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Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats

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Abstract

Cytochrome oxidase is the mitochondrial enzyme that catalyzes the utilization of oxygen for the electron transport chain during cellular respiration. Chronic subcutaneous infusion of sodium azide, an inhibitor of cytochrome oxidase, produced a spatial memory retention deficit in rats in a holeboard maze. Methylene blue, which has been shown to increase oxygen consumption in vitro, was used to restore mitochondrial electron transport in order to facilitate memory consolidation. Administration of 1 mg/kg methylene blue after training, during the memory consolidation period, completely restored the memory retention impaired by the inhibitor of cytochrome oxidase. This suggests that methylene blue may compensate for impaired mitochondrial respiration and improve spatial memory retention. Memory retention deficits found in some neurodegenerative diseases may be improved by drugs targeting impaired mitochondrial respiration. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cytochrome *c* oxidase; Electron transport chain; Holeboard; Memory consolidation; Mitochondrial respiration; Neurodegenerative diseases; Oxygen consumption; Sodium azide

Increasing evidence suggests that impaired mitochondrial respiration is associated with learning and memory difficulties in various diseases [1,5,8]. Inhibition of cytochrome *c* oxidase by a genetic defect such as in Leigh's disease (subacute necrotizing encephalopathy) or by a vascular impairment [19] produces similar symptoms. In addition, cytochrome *c* oxidase activity has been shown to decline in Alzheimer's disease [8,9,11,17]. Similar behavioral deficits resulted when comparable mitochondrial impairment has been induced in animal models. Sodium azide (SA) has been shown to decrease cytochrome *c* oxidase activity when chronically administered to rats [4,6], and this inhibition resulted in spatial memory deficits in rats in the Morris water maze [2,3].

Martinez et al. [14] showed that memory retention was enhanced in an inhibitory avoidance task in rats given 1 mg/kg methylene blue (MB) intraperitoneally following training sessions. Post-training MB may improve memory retention by increasing mitochondrial respiration during the critical period of memory consolidation. MB increases mitochondrial respiration in vitro [18] and hence it has the potential to increase oxygen consumption and compensate for decreased cytochrome *c* oxidase activity. MB acts as an electron shuttle to oxygen that bypasses cytochrome *c* oxidase in the electron transport chain in mitochondria [18].

If post-training MB can compensate for the decreased cytochrome *c* oxidase activity produced by SA by shuttling electrons in the electron transport chain, then it may improve memory consolidation. A baited holeboard maze was used to evaluate performance (training trials) and memory retention (probe trials) in subjects given chronic SA and those given chronic SA plus post-training MB (SA-MB).

Subjects were 39 adult male Sprague–Dawley rats (Harlan, Houston, TX) approximately 11 weeks old and weighing an average of 370 g. Twelve subjects were assigned to the control group, 14 to the SA group, and 13 to the SA-MB group. All subjects were doubly-housed 1 week prior to surgery on a 12 h light/12 h dark schedule and given water and food ad lib until the experiment began. All procedures followed guidelines from the American Association for the Accreditation of Laboratory Animal

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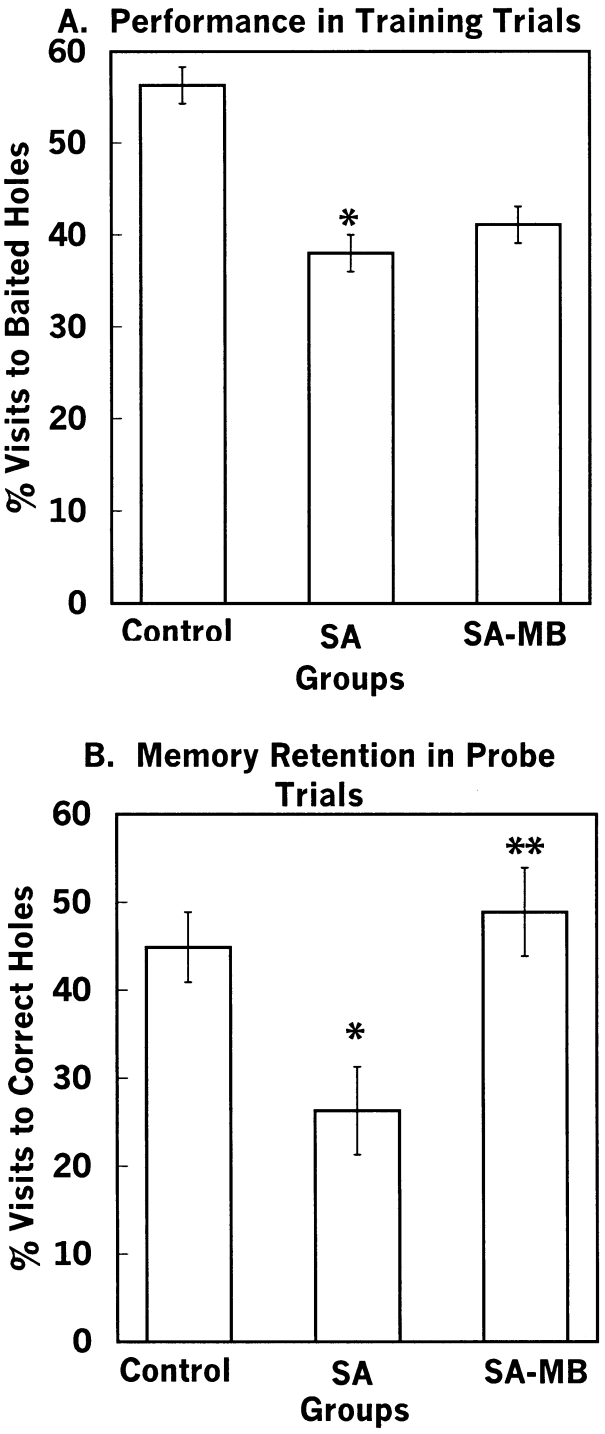

Fig. 1. Mean ± standard error (SE) bars of spatial learning performance in training trials (A) and memory retention in probe trials (B) in groups of control rats (C) and rats treated with sodium azide (SA) or sodium azide plus post-training methylene blue (SA-MB). *C vs. SA, $P < 0.05$, Bonferroni-corrected. **SA vs. SA-MB, $P < 0.05$, Bonferroni-corrected.

Care, and all protocols were approved by the institutional Animal Care and Use Committee.

The apparatus used for training was a holeboard maze made of a sheet of smoked acrylic (~1 m^2) with four rows of four holes (2.5 cm in diameter) [13]. Outer holes were 17.8 cm from the edge and rows were separated by 18.4 cm. Polyurethane cups (5 ml) 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. During the 2 days prior to surgery, all holes were baited with Fruit Loops® (a sweetened cereal), and subjects underwent familiarization trials until each subject consistently sought and consumed bait from the holes.

On the day of surgery, each rat received an intraperitoneal dose of 0.5–0.7 ml/kg of a 3:3:1 anesthetic mixture containing ketamine hydrochloride 100 mg/kg, xylazine hydrochloride 20 mg/kg, and acepromazine 10 mg/kg, respectively. If anesthesia failed to occur within 10–15 min, up to 20 mg/kg ketamine hydrochloride was administered intramuscularly. Because the goal of this project was to examine behavioral effects of changes in metabolic activity, anesthetics with a low potential for respiratory depression were selected. Surgery began when an individual subject failed to respond to the tail-pinch test. The incision area was shaved and disinfected with Betadine® solution. A horizontal incision approximately 3 cm long was made at midline on the dorsal surface of the rat, approximately 1 cm posterior to the ears. A subcutaneous pocket (5–6 cm) was formed inferior to the incision for the placement of an osmotic pump. A 2 ml Alzet osmotic pump (model 2ML4) filled with SA (in physiological saline) was implanted in each subject in the SA and SA-MB groups. This pump provided a subcutaneous infusion rate of 0.9 mg kg^{-1} h^{-1} [4] for 28 days. SA concentrations were individualized for each subject's weight. Sham pumps were implanted in each control subject. Incisions were closed with 9 mm wound clips. During recovery from anesthesia, each subject was placed in a clean cage on a heating pad and/or under a heat lamp for 20 min. The subjects were then placed in regular single housing. The rats recuperated from surgery for 2 weeks. Seven subjects died after surgery (two in the control group, two in the SA group and three in the SA-MB group) presumably due to the surgical intervention. To prevent alterations in the infusion-rate dosage of SA due to weight fluctuations, each subject was weighed daily and 5–25 g rat chow and nutritional supplement was given according to a schedule to maintain the same weight throughout the experiment.

Following recuperation from surgery, each subject was trained in the baited holeboard maze on post-surgical days 15 through 19 and 22 through 26 in five daily trials. Fifteen minutes after the end of each daily session, 1 mg/kg of MB (~0.04 ml Methylene Blue Injection, USP 1% (10 mg/ml) from American Regent Laboratories, Inc.) or a similar volume of normal saline was administered intraperitoneally. On post-surgical days 20 and 27, a probe test of one unbaited trial was conducted 24 h after the last training trial to assess the subject's memory retention of the baiting pattern. Experimenters were blind to subject group assignment.

Before each training trial, the holeboard surface was wiped with a 3:1 water/vinegar solution to mask odor cues and four holes were baited according to the same pattern throughout the experiment. Subjects were run individually in sets of three. Each subject in a set sequentially underwent the first trial, then each underwent the second trial, etc., until all three subjects had finished five trials. The average inter-trial interval was 12 min. Each trial began when the subject was placed on the center front of the maze surface and ended immediately after he had consumed all bait or when 5 min had elapsed.

Experimenters recorded trial duration and order of visits to the baited and unbaited holes. A correct response was defined as a visit to a baited hole in which bait was consumed, and an error as a nose-poke (nose lower than the horizontal plane of the maze surface) in an empty hole. The subjects' ability to learn the baiting pattern during training trials was evaluated by the percentage of baited holes visited compared to total visits per trial. Unbaited probe trials on days 20 and 27 were used to calculate a memory retention score defined as the percentage of training-baited holes visited compared to total visits per trial.

Chronic SA administration produced deficits in learning performance during training trials in addition to deficits in memory retention during probe trials (Fig. 1). As anticipated, post-training MB administration improved memory retention in probe trials (Fig. 1B) without improving training-trial performance (Fig. 1A). This behavior difference is visible only on the probe trials because there was no MB given during behavioral testing in the training trials. MB was administered post-training, that is, only after the end of trials each day. And the memory retention was tested the following day on the probe trial.

A split-plot analysis of variance performed on training scores was significant for a main effect of treatment group ($F(2, 29) = 5.20$, $P = 0.012$). Bonferroni-corrected comparisons showed significant differences in training scores only between the control (mean 56%, SE 2%) and SA (mean 38%, SE 2%) groups ($P = 0.015$). A main effect was also significant across training days ($F(9, 18) = 5.85$, $P < 0.0001$), but no significant differences in this effect were found among groups.

In probe trials, a split-plot analysis of variance performed on memory retention scores was significant for a main effect of treatment group ($F(2, 29) = 6.67$, $P = 0.004$). Bonferroni-corrected comparisons showed a significant difference between the control (mean 45%, SE 5%) and SA (mean 26%, SE 5%) groups ($P = 0.02$), and between the SA and SA-MB (mean 49%, SE 5%) groups ($P = 0.005$).

The time of MB administration is important because only post-training MB administration can affect the process of memory consolidation. Thus, successful memory retention is improved by post-training but not by pre-training MB administration [14]. In the Martinez et al. [14] study, 1 mg/kg MB administered intraperitoneally either 15 min before training, 6 h after training, or 15 min before testing had no effect on memory in an avoidance memory task. Furthermore, because MB was given after training, it could not have interfered with acquisition trials, but MB acted to enhance retention in probe trials, suggesting that MB acted mainly on aspects of memory consolidation. The memory retention score in the SA-MB group indicates that MB may compensate for the memory retention impairment produced by SA.

The effect of low-dose MB on memory retention in the SA-MB subjects may be due to enhanced oxygen consumption, by providing an alternate route of electron flow to oxygen that bypasses cytochrome *c* oxidase in the electron transport chain [18]. Therefore, MB's action in impaired mitochondrial respiration was probably compensatory in nature. In vitro studies also indicate that high amounts of MB inhibit superoxide generation [16] and Alzheimer's disease-like tau aggregation [20].

It was anticipated that MB would mainly affect memory consolidation rather than behavioral learning performance because MB was administered after each session and the probe tests for memory were done 24 h after MB administration. Peter et al. [15] have demonstrated that the plasma half-life of MB in humans resulting from an intravenous dose of 10 mg/kg was approximately 5 h. Thus, with the post-training MB administration in this study, MB was likely present during memory consolidation, but it was unlikely that an appreciable amount of MB was circulating during training and probe trials done 24 h after MB administration. There is the possibility that a metabolite of MB may have contributed to the behavioral effects by another mechanism. However, the available studies [7] do not support a significant metabolite involvement.

Traditional pharmacological intervention in neurodegenerative diseases has typically sought to increase or mimic the synaptic activity of specific neurotransmitters produced by the degenerating neurons. On the other hand, drugs successful at targeting impaired mitochondrial respiration, such as MB [12], could improve neuronal energy production and memory consolidation. Such drug intervention would not produce undesirable activity associated with a particular neurotransmitter, and would not be limited to one neuron type. MB is already an FDA-approved drug and has been used safely for many years as an antidote for some poisonings [12] and as a mild urinary tract analgesic [10]. The results from this study suggest that MB has the potential to positively affect the clinical outcome of memory retention difficulties in neurodegenerative disorders associated with mitochondrial dysfunction.

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