less time exploring the novel object in the NOR test (MuSK+ mice 36.4% ± 14.0 vs controls 52.4% ± 13.0, $p = .02$), unrelated to the muscle weakness and regardless of rodents' innate preference of novelty. In the BM test, control mice were more eager to use the direct strategy than the MuSK+ mice (MuSK+ 17.3% vs controls 29.5%, $p = .02$). Our findings shed new light on cognition dysfunction in human MuSK+ MG patients and indicate that recognition memory in the perirhinal cortex could be affected in MuSK+ MG.

1. Introduction

In addition to disturbed neuromuscular transmission and muscle fatigue, cognitive defects in myasthenia gravis (MG) patients were described already in 1988 [1]. Another study reported significant difficulties on several measures of cognitive function in MG patients seropositive for acetylcholine receptor antibodies (AChR+), including response fluency, information processing as well as verbal and visual learning [2]. Moreover, reports of REM sleep reduction [3] and detection of AChR antibodies as well as pathologically elevated proportion of enlarged lymphoid cells in the cerebrospinal fluid [4,5] support the hypothesis of central nervous system (CNS) involvement, at least in AChR+ MG.

Additionally, case reports have emerged on cognitive dysfunction in MG patients who are seropositive for muscle specific tyrosine kinase antibodies (MuSK+) [6,7]. Recent reports found that MuSK+ MG patients are cognitively more impaired than healthy subjects in the Free and Cued Selective Reminding Object Test, that involves the recognition and memory of objects and implies the functioning of hippocampus and perirhinal cortex [8]. In MG, the MuSK antibodies inhibit the essential role of the receptor in neuromuscular junction (NMJ) transmission, maintenance and integrity [9]. Nevertheless, in contrast to what is suggested by its name, MuSK is actually expressed throughout the body and in particular in the brain [10]. Two isoforms of MuSK can be detected in the CNS and inhibition of MuSK using receptor specific intrathecal antisense oligonucleotides results in memory deficits in rodents [10,11]; implying an important role for MuSK in long-term potentiation. It is thus conceivable that MuSK antibodies, in addition to the MG phenotype as that occurring in MuSK+ MG, could result in similar cognitive dysfunction in these patients.

In the light of the reported cognitive problems in patients with MuSK+ MG, the aim of our study was to investigate whether cognitive dysfunction can be observed in the MuSK+ PTMG mouse model, in addition to myasthenic muscle fatigue.

2. Methods

2.1. Subjects

Female wild type C57BL/6J mice were supplied from Taconic (Denmark). Mice aged 6 weeks at arrival, weighing approximately 19 g, were housed under standardized conditions (21 °C, humidity 45–65%) under a 12 h light/dark cycle with lights on at 06:00, 3 mice per cage. Food and water were provided ad libitum and soft food and water

* Corresponding author at: Department of Neuroscience, Uppsala University, BMC, Husargatan 3, 75237 Uppsala, Sweden.
E-mail address: liis.sabre@neuro.uu.se (L. Sabre).

https://doi.org/10.1016/j.jns.2019.02.001
Received 18 December 2018; Received in revised form 23 January 2019; Accepted 1 February 2019
Available online 04 February 2019

bottles with long nipples were provided after immunization start. The Local Ethical Committee in Uppsala, Sweden, approved the animal procedures (C41/15). All the procedures, behavioral tests as well as cleaning the cages were conducted by the same person (LS). The mice were exposed to daily handling before conducting the behavioral tests. The experimenter (ARP) was blinded to the immunization status of the mice when conducting neurophysiological and clinical evaluation.

2.2. Patient material

Plasma was obtained from therapeutic plasma exchange of four patients with MuSK+ MG at the Neurology Department, Catholic University, Rome. Their clinical characteristics are described in the Supplementary material (Supplemental Table 1). Patients signed informed consent for the experimental use of their plasma for immunization purposes.

2.3. IgG purification

Total IgG was purified from plasmapheresis material using a human total IgG-specific affinity resin (BAC BV, www.captureselect.com) on an AKTA explorer 900 (Pharmacia Biotech). The protocol has been described in detail previously [12]. In brief, the amount of plasma used per run was based on the IgG subtype concentrations, and the dynamic capacity of the column material was 15 mg/ml for the IgG total affinity resin. The flow rate for all steps was set at 13 ml/min. Before addition of patient material, the column (POROS™ GoPure™ MabCapture™ A Select Pre-packed Column, Thermofisher, Gothenburg, Sweden) was equilibrated with phosphate-buffered saline (PBS). Eluted fractions per purification containing high concentrations of IgG, based on OD280 measurements, were pooled and subsequently dialyzed to PBS (molecular weight cut-off 3.5 kD) and sterilized using a 0.22 μm filter (Millipore, Burlington, Massachusetts, MA). Samples from each dialysis fraction were loaded on an 8% sodium dodecyl sulphate–polyacrylamide (SDS) gel using sample buffer without a reducing agent. Separated proteins were either stained with Coomassie brilliant blue or transferred to PVDF membrane (Millipore, Burlington, Massachusetts, MA, USA). Transfer efficiency was checked by incubation in ponceau S buffer [12]. The protein concentration was measured with nanodrop (A280; mg/ml).

2.4. Cell based assays

Murine myoblast cells C2C12 (ATCC, Manassas, VA, USA) were plated at 5×10^3 density in Dulbecco's modified Eagle's medium (supplemented with 20% fetal bovine serum) and passaged when myoblasts reached 50% confluence. Myoblasts were plated at near-confluent density in collagen coated 24-well trays in 5% horse serum to induce differentiation. Incubation proceeded until large myotubes formed (5 days) and AChR cluster formation was induced by addition of recombinant neural agrin (100 pM) to the culture medium [13]. In addition to the experimental cultures exposed to MuSK-MG IgG (4.5 mg) ± neural agrin, control cultures were exposed to healthy donor IgG (4.5 mg) ± neural agrin. AChRs were labelled by adding TRITC-α-bungarotoxin to the culture medium (10 μg/ml). After 30 min, washing was done with warm medium and then the cells were fixed with 95% ethanol at −20 °C for 5 min.

The number of large AChR clusters (N 25 μm long) were counted per myotube segment on a fluorescence microscope. The operator (ARP) was blinded to the experimental groups.

2.5. Immunization

To induce MG, every mouse received the same amount (7.5 mg) of purified MuSK MG patient IgG through intraperitoneal (i/p) injection daily each morning for 14 consecutive days. Twelve mice were immunized with MuSK IgG from 3 MG patients (patient #1–3 mice, #2–6 mice, #4–3 mice), IgG from patient #3 was eventually not used because the concentration of IgG was not sufficient. Twelve control mice received daily injection of 300 μl PBS i/p. Cyclophosphamide was injected i/p (300 mg/kg) 24 h after the first IgG injection to prevent an active immune response to the human protein. Each morning the mice were weighed and evaluated for muscle weakness and fatigue.

2.6. Clinical evaluation

Mice were observed for visual scoring of onset of MG and tested for muscle strength before immunization [14]. At first, the mice were observed on a flat platform for 2 min and then exercised gently by dragging them suspended by the base of the tail across a grid 20 times as they attempted to grip the grid. After an inverted mesh hang-time test was performed (by placing the mouse on an upside-down grid for 180 s) and muscle fatigue in grip strength was evaluated. The mice were given 3 attempts and the longest hanging time was noted.

Clinical muscle weakness was graded as follows: grade 0.5: the mouse appears somewhat less active than normal after the standard exercise regime, hang-time > 60 s; grade 1: mouse appears normal before the exercise but lies prone and remains largely immobile after the test and/or hang-time < 60 s; grade 2: before and after the exercise the mouse lies prone, remains immobile and/or hang-test < 30 s; grade 3: severe weakness and appears dehydrated and paralyzed with > 15% weight loss and/or hang-time 0 s [9,14]. Mice with muscle weakness grade 2 were not included into the behavioral study and mice with grade 3 were euthanized to reduce the suffering. The clinical scoring was done by the blinded experimenter (ARP).

2.7. Neurophysiological protocol

The mice were neurophysiologically examined on day 4 and day 15 under inhalation anesthesia with isoflurane/oxygen, with the adjusted isoflurane concentration to suppress reflexes but maintain steady breathing. In order to maintain a physiological body temperature, the mice were kept on a heating pad at 37 °C during the entire procedure. Repetitive nerve stimulation (RNS) was performed with Keypoint equipment (Dantec Keypoint Focus, IEC 60601–1-1, Natus, Pleasanton, CA, USA). Monopolar needle electrodes with a recording area of 2 mm^2 each were inserted at 2 positions 2-3 mm along the nerve and recording with an identical monopolar electrode was made from the corresponding muscle belly. In each animal RNS was performed with 10 supramaximal stimulations (3 Hz) where an abnormal decrement was defined as ≥10% run-down of the amplitude between the 1st and the 4th response (performed by the blinded experimenter ARP). The protocol has previously been described in detail [15]. In brief, recording from the masseter muscle (right-sided) was performed upon stimulation of the trigeminal nerve close to the zygomatic process and recording of the medial gastrocnemius muscle (right-sided) upon stimulation of the sciatic nerve at the sciatic notch.

2.8. Antibody assays

To determine the reactivity and epitope specificity of the purified MuSK MG patient IgG, a MuSK ELISA was performed according to previously described protocol [16]. In short, the full-length extracellular domain of MuSK or ~100 amino acid long overlapping fragments of the extracellular domain were immobilized in a maxisorp plate. The plate was blocked with 1% casein, 0.05% tween in PBS and purified IgG was subsequently allowed to bind. Bound antibodies were detected with a mouse anti human IgG4 antibody (1:5000, NI315 Nordic Biosite, Täby, Sweden) and rabbit anti mouse conjugated to alkaline phosphate (1:750, Dako, Glostrup, Denmark). Plates were developed with *p*-Nitrophenyl Phosphate Substrate and imaged on a biotek plate reader at 405 nm.

2.9. Neuromuscular junction (NMJ) staining and fragmentation scoring

Mice were euthanized by CO_2 inhalation and the sternomastoid and omohyoid (left-sided) muscles were dissected. The methods for staining have been described in detail previously [17,18]. Briefly, the muscles were mounted on Sylgard in Petri dish, injected with 50 μl of tetramethylrhodamine-conjugated α-bungarotoxin (5 μg/ml; Sigma-Aldrich, Stockholm, Sweden) and incubated for 30 min. Incubation with primary antibodies against neurofilament (1:10,000, 200 kD, Abcam, Cambridge, UK) overnight at 4 °C followed by washing with 1% BSA and then incubated for 1 h with goat-anti-chicken secondary antibodies (1:1000; Alexa fluor 488, Abcam, Cambridge, UK) in blocking buffer. The muscles were examined with fluorescence microscope (Nikon Eclipse, Lvdia-N). Laser gain and intensity were equal for all images. Quantification of postsynaptic area was performed in ImageJ. The number of postsynaptic fragments was counted using fluorescence microscopy of AChRs in ≥30 NMJs per muscle from all the 12 immunized mice and 10 control mice.

2.10. MuSK IP of brain lysates of PTMG versus control mice

The hippocampus was removed and subsequently lysed. Protein concentration was measured with Bradford protein assay and the same amount of protein was used from every brain lysate. Protein A-agarose slurry was incubated with 1:1000 rabbit anti-MuSK antibody (abs549, Sigma Aldrich, Saint Louis, MO, USA) for 1–12 h at 4 °C under gentle rotation. Centrifugation at 2000 rpm for 2 min at 4 °C and the supernatant was discarded. Protein A beads were washed with lysis buffer (homogenization buffer), centrifuged at 1000–3000 × *g* for 2 min, 3×. The brain lysates were kept on ice and 50 μl of beads were added to each sample and incubated for 1 h at 4 °C. 40 μl of non-bound sample per sample was mixed with 40 μl 4xSB with dtt. Beads were spun down and transferred to cold 1.5 ml Eppendorf tubes to minimize the volume. Washed 3 × 10 min at 4 °C with 500 μl lysis buffer and supernatant removed. Added 50 μl 2xSDS buffer to the beads and cook for 5 min at 95 °C. 10% SDS-PAGE gel was run (10 μl sample per well). The gel was removed and the plates put into cold transfer buffer. The membrane was washed in transfer buffer before assembling the sandwich before transfer of the gel to the nitrocellulose membrane. Transfer for 2-3 h at 300 mA was controlled with ponceau S staining. The membrane was blocked 30 min-1 h at RT using blocking buffer (2% BSA in PBS). Addition of primary antibodies diluted in blocking buffer: 1:1000 rabbit anti-MuSK. Incubation at 4 °C overnight washing 3 times of the membranes with TTBS 5 min each. Incubation of the membranes with conjugated secondary antibodies in blocking solution (anti-rabbit and anti-mouse) for 1 h rolled at RT. Washing 3× PBS-T 5 min each and excessive reagents removed before the image was scanned.

C2C12 cells were prepared accordingly as positive controls: 7 days old C2C12 cells were trypsinized and spun down. (0.5–1 × 106 cells) at 3000 rpm 5′ at 4C. The supernatant was completely removed and the cell pellet was lyzed in 500 μl of homogenization buffer on ice for 30 min.

2.11. Behavioral tests

The behavioral tests generally used to assess various modes of cognition in mice include the Barnes maze (BM) and Novel Object Recognition (NOR) [19]. The BM and NOR tests investigate spatial learning as well as memory consolidation, which are critically influenced by the medial entorhinal cortex in the dorsal hippocampus [15].

2.11.1. Barnes maze

To investigate spatial learning and memory consolidation, 12 MuSK + MG mice and 12 control mice were tested in the Barnes maze (BM) using an adapted Nature Protocol [20] (doi:https://doi.org/10.1038/nprot.2007.390). The maze was a white circular table with 20 equally spaced holes around the outer border, 105 cm above the floor and diameter 92 cm. The escape box located at 9 o'clock direction during the familiarization and acquiring phase. Bright light (420 lx) was used as reinforcement. Two external visual cues were placed around the maze and the endogenous cues were the same during the whole study. All tests were performed by one experimenter (LS). On the first day (immunization day 5), all mice completed one habituation trial where they explored the maze for 5 min. If the mouse did not go to the hole within 5 min, pulling the base of the tail in the opposite direction or placing it directly into the escape box gently directed it. The mouse was allowed to stay there for 2 min. After the adaption period, each mouse underwent 4 spatial acquisition trials per day for 4 days with inter-trial interval of 15 min. The experimenter placed the mouse in a cylindrical black start chamber in the middle of the maze for 10 s. The mouse was then released from the chamber and allowed to explore the maze for 3 min. The trial ended after 3 min or when the mouse entered the escape box. If the mouse did not enter the escape box, it was placed into that and left for 1 min. After each trial the maze and the escape box were cleaned with 70% ethanol. On day 5 (immunization day 9) and 12 (1 day after immunization ended) probe trials were conducted. The escape box was removed and the test lasted 90 s. The EthoVision XT11 detection program was used to automatically analyze the video files and all the files were also manually analyzed. The main outcomes measured were primary and total errors and primary latency during the acquisition phase and primary errors, primary latency, number of pokes in each hole and path length to reach the virtual target hole during the test. The behavior of mice was also manually classified as direct, serial or random.

The best-known and utilized tests evaluating visual-spatial working memory in humans are the Corsi Block-Tapping Test (CBT) and Visual Pattern Span [21].

2.11.2. Novel object recognition

To test recognition memory the novel object recognition (NOR) test with familiarization phase on 10. immunization day and test phase 24 h later were used since this is considered the most appropriate test for memory in animals, as well as in humans [22]. Of the 24 mice, all 24 performed the familiarization and test phase of NOR. We adapted the protocol from Leger et al. [23]. Behavioral procedures were carried out in a circular arena with 43 cm in diameter and 30 cm high, 79 cm above the floor. To avoid any stress from bright light, lighting was adjusted to 50 lx in the center of the open field. Each mouse was presented with two similar objects (A + A) during the habituation phase for 3 min. Spatial cues were avoided. The intersession interval was 24 h. On the second day, one familiar (A) and one new object (B) were presented to the mouse (A + B or B + A). The amount of time taken to explore the familiar and the new object were measured and discrimination index (DI) calculated ($DI = (B_{time}\text{-}A_{time})/(B_{time} + A_{time})$). The maze and the objects were cleaned with 70% ethanol after each trial. The experiment was video recorded and analyzed by EthoVision XT11 and also analyzed manually. Object exploration was defined as nose contact with the object or directed toward it at a distance ≤2 cm. Climbing over the object was not considered an explorative behavior, unless it was accompanied by nose-directing behavior toward the object. There is certain connection to the Free and Cued Selective Reminding Object test, performed in human patients, since both test recognition and memory of objects [22].

2.12. Statistical analysis

Power calculation was done prior to the study. In order to achieve a power of 80% and alfa-error of 5% with an estimated weight difference of 5% between immunized and control mice, 12 PTMG mice and 12 control mice would be required. Paired or independent *t*-test was performed for parametric data. In case of non-parametric data, the Mann-Whitney *U* test was applied. Differences with *p*-values < .05 were

considered statistically significant. All analyses were performed using StatsDirect software version 3.1.4 or Graphpad version 6.07 for Windows (Graph Pad software, La Jolla, CA, www.graphpad.com).

3. Results

3.1. Characterization of MuSK antibodies and MuSK expression in the hippocampus

All purified IgG samples bound recombinant FL extracellular MuSK and had reactivity against the N terminal Ig-like 1 domain (aa 21–125) in ELISA (Supplemental Fig. 1). Sample # 1 also had additional low reactivity against the Fz domain. This material was subsequently used to induce MG in mice. Myotubes exposed to MuSK+ IgGs were smaller and with reduced number of AChR clusters than myotubes exposed to control IgGs (26.0 ± 7.0 vs 48.3 ± 8.7, $p = .004$; (Supplemental Fig. 2).

To assess whether exposure to MuSK MG IgG reduced the expression of MuSK in the hippocampus, MuSK protein concentration was determined in the brains (hippocampus) of the PTMG MuSK+ mice in comparison to the control mice. There was however no overall significant difference in the MuSK+ PTMG mice compared to the controls (Supplemental Fig. 3).

3.2. MuSK+ PTMG clinical and neurophysiological characteristics

Out of the 12 female mice immunized with human MuSK IgG, final PTMG grade 3 was observed in 1 mouse (8.3%), grade 2 in 1 mouse (8.3%), grade 1 in 4 mice (33.3%), grade 0.5 in 2 mice (16.7%) and grade 0 in 4 mice (33.3%) (Supplemental Table 2). As expected, RNS on immunization day 4 did not reveal any decrement in neither group of mice in any muscle. However, on the 15th day after the immunization initiation, four immunized mice revealed a decrement of 10–15% on RNS (Supplemental Fig. 4, Supplemental Table 2). The MuSK immunized mice did not gain weight during the study (from 19.6 ± 1.0 g to 19.6 ± 1.5 g, $p = .91$) in comparison to control mice, whose weight increased significantly (from 19.9 ± 0.9 g to 20.7 ± 1.1 g, $p = .0003$) and was significantly higher by the end of the study ($p = .04$).

Immunostaining of AChRs at the NMJs of the omohyoid muscle revealed that a significantly higher number of postsynapses of MuSK+ PTMG mice were fragmented and lost their pretzel-shape (3.3 ± 1.7) compared to control mice (2.7 ± 1.4; $p = .0001$). A similar picture was seen with higher number of fragmented NMJs in the sternomastoid of MuSK+ PTMG mice (3.7 ± 2.0) compared to control mice (3.2 ± 2.3, $p = .02$; Supplemental Fig. 4C).

3.3. Behavioral studies

3.3.1. Barnes maze test (BM)

Both the MuSK+ and control mice were able to learn the task with decreasing number of primary errors as well as shorter primary escape latency over the training phase (Fig. 1). Nevertheless, the number of primary errors was higher in the MuSK+ mice; although likely due to high variance this did not attain statistical significance (Table 1). The strategy to find the target hole was mostly mixed or serial in MuSK+ mice at the beginning of the acquisition, reaching to direct and serial strategy by the end of the learning phase (Fig. 2). Intriguingly, the control mice were more eager to use the direct strategy already in the beginning of the study after a short adaption period (MuSK+ 17.3% vs controls 29.5%, $p = .02$). In the probe trial, day 5, MuSK+ mice spent less time (51.2%) in the correct zone compared to the control mice (66.2%, $p = .02$). The difference did not remain significant by the second probe trial on day 12, suggesting that this finding was not strictly related to the induction of MG. We did not find significant differences between the groups in primary escape latency, number of wrong holes visited nor the whole path length to reach the hole where

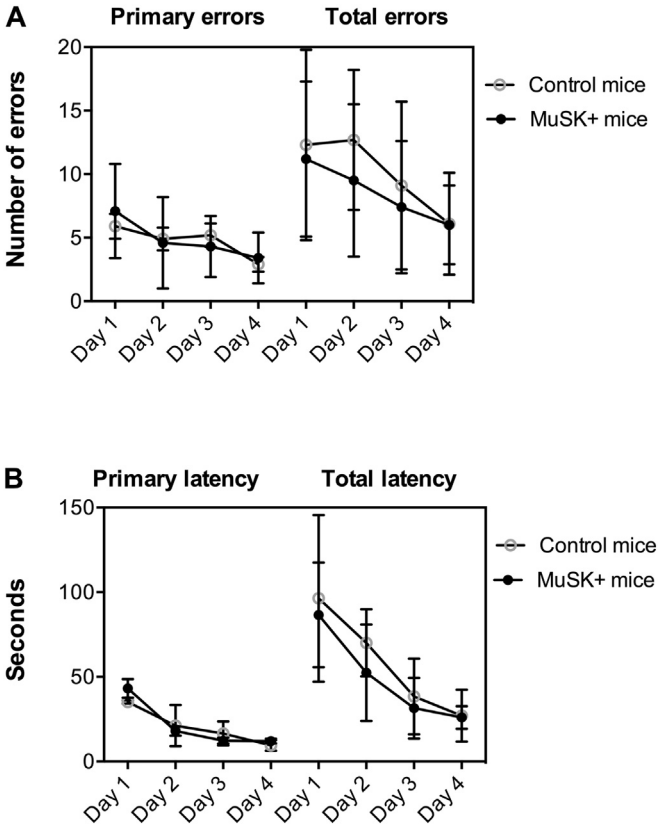

Fig. 1. Primary errors and total number of errors during the acquisition phase of the Barnes maze in MuSK+ mice and controls. (A) Primary and total escape latency during the acquisition phase of the Barnes maze in MuSK+ mice and controls (B). Data displayed as mean ± SD.

escape box should have been on neither trial days. Still, there was a tendency of MuSK+ PTMG mice to perform worse than the controls on day 5 (Table 1).

3.3.2. Novel object recognition test (NOR)

Four MuSK+ PTMG mice and two control mice were excluded from the final analysis because of their minimal activity and no interest of the objects (motivational or emotional reasons) [23]. The MuSK+ mice excluded were A1 (antibodies from pat#1), A2 (antibodies from pat#2), A3 (antibodies from pat#2) and C4 (antibodies from pat #4).

Time spent exploring individual objects during familiarization phase was not different among the groups (MuSK+ mice 11.3 ± 5.6 s vs controls 15.5 ± 8.6 s, $p = .23$). Still, 24 h later, MuSK+ mice spent less time in exploring the new object (MuSK+ 36.4% ± 14.0 vs controls 52.4% ± 13.0, $p = .02$) and the exploration time in MuSK+ group decreased significantly in comparison to the familiarization phase (Fig. 3). Such significant decrease in exploration time did not appear among the control mice.

4. Discussion

Several studies suggest that MG patients experience cognitive problems, especially AChR+ MG patients [4,5]. Camillo Marra and colleagues did not find differences in cognitive function between elderly MG patients (most of them AChR+ MG patients) and controls [24]. When the same research group concentrated on MuSK+ MG patients, they found that MuSK+ MG patients scored worse in linguistic tasks and memory recall [8]. MuSK+ MG using passive transfer of human MuSK antibodies to mice is a well-established model of MG, and therefore the passive transfer MuSK MG (PTMG) model could help advancing the knowledge on its possible cognitive defects [12,13]. Our

Table 1
The number of errors, primary latency and path length (mean ± SD; median (min; max)) of mice with muscle-specific tyrosine kinase (MuSK+) passive transfer myasthenia gravis (PTMG) and control mice on Barnes maze test probe trials on day 5 and 12.

		Day 5			Day 12		
		MuSK+ PTMG mice	Control mice	*p*- value	MuSK+ PTMG mice	Control mice	*p*-value
Primary number of errors	Mean ± SD	6.6 ± 8.5; 3	2.6 ± 2.5		5.0 ± 3.3	3.5 ± 2.5	.75
	Median (min; max)	3 (0; 29)	2.5 (0; 9)	.33	5 (1; 12)	4 (0; 7)	
Primary latency (*sec*)	Mean ± SD	15.5 ± 20.3	8.7 ± 6.4		16.7 ± 11.1	14.5 ± 9.0	.61
	Median (min; max)	9 (3; 74)	7 (2; 24)	.48	12 (4; 38)	11 (3, 29)	
Primary path length (cm)	Mean ± SD	152.5 ± 171.7	74.3 ± 43.5		110.6 ± 78.4	89.4 ± 46.7	.68
	Median (min; max)	80 (41; 629)	62 (41; 190)	.28	75 (43; 268)	83 (41; 161)	

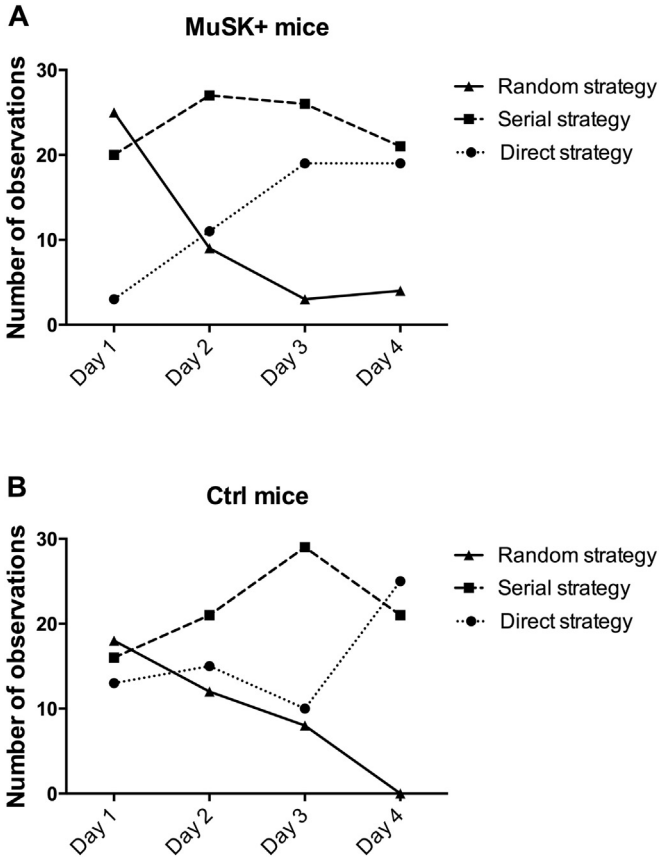

Fig. 2. Search strategies used by MuSK+ mice (A) and control mice (B) during the acquisition phase of the Barnes maze. Number of observations displayed.

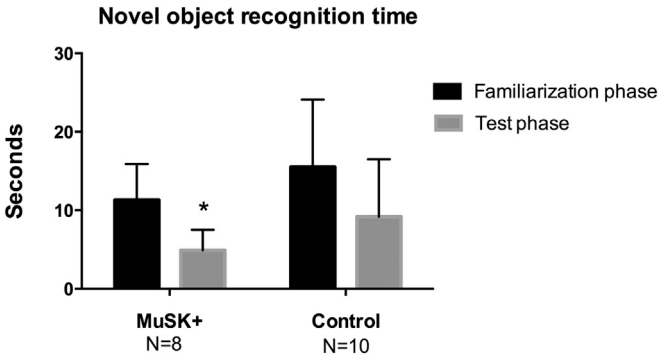

Fig. 3. Exploration time of the novel object in the novel object recognition test during habituation phase and 24 h later at test phase in MuSK+ and control mice. *$p < .05$.

finding that MuSK+ PTMG mice spent more time exploring old objects, regardless of the innate preference of novelty in rodents, indicates deficits in non-spatial memory required to remember selections already made. The MuSK+ PTMG mice also examined objects less in the NOR study and they learned slower than controls in the BM test. Nevertheless, no substantial differences were found in their overall spatial memory.

MuSK is crucial for the transmission and integrity of the NMJ and little is known about its possible role for cognitive symptoms. When MuSK was described, it was generally accepted as a protein expressed only in muscles [25,26]. Since then, more information on MuSK expression is available, highlighting that it is also expressed in the brain, in neurons and in several non-neuronal tissues [27,28]. Two MuSK isoforms were described in the hippocampus, cortex and cerebellum as well as in muscle and myotube extracts and they were expressed similarly in brain tissues and muscles [10]. Our study thus highlights the role of MuSK in memory consolidation, synaptic plasticity and mediating cholinergic responses. Further, they suggest that MuSK expression in the hippocampus is required for consolidation of inhibitory avoidance memory. Hippocampal activation is considered to be related to the detection and encoding of novel information [29,30] and it is required when task parameters include spatial or contextual factors [31]. In the present study, we used BM to study the spatial memory, however we did not find a significant difference between the groups. MuSK+ PTMG and control mice did not differ in finding the target hole, although MuSK mice made slightly more primary errors. In the initial learning phase, the control mice used a more direct strategy than MuSK+ PTMG mice, which suggests that control mice chose spatial navigation technique over a serial-search strategy [32]. The use of direct strategy increased in the MuSK+ group by the end of the learning phase, which implies slower learning, although no decline in spatial short- or long-term memory was obvious.

Similarly to Bohbot et al. who examined AChR+ MG patients, our findings would argue that the cognitive problems MG patients have might lie in remembering which selection they previously made not recalling spatial locations or objects per se [33]. We evaluated object recognition memory by the NOR test with the purpose to assess whether subjects remembered the previously presented object. In the test only one object was changed not the place of the objects or the arena. We used objects recommended from previous studies in our lab having different color, shape, and size but not material. So, the discrimination could not have been by olfactory cues [19]. Naturally rodents should spend more time in exploring novel objects [34] and thus remember which stimulus they previously encountered. These processes involve structures beyond the hippocampus [35]. As the MuSK+ mice did not prefer novel stimulus in the NOR test, this suggests that MuSK+ PTMG mice had poor memory of the familiar stimulus. The recognition memory, which was disturbed in MuSK+ mice, depends mostly on perirhinal and entorhinal cortices and has been investigated in different mouse models [19,31]. It could be questioned whether motor disturbance due to clinical weakness of PTMG could have influenced the object exploration in the NOR. To avoid this doubt, we excluded the mice with severe weakness. Further, although our experimental

manipulation altered basic motor behavior, required to explore the object, such an impairment should be expected to have the same effect on the exploration of the novel and familiar object [34]. In addition, depressive symptoms such as anxiety are common among patients with autoimmune diseases [36], therefore we can not rule out that cognitive performance may be related to a depressive state.

This study has a number of potential limitations that could have influenced the results. In line with previous studies [18,37], not all our immunized mice developed clinical myasthenia. Presumably this also could have had impact on the behavioral results, as the group could have become too small to draw reliable statistical conclusions in the BM. Two-thirds of immunized mice displayed clinical weakness and they did not gain weight as the control mice and one reason could be the mode of passive immunization of human MuSK antibodies. This was in line with previous findings that mice injected with IgG purified from MG patients did not gain overt weakness nor lose weight [37]. The amount of MuSK IgG used was not very high because too weak animals should have been excluded from the behavioral test due to their minimal activity and ethical reasons. Secondly, we injected control mice with PBS not with purified IgG from healthy persons and that could have had effect on the results. Thirdly, the reason for not detecting an abnormal decrement so often could be that the disease duration was limited at the RNS evaluation, and the possibility to detect decrement in the acute phase of MG is limited [38]. The time limitations that arose from the tight schedule of passive immunization further did not allow us to perform more behavioral tests. Initiation of testing too early after immunization onset could have also affected the results and suggested inter-trial interval periods restricted to perform more studies on 1 day. Further, in the BM test we used bright light as a motivator. Therefore, the behavior we noticed could have been exploratory instead of escape as they were not motivated to escape. In the NOR test we had to exclude six mice because their total object exploration was < 3 s [39] and they were “frozen” most of the time. This could have been stress- or anxiety related; however, this behavior was not observed in the BM test although the experiment conditions were almost the same and no motivation was used. Lastly, we know that memory processing in human differ between the left and right hippocampus. Lateralized memory processing in mice is not as clear [40,41] therefore we studied MuSK protein concentration from both hippocampuses. Despite these limitations, we believe that our study provides important evidence to support the cognitive dysfunction observed in human MuSK+ MG in the MuSK+ PTMG model.

In conclusion, this is the first study to provide support of the similar cognitive dysfunction in MuSK+ PTMG mice that have been observed in MuSK+ MG patients. Further, our results indicate that MuSK antibodies might affect the perirhinal cortex that is important in object recognition memory.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2019.02.001.

Acknowledgements

The study was funded by the Swedish Research Council (VR-523-2014-2048 to ARP). The authors are most grateful to Dr. Maartje Huijbers for valuable input and advice as well as to Dr. Mårten Larsson and Dr. Tanel Punga for help with the IgG purifications.

References

[1] D.M. Tucker, D.P. Roeltgen, P.D. Wann, R.I. Wertheimer, Memory dysfunction in myasthenia gravis: evidence for central cholinergic effects, Neurology 38 (1988) 1173–1177.
[2] R.H. Paul, R.A. Cohen, J.M. Gilchrist, M.S. Aloia, J.M. Goldstein, Cognitive dysfunction in individuals with myasthenia gravis, J. Neurol. Sci. 179 (2000) 59–64.
[3] G. Mennuni, M. Morante, C. Scoppetta, P. Bergonzi, First REM latency in patients with myasthenia gravis: an observation, an hypothesis, Acta Neurol. 5 (1983) 253–255.
[4] A.K. Lefvert, R. Pirskanen, Acetylcholine-receptor antibodies in cerebrospinal fluid of patients with myasthenia gravis, Lancet 2 (1977) 351–352.
[5] K.M. Muller, E. Taskinen, A.K. Lefvert, R. Pirskanen, M. Iivanainen, Immunoactivation in the central nervous system in myasthenia gravis, J. Neurol. Sci. 80 (1987) 13–23.
[6] S. Lanfranconi, S. Corti, P. Baron, G. Conti, L. Borellini, N. Bresolin, A. Bersano, Anti-MuSK-positive myasthenia gravis in a patient with parkinsonism and cognitive impairment, Neurol. Res. Int. 2011 (2011) 859802.
[7] S. Bhagavati, P.J. Maccabee, G. Chari, Is cerebral involvement an occasional feature of muscle-specific kinase antibody-positive syndrome? Eur. J. Neurol. 14 (2007) e21–e22.
[8] P.E. Alboini, C. Marra, A. Evoli, Cognitive impairment in myasthenia gravis with MuSK antibodies, 13th International Conference on Myasthenia Gravis and Related Disorders, 2017, p. 45 New York.
[9] A.R. Punga, S. Lin, F. Oliveri, S. Meinen, M.A. Ruegg, Muscle-selective synaptic disassembly and reorganization in MuSK antibody positive MG mice, Exp. Neurol. 230 (2011) 207–217.
[10] A. Garcia-Osta, P. Tsokas, G. Pollonini, E.M. Landau, R. Blitzer, C.M. Alberini, MuSK expressed in the brain mediates cholinergic responses, synaptic plasticity, and memory formation, J. Neurosci. 26 (2006) 7919–7932.
[11] A.M. Gomez, R.C. Froemke, S.J. Burden, Synaptic plasticity and cognitive function are disrupted in the absence of Lrp4, elife 3 (2014) e04287.
[12] J.J. Plomp, M.G. Huijbers, S.M. van der Maarel, J.J. Verschuuren, Pathogenic IgG4 subclass autoantibodies in MuSK myasthenia gravis, Ann. N. Y. Acad. Sci. 1275 (2012) 114–122.
[13] S.T. Ngo, C. Balke, W.D. Phillips, P.G. Noakes, Neuregulin potentiates agrin-induced acetylcholine receptor clustering in myotubes, Neuroreport 15 (2004) 2501–2505.
[14] W.D. Phillips, P. Christadoss, M. Losen, A.R. Punga, K. Shigemoto, J. Verschuuren, A. Vincent, Guidelines for pre-clinical animal and cellular models of MuSK-myasthenia gravis, Exp. Neurol. 270 (2015) 29–40.
[15] E. Chroni, A.R. Punga, Neurophysiological characteristics of MuSK antibody positive myasthenia gravis mice: focal denervation and hypersensitivity to acetylcholinesterase inhibitors, J. Neurol. Sci. 316 (2012) 150–157.
[16] M.G. Huijbers, A.F. Vink, E.H. Niks, R.H. Westhuis, E.W. van Zwet, R.H. de Meel, R. Rojas-Garcia, J. Diaz-Manera, J.B. Kuks, R. Klooster, K. Straasheijm, A. Evoli, I. Illa, S.M. van der Maarel, J.J. Verschuuren, Longitudinal epitope mapping in MuSK myasthenia gravis: implications for disease severity, J. Neuroimmunol. 291 (2016) 82–88.
[17] G. Bezakova, T. Lomo, Muscle activity and muscle agrin regulate the organization of cytoskeletal proteins and attached acetylcholine receptor (AchR) aggregates in skeletal muscle fibers, J. Cell Biol. 153 (2001) 1453–1463.
[18] A.R. Punga, M. Maj, S. Lin, S. Meinen, M.A. Ruegg, MuSK levels differ between adult skeletal muscles and influence postsynaptic plasticity, Eur. J. Neurosci. 33 (2011) 890–898.
[19] M. Antunes, G. Biala, The novel object recognition memory: neurobiology, test procedure, and its modifications, Cogn. Process. 13 (2012) 93–110.
[20] B. Sunyer, S. Patil, H. Höger, G. Lubec, Barnes maze, a useful task to assess spatial reference memory in the mice, Protoc. Exch. (2007), https://doi.org/10.1038/nprot.2007.390.
[21] L. Piccardi, F. Bianchini, O. Argento, A. De Nigris, A. Maialetti, L. Palermo, C. Guariglia, The walking Corsi Test (WalCT): standardization of the topographical memory test in an Italian population, Neurol. Sci. 34 (2013) 971–978.
[22] A. Brodziak, E. Kolat, A. Rozyk-Myrta, In search of memory tests equivalent for experiments on animals and humans, Med. Sci. Monit. 20 (2014) 2733–2739.
[23] M. Leger, A. Quiedeville, V. Bouet, B. Haelewyn, M. Boulouard, P. Schumann-Bard, T. Freret, Object recognition test in mice, Nat. Protoc. 8 (2013) 2531–2537.
[24] C. Marra, F. Marsili, D. Quaranta, A. Evoli, Determinants of cognitive impairment in elderly myasthenia gravis patients, Muscle Nerve 40 (2009) 952–959.
[25] C.G. Jennings, S.M. Dyer, S.J. Burden, Muscle-specific trk-related receptor with a kringle domain defines a distinct class of receptor tyrosine kinases, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 2895–2899.
[26] D.M. Valenzuela, T.N. Stitt, P.S. DiStefano, E. Rojas, K. Mattsson, D.L. Compton, L. Nunez, J.S. Park, J.L. Stark, D.R. Gies, et al., Receptor tyrosine kinase specific for the skeletal muscle lineage: expression in embryonic muscle, at the neuromuscular junction, and after injury, Neuron 15 (1995) 573–584.
[27] A.K. Fu, F.D. Smith, H. Zhou, A.H. Chu, K.W. Tsim, B.H. Peng, N.Y. Ip, Xenopus muscle-specific kinase: molecular cloning and prominent expression in neural tissues during early embryonic development, Eur. J. Neurosci. 11 (1999) 373–382.
[28] F.C. Ip, D.G. Glass, D.R. Gies, J. Cheung, K.O. Lai, A.K. Fu, G.D. Yancopoulos, N.Y. Ip, Cloning and characterization of muscle-specific kinase in chicken, Mol. Cell. Neurosci. 16 (2000) 661–673.
[29] E. Duzel, R. Habib, M. Rotte, S. Guderian, E. Tulving, H.J. Heinze, Human hippocampal and parahippocampal activity during visual associative recognition memory for spatial and nonspatial stimulus configurations, J. Neurosci. 23 (2003) 9439–9444.
[30] S.M. Taubenfeld, M.H. Milekic, B. Monti, C.M. Alberini, The consolidation of new but not reactivated memory requires hippocampal C/EBPbeta, Nat. Neurosci. 4 (2001) 813–818.
[31] B.D. Winters, L.M. Saksida, T.J. Bussey, Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval, Neurosci. Biobehav. Rev. 32 (2008) 1055–1070.
[32] F.E. Harrison, R.S. Reiserer, A.J. Tomarken, M.P. McDonald, Spatial and nonspatial escape strategies in the Barnes maze, Learn. Mem. 13 (2006) 809–819.
[33] V.D. Bohbot, R. Jech, J. Bures, L. Nadel, E. Ruzicka, Spatial and nonspatial memory involvement in myasthenia gravis, J. Neurol. 244 (1997) 529–532.
[34] E. Dere, J.P. Huston, M.A. De Souza Silva, The pharmacology, neuroanatomy and

neurogenetics of one-trial object recognition in rodents, Neurosci. Biobehav. Rev. 31 (2007) 673–704.
[35] E. Tulving, H.J. Markowitsch, Memory beyond the hippocampus, Curr. Opin. Neurobiol. 7 (1997) 209–216.
[36] C.R. Pryce, A. Fontana, Depression in Autoimmune Diseases, Curr. Top. Behav. Neurosci. 31 (2017) 139–154.
[37] S. Viegas, L. Jacobson, P. Waters, J. Cossins, S. Jacob, M.I. Leite, R. Webster, A. Vincent, Passive and active immunization models of MuSK-Ab positive myasthenia: electrophysiological evidence for pre and postsynaptic defects, Exp. Neurol. 234 (2012) 506–512.
[38] M. Liik, A.R. Punga, Repetitive nerve stimulation often fails to detect abnormal decrement in acute severe generalized myasthenia gravis, Clin. Neurophysiol. 127 (2016) 3480–3484.
[39] A. Vogel-Ciernia, M.A. Wood, Examining object location and object recognition memory in mice, Curr. Protoc. Neurosci. 69 (2014) 1–17.
[40] R.T. Gerlai, A. McNamara, S. Williams, H.S. Phillips, Hippocampal dysfunction and behavioral deficit in the water maze in mice: an unresolved issue? Brain Res. Bull. 57 (2002) 3–9.
[41] O.A. Shipton, M. El-Gaby, J. Apergis-Schoute, K. Deisseroth, D.M. Bannerman, O. Paulsen, M.M. Kohl, Left-right dissociation of hippocampal memory processes in mice, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 15238–15243.