Magnesium supplement enhances spatial-context pattern separation and prevents fear overgeneralization

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Enhancement of pattern separation could be helpful in improving the quality of normal daily learning and in treating individuals with cognitive impairment and certain psychiatric disorders. Previously, we have shown that elevating brain magnesium, by a novel magnesium compound (magnesium-L-threonate; MgT), enhances extinction of fear memory without enhancing amygdala-dependent fear memory. Here, we investigated the effects of MgT treatment on contextual-fear memory and subsequent pattern separation. Sprague–Dawley male rats were treated with MgT for 4 weeks and memory was evaluated using a spatial-context fear conditioning task. The pattern separation ability of MgT-treated rats was assessed using a spatial-context-discrimination task. MgT treatment did not enhance the retention of contextual-fear memory. Interestingly, the ability to discriminate between two, more or less distinct, contexts was enhanced in MgT-treated rats. Our results suggest that elevation of brain magnesium might be helpful in enhancing spatial-context discrimination and/or pattern separation besides preventing aversive-event-induced overgeneralization of fear. *Behavioural Pharmacology* 00:000–000 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Pattern separation is defined as the cognitive processes during which overlapping or similar representations are transformed into two separate memory engrams. It is critical for determining distinct features of different episodes of daily life. Identification of a cognitive enhancer to boost pattern separation is important not only to improve the quality of normal daily learning but also to treat individuals with cognitive impairment and certain psychiatric disorders. For example, pattern separation decreases during normal aging and is impaired in individuals with mildcognitive impairment (Toner *et al.*, 2009; Stark *et al.*, 2010). Patients with post-traumatic stress disorder re-experience symptoms in response to novel contexts that are reminiscent of the original one associated with the traumatic event (Ehlers *et al.*, 2004). Furthermore, depressed patients often negatively overgeneralize autobiographical memories in response to emotion-related cues (Williams and Broadbent, 1986; Sumner *et al.*, 2011).

Contextual-fear conditioning and subsequent spatial-context discrimination tasks are among the experimental paradigms used to evaluate pattern separation (McHugh *et al.*, 2007). At the anatomical level, contextual representations in such paradigms are believed to be assembled by the hippocampus (Maren and Holt, 2000; Anagnostaras *et al.*, 2001). The hippocampus is also involved in pattern separation, as supported by theoretical (Marr, 1971; McNaughton and Morris, 1987) and experimental data (Gilbert *et al.*, 2001, for a human study Yassa *et al.*, 2011; Nakashiba *et al.*, 2012). Thus, enhancing hippocampal functions might improve pattern separation. Indeed, the enhancement of hippocampal neurogenesis, synaptic plasticity/density, and brain-derived neurotropic factor (BDNF) expression triggered by environmental enrichment enhances pattern separation in rodents (Van Praag *et al.*, 2000; Creer *et al.*, 2010; Bekinschtein *et al.*, 2011).

We found previously that elevating brain magnesium by a novel magnesium compound, magnesium-L-threonate (MgT), enhances hippocampus-dependent spatial learning and pattern completion in rats (Slutsky *et al.*, 2010). Recently, we also showed that MgT treatment enhances fear extinction without affecting the amygdala-dependent delay-cued fear memory (Abumaria *et al.*, 2011). Mechanistically, MgT treatment upregulates signaling of NMDA receptors (NMDAR), BDNF expression, and enhances synaptic plasticity/density in the hippocampus (Slutsky *et al.*, 2010) and prefrontal cortex, but not in the amygdala (Abumaria *et al.*, 2011).

Here, we used MgT treatment as a tool to study whether elevation of brain magnesium may affect contextual-fear memory and contextual pattern separation. Unexpectedly, MgT treatment did not enhance the contextual-fear memory. These findings were confirmed using several conditioning protocols involving modifications of context exposure times, footshock (number, intensity, and duration), and following repeated conditioning. We also conducted two experiments to explore the effects of

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MgT treatment on contextual pattern separation using a spatial-context discrimination task (as described before, McHugh *et al.*, 2007). Interestingly, following strong conditioning protocols, we found that MgT treatment enhanced spatial-context discrimination and/or pattern separation in rats despite its lack of effect on spatial-contextual fear (irrespective of the strength of conditioning).

Methods

Experimental animals

Male Sprague–Dawley rats (4–6 months old) were obtained from Vital River Laboratory (Animal Technology Co. Ltd, Beijing, China). All rats were individually housed with free access to food and water under a 12:12 h reversed light–dark cycle. The individual housing of rats allowed monitoring of the MgT dose to ensure that every rat consumed the target dose as much as possible (MgT dose was given through drinking water; see below). To reduce any potential stress that might be induced by individual housing, rats were housed in transparent Plexiglas cages that were kept close to each other so each rat remained in visual, olfactory, and auditory contact with neighboring rats. Furthermore, over the experimental time course, each rat was handled every second day for 2 min. Behavioral experiments were conducted during the dark phase. All experiments involving animals were approved by the Tsinghua University Committee on Animal Care and Use.

Magnesium-L-threonate treatment

Eighty-three rats were assigned randomly to control and MgT-treated groups. MgT (Magceutics Inc., Hayward, California, USA) was administered through drinking water (604 mg/kg/day, MgT; 50 mg/kg/day elemental magnesium) as described before (Slutsky *et al.*, 2010; Abumaria *et al.*, 2011). Briefly, the water intake (on a daily basis) and body weight (every 3 days) were measured. The concentration of MgT in drinking water was determined and adjusted, on the basis of these parameters, to reach the target dose. Before all behavioral experiments were commenced, MgT treatment was provided for 4 weeks. We have shown previously that this course of treatment can effectively elevate brain magnesium (Slutsky *et al.*, 2010) without observing side effects or changes in locomotor activity (Slutsky *et al.*, 2010) and basal freezing behavior (Abumaria *et al.*, 2011). Rats received rat chow containing 0.15% elemental magnesium, which is broadly accepted as the basic nutritional magnesium concentration.

Conditioning apparatus

Experiments were conducted using a video-based fear-conditioning system (Coulbourn Instruments, Allentown, Pennsylvania, USA). Rats were conditioned in modular fear-conditioning chambers (rat test cage) with a metal stainless-steel rod floor connected to a shock generator. Each chamber is placed inside a sound-insulating cubicle. Stimulus presentation was automated using FreezeFrame2 software (Actimetrics, Evanston, Illinois, USA).

Contextual modifications

The conditioning chambers were modified to change the context from A to B or A′. Context A (the initial conditioning context) consisted of the original conditioning chamber with white light on the left side, background noise was ~54 dB, and 20% ethanol odor. In context B, the metal grid floor was replaced by a smooth plastic board, the door was covered with a white paper, background noise was lowered (from 54 to 42 dB), and the odor was changed (20% ethanol replaced by 2% acetic acid). In context A′, the door of the chamber was covered with a white paper, background noise was lowered (from 54 to 42 dB), and the odor was changed (20% ethanol replaced by 2% acetic acid). The stainless-steel grid floor was not removed from context A′.

Monitoring of freezing behavior

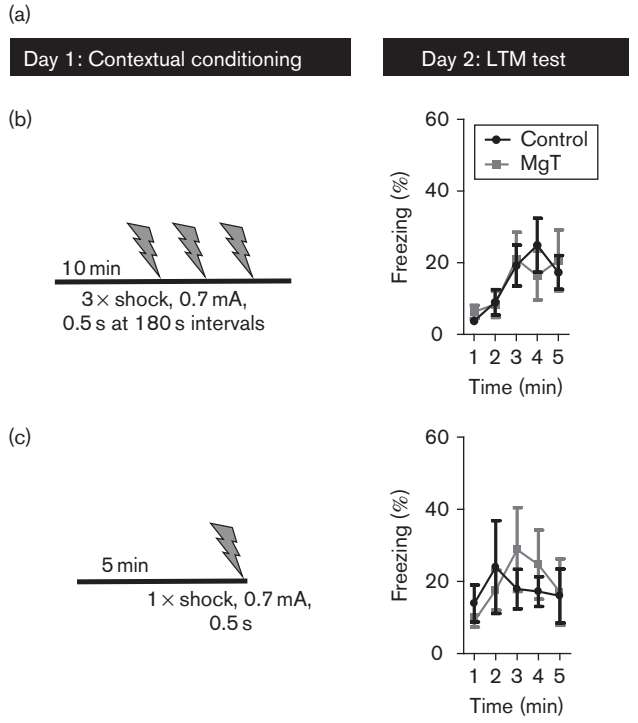
The total amount of context-induced freezing as a percentage of the total exposure time was used as a measure of fear. Freezing behavior was measured with an automated system using Freeze View 2 software (Actimetrics). The software measures changes in pixel luminance intensity across successive video frames (taken at 1–5 Hz) and computes changes in motion as these two parameters correlated linearly (Actimetrics). A threshold is then applied to the data to yield a percentage freezing score. In our case, frames were taken at a rate of 3.12 Hz.

Contextual-fear conditioning and evaluation of context memory

Two cohorts of rats (each included control and MgT-treated rats) were prepared to conduct the two independent experiments presented in Fig. 1b and c. In all experiments, the rats were conditioned and tested (24 h later) in the original context (context A). Pre-exposure (habituation) to context, 24 h before conditioning, was shown to induce ceiling effects on freezing behavior, which obscures the detection of enhancement of contextual-fear memory (Huff *et al.*, 2005). Therefore, to maximize the chance of detecting enhancement of contextual-fear memory by MgT treatment, the rats were not pre-exposed to contexts before the conditioning day.

In the experiments presented in Fig. 1b, we evaluated the effects of MgT treatment on contextual-fear memory. Because this evaluation of contextual-fear memory under MgT treatment has not been performed before, we began with a conditioning protocol that was identical to the delay-cued and trace-cued fear conditioning protocols, which we used before (Abumaria *et al.*, 2011), but without tone presentations. Each rat was placed in the conditioning chamber for 10 min, followed by three unsignaled electrical footshocks (0.7 mA for 0.5 s, intershock interval = 180 s). The rat was removed from the chamber 180 s

Fig. 1



Effects of magnesium-L-threonate (MgT) treatment on retention of contextual-fear memory. (a) Experimental design showing the contextual-fear conditioning (day 1) and a long-term contextual-fear memory (LTM) test 24 h later (day 2). (b) Left: illustration of the contextual-fear conditioning protocol. Right: freezing behavior of control and MgT-treated rats during the 5 min contextual LTM test performed 24 h after conditioning. (c) Same as (b) but using a weaker contextual conditioning protocol. Data were analyzed using two-way repeated-measures analysis of variance. Data presented as mean±SEM.

after the last footshock (Fig. 1b, left). In the second experiment (Fig. 1c), we evaluated the effects of MgT treatment on contextual-fear memory using a weaker conditioning protocol to exclude the possibility that the lack of effects of MgT treatment on contextual-fear memory (Fig. 1b) might stem from overtraining. Therefore, the exposure to context was reduced (5 min instead of 10 min) and only a single and brief footshock was used as the unconditional stimulus. Each rat was placed in the conditioning chamber for 5 min, followed by one unsignaled electrical footshock (0.7 mA for 0.5 s) and then the rat was removed immediately (Fig. 1c, left).

Twenty-four hours after conditioning, long-term contextual-fear memory tests were performed in context A. During memory tests, the rats were exposed to the context for 5 min. Behavioral experiments were conducted by experimenters who were blinded to the treatment.

Spatial-context discrimination task and fear generalization tests

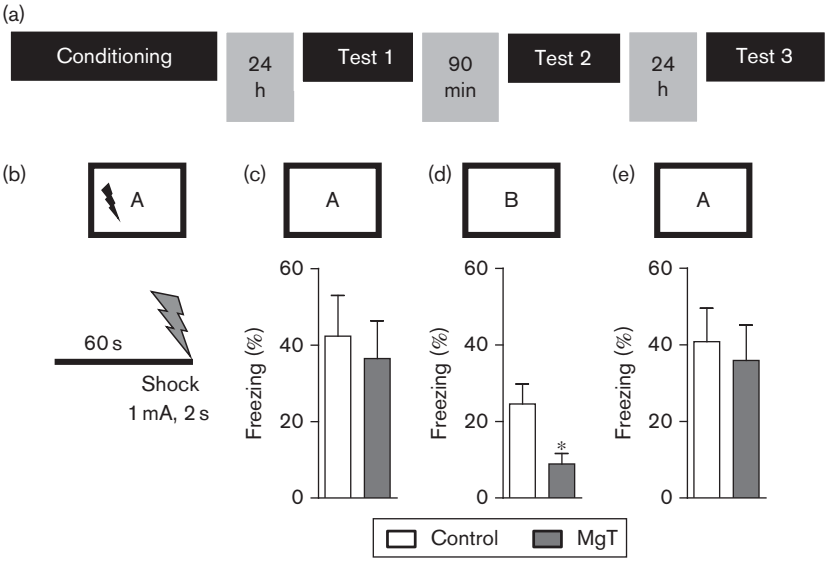
The experiments involving spatial-context discrimination between two, distinct (Fig. 2) or less distinct (Fig. 3), contexts were conducted as described before (McHugh *et al.*, 2007), with modifications. In the present study, we increased the difficulty of the discrimination task by introducing fewer modifications in the original conditioning context (see the Contextual modifications section). Furthermore, we used stronger conditioning protocols than those used in Fig. 1. Such stronger conditioning protocols were adopted to increase the freezing behavior to levels higher than those observed following the above-described protocols (Fig. 1), which should facilitate the detection of attenuating fear responses in the safe contexts.

In the experiments presented in Fig. 2, we evaluated the rats' ability to distinguish between two distinct contexts (A vs. B). Each rat was placed in the conditioning context A and received a single electrical footshock (1 mA for 2 s) 60 s later, whereupon the rat was taken out of the context (Fig. 2a and b). This protocol was shown to increase generalization of contextual fear to a novel and distinct context (Baldi *et al.*, 2004). Furthermore, it allowed us to evaluate the contextual-fear memory in MgT-treated rats (for the third time) following a longer and stronger electrical footshock. Long-term contextual-fear memory tests were performed in context A, context B (90 min later), and again in context A (24 h after the first test, Fig. 2). During memory tests, the rats were exposed to the context for 5 min.

In an independent cohort of control and MgT-treated rats, we evaluated the rats' ability to learn to distinguish between two less distinct contexts (A vs. A′, Fig. 3). A conditioning protocol similar to that in Fig. 2 was used; however, conditioning was repeated over three consecutive days (Fig. 3a and b). This conditioning protocol was shown to enhance fear generalization in this discrimination task (McHugh *et al.*, 2007). Furthermore, it allowed us to evaluate the effects of MgT treatment on contextual-fear memory following repeated conditioning.

During the fear generalization test (Fig. 3a and c), the rats from each group were divided into two halves: one half from each group visited context A and then visited context A′ (morning session), whereas the second half visited context A′ first and then visited context A (afternoon session). No footshock was delivered during this test. The freezing behavior was assessed during a 3-min test and the ratio of freezing in context A′/context A was used as an index for fear generalization. To evaluate the contextual pattern separation abilities in the same rats, we tested their ability to learn to distinguish between the two less distinct pair of contexts (Fig. 3a and d) as described before (McHugh *et al.*, 2007). During this task, the rats visited the two contexts for the next 12 days (3 min each, Fig. 3a, right). The starting test context (whether to start with A and then A′ or vice versa) was determined randomly. The rats received a footshock 3 min after exposure to context A only. Freezing behavior

Fig. 2



Effects of magnesium-L-threonate (MgT) treatment on fear generalization and pattern separation between two distinct contexts (A and B). (a) Experimental design showing the contextual-fear conditioning (in context A, day 1) and long-term memory tests. Test 1 was performed 24 h after conditioning (in context A), 90 min later test 2 was commenced (in context B), and 24 h after test 1 a third test was performed (test 3 in context A). (b) Illustration of the contextual-fear conditioning protocol conducted in the original chamber (context A). (c–e) Freezing behavior of control and MgT-treated rats during 5 min contextual-fear memory tests performed in context A (c), context B (d), and again in context A (e). Two-tailed unpaired *t*-test; $*P<0.05$. Data presented as mean±SEM.

was monitored in each context over the 3-min test. Behavioral experiments were conducted by experimenters who were blinded to the treatment.

Statistical analysis

Data were first tested for normality before applying statistical analysis to detect significant differences. Experiments were analyzed statistically using two-tailed (unpaired and/or paired) Student's *t*-test or two-way repeated-measures analysis of variance (ANOVA). ANOVA was followed by Fisher's post-hoc test. Before applying ANOVA tests, data were tested for sphericity using Mauchly's test to ensure that the important assumption of ANOVA (homogeneity of variances) was not violated. Data presented in Fig. 3c were analyzed using an unpaired *t*-test with Welch's correction because of the difference in variances between the two groups. The difference was considered significant if the *P*-value was less than 0.05. Data are presented as mean ± SEM.

Results

Effects of MgT treatment on contextual-fear memory

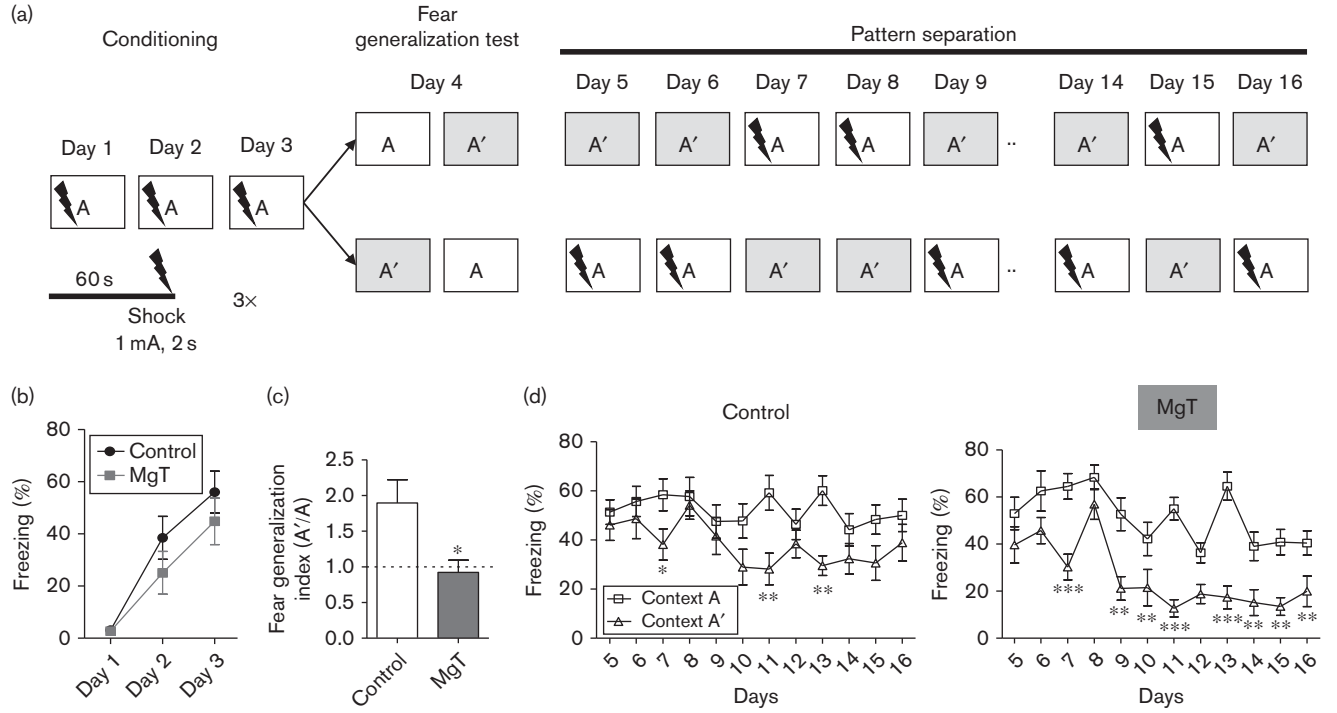
First, we explored the effect of MgT treatment on contextual-fear memory. Control and MgT-treated rats were conditioned in context A (original context). Twenty-four hours later, their long-term contextual-fear memory (LTM) was evaluated in the same context (for the experimental design, see Fig. 1a). During the conditioning session, each rat was placed in the conditioning chamber for 10 min, followed by three unsignaled electrical footshocks (0.7 mA for 0.5 s, intershock interval = 180 s). The rat was removed from the chamber 180 s after the last footshock (Fig. 1b, left). This protocol is identical to our delay-cued fear conditioning protocol (Abumaria *et al.*, 2011) but without tone presentations. Surprisingly, both control and MgT-treated rats showed equivalent freezing behavior during the LTM test ($n = 10$/group, two-way ANOVA repeated measure, effect of time: $F_{4,72} = 6.2$, $P = 0.0002$; effect of drug: $F_{1,72} = 0.0019$, $P = 0.97$; effect of time × drug interaction: $F_{4,72} = 0.67$, $P = 0.61$; Fig. 1b, right).

To ensure that the lack of effect was not because of overtraining, we exposed new groups of rats to a weaker conditioning protocol (Fig. 1c, left, see the Methods section). Again, the freezing behavior in both groups was similar during the LTM test (control: $n = 7$; MgT-treated: $n = 9$; ANOVA showed no significant differences: effect of time × drug interaction: $F_{4,56} = 0.88$, $P = 0.48$; Fig. 1c, right), suggesting that the plausible hippocampus-dependent contextual-fear memory was not enhanced in MgT-treated rats. These findings were unexpected as MgT treatment enhances hippocampus synaptic plasticity and spatial learning in rats (Slutsky *et al.*, 2010).

Effects of MgT treatment on fear generalization and spatial-context discrimination

Next, we conducted two series of experiments in two different cohorts of rats (Figs 2 and 3) to evaluate the context specificity of conditioning in control and

Fig. 3



Effects of magnesium-L-threonate (MgT) treatment on fear generalization and pattern separation between two less distinct contexts (A and A′). (a) Experimental design showing the conditioning protocol (left), contextual-fear generalization test (middle), and pattern separation training (right). (b) Freezing behavior (during 60 s) of control and MgT-treated rats over 3 days of repeated conditioning in context A. (c) Fear generalization index of control and MgT-treated rats during the fear generalization test. Fear generalization index was calculated as the ratio of freezing behavior during a 3-min test in context A′ over that in context A. Two-tailed unpaired *t*-test with Welch's correction; $*P<0.05$. (d) Freezing behavior of control (left) and MgT-treated (right) rats during 3-min tests in context A and context A′ over the 12 days of pattern separation training. Two-way repeated-measures analysis of variance was followed by Fisher's post-hoc test; $*P<0.05$, $**P<0.01$, $***P<0.001$. Data presented as mean±SEM.

MgT-treated rats. The rationale behind these experiments was to compare the hippocampal ability to assemble distinct contextual representations between control and MgT-treated rats.

New groups of rats were conditioned in context A. During the conditioning session, each rat was placed in the context and received a single electrical footshock (1 mA for 2 s) 60 s later, whereupon the rat was taken out of the context (Fig. 2a and b). This protocol allowed us to evaluate the contextual-fear memory following a conditioning protocol different from those described above (difficulty was adjusted by minimizing exposure to the context and increasing shock duration and intensity). Furthermore, this stronger conditioning protocol (than those used in Fig. 1) was adopted to induce higher freezing behavior than that observed in Fig. 1, which should facilitate the detection of attenuating fear responses, if any, during subsequent context specificity tests (see below).

Twenty-four hours later, both control ($n = 10$) and MgT-treated ($n = 12$) rats showed equivalent freezing behavior during a 5 min LTM test (two-tailed unpaired *t*-test, $t_{(20)} = 0.4$, $P = 0.69$; Fig. 2a and c). These results, again, confirm that contextual-fear memory was not enhanced in MgT-treated rats.

Ninety minutes after LTM test 1, we examined the context specificity of the conditioning by assessing the freezing behavior of the same rats in another context (context B, LTM test 2, Fig. 2a and d), in which the metal grid floor was replaced by a smooth plastic board, the door was covered by a white paper, background noise was lowered (from 54 to 42 dB), and the odor was changed (20% ethanol replaced by 2% acetic acid). Context B induced a nonsignificant reduction in freezing behavior (by ~42%) in control rats (paired *t*-test, $t_{(9)} = 1.73$, $P = 0.12$; compared with their freezing in context A, Fig. 2d). Interestingly, exposure to context B induced a significant reduction in freezing behavior of MgT-treated rats (paired *t*-test, $t_{(11)} = 2.99$, $P = 0.012$; compared with their freezing in context A), which was also significantly lower than the freezing of control rats in context B (unpaired *t*-test, $t_{(20)} = 2.78$, $P = 0.012$; Fig. 2d). The data suggest that MgT-treated rats had a better ability to distinguish between the two contexts, which helped in reducing generalization of fear to a context containing features reminiscent of the original context.

Our previous data show that MgT treatment enhances the retention of extinction of fear memory (Abumaria *et al.*, 2011). To verify that the reduction in freezing of MgT-treated rats was because of the context specificity, but not because of extinction of contextual-fear memory, we re-evaluated the contextual-fear memory of the same rats in context A (LTM test 3, Fig. 2a). Again, both control and MgT-treated rats showed higher and equivalent freezing ($t_{(20)} = 0.38$, $P = 0.71$; Fig. 2e), indicating that the original fear memory was not extinguished.

The above data suggest that MgT treatment might enhance pattern separation without enhancing or impairing contextual-fear memory. However, in these experiments, one of the most relevant contextual elements associated with the footshock was removed from context B (the metal grid floor). We have shown that MgT treatment enhances prefrontal cortex functions (Abumaria *et al.*, 2011). The prefrontal cortex is known to be involved in attention processes during fear conditioning (Han *et al.*, 2003). Therefore, the reduction in freezing of MgT-treated rats in context B (Fig. 2d) can be interpreted as enhancement of attention to the absence of the metal grid floor, the element that would be assigned as the major source of the electrical footshock.

To exclude this possibility, we subjected a new cohort of rats (control and MgT-treated) to contextual-fear conditioning and then evaluated their pattern separation abilities using a less distinct pair of contexts (referred to as A and A′) as described before (McHugh *et al.*, 2007), with modifications. This pair of contexts shared all the features, including the metal grid floor, except that context A′ had the door of the chamber covered with a white paper, had lower background noise (from 54 to 42 dB), and a different smell (20% ethanol replaced by 2% acetic acid).

Contextual-fear conditioning was conducted as in Fig. 2b; however, it was repeated over 3 days (days 1–3, Fig. 3a, left). This protocol allowed us to evaluate the contextual-fear memory in MgT-treated rats after repeated conditioning. Repeated conditioning resulted in significant, but equivalent, increases in freezing behavior in both control ($n = 13$) and MgT-treated ($n = 12$) rats (ANOVA effect of repeated training: $F_{2,46} = 33.45$, $P < 0.0001$; effect of drug: $F_{1,46} = 1.44$, $P = 0.24$; effect of repeated training × drug interaction: $F_{2,46} = 0.71$, $P = 0.49$; Fig. 3b), indicating that contextual-fear memory was not affected by the treatment even after repeated conditioning.

On day 4, the context specificity of conditioning was assessed by a fear generalization test (Fig. 3a, middle). The rats from each group were divided into two halves; one half from each group visited context A and then visited context A′ (morning session), whereas the second half visited context A′ first and then visited context A (afternoon session). No footshock was delivered during this test. The freezing behavior was assessed during a 3-min test and the ratio of freezing in context A′/context A was used as an index for fear generalization. Interestingly, the fear generalization index of MgT-treated rats was significantly lower than that of the controls (by ~50%, percentage of control, unpaired *t*-test with Welch's correction, $t_{(18)} = 2.65$, $P = 0.016$; Fig. 3c).

It is important to note that exposure to context A induced similarly high freezing behavior in both groups (mean ± SD, control: 36.3 ± 18.4, MgT: 41.0 ± 22.8). Meanwhile, exposure to context A′ led to a significant increase in the freezing behavior of control rats compared with that in context A (mean ± SD: 54.0 ± 22.8, paired *t*-test, $t_{(12)} = 2.20$, $P = 0.04$), but not in MgT-treated rats (mean ± SD: 40.0 ± 27.1). Therefore, the significant difference in the fear generalization index between control and MgT-treated rats in Fig. 3c does not indicate better pattern separation in MgT-treated rats as their freezing behavior in both contexts remained similar. The data rather suggest that MgT treatment prevented overgeneralization of fear that was shown by control rats. Such fear overgeneralization in controls might stem from the repeated exposure to the aversive event (i.e. the repeated electrical footshock during conditioning) and the high similarity between both contexts.

We proceeded with evaluation of the control and MgT-treated rats' abilities to learn to discriminate between those two, less distinct, contexts. During the subsequent context discrimination training, the rats visited the two contexts for the next 12 days (3 min each, Fig. 3a, right). The rats received a footshock only in context A. Over the 12 days of training, each group learned to discriminate between the two similar contexts (control: ANOVA effects of time: $F_{11,264} = 2.43$, $P = 0.0069$, context: $F_{1,264} = 4.9$, $P = 0.036$, and time × context: $F_{11,264} = 1.85$, $P = 0.047$; MgT-treated: ANOVA effects of time: $F_{11,242} = 12.65$, $P < 0.0001$, context: $F_{1,242} = 21.67$, $P = 0.0001$, and time × context: $F_{11,242} = 2.98$, $P = 0.001$; Fig. 3d, left and right, respectively). However, MgT-treated rats reduced their freezing behavior to almost the baseline level (i.e. <20%) in context A′ over the time course of the training phase (days 9–16). In contrast, at no point during the training phase did the control rats show such reductions in freezing behavior under our experimental conditions (Fig. 3d).

Discussion

The key finding of the present study was that a treatment, MgT, that has been reported to elevate the brain magnesium ~~test~~ (Slutsky *et al.*, 2010) enhanced spatial-context discrimination and/or pattern separation without enhancing or impairing contextual-fear memory, which might lead to a reduction in contextual-fear generalization.

The hippocampus is well known to be a major brain region involved in contextual learning (Maren and Holt, 2000; Anagnostaras *et al.*, 2001). Hippocampal lesions (Kim and Fanselow, 1992) or intrahippocampal infusions

of NMDAR blockers attenuate the acquisition of contextual-fear memory (Young *et al.*, 1994). ~~However,~~ infusion of a cognitive enhancer, HDAC2 inhibitor, into the hippocampus of aging mice enhances contextual fear (Guan *et al.*, 2009). Overexpression of the NR2B subunit of NMDAR in the entire forebrain also enhances hippocampal synaptic plasticity and contextual-fear memory (Tang *et al.*, 1999). MgT treatment has been shown to enhance synaptic plasticity/density and upregulate NR2B-containing NMDAR, p-CREB, and BDNF expression in the hippocampus, which lead to improvement in the hippocampus-dependent spatial learning, pattern completion, and trace-cued fear conditioning in rats (Slutsky *et al.*, 2010; Abumaria *et al.*, 2011). Therefore, it is logical to expect that contextual-fear memory would also be enhanced by MgT treatment.

The present study, however, shows that MgT treatment did not enhance contextual-fear memory. Thus, our data raise the possibility that enhancing synaptic transmission and plasticity in the hippocampus may not be sufficient to enhance contextual-fear memory. In support of this possibility, pretraining infusions of BDNF into the hippocampus of young rats enhance neither LTM (Lee *et al.*, 2004) nor memory for inhibitory avoidance, another context-based/fear-based memory task that requires an intact hippocampus (Alonso *et al.*, 2002). Therefore, coenhancement of synaptic plasticity in other brain regions, besides the hippocampus, might be necessary to enhance the contextual-fear memory.

The amygdala is believed to be the structure where associative fear memories are formed and fear responses are initiated (LeDoux, 2007). Infusions of NMDAR blockers in the amygdala attenuate contextual-fear memory (Fanselow and Kim, 1994). Blocking mTOR-dependent protein synthesis by infusing rapamycin into the amygdala impairs inhibitory avoidance memory (Jobim *et al.*, 2011). It was proposed that the amygdala might contribute toward the consolidation of contextual-fear memory by associating elemental features of the context with the aversive electrical footshock (Anagnostaras *et al.*, 2001). Given these roles of the amygdala in fear memory processes, it is possible that coenhancement of the amygdala synaptic plasticity is also critical for enhancing contextual-fear memories. Therefore, we postulate that MgT treatment did not enhance contextual-fear memory (Figs 1, 2c, and 3b) because the treatment does not enhance synaptic plasticity and NMDAR signaling in the amygdala as we have shown before (Abumaria *et al.*, 2011). However, we cannot exclude the possibility that MgT treatment might influence other brain regions leading to the observed behavioral readouts of contextual-fear memory. Nevertheless, the lack of enhancement of contextual-fear memory following MgT treatment (this study) and following BDNF infusions into the hippocampus (Lee *et al.*, 2004) requires further investigations to advance our knowledge on the hippocampal role in contextual-fear memory.

Although MgT treatment did not enhance contextual-fear memory *per se*, MgT-treated rats showed a better ability to discriminate between two, more or less distinct, contexts and had less contextual-fear generalization than control rats. Interestingly, following repeated conditioning and during the fear generalization test (Fig. 3c), control rats showed fear overgeneralization to a context highly similar to the conditioning one, in which the rats were exposed to repeated conditioning. This overgeneralization was attenuated after the first exposure as shown by the reduction in their freezing in context A′ following the first exposure (Fig. 3d, left, day15). Meanwhile, MgT treatment prevented fear overgeneralization from the first exposure to context A′ (Fig. 3c). The data (Figs 2 and 3) suggest that the ability of the hippocampus to form subtly different spatial-contextual representations might be enhanced by MgT treatment.

It is worth noting that our previous studies have reported that MgT-treated and control rats showed equivalently low freezing behavior when the rats were placed in a novel and completely different context than the conditioning context, 24 h after fear conditioning (Abumaria *et al.*, 2011). Furthermore, MgT treatment does not increase the exploratory activity in the open-field test (Slutsky *et al.*, 2010). Therefore, the reductions in freezing behavior of MgT-treated rats reported in the present study are less likely to stem from modifications of the rats' behavioral reactivity by MgT treatment (i.e. increase in exploratory activity or attenuation of freezing behavior in a novel context).

A lesion study provided the first experimental evidence on the role of hippocampal dentate gyrus (DG) in pattern separation (Gilbert *et al.*, 2001). Furthermore, a study by McHugh *et al.* (2007) shows that genetic ablation of NMDAR in DG impairs contextual pattern separation without affecting contextual-fear memory. ~~However,~~ environmental enrichment or voluntary exercise, known to increase synaptic plasticity/density in the hippocampus (Van Praag *et al.*, 2000), improves pattern separation in rodents (Creer *et al.*, 2010). The increase in hippocampal BDNF expression *per se* is hypothesized to provide a major contribution to the enhancing effects of environmental enrichment on pattern separation (Bekinschtein *et al.*, 2011). We have shown that MgT treatment increases synaptic density in DG, promotes activation of NMDAR signaling, and enhances BDNF expression and synaptic plasticity in the hippocampus (Slutsky *et al.*, 2010). These cellular and molecular mechanisms, induced by MgT treatment, might contribute toward the effects of MgT treatment reported in the present study.

It is important to note that when control and MgT-treated rats were tested in a more distinct context (a test that is DG independent, McHugh *et al.*, 2007), both groups dropped their freezing behavior in this context but MgT-treated rats showed significantly lower freezing behavior than control rats

(Fig. 2d). Therefore, although MgT treatment might enhance DG-dependent pattern separation (Fig. 3d), the treatment might also enhance DG-independent spatial-context discrimination (Fig. 2d). To exclude the latter possibility, the freezing behavior of control and MgT-treated rats should have been evaluated in a context completely different from the conditioning one to check whether both groups drop their freezing behavior equally. Our previous studies have reported that MgT-treated and control rats showed equivalently low freezing behavior when the rats were placed in a novel and completely different context 24 h after fear conditioning (Abumaria *et al.*, 2011), suggesting no effects of MgT treatment on DG-independent spatial-context discrimination. However, in these experiments, the conditioning protocol differs from the one used in the present study (Fig. 3). Therefore, it remains to be determined whether the treatment might affect other brain regions/subregions leading to the behavioral readouts reported in the present study.

Recently, compelling evidence suggests that hippocampal neurogenesis is a critical factor determining the competence of pattern separation (Aimone *et al.*, 2011; Yassa and Stark, 2011; Sahay *et al.*, 2011a; Nakashiba *et al.*, 2012). Ablation of neurogenesis in DG impairs spatial pattern separation (Clelland *et al.*, 2009), whereas genetic (Sahay *et al.*, 2011b) or pharmacological (Wang *et al.*, 2012) enhancement of neurogenesis in DG improves pattern separation in rodents. Hence, in the light of the current findings, future studies on the relationship between the level of brain magnesium and neurogenesis in DG are warranted. In this context, in-vitro studies suggest a critical role for the magnesium ion in cell proliferation and survival (Maguire, 1988; Wolf and Cittadini, 1999).

Finally, physical exercise (Creer *et al.*, 2010) and certain medicines (e.g. metformin, Wang *et al.*, 2012) have been shown to be clinically applicable approaches to enhance pattern separation. The present study suggests a role for a dietary factor, namely high magnesium intake, in enhancing spatial-context discrimination and/or pattern separation besides preventing fear overgeneralization. Future studies should investigate whether the current results of MgT treatment are extendable to other discrimination tasks involving appetitive rewarding stimuli.

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Conflicts of interest

G.L. declares that he is a cofounder of Magceutics, a company whose goal is to develop drugs to treat age-dependent memory decline and Alzheimer's disease. He also reports his United States patent application on 'Magnesium-L-Threonate'. For the remaining authors there are no conflicts of interest.

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