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Therapeutic Benefits of Methylene Blue on Cognitive Impairment during Chronic Cerebral Hypoperfusion

Allison Auchter, Justin Williams, Bryan Barksdale, Marie H. Monfils and Francisco Gonzalez-Lima*
The University of Texas at Austin, Department of Psychology, Austin, TX, USA

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Abstract. Chronic cerebral hypoperfusion, a risk factor for mild cognitive impairment and Alzheimer's disease, affects mitochondrial respiration and memory consolidation. Therefore, drugs that improve mitochondrial function may be appropriate cognitive treatments for cerebral hypoperfusion. Methylene blue (MB) crosses the blood-brain barrier and at low doses serves as an electron cycler in the mitochondrial electron transport chain. Previous studies implicate MB in both memory enhancement and neuroprotection. We treated rats that underwent permanent bilateral carotid occlusion (2VO) or sham surgery with daily 4 mg/kg USP MB or saline for one month. Animals went through a battery of behavioral tests, including open field, visual water maze, and odor-recognition tasks. 2VO rats showed worse performance in the visual water task without showing differences in general motor activity, visually guided swimming ability or odor recognition. Daily MB attenuated the deficits in visual learning and memory that resulted from cerebrovascular insufficiency. During training on three different discrimination problems in the visual water task, all animals were able to reach a criterion of 8/10 correct trials, but 2VO animals took longer to learn each problem and showed lower performance in a challenging memory probe. However, animals that received daily post-session MB performed significantly better than saline-treated subjects both during training and during the memory probe. This is the first study to demonstrate that MB attenuates learning and memory deficits caused by carotid occlusion. The results suggest that MB may be beneficial for conditions involving chronic cerebral hypoperfusion, such as mild cognitive impairment, vascular dementia, and Alzheimer's disease.

Keywords: Carotid occlusion, cerebral hypoperfusion, cognitive impairment, memory enhancement, methylene blue

INTRODUCTION

There is a great need to develop effective early interventions to treat memory deficits associated with mild cognitive impairment, Alzheimer's disease (AD), vascular dementia, and other related neurocognitive disorders. For example, in the US alone, over 4 million people are living with AD, resulting in continued intellectual loss to society, an annual cost of over $100 billion (http://report.nih.gov/nihfactsheets/ViewFactSheet.aspx?csid=107) and a great deal of suffering for both the inflicted and their caregivers. A compelling body of evidence supports a role of compromised cerebral blood supply and mitochondrial dysfunction in memory-related neurodegenerative disorders [1–6]. With an aging population, it is critical to treat deficits in brain energy metabolism that occur before the onset of dementia. Treatments such as low-dose USP methylene blue (MB) designed to target the effectiveness of mitochondrial respiration may be able to ameliorate these deficits, thereby delaying or preventing the development of cognitive impairment and dementia. The present preclinical study addressed this possibility by determining whether a chronic intervention with low-dose MB that targets cytochrome oxidase and enhances mitochondrial respiration can improve memory retention in a rat model exhibiting chronic cerebral hypoperfusion and mitochondrial dysfunction [7, 8].

*Correspondence to: Francisco Gonzalez-Lima, The University of Texas at Austin, Department of Psychology, 108 E. Dean Keeton Stop A8000, Austin, TX 78712, USA. Tel.: +1 512 471 5895; E-mail: gonzalezlima@utexas.edu.

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MB (also known as methylthioninium chloride) is an FDA-grandfathered and inexpensive drug that has been used safely in humans for over 120 years. Indeed, MB was the first synthetic chemical used therapeutically in humans [9]. Pharmaceutical grade (USP purity) MB is available in every emergency room in the United States, but its use does not currently extend to neuroprotection or cognitive enhancement. Low-dose (1–4 mg/kg) MB currently has many human applications, including treatment for various metabolic poisonings such as methemoglobinemia, cyanide poisoning, and toxic encephalopathy [10]. In a similar way as MB compensates for cytochrome oxidase inhibition from poisons, MB may also improve memory retention in conditions characterized by impaired oxidative energy metabolism.

MB is an autoxidizable compound with unique redox mechanisms that are ideal for targeting neural tissue oxidative metabolism. After administration, MB passes through the blood-brain barrier and accumulates in the human brain within hours [11]. MB is a relatively small lipophilic compound, so it diffuses readily into neuronal mitochondria. At low doses, MB enhances electron flow in the mitochondrial respiratory chain and thereby, stimulates mitochondrial cytochrome oxidase metabolism and oxygen consumption [12–15]. MB can even replace oxygen as an oxidant in anoxic conditions [16]. Moreover, low-dose MB displays potent antioxidant effects. For example, low-dose MB prevents superoxide free radical formation during reperfusion after ischemia [17] and we have also shown that it decreases free radical-induced lipid peroxidation [18]. We have used low-dose MB successfully *in vivo* as an artificial electron donor and established that it can donate electrons to the mitochondrial electron transport chain in its reduced form, thus increasing cytochrome oxidase activity and brain oxygen consumption [18, 19]. Adverse reactions to MB have only been observed with doses much larger than our range of interest and/or with non-pharmaceutical "chemical grade" MB that contains toxic impurities not found in purified USP MB [14, 20].

MB's ability to target cytochrome oxidase and neuronal energy metabolism at the mitochondrial level provides us with a novel approach to attenuate learning and memory deficits [20, 21]. Because MB has been proven effective at enhancing memory in a large number of experimental paradigms [13, 22–30], including humans [31], we hypothesized that MB could also effectively attenuate memory deficits in an amnestic model of cerebral hypoperfusion. Therefore, our objective was to investigate whether chronic administration of MB could be used to attenuate learning and memory deficits caused by chronic cerebral hypoperfusion. A 4 mg/kg i.p. MB dose was selected based on our findings that 1–4 mg/kg doses of MB improve memory retention in various tasks (with optimal memory effects at 4 mg/kg i.p. [14]; for review, see [20]), and that doses above and below this hormetic range are behaviorally less effective [9]. The beneficial effects of chronic low-dose MB on cognitive deficits caused by cerebral hypoperfusion represent a proof-of-principle and the basis for the possible development of early effective interventions against mild cognitive impairment and AD. This also should have translational value for other memory disorders and contribute to the study of new neurometabolic mechanisms to enhance memory.

MATERIALS AND METHODS

Subjects

Subjects were 39 adult male Long-Evans rats weighing approximately 500–600 g at the time of surgery. Two rats died from surgical complications and one rat died of a respiratory infection during visual water task training, making the final $n = 36$. Rats were raised from birth in AALAC-approved facilities under standard laboratory conditions (12 h: 12 h, light: dark cycle) with *ad libitum* access to food and water. Animals were housed in triads until the day of surgery, after which they were housed individually for the remainder of the experiment. Rats were housed singly after surgery to prevent animals from interfering with wound healing, such as removal of sutures. In pilot subjects, when several subjects were housed together after surgery, they would remove each other's sutures, resulting in reopening of the wounds. Subjects came from a selective breeding project that involved selecting animals for their propensity to extinguish conditioned fear. Each animal came from one of three possible lineages: animals phenotypically selected for effective extinction of conditioned fear (high extinguishers), animals selected for ineffective extinction of conditioned fear (low extinguishers), or animals from a line not bred for an extinction phenotype (randomly-bred) [32]. All subjects were behaviorally tested at approximately 60 days of age with 3 trials of paired tone-shock conditioning, 19 tone alone extinction trials and a memory probe. They were subsequently used as breeders for the in-house colony. Hence these animals were phenotypically selected and sexually experienced mature rats before inclusion in this experiment. However, in our behavioral experiments, there were no significant dif-

ferences based on lineage, so animals from all lineages were analyzed together. All animal care and experimental procedures were approved by the University of Texas at Austin's Institutional Animal Care and Use Committee.

Surgical and drug treatments

A timeline of the procedures is show in Fig. 1. Rats were randomized and subjected to a bilateral carotid occlusion surgical model known as 2-vessel occlusion (2VO; $n = 20$) or to sham surgery without vessel occlusion (sham; $n = 19$) as previously described by de la Torre and Fortin [33, 34]. Prior to surgery, rats were anesthetized via isoflurane inhalation at 4% for induction, and maintained under general anesthesia at 1.5–3% for the duration of the surgical procedure using an E-Z anesthesia vaporizer system (Euthanex, Corp., Palmer, PA, USA). For the surgery, a midline ventral incision was made in the neck. The carotid arteries were carefully exposed and dissected free from their sheath and vagal nerve [33]. In the 2VO condition, each artery was doubly ligated immediately posterior to the carotid bifurcation using 4-0 silk sutures. For the sham condition, the carotid arteries were dissected free from their sheaths, but no occlusion was performed. Prior to closing, each animal was injected subcutaneously with 1 mg/kg of the surgical analgesic meloxicam (TW Medical Veterinary Supply, Lago Vista, TX, USA), randomly assigned to MB or saline groups and injected intraperitoneally with either 4 mg/kg MB (USP Methylene Blue, Spectrum Chemical Manufacturing Corporation, New Brunswick, NJ, USA) or the same volume of physiological saline. The incision was sutured closed and each animal was transferred to a recovery cage and closely monitored for 30 min before returning to its home cage in the colony room. Rats were then injected daily i. p. with either 4 mg/kg MB dissolved in physiological saline (MB groups) or the saline vehicle alone (saline groups) for 30 days. MB was injected daily immediately after each training session, i.e., during the post-session memory consolidation phase, because MB has been shown to preferentially facilitate memory consolidation in other paradigms [13, 35]. Animals were allowed to recover for 7 days before behavioral testing began.

Behavioral testing

Overview

Behavioral testing for this experiment began on Day 8 after surgery and continued until Day 30. All animals went through the same behavioral tests in the same order. First, animals went through one day of open field, followed by 20 days of visual water task (VWT) training. Upon completion of the VWT task, animals went through 2 days of an odor recognition task (Fig. 1).

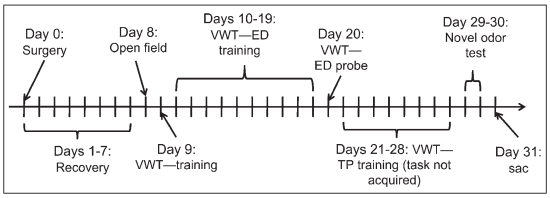

Fig. 1. Experimental timeline for behavioral testing: Visual water task (VWT), elemental discrimination (ED), transverse patterning (TP), sacrifice (sac).

Open field

Subjects were run through one day of open field activity to screen for differences in gross motor behavior following surgery. The test was conducted by placing each subject in an open field chamber (43.2 cm^2) with clear plastic sides (30.5 cm high) and a white Plexiglas floor. For 10 min, rats were allowed to freely explore the chamber. Movement was recorded using arrays of infrared light beam motion detectors (16×16 cm, 2.5 cm apart) controlled by the Activity Monitor program version 5.10 (Med Associates, St. Albans, VT, USA). Measures of motor activity included ambulatory distance, ambulatory time, rearing time, immobility time and average velocity, which were automatically scored by a computer using MED-PC software. To minimize odor-cued motor activity, chambers were wiped down with 70% ethanol between each session.

Visual water task

Apparatus and training procedure: The VWT was used to train rats on several discrimination problems, as previously described by [36]. Briefly, a trapezoidal metal tank (118 cm L×72 cm W×25 cm W and 41 cm H) was filled with water to a depth of 15 cm. The end wall of the tank was composed of clear Plexiglas. A computer monitor (30") was placed behind the transparent wall and was used to control stimulus presentation. The end of the tank was bisected by a midline divider (60 cm H) that extended 46 cm into the pool from the transparent wall. This divider separated the monitor such that one pattern was visible at the end of each "arm" created by the divider. For each trial, a transparent invisible platform (32 cm L x 13 cm

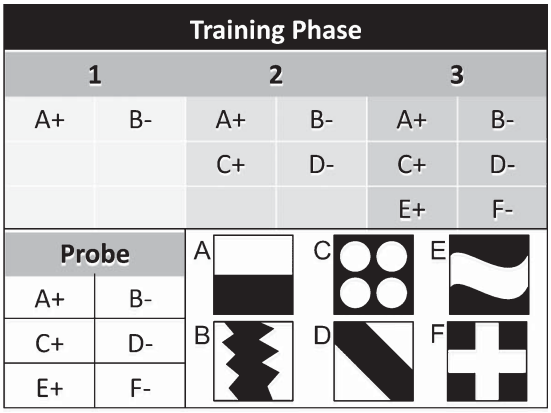

Fig. 2. Top: During Phase 1 of training, subjects were trained for 3 days (30 trials/day) on the Pair 1, in which Pattern A predicted escape (+) while Pattern B did not (-). During Phase 2, Pair 2 (C+, D-) was introduced and intermixed with Pair 1 for three days of training. Finally, during Phase 3, Pair 3 (E+, F-) was introduced and intermixed with Pairs 1 and 2. Bottom Left: The final probe day consisted of 30 random presentations of all 3 pairs. Bottom Right: The six patterns used during VWT.

W x 15 cm H) was completely submerged at the end of one of the arms, and was always paired with a pattern that predicted escape from the pool. Right/left randomization, behavioral responses, and parameter control were provided and recorded by a computer program (Acumen; http://www.cerebralmechanics.com).

To address the possibility that the 2VO reduces blood flow to the retina, thereby impairing subjects' ability to visually discriminate between the patterns presented in the VWT, a separate group of 15 naïve pilot subjects was pretested in the task with different iterations of 12 distinct discriminanda. The combinations of patterns that were most easily detected by pilot subjects (as indicated by shortest total number of trials to get 8/10 trials correct) were used for the experimental subjects in the VWT. The patterns selected for use in this experiment are shown in Fig. 2. Additionally, during the VWT training phase, all experimental subjects were able to reach the 8/10 criteria for each elemental discrimination problem, eliminating the possibility that 2VO hindered subjects' ability to discriminate between the patterns.

Rats were trained to associate escape from the water by swimming to a "correct" (+) pattern that predicted the presence of the invisible escape platform. At the end of the opposite arm was an "incorrect" (–) pattern that consistently predicted the absence of the platform. VWT training began 8 days following surgery and continued for 20 days. After an initial training day, all subjects were first trained to solve three visual elemental discrimination sets followed by three transverse pattern sets. Beginning with elemental discrimination training, at the start of each trial the subject was placed into the opposite end of the tank facing the transparent wall and allowed to swim to one of the stimuli presented by the monitor at the end of the arms. If the subject swam into the incorrect arm, it was blocked into the arm with a metal divider for 10 seconds and then allowed to swim to the opposite arm containing the hidden platform. Once the subject reached the platform, it was promptly removed and placed in a holding cage to await the next trial.

Task training and recording of swimming speed: On the first day of the VWT task, an insert was placed in the tank that completely dissected the tank into a left and right section such that subjects could only swim down one side of the tank without having the option of two separate arms. On this day, the screen at the end of the tank was left blank (i.e., no pattern) and the invisible platform was always present at the end of the tank nearest the screen. Each subject went through 15 trials in which they were placed at one end of the tank on the right or left side and allowed to swim to the other end to discover the platform. Upon reaching the platform, the subject was removed from the tank and placed in a heated recovery cage. There were two purposes to this task. The first was to acclimate subjects to the task by allowing them to associate swimming to the invisible platform with escape from the tank. The second purpose was to observe swimming ability for the subjects independently of pattern discrimination. Subjects learned of the presence of the platform within the first few trials, and by the tenth trial all subjects consistently swam directly to the platform immediately after being placed in the tank. Thus, we used the average latency of the final five trials to compute the swimming speed for each subject.

Elemental discrimination and transverse patterning: Training followed a stepwise approach that closely resembled previously published procedures [36–39]. Briefly, subjects were trained to solve three visual discriminations (Fig. 2; Problem 1: A+B-; Problem 2: C+D-; Problem 3: E+F-). Training consisted of 10 days (30 trials each day) in three phases, followed by a test a day later. Phase 1 consisted of training subjects on Problem 1 until subjects could reach a criterion of 8/10 correct trials. All subjects were able to reach the criterion within 4 days. During Phase 2, Problem 2 was introduced and intermixed with Problem 1 over a period of three days. Similarly, in Phase 3, Problem 3 was introduced and intermixed with Problems 1 and 2 over a period of 3 days. On day 11, subjects received

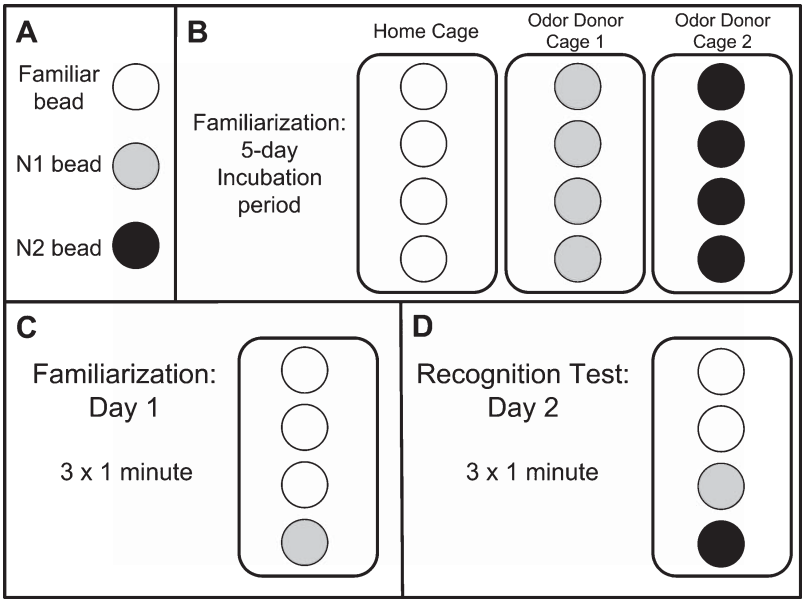

Fig. 3. Novel Odor Test experimental procedure. A) Color-coded representation of wooden beads. B) 5-day incubation period wherein beads absorbed odors. C) Familiarization–introduction to the 1st novel odor (N1). D) Recognition test: introduction of 2nd novel odor (N2).

a memory probe consisting of randomly alternating problems on each trial (3, 2, 1, 2, 1, 3, 2, 3, 1, 3, 1, 2, 1, 2, 3, ... x2) for a total 10 presentations of each problem [37, 38].

Transverse patterning followed the same stepwise procedure as the elemental discrimination, however the rats were presented with new, more difficult problem sets. In this case, instead of having three patterns that always predicted escape from the tank, whether the pattern was correct or not depended upon which pattern was presented alongside it (Problem 1: X+Y-; Problem 2: Y+Z-; Problem 3: Z+X-). Unfortunately, after 8 days of training, none of the subjects were able to acquire the task. As a consequence, this task was excluded from our analysis. However, since all animals went through the same training protocol in a similar time frame, the behavioral tasks performed subsequently were still incorporated in our analysis.

Novel odor test

Familiarization of wooden beads: Experimental protocol for the novel odor test was modified from related procedures [40–43]. Five days before novel odor testing, four 2.5 cm round wooden beads, each with a small hole bored through its diameter (http://www.craftparts.com) were introduced into each subject's cage in order to acquire the odor of the animal. Animals were tested in their home cages, so introducing the wooden beads 5 days prior to testing allowed for familiarization to both the testing environment and the presence of beads.

Several beads were also introduced into cages of previously selected singly-housed odor-donor rats. These rats were not subjects in the experiment, but instead served only as odor donors for the novel odor test. The wood beads were incubated in the cages for 5 days so as to provide equally salient novel odors for the upcoming task. For testing, the odor-donors were counterbalanced such that any one odor served as either a recently familiarized odor (N1) or a brand new novel odor (N2) during memory assessment for different experimental subjects.

Habituation to the first novel odor: To habituate rats to the first novel odor, after 5 days of familiarization to the four beads in the testing environment (home cage), all four beads were removed from the test cage. Subsequently, three familiar beads and one novel-odor bead (N1)—taken from one of the odor donor cages—were introduced back into the home cage and randomly arranged in a line approximately 5 cm from the front of the cage, as shown in Fig. 3. Subjects were allowed to freely explore the beads for three 1-min trials. The time the rat spent investigating each bead was recorded by an experimenter who was blind to which beads were familiar or novel. There was a 1-min inter-trial interval during which all beads were removed from the cage. For each trial, the spatial arrangement of the four beads was randomly altered.

Table 1
Locomotor activity in the open field test and swimming velocity in the visual water maze (VWT). There were no significant differences between groups at the $p < 0.05$ level

Measure	Sham-saline (Mean ± SEM)	Sham-MB (Mean ± SEM)	2VO-saline (Mean ± SEM)	2VO-MB (Mean ± SEM)
Open Field				
Ambulatory distance (cm)	2549 ± 351	2782 ± 342	2878 ± 394	3025 ± 374
Ambulatory time (s)	955 ± 121	1044 ± 137	1060 ± 136	1194 ± 154
Rearing time (s)	120 ± 17	130 ± 18	117 ± 19	118 ± 15
Immobility time (s)	397 ± 17	387 ± 18	376 ± 15	381 ± 21
Average velocity (cm/s)	3.22 ± 0.30	3.11 ± 0.12	3.44 ± 0.14	2.82 ± 0.24
VWT				
Swimming velocity (cm/s)	2.89 ± 0.21	3.02 ± 0.16	2.96 ± 0.13	2.68 ± 0.29

Odor-recognition assessment: The odor recognition test was administered 24 h after the N1 habituation phase. This assessment follows the same procedure as the habituation phase, however in this case each subject was presented with an N1 odor bead (which it thoroughly explored the previous day), an unfamiliar novel odor bead (N2), and two familiar home cage beads in random arrangement in the test cage (see Fig. 3). To prevent scent-marking effects, the bead used for the N1 odor on the habituation day was discarded, and a new bead from the same N1 odor donor cage was used for the memory assessment. Again, a blind experimenter recorded the amount of time subjects investigated each bead during each trial.

Statistical analysis

All statistical testing was carried out using the PASW 18.0 software package (SPSS, Inc., Chicago, IL). All differences were considered statistically significant at the two-tailed $p < 0.05$ level. Two-way ANOVAs were used to verify that there were no group differences for the following measures of general motor activity: ambulatory distance, ambulatory time, rearing time, immobility time, center time, average velocity, and swimming speed.

For the VWT, a repeated-measures ANOVA containing one within-subject variable (training day) and one between subjects variable (group) followed by pairwise comparisons using simple and repeated contrasts was used to verify task acquisition and to observe group differences in the rate of acquisition. Following training, a one-way ANOVA with planned comparisons was used to assess the effects of 2VO surgery and MB administration on performance.

Analysis of performance on the odor recognition test used a 2×2 AVOVA to explore main effects and interactions (between-groups comparisons), and a Wilcoxon matched-pair signed-rank test for each group (within-group comparisons). It was expected that subjects would spend more time investigating a novel odor over a familiar odor in the within-groups comparisons.

RESULTS

As previously mentioned, subjects included in this experiment initially came from one of three different selectively bred lines. However, there were no significant differences in any behavior due to lineage. Thus, subjects from all lineages were analyzed together.

General motor behavior

There were no significant group differences due to treatment condition in any general activity measures in the open field, including ambulatory distance, ambulatory time, rearing time, immobility time, and average velocity. Additionally, using a two-way analysis of variance (ANOVA) there were no group differences in swimming speed measured on the first day of the VWT. Thus, any observed group differences were not due to differences in general activity or swimming ability (Table 1).

Visual water task

Figure 4 shows the acquisition curves and test performance for each group. Repeated measures analysis of the task acquisition revealed a general increase in percent correct trials through all 9 days of training, $F(8, 208) = 20.064$, $p < 0.001$. By the third day, all groups reached an average performance of at least 70%, and all subjects reached the performance criterion of 8/10 correct trials. However, when comparing differences in performance between the first and second days of training, a one-way ANOVA revealed between-groups differences in rates of task acquisition, $F(3,31) = 9.434$, $p < 0.001$. Planned comparisons

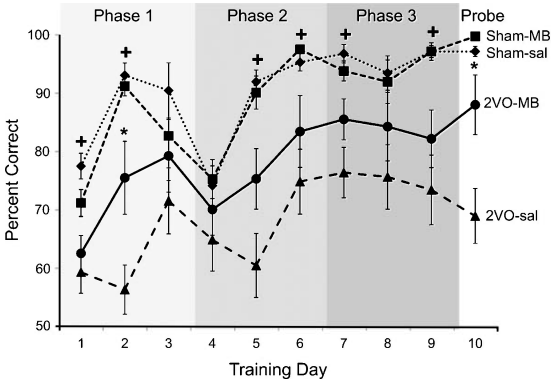

Fig. 4. Visual Water Task Results. During Phase 1, subjects were introduced to the first pattern. In Phase 2, the second pattern was introduced and intermixed with the first pattern. In Phase 3, the third pattern was introduced and intermixed with the first two patterns. On Probe day 10, subjects were given a random presentation of all three patterns. All groups significantly improved in performance from the beginning to the end of training, but the 2VO-sal group showed the least amount of progress during training compared to the 2VO-MB, sham-MB and sham-sal groups. Though all subjects were able to reach a minimum criteria of 8/10 correct trials during Phase 1, the 2VO-sal group took longer to acquire the task as evidenced by a lack of improvement from Day 1 to Day 2, while the 2VO-MB, sham-MB and sham-sal groups showed a significant increase in percent correct trials. On the Probe day 10, the 2VO-MB and 2VO-sal groups performed worse than the sham-MB and sham-sal groups, however the 2VO-MB group significantly outperformed the 2VO-sal group. + = sham versus 2VO ($p < 0.05$), * = 2VO-sal versus sham-sal.

showed that the 2VO groups had less improvement on the second day of training than the sham groups [$t(31) = 3.875$, $p < 0.001$], but the 2VO-MB group showed better acquisition than the 2VO-saline group [$t(31) = 3.348$, $p < 0.01$].

Using a 2-way ANOVA on the probe day, there was a significant interaction between MB treatment and surgical condition ($F(1, 32) = 5.82$, $p = 0.022$) which was further interpreted using a planned-comparisons analysis. Levene's test for equality of variance revealed that the groups did not show homogeneity of variance ($F(3,32) = 6.077$, $p = 0.002$). As such, contrasts tests were conducted without assuming equal variances. The planned-comparisons revealed that the subjects that underwent 2VO had worse performance in the elemental discrimination memory probe test than the sham subjects ($t(32) = 5.921$, $p < 0.001$), but that 2VO subjects treated with MB performed significantly higher than those treated with saline ($t(32) = 3.954$, $p < 0.001$).

Odor recognition test

A 2×2 ANOVA did not reveal main effects for surgery [$F(1,32) = 2.66$, $p = 0.11$], drug treatment [$F(1,32) = 0.014$, $p = 0.91$] or an interaction effect [$F(1,32) = 0.017$, $p = 0.90$] on percentage of time spent exploring the most novel odor (N2). The only discernable difference between 2VO and sham groups was in the amount of subject variability in the percent time exploring N1 versus N2, with the 2VO-saline group having a much higher variability than the other groups. As such, odor recognition was only mildly affected by 2VO. On average, all groups spent a significantly greater percentage of time exploring N2 over N1 except the 2VO-saline group using the Wilcoxon matched-pair signed-rank test for each group (Table 2). This means that MB treatment reduced the amount of variability introduced by 2VO, but it did not result in increased exploration time of N2.

DISCUSSION

The experiment showed that daily low-dose MB (4 mg/kg i.p.) after permanent bilateral carotid artery occlusion (2VO) enhanced discrimination learning and memory in a visual water maze task. It is unlikely that the observed therapeutic benefits produced by low-dose MB were due to non-specific effects on behavior because the 2VO surgery and MB treatments had no significant effects on open field motor performance, visually guided swimming or the familiar odor recognition test. One limitation of the study is that the findings are not based on naïve subjects, but on subjects that were behaviorally and sexually experienced. However, all the subjects had the same behavioral experience before the start of these experiments, and therefore, it is unlikely that pre-existing

Table 2
Novel odor recognition test. There were no between-groups differences in mean exploration time. Within-subjects analysis showed that groups spent more time exploring N2 over N1 beads, but this mean effect was not significant in the 2VO-saline group due to its high variance

Group ($n = 9$ per group)	N1 (Mean % exploration time ± SEM)	N2 (Mean % exploration time ± SEM)	Familiar beads (Mean % exploration time ± SEM)	N1-N2 paired Wilcoxon signed-ranks test
Sham-saline	35.6 ± 17.4	62.3 ± 17.6	2.1 ± 1.8	$p = 0.05$*
Sham-MB	32.4 ± 13.1	63.6 ± 14.5	4.0 ± 4.9	$p = 0.04$*
2VO-saline	43.1 ± 21.7	54.0 ± 21.0	2.9 ± 3.6	$p = 0.21$
2VO-MB	43.4 ± 13.2	53.9 ± 11.8	2.7 ± 2.5	$p = 0.05$*

differences among the groups could confound our findings. Therefore, the adverse effects of 2VO and the beneficial effects of MB were specific to the learning and memory of the visuospatial discrimination task. Rats receiving MB exhibited not only better learning over days, but also enhanced memory retention as demonstrated in the probe test. As predicted, mean scores for visual memory retention followed the order sham-MB>sham-saline>2VO-MB>2VO-saline, with MB groups performing better than their saline counterparts.

Visuospatial memory deficits after bilateral carotid artery occlusion

The occlusion of both carotid arteries (two vessel occlusion, or 2VO) is a well-established rat model of chronic cerebral hypoperfusion developed by de la Torre and colleagues [33, 34]. The 2VO model simulates some specific visuospatial memory changes observed in mild cognitive impairment and early in the progression of dementia, especially in sporadic AD. This model causes a mild form of chronic cerebral hypoperfusion that allows investigations of the consequent behavioral deficits and the testing of experimental treatments that may reduce these deficits. The 2VO involves a permanent occlusion found to reduce cerebral blood flow by 21% in the cortex and 32% in the hippocampus of middle-aged rats [44]. Occlusion of the two carotid arteries does not produce a complete loss of perfusion to any brain region due to the collateral circulation via the circle of Willis present in the arterial configuration of rats [45]. Therefore, the 2VO model is not a model of acute stroke or cerebrovascular infarction in humans. Instead, 2VO for one month models decrements in neuronal oxidative energy metabolism without the immediate loss of brain cells seen in cerebrovascular infarction or stroke, resembling more the age-related changes in brain hypoperfusion and visuospatial memory seen in mild cognitive impairment and early AD in humans. This 2VO model is characterized by impairment in visuospatial memory tasks such as the Morris water maze [7, 46]. The 2VO-induced hypoperfusion causes the largest metabolic reductions of cytochrome oxidase activity in hippocampal CA1 and posterior parietal cortex regions [46]. Hence after chronic brain hypoperfusion with 2VO for four weeks, regional cytochrome oxidase activity is primarily reduced in hippocampal and posterior parietal regions, which occurs in parallel with visuospatial memory dysfunction in the Morris water maze. The present results extend this 2VO finding to a different visuospatial task involving visual pattern discrimination learning in a Y-maze water tank [36]. Importantly, in previous studies as well as in the present study, 2VO rats were impaired in visually-guided learning and memory but not in visually-guided escape to the visible platform or in motor swimming speed. Furthermore, our findings indicate that 2VO rats are not impaired in the open field locomotion task or in the recognition of familiar odors. Together these findings are consistent with the conclusion that dysfunction in brain regions mediating visuospatial learning and memory may underlie much of the behavioral deficits seen in the one-month 2VO rat model.

MB reduced visual learning and memory deficits without affecting non-specific activity

MB was administered daily to improve progressively more challenging visual discrimination learning phases. MB was injected daily in the post-session period to improve the retention of the discrimination learning, as it has been shown before that MB is most effective at improving memory consolidation than other memory stages [24, 35]. The half-life of MB in rats is about 5-6.5 h [11], so it is not likely that the daily improvement of the visual discrimination seen 24 h after each injection is a result of the continued drug action. A more likely explanation is that MB given immediately after each training session acted to enhance brain oxidative energy metabolism during the critical stage of memory consolidation. Thus our rationale for the timing of injection was that MB would enhance brain metabolic processes mediating memory consolidation. Since MB was given immediately after each session it could not have directly interfered with the acquisition trials the next day. Instead, we conclude that MB acted on consolidation processes to facilitate memory retention after each daily session and on the probe trial.

One alternative interpretation may be that chronic MB administration simply increases levels of motor activity in a non-specific manner, and thereby may improve learning performance. But this alternative is highly unlikely because swimming speed during the visual water task was not influenced by MB administration. To further test the possibility that non-specific motor or sensory effects could account for the differences between MB and saline groups, we conducted additional behavioral tests before and after the visual water task. There were no significant group differences in any activity measures in the open field motor behavior or in the familiar odor recognition tests. These tests

together with the monitored swimming speed served to rule out the possibility that repeated MB injections simply increased general arousal or non-specific sensory and motor activity. Therefore, the results suggest that the effects of chronic MB administration were specific to the visual learning and memory tests.

The improvement of visual learning and memory following post-session MB administration is consistent with the results of many previous MB studies in other behavioral models, all of which suggest that post-session administration of low-dose (1–4 mg/kg) MB in different learning and memory tasks improves memory retention, both in healthy animals and in animals with compromised energy metabolism [13, 20, 22, 23, 35]. For example, repeated post-session administration of low-dose MB has also enhanced visuospatial memory retention in a holeboard food search task without any effect on general motor activity [13]. However, large doses of MB (50–100 mg/kg) impair both memory and sensory-motor performance because they lead to methemoglobinemia and consequently compromise oxygen delivery [14, 35].

Potential wide impact and therapeutic benefits of MB against neurocognitive impairment

The observed MB benefits in this model appear relevant and potentially translatable to patients with mild cognitive impairment and AD showing cerebrovascular hypoperfusion. The results could also potentially benefit patients with other memory disorders featuring impaired brain energy metabolism. The present model may be further investigated to provide insights on the *in vivo* mechanisms of action of MB interventions at behavioral and neurometabolic levels. Importantly, MB may provide a safe, highly bioavailable and affordable drug intervention. Most drug development efforts beyond basic research fail due to high toxicity and inadequate pharmacokinetics of candidate drugs. However, low-dose MB is an intervention with benign safety profiles and high bioavailability to nerve cells. MB constitutes an affordable and safe alternative to current treatment options for cognitive impairment. Memory functions are extremely sensitive to oxidative energy deficits [47]. The brain is especially vulnerable because it depends almost exclusively on electron transport-derived oxidative energy [48]. Thus it is likely that impairments in cerebral blood flow and oxidative energy metabolism may underlie memory deficits. For example, mild cognitive impairment and AD have been linked to mitochondrial dysfunction [1, 49] and to specific deficiencies in cytochrome oxidase activity [50–52]. The key observations that carotid artery disease and mild cognitive impairment are risk factors for AD [4, 53] present an opportunity to develop early metabolic interventions to impact these disorders. Low-dose MB can also successfully attenuate neurological and behavioral deficits in a model of mild traumatic brain injury [54].

CONCLUSIONS

Despite compelling evidence of a role of chronic brain hypoperfusion in the pathophysiology of mild cognitive impairment and AD [4–6], animal models that exhibit this metabolic deficit have not been used before to test new memory-enhancing interventions targeting mitochondrial respiration [21]. The present study is the first one to demonstrate that low-dose MB has beneficial therapeutic effects on an animal model of chronic brain hypoperfusion. This study served as a proof-of-concept to test the efficacy of a chronic metabolic intervention with low-dose MB in a hypoperfusion model relevant to mild cognitive impairment and AD. Thus if this intervention is proven effective in clinical trials, chronic low-dose MB treatment could be accomplished with relatively inexpensive and non-invasive means.

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