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Research report

Substrain and light regime effects on integrated anxiety-related behavioral *z*-scores in male C57BL/6 mice—Hypomagnesaemia has only a small effect on avoidance behavior☆

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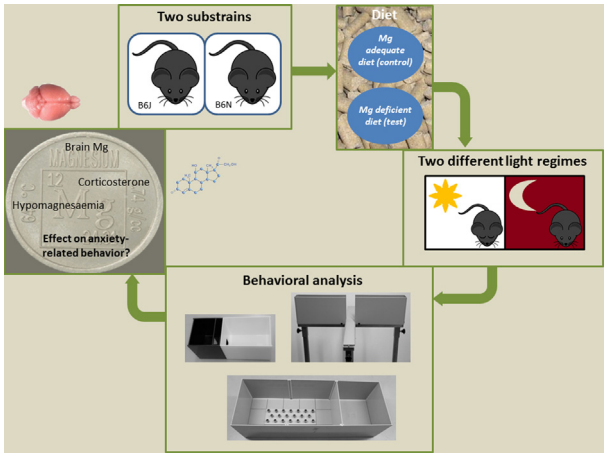
HIGHLIGHTS

- Magnesium deficient diet significantly reduced plasma magnesium in male C57BL/6.
- Integrated behavioral *z*-scores to combine results of three behavioral tests.
- Effect of light regime on behavioral and physiological parameters.
- Substrain differences in anxiety-related, exploration and locomotory behavior.
- An effect of magnesium deficiency on avoidance and exploration.

GRAPHICAL ABSTRACT



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ABSTRACT

Magnesium (Mg) has been described to possess an anxiolytic function, but a number of studies present inconsistent results on this matter. In this study the effect of Mg deficiency on anxiety-related behavior, brain and blood plasma Mg in young adult male C57BL/6JOlaHsd and C57BL/6NCrl mice was studied. The animals were put on a control or Mg deficient diet from day 0 and significant hypomagnesaemia was evident from day 12 onwards in the test animals. Housing and test conditions were under either

Abbreviations: AM, amygdala; B6J, C57BL/6JOlaHsd; B6N, C57BL/6NCrl; CB, cerebellum; CX, prefrontal/medial cortex; EPM, elevated plus maze; HC, hippocampus; HT, hypothalamus; *i.p.*, intraperitoneal; LD, light-dark box; ME, mesencephalon; Mg, Magnesium; mHB, modified Hole Board; SPF, Specified Pathogen Free.

☆ Prof. Dr. Frauke Ohl passed away on January 28th 2016. We remember Frauke as a dedicated scientist and an inspiring and powerful boss, colleague and friend.

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conventional light regime (white light behavioral test conditions) or reverse light regime (red light behavioral test conditions). The animals were tested in three tests for unconditioned anxiety: the modified Hole Board (day 14), the light-dark test (day 21) and the elevated plus maze (day 28). Overall integrated behavioral *z*-scores were calculated over these three behavioral tests. Mg showed a structure dependent distribution at the level of the brain, that differed between C57BL/6 substrain and light regime (conventional versus reverse), respectively. Likewise, total brain Mg did differ between substrain and light regime, but was not affected by the diet. Animals on the Mg deficient diet housed under conventional light regime had a higher final (day 28) blood plasma corticosterone level as compared to controls. Animals housed under reverse light regime exhibited no diet effect of plasma corticosterone levels. The significant hypomagnesaemia at blood plasma level resulted in an effect of Mg deficiency on avoidance, but not overall anxiety-related behavior. Significant differences regarding avoidance behavior were found between the two substrains and light regimes, respectively.

1. Introduction

Magnesium (Mg) is of high interest as potential anxiolytic since it can be applied as a dietary supplement and at a low dose shows no side effects [1]. Indeed, Mg has been used by man for many decades to relieve various emotional problems, including anxiety [2,3]. Since anxiety is an evolutionarily old emotion and neuronal circuits regulating anxiety are comparable in animals and humans, mice are regularly being used as models with considerable translational value in anxiety research [4]. Poleszak and colleagues [5] for example administered Mg hydroaspartate intraperitoneally (*i.p.*) to male albino Swiss mice and the serum Mg level significantly increased. In the elevated plus maze (EPM) the *i.p.* injection of Mg hydroaspartate produced anxiolytic-like effects in these outbred mice [5]. Orally administered Mg also resulted in anxiolytic effects in the EPM in two studies by Jahromy et al. [6,7]. Moreover, this Mg induced anxiolytic-like activity could be antagonized by D-serine (a selective agonist of the glycine$_B$ site of the *N*-methyl-D-aspartate receptor complex [8]) and flumazenil (a benzodiazepine receptor antagonist [9]).

Moreover, *in vivo* observations indicated that Mg deficiency is implicated in the pathophysiology of anxiety in mice, although inconsistent results have been reported. Mg deficiency in C57BL/6J and C57BL/6NCrl mice increased anxiety-related behavior in the light-dark (LD) and open field (OF) tests [10,11]. Similar findings were reported by Muroyama et al. [12]: Mg deficient feeding for three weeks to male C57BL/6NCrl mice significantly reduced serum Mg concentrations and mice receiving the Mg deficient diet showed elevated anxiety-related behavior in the LD after 14 and 21 days; no effect was found after 7 days. In contrast, Sartori et al. [13,14] found that Mg deficiency caused anxiogenic behavior in the EPM in BALB/cAnNCrl mice, but not in C57BL/6NCrl mice. Similarly, no effect of Mg deficiency was observed by Bardgett et al. when testing C57BL/6J mice in the LD after 10 days on the diet [15]. Also Winther et al. [16] found no difference in the number of centre entries in the OF between control and Mg deficient C57BL/6NBomTac mice. In contrast, Pyndt Jørgensen et al. [17] described that a Mg deficient diet for six weeks decreased anxiety-related behavior in the LD in C57BL/6NBomTac mice.

There are a variety of possible factors causing the inconsistency of previous results. We propose to clarify the contradictory messages of the effects of Mg with a test setup by exposing male mice of C57BL/6JOlaHsd and C57BL/6NCrl, two substrains of the widely used C57BL/6 mouse, to three unconditioned anxiety tests (modified Hole Board (mHB), LD and EPM). Magnesium deficiency was induced through the diet and blood plasma samples were assessed for Mg content to test whether hypomagnesaemia was established. We decided to not use erythrocyte but rather blood plasma Mg content as parameter to observe hypomagnesaemia, since in C57BL/6 mice serum or blood plasma Mg content responds better (*i.e.* higher absolute effect sizes) to both a high and low Mg diet than red blood cell Mg content [18,19]. Furthermore, circulating corticosterone levels were determined, since this endocrine measure has been found to induce anxiety-related behavior in laboratory animals [20]. Additional information could be found in the Mg levels in different brain areas which inclined us to study the brain Mg content of the cerebellum (CB), amygdala (AM), prefrontal cortex (CX), hypothalamus (HT), mesencephalon (ME) and hippocampus (HC). In a previous study using C57BL/6J and A/J mouse inbred strains, we found that blood plasma and brain Mg levels were significantly correlated with several anxiety-related behavioral parameters in the mHB [21]. This behavioral test offers the possibility to control for possible confounding motivational factors on anxiety-related behavior, which is essential for the behavioral charactertization of a potential anxiolytic, since anxiety-related behavior can strongly be influenced by other behavioral dimensions like locomotor activity or exploratory motivation [22,23].

For many years it has been accepted that behavior and physiology are influenced by the light/dark cycle [24]. However, the time of day and light conditions of behavioral testing vary greatly between studies and are therefore influencing the behavioral results [25]. Mice are nocturnal animals and are in their most active phase in the dark. Roedel et al. [26] found when testing DBA/2N mice in both dark and light phase conditions that the animals in the light phase showed more behavioral inhibition and even cognitive disruption. Thus testing mice during their active phase is more suitable for behavioral studies [27]. Since all the reported *in vivo* Mg deficiency studies [10–13,15–17] have been performed under white light test conditions it is important to perform a Mg deficiency experiment in both red and white light conditions in order to identify a possible effect of light regime (all the while keeping the time-of-day of testing constant). Therefore in order to exclude any effect of the light regime on anxiety-related behavior, were the male mice in this study housed under either conventional (*i.e.* behaviorally tested in white light conditions) or reversed (*i.e.* behaviorally tested in red light conditions) light-dark cycle.

Finally, Guilloux et al. proposed the use of integrated behavioral *z*-scores as a sensitive and reliable method to present behavioral results in mice phenotyping [28]. This validated method can be used to combine data from different behavioral tests that study the same behavioral dimension (in this case anxiety-related behavior) and can potentially strengthen some susceptible behavioral parameters. In this study data from the mHB, LD and EPM was assessed using this method and results in an overall *z*-score describing anxiety-related behavior, exploration and locomotion across the three behavioral tests.

2. Materials and methods

2.1. Ethical statement

The protocol of the experiment was peer-reviewed by the scientific committee of the Department of Animals in Science and Society (Utrecht University, The Netherlands) and approved by the Ethics Committee for Animal Experiments of the University Medical Center Utrecht and Utrecht University, Utrecht-The Netherlands (approval number: 2010.I.11.241). Further, the animal experiment followed the Dutch 'Code on Laboratory Animal Care and Welfare'. The present animal study is reported in accordance with the ARRIVE guidelines (Animal Research: Reporting of *in vivo* Experiments: http://www.nc3rs.org.uk/arrive-guidelines).

2.2. Animals and housing

This study consists of n = 57 naïve male C57BL/6JOlaHsd (hereafter referred to as B6J) and n = 56 male C57BL/6NCrl (B6N) mice housed under a conventional light regime (lights on: 7:00AM–7:00PM). Additionally, a group of n = 47 naïve male B6J and n = 48 B6N was housed under a reversed light regime (lights on: 7:00PM–7:00AM). The SPF (Specified Pathogen Free) animals were obtained from Harlan Laboratories BV (Horst, The Netherlands; B6J) and Charles River Laboratories (Saint-Germain-sur-l'Arbresle, France; B6N). Upon arrival, the animals were 4–6 weeks of age and were housed individually in a wire topped Euro-standard Type II L cage (Tecniplast, Buguggiate, Italy) provided with bedding material, tissues and a shelter. The animals were housed under standard conditions and had *ad libitum* access to food and demineralized water to avoid consumption of additional Mg from normal drinking water. Drinking water was refreshed two times a week.

2.3. Experimental procedure

During the habituation period (day of arrival until day 0; Fig. 1) all mice were placed on the control diet (Diet# 98341, Harlan Teklad, Madison, WI, USA). The animals were handled for a few minutes four times a week by the person who also performed the behavioral tests. Handling included picking up the animal at the tail base, placing it on the arm and restraining it by hand for a few seconds.

One day prior to start of the experimental period (day −1) and on days 12 and 19 blood samples of all mice were taken by tail incision and were collected in pre-chilled lithium-heparin-coated microvette tubes (CB300, Sarstedt, Nümbrecht, Germany) in order to determine plasma Mg level. On day 0 the mice were divided into a control (conventional light regime: B6J n = 24, B6N n = 23; reverse light regime: B6J n = 23, B6N n = 24) and test group (conventional light regime: B6J and B6N n = 33; reverse light regime: B6J and B6N n = 24) with randomized cage position. The control animals remained on the control diet (containing 1.7 g of MgO/kg food; *i.e.* ≈0.1% w/w Mg^{2+}), whereas the test animals were transferred to a Mg deficient diet (Diet# 93106, 0.0015–0.003% w/w Mg^{2+}, Harlan Teklad) similar to Bardgett et al. [15]. We decided to choose a purified control diet that was prepared on the basis of formulations suggested by the American Institute of Nutrition [29], but that offers two times more Mg than the minimum dietary Mg requirements. In fact our Mg adequate diet had a dietary Mg concentration between that of Refs. [10,11,13] and Ref. [12]. The accompanying Mg deficient diet was prepared with sucrose instead of MgO. By using these two diets we meet a basic rule of experimental design: all variables should be controlled except that due to the treatment [30]. Mice were selected by a computerized randomization program that ensured that the distributions of blood plasma Mg level and body weight at day −1 for control and test group were comparable.

Behavioral experiments were performed on days 14 (mHB), 21 (LD) and 28 (EPM) between 10:00AM and 2:00PM throughout to minimize any effects of a circadian activity rhythm. The weight gain of the animals was monitored. Following the last behavioral test (EPM), the animals were euthanized by decapitation thirty minutes after the test. Trunk blood was collected in lithium-heparin coated tubes (Minicollect tubes, Greiner Bio-One GmbH, Kremsmünster, Austria). The plasma was collected in Micro tubes and stored at −20 °C for Mg and corticosterone determination. The brains of the animals were removed and were manually dissected into six parts containing the following core structures: cerebellum (CB), hippocampus (HC), prefrontal/medial cortex (CX), amygdala (AM), hypothalamus (HT), and mesencephalon (ME). Dissections were performed based on a rapid method described by Gispen et al. [31]. Dissected structures were weighed, kept in separate pre-chilled Microtubes and stored at −80 °C until analysis. A schematic overview of the experimental protocol is shown in Fig. 1.

2.4. Biochemical analyses

Mg and corticosterone levels in lithium-heparin plasma were determined as described in Laarakker et al. [21]. Magnesium in the brain structures was determined by atomic absorption spectrometry as described before [21]. Total brain Mg concentration in each mouse was calculated as the sum of the products of Mg concentration (in μmol/g dry weight) and dry weight per structure divided by the total dry weight of all structures.

2.5. Behavioral testing

2.5.1. mHB (day 14)

The mHB combines the features of an OF, a hole board test and a LD. A detailed description of the test set-up can be found in Labots et al. [32]. In the conventional light regime, a stage light above the mHB lit the board to induce a light intensity difference of about 300 lx between the protected area (150 lx, box) and the unprotected area (450 lx, board). The light intensity difference was about 115 lx in the reverse light regime, where the board was lit with 120 lx and the Box 1–5 lx. The animals were free to explore the arena for 5 min. Several parameters being indicative for anxiety-related behavior, exploration and locomotion were scored by a trained observer using the program Observer 5.0 (Noldus Information Technology, Wageningen, The Netherlands). The tests were also videotaped for raw data storage. An overview of the parameters can be found in Supplementary Table A.1. The apparatus was cleaned with water and tissues between every trial to avoid a bias based on mice olfactory cues.

2.5.2. LD (day 21)

The apparatus was a Plexiglas box consisting of a small (15 × 20 × 25 cm, l × w × h) dark compartment with black walls and black floor, connected by a transparent tunnel (5.5 × 6 × 9.5 cm, l × w × h) to a large (30 × 20 × 25 cm, l × w × h) light compartment with white walls and floor, brightly illuminated by white stage light (~650 lx). The dark compartment had a light intensity of ~5 lx under red light conditions and ~25 lx under white light conditions. The equipment is without a ceiling. In the experiment, mice were transferred from their home cage to the LD and were placed into the dark compartment facing away from the opening (tunnel). The mice were allowed to move freely between the two chambers for 5 min. Behavior was immediately scored by a trained observer using the program Observer 5.0. The tests were also videotaped for raw data storage. An overview of the parameters measured can be found in Supplementary Table A.1. The apparatus was cleaned between

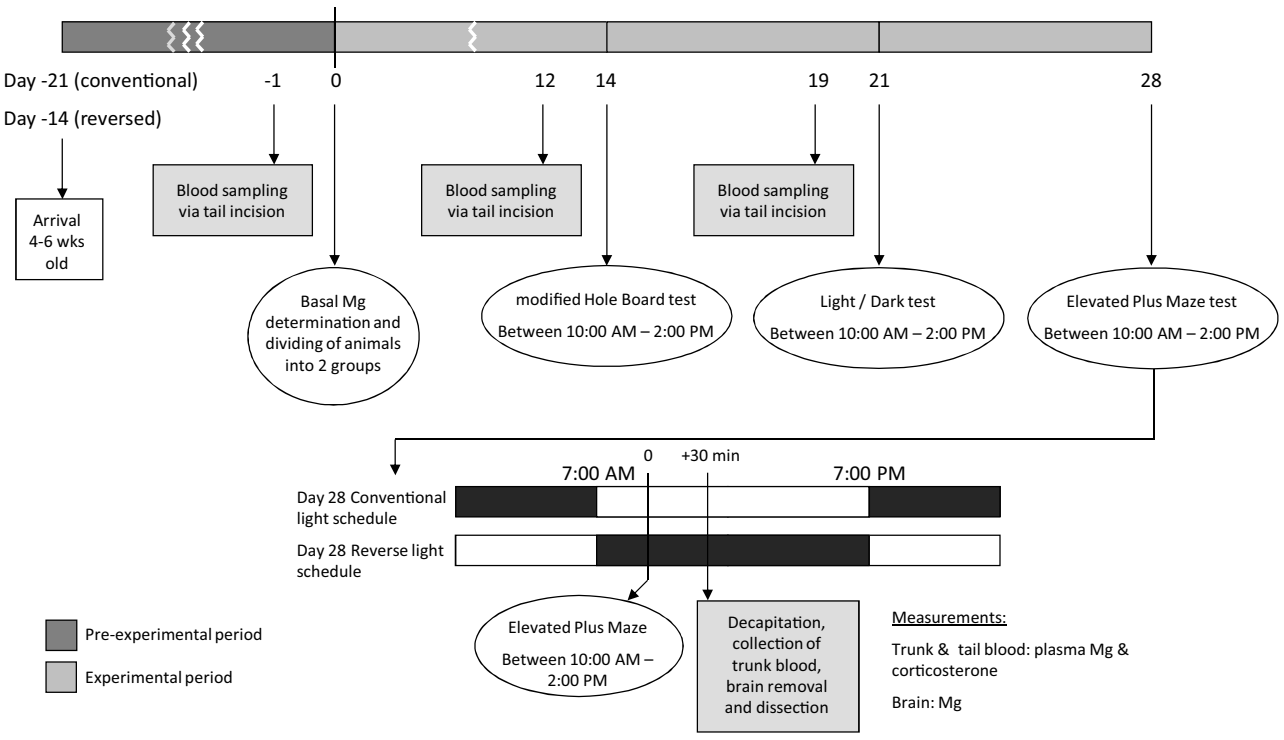

Fig. 1. Schematic overview of the experimental protocol. The animals housed under a conventional light regime underwent 21 days of habituation. The animals housed under reversed light regime underwent 14 days of habituation.

trials with tap water and tissues to avoid a bias on mice olfactory signals.

2.5.3. EPM (day 28)

The EPM was made of grey PVC and consisted of a central platform (6 × 6 cm), two open arms (28 × 6 cm) and two enclosed arms with side and end walls (28 × 6 × 16 cm). A stand base raised the whole apparatus 84 cm above the floor level. 75 lx illumination level by white light in the conventional light regime and 1–5 lx illumination level was provided by a red light for the reverse light condition on the EPM. Testing procedure was the same as described above for the LD test, except that mice were placed in the center of the EPM facing a closed arm as a starting position. When the animal placed all four paws within each arm or in the center the term "entry" was used. Several parameters were measured by the trained observer indicative for: avoidance behavior, risk assessment, locomotion, general exploration and arousal/de-arousal. Behavior was immediately scored by a trained observer using the program Observer 5.0. The tests were also videotaped for raw data storage. An overview of all EPM parameters can be found in Supplementary Table A.1. The apparatus was cleaned with water and tissues between every trial to avoid a bias based on mice olfactory cues.

2.6. Statistical analyses

All statistical analyses were carried out according to Field [33], using an IBM® SPSS® Statistics for Windows, version 22 (IBM Corp., Armonk, N.Y., USA) computer program, and paying attention to the assumptions that underlie the various statistical procedures. Two-sided, exact (*i.e.* for the nonparametric tests) probabilities were estimated throughout. The data were summarized by means and 95% confidence intervals (CI). To assess the consistency of behavioral performance over time and related assays and to obtain comprehensive and integrated measures in each group, the data for the different behavioral dimensions were normalized using a *z*-score methodology. Details and rationale have been described by Guilloux et al. [28]. However, for our data we used a slightly modified methodology: *i.e.* instead of normalizing to a comparison group we normalized to the pooled data (all the animals taken together). Thus, *z*-scores calculate how many standard deviations (σ) an observation (X) is above or below the mean of the pooled data (μ): $z = (X - \mu)/\sigma$; X represents the individual data for the observed parameter; μ and σ represent the mean and the standard deviation for the pooled data of that observed parameter, respectively. First we calculated *z*-scores for each behavior test measure. The directionality of scores was adjusted so that increased score values reflected increased values for that behavioral dimension. Table A.1 gives an overview of the different behavioral parameters per behavioral test. Second, each individual's *z*-score was calculated within behavioral tests (mHB, LD, or EPM) by averaging the *z*-scores of the behavior test measures. Third, an overall *z*-score was obtained for each animal by averaging values across behavioral tests.

The Kolmogorov-Smirnov one-sample test was used to check Gaussianity of the data. This was done per experimental group and led to the conclusion that several parameters were not normally distributed. All experimental groups of these non-normal distributed parameters were rank-transformed. Brain structure and blood plasma Mg data were tested for significant differences by repeated measures analysis of variance (ANOVA) with main between-subject factors *substrain*, *dietary treatment* and *light regime* and main within-subject factor *brain structure* (brain structure data) or *time* (blood plasma Mg data). The choice of a multivariate instead of a univariate statistic in the repeated measures ANOVA was based on the criteria given by Algina and Keselman [34]. In case of multivariate repeated measures ANOVA, tests of significance were derived using the Wilk's λ criterion. In case of univariate repeated measures ANOVA violations to sphericity were addressed with a Huynh-Feldt correction to degrees of freedom. Body weight, total brain Mg concentration and blood plasma corticosterone level were compared with a three-way ANOVA with main factors *substrain*, *dietary treatment* and *light regime*. Since the difference in body weight gain between the dietary groups can

have an effect on behavior, an analysis of covariance (ANCOVA) was performed with *substrain, dietary treatment* and *light regime* as main factors and *body weight gain* as covariate for the integrated behavioral z-scores. For all ANOVA's/ANCOVA's, homoscedasticity was tested by the Levene's test, which is a powerful and robust test based on the *F* statistic. The variances should be similar and the within-group data should be normally distributed. If it was not possible to fulfill these criteria, the parameter in question was rank-transformed. *Post hoc* comparisons for normally distributed data were performed with the paired (within-subject *post hoc* comparisons) or unpaired Student's *t* test (between-subject *post hoc* comparisons). The unpaired Student's *t* tests were performed using pooled (for equal variances) or separate (for unequal variances) variance estimates. The equality of variances was tested with the Levene's test. For the unpaired Student's *t* test with separate variance estimates, IBM® SPSS® Statistics uses the Welch-Satterthwaite correction. *Post hoc* comparisons for non-normally distributed data were performed with the Wilcoxon matched-pairs signed ranks test (within-subject *post hoc* comparisons) or Wilcoxon-Mann-Whitney test (between-subject *post hoc* comparisons). *Post hoc* comparisons for the behavioral data were performed with an ANCOVA between the different groups.

To estimate the relative magnitude of the differences between the control and Mg deficient diets, Cohen's *d* effect size coefficients may be used. The Cohen's *d* score is here defined as the difference between the means of a variable between the test (Mg deficient) and control (Mg adequate) mice divided by the pooled SD. Between the Cohen's *d* score for the LD-parameter *'latency until the first entrance to the light compartment'* and *'difference in dietary Mg concentration between control and test diet'* or *'day of behavioral testing'*, Spearman coefficient of rank correlation (R_S) was calculated; significance was assessed by a two-tailed test based on the *t* statistic. Selected associations (Spearman's R_S) were also calculated between integrated behavioral z-scores, blood plasma Mg and corticosterone levels, and brain (structure) Mg concentration.

To take the greater probability of a Type I error due to multiple hypotheses into account, a more stringent criterion should be used for statistical significance (*i.e.* for the ANCOVAs between two groups, paired and unpaired Student's *t* tests, Wilcoxon matched pairs signed ranks tests and Wilcoxon-Mann-Whitney tests). We approached this problem by calculating a so-called Dunn-Šidák correction ($\alpha = 1 - [1 - 0.05]^{1/\gamma}$; γ = times a group is used in a comparison). Calculating numerous correlations also increases the risk of a Type I error. To reduce this, the level of statistical significance of Spearman correlation coefficients were adjusted by using also the Dunn-Šidák method ($\alpha = 1 - [1 - 0.05]^{1/15} \approx 0.003414$; 15 = total number of parameters used for correlations). In all other cases – including the correlation between the *Cohen's d score for the LD-parameter 'latency until the first entrance to the light compartment'* and *'difference in dietary Mg concentration between control and test diet'* or *'day of behavioral testing'* – the probability of a Type I error < 0.05 was taken as the criterion of significance.

3. Results

3.1. Mortality

No mortality had occurred in this study until day 19. Six animals (B6J test: n = 1, B6N test: n = 5) housed under the conventional light regime died before LD testing and nine animals (B6J test: n = 3 and B6N test: n = 6) before EPM testing (after LD testing). Additionally, one animal (B6N test) under the conventional light regime showed epileptic-like seizures and could not perform in the LD experiment. Two animals (B6J test) housed under the reversed light scheme died before EPM testing (after LD testing). Some of the animals were found dead in their cages without any clinical indication, but others showed lethal tonic-clonic seizures. No mortality was observed in the control groups.

3.2. Body weight

3.2.1. Conventional light regime

During the habituation period and a part of the experimental period (until day 12) body weight of control and test animals increased in an identical fashion for both B6J and B6N (data not shown). On the last day of testing, the Mg deficient animals weighed 14.5% and 19.5% less for B6J and B6N respectively compared to the control animals (B6J: 22.4 g 95%CI [21.9–22.9], n = 32 *vs.* 26.2 g 95%CI [25.6–26.8], n = 24; $P < 0.0000005$ unpaired Student's *t* test and B6N: 24.4 g 95%CI [23.5–25.2], n = 22 *vs.* 30.3 g 95%CI [28.6–32.0], n = 23; $P < 0.0000005$ unpaired Student's *t* test with Welch-Satterthwaite correction).

3.2.2. Reverse light regime

During the habituation period and a large part of the experimental period (until day 21 for B6J and day 28 for B6N) body weight of control and test animals increased in an identical fashion for both B6J and B6N (data not shown). On the last day of testing, the Mg deficient animals weighed 13.2% and 8.2% less for B6J and B6N respectively compared to the control animals (B6J: 27.0 g 95%CI [25.9–28.1], n = 23 *vs.* 31.1 g 95%CI [30.1–32.0], n = 23; $P = 0.000001$ unpaired Student's *t* test and B6N: 27.9 g 95%CI [27.1–28.7], n = 24 *vs.* 30.4 g 95%CI [29.2–31.6], n = 24; $P = 0.000851$ unpaired Student's *t* test with Welch-Satterthwaite correction). Thus, as a consequence, body weight gain was significantly influenced by the dietary factor.

3.2.3. Conventional vs. reverse light regime

Panels A and B from Fig. 2 summarize the initial body weights (day 0) and weight gains over the 28 experimental days, respectively. The initial weights were different between substrains and light regimes (three-way ANOVA, Supplementary Table A.2). In addition, the weight gain was influenced by the Mg deficient diet. The animals on this diet had a significant lower weight gain compared to controls in both substrains and under both light regimes. However, the diet effect was more pronounced with the conventional than the reverse light regime (Supplementary Tables A.2 and A.3).

3.3. Physiological parameters

3.3.1. Brain wet weight

Under both light regimes, control and test groups demonstrated similar absolute brain wet weights in both B6J and B6N (Fig. 2, panel C; Supplementary Tables A.2 and A.3). In contrast, relative brain wet weights of Mg deficient mice were significantly higher. This was most likely caused by the significantly lower body weight of the test animals at the end of the experiment. Comparing the two light regimes showed a higher absolute and relative brain wet weight for the B6N animals housed under a reversed light regime (Fig. 2, panels C and D).

3.3.2. Brain Mg level

In the post hoc comparisons the concentration of Mg in the total brain was not significantly influenced by the diet in both B6J and B6N housed under conventional or reverse light regime, but the ANOVA revealed a dietary treatment effect (Fig. 3; Supplementary Tables A.4 and A.5). When housed under a reverse light regime, the B6N had a higher total brain Mg than the B6J mice. Brain Mg showed a structure dependent and light regime dependent distribution (Fig. 3). Under a reverse light regime Mg level in CB (B6N

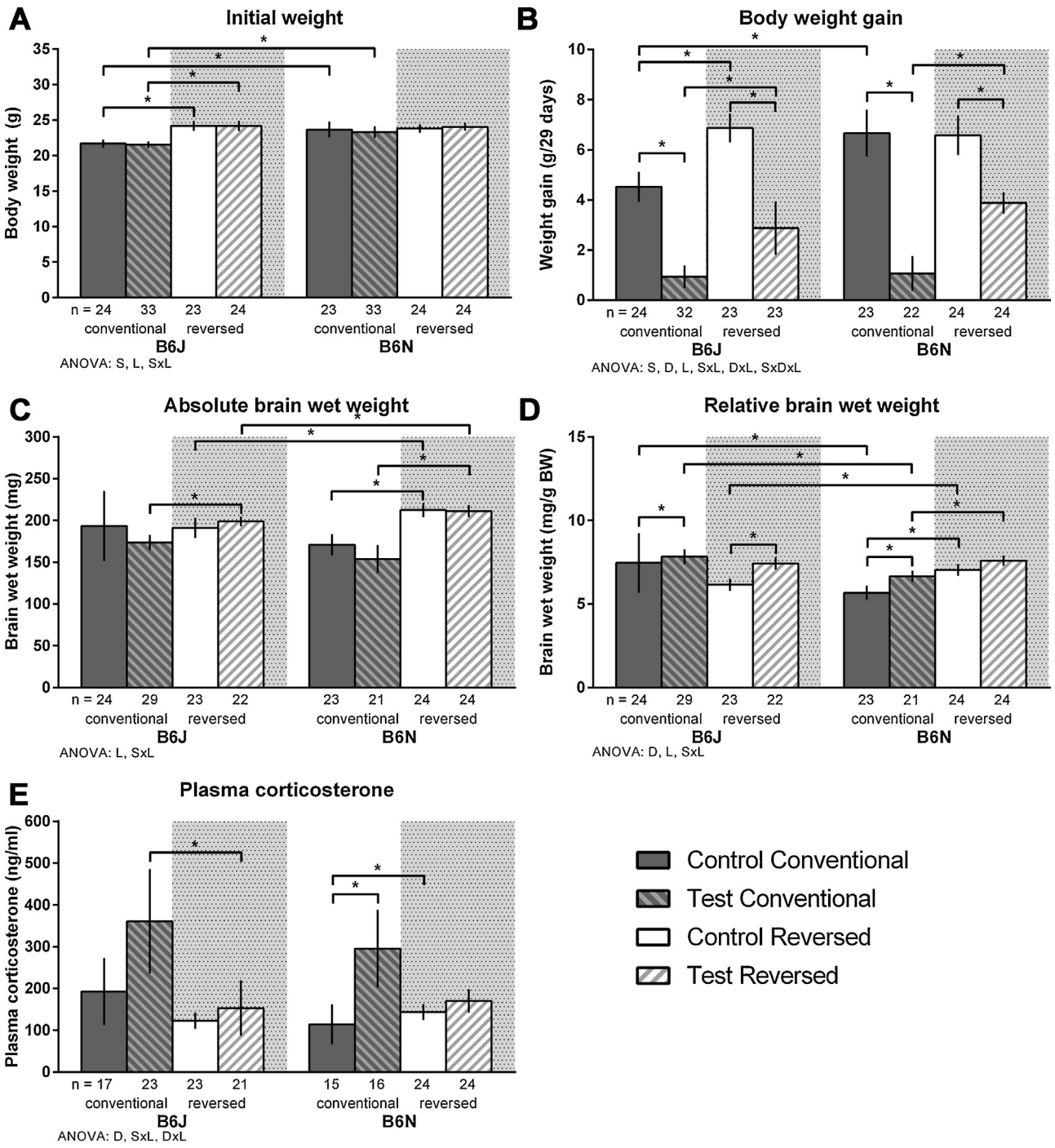

Fig. 2. Body weight, brain wet weight and plasma corticosterone of male B6J and B6N mice on either Mg normal (control) or Mg deficient (test) diet housed under conventional or reversed light regime. Results are presented as means with 95% CI. Bars in white area = conventional light regime, bars in grey area = reverse light regime. Effects were significant in the ANOVA when $P < 0.05$. S indicates significant effect of C57BL/6 substrain; D, significant effect of dietary treatment; L, significant effect of light regime; SxL, DxL and SxDxL, significant interaction. * = Significant difference ($P < 0.016952$) in *post hoc* comparison.

only), HC (all groups), CX (B6J control and B6N) and HT (all groups) were higher when compared to the conventional light regime counterparts. The opposite became evident in the AM (B6J only), where the Mg level was lower in the reversed light regime. Total brain Mg was significantly higher under a reverse compared to a conventional light regime. Additionally, the repeated measures ANOVA revealed a dietary treatment effect, albeit that this Mg effect was only significant in the HT and ME for the B6J mice housed under conventional lighting and AM in B6J under reversed light regime in the *post hoc* comparisons.

3.3.3. *Plasma parameters*

The effect of the Mg deficient diet fed for 28 days on the plasma Mg concentration is shown in Fig. 4. There were significant dietary treatment, time and dietary treatment by time interaction effects (Supplementary Table A.6). After twelve days (*i.e.* before the first behavioral test) the Mg concentration in blood plasma was on average significantly decreased by the Mg deficient diet to approximately 34% (B6J-conventional; Fig. 4, panel A), 29% (B6N-conventional; Fig. 4, panel B), 26% (B6J-reverse; Fig. 4, panel C) and 31% (B6N-reverse; Fig. 4, panel D) of control levels.

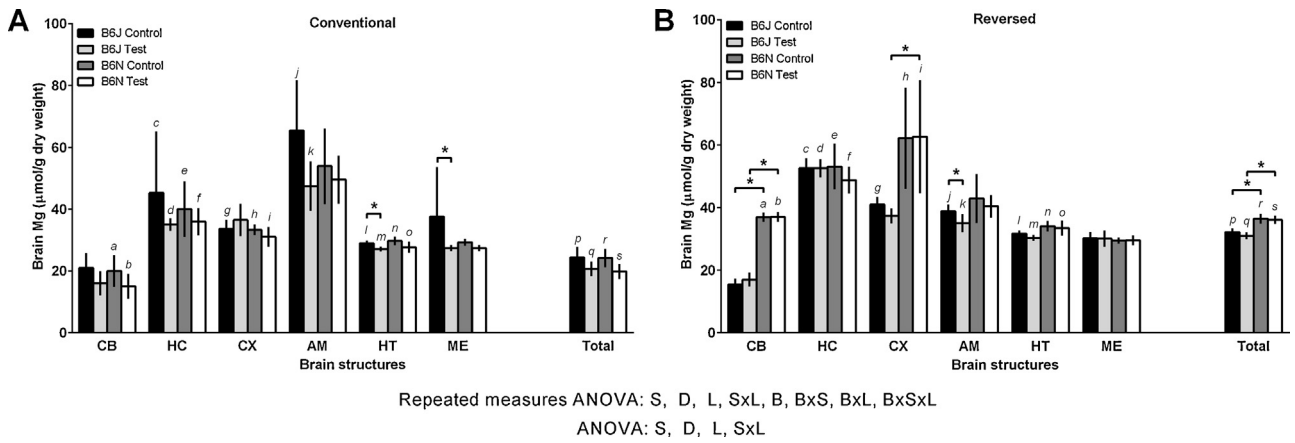

Fig. 3. Brain Mg concentration in male B6J and B6N mice on either Mg normal (control) or Mg deficient (test) diet housed under conventional (panel A) or reversed (panel B) light regime. Results are presented as means with 95% CI. Values based on conventional regime B6J control: n = 20, test n = 23, B6N control: n = 19, test n = 17; reverse light regime B6J control: n = 18, test n = 17, B6N control n = 24, test n = 24. Effects were significant in the (repeated measures) ANOVA when $P < 0.05$. S indicates significant effect of C57BL/6 substrain (brain structures and total brain); D, significant effect of dietary treatment (brain structures and total brain); L, significant effect of light regime (brain structures and total brain); SxL, significant interaction (brain structures and total brain); B, significant effect of brain structure (brain structures); BxS (brain structures) and BxSxL (brain structures), significant interaction. * = Significant difference ($P < 0.006391$) in *post hoc* comparison. Groups under a conventional light regime (panel A) and their counterparts under a reverse light regime (panel B) bearing the same lowercase letter are significantly different ($P < 0.006391$) in between-subject *post hoc* comparison. Within-subject *post hoc* comparisons (within brain comparison, between brain structure comparison) are presented in Supplementary Table A.5.

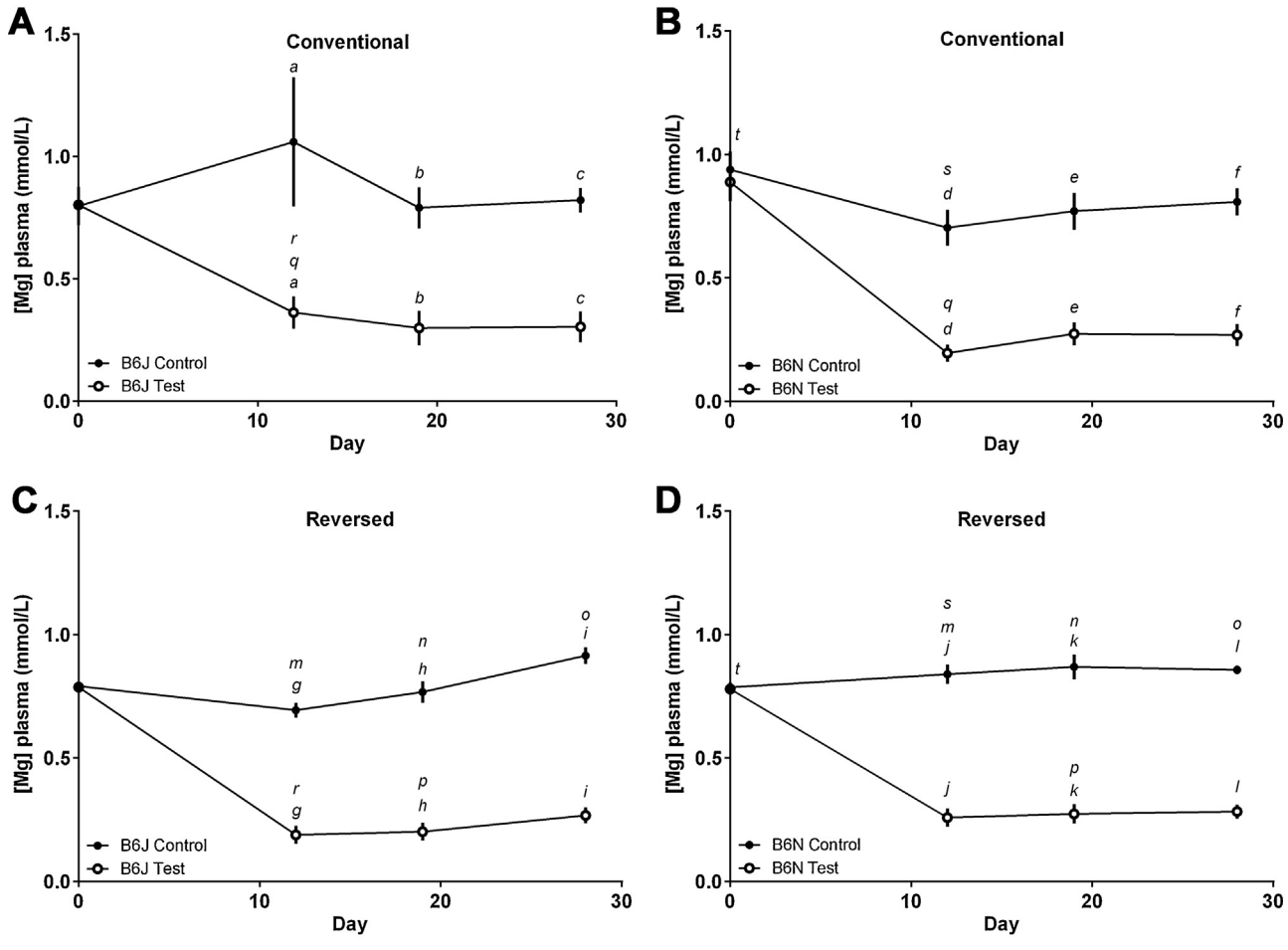

Fig. 4. Plasma Mg concentration over time in male B6J and B6N mice on either normal (control) or Mg deficient (test) diet housed under conventional or reversed light regime. Results are presented as means with 95% CI. Values based on conventional regime B6J control: n = 20, test n = 28, B6N control: n = 21, test n = 18, reverse light regime B6J control: n = 21, test n = 19, B6N control n = 24, test n = 24. Effects were significant in the repeated measures ANOVA when $P < 0.05$. D indicates significant effect of dietary treatment; L, significant effect of light regime; T, significant effect of time; TxS, TxD, TxL and TxSxL, significant interaction. Groups bearing the same lowercase letter are significantly different ($P < 0.007301$) in between-subject *post hoc* comparison. Within-subject *post hoc* comparisons (between day comparison) are presented in Supplementary Table A.6.

The endocrine stress response was evaluated by measurement of the plasma corticosterone level on the last day of the experiment. The data is presented in panel E from Fig. 2. For *P* values see Supplementary Tables A.2 and A.3. The animals housed under the conventional light regime on the Mg deficient diet had a higher corticosterone concentration in their plasma compared to the control counterparts, albeit that this treatment effect was only significant in the B6N mice. The effect of light regime was clearly reflected in the corticosterone levels in the test animals under both light regimes. The test animals under conventional light regime had a higher corticosterone level in their plasma compared to the test animals under reversed light regime. The control animals showed no difference between light regimes.

3.4. Integrated behavioral z-scores

The (adjusted) results for overall integrated behavioral *z*-scores for anxiety-related behavior, exploration and locomotion averaged from the behavioral tests are summarized in Fig. 5. Regarding anxiety-related behaviors (avoidance, risk assessment and arousal) there were substrain, light regime effects and dietary treatment effects. Table A.7 shows the adjusted and unadjusted means with the 95% CI. Tables A.8 and A.9 provide an overview of the results (*P* values) from the three-way ANCOVA (with main factors substrain, light regime and dietary treatment and covariate body weight gain) and *post hoc* comparisons. For avoidance, risk assessment, arousal and exploration there was a C57BL/6 substrain effect. Except for avoidance there was a light regime and substrain by light regime interaction effect for each behavioral dimension. B6N control animals showed a higher locomotion in the reversed light regime compared to their counterparts under the conventional light regime (Fig. 5, panel F). An overall diet effect was found for avoidance behavior (Fig. 5, panel B) and exploration (Fig. 5, panel E); there was a substrain by dietary treatment by light regime interaction for arousal (Fig. 5, panel D) and exploration (Fig. 5, panel E). In the *post hoc* comparisons, the diet effect in exploration behavior was evident in B6J mice housed under a conventional or a reverse light regime.

3.5. Correlations

For individual mice the associations between the integrated behavioral z-scores and Mg levels in blood plasma, total brain and the different brain structures were studied; as well as correlations between the z-scores and blood plasma corticosterone concentration. In addition the relationship was studied between plasma Mg or corticosterone level and Mg concentration in total brain and the different brain structures (Table 1). There were no significant correlations between the two blood plasma parameters and the integrated behavioral z-scores. Except for ME, there were significant correlations between brain Mg concentration and one or more integrated behavioral z-scores. The strongest correlations were found between anxiety and CB-Mg, avoidance and total brain-Mg, risk assessment and HC-Mg, arousal and HC-Mg, exploration and total brain-Mg, locomotion and CB-Mg. Blood plasma Mg and corticosterone levels were significantly negatively correlated.

4. Discussion

In order to explore potential causes for the inconsistencies regarding the role of Mg in anxiety-related research in mice, this experiment compared the animals in two different light regimes, induced Mg deficiency in a similar fashion as others did via the diet [15], and compared two substrains of the C57BL/6 mouse. As a result, a clear hypomagnesaemia was established in animals exposed to the Mg deficient diet from day 12 onwards (Fig. 4). Both basal levels and time course of plasma Mg of control and test C57BL/6 mice, respectively, were comparable to that reported by other researchers [10,15,35].

A number of animals on the Mg deficient diet died in the present study, showing signs of epilepsy-like seizures. Notably, it has been reported that Mg deficiency can be associated with sudden death in mice [36]. Also, from the first half of the previous century on, mice on a Mg deficient diet were described to show tremors, hyperreactivity, myoclonic spasms and tonic clonic seizures within two weeks of the start of administration [37]. Since then, Mg deficiency in mice was used as a model to study (audiogenic-) seizures [38]. More recent studies describing anxiety-related behavior in Mg deficient mice do not mention any mortality observed in their studies, except for one study: Singewald et al. [10] not only mentioned mortality, but signs of severe hypomagnesaemia and, therefore, induced a milder Mg deficiency (*i.e.* 45% reduction) as compared to Bardgett et al. [15] (76% reduction), Muroyama et al. [12] (65% after 21 days) and the current study (70–74%). The Mg content of the Mg adequate and Mg deficient diet of the different studies varies between 0.05–0.24% and 0.0015–0.005%, respectively (Table 2). This might give an explanation as to the inconsistent results found between the studies. Fig. 6 shows the results (*i.e.* the quantified relative difference, Cohen's *d*, between mice on a Mg deficient and Mg adequate diet) for the LD-parameter *'latency until the first entrance to the light compartment'* for the various studies; this parameter is independent from the duration of the behavioral test. However, this figure suggests that there is neither a clear relationship of *'the difference in dietary Mg concentration between control and test diet'* and the Cohen's *d* ($R_S = -0.173$, $P = 0.656$, $n = 9$) nor between *'day of behavioral testing'* and the Cohen's *d* ($R_S = 0.136$, $P = 0.728$, $n = 9$).

Interestingly, mortality in the present study mostly occurred in animals housed under a conventional light regime (23% vs. 4% mortality, Section 3.1). Further, corticosterone levels in the final blood sample revealed a treatment effect in animals housed under conventional light regime in that mice on a Mg deficient diet showed an increased corticosterone level as compared to control animals (Fig. 2, panel E). Since the light phase is the resting phase for mice, it may be suggested that Mg-deficient mice were especially sensitive to disruptions of their resting phase by behavioral testing.

Furthermore, Mg deficient animals had a lower body weight at the end of the experiment, which has also been described previously [10,12,16,35]. The weight gain reduction in the Mg deficient group (Fig. 2, panel B) may be due to suppressed appetite [39], a change in gut microbiota [16,40] and/or altered energy metabolism [41]. There also was a difference in weight gain between the two light regimes, in that animals gained less weight when housed under the conventional light regime (Fig. 2, panel B). This observation underlines the hypothesis that disruption of the animals' resting phase may have made them more receptive to the effects of Mg deficiency. Since the difference in weight gain due to the dietary treatment has been established and might have an influence on behavioral outcome, was weight gain used in the inferential statistical analysis as a covariate. In order to rule out this effect, future studies might make use of pair-fed controls [30,42]. Also, re-analysis of older studies using this covariate might give a better insight on the inconsistency in results.

In corroboration with Belknap et al. [43] we found that Mg deficiency had no effect on absolute total brain wet weight (Fig. 2, panel C). Further, in the present study, control and test mice had similar total brain Mg levels (Fig. 3), a finding that has been reported before for C57BL/6J mice [44]. Belknap et al. [43] and Chollet et al. [18] noted that Mg deficient C57BL/6J mice showed significantly lower total brain Mg concentrations as compared to control animals. The lack of effect of Mg deficiency on total brain Mg levels in our study might be due to the genetic background of the C57BL/6 substrains used: as described before for C57BL/6J mice [18], central Mg has

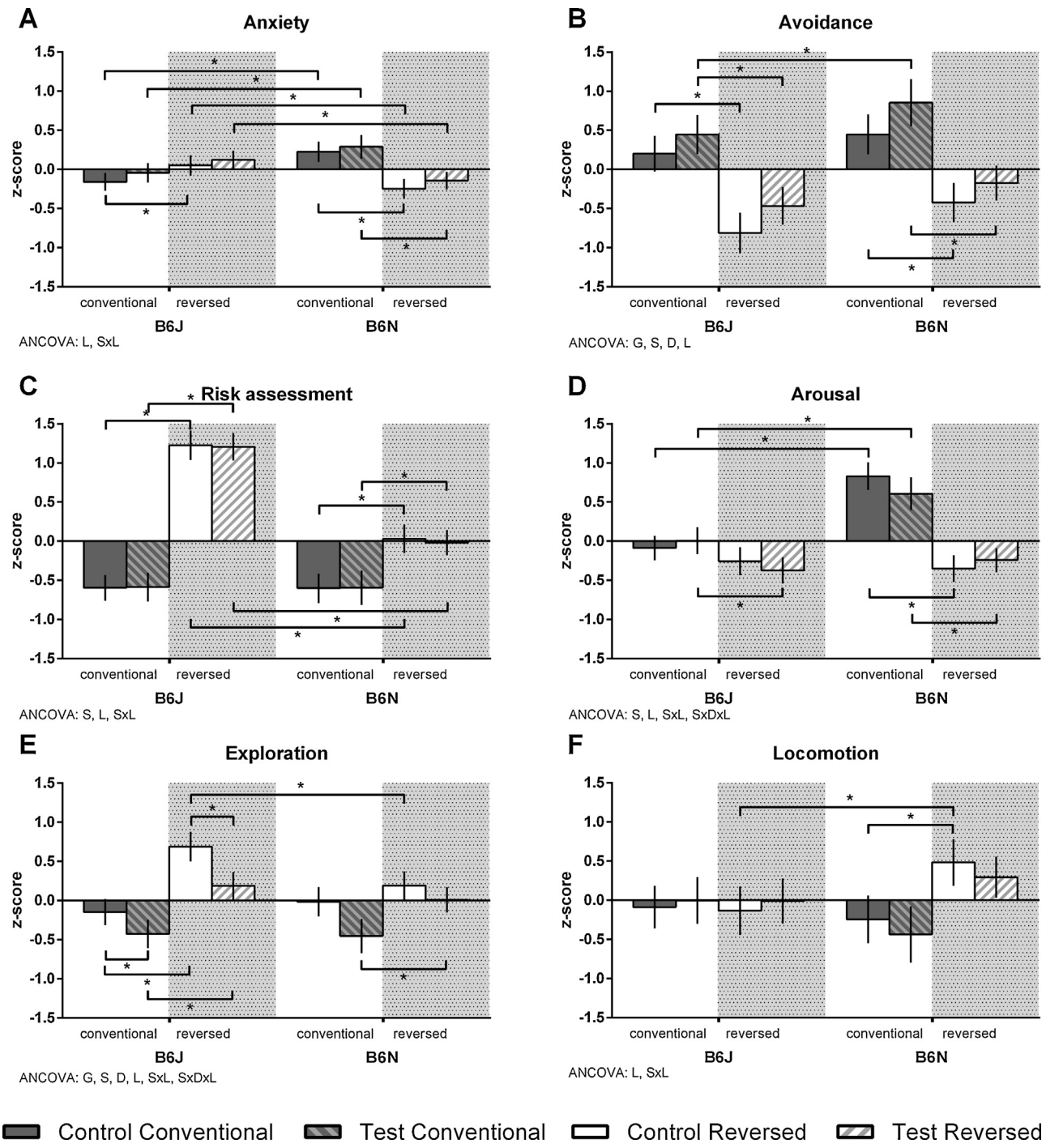

Fig. 5. Integrated behavioral z-scores for male B6J and B6N mice on either normal (control) or Mg deficient (test) diet housed under conventional or reversed light regime. Results are presented as means with 95% CI. Values based on conventional regime B6J control: n = 23, test n = 29, B6N control: n = 23, test n = 18, reverse light regime B6J control: n = 23, test n = 22, B6N control n = 24, test n = 24. Effects were significant in the ANCOVA when $P < 0.05$. G indicates significant effect of the covariate body weight gain, S, significant effect of C57BL/6 substrain; D, significant effect of dietary treatment; L, significant effect of light regime; SxL and SxDxL, significant interaction. * = Significant difference ($P < 0.016952$) in *post hoc* comparison.

also been found to display a highly structure-specific distribution in the B6J and B6N substrains. Moreover, we found evidence that this distribution is substrain specific (Fig. 3).

Mg deficient B6N mice, but not B6J animals, under a conventional light regime showed significantly increased plasma corticosterone levels compared with their counterparts fed a Mg adequate diet (Fig. 2, panel E). In contrast, Singewald et al. [10] found that plasma corticosterone levels in male B6N mice – also under a conventional light regime – did not differ significantly between control and test groups. There also was a significant, negative association between plasma Mg and corticosterone concentration (Table 1). However, since there were no significant associations between the integrated behavioral z-scores or central Mg content and blood plasma corticosterone level (Table 1), we feel that plasma corticosterone is not a valuable biomarker for Mg depleted induced changes. Since exogenous administration of corticosterone has been found to influence behavior in C57BL/6 mice [45,46] we feel that corticosterone is a media-

Table 1
Selected associations (Spearman's R_S) between integrated z-score mHB parameters, blood plasma Mg and corticosterone levels, and brain Mg concentration.

Integrated behavioral *z*-score/Blood plasma parameter	Brain Mg							Blood plasma	
	Brain structure								
	CB	HC	CX	AM	HT	ME	Total	corticosterone	Mg
Anxiety	***−0.290***	−0.046	−0.143	−0.046	−0.128	−0.171	***−0.256***	−0.153	−0.015
Avoidance	***−0.319***	***−0.423***	***−0.271***	0.204	***−0.271***	−0.145	***−0.465***	−0.007	−0.030
Risk assessment	0.211	***0.618***	***0.412***	***−0.322***	***0.371***	0.047	***0.444***	−0.207	0.135
Arousal	***−0.227***	***−0.409***	***−0.379***	0.175	***−0.313***	−0.069	***−0.348***	0.148	−0.106
Exploration	***0.333***	***0.408***	***0.220***	−0.105	***0.242***	0.184	***0.475***	−0.037	0.056
Locomotion	***0.287***	0.117	0.160	−0.159	0.137	0.062	***0.257***	−0.004	−0.059
Plasma Mg	−0.012	0.088	0.156	0.096	***0.296***	***0.224***	0.111	***−0.321***	–
Plasma corticosterone	0.166	−0.177	−0.064	−0.042	−0.282	−0.121	−0.007	–	–

Association based on 153–189 animals. Significant ($P < 0.003414$) Spearman's R_S are indicated in bolditalic characters. Blood plasma samples were obtained from trunk blood (day 28).

Table 2
Dietary magnesium concentration of the experimental diets used by different research groups.

Reference	Experimental diet (Mg content)		Name of the diet (supplier)	Dietary Mg concentration
Singewald et al. [10] & Sartori et al. [11,13]	Control	(High Mg)	ssniff® EF R/M Control (*Ssniff Spezialdiäten GmbH, Soest, Germany*)	≈0.21% Mg
	Test	(Low Mg)	ssniff® EF R/M Magnesium deficient diet (*Ssniff Spezialdiäten GmbH, Soest, Germany*)	≈0.005% Mg
Muroyama et al. [12]	Control	(Normal Mg)[a]	AIN-93G (*Oriental Yeast Co., Ltd., Tokyo, Japan*)	≈0.05% Mg
	Test	(Low Mg)	AIN-93G, but in mineral mix sucrose instead of magnesium oxide (*Oriental Yeast Co., Ltd., Tokyo, Japan*)	≈0.004% Mg
Bardgett et al. [15] & [This study]	Control	(Moderate Mg)	TD.98341 Magnesium Control Diet	≈0.1% Mg
	Test	(Low Mg)	TD.93106 Magnesium Deficient Diet (*Harlan Teklad, Madison, WI, USA*)	≈0.0015–0.003% Mg
Winther et al. [16] & Pyndt Jørgensen et al. [17]	Control	(High Mg)	Altromin Standard diet 1324 rats/mice—maintenance diet (*Altromin Spezialfutter GmbH & Co. KG, Lage, Germany*)	≈0.24% Mg
	Test	(Low Mg)	ssniff® EF R/M Magnesium deficient diet (*Ssniff Spezialdiäten GmbH, Soest, Germany*)	≈0.005% Mg

[a] Normal Mg content = recommended minimum requirement for dietary Mg [29].

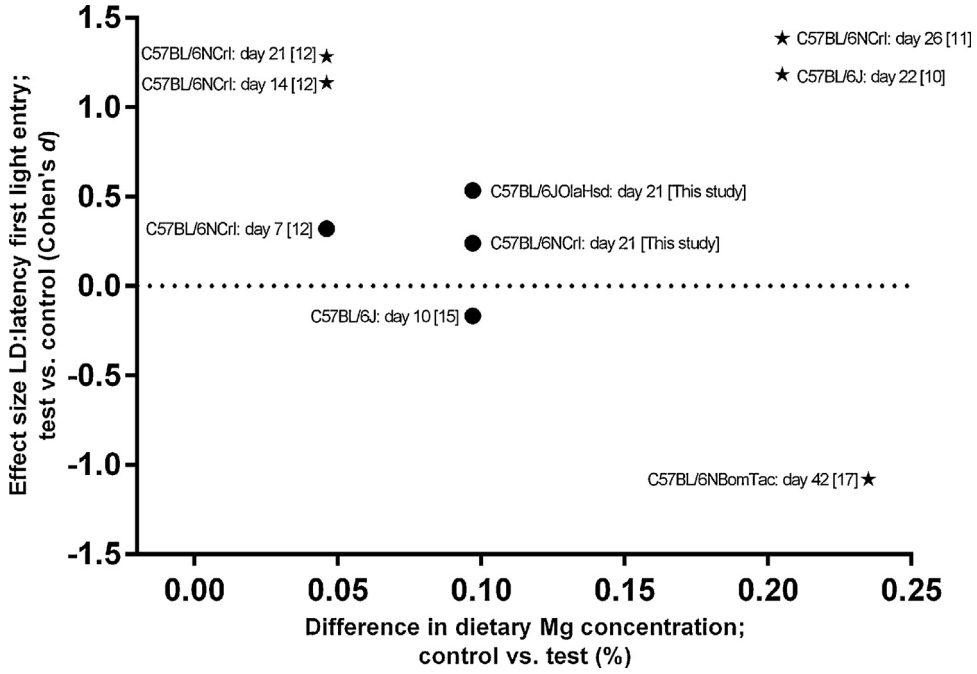

Fig. 6. Association between Cohen's *d* for avoidance in LD and difference in dietary Mg concentration between control and test group. The Cohen's *d* score is here defined as the difference between the means of *'latency until the first light compartment entry'* in LD for test (on a Mg deficient diet) and control (on a Mg adequate diet) C57BL/6 mice divided by the pooled SD. All mice were under a conventional light regime. The age of the animals at behavioral testing as well as the official substrain name and reference number are indicated. ★ = Significant difference between test and control group ($P < \alpha$); ● = non-significant difference between test and control group($P \geq \alpha$).

tor of behavioral effects. A close association of Mg with the renin-angiotensin-aldosterone system has been described [47,48] and Franklin et al. [49] suggest that plasma aldosterone levels or the plasma aldosterone to corticosterone ratio may represent an early indicator of the development of depression. Maybe plasma aldosterone could also serve as an early indicator for the development of anxiety-related behavior. Thus in future studies aldosterone should also be determined besides blood plasma corticosterone.

While in the literature anxiety-modulating mechanisms of magnesium has been hypothesized [5,8–12,50], our present results designated that a Mg deficient diet did affect avoidance behavior in mice when compared to a non-Mg deficient diet (Fig. 5, panel B), but not overall anxiety-related behavior (Fig. 5, panel A). We do consider the choice of mouse strains or general experimental design unlikely to be causal for this lack of treatment effect since both substrains responded in a similar fashion to the dietary treatment. Additionally, for our study we chose C57BL/6 mice (*i.e.* mice from the substrains C57BL/6JOlaHsd [B6J] and C57BL/6NCrl [B6N], respectively) as animal model, because they have been reported to belong to the fourth quartile ('low anxious') of the phenotypic anxiety spectrum [21] and have been used in previous Mg deficiency studies on anxiety-related behavior of mice [10–12,15]. Regarding the behavioral testing regime, the tests were conducted starting with the least invasive one, followed by one week rest between experiments in order to avoid any 'carry over effects' or have altered the results of subsequent testing [51].

The lack of alteration in other anxiety-related parameters in behavioral tests, however, is conceivably not accidental since the results for anxiety-related parameters of the three tests are in line with each other (data not shown). In this study the data is represented as integrated behavioral z-scores, combining parameters from behavioral dimensions over three different tests. For possible confounding factors concerning the inconsistency between the effect of Mg deficiency on anxiety-related behavior, we can now exclude the effect of the light regime. Even though this study shows large effects of light regime on anxiety-related behavior, exploration, locomotion and even weight gain (Figs. 2 and 5), the behavioral effect of diet was observed only in avoidance and exploration. Similarly, the two substrains gave different results for these behaviors, weight gain and Mg brain levels, but both responded – with the exception of avoidance and exploration – to the dietary treatment in a comparable fashion.

For the present study the effects of light regime are a combination of circadian, sleep-wake related, and illumination level (in combination with type of light: white or red) effects. The pure effects of these factors on behavior of C57BL/6 mice in anxiety paradigms have been studied before. Both circadian phase and behavioral test illumination effects have been studied for example by Post et al. [52] regarding OF and EPM behavior of C57BL/6 and BALB/c mice. Whether the mouse is awake or asleep when removed from the home cage for (behavioral) testing can influence the test results. Several studies have been performed to study the effects of sleep fragmentation or disruption. *E.g.* Ramesh et al. [53] described that male C57BL/6 mice exposed to sleep fragmentation were more anxious in the EPM than their counterparts having control sleep conditions. Interestingly, Chollet et al. [18,44] described a relationship between central Mg level and sleep quality. We found that the light regime has an effect on central Mg content *i.e.* on total brain-, HC-, CX-, AM- and ME-Mg level (Fig. 3).

Having ruled out substrain and light regime as possible confounding factors, the influence of body weight gain and two other possibilities explaining the inconsistencies remain: apparatus design and/or the microbiotic status of the animals. Most studies looking into Mg deficiency on behavior, performed the LD experiment [10–12,15]. However, there is no consensus concerning the apparatus design. For instance the size of the boxes varies between research groups [54]. In our study and Bardgett et al. [15] the dark versus light compartment ratio was 1:2, whereas in Singewald et al. [10], Muroyama et al. [12] and Sartori et al. [11] this ratio was 1:1. Kulesskaya and Voikar [55] showed that the difference in equipment and procedure indeed influences the approach-avoidance behavior of the animals. In the same line, it is not unlikely that these differences in apparatus dimensions influenced the LD data. Similarly, effects of apparatus design for the EPM have been described [56].

Strong pre-clinical evidence (reviewed in *e.g.* Refs. [57–59]) suggested that gut microbiota have an important role in bidirectional interactions between the gut and the nervous system and may in turn influence behaviors including anxiety. Diet is one of the most important factors shaping the microbial diversity of the gut [59]. For instance dietary magnesium deficiency affects gut microbiota composition in C57BL/6 mice [16,17,35], especially the concentration of bifidobacteria in the gut was affected [35]. Savignac et al. [60] reported that these bacteria influence anxiety-related behavior in male BALB/c mice. Sartori et al. [11], Muroyama et al. [12] and we [this study] tested male SPF mice of the B6N inbred strain and obtained these mice from Charles River Laboratories. Although the vendor was the same, the breeding facilities from where the mice have been obtained were not: Sartori et al. [11] Sulzfeld (Germany), Muroyama et al. [12] Atsugi (Japan), Labots et al. [this study] Saint-Germain-sur-l'Arbresle (France). As a consequence the starting (*i.e.* alpha) microbiota diversity will differ between the three sources of B6N mice and due to environmental factors the (beta) microbiota diversity will also differ between the three studies, which may result in non-reproducible findings.

5. Conclusions

A Mg deficient diet significantly reduced the plasma Mg level in male B6J and B6N mice. This hypomagnesaemia did have an effect on avoidance and exploration behavior, but not on overall anxiety-related behavior, risk assessment, arousal and locomotion. A clear effect of the light regime and substrain was observed in both the behavioral and physiological parameters. The inconsistency in previous studies might be due to the effect of body weight gain differences, the differences in the design of the LD apparatus and/or differences in composition of the microbiota.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2016.01.060.

References

[1] S.A. Abrams, S.A. Atkinson, Calcium, magnesium, phosphorus and vitamin D fortification of complementary foods, J. Nutr. 133 (2003) 2994S–2999S.
[2] G.A. Eby III, K.L. Eby, Magnesium for treatment-resistant depression: a review and hypothesis, Med. Hypotheses 74 (2010) 649–660, http://dx.doi.org/10.1016/j.mehy.2009.10.051.

[3] L.J. Black, K.L. Allen, P. Jacoby, G.S. Trapp, C.M. Gallagher, S.M. Byrne, W.H. Oddy, Low dietary intake of magnesium is associated with increased externalising behaviours in adolescents, Public Health Nutr. 18 (2015) 1824–1830, http://dx.doi.org/10.1017/S1368980014002432.
[4] C. Hohoff, Anxiety in mice and men: a comparison, J. Neural Transm. 116 (2009) 679–687, http://dx.doi.org/10.1007/s00702-009-0215-z.
[5] E. Poleszak, B. Szewczyk, E. Kedzierska, P. Wlaz, A. Pilc, G. Nowak, Antidepressant- and anxiolytic-like activity of magnesium in mice, Pharmacol. Biochem. Behav. 78 (2004) 7–12, http://dx.doi.org/10.1016/j.pbb.2004.01.006.
[6] M. Jahromy, G. Golbaghi, A. Mohajer, F. Poor, M. Riazi, Effects of buspirone on anxiolytic effects of magnesium in male mice, Pharmacol. Pharm. 5 (2014) 657–662, http://dx.doi.org/10.4236/pp.2014.57075.
[7] M. Jahromy, A. Shariatifar, S. Samiee, M. Vaziri, M. Shahraki, S. Dara, Involvement of serotonergic system and magnesium on anxiolytic effects of pomegranate in male mice, World J. Neurosci. 4 (2014) 293–298, http://dx.doi.org/10.4236/wjns.2014.44032.
[8] E. Poleszak, P. Wlaz, A. Wrobel, S. Fidecka, G. Nowak, NMDA/glutamate mechanism of magnesium-induced anxiolytic-like behavior in mice, Pharmacol. Rep. 60 (2008) 655–663, http://dx.doi.org/10.1016/j.pbb.2007.07.018.
[9] E. Poleszak, Benzodiazepine/GABA(A) receptors are involved in magnesium-induced anxiolytic-like behavior in mice, Pharmacol. Rep. 60 (2008) 483–489.
[10] N. Singewald, C. Sinner, A. Hetzenauer, S.B. Sartori, H. Murck, Magnesium-deficient diet alters depression- and anxiety-related behavior in mice—influence of desipramine and *Hypericum perforatum* extract, Neuropharmacology 47 (2004) 1189–1197, http://dx.doi.org/10.1016/j.neuropharm.2004.08.010.
[11] S.B. Sartori, N. Whittle, A. Hetzenauer, N. Singewald, Magnesium deficiency induces anxiety and HPA axis dysregulation: modulation by therapeutic drug treatment, Neuropharmacology 62 (2012) 304–312, http://dx.doi.org/10.1016/j.neuropharm.2011.07.027.
[12] A. Muroyama, M. Inaka, H. Matsushima, H. Sugino, Y. Marunaka, Y. Mitsumoto, Enhanced susceptibility to MPTP neurotoxicity in magnesium-deficient C57BL/6N mice, Neurosci. Res. 63 (2009) 72–75, http://dx.doi.org/10.1016/j.neures.2008.09.009.
[13] S.B. Sartori, A. Hetzenauer, O. Ladurner, N. Singewald, Magnesium-depletion as animal model of enhanced anxiety-related behaviour: diazepam sensitivity in two mouse strains, Naunyn Schmiedeberg's Arch. Pharmacol. 372 (2006) 77.
[14] S.B. Sartori, R. Landgraf, S. Singewald, The clinical implications of mouse models of enhanced anxiety, Future Neurol. 6 (2011) 531–571, http://dx.doi.org/10.2217/fnl.11.34.
[15] M.E. Bardgett, P.J. Schultheis, D.L. McGill, R.E. Richmond, J.R. Wagge, Magnesium deficiency impairs fear conditioning in mice, Brain Res. 1038 (2005) 100–106, http://dx.doi.org/10.1016/j.brainres.2005.01.020.
[16] G. Winther, B.M. Pyndt Jørgensen, B. Elfving, D.S. Nielsen, P. Kihl, S. Lund, D.B. Sørensen, G. Wegener, Dietary magnesium deficiency alters gut microbiota and leads to depressive-like behaviour, Acta Neuropsychiatr. 27 (2015) 168–176, http://dx.doi.org/10.1017/neu.2015.7.
[17] B. Pyndt Jørgensen, G. Winther, P. Kihl, D.S. Nielsen, G. Wegener, A.K. Hansen, D.B. Sørensen, Dietary magnesium deficiency affects gut microbiota and anxiety-like behaviour in C57BL/6N mice, Acta Neuropsychiatr. 27 (2015) 307–311, http://dx.doi.org/10.1017/neu.2015.10.
[18] D. Chollet, P. Franken, Y. Raffin, J.G. Henrotte, J. Widmer, A. Malafosse, M. Tafti, Magnesium involvement in sleep: genetic and nutritional models, Behav. Genet. 31 (2001) 413–425, http://dx.doi.org/10.1023/A:1012790321071.
[19] L. De Franceschi, C. Brugnara, Y. Beuzard, Dietary magnesium supplementation ameliorates anemia in a mouse model of β-thalassemia, Blood 90 (1997) 1283–1290.
[20] S.M. Korte, Corticosteroids in relation to fear, anxiety and psychopathology, Neurosci. Biobehav. Rev. 25 (2001) 117–142, http://dx.doi.org/10.1016/S0149-7634(01)00002-1.
[21] M.C. Laarakker, H.A. van Lith, F. Ohl, Behavioral characterization of A/J and C57BL/6J mice using a multidimensional test: association between blood plasma and brain magnesium-ion concentration with anxiety, Physiol. Behav. 102 (2011) 205–219, http://dx.doi.org/10.1016/j.physbeh.2010.10.019.
[22] F. Ohl, F. Holsboer, R. Landgraf, The modified hole board as a differential screen for behavior in rodents, Behav. Res. Methods Instrum. Comput. 33 (2001) 392–397, http://dx.doi.org/10.3758/BF03195393.
[23] F. Ohl, I. Sillaber, E. Binder, M.E. Keck, F. Holsboer, Differential analysis of behavior and diazepam-induced alterations in C57BL/6N and BALB/c mice using the modified hole board test, J. Psychiatr. Res. 35 (2001) 147–154, http://dx.doi.org/10.1016/S0022-3956(01)00017-6.
[24] U. Albrecht, H. Oster, The circadian clock and behavior, Behav. Brain Res. 125 (2001) 89–91, http://dx.doi.org/10.1016/S0166-4328(01)00288-1.
[25] C. Kopp, Locomotor activity rhythm in inbred strains of mice: implications for behavioural studies, Behav. Brain Res. 125 (2001) 93–96, http://dx.doi.org/10.1016/S0166-4328(01)00289-3.
[26] A. Roedel, C. Storch, F. Holsboer, F. Ohl, Effects of light or dark phase testing on behavioural and cognitive performance in DBA mice, Lab. Anim. 40 (2006) 371–381, http://dx.doi.org/10.1258/002367706778476343.
[27] S. Hossain, B. Wong, E. Simpson, The dark phase improves genetic discrimination for some high throughput mouse behavioral phenotyping, Genes Brain Behav. 3 (2004) 167–177, http://dx.doi.org/10.1111/j.1601-183x.2004.00069.x.
[28] J. Guilloux, M. Seney, N. Edgar, E. Sibille, Integrated behavioral z-scoring increases the sensitivity and reliability of behavioral phenotyping in mice: relevance to emotionality and sex, J. Neurosci. Methods 197 (2011) 21–31, http://dx.doi.org/10.1016/j.jneumeth.2011.01.019.
[29] P.G. Reeves, F.H. Nielsen, G.C. Fahey Jr., AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc Writing Committee on the reformulation of the AIN-76A rodent diet, J. Nutr. 123 (1993) 1939–1951.
[30] A.C. Beynen, M.E. Coates, Nutrition and experimental results, in: L.F.M. Van Zutphen, V. Baumans, A.C. Beynen (Eds.), Principles of Laboratory Animal Science. A Contribution to the Humane Use and Care of Animals and to the Quality of Experimental Results, 2nd ed. (revised), Elsevier Science Publishers, Amsterdam, The Netherlands, 2001, pp. 111–127 (Chapter 6).
[31] W.H. Gipsen, P. Schotman, E.R. de Kloet, Brain RNA and hypophysectomy; a topographical study, Neuroendocrinology 9 (1972) 285–296, http://dx.doi.org/10.1159/000122060.
[32] M. Labots, H.A. Van Lith, F. Ohl, S.S. Arndt, The modified hole board—measuring behavior, cognition and social interaction in mice and rats, J. Vis. Exp. 98 (2015) e52529, http://dx.doi.org/10.3791/52529.
[33] A. Field, Discovering Statistics Using SPSS, 3rd ed., SAGE Publications Ltd., London, U.K, 2009.
[34] J. Algina, H.J. Keselman, Detecting repeated measures effects with univariate and multivariate statistics, Psychol. Methods 2 (2) (1997) 208–218, http://dx.doi.org/10.1037/1082-989X.2.2.208.
[35] B.D. Pachikian, A.M. Neyrinck, L. Deldicque, F.C. De Backer, E. Catry, E.M. Dewulf, F.M. Sohet, L.B. Bindels, A. Everard, M. Francaux, Y. Guiot, P.D. Cani, N.M. Delzenne, Changes in intestinal bifidobacteria levels are associated with the inflammatory response in magnesium-deficient mice, J. Nutr. 140 (2010) 509–514, http://dx.doi.org/10.3945/jn.109.117374.
[36] M. Watanabe, A. Shinohara, T. Matsukawa, M. Chiba, J. Wu, T. Iesaki, T. Okada, Chronic magnesium deficiency decreases tolerance to hypoxia/reoxygenation injury in mouse heart, Life Sci. 88 (2011) 658–663, http://dx.doi.org/10.1016/j.lfs.2011.01.027.
[37] H. Kruse, E. Orent, E. McCollum, Studies on magnesium deficiency in animals: I. Symptomatology resulting from magnesium deprivation, Nutr. Rev. 96 (1932) 519–539, http://dx.doi.org/10.1111/j.1753-4887.1979.tb06654.x.
[38] P. Bac, P. Maurois, C. Dupont, N. Pages, J.P. Stables, P. Gressens, P. Evrard, J. Vamecq, Magnesium deficiency-dependent audiogenic seizures (MDDASs) in adult mice: a nutritional model for discriminatory screening of anticonvulsant drugs and original assessment of neuroprotection properties, J. Neurosci. 18 (1998) 4363–4373.
[39] A. Miyao, I. Ikegaki, A. Nishio, Comparative biochemical studies on the histamine metabolism in acute magnesium-deficient mice and rats, Mem. Fac. Arg. Kagoshima Univ. 20 (1984) 111–125.
[40] B. Pyndt Jørgensen, J.T. Hansen, L. Krych, C. Larsen, A.B. Klein, D.S. Nielsen, K. Josefsen, A.K. Hansen, D.B. Sørensen, A possible link between food and mood: dietary impact on gut microbiota and behavior in BALB/c mice, PLoS One 9 (2014) e103398, http://dx.doi.org/10.1371/journal.pone.0103398.
[41] N. Whittle, L. Li, W.Q. Chen, J.W. Yang, S.B. Sartori, G. Lubec, N. Singewald, Changes in brain protein expression are linked to magnesium restriction-induced depression-like behavior, Amino Acids 40 (2011) 1231–1248, http://dx.doi.org/10.1007/s00726-010-0758-1.
[42] J.H.A.J. Curfs, A. Chwalibog, B.S. Saneij, M. Ristkes-Hoitinga, Nutrient requirements, experimental design, and feeding schedules in animal experimentation, in: J. Hau, S.J. Shapiro (Eds.), Handbook of Laboratory Animal Science. Vol 1: Essential Principles and Practices, 3rd ed., CRC Press Taylor & Francis Group, Boca Raton, USA, 2011, pp. 307–342 (Chapter 11).
[43] J.K. Belknap, J.H. Berg, R. Cocke, A.N. Clancy, Induction and reversal of the magnesium deficiency syndrome in inbred mice, Exp. Neurol. 57 (1977) 506–515, http://dx.doi.org/10.1016/0014-4886(77)90085-1.
[44] D. Chollet, P. Franken, Y. Raffin, A. Malafosse, J. Widmer, M. Tafti, Blood and brain magnesium in inbred mice and their correlation with sleep quality, Am. J. Physiol. Regul. Integr. Comp. Physiol. 279 (2000) R2173–R2178.
[45] M. Sturm, A. Becker, A. Schroeder, A. Bilkei-Gorzo, A. Zimmer, Effect of chronic corticosterone application on depression-like behavior in C57BL/6N and C57BL/6J mice, Genes Brain Behav. 14 (2015) 292–300, http://dx.doi.org/10.1111/gbb.12208.
[46] T. Demuyser, L. Deneyer, E. Bentea, G. Albertini, J. Van Liefferinge, E. Merckx, A. De Prins, D. De Bundel, A. Massie, I. Smolders, In-depth behavioral characterization of the corticosterone mouse model and the critical involvement of housing conditions, Physiol. Behav. 156 (2015) 199–207, http://dx.doi.org/10.1016/j.physbeh.2015.12.018.
[47] H. Murck, Magnesium and affective disorders, Nutr. Neurosci. 5 (2002) 375–389, http://dx.doi.org/10.1080/1028415021000039194.
[48] H. Murck, P. Schüssler, A. Steiger, Renin-angiotensin-aldosterone system: the forgotten stress hormone system: relationship to depression and sleep, Pharmacopsychiatry 45 (2012) 83–95, http://dx.doi.org/10.1055/s-0031-1291346.
[49] M. Franklin, I. Bermudez, N. Hlavacova, S. Babic, H. Murck, C. Schmuckermair, N. Singewald, S. Gaburro, D. Jezova, Aldosterone increases earlier than corticosterone in new animal models of depression: is this an early marker? J. Psychiatr. Res. 46 (2012) 1394–1397, http://dx.doi.org/10.1016/j.jpsychires.2012.07.010.

[50] K. Mlyniec, C.L. Davies, I.G. de Aguero Sanchez, K. Pytka, B. Budziszewska, G. Nowak, Essential elements in depression and anxiety. Part I, Pharmacol. Rep. 66 (2014) 534–544, http://dx.doi.org/10.1016/j.pharep.2014.03.001.
[51] R. Paylor, C.M. Spencer, L.A. Yuva-Paylor, S. Pieke-Dahl, The use of behavioral test batteries, II: effect of test interval, Physiol. Behav. 87 (2006) 95–102, http://dx.doi.org/10.1016/j.physbeh.2005.09.002.
[52] A.M. Post, P. Weyers, P. Holzer, E. Painsipp, P. Pauli, T. Wultsch, A. Reif, K.P. Lesch, Gene-environment interaction influences anxiety-like behavior in ethologically based mouse models, Behav. Brain Res. 218 (2011) 99–105, http://dx.doi.org/10.1016/j.bbr.2010.11.031.
[53] V. Ramesh, D. Nair, S.X. Zhang, F. Hakim, N. Kaushal, F. Kayali, Y. Wang, R.C. Li, A. Carreras, D. Gozal, Disrupted sleep without sleep curtailment induces sleepiness and cognitive dysfunction via the tumor necrosis factor-alpha pathway, J. Neuroinflammation 9:91 (2012) 1–15, http://dx.doi.org/10.1186/1742-2094-9-91.
[54] M. Bourin, M. Hascoët, The mouse light/dark box test, Eur. J. Pharmacol. 463 (2003) 55–65, http://dx.doi.org/10.1016/S0014-2999(03)01274-3.
[55] N. Kulesskaya, V. Voikar, Assessment of mouse anxiety-like behavior in the light–dark box and open-field arena: role of equipment and procedure, Physiol. Behav. 133 (2014) 30–38, http://dx.doi.org/10.1016/j.physbeh.2014.05.006.
[56] N. Hagenbuch, J. Feldon, B.K. Yee, Use of the elevated plus-maze test with opaque or transparent walls in the detection of mouse strain differences and the anxiolytic effects of diazepam, Behav. Pharmacol. 17 (2006) 31–41, http://dx.doi.org/10.1097/01.fbp.0000189811.77049.3e.
[57] J.A. Foster, K. McVey Neufeld, Gut–brain axis: how the microbiome influences anxiety and depression, Trends Neurosci. 36 (2013) 305–312, http://dx.doi.org/10.1016/j.tins.2013.01.005.
[58] R.M. Stilling, T.G. Dinan, J.F. Cryan, Microbial genes, brain & behaviour—epigenetic regulation of the gut-brain axis, Genes Brain Behav. 13 (2014) 69–86, http://dx.doi.org/10.1111/gbb.12109.
[59] R.A. Luna, J.A. Foster, Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression, Curr. Opin. Biotechnol. 32 (2015) 35–41, http://dx.doi.org/10.1016/j.copbio.2014.10.007.
[60] H.M. Savignac, B. Kiely, T.G. Dinan, J.F. Cryan, Bifidobacteria exert strain-specific effects on stress-related behavior and physiology in BALB/c mice, Neurogastroenterol. Motil. 26 (2014) 1615–1627, http://dx.doi.org/10.1111/nmo.12427.