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MEMORY IMPROVEMENT WITH THE METABOLIC ENHANCER METHYLENE BLUE

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MEMORY IMPROVEMENT WITH THE METABOLIC ENHANCER METHYLENE BLUE

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2006

Dedication

To

My Hero,

and Loving Father,

Dennis Eugene Wrubel,

For Always Supporting and Believing in Me.

Acknowledgements

Without the love and unconditional support of my parents, Mary Beth and Dennis Wrubel, I could not have made it this far. I thank them both for everything they have done to support me throughout this journey. I want thank to my “best girl” and daughter, Eve, for her understanding and patience throughout my college experience and for making my life at home so special and fun. I also give special thanks to my boyfriend, and best friend, Jay Burns, for his emotional support, understanding, and help "sailing the ship" during the dissertation phase of my studies. I would like to thank Dr. Francisco Gonzalez-Lima for his leadership, training and expertise, and for modeling what it takes to be a truly successful scientist. I want to give special thanks to the members of my dissertation committee, Dr. Adriana Alcantara, Dr. Edwin Barea-Rodriguez, Dr. Theresa Jones, and Dr. Michael Telch for sharing their knowledge with me and pushing me to produce and defend a dissertation that I can be proud of. I also need to thank my previous academic mentors, Dr. Jim Todd and Dr. Ken Rusiniak, for their encouragement and advice over the years. I want to acknowledge my labmates and fellow graduate students for their friendship, with special thanks to Xian Zhang, Penny Riha, Alison Crane Tannenbaum, and Liz Wuehrmann who have been great friends and a support system during my studies at UT. Also thanks to my good friends Angie Dean, Ajax, Dario Dieguez, and Mark Petricca for their support and for providing humor during rough times. And finally, I want to thank all of the subjects, without whom we could not answer these questions in basic research and try to make this world a better place.

MEMORY IMPROVEMENT WITH THE METABOLIC ENHANCER METHYLENE BLUE

Publication No._____

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The University of Texas at Austin, 2006

Supervisor: Francisco Gonzalez-Lima

The goal of this dissertation was to investigate the memory retention effects of methylene blue (MB) in both appetitive and aversive memory tasks in rats. Methylene blue is a metabolic enhancer that improves memory retention in a variety of tasks including inhibitory avoidance, object recognition, spatial memory, and extinction of Pavlovian fear conditioning. Low dose MB has no side effects on behavior. MB works as a metabolic enhancer by increasing brain cytochrome oxidase activity and oxygen consumption. The first experiment was conducted to examine the effects of MB treatment in normal rats in the hole board spatial memory task, to determine if it could enhance memory of discrimination learning of rewarded versus non-rewarded trials. Subjects treated with MB discriminated better between rewarded and non-rewarded trials as compared to control subjects, indicated by a greater number of correct responses on

rewarded trials than non-rewarded trials. The second experiment was conducted to determine the effects of MB administered following acquisition trials in Pavlovian fear conditioning. Methylene blue-treated subjects demonstrated greater conditioned freezing to the tone conditioned stimulus (CS) without significant effects on context freezing. MB subjects also had higher freezing scores following extinction, indicating that they retained stronger fear-conditioning than controls. The third experiment was conducted to characterize extinction in the congenitally helpless (CH) rat, an animal model of post-traumatic stress disorder and depression, and to determine if low dose MB could facilitate fear extinction memory in these subjects. CH rats exhibited abnormally high conditioned freezing in response to the CS tone. They failed to show the gradual decrement in freezing characteristic of the normal extinction curves seen in control subjects. Administration of MB to CH rats significantly ameliorated their fear extinction deficit. It was concluded that MB is a very promising treatment for memory enhancement, and if administered post-training and in low doses, may be a potent memory-enhancing compound with little to no side effects. MB provides an important therapeutic advantage because it optimizes neuronal energy metabolism during memory formation, while avoiding side effects of drugs acting on synaptic transmission elsewhere in the brain.

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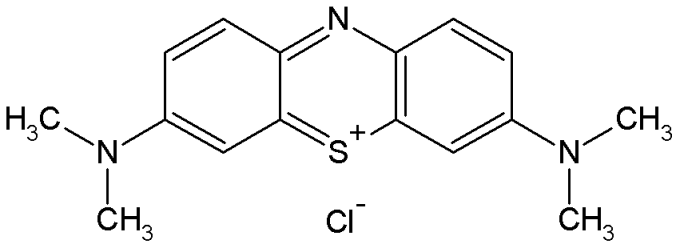
MEMORY IMPROVEMENT WITH THE METABOLIC ENHANCER METHYLENE BLUE

CHAPTER 1: INTRODUCTION

1.1 METHYLENE BLUE

Methylene blue (MB) is a redox dye that has cationic and lipophilic properties that attract it to the mitochondrial membrane (Visarius et al., 1997). It has been used as a selective supravital stain for neurons since the time of Ramon y Cajal. Its redox couple feature allows it to be an electron mediator between enzymes and substrates. Low dose methylene blue enters mitochondria, where it diverts a portion of the electron flow to molecular oxygen (Hassen & Fridovich, 1979). In its oxidized form MB retains its characteristic blue color, but becomes colorless in its reduced form, leucomethylene blue. The molecular formula of methylene blue is $C_{16}H_{18}ClN_3S.3H_2O$ (Figure 1A).

Figure 1A: The structural formula of methylene blue. (From http://en.wikipedia.org/wiki/Methylene_blue)



The half-life of methylene blue is 5-6.5 hours and it crosses the blood-brain barrier where it accumulates in the human brain at a concentration 10 to 20 times greater than in the circulation after 1 hour (Peter et al., 2000). Studies have found that MB is able to cross the blood-brain barrier when administered intraperitoneally to rats (O'Leary et al., 1968). It is commonly administered as an antidote for drug-induced, and some forms of idiopathic, methemoglobinemia; a condition in which (usually due to metabolic poisoning) the body is unable to convert methemoglobin to hemoglobin to allow oxygen transport (Bodansky & Gutmann, 1947; Bradberry, 2003; Clifton & Leiken, 2003; Etteldorf, 1951). MB is able to reverse this process, and has been safely used in humans for over a century. It is also used for treatment of drug-related encephalopathy in cancer chemotherapy with ifosfamide (Kupfer et al., 1996). Methylene blue is classified as a non-neuroleptic phenothiazine and has been implicated for treatment of malaria, schizophrenia, bipolar disorder, and depression (Atamna, 1996; Deutsch et al., 1997; Klamer et al., 2004; Naylor et al., 1986, 1987).

1.2 METHYLENE BLUE IMPROVES MEMORY RETENTION

The memory retention enhancing effects of MB were first reported by Martinez, Jr. and colleagues (1978), who discovered that low dose post-training administration of MB improved memory retention in an inhibitory avoidance task. Riha et al. (2005) found that MB increased memory retention of an object recognition task and aided in between-days habituation to a familiar environment. It was also reported that MB improved spatial memory retention in rats in the hole board maze, and that MB-treated subjects

performed better than controls in a reversed baiting pattern (Callaway et al., 2004). Recently, Gonzalez-Lima & Bruchey (2004) reported that memory retention of extinction of Pavlovian fear conditioning could be enhanced with post-extinction administration of MB. The authors proposed that MB could be administered as a therapeutic agent in conjunction with behavioral therapy to facilitate retention of extinction of conditioned fears or other traumatic memories.

Martinez, Jr. et al. (1978) found that low dose (1 mg/kg) MB produced retrograde enhancement of memory retention in an inhibitory avoidance response task. Mice administered 0.05 mg/kg of MB immediately following training exhibited slight retrograde enhancement of learning. A second experiment was conducted in rats administered 0.01, 0.1, or 1 mg/kg MB or saline I.P. (intraperitoneal injection) immediately following training. A retention test conducted 24 hours after training showed that 1 mg/kg MB produced significant retrograde enhancement of learning. Rats receiving a second injection of MB 15 minutes prior to the retention test performed significantly better than rats receiving only one injection. However, this study did not evaluate other behavioral effects of methylene blue that could have contributed to their findings.

Recently we began examining the memory retention effects of MB in normal rats in a spatial memory food search task using the hole board maze. The hole board maze is an appetitive memory task, which requires animals to rely on spatial cues in order to solve it. Callaway et al. (2004) found that low dose MB enhanced spatial memory retention in rats. Subjects were treated with 1 mg/kg MB daily following 5 training

sessions in the baited hole board maze. This was followed by an unbaited probe trial on the sixth day, and then 15 days later training sessions were conducted identical to the first sessions with a reversed baiting pattern, also followed by an unbaited probe trial on the final day of the study. Post-training MB administration enhanced memory retention in unbaited probe trials and MB-treated subjects performed significantly better than controls in the reversed baiting pattern.

Interestingly, Gonzalez-Lima & Bruchey (2004) found that memory retention of extinction of Pavlovian fear conditioning could be improved with the administration of 4 mg/kg MB. Saline or MB was administered daily for 5 days following extinction training of tone-foot shock conditioning. Total post-extinction freezing scores were lower in subjects receiving MB than saline, with saline administered rats freezing a total of twice as much as those treated with MB. Methylene blue subjects also had a longer lasting effect of extinction, freezing five times less than subjects receiving saline on the last two days of the 5 post-extinction probe trials. The authors proposed that MB may be a promising treatment in addition to exposure therapy in patients being treated for phobias.

1.3 THERE ARE NO SIDE EFFECTS OF LOW DOSE METHYLENE BLUE ON BEHAVIOR

MB has been used therapeutically in humans for decades with very little side effects. In high doses however, MB has opposite effects and can convert hemoglobin to methemoglobin resulting in a dangerous situation (Harvey, 1975; Martinez, Jr., et al., 1978). Therefore it was important to determine effective doses of MB on memory

retention improvement with little to no side effects that could alter behavior or the health of the subjects. Eroglu & Caglayan (1997) reported that intermediate doses of MB (7.5 mg/kg to 30 mg/kg) increased entries to open arms in the elevated plus maze in mice and doses of 15 and 30 mg/kg significantly decreased immobility time in the forced swim test. It is important that MB is enhancing memory retention without any anxiolytic or antidepressant effects that may influence behavioral measures. Therefore, a study conducted by Riha et al. (2005) examined the side effects of several doses (1, 4, 10, 50, and 100 mg/kg) of MB on locomotor, feeding, reward, and fear-related behaviors in rats. They found no differences between MB-treated and saline administered subjects at the 1, 4, and 10 mg/kg doses. This study also examined the effects of MB on memory retention of an object recognition task and the open field habituation test. Methylene blue, at the 4 mg/kg dose, increased memory retention in the object recognition task and also aided in between-days habituation to a familiar environment. The highest doses of MB (50 and 100 mg/kg) caused illness and decreased activity in a running wheel. A feeding test was conducted to determine if MB affected reward value or motivation, and no group differences were found.

Gonzalez-Lima and Bruchey (2004) examined motor activity in the open field in rats receiving either 4 mg/kg saline or MB (1 injection or 5 daily repeated injections) and found no group differences in locomotor activity measures or center avoidance, a measure of fearfulness. Therefore, studies have shown that the memory retention enhancing effects of low dose MB cannot be attributed to alterations in locomotor activity, motivation, reward value, or fearfulness. Despite these reports, in order to

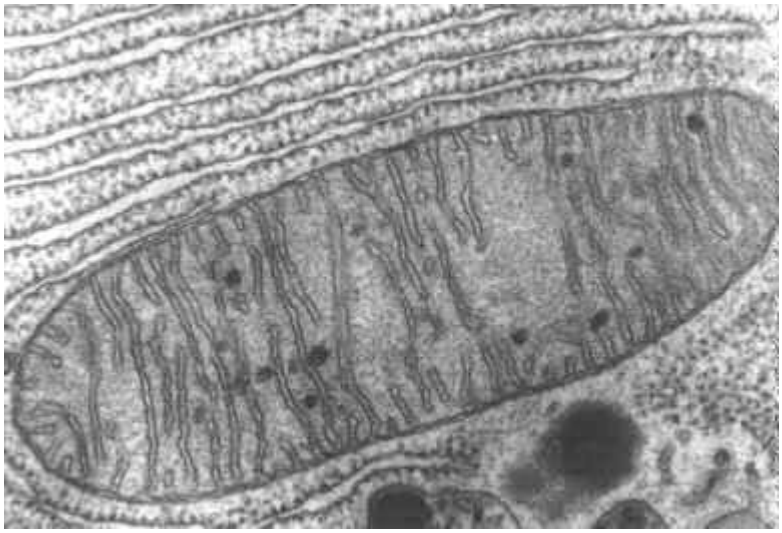
control for any side effects or state-dependent learning effects that may occur by giving pre-training MB, it has generally been administered following training in behavioral studies. Also, because MB enhances memory retention of the events preceding its administration, the time of injection follows the target memory task.

Previous studies demonstrated memory retention improvement with MB in the hole board maze and following inhibitory avoidance learning at the 1 mg/kg dose (Callaway et al, 2002; Callaway et al., 2004; Martinez et al., 1978). This was the dose chosen for administration in the experiments in chapters 2 and 3, which examined the effects of MB on discrimination learning and acquisition of Pavlovian fear conditioning. Later work suggested that the 4 mg/kg dose may be more effective for memory retention enhancement than the 1 mg/kg dose of MB, when it improved memory retention in an object recognition task and aided in between-days habituation to a familiar environment and the 1 mg/kg dose did not (Riha et al., 2005). Gonzalez-Lima & Bruchey (2004) also found that the 4 mg/kg dose improved memory retention of extinction of Pavlovian fear conditioning, and since we were also using this paradigm for the experiment in chapter 4 examining fear conditioning in congenitally helpless rats, the 4 mg/kg dose was chosen for this study.

1.4 METHYLENE BLUE WORKS AS A METABOLIC ENHANCER BY INCREASING CYTOCHROME OXIDASE ACTIVITY AND OXYGEN CONSUMPTION IN THE BRAIN

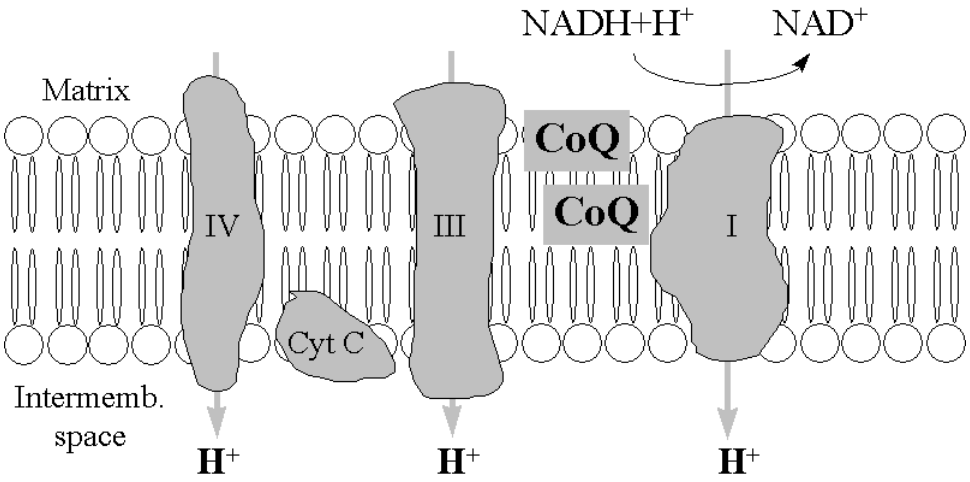
We hypothesize that MB works as a metabolic enhancer by increasing cytochrome oxidase (CO) activity (Callaway et al., 2002; Gonzalez-Lima & Bruchey, 2004; Riha et al., 2005). Cytochrome oxidase, also known as Complex IV, is the terminal enzyme of the electron transport chain. The electron transport chain is a series of four protein complexes, located in the mitochondrial cristae (the inner mitochondrial membrane) of mitochondria, the powerhouse of the neuron (Figure 1B), that drives mitochondrial respiration. Mitochondria are the primary source of ATP (adenosine triphosphate), meeting the energy demands of the cell.

Figure 1B: Electron micrograph of the mitochondria. The outer mitochondrial membrane encloses the organelle. The electron transport chain is located in the inner mitochondrial membrane, which has folds projecting inward. (From Fawcett, 1994)



NADH and FADH (products of food stuffs that we eat) donate electrons to the electron transport chain in Complexes I and II respectively. Electrons are shuttled from one complex to the next, finally being carried from Complex III to Complex IV (CO). CO then passes its electrons to molecular oxygen, the final electron acceptor (Figure 1C). An estimated 95% of the oxygen used by the neuron reacts in this single process (Wikstrom, 1981). Concurrently, protons are pumped into the inner mitochondrial membrane creating an electrochemical gradient that drives ATP synthesis. In this process, hydrogens from within the mitochondrial membrane are pumped back into the matrix through ATPase and it is this reaction that converts ADP (adenosine diphosphate) to ATP (adenosine triphosphate) and powers the cell. Because CO is the terminal enzyme of the electron transport chain, it is tightly coupled to neuronal metabolism and ATP production (Wong-Riley, 1989). It is a sensitive metabolic marker for neuronal functional activity, particularly in the dendrites, where mitochondria are heavily concentrated. CO increases are consistently correlated with increased energy demand (Sakata et al., 2005; Gonzalez-Lima, 1998). Interestingly, CO is bigenomically encoded, requiring both nuclear and mitochondrial gene expression to form all of its 13 subunits.

Figure 1C: The electron transport chain. Electrons are shuttled from one complex to the next, with the final transfer from complex IV (CO) to molecular oxygen. (From http://binfo.ym.edu.tw/bch/junk/e-tp.htm)



Methylene blue is an interesting compound for memory retention because of its unique mechanism of action**.** Electrons can be donated from reduced MB to enter the electron transport chain between Complexes II and III (Visarius et al., 1997), resulting in enzyme induction of CO (Callaway et al., 2004; Gonzalez-Lima & Bruchey, 2004) and elevated cellular oxygen consumption (Riha et al., 2005; Lindahl & Oberg, 1961; Visarius et al., 1997). Therefore, by increasing CO activity, MB can enhance the amount of ATP available in a neuron in order to improve memory retention and recall in certain cognitive tasks.

Using biochemical spectrophotometric analysis, Callaway et al. (2004) found that a low dose of MB enhanced cytochrome *c* oxidation *in vitro* in brain homogenates and after *in vivo* administration to rats, and that corresponding low dose MB enhanced spatial memory retention. The study found that there was a 25% increase in the rate of cytochrome *c* oxidation when 0.5 μM MB was added to brain homogenate. This 0.5 μM concentration of MB has also been found to stimulate respiration in rat liver mitochondria (Visarius et al., 1997). Following *in vivo* administration of 1 mg/kg MB administered I.P. after which subjects were sacrificed 1, 2, or 24 hours later, spectrophotometry was used to determine cytochrome *c* oxidase activity, and it was found that there was a 30% increase in cytochrome *c* oxidation 24 hours post-injection.

In another study, Callaway et al. (2002) found that 1 mg/kg MB administered I.P. 15 minutes after daily training trials completely restored memory retention impaired by sodium azide in the hole board, presumably by increasing mitochondrial oxygen consumption. Sodium azide has been shown to decrease cytochrome *c* oxidase activity

when chronically administered to rats (Cada et al., 1995; Berndt et al., 2001). After recuperation from surgery of subcutaneous implantation of an osmotic pump containing sodium azide, subjects were trained in the hole board maze for 5 days followed by a probe trial, which was followed by another 5 days of training and injections and a second probe trial. MB enhanced memory retention in probe trials (reference memory) without improving memory in baited training trials (working memory), and subjects receiving MB plus sodium azide performed as well as subjects administered saline in probe trials. The authors hypothesized that MB was acting as an electron shuttle to oxygen, or a bypass from the inhibited CO to oxygen, in order to compensate for the impaired mitochondrial respiration.

Recently Gonzalez-Lima & Bruchey (2004) found that memory retention of extinction in Pavlovian fear conditioning could be improved with the administration of 4 mg/kg MB following extinction. Spectrophotometric analysis of the rate of cytochrome *c* oxidation in brain homogenates of these subjects showed a 38% increase over controls administered saline. In order to determine if MB would have greater metabolic enhancing effects in regions implicated in memory of extinction, CO histochemistry was also performed. It was found that the regions most active in a mouse brain mapping study using CO histochemistry following extinction of conditioned freezing (Barrett et al., 2003) also demonstrated greater metabolic activity in subjects receiving MB in this study, namely prefrontal cortical regions. Also, MB-treated subjects had general relative metabolic increases in all brain regions examined.

Since CO activity and cellular oxygen consumption are tightly coupled, Riha et al. (2005) used an oxygen probe to examine oxygen consumption *in vitro* by adding reduced MB (favored for entry into the cell) to brain homogenates. Measurements were taken over a 5 minute period. It was found that 5 μM and 10 μM concentrations of MB increased brain oxygen utilization. The same paper also used an oxygen probe to examine oxygen consumption *in vivo* in rats sacrificed 1, 2, and 24 hours following I.P. injection of either 1 mg/kg MB or saline. It was found that MB-treated subjects whose brains were harvested 24 hours post-injection had a 60% difference (increase) from saline administered rats in oxygen consumption. Together these studies suggest that MB works as a metabolic enhancer, improving memory retention by enhancing CO activity and increasing oxygen consumption in the brain.

Another proposed mechanism of action of MB that could contribute to its positive effects on memory retention involves its antioxidant properties. MB inhibits superoxide by accepting electrons from tissue oxidases (Kelner et al., 1988; Salaris et al., 1991). It is also an inhibitor of nitric oxide synthase (Mayer et al., 1993) which can form free radicals after reacting with superoxide. Studies from our laboratory examined the effects of MB as an antioxidant to protect against rotenone-induced neurodegeneration in an animal model of optic neuropathy (Zhang, 2005). Neurodegeneration in the retinal ganglion cell layer 24 hours after rotenone injection was completely prevented by the injection of methylene blue plus rotenone. The author proposed that MB administration enhanced oxygen consumption and acted as an antioxidant in this study, preventing oxidative damage and cell death.

Since MB has a half-life of 5-6.5 hours (Peter et al., 2000), it is unlikely that an increase in memory retention observed more than 24 hours after the last injection of MB reflects a continued direct action of the drug. Rather, it is probably due to secondary brain metabolic effects occurring at a critical time in memory consolidation. Methylene blue increases CO enzymatic activity in a use-dependent manner, with brain regions with the highest metabolic demand during memory consolidation in a particular task showing the largest increases in CO activity (Gonzalez-Lima & Bruchey, 2004). Methylene blue administration leads to enzyme induction after a few hours, so there is enhanced metabolic capacity in the brain on the following day. This use-dependent regional enzyme induction persists even though MB is no longer active, which is why we don't see effects of MB on working memory (the memory is constantly being updated in working memory). We hypothesize that through metabolic enhancement, MB provides more fuel to task-related brain regions making them more functional where CO has the highest demand. Thus, MB does not show memory enhancing effects instantly, and MB-treated subjects tend to demonstrate day to day improvement in performance. Based on the current and preceding studies demonstrating the powerful memory enhancing effects of MB, it appears to be a useful compound not only in models of metabolic impairment, but also in normal subjects. It is a very promising drug for memory improvement, and if administered correctly and in low doses, could provide a potent resource for those looking for memory enhancing compounds with little to no side effects.

1.5 EXPERIMENTAL GOALS

The primary goal of the following experiments was to further investigate the memory retention effects of methylene blue in both appetitive and aversive memory tasks**.** First of all, we were interested in expanding on the effects of MB on memory retention in the hole board maze, an appetitive spatial memory task, this time using a discrimination learning paradigm. Second of all, knowing that MB enhanced memory retention of extinction in Pavlovian tone-foot shock fear conditioning (Gonzalez-Lima & Bruchey, 2004), we were interested in determining if MB could also enhance memory retention of acquisition in the same paradigm. If so, we wanted to examine the long-term effects of MB on memory retention of the aversive conditioned stimulus. Last of all, we wanted to determine any therapeutic effects MB may have during extinction of conditioned fear in congenitally helpless rats, an animal model of post-traumatic stress disorder (PTSD) and depression, hypothesized to show extinction deficits. We expected MB to improve memory retention in these tasks based on the general hypothesis that MB increases cytochrome oxidase activity, the terminal enzyme of the electron transport chain that utilizes oxygen to produce ATP, and that this metabolic action can be used to improve memory retention.

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CHAPTER 2: THE METABOLIC ENHANCER METHYLENE BLUE IMPROVES MEMORY RETENTION OF DISCRIMINATION LEARNING IN RATS

2.1 ABSTRACT

An experiment was conducted to investigate the effects of administration of low dose methylene blue (MB), a metabolic enhancer, on memory retention of discrimination learning of rewarded versus non-rewarded trials in an appetitive spatial memory task utilizing the hole board maze. Following habituation to the maze, 6 days of training ensued, consisting of 5 daily trials with maze baited in a fixed pattern. On the last day of the training phase, subjects were matched to groups based on performance, and injected with either MB (1 mg/kg) or saline (0.9% NaCl) I.P. post-training. This was followed by 6 days of discrimination training, consisting of one non-rewarded (unbaited) trial followed by four rewarded (baited) training trials, and daily injections. Subjects treated with MB reliably discriminated between rewarded and non-rewarded trials as indicated by a greater number of correct responses on rewarded trials than non-rewarded trials. No such discrimination effects were observed in the control group. Post-training MB may improve memory retention by enhancing mitochondrial respiration through an induction in the enzyme cytochrome oxidase, leading to increased enzymatic activity in brain regions requiring more ATP in discrimination learning in the hole board.

2.2 INTRODUCTION

Methylene blue (MB) is a metabolic enhancer that has been demonstrated to improve memory retention in a variety of tasks when administered post-training and in low doses (Martinez, Jr., et al., 1978; Callaway et al., 2002, 2004; Gonzalez-Lima & Bruchey, 2004; Riha et al., 2005). Previous studies have shown that post-training MB improves memory retention in the hole board maze, an appetitive spatial memory task. Callaway et al. (2004) found that daily post-training injections of 1 mg/kg MB enhanced spatial memory retention in rats in the hole board maze. Methylene blue-treated subjects showed enhanced retention of reference memory, and also performed better than control subjects following training in a reversed baiting pattern. In a second study by Callaway et al. (2002), also examining memory in the hole board maze, it was found that daily post-training administration of 1 mg/kg MB completely restored memory retention impaired by the drug sodium azide. In this study MB enhanced retention of reference memory, without improving retention of working memory.

No prior experiments had been conducted to determine the effects of MB administration on discrimination learning. We wanted to further examine the effects of post-training MB treatment on memory retention in a discrimination learning procedure in the hole board maze. Previous experiments in our laboratory examined the behavior of rats given regular alternation of rewarded and non-rewarded trials in order to determine reward related expectancies in appetitive memory tasks (Nair & Gonzalez-Lima, 1999; Lilliquist et al., 1999). Given the previous success with the 1 mg/kg dose of MB for memory retention improvement in the hole board maze, we chose to utilize this dose for

our study as well. Methylene blue has no side effects on behavior in rats at the 1 mg/kg dose chosen for this study (Riha et al., 2005).

2.3 HYPOTHESIS

It was hypothesized that subjects receiving MB would demonstrate improved discrimination between rewarded (baited) and non-rewarded (unbaited) trials, with more accuracy in rewarded than non-rewarded trials, as measured by reference memory scores. This hypothesis was based on previous studies demonstrating improvement of reference memory retention in the hole board maze following MB administration (Callaway et al., 2002, 2004), and previous work conducted in our laboratory showing subjects' ability to discriminate between rewarded and non-rewarded trials in appetitive memory tasks (Nair & Gonzalez-Lima, 1999; Lilliquist et al., 1999).

2.4 MATERIALS AND METHODS

2.4.1 Subjects

Subjects were 20 male Long-Evans hooded rats (Harlan, Indianapolis, IN) weighing between 148 and 165 g on the first day of the experiment. They were singly-housed under standard laboratory conditions with a 12 hr light/dark cycle. Rats were handled daily for 7 days prior to and throughout the experiment to habituate them to the experimenters. For motivational purposes in the food search task, subjects were food-restricted by administering 9-11 g of rat chow per day. Four subjects that did not demonstrate evidence of learning during the first 4 days of training in the hole board

were excluded from the study prior to the discrimination training phase of the experiment, leaving an N=16 for final data analysis. Subjects were housed and handled according to IACUC protocol.

2.4.2 Apparatus

Hole board floor inserts (MED Associates Inc., St. Albans, VT) were placed in 2 automated MED Associates ENV-515 Test Environments, each measuring 31 x 45 x 45 cm with 16 beam infrared arrays to scan for activity counts. Test chambers were connected to a single computer (Dell Optiplex). The hole board task floor inserts had 16 equidistant holes, with 4 rows of 4 holes, each measuring 1.25" in diameter. Inserts were placed on 3" centers with an underlying food tray so that reinforcers could be placed in the desired holes. Below each hole, pellets were placed under a screen in order to control for olfactory cues coming from baited holes. Infrared beams detected entry to the task floor holes, or nose pokes. Software (MED-PC for Activity Boxes) recorded holes nose-poked, novel or repeat entries to holes, working memory scores (re-visits to previously baited holes), and reference memory scores (number of nose pokes to baited holes divided by the total number of nose pokes). Hole boards were placed in a dimly lit (100 lumens) sound-attenuated behavioral testing room.

2.4.3 Behavioral Training

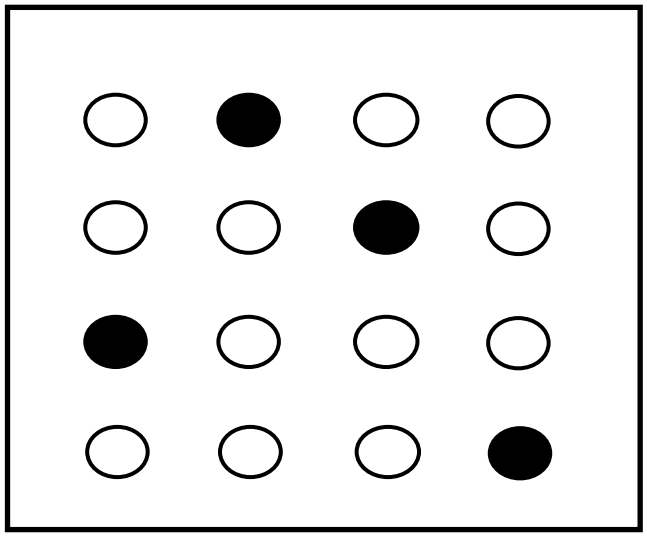
2.4.3.1 Habituation Phase

Prior to habituation trials in the hole board maze, rats were habituated to the novel bait used in the task. One Noyes 45 mg sucrose pellet was put in each subjects' home cage following daily handling sessions. Subjects were habituated to the hole board for 2 days with all 16 holes baited. During habituation, one pellet was placed on the hole board surface and all holes were baited with one sucrose pellet. Subjects were run in pairs with a black partition dividing the area between test environments. Trials lasted 15 minutes or until all 16 holes were nose-poked.

2.4.3.2 Training Phase (Days 1-6)

Habituation was followed by 6 days of training with 4 holes baited in a fixed pattern (Figure 2A).

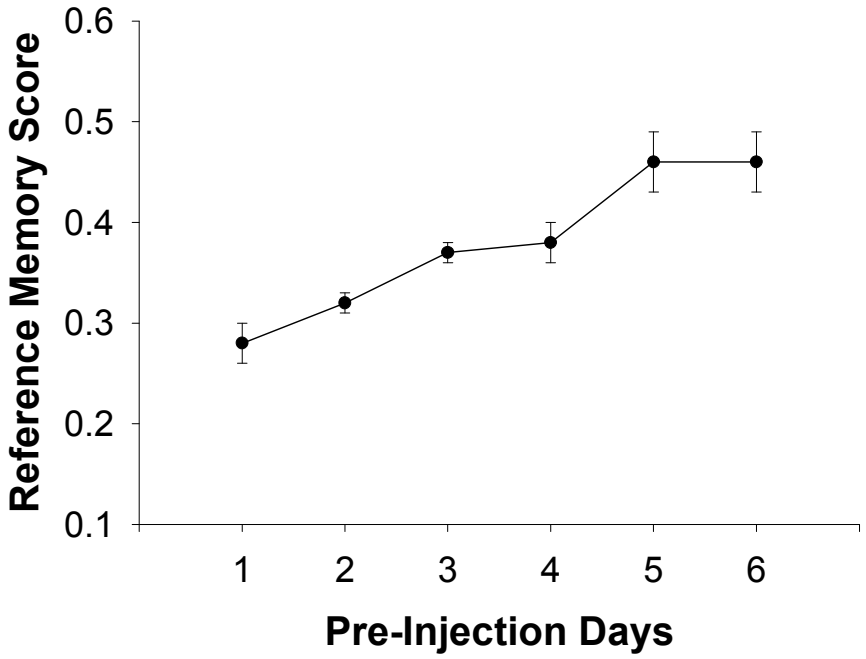
Figure 2A: The baiting pattern in hole board utilized for all trials. Dark circles are baited holes, and open circles are unbaited holes.



Each subject had 5 consecutive daily trials lasting 5 min each or until the fourth baited hole was nose-poked, with an average inter-trial interval of 2 minutes. In between trials, rats were put into a dark box and experimenters wiped down the test chambers with a mild detergent, rotated the hole board insert, and rebaited the maze in order to control for any olfactory cues that could affect performance in subsequent trials. Bait left over from previous trials was discarded and replaced. The measurement of interest in this study was the computer recorded reference memory score computed by adding the number of nose pokes to baited holes and dividing by the total number of nose pokes.

Experimenters waited until subjects performed relatively well in solving the maze but could still demonstrate improvement, in order to control for ceiling effects due to overtraining that could mask any drug effects (Figure 2B).

Figure 2B: Acquisition curve for all subjects showing means ± standard errors for reference memory scores (number of nose pokes to baited holes/total number of nose pokes) for the training phase of the experiment, prior to injections, in the hole board. During this phase there were 5 daily trials with 4 of the 16 holes baited in a fixed baiting pattern (Figure 2A).



On the 5th day of training, experimenters matched subjects into two groups according to training performance and on the 6th day subjects were injected with either MB (1 mg/kg) or saline (0.9% NaCl) I.P. immediately post-training. Blue food coloring was added to the saline so experimenters would be blind to group assignment.

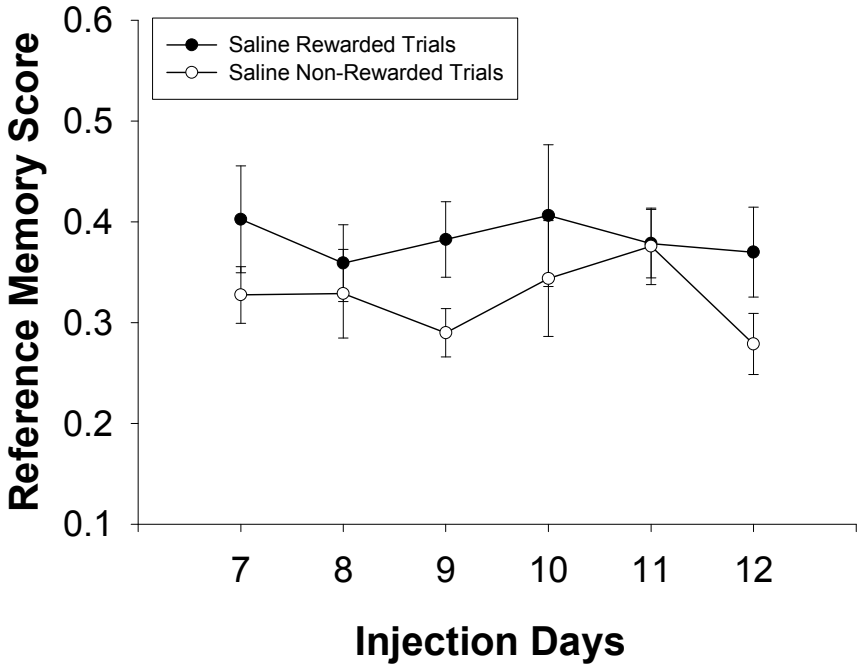
2.4.3.3 Discrimination Training Phase (Days 7-12)

The discrimination training phase of the experiment consisted of a memory-based daily alternation between rewarded and non-rewarded trials (Nair & Gonzalez-Lima, 1999). Beginning on the 7th day, discrimination training sessions began with one non-rewarded/unbaited trial lasting 5 min or until the fourth hole normally baited was nose-poked. This was followed by four rewarded/baited training trials (baited in the same pattern as on Days 1-6). The first trial of each day was the non-rewarded trial, so that performance on this first trial depended on the memory-based discrimination of the daily alternation schedule of reward-nonreward. Having more rewarded trials (4) than non-rewarded (1) per day helped prevent the rapid extinction of reward-seeking performance produced by presentation of the non-rewarded trials (Lilliquist et al., 1999). Discrimination training sessions lasted for 6 days. Subjects were injected with either MB (1 mg/kg) or saline (0.9% NaCl) I.P. immediately post-training following each daily discrimination training session. Methylene blue and control subjects were run in pairs to rule out any confounds that time of day may have caused on performance.

2.5 RESULTS

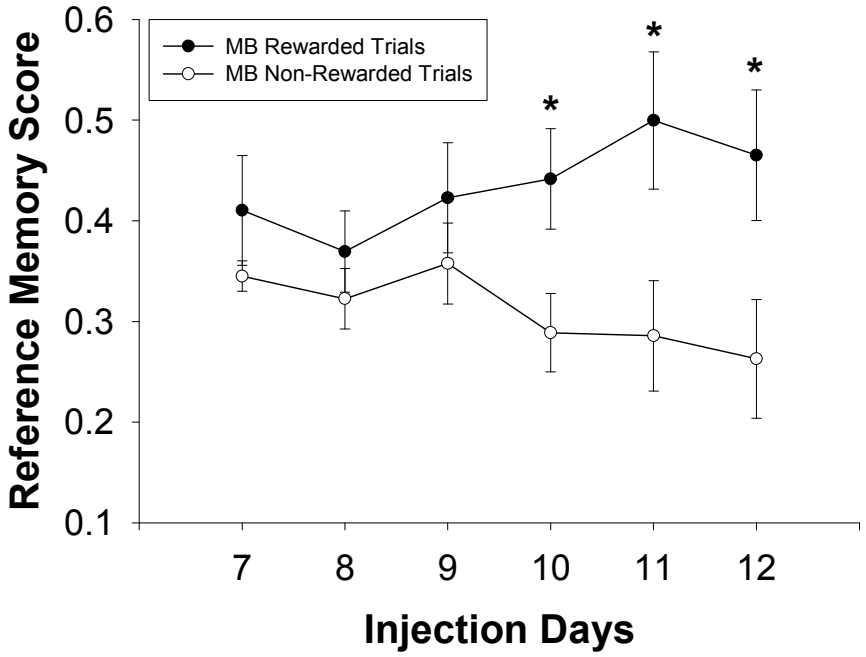
A within-subjects repeated measures ANOVA found a significant interaction between rewarded and non-rewarded trials by day in the MB group, $F(1,5)= 2.680$, $p = 0.028$, but not in the saline group. Performance in rewarded versus non-rewarded trials was not significantly different in subjects administered saline. Although they showed a trend for better performance in rewarded trials, they did not reliably discriminate between rewarded and non-rewarded trials in this task (Figure 2C).

Figure 2C: Means ± standard errors for reference memory scores (number of nose pokes to baited holes/total number of nose pokes) in rewarded versus non-rewarded trials in saline administered subjects for the discrimination training phase of the experiment. Saline-administered rats did not demonstrate a strong discrimination between rewarded and non-rewarded trials.



Independent t-tests for sessions on Days 10, 11, and 12 compared rewarded to non-rewarded reference memory scores in MB-treated subjects and the sharper Bonferonni correction was used (Hochberg, 1988). On Day 10, there was a significant difference between baited and unbaited trials in the MB group at t (14)= 3.038, corrected p=0.018. On Day 11, there was a significant difference between baited and unbaited trials in the MB group at t (14)= 3.080, corrected p=0.024. And on Day 12, there was a significant difference between baited and unbaited trials in the MB group at t (14)= 2.585, corrected p=0.022 (Figure 2D).

Figure 2D: Means ± standard errors for reference memory scores (number of nose pokes to baited holes/total number of nose pokes) in rewarded versus non-rewarded trials in methylene blue-treated subjects for the discrimination training phase of the experiment. Methylene blue-administered rats reliably discriminated between rewarded and non-rewarded trials on days 10-12 of the experiment. *$p < .05$



There were no significant differences or trends showing improvement in MB-treated and control subjects when comparing reference memory scores within each of the 5 daily trials, demonstrating that the memory improvement observed was in overall reference, not working memory. Saline and MB-treated groups did not statistically differ significantly in regards to reference memory scores in rewarded or non-rewarded trials. There were also no significant differences found in total number of nose pokes between MB-treated and control groups in rewarded or non-rewarded trials.

2.6 DISCUSSION

Methylene blue enhanced memory retention of discrimination learning of rewarded versus non-rewarded trials in a food search task in rats. Subjects treated with MB reliably discriminated between rewarded and non-rewarded trials and the control group did not. This was indicated by a greater number of correct responses on rewarded trials than non-rewarded trials. These results supported our original hypothesis. These effects were seen on the last three days of the study which is consistent with other behavioral data conducted in our laboratory showing that the 1 mg/kg dose of MB may require more than one memory consolidation period with MB on board to see an effect on memory retention (Gonzalez-Lima & Bruchey, 2004).

The results of this study also confirm Callaway et al.'s (2002, 2004) findings that MB improves reference memory, but not working memory. This could be the result of enzyme induction of CO following the period of memory consolidation of the discrimination learning, which takes longer than the half an hour (or less) it takes to complete the training trials. Saline and MB-treated groups did not differ significantly in regards to reference memory scores in the discrimination learning version of the hole board maze in rewarded trails, which also follows with the results of Callaway et al. (2002, 2004), who only found differences in reference memory in unbaited probe trials. Since no significant differences were found in the total number of nose pokes between MB-treated and saline administered groups in either rewarded or non-rewarded trials, the enhanced effects of MB on discrimination learning observed in this study were due to greater accuracy in overall reference memory in the MB-treated group, and were not a

confound of effects the drug may have had on motivation during this task. Overall, this study demonstrates that the metabolic enhancer MB has positive effects on discrimination learning, and that MB is a promising drug for the enhancement of memory.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health grant R01 NS37755 to Francisco Gonzalez-Lima and a Society for Neuroscience Minority Fellowship to Kathryn Wrubel. I would like to thank Monica Maldonado and David McCollum for their assistance with data collection and analysis.

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CHAPTER 3: THE LONG-TERM EFFECTS OF METHYLENE BLUE ON MEMORY RETENTION OF ACQUISITION AND EXTINCTION IN A PAVLOVIAN FEAR CONDITIONING PARADIGM IN RATS

3.1 ABSTRACT

Methylene blue (MB), a metabolic enhancer, has been shown to improve memory retention of extinction of Pavlovian fear conditioning in the rat. An experiment was designed to investigate the effects of MB administration on memory retention of an aversive memory task following post-acquisition administration of MB in Pavlovian fear tone-foot shock conditioning. The effects of MB on conditioned freezing to a tone CS were observed for 2 months. Subjects were injected with either 1 mg/kg MB or saline (0.9% NaCl) I.P immediately following 4 daily acquisition sessions consisting of 1 tone-shock pairing per day. Post-acquisition probe trials were conducted on Days 2, 12, and 22 post-acquisition. An out-of-context probe trial was conducted on Day 23 post-acquisition, in order to examine the influence of context on conditioned freezing scores. The long-term memory retention of the CS-US association was also examined following an extinction session which was conducted 22 days after the out-of-context probe trial. This was followed by post-extinction probe trials on Days 2 and 23 post-extinction. Subjects in the MB group froze significantly more than controls to the tone CS on Day 12 post-acquisition. They also demonstrated more resistance to extinction than controls in post-extinction probe trials, even though MB administration had been discontinued for 45 days prior to the extinction session. Post-training MB improved memory consolidation

of acquisition of Pavlovian fear conditioning, demonstrating that it not only improves memory of extinction in this paradigm, and that it can enhance memory of aversive, as well as appetitive tasks.

3.2 INTRODUCTION

Methylene blue (MB) is a redox dye that has been reported to enhance memory retention of spatial memory (Callaway et al., 2002, 2004), habituation (Riha et al., 2005), and inhibitory avoidance training (Martinez, Jr., et al., 1978). Methylene blue is hypothesized to work as a metabolic enhancer through enzyme induction of cytochrome oxidase, which is tightly coupled to metabolism and oxygen consumption in the neuron (Wong-Riley, 1989). Gonzalez-Lima & Bruchey (2004) recently reported that memory retention of extinction of Pavlovian fear tone-foot shock conditioning could be enhanced with post-extinction administration of MB. Total post-extinction freezing scores were lower in subjects receiving MB, with control subjects freezing a total of twice as much as those treated with MB. Methylene blue-treated subjects froze significantly less than subjects receiving saline on the last two days of the post-extinction probe trials, demonstrating that they also had a longer lasting effect of extinction, and that they did not show the spontaneous recovery observed in control subjects.

No experiments had been conducted to determine the effects of MB on memory retention of acquisition of Pavlovian fear conditioning, including long-term effects, if any, MB has on behavior. The present experiment was conducted in order to further examine MB's role in memory retention, this time utilizing it for enhanced memory consolidation of an aversive memory task. It was of interest to determine if MB could improve memory of the tone-foot shock pairings if administered post-acquisition. We also wanted to determine how long-lasting these effects on memory retention would be, and if MB administration following acquisition sessions could retard extinction

conducted weeks later. The duration of action of the memory retention effects of low dose MB in rats was examined for 2 months following post-acquisition treatment. Conditioned suppression of drinking was also monitored in the study. In order to determine if there was a drug effect (by controlling for ceiling effects in freezing or floor effects in conditioned suppression ratios) the standard lab protocol of two days of acquisition with 4 tone-shock pairings per day was not utilized. Instead subjects were administered only 1 tone-shock pairing per day for four consecutive days. This study was conducted to further examine the effects of MB in a fear conditioning paradigm, and to see its effects on retention of aversive memory tasks.

3.3 HYPOTHESIS

We hypothesized that MB-treated subjects would show greater fear responses in probe trials following acquisition, as measured by both freezing to the CS tone and conditioned suppression of drinking. It was predicted that MB would improve retention of the memory of acquisition since it was present during memory consolidation of the tone-shock pairings. We also hypothesized that subjects would be less likely to extinguish fear responses than saline administered controls following extinction. Since MB has been shown to improve memory retention of various appetitive memory tasks following post-session administration, it seemed likely that it could improve memory retention of aversive tasks as well.

3.4 MATERIALS AND METHODS

3.4.1 Subjects

Subjects were 16 male Long-Evans hooded rats (Harlan, Indianapolis, IN) weighing between 156 and 173 g on the first day of habituation. For motivational purposes in the conditioned suppression of drinking paradigm, subjects were water-deprived prior to any behavioral testing day. Two hours after each daily training session, subjects were given free access to normal drinking water for 45 min in the home cage. Subjects were individually housed under standard laboratory conditions with a 12 hr light/dark cycle. They had free access to food in the home cage at all times and were housed and handled according to IACUC protocol. Subjects were handled for 7 days prior to and throughout the experiment to habituate them to the experimenters.

3.4.2 Apparatus

The apparati were two modular test chambers measuring 22 x 25 x 32 cm (MED Associates Inc., Georgia, VT) placed in sound-attenuating boxes and illuminated by a red light. Tones were produced by two Wavetek Sweep/Modulation generators (Wavetek, San Diego, CA) and presented through speakers mounted on the chambers. The conditioned stimulus (CS) was a frequency-modulated tone of 1-2 kHz, with 2 sweeps per second, 15 sec in duration, with an intensity of 65 dB. The unconditioned stimulus (US) was a 0.75-sec 0.4 mA foot shock delivered through the grid floors of the chambers (consisting of nineteen 4.8 mm rods placed 1.6 cm apart). Rods were individually wired to a Lafayette Instruments Master Shocker (Lafeyette Instrument Company, Lafayette,

IN). A water bottle hooked on the outside of each of the chambers and a lickometer recorded infrared beam breaks to the drinking spout. Lights were kept on in the behavioral testing room during the habituation, acquisition, and extinction phases of the experiment as a contextual variable. Test chambers were wiped down with a mild detergent and gloves were changed between subjects to control for any olfactory cues left behind by other subjects that could interfere with learning the task.

3.4.3 Freezing Scores and Drinking Measurements

Freezing behavior was scored during the 15 sec pre-CS period, and during the 15 sec CS tone by experimenters trained to have high inter-rater reliability. Freezing was defined as the subject having all four paws on the floor, with minimal head movements, and shallow, rapid breathing. Freezing scores were recorded in five 3-second bins with 1 point assigned if a subject froze for the majority of a bin (minimum score= 0, maximum score= 5). Thus a subject who froze for the majority of each of the five 3-second bins received a score of 5.

Computer programs were created using MED Associates MED-PC software to generate the tones and shocks. The computer also recorded latency to initiate a drink, pre-CS drink time, CS drink time, total drink time, latency to resume drinking after CS onset, and minute by minute drinking bouts during the 15 min post-session drinking period in the chamber. For each session, a computer program calculated a drinking suppression ratio (Conditioned Suppression Ratio = $CS_{(drink\ time)}$ /(Pre-$CS_{(drink\ time)}$ + $CS_{(drink\ time)}$). Suppression values ranged from 0-0.5, with 0.5 indicating no change in

drinking behavior caused by the CS tone presentation and 0 signifying complete conditioned suppression.

3.4.4 Behavioral Training

Table 3.1 shows the experimental design.

Table 3.1: Experimental design.

Procedure	Info	MB or Saline	Days Since Previous Test
Habituation 1-4 Days	15 min		
Acquisition 1-4 Days	1 Tone - Shock	Inject 1 mg/kg	
Day 1 Probe	1 Tone		2 Days
Day 2 Probe	1 Tone		10 Days
Day 3 Probe	1 Tone		10 Days
Out of Context Probe 4	1 Tone		1 Day
Extinction #1- Scored 1st and 60th Tones	60 Tones in 60 min		22 Days
Day 5 Probe	1 Tone		2 Days
Day 6 Probe	1 Tone		23 Days

3.4.4.1 Habituation Phase

Subjects were habituated to the operant chambers for 15 min per day for 4 days. During this time the water-deprived subjects had free access to a 10% sucrose solution delivered to the chamber through a drinking tube. The total amount of time spent drinking during the habituation sessions was averaged across all four days for each subject and used to match them for group assignment.

3.4.4.2 Acquisition Phase, Post-Acquisition Probe Trials, and Out-of-Context Probe Trials

Habituation was followed by 4 days of acquisition consisting of 1 tone-shock pairing per day. A 15-sec pre-CS period was initiated after subjects drank the sucrose solution continuously for a 5-sec latency period criterion and was followed by a 15-sec tone CS co-terminating in a shock. Subjects then had a 15 min period in the chamber in which they could drink freely. Immediately following each daily acquisition session, subjects were injected with either 1 mg/kg MB or saline (0.9% NaCl) I.P. Blue food coloring was added to the saline solution so experimenters would be blind to group assignment. After the first two days of acquisition, it was clear that the operant chambers had become a strong excitatory context (positive associative strength) since conditioned suppression ratios dropped to almost zero, so on the third day of acquisition experimenters began recording freezing scores during pre-CS and CS periods.

Two days after acquisition, a probe trial consisting of one tone was initiated after subjects fulfilled the 5-sec latency drinking period. Freezing was scored during the pre-

CS and CS period by the experimenters and no injections were given following the probe trial. Identical second and third post-acquisition probe trials were each run 10 days following the previous trial. An out-of-context probe trial was conducted on the day following the third probe trial in order to examine the influence of context on subjects' freezing scores. In order to alter contextual cues for the out-of-context probe, subjects were placed in a plastic transfer cage lined with black contact paper which had been cleaned with a betadine solution, and lights were turned off in the room with red lights illuminating the test cages. One tone was played, initiated by the experimenters, through speakers above the cages. The out-of-context probe trial lasted 1 min and pre-CS and CS freezing scores were recorded by the experimenters.

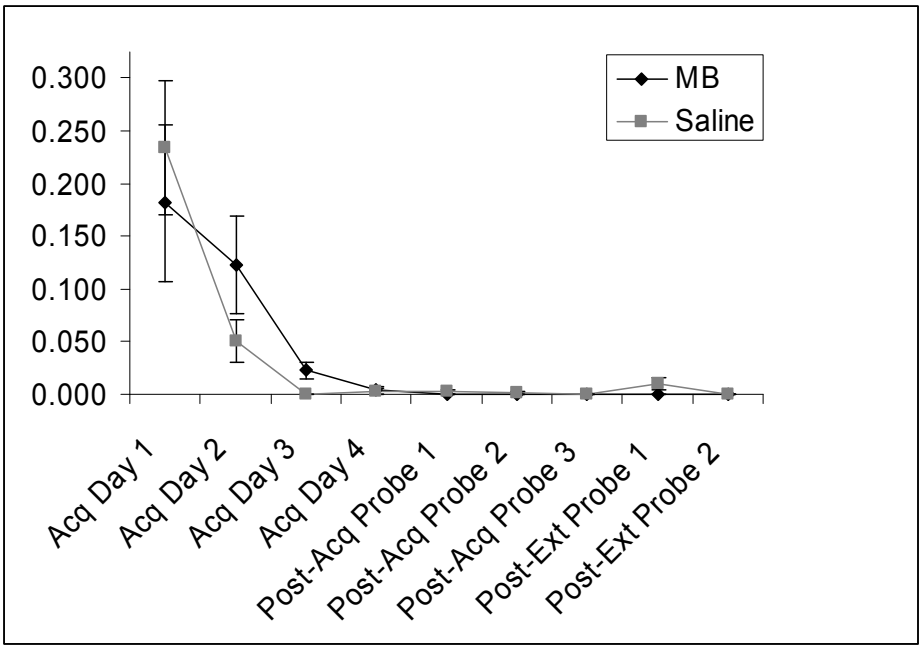
3.4.4.3 Extinction Phase and Post-Extinction Probe Trials

The long-term memory retention of the CS-US association was also tested following extinction training. In order to determine if MB administration during acquisition could retard future extinction training, 22 days after the out-of-context probe trial, an extinction session consisting of 60 tones in 60 minutes was run in the test chambers used for acquisition training. Subjects were also water-deprived during this phase of the experiment. Two days after the extinction session, a post-extinction probe trial was conducted in the acquisition context, and a second post-extinction probe trial was run 23 days later in the same context.

3.5 RESULTS

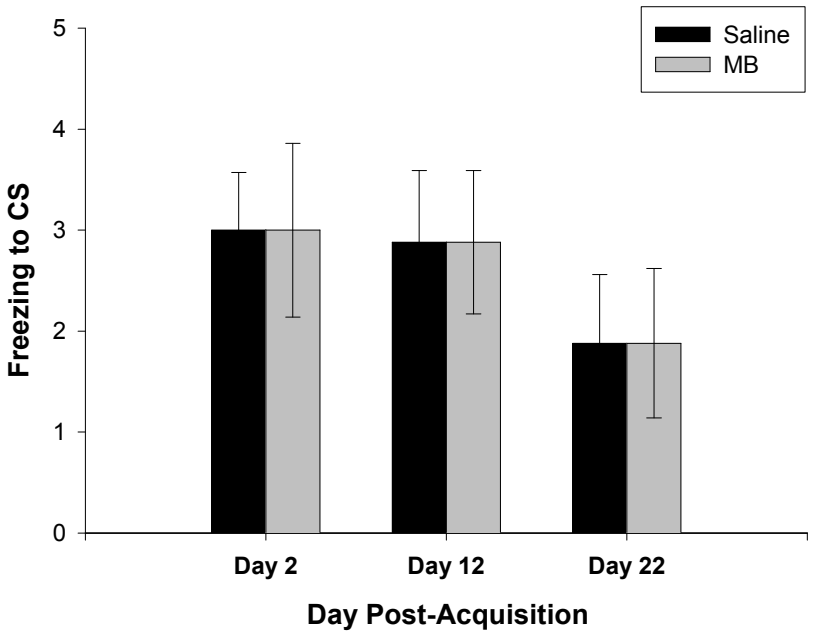
Conditioned suppression ratios, latency to initiate drinking, duration of drinking bouts (during pre-CS, CS, and the 15-min drink period), and freezing scores were calculated for each experimental session. Suppression ratios on the fourth day of acquisition demonstrated that subjects in both groups were almost completely suppressing drinking during the tone CS (saline mean= 0.004, MB mean= 0.003) and ratios stayed below these values for the duration of the study (even though probes were conducted over two months following the initial acquisition phase), demonstrating that the training context had powerful excitatory effects (Figure 3A). Latency to initiate drinking measures were not significantly different between groups.

Figure 3A: Conditioned suppression ratios in both MB and saline administered subjects. Suppression ratios on the fourth day of acquisition demonstrated that subjects in both groups were almost completely suppressing drinking during the tone CS and ratios stayed below these values for the duration of the study.



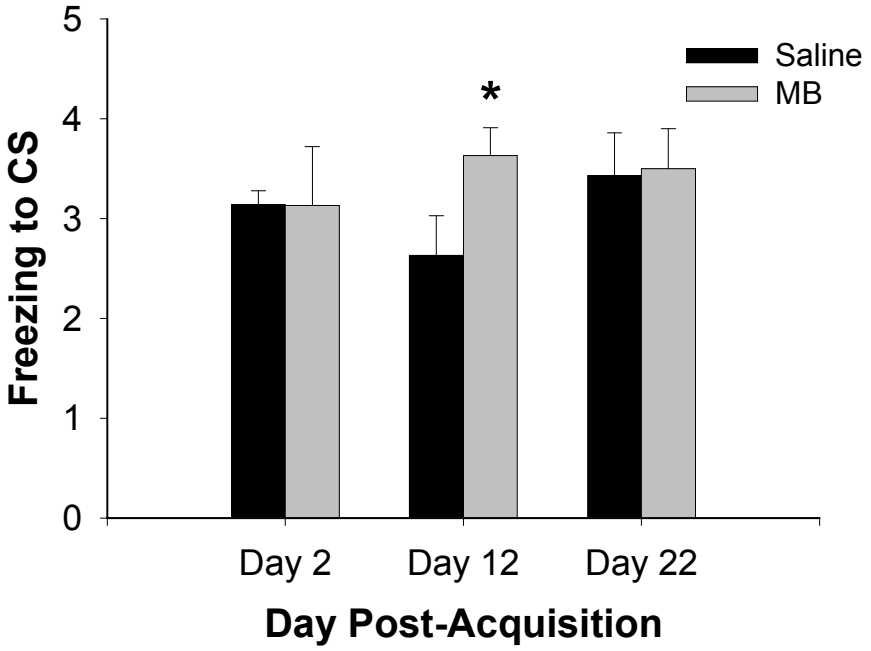
Nonparametric freezing scores were evaluated using Mann-Whitney U tests and Wilcoxon signed-rank comparisons. Post-acquisition probes demonstrated no difference in pre-CS freezing between groups, so any significant effects observed between groups during probes were not context-specific (Figure 3B).

Figure 3B: Means ± standard errors for post-acquisition freezing in the pre-CS period in saline administered and methylene blue-treated subjects. There were no significant differences between groups on Day 2, 12, or 22 post-acquisition.



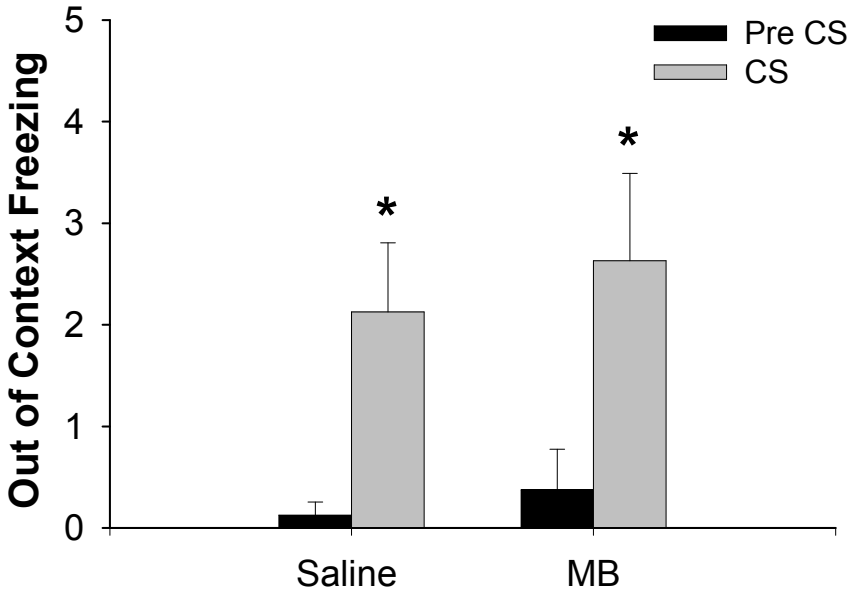
Results of the post-acquisition probes demonstrated that while both groups had similar conditioned performance by post-acquisition Day 2, the MB group demonstrated a significantly higher freezing score to the CS tone in the second probe trial conducted on post-acquisition Day 12 ($U_{(14)} = 14.5$, $p = 0.047$). This group difference was not present on post-acquisition Day 22 (Figure 3C). There were no differences found between groups in total freezing scores (CS + pre-CS) on any of the post-acquisition probe days.

Figure 3C: Means ± standard errors for post-acquisition freezing to the tone CS in saline administered and methylene blue-treated subjects. *$p < .05$



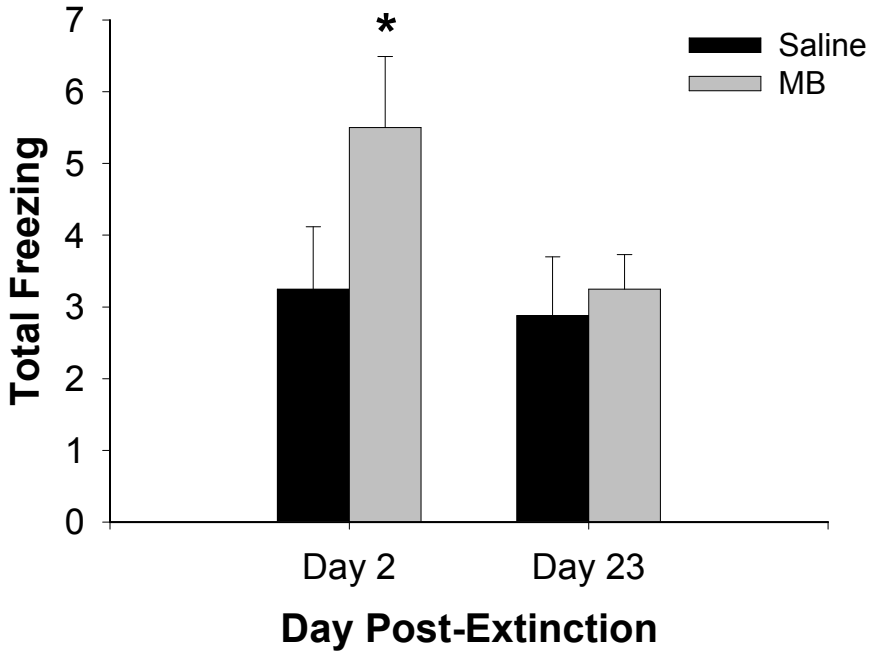
The out-of-context probe trial conducted on post-acquisition Day 23 showed that both groups were freezing to the CS tone (saline Z = -2.121, p= 0.034; MB Z= -2.032, p= 0.042) and that the pre-CS values in the probes were affected by some context freezing (Figure 3D).

Figure 3D: Means ± standard errors for the out-of-context probe trial, showing freezing to CS in saline administered and methylene blue-treated subjects. The out-of-context probe trial was conducted on Day 23. *$p < .05$



Rats treated with MB during acquisition also demonstrated greater resistance to extinction than controls, as shown by greater post-extinction total freezing scores. This group difference was seen on the Day 2 post-extinction probe trial ($p < 0.05$, one-tailed t-test). No significant differences were found between groups on Day 23 post-extinction (Figure 3E).

Figure 3E: Means ± standard errors for post-extinction total freezing (Pre CS + CS) in saline administered and methylene blue-treated subjects. *$p < .05$



3.6 DISCUSSION

Low dose MB (1 mg/kg) administered following acquisition sessions improved retention of the acquisition memory in rats. Freezing scores were recorded because drinking suppression scores were near 0 in both groups, resulting in a floor effect. This effect occurred after only 2 days of one daily tone-shock pairing and lasted throughout the entire study. This was surprising to us, since we did not expect the water-deprived subjects to suppress drinking behavior so rapidly.

There were no group differences in pre-CS freezing in post-acquisition probes but a group difference was found in CS freezing, with the MB-treated group demonstrating greater freezing to the CS tone on the second post-acquisition probe trial. This data suggests that MB enhanced memory for the tone-shock association without significantly affecting contextual freezing. It was originally expected that subjects in the MB condition would demonstrate greater freezing behavior in all three post-acquisition sessions, yet this was not the case. Subjects in both groups froze equally to the tone CS on Day 2 post-acquisition, and it was only on the second probe trial that a difference in freezing to the CS tone was observed. Perhaps the passage of time contributed to saline subjects memory of the original tone-shock association deteriorating, while subjects in the MB group still retained it. Subjects in both groups froze almost equally to the CS tone on Day 22 post-acquisition. They still remembered the tone-shock association, but MB-treated subjects did not show better memory retention at this point in time. Subjects in the saline group showed strong freezing to the CS tone in all of the post-acquisition probes, which could have resulted in a ceiling effect that may have masked any drug

effects methylene blue had on memory retention. This is the first study demonstrating that low dose MB enhances memory retention of fear conditioning, and that this effect can persist for 12 days after MB administration. Methylene blue-treated subjects had higher total freezing scores on post-extinction Day 2 indicating that they retained stronger fear-conditioning than controls, demonstrated by an impairment in extinction, as predicted in our hypothesis. This was very interesting since the extinction training was conducted 45 days after the final post-acquisition injection of MB. We had not conducted any studies examining the long-term effects of MB administration on memory, and this was a very significant finding. Perhaps at the 4 mg/kg dose, which we now know to have greater effects on memory retention, the outcome of this study would have been different. This study demonstrates that memory retention can be enhanced in both appetitive and aversive memory tasks, and that these effects can be long lasting. These findings suggest that the timing of MB administration is very important during studies utilizing a fear conditioning paradigm.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health grant R01 NS37755 to Francisco Gonzalez-Lima and a Society for Neuroscience Minority Fellowship to Kathryn Wrubel. I would like to thank Dr. Douglas Barrett for his assistance with computer programming and troubleshooting with MED-PC, and Amir Gafur for his help with data collection and analysis.

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CHAPTER 4: METHYLENE BLUE FACILITATES FEAR EXTINCTION IMPAIRED IN AN ANIMAL MODEL OF POST-TRAUMATIC STRESS DISORDER

4.1 ABSTRACT

A study was conducted in order to characterize the behavior of congenitally helpless (CH) rats, an animal model of post-traumatic stress disorder (PTSD), in extinction of Pavlovian fear conditioning. In the first experiment, acquisition, consisting of 4 daily tone-shock pairings in an operant chamber, was followed by 2 days of extinction in a different context. Conditioned freezing to the CS tone was scored during extinction training, and one week later tone-alone probe trials were conducted in the acquisition context to examine renewal effects. Another session consisting of tone-alone probe trials was run in the extinction context on the following day. One month later, a session consisting of tone-alone probe trials was conducted in the acquisition context in order to observe any long term effects of the extinction training. The CH rats demonstrated a dramatic extinction deficit as compared to control subjects, and did not display the gradual extinction curves observed in controls. Congenitally helpless rats demonstrated greater tone-evoked freezing as compared to controls in both the acquisition and extinction contexts one week after extinction training, and also in the long-term extinction probe conducted one month later. A recent study found that methylene blue (MB), a metabolic enhancer, improved memory retention of extinction of fear conditioning. Due to the extinction deficits observed in CH subjects, it was of

interest to determine if MB could facilitate extinction in these subjects. In the second experiment (which began the day after the long term extinction probe session) CH subjects had extinction probe sessions in the extinction context for 5 days, each followed by either 4 mg/kg methylene blue or saline administered I.P. On the sixth day, subjects were returned to the acquisition context for a tone-alone probe trial session and these freezing scores were compared with the pre-treatment freezing scores obtained in the long-term extinction probe session. Methylene blue administration improved retention of the extinction memory in treated CH subjects as demonstrated by significant decreases in freezing as compared to saline-administered CH subjects. The impaired ability to extinguish fear to a traumatic memory in CH rats supports the validity of this strain as an animal model for vulnerability to PTSD, and this study further demonstrates that MB may be a promising therapeutic candidate for the facilitation of extinction.

4.2 INTRODUCTION

Congenitally helpless (CH) rats are a genetic strain of animals developed from the learned helpless model of depression which states that repeated uncontrollable stress influences how an animal copes with stress later on in life (Overmier & Seligman, 1967). CH rats are produced by selectively breeding unrelated generations of animals who, after exposure to several trials of inescapable shock, do not try to escape in a situation presented later when they have an opportunity to lever-press to avoid the shock. They have not been shocked themselves but CH rats demonstrate behaviors typical of people with depression and post-traumatic stress disorder (PTSD) such as sleep disruption, reward insensitivity, reduced sexual activity, and attention deficits (Nestler et al., 2002; Shumake & Gonzalez-Lima, 2003b). Interestingly, antidepressants have been shown to reverse this condition (Henn & Edwards, 1994). Studies show that over 95% of the CH offspring typically show the learned helpless phenotype, as opposed to the 5-20% of rats that demonstrate learned helplessness in the escape paradigm (Henn et al., 1985; Lachman et al., 1992). Since epidemiological studies show that 40-50% of the risk of susceptibility to depression and 30% of the risk of susceptibility to PTSD is genetic (Fava et al., 2000; Sanders et al., 1999; True et al., 1993), an appropriate animal model of congenital vulnerability to these diseases could be very useful.

A recent paper reviewed behavioral characteristics of rats predisposed to learned helplessness (Shumake et al., 2005). It compared CH rats to normal rats in behaviors reflecting three temperament dimensions outlined in Cloninger's theory of personality: reward dependence, novelty seeking, and harm avoidance (Cloninger, 1987).

Congenitally helpless subjects demonstrated reduced reward sensitivity, high novelty seeking, an increase in conditioned fear, and a deficit in fear extinction. These measures correlate best with findings from the human literature on PTSD. For example patients with PTSD show stronger acquisition and reduced extinction of aversively conditioned responses (Orr et al., 2000), and the same profile of low reward dependence, high novelty seeking, and high behavioral inhibition as CH rats (Richman & Frueh, 1996; Shumake et al., 2005; Wang et al., 1997).

The brains of CH rats have been comprehensibly mapped using metabolic brain imaging techniques, and these subjects show extensive alterations in regional brain metabolism as compared to non-helpless subjects (Shumake et al., 2000, 2001, 2002, 2003a, 2004; Shumake & Gonzalez-Lima, 2003b). For example, CH rats have reduced metabolism in prefrontal cortical regions (Shumake et al., 2000), which are highly engaged in behavioral extinction (Barrett et al., 2003; Nair et al., 2001a, 2001b; Milad & Quirk, 2002). If CH subjects demonstrate deficits in extinction of Pavlovian fear conditioning, they may be a good animal model for studying PTSD. Since methylene blue (MB), a metabolic enhancer, has been shown to improve memory of extinction of fear conditioning (Gonzalez-Lima & Bruchey, 2004), it was of interest to determine if MB could facilitate extinction in CH subjects, which were hypothesized to show extinction deficits.

4.3 HYPOTHESIS

Since CH rats have decreased metabolism in prefrontal cortical regions known to mediate extinction (Shumake et al., 2000), we predicted that they would show impaired extinction following Pavlovian fear conditioning. We also expected them to have stronger fear responses to the CS tone as measured by increased freezing behavior compared to non-helpless controls. This could lead to persistent fear-related conditioned responses to the CS tone, which may be more resistant to extinction than in normal control rats. If so, we also predicted that post-extinction treatment with MB would enhance memory of extinction of fear conditioning, as demonstrated in normal rats by Gonzalez-Lima & Bruchey (2004), and that MB-treated CH rats would demonstrate less freezing to the CS tone following extinction training.

4.4 MATERIALS AND METHODS

4.4.1 Subjects

For the first experiment, subjects were 23 female Sprague-Dawley congenitally helpless rats (bred in our laboratory from breeding pairs obtained from the Central Institute for Mental Health in Mannheim, Germany, courtesy of Dr. Fritz Henn), and 12 female control rats, all weighing approximately 300 g at the beginning of the experiment. The CH subjects used in this study were bred in our laboratory and were not tested for susceptibility to learned helplessness. The control rats were Sprague-Dawley strain obtained from Harlan (Houston, TX). Subjects were housed 2-3 per cage under standard laboratory conditions with a 12 hr light/dark cycle and free access to food and water.

Animal experimentation was approved by the University of Texas Institutional Animal Care and Use Committee. Male rats would also have been used, but, because of the need for male subjects for another experiment and high infertility in our colony, we were unable to breed a sufficient number of male subjects for this purpose. Rats were given daily vaginal smears and began training on the first day of their estrus cycle in both experiments. For the second experiment, female congenitally helpless rats were subdivided into 2 groups: 12 were treated with MB and 11 were administered saline.

4.4.2 Apparati

Different apparati were utilized during extinction training and probes, in order to parse out any effects of contextual fear on freezing behavior. The acquisition session was conducted in standard operant chambers and extinction sessions were conducted in open field activity chambers in a different room. When possible, light and odor cues were also changed between contexts.

4.4.2.1 Acquisition Context (Context A)

Pavlovian tone-foot shock acquisition training was conducted in 4 operant conditioning chambers, each measuring 22 x 25 x 32 cm (MED Associates, St. Albans, VT) and enclosed in sound-attenuated boxes illuminated by red lights. The two sides of each chamber were aluminum, and the front, back, and top were made of clear plexiglas. Tones were generated by a Wavetek Sweep/Modulation Generator (Wavetek, San Diego, CA) and presented through speakers mounted at the top of each chamber. The acoustic

conditioned stimulus (CS) was a frequency-modulated tone of 1-2 kHz, 2 sweeps/sec, 15 sec in duration, with an intensity of 68 dB, measured at the center of the floor of the chamber with a decibel meter. The unconditioned stimulus (US) was a mild foot shock of 0.5 mA, 0.75 sec in duration, delivered through metal bars (separated by 1.2 cm) which formed the floor of the chamber, and were wired to shock generators (MED Associates). Stimulus presentations were controlled by computer programs, written by the experimenters using the MED-PC for Windows programming language (MED Associates). A Bioclean solution (Stanbio Laboratory, Boerne, TX) was placed in the tray beneath the chamber to provide a distinct olfactory cue for the acquisition context.

4.4.2.2 Extinction Context (Context B)

Extinction training occurred in a different context. Two open field activity boxes measuring 31 x 45 x 45 cm (MED Associates) with fiberglass bottoms, clear plexiglas sides, and an open top were utilized for extinction training. Horizontal activity was detected by arrays of infrared motion detectors (16 x 16, 1" apart), with two arrays located 1 cm above the floor of the chamber. Rearings were detected with a vertical-axis array positioned 13 cm above the surface of the floor to ensure that only those rearing movements in which the subject's forepaws left the ground would register as rearing counts. Open-field boxes were controlled by the MED Associates Activity Monitor program (Version 5.10), which records various parameters related to a subject's locomotion, resting, and rearing behaviors. The tone CS which was 1-2 kHz, 2 sweeps/sec, and 15 sec in duration, was digitally recorded from the tone generator used in

the acquisition context and presented through a computer speaker above the open fields (measured at 68 dB in the center of the floor of each open field with a decibel meter).

4.4.3 Freezing Scores

Freezing scores were utilized as an index of tone-evoked fear. Experimenters trained to score freezing with high inter-rater reliability recorded a freezing score during pre-CS and CS periods through direct observation. Freezing was operationally defined as the subject having all four feet on the floor, with minimal head movements, and shallow, rapid breathing. A score of 1 was recorded if the subject froze for the majority of a 3 sec bin, with a maximum freezing score of 5 for complete freezing during the 15-sec pre-CS and CS periods.

4.5 EXPERIMENT 1: THE CHARACTERIZATION OF EXTINCTION OF PAVLOVIAN FEAR CONDITIONING IN THE CONGENITALLY HELPLESS RAT

The congenitally helpless rat is a relevant animal model of PTSD, displaying several brain and behavioral correlates in both the animal model and human literature on the disease. Since humans with PTSD show deficits in extinction of conditioned fears (Peri et al., 2000; Orr et al., 2000) it was of interest to determine if CH rats would also demonstrate similar deficits when compared with normal control rats. This is the first such study to be conducted in the CH female rat.

4.5.1 Behavioral Training

Subjects were handled every day for 7 days prior the start of training. During this time, each subject was habituated to the acquisition context in the absence of tones or shocks for 1 hr a day. Vaginal smears were used to determine the estrus cycle phase for each individual subject before training began. An estrus cycle of 4 days was confirmed and training was staggered between subjects according to estrus cycle, such that the first day of acquisition training occurred while each subject was in estrus. Diestrus females received up to 3 additional days of habituation and handling.

The training procedure is presented in Table 4.1 under "Experiment 1".

Table 4.1: The experimental design.

Table 1. Experimental Design

Experiment 1	**Day 1**	**Days 2 - 3**	**Day 9**	**Day 10**
Session	Acquisition	Extinction	Probe-Acq	Probe-Ext
Context	Context A	Context B	Context A	Context B
Stimuli	4 T → S	30 T / Day	4 T	4 T
Experiment 2	**Day 1**	**Days 2 - 6**		**Day 7**
Session	Probe-Acq	Probe-Ext		Probe-Acq
Context	Context A	Context B		Context A
Stimuli	4 T	4 T / Day **+ MB or Saline**		4 T

T, 1-2 kHz 68 dB 15 sec tone CS; S, 0.5 mA 0.75 sec footshock US; MB, methylene blue

For acquisition training, all subjects were placed in the conditioning chambers (Context A) and received 4 tone-shock pairings over 15 min, with pseudorandom inter-trial intervals averaging 3 min Each 15-sec tone CS co-terminated with the foot shock US.

Extinction training occurred over 2 consecutive days while female subjects were in the diestrus phase, to control for any hyperactivity that could occur during estrus (Birke & Archer, 1975) and interfere with conditioned freezing measures in the open field (Context B, used for extinction training). Subjects were placed in the open field, and 30 tones were presented over 60 min, with 2 min between each tone onset. The first minute in the open field context served as a baseline measure of activity (prior to the first tone CS presentation).

Post-extinction probe trials occurred one week later in the acquisition context (when subjects were in estrus), in order measure context renewal. Each subject was returned to the operant chamber (Context A) and presented with four 15-sec tones (in the absence of foot shock) over 10 min, with 2 min intervals between each tone onset. Post-extinction probe trials (consisting of 4 tone CS presentations, as described above) occurred in the extinction context on the following day, when subjects were in diestrus, and served to measure retention of extinction learning. Experimenters recorded freezing scores during pre-CS and CS periods in both contexts.

4.5.2 Statistical Analysis

The SPSS 11.5 for Windows statistical software package was used for statistical analysis. All data were analyzed with analyses of variance (ANOVAs), and *p*-values are reported as Huynh-Feldt corrected values, with an alpha value of .05 regarded as significant. When warranted, simple-effects tests of significant interactions were performed and adjusted by Bonferroni correction.

4.5.3 RESULTS

Helpless and control subjects showed similar baseline open field activity prior to extinction training. In contrast, they showed dramatic group differences during extinction, with CH rats exhibiting a large extinction deficit.

4.5.3.1 Baseline Motor Activity in the Open Field

There were no differences in baseline motor activity measures between CH and control subjects during the first minute of Extinction Session 1 (prior to the onset of the first tone CS) in any of the parameters analyzed, which included ambulatory time, short movement time, immobility time, and rearing time.

There were no significant differences between groups in time spent in the center of the open field during the first minute of Extinction Session 1, which is considered a measure of fearfulness since timid subjects have been found to spend more time along the walls of the apparati (Treit & Fundytus, 1988). This indicates that the differences

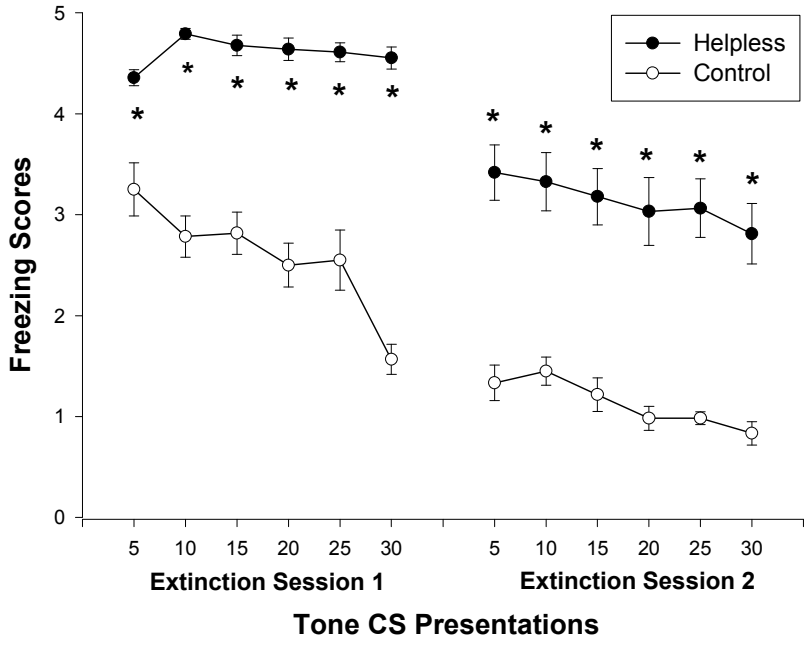
observed in extinction behavior between CH rats and control subjects cannot be attributed to baseline strain differences in general motor activity or fearfulness.

4.5.3.2 Short-term Extinction

Short-term extinction was assessed by comparing changes in freezing behavior within each hour and across both days of extinction training. Data were evaluated with a repeated measures ANOVA, with session and time serving as repeated measures. Behavioral measures from each 15-sec tone CS presentation were averaged into 6 bins representing 5 tone presentations every 10 min.

There was a significant group effect in freezing behavior during extinction training, $F(1,29)= 139.667$, $p < 0.001$, with CH subjects freezing much more than controls. While control rats showed similar rates of extinction in both sessions with smooth learning curves, CH subjects displayed no evidence of extinction in Extinction Session 1, and slowly began to extinguish freezing behavior in Extinction Session 2 (Figure 4A).

Figure 4A: Extinction curves (means ± standard errors). The conditioned response during Extinction Sessions 1 and 2 was measured in terms of freezing behavior. Each 1 hr extinction session consisted of 1 tone CS every 2 min, averaged by 5 tones for each 10-min bin for the repeated measures ANOVA. Significant differences ($p < .01$) between congenitally helpless and control groups were found at every time point in both sessions.
* $p < .001$



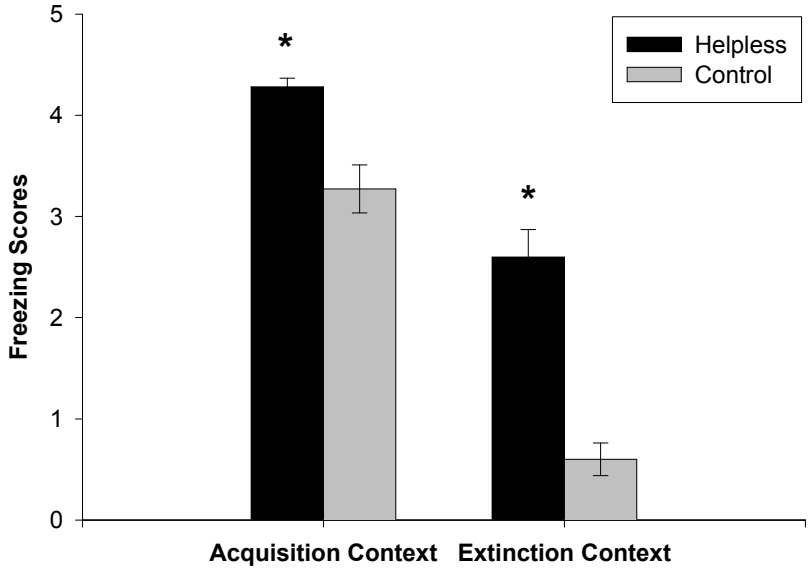
4.5.3.3 Context Renewal

In order to assess the effects of context renewal on extinction, data from the four tone probes delivered on Day 9 in the acquisition context, Context A, were analyzed. Congenitally helpless subjects showed significantly more freezing to context relative to controls, $F(1,33)= 230.936$, $p < 0.0001$ in this session (Figure 4B).

4.5.3.4 Long-term Extinction

Long-term extinction was assessed by comparing freezing behavior between groups during the four probe trials on Day 10, which were conducted in Context B, the extinction context. While both control and CH rats showed long-term reductions in freezing as a consequence of extinction training, CH subjects showed less long-term extinction than controls, $F(1,33)= 23.914$, $p < 0.0001$ (Figure 4B).

Figure 4B: Freezing scores from post-extinction probe trials (means ± standard errors) in both the acquisition (Context A) and extinction (Context B) contexts. Each probe test consisted of 4 tones in 10 min. Congenitally helpless subjects showed significantly more freezing to context relative to controls. While both control and CH rats showed long-term reductions in freezing as a consequence of extinction training, CH subjects showed less long-term extinction than controls. * $p < .0001$



4.6 EXPERIMENT 2: THE MEMORY RETENTION EFFECTS OF METHYLENE BLUE IN CONGENITALLY HELPLESS RATS ON EXTINCTION OF PAVLOVIAN FEAR CONDITIONING

Gonzalez-Lima & Bruchey (2004), demonstrated that treatment with 4 mg/kg MB following extinction of Pavlovian fear conditioning improved retention of extinction. Congenitally helpless rats are postulated to be an animal model of PTSD, demonstrating extinction deficits observed in patients with the disorder. Since humans with PTSD often go through extinction training to reduce their fears, we administered MB during extinction training to determine if it would facilitate retention of extinction in CH subjects. We hypothesized that CH subjects receiving MB during extinction probes would extinguish better than CH subjects administered saline.

4.6.1 Behavioral Training

Subjects were the same 23 female congenitally helpless rats used in the first experiment. The training procedure is presented in Table 4.1 under "Experiment 2". This experiment began one month after the context renewal probe trial conducted in Experiment 1. Congenitally helpless females were given daily vaginal smears to assess their estrus cycle. On their first day of estrus they were returned to the acquisition context (Context A) for probe trials consisting of four 15-sec tones (in the absence of foot shock) over 10 min, with 2 min intervals between each tone onset. The pre-treatment probe session conducted in the acquisition context verified that all CH subjects continued to show significant freezing in the acquisition context. Subjects were matched into

groups based on these freezing scores and assigned to either the MB treatment or saline control group.

For each of the next 5 days, subjects were presented with four 15-sec tones (in the absence of foot shock) over 10 min, with 2 min intervals between each tone onset in the extinction context (Context B). Extinction sessions were conducted in a different context in order to reduce confounds resulting from context conditioning. Thirty minutes following each daily probe session in the extinction context, subjects were injected with either MB (4 mg/kg) or saline I.P. Freezing scores recorded during pre-CS and CS presentations were recorded and analyzed as previously described.

On Day 7, subjects were returned to the acquisition context (Context A) for a post-treatment probe session consisting of four 15-sec tones (in the absence of foot shock) over 10 min, with 2 min intervals between each tone onset. Freezing scores were recorded by an experimenter blind to group assignment. Freezing scores to the tone CS in pre-treatment and post-treatment probe sessions recorded in the acquisition context were compared between groups to determine if MB aided in the facilitation of memory retention of extinction.

4.6.2 Statistical Analysis

The SPSS 11.5 for Windows statistical software package was used for statistical analysis. All data were analyzed with analyses of variance (ANOVAs), and *p*-values are reported as Huynh-Feldt corrected values, with an alpha value of .05 regarded as

significant. When warranted, simple-effects tests of significant interactions were performed and adjusted by Bonferroni correction.

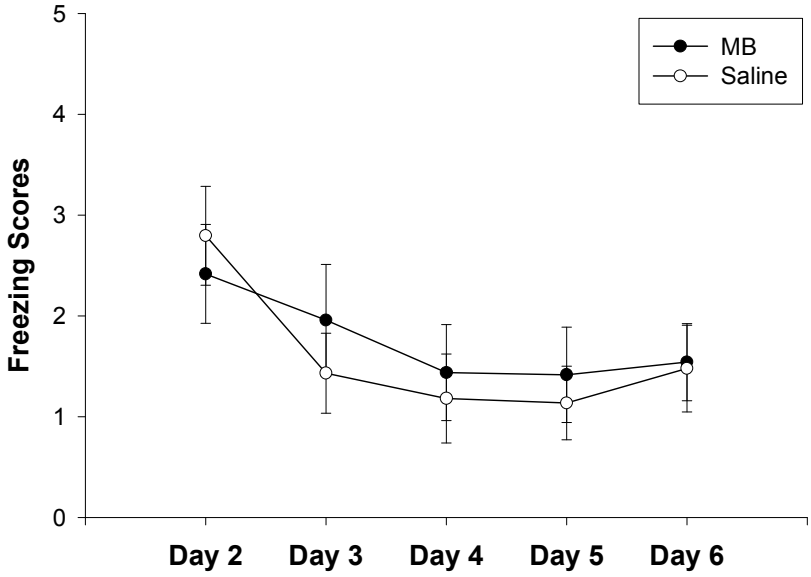
4.6.3 RESULTS

Methylene blue treatment following extinction training significantly improved memory retention of extinction in the acquisition context in CH subjects. While MB did not improve extinction learning in extinction (Context B) sessions, it did significantly improve the generalization of that learning to the acquisition context (Context A).

4.6.3.1 Effects of Methylene Blue Treatment in the Extinction Context Probe Sessions

Freezing to the tone CS in the extinction context (Context B) was analyzed with a repeated measures ANOVA, with the average of the 4 trials from each extinction session serving as the repeated measure. No significant effects related to treatment were obtained (Fs < 1), but a significant main effect of session indicated that both MB and saline administered subjects froze less with each session, $F(4,84) = 9.5$, $p < .001$. (Figure 4C).

Figure 4C: Conditioned freezing (mean ± standard errors) to the tone in Context B (extinction/open field) during the 5 days of MB and saline injections (4 mg/kg I.P.) in congenitally helpless rats. No significant effects related to treatment were obtained (Fs < 1), but a significant main effect of session indicated that both MB-treated and saline administered subjects froze less with each session. * $p < .001$



In addition to freezing, general activity measures recorded by the infrared arrays in Context B (the extinction context) were analyzed using the same repeated measures design. There were no significant treatment-related differences in ambulation, rearing, immobility, or short movement times, demonstrating that MB did not alter general motor activity (Table 4.2).

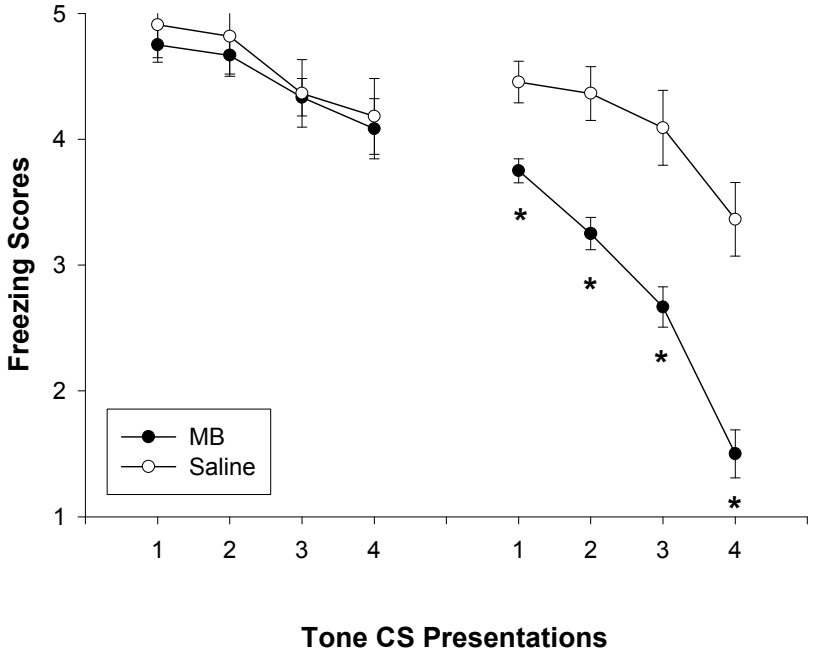
Table 4.2: Average post-drug administration activity measures (in sec) per session in the extinction context (Context B). The 4 sessions were 10 minutes for a total of 600 sec per session. Groups were not significantly different in any of these measures, demonstrating that there were no side effects of methylene blue on behavior.

	METHYLENE BLUE	**SALINE**	
	Mean ± S.E.	Mean ± S.E.	P-Values (ANOVA)
Ambulatory Time	29 ± 6	29 ± 5	p = 0.994
Short Movement Time	103 ± 12	108 ± 7	p = 0.715
Immobility Time	304 ± 43	282 ± 37	p = 0.696
Rearing Time	150 ± 25	171 ± 27	p = 0.568

4.6.3.2 Effects of Methylene Blue Treatment in the Acquisition Context Probe Sessions

Differences in freezing in the acquisition context (Context A) were analyzed with a repeated measures ANOVA, with pre- and post-treatment probes serving as repeated measures. A significant two-way (treatment x probe) interaction was obtained, $F(1,21)= 22$, $p < .001$, indicating that CH rats treated with MB showed significantly reduced freezing in the acquisition context after treatment with the metabolic enhancer MB, and that it did facilitate fear extinction impaired in these subjects (Figure 4D).

Figure 4D: Effect of methylene blue on extinction in the acquisition context (Context A). Pre- and post-treatment freezing scores can be seen in the CH subjects. Conditioned freezing (mean ± standard errors) to the tone before and after injections of 4 mg/kg I.P. of MB or saline is shown. Subjects were matched into groups with respect to pre-injection freezing scores, then tested again after 5 days of tone presentations in the extinction context with injections of MB or saline. Conditioned freezing was significantly reduced in CH subjects receiving MB injections. * $p < .001$



4.7 GENERAL DISCUSSION

Congenitally helpless rats exhibited abnormally high freezing in response to a tone formerly predictive of electric shock, even after 60 presentations of the tone in the absence of shock. They failed to show the gradual decrement in freezing characteristic of the normal extinction curves seen in control subjects, instead, continuing to show significantly greater tone-evoked fear one week after extinction training, when tested in both the acquisition and extinction contexts as we hypothesized. Congenitally helpless subjects also showed enhanced acquisition following fear conditioning, freezing much more to the CS tone than controls in Experiment 1. The extinction deficit in CH subjects was successfully ameliorated with MB treatment (a metabolic enhancer) in the acquisition context, suggesting that a difference in brain metabolic activity may be involved in CH rats' impaired extinction.

The behavioral deficit observed in CH rats cannot be explained by a general suppression of motor activity or by an overall increase in non-specific fearfulness, which can be indicated by thigmotaxis, or time spent in the periphery versus the center of the open field (Treit & Fundytus, 1988). There were no baseline group differences in open field behavior prior to the onset of the fear-associated tones, in terms of motor activity or thigmotaxis.

Several convergent lines of evidence led to the hypothesis that CH rats would show deficits in Pavlovian extinction. This experiment demonstrated that CH rats show deficits in both extinction performance and retention: an inherently neutral tone CS is capable of evoking maladaptive fearful responses in the helpless rats, even outside of the

context in which they experienced the aversive shock US. Post-traumatic stress disorder is likewise characterized by the formation and persistence of traumatic memories that evoke fear across multiple contexts. Post-traumatic stress disorder patients show increases in autonomic arousal (heart rate, skin conductance) in response to aversive Pavlovian conditioning, and these conditioned responses are resistant to extinction (Peri et al., 2000; Orr et al., 2000). This phenomenon is known as paradoxical enhancement, and was demonstrated by CH subjects in the first extinction session, making an even stronger argument for their role as a model of PTSD.

Medial prefrontal cortex may be crucial for the inhibition of the conditioned response during extinction (Barrett et al., 2003; Milad & Quirk, 2002), and PTSD patients show decreased metabolic activity in this region during extinction (Bremner et al., 1999). In newborn CH rats, brainstem regions are metabolically uncoupled from networks of frontal and limbic regions (Shumake et al., 2004). This functional decoupling between brainstem and forebrain regions may be indicative of a developmental disorder in which brainstem regions are removed from the inhibition provided by the frontal cortex during successful Pavlovian extinction. Such a disruption could selectively impair Pavlovian extinction while leaving acquisition relatively intact.

Successful fear extinction training in PTSD patients may be facilitated by pharmacotherapy. Methylene blue is an FDA-approved drug which can be safely used in humans. It acts as an electron carrier in mitochondria, improving cellular respiration (Visarius et al., 1997), and enhancing neuronal metabolism. Administration of MB to CH rats significantly ameliorated their extinction deficit in the fear-evoking acquisition

context, as predicted in our hypothesis. It did not enhance memory retention of extinction during extinction sessions in Context B, only showing positive effects on extinction retention in context-based extinction in this study. Our recent study (Gonzalez-Lima & Bruchey, 2004) confirmed the efficacy of MB in Pavlovian fear extinction and on regional brain metabolism. MB successfully improved Pavlovian fear extinction memory, and resulted in higher CO activity in the frontal cortical regions implicated in extinction (Barrett et al., 2003). In addition, this study found that the regional metabolic increases were correlated with the degree of extinction retention. The effects of MB on the CH subjects seen here, together with our prior findings, suggest that MB may be therapeutically useful in PTSD patients. MB's metabolic effect may facilitate the adaptive inhibition of the conditioned fear response provided by frontal cortical regions during successful Pavlovian extinction.

Congenitally helpless rats showed somewhat enhanced acquisition and dramatically impaired extinction of fear evoked by an aversive memory- a crucial part of the behavioral phenotype associated with PTSD (Charney et al., 1993). The CH rat also shows differences in regional brain metabolism which resemble biological abnormalities detected in PTSD patients. Thus, CH rats may be a useful model for studying those biological factors which render a subset of individuals vulnerable to PTSD, and for testing novel treatments such as methylene blue, which may facilitate the extinction of conditioned fear.

ACKNOWLEDGEMENTS

This research was supported by National Institutes of Health grant R01 NS37755 to Francisco Gonzalez-Lima and a Texas Consortium in Behavioral Neuroscience Fellowship to Kathryn Wrubel. The original CH rat breeding pairs which produced the offspring used for this study were obtained from the Central Institute for Mental Health (Mannheim, Germany) courtesy of Dr. Fritz Henn. I would like thank Elizabeth Johnson and Abby Schindler for their assistance with data collection and analysis. A special thanks to Dr. Douglas Barrett for assisting with conduction of Experiment 1, and for programming the computers, and troubleshooting MED-PC. Also thanks to Dr. Jason Shumake for his previous work with the CH rats.

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CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

5.1 GENERAL DISCUSSION

These studies were undertaken to further understand the effects of methylene blue (MB) on behavior and memory retention, to determine its duration of action on memory, and to see if it may be a useful adjunct to treatment in certain types of behavior therapy. It was found that MB could enhance discrimination learning of rewarded versus non-rewarded trials, that it has long-lasting effects on memory retention of an aversive experience, and that it can enhance memory of extinction in an animal model of post-traumatic stress disorder.

The first experiment was conducted to examine the effects of MB treatment in normal rats in discrimination learning of rewarded versus non-rewarded trials in the hole board maze. Methylene blue-treated subjects reliably discriminated between rewarded and non-rewarded trials, whereas control subjects did not (indicated by greater reference memory scores in rewarded than in non-rewarded trials). Given our laboratory's history studying discrimination learning between rewarded and non-rewarded trials (Nair & Gonzalez-Lima, 1999; Lilliquist et al., 1999), it was of interest to us to find a compound that could have positive effects on discrimination learning. These previous studies examined discrimination learning in the infant rat in a runway apparatus, and the age at which rats could learn to discriminate between rewarded and non-rewarded trials. Perhaps MB could be administered in such a developmental study to see if we could enhance memory retention at a younger age than previously determined (in experiments

not using a memory enhancer). Given the low risk of side effects of low dose MB, this drug would be ideal for such a study.

The second experiment examined the effects of post-acquisition administration of MB in a Pavlovian fear conditioning paradigm. The duration of these effects was also studied, as well as any effects MB administration following acquisition had on extinction training conducted weeks later. Low dose MB (1 mg/kg) administered following acquisition sessions improved retention of the acquisition memory in rats without significantly affecting contextual freezing. This is the first study demonstrating that MB enhanced memory retention of acquisition of fear conditioning. Interestingly, MB subjects also had higher total freezing scores on post-extinction Day 2, indicating that MB-treated rats retained stronger fear-conditioning than controls following an extinction session. The results were clear, that MB can enhance memory of an aversive task and that the effects of MB are long-lasting, months even, which is a significant portion of a rat's lifespan. If we want to use MB as an adjunct to behavior therapy for phobias or PTSD, we need to know the ideal time of administration to facilitate the extinction memory. If administered improperly, MB could lead to increase in fear sensitization, as opposed to having therapeutic effects.

The third, and final, experiment was conducted to characterize extinction in the congenitally helpless (CH) rat, an animal model of post-traumatic stress disorder and depression, hypothesized to demonstrate deficits in extinction of fear conditioning. If a deficit was present, we wanted to determine if it could be facilitated with low dose MB treatment. Congenitally helpless rats exhibited more freezing to the tone CS than

controls, and did not show normal extinction curves during extinction training. They also showed greater tone-evoked fear one week after extinction training in both the acquisition and extinction contexts. Methylene blue administration significantly ameliorated the extinction deficit observed in CH rats in the acquisition context. Since MB has the potential to increase metabolic activity in prefrontal cortical regions metabolically impaired in CH rats and hypothesized to contribute to their extinction deficits, it could be tested as a model therapeutic for patients undergoing behavioral treatment for phobias or PTSD.

5.2 MOLECULAR MECHANISMS OF MEMORY AND METHYLENE BLUE

There are several stages of memory including acquisition, consolidation, retention, and retrieval (Matynia et al., 2001). Most research on the molecular mechanisms of memory takes place during the acquisition phase. In Pavlovian tone-foot shock conditioning, acquisition (training) takes place during the initial learning of the tone-shock association. Following this, consolidation of the association occurs, which can take hours to days to complete, and this is when memory is moved from a labile to a more fixed state (Abel & Lattal, 2001). Memory retrieval occurs when subjects are tested following memory consolidation. Since we are interested in improving retention of memory consolidation with MB, we administer it immediately following acquisition.

There is some evidence that sleep (particularly REM, or rapid eye movement, sleep) plays an integral role in memory consolidation, but studies are conflicting on the issue. REM sleep deprivation causes memory deficits, and studies also show that REM

sleep increases during specific time periods following training, particularly in hippocampal dependent tasks (Smith & Rose, 1996). During REM sleep there are increases in acetylcholine (Marrosu et al., 1995). Acetylcholine acts on muscarinic receptors, which have been found to be involved in fear conditioning (Rudy, 1996), and are known to modulate many signal transduction pathways. Also, acetylcholine applied to the hippocampus facilitates long-term potentiation (Auerbach & Segal, 1994). Decreased levels of serotonin, another neurotransmitter, occur in the hippocampus during REM sleep (Park et al., 1999), and serotonin antagonists facilitate memory (Izquierdo et al., 1998). Human studies suggest that REM sleep may not be important for explicit or declarative memory- including language, rote, and conceptual memory (Siegel, 2001). Procedural memory (performance on perceptual or perceptuo-motor skills), however, has been shown to be impaired by sleep disruption (Stickgold et al., 2000). Studies examining the role of memory consolidation in REM sleep often do not address stress, frustration, or emotional aspects of the learning situation, even though moderate stress produces increases in REM sleep, which could be a confound in certain studies. Also, MAO (monoamine oxidase) inhibitors suppress REM sleep, yet these drugs are not known to cause memory impairments in humans, so the literature is conflicting in this area (Siegel, 2001).

In 1949, Donald Hebb suggested that changes occurred in the synapse during learning, that there could be use-dependent neural plasticity, and that theses changes can be permanent. In his book, *The Organization of Behavior*, Hebb wrote "When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it,

some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased" (p. 62). Although he did not have conclusive evidence at the time, we now know this to be likely. Long-term potentiation, or LTP, is an experimental model of synaptic changes thought to be relevant to learning and memory. LTP is a long-term increase in the excitability of a neuron to a particular synaptic input caused by repeated high-frequency activity of that input. It leads to synaptic plasticity, in which changes occur in the structure of the synapse following gene expression, causing perforations that lead to the production of new synapses, and synaptic growth and remodeling.

Several molecular cascades have been implicated in the formation of acquisition and consolidation of memory. A key player implicated in the acquisition of memory is the NMDA (N-methyl-D-aspartate) receptor. The NMDA receptor is a neurotransmitter and voltage-dependent ion channel that is normally blocked with a Mg2+ (magnesium) ion. Only when the postsynaptic cell membrane is both depolarized and glutamate attaches to the binding site will the NMDA receptor allow Ca2+ (calcium) to enter the dendritic spine. Since NMDA receptors are sensitive to both presynaptic transmitter release, and postsynaptic depolarization, they can act as a sort of Hebbian coincidence detector (Martinez, Jr. & Derrick, 1996). Learning causes activation of postsynaptic NMDA receptors, which in turn causes an influx of calcium. This influx causes a number of calcium sensitive second-messenger processes. Ca2+ is buffered by Ca2+ calmodulin, after which it activates kinases such as CaMKII (Ca2+ calmodulin-dependent kinase II) which phosphoralates cyclic AMP-response-element-binding protein

also known as CREB (a mediator of gene expression). Ca2+ calmodulin also activates adenylyl cyclase which makes cyclic AMP, which activates PKA (another kinase) which also phosphoralates CREB. CREB binds to CRE (cyclic AMP-response element), which is a specialized stretch of DNA and gene promoter, triggering its transcriptional activity and resulting in gene expression (Carlezon et al., 2005). Then effector and regulator genes can increase the number of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) molecules, enhance protein synthesis, and induce long-term plasticity in the brain. The number of AMPA molecules in the postsynaptic membrane is a function of synaptic history and these molecules are critical in strengthening individual synapses (Dudai, 2002). This long-term plasticity also can include gene transcription for new dendrites.

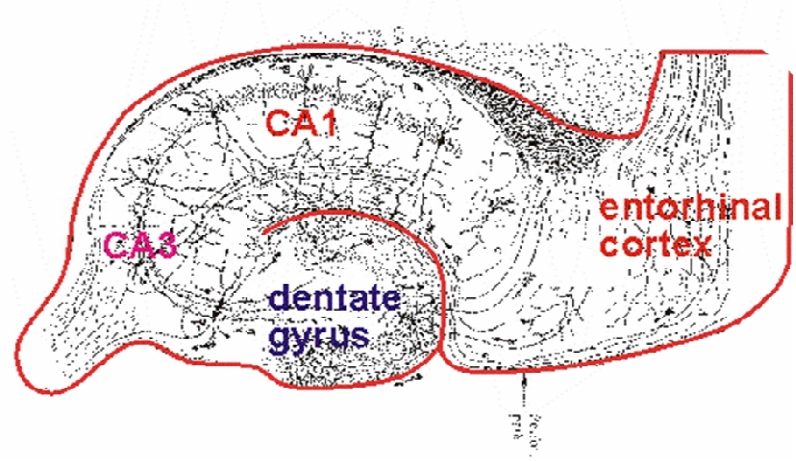
Short term memory is hypothesized to last for minutes or hours post-training, whereas long term memory lasts from hours to days, or even weeks and years. Protein synthesis causes cellular growth and remodeling, and is required soon after training if long term memory is to occur, although short term memory does not require protein synthesis (Cavallaro et al., 2001; Pinter et al., 2005). A few hours after the CS-US pairing in fear conditioning, there are significant changes in the expression level of genes thought to be involved in long-term storage of conditioned fear memories (Ressler et al., 2002). Blocking NMDA receptors interferes with extinction, a *new* learning process that does not erase the original CS-US association in fear conditioning (Santini et al., 2001). Other studies show that blocking protein synthesis, protein kinase A, and MAP kinase

activity (kinases involved memory consolidation) also interfere with memory consolidation of fear conditioning (Schafe et al., 1999).

There are links between methylene blue, LTP, and memory consolidation that may explain the memory enhancing action of MB on memory retention. We know that MB increases enzymatic activity of cytochrome oxidase (CO), the rate-limiting enzyme in the electron transport chain found in mitochondria (Callaway et al., 2002; Callaway et al., 2004; Gonzalez-Lima & Bruchey, 2004; Riha et al., 2005). Researchers have found that some mitochondrial genes, including transcripts for 3 subunits of the multi-subunit cytochrome *c* oxidase enzyme (Complex IV) are up-regulated and overexpressed during memory consolidation (Pinter et al., 2005). A study examined gene expression during memory consolidation of visual-olfactory conditioning in the American cockroach, which simulated an aspect of its foraging behavior. A food odor unconditioned stimulus (US) was paired with an LED visual cue conditioned stimulus (CS). Antennal projections toward the CS in the absence of the US following conditioning were recorded. Following the period of memory consolidation (determined to be 21-23 hours after training), control and trained subjects' brains were extracted, and subtractive hybridization was used to identify up-regulated and down-regulated gene transcripts. Of the 6 genes found to be up-regulated in the roach brain, 3 were transcripts for CO. The 3 roach complementary DNA clones for cytochrome oxidase that were found are 60-71% identical to human subunits. One subunit of mitochondrial ATPase (also mitochondrial DNA encoded) was also found to be up-regulated. According to the authors, these mitochondrial genes could be overexpressed in order to meet the energy demands of memory consolidation, which

could include structural changes in the synapse due to mitochondria in the neuronal terminals providing local autonomous DNA. Interestingly, up-regulation of CO gene expression was also found following induction of LTP in the mossy fiber pathway of the hippocampus (Lingala et al., 2005, unpublished). In-situ hybridization was performed for CO to verify its anatomical location, which was determined to be in the CA3 region of the hippocampus (Figure 5A). The authors hypothesized that CO may play a role in maintaining synaptic plasticity through the mossy fiber-LTP pathway by facilitating energy generation.

Figure 5A: The hippocampus. Following LTP induction in the hippocampus, increases in CO and pCREB were found in regions CA3 and CA1 respectively. (Cajal drawing from http://www.neuroscience.bham.ac.uk/neurophysiology/research/hippocampus.htm)



Studies implicating a role of CO in memory consolidation and LTP are not surprising, since we know that CO inhibition results in memory deficits (Bennett & Rose, 1992; Bennett et al., 1992; Callaway et al., 2002). During LTP and memory consolidation, increased energy demand in the brain may lead to an increase in CO and greater expression of CO genes. Also, by enhancing both nuclear and mitochondrial DNA transcription, more CO is synthesized, due to its bigenomic regulation. A study by Fride et al. (1989) examined cerebral protein synthesis during the memory formation of an active avoidance task in rats. The authors found that two stages of protein synthesis occurred during the formation of long term memories. The first one occurred in the cytoplasm, began with the commencement of training, and was independent of newly synthesized mitochondrial RNA. The second stage took place in mitochondria, began approximately 25 min after training, and was dependent upon newly synthesized mitochondrial RNA. Perhaps CO is the link to these findings.

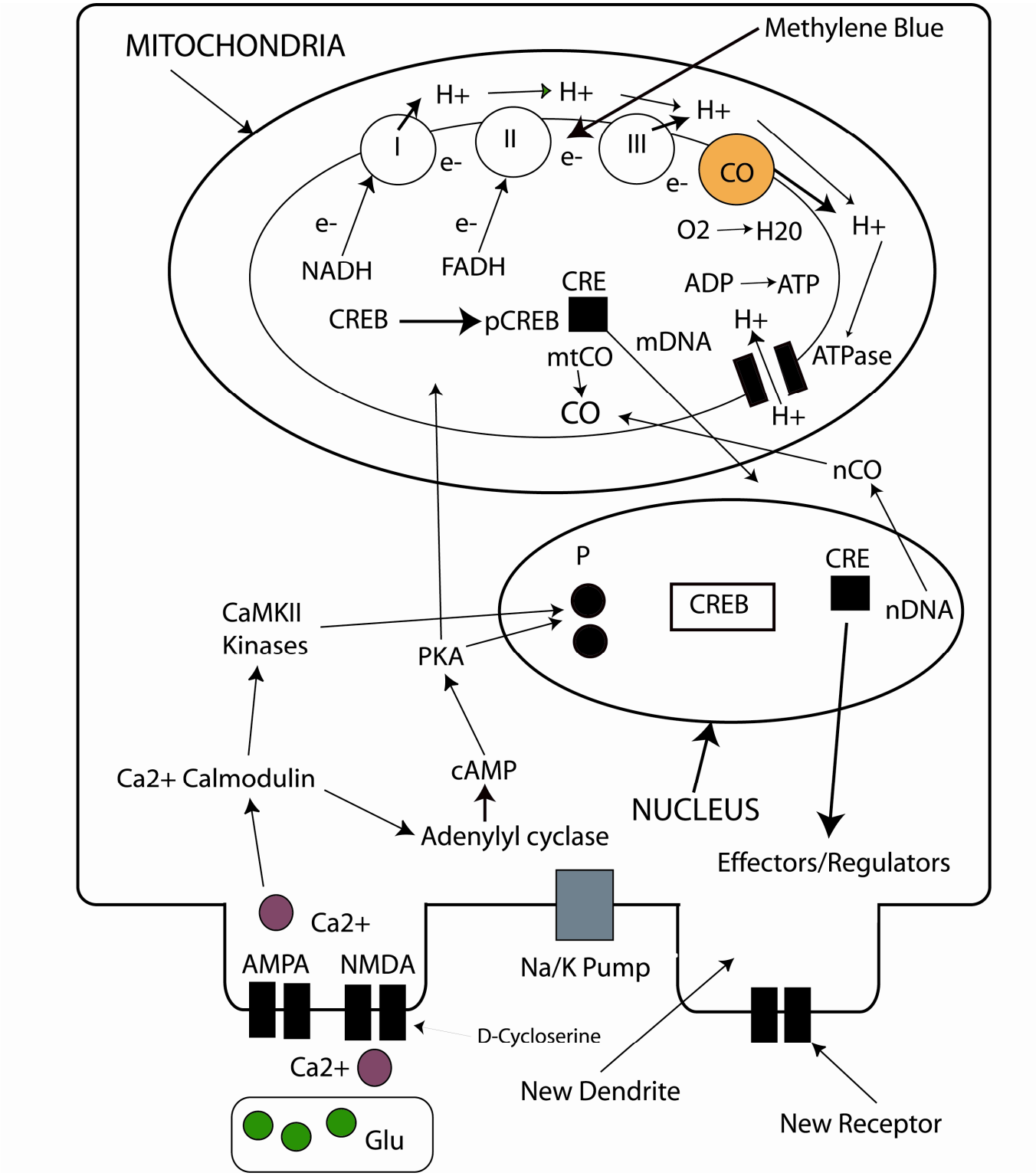
Another mechanism that could explain some of the memory retention enhancing effects of MB is that CREB has been found in mitochondria as well as in the nucleus of neurons. Cammarota et al. (1999) found immunoreactivity to pCREB in both synaptic and nonsynaptic mitochondria from hippocampus and cerebral cortex in the rat. This study also utilized immunoelectron microscopy analysis of hippocampal mitochondrial fractions and found the immunoreactivity to be localized to the inner mitochondrial membrane. Also, the CREB extracted from synaptic mitochondria was able to be phosphorylated *in vitro* by PKA (a kinase implicated in the molecular cascade occurring

in memory consolidation). The authors hypothesized that transcription factors in the dendrite may modulate gene expression that is transported to the nucleus.

A study by Bernabeu et al. (1997) found increased phosphorylation of CREB in CA1 hippocampal cells following memory formation of an inhibitory avoidance response in rats (Figure 5A). This is particularly interesting since the original work demonstrating that MB improved memory retention was in rats in an inhibitory avoidance paradigm (Martinez, Jr. et al., 1978). Also, Bevilaqua et al. (1999) found that rats killed 2 hours after training showed a significant increase in both pCREB and CREB immunoreactivities in synaptic mitochondria from the hippocampus following inhibitory avoidance learning. Figure 5B shows the many molecular cascades implicated in memory consolidation and how MB may be interacting with them.

Figure 5B: Learning causes activation of postsynaptic NMDA receptors, resulting in an influx of Ca2+, which is buffered by Ca2+ calmodulin. Ca2+ calmodulin activates kinases such as CaMKII which phosphoralate CREB, resulting in gene expression, as well as adenylyl cyclase which makes cyclic AMP, and activates PKA also phosphorylating CREB. Effector and regulator genes increase the number of AMPA receptors, enhance protein synthesis, and induce long-term plasticity in the brain. LTP is linked to g gene expression of mtDNA and there are increases in CO gene expression in memory consolidation. Both nuclear (nDNA) and mitochondrial DNA (mDNA) are required for CO synthesis. There is also evidence that pCREB and CREB are found in mitochondria. Abbreviations are as follows: Glu, glutamate; NMDA, N-methyl-D-aspartate receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; Ca2+, calcium; Ca2+ Calmodulin, calcium calmodulin; CaMKII Kinases, calcium calmodulin-dependent kinase II; cAMP, cyclic AMP; PKA, protein kinase A; P, phosphorylate; CREB, cyclic AMP response element binding protein; CRE, cyclic AMP response element; nDNA, nuclear DNA; nCO, nuclear cytochrome oxidase subunits; NADH, nicotinamide adenine dinucleotide; e-, electron; FADH, reduced flavin adenine dinucleotide; O2, oxygen; H2O, water; H+, hydrogen; ADP, adenosine diphosphate; ATP, adenosine triphosphate; pCREB, phosphorylated cyclic AMP response element binding protein; mDNA, mitochondrial DNA; mtCO, mitochondrial cytochrome oxidase subunits; Na/K Pump, sodium potassium pump. (Figure modified from Abel & Lattal, 2001)

Figure 5B: Caption on previous page.



5.3 THE NEURAL SUBSTRATES AND BRAIN CIRCUITRY IMPLICATED IN MEMORY OF FEAR CONDITIONING

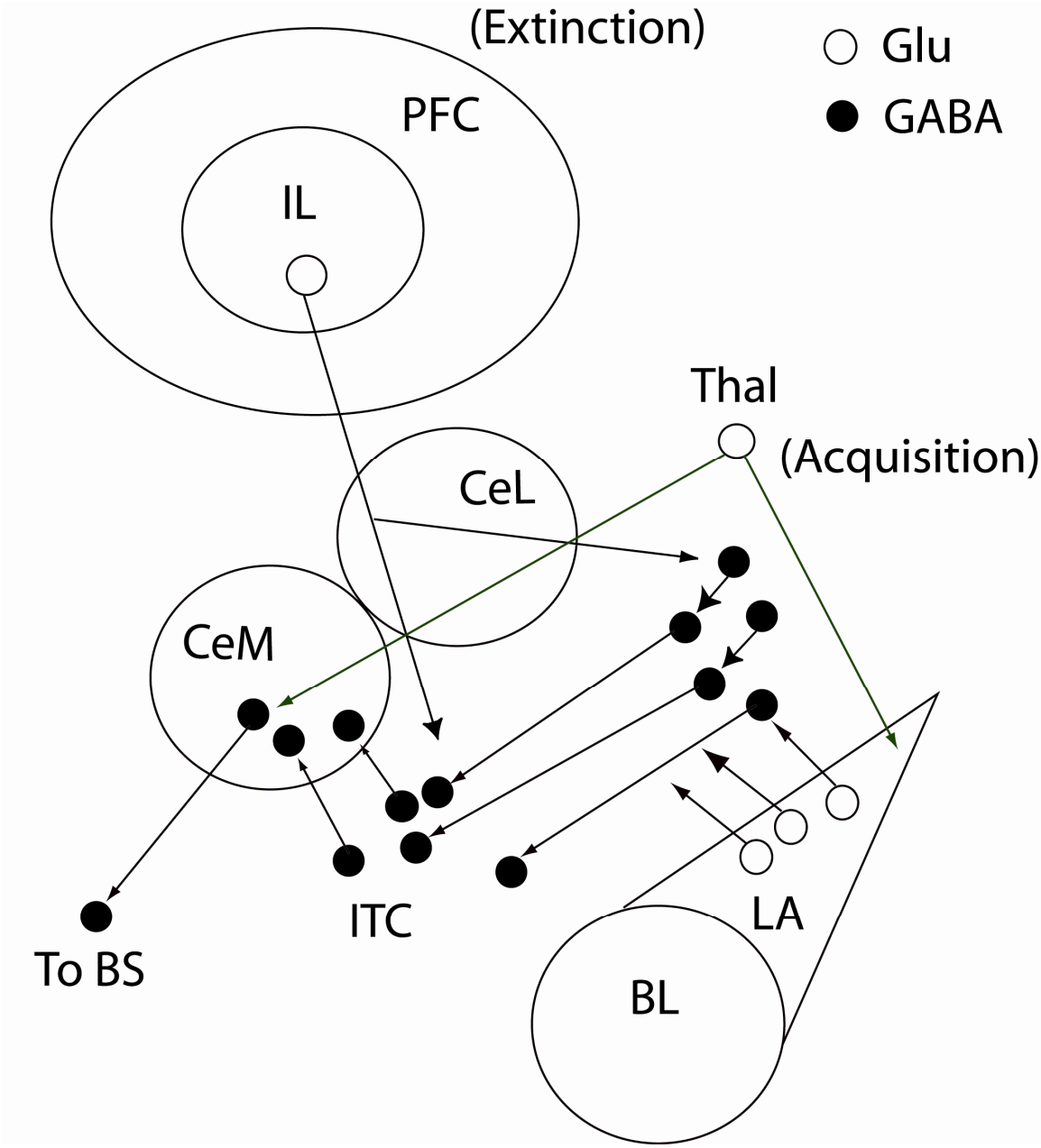
Many brain structures, pathways, and neurotransmitters have been implicated in the acquisition and extinction of Pavlovian fear conditioning. The hippocampus, located in the limbic system of the temporal lobe, is integral in acquisition learning of context and spatial memory. However, as memories consolidate and are retrieved, they become independent of the hippocampus (termed system consolidation), and are mostly stored in the prefrontal cortex (Matynia et al., 2001). Studies show that lesions of the hippocampus disrupt contextual fear conditioning if present during acquisition learning, but they do not affect memory if lesions are given weeks after training (Anagnostaras et al., 2001). Projections from the CA1 and subiculum regions of the hippocampus to the amygdala also mediate contextual fear conditioning. Cortical lesions greatly impair autobiographical and semantic memories (facts) with little impairment of recent memories (Graham & Hodges, 1997; Hodges & Graham, 1998). Imaging studies implicate that the left prefrontal cortex is more involved in memory encoding, whereas the right prefrontal cortex is more involved in memory retrieval (Poldrack & Gabrieli, 1998).

Several brain regions are thought to be involved in the acquisition and extinction of Pavlovian fear conditioning (for a good review see Sotres-Bayon et al., 2006). There is overwhelming evidence that the amygdala has integral involvement in the process of forming and storing memories for emotional events. The amygdala is located in the medial temporal lobe, and is composed of a group of subcortical nuclei. The lateral

nucleus of the amygdala (LA) is one site of plasticity in fear conditioning (LeDoux, 2000), since both CS and US inputs converge in it. Lesions of LA (Maren, 2001) interfere with acquisition of fear conditioning. LA receives CS input from the medial geniculate nucleus and posterior intralaminar nucleus (which are targets of inferior colliculus projections), and US somatosensory input from the thalamus (in regions targeted by the spinothalamic tract). The LA projects to the medial section of the central nucleus (CeM) by way of intercalated cell masses (ITC), which receive glutamatergic inputs from the LA. ITC cells are clusters of GABAergic neurons that generate feed-forward inhibition in the CeM (Pare & Smith, 1993). The CeM receives inputs from the spinal cord and trigeminal nucleus regarding nociceptive US inputs, as well as thalamic input on the CS from the posterior thalamic nuclei. Lesions of the central nucleus cause deficits in aversive conditioning (Amorapanth et al., 2000). The CeM also projects to brain stem nuclei such as the periaqueductal grey, involved in freezing behavior, as well as hypothalamic sites involved in regulating other aspects of the fear response (elevated heart rate, etc.). Activation of LA excites ITC cells, disinhibiting CeM neurons, and facilitating output of CeM, resulting in freezing behavior during acquisition of fear conditioning. We know that extinction does not erase the fear memory, rather it inhibits the expression of conditioned fear (Pavlov, 1927). This is thought to be mediated by the infralimbic region of the medial prefrontal cortex (IL) which sends glutamatergic projections to the ITC cells. IL lesions impair extinction (Quirk et al., 2000), and electrical stimulation of IL reduces expression of conditioned fear, overriding the former traumatic memory (Milad & Quirk, 2002). Also, in a metabolic brain mapping study of

extinction of fear conditioning, subjects who were extinguished to the tone CS showed significantly greater activity in the IL region than controls (Barrett et al., 2003). Recently it was also found that chemical stimulation of IL increased c-Fos expressions in amygdala ITC cells (Royer & Pare, 2002). Figure 5C shows the pathways thought to be involved in acquisition and extinctions of fear conditioning.

Figure 5C: Diagram of the pathways involved in acquisition and extinction of fear conditioning. Abbreviations are as follows: LA, lateral amygdala; BL, basolateral amygdala; ITC, intercalated cell mass; Thal, thalamus; CeL, lateral central nucleus of the amygdala; CeM, medial central nucleus of the amygdala; BS, brain stem; IL, infralimbic region; PFC, prefrontal cortex. (Adapted from Pare et al., 2004)



Several neurotransmitters have been implicated in memory retention in the amygdala. Cholinergic projections from the basal forebrain to the cerebral cortex have been implicated in cortical plasticity (Pare, 2003), and the LA has connections to the basal forebrain. Post-training infusions of cholinergic agonists in the LA enhance memory for inhibitory avoidance, and fear conditioning (Power et al., 2002; Vazdarjanova & McGaugh, 1999). Through its connections to the basal forebrain, LA may improve memory by stimulating cholinergic activation of the cortex. Also, drugs and stress hormones activate release of norepinephrine in the LA (McGaugh et al., 2002). Amygdala norepinephrine levels are increased following inhibitory avoidance training and are correlated with memory retention in the task, and studies show that epinephrine (which enhances memory) increases norepinephrine release in the amygdala (McGaugh, 2002) leading some investigators to hypothesize that norepinephrine release in the amygdala may play an important role in memory consolidation.

Prefrontal dopamine is implicated in attention and arousal in working memory (Robbins, 2005). A study of discrimination learning in the rat found increased levels of dopamine and its metabolites (during learning sessions) in the amygdala showing the importance of the neurotransmitter in this task (Hori et al., 1993). Together, these findings demonstrate that memory is a distributive process located throughout the brain and is mediated by many processes.

5.4 FUTURE DIRECTIONS

5.4.1. Testing the Memory Enhancing Effects of MB in Humans with Anxiety Disorders

Anxiety disorders are among the most common mental health problems, affecting approximately 19 million Americans. Only 30% of these people receive psychological or pharmacological treatment (Barlow, 1998). Anxiety disorders usually include affective components such as feelings of fear, tension, and apprehension, as well as physiological responses such as increased blood pressure and heart rate. Humans treated for anxiety disorder often undergo exposure therapy using the principles of extinction. Extinction does not erase the initial CS-US association; rather it forms a new memory, which competes with the conditioned memory for control of fear expression (Milad et al., 2006). Exposure therapy requires repeated confrontation with the phobic stimuli. In flooding, or implosion therapy, exposure to phobic stimuli either through imagery or reality, is at an intense level for a prolonged period of time (studies have shown that the faster fear reduces during exposure therapy, the less likely it is that there will be return of fear). Flooding is defined as exposure to the traumatic cues to promote the experience of anxiety (or other aversive emotions) in the context of therapy. Patients experience the emotions associated with the traumatic cues, until the event or cues become increasingly less aversive. Interestingly, it has been found that patients who are encouraged to use safety signals, or props to cope with the feared situation, report more fear following exposure treatment than patients encouraged to focus on their fear during exposure (Sloan

& Telch, 2002). In patients with phobias and PTSD, exposure therapy is conducted to extinguish the fear connected to the traumatic memories, it does not however extinguish the memory itself. In doing so, it inhibits the fear associated with the traumatic memory, but the memory of the fear is still intact. This is why subjects who have gone through extinction of conditioned fear, sometimes show spontaneous recovery, or the reappearance of a CR over time that was thought to be extinguished. Renewal (the return of conditioned responding when a subject is tested in the original acquisition context following extinction in a different context), and reinstatement (the return of conditioned responding to the CS following extinction when US alone presentations are made) are also issues that lead to difficulties in treatment of phobias and PTSD. Some studies show that if exposure therapy is done in multiple contexts, renewal effects can be reduced (Hermans et al., 2006). Due to patients' avoidance of the fear evoking stimuli and the anxiety they cause, compliance is a big issue in this field. Return of fear is also a critical issue, which could result from insufficient initial exposure (Rachman, et al., 1988), distraction during treatment (Kamphuis & Telch, 2000), and failure to extinguish fear in relevant contexts (Bouton, 2000). Due to the nature of the complications of compliance issues and causes for return of fear, finding a pharmacological treatment that could reduce the number of sessions required for exposure therapy while increasing their effectiveness, would be an ideal situation.

Methylene blue is a promising candidate for such a treatment. Based on our current findings that MB facilitates extinction memory in the congenitally helpless (CH) rat, an animal model of post-traumatic stress disorder, and earlier findings that MB

improves extinction memory when administered post-extinction in normal rats (Gonzalez-Lima & Bruchey, 2004), it is of interest to test this compound in human subjects undergoing exposure therapy for anxiety disorders. Benzodiazepines, selective serotonin reuptake inhibitors, and beta-blockers have shown some effectiveness in this regard (Lydiard & Falsetti, 1995; Meyer & Quenzer, 2005), but they generally do not enhance the effectiveness of exposure therapy. Also, studies show that mismatch of internal states pre- and post-treatment, if manipulated by drug effects, can lead to return of fear (Bouton & Swartzentruber, 1991).

D-cycloserine, a partial NMDA agonist (shown in the bottom of Figure 5B), has recently been proposed to improve extinction memory and reduce fear relapse (Ressler et al., 2004; Richardson et al., 2004). It has been reported to decrease social anxiety, as well as fear of heights, in humans when administered in addition to exposure therapy (Hofmann et al., 2006; Ressler et al., 2004). D-cycloserine has several limitations however, such as CS non-specificity and subjects' tolerance to repeated administrations (Ledgerwood et al., 2005; Parnas et al., 2005; Quartermain et al., 1994). Rats extinguished to a light CS and injected with D-cycloserine also show reduced fear to a tone CS that has not been extinguished (Ledgerwood et al., 2005), suggesting generalization of extinction effects produced by the drug, which could result in a maladaptive reduction of fear to unrelated but real threats. Also, repeated exposure to D-cycloserine results in loss of its effectiveness as a facilitator of extinction (Parnas et al., 2005). If administered intermittently, and in the short-term, however, D-cycloserine has

been shown to be very therapeutic in conjunction with exposure therapy (Hofmann et al., 2006; Ressler et al., 2004).

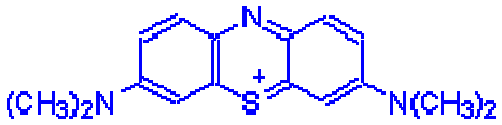
L-type voltage-gated calcium channels (LVGCCs) also play a role in fear conditioning. Studies have demonstrated that the LVGCC blocker nifedipine enhances memory retention when infused into the hippocampus (Quevedo et al., 1998). LVGCCs are hypothesized to mediate long term changes in neuronal activity. Studies have shown that LVGCCs are essential for the extinction, but not for the acquisition or expression, of conditioned fear in mice (Cain et al., 2002).

Unlike other treatments proposed for treatment of anxiety disorders, MB does not appear to be working as an anxiolytic compound or on a neurotransmitter system. Drugs working on pharmacologically specific synapses have actions distributed throughout the brain, in relevant regions, as well as other regions that could lead to unwanted side effects. Methylene blue selectively enhances metabolism in the brain during memory formation, in regions related to the task, without the side effects of drugs acting on synaptic transmission elsewhere in the brain. It can be used to facilitate extinction memory formation for a specific fear-evoking stimulus, and can facilitate extinction training through repeated administrations without losing its effectiveness. Also, since patients with PTSD show decreased activity in medial prefrontal cortex (Bremner et al., 1999), which is implicated in memory of extinction, MB could provide increased metabolic activity in this region through CO enzyme induction, leading to facilitation of extinction.

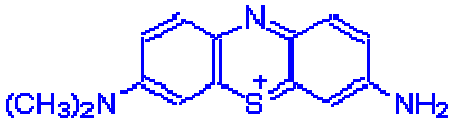
5.4.2 More Therapeutic Interventions

Oxidation of methylene blue results in 3 strongly metachromatic dyes: Azure A, Azure B, and Azure C (Marshall, 1976). (Figure 5D).

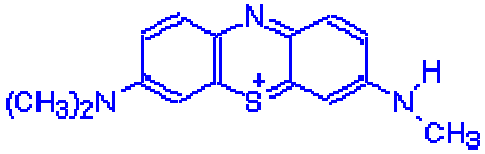
Figure 5D: Comparison of MB and its derivatives following oxidation. (From http://stainsfile.info/StainsFile/dyes/52015.htm)



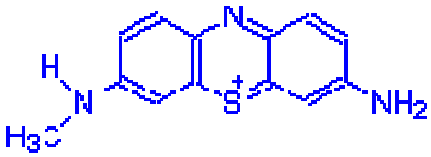
Methylene blue



Azure A



Azure B



Azure C

Taniguchi et al. (2005) reported that several phenothiazines such as MB (and its derivatives azures A, B, and C) are able to inhibit tau filament formation, which is mostly made up of tau protein, considered to be a defining neuropathological characteristic of Alzheimer's disease. Thus, these compounds may be useful for treatment in animal models of Alzheimer's disease as a way to inoculate against the formation of neurofibrillary tangles. Also, MB and azures A, B, and C are photoactive and have been proposed for virus inactivation of blood products containing blood borne viruses Hepatitis B and C, HIV, and Parvovirus B19 (Mohr et al., 1997; Specht, 1994). This is because, in combination with light, these compounds create oxidative stress in DNA (8-hydroxy-guanine). This study found that using light emitting diodes can improve virus kill and reduce damage to plasma proteins.

It would be of interest to examine the effects of azures A, B, and C on memory retention in various behavioral paradigms, to see if one derivative or another may be more useful in the facilitation of memory retention. There have been no behavioral studies examining the effects of these compounds to date. It could be that one of the azure stains would be even more effective than MB if they were tested in similar experiments. If so, they could be very exciting compounds to study for the facilitation of memory retention and may contribute to a better understanding of the effects we already observed in methylene blue treatment as a metabolic enhancer.

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Vita

Kathryn Marigrace Wrubel was born on October 17, 1976 in Wayne, Michigan. Her parents are Mary Beth (Gillespie) Wrubel and Dennis Eugene Wrubel and she has one sibling, James Clay Wrubel, 3 years her senior. Kathryn attended Canton High School where she graduated in 1994. She continued her education at Eastern Michigan University (E.M.U.) where she graduated with honors with a B.S. in psychology and biology minor in 1999. While there, Kathryn was the recipient of many scholarships including the Michigan Association of Governing Boards Outstanding Student Award for the top student at E.M.U., the Robert Anderson Award for the top student in Psychology, the Golden Key National Honor Society Scholarship for Junior Initiate, the Pursell Scholarship for the College of Arts and Sciences, a Michigan Competitive Scholarship, and an E.M.U. Campus Leader Award. Kathryn also attended the University of Texas at Houston Summer Research Program where she spent the summer of 1998 studying impulsivity in sociopaths. She attended the E.M.U. Undergraduate Symposium in Excellence for many years and in 2005 the school brought her back for the 25th Annual Undergraduate Symposium as an example of excellence for the Psychology Department. Kathryn began doctoral study in Behavioral Neuroscience at the University of Texas at Austin with Dr. Francisco Gonzalez-Lima in the fall of 2000. She received two nationally competitive academic fellowships while studying there, a Society for Neuroscience Minority Graduate Fellowship and a Texas Consortium in Behavioral Neuroscience Fellowship. Kathryn has a 10 year-old daughter, Eve Marie Wrubel, who means the world to her. She also owns a small online business selling and breeding crested geckos (www.katiscresteds.com), and enjoys live music, dogs, black & white photography, and collecting antiques from the 1950s and 1960s.

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