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Targeting mitochondria via methylene blue: Implications in memory enhancement and neuroprotection

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Targeting mitochondria via methylene blue: implications in memory enhancement and neuroprotection

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Dedication

To Kenneth and Linda Auchter for cultivating my inquisitive mind.

To Kristin Stewart for preventing me from losing it.

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I would like to thank several people, without whom this dissertation would not be possible. Firstly, I would like to thank my partner, Kristin Stewart, who has not only supported me through my academic pursuits, but has navigated the PhD process along with me. Obtaining our doctorates from the University of Texas at Austin together is an indescribable experience and an extraordinary accomplishment. Secondly, I'd like to thank my immediate family—Ken, Linda and Becca Auchter—who has supported me tremendously through this process. I would also like to thank my family-in-law—the rest of the Stewart clan—who has been a fantastic source of encouragement. Thirdly, I would like to thank my advisors, committee members and fellow graduate students, who together created a supportive, nurturing and collaborative environment. Finally, I would like to thank all the rats who served as subjects for this dissertation, without whom I could not have conducted the following research—an effort aimed to contribute to scientific understanding for the betterment of society.

Targeting mitochondria via methylene blue: implications in memory enhancement and neuroprotection

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The University of Texas at Austin, 2015

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Memory—though seemingly simple in concept—is altogether a notoriously elusive and difficult process to understand. While some memories are fragile and changeable under certain circumstances, others are persistent and difficult to erase. This work is a comprehensive investigation into memories: how to change persistent ones, enhance complicated ones and protect delicate ones.

The first objective of this work was to explore a strategy for manipulating fear memories, which are notoriously difficult to erase. This was done by testing different parameters for attenuation of fear using extinction as a strategy for interrupting fear memory reconsolidation. Briefly, by manipulating extinction parameters such that subjects were unable to

predict the occurrence of fearful stimuli, we were able to maximize the degree of memory updating.

Since memory processing is contingent upon specific neuronal activity, and neuronal activity is primarily fueled by products of mitochondrial respiration, the second and third objectives of this work focused largely on how manipulating mitochondrial activity enhances and protects memory processes respectively. This was done using mitochondrial enhancer methylene blue (USP grade). Methylene blue (MB) is a synthetic dye with a unique ability to cross the blood-brain barrier and diffuse into neuronal mitochondria. There, it serves as a redox electron cycler, increasing neurons' capacity for mitochondrial respiration.

The second objective of this work examined the effect of post-extinction administration of MB and its interaction with reconsolidation update mechanisms in the persistent attenuation of fear memories. Building on findings from the first objective, we found that administering MB after extinction helped subjects maintain the fear attenuation induced by extinction.

The third objective explored the ability of MB to prevent the cognitive deficits arising from chronic cerebral hypoperfusion, a risk factor for mild cognitive impairment and Alzheimer's disease. Finally, quantitative

cytochrome oxidase histochemistry (a marker for mitochondrial activity) was used to map the effects of MB on brain mitochondria in the hypoperfusion model. Methylene blue attenuated some of the cognitive deficits that arose from chronic cerebral hypoperfusion, which was reflected in enhanced brain mitochondrial activity.

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Targeting mitochondria via methylene blue: implications in memory enhancement and neuroprotection

Chapter 1: Introduction

Memory formation is a highly dynamic process. Though our understanding of memory has advanced tremendously over the past few decades, learning and remembering have proven to be some of the most complex and elusive biological phenomena. This work is a compilation of experiments aimed to explore how memories are changed and how they can be protected. I begin with an introduction to the concepts addressed in this dissertation. In the following chapters, I will detail the ideas and experiments I have conducted as a part of my graduate work, seeking to understand how maladaptive memories can be modified and how advantageous ones can be enhanced and protected. The final chapter serves as an overall discussion of my findings.

1.1 A brief history of the study of learning and memory

The study of human memory spans back at least 2,000 years to Aristotle's comparison of the human mind to a "blank slate", theorizing that all humans are born free of any knowledge and are merely the sum of their experiences. However, a scientific approach to studying memory was not

developed until the late-1800s when Herman Ebbinghaus described the learning curve and the forgetting curve after performing experiments on himself (Ebbinghaus, 1885). Ivan Pavlov (1904) later became a spokesman for the scientific method, stating in his Nobel address

> *Essentially, only one thing in life is of real interest to us—our psychical experience. Its mechanism, however, was and still is shrouded in profound obscurity. All human resources—art, religion, literature, philosophy, and the historical sciences—all have joined in the attempt to throw light upon this darkness. But humanity has at its disposal yet another powerful resource—natural science with its strict objective methods. (Nobel lecture)*

Undoubtedly, though Pavlov's original focus was in physiology, Pavlov contributed substantially to our understanding of memory, and his legacy is his development of the understanding of "conditioned reflexes"[1]. In Pavlov's

[1] Actually, professor of the history of medicine at John's Hopkins University Daniel P. Todes (2014) points out that Pavlov never actually used the term "conditioned reflexes". This term comes from a bad translation of the Russian *uslovnyi*, or "conditional" reflex. For Pavlov, the emphasis fell on the contingent nature of the reflexes, which he believed involved a connection between a subcortical brain region (which supported instincts) and a cortical one (where associations were built). Interestingly, at that time such conjectures about brain circuitry flew in the face of behaviorist theory, which viewed the mind as a "black box". As such, this mistranslation is serendipitous, as it is the behaviorists who adopted and popularized Pavlov's classical conditioning as an important behavior-analytic process.

(Pavlov, 1927) original experiments, a neutral stimulus (a ticking metronome; conditioned stimulus; CS) was paired with a biologically relevant stimulus (meat powder; unconditioned stimulus; US) which—on it's own—elicited an unconditioned response (salivating; UR). This pairing caused an association to form between the stimuli, such that subsequent presentations of only the CS induced a conditioned response (salivating;[2] CR). If the CS is presented repeatedly in absence of the US (such that the CS no longer predicts the US), then eventually the CR will diminish in a process called extinction. Interestingly, the finding that conditioned responses extinguished was not particularly surprising or interesting to Pavlov. What interested him was that when subjects were presented with the CS again after a delay, the conditioned response spontaneously recovered.

Pavlov's findings shed light on how associations are formed and changed, which—as will be discussed later—are critical concepts used not only to understand how memories are stored and maintained, but also to understand and treat psychiatric disorders thought to arise from maladaptive responses to memories (e.g. phobias and post-traumatic stress disorder). Undeniably, Pavlov's devotion to dogs' saliva drops and carefully

[2] Though both the UR and CR in this case is salivation, Pavlov noted that the saliva produced by the CS differed in composition from the saliva produced by the US. For something to be considered true classical conditioning, the CR and UR should be distinct from each other. The CR should be any new response to the previously neutral CS that can be clearly linked to experience with the conditional relationship of CS and US (Bouton, 2007; Rescorla, 1988).

controlled laboratory experiments has not only motivated scientific practice, but also provided us with a foundation for subsequent memory research.

Also of note in the field of memory research are the pioneering studies of Müller and Pilzecker in the early 1900s, in which they discovered that memory for newly learned information was disrupted by learning of other information shortly after the original learning (Müller & Pilzecker, 1900). From this finding, they deduced that processes underlying new memories are initially fragile and consolidate over time. Even now, the consolidation hypothesis of memory still guides research into the time-dependent involvement of neural systems enabling lasting memory (Dundai, 1996; Glickman, 1961; Herz, 1972; Lechner, Squire & Byrne, 1999; McGaugh, 1966; Polster, Nadel & Schacter, 1991 Weingartner & Parker, 2014).

As technology advanced in the 1940's, theories of memory encoding and storage began to emerge. Karl Lashley dedicated his life to finding where "engrams", theoretical memory traces, are formed in the brain. By systematically lesioning different areas of cortex in rats and then testing the rats in mazes, Lashley attempted to pinpoint where these memory traces were kept. Lashley never pinpointed one specific brain region, concluding after 25 years of research that memories are not localized in one brain region, but rather are widely distributed throughout the cortex, and that if certain parts of the brain are damaged, other parts of the brain may take on the role of the damaged portion (Lashley, 1950). This was an important finding for neuroscience, as dogma at that time suggested that once developed, the brain was physiologically static.

Later, Richard F. Thompson continued the search for the engram in the cerebellum, rather than the cerebral cortex. Using classical conditioning of eyelid responses in rabbits (whereby a tone CS was paired with a puff of air to the cornea US such that the tone alone elicited an eyeblink conditioned response) and examining several brain regions, Thomson found that the lateral interpositus nucleus in the cerebellum was a key element for the conditioned eyeblink response (McCormick & Thompson, 1984). This approach, though successful, examined only basic automatic responses, which almost all animals possess as defense reflexes.

The works of Pavlov, Lashley and Thompson are only a few examples of learning and memory research in the 20th century. Subsequent studies have provided a framework for memory that falls somewhere between Lashley's interpretation of whole-brain memory representation and the idea that memories are housed within a specific brain region or cell. Though there is still some debate among scientists about how memories are encoded and stored (i.e. the nature of the engram), we now understand that most memories are more comprehensive than the simplistic, reflexive[3] mechanisms of the eyeblink conditioning, but more focused than whole-brain

[3] I refer to cerebellar eyeblink responses as "simplistic" and "reflexive" because the stimulus-response mechanisms in this case are largely contained between direct sensory inputs (e.g. cochlear nuclei in the case of a tone CS), brainstem nuclei (e.g. pontine nuclei) and cranial nerves that control various eye muscles (Lewis et al., 1987; Steinmetz et al., 1987; Steinmetz & Sengelaub, 1992; Thompson et al., 1997). The sensory information does not typically "reach out" to other brain structures for assistance in forming these associations.

representation. A plethora of studies have demonstrated that declarative, or explicit, memories are particularly represented in limbic system structures (e.g. the hippocampal formation and amygdala) and outer cortical regions (e.g. prefrontal cortex). The literature on this particular topic is extensive, and will be discussed further in the following sections and chapters (for a review, see Eichenbaum, 2000).

1.2 Understanding memory mechanisms

1.2.1 Consolidation and reconsolidation

Memory consolidation is the process of stabilizing or storing memories after acquisition (Burnham, 1903; Müller & Pilzecker, 1900; Ribot, 1882). Though it was once believed that memories are plastic during formation but stable once established (Dundai, 2004; Kandel, 2001; Martin, Grimwood & Morris, 2000; Müller and Pilzecker, 1900), it is now firmly established that memory retrieval is not simply a passive readout of a previously consolidated memory. Rather, is a more complex process whereby upon recollection, the original memory enters into a destabilized state, and maintenance of the memory requires restabilization by means of reconsolidation (Misanin, Miller, & Lewis, 1968; Nader & Einarsson, 2010; Nader, Schafe, & LeDoux, 2000a; Nader, Schafe, & LeDoux, 2000b). Evidence from several species suggests that reconsolidation blockade via pharmacological (Alberini, 2005; Dębiec & LeDoux, 2004; Dębiec & LeDoux, 2013; Duvarci & Nader, 2004; Fricks-Gleason & Marshall, 2008; Lee, Milton & Everitt, 2006; Nader, Schafe, & LeDoux, 2000; Stern et al., 2012) or

behavioral (Jones & Monfils, 2013; Kindt & Soeter, 2013; Millan, Milligan-Saville & McNally, 2013; Monfils et al., 2009; Olshavsky et al., 2013; Oyarzún et al., 2012; Pedriera & Maldonado, 2003; Quirk et al., 2010; Schiller et al., 2010; Xue et al., 2012) intervention during a specific post-retrieval reconsolidation window alters the memory. Not only does this understanding shed light on the way we compile our experiences into memories throughout our lives, but the ability to update memories after they have been consolidated has tremendous clinical relevance for treatment of fear and anxiety disorders.

From an evolutionary perspective, experiencing fear and anxiety in response to particular learned cues is crucial for survival. An organism must be able to form associations between environmental predictors and aversive stimuli in order to avoid dangerous situations. However, it is equally important to extinguish fear when cues that once predicted danger are no longer predictive of aversive stimuli. It has been postulated many times (and is now widely accepted) that failure to extinguish fear responses is an important mechanism in the pathogenesis of fear and anxiety disorders (Hoffman, 2008; Milad et al., 2006; Mineka et al., 1996; 2008; Myers & Davis, 2002).

In conditioned fear paradigms, extinction involves exposing a previously conditioned subject repeatedly to the fear-eliciting—but not inherently aversive—stimulus (CS) in the absence of the aversive stimulus (US). As described previously, this leads to gradual reduction in the emotional response to the CS (Pavlov, 1927). Interestingly, though extinction

leads to suppression of the conditioned response, extinction itself does not change the original fear memory. If that were the case, then Pavlov would not have observed spontaneous recovery of the conditioned response after a time delay. Rather, evidence suggests that extinction results in the formation of a new inhibitory memory as the predictive value of the CS preceding the US lessens (Barrett et al., 2003; Effting & Kindt, 2007; Morgan, Romanski & LeDoux, 1993; Pavlov, 1927; Quirk et al., 2000; Quirk & Mueller, 2008; Robbins, 1990).

1.2.2 The challenges of reconsolidation updating in regard to fear memories

Unfortunately, using reconsolidation updating to reduce fearful responses is challenging, as fear memories are particularly resistant to change. This is problematic, as lingering fear memories leave organisms vulnerable to return of fear after extinction through the passage of time (spontaneous recovery), CS exposure in a new context (renewal), or additional US exposure (reinstatement). Fortunately, Monfils et al. (2009) devised an approach to fear attenuation that combines the principles of reconsolidation updating and extinction. They were able to use extinction to interfere with reconsolidation by giving extinction training during a retrieval-induced labile period. This manipulation persistently attenuated fear responses in spontaneous recovery, renewal and reinstatement paradigms, implicating retrieval + extinction as a novel and non-invasive approach to fear memory updating.

The retrieval + extinction paradigm has been replicated several times in both animals (Auber et al., 2013; Ma, Zhang & Yu, 2012; Millan, Milligan-Saville & McNally, 2013; Olshavsky et al., 2013; Quirk et al., 2010; Xue et al., 2012) and humans (Kindt, Soeter & Vervliet, 2009; Schiller et al., 2010; 2013). However, there are several questions that still need to be answered in order to fully utilize post-retrieval strategies to disrupt or weaken pathogenic memories. For instance, it still remains somewhat unclear under what extinction conditions memories are updated (Hutton-Bedbrook & McNally, 2013).

1.2.3 A potential role for unpredictability in fear memory updating

It has been suggested that the functional role of reconsolidation is to keep older memories up to date with new learning, and that reconsolidation is triggered only when there is an opportunity for new learning to take place during reactivation (Morris et al., 2006; Pedriera, Perez-Cuesta & Maldonado, 2004; Sevenster, Beckers & Kindt, 2012). This line of thought is rooted in the evidence that associative learning requires prediction error (Rescorla & Wagner, 1972). However, newer models of learning and memory suggest that too much prediction error actually induces new learning rather than memory updating (Gershman et al., 2010; Gershman & Niv, 2012; Redish et al., 2007). This evidential discrepancy inspired the first objective of this dissertation—to explore how the element of unpredictability or randomness during extinction influences prediction error and consequentially modulates memory updating.

1.2.4 Other potentially important factors in fear memory updating

Additionally, there is a large degree of individual variability in extinction alone, and equally as much (if not more) variation in the retrieval + extinction paradigm. Thus, it is important not only to describe the optimal parameters for the retrieval + extinction paradigm, but also to investigate the implications such individual variability. This observation inspired the second objective of this dissertation—to explore the relationship between individual responsiveness to extinction and fear memory updating. To add another layer to this idea, I also combined the previously described conceptualization of the retrieval + extinction paradigm with a current strategy for memory enhancement via upregulation of the brain's metabolic energy mechanisms.

1.3 The brain's need for energy

Though the human brain accounts for only 2% of the body's weight, it consumes about 20% of its resting metabolism (Kety, 1957; Rolfe & Brown, 1997; Sokoloff 1960). Since no organism more neurally complex than an arthropod can survive without a brain, it is supremely important that its metabolic machinery is operational, and that the neurons containing the organelles operating said machinery—mitochondria—are supplied with the necessary substrates.

Disturbances in the normal supply of glucose and oxygen to brain tissue (e.g. via ischemic attack or cerebral hypoperfusion) result in mitochondrial (Lin & Beal, 2006; Moreira et al., 2007), cognitive and

behavioral dysfunction (Marcus et al., 1989; Meier-Ruge & Bertoni-Freddari, 1996). In the absence of glucose and oxygen, electrons from the mitochondrial electron transport chain are taken up by other molecules to form harmful reactive oxygen species (ROS), which in excess can result in damage to mitochondrial DNA, RNA, proteins and lipids and activation of cysteine proteases that trigger neural apoptosis (Simon et al., 2000).

Conversely, when mitochondria are supplied with enough glucose and oxygen, glucose is broken down into coenzymes that are used by the electron transport chain to produce ATP—the neural energy currency—at the expense of oxygen. When this mechanism is maximized or enhanced, so is the functionality of the brain's neural networks (Callaway et al., 2004). One potential end result of increased brain functionality is increased learning and memory, and thus a promising strategy for enhancement of extinction (Wrubel et al., 2007).

1.4 Methylene blue

Methylene blue (MB) is a synthetic blue dye with neurometabolic enhancing properties. Because MB is small and aromatic with both cationic and lipophilic properties, it crosses the blood brain barrier and is attracted to the mitochondrial membrane (Visarius et al., 1997). It diffuses into the mitochondrial matrix, where at low doses it forms a redox equilibrium with the enzymes of the electron transport chain (Bruchey & Gonzalez-Lima, 2008; Riha, Rojas & Gonzalez-Lima, 2011; Visarius et al., 1997). MB has been shown to enhance mitochondrial respiration, chiefly by increasing activity in

cytochrome oxidase (CO), the terminal enzyme of the electron transport chain (Wong-Riley, 1989); this is coupled with increases in ATP production and oxygen consumption (Atamna et al., 2008; Bruchey & Gonzalez-Lima, 2008; Lindahl & Oberg, 1961; Riha et al., 2005). Through enhanced mitochondrial efficiency, MB also reduces the incidence of ROS formation (Kelner et al., 1988; Salaris, Babbs & Voorhees, 1991) and consequently delays cellular senescence (Atamna et al., 2008).

1.4.1 Methylene blue improves memory

The memory enhancing effects of MB were first reported by Martinez Jr. and colleagues (1978), who found that administration of low dose MB (1 mg/kg) post training improved memory retention in an inhibitory avoidance task. Since then, MB has been shown to enhance both spatial (Callaway et al., 2002; 2004; Riha et al., 2005) and non-spatial (Auchter et al., 2014; Gonzalez-Lima & Bruchey, 2004; Wrubel et al., 2007) memories, including enhancement of memory for fear extinction (Gonzalez-Lima & Bruchey, 2004; Wrubel et al., 2007). Such memory enhancement is also marked by increases in CO activity (Atamna & Kumar, 2010; Callaway et al., 2002; 2004).

Though MB's memory-enhancing properties have been well documented, a few aspects remain to be explored. For instance, though we know MB enhances memory for fear extinction, how MB interacts with the reconsolidation of fear memories is unclear. Further, in the case of extinction memory enhancement, post-extinction administration of MB enhances mitochondrial respiration during memory consolidation, thus enhancing

consolidation (Wrubel et al., 2007). One caveat to this approach is the fact that MB enhances whichever neural network is active in the time period MB is on board. However, if MB is administered to a subject after a learning task in which the subject did not learn, then MB may be theoretically detrimental, as one would be enhancing a network that does not predict task learning. Along with investigating variability in responsiveness to extinction, the second objective of this dissertation also aims to investigate how the effectiveness of learning during extinction moderates MB's therapeutic effectiveness.

1.4.2 Methylene blue is neuroprotective

MB has also been shown to be neuroprotective in rodent models of stroke (Miclescu et al., 2010; Kelner et al., 1988; Salaris, Babbs & Voorhees, 1991; Shen et al., 2013; Watts et al., 2013), Alzheimer's Disease (Atamna & Kumar, 2010; Callaway et al., 2002; Oz, Lorke, & Petroianu, 2009; Riha, Rojas & Gonzalez-Lima, 2005), Parkinson's Disease (Rojas et al., 2009b) and optic neuropathy (Rojas et al., 2009a; Zhang, Rojas & Gonzalez-Lima, 2006). However, MB's whole brain effects on mitochondrial functional connectivity in models of global metabolic challenge—and how this functional connectivity impacts learning and memory—have not been described.

The final objective of this dissertation is to investigate how daily administration of MB during chronic cerebral hypoperfusion—a model for age-related memory decline—affects learning and memory performance in a visual discrimination task. This investigation is furthered by histochemically

staining brains of subjects who were trained on the visual discrimination task (with or without hypoperfusion and with or without MB treatment) for CO activity, using a well-established protocol for quantitative metabolic mapping (Gonzalez-Lima & Cada, 1994). Changes in functional connectivity as a result of chronic cerebral hypoperfusion are described, along with how these changes are affected by MB treatment.

1.5 Summary of experimental goals

The following dissertation is an exploration into memories: how to change persistent ones, enhance complicated ones and protect delicate ones. I start in Chapter 2 by exploring a strategy for manipulating fear memories, which are notoriously difficult to change. The goal is to further characterize the retrieval + extinction paradigm by manipulating extinction parameters such that subjects were unable to predict the occurrence of fearful stimuli.

Since memory processing is contingent upon specific neuronal activity, and neuronal activity is primarily fueled by products of mitochondrial respiration, the second and third objectives of this work focused largely on how manipulating mitochondrial activity via MB administration enhances and protects memory processes respectively. The second objective is explored in Chapter 3, in which I examine the effect of post-extinction administration of MB and its interaction with responsiveness to extinction and reconsolidation update mechanisms in the persistent attenuation of fear memories.

The third objective, investigating the ability of MB to prevent the cognitive deficits arising from chronic cerebral hypoperfusion, is explored in Chapter 4. Finally, quantitative cytochrome oxidase histochemistry (a marker for mitochondrial activity) is used in Chapter 5 to map the effects of MB on brain mitochondria in the hypoperfusion model. Finally, the results from each experiment are summarized and discussed in Chapter 6, the concluding chapter of this work.

1.6 Literature Citations

Alberini, C. M. (2005). Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends in Neurosciences*, 28(1), 51–6.

Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., & Ames, B. N. (2008). Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. *The FASEB Journal*, 22(3), 703-712.

Atamna, H., & Kumar, R. (2010). Protective role of methylene blue in Alzheimer's disease via mitochondria and cytochrome c oxidase. *Journal of Alzheimer's Disease*, 20, 439-452.

Auber, A., Tedesco, V., Jones, C. E., Monfils, M. H., & Chiamulera, C. (2013). Post-retrieval extinction as reconsolidation interference: methodological issues or boundary conditions?. *Psychopharmacology*, 226(4), 631-647.

Bouton, M. E. (2007). Learning and behavior: A contemporary synthesis. *Sinauer Associates*.

Bruchey, A. K., & Gonzalez-Lima, F. (2008). Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. *American journal of pharmacology and toxicology,* 3(1), 72.

Burnham, W. H. (1903). Retroactive amnesia: illustrative cases and a tentative explanation. *The American Journal of Psychology*, 118-132.

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired

by an inhibitor of cytochrome oxidase in rats. *Neuroscience letters*, 332(2), 83-86.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, 77(1), 175-181.

Debiec, J., & LeDouxy, J. E. (2013). Reconsolidation of Pavlovian Conditioned Defense Responses in the Amygdala. *Memory Reconsolidation*, 69.

Dudai, Y. (1996). Consolidation: fragility on the road to the engram. *Neuron*, 17(3), 367-370.

Ebbinghaus, H. (1885). Über das gedächtnis: untersuchungen zur experimentellen psychologie. *Duncker & Humblot*.

Effting, M., & Kindt, M. (2007). Contextual control of human fear associations in a renewal paradigm. *Behaviour Research and Therapy*, 45(9), 2002-2018.

Dębiec, J., & Ledoux, J. E. (2004). Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience*, 129(2), 267-272.

Duvarci, S., & Nader, K. (2004). Characterization of fear memory reconsolidation. *The Journal of neuroscience*, 24(42), 9269-9275.

Eichenbaum, H. (2000). A cortical–hippocampal system for declarative memory. *Nature Reviews Neuroscience*, 1(1), 41-50.

Fricks-Gleason, A. N., & Marshall, J. F. (2008). Post-retrieval β-adrenergic receptor blockade: Effects on extinction and reconsolidation of

cocaine-cue memories. *Learning & memory*, 15(9), 643-648.

Gershman, S. J., Blei, D. M., & Niv, Y. (2010). Context, learning, and extinction. *Psychological Review*, 117(1), 197–209.

Gershman, S. J., Jones, C. E., Norman, K. A., Monfils, M. H., & Niv, Y. (2013). Gradual extinction prevents the return of fear: implications for the discovery of state. *Frontiers in behavioral neuroscience*, *7*.

Gershman, S. J., & Niv, Y. (2012). Exploring a latent cause theory of classical conditioning. *Learning & Behavior*, *40*(3), 255–68.

Glickman, S. E. (1961). Perseverative neural processes and consolidation of the memory trace. *Psychological Bulletin*, 58(3), 218.

Gonzales-Lima, F., & Jones, D. (1994). Quantitative mapping of cytovhrome oxidase activity in the central auditory system of the gerbil: a study with calibrated activity standards and metal-intensified histochemistry. *Brain research*, 660(1), 34-49.

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, 11(5), 633-640.

Hofmann S.G. (2008). Cognitive processes during fear acquisition and extinction in animals and humans: implications for exposure therapy of anxiety disorders. *Clin Psychol Rev*; 28, 199–210.

Hutton-Bedbrook, K., & McNally, G. P. (2013). The promises and pitfalls of retrieval-extinction procedures in preventing relapse to drug seeking. *Frontiers in psychiatry*, 4.

Jones, C. E., & Monfils, M. H. (2013). Using reconsolidation and extinction to weaken fear memories in animal models. *Memory Reconsolidation*, 165.

Kaplan G.B., Heinrichs S.C., Carey R.J. (2011). Treatment of addiction and anxiety using extinction approaches: neural mechanisms and their treatment implications. *Pharmacol Biochem Behav*, 97: 619–625.

Kelner, M. J., Bagnell, R., Hale, B., & Alexander, N. M. (1988). Methylene blue competes with paraquat for reduction by flavo-enzymes resulting in decreased superoxide production in the presence of heme proteins. *Archives of biochemistry and biophysics*, 262(2), 422-426.

Kety, S. S. (1957). The general metabolism of the brain in vivo. *Metabolism of the nervous system*, 221-237.

Kindt, M., Soeter, M., & Vervliet, B. (2009). Beyond extinction: erasing human fear responses and preventing the return of fear. *Nature neuroscience*, *12*(3), 256-258.

Kindt, M., & Soeter, M. (2013). Reconsolidation in a human fear conditioning study: a test of extinction as updating mechanism. *Biological psychology*, *92*(1), 43-50.

Lashley, KS. (1950). In search of the engram. Oxford, England: Academic Press.

Lechner, H. A., Squire, L. R., & Byrne, J. H. (1999). 100 years of consolidation—remembering Müller and Pilzecker. *Learning & Memory*, 6(2), 77-87.

Lee, J. L., Milton, A. L., & Everitt, B. J. (2006). Cue-induced cocaine seeking and relapse are reduced by disruption of drug memory reconsolidation. *The Journal of neuroscience*, *26*(22), 5881-5887.

Lewis, J. L., LoTurco, J. J., & Solomon, P. R. (1987). Lesions of the middle cerebellar peduncle disrupt acquisition and retention of the rabbit's classically conditioned nictitating membrane response. *Behavioral neuroscience*, *101*(2), 151.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, 443(7113), 787-795.

Lindahl, P. E., & Öberg, K. E. (1961). The effect of rotenone on respiration and its point of attack. *Experimental Cell Research*, 23(2), 228-237.

Marcus, D. L., de Leon, M. J., Goldman, J., Logan, J., Christman, D. R., Wolf, A. P., ... & Freedman, M. L. (1989). Altered glucose metabolism in microvessels from patients with Alzheimer's disease. *Annals of neurology*, *26*(1), 91-94.

Ma, X., Zhang, J. J., & Yu, L. C. (2012). Post-retrieval extinction training enhances or hinders the extinction of morphine-induced conditioned place preference in rats dependent on the retrieval-extinction interval. *Psychopharmacology*, *221(*1), 19-26.

Martinez Jr, J. L., Jensen, R. A., Vasquez, B. J., McGuinness, T., & McGaugh, J. L. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, 6(3), 387-390.

McCormick, D. A., & Thompson, R. F. (1984). Cerebellum: essential involvement in the classically conditioned eyelid response. *Science*, 223(4633), 296-299.

McGaugh, J. L. (1966). Time-dependent processes in memory storage. *Science*, 153(3742), 1351-1358.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science*, 287(5451), 248-251.

Meier-Ruge, W., & Bertoni-Freddari, C. (1996). The significance of glucose turnover in the brain in the pathogenetic mechanisms of Alzheimer's disease. *Reviews in the Neurosciences*, 7(1), 1-20.

Miclescu, A., Sharma, H. S., Martijn, C., & Wiklund, L. (2010). Methylene blue protects the cortical blood–brain barrier against ischemia/reperfusion-induced disruptions. *Critical care medicine*, 38(11), 2199-2206.

Milad M.R., Rauch S.L., Pitman R.K., Quirk G.J. (2006). Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biological Psychology*, 73, 61–71.

Millan, E. Z., Milligan-Saville, J., & McNally, G. P. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiology of learning and memory*, *101*, 26-32.

Mineka S, Zinbarg R. (1996). Conditioning and ethological models of anxiety disorders: stress-in-dynamic-context anxiety models. *Nebraska Symposium on Motivation*, 43, 135–210.

Mineka S, Oehlberg K. (2008). The relevance of recent developments in classical conditioning to understanding the etiology and maintenance of anxiety disorders. *Acta Psychology*, 127, 567–580.

Misanin, J. R., Miller, R. R., & Lewis, D. J. (1968). Retrograde Amnesia Produced by Electroconvulsive Shock after Reactivation of a Consolidated Memory Trace. *Science*, 160(3827), 554–555.

Monfils, M.-H., Cowansage, K. K., Klann, E., & LeDoux, J. E. (2009). Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *Science*, *324*(5929), 951–5.

Moreira, P. I., Santos, M. S., Seiça, R., & Oliveira, C. R. (2007). Brain mitochondrial dysfunction as a link between Alzheimer's disease and diabetes. *Journal of the neurological science*s, 257(1), 206-214.

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience letters*, 163(1), 109-113.

Morris, R. G., Inglis, J., Ainge, J. A., Olverman, H. J., Tulloch, J., Dudai, Y., & Kelly, P. A. (2006). Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron*, 50(3), 479-489.

Müller, G., & Pilzecker, A. (1900). Experimentelle beiträge zur lehre vom gedächtniss, *1*.

Myers KM, Davis M. (2002). Behavioral and neural analysis of extinction. *Neuron*, 36, 567–584.

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000a). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722–6.

Nader, K., & Einarsson, E. O. (2010). Memory reconsolidation: an update. *Annals of the New York Academy of Sciences*, 1191, 27–41.

Nader, K., Schafe, G. E., & LeDoux, J. E. (2000b). The labile nature of consolidation theory. *Nature Reviews. Neuroscience*, *1*(3), 216–9.

Olshavsky, M. E., Song, B. J., Powell, D. J., Jones, C. E., Monfils, M. H., & Lee, H. J. (2013). Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus. *Frontiers in behavioral neuroscience*, 7.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *The Journal of Neuroscience*, 20(16), 6225-6231.

Oyarzún, J. P., Lopez-Barroso, D., Fuentemilla, L., Cucurell, D., Pedraza, C., Rodriguez-Fornells, A., & de Diego-Balaguer, R. (2012). Updating fearful memories with extinction training during reconsolidation: a human study using auditory aversive stimuli. *PLoS One*, 7(6), e38849.

Oz, M., Lorke, D. E., & Petroianu, G. A. (2009). Methylene blue and Alzheimer's disease. *Biochemical pharmacology*, 78(8), 927-932.

Pavlov, I (1904). *Physiology of Degestion*. Nobel Lecture. Published in *Nobel Lectures, Physiology or Medicine 1901-1921*, Elsevier Publishing Company, Amsterdam.

Pavlov, I. P. (1927). Conditioned reflexes: an investigation of the

physiological activity of the cerebral cortex. New York, *Boyer*.

Pedreira, M. E., & Maldonado, H. (2003). Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron*, *38*(6), 863-869.

Pedreira, M. E., Pérez-Cuesta, L. M., & Maldonado, H. (2004). Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. *Learning & Memory*, 11(5), 579-585.

Polster, M. R., Nadel, L., & Schacter, D. L. (1991). Cognitive neuroscience analyses of memory: A historical perspective. *Journal of Cognitive Neuroscience*, 3(2), 95-116.

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, 33(1), 56–72.

Quirk, G. J., Paré, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., & Vicentic, A. (2010). Erasing fear memories with extinction training. *The Journal of Neuroscience*, *30*(45), 14993-14997.

Redish, A. D., Jensen, S., Johnson, A., & Kurth-Nelson, Z. (2007). Reconciling reinforcement learning models with behavioral extinction and renewal: implications for addiction, relapse, and problem gambling. *Psychological Review*, *114*(3), 784–805.

Rescorla, RA. (1988). Pavlovian conditioning: It's not what you think it is. *American Psychologist*.

Ribot, T. (1882). Diseases of memory, an essay in the positive psychology, *43*.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005).

Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, 511(2), 151-158.

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *Neuroimage*, 54(4), 2623-2634.

Robbins, S. J. (1990). Mechanisms underlying spontaneous recovery in autoshaping. Journal of Experimental Psychology: Animal Behavior Processes, 16(3), 235.

Rojas, J. C., John, J. M., Lee, J., & Gonzalez-Lima, F. (2009a). Methylene blue provides behavioral and metabolic neuroprotection against optic neuropathy. *Neurotoxicity research,* 15(3), 260-273.

Rojas, J. C., Simola, N., Kermath, B. A., Kane, J. R., Schallert, T., & Gonzalez-Lima, F. (2009b). Striatal neuroprotection with methylene blue. *Neuroscience*, 163(3), 877-889.

Rolfe, D. F., & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological reviews*, *77*(3), 731-758.

Salaris, S. C., Babbs, C. F., & Voorhees, W. D. (1991). Methylene blue as an inhibitor of superoxide generation by xanthine oxidase: a potential new drug for the attenuation of ischemia/reperfusion injury. *Biochemical pharmacology,* 42(3), 499-506.

Schiller, D., Monfils, M.-H., Raio, C. M., Johnson, D. C., Ledoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature*, *463*(7277), 49–53.

Sevenster, D., Beckers, T., & Kindt, M. (2012). Retrieval per se is not sufficient to trigger reconsolidation of human fear memory. *Neurobiology of learning and memory*, *97*(3), 338-345.

Shen, Q., Du, F., Huang, S., Rodriguez, P., Watts, L. T., & Duong, T. Q. (2013). Neuroprotective efficacy of methylene blue in ischemic stroke: an MRI study. *PloS one*, 8(11), e79833.

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, 5(5), 415-418.

Sokoloff, L. (1960). The metabolism of the central nervous system in vivo. *Handbook of physiology-neurophysiology*, 3, 1843-1864.

Steinmetz, J. E., Logan, C. G., Rosen, D. J., Thompson, J. K., Lavond, D. G., & Thompson, R. F. (1987). Initial localization of the acoustic conditioned stimulus projection system to the cerebellum essential for classical eyelid conditioning. *Proceedings of the National Academy of Sciences*, *84*(10), 3531-3535.

Steinmetz, J. E., & Sengelaub, D. R. (1992). Possible conditioned stimulus pathway for classical eyelid conditioning in rabbits. I. Anatomical evidence for direct projections from the pontine nuclei to the cerebellar interpositus nucleus. *Behavioral and neural biology*, *57*(2), 103-115.

Stern, C. A., Gazarini, L., Takahashi, R. N., Guimaraes, F. S., & Bertoglio, L. J. (2012). On disruption of fear memory by reconsolidation blockade: evidence from cannabidiol treatment. *Neuropsychopharmacology*, *37*(9), 2132-2142.

Thompson, R. F., Bao, S., Chen, L., Cipriano, B. D., Grethe, J. S., Kim, J. J., ... & Krupa, D. J. (1997). Associative learning. *International review of neurobiology*, *41*, 151-189.

Todes, D. P. (2014). Ivan Pavlov: A Russian Life in Science. *Oxford University Press.*

Visarius, T. M., Stucki, J. W., & Lauterburg, B. H. (1997). Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS letters,* 412(1), 157-160.

Watts, L. T., Lloyd, R., Garling, R. J., & Duong, T. (2013). Stroke Neuroprotection: Targeting Mitochondria. *Brain sciences*, 3(2), 540-560.

Weingartner, H., & Parker, E. S. (Eds.). (2014). Memory consolidation: Psychobiology of cognition. *Psychology Press.*

Wong-Riley, M. T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends in neurosciences*, 12(3), 94-101.

Wrubel, K. M., Riha, P. D., Maldonado, M. A., McCollum, D., & Gonzalez-Lima, F. (2007). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology Biochemistry and Behavior*, 86(4), 712-717.

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., ... & Lu, L. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science*, *336*(6078), 241-245.

Zhang, X., Rojas, J. C., & Gonzalez-Lima, F. (2006). Methylene blue prevents neurodegeneration caused by rotenone in the retina. *Neurotoxicity research*, *9*(1), 4757.

Chapter 2: Reconsolidation-extinction interactions in fear memory attenuation: examining the role of inter-trial interval variability

2.1 Abstract

Fear extinction typically results in the formation of a new inhibitory memory that suppresses the original fear memory, leading to attenuation of the conditioned response. Evidence also suggests that administration of extinction during a retrieval induced labile period results in integration of the extinction memory into the original fear memory, rendering the fear memory less susceptible to reinstatement. Here we investigate the parameters by which the retrieval-extinction paradigm is most effective in memory updating. Specifically, we manipulate the inter trial intervals between conditioned stimuli presentation during extinction, examining how having interval lengths with different degrees of variability affect the strength of memory updating. We show that randomizing the inter trial intervals (thus randomizing CS presentations) during extinction leads to less return of fear via reinstatement than extinction with fixed inter trial intervals. Subjects who received variable inter trial intervals during extinction also showed higher PreCS freezing during the inter trial intervals, indicating that the randomization of CS presentation led to a higher general reactivity during extinction, which may be one potential mechanism for potentiated memory updating.

Key words: reconsolidation, extinction, fear attenuation, inter-trial interval

2.2 Introduction

Over the past several decades, the field of learning and memory has transitioned away from the once dogmatic view that memories are plastic during formation but stable once established (Dundai, 2004; Kandel, 2001; Martin, Grimwood & Morris, 2000; Müller and Pilzecker, 1900). We have evolved into a new and exciting era in which we are confident that memory retrieval and maintenance are also active and plastic processes. It is now firmly established that memory retrieval is not simply a passive readout of a previously consolidated memory. Rather, is a more complex process whereby upon recollection, the original memory can enter into a destabilized state, and maintenance of the memory requires restabilization by means of reconsolidation (Misanin, Miller, & Lewis, 1968; Nader & Einarsson, 2010; Nader, Schafe, & LeDoux, 2000a; Nader, Schafe, & LeDoux, 2000b). Evidence from several species suggests that reconsolidation blockade via pharmacological (Alberini, 2005; Dębiec & LeDoux, 2004; Dębiec & LeDoux, 2013; Duvarci & Nader, 2004; Fricks-Gleason & Marshall, 2008; Lee, Milton & Everitt, 2006; Nader, Schafe, & LeDoux, 2000a; Stern et al., 2012) or behavioral (Jones & Monfils, 2013; Kindt & Soeter, 2013; Millan, Milligan-Saville & McNally, 2013; Monfils et al., 2009; Olshavsky et al., 2013; Oyarzún et al., 2012; Pedriera & Maldonado, 2003; Quirk et al., 2010; Schiller et al., 2010; Xue et al., 2012) intervention during a specific post-retrieval reconsolidation window alters the original memory. The ability to update memories after they have been consolidated has tremendous clinical relevance for treatment of fear and anxiety disorders. However, it is has

become clear over the past decade or so that reconsolidation is not a universal feature of memory retrieval, and that fear memories are notoriously difficult to change (Eisenberg et al., 2003; Nader et al., 2005; Pedreira & Maldonado, 2003).

Historically, the most widely used treatment for fear and anxiety disorders has been extinction (clinically referred to as exposure therapy). In conditioned fear paradigms, extinction involves repeatedly exposing subjects conditioned to a fear-eliciting—but not inherently aversive—stimulus (conditioned stimulus; CS) to the CS in the absence of the aversive stimulus (unconditioned stimulus; US). This leads to gradual reduction in the emotional response to the CS (Pavlov, 1927). This experience is not incorporated into the original fear memory, but instead results in the formation of a new inhibitory memory as the predictive value of the CS preceding the US lessens (Barrett et al., 2003; Effting & Kindt, 2007; Morgan, Romanski & LeDoux, 1993; Pavlov, 1927; Quirk et al., 2000; Quirk & Mueller, 2008; Robbins, 1990).

Inhibitory memory formation is distinct from reconsolidation updating. This difference is important, as the remaining fear memory leaves an organism vulnerable to return of fear through the passage of time (spontaneous recovery), CS exposure in a new context (renewal), or additional US exposure (reinstatement). Reconsolidation updating reduces the susceptibility of the organism to fear return. Monfils et al. (2009) devised an approach to fear attenuation that combines the principles of reconsolidation updating and extinction. They were able to use extinction to

interfere with reconsolidation by giving extinction training after the fear memory retrieved, during a period in which the memory is being reconsolidated and is susceptible to interference. Such a unique window of opportunity for memory updating via extinction was fittingly deemed "the reconsolidation window" (Monfils et al., 2009). Extinction training during the reconsolidation window persistently attenuated fear responses in spontaneous recovery, renewal and reinstatement paradigms, implicating retrieval + extinction as a novel and non-invasive approach to memory updating.

There are several questions that still need to be answered in order to fully utilize post-retrieval strategies to disrupt or weaken pathogenic memories. For instance, it still remains somewhat unclear under what extinction conditions memories are updated (Hutton-Bedbrook & McNally, 2013). Here, we explore how the element of unpredictability during extinction can modulate memory updating.

It has been suggested that the functional role of reconsolidation is to keep older memories up to date with new learning, and that reconsolidation is triggered only when there is an opportunity for new learning to take place during reactivation (Morris et al., 2006; Pedriera, Perez-Cuesta & Maldonado, 2004; Sevenster, Beckers & Kindt, 2012). This line of thought is rooted in the evidence that associative learning requires prediction error (Rescorla & Wagner, 1972). According to these models, prediction error is not determined by the mere co-occurrence of the CS and US, but by the discrepancy between what has already been learned and what can be learned

on a given trial. In this case, memory retrieval via an unreinforced CS (assuming that all CSs were reinforced during training) signals a need for new learning, and thus results in memory destabilization.

Recently, the emergence of new normative learning models that integrate seasoned learning models (e.g., Pearce & Hall, 1980; Rescorla & Wagner, 1972) with more complex principles of computational learning theory (e.g. Bayesian statistical inference) has shed new light on the field of learning and memory. Courville and colleagues (2006; 2003; 2005) were among the first to describe classical conditioning in terms Bayesian inference and uncertainty. According to this statistical model, surprise signals change and therefore uncertainty and the need for new learning (Courville et al., 2006). Gershman and Niv (2012) combined these principles with reinforcement learning models proposed by Redish, Jensen, Johnson and Kurth-Nelson (2007) and human rational categorization models proposed by Anderson (1991) to come up with a latent cause theory of classical conditioning (see Gershman, Blei, & Niv, 2010 for a detailed comparison of these models). According to this theory, an animal combines its *a priori* beliefs about the structure of the world with its current observations (1) to make inferences about how CSs and USs are linked and (2) to make predictions about possible future occurrences of a US. In regard to extinction learning, the theory posits that the subject discovers a new state of the world during extinction training—distinct from the state it experienced during fear memory acquisition—which warrants the formation of an entirely new memory (Gershman et al., 2010; Gershman & Niv, 2012; Redish et al., 2007).

If this is the case, then perhaps preventing the subject from inferring a new state of the world during extinction will lead to memory updating over new memory formation. In accordance with this idea, recent evidence suggests that large prediction errors promote new memory formation over memory updating, and that reducing prediction error during extinction training prevents the formation of a new memory while still modifying the old memory (Gershman, Jones, Norman, Monfils, & Niv, 2013). Based on these results, one may consider the likelihood of memory updating on a spectrum: the less the subjective experience of extinction training resembles fear conditioning, the more prediction error—and consequentially, the more likely a new memory will be formed.

In addition to non-paired CSs evoking prediction error during extinction, any number of contextual and parametric variables may also serve as latent variables, important for discriminating between expected and observed outcomes. Prediction error could come from disparities in contextual stimuli, or perhaps from parameters such as the number of within-session CS presentations and the time elapsed between the stimuli. Typically, fear conditioning consists of only a few trials of CS-US pairings, while extinction consists of many more exposures to the CS only. Additionally, if the intervals between CSs during extinction is fixed (i.e. occurring at regular, predictable intervals), then not only does extinction training contain many more stimulus presentations, but the consistent time intervals between CSs may become additional within-session cues that are not so apparent during fear conditioning. We posit that these fixed inter-trial

intervals (ITIs) promote new learning over memory updating by introducing this new predictable timing cue during extinction.

There is also a plausible second scenario that could explain the ITI's influence on memory updating. Since we are defining fixed ITIs as "predictable" during extinction, then fixed ITIs could actually reduce the amount of surprise that is experienced when the CSs occur, and thus according to Bayesian learning models could also result in smaller uncertainty and less new memory consolidation. However, both the Rescorla-Wagner model and Bayesian learning models only speak about "surprise" in terms of expecting a reinforced CS and observing a non-reinforced CS. Instead, we are curious about whether an animal's expectation (or lack thereof) to receive any stimulus at all affects how the animal subsequently makes and integrates inferences about the latent causes of the US. Since presumably the animal does not expect a CS during fear conditioning, then ensuring the animal cannot expect a CS during extinction may be one way to prevent the animal from inferring new latent causes. Stated differently, we are suggesting that induction of new memory formation as a result of surprise is limited to CS-US prediction error. However, since subjects are unable to predict when the CS-US occurs during fear conditioning, then memory updating is more likely when they are unable to predict CSs during extinction.

In the following experiments, we examined the interaction between memory destabilization via retrieval and variability of the extinction ITIs on memory updating of previously conditioned CS-US (tone-shock) association.

We gave fear-conditioned subjects either a retrieval CS or no retrieval CS, followed by extinction with either fixed ITIs or ITIs with some degree of random variability. We tested whether our experimental manipulation had an effect on reinstatement by re-exposing all subjects to the US 24 hrs after extinction, and testing for return of fear the next day. In Experiment 1, we simply determined whether variable (random) extinction ITIs were more effective than fixed extinction ITIs at promoting reconsolidation updating using a 1-minute mean extinction ITI. Though randomizing CS presentation allowed us to test the effects of adding objective variability to the ITIs, using such a short interval does not leave much time for subjects to experience the variability subjectively, since CSs are occurring at a rather rapid pace regardless of objective randomness. In Experiment 2, we expanded the extinction session, giving it an average ITI length of 2 minutes. This not only allowed us to introduce more subjective variability (i.e. the ITI could range anywhere from 20 to 240 seconds; Table 2.1), but we could also include an intermediate condition resembling the constrained amount of variability allowed by Experiment 1 (see Figure 2.1 for full experimental design). In other words, though each experiment included an objectively random ITI condition, including both a "small variability" (i.e. the amount of variability allowed by a short time frame) and "large variability" (i.e. the amount of variability allowed by a longer time frame) condition in Experiment 2 allowed us to compare the subjective experience of randomness.

2.3 Hypothesis

Random, unpredictable time periods between CSs during extinction hinders a subject's ability to use the inter trial intervals as predictors (unique to extinction) of CS occurrences. Such unpredictability will promote fear memory updating over new extinction memory formation.

2.4 Materials and Methods

2.4.1 Subjects

All subjects were male Sprague-Dawley rats (250-300 g; Harlan Laboratories, Inc.). Sixty-four rats were used in Experiment 1 and 60 rats were used in Experiment 2. All procedures were conducted in compliance with the National Institute of Health Guide for the Care and Use of Experimental Animals and were approved by the University of Texas at Austin Institutional Animal Care and Use Committee. Rats were housed in pairs and maintained on a 12-h light/dark cycle with ad libitum availability of food and water.

2.4.2 Apparatus and Stimuli

All behavioral procedures were conducted in standard conditioning chambers (Coulbourn Instruments) equipped with stainless-steel rod floors connected to shock generators. Each chamber was encased in an acoustic isolation box (Coulbourn Instruments) and illuminated by a red house light. Each chamber was wiped down with Windex between sessions. Delivery of the stimuli was controlled by Freeze Frame software, and behavior was

recorded using infrared digital cameras mounted on the ceiling of each chamber (Coulbourn Instruments). Each experiment used a tone (5 kHz, 80 dB) conditioned stimulus (CS) played for 20 seconds and a footshock (0.7 mA) uncondition stimulus (US) that lasted for 500 milliseconds.

2.4.3 Behavioral Procedures

2.4.3.1 Habituation

The day prior to fear conditioning, subjects were introduced to the context and allowed to explore for 10 minutes. The purpose of this exposure was to minimize fear conditioning to the context.

2.4.3.2 Fear Conditioning

On the day of fear conditioning, rats were habituated to the context for 3 minutes prior to receiving three 20-second presentations of the tone CS. Each CS co-terminated with a 0.7 mA footshock. For Experiment 1, the inter-trial interval (ITI) between CS presentations was 1 minute, similar to the average ITI used subsequently during extinction. Likewise, for Experiment 2, the ITI during fear conditioning was 2 minutes (Figure 2.1).

2.4.3.3 Retrieval and Extinction

Experiment 1

The day after fear conditioning, subjects were placed back into the same context. Subjects were given 3 minutes to habituate and then received either extinction training alone (no retrieval condition), or a single retrieval

CS followed 60 minutes later by extinction (retrieval condition). Following the retrieval CS in the retrieval condition, subjects were placed back into their home cages and returned to the colony for the 60-minute interval between retrieval and extinction. Extinction consisted of 18 (retrieval condition) or 19 (no retrieval condition) CSs separated by either exactly 60 seconds (fixed ITI condition) or an interval that varied from 20 to 100 seconds (variable ITI condition). The ITIs used for the variable extinction condition were chosen randomly from a normal distribution with a 60 second mean interval and a standard deviation of 30 seconds (Table 2.1). The total length of extinction and number of CSs were the same in all groups, since the mean interval was 60 seconds in all conditions and subjects that did not receive a retrieval CS received one additional extinction CS.

Experiment 2

Once it was determined that introducing variability to (i.e. randomizing) 1-minute average extinction ITIs helped attenuate fear memory, we wanted to broaden our understanding of how variability affects the subjective experience of randomness by introducing even more variability over a longer extinction session. Like in Experiment 1, the day after fear conditioning, subjects were placed back into the same context, given 3 minutes to habituate followed by either extinction alone or an isolated retrieval CS. In the retrieval condition, subjects were transported back to their home cages for 60 minutes, then returned to the chamber for extinction. In this experiment, subjects were given one of three ITI conditions

during extinction: exactly 120 seconds (fixed ITI condition), an interval that varied from 90 to 150 seconds (small variability ITI), or an interval that varied from 20 to 240 seconds (large ITI variability condition; Table 2.1). The intervals used in the small and large variability conditions were chosen using a similar method as Experiment 1—by randomly selecting intervals from a normal distribution with a mean of 120 seconds. For the small variability condition, the distribution from which intervals were selected had a standard deviation of 15 seconds, which resulted in a *range* of intervals similar to Experiment 1. For the large variability condition, the distribution from which intervals were selected had a standard deviation of 60 seconds (which is essentially equivalent to random presentation of CSs), and resulted in a variability:ITI ratio similar to Experiment 1 (Table 2.1). This way, we could determine whether the variability effect we observed in Experiment 1 could be explained by the absolute deviation of ITIs from the mean or if the effect was driven by the relationship between the standard deviation of the ITIs with the mean ITI.

2.4.3.4 Reinstatement

Twenty-four hours after extinction, subjects were placed back into the chambers, allowed to habituate for 5 minutes, followed by 2 exposures to the US alone, separated by 1 minute in Experiment 1 and 2 minutes in Experiment 2. Subjects were also kept in the context for 5 minutes following the last US before being put back into their home cages. The following day, subjects were placed back into the context and given 3 presentation of the CS

alone to probe for reinstatement-induced return of fear. The timing between the CS during the reinstatement probe was 1 minute for Experiment 1 and 2 minutes for Experiment 2.

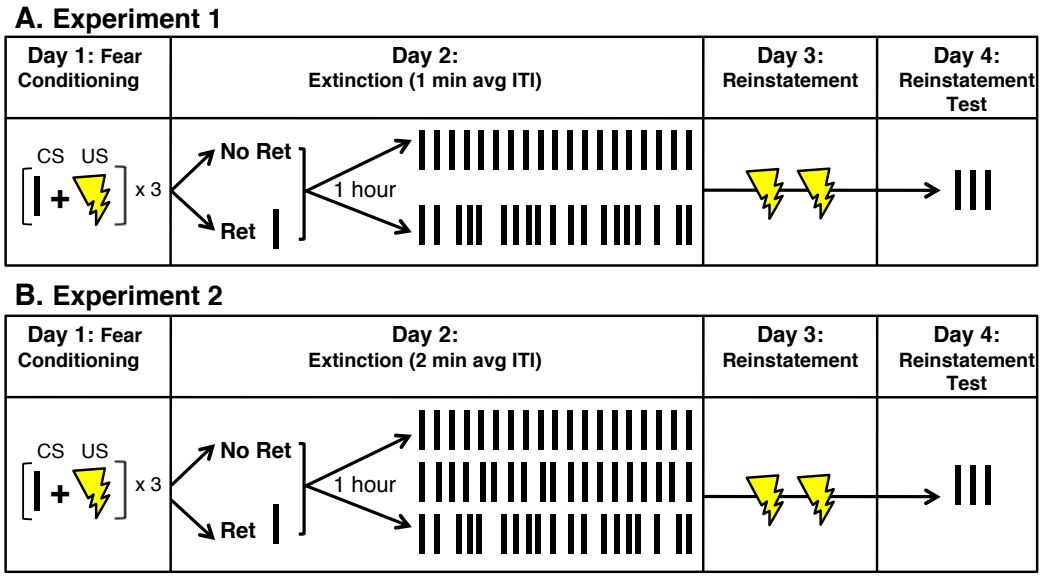

Figure 2.1 Experimental timeline. Subjects received fear conditioning on Day 1, which consisted of 3 CS-US pairings. On Day 2, subjects received either a retrieval CS or no retrieval CS, followed one hour later by extinction (19 total CSs) with either fixed ITIs or variable ITIs. Average ITIs were 1 minute in Experiment 1 and 2 minutes in Experiment 2. In Experiment 2, the ITI variability was divided into 3 different categories: no variability (fixed), small variability and large variability. On Day 3, subjects received 2 unpaired USs (reinstatement). On Day 4, subjects received 3 unpaired CSs as a probe for reinstatement of fear. CS = conditioned stimulus, US = unconditioned stimulus, ITI = inter trial interval, ret = retrieval min = minute.

Table 2.1 Extinction ITI parameters.

Experiment 1	ITI average (sec)	Min ITI (sec)	Max ITI (sec)	SD	SD:ITI
Not variable (fixed)	60	60	60	0	0
Variable	60	20	110	30	0.5
Experiment 2					
Not variable (fixed)	120	120	120	0	0
"Small" variability	120	90	150	15	0.125
"Large" variability	120	20	240	60	0.5

2.4.4 Scoring—freezing

Freezing was defined as the cessation of movement while in the crouched position, excluding breathing, whisker twitching and resting/sleeping. The total number of seconds spent freezing during the 20 seconds prior to the CS (pre-CS) as well during the 20-second CS is expressed as a percentage of pre-CS/CS duration.

2.4.5 Statistical Analyses

Statistical analyses were carried out using PASW Statistics software version 18.0 and R Statistical Computing software. Mixed factor ANOVAs with the CS cue as a repeated measure, and retrieval group and variability group membership as between subject factors. Where appropriate, post-hoc tests were performed with Tukey's honestly significant difference mean comparison. Percent freezing at the end of extinction is expressed as the

mean percentage over the final 3 CSs during extinction training; percent freezing during reinstatement tests is expressed as the mean percentage over three CS presentations.

2.5 Results

2.5.1 Experiment 1: Variable extinction ITIs lead to reduced freezing at reinstatement test

To examine the effect of fixed vs. variable ITIs, we used a 2x2 factorial design, whereby fear-conditioned subjects received either extinction only (i.e. no retrieval CS) or a retrieval CS followed 60 minutes later by extinction with either fixed or "variable" (random) ITIs. The day after extinction, all subjects were re-exposed to the US only and probed the following day for CS-induced return of fear (Figure 2.2). All groups showed a significant within-subjects reduction in freezing during extinction, as revealed by a repeated-measures analysis of variance (ANOVA) using the average freezing during the first three and last three CSs as the repeated factor and the retrieval condition and the ITI variability condition as the between-subjects factors ($F(1,60)=187.2$, $p<0.001$).

We then assessed the return of fear via reinstatement. A univariate ANOVA with the retrieval condition and the ITI variability condition as the between subjects factors and co-varying for freezing at the end of extinction revealed significant main effects of both retrieval ($F(1,59)=5.19$, $p<0.05$) and ITI variability ($F(1,59)=4.00$, $p<0.05$), with no significant effect of post-extinction freezing ($F(1,59)=3.12$, $p=.08$) and no significant interaction

($F(1,59)=0.04$, $p=.95$). Post-hoc analysis revealed that on average, subjects who received variable ITIs during extinction showed lower post-reinstatement freezing than subjects that received fixed ITIs. Likewise, subjects who received a retrieval CS one hour before extinction showed less post-reinstatement freezing than subjects who did not receive the retrieval cue. Subjects who received both retrieval cue and variable extinction ITIs showed the least post-reinstatement freezing; but interestingly, all groups showed post-reinstatement return of fear, as revealed by significant increases in freezing from the end of extinction to post-reinstatement using paired-samples t-tests (all $p's<0.05$).

Results indicate that receiving the retrieval CS one hour before extinction resulted in a significant less return of fear after reinstatement than groups that received extinction alone, without influencing the degree of extinction within-session (Figure 2.2). Also, receiving extinction with variable ITIs resulted in less return of fear. The lack of interaction between the two factors suggests that both retrieval and variable ITIs operated independently to attenuate fear. However, since all groups still showed some degree of return of fear, we cannot conclude from these data that our manipulations resulted in complete memory updating.

2.5.2 Experiment 2: Greater ITI variability during extinction leads to reduced freezing at reinstatement test

Once we determined that introducing variability to 1-minute average extinction ITIs helped attenuate the fear memory, we wanted to broaden our

understanding of how variability affects the subjective experience of randomness, and how that subjective experience effects persistent fear reduction. We tested this by introducing more variability over a longer extinction session and directly comparing it to a constrained (small variability) condition. We extended the extinction session such that the average ITI was 2 minutes to allow for more random variability. We used a 3x2 factorial design, whereby fear-conditioned subjects received either extinction only (i.e. no retrieval CS) or a retrieval CS followed 60 minutes later by extinction with no variability (fixed ITIs), "small variability" (equivalent to the amount of variability present in Experiment 1), or "large variability" (random) ITIs[4]. The day after extinction, all subjects were re-exposed to the US only and probed the following day for CS-induced return of fear (Figure 2.1). Again, all groups showed a significant within-subjects reduction in freezing during extinction training, as revealed by a repeated-measures analysis of variance (ANOVA) using the average freezing during the first three and last three CSs as the repeated factor and the retrieval condition and the ITI variability condition as the between-subjects factors ($F(1,51)=199.8$, $p<0.001$).

[4] It is important to note that though the "variable" condition in Experiment 1 and the "large variability" condition in Experiment 2 both consist of random ITIs, the *variability* in Experiment 2 is greater than in Experiment 1. In other words, the *range* of possible time intervals from which to randomly draw is larger in Experiment 2, though the *number* of intervals is technically infinite in both cases. Thus, the discernible variability in time intervals is larger in magnitude in Experiment 2 than it is in Experiment 1.

After controlling for post-extinction freezing—which was a significant covariate ($F(1,50)=5.19$, $p<0.05$)—a univariate ANOVA with the retrieval condition and the ITI variability condition as the between subjects factors revealed no significant main effects for either retrieval ($F(1,50)=0.01$, $p>0.05$) or ITI variability ($F(2,50)=1.78$, $p>0.05$). However, there was a significant interaction between retrieval and ITI variability ($F(2,50)=3.31$, $p<0.05$). Post-hoc planned comparisons analysis revealed that compared to groups that had no variability and small variability, the large variability group showed less post-reinstatement freezing than the fixed and small variability groups *only* in the retrieval condition (Figure 2.3; $F(2,31)=3.41$, $p<0.05$). The large variability group did not show reduced post-reinstatement percent freezing in the no retrieval condition ($F(2,31)=1.42$, $p>0.05$).

Interestingly, the "small" variability condition was no different from the fixed condition in freezing behavior at any stage of the experiment. Also, unlike Experiment 1—which showed significant main effect of both independent variables, but no interaction—this experiment showed a significant interaction, with no main effects. This suggests that when a 2-minute average extinction ITI, memory updating via extinction training is contingent upon both memory destabilization via the retrieval CS and a largely variable ITI.

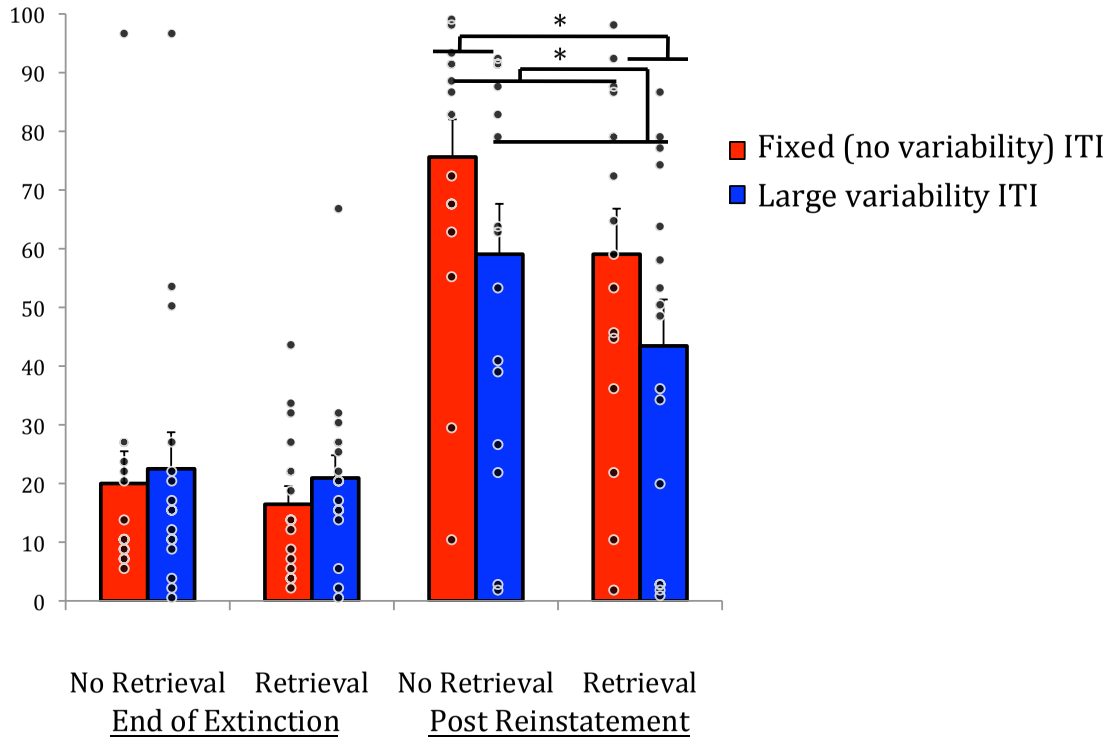

Figure 2.2 Percent freezing at the end of extinction and post reinstatement for Experiment 1 (1-minute average ITI). All groups showed a significant increase in freezing from the end of extinction to reinstatement, but there was a main effect of both ITI variability and retrieval. Groups who received a retrieval CS before extinction showed less reinstatement than groups who did not. Groups who had variable extinction ITIs showed less reinstatement than groups who did not. ITI = inter trial interval, black dots represent individual data points, *p<.05

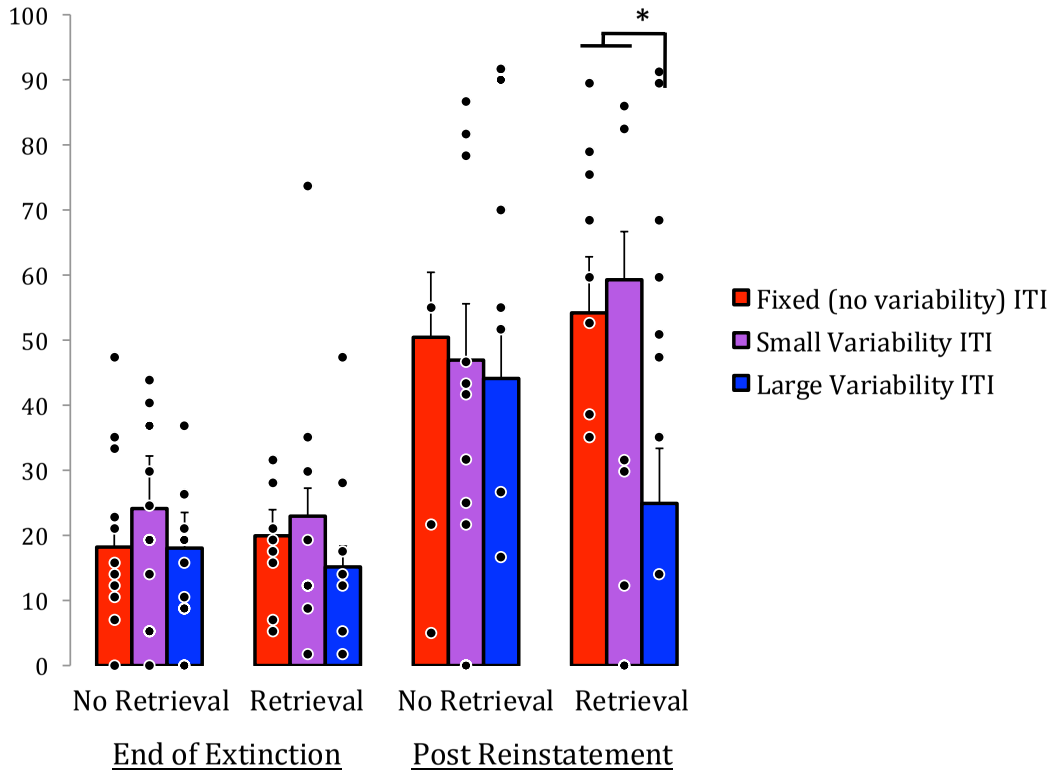

Figure 2.3 Percent freezing at the end of extinction and post reinstatement for Experiment 2 (2-minute average ITI). There was a variability by retrieval interaction. The group who received both a retrieval CS and largely variable ITIs showed less post reinstatement freezing, and also did not show a significant increase in freezing from the end of extinction to post reinstatement. All other groups showed significant increases on freezing from the end of extinction to post reinstatement. ITI = inter trial interval, black dots represent individual data points, *p<.05

2.5.3 PreCS freezing

We see from Experiments 1 and 2 that variable ITIs during extinction lead to greater attenuation of fear memory, with notable memory updating using largely variable ITIs with a mean of 2 minutes. By introducing variability to the extinction ITI, we undoubtedly also introduce a degree of unpredictability to the CS presentation. This could potentially provide some explanation for the mechanism of memory updating. Congruent with our original hypothesis, perhaps a fixed ITI adds a new cue to extinction (predictable timing) that makes the likeness between the fear conditioning and extinction contexts diverge enough to induce new learning. However, we cannot directly measure "predictability" of the CS using freezing during CSs.

We were able to gain some insight indirectly by analyzing the amount of freezing subjects displayed in the 20 seconds preceding each extinction CS. Our logic is that subjects who freeze during the interval preceding the CS are unable to anticipate the next CS occurrence, and thus freeze more in anticipation (rather than viewing the ITI as a predictor of safety and ceasing to freeze). In Experiment 1 (in which we saw more persistent reduction of fear with variable ITIs, but all groups showed some degree of return of fear and thus not a complete memory update), we did not see any group differences in preCS freezing. However, in Experiment 2 (in which the group who received both a retrieval CS and largely variable ITIs showed no significant return of fear), preCS freezing was the same in all groups in the first half of extinction, but preCS freezing for the retrieval + largely variable ITI group remained higher than the other groups as extinction progressed

(Figure 2.4). Such differences in preCS freezing did not persist through reinstatement, indicating that higher freezing during preCSs could not be explained by generalized freezing. Rather, preCS freezing likely indicated that subjects in the large variability extinction condition did not know when to expect the CS, and thus froze more between CSs. The effect happened only after retrieval-induced memory destabilization, and thus updating was mediated by stimulus expectation during extinction. In other words, retrieval destabilized the fear memory, largely variable ITIs led to uncertainty of CS presentation, and the combination of the two led to more persistent fear reduction.

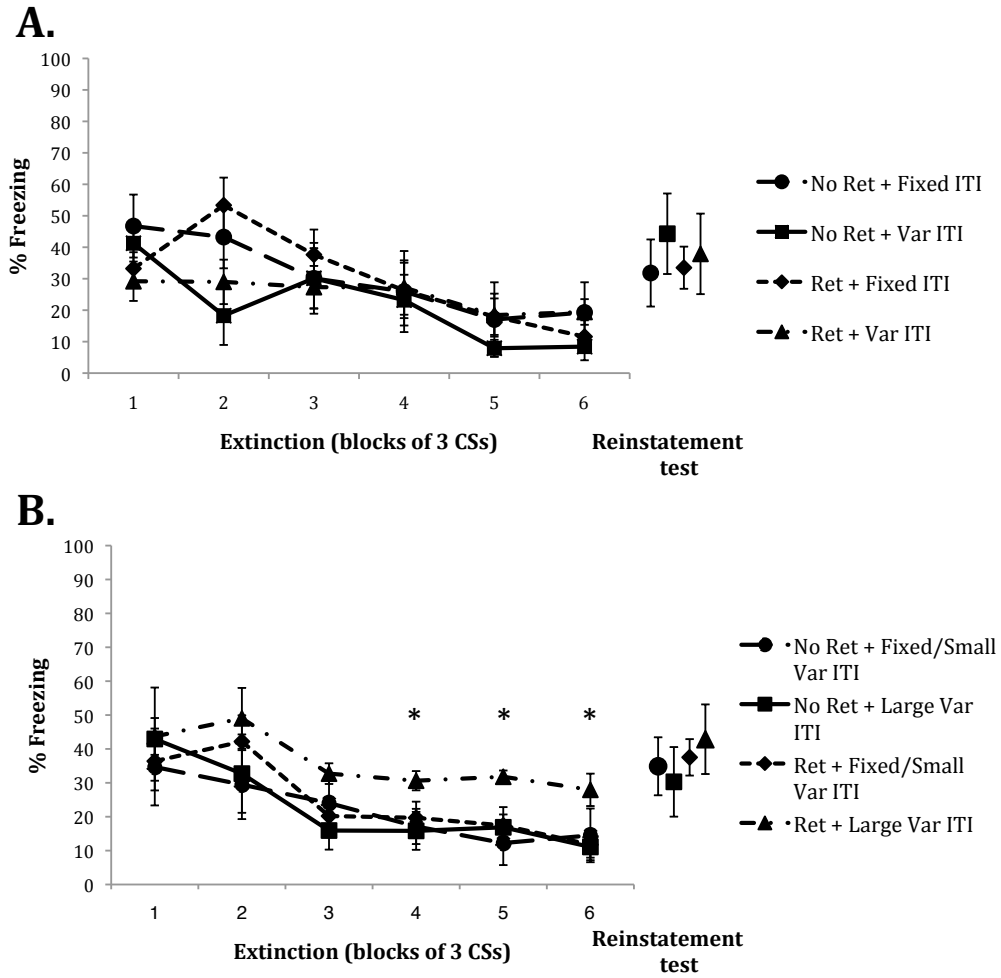

Figure 2.4 Freezing during the 20 seconds prior to CS presentation during extinction and before the reinstatement test**. A.** In Experiment 1, there were no group differences in preCS freezing during extinction or prior to the reinstatement test. **B**. In Experiment 2, all groups showed similar preCS freezing during the first half of extinction, but the group that received the retrieval CS and largely variable ITIs showed more preCS freezing during the second half of extinction than the other groups, indicating uncertainty for when the CS would appear. These group differences in preCS freezing did not persist through reinstatement, and thus are not due to generalized freezing, but instead are in response to ITI variability. Ret = retrieval, ITI = intertrial interval.

2.6 Discussion

Behavioral interference via extinction training during a post-retrieval reconsolidation window has been shown to persistently attenuate fear responses (Monfils et al., 2009). To maximize the degree of fear attenuation, we hypothesized that exposing subjects to random, unpredictable CSs during post-retrieval extinction would promote greater memory updating than CSs with fixed ITIs. We showed that this was indeed the case, as subjects in Experiments 1 and 2 who received a retrieval cue followed by extinction with a variable ITI showed more persistent reduction of fear than subjects who received fixed extinction ITIs.

In Experiment 1, extinction consisted of 18 CSs, with an average ITI of 1 minute. In this case, we saw main effects of the two treatment conditions: subjects who received an isolated retrieval CS prior to extinction showed less post-reinstatement return of fear on average than subjects who did not receive the retrieval cue; subjects who received variable extinction ITIs showed less post-reinstatement return of fear than subjects who received fixed ITIs. We saw no interactions and no differences in PreCS freezing, indicating that memory destabilization via retrieval and increased memory updating via a variable ITI acted independently.

In Experiment 2, we repeated the procedures of Experiment 1, but we (1) extended the 18-CS extinction such that the average ITI was 2 minutes and (2) tested two levels of variability of ITIs, such that the "small" variablility ITI condition resembled the variability introduced in Experiment 1, while the "large" variability ITI condition was completely randomized. In

this experiment, we observed a significant interaction between the retrieval and variability conditions, indicating that increased variability in ITI increased memory updating *only* when the memory was already destabilized via an isolated retrieval CS.

The discrepancy in the interaction between the retrieval and variability conditions in Experiments 1 and 2 may be partially due to the limited subjective variability in ITI that was allowed by shorter extinction session (i.e. Experiment 1). Though the "variable" condition in Experiment 1 and the "large variability" condition in Experiment 2 both consisted of random CS presentations, the extended length of extinction in Experiment 2 allowed for a larger range of potential ITIs, thus a higher likelihood that subjects had a subjective experience of CS "randomness".

This explanation is supported by group differences in PreCS freezing in Experiment 2 that were not present in Experiment 1. In Experiment 2, the group that received a retrieval CS + large variability extinction ITI (which is also the only group in either experiment that showed no increase whatsoever in freezing from post-extinction to post-reinstatement) froze more in the 20 seconds preceding each CS during the second half of extinction. This demonstrates that the subjects in this group likely believed that something aversive could happen at any given moment more so than the other groups, and thus froze more during the ITI. Since we did not observe the same group differences in freezing during the CSs (which indicates anticipation for USs), we can reasonably assume that all groups experienced the same reduction in expectation for a paired CS-US. Consequently, by using a variable ITI and thus

manipulating subjects' expectations for any stimulus at all, we effectively mediated the amount of memory updating induced by extinction. Evidence suggests that stress can be detrimental to memory reconsolidation (Maroun & Akirav, 2008; Wang et al., 2008), so perhaps the explanation for this finding lies in the possibility that by reducing a subject's ability to predict when the CS occurs during extinction, we generally increased the amount of stress the subject experienced during a time period critical for reconsolidation of the original memory elicited by the isolated retrieval CS.

New evidence supports that the larger the discrepancy between expected and observed outcomes, the more likely the animal is to infer a new state of the world, and thus is more likely to form a new memory for the experience rather than update and old one (Gershman, Blei, & Niv, 2010; Gershman & Niv, 2012; Redish, Jensen, Johnson, & Kurth-Nelson, 2007). Though Bayesian learning theories suggest that surprise signals uncertainty and thus the need for new learning, our results support that this phenomenon may not apply in the same way to uncertainty of CS timing. On the contrary, we observed new learning when predictability of the CS during extinction was high (as evidenced by higher return of fear in the fixed ITI conditions). Though this seems at odds with normative statistical models of learning, it is actually not so incongruous. We posit that because CS-US presentation during fear conditioning was surprising from the perspective of the animal, that it is less likely to infer a new latent cause when CS-only presentation during extinction is also surprising. In other words, the animal learns to expect unpredictability in stimulus timing; thus, when timing

becomes predictable, a new latent cause emerges leading to new memory formation.

In summary, we understand now that memories change often; however situations and mechanisms that instigate memory updating are still not fully characterized. One theoretically simplistic, yet mathematically complex explanation for why memories update is that experiences that greatly resemble each other (i.e. possess the same latent causes) are more likely to be (re)consolidated together (Gershman et al., 2013). Conversely, experiences that are similar enough to evoke a memory but also possess unique elements (i.e. new latent causes) qualify a different situation and require new encoding and differential consolidation. In these experiments, extinction training administered during the post-retrieval reconsolidation window resulted in maximal fear memory attenuation when extinction consisted of CSs presented at variable (random) intervals, rather than fixed (predictable) intervals. We posit that this randomness created a situation whereby extinction training maximally resembled the unpredictable nature of fear conditioning, and thus promoted memory updating over new memory formation. This paradigm shows promise in translational research on treatments for fear and anxiety disorders, as unpredictability and random exposure is not typically considered in current treatments.

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2.7 Literature Citations

Alberini, C. M. (2005). Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends in Neurosciences*, *28*(1), 51–6.

Anderson, J. R. (1991). The adaptive nature of human categorization. *Psychological Review*, *98*, 409–429.

Barrett, D., Shumake, J., Jones, D., & Gonzalez-Lima, F. (2003). Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *The Journal of neuroscience*, *23*(13), 5740-5749.

Courville, A. C. (2005). Similarity and discrimination in classical conditioning: A latent variable account. *Advances in Neural Information Processing Systems*, 12, 313–320.

Courville, A. C., Daw, N. D., & Touretzky, D. S. (2006). Bayesian theories of conditioning in a changing world. *Trends in Cognitive Sciences*, *10*(7), 294–300.

Courville, A. C., Gordon, G., Touretzky, D. S., & Daw, N. D. (2003). Model uncertainty in classical conditioning. *Advances in Neural Information Processing Systems*, 16, 977–984.

Dębiec, J., & Ledoux, J. E. (2004). Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience*, 129(2), 267-272.

Duvarci, S., & Nader, K. (2004). Characterization of fear memory reconsolidation. *The Journal of neuroscience*, *24*(42), 9269-9275.

Debiec, J., & LeDouxy, J. E. (2013). Reconsolidation of Pavlovian Conditioned Defense Responses in the Amygdala. *Memory Reconsolidation*, 69.

Dudai, Y . (2004). The neurobiology of consolidations, or, how stable is the engram? *Annual Review of Psychology*, 55, 51e86.

Effting, M., & Kindt, M. (2007). Contextual control of human fear associations in a renewal paradigm. *Behaviour Research and Therapy*, 45(9), 2002-2018.

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience letters*, 163(1), 109-113.

Fricks-Gleason, A. N., & Marshall, J. F. (2008). Post-retrieval β-adrenergic receptor blockade: Effects on extinction and reconsolidation of cocaine-cue memories. *Learning & memory*, *15*(9), 643-648.

Gershman, S. J., Blei, D. M., & Niv, Y. (2010). Context, learning, and extinction. *Psychological Review*, *117*(1), 197–209.

Gershman, S. J., Jones, C. E., Norman, K. A., Monfils, M. H., & Niv, Y. (2013). Gradual extinction prevents the return of fear: implications for the discovery of state. *Frontiers in behavioral neuroscience*, *7*.

Gershman, S. J., & Niv, Y. (2012). Exploring a latent cause theory of classical conditioning. *Learning & Behavior*, *40*(3), 255–68.

Hutton-Bedbrook, K., & McNally, G. P. (2013). The promises and pitfalls of retrieval-extinction procedures in preventing relapse to drug seeking. *Frontiers in psychiatry*, 4.

Jones, C. E., & Monfils, M. H. (2013). Using reconsolidation and extinction to weaken fear memories in animal models. *Memory Reconsolidation*, 165.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294(5544), 1030e1038.

Kindt, M., & Soeter, M. (2013). Reconsolidation in a human fear conditioning study: a test of extinction as updating mechanism. *Biological psychology*, *92*(1), 43-50.

Lee, J. L., Milton, A. L., & Everitt, B. J. (2006). Cue-induced cocaine seeking and relapse are reduced by disruption of drug memory reconsolidation. *The Journal of neuroscience*, *26*(22), 5881-5887.

Martin, S. J., Grimwood, P. D., & Morris, R. G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annual Review of Neuroscience*, 23, 649e711.

Millan, E. Z., Milligan-Saville, J., & McNally, G. P. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiology of learning and memory*, 101, 26-32.

Misanin, J. R., Miller, R. R., & Lewis, D. J. (1968). Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science*, 160(3827), 554-555.

Monfils, M. H., Cowansage, K. K., Klann, E., & LeDoux, J. E. (2009). Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *Science*, 324(5929), 951-955.

Müller, G. E., & Pilzecker, A. (1900). Experimentelle beiträge zur lehre vom gedächtniss (Vol. 1). JA Barth.

Nader, K., & Einarsson, E. Ö. (2010). Memory reconsolidation: an update. *Annals of the New York Academy of Sciences*, *1191*(1), 27-41.

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000a). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722-726.

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000b). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722-726.

Olshavsky, M. E., Song, B. J., Powell, D. J., Jones, C. E., Monfils, M. H., & Lee, H. J. (2013). Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus. *Frontiers in behavioral neuroscience*, 7.

Oyarzún, J. P., Lopez-Barroso, D., Fuentemilla, L., Cucurell, D., Pedraza, C., Rodriguez-Fornells, A., & de Diego-Balaguer, R. (2012). Updating fearful memories with extinction training during reconsolidation: a human study using auditory aversive stimuli. *PLoS One*, *7*(6), e38849.

Pavlov, I. (1927). Conditioned reflexes. *An Investigation of the Physiological Activity of the Cerebral Cortex*.

Pearce, J. M., & Hall, G. (1980). A model for Pavlovian learning: Variations in the effectiveness of conditioned but not of unconditioned stimuli. *Psychological Review*, *87*(6), 532–552.

Pedreira, M. E., & Maldonado, H. (2003). Protein synthesis subserves

reconsolidation or extinction depending on reminder duration. *Neuron*, *38*(6), 863-869.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *The Journal of Neuroscience*, 20(16), 6225-6231.

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology*, *33*(1), 56-72.

Quirk, G. J., Paré, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., & Vicentic, A. (2010). Erasing fear memories with extinction training. *The Journal of Neuroscience*, *30*(45), 14993-14997.

Redish, A. D., Jensen, S., Johnson, A., & Kurth-Nelson, Z. (2007). Reconciling reinforcement learning models with behavioral extinction and renewal: implications for addiction, relapse, and problem gambling. *Psychological review*, *114*(3), 784.

Rescorla, R. A., & Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In *Classical conditioning: current research and theory*.

Robbins, S. J. (1990). Mechanisms underlying spontaneous recovery in autoshaping. Journal of Experimental Psychology: Animal Behavior Processes, 16(3), 235.

Schiller, D., Monfils, M. H., Raio, C. M., Johnson, D. C., LeDoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature*, *463*(7277), 49-53.

Stern, C. A., Gazarini, L., Takahashi, R. N., Guimaraes, F. S., & Bertoglio, L. J. (2012). On disruption of fear memory by reconsolidation blockade: evidence from cannabidiol treatment. *Neuropsychopharmacology*, *37*(9), 2132-2142.

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., ... & Lu, L. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science*, *336*(6078), 241-245.

Chapter 3: Reconsolidation updating, genetic variability and memory enhancement interact with extinction to predict reinstatement of fear memories

3.1 Abstract

Many factors account for how well an individual extinguishes conditioned fear, such as genetic variability, learning capacity and the parameters under which extinction is administered. Correspondingly, many strategies have been proposed to enhance the effectiveness of fear extinction by targeting these factors. For instance, administering extinction during the reconsolidation window attenuates fear and prevents it from returning. Additionally, enhancing the extinction memory is a promising strategy for preventing the return of fear. Here we assess how within-session extinction during the reconsolidation window and post-extinction enhancement of consolidation interact to prevent fear reinstatement. Subjects selectively bred for extinction phenotype and fear conditioned to a tone stimulus were administered extinction one hour after either a retrieval conditioned stimulus or no retrieval stimulus. Following extinction, subjects received injections of either saline or mitochondrial enhancer USP methylene blue (MB), shown previously to enhance extinction memory. Results show that both reconsolidation updating and MB administration prevented fear memory reinstatement, but that prevention depended on the level of fear extinction. MB administration ensured that subjects remained at the level of freezing that remained at the end of extinction. Thus, only subjects who

extinguished effectively benefitted from MB. This phenomenon suggests that metabolic enhancement via MB is an effective clinical strategy for persistent reduction of fear in subjects who are responsive to extinction. Likewise, reconsolidation updating in these subjects was effective for subjects who were responsive to extinction, with limited effect on extinction-resistant subjects.

3.2 Introduction

As fear and anxiety-related disorders have become among the most prevalent psychiatric disorders, strategies for attenuating persistent fear have become important topics of discussion. Historically, the most widely used behavioral intervention for reducing fear responses is extinction (clinically referred to as exposure therapy), whereby a subject is repeatedly exposed to a fearful stimulus in a safe environment, such that the emotional reaction to the stimulus diminishes. However, extinction is an incredibly complex process, and its long-term efficacy for reduction of fear is influenced by innumerable factors, including genetic variability (see Londsdorf & Kalisch, 2011 for review), strength of the fear memory (Wang, Alvares & Nader, 2009) length of time since extinction (Myers, Ressler & Davis, 2006), parameters of extinction (Baum, 1969; Cain, Blouin & Barad, 2003) and a plethora of contextual stimuli (Bouton, 1988; 2002; Bouton et al. 2006; Effting & Kindt, 2007; Ji & Maren, 2007; Milad et al., 2005). The following experiment assesses how genetic variability, reconsolidation updating, responsiveness to extinction and metabolic enhancement contribute to resilient fear reduction.

3.2.1 Fear extinction

In the realm of non-human animal research, fear is typically modeled by some iteration of classical (i.e. Pavlovian) conditioning and extinction (Bouton, Mineka, & Barlow, 2001; Davis et al., 2006) . In rodent fear conditioning, a previously neutral stimulus (conditioned stimulus; usually a

tone or light) is paired with an aversive stimulus (unconditioned stimulus; usually a mild electric footshock). During extinction, the conditioned stimulus (CS) is presented repeatedly in absence of the unconditioned stimulus (US). This leads to a state in which the CS is no longer predictive of an aversive event, thus the conditioned response (CR; freezing) declines (Bouton, 1993; 2004; Myers & Davis, 2002; 2006; Rescorla, 1996; Sotres-Bayon, Bush, & LeDoux, 2004). While this strategy for reduction of fear has shown some clinical efficacy, current behavioral models suggest that extinction alone does not result in lasting changes to the original blueprint of the fear memory itself (Bouton & Bolles, 1979; Effting & Kindt, 2007; Pavlov, 1927; Rescorla & Heth, 1975; Robbins, 1990; Westbrook et al., 2002). Instead, extinction triggers the formation of a new inhibitory memory (Barrett et al., 2003; Effting & Kindt, 2007; Morgan, Romanski & LeDoux, 1993; Pavlov, 1927; Quirk et al., 2000; Quirk & Mueller, 2008; Robbins, 1990). The result of this process is two memories that compete for expression upon future CS induced memory retrieval (Rescorla & Heth, 1975; Rescorla, 2001; Robbins, 1990). If upon memory retrieval the memory for extinction is stronger than the original fear memory, then the extinction memory suppresses the fear memory, and the animal will not respond in a fearful manner. If upon retrieval the extinction memory is not strong enough to suppress the fear memory (e.g. due to weak extinction or strong re-emergence of the original fear), then the animal will respond fearfully (e.g. freeze). This phenomenon leaves the subject susceptible to fear return due to the passage of time (spontaneous recovery; Robbins, 1990), return to the

fearful context (renewal; Bouton & Bolles, 1979) or re-exposure to the US (reinstatement; Bouton, 1993; 2004; Myers & Davis, 2002; 2006; Rescorla, 1996; Sotres-Bayon, Bush, & LeDoux, 2004). The subject would be less vulnerable to fear return if, upon memory retrieval, the original fear memory was altered.

3.2.2 Reconsolidation updating

Manipulate or updating the original blueprint for a predictive cue is a more strategic approach to alleviating fear. If the original memory that a CS predicts an aversive event is altered, then the anxiety elicited by that memory is also permanently altered. As proof of principle for this concept, inhibitors of protein synthesis (which are necessary for memory consolidation and reconsolidation) shortly after training or after retrieval of a memory have been shown to alter (re)consolidation (Davis & Squire, 1984; Dundai, 1996; Flexner et al., 1965; Goelet et al., 1986; McGaugh, 2000; Nader, Schafe & Ledoux, 2000; Sara, 2000; Schafe & Ledoux, 2000). However, though this line of research has been tremendously helpful in understanding mechanisms of memory, consolidation and reconsolidation, the toxic nature of protein synthesis inhibitors abolishes its translational clinical value. However, a burgeoning line of research is describing ways to modify previous memories through a behavioral approach. Monfils et al. (2009) devised a behavioral paradigm whereby rats showed more persistent reduction in fear responses when extinction was administered during a post retrieval labile window as compared with extinction alone. This paradigm

has been replicated several times in both non-human animals (Auber et al., 2013; Ma, Zhang & Yu, 2012; Millan, Milligan-Saville & McNally, 2013; Olshavsky et al., 2013; Quirk et al., 2010; Xue et al., 2012) and humans (Kindt, Soeter & Vervliet, 2009; Schiller et al., 2010; 2013). However, there is a large degree of individual variability in extinction alone, and equally as much (if not more) variation in the retrieval + extinction paradigm. Thus, it is important not only to describe the optimal parameters for the retrieval + extinction paradigm (as investigated in Chapter 2), but also to investigate the implications such individual variability.

3.2.3 Individual differences in fear extinction: the role of genetic variability

One major consideration of extinction that is often overlooked is that there is considerable individual variation in responsiveness to extinction, and that genetic factors represent a significant source of this variation (Lonsdorf & Kalisch, 2011). Indeed, there are significant individual differences in conditionability (Hettema et al., 2003) and extinction (Soliman et al., 2010) in both humans (Fava, Rafanelli, Grandi, & Conti, 2001; Fava et al., 1995; Foa & Kozak, 1986) and other animals (Bush, Sotres-Bayon, & LeDoux, 2007; Shumake, Furgeson-Moreira, & Monfils, 2014; Yehuda & LeDoux, 2007) . Additionally, anxiety disorders show strong heritability (Hettema et al., 2001). Specifically, one-third of the variance in human fear conditioning (Hettema, 2003; Lonsdorf & Lalisch, 2011) and in the

vulnerability for anxiety disorders (Gordon & Hen, 2004) is attributed to genetic factors.

Such factors may also play a role in memory processing during extinction, and should be considered when evaluating extinction's effectiveness. However the large number of potential genetic variants that could influence fear and extinction makes a comprehensive genetic assessment difficult. Instead of investigating the role of specific genes, we assessed extinction memory interactions using rats selectively bred for extinction behavior: high extinguishers (a lineage particularly responsive to extinction), low extinguishers (a lineage particularly unresponsive to extinction), and a randomly bred lineage.

3.2.4 Memory enhancement via metabolic enhancer methylene blue

Most pharmacological treatments for fear and anxiety related disorders alter the action of specific neurotransmitter systems at the level of the neuronal synapse. These agents include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinepherine reuptake inhibitors (SNRIs), tricyclic antidepressants and benzodiazepines. Each of these classes of drugs are thoughts to exert their anxiolytic effects through targeting pathways in the amygdala and other related brain structures thought to play a role in fear memory consolidation and expression. A recent review from (Nandhra, Murphy, & Sule, 2013) describes recent research findings suggesting that targeting memory and enhancing cognition may represent an alternative approach to the reduction of anxiety symptoms.

One potential strategy for this is to use pharmacological agents that do not alter neurotransmission, but instead target general neuronal functioning to boost the overall action of a neuronal network, rather than a specific receptor system. Methylene blue (MB) is a historical blue dye with the ability to do just that. Diverging greatly from typical pharmacological treatments, MB's unique molecular structure allows it to cross the blood brain barrier and accumulates in nervous tissue, particularly in the matrices of mitochondria (Gabrielli et al., 2004). There, at low doses MB forms a redox equilibrium with electron transport chain complexes, resulting in increased oxygen consumption and ATP production, as well as decreased formation of damaging reactive oxygen species (Atamna et al., 2008; Ohlow & Moosmann, 2011; Riha et al., 2005; Rodriguez et al., 2014; Rojas & Gonzalez-Lima, 2010; Rojas et al., 2012; Wen et al., 2011). MB's ability to enhance mitochondrial respiration has been demonstrated as a unique strategy for memory enhancement (Callaway et al., 2004; Martinez, 1978; Riha et al., 2005; Riha, Rojas & Gonzalez-Lima, 2011), including enhancement of memory for extinguished fear (Gonzalez-Lima & Bruchey, 2004; Wrubel et al., 2007). Methylene blue's action in these cases is to enhance mitochondrial respiration during memory consolidation, thus enhancing consolidation. One caveat to this approach is the fact that MB enhances whichever neural network is active in the time period MB is on board. However, if MB is administered to a subject after a learning task in which the subject did not learn, then MB may be theoretically detrimental, as one would be enhancing a network that does not predict task learning (Telch et al., 2014). This

experiment is the first investigation in rats in regard to how effectiveness of learning during the task can moderate MB's therapeutic effectiveness. It is our hypothesis that MB administration will be therapeutically beneficial in extinction learning for subjects who respond favorably to extinction. Conversely, we predict that MB will not be therapeutic—and may in fact be detrimental—in extinction learning for subjects who do not respond to extinction.

3.2.5 Combining fear extinction, genetic variability, reconsolidation updating and memory enhancement in a model of persistent fear reduction

Undeniably, fear reduction via extinction is a complex process that can be influenced by many factors. In this experiment, we test several of these factors—genetic variability, reconsolidation updating and memory enhancement via MB—in combination in order to clarify which factors are the most important in regard to persistent fear reduction.

3.3 Hypothesis

Subjects selectively bred for high or low extinction behavior will show differential effects of both the retrieval + extinction manipulation and post extinction treatment with MB. Specifically, subjects who respond optimally to extinction (i.e. demonstrate large decreases in freezing during extinction) will benefit the most from both the memory updating paradigm and MB treatment. Those subjects who do not respond well to extinction (i.e

demonstrate no decreases or small decreases in freezing during extinction) will not benefit from the memory updating manipulation or MB treatment.

3.4 Materials and Methods

3.4.1 Subjects

3.4.1.1 Selective breeding—parent generations

Subjects were selected from the third and fourth generations of a population of Long-Evans rats bred specifically for extinction phenotype (see Shumake et al., 2014 for a detailed description of selection criteria and breeding procedures). Briefly, for four generations, Long-Evans rats were fear conditioned to a pure tone (20s, 5 kHz, 80 dB) co-terminating with a footshock (0.7 mA for 0.5 s). The following day, subjects received 18 extinction trials of tone-alone presentations of the same duration as experienced in acquisition. On the third day, rats received 3 memory-recall trials of tone-alone presentations, again with the same time parameters.

Founding breeders for the two selected lines were chosen by a two-step procedure that ensured selection for differences in extinction in the absence of differences in acquisition (Shumake et al., 2014) . First, selection for both lines was restricted to subjects showing robust freezing 24 hours after acquisition. Second, subjects from this subsample were ranked according to their freezing scores 24 hours after extinction training, and the top and bottom 10 males and females (each comprising 20% of the screened sample) were chosen as the founders of the low extinguisher (LE) and high extinguisher (HE) lines. The optimal cutoff criterion for ‘‘robust freezing’’

was set at 75 %, the value which achieved minimal differences between groups post-conditioning relative to maximal differences between groups post-extinction (determined by a cost function).

A randomly bred (RB) control line was started from a random selection of 10 males and 10 females from the remaining 60% of the initially screened sample that were not chosen for the selected lines. Since it excluded population extremes, the selection of Generation 0 of the RB line was only partially random, but the selection of subsequent generations was entirely random.

3.4.1.2 Husbandry

Breeding pairs were housed together (1 male to 1 female) for 10 days (i.e., the average length of 2 estrous cycles). Females were then re-housed with their former female cage mate for the next 10 days, and then single housed until giving birth. On the day after birth, newborns were briefly separated from their mothers for sexing and culling. The number of males and females were counted, and litters were reduced in size to 12 pups (ideally to 6 males and 6 females or to the most equal sex ratio possible). The litters were then left undisturbed except for weekly cage changes until weaning at 21 days.

3.4.1.3 Experimental subjects

Subjects consisted of 96 male Long-Evans rats (approximately 60 days of age) selected from the third and fourth generations of the selectively bred

lines described above. From weaning until 41 days, rats were group housed with their same-sex siblings, usually in groups of 6. Thereafter, rats were housed in pairs. Throughout all breeding and experimental procedures, subjects were housed in temperature and humidity-controlled transparent polyethylene cages and were maintained on a 12h/12h light/dark cycle with food and water available ad libitum. Procedures were conducted in compliance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by the Animal Care and Use Committee of the University of Texas at Austin.

3.4.2 Apparatus

All experimental manipulations (fear conditioning, retrieval, extinction, reinstatement, reinstatement probe) were administered in the same context (operant conditioning chambers; Coulbourn Instruments). Each chamber was equipped with a stainless-steel rod flooring connected to a shock generator (Model H10-11R-TC-SF; Coulbourn Instruments) and individually enclosed in a sound-insulated box (Isolation Cubicle, Model H10-24T; Coulbourn Instruments). Chambers were illuminated with a red light. Behavior was recorded by infrared digital cameras (Panasonic, model wvBP334) mounted on the ceiling of each unit. Stimulus presentation was automated using FreezeFrame2 software (Coulbourn Instruments). All equipment was cleaned with Windex (SC Johnson) between each session.

3.4.3 Habituation to the context

To minimize contextual fear conditioning, on the day before fear conditioning, subjects were placed in the conditioning chambers for 15 minutes and allowed to freely explore. Neither the CS nor the US was given during this session.

3.4.4 Experimental procedures

3.4.4.1 Fear conditioning

A pictorial representation of the experimental timeline is illustrated in Figure 3.1. Subjects were placed in the conditioning chambers, allowed to habituate for 3 minutes, and then fear conditioned with 3, 20-s, 5 kHz, 80 dB tones (CS). Each co-terminated with a 0.5-s, 0.7 mA footshock (US). The interval between each CS was 120 s. After conditioning, each subject remained in the chamber for 3 minutes and was then returned to its home cage.

3.4.4.2 Retrieval and extinction

The day after conditioning, subjects were returned to the conditioning chambers and allowed to habituate for 3 minutes. Following habituation subjects received a retrieval (CS) trial. After retrieval, subjects either remained in the chambers and received 18 more CSs (extinction alone group) or were placed back in their home cages, then returned to the conditioning chambers 60 min later to complete the 18-CS extinction (retrieval +

extinction group)[5]. The interval between CSs was variable, ranging from 20 sec to 240 sec with an average of 120 sec. Upon completion of extinction, subjects remained in the conditioning chambers for 3 minutes. All subjects spent the same total amount of time in the context and were exposed to the same number of CSs (19 total). Subjects in the retrieval + extinction group spent the 60-minute duration between the retrieval CS and extinction with their cage-mates in the colony room.

3.4.4.3 Drug administration

MB was dissolved in sterile saline to a concentration of 5 mg/mL. Prior to returning to their home cages after extinction, subjects received an intraperitoneal (IP) injection of either 4 mg/kg USP MB or an equivalent volume of sterile saline.

3.4.4.4 Reinstatement and reinstatement test

The day after extinction, subjects habituated to the fear conditioning chambers for 5 minutes and then received two footshocks (0.5 s, 0.7 mA). The interval between footshocks was 120 s. Following the shocks, subjects remained in the chambers for 5 minutes. The following day, rats were placed back into the chambers, habituated for 5 minutes. To test for reinstatement

[5] The extinction parameters used in this experiment were taken from the most optimal extinction parameters found from Chapter 2—19 total CSs with a largely variable inter-trial interval averaging 2 minutes.

of the tone-shock fear association, subjects heard 3 presentations of the CS alone.

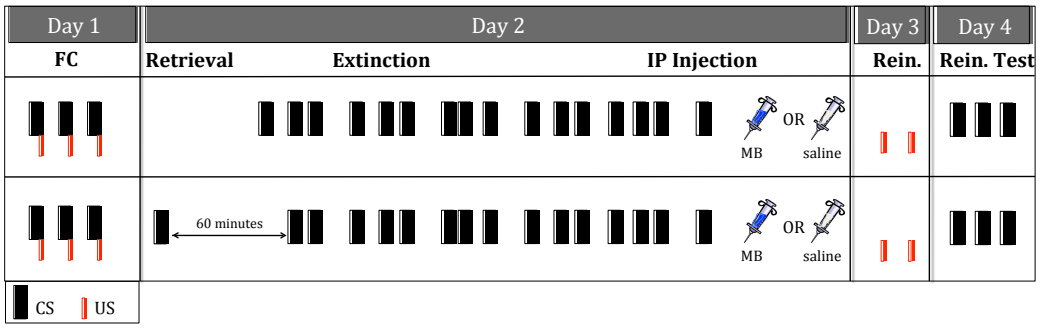

Figure 3.1 Experimental timeline. On Day 1, subjects received three tone-shock pairings. On Day 2, subjects received either a retrieval CS followed by extinction or extinction alone. Immediately following extinction, subjects received injections of either saline or MB. On Day 3 subjects were presented with two unpaired USs, followed by three unpaired CSs the following day to test for reinstatement. FC = fear conditioning, Rein = reinstatement, CS = conditioned stimulus, US = unconditioned stimulus, MB = methylene blue

3.4.5 Behavioral scoring—freezing

Freezing was defined as the absence of all movement aside from breathing and ear twitching, not including sleeping or resting. Behavioral was scored manually from videos by an experimenter blind to experimental conditions. The total amount of CS-induced freezing was expressed as a percentage of total time spent freezing during each 20-s CS. Freezing during the PreCS is expressed as percent freezing during the 20 seconds immediately preceding the first CS. Freezing during the first four extinction CSs and last four extinction CSs is expressed as mean freezing over four CS presentations at the beginning and end of extinction respectively. Post

reinstatement freezing is expressed as the mean over three CS presentations 24 hours after reinstatement.

3.4.6 Statistical analyses

Data was analyzed using SPSS Statistics software version 22 using mixed factor ANOVAs, univariate ANOVAs, hierarchical linear regression analyses and bivariate correlation comparisons. A mixed factor ANOVA was used to verify similar acquisition of the fear memory across groups using contextual freezing (freezing during the 20 seconds prior to the first CS) and freezing during the three fear conditioning CSs as repeated measures and group membership (lineage, extinction type and drug treatment) as between subjects factors. A mixed factor ANOVA was used similarly to assess extinction, with average freezing during the first four extinction CSs and the last four extinction CSs as repeated measures and group membership as between subjects factors. A hierarchical linear regression was used to explore how much post reinstatement freezing was accounted for by lineage, extinction type, within-session extinction and drug treatment. Subsequently, separate univariate analyses were used to assess the effects of extinction type and drug treatment in each lineage, with within-session extinction as a covariate. Where appropriate, post-hoc tests were performed using Bonferroni corrected mean comparisons.

3.5 Results

3.5.1 General comments

Subjects were sampled from the third and fourth generations of a population of rats selectively bred for extinction phenotype. Preliminary analyses showed that there were no differences ($p > .05$) in any behavioral measure between subjects from the third and fourth generations, and including generation as an independent variable did not explain more variance or increase the overall fit of our models. Consequently, subjects from both generations were analyzed together in all of our analyses. Also, all groups displayed similar fear conditioning, showing no significant differences in freezing during the pre CS interval, fear conditioning or the first four CSs of extinction the following day. Thus, any observed differences in extinction and reinstatement are independent of generation and acquisition of the conditioned fear memory.

3.5.2 Extinction

A repeated measures ANOVA revealed significant within-session differences in average freezing during the first four and last four extinction CSs, $F(1, 84)=259.01$, $p=.000$, $\eta^2 =.755$, indicating that all groups showed attenuation of freezing during extinction. There were no significant interactions ($p > .05$) between extinction time point and extinction type, drug treatment, retrieval by drug treatment, retrieval x lineage, drug treatment x lineage, or retrieval by drug treatment by lineage (Table 3.1; Figure 3.2). There was a significant interaction between extinction time point and lineage, $F(2, 81)=5.62$, $p=.005$, $\eta^2 =.118$ (Table 3.1). Post hoc comparisons using Bonferroni correction revealed that though all lineages showed similar

freezing during the first 4 CSs during extinction, F(2, 92)=0.47, p=.625, η^2 =.010, the high extinction line (M=30.88, SD=21.40) froze less at the end of extinction than the low extinction line (M=51.34, SD=18.09). The randomly bred line (M=37.61, SD=24.00) froze significantly less than the low extinction line at the end of extinction, but did not differ from the high extinction line (Figure 3.2a). This indicates that the low extinction line did in fact show less within session extinction than the high extinction line, without nonspecific effects on fear conditioning, and in absence of any effects of extinction type or drug treatment (Figure 3.2b, c and d).

Table 3.1. Within-subjects extinction.

Variable	*df*	*F*	η^2	*p*
Ext time point (first 4 CSs and last 4 CSs)	1	259.012	.755	.000***
Ext time point * ext type	1	2.417	.028	.124
Ext time point * drug treatment	1	3.397	.039	.069
Ext time point * lineage	2	5.624	.118	.005**
Ext time point * ext type * drug treatment	1	.112	.001	.739
Ext time point * ext type * lineage	2	.041	.001	.960
Ext time point * drug treatment * lineage	2	1.153	.027	.321
Ext time point * ext type * drug treatment * lineage	2	.813	.019	.447
Error	84			

Note. ***p<.001, **p<.01, Ext = extinction

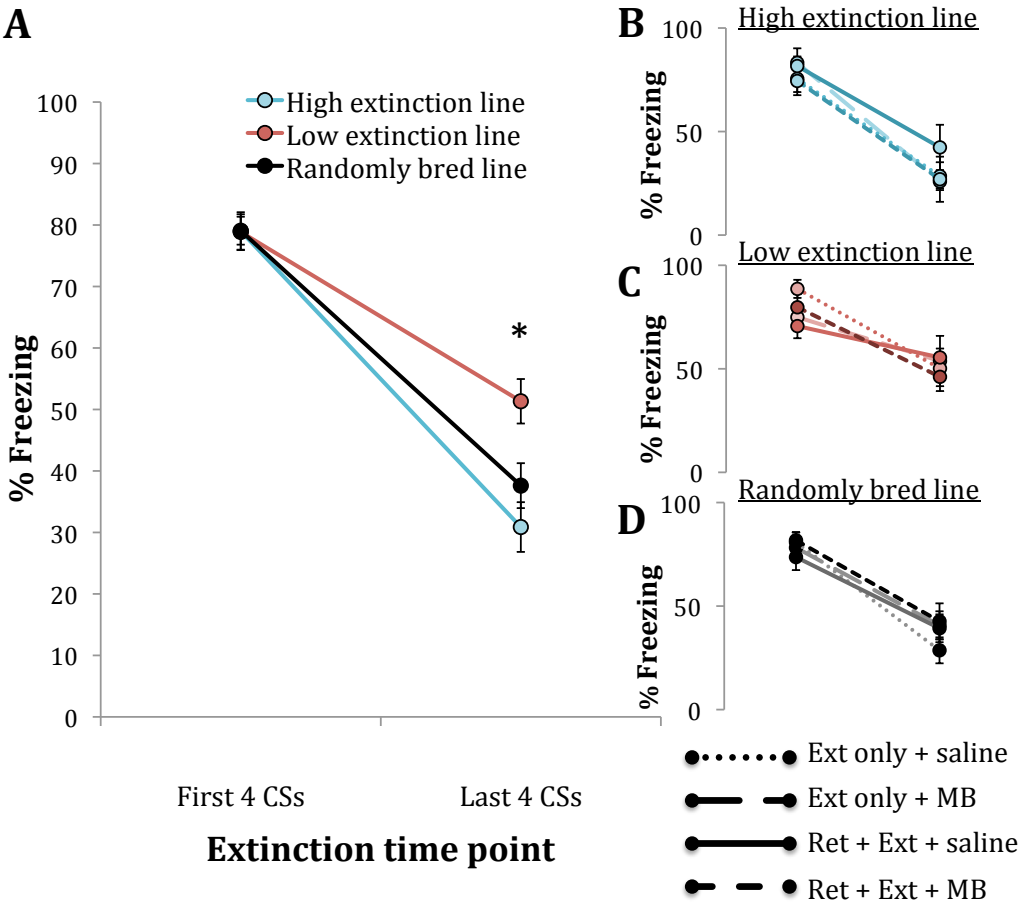

Figure 3.2 Percent freezing during extinction. All groups froze similarly at the beginning of extinction, indicating similar levels of fear conditioning. Additionally, all groups showed significant within-session decreases in freezing, indicating that all groups extinguished **A.** The low extinction lineage froze significantly more at the end of extinction than the high extinction line and the randomly bred line. There were no effects of the experimental manipulations (extinction condition or drug administration) on within-session extinction for the low extinction line **(B)**, the high extinction line **(C)** or the randomly bred line **(D)**. *p < .05, CS = conditioned stimulus, MB = methylene blue, Ext = extinction, Ret = retrieval

3.5.2 Post reinstatement

3.5.2.1 Predicting reinstatement from lineage, extinction type, within-session extinction and drug treatment

To assess how much of the variance in post reinstatement freezing was accounted for by lineage, extinction type, degree of fear extinction and drug treatment, hierarchical regression analyses were performed. This type of analyses allowed us to determine if extinction type and drug treatment predicted reinstatement (controlling for lineage), as well as if within-session extinction moderated the effect of methylene blue. We used a three stage hierarchical regression, with post reinstatement freezing as the dependent variable. Lineage was entered in the first stage; within-session extinction, extinction type and drug treatment were entered in the second stage; and within-session extinction by drug treatment interaction was entered in the final stage. The interaction variable was entered in the final stage because we initially predicted that the therapeutic effect of MB treatment would depend on the level of extinction, and such a relationship was revealed by the drug treatment by within-session extinction interaction.

The hierarchical multiple regression (Table 3.2) revealed that at stage 1, lineage contributed significantly to the regression model, $F(1,94)=9.336$, $p=.003$, and accounted for 8.1% of the variation in post reinstatement freezing. Introducing within-session extinction, drug treatment and type of extinction explained an additional 11.0% of the variation in reinstatement and this change in R^2 was significant, $F(4,91)=6.590$, $p=.000$. Finally, adding the within-session extinction by drug treatment interaction to the model

explained an additional 3.3% of variation in reinstatement, and this change in R^2 was also significant, $F(5,90)=6.477$, $p=.000$. When all five independent variables were included in stage 3 of the regression model, neither drug treatment nor extinction type were significant predictors of reinstatement. However, extinction type was very close to reaching significance and there was a significant drug treatment by extinction interaction (Table 3.2). The most important predictor of reinstatement was within-session extinction, followed by lineage and within-session extinction x drug treatment, accounting for 9%, 6% and 5% of the variance in post reinstatement freezing respectively. Together, the five independent variables accounted for 22.4% of the variance in post reinstatement freezing. Since lineage and within-session extinction were the most important predictors of reinstatement, their interactions with the other variables were further assessed.

Table 3.2. Summary of hierarchical regression analysis for variables predicting post reinstatement freezing.

Variable	®	*t*	sr^2	*r*	r^2	Δr^2
Step 1				.301	.090	.090
Lineage	-.301	-3.056**	.09			
Step 2				.474	.225	.134
Lineage	-.219	-2.272*	.05			
Within-session extinction	.313	3.190**	.10			
Extinction type	-.192	-2.054*	.04			
Drug treatment	-.136	-1.461	.02			
Step 3				.514	.265	.040
Lineage	-.220	-2.321*	.06			
Within-session extinction	.284	2.927**	.09			
Extinction type	-.178	-1.940^	.04			
Drug treatment	-.139	-1.531	.03			
Within-session extinction * drug treatment	.202	2.213*	.05			

Note. N=96; **p < .01, *p < .05, ^p = .055 (trending toward significance)

3.5.2.2 Assessment of within-session extinction's effect on reinstatement

Since within-session extinction was a strong predictor of reinstatement, we first looked at the correlation between the two for each treatment condition (type of extinction and drug treatment; Figure 3.3). We then used bivariate correlation comparisons to assess differences in these correlations for each condition. Since our groups were independent of each other, we used Fischer's z-transformation for correlation coefficients for each of our groups and then tested the null hypothesis that $p_1 - p_2 = 0$ using the Student *t*-test, as described by Fisher (1921). A summary of bivariate correlation comparisons is shown in Table 3.3.

Results show that in conditions in which subjects were treated with MB, there was a significant linear correlation between freezing at the end of extinction and freezing post reinstatement. This linear relationship was not present in subjects treated with saline. This indicates that subjects treated with MB were likely to retain their post-extinction levels of freezing, regardless of whether they received extinction alone or retrieval + extinction; and conversely within-session extinction was not significantly predictive of post reinstatement freezing in conditions in which subjects were treated with saline. When collapsing across drug treatment conditions, there was a linear relationship between freezing at the end of extinction and reinstatement for both extinction alone and retrieval extinction groups, but this relationship is likely driven by the inclusion of subjects who received MB. This is further evidenced by comparing group correlations collapsed across extinction type. The extinction-reinstatement correlations between MB- and saline-treated subjects were the only correlations that were significantly different from each other (Table 3.3; Figure 3.3). Importantly, this finding should not be interpreted as strictly a therapeutic effect of MB. Instead, this suggests that MB helps retain post extinction freezing levels. Thus, MB is indeed therapeutic for subjects who show low levels of freezing at the end of extinction; but for subjects not responsive to extinction (i.e. still show high freezing at the end of extinction), MB is likely to reinforce fear rather than attenuate it.

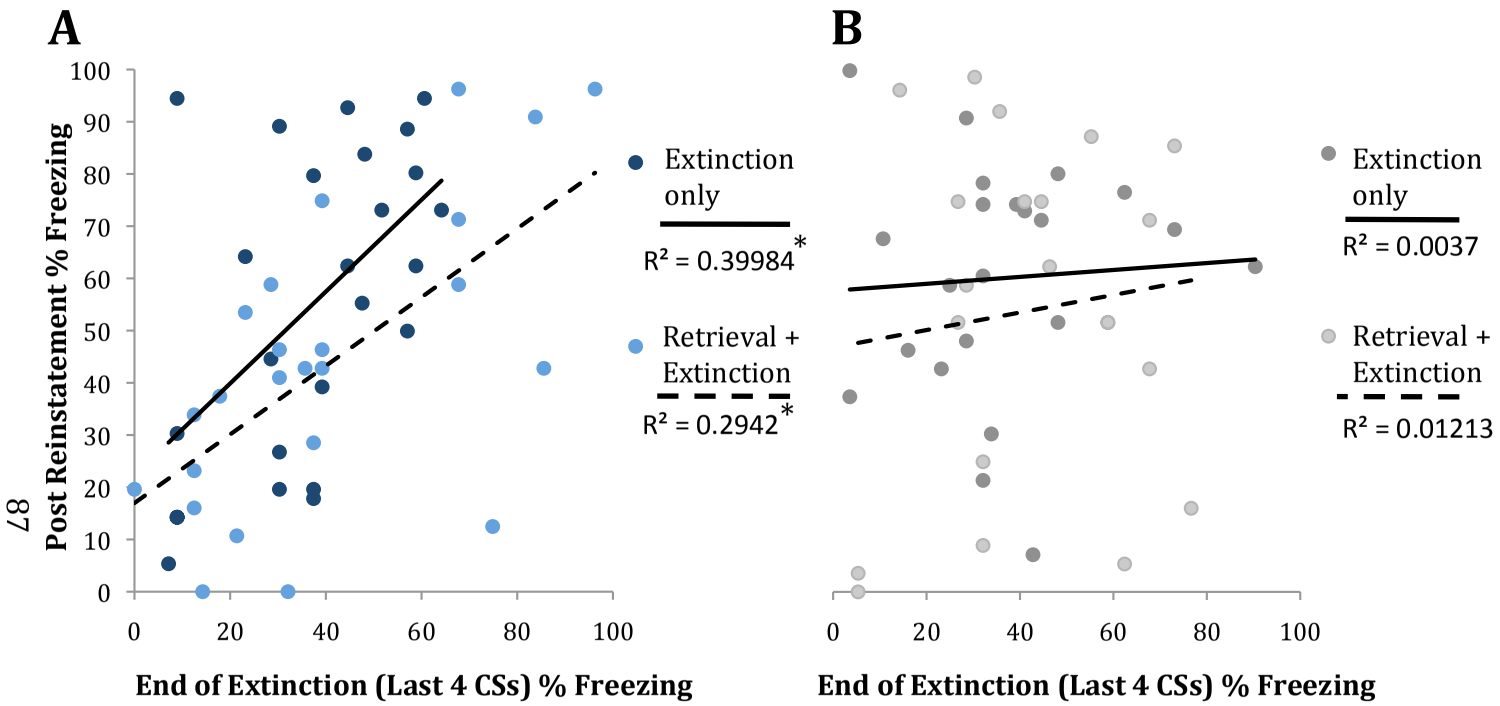

Figure 3.3 End of extinction-post reinstatement freezing correlations. Groups that received post extinction MB treatment showed a linear relationship between the end of extinction and post reinstatement. Similar linear relationships were not observed in subjects treated with saline.

Table 3.3. Bivariate correlation comparisons for freezing during last 4 extinction CSs and reinstatement.

Comparison	r_1	r_2	r'_1	r'_2	*r' diff*	*SE diff*	*z*	*p*
Sal vs. MB (both ext conditions)	.089	.539***	.089	.603	-.514	.211	-2.433	.015*
ExtSal vs. ExtMB	.080	.575**	.080	.655	-.575	.298	-1.927	.054^
RetExtSal vs. RetExtMB	.155	.576**	.156	.656	-.500	.321	-1.557	.119
Ext vs. RetExt (both drug treatment conditions)	.372**	.399**	.391	.422	-.032	.211	-.150	.881
ExtSal vs. RetExtSal	.080	.155	.080	.156	-.076	.318	-.239	.811
ExtMB vs RetExtMB	.575**	.576**	.655	.656	-.001	.302	-.005	.996

Note. Sal = saline. MB = methylene blue. Ext = extinction. RetExt = retrieval + extinction. r_1 and r_2 are the Pearson's correlation coefficients for two sample correlations (r_1 corresponds to the first group listed under "Comparison" and r_2 corresponds to the second group). r'_1 and r'_2 are values used in the Fisher's r-to-z transformation, calculated by $r' = \{\ln[(1+r)/(1-r)]/2\}$. *r' diff* = $r'_1 - r'_2$. *SE diff* = standard error difference. *z* = r' diff/SE diff. p-values calculated from 2-tail *z* distribution. ***p < .001, **p < .01, *p < .05, ^p = .054 (trending toward significance)

3.5.2.3 Assessment of lineage's effect on reinstatement

A repeated measures ANOVA with average freezing during the last 4 extinction CSs and post reinstatement as within-subjects variables and lineage as the between subjects variable confirmed a significant effect of lineage on reinstatement, $F(2, 93)=6.876$, $p=.002$, $\eta^2 =.915$, as well as an overall increase in freezing from the end of extinction to post reinstatement, $F(1, 93)=26.769$, $p=.000$, $\eta^2 =.999$. There was not a significant ($p > .05$) interaction between time point and lineage. Bonferroni corrected post hoc comparisons show that mean post reinstatement freezing for the high extinction line (M=43.91, SD=27.23) was significantly lower than the low extinction line (M=66.08, SD=19.55). The mean post reinstatement freezing for the randomly bred group (M=53.15, SD=29.12) fell in between the low and high extinction lines, and was not significantly different from either (Figure 3.4a). Additionally, paired samples t-tests for each line show significant increases in freezing from the last 4 extinction CSs to post reinstatement for the low extinction line, $t(24)=-5.262$, $p=.000$, and randomly bred line, $t(42)=-3.824$, $p=.000$, but not for the high extinction line ($p > .05$). This demonstrates that the high extinction line was more resistant to reinstatement than the other two lines.

3.5.2.4 Effects of extinction type and drug treatment

To assess the effects of extinction type and drug treatment within the selectively bred lines, separate univariate ANOVAs were conducted for each extinction line with average freezing during post reinstatement as the

dependent variable, extinction type and drug treatment as between subjects variables and average freezing during the last 4 extinction CSs as a covariate. Results for each lineage are summarized in Table 3.4 and Figure 3.4b, c and d. Figure 3.5 provides an alternative visualization of the results. Though both figures are contrived from the same analysis, Figure 3.4 (b, c and d) allows for visual comparison between experimental conditions within each lineage, while Figure 3.5 allows for comparison between lineages within each experimental condition.

High extinction line

In the high extinction line, there was a between subjects main effect for drug treatment, $F(1,23)=5.151$, $p=.033$, $\eta^2 =.183$, but not for extinction type ($p > .05$), and no interaction ($p > .05$). Additionally, average freezing during the last 4 extinction CSs was not a significant covariate ($p > .05$). Post hoc paired samples t-tests revealed that the extinction only + saline group, showed significant reinstatement, $t(7)=-2.567$, $p=.037$, while the rest of the groups did not (extinction only + MB group, $t(6)=-.883$, $p=.411$; retrieval + extinction + saline, $t(6)=-.102$, $p=.922$; retrieval + extinction + MB group, $t(5)=-.251$, $p=.812$). In summary, for the high extinction line, reinstatement-resistant extinction occurred in subjects who received retrieval + extinction and/or post-extinction methylene blue injections (Figure 3.4b).

Low extinction line

In the low extinction line, there were no significant main effects of extinction type or drug treatment (though drug treatment trended toward significance; see Table 4), nor was their interaction significant ($p>.05$). Freezing during the last 4 extinction CSs was a significant covariate $F(1,20)=7.361$, $p=.013$, $\eta^2=.269$. Post hoc paired samples t-tests revealed that the extinction only + saline group, $t(5)=-3.005$, $p=.030$, the extinction only + MB group, $t(6)=-2.681$, $p=.036$, and the retrieval + extinction + saline group, $t(5)=-5.459$, $p=.003$, showed significant reinstatement, while the retrieval + extinction + MB group did not, $t(5)=-.908$, $p=.405$ (Figure 3.4c). In summary, for the low extinction line, reinstatement-resistant extinction occurred only in subjects who received both retrieval + extinction and post-extinction methylene blue injections, however this effect was only revealed through paired samples t-tests, and was not evident in an overall extinction type by drug treatment interaction.

Randomly bred line

In the randomly bred line, there was a main effect of extinction type, $F(1, 38)=4.445$, $p=.042$, $\eta^2=.105$, but not of drug treatment, nor was there a significant interaction between extinction type and drug treatment ($p>.05$). Freezing during the last 4 exinction CSs was a significant covariate, $F(1,38)=17.402$, $p=.000$, $\eta^2=.314$. Post hoc paired samples t-tests revealed that the extinction only + saline group, $t(10)=-2.637$, $p=.025$, and the extinction only + MB group, $t(11)=-3.609$, $p=.004$, showed significant reinstatement, while the retrieval + extinction + saline group, $t(7)=-.545$,

p=.603, and the retrieval + extinction + MB group did not, t(11)=-.858, p=.409. In summary, for the randomly bred line, reinstatement-resistant extinction occurred only in subjects who received retrieval + extinction, regardless of drug treatment (Figure 3.4d).

Table 3.4. Between-subjects assessment of reinstatement for each lineage. Drug treatment was the only significant predictor of reinstatement in the high extinction line, while both freezing at the end of extinction and drug treatment predicted reinstatement in the low extinction line. In the randomly bred line, freezing at the end of extinction and extinction type predicted reinstatement.

	High Extinction Line				Low Extinction Line				Randomly Bred Line			
Variable	*df*	*F*	η^2	*p*	*df*	*F*	η^2	*p*	*df*	*F*	η^2	*p*
Last 4 Ext CSs	1	1.694	.069	.206	1	7.361	.269	.013*	1	17.402	.314	.000**
Extinction type	1	.777	.033	.387	1	.068	.003	.798	1	4.445	.105	.042*
Drug treatment	1	5.151	.183	.033*	1	3.965	.165	.060^	1	.328	.009	.570
Extinction type * drug treatment	1	.114	.005	.738	1	2.342	.105	.142	1	.071	.002	.792
Error	23				20				38			

Note. **p<.001, *p < .05, ^p = .06 (trending toward significance)

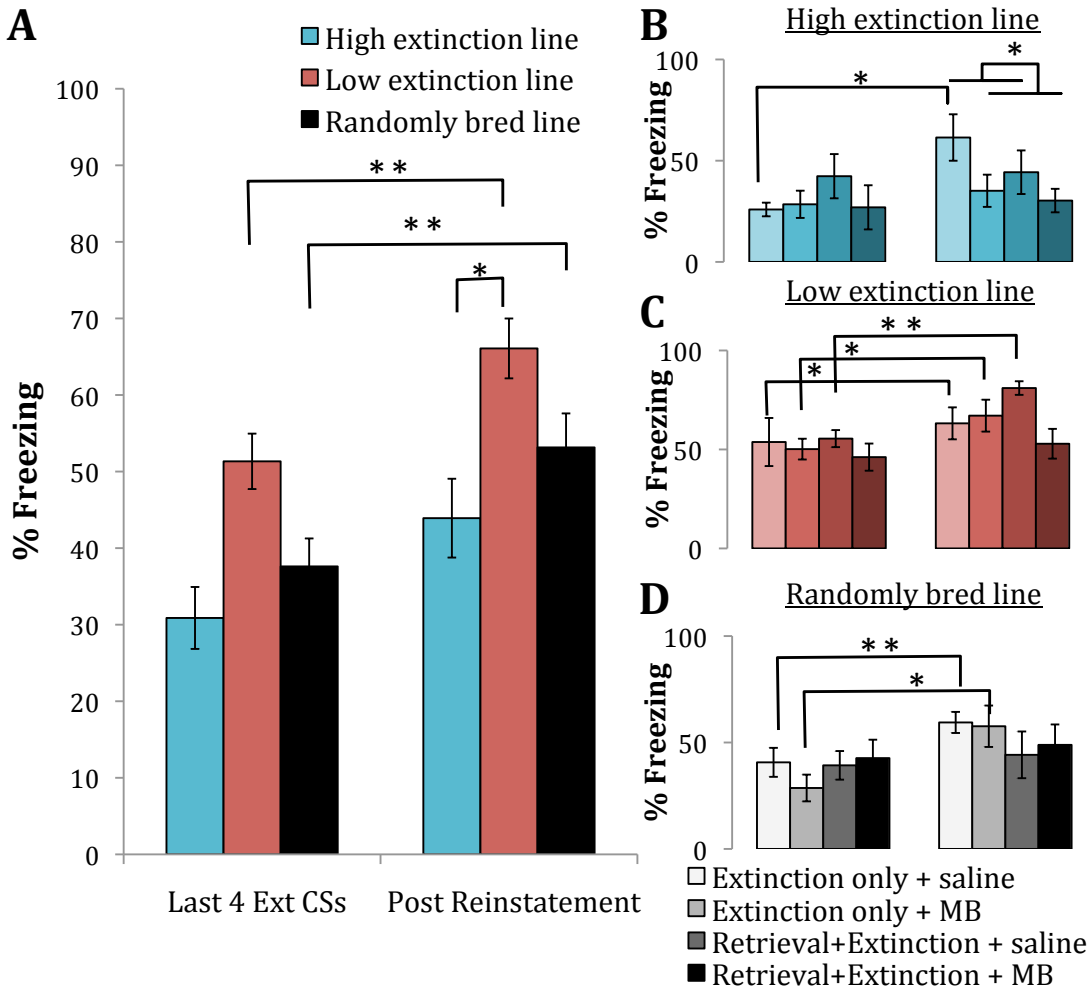

Figure 3.4 End of extinction vs. post reinstatement percent freezing. **A.** Overall effects of lineage. Only the high extinction line showed no significant reinstatement as a whole. **B.** High extinction line. Only the extinction only + saline group showed significant reinstatement. **C.** Low extinction line**.** Only the retrieval + extinction + MB group did not show significant reinstatement. **D.** Randomly bred line. Regardless of drug treatment, groups who received retrieval + extinction showed no reinstatement, while groups who received extinction only showed significant reinstatement. CS = conditioned stimulus, MB = methylene blue, *p < .05, **p < .01

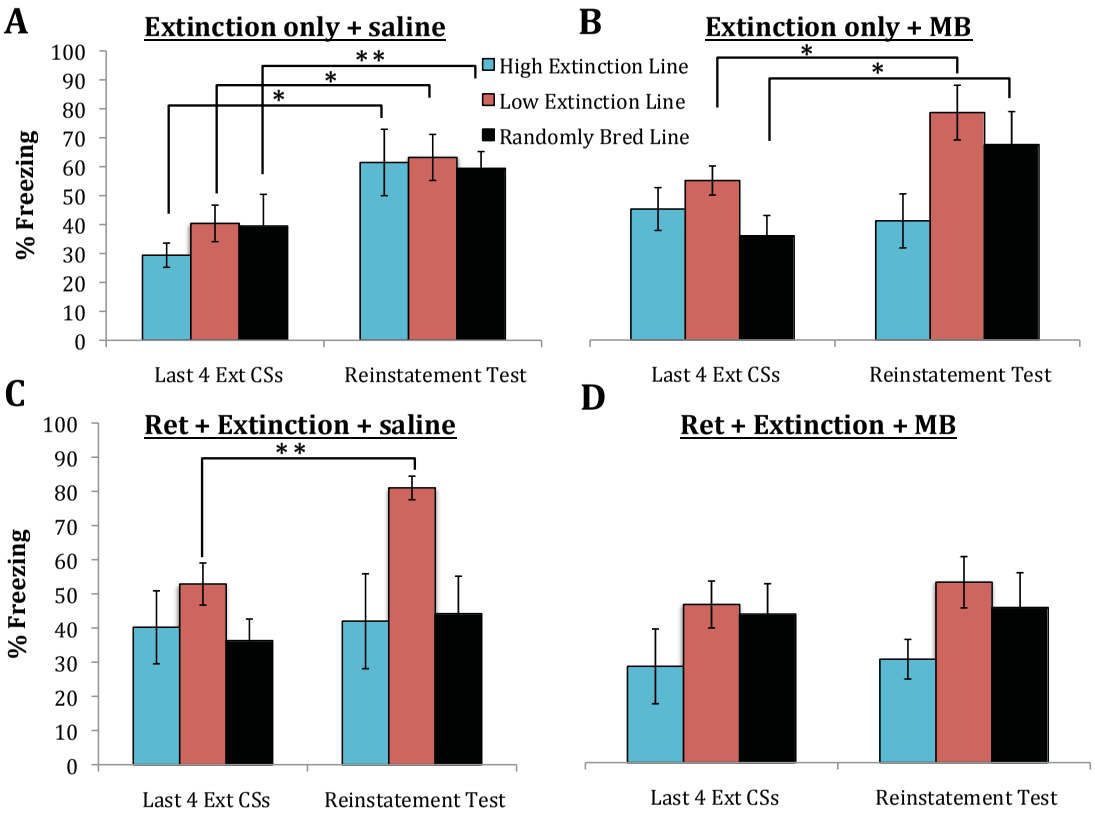

Figure 3.5 Comparison of lineage differences within each experimental condition. A. In the extinction only + saline group, all three lineages showed significant reinstatement. B. In the extinction only + MB group, only the low extinction line and the randomly bred group showed significant reinstatement. C. In the retrieval + extinction + saline group, only the low extinction line showed significant reinstatement. D. In the retrieval + extinction + MB group, no lineage showed significant reinstatement. Ext = extinction, ret = retrieval, MB = methylene blue, CS = conditioned stimulus, * $p < .05$, **$p < .01$

3.6 Discussion

This experiment was a comprehensive analysis of how two potentially therapeutic strategies for persistent fear attenuation—reconsolidation updating and metabolic enhancer methylene blue—can be utilized concurrently. We also used subjects selectively bred for extinction phenotype and observed how their genetic predisposition for extinction along with their actual extinction behavior modulated the effects of our two therapeutic strategies.

3.6.1 Selectively bred extinction phenotype affects both extinction and reinstatement

3.6.1.1 Phenotypic differences in extinction behavior

For this project, we selected subjects from three lineages selectively bred for either high extinction phenotype, low extinction phenotype or a randomly bred line. The selectively bred phenotype was reflected in extinction freezing. All three lineages froze similarly at the beginning of extinction (the first 4 extinction CSs), but subjects from the high extinction line froze less at the end of extinction (the last 4 extinction CSs) than subjects from the low extinction line, indicating a greater within-session attenuation of fear (Figure 3.2a). The randomly bred line showed similar behavior as the high extinction line, which indicates that subjects in both groups reached floor levels of freezing during extinction.

3.6.1.2 Phenotypic differences in reinstatement

Though freezing at the beginning and end of extinction was similar for both the randomly bred and high extinction lines, differences in reinstatement between the two lines suggest that selective breeding did indeed result in distinct behavioral differences between randomly bred and high extinction subjects (Figure 3.4a,c). More specifically, as a whole both the low extinction line and the randomly bred group showed significant increases in freezing from the end of extinction to post-reinstatement, indicating successful reinstatement of fear. On the other hand, subjects from the high extinction line did not show significant reinstatement as indicated by and increase in freezing from the end of extinction and post reinstatement.

3.6.2 Extinction moderates MB's effect on reinstatement

Results suggest that methylene blue was effective only in subjects who were responsive to behavioral therapy. This makes sense, as methylene blue's action as a neurometabolic enhancer does not discriminate between successful and unsuccessful treatments. In other words, methylene blue enhances the consolidation of whatever "brain state" is active during a task. Thus, if a subject remained fearful through extinction, then treatment with methylene blue enhanced the consolidation of that fearful brain state. On the other hand, if fear was reduced during extinction, then MB administration was therapeutic, as we enhanced the "less fearful brain state." Thus, extinction acted as a moderator for the effect of MB. This observation is in

accordance with recent findings of methylene blue's effect on extinction in humans with claustrophobia (Telch et al., 2014). Stated broadly, MB can be utilized as an alternative to (or potentially in conjunction with, though this would required further investigation) traditional pharmacological treatments in cases of successful cognitive behavioral therapy.

3.6.3 Prevention of return of fear via retrieval + extinction depends on lineage

Monfils et al. (2009) demonstrated that extinction presented during the reconsolidation window prevents reinstatement of fear. We replicated this finding in the randomly bred extinction line by showing a main effect of extinction type (retrieval + extinction vs. extinction alone) on reinstatement freezing, with the retrieval + extinction condition freezing less than the extinction alone condition during the post reinstatement test. Similarly, randomly bred subjects who received the retrieval + extinction manipulation did not show significant reinstatement of freezing when comparing the end of extinction freezing to post reinstatement freezing, regardless of whether they received post extinction MB or not. Reinstatement of freezing from the end of extinction to post reinstatement was, however, apparent in subjects from the randomly bred line given extinction alone.

The effect of retrieval + extinction was more complex in the high and low extinction lines. There was no main effect of extinction type and no extinction type by drug treatment interaction for the high extinction line. When comparing freezing at the end of extinction and post reinstatement for

each treatment group in the high extinction line, it is revealed that only the group that received extinction alone + saline showed significant reinstatement of fear (Figure 3,4b). Additionally, since the high extinction line as a whole did not show significant reinstatement, we can reasonably conclude that the lack of a main effect for extinction type in this case is explained by the fact that subjects in the high extinction line were already somewhat resilient to reinstatement, and thus treatment via either reconsolidation updating or MB resulted in a lack of reinstatement of fear.

We see a different effect in the low extinction line. Like for the high extinction line, there is no overall main effect of extinction type and no extinction type by drug treatment interaction. However, when comparing freezing at the end of extinction and post reinstatement for each group, we see that only the group who received both retrieval + extinction and MB showed no reinstatement of freezing (Figure 3.4c). Thus, for the high extinction line—in which subjects were less susceptible to reinstatement—treatment with *either* retrieval + extinction *or* MB prevented reinstatement; whereas for the low extinction line—in which subjects were more susceptible to reinstatement—only treatment with *both* retrieval + extinction *and* MB prevented reinstatement.

3.6.4 Conclusions

Though we did observe some differences in both extinction and reinstatement as a result of lineage, the majority of our findings support two major points: (1) The retrieval + extinction behavioral manipulation

prevented reinstatement for every lineage except the low extinction line (Figure 3.5c); and (2) MB was effective at preventing reinstatement when subjects responded favorably to extinction (Figure 3.3a). The first point suggests that memory updating is not universal across all subjects, but is instead at least partially influenced by the degree of extinction. However, unlike with MB treatment, extinction did not moderate memory updating, since there was not a linear relationship between freezing at the end of extinction and post reinstatement in subjects who did not receive MB (Figure 3.3b). Rather, it seems that subjects who are genetically predisposed to lower extinction are also less likely to show memory updating via extinction training. However, it is interesting to note that the combination of retrieval + extinction and MB treatment prevented reinstatement in subjects predisposed to lower extinction (Figure 3.5d). The second point confirms that MB is effective at enhancing memory consolidation. However, it's use as a clinical treatment for fear and anxiety disorders should be limited to subjects who respond favorably to extinction. Stated differently, regardless of whether we observe extinction memory formation or fear memory updating as a result of extinction training, if subjects do not extinguish conditioned responses, then post retrieval MB can only act to (re)consolidate fear memories, as no other alternative exists. Conversely, when subjects extinguish conditioned responses effectively, MB is therapeutic regardless of extinction condition.

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3.7 Literature Citations

Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., & Ames, B. N. (2008). Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. *The FASEB Journal*, *22*(3), 703-712.

Auber, A., Tedesco, V., Jones, C. E., Monfils, M. H., & Chiamulera, C. (2013). Post-retrieval extinction as reconsolidation interference: methodological issues or boundary conditions? *Psychopharmacology*, *226*(4), 631-647.

Baum, M. (1969). Extinction of an avoidance response following response prevention: Some parametric investigations. *Canadian Journal of Psychology/Revue canadienne de psychologie*, 23(1), 1.

Bouton, M. E. (1988). Context and ambiguity in the extinction of emotional learning: Implications for exposure therapy. *Behaviour research and therapy*, 26(2), 137-149.

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychological bulletin*, *114*(1), 80.

Bouton, M. E. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biological psychiatry*, 52(10), 976-986.

Bouton, M. E. (2004). Context and behavioral processes in extinction. *Learning & memory*, *11*(5), 485-494.

Bouton, M. E., & Bolles, R. C. (1979). Role of conditioned contextual stimuli in reinstatement of extinguished fear. *Journal of Experimental Psychology: Animal Behavior Processes*, *5*(4), 368.

Bouton, M. E., Mineka, S., & Barlow, D. H. (2001). A modern learning theory perspective on the etiology of panic disorder. *Psychological review*, *108*(1), 4.

Bouton, M. E., Westbrook, R. F., Corcoran, K. A., & Maren, S. (2006). Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biological psychiatry*, *60*(4), 352-360.

Bush, D. E., Sotres-Bayon, F., & LeDoux, J. E. (2007). Individual differences in fear: isolating fear reactivity and fear recovery phenotypes. *Journal of traumatic stress*, *20*(4), 413-422.

Cain, C. K., Blouin, A. M., & Barad, M. (2003). Temporally massed CS presentations generate more fear extinction than spaced presentations. *Journal of Experimental Psychology: Animal Behavior Processes*, 29(4), 323.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, *77*(1), 175-181.

Davis, M., Ressler, K., Rothbaum, B. O., & Richardson, R. (2006). Effects of D-cycloserine on extinction: translation from preclinical to clinical work. *Biological psychiatry*, *60*(4), 369-375.

Davis, H. P., & Squire, L. R. (1984). Protein synthesis and memory: a review. *Psychological bulletin*, *96*(3), 518.

Dudai, Y. (1996). Consolidation: fragility on the road to the engram. *Neuron*, *17*(3), 367-370.

Effting, M., & Kindt, M. (2007). Contextual control of human fear associations in a renewal paradigm. *Behaviour Research and Therapy*, 45(9), 2002-2018.

Fani, N., Tone, E. B., Phifer, J., Norrholm, S. D., Bradley, B., Ressler, K. J., ... & Jovanovic, T. (2012). Attention bias toward threat is associated with exaggerated fear expression and impaired extinction in PTSD. *Psychological medicine*, *42*(03), 533-543.

Fava, G. A., Rafanelli, C., Grandi, S., Conti, S., Ruini, C., Mangelli, L., & Belluardo, P. (2001). Long-term outcome of panic disorder with agoraphobia treated by exposure. *Psychological medicine*, *31*(05), 891-898.

Fava, G. A., Zielezny, M., Savron, G., & Grandi, S. (1995). Long-term effects of behavioural treatment for panic disorder with agoraphobia. *The British Journal of Psychiatry*, *166*(1), 87-92.

Fisher, R. A. (1921) On the probable error of a coefficient of correlation deduced from a small sample. *Metron*, 1, 3-32.

Flexner, L. B., Flexner, J. B., & Stellar, E. (1965). Memory and cerebral protein synthesis in mice as affected by graded amounts of puromycin. *Experimental neurology*, *13*(3), 264-272.

Foa, E. B., & Kozak, M. J. (1986). Emotional processing of fear: exposure to corrective information. *Psychological bulletin*, *99*(1), 20.

Gabrielli, D., Belisle, E., Severino, D., Kowaltowski, A. J., & Baptista, M. S. (2004). Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. *Photochemistry and photobiology*, *79*(3), 227-232.

Goelet, P., Castellucci, V. F., Schacher, S., & Kandel, E. R. (1986). The long and the short of long-term memory: A molecular framework. *Nature*, *322*, 419–422

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, *11*(5), 633-640.

Gordon, J. A., Hen, R. (2004). Genetic approaches to the study of anxiety. *Annual Reviews Neuroscience*, 27, 193–222.

Hermans, D., Dirikx, T., Vansteenwegenin, D., Baeyens, F., Van den Bergh, O., & Eelen, P. (2005). Reinstatement of fear responses in human aversive conditioning. *Behaviour research and therapy*, *43*(4), 533-551.

Hettema, J. M., Neale, M. C., & Kendler, K. S (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry*, 158: 1568–1578.

Hettema, J. M., Annas, P., Neale, M. C., Kendler, K. S., & Fredrikson M. (2003). A twin study of the genetics of fear conditioning. *Arch Gen Psychiatry*, 60: 702–708.

Ji, J., & Maren, S. (2007). Hippocampal involvement in contextual modulation of fear extinction. *Hippocampus*, 17(9), 749-758.

Kindt, M., Soeter, M., & Vervliet, B. (2009). Beyond extinction: erasing human fear responses and preventing the return of fear. *Nature neuroscience*, *12*(3), 256-258.

Lonsdorf, T. B, Kalisch, R. (2011). A review on experimental and clinical genetic associations studies on fear conditioning, extinction and

cognitive-behavioral treatment. *Transl Psychiatry*, 1: e41.

Ma, X., Zhang, J. J., & Yu, L. C. (2012). Post-retrieval extinction training enhances or hinders the extinction of morphine-induced conditioned place preference in rats dependent on the retrieval-extinction interval. *Psychopharmacology*, *221*(1), 19-26.

Martinez Jr, J. L., Jensen, R. A., Vasquez, B. J., McGuinness, T., & McGaugh, J. L. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, *6*(3), 387-390.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science*, *287*(5451), 248-251.

Merrill, K. A., Steinmetz, J. E., Viken, R. J., Rose, R. J. (1999). Genetic influences on human conditionability: a twin study of the conditioned eyeblink response. *Behavioral Genetics*, 29, 95–102.

Milad, M. R., Orr, S. P., Pitman, R. K., & Rauch, S. L. (2005). Context modulation of memory for fear extinction in humans. Psychophysiology, 42(4), 456-464.

Millan, E. Z., Milligan-Saville, J., & McNally, G. P. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiology of learning and memory*, *101*, 26-32.

Monfils, M. H., Cowansage, K. K., Klann, E., & LeDoux, J. E. (2009). Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *science*, *324*(5929), 951-955.

Myers, K. M., & Davis, M. (2002). Behavioral and neural analysis of extinction. *Neuron*, *36*(4), 567-584.

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. Molecular psychiatry, 12(2), 120-150.

Myers, K. M., Ressler, K. J., & Davis, M. (2006). Different mechanisms of fear extinction dependent on length of time since fear acquisition. *Learning & Memory*, 13(2), 216-223.

Nandhra, H. S., Murphy, C. L., & Sule, A. (2013). Novel pharmacological agents targeting memory and cognition in the treatment of anxiety disorders. *Human Psychopharmacology: Clinical and Experimental*, *28*(6), 538-543.

Ohlow, M. J., & Moosmann, B. (2011). Phenothiazine: the seven lives of pharmacology's first lead structure. *Drug discovery today*, *16*(3), 119-131.

Olshavsky, M. E., Song, B. J., Powell, D. J., Jones, C. E., Monfils, M. H., & Lee, H. J. (2013). Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus. *Frontiers in behavioral neuroscience*, *7*.

Pavlov, I. P. (1927). Conditioned reflexes. *An Investigation of the physiological activity of the cerebral cortex*.

Quirk, G. J., Paré, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., & Vicentic, A. (2010). Erasing fear memories with extinction training. *The Journal of Neuroscience*, *30*(45), 14993-14997.

Rescorla, R. A. (1996). Preservation of Pavlovian associations through extinction. *The Quarterly Journal of Experimental Psychology: Section B*, *49*(3), 245-258.

Rescorla RA. (2001) Experimental extinction. In: *Handbook of contemporary learning theories* (Mowrer RR, Klein S, eds), pp 119–154. Mahwah, NJ: Erlbaum

Rescorla, R. A., & Heth, C. D. (1975). Reinstatement of fear to an extinguished conditioned stimulus. *Journal of Experimental Psychology: Animal Behavior Processes*, *1*(1), 88.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, *511*(2), 151-158.

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *Neuroimage*, *54*(4), 2623-2634.

Robbins, S. J. (1990). Mechanisms underlying spontaneous recovery in autoshaping. *Journal of Experimental Psychology: Animal Behavior Processes*, 16(3), 235.

Rodriguez, P., Jiang, Z., Huang, S., Shen, Q., & Duong, T. Q. (2014). Methylene blue treatment delays progression of perfusion–diffusion mismatch to infarct in permanent ischemic stroke. *Brain research*, *1588*, 144-149.

Rojas, J. C., & Gonzalez-Lima, F. (2010). Mitochondrial optic neuropathy: in vivo model of neurodegeneration and neuroprotective strategies. *Eye and brain*, *2*, 21-37.

Rojas, J. C., Bruchey, A. K., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of

methylene blue. *Progress in neurobiology*, *96*(1), 32-45.

Royce, J. R. (1972). Avoidance conditioning in nine strains of inbred mice using optimal stimulus parameters. *Behavioral Genetics*, 2: 107–110.

Sara, S. J. (2000). Retrieval and reconsolidation: toward a neurobiology of remembering. *Learning & Memory*, *7*(2), 73-84.

Schafe, G. E., & LeDoux, J. E. (2000). Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J Neurosci*, *20*(18), RC96.

Schiller, D., Monfils, M. H., Raio, C. M., Johnson, D. C., LeDoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature*, *463*(7277), 49-53.

Schiller, D., Kanen, J. W., LeDoux, J. E., Monfils, M. H., & Phelps, E. A. (2013). Extinction during reconsolidation of threat memory diminishes prefrontal cortex involvement. *Proceedings of the National Academy of Sciences*, *110*(50), 20040-20045.

Shumake, J., Furgeson-Moreira, S., & Monfils, M. H. (2014). Predictability and heritability of individual differences in fear learning. *Animal cognition*, *17*(5), 1207-1221.

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S.S. et al (2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science*, 327: 863–866.

Sotres-Bayon, F., Bush, D. E., & LeDoux, J. E. (2004). Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction. *Learning & Memory*, 11(5), 525-535.

Telch, M. J., Bruchey, A. K., Rosenfield, D., Cobb, A. R., Smits, J., Pahl, S., & Gonzalez-Lima, F. (2014). Effects of post-session administration of methylene blue on fear extinction and contextual memory in adults with claustrophobia. *American Journal of Psychiatry*, *171*(10), 1091-1098.

Wang, S. H., de Oliveira Alvares, L., & Nader, K. (2009). Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. *Nature neuroscience*, 12(7), 905-912.

Wen, Y., Li, W., Poteet, E. C., Xie, L., Tan, C., Yan, L. J., ... & Yang, S. H. (2011). Alternative mitochondrial electron transfer as a novel strategy for neuroprotection. *Journal of Biological Chemistry*, *286*(18), 16504-16515.

Westbrook, R. F., Iordanova, M., McNally, G., Richardson, R., & Harris, J. A. (2002). Reinstatement of fear to an extinguished conditioned stimulus: two roles for context. *Journal of Experimental Psychology: Animal Behavior Processes*, 28(1), 97.

Wrubel, K. M., Riha, P. D., Maldonado, M. A., McCollum, D., & Gonzalez-Lima, F. (2007). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology Biochemistry and Behavior*, *86*(4), 712-717.

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., ... & Lu, L. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science*, *336*(6078), 241-245.

Yehuda, R., & LeDoux, J. (2007). Response variation following trauma: a

translational neuroscience approach to understanding PTSD. Neuron, 56(1), 19-32.

Yehuda, R., Flory, J. D., Southwick, S., & Charney, D. S. (2006). Developing an agenda for translational studies of resilience and vulnerability following trauma exposure. *Annals of the New York Academy of Sciences*, *1071*(1), 379-396.

Chapter 4: Therapeutic benefits of methylene blue on cognitive impairment during chronic cerebral hypoperfusion[6]

4.1 Abstract

Chronic cerebral hypoperfusion, a risk factor for mild cognitive impairment and Alzheimer's disease, affects mitochondrial respiration and memory consolidation. Therefore, drugs that improve mitochondrial function may be appropriate cognitive treatments for cerebral hypoperfusion. Methylene blue (MB) crosses the blood-brain barrier and at low doses serves as an electron cycler in the mitochondrial electron transport chain. Previous studies implicate MB in both memory enhancement and neuroprotection. We treated rats that underwent permanent bilateral carotid occlusion (2VO) or sham surgery with daily 4 mg/kg USP MB or saline for one month. Animals went through a battery of behavioral tests, including open field, visual water maze and odor-recognition tasks. 2VO rats showed worse performance in the visual water task without showing differences in general motor activity, visually guided swimming ability or odor recognition. Daily MB attenuated the deficits in visual learning and memory that resulted from

[6] Auchter, A., Williams, J., Barksdale, B., Monfils, M. H., & Gonzalez-Lima, F. (2014). Therapeutic benefits of methylene blue on cognitive impairment during chronic cerebral hypoperfusion. Journal of Alzheimer's disease, 42, S525–35. J. Williams and B. Barksdale contributed to experimental design and execution. M.H. Monfils and F. Gonzalez-Lima were co-principle investigators on the manuscript.

cerebrovascular insufficiency. During training on three different discrimination problems in the visual water task, all animals were able to reach a criterion of 8/10 correct trials, but 2VO animals took longer to learn each problem and showed lower performance in a challenging memory probe. However, animals that received daily post-session MB performed significantly better than saline-treated subjects both during training and during the memory probe. This is the first study to demonstrate that MB attenuates learning and memory deficits caused by carotid occlusion. The results suggest that MB may be beneficial for conditions involving chronic cerebral hypoperfusion, such as mild cognitive impairment, vascular dementia and Alzheimer's disease.

Keywords: Methylene blue, memory enhancement, cerebral hypoperfusion, carotid occlusion, cognitive impairment

4.2 Introduction

There is a great need to develop effective early interventions to treat memory deficits associated with mild cognitive impairment, Alzheimer's disease (AD), vascular dementia and other related neurocognitive disorders. For example, in the US alone, over 4 million people are living with AD, resulting in continued intellectual loss to society, an annual cost of over $100 billion (http://report.nih.gov/nihfactsheets/ViewFactSheet.aspx?csid=107) and a great deal of suffering for both the inflicted and their caregivers. A compelling body of evidence supports a role of compromised cerebral blood supply and mitochondrial dysfunction in memory-related neurodegenerative disorders (de la Torre, 2010, 2012a, 2012b; Hauptmann et al., 2009; Hirai et al., 2001; Velliquette et al., 2005) . With an aging population, it is critical to treat deficits in brain energy metabolism that occur before the onset of dementia. Treatments such as USP methylene blue (MB) designed to target the effectiveness of mitochondrial respiration may be able to ameliorate these deficits, thereby delaying or preventing the development of cognitive impairment and dementia. The present preclinical study addressed this possibility by determining whether a chronic intervention with low-dose MB that targets cytochrome oxidase and enhances mitochondrial respiration can improve memory retention in a rat model exhibiting chronic cerebral hypoperfusion and mitochondrial dysfunction (Cada, de la Torre, & Gonzalez-Lima, 2000; Obrenovich et al., 2006) .

MB (also known as methylthioninium chloride) is an FDA-grandfathered and inexpensive drug that has been used safely in humans for

over 120 years. Indeed, MB was the first synthetic chemical used therapeutically in humans (Bruchey & Gonzalez-Lima, 2008). Pharmaceutical grade (USP purity) MB is available in every emergency room in the United States, but its use does not currently extend to neuroprotection or cognitive enhancement. Low-dose (1-4 mg/kg) MB currently has many human applications, including treatment for various metabolic poisonings such as methemoglobinemia, cyanide poisoning and toxic encephalopathy (Wainwright & Crossley, 2002). In a similar way as MB compensates for cytochrome oxidase inhibition from poisons, MB may also improve memory retention in conditions characterized by impaired oxidative energy metabolism.

MB is an autoxidizable compound with unique redox mechanisms that are ideal for targeting neural tissue oxidative metabolism. After administration, MB passes through the blood-brain barrier and accumulates in the human brain within hours (Peter, Hongwan, Kupfer, & Lauterburg, 2000). MB is a relatively small lipophilic compound, so it diffuses readily into neuronal mitochondria. At low doses, MB enhances electron flow in the mitochondrial respiratory chain and thereby, stimulates mitochondrial cytochrome oxidase metabolism and oxygen consumption (Callaway, et al., 2004; Huang et al., 2013; Riha, et al., 2005; Visarius, Stucki & Lauterburg, 1997). MB can even replace oxygen as an oxidant in anoxic conditions (Lee & Urban, 2002). Moreover, low-dose MB displays potent antioxidant effects. For example, low-dose MB prevents superoxide free radical formation during reperfusion after ischemia (Salaris, Babbs & Voorhees, 1991) and we have

also shown that it decreases free radical-induced lipid peroxidation (Zhang & Rojas, 2006). We have used low-dose MB successfully *in vivo* as an artificial electron donor and established that it can donate electrons to the mitochondrial electron transport chain in its reduced form, thus increasing cytochrome oxidase activity and brain oxygen consumption(Rojas & Gonzalez-lima, 2010; Zhang & Rojas, 2006). Adverse reactions to MB have only been observed with doses much larger than our range of interest and/or with "chemical grade" MB that contains toxic impurities not found in USP MB (Riha et al., 2005; Rojas, Bruchey, & Gonzalez-Lima, 2012).

MB's ability to target cytochrome oxidase and neuronal energy metabolism at the mitochondrial level provides us with a novel approach to attenuate learning and memory deficits (Gonzalez-Lima, Barksdale, & Rojas, 2014; Rojas et al., 2012). Because MB has been proven effective at enhancing memory in a large number of experimental paradigms (Callaway et al., 2004; 2002; Deiana et al., 2009; Gonzalez-Lima & Bruchey, 2004; Medina et al., 2011; O'Leary et al., 2010; Paban et al., 2014; Riha et al., 2011; Wrubel et al., 2007a; Wrubel, et al., 2007b), including humans (Telch et al., 2014), we hypothesized that MB could also effectively attenuate memory deficits in an amnestic model of cerebral hypoperfusion. Therefore, our objective was to investigate whether chronic administration of MB could be used to attenuate learning and memory deficits caused by chronic cerebral hypoperfusion. A 4 mg/kg i.p. MB dose was selected based on our findings that 1–4 mg/kg doses of MB improve memory retention in various tasks, with optimal memory effects at 4 mg/kg i.p. (Riha et al., 2005; for review, see Rojas et al., 2012),

and that doses above and below this hormetic range are behaviorally less effective (Bruchey & Gonzalez-Lima, 2008). The beneficial effects of chronic low-dose MB on cognitive deficits caused by cerebral hypoperfusion represent a proof-of-principle and the basis for the possible development of early effective interventions against mild cognitive impairment and AD. This also should have translational value for other memory disorders and contribute to the study of new neurometabolic mechanisms to enhance memory.

4.3 Hypothesis

Chronic cerebral hypoperfusion will lead to learning and memory deficits in visual and odor discrimination tasks. Such deficits will be prevented or reduced by daily i.p. administration of 4 mg/kg methylene blue.

4.4 Materials and Methods

4.4.1 Subjects

Subjects were 39 adult male Long-Evans rats weighing approximately 500-600 g at the time of surgery. Two rats died from surgical complications and one rat died of a respiratory infection during visual water task training, making the final N = 36. Rats were raised from birth in AALAC-approved facilities under standard laboratory conditions (12 h: 12 h, light: dark cycle) with *ad libitum* access to food and water. Animals were housed in triads until the day of surgery, after which they were housed individually for the remainder of the experiment. Rats were housed singly after surgery to

prevent animals from interfering with wound healing, such as removal of sutures. In pilot subjects, when several subjects were housed together after surgery, they would remove each other's sutures, resulting in reopening of the wounds. Subjects came from a selective breeding project that involves selecting animals for their propensity to extinguish conditioned fear. Each animal came from one of three possible lineages: animals phenotypically selected for effective extinction of conditioned fear (high extinguishers), animals selected for ineffective extinction of conditioned fear (low extinguishers) or animals from a line not bred for an extinction phenotype (randomly-bred) (Shumake, Furgeson-Moreira, & Monfils, 2014). All subjects were behaviorally tested at approximately 60 days of age with 19 trials of paired tone-shock conditioning, 19 tone alone extinction trials and a memory probe. They were subsequently used as breeders for the in-house colony. Hence these animals were phenotypically selected and sexually experienced mature rats before inclusion in this experiment. However, in our behavioral experiments, there were no significant differences based on lineage, so animals from all lineages were analyzed together. All animal care and experimental procedures were approved by the University of Texas at Austin's Institutional Animal Care and Use Committee.

4.4.2 Surgical and drug treatments

A timeline of the procedures is show in Fig 1. Rats were randomized and subjected to a bilateral carotid occlusion surgical model known as 2-vessel occlusion (2VO; n=20) or to sham surgery without vessel occlusion

(sham; n=19) as previously described by de la Torre and Fortin (de la Torre, 2010, 2012a, 2012b; Hauptmann et al., 2009; Hirai et al., 2001; Velliquette, O'Connor, & Vassar, 2005). Prior to surgery, rats were anesthetized via isoflurane inhalation at 4% for induction, and maintained under general anesthesia at 1.5-3% for the duration of the surgical procedure using an E-Z anesthesia vaporizer system (Euthanex, Corp., Palmer, PA, USA). For the surgery, a midline ventral incision was made in the neck. The carotid arteries were carefully exposed and dissected free from their sheath and vagal nerve (de la Torre & Fortin, 1991). In the 2VO condition, each artery was doubly ligated immediately posterior to the carotid bifurcation using 4-0 silk sutures. For the sham condition, the carotid arteries were dissected free from their sheaths, but no occlusion was performed. Prior to closing, each animal was injected subcutaneously with 1 mg/kg of the surgical analgesic meloxicam (TW Medical Veterinary Supply, Lago Vista, TX, USA), randomly assigned to MB or saline groups and injected intraperitoneally with either 4 mg/kg MB (USP Methylene Blue, Spectrum Chemical Manufacturing Corporation, New Brunswick, NJ, USA) or the same volume of physiological saline. The incision was sutured closed and each animal was transferred to a recovery cage and closely monitored for 30 minutes before returning to its home cage in the colony room. Rats were then injected daily i.p. with either 4 mg/kg MB dissolved in physiological saline (MB groups) or the saline vehicle alone (saline groups) for 30 days. MB was injected daily immediately after each training session, i.e. during the post-session memory consolidation phase, because MB has been shown to preferentially facilitate memory

consolidation in other paradigms (Callaway et al., 2004; Martinez et al., 2013). Animals were allowed to recover for 7 days before behavioral testing began.

4.4.3 Behavioral testing

Behavioral testing for this experiment began on Day 8 after surgery and continued until Day 30. All animals went through the same behavioral tests in the same order. First, animals went through one day of open field, followed by 20 days of visual water task (VWT) training. Upon completion of the VWT task, animals went through 2 days of an odor recognition task (Fig. 4.1).

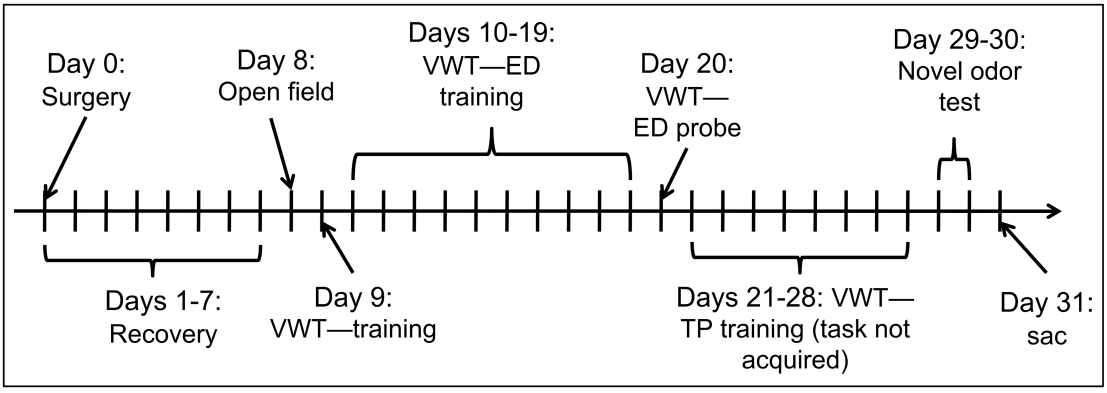

Figure 4.1 Experimental timeline for behavioral testing. Visual water task (VWT), elemental discrimination (ED), transverse patterning (TP), sacrifice (sac).

4.4.3.1 Open field

Subjects were run through one day of open field activity to screen for differences in gross motor behavior following surgery. The test was conducted by placing each subject in an open field chamber (43.2 cm^2) with clear plastic sides (30.5 cm high) and a white Plexiglas floor. For 10 minutes, rats were allowed to freely explore the chamber. Movement was recorded using arrays of infrared light beam motion detectors (16x16 cm, 2.5 cm apart) controlled by the Activity Monitor program version 5.10 (Med Associates, St. Albans, VT, USA). Measures of motor activity included ambulatory distance, ambulatory time, rearing time, immobility time and average velocity, which were automatically scored by a computer using MED-PC software. To minimize odor-cued motor activity, chambers were wiped down with 70% ethanol between each session.

4.4.3.2 Visual water task

Apparatus and training procedure

The visual water task (VWT) was used to train rats on several discrimination problems, as previously described by (Prusky, West, & Douglas, 2000). Briefly, a trapezoidal metal tank (118 cm L ×72 cm W × 25 cm W and 41 cm H) was filled with water to a depth of 15 cm. The end wall of the tank was composed of clear Plexiglas. A computer monitor (30”) was placed behind the transparent wall and was used to control stimulus presentation. The end of the tank was bisected by a midline divider (60 cm H) that extended 46 cm into the pool from the transparent wall. This divider

separated the monitor such that one pattern was visible at the end of each "arm" created by the divider. For each trial, a transparent invisible platform (32 cm L x 13 cm W x 15 cm H) was completely submerged at the end of one of the arms, and was always paired with a pattern that predicted escape from the pool. Right/left randomization, behavioral responses and parameter control were provided and recorded by a computer program (Acumen; http://www.cerebralmechanics.com).

To address the possibility that the 2VO reduces blood flow to the retina, thereby impairing subjects' ability to visually discriminate between the patterns presented in the VWT, a separate group of 15 naïve pilot subjects was pretested in the task with different iterations of 12 distinct discriminanda. The combinations of patterns that were most easily detected by pilot subjects (as indicated by shortest total number of trials to get 8/10 trials correct) were used for the experimental subjects in the VWT. The patterns selected for use in this experiment are shown in Fig 2. Additionally, during the VWT training phase, all experimental subjects were able to reach the 8/10 criteria for each elemental discrimination problem, eliminating the possibility that 2VO hindered subjects' ability to discriminate between the patterns.

Rats were trained to associate escape from the water by swimming to a "correct" (+) pattern that predicted the presence of the invisible escape platform. At the end of the opposite arm was an "incorrect" (-) pattern that consistently predicted the absence of the platform. VWT training began 8 days following surgery and continued for 20 days. After an initial training

day, all subjects were first trained to solve three visual elemental discrimination sets followed by three transverse pattern sets. Beginning with elemental discrimination training, at the start of each trial the subject was placed into the opposite end of the tank facing the transparent wall and allowed to swim to one of the stimuli presented by the monitor at the end of the arms. If the subject swam into the incorrect arm, it was blocked into the arm with a metal divider for 10 seconds and then allowed to swim to the opposite arm containing the hidden platform. Once the subject reached the platform, it was promptly removed and placed in a holding cage to await the next trial.

Task training and recording of swimming speed

On the first day of the VWT task, an insert was placed in the tank that completely dissected the tank into a left and right section such that subjects could only swim down one side of the tank without having the option of two separate arms. On this day, the screen at the end of the tank was left blank (i.e. no pattern) and the invisible platform was always present at the end of the tank nearest the screen. Each subject went through 15 trials in which they were placed at one end of the tank on the right or left side and allowed to swim to the other end to discover the platform. Upon reaching the platform, the subject was removed from the tank and placed in a heated recovery cage. There were two purposes to this task. The first was to acclimate subjects to the task by allowing them to associate swimming to the invisible platform with escape from the tank. The second purpose was to

observe swimming ability for the subjects independently of pattern discrimination. Subjects learned of the presence of the platform within the first few trials, and by the tenth trial all subjects consistently swam directly to the platform immediately after being placed in the tank. Thus, we used the average latency of the final five trials to compute the swimming speed for each subject.

Elemental discrimination and transverse patterning

Training followed a stepwise approach that closely resembled previously published procedures (Driscoll et al., 2005; Driscoll et al., 2004; Prusky et al., 2000). Briefly, subjects were trained to solve three visual discriminations (Fig 4.2; Problem 1: A+B-; Problem 2: C+D-; Problem 3: E+F-). Training consisted of 10 days (30 trials each day) in three phases, followed by a test a day later. Phase 1 consisted of training subjects on Problem 1 until subjects could reach a criterion of 8/10 correct trials. All subjects were able to reach the criterion within 4 days. During Phase 2, Problem 2 was introduced and intermixed with Problem 1 over a period of three days. Similarly, in Phase 3, Problem 3 was introduced and intermixed with Problems 1 and 2 over a period of 3 days. On day 11, subjects received a memory probe consisting of randomly alternating problems on each trial (3, 2, 1, 2, 1, 3, 2, 3, 1, 3, 1, 2, 1, 2, 3, …x2) for a total 10 presentations of each problem (Driscoll et al., 2005, 2004).

Transverse patterning followed the same stepwise procedure as the elemental discrimination, however the rats were presented with new, more

difficult problem sets. In this case, instead of having three patterns that always predicted escape from the tank, whether the pattern was correct or not depended upon which pattern was presented alongside it (Problem 1: X+Y-; Problem 2: Y+Z-; Problem 3: Z+X-). Unfortunately, after 8 days of training, none of the subjects were able to acquire the task. As a consequence, this task was excluded from our analysis. However, since all animals went through the same training protocol in a similar time frame, the behavioral tasks performed subsequently were still incorporated in our analysis.

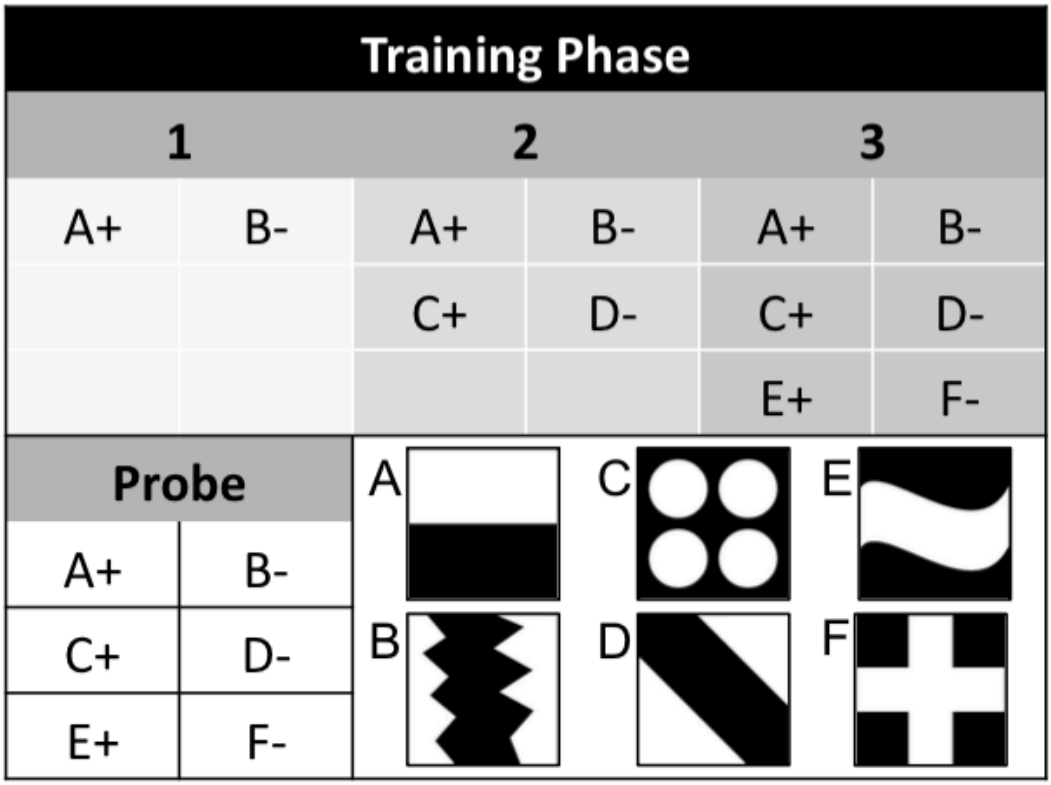

Figure 4.2 VWT training. **Top:** During Phase 1 of training, subjects were trained for 3 days (30 trials/day) on the Pair 1, in which Pattern A predicted escape (+) while Pattern B did not (-). During Phase 2, Pair 2 (C+, D-) was introduced and intermixed with Pair 1 for three days of training. Finally, during Phase 3, Pair 3 (E+, F-) was introduced and intermixed with Pairs 1 and 2. **Bottom Left:** The final probe day consisted of 30 random presentations of all 3 pairs. **Bottom Right:** The six patterns used during VWT.

4.4.3.3 Novel odor test

Familiarization of wooden beads

Experimental protocol for the novel odor test was modified from related procedures (Crews et al., 2007; Ennaceur, Neave & Aggleton, 1997; Spinetta et al., 2008; Tillerson et al., 2006). Five days before novel odor testing, four 2.5 cm round wooden beads, each with a small hole bored

through its diameter (http://www.craftparts.com) were introduced into each subject's cage in order to acquire the odor of the animal. Animals were tested in their home cages, so introducing the wooden beads 5 days prior to testing allowed for familiarization to both the testing environment and the presence of beads.

Several beads were also introduced into cages of previously selected singly-housed odor-donor rats. These rats were not subjects in the experiment, but instead served only as odor donors for the novel odor test. The wood beads were incubated in the cages for 5 days so as to provide equally salient novel odors for the upcoming task. For testing, the odor-donors were counterbalanced such that any one odor served as either a recently familiarized odor (N1) or a brand new novel odor (N2) during memory assessment for different experimental subjects.

Habituation to the first novel odor

To habituate rats to the first novel odor, after 5 days of familiarization to the four beads in the testing environment (home cage), all four beads were removed from the test cage. Subsequently, three familiar beads and one novel-odor bead (N1)—taken from one of the odor donor cages—were introduced back into the home cage and randomly arranged in a line approximately 5 cm from the front of the cage, as shown in Fig 4.3. Subjects were allowed to freely explore the beads for three 1-minute trials. The time the rat spent investigating each bead was recorded by an experimenter who was blind to which beads were familiar or novel. There was a 1-minute inter-

trial interval during which all beads were removed from the cage. For each trial, the spatial arrangement of the four beads was randomly altered.

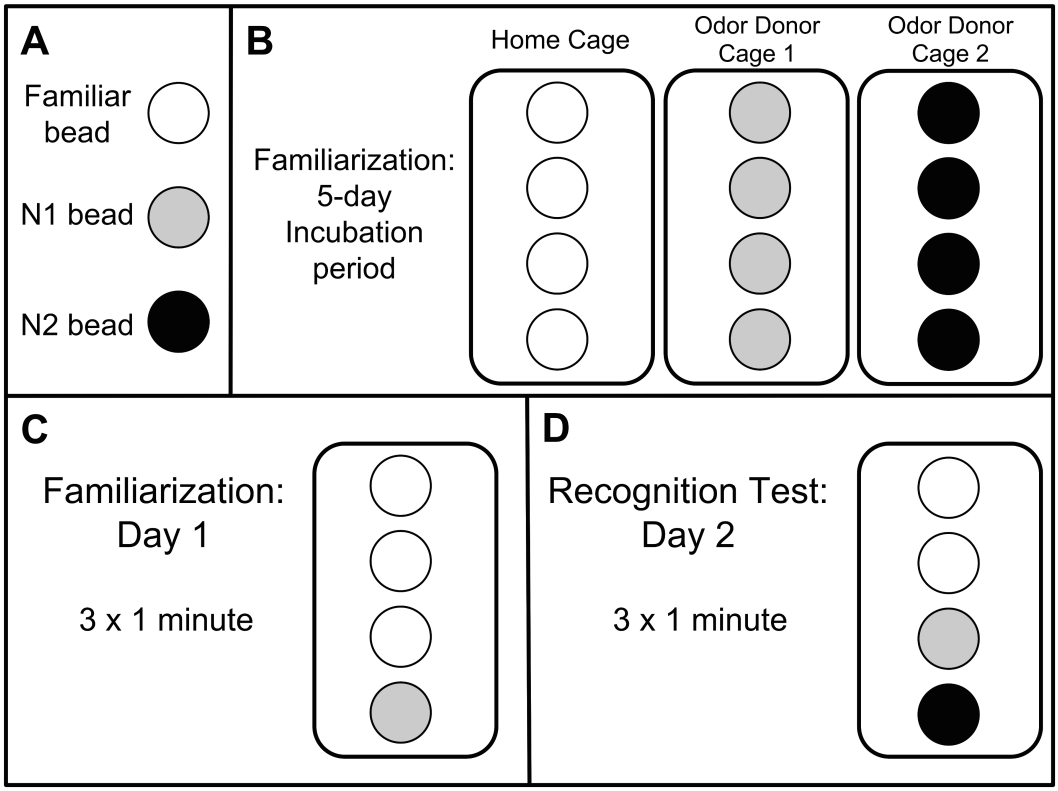

Figure 4.3 Novel odor test experimental procedure. A. Color-coded representation of wooden beads. B. 5-day incubation period wherein beads absorbed odors. C. Familiarization--introduction to the 1st novel odor (N1). D. Recognition test: introduction of 2nd novel odor (N2).

Odor-recognition assessment

The odor recognition test was administered 24 hours after the N1 habituation phase. This assessment follows the same procedure as the habituation phase, however in this case each subject was presented with an N1 odor bead (which it thoroughly explored the previous day), an unfamiliar

novel odor bead (N2) and 2 familiar home cage beads in random arrangement in the test cage (see Fig 4.3). To prevent scent-marking effects, the bead used for the N1 odor on the habituation day was discarded, and a new bead from the same N1 odor donor cage was used for the memory assessment. Again, a blind experimenter recorded the amount of time subjects investigated each bead during each trial.

4.4.4 Statistical analyses

All statistical testing was carried out using the PASW 22 software package (SPSS, Inc., Chicago, IL). All differences were considered statistically significant at the two-tailed $p<.05$ level. Two-way ANOVAs were used to verify that there were no group differences for the following measures of general motor activity: ambulatory distance, ambulatory time, rearing time, immobility time, center time, average velocity and swimming speed.

For the VWT, a repeated-measures ANOVA containing one within-subject variable (training day) and one between subjects variable (group) followed by pair-wise comparisons using simple and repeated contrasts was used to verify task acquisition and to observe group differences in the rate of acquisition. Following training, a one-way ANOVA with planned comparisons was used to assess the effects of 2VO surgery and MB administration on performance.

Analysis of performance on the odor recognition test used a 2x2 AVOVA to explore main effects and interactions (between-groups comparisons), and a Wilcoxon matched-pair signed-rank test for each group

(within-group comparisons). It was expected that subjects would spend more time investigating a novel odor over a familiar odor in the within-groups comparisons.

4.5 Results

As previously mentioned, subjects included in this experiment initially came from one of three different selectively bred lines. However, there were no significant differences in any behavior due to lineage. Thus, subjects from all lineages were analyzed together.

4.5.1 General Motor Behavior

There were no significant group differences due to treatment condition in any general activity measures in the open field, including ambulatory distance, ambulatory time, rearing time, immobility time and average velocity. Additionally, using a two-way analysis of variance (ANOVA) there were no group differences in swimming speed measured on the first day of the VWT. Thus, any observed group differences were not due to differences in general activity or swimming ability (Table 4.1).

Table 4.1 Locomotor activity in the open field test and swimming velocity in the visual water task (VWT). There were no significant differences between groups at the p<.05 level.

	Measure	**Sham-saline** (Mean ± SEM)	**Sham-MB** (Mean ± SEM)	**2VO-saline** (Mean ± SEM)	**2VO-MB** (Mean ± SEM)
Open Field	Ambulatory distance (cm)	2549 ± 351	2782 ± 342	2878 ± 394	3025 ± 374
	Ambulatory time (sec)	955 ± 121	1044 ± 137	1060 ± 136	1194 ± 154
	Rearing time (sec)	120 ± 17	130 ± 18	117 ± 19	118 ± 15
	Immobility time (sec)	397 ± 17	387 ± 18	376 ± 15	381 ± 21
	Average velocity (cm/sec)	3.22 ± 0.30	3.11 ± 0.12	3.44 ± 0.14	2.82 ± 0.24
VWT	Swimming velocity (cm/sec)	2.89 ± 0.21	3.02 ± 0.16	2.96 ± 0.13	2.68 ± 0.29

4.5.2 Visual Water Task

Figure 4.4 shows the acquisition curves and test performance for each group. Repeated measures analysis of the task acquisition revealed a general increase in percent correct trials through all 9 days of training, $F(8, 208)=20.064$, $p<.001$. By the third day, all groups reached an average performance of at least 70%, and all subjects reached the performance criterion of 8/10 correct trials. However, when comparing differences in performance between the first and second days of training, a one-way ANOVA revealed between-groups differences in rates of task acquisition, $F(3,31)=9.434$, $p<.001$. Planned comparisons showed that the 2VO groups had less improvement on the second day of training than the sham groups, $t(31)=3.875$, $p<.001$, but the 2VO-MB group showed better acquisition than the 2VO-saline group, $t(31)=3.348$, $p<.01$.

Using a 2-way ANOVA on the probe day, there was a significant interaction between MB treatment and surgical condition, $F(1, 32)=5.82$, $p=.022$ which was further interpreted using a planned-comparisons analysis. Levene's test for equality of variance revealed that the groups did not show homogeneity of variance, $F(3,32)=6.077$, $p=.002$. As such, contrasts tests were conducted without assuming equal variances. The planned-comparisons revealed that the subjects that underwent 2VO had worse performance in the elemental discrimination memory probe test than the sham subjects, $t(32)=5.921$, $p<.001$, but that 2VO subjects treated with MB performed significantly higher than those treated with saline, $t(32)=3.954$, $p<.001$.

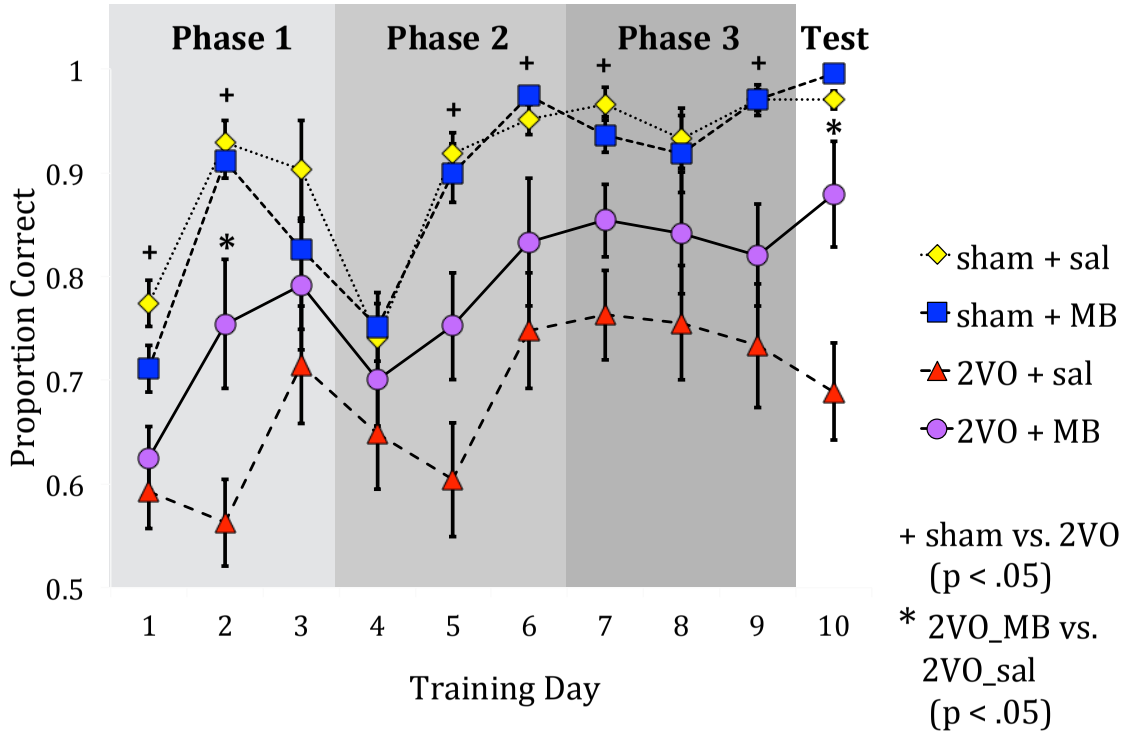

Figure 4.4 Visual water task results. During Phase 1, subjects were introduced to the first pattern. In Phase 2, the second pattern was introduced and intermixed with the first pattern. In Phase 3, the third pattern was introduced and intermixed with the first two patterns. On Probe day 10, subjects were given a random presentation of all three patterns. All groups significantly improved in performance from the beginning to the end of training, but the 2VO-sal group showed the least amount of progress during training compared to the 2VO-MB, sham-MB and sham-sal groups. Though all subjects were able to reach a minimum criteria of 8/10 correct trials during Phase 1, the 2VO-sal group took longer to acquire the task as evidenced by a lack of improvement from Day 1 to Day 2, while the 2VO-MB, sham-MB and sham-sal groups showed a significant increase in percent correct trials. On the Probe day 10, the 2VO-MB and 2VO-sal groups performed worse than the sham-MB and sham-sal groups, however the 2VO-MB group significantly outperformed the 2VO-sal group. + = sham vs. 2VO ($p<.05$), * = 2VO-sal vs. sham-sal

4.5.3 Odor Recognition Test

A 2x2 ANOVA did not reveal main effects for surgery, $F(1,32)=2.66$, $p=.11$, drug treatment, $F(1,32)=.014$, $p=.91$ or an interaction effect, $F(1,32)=.017$, $p=.90$ on percentage of time spent exploring the most novel odor (N2). The only discernable difference between 2VO and sham groups was in the amount of subject variability in the percent time exploring N1 vs N2, with the 2VO-saline group having a much higher variability than the other groups. As such, odor recognition was only mildly affected by 2VO. On average, all groups spent a significantly greater percentage of time exploring N2 over N1 except the 2VO-saline group using the Wilcoxon matched-pair signed-rank test for each group (Table 4.2). This means that MB treatment reduced the amount of variability introduced by 2VO, but it did not result in increased exploration time of N2.

Table 4.2 Novel odor recognition test. There were no between-groups differences in mean exploration time. Within-subjects analysis showed that groups spent more time exploring N2 over N1 beads, but this mean effect was not significant in the 2VO-saline group due to its high variance.

Group (n=9 per group)	**N1** (Mean % exploration time ± SEM)	**N2** (Mean % exploration time ± SEM)	**Familiar beads** (Mean % exploration time ± SEM)	**N1-N2 paired Wilcoxon signed-ranks test**
Sham-saline	35.6 ± 17.4	62.3 ± 17.6	2.1 ± 1.8	P = .05*
Sham-MB	32.4 ± 13.1	63.6 ± 14.5	4.0 ± 4.9	P = .04*
2VO-saline	43.1 ± 21.7	54.0 ± 21.0	2.9 ± 3.6	P = .21
2VO-MB	43.4 ± 13.2	53.9 ± 11.8	2.7 ± 2.5	P = .05*

4.6 Discussion

The experiment showed that daily low-dose MB (4 mg/kg i.p.) after permanent bilateral carotid artery occlusion (2VO) enhanced discrimination learning and memory in a visual water maze task. It is unlikely that the observed therapeutic benefits produced by low-dose MB were due to non-specific effects on behavior because the 2VO surgery and MB treatments had no significant effects on open field motor performance, visually guided swimming or the familiar odor recognition test. One limitation of the study is that the findings are not based on naïve subjects, but on subjects that were behaviorally and sexually experienced. However, all the subjects had the same behavioral experience before the start of these experiments, and therefore, it is unlikely that pre-existing differences among the groups could confound our findings. Therefore, the adverse effects of 2VO and the beneficial effects of MB were specific to the learning and memory of the visuospatial discrimination task. Rats receiving MB exhibited not only better learning over days, but also enhanced memory retention as demonstrated in

the probe test. As predicted, mean scores for visual memory retention followed the order sham-MB > sham-saline > 2VO-MB > 2VO-saline, with MB groups performing better than their saline counterparts.

4.6.1 Visuospatial memory deficits after bilateral carotid artery occlusion

The occlusion of both carotid arteries (two vessel occlusion, or 2VO) is a well-established rat model of chronic cerebral hypoperfusion developed by de la Torre and colleagues (de la Torre & Fortin, 1991; 1994). The 2VO model simulates some specific visuospatial memory changes observed in mild cognitive impairment and early in the progression of dementia, especially in sporadic AD. This model causes a mild form of chronic cerebral hypoperfusion that allows investigations of the consequent behavioral deficits and the testing of experimental treatments that may reduce these deficits. The 2VO involves a permanent occlusion found to reduce cerebral blood flow by 21% in the cortex and 32% in the hippocampus of middle-aged rats (de la Torre, Fortin et al., 1993). Occlusion of the two carotid arteries does not produce a complete loss of perfusion to any brain region due to the collateral circulation via the circle of Willis present in the arterial configuration of rats (Paxinos & Watson, 2006). Therefore, the 2VO model is not a model of acute stroke or cerebrovascular infarction in humans. Instead, 2VO for one month models decrements in neuronal oxidative energy metabolism without the immediate loss of brain cells seen in cerebrovascular infarction or stroke, resembling more the age-related changes in brain

hypoperfusion and visuospatial memory seen in mild cognitive impairment and early AD in humans. This 2VO model is characterized by impairment in visuospatial memory tasks such as the Morris water maze (Cada et al., 2000; de la Torre et al., 1997). The 2VO-induced hypoperfusion causes the largest metabolic reductions of cytochrome oxidase activity in hippocampal CA1 and posterior parietal cortex regions (de la Torre et al., 1997). Hence after chronic brain hypoperfusion with 2VO for four weeks, regional cytochrome oxidase activity is primarily reduced in hippocampal and posterior parietal regions, which occurs in parallel with visuospatial memory dysfunction in the Morris water maze. The present results extend this 2VO finding to a different visuospatial task involving visual pattern discrimination learning in a Y-maze water tank (Prusky et al., 2000). Importantly, in previous studies as well as in the present study, 2VO rats were impaired in visually-guided learning and memory but not in visually-guided escape to the visible platform or in motor swimming speed. Furthermore, our findings indicate that 2VO rats are not impaired in the open field locomotion task or in the recognition of familiar odors. Together these findings are consistent with the conclusion that dysfunction in brain regions mediating visuospatial learning and memory may underlie much of the behavioral deficits seen in the one-month 2VO rat model.

4.6.2 MB reduced visual learning and memory deficits without affecting non-specific activity

MB was administered daily to improve progressively more challenging visual discrimination learning phases. MB was injected daily in the post-session period to improve the retention of the discrimination learning, as it has been shown before that MB is most effective at improving memory consolidation than other memory stages (Gonzalez-Lima & Bruchey, 2004; Martinez et al., 2013). The half-life of MB in rats is about 5-6.5 hours (Peter et al., 2000), so it is not likely that the daily improvement of the visual discrimination seen 24 hours after each injection is a result of the continued drug action. A more likely explanation is that MB given immediately after each training session acted to enhance brain oxidative energy metabolism during the critical stage of memory consolidation. Thus our rationale for the timing of injection was that MB would enhance brain metabolic processes mediating memory consolidation. Since MB was given immediately after each session it could not have directly interfered with the acquisition trials the next day. Instead, we conclude that MB acted on consolidation processes to facilitate memory retention after each daily session and on the probe trial.

One alternative interpretation may be that chronic MB administration simply increases levels of motor activity in a non-specific manner, and thereby may improve learning performance. But this alternative is highly unlikely because swimming speed during the visual water task was not influenced by MB administration. To further test the possibility that non-specific motor or sensory effects could account for the differences between

MB and saline groups, we conducted additional behavioral tests before and after the visual water task. There were no significant group differences in any activity measures in the open field motor behavior or in the familiar odor recognition tests. These tests together with the monitored swimming speed served to rule out the possibility that repeated MB injections simply increased general arousal or non-specific sensory and motor activity. Therefore, the results suggest that the effects of chronic MB administration were specific to the visual learning and memory tests.

The improvement of visual learning and memory following post-session MB administration is consistent with the results of many previous MB studies in other behavioral models, all of which suggest that post-session administration of low-dose (1-4 mg/kg) MB in different learning and memory tasks improves memory retention, both in healthy animals and in animals with compromised energy metabolism (Callaway et al., 2004; Callaway et al., 2002; Martinez et al., 2013; Riha et al., 2011; Rojas et al., 2012). For example, repeated post-session administration of low-dose MB has also enhanced visuospatial memory retention in a holeboard food search task without any effect on general motor activity (Callaway et al., 2004). However, large doses of MB (50-100 mg/kg) impair both memory and sensory-motor performance because they lead to methemoglobinemia and consequently compromise oxygen delivery (Martinez et al., 2013; Riha et al., 2005).

4.6.3 Potential wide impact and therapeutic benefits of MB against neurocognitive impairment

The observed MB benefits in this model appear relevant and potentially translatable to patients with mild cognitive impairment and AD showing cerebrovascular hypoperfusion. The results could also potentially benefit patients with other memory disorders featuring impaired brain energy metabolism. The present model may be further investigated to provide insights on the *in vivo* mechanisms of action of MB interventions at behavioral and neurometabolic levels. Importantly, MB may provide a safe, highly bioavailable and affordable drug intervention. Most drug development efforts beyond basic research fail due to high toxicity and inadequate pharmacokinetics of candidate drugs. However, low-dose MB is an intervention with benign safety profiles and high bioavailability to nerve cells. MB constitutes an affordable and safe alternative to current treatment options for cognitive impairment. Memory functions are extremely sensitive to oxidative energy deficits (Whitmer et al., 2009). The brain is especially vulnerable because it depends almost exclusively on electron transport-derived oxidative energy (Clarke & Sokoloff, 1999). Thus it is likely that impairments in cerebral blood flow and oxidative energy metabolism may underlie memory deficits. For example, mild cognitive impairment and AD have been linked to mitochondrial dysfunction (de la Torre, 2010; Mosconi et al., 2008) and to specific deficiencies in cytochrome oxidase activity (Valla, Berndt & Gonzalez-Lima, 2001; Valla et al., 2007; 2010). The key observations that carotid artery disease and mild cognitive impairment are

risk factors for AD (Hirai et al., 2001; Morris et al., 2001) present an opportunity to develop early metabolic interventions to impact these disorders. Low-dose MB can also successfully attenuate neurological and behavioral deficits in a model of mild traumatic brain injury (Watts et al., 2014).

4.6.4 Conclusions

Despite compelling evidence of a role of chronic brain hypoperfusion in the pathophysiology of mild cognitive impairment and AD (Hauptmann et al., 2009; Hirai et al., 2001; Velliquette et al., 2005), animal models that exhibit this metabolic deficit have not been used before to test new memory-enhancing interventions targeting mitochondrial respiration (Gonzalez-Lima et al., 2014). The present study is the first one to demonstrate that low-dose MB has beneficial therapeutic effects on an animal model of chronic brain hypoperfusion. This study served as a proof-of-concept to test the efficacy of a chronic metabolic intervention with low-dose MB in a hypoperfusion model relevant to mild cognitive impairment and AD. Thus if this intervention is proven effective in clinical trials, chronic low-dose MB treatment could be accomplished with relatively inexpensive and non-invasive means.

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4.7 Literature Citations

Bruchey, A., & Gonzalez-Lima, F. (2008). Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. *American journal of pharmacology and toxicology*, *3*(1), 72–79. doi:10.3844/ajptsp.2008.72.79

Cada, A., Torre, J., & Gonzalez-Lima, F. (2000). Chronic cerebrovascular ischemia in aged rats: effects on brain metabolic capacity and behavior. *Neurobiology of aging*, *21*(2), 225–33.

Callaway, N., Riha, P., Bruchey, A., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, *77*(1), 175–181. doi:10.1016/j.pbb.2003.10.007

Callaway, N., Riha, P., Wrubel, K., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neuroscience letters*, *332*(2), 83–6. doi:10.1016/S0304-3940(02)00827-3

Clarke, D. D., & Sokoloff, L. (1999). Circulation and energy metabolism of the brain. *Basic neurochemistry: molecular, cellular and medical aspects*, *6*, 637-669.

Crews, D., Gore, A., Hsu, T., Dangleben, N., Spinetta, M., Schallert, T., Skinner, M. (2007). Transgenerational epigenetic imprints on mate preference. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 5942–5946. doi:10.1073/pnas.0610410104

de la Torre, J., & Fortin, T. (1991). Partial or global rat brain ischemia: the

SCOT model. *Brain research bulletin*, *26*, 365–372. doi:10.1016/0361-9230(91)90008-8

de la Torre, J., & Fortin, T. (1994). A chronic physiological rat model of dementia. *Behavioural brain research*, *63*, 35–40. doi:10.1016/0166-4328(94)90048-5

de la Torre, J., Fortin, T., Park, G., Pappas, B., & Richard, M. (1993). Brain blood flow restoration "rescues" chronically damaged rat CA1 neurons. *Brain research*, *623*, 6–15. doi:10.1016/0006-8993(93)90003-6

de la Torre, J. C., Čada, A., Nelson, N., Davis, G., Sutherland, R. J., & Gonzalez-Lima, F. (1997). Reduced cytochrome oxidase and memory dysfunction after chronic brain ischemia in aged rats. *Neuroscience letters*, *223*(3), 165-168. doi:10.1016/S0304-3940(97)13421-8

de la Torre, J. C. (2010). Vascular risk factor detection and control may prevent Alzheimer's disease. *Ageing research reviews*, *9*(3), 218-225.

de la Torre, J. C. (2012). Cardiovascular risk factors promote brain hypoperfusion leading to cognitive decline and dementia. *Cardiovascular psychiatry and neurology*, *2012*.

de la Torre, J. C. (2012b). Cerebral hemodynamics and vascular risk factors: setting the stage for Alzheimer's disease. *Journal of Alzheimer's Disease*, *32*(3), 553-567.

Deiana, S., Harrington, C., Wischik, C., & Riedel, G. (2009). Methylthioninium chloride reverses cognitive deficits induced by scopolamine: comparison with rivastigmine. *Psychopharmacology*, *202*(1-3), 53–65.

doi:10.1007/s00213-008-1394-2

Driscoll, I., Howard, S., Prusky, G., Rudy, J., & Sutherland, R. (2005). Seahorse wins all races: hippocampus participates in both linear and non-linear visual discrimination learning. *Behavioural brain research*, *164*(1), 29–35. doi:10.1016/j.bbr.2005.05.006

Driscoll, I., Sutherland, R., Prusky, G., & Rudy, J. (2004). Damage to the hippocampal formation does not disrupt representational flexibility as measured by a novelty transfer test. *Behavioral neuroscience*, *118*, 1427–1432. doi:10.1037/0735-7044.118.6.1427

Ennaceur, A., Neave, N., & Aggleton, J. (1997). Spontaneous object recognition and object location memory in rats: The effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. *Experimental Brain Research*, *113*, 509–519. doi:10.1007/PL00005603

Gonzalez-Lima, F., Barksdale, B. R., & Rojas, J. C. (2014). Mitochondrial respiration as a target for neuroprotection and cognitive enhancement. *Biochemical pharmacology*, *88*(4), 584–93. doi:10.1016/j.bcp.2013.11.010

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, *11*(5), 633-640. doi:10.1101/lm.82404

Hauptmann, S., Scherping, I., Drose, S., Brandt, U., Schulz, K., Jendrach, M., Muller, W. (2009). Mitochondrial dysfunction: An early event in Alzheimer pathology accumulates with age in AD transgenic mice.

Neurobiology of Aging, *30*, 1574–1586. doi:10.1016/j.neurobiolaging.2007.12.005

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R. L., Atwood, C. S., ... & Smith, M. A. (2001). Mitochondrial abnormalities in Alzheimer's disease. *The Journal of Neuroscience*, *21*(9), 3017-3023.

Huang, S., Du, F., Shih, Y., Shen, Q., Gonzalez-Lima, F., & Duong, T. (2013). Methylene blue potentiates stimulus-evoked fMRI responses and cerebral oxygen consumption during normoxia and hypoxia. *NeuroImage*, *72*, 237–242. doi:10.1016/j.neuroimage.2013.01.027

Lee, R., & Urban, J. (2002). Functional replacement of oxygen by other oxidants in articular cartilage. *Arthritis and rheumatism*, *46*(12), 3190–200. doi:10.1002/art.10686

Martinez, J., Jensen, R., Vasquez, B., McGuinness, T., & McGaugh, J. (2013). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, *6*(3), 387–390. doi:10.3758/BF03326744

Medina, D., Caccamo, A., & Oddo, S. (2011). Methylene blue reduces aβ levels and rescues early cognitive deficit by increasing proteasome activity. *Brain pathology (Zurich, Switzerland)*, 21(2), 140–9. doi:10.1111/j.1750-3639.2010.00430.x

Morris, J., Storandt, M., Miller, J., McKeel, D., Price, J., Rubin, E., & Berg, L. (2001). Mild cognitive impairment represents early-stage Alzheimer disease. *Archives of neurology*, 58, 397–405. doi:10.1001/archneur.58.3.397

Mosconi, L., Tsui, W., Herholz, K., Pupi, A., Drzezga, A., Lucignani, G., … Leon,

M. (2008). Multicenter standardized 18F-FDG PET diagnosis of mild cognitive impairment, Alzheimer's disease, and other dementias. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine*, *49*, 390–398. doi:10.2967/jnumed.107.045385

O'Leary, J., Li, Q., Marinec, P., Blair, L., Congdon, E., Johnson, A., Dickey, C. (2010). Phenothiazine-mediated rescue of cognition in tau transgenic mice requires neuroprotection and reduced soluble tau burden. *Molecular neurodegeneration*, *5*, 45. doi:10.1186/1750-1326-5-45

Obrenovich, M., Smith, M., Siedlak, S., Chen, S., Torre, J., Perry, G., & Aliev, G. (2006). Overexpression of GRK2 in Alzheimer disease and in a chronic hypoperfusion rat model is an early marker of brain mitochondrial lesions. *Neurotoxicity Research*, *10*, 43–56. doi:10.1007/BF03033333

Paban, V., Manrique, C., Filali, M., Maunoir-Regimbal, S., Fauvelle, F., & Alescio-Lautier, B. (2014). Therapeutic and preventive effects of methylene blue on Alzheimer's disease pathology in a transgenic mouse model. *Neuropharmacology*, *76*, 68–79. doi:10.1016/j.neuropharm.2013.06.033

Peter, C., Hongwan, D., Kupfer, A., & Lauterburg, B. (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *European Journal of Clinical Pharmacology*, *56*, 247–250. doi:10.1007/s002280000124

Prusky, G. T., West, P. W., & Douglas, R. M. (2000). Behavioral assessment of visual acuity in mice and rats. *Vision research*, *40*(16), 2201-2209.

Riha, P., Bruchey, A., Echevarria, D., & Gonzalez-Lima, F. (2005). Memory

facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, *511*(2-3), 151–8. doi:10.1016/j.ejphar.2005.02.001

Riha, P., Rojas, J., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *NeuroImage*, *54*(4), 2623–34. doi:10.1016/j.neuroimage.2010.11.023

Rojas, J., Bruchey, A., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of methylene blue. *Progress in neurobiology*, *96*(1), 32–45. doi:10.1016/j.pneurobio.2011.10.007

Rojas, J., & Gonzalez-lima, F. (2010). Mitochondrial optic neuropathy : In vivo model of neurodegeneration and neuroprotective strategies. *Eye and Brain*, 21–37. doi:10.2147/EB.S9363

Salaris, S., Babbs, C., & Voorhees, W. (1991). Methylene blue as an inhibitor of superoxide generation by xanthine oxidase. *Biochemical Pharmacology*, *42*(3), 499–506. doi:10.1016/0006-2952(91)90311-R

Shumake, J., Furgeson-Moreira, S., & Monfils, M. H. (2014). Predictability and heritability of individual differences in fear learning. *Animal cognition*, *17*(5), 1207–21. doi:10.1007/s10071-014-0752-1

Spinetta, M., Woodlee, M., Feinberg, L., Stroud, C., Schallert, K., Cormack, L., & Schallert, T. (2008). Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. *Psychopharmacology*, *201*(3), 361–71. doi:10.1007/s00213-008-1294-5

Telch, M. J., Bruchey, A. K., Rosenfield, D., Cobb, A. R., Smits, J., Pahl, S., &

Gonzalez-Lima, F. (2014). Effects of post-session administration of methylene blue on fear extinction and contextual memory in adults with claustrophobia. *American Journal of Psychiatry*, *171*(10), 1091-1098.

Tillerson, J., Caudle, W., Parent, J., Gong, C., Schallert, T., & Miller, G. (2006). Olfactory discrimination deficits in mice lacking the dopamine transporter or the D2 dopamine receptor. *Behavioural Brain Research*, *172*, 97–105. doi:10.1016/j.bbr.2006.04.025

Valla, J., Berndt, J., & Gonzalez-Lima, F. (2001). Energy hypometabolism in posterior cingulate cortex of Alzheimer's patients: Superficial laminar cytochrome oxidase associated with disease duration. *J. Neurosci.*, *21*(13), 4923–4930.

Valla, J., Schneider, L., Small, A., & Gonzalez-Lima, F. (2007). Quantitative cytochrome oxidase histochemistry: Applications in human Alzheimer's Disease and animal models. *Journal of Histotechnology*, *30*, 235–247. doi:10.1179/his.2007.30.4.235

Valla, J., Yaari, R., Wolf, A., Kusne, Y., Beach, T., Roher, A., Reiman, E. (2010). Reduced posterior cingulate mitochondrial activity in expired young adult carriers of the APOE ε Allele, the major late-onset Alzheimer's susceptibility gene. *Journal of Alzheimer's Disease*, *22*, 307–313. doi:10.3233/JAD-2010-100129

Velliquette, R., O'Connor, T., & Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease

pathogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *25*, 10874–10883. doi:10.1523/JNEUROSCI.2350-05.2005

Visarius, T., Stucki, J., & Lauterburg, B. (1997). Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS Letters*, *412*(1), 157–160. doi:10.1016/S0014-5793(97)00767-9

Wainwright, M., & Crossley, K. (2002). Methylene Blue - a Therapeutic Dye for All Seasons? *Journal of Chemotherapy*, *14*(5), 431–443.

Watts, L., Long, J., Chemello, J., & Koughnet, {Van. (2014). Methylene Blue Is Neuroprotective against Mild Traumatic Brain Injury. *Journal of neurotrauma*, *9*, 1–9. doi:10.1089/neu.2013.3193

Whitmer, R., Karter, A., Yaffe, K., Quesenberry, C., & Selby, J. (2009). Hypoglycemic episodes and risk of dementia in older patients with type 2 diabetes mellitus. *JAMA : the journal of the American Medical Association*, *301*, 1565–1572. doi:10.1001/jama.2009.460

Wrubel, K., Barrett, D., Shumake, J., Johnson, S., & Gonzalez-Lima, F. (2007a). Methylene blue facilitates the extinction of fear in an animal model of susceptibility to learned helplessness. *Neurobiology of learning and memory*, *87*(2), 209–17. doi:10.1016/j.nlm.2006.08.009

Wrubel, K., Riha, P., Maldonado, M., McCollum, D., & Gonzalez-Lima, F. (2007b). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology, biochemistry, and behavior*, *86*(4), 712–7. doi:10.1016/j.pbb.2007.02.018

Zhang, X., & Rojas, {C. (2006). Methylene blue prevents neurodegeneration

caused by rotenone in the retina. *Neurotoxicity Research*, *9*(1), 47–57. doi:10.1007/BF03033307

Chapter 5: Methylene blue enhances mitochondrial activity, functional connectivity and memory in rats experiencing chronic cerebral hypoperfusion.

5.1 Abstract

Chronic cerebral hypoperfusion, one of the risk factors for mild cognitive impairment (MCI) and Alzheimer's disease (AD), has been shown to diminish mitochondrial respiration and impair memory. As such, drugs that improve mitochondrial function may be appropriate treatments for cerebral hypoperfusion. Methylene blue (MB) is a historical blue dye that crosses the blood-brain barrier and serves as an electron cycler in the mitochondrial electron transport chain. Previous studies implicate MB in both memory enhancement and neuroprotection. This chapter is an extension of the previous experiment, whereby rats underwent either permanent bilateral carotid occlusion (2VO) or sham surgery, followed by daily intraperitoneal injections of either 4 mg/kg USP MB or saline. Following recovery from surgery, subjects were trained on the visual water task (VWT). Upon completion of behavioral analyses, brains were histochemically stained for cytochrome oxidase (CO) activity, an index of mitochondrial function. Whole brain quantitative CO mapping revealed that 2VO surgery resulted in (1) decreased CO activity in visual cortex, prefrontal cortex, perirhinal cortex, hippocampus and amygdala, and (2) weaker functional connectivity between the midbrain, visual cortices and hippocampus. Treatment with MB increased CO activity in regions affected by 2VO and strengthened functional

connectivity between cortical, midbrain and hippocampal brain regions, as well as between the amygdala and prefrontal cortex. Brain-behavior correlations revealed positive correlations between VWT performance and brain regions enhanced by MB treatment. These results are the first to demonstrate that (1) 2VO weakens metabolic functional connectivity in regions specific to a particular task and (2) MB restores both regional mitochondrial activity and functional connectivity in brain regions susceptible to 2VO and correlated with task-specific learning.

5.2 Introduction

Disturbances in the normal blood supply of glucose and oxygen to the brain result in cognitive and behavioral dysfunction (Marcus et al., 1989; Meier-Ruge & Bertoni-Freddari, 1996). Additionally, it has been firmly established that aging and dementia are accompanied by progressive reduction in cerebral blood flow and energy metabolism (de la Torre, 2012; Farkas & Luiten, 2001; Kalaria, 1991; Lassen & Ingvar, 1980). Patients with cerebrovascular disorders and late-onset Alzheimer's disease (AD) show marked cerebral hypoperfusion (de la Torre, 1999; Obrist et al., 1970; Rogers et al., 1986; Tachibana et al., 1984a; Yamaguchi et al., 1980). Even normal age-related reduction in cerebral blood flow results in significant functional pathology when combined with other risk factors such as cardiovascular disease (Cohen et al., 2009; Haley et al., 2007; Sparks, 1990), head injury (Graham et al., 1995) and/or cerebrovascular ischemia (Cada, de la Torre & Gonzalez-Lima, 2000; de la Torre et al., 1997). Interestingly, the pathology of cerebral hypoperfusion differs from cerebrovascular disease (e.g. stroke). In AD patients who have not suffered cerebral infarction, cerebral hypoperfusion is reduced more symmetrically, globally and chronically (Tachibana et al., 1984b; Zemcor et al., 1984), implicating chronic cerebral hypoperfusion in the pathogenesis of AD independently of cerebrovascular disease.

Permanent bilateral carotid artery occlusion (2-vessel occlusion; 2VO) is a useful model for the reproduction of chronic cerebral hypoperfusion as it occurs in human aging and AD (Farkas, Luiten & Bari, 2007). Typically in

rats, permanent vessel occlusion does not result in reperfusion injury (as a result of instant recovery of perfusion). Additionally, cerebral hypoperfusion is global, damage to nervous tissue is less dramatic and there are no obvious signs of motor dysfunction or seizures (Farkas, Luiten & Bari, 2007).

Neuropathological changes that result from 2VO resemble those of late-onset AD, strengthening its use as an experimental model (de la Torre, 1999; 2000). Since hippocampal damage is a feature of AD, and the hippocampus is also particularly sensitive to ischemia, the time course of damage to the hippocampus as a result of 2VO surgery has been highly characterized. While conspicuous hippocampal damage is not seen during the first week after 2VO (Ohtaki et al., 2006), damage to the CA1 subfield is observed in 6-29% of animals at 2 weeks (Farkas et al., 2006; Schmidt-Kastner et al., 2001), 55% at 4 weeks (Ohtaki et al., 2006), and total hippocampal destruction was observed in 67% of 2VO rats at 8-13 weeks (Farkas et al., 2004b; Liu et al., 2006).

2VO also serves as a good model for aging and dementia because it not only results in global neurological changes, but also results in learning and memory impairment (Cada, de la Torre & Gonzalez-Lima, 1997; de la Torre, 2000). It has been firmly established that experimental cerebral hypoperfusion compromises spatial learning in rats (Farkas & Luiten, 2001; Liu et al., 2005; Murakami et al., 2000; Shang et al., 2005; Sopala & Danysz, 2001). Non-spatial object recognition deficits have also been observed 60 and 90 days following 2VO surgery (Sarti et al., 2002), but we did not observe

deficits in odor recognition after 30 days (see Chapter 4; Auchter et al., 2014).

When neurons are starved of glucose and oxygen via chronic cerebral hypoperfusion, the impending result is mitochondrial dysfunction. This is reflected by ATP that is rapidly depleted in the initial phases after 2VO surgery (Ueda & Fujita, 2004). If mitochondria do not receive enough glucose and oxygen, electrons from the electron transport chain used to drive ATP synthesis are taken up by other molecules, resulting in the formation of damaging reactive oxygen species (ROS; Murphey, 2009; Turrens, 2003). These molecules are as their name suggests—extremely reactive. Their excessive presence in neurons can lead to damage in mitochondrial DNA, and ultimately to programmed cell death (Circu & Aw, 2010; Simon, Haj-Yehia & Levi-Schaffer, 2000).

Methylene blue (MB) is a blue dye with neurometabolic enhancing properties at low doses (Rojas, Bruchey and Gonzalez-Lima, 2012). MB crosses the blood-brain barrier and diffuses into the mitochondrial matrix, where at low doses it forms a redox equilibrium with the enzymes of the electron transport chain (Bruchey & Gonzalez-Lima, 2008; Riha, Rojas & Gonzalez-Lima, 2011; Visarius et al., 1997). MB has been shown to enhance mitochondrial respiration, chiefly by increasing activity in cytochrome oxidase (CO) (Rojas, Bruchey and Gonzalez-Lima, 2012). CO is the terminal enzyme of the electron transport chain and its activity reflects neuronal activity (Wong-Riley, 1989).MB enhancement of CO activity is coupled with increases in ATP production and oxygen consumption (Atamna et al., 2008;

Bruchey & Gonzalez-Lima, 2008; Huang et al., 2013; Lindahl & Oberg, 1961; Riha et al., 2005). Through enhanced mitochondrial efficiency, MB also reduces the incidence of ROS formation (Kelner et al., 1988; Salaris, Babbs & Voorhees, 1991) and consequently delays cellular senescence (Atamna et al., 2008).

MB at low doses has been shown to enhance both spatial (Callaway et al., 2002; Riha et al., 2005; Wrubel et al., 2007) and non-spatial (Gonzalez-Lima & Bruchey, 2004) memories, and such enhancement is also marked by increases in CO activity (Atamna & Kumar, 2010; Callaway et al., 2002; 2004). MB has also been shown to be neuroprotective in rodent models of stroke (Kelner et al., 1988; Miclescu et al., 2010; Salaris, Babbs & Voorhees, 1991; Shen et al., 2013; Watts et al., 2013), Alzheimer's Disease (Atamna & Kumar, 2010; Callaway et al., 2002; Oz, Lorke, & Petroianu, 2009; Riha, Rojas & Gonzalez-Lima, 2005), Parkinson's Disease (Rojas et al., 2009) and optic neuropathy (Rojas et al., 2009a; Zhang, Rojas & Gonzalez-Lima, 2006). However, MB effects have not been described during chronic cerebral hypoperfusion.

The objectives of this experiment were to describe (1) how 2VO weakens brain regional mitochondrial activity as measured by CO histochemistry, metabolic functional connectivity and performance on a visual discrimination task, (2) how MB strengthens performance and restores both regional mitochondrial activity and functional connectivity and (3) how specific changes in brain regional mitochondrial activity are correlated with behavioral performance.

5.3 Hypothesis

Chronic cerebral hypoperfusion results in reduction in regional CO activity in brain regions susceptible to hypoxia and a reduction in functional connectivity between regions involved in visual discrimination learning. Treatment of chronic cerebral hypoperfusion with daily low-dose methylene blue (MB) injections will restore both brain regional activity and functional connectivity.

5.4 Materials and Methods

5.4.1 Subjects

Subjects for this analysis were the same subjects used in Chapter 4. Briefly, subjects were 39 adult male Long-Evans rats weighing approximately 500-600 g at the time of surgery. Two rats died from surgical complications and one rat died of a respiratory infection during visual water task training. Additionally, it was determined upon brain extraction that one subject suffered a hemorrhagic stroke, and thus was excluded from the analysis, making the final N = 35. Rats were raised from birth in AALAC-approved facilities under standard laboratory conditions (12 h: 12 h, light: dark cycle) with *ad libitum* access to food and water.

5.4.2 Summary of behavioral procedures

Since subjects were the same as in Chapter 4, detailed explanations of behavioral procedures can be found in Section 4.4.3, and an experimental

timeline is shown in Figure 4.1. Briefly, the experiment followed a 2x2 experimental design, whereby subjects were given either 2VO surgery or a sham control, followed by daily intraperitoneal injections of either 4 mg/kg MB or an equivalent volume of saline. Upon recovery from surgery, subjects were trained on two variations of the visual water task (VWT), whereby subjects were placed at one end of a Y-shaped water tank and allowed to swim down one of two arms to find an invisible escape platform. Distinct patterns were displayed at the end of each arm, and the location of the platform was indicated by the patterns.

VWT protocols were adapted from those described by Driscoll et al. (2005; 2006). In the first variation of the VWT, subjects were trained for 10 days on three elemental pattern discriminations (A+B-, C+D-, E+F-; see Figure 4.2). In the second variation of the task, subjects were trained on a more difficult transverse pattern discrimination (X+Y-, Y+Z-, Z+X-). After completion of the VWT, subjects were also tested on an odor discrimination task (see Figure 4.3).

5.4.3 Quantitative cytochrome oxidase histochemistry

Cytochrome oxidase brain mapping was conducted by an investigator blind to experimental condition through a well-established method of optical densitometry of brain sections as detailed by Gonzalez-Lima and Cada (1994). Following behavioral procedures, animals were sacrificed, and brains were quickly removed and frozen in isopentane. Coronal brain sections (40 μm thick) were obtained and mounted on slides using a cryostat microtome

(Microm HM-505E, Heidelberg, Germany). To obtain increasing gradients of CO activity, a frozen brain homogenate paste was sectioned into different tissue thicknesses (10, 20, 40, 60 and 80 μm) and mounted on separate slides. CO activity of the homogenate was spectrophotometrically assessed and used to calculate CO values for the different section thicknesses. The brain homogenate paste sections were used as calibration standards for each CO staining batch.

Each CO staining batch consisted of representative slides from two brain levels for each subject along with two slides with a set of 5 standards each. Each batch was processed for CO histochemistry following the method previously described by Gonzalez-Lima and Jones (1994). Briefly, slides were fixed for 5 minutes with 1.5% glutaraldehyde, rinsed three times in phosphate buffer with sucrose and preincubated in a solution containing cobalt chloride and DMSO dissolved in Tris buffer. After rinsing sections with phosphate buffer (pH 7.6; 0.1 M), they were incubated at 37 °C for 1 hour in the dark and with continuous stirring in a solution containing diaminobenzidine, sucrose, cytochrome c and catalase (Sigma-Aldrich, Barcelona, Spain) dissolved in phosphate buffer (pH 7.6; 0.1 M). The slides were then dehydrated in increasing concentrations of ethanol, coverslipped with permount (Merck, Darmstadt, Germany) and allowed to dry for 48 hours.

To obtain CO activity values for regions of interest (ROIs), a calibration step tablet (Kodak, density range 0.06-3.05) and CO-stained slides were placed on a high-precision illuminator and digitized using a CCD digital

microscope camera (Leica Microsystems DFC450, Wetzlar, Germany). Images were then analyzed using ImageJ software, which allows creating a logarithmic calibration curve of optical density units as a function of pixel (gray level) values in each image. Before analysis, images were corrected for slide and light box artifacts using background subtraction. For brain structure analyses, 44 ROIs were located and outlined in each brain hemisphere in each subject by an experimenter blind to experimental conditions using a rat brain atlas (Paxinos & Watson, 3rd edition; see Table 5.1 for a complete list of ROIs and abbreviations). The measurement obtained was an average optical density (OD) value for the outlined ROI. To maximize the accuracy of OD values, this procedure was repeated in three adjacent brain sections for each ROI, and the mean of the three sections in both cerebral hemispheres was used as the overall mean OD value for that ROI. Mean OD values were later converted into mean CO using calibration curves based on tissue standards and spectrophotometrically-determined cytochrome oxidase activity. This method yielded a linear relationship ($r>0.95$) between biochemical cytochrome oxidase activity measured spectrophotometrically and histochemical cytochrome oxidase reactivity measured by optical density.

For regional CO activity measures, when 2VO-induced lesions were present within the ROI, both the affected and surviving tissue were included in the outlined ROI (i.e. the ROI was defined using the brain atlas, not the lesion). The reason for doing this was two-fold. First, it allowed us to index general CO activity in each ROI, taking into account both lesions and

compensatory increases in CO in surviving tissue (if such compensatory phenomena existed). Second, since some lesions did not have clearly-defined borders, including the entire ROI gave us measures of CO activity that were unbiased by experimenter evaluation of infarct vs. penumbra vs. healthy tissue. An assessment of lesion volume was conducted in a separate analysis described below.

5.4.4 Statistical analyses

All statistical testing was carried out using the PASW 22 software package (SPSS, Inc., Chicago, IL). All differences were considered statistically significant at the two-tailed $p<.05$ level. For univariate group comparisons, average CO values for each ROI were used as dependent variables in separate analyses, with surgery and drug treatment as independent variables.

Since subjects went through two iterations of the visual water task (elemental discrimination and transverse pattern discrimination), a composite "VWT performance score" was calculated by averaging the scores from the final probe for each task. Though subjects did not learn the transverse task (as indicated by the inability to reach and 8/10 criteria for pattern discriminations), their performance in the task likely still influenced cytochrome oxidase activity. Thus, brain-behavior correlations were conducted using two-tailed bivariate correlations between the VWT performance score and mean CO activity for each ROI.

Functional connectivity was analyzed in two ways. First, two-tailed bivariate correlations were performed between all brain ROIs within each

experimental condition. Then, to detect which regional correlations showed significant differences between groups, correlation coefficients were compared using Fisher's z-transformations, followed by independent samples t-tests.

In 2VO subjects that showed lesions (n=6), volumetric analysis was conducted on affected areas. Infarct volumes (V) were derived from the lesion area per slice (A) and distance between collected sections (d), using the formula $V = \sum A \times d$. The value d was calculated as $d = (T) \times$ (number of designated sections-1), where T is the distance between every designated section (80 μm). Lesion area per slice was measured by setting a conservative optical density threshold aimed to prevent the inclusion of any white matter and tissue artifacts as affected tissue. This was especially important since we observed lesions in the striatum, which in rats is speckled with white matter tracts. To ensure the threshold was unbiased, for each subject the minimum and maximum threshold values were set to 85% and 98%[6] of the measured mean OD value for white matter (i.e. "more OD" than white matter, but "less OD" than healthy gray matter tissue). Figure 5.1 demonstrates that by using this threshold and outlining the ROI, we could obtain a conservative measure of lesion area per section.

[6] A few subjects showed tissue loss where lesions occurred. In these cases, the maximum value for the threshold was set equal to white matter so that regions with missing tissue were still considered in the calculation of lesion area.

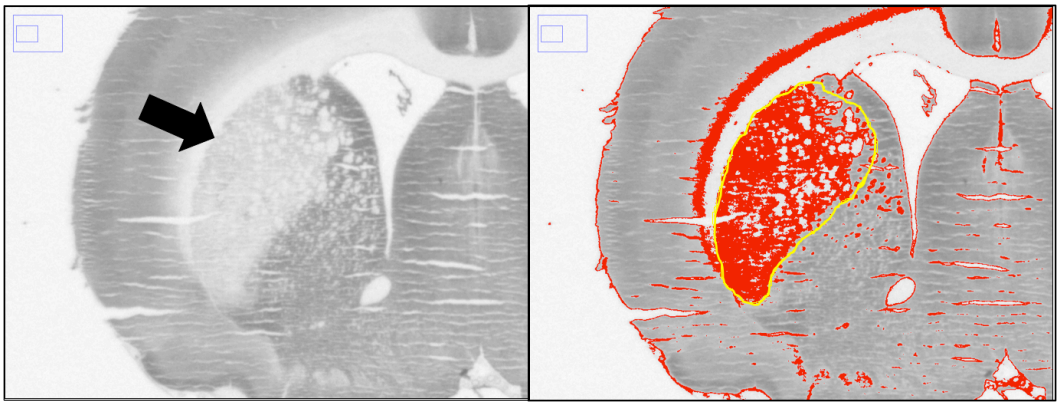

Figure 5.1 Determination of lesion volume. For every section showing a lesion (e.g., arrow pointing to **lateral side of striatum**), a threshold was applied to highlight (in red) tissue with OD values between that of white matter and healthy gray matter tissue (**medial side of striatum**). For each section showing a lesion, the area of the highlighted tissue within the approximate lesion outline (in yellow) was used as A in the equation to calculate lesion volume, V = ∑A x d, where d is the distance between sections.

5.5 Results

5.5.1 Univariate ROI analysis

Table 5.1 shows mean CO values and standard errors for each ROI in each experimental condition. Only V2 showed a significant main effect of surgery, $F(1,31)=4.491$, $p=.047$. There were no main effects for drug treatment. Several regions showed significant surgery by drug treatment interactions, including perirhinal cortex, $F(1,31)=5.667$, $p=.024$, secondary motor cortex, $F(1,31)=5.883$, $p=.022$, primary visual cortex, $F(1,31)=4.606$, $p=.045$, basolateral amygdala, $F(1,31)=5.350$, $p=.028$, medial amygdala, $F(1,31)=5.189$, $p=.030$ and the anterior portion of CA1, $F(1,31)=4.951$, $p=.034$, CA2, $F(1,31)=4.650$, $p=.039$, and CA3, $F(1,31)=4.940$, $p=.034$, hippocampal subfields. These significant surgery by drug treatment interactions are shown in Figure 5.2.

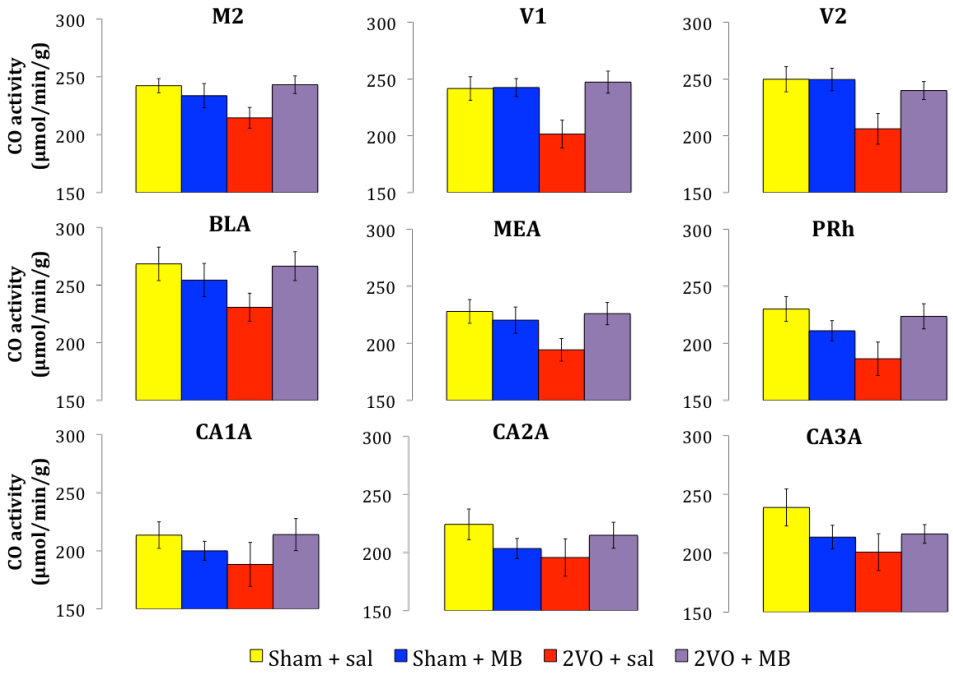

Figure 5.2 ROIs showing significant univariate effects. In these regions, 2VO surgery (red) resulted in a decrease in cytochrome oxidase activity, which was restored by MB treatment (violet).

Table 5.3 Mean CO activity values (µmol/min/g) ± standard errors (SEM) for each ROI and each experimental condition. Means showing an individual group effect are boldfaced.

ROI	Sham + saline	SEM	Sham + MB	SEM	2VO + saline	SEM	2VO + MB	SEM
Prelimbic cortex (PLC)	246.43	± 9.88	243.80	± 13.63	225.09	± 16.21	247.76	± 9.69
Medial orbital cortex (MO)	245.24	± 11.49	239.99	± 11.85	221.16	± 15.31	248.61	± 9.82
Ventral orbital cortex (VO)	254.38	± 9.57	248.35	± 12.49	235.53	± 12.42	254.43	± 8.99
Lateral orbital cortex (LO)	251.27	± 8.88	249.62	± 11.52	233.50	± 11.32	252.09	± 7.87
Agranular insular cortex (AI)	227.63	± 7.31	220.18	± 8.90	211.33	± 10.82	229.76	± 8.69
Infralimbic cortex (IL)	281.87	± 9.58	270.41	± 14.29	269.97	± 15.16	272.44	± 5.28
Primary motor cortex (M1)	237.17	± 4.92	230.15	± 10.02	222.54	± 13.23	232.86	± 3.74
Secondary motor cortex (M2)	242.28	± 6.10	233.62	± 10.50	**214.49**	± 8.99	243.12	± 7.61
Primary somatosensory cortex (S1)	214.27	± 13.65	214.25	± 7.76	222.05	± 11.91	212.94	± 7.10
Secondary somatosensory cortex (S2)	226.35	± 7.55	218.51	± 10.34	210.28	± 7.41	230.64	± 8.22
Cingulate cortex (Cin)	259.93	± 8.32	244.58	± 10.16	258.77	± 15.84	257.88	± 9.85
Perirhinal cortex (PRh)	230.05	± 10.87	210.98	± 8.89	**186.46**	± 14.63	223.64	± 10.99
Primary visual cortex (V1)	241.59	± 10.36	242.43	± 7.87	**201.47**	± 12.24	247.19	± 9.66
Secondary visual cortex (V2)	249.72	± 11.05	249.49	± 9.86	**206.10**	± 13.52	**239.80**	± 7.90
Medial septal nucleus (MS)	203.40	± 8.33	197.97	± 12.39	202.64	± 10.36	197.97	± 6.04
Lateral septal nucleus (LS)	272.30	± 8.28	262.01	± 12.81	273.92	± 11.81	268.67	± 5.58
Acumbens core (AcbC)	313.40	± 9.76	304.24	± 10.43	315.15	± 17.36	307.88	± 7.59
Acumbens shell (AcbS)	241.13	± 10.53	223.67	± 8.36	233.98	± 8.35	224.90	± 7.96
Caudate/Putamen (CPu)	198.87	± 13.32	199.51	± 8.52	191.14	± 11.26	191.27	± 6.12
Globus palidus (GP)	115.97	± 6.57	108.53	± 4.83	115.23	± 5.74	112.64	± 4.92
Ventral pallidum (VP)	179.11	± 5.82	169.26	± 6.40	168.38	± 8.74	168.31	± 4.50
Lateral hypothalamus (LH)	160.75	± 9.32	157.53	± 9.78	154.51	± 5.90	163.81	± 8.06
Paraventricular nucleus (PVH)	191.74	± 6.94	183.50	± 8.14	184.33	± 8.43	203.21	± 6.86
Medial preoptic area (MPO)	206.22	± 6.62	198.81	± 7.51	210.35	± 10.84	208.42	± 5.72
Bed nucleus stria terminalis (BST)	233.80	± 7.98	218.64	± 9.49	237.61	± 14.51	224.12	± 6.61
Subthalamic nucleus (Sub)	273.20	± 6.04	249.10	± 10.74	249.36	± 14.37	248.75	± 10.91
Medial geniculate nucleus (MGN)	255.28	± 8.38	245.48	± 5.66	240.78	± 12.86	235.70	± 5.45
Basomedial amygdala (BMA)	186.21	± 7.73	180.59	± 10.02	175.09	± 4.88	195.70	± 9.16

Table 5.3 cont.

Medial amygdala (MEA)	227.95	±	10.39	220.31	±	11.47	**194.29**	±	9.88	226.00	±	9.75
Basolateral amygdala (BLA)	268.46	±	14.51	254.36	±	14.44	**230.77**	±	12.21	266.47	±	12.53
Central amygdala (CEA)	223.13	±	8.63	219.37	±	10.03	206.30	±	9.63	234.55	±	9.36
Anterior hippocampus CA1 (CAIA)	213.48	±	11.42	199.98	±	8.20	**188.32**	±	18.91	213.97	±	13.79
Anterior hippocampus CA2 (CA2A)	224.26	±	13.21	203.44	±	8.69	**195.70**	±	16.03	214.87	±	11.19
Anterior hippocampus CA3 (CA3A)	238.88	±	15.71	213.68	±	10.05	**200.94**	±	15.59	216.38	±	8.13
Anterior dentate gyrus (DGA)	319.76	±	26.01	294.90	±	15.21	306.25	±	31.40	297.03	±	19.93
Posterior hippocampus CA1 (CA1P)	298.13	±	13.98	277.27	±	12.72	266.56	±	14.28	277.08	±	13.51
Posterior hippocampus CA2 (CA2P)	275.84	±	11.87	249.69	±	11.82	236.40	±	17.96	248.44	±	10.82
Posterior hippocampus CA3 (CA3P)	292.89	±	9.91	272.22	±	13.02	255.42	±	16.45	269.08	±	9.99
Posterior dentate gyrus (DGP)	269.60	±	15.75	256.92	±	10.66	258.72	±	12.20	255.88	±	7.02
Subiculum (S)	239.98	±	12.04	226.65	±	10.53	221.74	±	11.20	217.73	±	6.72
Superior colliculus (SC)	254.18	±	13.53	246.46	±	8.80	240.89	±	10.46	249.47	±	8.05
Substantia nigra (SN)	214.80	±	8.68	207.29	±	8.24	211.66	±	11.57	209.81	±	6.72
Red nucleus (R)	250.37	±	12.02	251.90	±	13.05	234.69	±	13.86	257.29	±	12.34
Ventral tegmental area (VTA)	97.29	±	1.32	99.91	±	5.86	88.06	±	3.55	96.05	±	5.11

5.5.2 Functional connectivity

To analyze functional connectivity, CO values for each ROI were correlated with each other for each group. Functional heat maps for these correlation matrices are shown in Figures 5.3 and 5.4. Upon visual comparison of these maps, one can qualitatively deduce that MB treatment generally increased functional connectivity, specifically in a positive direction (i.e. MB resulted in more "redness" in the heat maps). This is true particularly in the sham condition (Figure 5.3). The result of MB treatment in the 2VO condition is not as obvious, though still apparent (Figure 5.4). One can also compare the left side of Figure 5.3 and the left side of Figure 5.4 to observe an apparent decrease in functional connectivity as a result of 2VO surgery. The most dramatic differences in functional connectivity appear in the outlined boxes in Figures 5.3 and 5.4 (also represented in larger form in Figure 5.5), which represent connectivity specifically between prefrontal and visual cortex ROIs, and amygdala and hippocampal ROIs. Specifically, it appears that the connectivity between cortical, amygdala and hippocampal ROIs is decreased in subjects that received 2VO, but connectivity is restored in 2VO subjects treated with MB.

To further explore the effects of cerebral hypoperfusion and MB treatment on functional connectivity, bivariate correlation comparisons for each ROI were conducted (1) between the sham + saline group (control subjects) and 2VO + saline group, and (2) between the 2VO + saline group and the 2VO + MB group (Figure 5.6). The first group of comparisons essentially reveals which interregional correlations were significantly

strengthened or weakened as a result of cerebral hypoperfusion. The second group of comparisons reveals which interregional correlations were significantly strengthened or weakened as a result of MB treatment during cerebral hypoperfusion.

As shown in the left panel of Figure 5.6, functional connectivity between visual cortices and basal forebrain, hippocampal and midbrain ROIs was weakened as a result of cerebral hypoperfusion. However, functional connectivity between cortical regions—particularly between secondary somatosensory cortex and prefrontal ROIs—was strengthened as a result of cerebral hypoperfusion. This could potentially be evidence of a compensatory mechanism activated in response to the functional disconnection between regions involved in the visual network.

As shown in the right panel of Figure 5.6, functional connectivity between visual cortices and hippocampal ROIs—which was weakened in 2VO subjects—was strengthened as a result of MB treatment. Additionally, MB treatment resulted in increased functional connectivity between amygdala and cortical ROIs, and in midbrain ROIs with visual cortices and hippocampus.

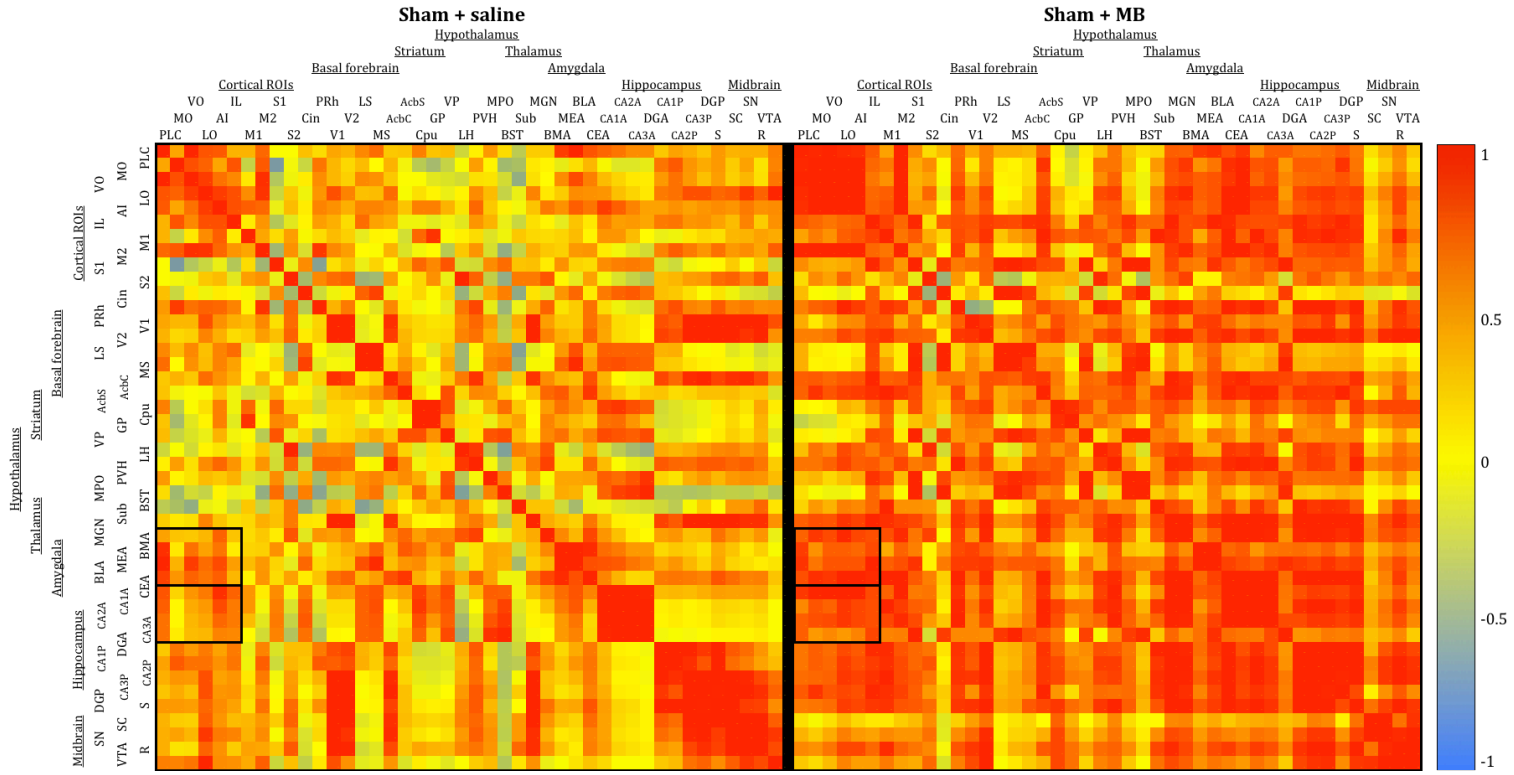

Figure 5.3 Functional heat maps showing interregional correlations for the sham + saline group **(left)** and sham + MB group **(right)**. The purpose of these diagrams is to show the differences in the overall pattern of correlation, rather than individual pairs. Colors correspond to Pearson's r correlation coefficients, with red indicating strong positive correlation and blue indicating strong negative correlations. Generally, the MB group showed a greater number of positive correlations (red) than the saline group. Black boxes outline correlations between prefrontal ROIs and amygdala ROIs and prefrontal ROIs and hippocampal ROIs.

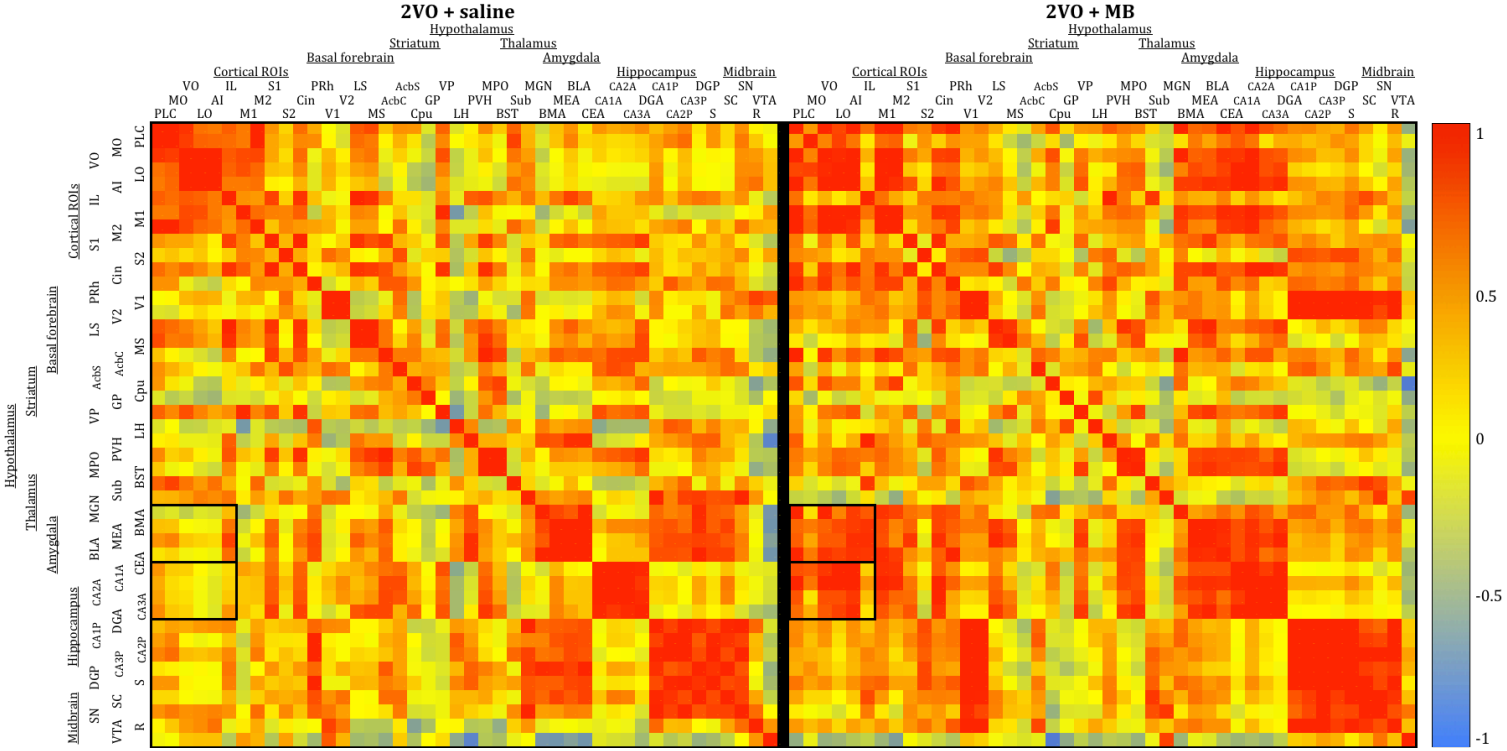

Figure 5.4 Functional heat maps showing interregional correlations for the 2VO + saline group **(left)** and 2VO + MB group **(right)**. Colors correspond to Pearson's r correlation coefficients, with red indicating strong positive correlations and blue indicating strong negative correlations. Black boxes outline correlations between prefrontal ROIs and amygdala ROIs and prefrontal ROIs and hippocampal ROIs.

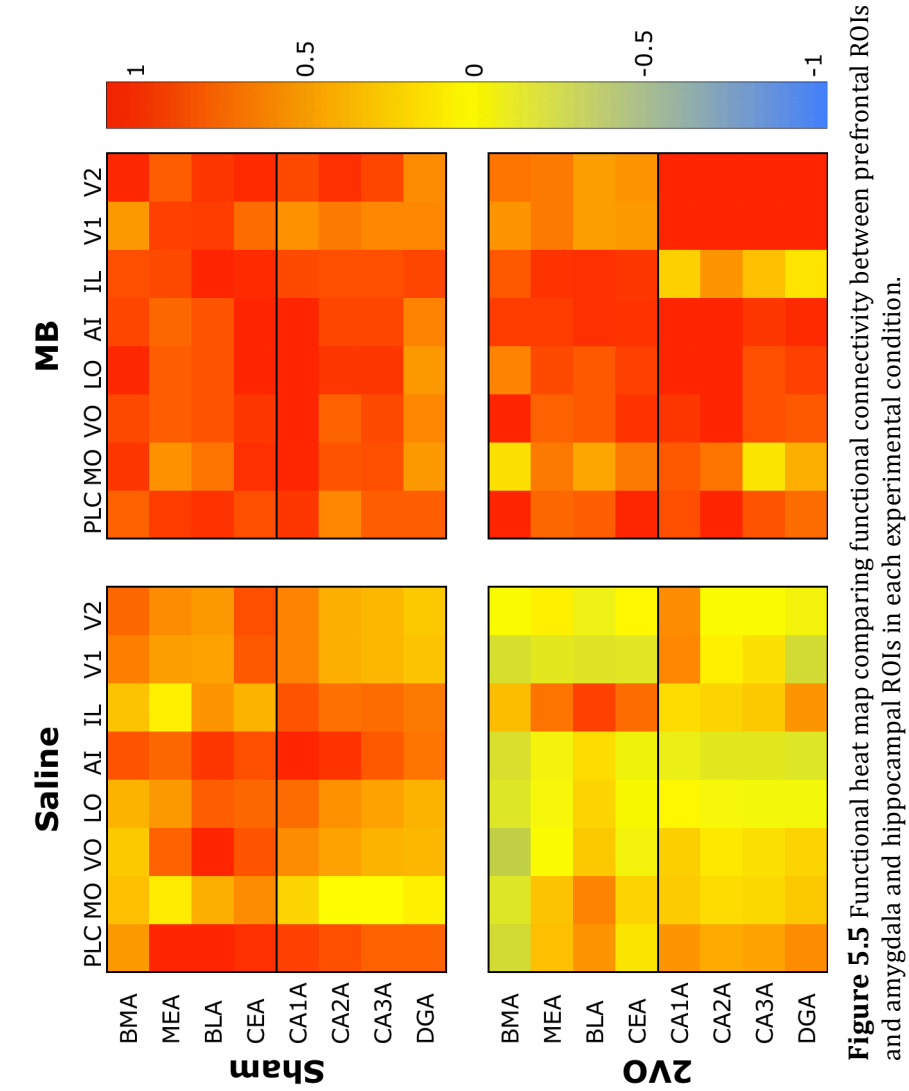

Figure 5.5 Functional heat map comparing functional connectivity between prefrontal ROIs and amygdala and hippocampal ROIs in each experimental condition.

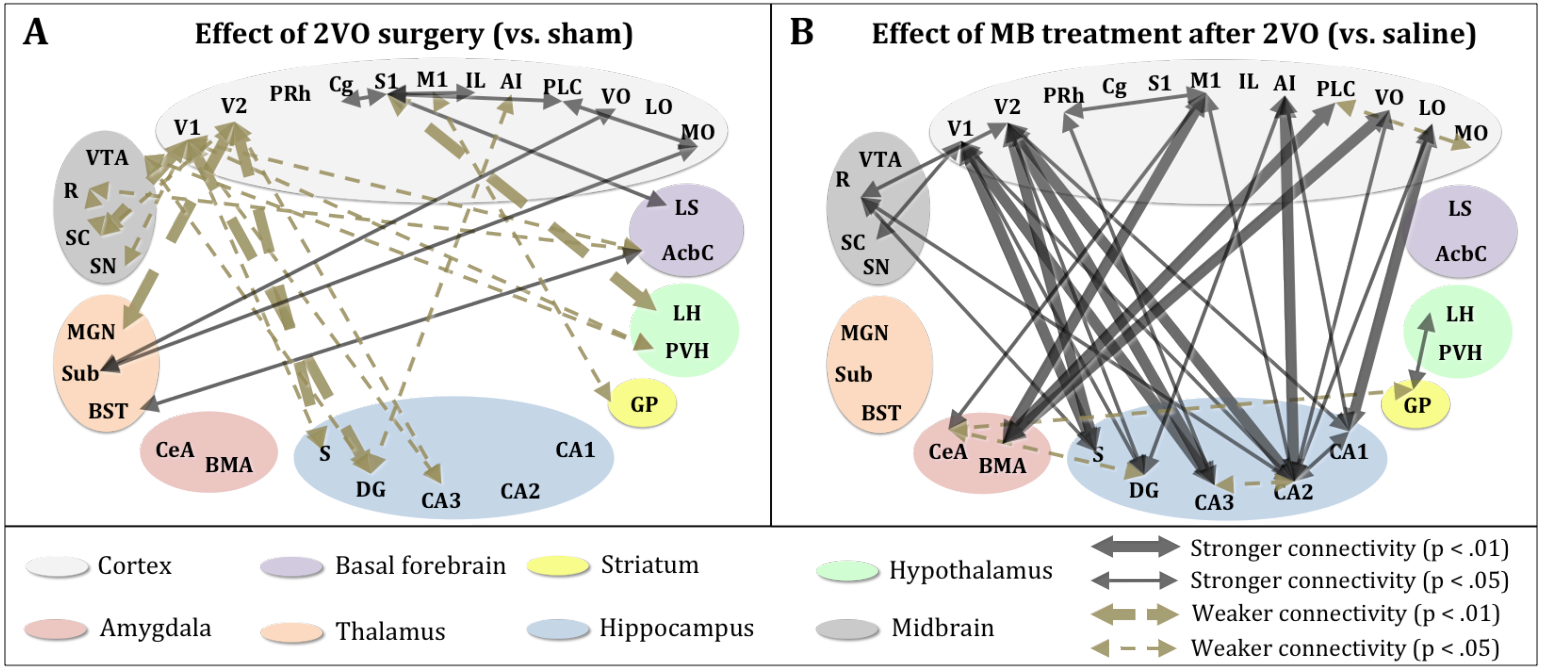

Figure 5.6 Significant differences in functional connectivity due to 2VO surgery and MB treatment. **A.** Comparison between control subjects (sham + saline group) and 2VO subjects (2VO + saline). 2VO surgery resulted in weaker functional connectivity (1) within ROIs involved in the visual pathway (V1, V2, SC), and (2) between visual ROIs and hippocampus (CA3, DG, S). Stronger interregional functional connectivity was observed within cortical ROIs (MO, VO, PLC, IL, S1, Cg) **B.** Comparison between 2VO subjects treated with saline and those treated with MB. In 2VO subjects, treatment with MB resulted in stronger functional connectivity (1) within ROIs involved in the visual pathway (V1, V2, SC), (2) between visual ROIs and hippocampus (CA1, CA2, CA3, DG, S), (3) between cortical ROIs (LO, VO, AI, M1) and hippocampus (CA1, CA2, DG), and (4) between cortical ROIs (VO, PLC, M1) and amygdala (BMA, CeA). Mildly weaker functional connectivity was observed between the amygdala (CeA), hippocampus (DG) and striatum (GP).

5.5.3 Lesions

About one third of subjects who received 2VO surgery (n=3/9 in the 2VO + saline group and n=3/8 in the 2VO + MB group) showed conspicuous anatomical lesions (Figure 5.7). Though there was quite a bit of heterogeneity in lesion size between subjects, the location of the lesions were categorically delimited, and thus were classified into 4 types: neocortical (occurring unilaterally or bilaterally in prefrontal regions and extending into primary motor, somatosensory and visual cortices; n=2), striatal (occurring unilaterally in the caudate and putamen n=6), hippocampal (occurring unilaterally through the rostrocaudal extent of CA1, CA2 and CA3 subfields; n=2), and/or perirhinal (occurring unilaterally or bilaterally in perirhinal cortex; n=3). Qualitative and quantitative lesion comparisons reveal several interesting phenomena. First, there was a negative linear correlation between lesion size and VWT performance, indicating that the greater the overall infarct volume, the worse subjects performed on the VWT (Figure 5.7). Second, though the number of subjects displaying lesions is small, it appears that the emergence of the lesions followed a specific pattern. In subjects with the smallest lesion volumes, lesions were limited to striatum. In more severe cases, neocortical and perirhinal lesions appeared (Figure 5.7). Subjects with the largest infarct volumes showed striatal, neocortical, perirhinal and hippocampal lesions. Finally, though both carotid arteries were ligated in the 2VO surgery, when subjects had smaller lesions, the

lesions were limited to either the right or the left cerebral hemisphere. Bilateral lesions only appeared in the most severe cases (in which subjects showed infarcts in multiple locations, including hippocampus). Perhaps the most interesting observation in subjects displaying lesions was the impact of MB treatment. Though the same number of subjects showed lesions in each 2VO group, lesions in subjects treated with MB were smaller and more localized (i.e. only unilateral and only in/near striatum) than lesions in saline-treated 2VO subjects.

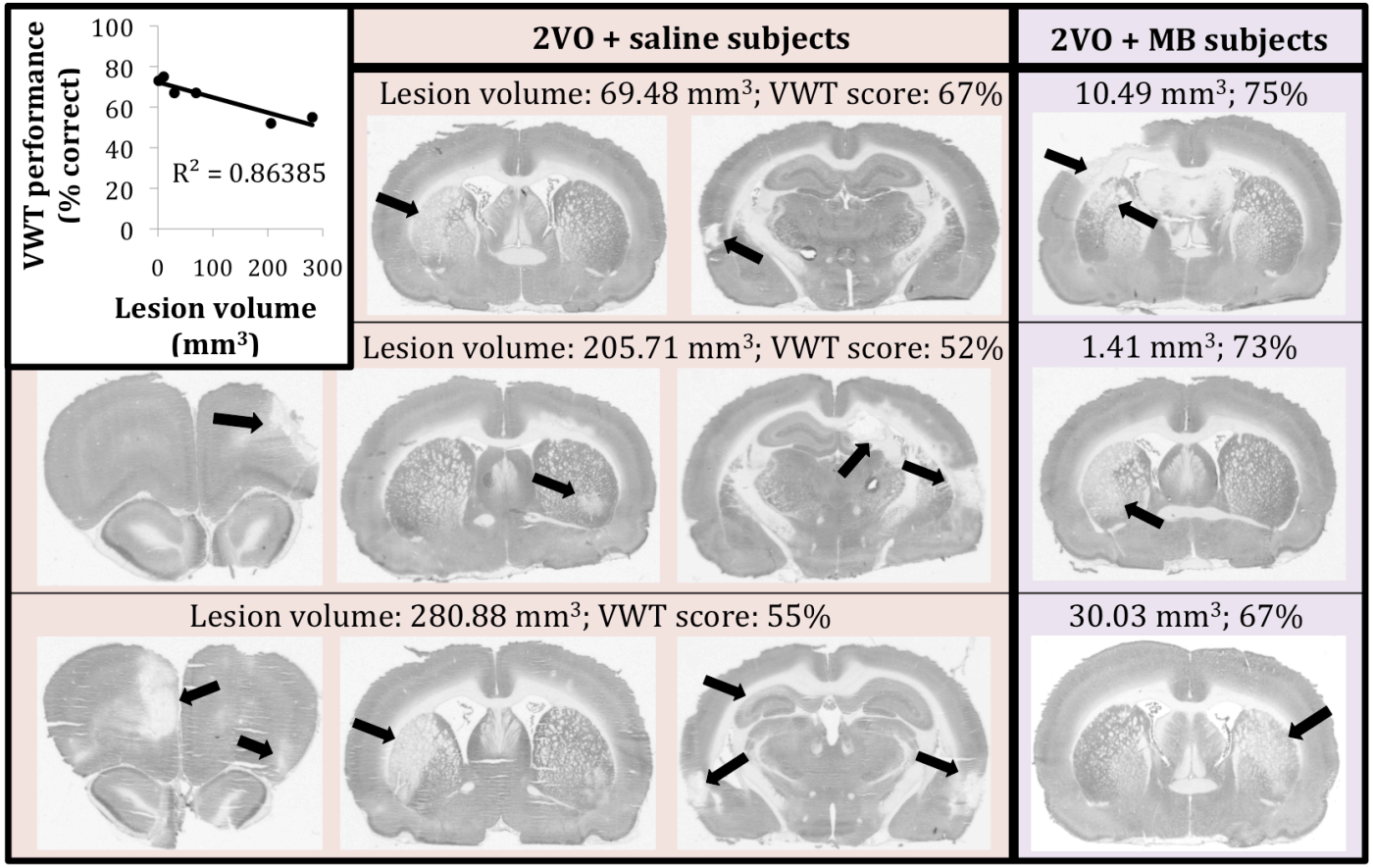

Figure 5.7 Subjects showing lesions. Six 2VO subjects showed lesions (n=3 saline-treated [pink background], n=3 MB-treated [purple background]). There was a negative linear correlation between lesion size and VWT performance (top left).

5.5.4 Brain-behavior correlations

Like the behavior performance for the elemental discrimination alone (see Chapter 4), a univariate ANOVA—with the composite VWT performance score as the dependent variable and the surgery and drug treatment conditions as independent variables—revealed significant main effects of surgery, $F(1,31)=7.480$, $p=.01$, and of drug treatment, $F(1,31)=7.314$, $p=.011$, as well as a surgery by drug treatment interaction, $F(1,31)=8.961$, $p=.006$. Post hoc pairwise comparisons demonstrated that subjects who received 2VO surgery performed worse than sham subjects, but such differences were ameliorated by MB treatment (Figure 5.8).

VWT performance scores were correlated with CO activity for a number of individual ROIs. Regions that showed significant positive brain-behavior correlations were perirhinal cortex, $r(35)=0.526$, $p=.001$, prelimbic cortex, $r(35)=0.353$, $p=.038$, secondary motor cortex, $r(35)=0.461$, $p=.005$, primary visual cortex, $r(35)=0.432$, $p=.035$, secondary visual cortex, $r(35)=0.410$, $p=.047$, caudate/putamen $r(35)=0.343$, $p=.043$, medial amygdala, $r(35)=.368$, $p=.030$, anterior CA1, $r(35)=0.455$, $p=.006$, anterior CA2, $r(35)=0.390$, $p=.021$, and anterior CA3, $r(35)=0.435$, $p=.009$. There were no significant negative correlations between CO activity and VWT performance.

To assess whether correlations between regional CO activities and VWT performance were specifically driven by subjects showing lesions, brain-behavior correlations were also conducted excluding subjects who displayed lesions. When lesioned subjects were excluded, perirhinal cortex,

r(29)=.397, p=.033, secondary motor cortex, r(29)=.409, p=.027, medial amygdala, r(29)=.424, p=.022, primary visual cortex, r(29)=.533, p=.0323, and secondary visual cortex, r(29)=.471, p=.049 still showed significant positive correlations between CO activity and behavioral performance. Hippocampal regions, prelimbic cortex and caudate/putamen no longer showed significant correlations. Interestingly, significant correlations emerged in agranular insular cortex, r(29)=.392, p=.035, and lateral orbital cortex, r(29)=.435, p=.018, when lesioned subjects were excluded. That many correlations persisted when lesions were absent suggests that the lesions are not entirely responsible for the observed brain-behavior correlations. However, correlations were not completely independent of lesions either, since some correlations disappeared when lesioned subjects were excluded. Further, since additional correlations appeared in non-lesions subjects (in ROIs that did not show lesions in lesioned subjects), the functional impact of lesions extended beyond the lesioned areas themselves.

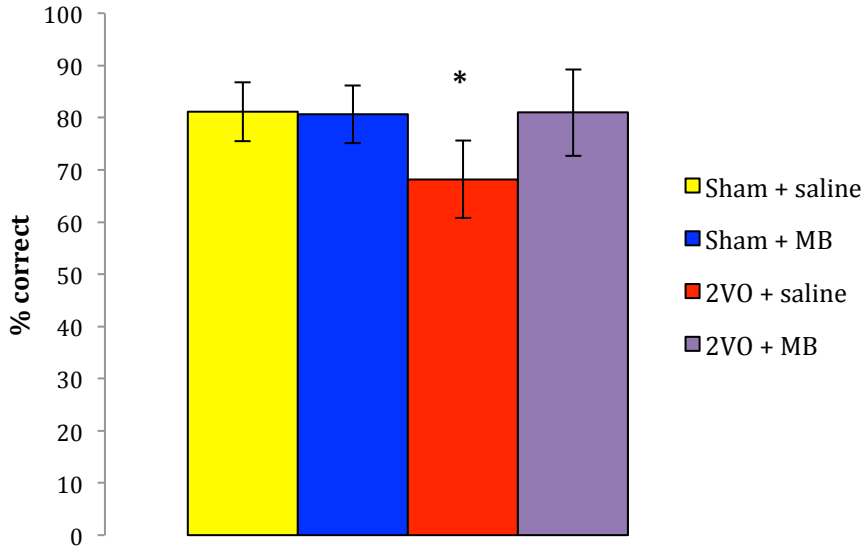

Figure 5.8 Composite VWT performance scores. 2VO surgery resulted in worse overall scores on the VWT. However, 2VO subjects treated with MB performed no different from sham groups. *p < .05

5.6 Discussion

Brain neurons and glia rely on the circulatory system to supply the necessary glucose and oxygen needed for mitochondrial oxidative phosphorylation and ATP synthesis (Erecinska & Silver, 1989). Cytochrome oxidase (CO)—a transmembrane protein localized in the inner mitochondrial membrane—is the terminal enzyme of the electron transport chain (Wong-Riley, 1989). It is well-known that chronic cerebral hypoperfusion results in impaired ATP synthesis (de la Torre et al., 1997), mitochondrial dysfunction and a reduction in CO activity (Cada, de la Torre & Gonzalez-Lima, 2000; de la Torre et al., 1997). There is also evidence that this mitochondrial dysfunction is paralleled by deficits in learning and memory tasks (Cada, de la Torre & Gonzalez-Lima, 2000; de la Torre et al, 1997). Here, we used a model of

chronic cerebral hypoperfusion, bilateral carotid occlusion (2VO), the neuropathological effects of which have been well-characterized and resemble those of age-related progression of mild cognitive impairment (MCI) and Alzheimer's disease (AD). We aimed to (1) characterize the whole-brain regional and functional connectivity changes in cytochrome oxidase activity that result from 2VO surgery, (2) describe how these changes are ameliorated via treatment with the neurometabolic enhancer USP methylene blue (MB), and (3) correlate brain metabolic activity with performance on the visual water task (VWT).

5.6.1 Mitochondrial dysfunction during chronic cerebral hypoperfusion

5.6.1.1 Regional changes in cytochrome oxidase activity

Regional changes in cytochrome oxidase activity as revealed by univariate analyses for each ROI were observed in cortical regions (secondary motor cortex, perirhinal cortex, primary visual cortex and secondary visual cortex), amygdala (basolateral and medial) and hippocampus (the anterior portions of CA1, CA2 and CA3). Though the neuropathological changes in hippocampus (Liu et al., 2006; Ohtaki et al., 2006; Shang et al., 2005), retina (Lavinsky et al., 2006) and optic tract (Farkas et al., 2004a) as a result of 2VO have been documented, regional differences in cortical areas specific to visually-guided movements have only been documented one other time (Cada, de la Torre & Gonzalez-Lima, 2000); and regional differences in the amygdala are novel findings.

Additionally, we show here that chronic cerebral hypoperfusion can affect regional mitochondrial activity in two ways. First, regions susceptible to hypoperfusion show decreases in CO activity without developing anatomical lesions. This is evidenced by the fact that we observed univariate differences in CO activity as a result of 2VO surgery in regions that did not show lesions (e.g. in basolateral and medial amygdala). Functional connectivity changes observed in response to 2VO surgery in regions that do not show anatomical lesions further support this notion. Second, in about one third of subjects, 2VO surgery resulted in tissue infarcts, as indicated by absence of histological CO staining. Studies comparing the CO method and other histological methods show that CO activity is a good proxy for cell density (Riha et al., 2005; Nelson & Silverstein, 1994; Wong-Riley & Welt, 1980). Thus we can infer that regions showing complete absence of CO activity in discrete identifiable regions are in fact infarcts. Since this happens in less than half of the subjects and that infarct sizes vary so widely suggests that subjects are differentially susceptible to developing lesions during cerebral hypoperfusion after 2VO. This differential susceptibility not only results in variable lesion sizes, but greater lesion sizes are also highly correlated to impaired behavioral performance in the VWT.

Additionally, that lesions appear in regions where mitochondrial activity is particularly involved in visual discrimination learning (Fidalgo et al., 2014)—and that CO activity in these regions were also correlated with VWT performance—brings about the question of whether the lesions caused the behavioral deficits, or whether the intensive behavioral training

facilitated the lesions. In one likely scenario, we see the appearance of lesions as a result of certain regions being particularly vulnerable to cerebral hypoperfusion. The infarcts would then lead to an inability of these subjects to master the VWT task. However, in an alternative scenario, the VWT itself may have resulted in increased mitochondrial demand in particular regions preceding neurodegeneration. In this case, cerebral hypoperfusion could result in increased oxidative stress in regions involved in learning the VWT task, and thus would result in "customized" lesions in brain regions involved in a particular task. Though both scenarios may have contributed in some way to the appearance of lesions, we believe that the former scenario is the most plausible, primarily because similar regions have been previously identified as susceptible to cerebrovascular ischemia. Additionally, since CO activity in the medial amygdala is correlated with VWT performance, if lesions resulted from the demanding VWT training, then we should also expect to see amygdala lesions, which we did not. The potential emergence of task-specific lesions can be further addressed in future investigations by (1) comparing mitochondrial lesions in trained vs. untrained subjects experiencing cerebral hypoperfusion, and/or (2) training subjects on a different task involving different brain regions (e.g. an auditory rather than visual task), and exploring whether the lesion location changes (e.g. to auditory rather than visual cortices).

5.6.1.2 Functional connectivity changes in cytochrome oxidase activity

2VO surgery resulted in weakened functional connectivity in several regions. The most obvious functional decoupling occurred between visual cortices (primary and secondary) and hippocampus (CA3, dentate and subiculum). 2VO subjects also showed weaker functional connectivity between visual cortex and the superior colliculus, further implicating 2VO surgery in the pathogenesis of the visual system. There were also some noteworthy increases in functional connectivity, namely between the subthalamic nucleus and prefrontal cortex (ventral and medial orbital cortex), between the bed nucleus of the stria terminalis and nucleus accumbens, and between several cortical regions (medial orbital cortex, prelimbic cortex, infralimbic cortex, cingulate, and primary somatosensory cortex). Because these increases in functional connectivity seemed to appear mostly in regions that were not significantly affected by 2VO surgery (as indicated by univariate analyses), this strengthened functional connectivity may indicate a compensatory mechanism that occurred in response to the weakened functional connectivity in visual and hippocampal regions. There is some evidence of compensatory mechanisms in 2VO, however most of this evidence concerns global normalization of cerebral blood flow via vascular changes, such as collateral perfusion and angiogenesis (Choy et al., 2006; Keynes & Garthwaite, 2004; Oldendorf, 1989). To verify the existence of compensatory mechanisms in metabolic activity, further investigation is needed.

5.6.2 Methylene blue restored mitochondrial activity during chronic cerebral hypoperfusion

When administered during chronic cerebral hypoperfusion, MB restored mitochondrial activity in affected brain regions (secondary motor cortex, perirhinal cortex, primary visual cortex, basolateral amygdala, medial amygdala and hippocampus). This effect was so robust that only one region (secondary visual cortex) that showed decreased CO activity as a result of 2VO was not restored by MB treatment. Additionally, though the same number of subjects showed lesions as a result of 2VO surgery, subjects treated with MB displayed smaller, more localized lesions than subjects treated with saline.

One potential explanation for the appearance of lesions in some subjects and not in others is the induction of necrotic vs. apoptotic mechanisms in response to chronic cerebral hypoperfusion. Necrosis is a form of neuronal cell injury caused by external cellular factors (e.g. toxins or trauma) that result in autolysis, unregulated and detrimental digestion of cellular components. Apoptosis is programmed or targeted cell death, the results of which can potentially benefit the organism. A key determinant of necrosis vs. apoptosis is the presence of intracellular ATP. Necrosis typically results in absence of intracellular ATP, while apoptosis occurs in the presence of ATP (Ueda & Fujita, 2004). In the weeks following 2VO surgery, there is a rapid depletion of ATP (Brīede & Dubers, 2007; Plaschke, 2005). This may lead to an induction of necrotic mechanisms in cells more vulnerable to ATP depletion. If this is indeed the case, then increases in ATP

production as a result of MB treatment may explain why lesions were smaller and more localized in MB-treated subjects.

MB treatment in 2VO surgery also resulted in stronger functional connectivity between the same regions that were weakened in saline-treated subjects (i.e. between visual cortex and hippocampus and visual cortex and midbrain). MB treatment also resulted in widespread strengthened connectivity between hippocampus (CA1, CA2 and dentate) and prefrontal cortex (lateral orbital cortex, ventral orbital cortex and anterior insular cortex), as well as between amygdala (central and medial) and frontal cortex areas (ventral orbital cortex, prelimbic cortex and primary motor cortex).

Though amygdala-cortical functional connectivity was not significantly affected by 2VO surgery, the strengthened functional connectivity between these regions likely assisted in the learning of the VWT. The amygdala has a modulatory role in both spatial and cued water maze tasks (Packard, Cahill & McGaugh, 1994), and dopamine signaling in the amygdala is required for conditioned escape tasks (Darvas, Fadok & Palmiter 2011). The medial amygdala was also positively correlated with VWT performance, strengthening the likelihood that the increased amygdala-cortical functional connectivity we saw as a result of MB treatment was related to learning the VWT escape task.

It has been demonstrated before that low doses (1-4 mg/kg) of methylene blue (1) enhance mitochondrial respiration, (2) are neuroprotective, and (3) enhance learning and memory in animals and humans (Rojas, Bruchey and Gonzalez-Lima, 2012; Telch et al 2014).

However, this is the first study to integrate all three of these implications to show that low-dose MB is neuroprotective by enhancing mitochondrial activity in regions specifically vulnerable to hypoperfusion. Additionally, functional networks that were specifically weakened by 2VO surgery were restored by daily MB treatment.

5.6.3 Conclusions

This is the first study to characterize the functional connectivity changes in brain cytochrome oxidase activity that result from chronic cerebral hypoperfusion, and to show how treatment with MB ameliorates these changes. Functional connectivity was weakened between regions specifically involved in visual discrimination learning, which likely resulted in decreased performance in the visual water task. Treatment with MB not only restored task-specific functional connectivity, but also restored average mitochondrial cytochrome oxidase activity in regions specifically affected by 2VO surgery. These findings implicate chronic low-dose MB as a potential prophylactic or therapeutic treatment for people at a high risk for, or symptomatic of, neurodegenerative disorders whose pathology is accompanied by cardiovascular disease or cerebrovascular hypoxia, including mild cognitive impairment, multi-infarct/vascular dementia, stroke, traumatic brain injury and late-onset AD.

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5.7 Literature Citations

Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., & Ames, B. N. (2008). Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. *The FASEB Journal*, 22(3), 703-712.

Atamna, H., & Kumar, R. (2010). Protective role of methylene blue in Alzheimer's disease via mitochondria and cytochrome c oxidase. *Journal of Alzheimer's Disease*, 20, 439-452.

Auchter, A., Williams, J., Barksdale, B., Monfils, M. H., & Gonzalez-Lima, F. (2014). Therapeutic benefits of methylene blue on cognitive impairment during chronic cerebral hypoperfusion. *Journal of Alzheimer's disease*, 42, S525–35.

Brīede, J., & Duburs, G. (2007). Protective effect of cerebrocrast on rat brain ischaemia induced by occlusion of both common carotid arteries. *Cell biochemistry and function,* 25(2), 203-210.

Bruchey, A. K., & Gonzalez-Lima, F. (2008). Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. *American journal of pharmacology and toxicology*, 3(1), 72.

Cada, A., de la Torre, J. C., & Gonzalez–Lima, F. (2000). Chronic cerebrovascular ischemia in aged rats: effects on brain metabolic capacity and behavior. *Neurobiology of aging*, 21(2), 225-233.

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired

by an inhibitor of cytochrome oxidase in rats. *Neuroscience letters*, 332(2), 83-86.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, 77(1), 175-181.

Choy, M., Ganesan, V., Thomas, D.L., Thornton, J.S., Proctor, E., King, M.D., van der Weerd, L., Gadian, D.G., Lythgoe, M.F. (2006). The chronic vascular and haemodynamic response after permanent bilateral common carotid occlusion in newborn and adult rats. J*. Cereb. Blood Flow Metab*. 26, 1066–1075.

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radical Biology and Medicine*, 48(6), 749-762.

Cohen, R. A., Poppas, A., Forman, D. E., Hoth, K. F., Haley, A. P., Gunstad, J., ... & Gerhard-Herman, M. (2009). Vascular and cognitive functions associated with cardiovascular disease in the elderly. *Journal of Clinical and Experimental Neuropsychology*, 31(1), 96-110.

Darvas, M., Fadok, J. P., & Palmiter, R. D. (2011). Requirement of dopamine signaling in the amygdala and striatum for learning and maintenance of a conditioned avoidance response. *Learning & memory*, *18*(3), 136-143.

de la Torre, J. C., Čada, A., Nelson, N., Davis, G., Sutherland, R. J., & Gonzalez-Lima, F. (1997). Reduced cytochrome oxidase and memory

dysfunction after chronic brain ischemia in aged rats. *Neuroscience letters*, *223*(3), 165-168.

de la Torre, J. C. (1999). Critical threshold cerebral hypoperfusion causes Alzheimer's disease. *Acta neuropathologica*, *98*(1), 1-8.

de la Torre, J.C. (2000). Impaired cerebrovascular perfusion. Summary of evidence in support of its causality in Alzheimer's disease. *Ann. N. Y. Acad. Sci*. 924, 136–152.

de la Torre, J. C. (2012). Cardiovascular risk factors promote brain hypoperfusion leading to cognitive decline and dementia. *Cardiovascular psychiatry and neurology*, 2012.

Driscoll, I., Howard, S., Prusky, G., Rudy, J., & Sutherland, R. (2005). Seahorse wins all races: hippocampus participates in both linear and non-linear visual discrimination learning. *Behavioural brain research,* 164(1), 29–35.

Driscoll, I., Howard, S., Stone, J., Monfils, M., Tomanek, B., Brooks, W., & Sutherland, R. (2006). The aging hippocampus: a multi-level analysis in the rat. *Neuroscience*, 139(4), 1173–85.

Erecinska, M. and Silver, I.A., ATP and brain function, *J. Cerebral Blood Flow Metab.,* 9 (1989) 2–19.

Farkas, E., Luiten, P.G.M. (2001). Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog. Neurobiol*. 64, 575–611.

Farkas, E., Donka, G., de Vos, R.A.I., Mihály, A., Bari, F., Luiten, P.G. M. (2004a). Experimental cerebral hypoperfusion imposes white matter injury

and microglial activation in the rat brain. *Acta Neuropathol*. (Berl.) 108, 57–64.

Farkas, E., Institóris, Á., Domoki, F., Mihály, A., Luiten, P.G.M., Bari, F. (2004b). Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion*. Brain Res*. 1008, 252–258.

Farkas, E., Institóris, Á., Domoki, F., Mihály, A., Bari, F. (2006). The effect of pre- and post-treatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat. *Brain Res*. 1087, 168–174.

Farkas, E., Luiten, P., & Bari, F. (2007). Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain research reviews*, 54(1), 162–80.

Fidalgo, C., Conejo, N. M., González-Pardo, H., & Arias, J. L. (2014). Dynamic functional brain networks involved in simple visual discrimination learning. *Neurobiology of learning and memory*, 114, 165-170.

Gonzalez-Lima, F., & Jones, D. (1994). Quantitative mapping of cytochrome oxidase activity in the central auditory system of the gerbil: a study with calibrated activity standards and metal-intensified histochemistry. *Brain research*, 660(1), 34-49.

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, 11(5), 633-640.

Haley, A. P., Forman, D. E., Poppas, A., Hoth, K. F., Gunstad, J., Jefferson, A. L., ... & Cohen, R. A. (2007). Carotid artery intima-media thickness and cognition in cardiovascular disease. *International journal of cardiology*, *121*(2), 148-154.

Huang, S., Du, F., Shih, Y. Y. I., Shen, Q., Gonzalez-Lima, F., & Duong, T. Q. (2013). Methylene blue potentiates stimulus-evoked fMRI responses and cerebral oxygen consumption during normoxia and hypoxia. *NeuroImage*, *72*, 237-242.

Kalaria, R. N. (1991). The blood-brain barrier and cerebral microcirculation in Alzheimer disease. *Cerebrovascular and brain metabolism reviews*, *4*(3), 226-260.

Kelner, M. J., Bagnell, R., Hale, B., & Alexander, N. M. (1988). Methylene blue competes with paraquat for reduction by flavo-enzymes resulting in decreased superoxide production in the presence of heme proteins. *Archives of biochemistry and biophysics*, 262(2), 422-426.

Keynes, R.G., Garthwaite, J., 2004. Nitric oxide and its role in ischaemic brain injury. *Curr. Mol. Med*. 4, 179–191.

Lassen, N. A., & Ingvar, D. H. (1980). Blood flow studies in the aging normal brain and in senile dementia. *Aging of the Brain and Dementia*, 91-98.

Lavinsky, D., Sarmento Arterni, N., Achaval, M., Netto, C.A., (2006). Chronic bilateral common carotid artery occlusion: a model for ocular ischemic syndrome in the rat. *Graefe's Arch. Clin. Exp. Ophthalmol*. 244, 199–204.

Lindahl, P. E., & Öberg, K. E. (1961). The effect of rotenone on respiration and its point of attack. *Experimental Cell Research*, 23(2), 228-237.

Liu, H.X., Zhang, J.J., Zheng, P., Zhang, Y. (2005). Altered expression of MAP-2, GAP-43, and synaptophysin in the hippocampus of rats with chronic cerebral hypoperfusion correlates with cognitive impairment. *Brain Res. Mol. Brain Res*. 139, 169–177.

Liu, J., Jin, D.Z., Xiao, L., Zhu, X.Z. (2006). Paeoniflorin attenuates chronic cerebral hypoperfusion-induced learning dysfunction and brain damage in rats. *Brain Res.* 1089, 162–170.

Marcus, D. L., de Leon, M. J., Goldman, J., Logan, J., Christman, D. R., Wolf, A. P., ... & Freedman, M. L. (1989). Altered glucose metabolism in microvessels from patients with Alzheimer's disease. *Annals of neurology*, *26*(1), 91-94.

Meier-Ruge, W., & Bertoni-Freddari, C. (1996). The significance of glucose turnover in the brain in the pathogenetic mechanisms of Alzheimer's disease. *Reviews in the Neurosciences*, *7*(1), 1-20.

Miclescu, A., Sharma, H. S., Martijn, C., & Wiklund, L. (2010). Methylene blue protects the cortical blood–brain barrier against ischemia/reperfusion-induced disruptions. *Critical care medicine*, 38(11), 2199-2206.

Murakami, Y., Ikenoya, M., Matsumoto, K., Li, H., Watanabe, H. (2000). Ameliorative effect of tacrine on spatial memory deficit in chronic two-vessel occluded rats is reversible and mediated by muscarinic M1 receptor stimulation. *Behav. Brain Res*. 109, 83–90.

Murphy, M. (2009). How mitochondria produce reactive oxygen species. *Biochem. J*, 417, 1-13.

Nelson, C., & Silverstein, F. S. (1994). Acute disruption of cytochrome oxidase activity in brain in a perinatal rat stroke model. *Pediatric research*, *36*, 12-19.

Obrist, W. D., Chivian, E., Cronqvist, S., & Ingvar, D. H. (1970). Regional cerebral blood flow in senile and presenile dementia. *Neurology*, *20*(4), 315-315.

Ohtaki, H., Fujimoto, T., Sato, T., Kishimoto, K., Fujimoto, M., Moriya, M., Shioda, S. (2006). Progressive expression of vascular endothelial growth factor (VEGF) and angiogenesis after chronic ischemic hypoperfusion in rat. *Acta Neurochir., Suppl*. 96, 283–287.

Oldendorf, W.H. (1989). Trophic changes in the arteries at the base of the brain in response to bilateral common carotid ligation, *J. Neuropathol*. Exp. Neurol. 48, 534–547.

Oz, M., Lorke, D. E., & Petroianu, G. A. (2009). Methylene blue and Alzheimer's disease. *Biochemical pharmacology*, 78(8), 927-932.

Packard, M. G., Cahill, L., & McGaugh, J. L. (1994). Amygdala modulation of hippocampal-dependent and caudate nucleus-dependent memory processes. *Proceedings of the National Academy of Sciences*, *91*(18), 8477–8481.

Plaschke, K. (2005). Aspects of ageing in chronic cerebral oligaemia. Mechanisms of degeneration and compensation in rat models. *J. Neural Transm*. 112, 393–413.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, 511(2), 151-158.

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *Neuroimage*, 54(4), 2623-2634.

Rogers, R. L., Meyer, J. S., Mortel, K. F., Mahurin, R. K., & Judd, B. W. (1986). Decreased cerebral blood flow precedes multi-infarct dementia, but follows senile dementia of Alzheimer type. *Neurology*, *36*(1), 1-1.

Rojas, J. C., John, J. M., Lee, J., & Gonzalez-Lima, F. (2009a). Methylene blue provides behavioral and metabolic neuroprotection against optic neuropathy. *Neurotoxicity research*, 15(3), 260-273.

Rojas, J. C., Simola, N., Kermath, B. A., Kane, J. R., Schallert, T., & Gonzalez-Lima, F. (2009b). Striatal neuroprotection with methylene blue. *Neuroscience*, 163(3), 877-889.

Rojas, J. C., & Gonzalez-Lima, F. (2010). Mitochondrial optic neuropathy: in vivo model of neurodegeneration and neuroprotective strategies. *Eye and brain*, *2*, 21-37.

Rojas, J. C., Bruchey, A. K., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of methylene blue. *Progress in neurobiology*, *96*(1), 32-45.

Rolfe, D. F., & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological reviews*, *77*(3), 731-758.

Royce, J. R. (1972). Avoidance conditioning in nine strains of inbred mice using optimal stimulus parameters. *Behavioral Genetics*, 2: 107–110.

Salaris, S. C., Babbs, C. F., & Voorhees, W. D. (1991). Methylene blue as an inhibitor of superoxide generation by xanthine oxidase: a potential new drug for the attenuation of ischemia/reperfusion injury. *Biochemical pharmacology*, 42(3), 499-506.

Sarti, C., Pantoni, L., Bartolini, L., Inzitari, D. (2002). Persistent impairment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav. Brain Res*. 136, 13–20.

Schmidt-Kastner, R., Truettner, J., Lin, B., Zhao, W., Saul, I., Busto, R., Ginsberg, M.D. (2001). Transient changes of brain-derived neurotrophic factor (BDNF) mRNA expression in hippocampus during moderate ischemia induced by chronic bilateral common carotid artery occlusion in the rat. *Brain Res. Mol. Brain Res*. 92, 157–166.

Shang, Y., Cheng, J., Oi, J., Miao, H. (2005). Scutellaria flavonoid reduced memory dysfunction and neuronal injury caused by permanent global ischemia in rats. *Phamacol. Biochem. Behav*. 82, 67–73.

Shen, Q., Du, F., Huang, S., Rodriguez, P., Watts, L. T., & Duong, T. Q. (2013). Neuroprotective efficacy of methylene blue in ischemic stroke: an MRI study. *PloS one*, 8(11), e79833.

Shumake, J., Furgeson-Moreira, S., & Monfils, M. H. (2014). Predictability and heritability of individual differences in fear learning. *Animal cognition*, *17*(5), 1207-1221.

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, 5(5), 415-418.

Sopala, M., Danysz, W. (2001). Chronic cerebral hypoperfusion in the rat enhances age-related deficits in spatial memory *J. Neural Transm*. 108, 1445–1456.

Sparks, D. L., Hunsaker, J. C., Scheff, S. W., Kryscio, R. J., Henson, J. L., & Markesbery, W. R. (1990). Cortical senile plaques in coronary artery disease, aging and Alzheimer's disease. *Neurobiology of aging*, *11*(6), 601-607.

Tachibana, H., Meyer, J. S., Kitagawa, Y., Rogers, R. L., Okayasu, H., & Mortel, K. F. (1984a). Effects of aging on cerebral blood flow in dementia. *Journal of the American Geriatrics Society*, *32*(2), 114-120.

Tachibana, H., Meyer, J. S., Okayasu, H., Shaw, T. G., Kandula, P., & Rogers, R. L. (1984b). Xenon Contrast CT–CBF Scanning of the Brain Differentiates Normal Age-Related Changes from Multi-infarct Dementia and Senile Dementia of Alzheimer Type. *Journal of gerontology*, *39*(4), 415-423.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, 552(2), 335-344.

Ueda, H., Fujita, R., (2004). Cell death mode switch from necrosis to apoptosis in brain, *Biol. Pharm. Bull*. 27, 950–955.

Visarius, T. M., Stucki, J. W., & Lauterburg, B. H. (1997). Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS letters*, 412(1), 157-160.

Watts, L. T., Lloyd, R., Garling, R. J., & Duong, T. (2013). Stroke Neuroprotection: Targeting mitochondria. *Brain sciences*, 3(2), 540-560.

Wong-Riley, M. T., & Welt, C. (1980). Histochemical changes in cytochrome oxidase of cortical barrels after vibrissal removal in neonatal and adult mice. *Proceedings of the National Academy of Sciences*, *77*(4), 2333-2337.

Wong-Riley, M. T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends in neurosciences*, 12(3), 94-101.

Wrubel, K. M., Riha, P. D., Maldonado, M. A., McCollum, D., & Gonzalez-Lima, F. (2007). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology Biochemistry and Behavior,* 86(4), 712-717.

Yamaguchi, F., Meyer, J. S., Yamamoto, M., Sakai, F., & Shaw, T. (1980). Noninvasive regional cerebral blood flow measurements in dementia. *Archives of neurology*, *37*(7), 410-418.

Zemcor, A., Risberg, J., Barclay, L. L., & Blass, J. P. (1984). Diagnosis of Alzheimer's dementia and multi-infarct dementia by rCBF compared to clinical classification. *Monogr Neural Sci*, *2*, 104-6.

Zhang, X., Rojas, J. C., & Gonzalez-Lima, F. (2006). Methylene blue prevents neurodegeneration caused by rotenone in the retina. *Neurotoxicity research*, *9*(1), 4757.

Chapter 6: General discussion, limitations and future directions

Memory formation and maintenance are highly complex and dynamic processes. The way we update and maintain memories has tremendous clinical relevance to how we approach treating both psychiatric and neurodegenerative disorders. This dissertation is a compilation of several experiments that serve as a proof of principle for clinical treatment of memory—namely, how to attenuate pathological memories and how to protect vulnerable ones via behavioral manipulation and mitochondrial enhancement. I began with an introduction to our current understanding of memory processes. Chapter 2 explored how unpredictability of stimulus presentation during extinction of conditioned fear can be implemented to induce memory updating mechanisms. Chapter 3 combined this strategy with post-extinction administration of metabolic enhancer, methylene blue (MB), to maximize fear attenuation. In Chapter 4, MB was administered to subjects experiencing chronic cerebral hypoperfusion—a condition that results in memory impairment—to ameliorate memory deficits in a visual discrimination task. Brain metabolic activity in these subjects was analyzed in Chapter 5, which verified that MB increased metabolic activity as well as functional connectivity during chronic cerebral hypoperfusion. In addition, brain metabolic activity was correlated with behavioral performance on the visual water task, tying together the findings from Chapters 4 and 5. That MB administration was correlated with behavioral performance in both the fear

memory updating paradigm and during cerebral hypoperfusion implicates MB as a memory enhancer and neuroprotective agent.

6.1 Memory consolidation and reconsolidation applied to fearful memories

Our knowledge about how the brain organizes learning and memory has evolved tremendously over the past century. The idea that memories take time to "consolidate" after first learning was first described by Müller and Pilzecker (1900). Since then, a plethora of evidence has revealed that memories are not just consolidated after initial encoding, but also reconsolidated after retrieval (see Keil et al., 1998; Murphy, 2002; Nader & Einarsson, 2010 for reviews). During the process of reconsolidation, the memory enters a labile state, and is open to change in a way that non-reactivated memories are not (Hupbach et al., 2007; Lewis et al., 1968; Misanin et al., 1968; Nader, Schafe & Le Doux, 2000; Walker et al., 2003). However, evidence has revealed that this does not happen in the same way for all memories; particularly, emotional and fearful memories, once encoded and consolidated, are notoriously difficult to change (Foa & Kozak, 1986; LeDoux, 1993; LeDoux et al., 1989).

This phenomenon does have an evolutionary explanation, as arousing situations—particularly situations involving fear—have particular relevance in terms of avoiding potentially harmful situations (Kindt et al., 2009; Schiller et al., 2010). However, it is equally important that we learn when cues that once predicted danger are no longer predictive of aversive stimuli. It has

been postulated many times (and is now widely accepted) that failure to extinguish fear responses is an important mechanism in the pathogenesis of fear and anxiety disorders (Hofman, 2008; Milad et al., 2006; 2009; Mineka et al., 1996; 2008; Myers & Davis, 2002; VanElzakker et al., 2014; Wessa, 2007).

6.2 Unpredictable extinction CSs promote memory updating

During extinction, a subject is repeatedly exposed to a fearful stimulus in absence of an aversive event, with the hope that the subject learns that the stimulus is not actually harmful. The problem with this approach is that usually the result is formation of a new inhibitory memory rather than change to an old one (Barrett et al., 2003; Effting & Kindt, 2007; Morgan, Romanski & LeDoux, 1993; Pavlov, 1927; Quirk et al., 2000; Quirk & Mueller, 2008; Robbins, 1990), which leaves the old memory susceptible to return via spontaneous recovery, renewal or reinstatement. Monfils et al. (2009) devised a novel approach to updating fear memories behaviorally by using extinction to interrupt reconsolidation of the fear memory during a retrieval-induced labile window. The retrieval + extinction paradigm has been replicated several times in both animals (Auber et al., 2013; Ma, Zhang & Yu, 2012; Millan, Milligan-Saville & McNally, 2013; Olshavsky et al., 2013; Quirk et al., 2010; Xue et al., 2012) and humans (Kindt, Soeter & Vervliet, 2009; Schiller et al., 2010; 2013). However, there are several questions that still need to be answered in order to fully utilize post-retrieval strategies to disrupt or weaken pathogenic memories. For instance, it still remains

somewhat unclear under what extinction conditions memories are updated (Hutton-Bedbrook & McNally, 2013).

Chapter 2 provided further clarification of this paradigm by demonstrating that this phenomenon was contingent on having variable inter-trial intervals (ITIs) during extinction. Without some degree of predictability, memories remained susceptible to return via reinstatement. However, random CS occurrences resulted in reinstatement updating. Further, we discovered that the subjective experience of randomness—which could be increased via a larger average ITI—was important for memory updating.

One theoretically simplistic, yet mathematically complex explanation for why memories update is that experiences that greatly resemble each other are more likely to be (re)consolidated together. Conversely, experiences that are similar enough to evoke a memory but also possess unique elements (i.e. new latent causes) qualify a different situation and require new encoding and differential consolidation. In these experiments, extinction training administered during the post-retrieval reconsolidation window resulted in maximal fear memory attenuation when extinction consisted of CSs presented at variable (random) intervals, rather than fixed (predictable) intervals. We posit that this randomness created a situation whereby extinction training maximally resembled the unpredictable nature of fear conditioning, and thus promoted memory updating over new memory formation. Based on these results, one may consider the likelihood of memory updating on a spectrum: the less the subjective experience of

extinction training resembles fear conditioning, the more prediction error—and consequentially, the more likely a new memory will be formed. This paradigm shows promise in translational research on treatments for fear and anxiety disorders, as unpredictability and random exposure is not typically considered in current treatments.

6.3 Methylene blue—mechanism of action

Methylene blue (MB) is a blue dye with metabolic enhancing properties. Its small aromatic structure causes MB to have a high bioavailability, cross the blood brain barrier, diffuse through both mitochondrial membranes and enter into the mitochondrial matrix, where ATP is produced. MB is also a redox agent, meaning it donates and accepts electrons. Additionally, the reduction potential of MB is similar to the reduction potential of electron transport chain enzymes. This means that MB acts as an electron cycler with electron transport chain enzymes, resulting in an increase in oxygen consumption and ATP production. Since MB also accepts electrons that would otherwise be accepted by miscellaneous molecules to form reactive oxygen species (ROS), MB also acts as an antioxidant.

6.4 Genetic variability and memory enhancement interact with responsiveness to extinction to influence persistent fear reduction

One major consideration of extinction that is often overlooked is that there is considerable individual variation in responsiveness to extinction, and that genetic factors represent a significant source of this variation (Lonsdorf & Kalisch, 2011). Indeed, there are significant individual differences in conditionability (Hettema et al., 2003) and extinction (Soliman et al., 2010) in both humans (Fava et al., 2001; Fava et al., 1995; Foa & Kozak, 1986) and animals (Bush, Sotres-Bayon & LeDoux, 2007; Shumake, Furgeson-Moreira, & Monfils, 2014; Yehuda & LeDoux, 2007) . Additionally, anxiety disorders show strong heritability (Hettema et al., 2001). Thus, after we addressed the importance of unpredictability in the retrieval + extinction paradigm, we expanded on these findings in Chapter 3 by assessing extinction memory interactions using rats selectively bred for extinction behavior: high extinguishers (a lineage particularly responsive to extinction), low extinguishers (a lineage particularly unresponsive to extinction), and a randomly bred lineage. To add another layer to this idea, the previously described conceptualization of the retrieval + extinction paradigm was combined with a current strategy for memory enhancement via upregulation of the brain's metabolic energy mechanisms via MB.

MB's ability to enhance mitochondrial respiration has been demonstrated as a unique strategy for memory enhancement (Callaway et al., 2004; Martinez et al, 1978; Riha et al., 2005; Riha, Rojas & Gonzalez-Lima, 2011), including enhancement of memory for extinguished fear (Gonzalez-Lima & Bruchey, 2004; Wrubel et al., 2007). Its action in these cases is to enhance mitochondrial respiration during memory consolidation, thus

enhancing consolidation. One caveat to this approach is the fact that MB enhances whichever neural network is active in the time period MB is on board. However, if MB is administered to a subject after a learning task in which the subject did not learn, then MB may be detrimental, as one would be enhancing a network that does not predict task learning (Telch et al., 2014). In addition to exploring the role of genetic variability in extinction responsiveness, Chapter 3 is also an investigation into how effectiveness of learning during extinction can moderate MB's therapeutic effectiveness. We predicted that MB administration would be therapeutically beneficial in extinction learning only for subjects who respond favorably to extinction.

Though we did observe some differences in both extinction and reinstatement as a result of lineage, the majority of our findings from this experiment support two major points: (1) The retrieval + extinction behavioral manipulation prevented reinstatement for subjects who were not genetically susceptible to extinction non-responsiveness; and (2) MB was effective at preventing reinstatement when subjects responded favorably to extinction. The first point suggests that memory updating is not universal across all subjects, but is instead at least partially influenced by the degree of extinction. However, unlike with MB treatment, extinction itself did not moderate memory updating. Rather, it seems that subjects who were genetically predisposed to lower extinction were also less likely to show memory updating via extinction training. However, we were able to prevent reinstatement in subjects predisposed to lower extinction by combining retrieval + extinction with MB treatment.

The second point confirms that MB is effective at enhancing memory consolidation. MB ensured that subjects remained at post-extinction levels of freezing—suggesting that MB effectively aided in attenuating fear memories in subjects who were responsive to extinction, but was not beneficial in subjects who did not extinguish. In other words, when administered immediately after completion of a task, MB enhances the consolidation of whatever "brain state" is active during the task. Stated differently, MB enhances memory for recently encoded events, regardless whether the events resulted in the desired behavioral change or not. This makes intuitive sense, as MB's action as a neurometabolic enhancer does not discriminate between successful and unsuccessful treatments. This also explains why evidence suggests that MB is more effective at improving memory consolidation than other memory stages (Gonzalez-Lima & Bruchey, 2004; Martinez et al., 1978). As a consequence, if a subject remained fearful through extinction, then treatment with methylene blue only enhanced the consolidation of that fearful brain state. On the other hand, if fear was reduced during extinction, then MB administration became therapeutic, as we were enhancing the "less fearful brain state." Thus, extinction acted as a moderator for the effect of MB. This observation is in accordance with recent findings of MB's effect on extinction in humans with claustrophobia (Telch et al., 2014). Stated broadly, MB can be utilized as an alternative to (or potentially in conjunction with, though this would require further investigation) traditional pharmacological treatments in cases of successful cognitive behavioral therapy. However, MB's post-exposure use as a clinical

treatment for fear and anxiety disorders should be limited to subjects who respond favorably to extinction.

6.5 Memory impairment induced by chronic cerebral hypoperfusion is attenuated with MB treatment

A compelling body of evidence supports a role of compromised cerebral blood supply and mitochondrial dysfunction in memory-related neurodegenerative disorders (de la Torre, 2010, 2012a, 2012b; Hauptmann et al., 2009; Hirai et al., 2001; Velliquette et al., 2005). With an aging population, it is critical to treat deficits in brain energy metabolism that occur before the onset of dementia. Treatments such as MB, designed to target the effectiveness of mitochondrial respiration, may be able to ameliorate these deficits, thereby delaying or preventing the development of cognitive impairment and dementia.

Chapter 4 explored whether chronic intervention with low-dose MB would improve memory retention in an established rat model of chronic cerebral hypoperfusion (Cada, de la Torre & Gonzalez-Lima, 2000; Obrenovich et al., 2006). It was confirmed that chronic cerebral hypoperfusion resulted in marked deficits in visual discrimination learning, which were ameliorated by daily post-training MB treatment. MB was injected in the post-session period to improve the retention of the discrimination learning, consistent with prior evidence that MB is most effective at improving memory consolidation than other memory stages (Gonzalez-Lima & Bruchey, 2004; Martinez et al., 1978). The half-life of MB

is about 5-6.5 hours (Peter et al., 2000), so it is not likely that the daily improvement of the visual discrimination seen 24 hours after each injection is a result of the continued drug action. A more likely explanation is that MB given immediately after each training session acted to enhance brain oxidative energy metabolism during the critical stage of memory consolidation. This was explored further in Chapter 5.

6.6 Mitochondrial dysfunction induced by chronic cerebral hypoperfusion is attenuated with MB treatment

The idea that mitochondrial function and memory are related is not a novel idea. The role of mitochondrial dysfunction in disease pathology has garnered quite a lot of attention in the previous decade (for a review, see Lin & Beal, 2006). Mitochondrial dysfunction is implicated in a tremendous number of memory-related neurodegenerative conditions including vascular dementia, Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis, multiple sclerosis and mild cognitive impairment (for review, see Lin & Beal, 2006). As such, protecting mitochondrial respiration in subjects under oxidative stress can prevent mitochondrial dysfunction and subsequent pathology (for review, see Gonzalez-Lima et al., 2014).

Chapter 5 explored (1) how chronic cerebral hypoperfusion weakened regional mitochondrial activity, metabolic functional connectivity and performance on a visual discrimination task, (2) how MB strengthened performance and restored both regional mitochondrial activity and functional connectivity and (3) how specific changes in brain regional

mitochondrial activity were correlated with performance. Functional connectivity was weakened between visual cortices (primary and secondary) and hippocampus (CA3, dentate and subiculum), regions that are specifically involved in visual discrimination learning. This functional decoupling along with additional mitochondrial dysfunction in secondary motor cortex, perirhinal cortex and amygdala likely resulted in decreased performance in the visual water task, as there were significant brain-behavior correlations in these regions. Treatment with MB not only restored task-specific functional connectivity, but also restored average mitochondrial activity in regions specifically affected by 2VO surgery. The combined findings from Chapters 4 and 5 implicate MB as a potential prophylactic or therapeutic treatment for people at a high risk for or symptomatic of neurodegenerative disorders whose pathology is accompanied by cardiovascular disease or cerebrovascular hypoxia.

6.7 Challenges of mitochondrial enhancement

Unfortunately, mitochondrial enhancement, a seemingly simple approach to treating mitochondrial dysfunction, is not as straightforward as it seems. Over the past four or five decades, researchers around the world have searched for the ultimate way to "boost" mitochondrial function to protect neurons and improve cognition. This field received considerable attention in the 1980s, with the identification of several potential

"smart drugs"[7]. These strategies often show promise in basic research, and some even do well in clinical trials. However, the clinical benefits in regard to human memory and cognition have not been impressive. One example is co-dergocrine mesilate (Hydergine), an anti-hypertensive ergot extract that was once claimed to "increase intelligence, memory, learning and recall" (Dean, Morgenthaler & Fowkes, 1990). However, after a decade of clinical research, it was concluded that the drug did not show much clinical benefit (Olin et al., 2001). Another approach followed with the hypothesis that cerebral metabolism was affected by changes in calcium homeostasis during aging (Hartmann, Eckert & Müller, 1994). Treatment with L-type calcium channel blockers (e.g. nimodipine, nifedipine) showed promise for enhancing memory in several animal models (Deyo et al., 1989; Quevedo et al., 1998; Scriabine et al., 1989); however, as with other attempts to increase memory pharmacologically, clinical trials of these drugs in humans have proved to be ineffective at enhancing learning or memory (Rose, 2002).

The other popular approach to enhancing mitochondrial health is via antioxidant defenses. Much of the cellular damage that is incurred via mitochondrial dysfunction is a result of formation of damaging reactive oxygen species (ROS), or "free radicals", as a byproduct of mitochondria

[7] Research into smart drugs, or nootropics, is actually broader than just mitochondrial enhancement—exploring strategies for increasing memory by enhancing attention, decreasing anxiety, increasing the salience of an experience or modulating hormones and/or neurotransmitters—however, such strategies are beyond the scope of this text (see Rose, 2002 for a review).

under oxidative stress (De La Fuente, 2002; Gutteridge, 1993). In fact, many researchers have attributed most or even all age-related pathologies to oxidative stress, spawning the popular oxidative stress theory of aging (De La Fuente, 2002; Finkel & Holbrook, 2000; Harman, 1957). This theory has become a popular hypothesis for the underlying causes of aging, implicating the production of ROS from mitochondria as the primary contributor to the aging process. As such, a large number of antioxidants have been proposed and tested in the hopes of finding a compound that slows aging and all the associated age-related diseases. Unfortunately, these compounds have run into the same difficulties as many smart drugs—namely, that they have low digestive bioavailability and do not easily cross the blood brain barrier (Gilgun-Sherki, Melemed & Offen, 2001; Pappert et al., 1996).

Though many proposed compounds have had limited success in in enhancing memory, MB still shows great clinical promise. Not only does MB have good bioavailability (Schirmer et al., 2011; Walter-Sack et al., 2009) and cross the blood brain barrier (Sweet & Standiford, 2007), but unlike any other proposed mitochondrial enhancer, it specifically accumulates in neural mitochondria (Gabrielli et al., 2004). There, it has been shown to increase oxygen consumption and ATP production, as well as act as an antioxidant by decreasing ROS production. None of the other aforementioned compounds have demonstrated such unique and promising mechanisms of action. MB has also been shown to stimulate the glucose oxidative pathway (Brin & Yonemoto, 1958) and act as a neuroprotective metabolic agent for treatment

of dementia, depression and drug-induced encephalopathy (Naylor et al., 1986; Wainwright & Crossley, 2002).

6.8 Limitations and future directions

Though this dissertation provides some good examples of how memories can be changed and protected, there are some limitations to the findings. In Chapters 2 and 3, it was demonstrated that retrieval + extinction does in fact lead to more persistent reduction in fear, but it does not go so far as to provide neurological mechanisms for the phenomenon. Many neural mechanisms of memory consolidation and reconsolidation have been characterized. For example, though it is well established that both consolidation and reconsolidation are dependent on protein synthesis (Davis & Squire, 1984; Dundai, 1996; 2004; Judge & Quartermain, 1982; McGaugh, 2000; Misanin et al., 1968; Nader, Schafe & Le Doux, 2000; Sara, 2000), the processes can be distinguished because they require, with some degree of overlap, the activation of different brain areas and circuits (Auber et al., 2013; Alberini, 2005; Bahar et al., 2004; Hernandez et al., 2002; Kelly et al., 2003; Lee et al., 2004; Nyberg et al., 1996; Salinska et al., 2004; Tronel & Sara, 2002). However, how randomness and unpredictability influence these mechanisms remains a black box—a potential line of inquiry for future research.

Additionally, though this study uncovered a few of the parameters that are necessary to induce reconsolidation updating mechanisms in fear memories, these particular findings were not applied to other types of

memories. Presumably, the subjective experience of randomness would serve to destabilize many types of memories, but such hypotheses require further investigation.

Though we were able to describe some behavioral and neurological effects of chronic cerebral hypoperfusion and concurrent treatment with MB in Chapters 3 and 4, our results leave much to be desired. For instance, though we tested the effects of 2VO and MB treatment in three types of nonspatial memory tasks (elemental visual discrimination, transverse pattern discrimination and odor discrimination), we only observed significant behavioral effects in elemental visual discrimination. However, we conducted CO brain mapping on subjects who underwent all three tasks, and thus our interpretation of the how specific aspects of task learning correlated with quantitative CO mapping is limited, since CO activity was likely affected by all three tasks.

Additionally, the emergence of structural lesions added a layer of complexity to these experiments. Though the lesions did provide us with some insight into how functional connectivity was weakened by 2VO surgery and how MB strengthened it, the number of subjects who presented with lesions was too small to allow us to meaningfully characterize them. In subjects who showed lesions, there appeared to be a pattern in the location of the lesions, such that less severe lesions were limited to the striatum in one cerebral hemisphere, whereas more severe lesions presented in multiple locations and in both cerebral hemispheres. However, at this point these descriptions are only qualitative. A more rich quantitative analyses of lesions

in 2VO surgery could be accomplished by added more subjects, and observing the size and locations of the lesions at different time periods following surgery. Additionally, subjects who showed lesions and were also treated with MB had much smaller lesions, but again, the sample size was too small to make this claim statistically.

Another minor caveat to Chapters 4 and 5 is that the subjects used in the experiment were not naïve. They were originally used in another experiment, and went through fear conditioning and extinction and were sexually experienced prior to 2VO surgery. However, all the subjects had the same behavioral experience before the start of these experiments, and therefore, it is unlikely that pre-existing differences among the groups could confound our findings. Therefore, the adverse effects of 2VO and the beneficial effects of MB were specific to the learning and memory of the visuospatial discrimination task.

6.9 Conclusions

Albert Einstein once said, "The only source of knowledge is experience." While this statement is partially true, Dr. Einstein did not acknowledge the role of memory as the middleman between experience and knowledge[8]. Without memory, meaningful information about ourselves and our environment cannot be accessed, and thus knowledge cannot exist.

[8] Although, "The only source of knowledge is experience, which is encoded, consolidated, retrieved, re-encoded and reconsolidated over and over again until the state of overall knowing is obtained" does not roll off the tongue quite as easily.

Memory is a very complex process that continues to be very difficult to characterize. While certain memories (e.g. fear memories) are very difficult to forget, others escape our consciousness far too easily. The overarching goal of this dissertation was to explore ways to change stubborn memories and protect vulnerable ones. The first objective was to further characterize the retrieval + extinction paradigm, which was done by showing that the subjective experience of randomness during extinction helps to destabilize and promote reconsolidation of fear memories.

The next objective was to manipulate memories via mitochondrial enhancement. Memory consolidation and retrieval are reliant on mitochondrial activity. This was evidenced in this dissertation by observing that (1) cerebral hypoperfusion resulted in mitochondrial dysfunction, weakened functional connectivity and memory dysfunction, and (2) mitochondrial enhancement led to memory enhancement and increased functional connectivity.

This dissertation also provides evidence that methylene is unlike any other molecule in that through its unique mechanism of action, it can both protect unhealthy mitochondria (and thus the neurons that contain them) and enhance healthy ones. No other currently existing (and tested) compound has so many unique and widespread potential clinical applications for all types of pathological brain and memory disorders.

6.10 Literature citations

Alberini, C. M. (2005). Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes?. *Trends in neurosciences*, *28*(1), 51-56.

Auber, A., Tedesco, V., Jones, C. E., Monfils, M. H., & Chiamulera, C. (2013). Post-retrieval extinction as reconsolidation interference: methodological issues or boundary conditions?. *Psychopharmacology*, *226*(4), 631-647.

Bahar, A., Dorfman, N., & Dudai, Y. (2004). Amygdalar circuits required for either consolidation or extinction of taste aversion memory are not required for reconsolidation. *European Journal of Neuroscience*, *19*(4), 1115-1118.

Barrett, D., Shumake, J., Jones, D., & Gonzalez-Lima, F. (2003). Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *The Journal of neuroscience*, *23*(13), 5740-5749.

Brin, M., & Yonemoto, R. H. (1958). Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. *Journal of Biological Chemistry*, *230*(1), 307-318.

Budson, A. E., & Price, B. H. (2005). Memory dysfunction. *New England Journal of Medicine*, *352*(7), 692-699.

Bush, D. E., Sotres-Bayon, F., & LeDoux, J. E. (2007). Individual differences in fear: isolating fear reactivity and fear recovery phenotypes. *Journal of traumatic stress*, *20*(4), 413–22. doi:10.1002/jts.20261

Cada, A., de la Torre, J., & Gonzalez-Lima, F. (2000). Chronic cerebrovascular

ischemia in aged rats: effects on brain metabolic capacity and behavior. *Neurobiology of aging*, *21*(2), 225–33.

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neuroscience letters*, *332*(2), 83-86.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, *77*(1), 175-181.

Davis, H. P., & Squire, L. R. (1984). Protein synthesis and memory: a review. *Psychological bulletin*, *96*(3), 518.

De la Fuente, M. (2002). Effects of antioxidants on immune system ageing. *European journal of clinical nutrition*, *56*, S5-8.

de la Torre, J.C. (2010). Vascular risk factor detection and control may prevent Alzheimer's disease. *Ageing research reviews*.

de la Torre, J.C. (2012a). Cardiovascular risk factors promote brain hypoperfusion leading to cognitive decline and dementia. *Cardiovascular psychiatry and neurology*.

de la Torre, J.C. (2012b). Cerebral hemodynamics and vascular risk factors: setting the stage for Alzheimer's disease. *Journal of Alzheimer's Disease*.

Dean, W., Morgenthaler, J., & Fowkes, S. W. (1990). Smart Drugs & Nutrients. *B & J Publishing (US).*

Deyo, R. A., Straube, K. T., & Disterhoft, J. F. (1989). Nimodipine facilitates associative learning in aging rabbits. *Science*, *243*(4892), 809-811.

Dudai, Y. (1996). Consolidation: fragility on the road to the engram. *Neuron*, *17*(3), 367-370.

Dudai, Y. (2004). The neurobiology of consolidations, or, how stable is the engram?. *Annu. Rev. Psychol.*, *55*, 51-86.

Effting, M., & Kindt, M. (2007). Contextual control of human fear associations in a renewal paradigm. *Behaviour Research and Therapy*, 45(9), 2002-2018.

Fava, GA, Rafanelli, C, Grandi, S, & Conti, S. (2001). Long-term outcome of panic disorder with agoraphobia treated by exposure. *Psychological* doi:10.1017/S0033291701003592

Fava, GA, Zielezny, M, Savron, G, & Grandi, S. (1995). Long-term effects of behavioural treatment for panic disorder with agoraphobia. *The British Journal of Psychiatry*.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239-247.

Foa, E. B., & Kozak, M. J. (1986). Emotional processing of fear: exposure to corrective information. *Psychological bulletin*, *99*(1), 20.

Gabrielli, D., Belisle, E., Severino, D., Kowaltowski, A. J., & Baptista, M. S. (2004). Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. *Photochemistry and photobiology*, *79*(3), 227-232.

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology*, *40*(8), 959-975.

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, *11*(5), 633-640.

Gonzalez-Lima, F., Barksdale, B. R., & Rojas, J. C. (2014). Mitochondrial respiration as a target for neuroprotection and cognitive enhancement. *Biochemical pharmacology*, 88(4), 584-593.

Gutteridge, J. M., & Halliwell, B. (1993). Invited review free radicals in disease processes: a compilation of cause and consequence. *Free Radical Research*, *19*(3), 141-158.

Hartmann, H., Eckert, A., & Müller, W. E. (1994). Disturbances of the neuronal calcium homeostasis in the aging nervous system. *Life sciences*, *55*(25), 2011-2018.

Hauptmann, S., Scherping, I., Drose, S., Brandt, U., Schulz, K., Jendrach, M., Muller, W. (2009). Mitochondrial dysfunction: An early event in Alzheimer pathology accumulates with age in AD transgenic mice. *Neurobiology of Aging*, *30*, 1574–1586. doi:10.1016/j.neurobiolaging.2007.12.005

Hernandez, P. J., Sadeghian, K., & Kelley, A. E. (2002). Early consolidation of instrumental learning requires protein synthesis in the nucleus accumbens. *Nature neuroscience*, *5*(12), 1327-1331.

Hettema, J. M., Neale, M. C., & Kendler, K. S (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry*, 158: 1568–1578.

Hettema, J. M., Annas, P., Neale, M. C., Kendler, K. S., & Fredrikson, M. (2003). A twin study of the genetics of fear conditioning. *Archives of General Psychiatry*, *60*(7), 702-708.

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R. L., Atwood, C. S., ... & Smith, M. A. (2001). Mitochondrial abnormalities in Alzheimer's disease. *The Journal of Neuroscience*, *21*(9), 3017-3023.

Hoffman, K. L., & McNaughton, B. L. (2002). Coordinated reactivation of distributed memory traces in primate neocortex. *Science*, *297*(5589), 2070-2073.

Hofmann S.G. (2008). Cognitive processes during fear acquisition and extinction in animals and humans: implications for exposure therapy of anxiety disorders. *Clin Psychol Rev*; 28, 199–210.

Hupbach, A., Gomez, R., Hardt, O., & Nadel, L. (2007). Reconsolidation of episodic memories: a subtle reminder triggers integration of new information. *Learning & Memory*, *14*(1-2), 47-53.

Hutton-Bedbrook, K., & McNally, G. P. (2013). The promises and pitfalls of retrieval-extinction procedures in preventing relapse to drug seeking. *Frontiers in psychiatry*, *4*.

Judge, M. E., & Quartermain, D. (1982). Characteristics of retrograde amnesia following reactivation of memory in mice. *Physiology & Behavior*, *28*(4), 585-590.

Keil, F. C., Smith, W. C., Simons, D. J., & Levin, D. T. (1998). Two dogmas of conceptual empiricism: Implications for hybrid models of the structure of knowledge. *Cognition*, *65*(2), 103-135.

Kelly, Á., Laroche, S., & Davis, S. (2003). Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory. *The Journal of neuroscience*, *23*(12), 5354-5360.

Kindt, M., Soeter, M., & Vervliet, B. (2009). Beyond extinction: erasing human fear responses and preventing the return of fear. *Nature neuroscience*, *12*(3), 256-258.

Lashley, K. S. (1950). In search of the engram.

Lashley, K. S. (1951). *The problem of serial order in behavior* (pp. 112-136). Bobbs-Merrill.

LeDoux, J. E. (1993). Emotional memory systems in the brain. *Behavioural brain research*, *58*(1), 69-79.

LeDoux, J. E., Romanski, L., & Xagoraris, A. (1989). Indelibility of subcortical emotional memories. *Journal of Cognitive Neuroscience*, *1*(3), 238-243.

Lee, J. L., Everitt, B. J., & Thomas, K. L. (2004). Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science*, *304*(5672), 839-843.

Lewis, D. J., Misanin, J. R., & Miller, R. R. (1968). Recovery of memory following amnesia, 704-705.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787-795.

Lonsdorf, T. B, Kalisch, R. (2011). A review on experimental and clinical genetic associations studies on fear conditioning, extinction and cognitive-behavioral treatment. *Transl Psychiatry*, 1: e41.

Ma, X., Zhang, J. J., & Yu, L. C. (2012). Post-retrieval extinction training enhances or hinders the extinction of morphine-induced conditioned place preference in rats dependent on the retrieval-extinction interval. *Psychopharmacology*, *221*(1), 19-26.

Martinez Jr, J. L., Jensen, R. A., Vasquez, B. J., McGuinness, T., & McGaugh, J. L. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, *6*(3), 387-390.

McClelland, J. L., & Rumelhart, D. E. (1985). Distributed memory and the representation of general and specific information. *Journal of Experimental Psychology: General*, *114*(2), 159.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science*, *287*(5451), 248-251.

Milad M.R., Rauch S.L., Pitman R.K., Quirk G.J. (2006). Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biol Psychol*, 73: 61–71.

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., ... & Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biological psychiatry, 66(12), 1075-1082.

Millan, E. Z., Milligan-Saville, J., & McNally, G. P. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiology of learning and memory*, *101*, 26-32.

Mineka S, Zinbarg R. (1996). Conditioning and ethological models of anxiety disorders: stress-in-dynamic-context anxiety models. *Nebr Symp Motiv*, 43, 135–210.

Mineka S, Oehlberg K. (2008). The relevance of recent developments in classical conditioning to understanding the etiology and maintenance of anxiety disorders. *Acta Psychol*, 127, 567–580.

Misanin, J. R., Miller, R. R., & Lewis, D. J. (1968). Retrograde amnesia produced by electroconvulsive shock after reactivation of a consolidated memory trace. *Science*, *160*(3827), 554-555.

Monfils, M. H., Cowansage, K. K., Klann, E., & LeDoux, J. E. (2009). Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *science*, *324*(5929), 951-955.

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience letters*, *163*(1), 109-113.

Müller, G. E., & Pilzecker, A. (1900). Experimentelle beiträge zur lehre vom gedächtniss, *JA Barth*, 1.

Murphy, G. L. (2002). The big book of concepts. *MIT press*.

Myers KM, Davis M. (2002). Behavioral and neural analysis of extinction. *Neuron*, 36: 567–584.

Nader, K., & Einarsson, E. Ö. (2010). Memory reconsolidation: an update. *Annals of the New York Academy of Sciences*, *1191*(1), 27-41.

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722-726.

Naylor, G. J., Martin, B., Hopwood, S. E., & Watson, Y. (1986). A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manicdepressive psychosis. *Biological psychiatry*, *21*(10), 915-920.

Nyberg, L., McIntosh, A. R., Cabeza, R., Habib, R., Houle, S., & Tulving, E. (1996). General and specific brain regions involved in encoding and retrieval of events: what, where, and when. *Proceedings of the National Academy of Sciences*, *93*(20), 11280-11285.

Obrenovich, M., Smith, M., Siedlak, S., Chen, S., Torre, J., Perry, G., & Aliev, G. (2006). Overexpression of GRK2 in Alzheimer disease and in a chronic hypoperfusion rat model is an early marker of brain mitochondrial lesions. *Neurotoxicity Research*, *10*, 43–56. doi:10.1007/BF03033333

Olin, J., Schneider, L., Novit, A., & Luczak, S. (2001). Hydergine for dementia. *Cochrane Database Syst Rev*, *2*, CD000359.

Olshavsky, M. E., Jones, C. E., Lee, H. J., & Monfils, M. H. (2013). Appetitive behavioral traits and stimulus intensity influence maintenance of conditioned fear. *Frontiers in behavioral neuroscience*, *7*.

Pappert, E. J., Tangney, C. C., Goetz, C. G., Ling, Z. D., Lipton, J. W., Stebbins, G. T., & Carvey, P. M. (1996). Alpha-tocopherol in the ventricular

cerebrospinal fluid of Parkinson's disease patients Dose-response study and correlations with plasma levels. *Neurology*, *47*(4), 1037-1042.

Pavlov, I. P. (1927). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. New York, *Boyer*.

Pearce, J. M., & Hall, G. (1980). A model for Pavlovian learning: Variations in the effectiveness of conditioned but not of unconditioned stimuli. *Psychological Review*, *87*(6), 532–552.

Pedreira, M. E., & Maldonado, H. (2003). Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron*, *38*(6), 863-869.

Pedreira, M. E., Pérez-Cuesta, L. M., & Maldonado, H. (2004). Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. *Learning & Memory*, 11(5), 579-585.

Peter, C., Hongwan, D., Kupfer, A., & Lauterburg, B. (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *European Journal of Clinical Pharmacology*, *56*, 247–250. doi:10.1007/s002280000124

Plaschke, K. (2005). Aspects of ageing in chronic cerebral oligaemia. Mechanisms of degeneration and compensation in rat models. *J. Neural Transm*. 112, 393–413.

Polster, M. R., Nadel, L., & Schacter, D. L. (1991). Cognitive neuroscience analyses of memory: A historical perspective. *Journal of Cognitive Neuroscience*, 3(2), 95-116.

Prusky, G. T., West, P. W., & Douglas, R. M. (2000). Behavioral assessment of visual acuity in mice and rats. *Vision research*, *40*(16), 2201-2209.

Quevedo, J., Vianna, M., Daroit, D., Born, A. G., Kuyven, C. R., Roesler, R., & Quillfeldt, J. A. (1998). L-type voltage-dependent calcium channel blocker nifedipine enhances memory retention when infused into the hippocampus. *Neurobiology of learning and memory*, *69*(3), 320-325.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *The Journal of Neuroscience*, *20*(16), 6225-6231.

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology*, *33*(1), 56-72.

Quirk, G. J., Paré, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., & Vicentic, A. (2010). Erasing fear memories with extinction training. *The Journal of Neuroscience*, *30*(45), 14993-14997.

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *Neuroimage*, *54*(4), 2623-2634.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, *511*(2), 151-158.

Robbins, S. J. (1990). Mechanisms underlying spontaneous recovery in autoshaping. *Journal of Experimental Psychology: Animal Behavior Processes*, 16(3), 235.

Rojas, J. C., Bruchey, A. K., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of methylene blue. *Progress in neurobiology*, *96*(1), 32-45.

Rose, S. P. (2002). 'Smart drugs': do they work? Are they ethical? Will they be legal?. *Nature reviews neuroscience*, *3*(12), 975-979.

Salinska, E., Bourne, R. C., & Rose, S. P. (2004). Reminder effects: the molecular cascade following a reminder in young chicks does not recapitulate that following training on a passive avoidance task. *European Journal of Neuroscience*, *19*(11), 3042-3047.

Sara, S. J. (2000). Retrieval and reconsolidation: toward a neurobiology of remembering. *Learning & Memory*, *7*(2), 73-84.

Schiller, D., Monfils, M. H., Raio, C. M., Johnson, D. C., LeDoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. Nature, 463(7277), 49-53.

Schiller, D., Kanen, J. W., LeDoux, J. E., Monfils, M. H., & Phelps, E. A. (2013). Extinction during reconsolidation of threat memory diminishes prefrontal cortex involvement. *Proceedings of the National Academy of Sciences*, *110*(50), 20040-20045.

Schirmer, R. H., Adler, H., Pickhardt, M., & Mandelkow, E. (2011). Lest we forget you—methylene blue…. *Neurobiology of aging*, *32*(12), 2325-e7.

Scriabine, A., Schuurman, T., & Traber, J. (1989). Pharmacological basis for the use of nimodipine in central nervous system disorders. *The FASEB journal*, *3*(7), 1799-1806.

Shumake, J., Furgeson-Moreira, S., & Monfils, M. H. (2014). Predictability and

heritability of individual differences in fear learning. *Animal cognition*, *17*(5), 1207–21. doi:10.1007/s10071-014-0752-1

Smith, D. H., Okiyama, K., Thomas, M. J., Claussen, B., & McIntosh, T. K. (1991). Evaluation of memory dysfunction following experimental brain injury using the Morris water maze. *Journal of neurotrauma*, *8*(4), 259-269.

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S.S. et al (2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science*, 327: 863–866.

Sweet, G., & Standiford, S. B. (2007). Methylene-blue–associated encephalopathy. *Journal of the American College of Surgeons*, *204*(3), 454-458.

Tronel, S., & Sara, S. J. (2002). Mapping of olfactory memory circuits: region-specific c-fos activation after odor-reward associative learning or after its retrieval. *Learning & memory*, *9*(3), 105-111.

VanElzakker, M. B., Dahlgren, M. K., Davis, F. C., Dubois, S., & Shin, L. M. (2014). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiology of learning and memory*, *113*, 3-18.

Velliquette, R., O'Connor, T., & Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *25*, 10874–10883.

doi:10.1523/JNEUROSCI.2350-05.2005

Wainwright, M., & Crossley, K. B. (2002). Methylene Blue-a therapeutic dye for all seasons?. *Journal of chemotherapy*, *14*(5), 431-443.

Walker, M. P., Brakefield, T., Hobson, J. A., & Stickgold, R. (2003). Dissociable stages of human memory consolidation and reconsolidation. *Nature*, *425*(6958), 616-620.

Walter-Sack, I., Rengelshausen, J., Oberwittler, H., Burhenne, J., Mueller, O., Meissner, P., & Mikus, G. (2009). High absolute bioavailability of methylene blue given as an aqueous oral formulation. *European journal of clinical pharmacology*, *65*(2), 179-189.

Wessa, M., & Flor, H. (2007). Failure of extinction of fear responses in posttraumatic stress disorder: evidence from second-order conditioning. *The American Journal of Psychiatry*, 164 (11), 1684-1692.

Wrubel, K. M., Riha, P. D., Maldonado, M. A., McCollum, D., & Gonzalez-Lima, F. (2007). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology Biochemistry and Behavior*, *86*(4), 712-717.

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., ... & Lu, L. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science*, *336*(6078), 241-245.

Yehuda, R., & LeDoux, J. (2007). Response variation following trauma: a translational neuroscience approach to understanding PTSD. Neuron, 56(1), 19-32.

Bibliography

Alberini, C. M. (2005). Mechanisms of memory stabilization: are consolidation and reconsolidation similar or distinct processes? *Trends in Neurosciences*, 28(1), 51–6.

Anderson, J. R. (1991). The adaptive nature of human categorization. *Psychological Review*, *98*, 409–429.

Atamna, H., Nguyen, A., Schultz, C., Boyle, K., Newberry, J., Kato, H., & Ames, B. N. (2008). Methylene blue delays cellular senescence and enhances key mitochondrial biochemical pathways. *The FASEB Journal*, 22(3), 703-712.

Atamna, H., & Kumar, R. (2010). Protective role of methylene blue in Alzheimer's disease via mitochondria and cytochrome c oxidase. *Journal of Alzheimer's Disease*, 20, 439-452.

Auber, A., Tedesco, V., Jones, C. E., Monfils, M. H., & Chiamulera, C. (2013). Post-retrieval extinction as reconsolidation interference: methodological issues or boundary conditions?. *Psychopharmacology*, 226(4), 631-647.

Auchter, A., Williams, J., Barksdale, B., Monfils, M. H., & Gonzalez-Lima, F. (2014). Therapeutic benefits of methylene blue on cognitive impairment during chronic cerebral hypoperfusion. *Journal of Alzheimer's disease*, 42, S525–35.

Bahar, A., Dorfman, N., & Dudai, Y. (2004). Amygdalar circuits required for either consolidation or extinction of taste aversion memory are not

required for reconsolidation. *European Journal of Neuroscience*, *19*(4), 1115-1118.

Barrett, D., Shumake, J., Jones, D., & Gonzalez-Lima, F. (2003). Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *The Journal of neuroscience*, *23*(13), 5740-5749.

Baum, M. (1969). Extinction of an avoidance response following response prevention: Some parametric investigations. *Canadian Journal of Psychology/Revue canadienne de psychologie*, 23(1), 1.

Bouton, M. E. (1988). Context and ambiguity in the extinction of emotional learning: Implications for exposure therapy. *Behaviour research and therapy*, 26(2), 137-149.

Bouton, M. E. (1993). Context, time, and memory retrieval in the interference paradigms of Pavlovian learning. *Psychological bulletin*, *114*(1), 80.

Bouton, M. E. (2002). Context, ambiguity, and unlearning: sources of relapse after behavioral extinction. *Biological psychiatry*, 52(10), 976-986.

Bouton, M. E. (2004). Context and behavioral processes in extinction. *Learning & memory*, *11*(5), 485-494.

Bouton, M. E. (2007). Learning and behavior: A contemporary synthesis. *Sinauer Associates*.

Bouton, M. E., & Bolles, R. C. (1979). Role of conditioned contextual stimuli in reinstatement of extinguished fear. *Journal of Experimental Psychology: Animal Behavior Processes*, *5*(4), 368.

Bouton, M. E., Mineka, S., & Barlow, D. H. (2001). A modern learning theory perspective on the etiology of panic disorder. *Psychological review*,

108(1), 4.

Bouton, M. E., Westbrook, R. F., Corcoran, K. A., & Maren, S. (2006). Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biological psychiatry*, *60*(4), 352-360.

Brīede, J., & Dubur, G. (2007). Protective effect of cerebrocrast on rat brain ischaemia induced by occlusion of both common carotid arteries. *Cell biochemistry and function,* 25(2), 203-210.

Brin, M., & Yonemoto, R. H. (1958). Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. *Journal of Biological Chemistry*, *230*(1), 307-318.

Bruchey, A. K., & Gonzalez-Lima, F. (2008). Behavioral, physiological and biochemical hormetic responses to the autoxidizable dye methylene blue. *American journal of pharmacology and toxicology,* 3(1), 72.

Budson, A. E., & Price, B. H. (2005). Memory dysfunction. *New England Journal of Medicine*, *352*(7), 692-699.

Burnham, W. H. (1903). Retroactive amnesia: illustrative cases and a tentative explanation. *The American Journal of Psychology*, 118-132.

Bush, D. E., Sotres-Bayon, F., & LeDoux, J. E. (2007). Individual differences in fear: isolating fear reactivity and fear recovery phenotypes. *Journal of traumatic stress*, *20*(4), 413-422.

Cada, A., de la Torre, J., & Gonzalez-Lima, F. (2000). Chronic cerebrovascular ischemia in aged rats: effects on brain metabolic capacity and behavior. *Neurobiology of aging*, *21*(2), 225–33.

Cain, C. K., Blouin, A. M., & Barad, M. (2003). Temporally massed CS

presentations generate more fear extinction than spaced presentations. *Journal of Experimental Psychology: Animal Behavior Processes*, 29(4), 323.

Callaway, N. L., Riha, P. D., Wrubel, K. M., McCollum, D., & Gonzalez-Lima, F. (2002). Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neuroscience letters*, 332(2), 83-86.

Callaway, N. L., Riha, P. D., Bruchey, A. K., Munshi, Z., & Gonzalez-Lima, F. (2004). Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacology Biochemistry and Behavior*, 77(1), 175-181.

Choy, M., Ganesan, V., Thomas, D.L., Thornton, J.S., Proctor, E., King, M.D., van der Weerd, L., Gadian, D.G., Lythgoe, M.F. (2006). The chronic vascular and haemodynamic response after permanent bilateral common carotid occlusion in newborn and adult rats. J*. Cereb. Blood Flow Metab*. 26, 1066–1075.

Circu, M. L., & Aw, T. Y. (2010). Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radical Biology and Medicine*, 48(6), 749-762.

Clarke, D. D., & Sokoloff, L. (1999). Circulation and energy metabolism of the brain. *Basic neurochemistry: molecular, cellular and medical aspects*, *6*, 637-669.

Cohen, R. A., Poppas, A., Forman, D. E., Hoth, K. F., Haley, A. P., Gunstad, J., ... & Gerhard-Herman, M. (2009). Vascular and cognitive functions

associated with cardiovascular disease in the elderly. *Journal of Clinical and Experimental Neuropsychology*, 31(1), 96-110.

Courville, A. C. (2005). Similarity and discrimination in classical conditioning: A latent variable account. *Advances in Neural Information Processing Systems*, 12, 313–320.

Courville, A. C., Daw, N. D., & Touretzky, D. S. (2006). Bayesian theories of conditioning in a changing world. *Trends in Cognitive Sciences*, *10*(7), 294–300.

Courville, A. C., Gordon, G., Touretzky, D. S., & Daw, N. D. (2003). Model uncertainty in classical conditioning. *Advances in Neural Information Processing Systems*, 16, 977–984.

Crews, D., Gore, A., Hsu, T., Dangleben, N., Spinetta, M., Schallert, T., Skinner, M. (2007). Transgenerational epigenetic imprints on mate preference. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 5942–5946. doi:10.1073/pnas.0610410104

Darvas, M., Fadok, J. P., & Palmiter, R. D. (2011). Requirement of dopamine signaling in the amygdala and striatum for learning and maintenance of a conditioned avoidance response. *Learning & memory*, *18*(3), 136-143.

Davis, H. P., & Squire, L. R. (1984). Protein synthesis and memory: a review. *Psychological bulletin*, *96*(3), 518.

Davis, M., Ressler, K., Rothbaum, B. O., & Richardson, R. (2006). Effects of D-cycloserine on extinction: translation from preclinical to clinical work. *Biological psychiatry*, *60*(4), 369-375.

De la Fuente, M. (2002). Effects of antioxidants on immune system ageing. *European journal of clinical nutrition*, *56*, S5-8.

de la Torre, J. C. (1999). Critical threshold cerebral hypoperfusion causes Alzheimer's disease. *Acta neuropathologica*, *98*(1), 1-8.

de la Torre, J.C. (2000). Impaired cerebrovascular perfusion. Summary of evidence in support of its causality in Alzheimer's disease. *Ann. N. Y. Acad. Sci*. 924, 136–152.

de la Torre, J. C. (2010). Vascular risk factor detection and control may prevent Alzheimer's disease. *Ageing research reviews*, *9*(3), 218-225.

de la Torre, J. C. (2012a). Cardiovascular risk factors promote brain hypoperfusion leading to cognitive decline and dementia. *Cardiovascular psychiatry and neurology*, 2012.

de la Torre, J. C. (2012b). Cerebral hemodynamics and vascular risk factors: setting the stage for Alzheimer's disease. *Journal of Alzheimer's Disease*, *32*(3), 553-567.

de la Torre, J., & Fortin, T. (1991). Partial or global rat brain ischemia: the SCOT model. *Brain research bulletin*, *26*, 365–372. doi:10.1016/0361-9230(91)90008-8

de la Torre, J., & Fortin, T. (1994). A chronic physiological rat model of dementia. *Behavioural brain research*, *63*, 35–40. doi:10.1016/0166-4328(94)90048-5

de la Torre, J., Fortin, T., Park, G., Pappas, B., & Richard, M. (1993). Brain blood flow restoration "rescues" chronically damaged rat CA1 neurons. *Brain research*, *623*, 6–15. doi:10.1016/0006-

8993(93)90003-6

de la Torre, J. C., Čada, A., Nelson, N., Davis, G., Sutherland, R. J., & Gonzalez-Lima, F. (1997). Reduced cytochrome oxidase and memory dysfunction after chronic brain ischemia in aged rats. *Neuroscience letters*, *223*(3), 165-168. doi:10.1016/S0304-3940(97)13421-8

Dean, W., Morgenthaler, J., & Fowkes, S. W. (1990). Smart Drugs & Nutrients. *B & J Publishing (US).*

Dębiec, J., & Ledoux, J. E. (2004). Disruption of reconsolidation but not consolidation of auditory fear conditioning by noradrenergic blockade in the amygdala. *Neuroscience*, 129(2), 267-272.

Debiec, J., & LeDouxy, J. E. (2013). Reconsolidation of Pavlovian Conditioned Defense Responses in the Amygdala. *Memory Reconsolidation*, 69.

Deiana, S., Harrington, C., Wischik, C., & Riedel, G. (2009). Methylthioninium chloride reverses cognitive deficits induced by scopolamine: comparison with rivastigmine. *Psychopharmacology*, *202*(1-3), 53–65.

Deyo, R. A., Straube, K. T., & Disterhoft, J. F. (1989). Nimodipine facilitates associative learning in aging rabbits. *Science*, *243*(4892), 809-811.

Driscoll, I., Howard, S., Prusky, G., Rudy, J., & Sutherland, R. (2005). Seahorse wins all races: hippocampus participates in both linear and non-linear visual discrimination learning. *Behavioural brain research*, *164*(1), 29–35. doi:10.1016/j.bbr.2005.05.006

Driscoll, I., Sutherland, R., Prusky, G., & Rudy, J. (2004). Damage to the hippocampal formation does not disrupt representational flexibility as measured by a novelty transfer test. *Behavioral neuroscience*, *118*,

1427–1432. doi:10.1037/0735-7044.118.6.1427

Dudai, Y. (1996). Consolidation: fragility on the road to the engram. *Neuron*, 17(3), 367-370.

Dudai, Y . (2004). The neurobiology of consolidations, or, how stable is the engram? *Annual Review of Psychology*, 55, 51e86.

Duvarci, S., & Nader, K. (2004). Characterization of fear memory reconsolidation. *The Journal of neuroscience*, 24(42), 9269-9275.

Ebbinghaus, H. (1885). Über das gedächtnis: untersuchungen zur experimentellen psychologie. *Duncker & Humblot*.

Effting, M., & Kindt, M. (2007). Contextual control of human fear associations in a renewal paradigm. *Behaviour Research and Therapy*, 45(9), 2002-2018.

Eichenbaum, H. (2000). A cortical–hippocampal system for declarative memory. *Nature Reviews Neuroscience*, 1(1), 41-50.

Ennaceur, A., Neave, N., & Aggleton, J. (1997). Spontaneous object recognition and object location memory in rats: The effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. *Experimental Brain Research*, *113*, 509–519. doi:10.1007/PL00005603

Erecinska, M. and Silver, I.A., ATP and brain function, *J. Cerebral Blood Flow Metab.,* 9 (1989) 2–19.

Fani, N., Tone, E. B., Phifer, J., Norrholm, S. D., Bradley, B., Ressler, K. J., ... & Jovanovic, T. (2012). Attention bias toward threat is associated with exaggerated fear expression and impaired extinction in PTSD.

Psychological medicine, *42*(03), 533-543.

Farkas, E., Luiten, P.G.M. (2001). Cerebral microvascular pathology in aging and Alzheimer's disease. *Prog. Neurobiol*. 64, 575–611.

Farkas, E., Donka, G., de Vos, R.A.I., Mihály, A., Bari, F., Luiten, P.G. M. (2004a). Experimental cerebral hypoperfusion imposes white matter injury and microglial activation in the rat brain. *Acta Neuropathol*. (Berl.) 108, 57–64.

Farkas, E., Institóris, Á., Domoki, F., Mihály, A., Luiten, P.G.M., Bari, F. (2004b). Diazoxide and dimethyl sulphoxide prevent cerebral hypoperfusion-related learning dysfunction and brain damage after carotid artery occlusion. *Brain Res*. 1008, 252–258.

Farkas, E., Institóris, Á., Domoki, F., Mihály, A., Bari, F. (2006). The effect of pre- and post-treatment with diazoxide on the early phase of chronic cerebral hypoperfusion in the rat. *Brain Res*. 1087, 168–174.

Farkas, E., Luiten, P., & Bari, F. (2007). Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. *Brain research reviews*, 54(1), 162–80.

Fava, G. A., Rafanelli, C., Grandi, S., Conti, S., Ruini, C., Mangelli, L., & Belluardo, P. (2001). Long-term outcome of panic disorder with agoraphobia treated by exposure. *Psychological medicine*, *31*(05), 891-898.

Fava, G. A., Zielezny, M., Savron, G., & Grandi, S. (1995). Long-term effects of behavioural treatment for panic disorder with agoraphobia. *The British Journal of Psychiatry*, *166*(1), 87-92.

Fidalgo, C., Conejo, N. M., González-Pardo, H., & Arias, J. L. (2014). Dynamic functional brain networks involved in simple visual discrimination learning. *Neurobiology of learning and memory*, 114, 165-170.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239-247.

Fisher, R. A. (1921) On the probable error of a coefficient of correlation deduced from a small sample. *Metron*, 1, 3-32.

Flexner, L. B., Flexner, J. B., & Stellar, E. (1965). Memory and cerebral protein synthesis in mice as affected by graded amounts of puromycin. *Experimental neurology*, *13*(3), 264-272.

Foa, E. B., & Kozak, M. J. (1986). Emotional processing of fear: exposure to corrective information. *Psychological bulletin*, *99*(1), 20.

Fricks-Gleason, A. N., & Marshall, J. F. (2008). Post-retrieval β-adrenergic receptor blockade: Effects on extinction and reconsolidation of cocaine-cue memories. *Learning & memory*, 15(9), 643-648.

Gabrielli, D., Belisle, E., Severino, D., Kowaltowski, A. J., & Baptista, M. S. (2004). Binding, aggregation and photochemical properties of methylene blue in mitochondrial suspensions. *Photochemistry and photobiology*, *79*(3), 227-232.

Gershman, S. J., Blei, D. M., & Niv, Y. (2010). Context, learning, and extinction. *Psychological Review*, 117(1), 197–209.

Gershman, S. J., Jones, C. E., Norman, K. A., Monfils, M. H., & Niv, Y. (2013). Gradual extinction prevents the return of fear: implications for the discovery of state. *Frontiers in behavioral neuroscience*, *7*.

Gershman, S. J., & Niv, Y. (2012). Exploring a latent cause theory of classical conditioning. *Learning & Behavior*, *40*(3), 255–68.

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology*, *40*(8), 959-975.

Glickman, S. E. (1961). Perseverative neural processes and consolidation of the memory trace. *Psychological Bulletin*, 58(3), 218.

Goelet, P., Castellucci, V. F., Schacher, S., & Kandel, E. R. (1986). The long and the short of long-term memory: A molecular framework. *Nature*, *322*, 419–422

Gonzales-Lima, F., & Jones, D. (1994). Quantitative mapping of cytovhrome oxidase activity in the central auditory system of the gerbil: a study with calibrated activity standards and metal-intensified histochemistry. *Brain research*, 660(1), 34-49.

Gonzalez-Lima, F., & Bruchey, A. K. (2004). Extinction memory improvement by the metabolic enhancer methylene blue. *Learning & Memory*, 11(5), 633-640.

Gonzalez-Lima, F., Barksdale, B. R., & Rojas, J. C. (2014). Mitochondrial respiration as a target for neuroprotection and cognitive enhancement. *Biochemical pharmacology*, *88*(4), 584–93. doi:10.1016/j.bcp.2013.11.010

Gordon, J. A., Hen, R. (2004). Genetic approaches to the study of anxiety. *Annual Reviews Neuroscience*, 27, 193–222.

Gutteridge, J. M., & Halliwell, B. (1993). Invited review free radicals in disease processes: a compilation of cause and consequence. *Free Radical Research*, *19*(3), 141-158.

Haley, A. P., Forman, D. E., Poppas, A., Hoth, K. F., Gunstad, J., Jefferson, A. L., ... & Cohen, R. A. (2007). Carotid artery intima-media thickness and cognition in cardiovascular disease. *International journal of cardiology*, *121*(2), 148-154.

Hartmann, H., Eckert, A., & Müller, W. E. (1994). Disturbances of the neuronal calcium homeostasis in the aging nervous system. *Life sciences*, *55*(25), 2011-2018.

Hauptmann, S., Scherping, I., Drose, S., Brandt, U., Schulz, K., Jendrach, M., Muller, W. (2009). Mitochondrial dysfunction: An early event in Alzheimer pathology accumulates with age in AD transgenic mice. *Neurobiology of Aging*, *30*, 1574–1586. doi:10.1016/j.neurobiolaging.2007.12.005

Hermans, D., Dirikx, T., Vansteenwegenin, D., Baeyens, F., Van den Bergh, O., & Eelen, P. (2005). Reinstatement of fear responses in human aversive conditioning. *Behaviour research and therapy*, *43*(4), 533-551.

Hernandez, P. J., Sadeghian, K., & Kelley, A. E. (2002). Early consolidation of instrumental learning requires protein synthesis in the nucleus accumbens. *Nature neuroscience*, *5*(12), 1327-1331.

Hettema, J. M., Neale, M. C., & Kendler, K. S (2001). A review and meta-analysis of the genetic epidemiology of anxiety disorders. *Am J Psychiatry*, 158: 1568–1578.

Hettema, J. M., Annas, P., Neale, M. C., Kendler, K. S., & Fredrikson M. (2003). A twin study of the genetics of fear conditioning. *Arch Gen Psychiatry*, 60: 702–708.

Hirai, K., Aliev, G., Nunomura, A., Fujioka, H., Russell, R. L., Atwood, C. S., ... & Smith, M. A. (2001). Mitochondrial abnormalities in Alzheimer's disease. *The Journal of Neuroscience*, *21*(9), 3017-3023.

Hoffman, K. L., & McNaughton, B. L. (2002). Coordinated reactivation of distributed memory traces in primate neocortex. *Science*, *297*(5589), 2070-2073.

Hofmann S.G. (2008). Cognitive processes during fear acquisition and extinction in animals and humans: implications for exposure therapy of anxiety disorders. *Clin Psychol Rev*; 28, 199–210.

Huang, S., Du, F., Shih, Y., Shen, Q., Gonzalez-Lima, F., & Duong, T. (2013). Methylene blue potentiates stimulus-evoked fMRI responses and cerebral oxygen consumption during normoxia and hypoxia. *NeuroImage*, *72*, 237–242. doi:10.1016/j.neuroimage.2013.01.027

Hupbach, A., Gomez, R., Hardt, O., & Nadel, L. (2007). Reconsolidation of episodic memories: a subtle reminder triggers integration of new information. *Learning & Memory*, *14*(1-2), 47-53.

Hutton-Bedbrook, K., & McNally, G. P. (2013). The promises and pitfalls of retrieval-extinction procedures in preventing relapse to drug seeking. *Frontiers in psychiatry*, 4.

Ji, J., & Maren, S. (2007). Hippocampal involvement in contextual modulation of fear extinction. *Hippocampus*, 17(9), 749-758.

Jones, C. E., & Monfils, M. H. (2013). Using reconsolidation and extinction to weaken fear memories in animal models. *Memory Reconsolidation*, 165.

Judge, M. E., & Quartermain, D. (1982). Characteristics of retrograde amnesia following reactivation of memory in mice. *Physiology & Behavior*, *28*(4), 585-590.

Kalaria, R. N. (1991). The blood-brain barrier and cerebral microcirculation in Alzheimer disease. *Cerebrovascular and brain metabolism reviews*, *4*(3), 226-260.

Kandel, E. R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. *Science*, 294(5544), 1030e1038.

Kaplan G.B., Heinrichs S.C., Carey R.J. (2011). Treatment of addiction and anxiety using extinction approaches: neural mechanisms and their treatment implications. *Pharmacol Biochem Behav*, 97: 619–625.

Keil, F. C., Smith, W. C., Simons, D. J., & Levin, D. T. (1998). Two dogmas of conceptual empiricism: Implications for hybrid models of the structure of knowledge. *Cognition*, *65*(2), 103-135.

Kelly, Á., Laroche, S., & Davis, S. (2003). Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for consolidation and reconsolidation of recognition memory. *The Journal of neuroscience*, *23*(12), 5354-5360.

Kelner, M. J., Bagnell, R., Hale, B., & Alexander, N. M. (1988). Methylene blue competes with paraquat for reduction by flavo-enzymes resulting in

decreased superoxide production in the presence of heme proteins. *Archives of biochemistry and biophysics*, 262(2), 422-426.

Kety, S. S. (1957). The general metabolism of the brain in vivo. *Metabolism of the nervous system*, 221-237.

Keynes, R.G., Garthwaite, J., 2004. Nitric oxide and its role in ischaemic brain injury. *Curr. Mol. Med*. 4, 179–191.

Kindt, M., Soeter, M., & Vervliet, B. (2009). Beyond extinction: erasing human fear responses and preventing the return of fear. *Nature neuroscience*, *12*(3), 256-258.

Kindt, M., & Soeter, M. (2013). Reconsolidation in a human fear conditioning study: a test of extinction as updating mechanism. *Biological psychology*, *92*(1), 43-50.

Lashley, KS. (1950). In search of the engram. Oxford, England: Academic Press.

Lashley, K. S. (1951). *The problem of serial order in behavior* (pp. 112-136). Bobbs-Merrill.

Lassen, N. A., & Ingvar, D. H. (1980). Blood flow studies in the aging normal brain and in senile dementia. *Aging of the Brain and Dementia*, 91-98.

Lavinsky, D., Sarmento Arterni, N., Achaval, M., Netto, C.A., (2006). Chronic bilateral common carotid artery occlusion: a model for ocular ischemic syndrome in the rat. *Graefe's Arch. Clin. Exp. Ophthalmol*. 244, 199–204.

LeDoux, J. E. (1993). Emotional memory systems in the brain. *Behavioural brain research*, *58*(1), 69-79.

LeDoux, J. E., Romanski, L., & Xagoraris, A. (1989). Indelibility of subcortical emotional memories. *Journal of Cognitive Neuroscience*, *1*(3), 238-243.

Lechner, H. A., Squire, L. R., & Byrne, J. H. (1999). 100 years of consolidation—remembering Müller and Pilzecker. *Learning & Memory*, 6(2), 77-87.

Lee, R., & Urban, J. (2002). Functional replacement of oxygen by other oxidants in articular cartilage. *Arthritis and rheumatism*, *46*(12), 3190–200. doi:10.1002/art.10686

Lee, J. L., Everitt, B. J., & Thomas, K. L. (2004). Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science*, *304*(5672), 839-843.

Lee, J. L., Milton, A. L., & Everitt, B. J. (2006). Cue-induced cocaine seeking and relapse are reduced by disruption of drug memory reconsolidation. *The Journal of neuroscience*, *26*(22), 5881-5887.

Lewis, J. L., LoTurco, J. J., & Solomon, P. R. (1987). Lesions of the middle cerebellar peduncle disrupt acquisition and retention of the rabbit's classically conditioned nictitating membrane response. *Behavioral neuroscience*, *101*(2), 151.

Lewis, D. J., Misanin, J. R., & Miller, R. R. (1968). Recovery of memory following amnesia, 704-705.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, 443(7113), 787-795.

Lindahl, P. E., & Öberg, K. E. (1961). The effect of rotenone on respiration and its point of attack. *Experimental Cell Research*, 23(2), 228-237.

Liu, H.X., Zhang, J.J., Zheng, P., Zhang, Y. (2005). Altered expression of MAP-2, GAP-43, and synaptophysin in the hippocampus of rats with chronic cerebral hypoperfusion correlates with cognitive impairment. *Brain Res. Mol. Brain Res*. 139, 169–177.

Liu, J., Jin, D.Z., Xiao, L., Zhu, X.Z. (2006). Paeoniflorin attenuates chronic cerebral hypoperfusion-induced learning dysfunction and brain damage in rats. *Brain Res.* 1089, 162–170.

Lonsdorf, T. B, Kalisch, R. (2011). A review on experimental and clinical genetic associations studies on fear conditioning, extinction and cognitive-behavioral treatment. *Transl Psychiatry*, 1: e41.

Ma, X., Zhang, J. J., & Yu, L. C. (2012). Post-retrieval extinction training enhances or hinders the extinction of morphine-induced conditioned place preference in rats dependent on the retrieval-extinction interval. *Psychopharmacology*, *221(*1), 19-26.

Marcus, D. L., de Leon, M. J., Goldman, J., Logan, J., Christman, D. R., Wolf, A. P., ... & Freedman, M. L. (1989). Altered glucose metabolism in microvessels from patients with Alzheimer's disease. *Annals of neurology*, *26*(1), 91-94.

Martin, S. J., Grimwood, P. D., & Morris, R. G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annual Review of Neuroscience*, 23, 649e711.

Martinez, J., Jensen, R., Vasquez, B., McGuinness, T., & McGaugh, J. (2013). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, *6*(3), 387–390. doi:10.3758/BF03326744

Martinez Jr, J. L., Jensen, R. A., Vasquez, B. J., McGuinness, T., & McGaugh, J. L. (1978). Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology*, 6(3), 387-390.

McClelland, J. L., & Rumelhart, D. E. (1985). Distributed memory and the representation of general and specific information. *Journal of Experimental Psychology: General*, *114*(2), 159.

McCormick, D. A., & Thompson, R. F. (1984). Cerebellum: essential involvement in the classically conditioned eyelid response. *Science*, 223(4633), 296-299.

McGaugh, J. L. (1966). Time-dependent processes in memory storage. *Science*, 153(3742), 1351-1358.

McGaugh, J. L. (2000). Memory--a century of consolidation. *Science*, 287(5451), 248-251.

Medina, D., Caccamo, A., & Oddo, S. (2011). Methylene blue reduces aβ levels and rescues early cognitive deficit by increasing proteasome activity. *Brain pathology (Zurich, Switzerland)*, 21(2), 140–9. doi:10.1111/j.1750-3639.2010.00430.x

Meier-Ruge, W., & Bertoni-Freddari, C. (1996). The significance of glucose turnover in the brain in the pathogenetic mechanisms of Alzheimer's disease. *Reviews in the Neurosciences*, 7(1), 1-20.

Merrill, K. A., Steinmetz, J. E., Viken, R. J., Rose, R. J. (1999). Genetic influences on human conditionability: a twin study of the conditioned eyeblink response. *Behavioral Genetics*, 29, 95–102.

Miclescu, A., Sharma, H. S., Martijn, C., & Wiklund, L. (2010). Methylene blue protects the cortical blood–brain barrier against ischemia/reperfusion-induced disruptions. *Critical care medicine*, 38(11), 2199-2206.

Milad, M. R., Orr, S. P., Pitman, R. K., & Rauch, S. L. (2005). Context modulation of memory for fear extinction in humans. Psychophysiology, 42(4), 456-464.

Milad M.R., Rauch S.L., Pitman R.K., Quirk G.J. (2006). Fear extinction in rats: implications for human brain imaging and anxiety disorders. *Biological Psychology*, 73, 61–71.

Milad, M. R., Pitman, R. K., Ellis, C. B., Gold, A. L., Shin, L. M., Lasko, N. B., ... & Rauch, S. L. (2009). Neurobiological basis of failure to recall extinction memory in posttraumatic stress disorder. Biological psychiatry, 66(12), 1075-1082.

Millan, E. Z., Milligan-Saville, J., & McNally, G. P. (2013). Memory retrieval, extinction, and reinstatement of alcohol seeking. *Neurobiology of learning and memory*, *101*, 26-32.

Mineka S, Zinbarg R. (1996). Conditioning and ethological models of anxiety disorders: stress-in-dynamic-context anxiety models. *Nebraska Symposium on Motivation*, 43, 135–210.

Mineka S, Oehlberg K. (2008). The relevance of recent developments in classical conditioning to understanding the etiology and maintenance of anxiety disorders. *Acta Psychology*, 127, 567–580.

Misanin, J. R., Miller, R. R., & Lewis, D. J. (1968). Retrograde Amnesia Produced by Electroconvulsive Shock after Reactivation of a Consolidated Memory Trace. *Science*, 160(3827), 554–555.

Monfils, M.-H., Cowansage, K. K., Klann, E., & LeDoux, J. E. (2009). Extinction-reconsolidation boundaries: key to persistent attenuation of fear memories. *Science*, *324*(5929), 951–5.

Moreira, P. I., Santos, M. S., Seiça, R., & Oliveira, C. R. (2007). Brain mitochondrial dysfunction as a link between Alzheimer's disease and diabetes. *Journal of the neurological science*s, 257(1), 206-214.

Morgan, M. A., Romanski, L. M., & LeDoux, J. E. (1993). Extinction of emotional learning: contribution of medial prefrontal cortex. *Neuroscience letters*, 163(1), 109-113.

Morris, R. G., Inglis, J., Ainge, J. A., Olverman, H. J., Tulloch, J., Dudai, Y., & Kelly, P. A. (2006). Memory reconsolidation: sensitivity of spatial memory to inhibition of protein synthesis in dorsal hippocampus during encoding and retrieval. *Neuron*, 50(3), 479-489.

Morris, J., Storandt, M., Miller, J., McKeel, D., Price, J., Rubin, E., & Berg, L. (2001). Mild cognitive impairment represents early-stage Alzheimer disease. *Archives of neurology*, 58, 397–405. doi:10.1001/archneur.58.3.397

Mosconi, L., Tsui, W., Herholz, K., Pupi, A., Drzezga, A., Lucignani, G., … Leon, M. (2008). Multicenter standardized 18F-FDG PET diagnosis of mild cognitive impairment, Alzheimer's disease, and other dementias. *Journal of nuclear medicine : official publication, Society of Nuclear*

Medicine, *49*, 390–398. doi:10.2967/jnumed.107.045385

Müller, G., & Pilzecker, A. (1900). Experimentelle beiträge zur lehre vom gedächtniss, *1*.

Murakami, Y., Ikenoya, M., Matsumoto, K., Li, H., Watanabe, H. (2000). Ameliorative effect of tacrine on spatial memory deficit in chronic two-vessel occluded rats is reversible and mediated by muscarinic M1 receptor stimulation*. Behav. Brain Res*. 109, 83–90.

Murphy, G. L. (2002). The big book of concepts*. MIT press*.

Murphy, M. (2009). How mitochondria produce reactive oxygen species. *Biochem. J*, 417, 1-13.

Myers KM, Davis M. (2002). Behavioral and neural analysis of extinction. *Neuron*, 36, 567–584.

Myers, K. M., & Davis, M. (2007). Mechanisms of fear extinction. Molecular psychiatry, 12(2), 120-150.

Myers, K. M., Ressler, K. J., & Davis, M. (2006). Different mechanisms of fear extinction dependent on length of time since fear acquisition. *Learning & Memory*, 13(2), 216-223.

Nader, K., & Einarsson, E. O. (2010). Memory reconsolidation: an update. *Annals of the New York Academy of Sciences*, 1191, 27–41.

Nader, K., Schafe, G. E., & Le Doux, J. E. (2000a). Fear memories require protein synthesis in the amygdala for reconsolidation after retrieval. *Nature*, *406*(6797), 722–6.

Nader, K., Schafe, G. E., & LeDoux, J. E. (2000b). The labile nature of consolidation theory. *Nature Reviews. Neuroscience*, *1*(3), 216–9.

Nandhra, H. S., Murphy, C. L., & Sule, A. (2013). Novel pharmacological agents targeting memory and cognition in the treatment of anxiety disorders. *Human Psychopharmacology: Clinical and Experimental*, *28*(6), 538-543.

Naylor, G. J., Martin, B., Hopwood, S. E., & Watson, Y. (1986). A two-year double-blind crossover trial of the prophylactic effect of methylene blue in manicdepressive psychosis. *Biological psychiatry*, *21*(10), 915-920.

Nelson, C., & Silverstein, F. S. (1994). Acute disruption of cytochrome oxidase activity in brain in a perinatal rat stroke model. *Pediatric research*, *36*, 12-19.

Nyberg, L., McIntosh, A. R., Cabeza, R., Habib, R., Houle, S., & Tulving, E. (1996). General and specific brain regions involved in encoding and retrieval of events: what, where, and when. *Proceedings of the National Academy of Sciences*, *93*(20), 11280-11285.

O'Leary, J., Li, Q., Marinec, P., Blair, L., Congdon, E., Johnson, A., Dickey, C. (2010). Phenothiazine-mediated rescue of cognition in tau transgenic mice requires neuroprotection and reduced soluble tau burden. *Molecular neurodegeneration*, *5*, 45. doi:10.1186/1750-1326-5-45

Obrenovich, M., Smith, M., Siedlak, S., Chen, S., Torre, J., Perry, G., & Aliev, G. (2006). Overexpression of GRK2 in Alzheimer disease and in a chronic hypoperfusion rat model is an early marker of brain mitochondrial lesions. *Neurotoxicity Research*, *10*, 43–56. doi:10.1007/BF03033333

Obrist, W. D., Chivian, E., Cronqvist, S., & Ingvar, D. H. (1970). Regional cerebral blood flow in senile and presenile dementia. *Neurology*, *20*(4), 315-315.

Ohlow, M. J., & Moosmann, B. (2011). Phenothiazine: the seven lives of pharmacology's first lead structure. *Drug discovery today*, *16*(3), 119-131.

Ohtaki, H., Fujimoto, T., Sato, T., Kishimoto, K., Fujimoto, M., Moriya, M., Shioda, S. (2006). Progressive expression of vascular endothelial growth factor (VEGF) and angiogenesis after chronic ischemic hypoperfusion in rat. *Acta Neurochir., Suppl*. 96, 283–287.

Oldendorf, W.H. (1989). Trophic changes in the arteries at the base of the brain in response to bilateral common carotid ligation, *J. Neuropathol*. Exp. Neurol. 48, 534–547.

Olin, J., Schneider, L., Novit, A., & Luczak, S. (2001). Hydergine for dementia. *Cochrane Database Syst Rev*, *2*, CD000359.

Olshavsky, M. E., Song, B. J., Powell, D. J., Jones, C. E., Monfils, M. H., & Lee, H. J. (2013). Updating appetitive memory during reconsolidation window: critical role of cue-directed behavior and amygdala central nucleus. *Frontiers in behavioral neuroscience*, 7.

Oyarzún, J. P., Lopez-Barroso, D., Fuentemilla, L., Cucurell, D., Pedraza, C., Rodriguez-Fornells, A., & de Diego-Balaguer, R. (2012). Updating fearful memories with extinction training during reconsolidation: a human study using auditory aversive stimuli. *PLoS One*, 7(6), e38849.

Oz, M., Lorke, D. E., & Petroianu, G. A. (2009). Methylene blue and Alzheimer's disease. *Biochemical pharmacology*, 78(8), 927-932.

Paban, V., Manrique, C., Filali, M., Maunoir-Regimbal, S., Fauvelle, F., & Alescio-Lautier, B. (2014). Therapeutic and preventive effects of methylene blue on Alzheimer's disease pathology in a transgenic mouse model. *Neuropharmacology*, *76*, 68–79. doi:10.1016/j.neuropharm.2013.06.033

Packard, M. G., Cahill, L., & McGaugh, J. L. (1994). Amygdala modulation of hippocampal-dependent and caudate nucleus-dependent memory processes. *Proceedings of the National Academy of Sciences*, *91*(18), 8477–8481.

Pappert, E. J., Tangney, C. C., Goetz, C. G., Ling, Z. D., Lipton, J. W., Stebbins, G. T., & Carvey, P. M. (1996). Alpha-tocopherol in the ventricular cerebrospinal fluid of Parkinson's disease patients Dose-response study and correlations with plasma levels. *Neurology*, *47*(4), 1037-1042.

Pavlov, I (1904). *Physiology of Degestion*. Nobel Lecture. Published in *Nobel Lectures, Physiology or Medicine 1901-1921*, Elsevier Publishing Company, Amsterdam.

Pavlov, I. P. (1927). Conditioned reflexes: an investigation of the physiological activity of the cerebral cortex. New York, *Boyer*.

Pearce, J. M., & Hall, G. (1980). A model for Pavlovian learning: Variations in the effectiveness of conditioned but not of unconditioned stimuli. *Psychological Review*, *87*(6), 532–552.

Pedreira, M. E., & Maldonado, H. (2003). Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron*, *38*(6), 863-869.

Pedreira, M. E., Pérez-Cuesta, L. M., & Maldonado, H. (2004). Mismatch between what is expected and what actually occurs triggers memory reconsolidation or extinction. *Learning & Memory*, 11(5), 579-585.

Peter, C., Hongwan, D., Kupfer, A., & Lauterburg, B. (2000). Pharmacokinetics and organ distribution of intravenous and oral methylene blue. *European Journal of Clinical Pharmacology*, *56*, 247–250. doi:10.1007/s002280000124

Plaschke, K. (2005). Aspects of ageing in chronic cerebral oligaemia. Mechanisms of degeneration and compensation in rat models. *J. Neural Transm*. 112, 393–413.

Polster, M. R., Nadel, L., & Schacter, D. L. (1991). Cognitive neuroscience analyses of memory: A historical perspective. *Journal of Cognitive Neuroscience*, 3(2), 95-116.

Prusky, G. T., West, P. W., & Douglas, R. M. (2000). Behavioral assessment of visual acuity in mice and rats. *Vision research*, *40*(16), 2201-2209.

Quevedo, J., Vianna, M., Daroit, D., Born, A. G., Kuyven, C. R., Roesler, R., & Quillfeldt, J. A. (1998). L-type voltage-dependent calcium channel blocker nifedipine enhances memory retention when infused into the hippocampus. *Neurobiology of learning and memory*, *69*(3), 320-325.

Quirk, G. J., & Mueller, D. (2008). Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology : Official Publication of the American College of Neuropsychopharmacology*, 33(1), 56–72.

Quirk, G. J., Paré, D., Richardson, R., Herry, C., Monfils, M. H., Schiller, D., & Vicentic, A. (2010). Erasing fear memories with extinction training. *The Journal of Neuroscience*, *30*(45), 14993-14997.

Quirk, G. J., Russo, G. K., Barron, J. L., & Lebron, K. (2000). The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *The Journal of Neuroscience*, 20(16), 6225-6231.

Redish, A. D., Jensen, S., Johnson, A., & Kurth-Nelson, Z. (2007). Reconciling reinforcement learning models with behavioral extinction and renewal: implications for addiction, relapse, and problem gambling. *Psychological Review*, *114*(3), 784–805.

Rescorla, RA. (1988). Pavlovian conditioning: It's not what you think it is. *American Psychologist*.

Rescorla, R. A., & Wagner, A. R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and nonreinforcement. In *Classical conditioning: current research and theory*.

Rescorla, R. A. (1996). Preservation of Pavlovian associations through extinction. *The Quarterly Journal of Experimental Psychology: Section B*, *49*(3), 245-258.

Rescorla RA. (2001) Experimental extinction. In: *Handbook of contemporary learning theories* (Mowrer RR, Klein S, eds), pp 119–154. Mahwah, NJ:

Erlbaum

Rescorla, R. A., & Heth, C. D. (1975). Reinstatement of fear to an extinguished conditioned stimulus. *Journal of Experimental Psychology: Animal Behavior Processes*, *1*(1), 88.

Ribot, T. (1882). Diseases of memory, an essay in the positive psychology, *43*.

Riha, P. D., Bruchey, A. K., Echevarria, D. J., & Gonzalez-Lima, F. (2005). Memory facilitation by methylene blue: dose-dependent effect on behavior and brain oxygen consumption. *European journal of pharmacology*, 511(2), 151-158.

Riha, P. D., Rojas, J. C., & Gonzalez-Lima, F. (2011). Beneficial network effects of methylene blue in an amnestic model. *Neuroimage*, 54(4), 2623-2634.

Robbins, S. J. (1990). Mechanisms underlying spontaneous recovery in autoshaping. Journal of Experimental Psychology: Animal Behavior Processes, 16(3), 235.

Rodriguez, P., Jiang, Z., Huang, S., Shen, Q., & Duong, T. Q. (2014). Methylene blue treatment delays progression of perfusion–diffusion mismatch to infarct in permanent ischemic stroke. *Brain research*, *1588*, 144-149.

Rogers, R. L., Meyer, J. S., Mortel, K. F., Mahurin, R. K., & Judd, B. W. (1986). Decreased cerebral blood flow precedes multi-infarct dementia, but follows senile dementia of Alzheimer type. *Neurology*, *36*(1), 1-1.

Rojas, J. C., John, J. M., Lee, J., & Gonzalez-Lima, F. (2009a). Methylene blue provides behavioral and metabolic neuroprotection against optic neuropathy. *Neurotoxicity research,* 15(3), 260-273.

Rojas, J. C., Simola, N., Kermath, B. A., Kane, J. R., Schallert, T., & Gonzalez-Lima, F. (2009b). Striatal neuroprotection with methylene blue. *Neuroscience*, 163(3), 877-889.

Rojas, J. C., & Gonzalez-Lima, F. (2010). Mitochondrial optic neuropathy: in vivo model of neurodegeneration and neuroprotective strategies. *Eye and brain*, *2*, 21-37.

Rojas, J. C., Bruchey, A. K., & Gonzalez-Lima, F. (2012). Neurometabolic mechanisms for memory enhancement and neuroprotection of methylene blue. *Progress in neurobiology*, *96*(1), 32-45.

Rolfe, D. F., & Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiological reviews*, *77*(3), 731-758.

Rose, S. P. (2002). 'Smart drugs': do they work? Are they ethical? Will they be legal?. *Nature reviews neuroscience*, *3*(12), 975-979.

Royce, J. R. (1972). Avoidance conditioning in nine strains of inbred mice using optimal stimulus parameters. *Behavioral Genetics*, 2: 107–110.

Salaris, S. C., Babbs, C. F., & Voorhees, W. D. (1991). Methylene blue as an inhibitor of superoxide generation by xanthine oxidase: a potential new drug for the attenuation of ischemia/reperfusion injury. *Biochemical pharmacology,* 42(3), 499-506.

Salinska, E., Bourne, R. C., & Rose, S. P. (2004). Reminder effects: the molecular cascade following a reminder in young chicks does not recapitulate that following training on a passive avoidance task. *European Journal of Neuroscience*, *19*(11), 3042-3047.

Sara, S. J. (2000). Retrieval and reconsolidation: toward a neurobiology of remembering. *Learning & Memory*, *7*(2), 73-84.

Sarti, C., Pantoni, L., Bartolini, L., Inzitari, D. (2002). Persistent impairment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav. Brain Res*. 136, 13–20.

Schafe, G. E., & LeDoux, J. E. (2000). Memory consolidation of auditory pavlovian fear conditioning requires protein synthesis and protein kinase A in the amygdala. *J Neurosci*, *20*(18), RC96.

Schiller, D., Monfils, M.-H., Raio, C. M., Johnson, D. C., Ledoux, J. E., & Phelps, E. A. (2010). Preventing the return of fear in humans using reconsolidation update mechanisms. *Nature*, *463*(7277), 49–53.

Schiller, D., Kanen, J. W., LeDoux, J. E., Monfils, M. H., & Phelps, E. A. (2013). Extinction during reconsolidation of threat memory diminishes prefrontal cortex involvement. *Proceedings of the National Academy of Sciences*, *110*(50), 20040-20045.

Schirmer, R. H., Adler, H., Pickhardt, M., & Mandelkow, E. (2011). Lest we forget you—methylene blue…. *Neurobiology of aging*, *32*(12), 2325-e7.

Schmidt-Kastner, R., Truettner, J., Lin, B., Zhao, W., Saul, I., Busto, R., Ginsberg, M.D. (2001). Transient changes of brain-derived neurotrophic factor (BDNF) mRNA expression in hippocampus during moderate ischemia induced by chronic bilateral common carotid artery occlusion in the rat. *Brain Res. Mol. Brain Res*. 92, 157–166.

Scriabine, A., Schuurman, T., & Traber, J. (1989). Pharmacological basis for the use of nimodipine in central nervous system disorders. *The FASEB journal*, *3*(7), 1799-1806.

Sevenster, D., Beckers, T., & Kindt, M. (2012). Retrieval per se is not sufficient to trigger reconsolidation of human fear memory. *Neurobiology of learning and memory*, *97*(3), 338-345.

Shang, Y., Cheng, J., Oi, J., Miao, H. (2005). Scutellaria flavonoid reduced memory dysfunction and neuronal injury caused by permanent global ischemia in rats. *Phamacol. Biochem. Behav*. 82, 67–73.

Shen, Q., Du, F., Huang, S., Rodriguez, P., Watts, L. T., & Duong, T. Q. (2013). Neuroprotective efficacy of methylene blue in ischemic stroke: an MRI study. *PloS one*, 8(11), e79833.

Shumake, J., Furgeson-Moreira, S., & Monfils, M. H. (2014). Predictability and heritability of individual differences in fear learning. *Animal cognition*, *17*(5), 1207-1221.

Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, 5(5), 415-418.

Smith, D. H., Okiyama, K., Thomas, M. J., Claussen, B., & McIntosh, T. K. (1991). Evaluation of memory dysfunction following experimental brain injury using the Morris water maze. *Journal of neurotrauma*, *8*(4), 259-269.

Sokoloff, L. (1960). The metabolism of the central nervous system in vivo. *Handbook of physiology-neurophysiology*, 3, 1843-1864.

Soliman, F., Glatt, C. E., Bath, K. G., Levita, L., Jones, R. M., Pattwell, S.S. et al

(2010). A genetic variant BDNF polymorphism alters extinction learning in both mouse and human. *Science*, 327: 863–866.

Sopala, M., Danysz, W. (2001). Chronic cerebral hypoperfusion in the rat enhances age-related deficits in spatial memory *J. Neural Transm*. 108, 1445–1456.

Sotres-Bayon, F., Bush, D. E., & LeDoux, J. E. (2004). Emotional perseveration: an update on prefrontal-amygdala interactions in fear extinction. *Learning & Memory*, 11(5), 525-535.

Sparks, D. L., Hunsaker, J. C., Scheff, S. W., Kryscio, R. J., Henson, J. L., & Markesbery, W. R. (1990). Cortical senile plaques in coronary artery disease, aging and Alzheimer's disease. *Neurobiology of aging*, *11*(6), 601-607.

Spinetta, M., Woodlee, M., Feinberg, L., Stroud, C., Schallert, K., Cormack, L., & Schallert, T. (2008). Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. *Psychopharmacology*, *201*(3), 361–71. doi:10.1007/s00213-008-1294-5

Steinmetz, J. E., Logan, C. G., Rosen, D. J., Thompson, J. K., Lavond, D. G., & Thompson, R. F. (1987). Initial localization of the acoustic conditioned stimulus projection system to the cerebellum essential for classical eyelid conditioning. *Proceedings of the National Academy of Sciences*, *84*(10), 3531-3535.

Steinmetz, J. E., & Sengelaub, D. R. (1992). Possible conditioned stimulus pathway for classical eyelid conditioning in rabbits. I. Anatomical evidence for direct projections from the pontine nuclei to the

cerebellar interpositus nucleus. *Behavioral and neural biology*, *57*(2), 103-115.

Stern, C. A., Gazarini, L., Takahashi, R. N., Guimaraes, F. S., & Bertoglio, L. J. (2012). On disruption of fear memory by reconsolidation blockade: evidence from cannabidiol treatment. *Neuropsychopharmacology*, *37*(9), 2132-2142.

Sweet, G., & Standiford, S. B. (2007). Methylene-blue–associated encephalopathy. *Journal of the American College of Surgeons*, *204*(3), 454-458.

Tachibana, H., Meyer, J. S., Kitagawa, Y., Rogers, R. L., Okayasu, H., & Mortel, K. F. (1984a). Effects of aging on cerebral blood flow in dementia. *Journal of the American Geriatrics Society*, *32*(2), 114-120.

Tachibana, H., Meyer, J. S., Okayasu, H., Shaw, T. G., Kandula, P., & Rogers, R. L. (1984b). Xenon Contrast CT–CBF Scanning of the Brain Differentiates Normal Age-Related Changes from Multi-infarct Dementia and Senile Dementia of Alzheimer Type. *Journal of gerontology*, *39*(4), 415-423.

Telch, M. J., Bruchey, A. K., Rosenfield, D., Cobb, A. R., Smits, J., Pahl, S., & Gonzalez-Lima, F. (2014). Effects of post-session administration of methylene blue on fear extinction and contextual memory in adults with claustrophobia. *American Journal of Psychiatry*, *171*(10), 1091-1098.

Thompson, R. F., Bao, S., Chen, L., Cipriano, B. D., Grethe, J. S., Kim, J. J., ... & Krupa, D. J. (1997). Associative learning. *International review of neurobiology*, *41*, 151-189.

Tillerson, J., Caudle, W., Parent, J., Gong, C., Schallert, T., & Miller, G. (2006). Olfactory discrimination deficits in mice lacking the dopamine transporter or the D2 dopamine receptor. *Behavioural Brain Research*, *172*, 97–105. doi:10.1016/j.bbr.2006.04.025

Todes, D. P. (2014). Ivan Pavlov: A Russian Life in Science. *Oxford University Press.*

Tronel, S., & Sara, S. J. (2002). Mapping of olfactory memory circuits: region-specific c-fos activation after odor-reward associative learning or after its retrieval. *Learning & memory*, *9*(3), 105-111.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, 552(2), 335-344.

Ueda, H., Fujita, R., (2004). Cell death mode switch from necrosis to apoptosis in brain, *Biol. Pharm. Bull*. 27, 950–955.

Valla, J., Berndt, J., & Gonzalez-Lima, F. (2001). Energy hypometabolism in posterior cingulate cortex of Alzheimer's patients: Superficial laminar cytochrome oxidase associated with disease duration. *J. Neurosci.*, *21*(13), 4923–4930.

Valla, J., Schneider, L., Small, A., & Gonzalez-Lima, F. (2007). Quantitative cytochrome oxidase histochemistry: Applications in human Alzheimer's Disease and animal models. *Journal of Histotechnology*, *30*, 235–247. doi:10.1179/his.2007.30.4.235

Valla, J., Yaari, R., Wolf, A., Kusne, Y., Beach, T., Roher, A., Reiman, E. (2010). Reduced posterior cingulate mitochondrial activity in expired young adult carriers of the APOE ε Allele, the major late-onset Alzheimer's

susceptibility gene. *Journal of Alzheimer's Disease*, *22*, 307–313. doi:10.3233/JAD-2010-100129

VanElzakker, M. B., Dahlgren, M. K., Davis, F. C., Dubois, S., & Shin, L. M. (2014). From Pavlov to PTSD: The extinction of conditioned fear in rodents, humans, and anxiety disorders. *Neurobiology of learning and memory*, *113*, 3-18.

Velliquette, R., O'Connor, T., & Vassar, R. (2005). Energy inhibition elevates beta-secretase levels and activity and is potentially amyloidogenic in APP transgenic mice: possible early events in Alzheimer's disease pathogenesis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *25*, 10874–10883. doi:10.1523/JNEUROSCI.2350-05.2005

Visarius, T. M., Stucki, J. W., & Lauterburg, B. H. (1997). Stimulation of respiration by methylene blue in rat liver mitochondria. *FEBS letters,* 412(1), 157-160.

Wainwright, M., & Crossley, K. (2002). Methylene Blue - a Therapeutic Dye for All Seasons? *Journal of Chemotherapy*, *14*(5), 431–443.

Walker, M. P., Brakefield, T., Hobson, J. A., & Stickgold, R. (2003). Dissociable stages of human memory consolidation and reconsolidation. *Nature*, *425*(6958), 616-620.

Walter-Sack, I., Rengelshausen, J., Oberwittler, H., Burhenne, J., Mueller, O., Meissner, P., & Mikus, G. (2009). High absolute bioavailability of methylene blue given as an aqueous oral formulation. *European journal of clinical pharmacology*, *65*(2), 179-189.

Wang, S. H., de Oliveira Alvares, L., & Nader, K. (2009). Cellular and systems mechanisms of memory strength as a constraint on auditory fear reconsolidation. *Nature neuroscience*, 12(7), 905-912.

Watts, L. T., Lloyd, R., Garling, R. J., & Duong, T. (2013). Stroke Neuroprotection: Targeting Mitochondria. *Brain sciences*, 3(2), 540-560.

Watts, L., Long, J., Chemello, J., & Koughnet, {Van. (2014). Methylene Blue Is Neuroprotective against Mild Traumatic Brain Injury. *Journal of neurotrauma*, *9*, 1–9. doi:10.1089/neu.2013.3193

Weingartner, H., & Parker, E. S. (Eds.). (2014). Memory consolidation: Psychobiology of cognition. *Psychology Press.*

Wen, Y., Li, W., Poteet, E. C., Xie, L., Tan, C., Yan, L. J., ... & Yang, S. H. (2011). Alternative mitochondrial electron transfer as a novel strategy for neuroprotection. *Journal of Biological Chemistry*, *286*(18), 16504-16515.

Wessa, M., & Flor, H. (2007). Failure of extinction of fear responses in posttraumatic stress disorder: evidence from second-order conditioning. *The American Journal of Psychiatry*, 164 (11), 1684-1692.

Westbrook, R. F., Iordanova, M., McNally, G., Richardson, R., & Harris, J. A. (2002). Reinstatement of fear to an extinguished conditioned stimulus: two roles for context. *Journal of Experimental Psychology: Animal Behavior Processes*, 28(1), 97.

Whitmer, R., Karter, A., Yaffe, K., Quesenberry, C., & Selby, J. (2009).

Hypoglycemic episodes and risk of dementia in older patients with type 2 diabetes mellitus. *JAMA : the journal of the American Medical Association*, *301*, 1565–1572. doi:10.1001/jama.2009.460

Wong-Riley, M. T. (1989). Cytochrome oxidase: an endogenous metabolic marker for neuronal activity. *Trends in neurosciences*, 12(3), 94-101.

Wong-Riley, M. T., & Welt, C. (1980). Histochemical changes in cytochrome oxidase of cortical barrels after vibrissal removal in neonatal and adult mice. *Proceedings of the National Academy of Sciences*, *77*(4), 2333-2337.

Wrubel, K., Barrett, D., Shumake, J., Johnson, S., & Gonzalez-Lima, F. (2007a). Methylene blue facilitates the extinction of fear in an animal model of susceptibility to learned helplessness. *Neurobiology of learning and memory*, *87*(2), 209–17. doi:10.1016/j.nlm.2006.08.009

Wrubel, K., Riha, P., Maldonado, M., McCollum, D., & Gonzalez-Lima, F. (2007b). The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacology, biochemistry, and behavior*, *86*(4), 712–7. doi:10.1016/j.pbb.2007.02.018

Xue, Y. X., Luo, Y. X., Wu, P., Shi, H. S., Xue, L. F., Chen, C., ... & Lu, L. (2012). A memory retrieval-extinction procedure to prevent drug craving and relapse. *Science*, *336*(6078), 241-245.

Yamaguchi, F., Meyer, J. S., Yamamoto, M., Sakai, F., & Shaw, T. (1980). Noninvasive regional cerebral blood flow measurements in dementia. *Archives of neurology*, *37*(7), 410-418.

Yehuda, R., Flory, J. D., Southwick, S., & Charney, D. S. (2006). Developing an

agenda for translational studies of resilience and vulnerability following trauma exposure. *Annals of the New York Academy of Sciences*, *1071*(1), 379-396.

Yehuda, R., & LeDoux, J. (2007). Response variation following trauma: a translational neuroscience approach to understanding PTSD. Neuron, 56(1), 19-32.

Zemcor, A., Risberg, J., Barclay, L. L., & Blass, J. P. (1984). Diagnosis of Alzheimer's dementia and multi-infarct dementia by rCBF compared to clinical classification. *Monogr Neural Sci*, *2*, 104-6.

Zhang, X., & Rojas, J. C. (2006). Methylene blue prevents neurodegeneration caused by rotenone in the retina. *Neurotoxicity Research*, *9*(1), 47–57. doi:10.1007/BF03033307