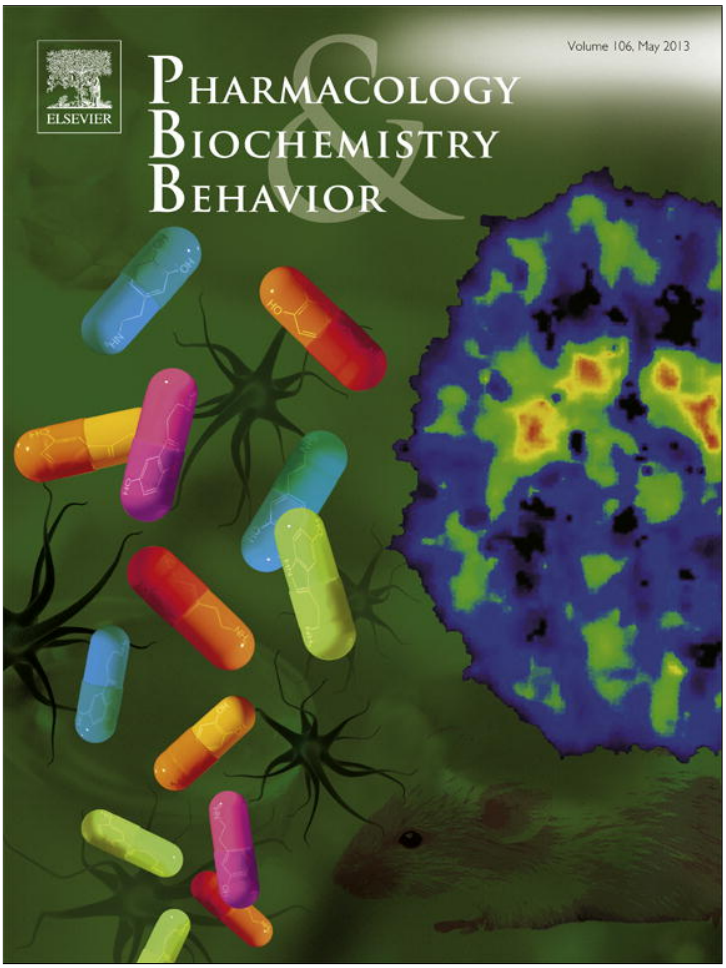

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Chronic dietary magnesium-L-threonate speeds extinction and reduces spontaneous recovery of a conditioned taste aversion

G. Andrew Mickley *, Nita Hoxha, Joseph L. Luchsinger, Morgan M. Rogers, Nathanael R. Wiles

The Neuroscience Program, Baldwin Wallace University, 275 Eastland Rd., Berea, OH, 44017, USA

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ABSTRACT

Elevation of brain magnesium enhances synaptic plasticity and extinction of conditioned fear memories. This experiment examined the generalizability of this phenomenon by studying the effects of a novel magnesium compound, magnesium-L-threonate (MgT), on conditioned taste aversion (CTA) extinction and spontaneous recovery (SR). Adult male Sprague–Dawley rats were maintained on a 23-hour water deprivation cycle and acquired a CTA following the taste of a CS [0.3% saccharin + 16 mg/ml MgT (SAC + MgT)] paired with a US [81 mg/kg (i.p.) lithium chloride (LiCl)]. Following CTA acquisition, rats drank a water + MgT solution for up to 1 hour/day over the next 31 days. For 14 additional days, some animals continued water + MgT treatment, but others drank water only to allow MgT to be eliminated from the body. We then employed 2 different extinction paradigms: (1) CS-Only (CSO), in which SAC was presented, every-other day, or (2) Explicitly Unpaired (EU), in which both SAC and LiCl were presented, but on alternate days. EU extinction procedures have been shown to speed CTA extinction and reduce spontaneous recovery of the aversion. Throughout extinction, half of the rats in each group continued to drink MgT (now in SAC or supplemental water + MgT solution), whereas the other half drank SAC only/water only until SAC drinking reached ≥90% of baseline (asymptotic extinction). Rats receiving MgT just before/during extinction drank less SAC on the first day of extinction suggesting that they had retained a stronger CTA. MgT enhanced the rate of extinction. Furthermore, the MgT-treated rats showed a relatively modest SR of the CTA 30 days later — indicating that the extinction procedure was more effective for these animals. Our data suggest that long-term dietary MgT may enhance the consolidation/retention of a CTA, speed extinction, and inhibit SR of this learned aversion.

1. Introduction

Magnesium (Mg^{2+}) is known to play a major role in cellular metabolism (Lin et al., 2002) and is critical for nervous system functioning (Paymaster, 1976; Furukawa et al., 2009). Aberrations in Mg^{2+} homeostasis leads to biochemical dysregulation and may contribute to psychological and neurological disorders such as depression (Whittle et al., 2011; Murck, 2002; Rasmussen et al., 1989; Singewald et al., 2011), Parkinson's Disease (Shindo et al., 2011) and glaucoma (Crish et al., 2012). Magnesium deficiency impairs fear conditioning in mice (Bardgett et al., 2005). Moreover, treatment with magnesium sulfate ($MgSO_4$) may produce therapeutic benefits as they enhance the metabolic response to energetic stresses induced by hypoxia, ischemia and traumatic brain injury (Wang et al., 2012; Vink et al, 2003; Goni-de-Cerio et al., 2012).

Mg^{2+} also modulates the voltage-dependent block of N-methyl-D-aspartate (NMDA) receptors, controlling their opening during coincidence detection — a function that is critical for synaptic plasticity (Mayer et al., 1984; Nowak et al., 1984). *In vitro* studies have shown that increasing Mg^{2+} concentrations in extracellular fluids can enhance synaptic plasticity of cultured hippocampal neurons (Slutsky et al., 2004). Subsequent *in vivo* experiments revealed that increases in brain Mg^{2+} enhanced short-term synaptic facilitation and long-term potentiation as well as spatial memory (Slutsky et al., 2010). The underlying mechanisms of these physiological and cognitive changes are still being investigated but current evidence suggests that chronic increases in extracellular Mg^{2+} cause a compensatory upregulation of NR2B NMDA receptors to counterbalance the sustained blockade of NMDA receptor channels (Slutsky et al., 2010). Similar mechanisms of homeostatic plasticity have been reported in other neural systems (for review, see Turrigiano, 2008).

These benefits in cognition and control of emotions follow chronic enhancements of brain Mg^{2+} (Abumaria et al., 2009, 2011). However, there are several practical challenges of simply providing increased levels of elemental Mg^{2+} in diet. High levels of Mg^{2+} intake can interfere with a variety of physiological functions and induce diarrhea and lethargy (Chester-Jones et al., 1990). Moreover, central nervous system regulation of brain cerebrospinal fluid (CSF) Mg^{2+} concentrations limits blood-brain barrier penetration of peripherally administered $MgSO_4$ (for

* Corresponding author. Tel.: +1 330 603 3839.
E-mail addresses: amickley@bw.edu (G.A. Mickley), nhoxha@mail.bw.edu (N. Hoxha), jluchsin@mail.bw.edu (J.L. Luchsinger), mrogers10@mail.bw.edu (M.M. Rogers), nwiles@mail.bw.edu (N.R. Wiles).

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review, see McKee et al., 2005). However, a newly developed compound, Magnesium-L-threonate (MgT; brand name Magtein™) has been shown to significantly enhance bioavailability and produce 7–15% increases in rat CSF Mg^{2+} while other magnesium compounds tested failed to significantly elevate Mg^{2+} in CSF when compared to controls (Slutsky et al., 2010).

Thus far, two behavioral investigations have studied the effects of MgT on learning and memory. Slutsky et al. (2010) reported that MgT treatment benefits performance on working, spatial and recognition memory tasks. MgT has also been evaluated for its ability to enhance extinction of conditioned fear responses in rodents. In particular, Abumaria et al. (2011) found that increased levels of Mg^{2+} in the brain enhanced the retention of fear extinction without impairing the initial fear memory. Abumaria et al. further suggested that the retention of this extinction memory is stronger in animals with increased levels of brain Mg^{2+} due to a corresponding increase in synaptic plasticity in the hippocampus and infralimbic prefrontal cortex that accompanies activation of NMDA receptor signaling and brain-derived neurotrophic factor expression in the prefrontal cortex (Abumaria et al., 2011). If these findings are verified, effective magnesium supplements may be used to enhance the efficacy of therapy for anxiety disorders such as post-traumatic stress disorder (PTSD) or phobias, as relapse to the original fear is a common problem after therapy (Rauhut et al., 2001). To gain a full appreciation for the potential of MgT and its ability to affect learning and memory, more pre-clinical research needs to be performed to determine its effects on different types of defensive reactions to learned fears.

The purpose of the current study was to examine the ability of MgT to affect the extinction and spontaneous recovery of a conditioned taste aversion (CTA). The pairing of a novel taste (conditioned stimulus; CS) with malaise or noxious sensation (unconditioned stimulus; US) results in the formation of a CTA (Garcia et al., 1955, 1961, 1968). Although somewhat resistant to extinction, a CTA may be reduced by the repeated, nonreinforced presentation of the CS (Nolan et al., 1997; Mickley et al., 2004). Further, spontaneous recovery (SR) of the CTA (i.e., a re-occurring suppression of CS consumption) appears when the CS is presented following a sufficiently long delay after extinction (Kraemer and Spear, 1992; Rosas and Bouton, 1996; Berman et al., 2003; Mickley et al., 2007).

Here we created an aversive memory that caused our animals to refuse the conditioned stimulus of saccharin (Houpt et al., 1996; Mickley et al., 2004). This CTA was extinguished by either repeated exposure to the CS alone (CS-Only; CSO-EXT) or through the use of an Explicitly Unpaired extinction procedure (EU-EXT) which has been shown to speed up extinction and attenuate SR of the CTA (Mickley et al., 2009; Mickley et al., 2011). We hypothesized that MgT-treated rats would exhibit a faster rate of extinction than controls and show a reduced SR of the CTA.

2. Materials and methods

2.1. Subjects

This study employed 36 adult male Sprague–Dawley rats (mean weight at the start of the study ± SEM = 282.3 ± 2.7 g) obtained from Charles River Laboratories (Wilmington, MA). All subjects were handled and maintained in accordance with the Animal Welfare Act and *The Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996). The study was approved by the Baldwin Wallace University Institutional Animal Care and Use Committee. Animals were individually housed in plastic tub cages (20 cm × 22 cm × 20 cm deep) with wire cage tops. Each cage bottom contained corncob bedding (The Andersons, Inc., Maumee, OH). Drinking water and other fluids were administered in 50 ml sipper (ball spout) bottles. Rats had access to food *ad libitum* (Lab Diet, No. 5001, containing 0.21% Magnesium, PMI Nutrition International, Richmond, IN) (see: http://labdiet.com/pdf/5001.pdf) and were housed in a temperature-controlled room between 23 and 26 °C with a 12-hour light/dark cycle (lights on at 0600 h; off at 1800 h).

2.2. Materials

Magnesium-L-threonate powder (brand name Magtein™; MgT) was obtained from AIDP (City of Industry, CA; http://www.magtein.com/) and mixed in the rats' drinking water. Reverse osmosis (RO) water was used to mix all MgT solutions to avoid administering additional Mg^{2+} in local tap water. MgT concentrations varied from 10 to 16 mg/ml depending on the phase of the study (see below and Table 2). Fluids were consumed at will by our animals during a 1-hour period each day and the concentration of MgT liquids was adjusted based on body weights of the rats and average volume consumed in order to get as close as possible to the target dosage of 604 mg/kg/day employed by other investigators (Slutsky et al., 2010; Abumaria et al., 2011). Similar doses and time courses of MgT exposure have been shown to be effective in elevating brain magnesium and enhancing hippocampal-dependent learning and memory in rats (Slutsky et al., 2010) as well as extinction of conditioned fears (Abumaria et al., 2011). Saccharin (SAC) and lithium chloride (LiCl) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

2.3. Pilot studies evaluating the acceptability of water + MgT, Saccharin + MgT and the ability of rats to distinguish between the tastes of SAC-only and SAC + MgT

In the main studies reported here we exposed rats to various water + MgT, SAC + MgT, and SAC-only solutions and we made adjustments in the MgT concentrations as the rats' weights changed during certain phases of our experiments. MgT is colorless, tasteless, and odorless to humans (http://www.magtein.com/thequality.html). However, it was important to determine if rats would attend to the tastes of these various solutions and adjust their consumption of them. Therefore, we performed 2 pilot studies to determine if rats were equally accepting of a range of concentrations of (1) water + MgT, and (2) SAC + MgT. Moreover, in a 3rd pilot study we attempted to evaluate the extent to which our animals could distinguish between the tastes of SAC-only vs. SAC + MgT by creating a CTA to SAC only and then assessing their consumption of both solutions. Finally, we wanted to assess the baseline consumption of SAC + MgT solutions with the goal of determining if this baseline was similar/different from the SAC-only baseline consumption we established in our previous studies (see for example, Mickley et al., 2009).

In the first pilot study, 23-hour fluid-deprived naïve male Sprague–Dawley rats (N = 8/group) were given either water + MgT (10 mg/ml) or water + MgT (16 mg/ml) on two successive days. These concentrations represent the lowest and highest concentrations of MgT that we employed in our main experiment. The animals drank approximately equal volumes of each [water + MgT (10 mg/ml) = 19.67 ± 0.82 ml (Mean ± SEM); water + MgT (16 mg/ml) = 20.81 ± 0.85 ml (Mean ± SEM)] — indicating no preference for the taste of either solution. Likewise, the average water-only drinking over 2 days [19.50 ± 3.09 ml (Mean ± SEM)] was not significantly different from the water + MgT averages (see above).

This first pilot study confirmed that rats will drink essentially equal amounts of two water + MgT solutions (10 mg/ml and 16 mg/ml) and water alone. Thus MgT, in the concentrations employed in this study, appeared to be neither aversive nor more desirable to our animals than was water alone. This gave us some confidence that if MgT was combined with another more-distinctive tastant (i.e., SAC) it might not alter the salience of that stimulus (see second pilot study described below). Further, since we needed to adjust the concentration of our MgT solutions as the study progressed (in order to continue to deliver the desired dose to our growing animals throughout the main experiment described below), this reassured us that the rats would not find

the highest dose we employed (16 mg/ml MgT) either more-aversive or more-desirable than water and adjust their consumption of it based on its hedonic value alone.

In the second pilot study we determined if animals would distinguish between 0.3% SAC only and 0.3% SAC + MgT and show a preference. Using a paradigm similar to that described above, 23-hour fluid-deprived naïve male Sprague–Dawley rats were offered either 0.3% SAC only (N = 10) or 0.3% SAC + MgT (16 mg/ml); i.e., the highest concentration we used in our main study) (N = 11) over 3 successive days. Similar amounts of the liquids were consumed by the rats on the third day measured: SAC only = 17.57 ± 1.29 ml (Mean ± SEM); SAC + MgT = 17.84 ± 1.25 ml (Mean ± SEM).

Therefore our first 2 pilot studies indicated that, within the concentrations tested, rats do not favor the taste of water vs. water + MgT nor SAC-only vs. SAC + MgT and drink similar amounts of the MgT-containing liquids as they do vehicles. This was important because we wanted to make sure that we did not expect to see, for example, a native preference for SAC only above and beyond that for SAC + MgT. If such a preference had been detected, then the measure of SAC only consumed by our controls on the first day of extinction would have been an indecipherable combination of the CTA and the animals' natural taste preferences. Thus, the first 2 pilot studies provided information that could alert us to potential influences of natural preferences and aversions on our findings.

A third pilot study attempted to evaluate the similarity of the tastes of SAC + MgT vs. SAC only in order to determine the likelihood that, within the context of our study, rats could/could not taste the difference between these 2 solutions. This study was important to the interpretation of the data derived from our main experiment (see below). If the SAC only and SAC + MgT tastants we employed in our study were not perceived as very similar, the control animals tasting SAC only on the first day of extinction would not recognize it as the same CS (i.e., SAC + MgT) they experienced on the conditioning day. Presumably this would make them avoid SAC to a lesser extent given that the training and testing stimuli were different. The animals trained with the same stimulus with which they are tested on the first day of extinction (SAC + MgT) would be expected to have no generalization decrement and demonstrate a stronger aversion on the first day of extinction. Likewise, when SAC + MgT was experienced at the spontaneous recovery (SR) test, by the control rats that were extinguished with SAC only, a greater SR would be expected since the tastants presented during extinction and SR test were different. Thus, it was central to the interpretation of our data that we learn the extent to which the tastes of SAC only and SAC + MgT were generalizable.

In the third pilot study 23-hour fluid-deprived rats (N = 9) were initially treated as described in the "Water Deprivation Acclimation and Preconditioning exposure to MgT" section below (see also Table 2). The only difference was that, instead of being offered 1 bottle/day, 2 bottles were placed on each cage. Thirsty rats will often drink voraciously the first liquid they encounter. Therefore, bottle positions were switched after 1, 5, and 10 min into the first 30-minute drinking period throughout the study in order to force the animals to sample the liquid in both bottles. This was less important when both bottles contained the same solution. However, it was important to habituate the rats to this procedure before the 2-bottle test day (see below). The next day (Experimental Day 9), rats had 30-minutes access to 0.3% SAC + MgT (16 mg/ml) followed by an i.p. injection of physiological saline (volume = 1 ml/kg). The following day (Day 10), the animals had 30 min to drink 0.3% SAC only and then received an injection of LiCl (81 mg/kg, i.p.). During a 30-minute period on Experimental Day 11 the rats had an opportunity to choose between drinking 0.3% SAC only or 0.3% SAC + MgT (16 mg/ml) during a two-bottle test wherein both solutions were presented simultaneously.

The preponderance of data from this third pilot study are consistent with the interpretation that rats perceived the tastes of SAC only and SAC + MgT as quite similar. Our second pilot study (see above) indicated that SAC only and SAC + MgT were equally preferred. Therefore it is noteworthy that the consumption of SAC only on Experimental Day 10 of the third pilot study was significantly higher than the SAC + MgT drunk on Experimental Day 9 [$t(8) = -9.54$, $p < 0.05$] suggesting a reduction of neophobia (Gillan and Domjan, 1977) to what were perceived as similar, sweet tastes. Paired t-tests indicated that, compared to the initial levels of consumption of the 2 solutions on Experimental Day 9 (when SAC + MgT was presented before a control saline injection) and Day10 (when SAC only was presented before LiCl), the consumption of BOTH solutions declined substantially during the 2-bottle test on Day 11 [SAC + MgT: $t(8) = 4{,}48$; $p < 0.05$; SAC Only: $t(8) = 22.64$; $p < 0.05$ (2-tail tests)] and fell to low levels typical of CTAs (means of both solutions < 4mls consumed). This suggests that the animals had a difficult time differentiating between the tastes. Rats drank less of the SAC-only solution (the one associated with malaise the day before) on the 2-bottle test day. However, a repeated measures Analysis of Covariance indicated that this difference between the volumes of SAC only and SAC + MgT consumption was not statistically significant on the 2-bottle test day. [Note: the extent of CS exposure can affect the strength of conditioning. Therefore we used the volume of SAC consumed before the LiCl was given on the CTA training day as the covariate.]

In summary, exposure to SAC + MgT reduced the neophobia normally seen to the taste of SAC only. There was a significant decrease in consumption of both SAC only and SAC + MgT despite the fact that SAC alone was paired with the aversive LiCl. Further, there was not a reliable difference in the amount of SAC only consumed and SAC + MgT consumed following the pairing of SAC only with LiCl. Thus, these data are consistent with the conclusion that rats found it difficult to differentiate between the SAC only and SAC + MgT solutions we employed in the main study reported below.

In a final pilot study, we estimated the levels of baseline/familiar SAC + MgT rats consume so that we could evaluate the degree to which the rats in this study had extinguished their CTA. This pilot study was performed separate from our main experiment since recording several days of baseline SAC + MgT pre-exposure in our animals would have impeded future CTA training, due to latent inhibition effects (Bakner et al., 1991). Further, we also wished to avoid the bias associated with the rats' initial hesitation to consume novel substances, referred to as neophobia (Gillan and Domjan, 1977). Baseline SAC + MgT consumption was determined by averaging consumption on the third day of exposure from a separate group (N = 11) of similarly-sized rats not used in the current study (see pilot studies described above). The average volume consumed by our rats drinking 0.3% SAC + MgT (16 mg/ml; i.e., the same MgT concentration employed during CTA acquisition and extinction) was 17.84 ml and this volume was used as the criterion for asymptotic extinction in this study (see below). Note: This was virtually identical to the baseline/familiar SAC-only consumed by rats in previous studies (Mickley et al., 2011).

2.4. Experimental design

The main experiment we describe here employed a 2 × 2 factorial design (see Table 1). Rats in all 4 groups were treated similarly during the early phases of the study (H_2O deprivation acclimation, Pre-Conditioning, CTA Acquisition, and Extended MgT Treatment; see Table 2). However, during the Wash-Out and Extinction phases of the experiment, animals received MgT or were designated as controls, and received no MgT. Rats also underwent extinction (EXT) procedures that involved either being exposed to the CS-Only (CSO) or an Explicitly Unpaired (EU) procedure during which both the CS and US were administered on a schedule that produced a disassociation between the two stimuli. See a summary of the study timeline in Table 2.

Table 1
Experimental design, group nomenclature and Ns.

		MgT treatment	
Extinction treatment	CS-Only extinction	MgT	Controls
		CSO MgT	CSO controls
		N[a] = 9/9/9	N = 9/9/8
	EU extinction	EU MgT	EU controls
		N = 9/8/8	N = 9/9/9

[a] The Ns represent the number of animals in the study during the conditioning/extinction/SR phases, respectively. There was 1 sick rat removed from the study during the extinction phase and an equipment malfunction caused the loss of data from another rat at the SR test.

2.5. Water deprivation acclimation and preconditioning exposure to MgT

Animals were acclimated to a 23-hour/day water deprivation schedule for 5 days and this schedule was maintained throughout the study. Each day, rats had access to fluids during two 30-minute drinking periods separated by 15 min (1200–1230 h; 1245–1325 h). This acclimation period was followed by a pre-conditioning phase (see Table 2) in which animals received water containing MgT (water + MgT; 10 mg/ml) for a total of 3 days during the first 30-minute drinking period. [Note: Although water + MgT (10 mg/ml) apparently tastes similar to water alone (see pilot data above) we allowed the rats to habituate to any MgT taste cues so that on the day of CTA acquisition the SAC + MgT would appear as a singular novel stimulus similar to SAC only. We selected this low concentration of MgT because our animals had the smallest body mass in the beginning of the study].

2.6. CTA acquisition

The day following MgT pre-exposure, rats received 0.3% saccharin containing 16 mg/ml MgT from 1200 h to 1230 h. This concentration of MgT was employed because we estimated it to be the maximal concentration that would be needed to maintain the target dose near the end of the study, when the rats were larger and underwent CTA extinction and spontaneous recovery testing. After the first drinking period, rats received an injection of the unconditioned stimulus (US) lithium chloride (LiCl, 81 mg/kg; i.p.) (Mickley et al., 2004). From 1245 to 1315 h animals were given water only to help the animals maintain good hydration.

2.7. Extended MgT treatment

Orally ingested MgT has been shown to take at least one month to raise brain magnesium levels to the extent required to have an effect on memory formation (Slutsky et al., 2010). Therefore, following the CTA acquisition day, all animals received 31 days of water + MgT exposure during the first half hour of drinking each day. As rats gained weight, we adjusted the concentration of water + MgT offered to the rats as we attempted to reach the target dose of 604 mg/kg/day employed by other investigators (Slutsky et al., 2010; Abumaria et al., 2011). See Table 2 for details.

2.8. MgT Wash-Out phase

During this phase of the study, we sought to reduce or eliminate elevated brain magnesium levels in animals that were randomly

Table 2
Timeline, phases, and stages of the experiment.

Phases	H_2O Deprivation Acclimation	Pre-Conditioning	CTA Acquisition	Extended MgT Treatment	MgT Wash-Out	Extinction	H_2O Only Latency	Spontaneous Recovery Test
Total # of days	5 days	3 days	1 day	31 days	14 days	Up to15 days	30 days	1 day
Magtein (MgT) Concentration	None	Water+MgT[a] (10mg/ml)	SAC[b]+MgT (16mg/ml) + LiCl[c]	Water+MgT: Concentration adjusted weekly according to mean body weight [Range = 10mg/ml – 12.1mg/ml]	Water+MgT: Concentration adjusted weekly according to mean body weight [Range = 12.2mg/ml – 13.4mg/ml]	SAC+MgT (16mg/ml)	None	SAC+MgT (16mg/ml)
STAGES		**STAGE ONE** ⟷ All animals received the same treatment. Mean±SEM MgT dose = 558.87 ± 10.33mg/kg/day			**STAGE TWO** ⟷ **Wash Out:** EU Control and CSO Controls received water only; EU MgT and CSO MgT groups received Magtein in water: Mean±SEM MgT dose = 595.90±19.02 mg/kg/day **Extinction:** Animals in EU MgT and CSO MgT groups received Magtein in 0.3% saccharin: Mean±SEM MgT dose = 639.69±9.74mg/kg/day; EU Control and CSO Controls received only 0.3% saccharin		**STAGE THREE** ⟷ All animals received the same treatment	

[a] MgT = Magnesium-L-threonate
[b] SAC = 0.3% saccharin solution
[c] LiCl = 81mg/kg lithium chloride, i.p.

assigned to our control groups (CSO Control; EU Control). Compared to experimental animals receiving water + MgT in the first half hour of drinking each day, these control rats received 14 days of water only exposure before the CTA extinction trials began (see below). [Note: The decision about the length of the wash-out phase was influenced by the work of Slutsky et al. (2010) who reported that, 2 weeks after termination of MgT treatment, the positive effects of MgT on working memory were diminished in aged rats (22–24 months old). However in the same report, the enhancement of working memory by MgT persisted for 30 days after termination of the treatment in young rats (2 months old). The current experiment used rats that were 3–4 months old at the start of the study and 6–7 months old at the end of the study. Therefore their age fell between the "young" and "aged" rats tested by Slutsky et al. (2010).] The elevation, then depletion, of brain magnesium levels in control subjects allowed for the specific exploration of the effects of dietary magnesium on only the extinction and SR processes. We made concentration adjustments each week based on the average body weight of the rats in the CSO MgT and EU MgT groups. See Table 2 for details.

2.9. Extinction

We used two different extinction training methods: (1) CS-Only exposures (CSO), or (2) Explicitly Unpaired exposures (EU; CS and US given on alternate days). CSO MgT animals received a 30-minute (1200–1230 h) exposure to SAC + MgT (16 mg/ml), followed by a 30-minute (1245–1315 h) opportunity to drink water. These rats were given SAC + MgT every-other day beginning the day following the MgT Wash-Out phase. On alternate days (even days), water only was administered during both drinking periods. EU MgT animals received 30 min of exposure to SAC + MgT (16 mg/ml), followed by 30 min of water only in the same time frame as CSO MgT animals. They received two 30-minute water exposures on even days but were injected with the US (81 mg/kg LiCl; i.p.) between 1230 and 1245 h on each of these days. Our previous studies have indicated that rats experiencing the EU extinction procedure achieve asymptotic extinction of a CTA more rapidly and show less SR of the CTA than do rats undergoing the CSO extinction (Mickley et al., 2009).

The main goal of this study was to understand the effects of MgT administration during CTA extinction. Therefore in this phase of the study, we utilized special procedures in an attempt to achieve the target MgT dose (Slutsky et al., 2010; Abumaria et al., 2011). In order to maintain the same taste stimulus as was employed during CTA acquisition, we again used 16 mg/ml SAC + MgT in this extinction phase of the study. Keeping the concentration of SAC + MgT constant necessitated us controlling the volume of MgT solution consumed each day while not restricting the amount of SAC solution consumed by the rats. As expected, animals drank very small amounts of the CS at the beginning of extinction. Therefore, the necessary volume of SAC + MgT to reach the target MgT dose was calculated for each animal based on body weight, and a line was drawn on the drinking bottle representing that target volume. An observer monitored the SAC + MgT (16 mg/ml) consumption from 1200 to 1230 h. When the target volume was consumed, the bottle was removed and immediately replaced with a drinking bottle containing 0.3% SAC only. [Note: rats apparently generalize between the tastes of SAC only and SAC + MgT — see pilot data above.] This allowed the MgT target dose to be reached without disrupting SAC consumption. If an animal did not drink the desired dose of MgT between 1200 and 1230 h, Water + MgT (16 mg/ml) was again offered from 1245 to 1315 h until the targeted MgT dose/day was achieved. SAC or SAC + MgT was only offered during the first drinking period of the day. Water alone or water + MgT was only offered during the second drinking period. In summary, during the CTA extinction process, we provided our animals free access to SAC + MgT and then switched to SAC-only when they reached the target MgT dose/day. This allowed us to continue to evaluate the level of extinction that each rat achieved (by measuring the volume of total SAC consumed; our dependent variable) while not over-dosing the animal on MgT. See Table 2 for the average MgT doses received during the extinction phase of the study.

CSO Control animals followed a fluid exposure schedule identical to that of the CSO MgT group. EU Control animals followed a fluid exposure schedule identical to animals in the EU MgT group. However, the rats in these control groups did not have MgT in their SAC solutions. All animals received every-other-day CS exposures until they reached asymptotic extinction of the CTA (i.e., ≥90% of SAC and SAC + MgT baseline volume; Nolan et al., 1997).

2.10. Latency and spontaneous recovery

After achieving asymptotic extinction, rats in all 4 groups entered a 30-day latency phase during which they received daily water exposure during both drinking periods. Rats received no injections during the latency period. On the day immediately following the 30-day latency period, animals were presented again with 0.3% SAC + MgT (16 mg/ml; i.e., the same concentration employed during CTA acquisition and extinction) from 1200 to 1230 h.

2.11. Statistical analyses

Unless otherwise specified, data were analyzed using 2-way ANOVAs [Extinction procedure (CSO or EU) × MgT treatment (MgT or Control)] followed, when appropriate with pairwise comparisons using Bonferroni-corrected t-tests. An $\alpha = 0.05$ was employed throughout.

The extinction data presented significant variability in the number of days for different rats to reach asymptotic extinction. Some rats extinguished after 4 days and others extinguished after 15 days. This fact shaped the selection of our inferential statistical analysis. For example, a repeated-measures ANOVA covering all the days required for all the rats to achieve asymptotic extinction was not feasible since there would be many "missing" data points as CTA extinction proceeded. Instead, we used linear regression methods to calculate the slope of the extinction curve (mls total SAC consumed across the days during the extinction phase of the study) for each rat in the study as a means of capturing the rate of extinction/subject. We then used these data in a 2-way ANOVA aimed at determining the extent to which MgT treatment and/or EU vs. CSO extinction methods changed the rate of CTA extinction.

The slope of extinction curves has been an important component in the evaluation of extinction learning — especially in studies of appetitive conditioning (Scheiner et al., 2001) and experiments demonstrating the efficacy of therapies to diminish conditioned fear responses (Robles, 2010). Slope data go beyond simply counting the days to achieve an extinction criterion and, instead take into account the start point, duration, and endpoint of the extinction process. This may be important since, in the clinic, the pace of early fear reduction following trauma can be predictive of persistence and success of therapy (Tarrier et al., 1999).

3. Results

MgT treatment sped up the rate of extinction and reduced spontaneous recovery of the CTA. The effects of MgT on CTA retention were more equivocal and extinction-group dependent; but rats that had received MgT during the washout period drank less SAC on the first day of extinction, suggesting a general enhancement of CTA consolidation or retrieval. The average daily dose of MgT consumed by our rats throughout the course of the study (577.16 + 14.14 mg/kg/day; Mean ± SEM; See Table 2 for details on the doses of MgT consumed during specific phases of the study) was similar to that administered in the other labs (Slutsky et al., 2010; Abumaria et al., 2011).

3.1. MgT effects on CTA consolidation or retrieval

As expected, rats in all 4 treatment groups drank equivalent volumes of MgT + SAC on the conditioning day before they received the LiCl treatment (US). However, when we compared the total SAC consumed (a combination of MgT + SAC and SAC-only solutions) on the first day of extinction training in order to evaluate the strength of the original CTA, a 2-way ANOVA revealed that MgT treatment significantly intensified the expression of the CTA (indicated by a suppression of SAC consumption) [$F(1,32) = 9.36$, $p = 0.004$] (See Fig. 1A). A more-detailed analysis that took into account extinction group assignment revealed a high degree of variability in SAC consumption between the EU and CSO control animals. Rats that were beginning the EU-EXT procedure drank more SAC than did animals beginning the CSO procedure [$F(1,32) = 4.17$, $p = 0.05$]. The analysis also revealed a marginally significant interaction between the MgT treatment and the EXT procedure employed [$F(1,32) = 3.95$, $p = 0.055$]. The animals in the EU-Control group drank significantly more SAC on the first day of extinction than did rats in the other 3 treatment groups — indicating that they had a weaker CTA (see Fig. 1B).

Before this first day of extinction, rats in the Control groups had not received MgT during the 2 weeks prior whereas animals in the MgT groups had received the compound for the 45 days before the measure was taken. A look at the amount of SAC consumed on day 1 of extinction by all the controls (combining future CSO- and EU-extinguished rats) might lead us to conclude that extended MgT treatment following CTA acquisition may enhance memory consolidation or retrieval. However, this conclusion is weakened by the variability in the SAC consumption of the Controls (no recent MgT).

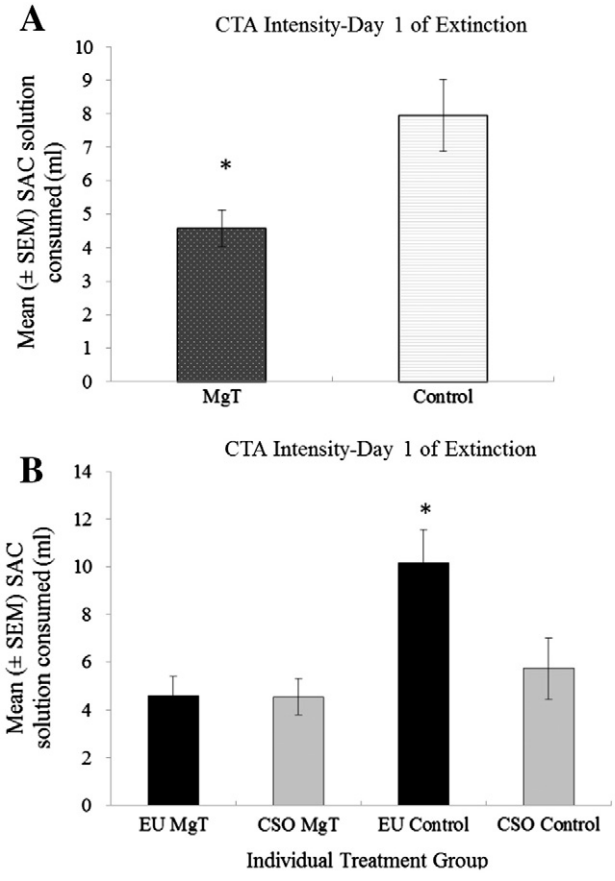

Fig. 1. Total saccharin solution consumed on the first day of extinction training. Panel A: Rats that received 45 days of MgT in their drinking water after a CTA conditioning trial exhibited a stronger aversion to the novel taste than did control animals (* = significantly less SAC consumed than controls; $p < 0.05$). Panel B: The CTA of EU Controls not receiving MgT during the Wash Out phase of the study was less potent than that of animals in the other treatment groups (* = significantly less SAC consumed than animals in all other treatment groups; $p < 0.05$).

3.2. MgT speeds CTA extinction

As described above (see Statistical analyses), we used linear regression methods to calculate the slope of the extinction curve (mls total SAC consumed across the days during the extinction phase of the study) for each rat in the study as a means of capturing the rate of extinction/subject. We then used these data in a 2-way ANOVA aimed at determining the extent to which MgT treatment and/or EU vs. CSO extinction methods changed the rate of CTA extinction. This analysis indicated that the average slopes of the MgT-treated rats' extinction curves (3.34 ± 0.44; Mean ± SEM) were steeper than those of the animals that received SAC only (Mean ± SEM = 2.40 ± 0.28) [$F(1,31) = 4.33$, $p = 0.05$]. See representatives of these curves in Fig. 2. Employing EU-EXT methods did not increase the rate of extinction (slope of extinction curves) as compared to rats that experienced the CSO extinction procedure.

Perhaps because the CTA extinction process was relatively rapid following a single SAC + LiCl pairing as compared to when there are multiple CS + US pairings (see Mickley et al., 2011, 2012), neither MgT treatment nor the method of extinction employed (CSO or EU) affected the number of days it took rats to reach asymptotic extinction. We stopped the CTA extinction procedure when the rats achieved baseline/asymptotic SAC drinking levels (see Materials and methods for information about how this was determined). Therefore, the volume of SAC consumed on the day animals achieved the criterion for asymptotic extinction was not significantly different for rats in the 4 treatment groups.

3.3. MgT reduces spontaneous recovery of a CTA

Both MgT treatment and method of extinction had a significant influence on the SR of the CTA. We computed a 2-way ANOVA [Extinction procedure (CSO-EXT or EU-EXT) × MgT treatment (MgT or no-MgT)] which evaluated the volume of liquid consumed (SAC or MgT + SAC) on the day of asymptotic extinction and on the SR test day as a repeated measures factor. The analysis revealed a significant reduction in SAC

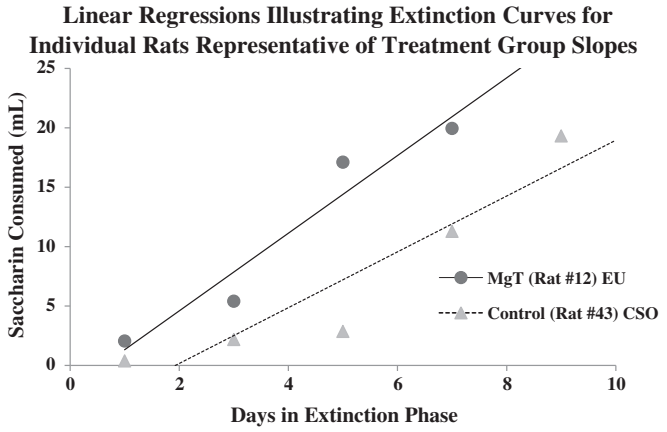

Fig. 2. Linear regressions for the SAC consumption of individual rats during the extinction phase of the study. The lines plotted are representative of animals in the 2 treatment groups either receiving SAC + MgT during extinction or receiving SAC only (Controls). The average slopes of the extinction curves for MgT-treated rats were significantly ($p < 0.05$) steeper than those of rats that were not drinking MgT in their SAC solution (see text). Although there was a significant difference between the slopes of the extinction curves of rats in the MgT and control groups, there was no significant difference between the slopes of the EU and CSO groups (one from each extinction group illustrated here). The ratio between these 2 representative slopes (slope of MgT Rat #12/slope of control Rat #43 = 1.39) is the same as that of the mean slopes for these 2 groups of animals — indicating that these individuals represent the groups from which they were selected.

consumption during the SR test as compared to the SAC consumed on the day of asymptotic extinction [$F(1,30) = 151.63$, $p < 0.001$] (see Figs. 3 and 4). The MgT treatments given during the washout and extinction phases of the study also significantly reduced SR of the CTA [$F(1,30) = 104.10$, $p = 0.009$]. Further, rats that experienced the EU-EXT technique showed less of a SR of the taste aversion than did rats that underwent the CSO-EXT procedure [$F(1,30) = 73.73$, $p = 0.025$].

4. Discussion

4.1. General summary

We hypothesized that rats exposed to MgT continuously for 45 days before the extinction procedures would exhibit a faster rate of extinction than controls and show a reduced SR of their CTA. In support of these hypotheses, we discovered that the slopes of the inter-session extinction curves were significantly steeper for rats treated with MgT. Furthermore, and also consistent with our hypothesis, rats belonging to the MgT group had a significantly reduced tendency to spontaneously recover their CTA when tested after the 30 day latency period following extinction. This was evidenced by MgT-treated rats drinking more SAC than controls on the SR test day. Although not part of our original hypotheses, our behavioral data also indicated that MgT may be capable of enhancing the consolidation or retrieval of a CTA. Rats exposed to MgT continuously for 45 days before extinction procedures were begun, displayed a more intense CTA (i.e., lower consumption of SAC on the first day of extinction training) than did rats that did not consume MgT in the 14 days before extinction training was initiated.

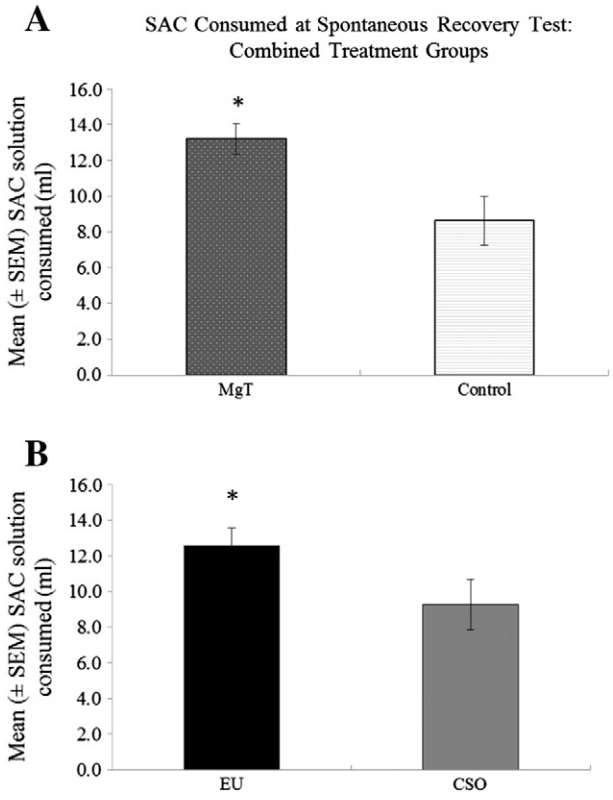

Fig. 3. Mean total SAC + Mgt (16 mg/ml) consumed on the day of the SR test. Panel A: Rats continuing to receive MgT during the "Wash Out" and extinction phases of the study (see Table 2) showed a significantly reduced SR of the CTA (i.e., they drank more SAC) than did controls. Likewise, rats going through the EU extinction procedure drank more SAC than CSO-EXT animals at the SR test — indicating that this extinction procedure reduced SR of the CTA (see Panel B). * = significantly more SAC consumed that the other group ($p < 0.05$).

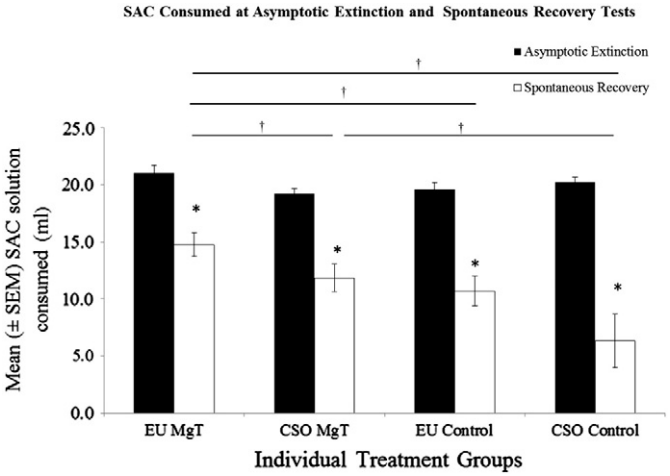

Fig. 4. Mean total SAC solution consumed on the day of asymptotic extinction and SR tests. Animals in all groups reached asymptotic extinction but they exhibited varying amounts of SR. Rats receiving MgT during the "Wash Out" and extinction phases of the study (see Table 2) and underwent EU-EXT showed the mildest SR; while those in the control group and undergoing CSO-EXT experienced the most severe SR. * = significantly reduced SAC consumption during SR as compared to the same animal's SAC drinking when they achieved asymptotic extinction. † = significant differences in the SAC consumption of the groups indicated at SR test; $p < 0.05$.

4.2. Does MgT intensify CTA consolidation or retrieval?

The current experimental procedures were aimed at testing the effects of MgT on CTA extinction and spontaneous recovery and called for one CS + US pairing before our animals experienced an extended MgT treatment period (31 days), followed by multiple CS exposures during extinction training. Orally ingested MgT has been shown to take at least one month to raise brain magnesium levels to the extent required to have an effect on memory formation (Slutsky et al., 2010). Therefore, we did not expect to discover changes in CTA acquisition in rats treated with MgT just 3 days before the conditioning day. Consequently, we did not evaluate the SAC consumption of rats in our 4 treatment groups immediately following the conditioning day. It was with some surprise that on the first day of extinction training, we observed less SAC consumption by the rats that had received MgT through the washout phase of the study (MgT group) than did those rats that stopped receiving MgT during the washout phase (control group).

While we cannot eliminate the possibility entirely, it is unlikely that this difference can be explained by MgT-induced enhancement of the CTA acquisition process. Rats in our previous studies exhibited a significant decline in CS consumption (often approaching total abstinence) following even a single CS + US pairing (see Mickley et al., 2004, 2007, 2009, 2012). Since rats in all 4 of our groups had received exactly the same treatment before (and immediately following) CTA acquisition, we have no reason to expect that there were group differences in CTA strength immediately after the conditioning day. It is well known that conditioned taste aversion is a robust and long-lasting form of aversive learning (Bernstein, 1991; Yamamoto et al., 1994).

If there was equivalency in the original CTA memory trace, then we may conclude that the MgT-induced differences in CTA retention we observed on the first day of the extinction phase was due to as-yet-unidentified neural alterations that took place in those rats receiving the compound for 45 days before extinction. There is evidence that the CTA consolidation process is protracted, taking several days (Ivanova and Bures, 1990; Shema et al., 2009), during which MgT might have exerted its effects. The cellular mechanisms of CTA acquisition and consolidation are different from memory retrieval (Bi et al., 2010) and so retrieval of the CTA on the first day of extinction could be another point at which MgT might have modulated this aversive memory.

Unfortunately, our conclusions about the effects of MgT on CTA consolidation or retrieval must be equivocal due to the high degree of variability in the SAC consumption by rats within our Control group (receiving no recent MgT). Inexplicably, rats that just started the EU extinction procedure drank more SAC than those beginning the CSO extinction procedure. Futures studies should address this question of the reliability and the neural mechanisms of MgT-induced CTA enhancement.

Previous research (Abumaria et al., 2011) using a fear conditioning paradigm found that MgT had no effect on an original fear memory. However, our data indicate that there may have been an MgT-induced enhancement of consolidation or retrieval of a CTA. What might explain these different findings? There are established differences in the neural mechanisms responsible for taste aversion learning and for fear learning using a tone + shock conditioned emotional response (CER) paradigm. Abumaria et al. (2011) suggested that MgT differentially affects brain physiology by enhancing hippocampus-dependent fear memory but not amygdala-dependent fear-memory in rats. Significant increases in prefrontal cortex-dependent retention of extinction were also found (Abumaria et al., 2009). Furthermore, Slutsky et al. (2010) showed chronic MgT treatment upregulated NMDA receptor activation and expression in the hippocampus. Additional research has noted that the hippocampus and prefrontal cortex modulate the expression of fear after a CER has been formed through involvement of the amygdala (Morgan and LeDoux, 1995; McEchron et al., 1998; LeDoux, 2000; Milad and Quirk, 2002; Sanders et al., 2003; Farinelli et al., 2006; Hobin et al., 2006; Herry et al., 2008). However, CTA differs from CERs in terms of the brain structures that encode these aversive memories. Apparently, the parabrachial nucleus and the nucleus of the solitary tract are the points of CS + US association and the CTA memory is subsequently transferred to the basolateral amygdala and gustatory neocortex for behavioral expression and long-term retention, respectively (for review, see Yamamoto et al., 1994). Furukawa et al. (2009) have reported brain-area-specific effects of Mg^{2+} on intracellular Ca^{2+} concentrations in rat hippocampus and cortex. Thus, MgT may differentially modulate the specific neural substrates of CER and CTA acquisition.

4.3. MgT speeds CTA extinction

The rate of extinction was calculated for each animal individually by measuring the change in saccharin consumption as a function of time until they reached ≥90% of their original saccharin consumption (asymptotic value). The MgT group had a steeper slope than did the control group, indicating that they reached asymptotic extinction at a faster rate. Abumaria et al. (2011) have reported stronger retention of CER extinction but, as far as we know, this is the first study demonstrating that chronic MgT treatment can significantly impact the rate of extinction of an aversive memory. In both Pavlovian and operant preparations, the speed and extent of response elimination in extinction depends on the similarity between stimulus conditions in effect before extinction and those in effect during extinction (for review see, Lattal and Lattal, 2012). Since MgT was consumed both immediately before and during extinction in MgT groups but not our controls, this may help explain the drug-induced enhanced rate of extinction we observed.

We employed a 14-day MgT "Wash Out" period immediately before our CTA extinction procedures as a way of differentiating our experimental rats (receiving MgT 2 weeks before and throughout extinction) from our controls (MgT treatment stopped 2 weeks before extinction began). Slutsky et al. (2010) demonstrated that 2 weeks after termination of MgT treatment the positive effects of MgT on working memory were diminished in aged rats (22–24 months old). However, in young rats (2 months old), the enhancement of working memory by MgT remained up to 30 days after termination of the treatment. These data suggest that magnesium can be depleted from the body of aged rats, but not young rats, within 2 weeks of termination of the treatment. The current study employed young-adult rats (3–4 months old at the start of our experiment; 6–7 months old at the end of the study) whose age fell between the young and aged rats tested by Slutsky et al. (2010). Relative to the animals that continued to receive MgT before and during CTA extinction, the control rats exhibited a slower rate of extinction (while still reaching asymptote) and a stronger spontaneous recovery of the aversion. Thus, the current study suggests that, like aged rats (Slutsky et al., 2010), young-adult rats are also sensitive to effects of MgT withdrawal. The phenomenon may depend on the type of behavioral paradigm employed. We tested emotional/aversive memories whereas Slutsky et al. (2010) employed spatial and working memory tasks. It should be noted that Abumaria et al. (2011) reported enhanced efficacy of fear extinction in 4–8-month old rats treated with MgT. It is possible that brain circuitries responsible for processing CTA-related memories (perhaps gustatory neocortex or amygdala; see Mickley et al., 2007, 2009), are sensitive to magnesium depletion even in young-adult rats.

Previously we have shown that the EU-EXT procedure more rapidly extinguishes a CTA than does a CSO extinction procedure (Mickley et al., 2009). However, perhaps because the CTA extinction process was relatively rapid following a single SAC + LiCl pairing as compared to when there are multiple CS + US pairings (see Mickley et al., 2011, 2012), neither MgT treatment nor the method of extinction employed (CSO or EU) affected the number of days it took rats to reach asymptotic extinction. Multiple SAC + LiCl pairings create a more-intense CTA than does a single CS + US pairing. For example, reaching asymptotic extinction following 3 CS + US associations requires 30–43 days (Mickley et al., 2012). However, following the single SAC + LiCl pairing used in this study, asymptotic extinction was achieved by all rats in ≤15 days. Since all animals extinguished quickly, it may have been difficult to detect significant differences in the number of days required to extinguish the CTA of rats differentiated by either MgT treatment or extinction method.

Our presentations of SAC were spaced over 48-hour intervals during extinction. This raises a question regarding the extent to which our data represent the rate of extinction versus the retention of extinction. The dynamics of CR reduction during the process of fear extinction have been the subject of several studies (Milad and Quirk, 2002; Phelps et al., 2004) and reviews (Myers and Davis, 2007). Within the CER paradigm, near-asymptotic levels of extinction can be achieved in a single session, over minutes, and consolidation can take place within 24 h. Diminution of CRs over longer intervals may reflect the extent to which an extinction memory is retained.

There is less known about the dynamics of CTA extinction. Even when a CTA is formed after a single CS + US pairing, asymptotic extinction of the association is not typically achieved in 1 training session (see the current data and Eisenberg et al., 2003). This may be due, in part, to the fact that the duration of CS exposure is frequently determined by the subject's voluntary consumption of the CS rather than being under control of the experimenter. This means that the behavioral outcomes of a single extinction session may reflect partial extinction of the CTA. Subsequent extinction trials likely build on a retained memory trace resulting in greater acceptance of the CS. Extinction is regarded as the sum of multiple time-dependent processes involving the competition of an excitatory CS + US trace and an inhibitory CS-US trace (Eisenberg et al. 2003). Thus, the extinction curves reported here may reflect a combination of rate of CTA extinction learning and retention of CTA extinction over the 48-hour period between extinction trials.

4.4. MgT reduces spontaneous recovery of a CTA

Both MgT treatment and method of extinction provided significant benefits in terms of reducing SR of a CTA. Rats that had continued to receive MgT treatment during the 14 day washout period and extinction phase (MgT group) drank considerably more saccharin at the SR test

than did rats that did not receive MgT during the 14 day washout period (control group). Furthermore, compared against themselves, rats in the MgT group consumed levels of SAC during their SR test that were closer to the amount of their saccharin consumption at asymptotic extinction than did rats in the control group. This indicates that the MgT treatment attenuated the severity of SR. These data are consistent with others who have demonstrated MgT treatment to be effective in enhancing extinction of fear and preventing SR of extinguished fear memories (Abumaria et al., 2011).

Additionally, rats that experienced the EU-EXT technique showed less of a SR of the taste aversion than did rats that achieved the same level of asymptotic extinction through use of the CSO-EXT procedure. This finding is consistent with others (Mickley et al., 2009; Rauhut et al., 2001; Thomas et al., 2005). Rats that received both MgT treatment during the washout phase and underwent the EU-EXT procedure showed the least SR when compared to other groups. Rats that did not receive MgT treatment during the washout phase and that underwent the CSO-EXT procedure displayed the greatest SR. This suggests that MgT may be especially efficacious when combined with behavioral methodologies aimed at reducing SR following fear extinction.

Initially, it seemed puzzling that MgT rats experienced both a stronger CTA acquisition and a weaker SR of the CTA. It has been well established that the strength of a CTA is dependent on the CS and US "dosages". Multiple pairings of a US with a CS, or higher doses of the US, will induce a CTA that requires more time to extinguish (Garcia et al., 1955, 1956a, 1956b). However, we know of no published literature indicating that rats reaching the same level of asymptotic extinction may differ in their spontaneous recovery of the CTA because of an initial difference in the intensity of the CTA. In fact, some early experiments reported that an increase in acquisition training of a conditioned eye-blink response caused a decrease in responding during SR test trials (Prokasy, 1958). Therefore, it may be appropriate to consider the development of a stronger CTA and the occurrence of a weaker SR of the CTA as two distinct effects of chronic MgT treatment that do not necessarily influence one another.

4.5. A possible alternative explanation for the extinction and SR data

A potential alternative explanation for our findings relies on the phenomenon of stimulus generalization. Our animals treated chronically with MgT (both prior to conditioning and during extinction) were trained and tested with the same combination of stimulus elements throughout, i.e., 0.3% saccharin + 16 mg/kg MgT. Animals that were not treated with MgT during extinction received the same concentration of saccharin but no MgT. Since the conditioning (SAC + MgT) and extinction (SAC + MgT) solution was the same for the experimental groups but was different for the controls (conditioning: SAC + MgT; extinction: SAC), could our data be interpreted as reflecting differences in stimulus generalization? Likewise, when SAC + MgT was experienced at the spontaneous recovery (SR) test, by the control rats that were extinguished with SAC only, might the greater SR we observed be expected since the tastants presented during extinction and SR test were different? Resolution of this issue depends heavily on the extent to which our rats distinguished between the tastes of SAC only and SAC + MgT. MgT (in the concentrations we used) has been described as colorless, tasteless, and odorless to humans (http://www.magtein.com/thequality.html). However, we needed to test the generalizability of the gustatory stimuli, SAC only and SAC + MgT, in our rats. Our third pilot study spoke to this issue directly.

To the best of our knowledge, there are no published studies that have looked at the generalizability/discriminability of SAC only vs. SAC + MgT. However, there is an established literature that investigated similar issues but employed NaCl and LiCl solutions (for review, see Ossenkopp et al., 1997). Despite the distinct physiological and behavioral effects of these 2 compounds, the tastes of the two salts are apparently quite similar (Scott and Giza, 1987). In citing evidence supporting this contention of taste similarity, Ossenkopp et al. (1997) point to a series of studies indicating that rats taught to avoid LiCl will also avoid NaCl (Nachman, 1963; Balagura and Smith, 1970). This is a paradigm similar to the one we employed in our third pilot study. Further support for the similarity of the LiCl and NaCl tastes has been noted by that fact that, in repeated non-reinforced tests with NaCl, a LiCl CTA was extinguished (i.e., the extinction, generalized so that animals would again drink LiCl; Nachman, 1963). Despite this strong similarity of the tastes of LiCl and NaCl, animals can be forced to discriminate between the 2 compounds through repeated experiences with ingestion. Because the postingestional cues associated with LiCl are aversive and those associated with NaCl are not, animals will ultimately avoid LiCl while continuing to accept NaCl (Balagura et al., 1972; Ossenkopp et al., 1997).

There are 2 important points that one can derive from this literature. First, the similarity of tastes has typically been evaluated in a manner similar to the procedure we used in our 3rd pilot study wherein one of the tastants was associated with malaise and the other was not and an opportunity was provided to detect a generalization of the aversion. Second, if training is sufficiently extensive and animals are given repeated experiences with 2 gustatory stimuli that are quite similar in taste, they may be able to learn to differentiate between them if one is continued to be reinforced while the other is not. In fact, extremely small differences in concentrations (e.g., 2.4×10^{-6} M) of the same compound (HCl) can be detected when similar taste discrimination methodologies are employed (Scott and Giza, 1987).

In order to interpret our findings as being consistent with MgT-induced enhancement of memory, it is important to confirm that SAC only and SAC + MgT are similar enough so that, in the context of our study, rats were unlikely to detect/recognize subtle taste differences between the 2 stimuli during extinction and our SR test. However, it must be acknowledged that it was not the intent of our third pilot experiment to do a full taste discrimination study. Taste discrimination is learned and thereby modified by experience. The use of a true taste discrimination paradigm as a control is far afield from the procedures used in our main experiment and therefore not very relevant. In our main study, there was little opportunity for discrimination learning (Nowlis, 1974) given that we used only one conditioning trial. Instead, the main point to be addressed here was the extent to which rats respond similarly to SAC only when it is used as a substitute for SAC + MgT in our control rats during the extinction and SR phases of our study. In our paradigm, we did not set up procedures wherein over a series of days the taste of one compound was followed by illness and the other was not in order to cause a taste discrimination. In fact, we intentionally tried to reduce the opportunity for our animals to discern differences (if any existed) between the tastes of the 2 compounds.

First, we allowed a long interval (45 days) between the conditioning day (when all rats tasted SAC + MgT) and the first day of extinction (when our control rats first tasted SAC only). Memory loss for specific characteristics of a stimulus is a robust phenomenon reported in a variety of both animal and human studies (for review, see Metzger and Riccio, 2009). Typically, this class of memory loss is reflected in the flattening of a generalization gradient. While CTAs are known to be long lasting, forgetting of stimulus attributes has been demonstrated in an aversive gustatory conditioning paradigm (Metzger and Riccio, 2009). In our third pilot study we reported generalizability of SAC only and SAC + MgT just one day after SAC only was associated with LiCl. If rats were not responding to any taste differences between our 2 compounds 24 h after conditioning, it seems unlikely that they would be able to recall specific stimulus attributes of SAC + MgT from 45 days earlier and compare them to the SAC only tasted on the first day of extinction.

Second, our study was designed to reduce the likelihood that the taste of MgT (if any) would be given predictive salience. All rats were exposed to 3 days of water + MgT before they experienced the

SAC + MgT tastant that served as the CS. This procedure is consistent with a typical latent inhibition paradigm (Bakner et al., 1991) and methods similar to this have been used to inhibit the potency of a stimulus to act as an effective CS (Best, 1975). Experiments aimed at determining the extent to which a compound stimulus can serve as an effective CS when a component has undergone pre-exposure indicate that the flavor aversion to the compound is not attenuated (Misanin and Hinderliter, 1990) while the unique/new features of the compound take on greater perceptual effectiveness (Blair and Hall, 2003). Further, behavioral work with hamsters has shown that if a taste aversion is conditioned to a two-component mixture, this aversion generalizes to the two components presented unmixed (Nowlis and Frank, 1977, 1981) but if one component is rendered ineffective for taste-aversion learning by making it thoroughly familiar to the animal prior to conditioning, the aversion generalizes to the novel component and to the mixture, but not to the familiar component (Nowlis and Frank, 1981). Although we have no data from the current study to confirm it, this literature would lead us to the prediction that our rats pre-exposed to water + MgT, followed later by SAC + MgT and a LiCl pairing may have tended to reduce the salience of MgT as a CS and raise the potency of SAC. If this is the case, it increases the likelihood of an effective generalization between SAC + MgT and SAC only during CTA extinction.

Finally, studies evaluating the generalization of CTAs indicate that generalization is most effective when the concentration of the CS paired with the US matches the one later tested. This holds for compound stimuli such that animals avoided mixtures containing the CS in proportion to its concentration in the mixture (Smith and Theodore, 1984). The SAC concentration employed in our study (0.3%) was consistent throughout the experiment and also consistent independent of whether it was offered alone or in combination with MgT. These features of our methods aimed to increase the likelihood that rats conditioned to avoid the taste of the SAC + MgT CS would also avoid the taste of SAC only.

In addition to citing the published literature, we can also offer evidence from our own observations indicating that an alternative explanation of lack of generalizability between SAC only and SAC + MgT fails to fully explain our data. If our control rats that received SAC + MgT during the conditioning procedure and SAC only during extinction were able to detect a difference between these 2 sweet stimuli, one would expect that the SAC only would appear as safer than the SAC + MgT and lead to more-rapid acceptance of the taste (see extinction curves). However, our control rats drinking SAC alone actually exhibited a slower rate of acceptance of the sweet taste than did the rats drinking SAC + MgT. If we consider SAC only as a novel taste, could this slower acceptance of it be attributed to neophobia? This is unlikely since taste neophobia is overcome much more rapidly than is a CTA (Mickley et al., 2004). Thus, these data do not seem to be consistent with the hypothesis that SAC only is being differentiated from SAC + MgT.

Rats are acute observers of their gustatory environment and it is likely that, given sufficient exposure to tastes of SAC only and SAC + MgT, and selective reinforcement of one of these tastes, they would be able to discriminate between the two. However, the published literature and the data provided by our pilot studies lead us to the conclusion that it is unlikely that stimulus generalization is the sole mediator of the effects we report here.

4.6. Conclusions

Our data are consistent with a growing animal literature suggesting that chronic MgT treatment may have clinical relevance since it has been shown to reduce learned helplessness (a model of human depression; Abumaria et al., 2009), enhance the efficacy of fear extinction (Abumaria et al., 2011), and reduce cognitive deficits in a mouse model of Alzheimer's Disease (Liu et al., 2009). Here we demonstrated that MgT increased the rate of CTA extinction, reduced SR of a CTA, and interacted with EU extinction procedures to further reduce SR of a CTA. Although $MgSO_4$ treatments have been reported to improve recovery following traumatic cortical damage in rats (Vink et al., 2003; Hoane et al., 2008) and have shown some promise as a pre-hospitalization treatment for acute stroke patients (Saver et al., 2004), there are limitations on the ability of this compound to move Mg^{2+} into the brain (McKee et al., 2005). Trials such as these may benefit from using MgT as a vehicle compound to deliver elemental magnesium to the central nervous system.

Acknowledgments

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