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Dietary magnesium deficiency induces the expression of neuroinflammation-related genes in mouse brain

Ryuhei Tsuji[1,2] | Hirofumi Inoue[3] | Mariko Uehara[3] | Satoshi Kida[1,2] (iD)

[1]Department of Applied Biological Chemistry, Graduate school of Agriculture and Life Sciences, The University of Tokyo, Tokyo, Japan

[2]Faculty of Applied Bioscience, Department of Bioscience, Tokyo University of Agriculture, Tokyo, Japan

[3]Faculty of Applied Bioscience, Department of Nutritional Science and Food Safety, Tokyo University of Agriculture, Tokyo, Japan

Correspondence
Satoshi Kida, Department of Applied Biological Chemistry, Graduate School of Agriculture and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo, Japan
Email: akida@g.ecc.u-tokyo.ac.jp

Abstract

Aims: Dietary Mg^{2+} deficiency (MgD) impairs hippocampus-dependent memory in mice; however, the molecular mechanisms underlying MgD-induced memory impairments are unclear. Here, we investigated the molecular signatures in the hippocampus of MgD mice by analyzing the hippocampal transcriptome.

Methods: We performed RNA-sequencing of the hippocampal transcriptome of MgD mice. We used gene ontology analyses and quantitative real-time PCR to validate the RNA-sequencing results.

Results: mRNAs for neuroinflammation-related genes were upregulated in the hippocampus and cortex of MgD mice.

Conclusion: MgD induces neuroinflammation in the mouse brain, including the hippocampus and cortex. Our findings suggest that MgD-induced neuroinflammation triggers the impairments of hippocampus-dependent memory.

KEYWORDS

hippocampus-dependent memory, magnesium deficiency, microglia, neuroinflammation, transcriptome

1 | INTRODUCTION

Magnesium (Mg²⁺) is an essential mineral for maintaining normal cellular functions by functioning as a cofactor in more than 300 enzymatic reactions.[1-3] Mg²⁺ deficiency (MgD) disturbs the homeostasis of numerous biological processes, causing chronic and acute diseases such as metabolic syndrome,[4] type 2 diabetes,[5] and hypertension.[6] Importantly, Mg²⁺ is required for the voltage-dependent blockade of N-methyl-D-aspartate-type glutamate receptors, thereby controlling their opening,[7–9] and also contributes to synaptic plasticity such as long-term potentiation.

Consistently, Mg has been shown to play an important role in learning and memory. Increasing brain Mg²⁺ concentration improves learning ability, working memory, and short- and long-term memory in rats,[10] while MgD impairs fear memory formation.[11,12] We previously investigated the effects of MgD on brain function and found that MgD diet-fed mice have deficits in hippocampus-dependent memories such as contextual fear, spatial, and social recognition memories, while they have normal amygdala- and insular cortex-dependent conditioned taste aversion memory, locomotor activity, and emotional behaviors.[13] Conversely, MgD mice have normal spine density and morphology of hippocampal neurons. Thus, previous studies have shown that MgD impairs hippocampus-dependent memory without affecting hippocampal

neuron morphology.[13] However, the molecular mechanisms underlying the impairments of hippocampus-dependent memory by MgD remain unclear. In this study, we analyzed the hippocampal transcriptome in MgD mice to identify the molecular signatures of MgD-induced deficits of hippocampus-dependent memory performance in mice.

2 | METHODS

2.1 | Animals

All experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, Japan Neuroscience Society and Tokyo University of Agriculture. All animal experiments performed in this study were approved by the Animal Care and Use Committee of Tokyo University of Agriculture. Male C57BL/6N mice were obtained from Charles River (Yokohama, Japan). The mice were housed in cages of 5 or 6, maintained on a 12 hours light/dark cycle, and allowed ad libitum access to pellet food and water. The mice were at least 12 weeks of age at the start of the experiments, and all samples were collected during the light phase of the cycle. All experiments were conducted blind to the treatment condition of the mice.
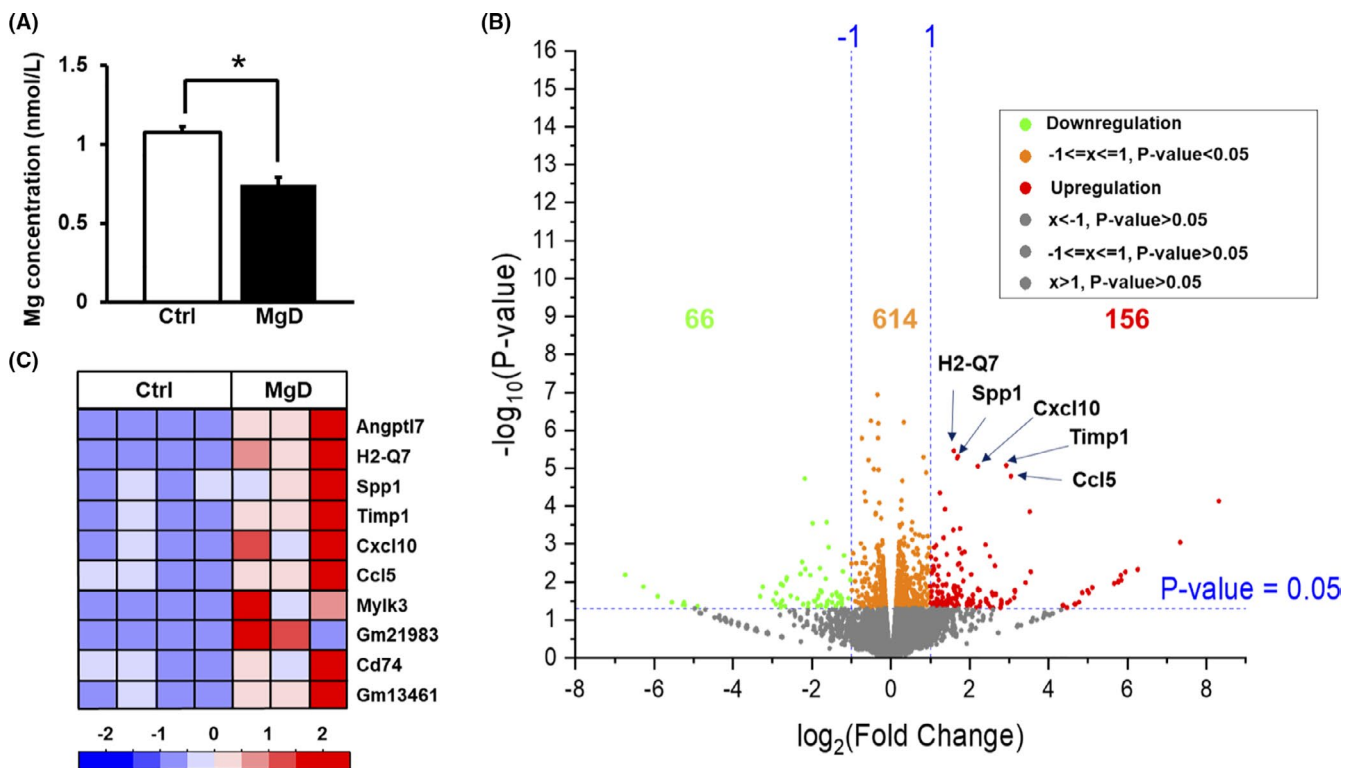
FIGURE 1 Serum Mg concentrations and overall mRNA expression changes in the hippocampus of Mg²⁺ deficiency (MgD) mice versus control (Ctrl) mice. (A) Serum Mg concentrations are decreased by MgD (Ctrl, n = 3; MgD, n = 4). *P <.05. Error bars indicate standard error of the mean. (B) Volcano plot showing 156 upregulated (log₂ fold change > 1, P < 0.05, red) and 66 downregulated (log₂ fold change < −1, P < 0.05, green) genes in the hippocampus of MgD mice compared to Ctrl mice. (C) Expression heatmap of the top 10 significantly upregulated genes in the hippocampus of MgD mice compared to Ctrl mice. Relative expression levels normalized by transcripts per million.

2.2 | MgD treatment

Dietary MgD was induced by using paired feeding procedures.[13-17] To habituate the mice to a powdered diet, both groups were given a powdered Mg^{2+}-containing diet ad libitum for 1 week. The mice were divided randomly into the MgD (n = 9) and control (Ctrl, n = 11) groups. The mice received a powdered MgD AIN-93 or Ctrl AIN-93 diet (Table S1), respectively, ad libitum during the treatment period (weeks 0-6). The Ctrl group was pair-fed with the MgD group.

2.3 | Mg concentration analysis

Blood samples were collected by the tail vein puncture; these samples were subsequently used to measure the serum Mg concentrations. Blood samples were incubated (16 hours, 4°C) and centrifuged (1200 g, 20 minutes, 4°C). The obtained supernatants were used to determine serum magnesium levels colorimetrically with a Wako Magnesium B Test Kit (Wako Pure Chemical Industries).

2.4 | Quantitative real-time PCR (qRT-PCR)

MgD and Ctrl mice were sacrificed by cervical dislocation. Regions of whole hippocampus and whole cortex were rapidly dissected and snap-frozen in liquid nitrogen. Total RNA was prepared using an RNeasy Mini Kit (QIAGEN) or acid guanidinium thiocyanate-phenol-chloroform extraction method.[18] Total RNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen) and an oligo dT primer. qRT-PCR was performed with the QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) using the Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). qRT-PCR was carried out at 50°C for 2 minutes 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The expression of target genes was normalized to that of *Hprt*. The primers for qRT-PCR are listed in Table S2.

2.5 | RNA-sequencing

Total RNA was isolated using an RNeasy Mini Kit (QIAGEN). After RNA quality check (all RIN values ≥ 8.0) with an RNA Nano Kit (Agilent
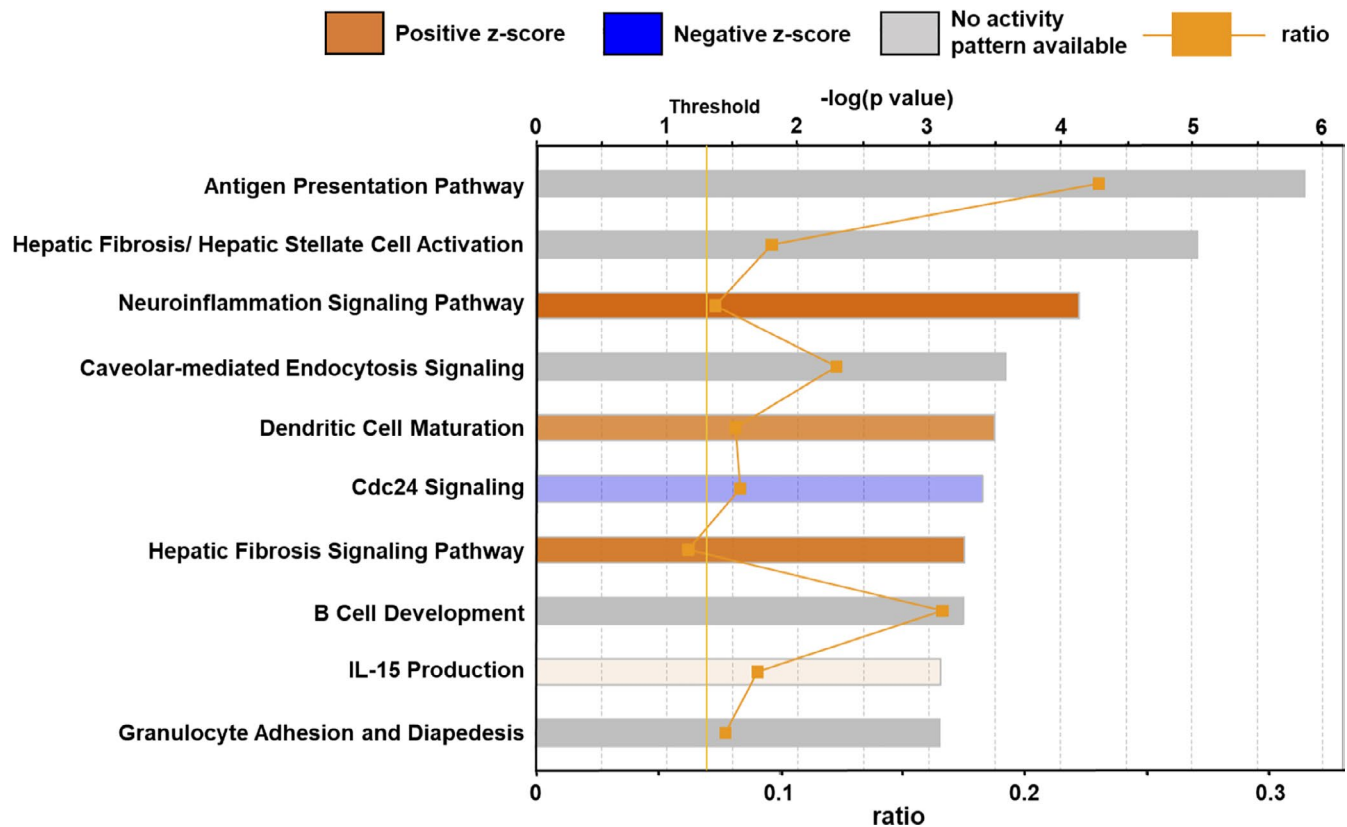

FIGURE 2 Top 10 biological pathways of Ingenuity Pathway Analysis. The significance score (negative log of the *P*-value calculated using Fisher's exact test) for each pathway is indicated by the bars. The orange points on each pathway bar represent the ratio of the number of genes in a given pathway that meet the cutoff criteria (*P* < 0.05) and the total number of genes that map to the pathway. An orange bar predicts an overall increase in the activity of the pathway. A blue bar predicts an overall decrease in the activity. A gray bar predicts no activity pattern.

Technologies) on an Agilent Bioanalyzer (Agilent Technologies), 1 µg total RNA was used to prepare cDNA libraries with a TruSeq RNA Sample Preparation Kit v2 (Illumina). The derived cDNA libraries were analyzed on an Agilent Bioanalyzer with a DNA 1000 Kit and quantified by qPCR using a KAPA Library Quantification Kit (KAPA Bio systems). cDNA libraries were pooled in lanes and clusters were generated on a cBot (Illumina) to obtain 100-bp single reads in a HiSeq 2500 sequencer (Illumina). Demultiplexed fastq files were generated using bcl2fastq ver. 2.18 (Illumina). The raw sequence reads were filtered to exclude adapter sequences, ambiguous nucleotides, and low-quality sequences, and the retained sequences were aligned against the mouse genome (mm10) using CLC Genomics Workbench software ver. 9.5 (Qiagen). Ingenuity Pathway Analysis (IPA, QIAGEN) was performed using 751 differentially expressed genes ($P < 0.05$). Pathway enrichment was examined through Fisher's exact test. Gene Set Enrichment Analysis (GSEA) was performed using GSEA ver. 2.0.3.[19,20] The C5 gene set collections (5917 gene ontology [GO] gene sets) were obtained from the Molecular Signature Database v6.0 (http://software.broadinstitute.org/gsea/msigdb/collections.jsp). The gene expression values were calculated as transcript per million (TPM).[21] Expressed genes were ranked according to their TPM values. Gene sets were considered to be highly enriched for a P-value < 0.05 and false discovery rate (FDR)-corrected q-value <0.25.[22] RNA-sequencing data have been deposited in the DDBJ Sequence Read Archive (accession number: DRA011148).

2.6 | Statistical analysis

Two-way repeated ANOVA was used to analyze an effect of body weight gain. Student's t test was used to analyze differences in the blood serum concentrations of Mg and mRNA expression levels. All values in the text and figures represent the mean ± standard error of the mean.

3 | RESULTS AND DISCUSSION

To examine the effects of dietary MgD on transcriptome profiles in the mouse brain, male C57BL/6N mice were fed an AIN-93-based MgD or Ctrl diet (Table S1). Importantly, MgD mice showed significantly lower blood serum Mg concentration than Ctrl mice (Figure 1A) without changing body weight gain (Figure S1), suggesting that the MgD diet lowers the Mg levels.
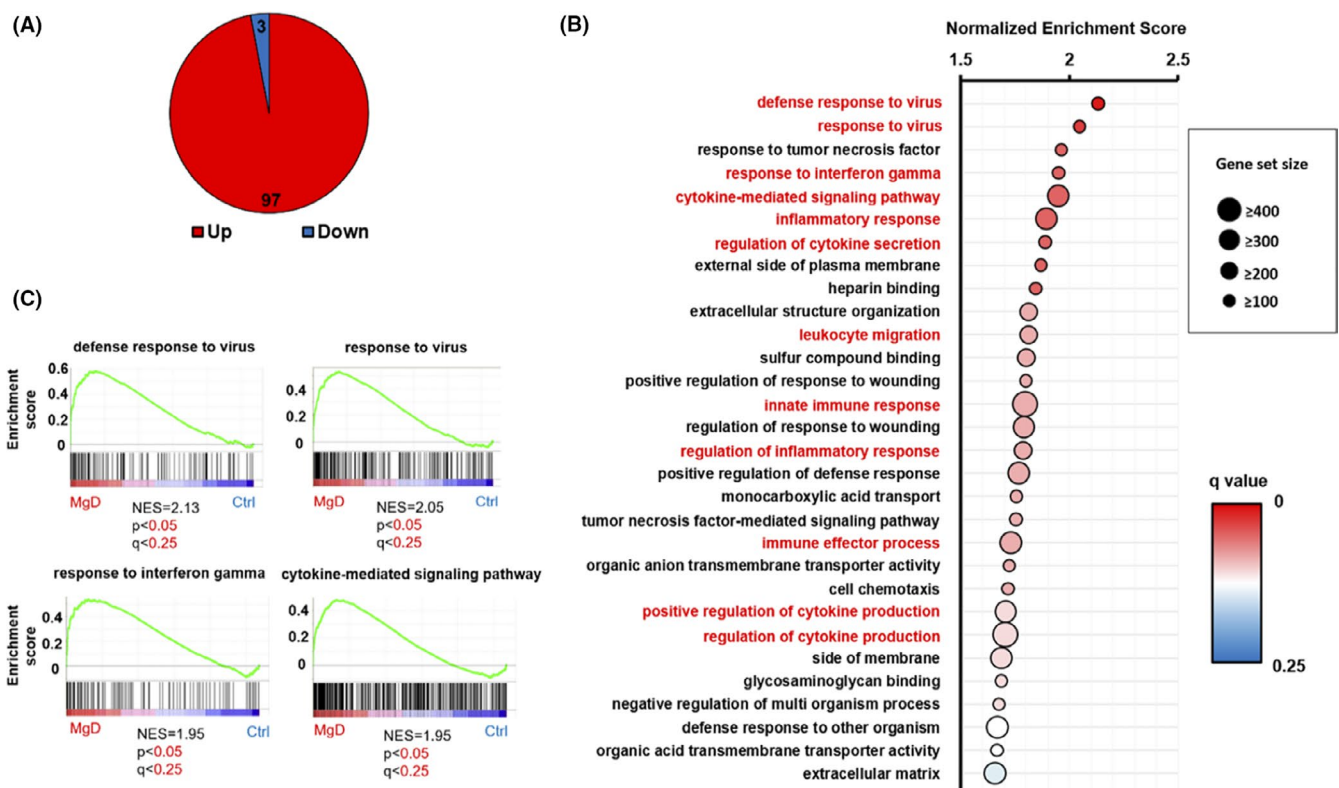

FIGURE 3 Gene Set Enrichment Analysis (GSEA) demonstrating the upregulation of immune/inflammation-associated gene sets in the hippocampus of Mg^{2+} deficiency (MgD) mice. (A) GSEA revealed that the expression levels of 97 and 3 gene sets were significantly increased or decreased, respectively, in the hippocampus of MgD mice (n = 3) compared to control (Ctrl) mice (n = 4) ($P < 0.05$ and FDR < 0.25, gene set size ≥ 100). (B) Twelve immune/inflammation-associated gene sets (in red) were observed in the top 30 gene sets showing upregulation of mRNA expressions in MgD mice. Normalized enrichment score (NES) for each gene set is indicated. (C) Representative enrichment plot showing significantly increased expression of inflammation-associated gene sets in the hippocampus of MgD mice. Genes included in the enrichment score are shown in Table S4.

We analyzed the hippocampal transcriptome by performing RNA-sequencing (Ctrl, n = 4; MgD, n = 3). We found that the expression of 156 and 56 genes was upregulated ($P < 0.05$, $\log_2 > 1$) or downregulated ($P < 0.05$, $\log_2 < -1$), respectively, in the hippocampus of MgD mice (Figure 1B). Interestingly, we observed inflammation-related genes including *Angptl7*, *H2-Q7*, *Spp1*, *Timp1*, *Cxcl10*, and *Ccl5* in the top 10 significantly upregulated genes in the hippocampus of MgD mice (Figure 1C). To explore the potential biological pathways affected by the MgD diet, we performed GO analysis using Ingenuity Pathway Analysis on the 751 differentially regulated genes in MgD mice compared to Ctrl mice. Interestingly, in the top 10 pathways, "neuroinflammation signaling" (z-score = 2.982, ratio = 0.0736), "dendritic cell maturation" (z-score = 2.138, ratio = 0.082), and "hepatic fibrosis signaling pathway" (z-score = 2.558, ratio = 0.0625) exhibited positive z-scores and are involved in immune/inflammation signaling pathways (Figure 2, Table S3). To clarify further the biological pathways affected by MgD, we performed GSEA (gene set size ≥ 100). Using $P < 0.05$ and FDR < 0.25, GSEA revealed that the expression of 97 and 3 gene sets was significantly increased or decreased, respectively, in the hippocampus of MgD mice (Figure 3A). Figure 3B shows the top 30 significantly upregulated gene sets in the hippocampus of MgD mice. Twelve immune/inflammation-associated gene sets were observed in the top 30 gene sets showing significant upregulation of mRNA expressions in MgD mice (Figure 3B,C). Collectively, these observations indicate that MgD upregulates the expression of genes related to immune/inflammation signaling pathways in the hippocampus, suggesting that MgD induces hippocampal neuroinflammation.

To validate the RNA-sequencing data, we measured the mRNA levels of inflammation-associated genes, which were included in the top 10 upregulated genes as above (Figure 1C) and/or are involved in gene sets whose upregulation was observed in GSEA (Table S4), in the hippocampus of MgD mice using qRT-PCR. *Angptl7*, *Spp1*, *Timp1*, *Cxcl10*, *Ccl5*, and *Ccl7* mRNA levels were significantly increased in the hippocampus of MgD mice compared to Ctrl mice (Figure 4A). These observations confirmed the results of RNA-sequencing and suggested that MgD increases the expression of neuroinflammation-associated genes in the hippocampus.

We next examined the expression levels of neuroinflammation-associated genes in the cortex of MgD mice. *Angptl7*, *Spp1*, *Timp1*, *Cxcl10*, *Ccl5*, and *Ccl7* mRNA levels were significantly increased in the cortex of MgD mice compared to Ctrl mice (Figure 4B). Thus, dietary MgD increases the expression of neuroinflammation-associated genes in the cortex as well as the hippocampus, suggesting that MgD induces neuroinflammation in the mouse brain.
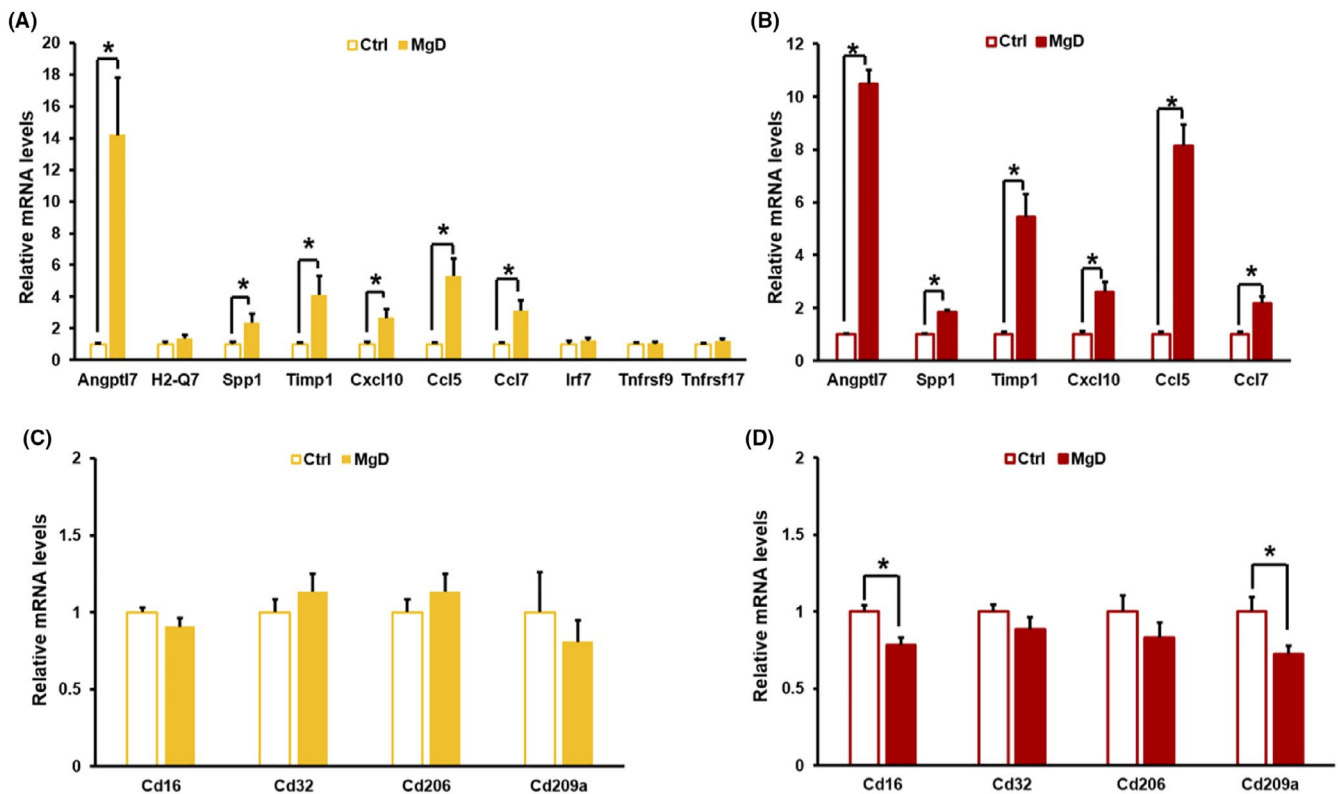

FIGURE 4 Changes in the mRNA levels of neuroinflammation-associated genes in the hippocampus and cortex of Mg^{2+} deficiency (MgD) mice. (A) Changes in mRNA levels in the hippocampus of MgD mice. *Angptl7*, *H2-Q7*, *Timp1*, *Cxcl10*, *Ccl5*, and *Ccl7* (control [Ctrl], n = 8; MgD, n = 6) and *Spp1* (Ctrl, n = 11; MgD, n = 9). (B) Increases in mRNA levels in the cortex of MgD mice (Ctrl, n = 8; MgD, n = 7). (C) No changes in the mRNA levels of M1 (Cd16, Cd32) and M2 (Cd206, Cd209a) microglia markers in the hippocampus of MgD mice (Ctrl, n = 11; MgD, n = 9). (D) Changes in the mRNA levels of microglia markers in the cortex of MgD mice (Ctrl, n = 8; MgD, n = 7). *$P < 0.05$. Error bars indicate standard error of the mean.

The activation of M1 microglia is related to the induction of neuroinflammation. To examine whether microglia are activated by dietary MgD, we examined the mRNA levels of activation markers for M1 and M2 microglia, in the hippocampus and cortex. The mRNA levels of Cd16 (M1 marker) and Cd209a (M2 marker), but not Cd32 (M1) and Cd206 (M2), mRNAs were significantly decreased in the cortex, while no changes of mRNAs were observed in the hippocampus (Figure 4C,D), suggesting that the activity of M1 and M2 microglia is negatively regulated in the cortex, but not in the hippocampus, by dietary MgD (Figure 4D).

Collectively, we found that MgD upregulates the expression of neuroinflammation-associated genes in the hippocampus and cortex, suggesting that MgD induces neuroinflammation in the mouse brain. Previous studies have shown that neuroinflammation in the brain impairs learning and memory.[23,24] Therefore, our findings raise the possibility that MgD-induced neuroinflammation impairs hippocampus-dependent memories such as contextual fear, spatial, and social recognition memories. Additionally, future studies are required to examine effects of MgD on cortex-dependent memories since upregulation of neuroinflammation-associated genes was observed in the cortex.

Similar to MgD, high-fat diet (HFD) feeding induces neuroinflammation and impairs hippocampus-dependent spatial memory.[25–27]. Importantly, a recent study showed that HFD feeding increases microglial activation and dendritic spine loss in the hippocampus.[26] In contrast, no obvious activation of microglia (Figure 4C,D) or spine loss[13] was observed in the hippocampus of MgD mice. Therefore, it is possible that dietary MgD induces weaker neuroinflammation compared to an HFD, although this level of neuroinflammation is sufficient for dysfunction of hippocampus-dependent memory performance. Further studies are required to investigate the relationship between the upregulation of neuroinflammation-associated genes and impaired hippocampus-dependent memory in MgD mice. Importantly, downregulation of makers of M1 (proinflammatory) and M2 (anti-inflammatory) microglia was observed in the cortex, raising the possibility that altered activities of M1 and M2 microglia have influences on the progression of neuroinflammation and memory function. It is important to further investigate effects of MgD on activities of M1 and M2 microglia in the cortex and cortex-dependent memory to understand mechanisms of MgD-induced neuroinflammation and memory impairments.

CONFLICT OF INTEREST

None.

AUTHORS CONTRIBUTION

SK is responsible for the hypothesis development and overall design of the research and experiment and supervised the experimental analyses. RT and SK cowrote the paper. RT performed MgD treatments, RNA-sequencing, and qRT-PCR. HI and MU contributed to the overall design of the experiment and prepared the control and MgD diets. All authors read and approved this paper.

ANIMAL STUDIES

All animal experiments were approved by the Animal Care and Use Committee of Tokyo University of Agriculture.

DATA AVAILABILITY STATEMENT

All relevant data are included in Supporting Information.

ORCID

Satoshi Kida https://orcid.org/0000-0002-8038-9583

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SUPPORTING INFORMATION

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