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ORIGINAL RESEARCH PAPER

# Intramuscular injection of mechano growth factor E domain peptide regulated expression of memory-related *sod*, miR-134 and miR-125b-3p in rat hippocampus under simulated weightlessness

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**Abstract**
*Objective* To investigate the expression of memory-related antioxidant genes and miRNAs under simulated weightlessness and the regulation of mechano growth factor (MGF) E domain, the peptide preventing nerve damage.
*Results* *Igf-iea* and *mgf* mRNA levels, expression of antioxidant genes *sod1* and *sod2* and levels of miR-134 and miR-125b-3p increased in rat hippocampus after 14 days tail suspension to simulate weightlessness which was inhibited with intramuscular injection of E domain peptide. Therefore, administration of MGF E domain peptide could reverse increased expressions of memory-related *igf-iea*, *mgf*, *sod1*, *sod2*, miR-134 and miR-125b-3p in rat hippocampus under simulated weightlessness.
*Conclusions* MGF may regulate the redox state and miRNA-targeted NR-CREB signaling, and intramuscular injection may be the alternative administration because of its safety, convenience and ability to pass through the blood brain barrier.

**Keywords** Hippocampus · Intramuscular injection · Mechano growth factor · miRNAs · Simulated weightlessness · SOD

## Introduction

Space microgravity environment has lasting effects on the nervous system, which can lead to space motion sickness, space adaptation syndrome and cognitive disorders. The cognitive decline is also found under simulated microgravity such as head-down bed rest (HDBR) in humans and tail suspension in animals. The model of tail suspension induces cephalic fluid shift thus generating fluid redistribution and balance to simulate the fluid shift under microgravity condition (Frigeri et al. 2008). For example, the executive functions and the high-order aspects of cognition functions, were significantly diminished (Liu et al. 2012), with a detrimental effect on individual physiology and working memory (Liu et al. 2015) during

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HDBR. Furthermore, hippocampus-dependent spatial memory was depressed in mice (Wu et al. 2000) and in rats (Chen et al. 2013) after tail suspension. However, the mechanism remains unclear and there is no effective protective measure until now.

Antioxidases, such as glutathione peroxidase (GPX), superoxide dismutase (SOD) as well as catalase (CAT), can improve memory in mice with brain injury, Alzheimer's disease or aging (Tsuru-Aoyagi et al. 2009; Massaad et al. 2009; Clausen et al. 2010; Lee et al. 2014). MiRNAs are small noncoding RNAs that regulate gene expression post-transcriptionally. Prior studies have shown that miRNAs played important role in memory formation-related synaptic plasticity (Aksoy-Aksel et al. 2014), neuronal activity (Sim et al. 2014) and brain development and function (Follert et al. 2014).

Insulin-like growth factor I (IGF-I), as a polypeptide, is a multipotent growth factor regulating cell proliferation, apoptosis (Yamahara et al. 2015), differentiation (Zhang et al. 2014), and so on. *Igf-i* gene has 6 exons. Splicing of *exon 4* to *exon 6* or *exon 5–6* leads three mRNAs, *igf-iea*, *igf-ieb* and *igf-iec*, encoding the common mature IGF-I peptide, from the highly conserved *exons 3* and *4*, and the different E peptides Ea (in humans and rats), Eb (in humans) and Ec (in humans) whose counterpart is the Eb peptide in rodents. Rodent Eb or human Ec peptide is also called mechano growth factor (MGF). In humans, the three E peptides all have a common constitutive sequence of 16 amino acids (aa) from *exon 4* and an active E domain, which of Ea, Eb and Ec contains 19 aa from *exon 6*, 61 aa from *exon 5* and 24 aa from *exons 5* and *8*, respectively (Vassilakos et al. 2014).

The E domain of pro-IGF-I may have a role in regulating cell growth and differentiation in neuroblastoma cells (Vassilakos et al. 2014; Matheny et al. 2010). The carboxy terminal sequence of MGF with 24 aa, when given by intramuscular injection, improves muscle function (Vassilakos et al. 2014; Matheny et al. 2010), slows down age-related loss of muscle mass (Kandalla et al. 2011), and attenuates myocardial infarction (Peña et al. 2015). Furthermore, MGF is a neuroprotective agent and can protect neurons against brain ischemia in gerbils with carotid artery administration (Dluzniewska et al. 2005), prevent dopaminergic cells loss from 6-hydroxydopamine (6-OHDA) (Quesada et al. 2009) and protect SH-SY5Y cells against 6-OHDA-induced cell death (Quesada et al. 2009, 2011). This suggests that MGF may improve memory impairment because of its neuroprotective effects. Otherwise, both gene expression and protein expression of MGF were decreased with memory damage in tail-suspended rats in our previous study (Chen et al. 2013). However, it is unclear how MGF may regulate the expression of memory-related antioxidases and miRNAs. Intramuscular injection of a growth factor such as IGF-I could both ameliorate muscle function and prevent neuronal apoptosis induced by brain ischemia (Chang et al. 2010, 2013). Whether MGF, or another growth factor, could regulate the expressions of memory-related molecules by intramuscular injection remains unclear.

Therefore, this study is aimed to investigate the expressions of antioxidant genes related with memory and miRNAs targeting memory-related proteins under simulated weightlessness and the regulation effects of intramuscular injection of MGF, which can be helpful to understand the vital effect of MGF on the memory and provide a more safe and convenient mode of administration because of its ability to pass through the blood brain barrier.

## Materials and methods

### Reagents

A 24 aa peptide (YQPPSTNKNTKSQRRKGSTFEEHK), a modified human Ec peptide, containing at the position 23 a histidine (H) instead of the arginine (R) of the original human Ec peptide, corresponding to the unique *C*-terminal E domain region of the human MGF was synthesized and purified to >90 % by HPLC (Genescript Corp, NJ). The peptide was stabilized by amidating the *C*-terminus and switching the arginines at positions 14 and 15 to the *D*-stereoisomer. All other general chemicals made in China were of analytical grade.

### Animals and treatment protocol

Male Sprague–Dawley rats (110–130 g) were housed at 23 ± 2 °C under 12-h light and dark cycles, were given a standard rodent unpurified diet (32 % protein, 14 % fat, and 54 % carbohydrate) and allowed access to food and water ad libitum. This study was carried out in strict accordance with the recommendations in

the Guide for the Care and Use of China Astronaut Research and Training Center. The protocol was approved by the Institutional Animal Care & Use Committee (IACUC) of China Astronaut Research and Training Center (Permit Number: ACC-IACUC-2012-003). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Six groups (n = 8 in each group) of rats were used (Fig. 1). The hindlimbs of the tail-suspended group were elevated to a spinal orientation of 30° above horizontal while the forelimbs were free to ambulate around the entire range of the cage. Rats in the groups with tail suspension plus MGF E domain peptide treatment were injected with 0.1 ml peptide (1 μg/0.1 ml saline) in muscle membrane of gastrocnemius on the second day after tail suspension for 7 or 14 days. The control and tail-suspended groups were received same volume, but saline alone. All rats were anaesthetised and the hippocampus and cerebral cortex were isolated from head and immediately stored in liquid nitrogen (−196 °C) until RT-qPCR detection.

RNA extraction

Total RNA was extracted from the hippocampus and cerebral cortex with TRIzol. The amount of RNA was quantified from the $A_{260}/A_{280}$ ratio. RNA quality was also determined by electrophoresis on an ethidium bromide-stained 1.5 % agarose gel.

Reverse transcription and qPCR for gene expression

One μg DNase-treated (Takara) total RNA was subsequently reverse transcribed to cDNA using random hexamers (Takara) and reverse transcriptase (Takara). Primer pairs were optimized for annealing temperature and specificity was confirmed by melting curve analysis. QPCR was carried out using SYBR Green Fluorescein mix (Takara), with an initial activation step of 95 °C for 1 min followed by 45 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 15 s) and extension (72 °C, 15 s), and final melting curve analysis. Expression levels were

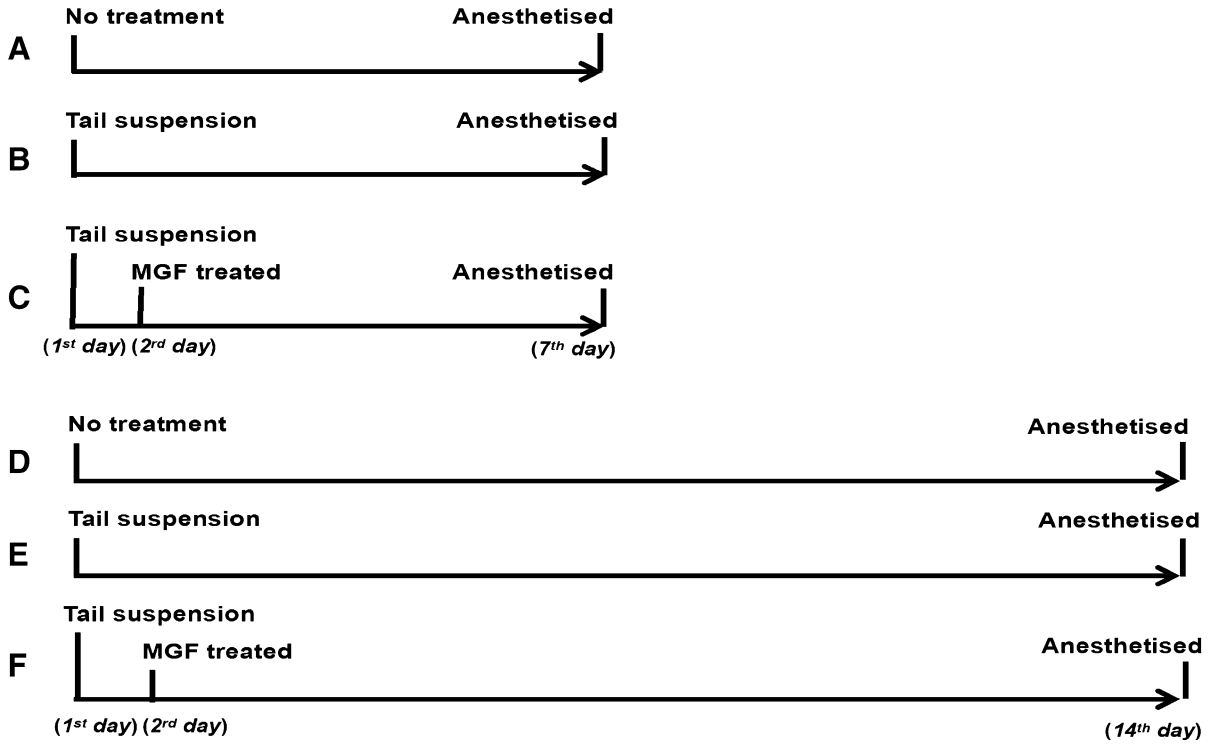



**Fig. 1** Animal groupings

normalized against the endogenous *gapdh* control. The primers sequences used for qPCR (*igf-iea*, *mgf*, *sod1*, *sod2*, *gpx1* and *cat*) were based on published sequences in GenBank Overview (http://www.ncbi.nlm.nih.gov/genbank/). They were designed using Primer premier 5 program version 5.0.0 and custom made by Invitrogen (Supplementary Table 1).

### Reverse transcription and qPCR for miRNA expression

One μg DNase-treated total RNA was added to Poly(A) tail with Poly(A) Polymerase and subsequently reverse transcribed to cDNA with PrimeScript RTase using One Step PrimeScript miRNA cDNA Synthesis Kit (Takara). Primers were optimized for annealing temperature and specificity was confirmed by melting curve analysis. QPCR was carried out using SYBR Green Fluorescein premix Ex Taq TM II (Takara) and Uni-miR qPCR primer (Takara), with an initial activation step of 95 °C for 1 min followed by 45 cycles of denaturation (95 °C, 15 s), annealing (60 °C, 15 s) and extension (72 °C, 15 s), and final melting curve analysis. Expression levels were normalized against the endogenous control (5 s rRNA). The forward primers sequences used for qPCR (miR-124, miR-134, miR-132, miR-138, miR-125b-3p, miR-125b-5p and miR-145) were designed using Primer premier 5 program version 5.0.0 and custom made by Invitrogen (Supplementary Table 1).

### Statistical analysis

Data are expressed as mean ± SEM. Differences in the means were tested using Student' s t-test and analysis of variance. Bonferroni post hoc tests were used when the p value indicated a significant difference between the groups. A *P*-value of 0.05 was considered statistically significant.

## Results

### Mgf mRNA expression increases in rat hippocampus after 14-day tail suspension but is inhibited by treatment with E domain peptide

The expressions of *igf-iea* and *mgf* were detected under simulated microgravity and treatment with MGF E domain peptide. After tail suspension for 7 days, expression of *igf-iea* and *mgf* increased by 0.4-fold and 0.7-fold, respectively, in the hippocampus ($P > 0.05$) and there was no effect of peptide treatment on the expressions of these isoforms (Fig. 2a); expression of *igf-iea* or *mgf* had no change in cerebral cortex but the peptide significantly inhibited *mgf* expression by 50 % ($P < 0.01$) (Fig. 2b). After tail suspension for 14 days, expression of *igf-iea* and *mgf* increased by 1.7-fold ($P < 0.01$) and 0.4-fold ($P < 0.05$), respectively in the hippocampus but the peptide inhibited the high levels of *igf-iea* ($P < 0.05$) and *mgf* ($P > 0.05$) (Fig. 2c); no change of *igf-iea* mRNA but excessive *mgf* mRNA level by 0.9 fold ($P < 0.05$) in cerebral cortex and no effects of peptide treatment were found (Fig. 2d).

### Expressions of sod1 and sod2 are slightly upregulated in rat hippocampus after 14-day tail suspension but recover after treatment with E domain peptide

The expressions of the antioxidant genes, *sod1*, *sod2*, *gpx1* and *cat,* were measured. After tail suspension for 7 days, expressions of *sod1* and *sod2*, in the hippocampus, had no change but, after E domain peptide treatment, reduced by 25 and 23 %, respectively ($P < 0.05$), but those of *gpx1* or *cat* were not affected after tail suspension or peptide treatment (Fig. 3a). Expressions of all antioxidant genes had no change after either tail suspension or peptide treatment (Fig. 3b). Analogously, after tail suspension for 14 days, expressions of *sod1* and *sod2* were slightly upregulated in the hippocampus ($P > 0.05$) but recovered with peptide treatment ($P < 0.05$), but those of *gpx1* or *cat* were not affected after tail suspension or peptide treatment (Fig. 3c). Expression of *sod1* in the cerebral cortex, lessened by 66 % and then partly recovered with peptide administration ($P < 0.05$) but the expressions of other antioxidant genes did not change with tail suspension or peptide treatment (Fig. 3d).

### Screen of miRNAs and their targeted memory-related proteins

MiRNAs targeting memory-related proteins were also screened according to previous reports. Six miRNAs (miR-124, miR-134, miR-132, miR-138, miR-125b

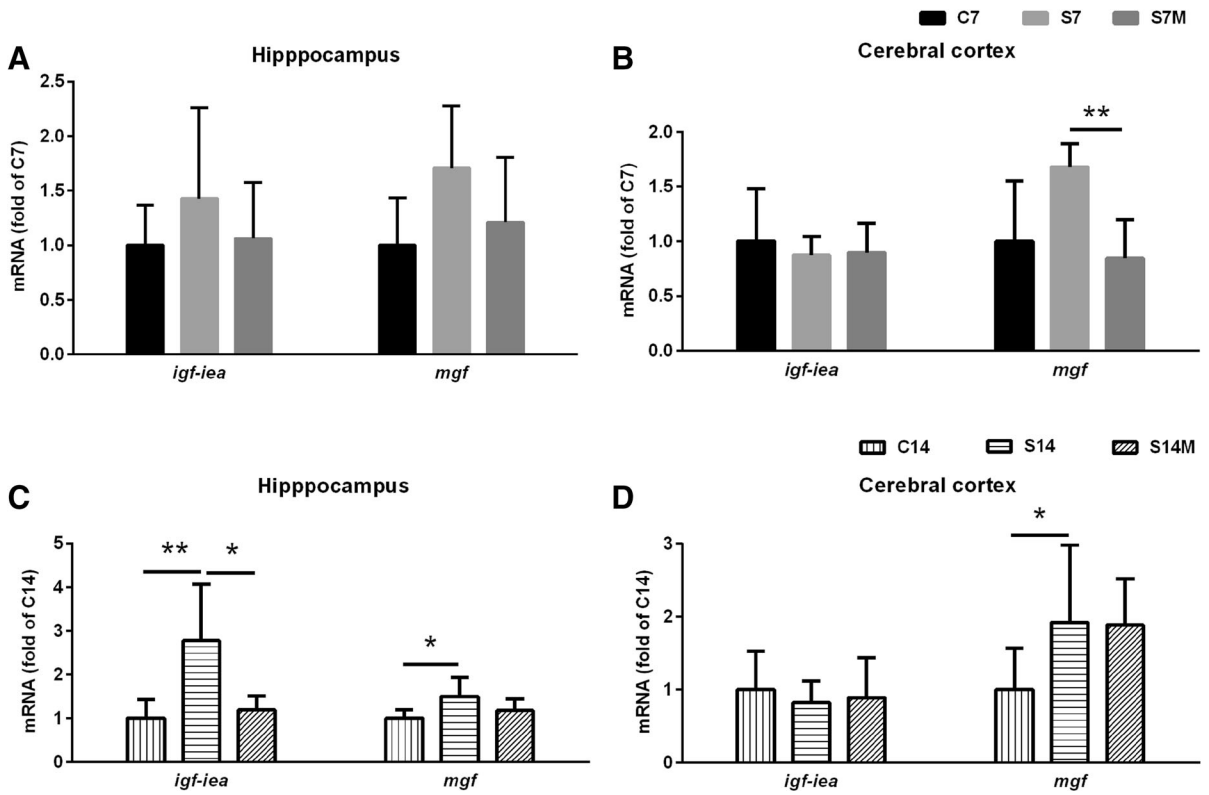



**Fig. 2** *Mgf* mRNA expression increases after 14-day tail suspension but is inhibited by E domain peptide treatment in rat hippocampus. Data are expressed as mean ± SEM, *P < 0.05, **P < 0.01. C7, control for 7 days; S7, tail suspension for 7 days; S7M, tail suspension with E domain peptide treatment for 7 days; C14, control for 14 days; S14, tail suspension for 14 days; S14M, tail suspension with E domain peptide treatment for 14 days

and miR-145) targeted memory-related proteins such as cAMP-response element binding protein (CREB), methyl CpG binding protein 2 (MeCP2), acyl protein thioesterase 1 (APT1), *N*-methyl-d-aspartate receptor subunit 2A (NR2A) and superoxide dismutase 2 (SOD2), and function in memory-related morphogenesis, plasticity and physiology of synapses and neuroprotection (Table 1).

## Expression of miRNAs targeting memory-related proteins in rat hippocampus and cerebral cortex

Expression of these miRNAs were analyzed in the rat hippocampus and cerebral cortex by smiRNAdb, a database containing expression information for human, mouse, rat, zebrafish, worm and fruitfly small RNAs. Mature miR-125b has two types, miR-125b-3p and miR-125b-5p. Expressions of most miRNAs were all moderate although those of miR-145, miR-125b-3p and miR-134 were low in the hippocampus and cerebral cortex. In addition, expression of all miRNAs in hippocampus was higher than that in cerebral cortex (Fig. 4a). To verify these results, we measured the levels of the miRNAs by RT-qPCR. All miRNAs in hippocampus were richer compared with that in cerebral cortex (Fig. 4b).

## Levels of miR-134 and miR-125b-3p rise in the rat hippocampus after 14-day tail suspension but are reversed after treatment with E domain peptide

The levels of these miRNAs were measured after tail suspension and peptide treatment. After 7 days, there was no change in miRNA expression in the hippocampus or in the cerebral cortex but elevated miR-125b-5p in cerebral cortex was found after tail suspension or E domain peptide treatment (Fig. 5a, b). After 14 days, only expressions of miR-134 and miR-125b-3p rose in the rat hippocampus ($P > 0.05$) but were reversed with peptide treatment ($P < 0.05$)

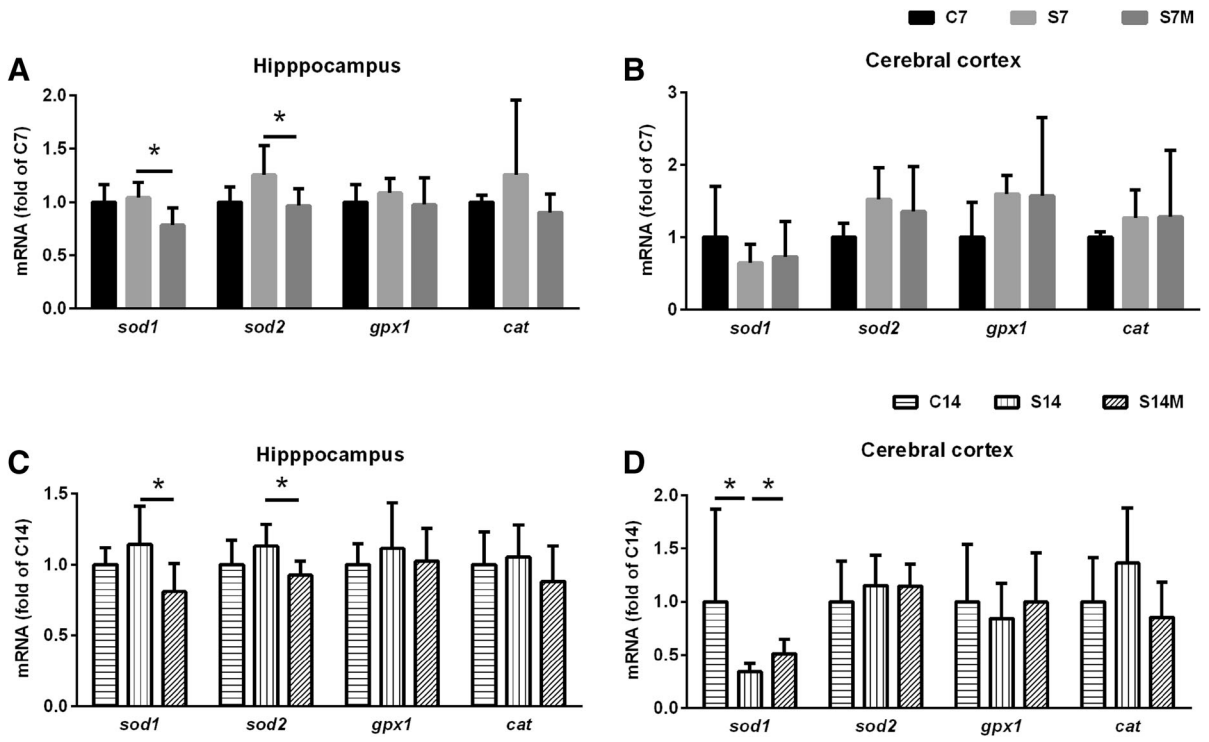



**Fig. 3** Expression of *sod1* and *sod2* are slightly upregulated after 14-day tail suspension but recover with E domain peptide treatment in the rat hippocampus. Data are expressed as mean ± SEM, *P < 0.05. C7, control for 7 days; S7, tail suspension for 7 days; S7M, tail suspension with E domain peptide treatment for 7 days; C14, control for 14 days; S14, tail suspension for 14 days; S14M, tail suspension with E domain peptide treatment for 14 days

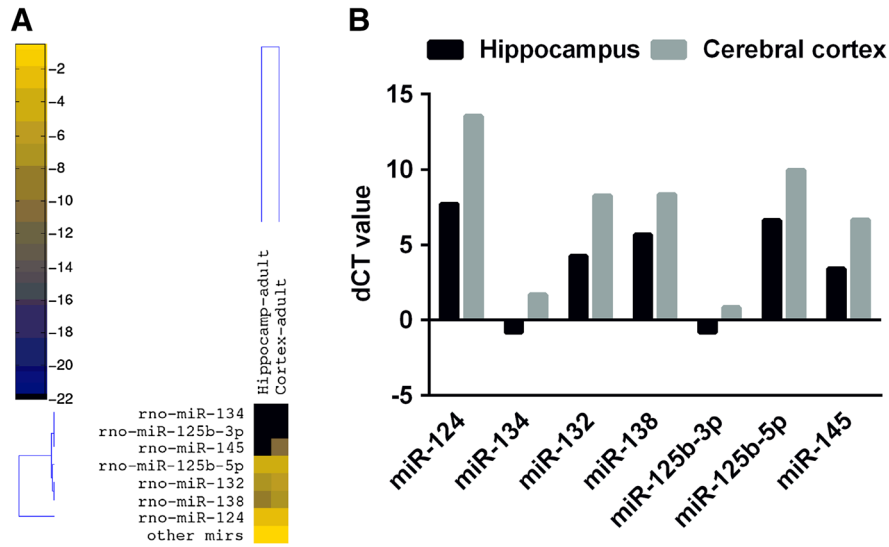
**Table 1** MiRNAs and their targeted memory-related proteins

| MiRNAs | Target proteins | Function related with memory | Reference |
|---|---|---|---|
| miR-124 | CREB | Long-term plasticity of synapses | Rajasethupathy et al. (2009) |
| miR-134 | CREB | Synaptic plasticity and memory formation | Gao et al. (2010) |
| miR-132 | MeCP2 | Synaptic plasticity | Lusardi et al. (2010) |
| miR-138 | APT1 | Dendritic spine morphogenesis | Siegel et al. (2009) |
| miR-125b | NR2A | Dendritic spine morphology and synaptic physiology | Edbauer et al. (2010) |
| miR-145 | SOD2 | Neuroprotection in the postischemic brain | Dharap et al. (2009) |

(Fig. 5c); no miRNA expression was changed with either tail suspension or peptide treatment in cerebral cortex, nevertheless, level of miR-134 rose after peptide treatment (P < 0.05) (Fig. 5d).

## Discussion

Hippocampus and cerebral cortex are critical for spatial memory (Griffin, 2015) which declines under microgravity. Therefore, the expressions of memory-related molecules such as *mgf*, *igf-iea*, antioxidases, and miRNAs were examined in tail-suspended rat hippocampus and cerebral cortex. However, expressions of *igf-iea*, *mgf*, *sod1*, *sod2*, miR-134 and miR-125b-3p changed with tail suspension in the hippocampus not in the cerebral cortex. Changes occurred in structural proteins and proteins involved in metabolism in hippocampus in response to the microgravity environment (Sarkar et al. 2006). Expressions of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) were also changed in



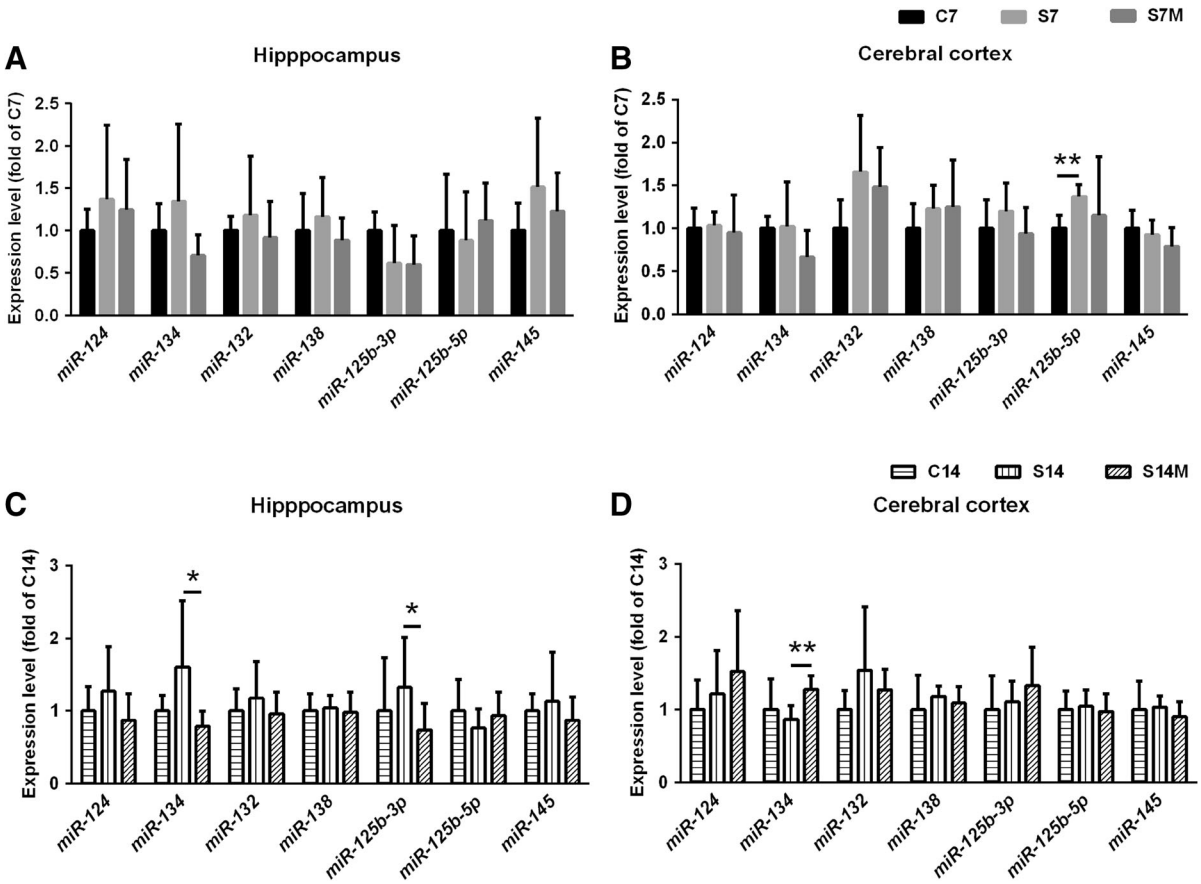




**Fig. 5** Levels of miR-134 and miR-125b-3p rise after 14-day tail suspension but are reversed with E domain peptide treatment in the rat hippocampus. Data are expressed as mean ± SEM, *P < 0.05, **P < 0.01. C7, control for 7 days; S7, tail suspension for 7 days; S7M, tail suspension with E domain peptide treatment for 7 days; C14, control for 14 days; S14, tail suspension for 14 days; S14M, tail suspension with E domain peptide treatment for 14 days

the hippocampus after space microgravity environment (Santucci et al. 2012). These results suggest that the hippocampus is relatively sensitive area in brain under microgravity.

Expressions of *mgf*, *sod1*, *sod2*, miR-134 targeting cAMP-response element binding protein (CREB) and miR-125b-3p targeting NMDA receptor (NR2A) were all upregulated in rat hippocampus after 14 days tail suspension. During neuron protection, MGF and SOD could protect neurons and prevent against oxidative stress, respectively. MGF may regulate the expression of SOD through activation of nuclear factor erythroid-2-related factor 2 (NRF2) (Quesada et al. 2011), an antioxidant protein and transcription factor, directly promoting the transcription of *sod* gene (Ruiz et al. 2013). During memory formation, NR2A-containing synaptic NMDA receptors leads to activation of CREB, long-term potentiation (LTP) and neuronal survival (Hardingham et al. 2002), which was inhibited by miR-134 and miR-125b-3p through post-transcriptional pathways. Moreover, no change of *gpx1*, *cat*, miR-124, miR-132, miR-138, miR-125b-5p or miR-145 was found in the hippocampus after 14 days. For those reasons, there may be two effects on hippocampus-dependent memory under simulated microgravity. The positive effect is MGF-NRF2-SOD mediated neuroprotective pathways and the negative is miR-134 and miR-125b-3p-mediated inhibition of NR2A-CREB pathway which were needed to be confirmed by the further research on the expressions of those proteins. Previously we had found a decrease in the expression of mRNA and protein for MGF in the hippocampus in the rat model of tail-suspension that was associated with a decrease in spatial memory. In the current work, however, as seen in Fig. 2c an increase in expression of IGF-IEa and MGF was seen in the hippocampus in the same model. The difference between these two studies may be related with that the decrease expression of MGF in previous work appeared after the training for spatial memory test.

Expression of *igf-iea* mRNA also increased in the rat hippocampus after 14 days tail suspension. MGF was more effective than insulin-like growth factor-I (IGF-I) Ea domain for protection of neurons (Aperghis et al. 2004). Different IGF-I isoforms Ea and MGF, probably indicate different and biological effects under various conditions (Philippou et al. 2014). There may be synergistic or complementary effects between IGF-I Ea and MGF under simulated microgravity which will be determined in the next study. Moreover, the responses to MGF E peptide treatments do not appear to be isoform (MGF)-specific, as might be expected.

No change of *igf-iea*, *mgf*, antioxidant genes or miRNAs was found in the rat hippocampus after 7 days tail suspension, which suggested that the expression of memory-related molecules was time specific in hippocampus under simulated microgravity. Expressions of other genes were unchanged except for increased *mgf* and decreased *sod1* after 14 days and increased miR-125b-5p after 7 days in rat cerebral cortex. This suggests the different effect of simulated microgravity and the various responses on cerebral cortex from hippocampus.

Interestingly, peripheral administration of MGF E domain peptide recovered the expressions of *igf-iea*, *mgf*, *sod1*, *sod2*, miR-134 and miR-125b-3p in hippocampus after 14 days tail suspension, as well as those of *mgf* after 7 days and *sod1* and miR-134 after 14 days in cerebral cortex. The positive and negative effects may play as adaptive responses for simulated microgravity, but, on the other hand, MGF may be able to maintain the steady regulation of memory process which is not excessive or inadequate because the effective and moderate neuroprotection of MGF (Dluzniewska et al. 2005) and there may also be negative feedback for MGF from periphery to central. Expressions of *sod1* and *sod2* were reversed in hippocampus with 7 days tail suspension plus peptide treatment which suggested an early effect of MGF on SOD level. These will be become clearer with further study.

The functional sequence of MGF, as well as its downstream signaling pathways remain unclear. Other research has shown that MGF may activate ERK through an IGF-IR-independent mechanism (Matheny et al. 2010).

## Conclusion

Intramuscular injection of MGF E-domain could reverse the increased expression of memory-related *sod1*, *sod2*, miR-134 and miR-125b-3p in rat hippocampus under simulated weightlessness. This suggests that MGF could regulate the antioxidase SOD and miR-134-targeted and miR-125b-3p-targeted NR-CREB signaling involved in memory formation in the

hippocampus under simulated microgravity and intramuscular injection may be the alternative administration because of its safety, convenience and ability to pass through the blood brain barrier. Further work will focus on whether the MGF E domain peptide plays a positive role in the memory process and expression of memory-related antioxidases and miRNAs.

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**Supporting information** Supplementary Table 1—sequences of qPCR primers for rats

**Compliance with ethical standards**

**Conflicts of interest** The authors report no conflicts of interest.

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