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Experimental murine non-alcoholic steatohepatitis is associated with behavioural, cognitive, and peripheral neuronal dysfunction

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Abstract

Introduction. Non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH), the leading causes of chronic liver disease worldwide, are associated with a wide range of psychoneurological complications and conditions. However, the causal relationship between liver and nervous system disease remains poorly understood, which warrants the development of clinically relevant and valid animal models thereof.

Aim. The objective of this work was to characterize the short- and long-term psychoneurological and peripheral neuronal deficits that complicate different stages of NAFLD/NASH in mice.

Materials and methods. 68 adult male C57Bl/6 mice were randomized into Control or NASH groups. NASH was induced over 3 (Experiment 1) or 6 (Experiment 2) mo using a combined model including a high-fat diet and low doses of carbon tetrachloride. Control group received standard chow, drinking water, and equivolume normal saline. Animal behaviour was assessed by the Open field (OF), Elevated plus maze (EPM), and Light/dark box (LDB) tests at 1, 2, 3, and 6 mo of NASH induction. Visuospatial memory was assessed by the Spontaneous alternation in the T-maze and Novel object recognition tests at 1, 2, and 3 mo of NASH modelling, and using the Barnes maze at 6 mo of NASH induction. Following 3 mo of NASH induction, needle electroneuromyography (ENMG) was performed on the gastrocnemius and biceps muscles with the electrical stimulation of the sciatic and musculocutaneous nerves, respectively. Liver pathology was confirmed by histomorphology. Statistical analysis was performed using Prism 10.2.3 and R 4.2.3 with RStudio 2024.09.0.

Results and discussion. Experimental modelling was associated with poorer overall survival ($p < 0.05$, $p < 0.01$) and substantial evidence of liver injury, i.e. cholestatic hepatitis, medio- and macrovesicular steatosis, focal necrosis and fibrosis of varying severity ($p < 0.05$, $p < 0.01$). Mice with NASH exhibited markers of elevated anxiety in the OF, EPM, and LDB tests ($p < 0.05$, $p < 0.01$), which were mostly specific to the very onset of liver disease (1 mo) as well as its later stages (6 mo). NASH was also associated with a significant decrease in spontaneous alternation at 3 mo ($p < 0.01$), negative object disrimination at 2 mo ($p < 0.05$), and poorer memory retention in the Barnes maze ($p < 0.05$, $p < 0.01$) compared with Control. ENMG data analysis revealed significantly lower peak M-wave amplitudes ($p < 0.01$) and threshold currents ($p < 0.05$) in the gastrocnemius, and increased peak latency in the biceps in the NASH group ($p < 0.05$).

Conclusion. Experimental alimentary/toxic NASH in male C57Bl/6 mice is associated with increased anxiety-like behaviour, visuospatial memory acquisition and retention impairment, and evidence of axonal and demyelinating peripheral motor neuropathy.

Keywords: cognitive deficit, memory deficit, behavioural disorders, neuromuscular joint dysfunction, non-alcoholic steatohepatitis, non-alcoholic fatty liver disease

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

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Экспериментальный неалкогольный стеатогепатит у мышей ассоциирован с поведенческими, когнитивными и периферическими нейрональными нарушениями

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Резюме

Введение. Неалкогольная жировая болезнь печени (НАЖБП) и неалкогольный стеатогепатит (НАСГ) – на сегодняшний день ведущая причина хронической патологии печени во всем мире – ассоциированы с широким спектром психоневрологических осложнений и заболеваний. Тем не менее в настоящий момент аспекты причинно-следственной связи между патологией печени и нервной системы изучены не до конца, что обусловливает необходимость разработки соответствующих клинически релевантных и валидных животных моделей.

Цель. Целью настоящей работы стала оценка кратко- и долгосрочных психоневрологических и периферических нейрональных нарушений, осложняющих течение различных стадий НАЖБП/НАСГ у мышей.

Материалы и методы. 68 взрослых мышей-самцов линии C57Bl/6 рандомизировали на группы «Контроль» и «НАСГ». НАСГ моделировали в течение 3 (эксперимент 1) или 6 (эксперимент 2) мес. при помощи комбинированной модели, включающей высокожировую диету и введение низких доз тетрахлорметана. Контрольные животные получали стандартный комбикорм, питьевую воду и физиологический раствор в эквивалентных объемах. Поведение животных оценивали через 1, 2, 3 и 6 мес индукции НАСГ в тестах «Открытое поле» (ОП), «Приподнятый крестообразный лабиринт» (ПКЛ) и «Черно-белая камера» (ЧБК). Визуально-пространственную память оценивали с помощью тестов «Спонтанное чередование в Т-лабиринте» и «Распознавание нового объекта» через 1, 2 и 3 мес. индукции НАСГ, а также с помощью теста «Лабиринт Барнс» через 6 мес. индукции НАСГ. Через 3 мес. моделирования НАСГ выполняли игольчатую электронейромиографию (ЭНМГ) икроножной мышцы и двуглавой мышцы плеча при электрической стимуляции седалищного и мышечнокожного нервов соответственно. Патологию печени верифицировали гистоморфологически. Статистическую обработку данных проводили в программе Prism 10.2.3 и среде R 4.2.3 с RStudio 2024.09.0.

Результаты и обсуждение. Экспериментальное моделирование сопровождалось снижением общей выживаемости животных ($p < 0,05$, $p < 0,01$) и признаками выраженного поражения печени, включая холестатический гепатит, средне- и крупнокапельный стеатоз, очаги некроза и фиброз различной степени тяжести ($p < 0,05$, $p < 0,01$). Мыши с НАСГ демонстрировали признаки повышения тревожности в тестах ОП, ПКЛ и ЧБК ($p < 0,05$, $p < 0,01$), которые были наиболее выражены на самых начальных (1 мес.) и более поздних (6 мес.) стадиях болезни. НАСГ был также ассоциирован со значительным снижением частоты спонтанного чередования в 3 мес. ($p < 0,01$), отрицательной дискриминацией объектов

в 2 мес. ($p < 0,05$) и ухудшением ретенции памятного следа в «Лабиринте Барнс» ($p < 0,05$, $p < 0,01$) по сравнению с контролем. При проведении ЭНМГ в икроножной мышце у мышей с НАСГ наблюдали значительное снижение максимальной амплитуды М-ответа ($p < 0,01$) и пороговой силы тока ($p < 0,05$), в двуглавой мышце плеча – увеличение латентности максимального ответа ($p < 0,05$).

Заключение. Экспериментальный алиментарно-токсический НАСГ у мышей-самцов линии C57Bl/6 ассоциирован с повышением тревожности, нарушением формирования и ретенции визуально-пространственного памятного следа, а также признаками аксонально-демиелинизирующей периферической моторной нейропатии.

Ключевые слова: когнитивный дефицит, мнестический дефицит, поведенческие нарушения, нейромышечные нарушения, неалкогольный стеатогепатит, неалкогольная жировая болезнь печени

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. С. В. Оковитый, В. А. Приходько – идея и планирование эксперимента. В. А. Приходько, Д. А. Орляхина, В. Д. Петрова, В. Е. Карев – проведение экспериментов и обработка данных. В. А. Приходько, В. Е. Карев – подготовка иллюстраций. В. А. Приходько, В. Е. Карев, Д. Ю. Ивкин, С. М. Напалкова, С. В. Оковитый – подготовка и редактура рукописи.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) (recently suggested to be replaced by metabolic-associated steatotic liver disease, MASLD) is an umbrella term including a range of chronic conditions characterized by excessive hepatic lipid accumulation, defined by the presence of steatosis in >5 % of hepatocytes, in the absence of excessive alcohol consumption or other plausible causes of toxic liver injury [1]. The global prevalence of NAFLD today exceeds 30 %, which makes it the leading cause of chronic liver disease worldwide [2]. About 20 % of NAFLD cases are classified as non-alcoholic steatohepatitis (NASH), which has a substantially higher risk of progression to liver fibrosis, cirrhosis, and end-stage liver disease as well as complication by hepatocellular carcinoma [3]. Due to the above, NAFLD and NASH are predicted to emerge as the main indication for liver transplantation in developed and developing countries in the next decade [2].

While NAFLD and NASH have long been known to correlate closely with cardiometabolic disease, their association with psychoneurological symptoms and conditions remains relatively underexplored. Mounting evidence suggests that NAFLD patients are at a greater risk of brain volume reduction and premature brain aging, exhibit signs of cognitive, memory, and psychomotor impairment, and are more likely to suffer from affective disorders, schizophrenia, vascular dementia and neurodegenerative conditions [4, 5]. Nevertheless, the underlying causal relationship between liver disorders and their neuronal complications remains ambiguous, which warrants the development of clinically relevant and valid animal models. We have previously reported distinct cognitive and behavioural deficits observed in murine NAFLD [6, 7]; however, to the best of our knowledge, their complex temporal patterns and dynamics have not yet been characterized in detail.

In view of the above, this work was carried out **in order to** characterize the short- and long-term psychoneurological and peripheral neuronal deficits that complicate different stages of NAFLD/NASH in mice, using behavioural testing and needle electroneuromyography.

MATERIALS AND METHODS

Animal experiments were conducted in full compliance with the principles of the Basel Declaration, European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (European Treaty Service No. 123, 18 March 1986), and the Order of the Ministry of Health of the Russian Federation No. 199n (1 April 2016) "On the approval of the Rules of Good Laboratory Practice", and was approved by the Bioethics Committee of the St. Petersburg

State Chemical and Pharmaceutical University of the Ministry of Health of the Russian Federation (protocol M-OE-02; 21 January 2020).

68 young adult (2 months old) male C57Bl/6 mice weighing 18-20 g were purchased from the Rappolovo laboratory animal supplier (Leningrad Oblast, Russia) in a single shipment, quarantined for 2 weeks, then housed in a standard animal facility with *ad libitum* access to standard chow (Laboratorkorm, Russia) and drinking water. In order to assess both the short- and long-term neurological complications of NASH, two experiments were conducted, with the animals randomized by random sequence generation into the following groups:

1. Control (Experiment 1: $n = 10$; Experiment 2: $n = 14$): standard chow + drinking water + 0.9 % NaCl intraperitoneally (i/p) weekly (q.wk.).
2. NASH (Experiment 1: $n = 30$; Experiment 2: $n = 14$): high-fat chow + 42 $g \cdot L^{-1}$ D-fructose (high-fat diet, HFD) + 0.32 $mg \cdot kg^{-1}$ b.w. carbon tetrachloride (CCl_4) i/p q.wk.

The experimental design is shown in Figure 1.

NASH was induced using the combined diet/chemical model described by Tsuchida et al. [8]. The high-fat chow was prepared *ex tempore* and consisted of 36.65 % standard chow, 21.10 % beef tallow, 41.00 % D-fructose, and 1.25 % cholesterol. In addition, the mice in the NASH group were offered a 42 $g \cdot L^{-1}$ aqueous D-fructose solution to drink, and injected i/p weekly with 0.32 $mg \cdot kg^{-1}$ CCl_4 as an accelerant. Mortality was recorded daily, and body weights were measured weekly throughout the experiment.

Animal locomotor activity and behaviour were assessed at 1, 2, 3, and 6 months of NASH induction using the Open field (OF), Elevated plus maze (EPM), and Light/dark box (LDB) tests (Open Science, Russia). Animal movement was video recorded for 3 min, then analyzed using VideoMot2 3.0.1 (TSE Systems GmbH, Germany) (OF and LDB) or RealTimer 1.30 (Open Science, Russia) (EPM).

In the OF test, distance covered (cm), mean velocity ($cm \cdot s^{-1}$), time in centre (s), total freezing duration (s), and the frequencies of freezing, line crossing, rearing, grooming, and hole peeking were registered [9]. In the EPM test, time spent in the open arms, closed arms, and centre (s), the number of entries into the open and closed arms, and the frequencies (min^{-1}) of rearing, grooming, head dipping, and peeking out were registered [10]. In the LDB test, time spent (s) and distance covered (cm) in the light chamber (LC), mean velocity ($cm \cdot s^{-1}$), total freezing duration (s), frequencies (min in the LC^{-1}) of freezing, grooming, rearing and peeking out (min in the dark chamber $(DC)^{-1}$), total number of transitions, latency to enter the DC for the 1st time (s), and the duration of the 1st DC visit (s) were registered [11].

Short-term visuospatial memory was assessed at 1, 2, and 3 months of NASH induction using the Spontaneous alternation in the T-maze (SATM) and Novel object

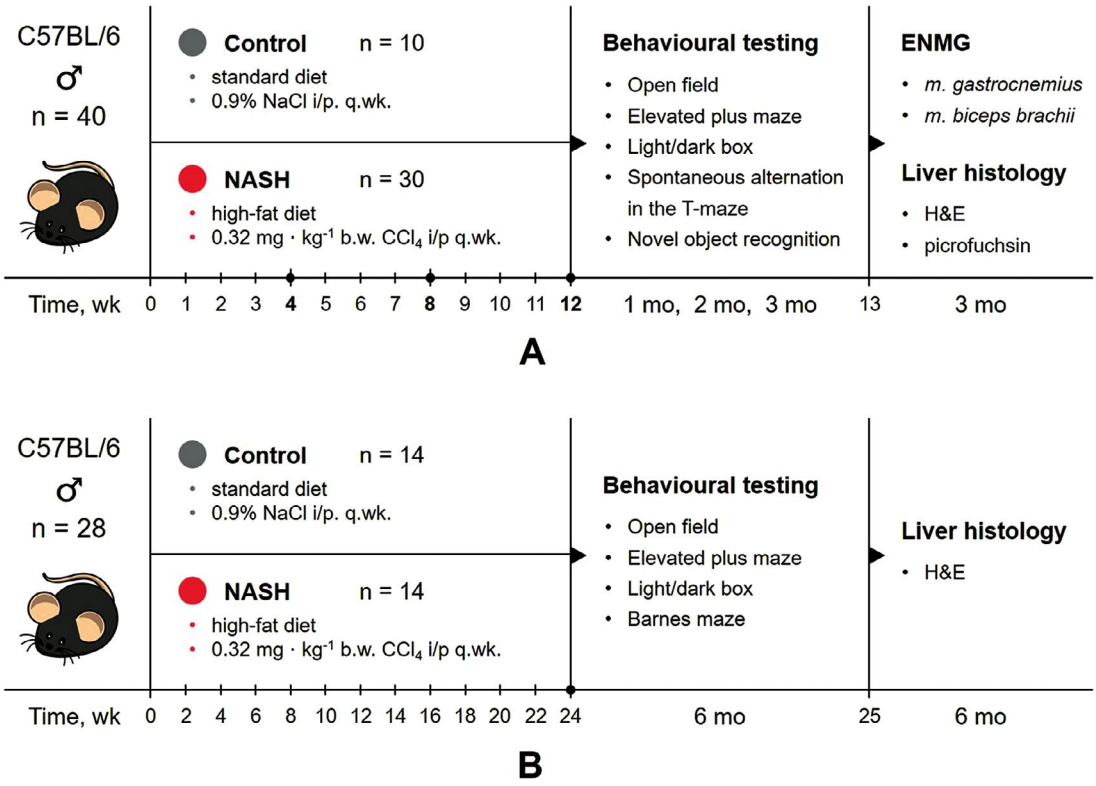

Figure 1. Design of Experiments 1 (A) and 2 (B). i/p, intraperitoneally; b.w., body weight; q.wk., weekly; ENMG, electroneuromyography; H&E, haematoxylin and eosin

recognition (NOR) tests (Open Science, Russia). In the SATM test, spontaneous alternation percentage was assessed live by an experienced researcher, in 3 trials per animal [12]. In the NOR test, total object exploration time (s) and novel object exploration time (s) were registered, and the discrimination index was calculated as described previously [13]. In addition, at 6 months of NASH induction, the Barnes maze test (Open Science, Russia) was conducted to assess short- and long-term visuospatial memory. During the 4-day training session, the mice were given 4 attempts (each 3 min long) per day to explore the maze and find the target hole, connected to the escape box. On days 5 and 12, a single trial (1.5 min long) was conducted, and total search time (s) and the number of errors (nose poking into the wrong holes) were registered [14].

Electroneuromyographic (ENMG) study was performed at 3 months of NASH induction under chloral hydrate (400 mg · kg^{-1} b.w.) anaesthesia using a Neuro-MEP-8 8-channel system with the Neuro-MEP.NETω 3.7.3.7 software (Neurosoft, Russia). M-waves were registered in the left *m. gastrocnemius* (MG) and right *m. biceps brachii* (MBB) following electrical stimulation (0.1 ms, square, from 1 to 10 mA in increments of 1 mA) of the left *n. ischiadicus* and right *n. musculocutaneus*, respectively [15]. Peak amplitude (mV), threshold and peak currents (mV), peak latency (ms), mean duration (mV), and mean area (mV · ms) were registered for each M-wave series.

Following the completion of all experiments, the animals were euthanized via carbon dioxide inhalation, and liver tissue samples were obtained for morphological analysis. The samples were fixed in 10 % neutral formalin, dehydrated, cleared in isopropyl alcohol, and embedded in paraffin according to conventional protocols. 4 µm-thick sections were prepared from paraffin blocks using a HM340 rotary microtome (Thermo Scientific, UK), mounted on slides, stained with haematoxylin and eosin or van Gieson's picrofuchsin, and cover-slipped. Whole slide imaging was performed using a Pannoramic MIDI automatic digital slide scanner with the Pannoramic Scanner Software for Research (3DHISTECH Kft, Hungary).

For each tissue sample, hepatitis activity (according to the METAVIR-A system), steatosis, hepatocellular ballooning (HCB), cholestasis, necrosis, periportal fibrosis (PPF), central vein fibrosis (CVF), perisinusoidal fibrosis (PSF), and bridging fibrosis (BF) were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Liver fibrosis was also staged according to the METAVIR-F system as F0 (no fibrosis), F1 (portal fibrosis without septa), F2 (portal fibrosis with few septa), or F3 (numerous septa without cirrhosis) [16].

Statistical analysis was performed using Prism 10.2.3 (GraphPad Software, USA) and R 4.2.3 (R Foundation for Statistical Computing, Austria) with RStudio 2024.09.0 (Posit Software PBC, USA). Schoenfeld residuals were calculated to check the proportional hazard assumption. Since the assumption was met ($p = 1$ for both experiments), the survival functions were compared using the log-rank (Mantel-Cox) test, and hazard ratios (HR) were calculated by the Mantel-Haenszel method using the 'survival' 3.6-4 package for R. Histological score analysis was carried out as described previously [17] by extended Fisher's exact test with the Bonferroni correction for pairwise comparisons, using the 'RVAideMemoire' 0.9-81-2 package for R. Median score totals were compared using Mood's test. Zone preference in the LDB test was analyzed using the chi-squared test.

Other numerical data were tested for normality using the Shapiro-Wilk W-test. For normally distributed data, the significance of differences between group means was tested using Student's t-criterion; otherwise, the Mann – Whitney test was used. The significance threshold was set at $p < 0.05$.

RESULTS AND DISCUSSION

By 3 months of Experiment 1, 100.0 % and 43.3 % of the Control and NASH mice were alive, respectively. By 6 months of Experiment 2, 100.0 % and 64.3 % of the Control and NASH mice were alive, respectively. HR values for the NASH group were calculated as 5.02 [95 % confidence interval (CI) 1.68; 14.96] and 6.47 [95 % CI 1.09; 38.38] for Experiments 1 and 2, respectively. Overall survival probability functions differed significantly ($p < 0.01$, $p < 0.02$ for Experiments 1 and 2, respectively). Survival analysis results are summarized in Table 1, and the survival curves are shown in Figure 2.

NASH, non-alcoholic steatohepatitis; RMST, restricted mean survival time; SEM, standard error of mean; HR, hazard ratio; CI, confidence interval.

On histology, all Control liver samples exhibited mild-to-moderate cholestatic granulomatous hepatitis. In several samples, micro- and macrovesicular steatosis involving less than 1 % of the hepatocytes, mild HCB, and mild intracellular cholestasis were observed. No signs of necrotic cell death were detected, and all samples were classified as METAVIR F0 (Figure 3).

Table 1. Survival analysis results

Group	Experiment 1 (3 mo)			Experiment 2 (6 mo)		
	RMST [SEM], wk	HR [95% CI]	p	RMST [SEM], wk	HR [95 % CI]	p
Control	12.0 [0.0]	–	–	24.0 [0.0]	–	–
NASH	8.1 [0.8]	5.02 [1.68; 14.96]	< 0.01	18.7 [2.0]	6.47 [1.09; 38.38]	< 0.05

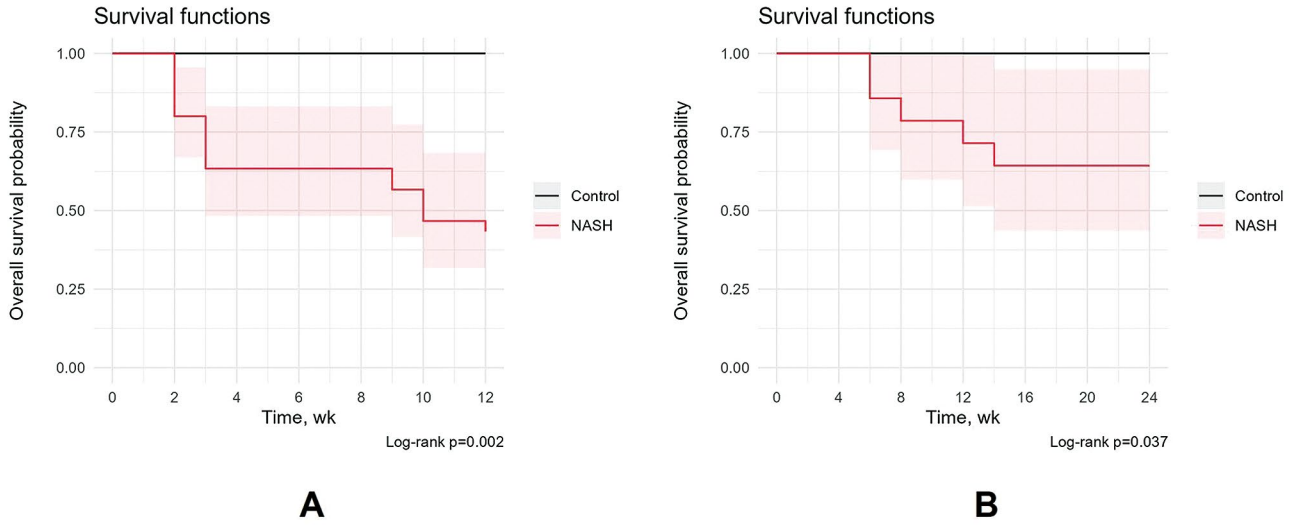

Figure 2. **Kaplan-Meier survival curves for Experiments 1 (A) and 2 (B). NASH, non-alcoholic steatohepatitis**

In the NASH group, substantial evidence of liver injury was found. All samples showed signs of moderate-to-severe cholestatic hepatitis, complicated by centrilobular and/or bridging necrosis in 90 % cases. 40 % samples exhibited mild medio- and macrovesicular steatosis involving up to 5 % of the hepatocytes. HCB varied from mild to severe, and intracellular cholestasis was either moderate or severe in degree. 90 % of the samples were staged as either F2 or F3 according to the METAVIR system; no signs of liver cirrhosis were detected (Figure 3). Based on the histological features described above, the presence of non-alcoholic stea-

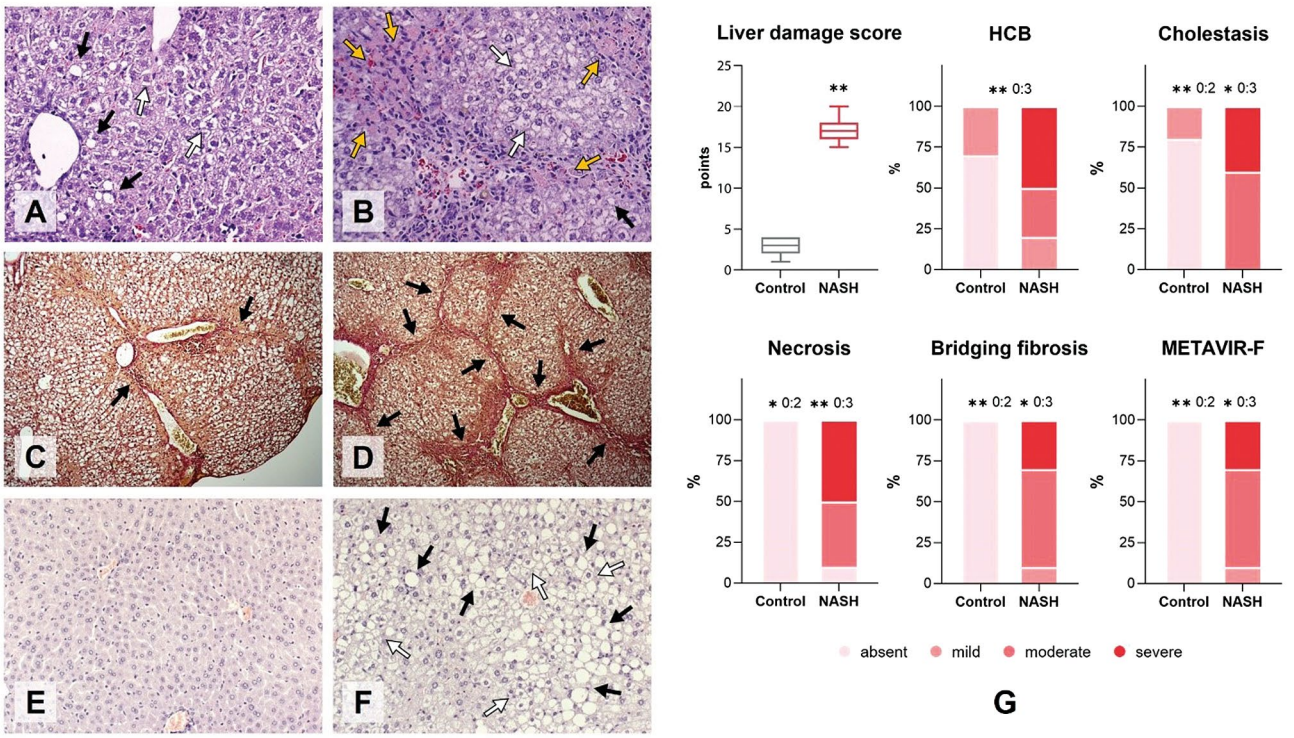

Figure 3. **(A–F) Microphotographs of representative liver samples from the experimental groups (A, C, E, Control; B, D, F, NASH) at 3 (A–D) and 6 (E, F) months of steatohepatitis induction. A, B, E, F, haematoxylin and eosin, ×200. C, D, van Gieson's picrofuchsin, ×100. Black arrows indicate steatosis (A–D) or fibrotic foci (C, D), white arrows, hepatocyte ballooning, yellow arrows, necrotic foci. (G) Liver damage score totals and individual parameter scores. NASH, non-alcoholic steatohepatitis; HCB, hepatocyte ballooning; * $p < 0.05$; ** $p < 0.01$**

tohepatitis, with liver fibrosis of varying severity in some of the cases, was confirmed.

Histological score totals were significantly ($p < 0.01$) higher in the NASH group compared to Control. According to score distribution analysis, NASH was associated with a significant increase in the prevalence of grade 3 HCB ($p < 0.01$), grade 2 and 3 cholestasis ($p < 0.01$ and $p < 0.05$, respectively), grade 2 and 3 necrosis ($p < 0.05$ and $p < 0.01$, respectively), F2 and F3 stage fibrosis ($p < 0.01$ and $p < 0.05$, respectively), and grade 2 and 3 bridging fibrosis ($p < 0.01$ and $p < 0.02$, respectively) (Figure 3).

In the NASH group, a decrease in total freezing duration and an increase in hole poking frequency were observed in the OF test at 1 month of liver disease induction, and an increase in mean velocity, at 2 months ($p < 0.05$ for all) (Figure 4). A decrease in total freezing duration, coupled with an unchanged freezing frequency, implies an irregular, intermittent nature of movement in the OF arena. Taken together with a slight decrease in the number of grooming bouts and an increase in rearing frequency, these findings could be interpreted as signs of agitation, indicative of high baseline anxiety levels and/or sensitization to the aversive stimulus [18, 19]. Although we did not observe increased thigmotaxis, commonly recognized as a universal marker for fear and anxiety [20], this does not necessarily exclude anxiety-like behaviour as the OF test results regarding anxiety states are mostly considered preliminary [21].

In the EPM test, Control mice exhibited an increase in both the time spent in the central zone ($p < 0.01$) and the number of entries into closed arms ($p < 0.05$) at 1 month with a subsequent steady decrease to Intact levels. Rearing frequency was increased at months 1 and 3 ($p < 0.05$ for both) but not 2 (Figure 5). Zone preference remained the same between the groups throughout the experimental period.

At 2 months, Control mice spent less time in the light chamber of the LDB, and at 3 months, had lower grooming frequency and longer duration of the 1st visit to the DC ($p < 0.05$ for all) (Figure 6). Zone preference shifted significantly in mice with experimental NASH towards the DC at months 2, 3, and 6 (Figure 7).

While high rearing frequency is usually interpreted as a sign of anxiety response [22, 23], the relationship between anxiety levels and grooming frequency can be seen in different perspectives. On the one hand, rodents prefer to groom while feeling safe and not experiencing fear and/or anxiety [24]; on the other hand, however, acute stress response can include frequent but short grooming bouts replacing other activities [25].

Head dipping on the open arms of the EPM may be considered not only directed exploration [26] but also a risk assessment behaviour [27]. Together with the increased number of both open and closed arm visits as well as the changes mentioned above, increased grooming and head dipping frequencies might also indicate elevated anxiety and agitation [25, 28]. Hyperlocomotion and hyperactivity have been described previously as characteristic aspects of depression- and anxiety-like behavioural changes accompanying diet-induced NAFLD in C57Bl/6J mice [29]. High-fat, high-sucrose diet has also been demonstrated to be associated with marked sensitization of rats to aversive stimuli in the OF and EPM tests [23]. Notably, the changes in mouse behaviour in the OF, EPM, and LDB tests mostly had a distinct biphasic nature, tending to invert towards the end of the study period compared to earlier time points, which may be indicative of underlying metabolic adaptation processes in the surviving animals.

In the SATM test, at all experimental time points, Control specimens alternated 70–73 % of the time, which is consistent with literature data for healthy mice [12]. NASH was associated with a steady decrease in the spontaneous alternation rate, which became statistically significant at 3 months ($p < 0.01$) (Figure 8). In the NOR test, Control mice spent over 50 % of the time exploring the novel object, indicating positive object discrimination and reflecting novelty preference, at all time points [13]. At 2 months, mice with NASH spent significantly ($p < 0.01$) less time exploring the novel object, resulting in a negative mean discrimination index (Figure 8).

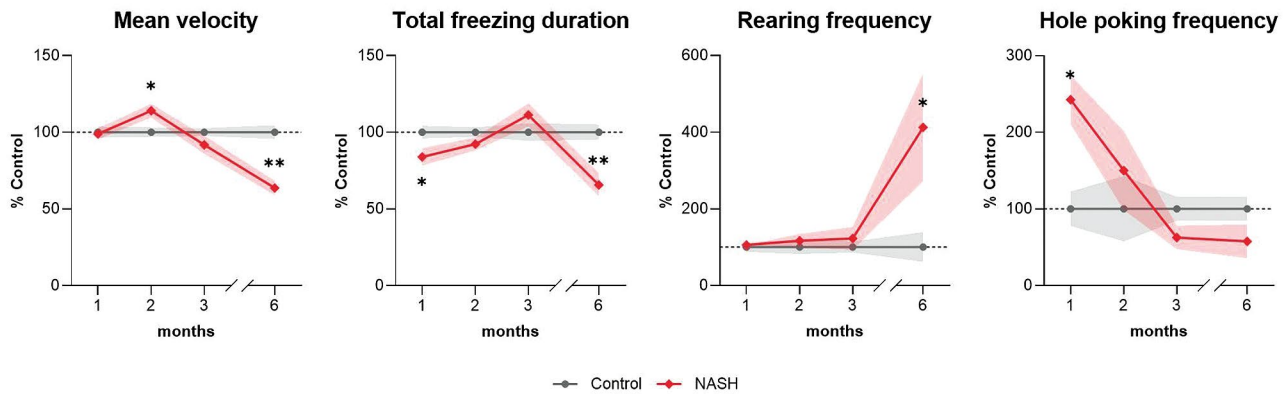

Figure 4. Open field test results at 1, 2, 3, and 6 months of steatohepatitis induction, normalized to respective Control values (mean; SEM). NASH, non-alcoholic steatohepatitis; * $p < 0.05$; ** $p < 0.01$

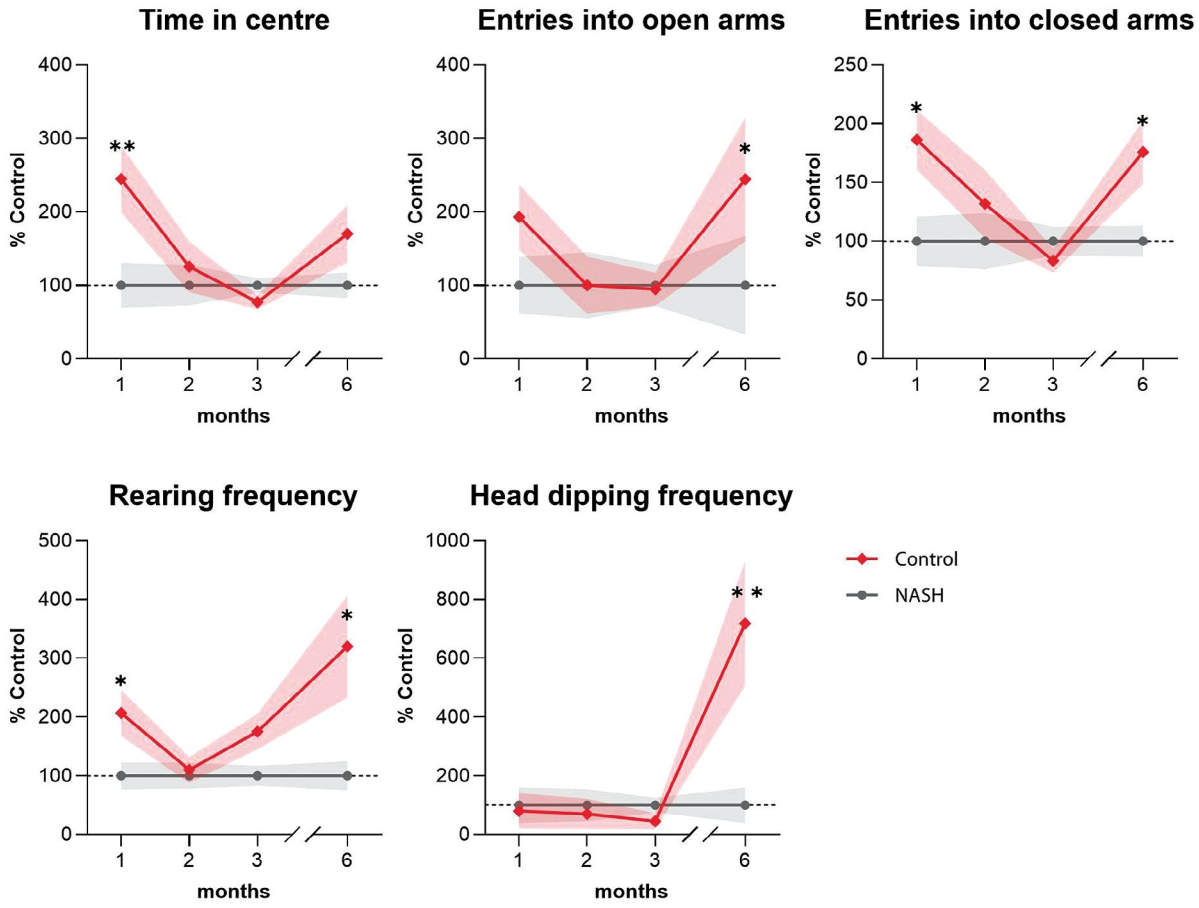

Figure 5. Elevated plus maze test results at 1, 2, 3, and 6 months of steatohepatitis induction, normalized to respective Control values (mean; SEM). NASH, non-alcoholic steatohepatitis; * $p < 0.05$; ** $p < 0.01$

In the Barnes maze, both groups of mice exhibited similar results during training, taking significantly less time to find the target hole and making less errors over time ($p < 0.05$, $p < 0.01$) (Figure 9, A). On trial day 5, mice with NASH managed to find the target hole with significantly ($p < 0.01$) less errors than Control mice, which generally reflects a better overall performance, but can also be a consequence of decreased exploration. On trial day 12, the NASH group demonstrated significantly ($p < 0.05$ for search time, $p < 0.01$ for errors) poorer performance compared with day 5, while Control specimens maintained their results (Figure 9, B). This evidences impaired retention of visuospatial information in mice with liver disease [14].

As observed in the SATM test, the short-term spatial memory steadily declined over the experimental period, with the difference from healthy animals reaching significance at 3 months of NASH induction. Selective impairment of allocentric spatial learning, processing, and memory retention without loss of visual reference memory is commonly observed in animals with hippocampal lesions [30, 31]. A study in rats with HFD-induced NASH, hyperammonaemia, and gut dysbiosis reported impaired learning and performance in the Barnes maze, possibly arising from metabolic and toxic damage to the prefrontal cortex [32].

In a close analogue of the Barnes maze, the Morris water maze, rats with a similar model of alimentary NASH exhibited poor memory retention while maintaining normal learning ability, possibly explained by the presence of additional non-visual cues [33]. Short-term spatial memory dysfunction in chronic liver disease has been shown to correlate with decreased metabolic activity in the entorhinal cortex, thalamus, hippocampus, amygdalae, and mammillary bodies as well as a general reduction of white and gray matter volume [32, 34, 35].

Progressive NAFLD induces endothelial dysfunction, microglial activation and cell death in the hippocampus and prefrontal cortex [36]. Elevated serum ammonia levels, besides causing direct neuronal and glial cell damage, potentiate dopamine catabolism, induce neurosteroid synthesis, repress γ-aminobutyric acid (GABA) production, and promote N-methyl-D-aspartate receptor expression, leading to general depression of the central nervous system (CNS) due to $GABA_A$ receptor sensitization [37].

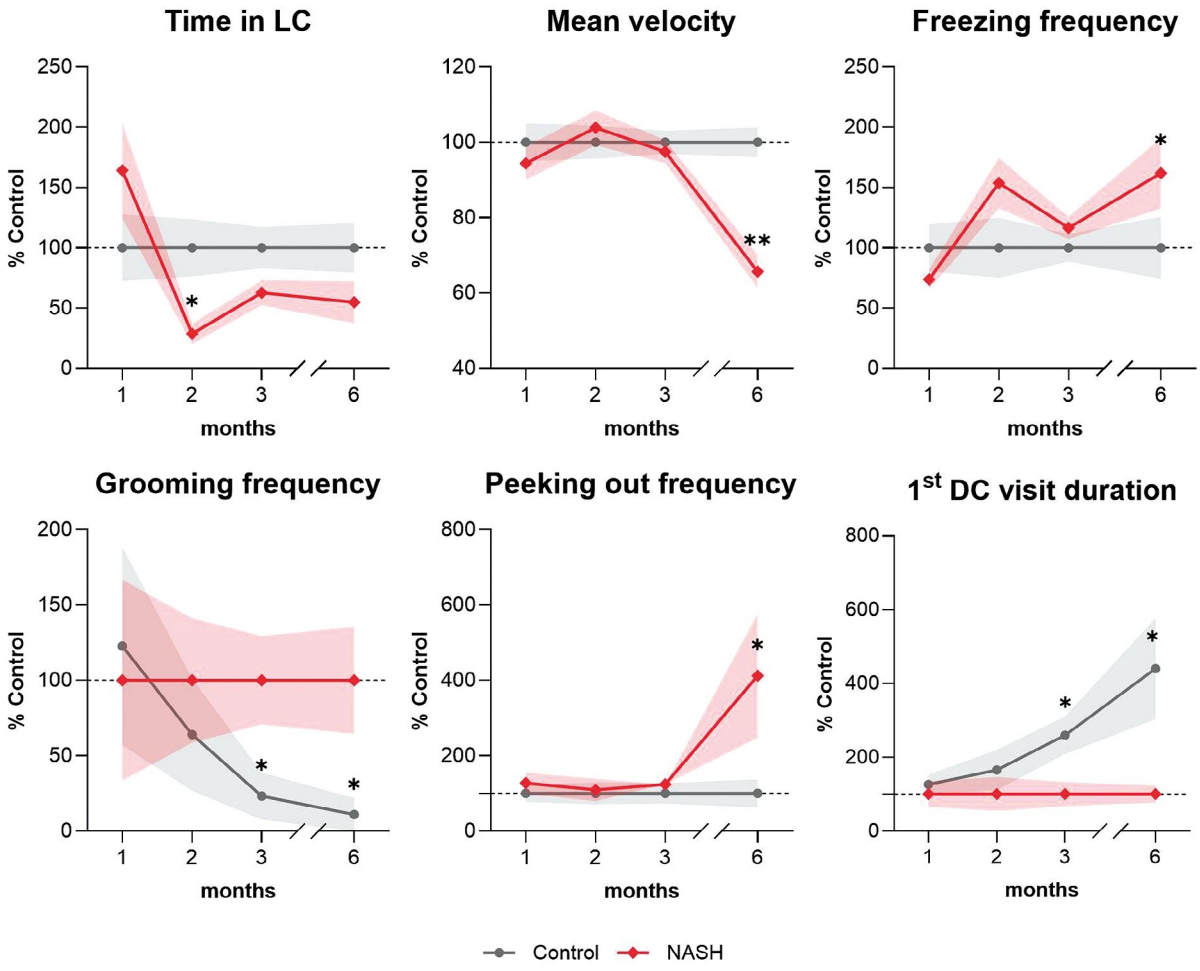

Figure 6. **Light/dark box test results at 1, 2, 3, and 6 months of steatohepatitis induction, normalized to respective Control values (mean; SEM). NASH, non-alcoholic steatohepatitis; LC, light chamber; DC, dark chamber; * $p < 0.05$; ** $p < 0.01$**

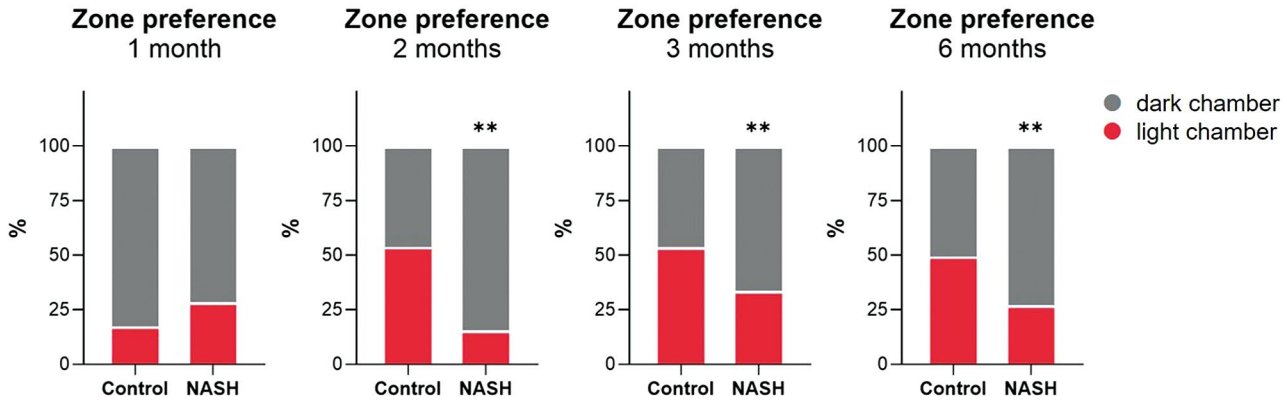

Figure 7. **Light/dark box zone preference at 2, 3, and 6 months of steatohepatitis induction. NASH, non-alcoholic steatohepatitis; ** $p < 0.01$**

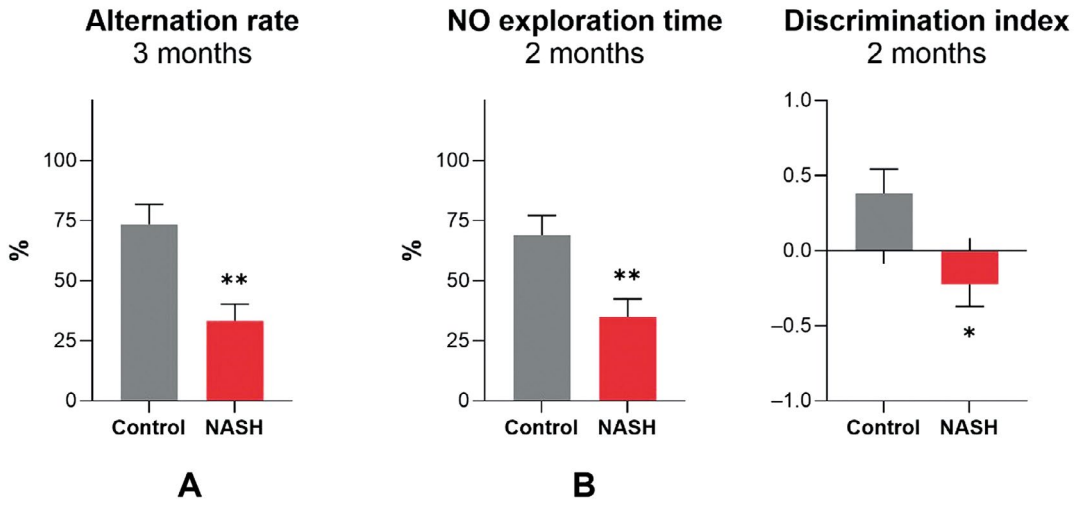

Figure 8. **(A) Rate of spontaneous alternation in the T-maze at 3 months of steatohepatitis induction. (B) Novel object recognition test results at 2 months of steatohepatitis induction (mean; SEM). NASH, non-alcoholic steatohepatitis; NO, novel object; * $p < 0.05$; ** $p < 0.01$**

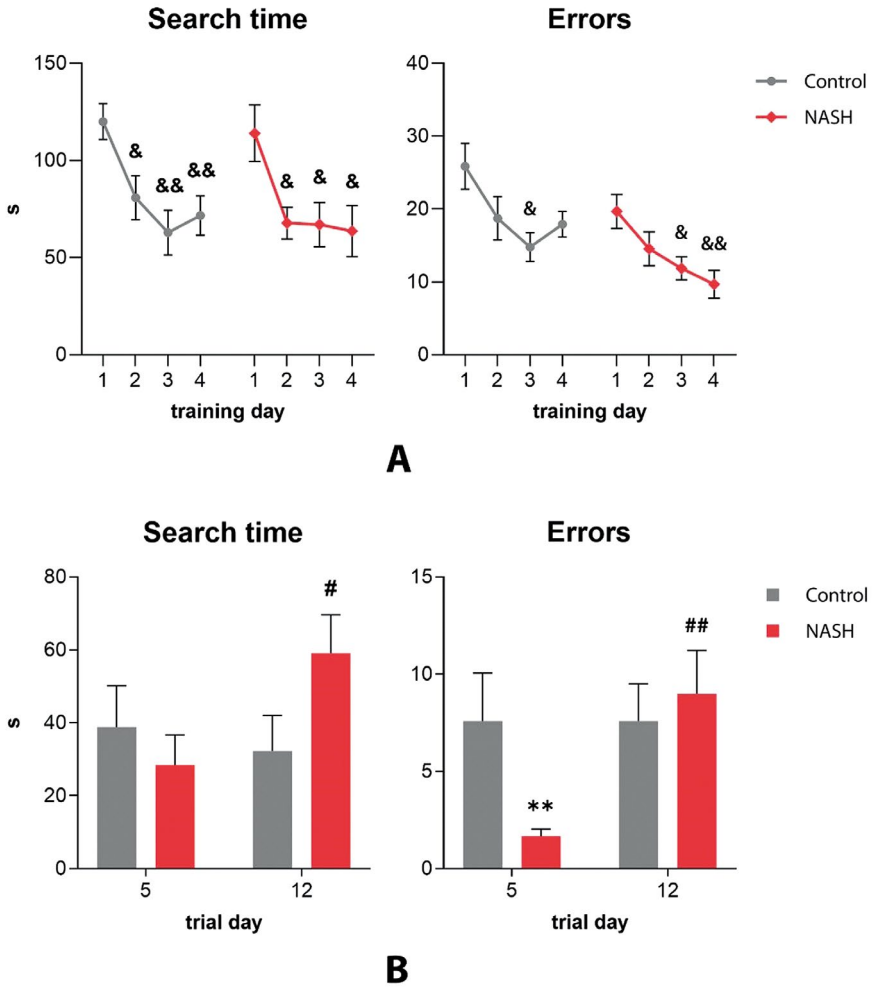

Figure 9. **(A) Results dynamics in the Barnes maze during the training period (mean; SEM). & $p < 0.05$ vs. own group on day 1; && $p < 0.01$ vs. own group on day 1. (B) Trial results in the Barnes maze (mean; SEM). NASH, non-alcoholic steatohepatitis; ** $p < 0.01$ vs. Control; # $p < 0.05$ vs. own group on day 5; ## $p < 0.01$ vs. own group on day 5**

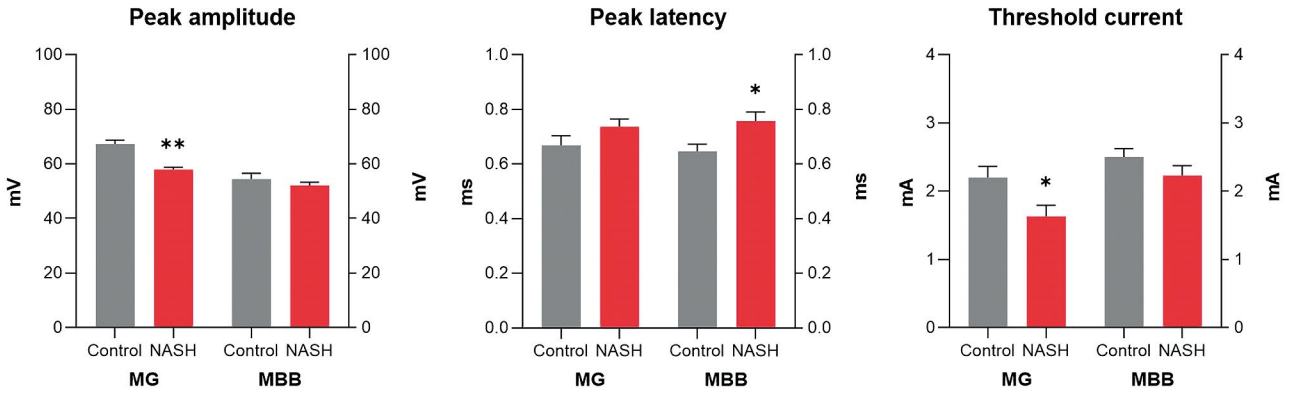

Figure 10. Parameters of M-waves induced by single-stimulus electrical nerve stimulation (mean; SEM). MG, *m. gastrocnemius*; MBB, *m. biceps brachii*; NASH, non-alcoholic steatohepatitis; * p < 0.05; *** p < 0.01.

Neurotransmitter metabolism alterations in the CNS that are observed in NASH and may be relevant to visuospatial memory decline include a decrease in dopamine levels in the prefrontal cortex and cerebellum and a decrease in noradrenaline in the striatum [32] as well as increased activity of acetylcholine esterase and monoamine oxidases [38]. Gut dysbiosis and the subsequent endotoxinaemia represent another link connecting NASH and memory deficits, possibly through hippocampal Toll-like receptor 4/brain-derived neurotrophic factor [39], insulin, insulin-like growth factor 1, and other signaling pathways [37].

Two small-scale observational clinical studies (n = 44, n = 213) reported visuospatial memory deficits in NAFLD subjects, according to MoCA (Montreal cognitive assessment) and RBANS (Repeatable battery for the assessment of neuropsychological status) scales [40, 41]. In a case-control study (n = 208), the results of the Line tracing test, which is commonly used to assess visuospatial function in minimal hepatic encephalopathy, were normal in patients with liver steatosis, but significantly poorer in those with NASH, liver fibrosis, or cirrhosis [42]. Visuospatial memory deficits can also be present in non-alcoholic liver cirrhosis in the absence of overt hepatic encephalopathy [43].

The transient nature of short-term recognition memory could possibly be explained by the adaptation of metabolic processes and CNS function developing over time. Both spatial and recognition memory are hippocampus-dependent, but spatial memory performance requires more hippocampal tissue and resource than does recognition memory. In an experimental study in rats, spatial memory declined after bilateral dorsal lesions that encompassed 30–50 % total volume of the hippocampus, while object recognition only became impaired when 75–100 % of the hippocampus was damaged [44].

ENMG data analysis revealed significantly lower peak M-wave amplitudes ($p < 0.01$) and threshold currents ($p < 0.05$) in *m. gastrocnemius*, and increased peak latency in *m. biceps brachii* in the NASH group ($p < 0.05$) (Figure 10).

The observed decrease in peak M-wave amplitudes in *m. gastrocnemius* might indicate partial loss and/or dystrophic alteration of motor units, reduced excitability and/or conductivity of motor nerve axons, possibly due to their demyelination and/or secondary axon loss. Increased M-wave latency, detected in *m. biceps brachii*, could also be a sign of pathologically reduced action potential propagation velocity, characteristic of demyelinating nerve disease [45].

As histology revealed no signs of myosteatosis, muscle hypotrophy, or sarcopenia (data not shown), the observed neuromuscular joint dysfunction can be concluded to most probably result from direct motor nerve damage accompanying chronic liver injury. Clinical studies have previously found different stages of NAFLD to induce predominantly axonal motor, sensory, and autonomic neuropathy [46–48] as well as promote peripheral polyneuropathy in type II diabetes mellitus [49, 50]. However, the aforementioned observations are likely indicative of combined axonal and demyelinating peripheral motor neuropathy associated with NASH and liver fibrosis.

CONCLUSION

Using behavioural testing and needle electroneuromyography, we analyzed a spectrum of central and peripheral neuronal deficits in mice with different stages of experimental NASH. Behavioural alterations were mostly represented by a biphasic increase in anxiety-like behaviour, observed both at the very onset and in later stages of liver disease. Working visuospatial memory deficit appeared to be positively correlated with liver disease severity, while object recognition memory only underwent a transient impairment with subsequent compensation. Abnormal ENMG results suggested the presence of combined axonal and demyelinating

peripheral motor neuropathy. These observations support the relevance and validity of combined alimentary and toxic murine NASH models for the research of psychoneurological complications of chronic liver disease.

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