Magnesium deficiency reduces fear-induced conditional lick suppression in mice

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Abstract. The consequences of broad-scale alterations in magnesium (Mg²⁺) levels on learning and memory are poorly understood. We have recently demonstrated that adult male mice maintained on an Mg²⁺-deficient diet exhibit reduced conditional freezing behavior. The purpose of the present study was to determine if the detrimental effect of Mg²⁺ deficiency in mice extended to another measure of conditional fear, conditioned lick suppression (CLS), as well as to another form of learning, spatial learning in the swim maze task. Adult male C57Bl/6J mice were provided with a normal or Mg²⁺-deficient diet and were trained and tested ten days later for conditional fear, using CLS and freezing as indicators of learning. Learning in the swim maze was tested in a separate cohort of mice during days 14-18 of diet exposure. Mg²⁺-deficient mice showed reduced CLS as well as conditional freezing behavior in comparison to control mice. However, learning in the swim maze task was normal in Mg²⁺-deficient mice. These studies indicate that the detrimental effects of Mg²⁺ deficiency extend to other measures of conditional fear but not to all forms of learning.

Key words: conditional fear, NMDA, glutamate, spatial learning, mice

Disease states, such as diabetes, atherosclerosis, and alcoholism, have all been linked to deficits in magnesium (Mg²⁺) [1-3]. Each disorder has also been associated with impairments in memory function [4-6], raising the possibility that Mg²⁺-deficiency contributes to such impairments. It is certainly clear that Mg²⁺ plays an important role in neuronal physiology [7], and it is perhaps best known for its role as a crucial gating factor in N-methyl-D-aspartate (NMDA)-type glutamate receptor function. Magnesium blocks the NMDA receptor ion channel when neurons are in a resting state and is removed from the channel during states of depolarization. Moreover, these receptors are necessary for some forms of learning and memory in mice, including fear conditioning [8]. Hypothetically, deficient brain Mg²⁺ levels should increase NMDA receptor sensitivity and alter forms of memory dependent on efficient NMDA receptor function.

While studies in animals have demonstrated the beneficial effects of Mg²⁺ supplementation on behavior and neuronal function [9-11], it cannot be completely assumed that Mg²⁺ deficiency would impair such phenomena. Indeed, little preclinical work has specifically addressed the effects of experimental Mg²⁺ deficiency on learning and memory in laboratory animals. We recently addressed this issue by demonstrating that mice maintained on an Mg²⁺-deficient diet for several days exhibit reduced contextual and cued fear conditioning [12]. This decrease in fear conditioning appeared to reflect a memory deficit and not a non-specific change in behavior, since Mg²⁺-deficient mice performed normally in tests of locomotor activity and anxiety, and demonstrated average to above-average sensitivity to footshock [12]. Mice maintained on an Mg²⁺-deficient diet also showed an increased sensitivity to the seizure-inducing effects of systemically administered NMDA, suggesting that NMDA receptors were hyper-responsive in Mg²⁺-deficient animals [12].

Most studies of fear conditioning, including ours [8, 12, 13], typically use freezing behavior as a...
measure of learning. In these studies, increased levels of freezing behavior upon presentation of the conditioned stimulus (CS) is taken to indicate better memory of the initial CS-footshock pairings. Immobility, however, may be prone to non-mnemonic influences. Animals that freeze less during CS presentations may nonetheless still express conditional fear if measured by avoidance of CS [14] or potentiation of startle by CS presentation [15]. Thus, it is worthwhile to corroborate changes in conditional freezing behavior with other measures of fear conditioning.

One powerful measure of fear conditioning is conditional lick suppression (CLS). In typical CLS studies [16-19], animals are water-deprived and trained to drink in a neutral environment. Conditioning involves the presentation of a novel stimulus (e.g., tone, light, odor) paired with footshock in the neutral environment with no water bottle present. The associative strength of the CS-footshock pairing is then measured by presenting the CS alone with the water bottle and recording the latency to lick for a specific period of time. Presentation of the CS should suppress licking behavior and thereby increase the latency to lick. While it is important to bear in mind that water deprivation alone may enhance some forms of fear conditioning [20, 21], this effect should be balanced across experimental conditions if all groups are water-deprived. The CLS procedure has not been extensively used in mouse behavioral studies, but it could serve as a useful addition to assessments of conditional fear in standard mouse behavioral phenotyping batteries.

In addition to determining if other measures of conditional fear are altered in Mg2+-deficient mice, it is also important to assess whether other forms of learning and memory are impaired by decreases in dietary Mg2+. One of the most widely studied learning tasks in laboratory mice is the Morris swim maze. This task can be used to test spatial learning and memory, and depends on the integrity of the hippocampus [22]. Moreover, it appears that NMDA receptors within the hippocampus are necessary for learning in this task [23, 24]. Since Mg2+ deficiency may disrupt NMDA receptor function, it could be expected that deficient mice would perform poorly in this task.

The purpose of the present studies was to determine if Mg2+ deficiency alters CLS and learning in the Morris swim maze. Male C57Bl/6J mice were maintained on a normal or Mg2+-deficient diet throughout behavioral testing. Mice from each group were trained and tested on the CLS task beginning on the tenth day of diet exposure. Separate groups of mice were tested in the swim maze during days 14-18 of diet exposure. These time points were chosen since our previous study [12] demonstrated fear conditioning deficits in mice maintained on an Mg2+-deficient diet for a similar amount of time.

Materials and methods

Animals

Young adult male (60 days of age) C57Bl/6J mice were purchased from the Jackson Laboratories (Bar Harbor, ME). Mice were handled for one week after arrival and were ear-clipped for identification. They were group-housed in sets of four and had free access to food, water, and bedding except during CLS testing (see below). Lights were on at 0600 and off at 1800. All testing was performed between 0900 and 1600. All procedures were approved by the Animal Care and Use Committee at Northern Kentucky University and followed the standards described in the Guide for the Care and Use of Laboratory Animals.

Diet

After one week of daily handling, mice were placed on an Mg2+-deficient (Diet # 90106, Harlan-Teklad, Madison, Wisconsin) or control (Diet #85341, Harlan-Teklad) diet. Each diet contained 0.003% and 0.1% Mg2+, respectively. Distilled water was provided for drinking. At the same time, wire mesh inserts were placed in the bottom of each cage above the wood chip bedding to prevent animals from eating the bedding or other matter. Our previous studies have shown that plasma Mg2+ is significantly reduced after three days on the Mg2+-deficient diet [12].

Conditional lick suppression

The CLS procedures were based on the work of White and Viaud [17-19]. Seven days after the beginning of the Mg2+-deficient (n = 26 mice) or control (n = 25 mice) diet, animals were placed on a water-restriction schedule and were allowed access to a water bottle for 20 minutes a day until the end of the CLS experiment. On days in which animals were tested in the operant chamber, mice were allowed access to the water bottle for 20 minutes in their home cage within 30 minutes of such testing. Mice maintained on this schedule appeared healthy throughout habituation, training, and testing trials.

After ten days of diet exposure, each mouse was placed into a standard operant chamber within a
sound attenuating cubicle (Med-Associates, St. Albans, VT) for 15 minutes a day on three consecutive days and allowed access to a water bottle filled with distilled water. The water bottle was placed into a lickometer (Med-Associates, St. Albans, VT), so that a photobeam was broken each time the animal licked from the sipper tube. The lickometer was connected to a PC running MED-PC software (Med Associates) that recorded licking at the sipper tube. During the three habituation days, the latency to the 5th lick was recorded and compared between the groups.

Thirteen days after being placed on the Mg²⁺-deficient or control diet, animals were placed into the operant chambers for five minutes without the water bottle present. After two minutes, an 80 dB, 2800 Hz tone was presented for 20 seconds. During the last second of the tone, animals received a 0.8 mA continuous footshock. This pairing was repeated every minute for the next three minutes. The presence or absence of freezing behavior, defined as no movement other than normal respiratory movements, was noted every 10 seconds during the entire five-minute training session. Animals were removed from the operant chamber 40 seconds after the third shock and returned to their home cage. The Med-PC software controlled the presentation of the tone and footshock.

On the following day, the animals were returned to the operant chamber for 10 minutes and allowed access to the water bottle. The latency to the 5th lick was recorded. Upon licking the sipper tube for the 5th time, the auditory stimulus that was paired with footshock on the previous day was presented continuously until the end of the trial. The latency to the 20th lick was recorded. In order to capture the freezing response to the auditory stimulus, the average amount of freezing over the two minutes following the presentation of the auditory stimulus was measured as described above for the training trial. By recording this behavior for two minutes, it became necessary to exclude any animal with a latency to the 5th lick that exceeded eight minutes since the animal would have been removed from the chamber at ten minutes and insufficient post-licking freezing data would have been generated. This exclusion criterion affected one animal in each group.

Swim maze task

A separate cohort of control and Mg²⁺-deficient mice (n = 20 per group) were tested in this task during days 14-18 of diet exposure. The maze was two meters in diameter and one meter tall. It was filled with water made opaque with non-toxic white and blue tempera paint. Extra-maze cues were present near the pool and two 100W lights were placed above the maze. On the first day of testing, animals were placed on or near a 10 cm diameter platform that was located one cm below the water’s surface in the center of the pool for three 15-second trials. After these trials, the platform was moved to a specific quadrant, and all mice were given four test trials a day with a five-minute intertrial interval. Testing was performed on five consecutive days. Mice were maintained under a 60W lamp between trials. Each trial began by placing the mouse in the water at a predetermined location that varied each day of testing. The latency to find the platform was recorded. This measure has been used by Morris [25] and others [26] as a stand-alone measure of memory and correlates well with other measures of performance in the swim maze task [27]. Furthermore, in our previous study [12], Mg²⁺-deficient mice did not exhibit general changes in motor activity or anxiety that could have interfered with swimming behavior. The mouse was allowed to remain on the platform for 10 seconds after locating it. If the mouse did not find the platform within 60 seconds, it was placed on the platform for 10 seconds.

Data analyses

The latency to the 5th lick on all three habituation days was compared between control and Mg²⁺-deficient mice over the three days. On the test day, latencies to the 5th and 20th licks were compared between experimental groups as a function of time in a similar manner. On the training day, freezing behavior was compared between groups during each minute of training. Freezing behavior was defined as the average amount of time spent freezing per minute. On the test day, each animal was observed for freezing behavior for two minutes after their fifth lick (or upon CS onset) and this data was compared between groups across the two-minute interval. Finally, the latency (in seconds) to find the platform in the swim maze was compared between the groups across test days. In all analyses, a two-way ANOVA was used to compare the data with diet as a betweengroup independent measure and time as a withingroup repeated measure. Fishers protected least squares difference (PLSD) test (one-tailed) was used to perform individual group comparisons when a statistical main effect was found. The alpha level for accepting statistical significance in all tests was set at p < 0.05.
Results

As we reported previously [12], animals maintained on the Mg²⁺-deficient diet for 10-20 days appeared normal and demonstrated home cage activity and behavior that was indistinguishable from mice maintained on the control diet. At days 14 and 21 of diet exposure, animals in the Mg²⁺-deficient group exhibited slightly yet significantly lower body weights in comparison to animals in the control diet group (table 1) (diet effect: F (1, 58) = 11.05, p < 0.002; between group differences at days 14 and 21, p < 0.02 and 0.03, respectively).

It should also be noted that there was significant weight gain observed in each group between the two time points (day effect: F (1, 58) = 12.3, p < 0.0009; differences in Mg²⁺-deficient and control groups between days, p < 0.05 and p < 0.008, respectively).

Over the three-day habituation period in the CLS experiment, the latency to the 5th lick did not differ between the two groups of mice (figure 1A). However, as expected, there was a significant decrease in the latency to drink over the three habituation days (day effect: F (2, 98) = 20.8, p < 0.0001). On the training day, both groups of mice spent little time freezing during the first two minutes prior to tone-footshock pairing (figure 1B). However, upon exposure to the three tone-footshock pairings over the last three minutes, both groups demonstrated a similar increase in freezing relative to the levels expressed during the first two minutes (time effect: F (4, 152) = 36.9, p < 0.0001).

On the test day, the tone was presented continuously after the 5th lick until the end of the 10-minute test session. The control and the Mg²⁺-deficient mice did not differ in the latency to the 5th lick in the test session. However, upon continuous presentation of the tone after the 5th lick, the Mg²⁺-deficient mice demonstrated a faster latency to the 20th lick in comparison to the control mice (time x group interaction: F (1, 49) = 4.18, p < 0.05) (figure 2A). The group difference in the

Table 1. Effect of Mg²⁺-deficient diet on body weight (g).

<table>
<thead>
<tr>
<th>Diet condition</th>
<th>Control</th>
<th>Mg²⁺-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day of exposure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td>21.4 ± 0.4 (16)</td>
<td>20.2 ± 0.3 (16)*</td>
</tr>
<tr>
<td>Day 21</td>
<td>23.2 ± 0.5 (16)</td>
<td>21.4 ± 0.5 (14)*</td>
</tr>
</tbody>
</table>

All data presented in average weight (g) per group ± S.E.M. The number of mice per group is in parentheses. Asterisks indicate statistically significant differences between groups. It should be noted that each group experienced significant weight gain between days 14 and 21.

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Figure 1. Effects of magnesium deficiency on the latency to lick during habituation trials and freezing behavior during tone-footshock training. A) Animals in each condition (control and Mg²⁺-deficient diets) demonstrated similar latencies to the 5th lick on all three habituation days. B) On the training day, all mice showed little freezing during the first two minutes of observation. After exposure to tone-footshock pairings during the 3rd, 4th, and 5th minute of training, all mice demonstrated similar levels of freezing. n = 26 in the Mg²⁺-deficient group and n = 25 in the control group.
latency to the 20th lick was supported by a subsequent planned post-hoc analysis (Fishers PLSD, p < 0.05, one-tailed). The Mg^{2+}-deficient group also showed significantly less freezing behavior for two minutes after tone onset in comparison to the control group (group effect: F (1, 49) = 5.3, p < 0.03) (figure 2B). Planned individual group comparisons at each time point revealed significant differences between the groups (Fishers PLSD, 1st minute, p < 0.05; 2nd minute, p < 0.02, one-tailed). Also the overall amount of freezing exhibited by both groups decreased over the two-minute interval (time effect: F (1, 49) = 9.1, p < 0.004).

In a separate cohort of Mg^{2+}-deficient and control mice, there was a clear effect of test day on average daily escape latency in the swim maze task (test day effect: F (4, 212) = 39.2, p < 0.0001) (figure 3). Latencies to find the platform dropped significantly between the first and second day of testing with smaller decreases observed over the remaining test days. The Mg^{2+}-deficient diet did not have a statistically significant effect on escape latency over the test days.

Figure 2. Magnesium deficiency reduces conditional lick suppression and freezing behavior in mice. A) On the test day, all animals exhibited similar latencies to the 5th lick, but after tone onset, the Mg^{2+}-deficient mice showed a faster latency to the 20th lick. All data represent mean latency to lick in seconds ± s.e.m. The asterisk represents a significant difference between the control and Mg^{2+}-deficient groups on the latency to the 20th lick measure. B) Mg^{2+}-deficient also exhibited significantly less freezing during the first two minutes after exposure to the tone alone. All data represent the mean % of time spent freezing per minute ± s.e.m. The asterisks represent significant differences between the control and Mg^{2+}-deficient groups on the freezing measure. n=26 in the Mg^{2+}-deficient group and n = 25 in the control group.

Figure 3. Magnesium deficiency does not alter performance in the swim maze spatial learning task. Mice in each condition (control and Mg^{2+}-deficient diets) demonstrated similar latencies to find the escape platform across days of testing. Data represent average latency to find the platform over four trials/day in seconds ± s.e.m. n = 20 per group.
Discussion

The results of the present study demonstrate that Mg²⁺-deficiency impairs conditional lick suppression. This is an important advance from our previous work [12] that demonstrated reduced conditional freezing behavior in Mg²⁺-deficient mice. Our new data show that the effects of Mg²⁺-restriction are not simply limited to idiosyncratic effects on freezing behavior but extend to other metrics of fear conditioning. Another important result from the study was that Mg²⁺-deficient mice do not demonstrate alterations in spatial learning in the swim maze task. These data suggest that some forms of learning and memory, such as fear conditioning, may be more sensitive to the detrimental effects of Mg²⁺-deficiency than other forms, such as spatial memory. Finally, our results are among the first to demonstrate CLS in laboratory mice and suggest that CLS can be used as a robust measure of fear conditioning in mice.

In our first study of Mg²⁺-deficient mice [12], we showed that such mice displayed reduced contextual and cued fear conditioning as assessed by measures of immobility. While a number of observations from that study ruled out non-specific factors as causes for reduced fear conditioning, it still seemed meritorious to consider whether other behavioral measures of conditional fear were altered by Mg²⁺-deficiency. The CLS paradigm was chosen for study since it had been used in earlier rat studies as a robust marker of conditional fear [16-19], employed an objective and explicit behavioral measure, and lent itself to easy comparison with changes in conditional freezing behavior (i.e., both measures could be done in the same apparatus at the same time). Our results show that Mg²⁺-deficient mice demonstrate deficits in CLS in addition to reductions in conditional freezing behavior. The reduced CLS found in Mg²⁺-deficient mice does not appear to be related to a change in licking behavior or a motivation to drink, since both groups of mice demonstrated similar latencies to the fifth lick prior to tone onset on the test trial in addition to similar latencies to the fifth lick on all three habituation trials. Nor could the change in CLS and freezing behavior be attributed to changes in shock sensitivity or altered immobility, since both groups of animals froze to a similar degree immediately after footshock presentation during the training trial. Overall, the CLS data strengthen the idea that Mg²⁺-deficiency plays an important role in fear conditioning and, to our knowledge, suggest for the first time the robustness of CLS as a measure of emotional learning in mice.

While the present study firmly supports a role for Mg²⁺ in fear conditioning, the results suggest that spatial learning, as reflected by performance in the swim maze, is not adversely affected by gross deficiency in dietary Mg²⁺. Mice from both conditions demonstrated similar decreases in their latency to find the submerged platform across the five training days. Given that fear conditioning is adversely affected by Mg²⁺ deficiency while learning in the swim maze is not, these findings suggest that such deficiency has a differential impact on the brain regions subserving such behavioral functions. Numerous research reports have linked fear conditioning to amygdala function [29] while learning in the swim maze appears to be supported by the hippocampus [22, 24]. It is not clear why the latter brain region would be less sensitive to Mg²⁺-deficiency than the former. Studies have shown that the adverse effects of traumatic brain injury on hippocampal cell loss and spatial learning are less sensitive to the ameliorative effects of Mg²⁺ supplementation [9, 28] (but see [11]). However, a recent review by Billard [7] indicates that changes in brain Mg²⁺ concentrations may contribute to hippocampal aging. Obviously, this issue of hippocampal sensitivity to Mg²⁺-deficiency merits further behavioral and physiological investigation.

If the amygdala serves as a source of the fear conditioning impairment in Mg²⁺-deficient mice, the molecular mediator of this effect within the amygdala (or elsewhere) is likely to be the NMDA receptor. NMDA receptors within the amygdala are necessary for fear conditioning [30]. In a Mg²⁺-deficient animal, it is probable that NMDA receptors allow more Ca²⁺ through the receptor channel, given the lack of Mg²⁺ blockade within the ion channel. This condition would likely yield neurons containing hypersensitive NMDA receptors and lead to noisy glutamatergic synapses that would interfere with efficient synaptic processing and new learning. In support of this hypersensitivity theory, we have shown that Mg²⁺-deficient mice have a lower threshold for NMDA-induced seizures [12]. While this hypersensitivity might be expected to produce excitotoxicity, our own cursory inspections of limbic system brain regions in Mg²⁺-deficient mice have not revealed overt neuropathology. Nonetheless, other work [31] has shown that Mg²⁺ deficiency can exacerbate brain injury.

While there are likely to be multiple factors that contribute to the cognitive impairment found in disease states associated with Mg²⁺ deficits, our research suggests that reduced Mg²⁺ itself may have untoward effects on memory. Our data complement...
preclinical and clinical work that demonstrates the beneficial effects of Mg²⁺ supplementation on adverse neurobehavioral outcomes in specific diseases and supports continued use of such an approach. At a basic level, however, further work is needed to reveal the mechanism(s) that accounts for the adverse effect of Mg²⁺ deficiency on fear-related learning and memory.

Conclusion

In mice, dietary deficiencies in Mg²⁺ can lead to specific impairments in fear conditioning but do not appear to affect spatial learning. The impairments in fear conditioning produced by decreased Mg²⁺ intake can be observed using multiple measures of such conditioning. Further work is needed to reveal the molecular mechanisms that account for conditioning deficits in Mg²⁺-deficient mice, but the present results support a possible role for Mg²⁺ deficiency in the cognitive impairment associated with disease states linked to low Mg²⁺ levels.

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