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Memory facilitation by methylene blue: Dose-dependent effect on behavior and brain oxygen consumption

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Abstract

Methylene blue administered post-training improves memory retention in avoidance and appetitive tasks, and restores spatial memory impaired by an inhibitor of cytochrome oxidase. Methylene blue may improve memory retention by increasing brain oxygen utilization. We investigated which doses improve memory without nonspecific behavioral effects, and whether methylene blue enhances brain oxygen consumption. Different doses were evaluated 24 h after administration in wheel running, feeding, open field habituation and object recognition tests. The 1–10 mg/kg methylene blue-treated rats were not different from saline-treated rats in locomotion or feeding behavior. The 50–100 mg/kg doses decreased running wheel behavior. The 4 mg/kg dose improved behavioral habituation and object memory recognition. Dose-dependent effects of methylene blue on brain oxygen consumption revealed that low concentrations increased brain oxygen consumption in vitro and 24 h after in vivo administration. Therefore, methylene blue doses that increase brain oxygen consumption also facilitate memory retention.

Keywords: Memory improvement; Brain energy metabolism; Habituation; Recognition memory; Mitochondrial respiration; (Rat)

1. Introduction

Memory difficulties in dementia are correlated with impaired mitochondrial oxidative metabolism and brain oxygen utilization (Beal et al., 1993; de la Torre, 2004). Hence a drug that increases brain mitochondrial respiration may improve memory. One candidate is methylene blue, a redox compound with cationic and lipophilic properties that attract it to the mitochondrial membrane (Visarius et al., 1999). Low-dose methylene blue acts on isolated mitochondria, where it increases oxygen consumption by a well-established mechanism of action that involves accepting electrons from oxygen (Lindahl and Oberg, 1961). After in vivo administration, methylene blue passes the blood-brain barrier and accumulates in the human brain within hours (Peter et al., 2000).

The memory-improving action of methylene blue in rats was first reported by Martinez et al. (1978). A low dose (1 mg/kg) injected post-training enhanced retention of an inhibitory avoidance response in rats when tested 24 h later (Martinez et al., 1978). However, this early study did not evaluate other behavioral effects of methylene blue which could explain their results. For example, methylene blue could simply modify levels of locomotor activity or fear and thereby increase inhibitory avoidance responses. Recent studies have examined the effects of 1 and 4 mg/kg doses in other appetitive and aversive tasks, and they have provided convincing evidence that methylene blue improves memory retention without affecting fearfulness

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or anxiety measures (Callaway et al., 2002, 2004; Gonzalez-Lima and Bruchey, 2004).

Although low doses improve memory in various tasks, methylene blue has not been evaluated for its effects on locomotor activity and feeding behavior that may contribute to the modification of behavior observed in previous avoidance and appetitive tasks. Our first objective was to investigate which methylene blue doses improve behavioral habituation and recognition memory 24 h after administration without nonspecific effects on behavior. Different doses were evaluated in wheel running, feeding, open field habituation and object recognition tests. The first two tests measured locomotion and feeding of a sweet cookie. The other two tests measured between-days memory retention of habituation of exploratory behavior (Cerbone and Sadile, 1994) and object recognition memory (Lebrun et al., 2000).

The brain depends mainly on the oxidation of glucose for its energy metabolism (Sokoloff, 1989). It is well established that glucose can improve memory, but its effect is highly dose-dependent and various candidate mechanisms have been proposed (Messier, 2004). Glucose is oxidized by mitochondrial enzymes to produce energy through oxidative phosphorylation, where cytochrome *c* oxidase is the terminal enzyme mediating oxygen utilization (Gonzalez-Lima and Cada, 1998). We previously demonstrated that brain cytochrome *c* oxidation increases 24 h after 1 mg/kg methylene blue administration in vivo (Callaway et al., 2004). If methylene blue can increase cellular respiration in the brain, it may explain the improved memory retention seen in animals after treatment with low-dose methylene blue (Martinez et al., 1978; Callaway et al., 2002, 2004; Gonzalez-Lima and Bruchey, 2004). However, there are no studies demonstrating a direct action of methylene blue on brain oxygen utilization.

Therefore, our second objective was to investigate brain oxygen consumption after in vitro administration of low concentrations of reduced methylene blue (0.5, 5, and 10 μM) to rat brain homogenates. These in vitro concentrations correspond to the 1–4 mg/kg doses (Peter et al., 2000) that improve memory retention (Martinez et al., 1978; Callaway et al., 2002, 2004; Gonzalez-Lima and Bruchey, 2004). A final experiment measured oxygen metabolism in rat brains 24 h after in vivo treatment with 1 mg/kg methylene blue. Oxygen consumption is an important indicator of neuronal activity (Sokoloff, 1989) and its measurement could provide insight into cellular mechanisms mediating methylene blue's effects on memory.

2. Materials and methods

2.1. Subjects

Experiments were done in accordance with NIH guidelines for the use of experimental animals and were approved by the University of Texas Institutional Animal Care and Use Committee. For the behavioral experiments, subjects were 36 male Long–Evans rats (Harlan, Houston, TX) weighing an average of 350 g at the beginning of behavioral testing. They were housed on a 12:12 light/dark cycle with food and water access ad libitum. An additional ten 7-month old male Long Evans rats were used for the in vitro oxygen assays.

In a previous study, Sprague Dawley rats showed an increase in cytochrome oxidase activity 24 h after administration of 1 mg/kg methylene blue (Callaway et al., 2004). To investigate if this cytochrome oxidase increase due to methylene blue treatment is related to an increase in oxygen consumption in vivo, 22 adult male Sprague Dawley rats (Harlan, Houston, TX) weighing an average of 270 g were used.

2.2. Behavioral procedures

2.2.1. Methylene blue administration and time of testing

Rats were counterbalanced into five treatment groups of saline, 1, 4, 10, or 100 mg/kg methylene blue (Faulding Pharmaceuticals, Paramus, NJ). All injections were performed intraperitoneally (i.p.). All behavioral experiments were conducted during the light phase of the cycle and behavior was measured 24 h after methylene blue administration, with the exception of the running wheel which lasted for a continuous 24-h period.

2.2.2. Running wheel

Rats were injected with methylene blue or saline, placed in the holding compartment of a standard activity wheel, and the number of revolutions was recorded 24 h later. The door between the holding cage and wheel was open to allow access to food and water ad libitum.

2.2.3. Feeding test

Before the feeding experiment, rats were pre-exposed to vanilla wafers (Nabisco Brands, Inc., East Hanover, NJ) in their home cage in order to overcome neophobia. Rats were injected with methylene blue or saline 24 h before testing. Trials were conducted in a box (43.2 cm×21.6 cm×30.5 cm) with clear plastic sides. One vanilla wafer softened with approximately 5 ml of distilled water was placed in a plastic weigh boat on a wire mesh floor. Subjects were placed in the box facing the wafer, and the experimenter left the room. After 5 min, the rats were removed, and the remaining wafer weight was recorded. All spillage was collected and included in the calculations.

2.2.4. Open field habituation test

An automated activity monitoring system from MED Associates (St. Albans, VT) recorded various motor indices of exploration. The 43.2 cm^2 chamber consisted of clear plastic sides 30.5 cm high and a plexiglass floor. Activity was detected by parallel beam arrays of infrared motion detectors (16×16, 1 in. apart) with two arrays located 2.5

cm above the floor, and another array 17.2 cm above the chamber floor to detect rearing. The measures included *ambulatory distance, time and counts* (number of horizontal beam breaks, and the calculated time and distance traveled); *stereotypic time and counts* (duration and frequency of short movements without ambulation of the rat, including grooming/turning/tailflick behavior); *vertical counts and time* (frequency and duration of vertical beam breaks, produced by rearing on hindlimbs); *resting time* (time spent with no new beam breaks); and *center crossings* (number of crossings into the central 38% area of the open field).

The chambers were cleaned with a mild detergent between animals. After a 5-min period in a dimly lit, sound-proofed testing room, rats were placed in the chamber facing one corner, and their behavior was recorded for 5 min. Immediately after the session, they were administered methylene blue or saline and returned to their home cage for 24 h. The following day, after another 5-min period in the testing room, they were placed in the open field chamber for a second 5-min session.

2.2.5. Object recognition test

The object recognition test (Ennaceur and Delacour, 1988) was conducted in the same chamber used for the open field test. Since all animals previously received 2 days of 5-min open field trials, they were already familiar with this environment and did not require more pre-exposure. Two cohorts were run, and the objects were counterbalanced for the second cohort. The first day consisted of a 5-min trial in which animals freely explored two identical objects that were suspended in opposite corners of the open field. The objects were unopened aluminum cola cans, large metal clamps, and blue paper-filled 50-ml centrifuge tubes. The objects were suspended so as not to interfere with the infrared beams or be displaced by the animals. The animals could only explore the objects by getting close to them but they could not climb the objects. The MED Associates activity monitoring program recorded time near the object, which was defined as the time spent in the corner (10×10 cm corner area) where the objects were suspended.

Between each trial, the arena was cleaned with diluted mild detergent, and the objects were wiped with 70% ethanol to minimize olfactory cues. Immediately following the exploratory session, animals were returned to their home room and injected with methylene blue or saline. After 24 h, animals were placed in the same open field, except this time one object was replaced with a novel object. Animals were allowed to explore both objects for 3 min. The difference in time spent in the corner near the familiar versus novel object was used as the measure of object recognition.

2.3. Brain oxygen assays

2.3.1. Fiber optic oxygen sensor

Dissolved oxygen concentration was measured with a fiber optic oxygen sensor system (Ocean Optics, Dunedin, FL). The system is as accurate as polarographic techniques; but it has a faster response time and does not consume oxygen or require frequent calibrations (Shaw et al., 2002). It consists of a fluorescence probe connected to a light source and spectrometer. Ocean Optics software converts partial pressure in the sample to mole percentage. Calibration was performed at Ocean Optics, and a single-point update was completed immediately before the study in a 37 °C incubator.

2.3.2. In vitro procedures

To study the dose-dependent effects of methylene blue on oxygen metabolism, different methylene blue concentrations were added to rat brain homogenates. Methylene blue enters cellular membranes in body tissues after it is reduced to leucomethylene blue by the capillary endothelium (Merker et al., 1997); therefore leucomethylene blue was used in vitro to facilitate entry into the cells in the brain homogenates. Methylene blue was reduced with 45 mM ascorbic acid in distilled water, and a minimal amount (<2 mM) of HCl was added to speed the reaction (Snehalatha et al., 1997; Mowry and Ogren, 1999). Three leucomethylene blue concentrations were used (0.5, 5.0, and 10.0 μM) to approximate the levels found in plasma after a single 100 mg IV injection in humans (Peter et al., 2000).

The brain homogenization and in vitro procedures were similar to those in a previous study (Callaway et al., 2004). Following decapitation, brains were quickly extracted, mixed thoroughly to form one homogenate, divided into centrifuge tubes, and frozen in isopentane. On the day of assay, homogenized brains were mixed in isolation buffer (0.32 M sucrose, 1 mM EDTA, 8.4 mM Trizma HCl and 1.6 mM Trizma base) to produce a 25% tissue mixture. To solubilize membranes, 10% deoxycholate was added for a final concentration of 0.25% tissue and 0.5% deoxycholate.

Leucomethylene blue (990 μL) was warmed to 37 °C. Ten microliters of tissue solution was added, inverted twice to mix, and placed in a 37 °C chamber for 5-min measurements. Control samples contained 10 μL tissue solution and 990 μL of 0.05 M potassium phosphate buffer (0.02 M KH_2PO_4 and 0.24 M Na_2HPO_4). All final solutions had a pH of 7.25±0.5.

2.3.3. In vivo procedures

Rats were injected with 1 mg/kg methylene blue i.p. and killed 1 h (n=5), 2 h (n=5), or 24 h (n=6) later. Controls subjects (n=6) were rats that received an equivalent volume of saline i.p. and killed 2 h later. Following decapitation, brains were quickly extracted, homogenized, frozen in isopentane, and stored at −40 °C. For each subject, approximately 50 μL of brain tissue homogenate was placed in a microcentrifuge tube, warmed to 37 °C in a water bath, and placed in a 37 °C incubator for analysis. Each sample was measured for 5 min with the oxygen sensor described above.

2.4. Statistical methods

SPSS software (version 11.5, SPSS, Chicago, IL) was used. Parametric data were analyzed using repeated-measures analysis of variance (ANOVA) to test the significance of mean group differences for the open field measures and oxygen probe data. This was followed by post-hoc *t*-tests using Bonferroni correction to reduce Type 1 error. Non-parametric data were analyzed with Mann–Whitney *U* tests for independent group mean comparisons, and the Wilcoxon signed-rank test was used for paired data in the object recognition test. The specific tests and two-tailed *P* levels are reported in the results.

3. Results

3.1. Behavioral effects of methylene blue

3.1.1. General observations after in vivo administration

Shortly after injection, the 100 mg/kg dose produced piloerection and uncoordinated movement in all of the first three subjects injected, and one of these three rats was found dead in the running wheel the following day. Therefore, this dose was discontinued from further use and the other two rats injected with 100 mg/kg were excluded from further testing. No further animals were injected with this dose. The first three subjects injected with 50 mg/kg group were examined in the running wheel and wafer feeding tests. However, because of decreased daily food consumption in these rats, they were excluded from the open field habituation and object recognition tests.

3.1.2. Running wheel and feeding tests

Group differences were examined with Mann–Whitney *U* tests, comparing each methylene blue group to saline-treated animals (Table 1). In the running wheel, rats given the highest doses of methylene blue (50 and 100 mg/kg) had significantly fewer median revolutions than saline-treated rats (35 versus 279, respectively) ($U_{(14)}$=9.0, P=0.038). Methylene blue-treated rats ate a similar amount of wafer in the feeding task as those treated with saline (P>0.05), except for the rats injected with 50 mg/kg that ate 0.03 g versus a 0.6–0.45 g mean range in the other groups. But since there were only 3 rats in the 50 mg/kg group, the significance level of this difference was $U_{(12)}$=5.0, P=0.055.

3.1.3. Open field habituation test

No significant group differences were found on day 1 using repeated-measures ANOVA. Previous studies indicate that there is a between-days habituation effect as measured by decreased exploratory activity in the familiar environment on day 2 (Cerbone and Sadile, 1994). On day 2, 24 h after methylene blue or saline treatment, 4 mg/kg treated rats showed less ambulatory time (15±3 s) as compared to saline-treated animals (27±2 s). Bonferroni-corrected comparisons verified that 4 mg/kg treated rats had significantly less ambulatory time than saline-treated rats (P=0.012), 1 mg/kg (P=0.031), and 10 mg/kg (P=0.001). This was also verified as between-days differences on ambulatory distance taking into account baseline performance on day 1 and comparing day 1 and day 2 ($F_{(3,24)}$=3.02, P<0.05). Days 1–2 percent decreases in ambulatory distance were 5%, 18% and 26%, for the 0, 1 and 4 mg/kg methylene blue groups respectively, whereas the 10 mg/kg methylene blue group showed a 25% increase that was not significant due to its high variance. No significant group differences were found in measures of rearing (vertical time or counts) on day 2. In addition, there were no group differences in the number of crossings into the center of the chamber, indicating that methylene blue did not affect center avoidance.

3.1.4. Object recognition test

As expected methylene blue improved memory for the familiar object on day 2. During the first 3 min of day 1, there were no significant differences in the amount of time rats spent exploring each identical object (P>0.05, paired *t*-test). All rats distinguished between the two objects as shown by a longer exploration time with the novel object ($F_{(1,24)}$=6.14, P=0.021, repeated-measures ANOVA). Day 2 group averages (Table 2) showed that methylene blue effects on memory recognition varied in a dose-dependent manner. On average, rats treated with 1 mg/kg methylene blue explored the novel object 2 s longer than the familiar object, the 4 mg/kg methylene blue-treated rats spent almost 10 s more exploring the novel object, and the 10 mg/kg

Table 1
Running wheel and feeding test results 24 h after saline or methylene blue (MB) treatment

MB dose (mg/kg)	*n*	Running wheel (revolutions)			*n*	Feeding test (g)		
		Mean±S.E.M.	Median	Range		Mean±S.E.M.	Median	Range
Saline	11	318±72	279	28–718	11	0.06±0.23	0.10	0–1.8
1	4	166±104	92	9–470	6	0.48±0.30	0.48	0–1.8
4	5	453±112	362	253–843	6	0.48±0.28	0.48	0–1.5
10	7	268±97	103	78–761	7	0.53±0.31	0.53	0–1.7
50	3	64±46	35*	3–154	3	0.03±0.04	0.03	0–0.1
100	2	116±111	116*	5–227	2	0.45±0.64	0.45	0–0.9

* P<0.05, 50–100 mg/kg versus saline.

Table 2
Dose-response effects of methylene blue (MB) on object recognition memory

MB dose (mg/kg, i.p.)	*n*	Familiar object (Mean±S.E.M.)	Novel object (Mean±S.E.M.)	%Difference (*N*/*F*×100)−100	D.I. (*N*−*F*/*N*+*F*)
0	10	5.01±1.53	10.05±2.58	101	0.34
1	6	6.65±1.54	8.65±3.41	30	0.13
4	6	3.62±1.48	13.16±5.01*	263	0.57
10	6	1.92±0.9	17.55±13.65	812	0.80

N—novel object; *F*—familiar object. D.I.—discrimination index.
* $P<0.05$ familiar versus novel.

methylene blue-treated group spent 15.6 s more in close proximity to the novel object. However due to subject variance, only the 4 mg/kg methylene blue-treated group of rats showed a statistically reliable group difference ($P=0.028$, Wilcoxon signed-ranks test).

3.2. Effects of methylene blue on brain oxygen consumption

3.2.1. Dose-dependent effects after in vitro administration

Values obtained from our control group indicated a partial pressure baseline measure of 28 mm Hg that is consistent with other studies of brain oxygen tension (Doppenberg et al., 1998; Duong et al., 2001). Oxygen concentration decreased over time in all groups ($n=9$, Fig. 1). This was expected because respiratory substrates were not added to the samples, and therefore consumption occurs only to the extent of the oxygen concentration in the samples. There was a main effect of leucomethylene blue ($F_{(3,32)}=10.18$, $P<0.001$, repeated-measures ANOVA). Independent group comparisons (Bonferroni-corrected) showed significant differences between control and 5 μM leucomethylene blue ($P=0.008$); control and 10 μM leucomethylene blue ($P<0.001$); and 0.5 μM and 10 μM leucomethylene blue ($P=0.025$). The difference in starting points suggests that leucomethylene blue increases oxygen metabolism within the first min post-administration.

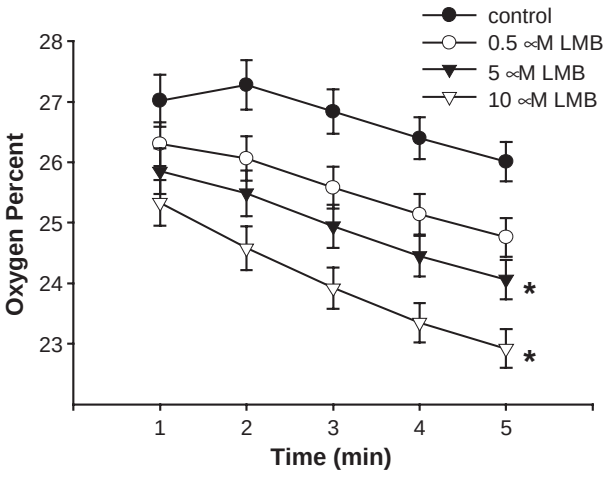

Fig. 1. Effect of in vitro administration of leucomethylene blue (LMB) in rat brain homogenate in a 5-min measurement period. *$P<0.05$ significantly different from untreated control.

3.2.2. Time-dependent effects after in vivo administration

There was a significant group effect of methylene blue administration ($F_{(3,18)}=9.59$, $P<0.001$, repeated-measures ANOVA). Brain oxygen concentration was significantly lower 24 h after treatment with methylene blue versus saline ($P=0.001$, Bonferroni-corrected comparison). But there were no differences 1 or 2 h following methylene blue administration (Fig. 2).

4. Discussion

This study is the first to determine which methylene blue doses improve between-days memory retention in habituation and object recognition tasks without nonspecific effects on motor activity, feeding, and fearfulness measures; and it is the first to directly demonstrate that methylene blue enhances brain oxygen consumption. Methylene blue has previously been shown to enhance memory retention of an inhibitory avoidance response (Martinez et al., 1978) and a baited holeboard pattern in normal rats (Callaway et al., 2004). However, these previous studies could also be explained by nonspecific drug effects on motor activity, fearfulness or feeding behavior rather than memory effects. These studies together with the present results suggest that

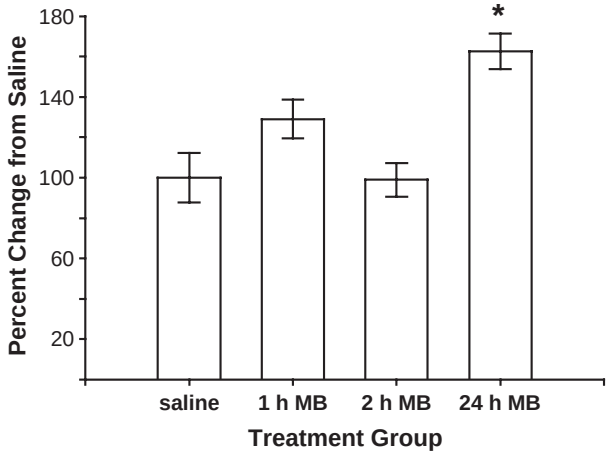

Fig. 2. Brain oxygen consumption in rats killed 1, 2, or 24 h after 1 mg/kg methylene blue (MB) or saline administration, expressed as percent difference from saline-treated rats. *$P<0.001$ significantly different from saline-treated animals.

1–4 mg/kg doses of methylene blue may improve memory retention without disrupting general activity, fearfulness, or sweet-seeking feeding behavior in rats. The 4 mg/kg dose was specifically selected for testing because humans have taken 300 mg/day of methylene blue (corresponding to 4.3 mg/kg for a 70 kg person) for one year without significant side effects (Naylor et al., 1986).

4.1. Behavioral effects of methylene blue

In the memory tests, rats were injected with 1–10 mg/kg methylene blue after the end of the training session in day 1 to improve memory retention tested in day 2. The reason was that post-training methylene blue administration was expected to enhance brain metabolic processes underlying the retention of the memory tested 24 h later. Therefore, methylene blue could not interfere with acquisition learning because it was administered after the training session.

The lower 1–4 mg/kg methylene blue doses generally improved the habituation effect in the open field test. However, the high variance produced by the 10 mg/kg dose did not result in significant group effects in both the open field habituation and object recognition tests. This suggests that methylene blue shows the same bell-shaped dose effects on memory as other "nootropic" agents.

The object recognition task revealed that methylene blue can also significantly improve this type of memory. Although all groups explored the novel object longer than the familiar one, the difference was significant only in those injected with 4 mg/kg methylene blue. One alternative interpretation of the memory studies is that methylene blue injections could simply modify locomotor activity and thus increase the habituation or object recognition effects. However, locomotor behavior during 24 h in the running wheel was not different in the 1–10 mg/kg methylene blue groups, suggesting that the 4 mg/kg methylene blue effect was specific to the memory tests and could not be explained by a nonspecific locomotor effect. Furthermore, a nonspecific modification of locomotor behavior could not account for the greater times spent exploring the novel object in the methylene blue-treated groups.

Another possibility is that 1–4 mg/kg doses of methylene blue may be anxiolytic and thus may stimulate exploratory behavior in the open field. To rule out this possibility, we measured fear-related behavior in the open field. Increased thigmotaxic behavior (more activity in periphery of chamber) and center avoidance (less crossings of center of chamber) are associated with fear or anxiety in rats (Treit and Fundytus, 1988). The open field data showed that there were no increases in center crossings in rats given 1–4 mg/kg doses of methylene blue, indicating no modification in fear-related behavior. Therefore, these measures suggest that methylene blue effects on the open field are more likely the result of improved memory retention.

The improvement in memory retention following post-training administration of 4 mg/kg methylene blue is consistent with a recent study using extinction of Pavlovian conditioning of tone-footshock in rats (Gonzalez-Lima and Bruchey, 2004). Conditioned freezing behavior was lower in rats given 4 mg/kg methylene blue during the post-extinction phase, suggesting that methylene blue improved retention of extinction memory. Since methylene blue was administered during the post-extinction period, methylene blue could only affect brain metabolism during that period as opposed to the prior acquisition period. In contrast, methylene blue administered after the acquisition phase would be expected to facilitate retention of the memory of the acquired behavior, as has been found in the present study and previous studies (Martinez et al., 1978; Callaway et al., 2002, 2004). Which memory benefits from the metabolic-enhancing effect of methylene blue would seem to depend on whether methylene blue is administered after acquisition or extinction (Gonzalez-Lima and Bruchey, 2004).

In addition, Gonzalez-Lima and Bruchey (2004) found no evidence of nonspecific effects on measures of motor activity and fearfulness in control rats similarly injected daily once or five times with 4 mg/kg methylene blue. Together these studies support the conclusion that single or repeated injections of 4 mg/kg methylene blue improve memory retention without significant effects on general motor activity or fear-related behavior.

4.2. Effects of methylene blue on brain oxygen consumption

Oxidative metabolism examined in vitro with an optical oxygen sensor revealed a dose-dependent effect, in which both 5 μM and 10 μM leucomethylene blue significantly increased oxygen consumption, although the effect of 0.5 μM leucomethylene blue did not reach statistical significance after Bonferroni correction. Recently, we demonstrated that 0.5 μM methylene blue increased cytochrome *c* oxidation while 5 μM had no effect and 10 μM decreased activity (Callaway et al., 2004). One important difference in the two methods employed is that the reduced form (leucomethylene blue) was used in the oxygen recordings but the oxidized form (methylene blue) was used in the cytochrome *c* oxidation measurements. The reduced form is favored for entry into the cell (Merker et al., 1997). Because there were only a few seconds of incubation with methylene blue or leucomethylene blue in both protocols, the amount entering the cell in the cytochrome *c* oxidase measurements was probably lower than that applied in the oxygen probe protocol and therefore not directly comparable. With a short incubation period, 5 μM methylene blue may enter at a concentration closer to 0.5 μM leucomethylene blue. At these concentrations, no differences were found in either measurement.

It is possible that increasing the amount of methylene blue changes its mechanism within the electron transport chain. Concentrations of 5–10 μM methylene blue effectively inhibit nitric oxide synthase (Mayer et al., 1993).

Nitric oxide and its derivative peroxynitrite can inhibit cytochrome *c* oxidase activity (Brown and Borutaite, 1999). Thus, methylene blue can have both direct and indirect actions on cytochrome *c* oxidation. Methylene blue may directly reduce oxygen at higher concentrations (10 μM methylene blue, 5 μM leucomethylene blue). This would prevent cytochrome *c* oxidase from donating its electrons to oxygen and inhibit cytochrome *c* oxidation. This may explain the decrease in the rate of cytochrome *c* oxidation with 10 μM methylene blue and the increase in oxygen consumption observed with 5 μM leucomethylene blue.

A low methylene blue concentration (2 μM) can be used to create a bypass in mitochondrial electron transport by donating electrons to a site before cytochrome *c* (Scott and Hunter, 1966). In rats, methylene blue (0.5–2 μM) stimulates cellular respiration in liver (Visarius et al., 1997) and increases cytochrome *c* oxidation in brain (Callaway et al., 2004). In vivo, 1 mg/kg methylene blue significantly increased liver fatty acid oxidation and energy production as compared to 10 mg/kg (Visarius et al., 1999), emphasizing the importance of low dose.

In the holeboard maze task, 1 mg/kg methylene blue administered post-training enhanced memory retention 24 h later in normal rats compared to saline-treated animals (Callaway et al., 2004). This dose of methylene blue also increased oxygen utilization 24 h after in vivo administration in our last experiment. This is consistent with a previous study (Callaway et al., 2004) showing that cytochrome *c* oxidase activity increases 24 h after a single 1 mg/kg methylene blue injection in rats, but not after 1 or 2 h, like in the present study. Taken together these studies suggest that low-dose methylene blue donates electrons to the mitochondrial respiratory chain which facilitates cytochrome *c* oxidase activity and increases oxygen consumption.

If methylene blue facilitates memory retention by improving cytochrome oxidase activity and consequently oxygen utilization, treatments that inhibit cytochrome oxidase activity and brain oxygen use should lead to impaired memory. Brain cytochrome oxidase activity can be partially inhibited by chronic sodium azide administration in rats (Cada et al., 1995; Berndt et al., 2001), which causes memory deficits without nonspecific motor effects in rats tested in the Morris water maze (Bennett et al., 1992) and the holeboard maze (Callaway et al., 2002). Similarly, rats with brain hypoperfusion and hypoxia resulting from ligation of the carotids show reduced cytochrome oxidase activity and memory impairment without sensorimotor deficits in the water maze test (de la Torre et al., 1997). More importantly, when methylene blue is administered to rats with decreased brain cytochrome oxidase activity, their impaired memory retention can be normalized to levels comparable to those of intact rats (Callaway et al., 2002). Together these studies suggest that an improved brain oxidative energy metabolism is a mechanism whereby methylene blue increases memory retention.

While traditional pharmacological treatments to improve memory focus on specific synaptic neurotransmitters, metabolic enhancers like methylene blue may improve overall brain energy production and memory retention by targeting mitochondrial oxidative metabolism, without producing the side effects associated with modifying a particular neurotransmitter system (Callaway et al., 2002). Methylene blue is an FDA-approved drug that has been used safely and without significant side effects for many years. It is used as an antidote for certain metabolic poisons (Bradberry, 2003) and as a neuroprotective redox agent to prevent drug-induced encephalopathy in cancer treatment (Kupfer et al., 1996). Based on our findings, it is possible that methylene blue may also be useful as a memory enhancer in humans with reduced brain oxidative metabolism, such as in individuals with Alzheimer's and vascular dementia (Valla et al., 2001; Aliev et al., 2003; de la Torre, 2004).

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