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Methylene blue improves brain oxidative metabolism and memory retention in rats

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Abstract

Methylene blue (MB) increases mitochondrial oxygen consumption and restores memory retention in rats metabolically impaired by inhibition of cytochrome *c* oxidase. This study tested two related hypotheses using biochemical and behavioral techniques: (1) that low-level MB would enhance brain cytochrome *c* oxidation, as tested in vitro in brain homogenates and after in vivo administration to rats and (2) that corresponding low-dose MB would enhance spatial memory retention in normal rats, as tested 24 h after rats were trained in a baited holeboard maze for 5 days with daily MB posttraining injections. The biochemical in vitro studies showed an increased rate of brain cytochrome *c* oxidation with the low but not the high MB concentrations tested. The in vivo administration studies showed that the corresponding MB low dose (1 mg/kg) increased brain cytochrome *c* oxidation 24 h after intraperitoneal injection, but not after 1 or 2 h postinjection. In the behavioral studies, spatial memory retention in probe trials (percentage of visits to training-baited holes compared to total visits) was significantly better for MB-treated than saline control groups (66% vs. 31%). Together the findings suggest that low-dose MB enhances spatial memory retention in normal rats by increasing brain cytochrome *c* oxidase activity.

Keywords: Cytochrome oxidase; Methylene blue; Mitochondria; Oxygen consumption; Holeboard maze; Memory retention; Spatial memory; Cerebral metabolism; Neurodegenerative diseases

1. Introduction

Methylene blue (MB) is a reduction–oxidation (redox) agent that improves mitochondrial respiration at low concentrations (0.5–2 μM) by shuttling electrons to oxygen in the electron transport chain, thereby increasing cellular oxygen consumption (Lindahl and Öberg, 1961; Visarius et al., 1997). The brain is the organ most dependent on oxygen consumption for the generation of metabolic energy through oxidative phosphorylation (Sokoloff, 1992). Cytochrome *c* oxidase is the terminal enzyme in the mitochondrial electron transport chain and is responsible for the utilization of oxygen for metabolic energy production in the brain (Wong-Riley, 1989).

Martinez et al. (1978) were the first to demonstrate that posttraining MB administration affects memory retention. Rats given intraperitoneal injections of 1 mg/kg MB after training showed enhanced memory retention 24 h later in an inhibitory avoidance test, whereas animals given a 50 mg/kg dose of MB showed impaired memory. Callaway et al. (2002) found that posttraining administration of the low dose of MB (1 mg/kg) reversed spatial memory deficits produced by impaired mitochondrial respiration in rats given sodium azide, an inhibitor of cytochrome *c* oxidase (Cada et al., 1995). Together these findings suggest that low-dose MB given after training may enhance memory retention 24 h later by increasing brain cytochrome *c* oxidation. This hypothesis was tested in the present study.

Impairments in mitochondrial respiration have been linked to various neurodegenerative diseases (Beal et al., 1993; Bowling and Beal, 1995) such as Alzheimer's disease in which a decrease in cytochrome *c* oxidase activity has been reported (Gonzalez-Lima and Cada,

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1998; Gonzalez-Lima et al., 1997; Kish et al., 1992; Valla et al., 2001). Moreover, a genetic defect in cytochrome *c* oxidase, the enzyme necessary for the rate-limiting step in mitochondrial respiration, causes Leigh's disease, a lethal subacute necrotizing encephalopathy (Cavanagh and Harding, 1994). Partial inhibition of cytochrome *c* oxidase, induced experimentally with sodium azide (Cada et al., 1995), produces spatial memory deficits in rats (Bennett et al., 1996; Bennett and Rose, 1992; Callaway et al., 2002). Therefore, impaired cytochrome *c* oxidation has been associated with memory deficits.

Since MB is a well-characterized redox agent that affects the electron transport chain (Lindahl and Öberg, 1961; Visarius et al., 1997), an increase in cytochrome *c* oxidation may be expected at low MB concentrations (similar to doses that improve memory in rats), and a decrease in cytochrome *c* oxidation may be found when using a high MB concentration (similar to doses that impair memory).

If low-dose MB increases cytochrome *c* oxidation 24 h after its administration, then MB may enhance memory retention tested at that time. The goal of this study was to determine if MB memory-enhancing effects could be explained by an increase in brain cytochrome *c* oxidase activity, as tested following in vitro and in vivo administration of MB using biochemical spectrophotometric analyses. The effect of MB on memory retention was tested in rats treated with 1 mg/kg MB daily after each of five training sessions using a baited holeboard maze to examine performance (daily training trials) and memory retention 24 h after the end of training (probe trials).

2. Methods

2.1. Biochemical methods

A more detailed explanation of the biochemical methods has been published by Gonzalez-Lima and Cada (1998).

2.1.1. Materials

For the spectrophotometric assay, the following reagents (all materials were purchased from Sigma, St. Louis, MO) were prepared: (1) isolation buffer (pH 7.0): 21.7 g sucrose, 0.076 g EDTA, 0.2644 g Trizma HCl, 0.039 g Trizma base, distilled water to 0.2 l; (2) dialysis buffer (pH 7.0): 1.405 g potassium phosphate monobasic, 2.082 g sodium phosphate dibasic, distilled water to 0.5 l; (3) 10% sodium deoxycholate in distilled water; (4) 1% solution of cytochrome *c* from the horse heart in the dialysis buffer; and (5) 500 μM MB in distilled water.

2.1.2. Procedure

(1) Sodium ascorbate (0.15 g) was added to 3 ml of a 1% solution of cytochrome *c* to reduce it. This solution was mixed by slowly inverting it three times, then the solution was stored on ice in the dark.

(2) A Sephadex PD-10 column (Amersham, Uppsala, Sweden) was equilibrated with 25 ml of the dialysis buffer, and then the reduced cytochrome *c* solution was added to the column to remove excess sodium ascorbate. Eluate was collected and stored on ice.

(3) Then 10 μl from the 1% solution of cytochrome *c* was added to 990 ml of the phosphate buffer and the absorbance at 550 nM was measured. Next, approximately 0.001 g of sodium hydrosulfite were added to the cuvette, and another reading was taken. The first reading should be no less than 95% of the second reading.

(4) By using the first reading from the previous step, the concentration of the cytochrome *c* in the solution was determined using a calibration curve. The calibration curve was done by measuring the absorbance of different concentrations (0.006% to 0.12%) of cytochrome *c* solution, and then the number of milliliters of buffer needed to make a 0.07% cytochrome *c* solution was calculated according to the formula: [0.07 × no. of milliliters desired]/[(absorbance/extinction coefficient)/100 × 12.384] = *x* μl cytochrome *c*. The extinction coefficient of cytochrome *c* oxidase was determined in our spectrophotometer (Shimadzu model UV-1201V) and was found to be 8.69.

(5) An aliquot of brain tissue homogenate was then homogenized in cold isolation buffer (1 g per 4 ml buffer).

(6) Fifty microliters of this solution was combined with 3.75 ml of isolation buffer and 200 μl of the 10% deoxycholate stock, the solution briefly mixed in the vortex and incubated for 5 min at room temperature. Afterwards, the solution was mixed in the vortex again and placed on ice. This working solution was used within 30 min.

(7) Diluted cytochrome *c* solution (990 μl) was placed in the cuvette and heated to 37 °C. Next, 10 μl of the tissue solution was added, mixed by turning once, and placed in the spectrophotometer.

(8) Change in the absorbance at 550 nm was recorded over a 2-min time period.

(9) Steps 7 and 8 were repeated, and for measurements including MB the volume of the solution was adjusted to yield a final 1 ml volume in the cuvette.

(10) The reduction of cytochrome *c* was calculated by dividing the change in the absorbance over 1 min by the extinction coefficient. To determine cytochrome oxidase activity in the brain homogenate, this number was then divided with 0.000025, which gave activity units. An activity unit is defined as 1 unit that oxidizes 1 μmol of reduced cytochrome *c* per minute per gram of tissue wet weight at pH 7 and 37 °C.

2.1.3. In vitro MB administration

Twelve Sprague–Dawley 3-month-old male rats were decapitated, and their unfixed brains were quickly harvested. Brains were then homogenized and the homogenate was stored at −40 °C.

The absorbance data were collected on four different days, in which five separate samples were measured for

each of four concentration levels of MB (0, 0.5, 5, and 10 μM). Our selected range of MB levels was based on data showing the concentration of MB in rat brain after systemic administration (Peter et al., 2000), so that the 0.5 μM concentration corresponded to the 1 mg/kg dose that improved memory retention (Callaway et al., 2002; Martinez et al., 1978). Because the goal of the spectrophotometric method was to measure the optimal cytochrome *c* oxidase enzymatic activity rate, we chose the three highest values from each data set for our final analysis. Data were reported as a percent change from the baseline activity (no MB) of each sample. Means and standard errors for each MB level were compared with the baseline values using ANOVA followed by Bonferroni-corrected, independent two-tailed Student *t* tests (Hochberg, 1988).

2.1.4. In vivo MB administration

Twenty four adult male Sprague–Dawley rats weighing an average of 270 g were given intraperitoneal injections of saline or 1 mg/kg MB dissolved in saline and then killed 1, 2, or 24 h later. Control animals received intraperitoneal saline injections of equivalent volume, and they were killed 2 h after the injection. Brains were quickly removed, homogenized, frozen in isopentane and stored at −40 °C. Spectrophotometry was used to determine cytochrome *c* oxidase enzymatic activity as described earlier.

2.2. Behavioral methods

2.2.1. Subjects

Subjects were 20 adult male Sprague–Dawley rats weighing an average of 270 g when experimental training began. They were housed two per cage on a 12-h light/dark schedule and given water ad lib. Before the experiment began, food was reduced to about 20 g daily until each subject's weight was approximately 80% of the original weight. Ten subjects were randomly assigned to the control group and 10 to the MB group. One control subject died of an unknown cause during the third week of the experiment. All procedures were conducted in agreement with the American Association for the Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee.

2.2.2. Apparatus

The apparatus used for training was a holeboard maze made from a sheet of smoked acrylic (~ 1 m^2) with four rows of four holes (2.5 cm in diameter) (Maldonado-Irizarry and Kelley, 1995). The outer holes were 17.8 cm from the edge and rows were 18.4 cm apart. Five-milliliter polyurethane cups were placed in all holes. The holeboard maze rested on 3-cm rubber risers in a clear acrylic box 45 cm high. This box was placed on a 60-cm-high table in a behavioral room where extra-maze cues remained constant throughout the experiment.

2.2.3. Procedure

In the 2 days prior to training, all holes were baited with a sweetened cereal (Froot Loops) and subjects underwent familiarization trials until each consistently consumed bait from the holes. Before each trial, the holeboard surface was wiped with a 3:1 water–vinegar solution to mask odor cues. However, it is probable that odor cues may have helped the rats to find the baited holes during the daily training trials. Therefore, the memory of the location of the baited holes was tested using a probe trial with unbaited holes and no odor cues after the end of 5 days of training. Before every training trial, four holes were baited according to Baiting Pattern 1 (BP1) or 2 (BP2), using the appropriate pattern for each run (Fig. 1). In the first of a two-run experiment, subjects were trained in the baited holeboard maze, five trials daily for 5 days. An unbaited probe trial was conducted on Day 6. Fifteen days after the first run ended, an identical second run was conducted, except the BP2 (reversal) was a mirror image of BP1. Since the two patterns were different, an increase in memory for BP2 could not be explained by an increase in memory for BP1.

Subjects were run individually in sets of two. Each subject was run alternately until both had finished five trials. The average intertrial interval was 10.5 min. Each trial began when the subject was placed on the center front of the maze surface and ended immediately after all bait was consumed or when 5 min had elapsed. Fifteen minutes after the end of each daily training session, 1 mg/kg of MB [~ 0.04 ml MB injection, USP 1% (10 mg/ml) from American Regent Laboratories, Shirley, NY] or a similar volume of normal saline was administered intraperitoneally.

2.2.4. Behavioral data analysis

An experimenter recorded trial duration and order of visits to baited and unbaited holes. A visit to a baited hole in which

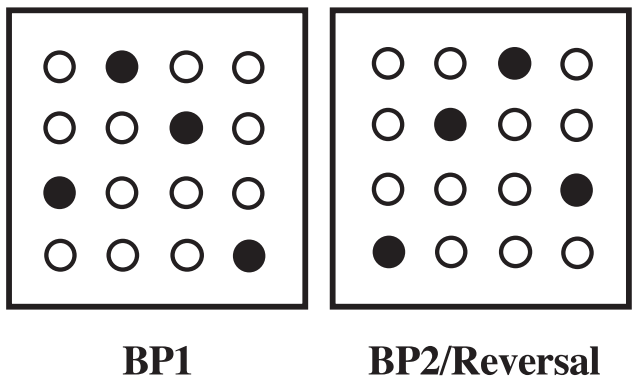

Fig. 1. Solid circles (●) correspond to holes baited and open circles (○) to holes unbaited in the baiting patterns used in training trials. The first baiting pattern (BP1) was used during the 5 days of training in the first run. The unbaited probe test on the sixth day was scored according to this same pattern. Fifteen days following the first run, the second run began using the second baiting pattern (BP2/reversal) for the 5 days of training trials. The unbaited probe test on the sixth day of the second run was scored according to BP2/reversal. To avoid any difference in difficulty between the two patterns, BP1 and BP2/reversal were mirror images.

bait was consumed was defined as a correct response, whereas an error was defined as a nose poke (nose lower than the horizontal surface of the maze) in an empty hole. The ability of the subjects to learn the baiting pattern during training was evaluated by the percentage of visits to training baited holes compared to total visits per trial. The daily percent increase in visits to baited holes was also calculated for Days 1–5. The unbaited probe trials on Day 6 were used to calculate a memory retention score defined as the percentage of training baited holes visited compared to total visits per trial. ANOVA was used to test for a main effect of treatment on behavioral scores. To verify the hypothesis that Day 6 means of MB-treated subjects were significantly ($P<.05$) greater than controls in BP1 and BP2 runs, a Bonferroni correction procedure was applied to independent two-tailed Student t tests (Hochberg, 1988).

3. Results

3.1. Biochemical results

Following in vitro MB administration (Fig. 2) there was a significant main effect for MB as compared to control, $F(3,44)=4.289$, $P=.01$. There was a 25% increase ($t=2.59$, $P=.025$) in the rate of cytochrome c oxidation when 0.5 μM MB was added to the brain homogenate. There was no significant change in the rate of cytochrome c oxidation when 5 μM MB was added ($t=1.13$, $P=.28$). Finally, when a higher MB concentration (10 μM) was added to the cuvette, a significant ($t=3.0$, $P=.024$) decrease occurred in the rate of the cytochrome c oxidation. Thus, the low MB concentration produced positive effects, whereas the high concentration produced negative effects relative to baseline without MB. MB absorbed light at 550 nM; however, since the change in the absorbance was measured over a fixed 60-s interval, MB's absorbance was included in our measurements and subtracted accordingly.

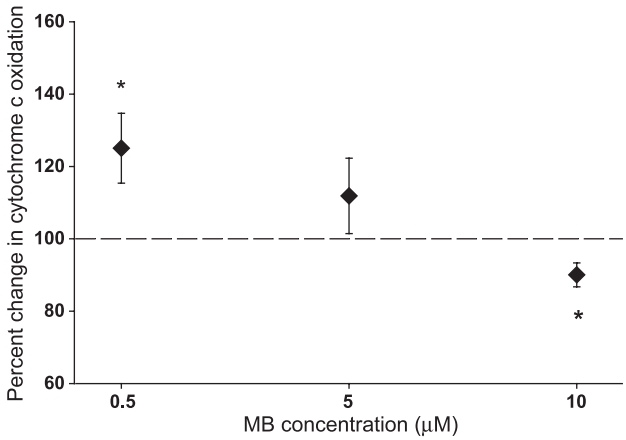

Fig. 2. Mean ± standard error bars for the effect of in vitro administration of MB to rat brain homogenate. The percent change in cytochrome c oxidation is shown after 0.5, 5, and 10 μM MB treatment. * $P=.02$.

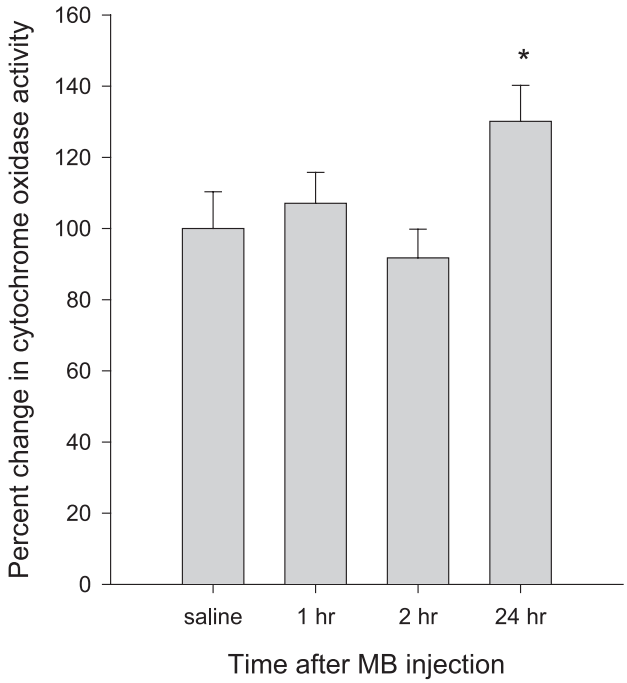

Fig. 3. Mean ± standard error bars for the effect of in vivo administration of MB. The percent change in cytochrome c oxidation is shown for animals killed 1, 2, and 24 h after 1 mg/kg MB administration, as well as for saline-treated animals. * $P=.0027$.

Following in vivo administration of 1 mg/kg of MB (Fig. 3) there was also a significant MB main effect, $F(3,66)=9.762$, $P<.001$. The increase in the rate of cytochrome c oxidation measured 24 h postinjection was significant ($t=3.93$, $P<.001$). However, there were no significant differences in the rate of cytochrome c oxidation after 1 or 2 h postinjection of MB as compared to saline-injected controls. The increase in activity in the 24-h group was about 30% as compared to the control group, similar to the 25% increase that was found when 0.5 μM MB was added to the brain homogenates directly.

3.2. Behavioral results

Posttraining MB treatment enhanced memory retention (percent visits to training baited holes compared to all visits) in probe trials (Fig. 4). The overall main effect for memory retention for MB-treated subjects was 66.4% (S.E. = 8.4), and for controls 31.4% (S.E. = 5.5), $F(1,17)=22.9$, $P=.00017$. In the first-run probe test, MB-treated subjects visited training baited holes 57.1% (S.E. = 9.3) of all visits, and control subjects 35.4% (S.E. = 6.4), $t=1.91$, $P=.037$. Memory retention in the probe test in the second run (using the BP2/reversal pattern) showed that MB-treated subjects had significantly greater reversal scores than controls. MB-treated subjects scored 75.8% (S.E. = 6.4) in memory retention and controls scored only slightly above chance with 27.4% (S.E. = 4.4), $t=6.20$, $P=.000014$.

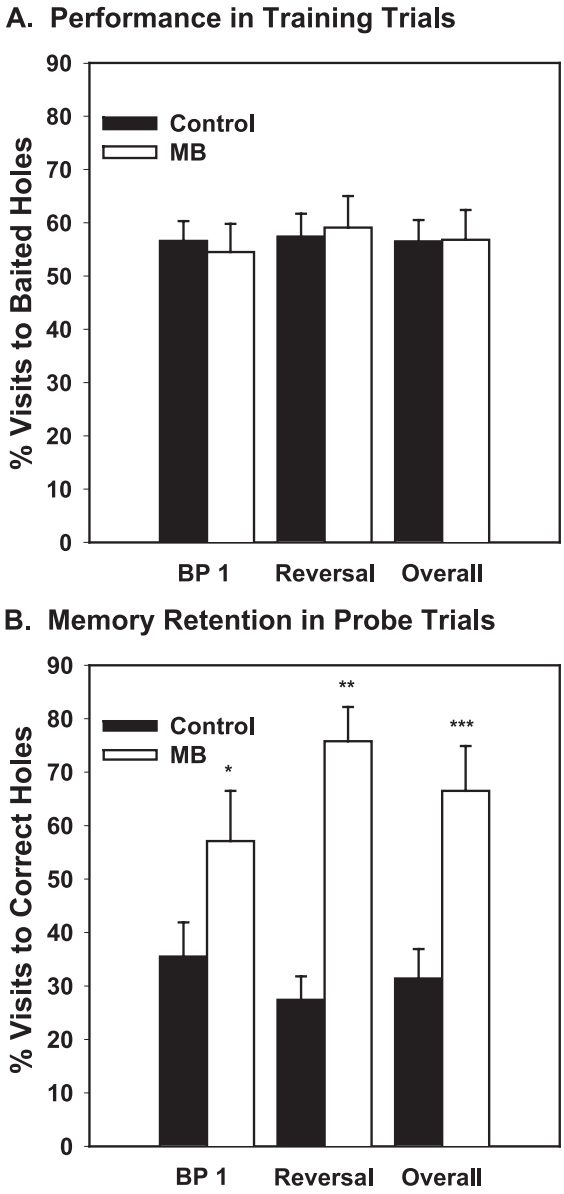

Fig. 4. Mean ± standard error bars for spatial learning performance (% visits to baited holes) in training trials (A) and memory retention scores in probe trials (B) in groups of control and MB-treated subjects. The percentage score is shown for the first baiting pattern (BP1), the second baiting pattern (BP2/reversal), and the averaged total (overall). * $P = 0.037$, * * $P = .000014$, * * * $P = .00017$.

In addition, in the second-run probe using the BP2/reversal pattern, control rats made more errors visiting the BP1 holes compared to MB-treated rats. MB-treated subjects made errors visiting the original BP1 baited holes only 14.9% (S.E. = 6.1), whereas control subjects visited BP1 baited holes 25.1% (S.E. = 5.6) of all visits.

Regarding learning performance during daily trials, the groups showed 77–80% increases in correct responses when comparing their scores before learning (first trial on Day 1 for control was 36.4 ± 6.3% of total visits, and for MB was 36.3 ± 4.8%) to the last session on Day 5 (average for the two runs was 65.4 ± 4.1% for control, and 64.4 ± 4.1% for MB). Furthermore, in a motor activity measure of holes visited per minutes, there were no group differences between control and MB groups during training, $F(1,17) = 1.11$, $P = .31$. Thus, while there was a learning curve across days for both groups, there were no group differences in overall performance in the Days 1–5 training sessions (Fig. 4).

4. Discussion

Callaway et al. (2002) found that rats whose cytochrome *c* oxidase activity was chronically inhibited by sodium azide showed impaired memory retention in the same holeboard test. Most importantly, when MB was administered to these rats, they raised their memory retention scores to the level of control subjects, presumably by compensating for their impaired mitochondrial respiration. These findings together with the present brain and memory measurements 24 h following in vivo MB administration suggest that an increased oxidative metabolic capacity in the brain is the mechanism whereby MB improves memory retention. An increase in cytochrome *c* oxidase activity increases the oxidative metabolic capacity of neurons by allowing more oxygen consumption and ATP formation in the brain (Gonzalez-Lima and Cada, 1998). Therefore, increased cytochrome *c* oxidase activity may enable an increased metabolic capacity for oxidative energy metabolism in the brain that improves memory retention.

4.1. Biochemical findings

By measuring the changes in cytochrome *c* oxidation in vitro using the spectrophotometric methods described above, we showed that MB is capable of donating as well as accepting electrons from the electron transport chain of the rat brain mitochondria. Depending on the concentration of MB added to the brain homogenate, the cytochrome *c* oxidation was either increased or decreased.

The low MB concentration (0.5 μM) used in the in vitro study was more effective than the intermediate concentration (5 μM) in enhancing enzymatic activity. A 0.5 μM concentration of MB has been also found to stimulate respiration in rat liver mitochondria, but a 5 μM MB concentration led to structural changes in rat liver mitochondria (Visarius et al., 1997). For example, swelling is observed in mitochondria exposed to 5 μM, but not to mitochondria exposed to 0.5, 1, or 2 μM (Visarius et al., 1997). These findings underscore the importance of using the optimal amounts of MB when applying this compound in vivo.

The in vivo administration of MB was conducted with a dose of 1 mg/kg because that dose leads to approximately 0.5 μM MB concentration in the rat brain, as estimated from the literature on the pharmacokinetics and organ distribution of MB after systemic administration (Burrows, 1984; Disanto and Wagner, 1972; Peter et al., 2000). Our direct brain

measurements of cytochrome *c* oxidation done at 1, 2, and 24 h after in vivo MB administration suggest that an increased oxidative metabolic capacity in the brain is the mechanism whereby MB improves memory retention tested 24 h postinjection.

Martinez et al. (1978) showed that the dose and time of MB administration were important for the memory-enhancing effect because successful memory retention in an inhibitory avoidance task was improved only 24 h after posttraining MB injection. For example, 1 mg/kg MB given intraperitoneally immediately after training sessions enhanced memory retention 24 h later, whereas administration either 6 h after training or 15 min before retention testing was ineffective in improving memory retention tested 24 h after training. A dose of 10 mg/kg of MB progressively accumulates in the brain, reaching a concentration of over 10 times greater in the brain than in blood, and after 24 h most of this dose of MB is trapped within body organs, with about 70% MB trapped after intravenous injection and 80% MB trapped after oral administration (Peter et al., 2000). An MB dose of 1 mg/kg administered intraperitoneally to rats is twice as effective as 10 mg/kg in the stimulation of liver mitochondrial metabolism in vivo (Visarius et al., 1997). Large doses of MB such as 50 mg/kg are detrimental because they lead to methemoglobin formation and memory deficits in mice (Martinez et al., 1978).

4.2. Behavioral findings

All subjects reached approximately the same overall performance at the end of training, but only those given posttraining MB showed better memory retention in the probe trials on Day 6. In addition, both groups showed an improvement in performance across training days, suggesting that learning was taking place, but the MB-treated rats showed better memory retention. A memory-retention effect of MB in the unbaited probe trials on Day 6 was anticipated because no olfactory cues were present in the probe trials, whereas olfactory cues may have influenced overall performance during the baited trials. Since the overall performance during training was similar among groups, the differences found in the unbaited probe trials on Day 6 can be best explained by better spatial memory retention after repeated posttraining MB administration.

In the second-run probe test, memory retention in MB-treated subjects was improved using the mirror-image baiting pattern (BP2/reversal), as shown by higher scores for BP2 holes and fewer errors visiting BP1 holes. Therefore, it is unlikely that the enhanced memory observed after both BP1 and BP2 runs was due to a nonspecific response bias for one of the patterns.

4.3. Conclusions

Together with our previous findings, the present results suggest that the mechanism of action of MB in memory retention may be related to enhancement of cytochrome *c* oxidation rather than any known metabolite involvement (Burrows, 1984; Disanto and Wagner, 1972). Therefore, MB could facilitate brain oxidative metabolism during the critical period for memory retrieval tested 24 h after training.

Traditionally, pharmacological treatments to facilitate memory have been designed to stimulate the synaptic activity of specific neurotransmitters that show dose sensitivity for increasing doses. MB, on the other hand, is a metabolic redox agent that only at low doses improves brain cytochrome *c* oxidation. Furthermore, this effect is manifested after a long postinjection interval that is presumably needed for the slow accumulation of MB in the brain. This effect of MB as a metabolic enhancer may facilitate memory retention when adequate MB levels are present in the brain during memory consolidation or retrieval at the time of memory testing. Furthermore, pharmacological interventions with MB and similar metabolic enhancers would not be limited to one neuron type, which may lead to undesirable side effects associated with stimulation or inhibition of a particular neurotransmitter system. MB has been safely used for many years as an antidote for some metabolic poisons and is a drug approved by the U.S. Food and Drug Administration that is well tolerated by humans (Harvey, 1970). Further studies will need to address the question of whether similar low doses of MB may improve human memory retention.

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