*Acta Neuropsychiatrica 2015*

DOI: 10.1017/neu.2015.10


*ACTA NEUROPSYCHIATRICA*

## Short Communication

# Dietary magnesium deficiency affects gut microbiota and anxiety-like behaviour in C57BL/6N mice

Pyndt Jørgensen B, Winther G, Kihl P, Nielsen DS, Wegener G, Hansen AK, Sørensen DB. Dietary magnesium deficiency affects gut microbiota and anxiety-like behaviour in C57BL/6N mice.

**Objective:** Magnesium deficiency has been associated with anxiety in humans, and rodent studies have demonstrated the gut microbiota to impact behaviour.
**Methods:** We investigated the impact of 6 weeks of dietary magnesium deficiency on gut microbiota composition and anxiety-like behaviour and whether there was a link between the two. A total of 20 C57BL/6 mice, fed either a standard diet or a magnesium-deficient diet for 6 weeks, were tested using the light-dark box anxiety test. Gut microbiota composition was analysed by denaturation gradient gel electrophoresis.
**Results:** We demonstrated that the gut microbiota composition correlated significantly with the behaviour of dietary unchallenged mice. A magnesium-deficient diet altered the gut microbiota, and was associated with altered anxiety-like behaviour, measured by decreased latency to enter the light box.
**Conclusion:** Magnesium deficiency altered behavior. The duration of magnesium deficiency is suggested to influence behaviour in the evaluated test.

**Bettina Pyndt Jørgensen[1], Gudrun Winther[2], Pernille Kihl[1], Dennis S. Nielsen[3], Gregers Wegener[2,4], Axel K. Hansen[1], Dorte B. Sørensen[1]**

[1]Section of Experimental Animal Models, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg C, Denmark; [2]Translational Neuropsychiatry Unit, Department of Clinical Medicine, Aarhus University, Risskov, Denmark; [3]Department of Food Science, Faculty of Science, University of Copenhagen, Frederiksberg C, Denmark; and [4]Pharmaceutical Centre of Excellence, School of Pharmacy, North West University, Potchefstroom, South Africa

Keywords: animal models; anxiety; gut microbiota; magnesium

Bettina Pyndt Jørgensen, Section of Experimental Animal Models, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Thorvaldsensvej 57, 1871 Frederiksberg C, Denmark.
Tel: +453 533 2724;
Fax: +453 533 2755;
E-mail: bmp@sund.ku.dk

Accepted for publication February 5, 2015

First published online March 16, 2015

**Significant outcomes**

- Dietary magnesium deficiency significantly alters the gut microbiota and reduces microbial diversity.
- Six weeks of dietary magnesium deficiency affects anxiety-like behaviour in the light/dark box.
- Duration of magnesium deficiency is suggested to influence behaviour in the evaluated test.

**Limitations**

- Future studies should address the impact of the length of the diet trial on anxiety-like behaviour, and should be supported by additional behavioural assays.

## Introduction

Neuropsychiatric disorders such as depression and anxiety have been linked with low levels of cerebral magnesium in humans (1,2). Animals with magnesium deficiency have been proposed as novel models of depression and anxiety, as some studies have shown magnesium deficiency to induce depressive- and anxiety-like behaviour, which mimic human symptoms of these disorders and impact the hypothalamo–pituitary–adrenal (HPA) axis within 3 weeks of dietary deficiency (3,4). Abnormalities in the HPA axis have been associated with anxiety disorders (5). Accumulating evidence demonstrates an impact of the gut microbiota (GM) on anxiety-like behaviour and development and regulation of the HPA axis (6–9). Several studies have shown dysregulation of the HPA axis and altered anxiety-like behaviour in germfree mice, which could be normalised by inoculation with faeces from specific pathogen-free mice (9–11). In addition, a study by Collins et al. (8) demonstrated transfer of the strain-specific anxiety-like behavioural phenotype by faecal transplantation from BALB/c mice to NIH Swiss mice and *vice versa*. Supporting this, a previous study in our laboratory showed stress-induced anxiety to be associated with an altered GM (6). Dietary magnesium deficiency has previously been shown to induce fluctuations in the intestinal abundance of *Bifidobacterium* spp. and increase the abundance of *Lactobacillus* spp. within 3 weeks of dietary deficiency. This effect may be a consequence of dietary nutrient availability affecting the GM directly, or a consequence of systemic magnesium deficiency affecting the alimentary tract (12). It can be hypothesised that these alterations of the GM composition influence behaviour.

### Aims of the study

We investigated the effect of 6 weeks of dietary magnesium deficiency on development of anxiety-like behaviour, the dietary impact on the GM and the association between this and behaviour.

## Materials and methods

The study was conducted in strict accordance with the EU directive 2010/63/EU and with the Danish Animal Experimentation Act (LBK 1306 from 23/11/2007 with 2011 amendments). The protocol was approved by the Danish Animal Experimentation Committee (j.no 2012-15-2934-00254). Efforts were made to improve animal welfare and minimise stress of the mice. Eight-week-old male C57BL/6NBomTac mice (Taconic Ltd., Ll, Skensved, Denmark) were standard housed with five mice per cage in a controlled 12-h light/dark cycle (light on at 7 a.m.), and were given free access to diet and water. The room temperature was set at 22°C and the humidity 50–60%. After 1 week of acclimatisation, mice were randomly assigned to either a standard diet (Altromin 1324; Brogaarden ApS, Lynge, Denmark) containing the usual 0.2% magnesium ($n = 10$), which corresponds to four times more than the daily minimum magnesium requirement of 500 mg/kg of food, or assigned to a magnesium-deficient (MgD) diet containing 0.02% magnesium ($n = 10$), providing 10% of the daily ration recommended (13) (Sniff Spezialdiäten, Soest, Germany). Mice were kept on their respective diets for 6 weeks, before being tested in the light/dark box (LDB) anxiety test. The LDB with the size $45 \times 30 \times 30$ cm was divided into a dark (lux 1) and a light (lux 400) compartment, with a $7 \times 7$ cm entry opening in between. Before the behavioural test, mice were allowed to get habituated to the test room for 24 h. All tests were carried out between 8 a.m. and 3 p.m. The mice were individually placed in the centre of the dark compartment, and their behaviour was video-recorded for 5 min, and subsequently scored by counting latency to enter the light compartment and the number of entries into and the time spent in the two compartments. Mice were scored as entering a compartment, once all four paws were placed inside it. After each test, the compartments were cleaned using 70% ethanol solution and were allowed to air-dry. Faecal samples were obtained from each of the mice before the diet trial and after exposure to the LDB. The day after behavioural testing, mice were euthanised by cervical dislocation, and caecal samples were obtained by dissection. Samples were collected directly into a sterile Eppendorf tube (Eppendorf®, Hamburg, Germany), stored on ice and transferred to −80°C within 30 min. Faecal and caecal samples were analysed as previously described by DNA extraction followed by polymerase chain reaction amplification of the bacterial 16S rRNA gene (V3 region) and separated by denaturation gradient gel electrophoresis (DGGE) (14). DGGE gels were analysed by cluster analysis using the dice similarity coefficient with a band position tolerance and optimisation of 1% using the unweighted pair group method with arithmetic mean clustering algorithm and by principal component analysis (PCA) in Bionumerics ver. 4.5 (Applied Maths, Belgium), as previously described and explained (15). The first three principal components (PC1, PC2 and PC3) of the PCAs were used to compare the GM composition of the treatment groups by analysis of variance (ANOVA), taking into account DGGE gel number and cage factor, and to create multiple linear regression models for analysing the correlation between GM composition and behaviour. The number of bands in each DGGE profile was used as a measure of GM diversity, as previously



https://doi.org/10.1017/neu.2015.10 Published online by Cambridge University Press

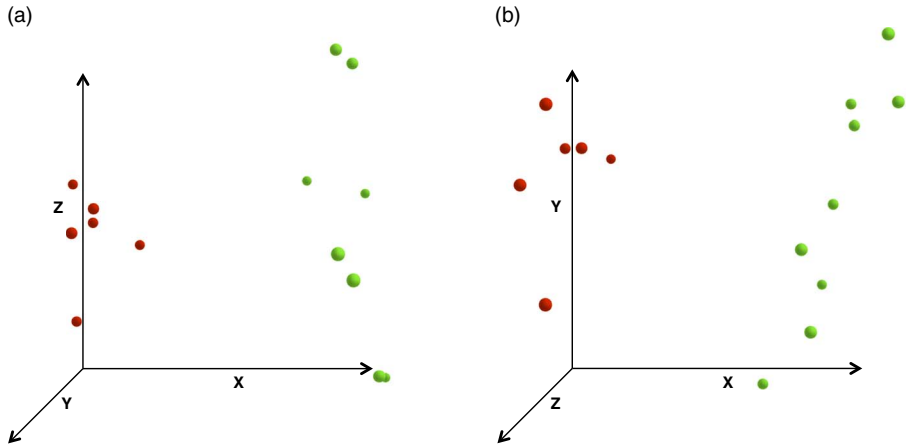


*Fig. 1.* Principal component analysis plots illustrating differences in gut microbiota composition between mice fed a control diet or an MgD diet in faeces ($p < 0.0001$) (a) and caecum ($p < 0.0001$) (b). Red: MgD mice, green: control mice.

described (16), also taking the DGGE gel number and cage into account. Due to technical issues, the number of samples in each group was as follows: faeces $n = 8$ (control) and $n = 6$ (MgD); and in caecum $n = 9$ (control) and $n = 6$ (MgD). Statistical analyses were performed using SAS JMP vers. 10 (SAS Institute Inc., Copenhagen, USA). Data were checked for adherence to a normal distribution by QQ-plots and the Shapiro–Wilks test for normality. Data were compared using the Welch *t*-test or the non-parametric Wilcoxon test when not normally distributed. For ANOVA tests, data were ranked if not normally distributed. A *p*-value <0.05 was considered significant. Robustness of the linear regression models was checked by removing a random sample twice, which did not affect significance.

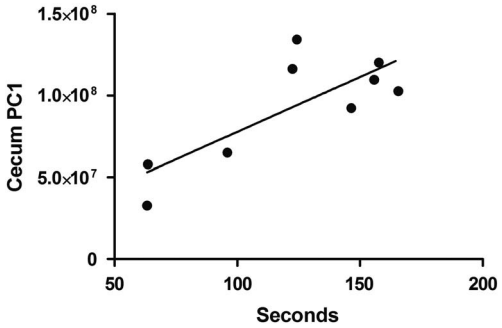


*Fig. 2.* Correlation between the gut microbiota of control mice and time spent in the light compartment. Time spent in the light compartment of the test is significantly correlated to the gut microbiota profile of mice on control diet (caecum PC1, $r^2 = 0.62$, $p = 0.011$). PC, principal component.

## Results

The MgD mice showed significantly shorter latency to enter the light compartment of the LDB than mice on the control diet ($7.89 \pm 2.54$ and $16.57 \pm 2.54$ s, respectively, $p = 0.027$), demonstrating an altered anxiety-like behaviour, with initially decreased aversion against the lit environment. No difference was observed between groups regarding entries into the light compartment ($14 \pm 4$ vs. $12 \pm 4$ entries for the magnesium-deficient and the control group, respectively, $p = 0.33$) and time spent in the light compartment ($127.8 \pm 43.7$ vs. $119.8 \pm 37.7$ s. for the magnesium-deficient and the control group, respectively, $p = 0.68$). Analysis of the gut showed that the MgD diet altered the GM composition of the mice significantly. No difference in GM composition was evident between the groups before the diet trial ($p > 0.05$ for PC1, PC2 and PC3); however, after 6 weeks on their respective diets, the GM of MgD mice differed significantly from mice fed a standard control diet (faeces PC1, $p = 0.0011$ and caecum PC1, $p = 0.0012$) (Fig. 1), which was also visualised by a distinct clustering of the treatment groups in the dendrogram of the cluster analysis of both faeces and caecum (not shown). Furthermore, the MgD diet decreased bacterial diversity of the gut significantly (faeces, $19.00 \pm 2.2$ and $26.16 \pm 2.0$ bands for MgD and control mice, respectively, $p = 0.045$). Linear regression analyses between behaviour and the individual GM profiles revealed a significant correlation between the GM composition and anxiety-like behaviour in the control group, in which the GM composition correlated with the time spent in the light box (caecum PC1, $p = 0.011$, $r^2 = 0.62$, Fig. 2).

## Discussion

The present study demonstrates altered anxiety-like behaviour in mice fed an MgD diet for 6 weeks,



https://doi.org/10.1017/neu.2015.10 Published online by Cambridge University Press

which is associated with a significantly changed GM profile and reduced bacterial diversity. In contrast to Singewald et al. (3), who found 3 weeks of dietary magnesium deficiency to increase the latency to enter the light compartment and to decrease the time spent in the light compartment, we did not observe an anxiogenic effect after 6 weeks of dietary magnesium deficiency. In line with Singewald et al., Sartori et al. also reported 3 weeks of dietary magnesium deficiency to induce anxiogenic behaviour, measured by increased latency to enter the light compartment. This was, however, not accompanied by a decrease in the time spent in the light compartment (4). It may be suggested that the duration of magnesium deficiency impacts behaviour in this version of the LDB test. This is supported by preliminary observations in our department, which demonstrated 3 and 6 weeks of magnesium deficiency to impact behaviour differently in a related anxiety assay (17). Poleszak et al. (18) demonstrated magnesium supplementation leading to high serum levels of magnesium to be associated with anxiolytic behaviour in mice, whereas supplementation leading to lower serum levels of magnesium did not affect behaviour. The magnesium stores of the body may very likely differ between mice fed an MgD diet for 3 and 6 weeks. It can be speculated that 6 weeks of dietary magnesium deficiency may impact the brain and behaviour in a different manner than 3 weeks of deficiency, further altering the behavioural outcome of the LDB test. Addressing this through behavioural testing at several time points up till 6 weeks of magnesium deficiency will clarify this. In accordance with previously reported alterations in the abundance of *Bifidobacterium* spp. and *Lactobacillus* spp. within 3 weeks of dietary magnesium deficiency (19), we observed a significant impact of the MgD diet on the GM. Feeding an MgD diet for 6 weeks altered both the GM and reduced the diversity significantly. A reduced microbial diversity has previously been associated with disease development (20), and altering the GM composition has been demonstrated to induce changes in anxiety-like behaviour (8). We did not observe major behavioural changes, and thus the impact of the observed GM changes on this aspect of anxiety-like behaviour is questionable. Interestingly, the GM composition correlated significantly with anxiety-like behaviour in the control group, and thus it may be speculated that inter-individual GM variation influence anxiety-like behaviour in the dietary unchallenged mice and that magnesium deficiency may disrupt a homoeostatic microbiota–gut–brain axis.

In conclusion, the duration of dietary magnesium deficiency may impact anxiety-like behaviour. This should be addressed through behavioural testing at several time points up till 6 weeks of magnesium deficiency in order to conclude further. Future studies should evaluate whether the induced GM alterations affect other aspects of anxiety.

#### Acknowledgement

Gu.W., G.W., D.B.S. and B.P.J. planned the experiment, Gu.W., B.P.J., P.K. performed the experiment, B.P.J., Gu.W. analysed the data and B.P.J., Gu.W., D.B.S., G.W., A.K.H. and D.S.N. wrote the manuscript.

#### Financial Support

This research received no specific grant from any funding agency, commercial or not-for-profit sectors.

#### Conflicts of Interest

There are no conflicts of interest between any of the authors. Gregers Wegener is editor-in-chief of Acta Neuropsychiatrica, but was not involved during the review and decision of this paper. Research material can be accessed by contacting the corresponding author.

#### Ethical Standards

The authors assert that all procedures contributing to this work comply with relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008, and also with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

#### References

1. Eby GA, Eby KL. Rapid recovery from major depression using magnesium treatment. Med Hypotheses 2006;**67**: 362–370.
2. Eby GA 3rd, Eby KL. Magnesium for treatment-resistant depression: a review and hypothesis. Med Hypotheses 2010;**74**:649–660.
3. Singewald N, Sinner C, Hetzenauer A et al. Magnesium-deficient diet alters depression- and anxiety-related behavior in mice – influence of desipramine and Hypericum perforatum extract. Neuropharmacology 2004;**47**:1189–1197.
4. Sartori SB, Whittle N, Hetzenauer A et al. Magnesium deficiency induces anxiety and HPA axis dysregulation: modulation by therapeutic drug treatment. Neuropharmacology 2012;**62**:304–312.
5. Faravelli C, Lo Sauro C, Godini L et al. Childhood stressful events, HPA axis and anxiety disorders. World J Psychiatry 2012;**2**:13–25.
6. Bangsgaard Bendtsen KM, Krych L, Sørensen DB et al. Gut microbiota composition is correlated to grid floor induced stress and behavior in the BALB/c mouse. PLoS One 2012;**7**:46231.
7. Bercik P, Denou E, Collins J et al. The intestinal microbiota affect central levels of brain-derived neurotrophic factor and behavior in mice. Gastroenterology 2011;**141**:599–U701.

8. Collins SM, Kassam Z, Bercik P. The adoptive transfer of behavioral phenotype via the intestinal microbiota: experimental evidence and clinical implications. Curr Opin Microbiol 2013;**16**:240–245.
9. Sudo N, Chida Y, Aiba Y et al. Postnatal microbial colonization programs the hypothalamic–pituitary–adrenal system for stress response in mice. J Physiol (Lond) 2004;**558**:263–275.
10. Heijtz RD, Wang S, Anuar F et al. Normal gut microbiota modulates brain development and behavior. Proc Nat Acad Sci 2011;**108**:3047–3052.
11. Neufeld KA, Kang N, Bienenstock J et al. Effects of intestinal microbiota on anxiety-like behavior. Commun Integr Biol 2011;**4**:492–494.
12. Zimowska W, Girardeau JP, Kuryszko J et al. Morphological and immune response alterations in the intestinal mucosa of the mouse after short periods on a low-magnesium diet. Br J Nutr 2002;**88**:515–522.
13. Crawley JN. Neuropharmacologic specificity of a simple animal model for the behavioral actions of benzodiazepines. Pharmacol Biochem Behav 1981;**15**:695–699.
14. Pyndt Jørgensen B, Hansen JT, Krych L et al. A possible link between food and mood: dietary impact on gut microbiota and behavior in BALB/c Mice. PLoS One 2014;**9**:e103398.
15. Lundberg R, Clausen SK, Pang W et al. Gastrointestinal microbiota and local inflammation during oxazolone-induced dermatitis in BALB/cA mice. Comp Med 2012;**62**:371–380.
16. Muyzer G, Smalla K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek 1998;**73**:127–141.
17. Winther G. Magnesium deficiency induces anxiety- and depression-like behavior and metabolic dysfunction in C57Bl/6 J mice, MSc Thesis, University of Aarhus, Denmark. 2012.
18. Poleszak E, Szewczyk B, Kędzierska E et al. Antidepressant- and anxiolytic-like activity of magnesium in mice. Pharmacol Biochem Behav 2004;**78**:7–12.
19. Pachikian BD, Neyrinck AM, Deldicque L et al. Changes in intestinal Bifidobacteria levels are associated with the inflammatory response in magnesium-deficient mice. J Nutr 2010;**140**:509–514.
20. Collins S, Verdu E, Denou E et al. The role of pathogenic microbes and commensal bacteria in irritable bowel syndrome. (IBD and IBS: Novel mechanisms and future practice.). Dig Dis 2009;**1**:85–89.



https://doi.org/10.1017/neu.2015.10 Published online by Cambridge University Press