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ScienceDirect
Neurobiology of Learning and Memory 87 (2007) 209–217
Neurobiology of Learning and Memory
www.elsevier.com/locate/ynlme

# Methylene blue facilitates the extinction of fear in an animal model of susceptibility to learned helplessness

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Received 29 June 2006; revised 22 August 2006; accepted 23 August 2006
Available online 2 October 2006

**Abstract**

The objectives were to (1) extend previous findings on fear extinction deficits in male congenitally helpless rats (a model for susceptibility to learned helplessness) to female congenitally helpless rats, and (2) attempt a therapeutic intervention with methylene blue, a metabolic enhancer that improves memory retention, to alleviate the predicted extinction deficits. In the first experiment, fear acquisition (four tone-shock pairings in operant chamber) was followed by extinction training (60 tones in open field). Congenitally helpless rats showed fear acquisition similar to controls but had dramatic extinction deficits, 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 extinction probe conducted one month later. In the second experiment (which began one month after the first experiment) congenitally helpless subjects were further exposed to tones for 5 days, each followed by 4 mg/kg methylene blue or saline IP, and had a fear renewal test in the acquisition context. Methylene blue administration improved retention of the extinction memory as demonstrated by significant decreases in fear renewal as compared to saline-administered congenitally helpless subjects. The impaired ability to extinguish fear to a traumatic memory in congenitally helpless rats supports the validity of this strain as an animal model for vulnerability to post-traumatic stress disorder, and this study further suggests that methylene blue may facilitate fear extinction as an adjunct to exposure therapy.


*Keywords:* Extinction; Post-traumatic stress disorder; Congenitally helpless rats; Methylene blue; Fear conditioning; Metabolic enhancer; Animal model; Disease susceptibility; Memory retention; Exposure therapy; Cytochrome oxidase

## 1. Introduction

Inescapable electric shock prevents animals from subsequently learning an escape response, a phenomenon termed learned helplessness (Overmier & Seligman, 1967). Vulnerability to learned helplessness is hereditable and can be enhanced through selective breeding, as evidenced by the creation of a strain of congenitally helpless rats (Henn & Vollmayr, 2005). Studies show that up to 95% of the congenitally helpless offspring typically show the learned helpless phenotype, as opposed to the 5–20% of randomly bred rats (Henn, Johnson, Edwards, & Anderson, 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 post-traumatic stress disorder (PTSD) is genetic (Fava & Kendler, 2000; Sanders, Detera-Wadleigh, & Gershon, 1999; True et al., 1993), an appropriate animal model of congenital vulnerability to these disorders could be very useful, especially in the development of therapeutic treatments.

A recent paper reported behavioral characteristics of male rats predisposed to learned helplessness (Shumake, Barrett, & Gonzalez-Lima, 2005). Compared to normal rats, congenitally helpless rats demonstrated reduced

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1074-7427/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.nlm.2006.08.009

reward sensitivity, high novelty seeking, an increase in conditioned fear, and a deficit in fear extinction. This behavioral phenotype appears similar in many respects to that of PTSD patients. For example, patients with PTSD show stronger acquisition and reduced extinction of aversively conditioned responses (Orr et al., 2000), and a personality profile of low reward dependence, high novelty seeking, and high behavioral inhibition (Richman & Frueh, 1996; Wang et al., 1997). Also, humans with PTSD show deficits in extinction of conditioned fears (Orr et al., 2000; Peri, Ben-Shakhar, Orr, & Shalev, 2000) and congenitally helpless rats also demonstrated similar deficits when compared with normal control rats (Shumake et al., 2005). Human studies have shown that females are more vulnerable to PTSD (with approximately twice the risk), and that their symptoms persist longer than those of males (Nemeroff et al., 2006). Based on the human studies, one would expect congenitally helpless females to show greater extinction deficits than males. The first objective of the present study was to extend our previous findings on fear extinction deficits to female congenitally helpless rats.

Methylene blue (MB), a metabolic enhancer, has been shown to improve memory for extinction of fear conditioning in normal rats (Gonzalez-Lima & Bruchey, 2004). The second objective of the present study was to determine if MB could facilitate extinction in congenitally helpless subjects. 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 congenitally helpless subjects. If so, MB may be a useful therapeutic adjunct to exposure therapy for patients with PTSD to aid in retention of extinction of conditioned fears to traumatic memories.

Methylene blue is a redox dye commonly administered as an antidote for 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 used safely in humans for over a century. 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. MB has also been shown to enhance memory retention in a spatial memory task, an object recognition task, and to aid in between-days habituation to a familiar environment (Callaway, Riha, Bruchey, Munshi, & Gonzalez-Lima, 2004; Riha, Bruchey, Echevarria, & Gonzalez-Lima, 2005). More relevant to the present study is that Gonzalez-Lima and Bruchey (2004) reported that memory retention of extinction of Pavlovian fear conditioning could be enhanced with post-extinction administration of MB in normal rats. Total post-extinction freezing scores were lower in subjects receiving MB than saline, and MB-treated subjects also had a longer lasting effect of extinction. Therefore, the current study was undertaken to determine if congenitally helpless rats, which are resistant to extinction, can likewise have their extinction learning improved by methylene blue.

## 2. Materials and methods

### 2.1. Subjects

For the first experiment, subjects were 23 female 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 Sprague–Dawley control rats obtained from Harlan (Houston, TX), all weighing approximately 300 g at the beginning of the experiment. The congenitally helpless subjects used in this study were bred in our laboratory and were not tested for susceptibility to learned helplessness, as this would involve administration of foot shocks prior to acquisition training. Subjects were housed 2–3 per cage under standard laboratory conditions with a 12 h 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.

### 2.2. 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.

#### 2.2.1. Acquisition context (Context A)

Pavlovian tone foot-shock acquisition training was conducted in four operant conditioning chambers, each measuring $22 \times 25 \times 32$ 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/s, 15 s 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 s 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.

#### 2.2.2. Extinction context (Context B)

Extinction training occurred in a different context. Two open-field activity boxes measuring $31 \times 45 \times 45$ 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 ($16 \times 16$, 2.54 cm apart), with two arrays located 1 cm above the floor of the chamber. Rearings were detected with a vertical-axis