Bacteria for the Brain: Subcutaneous immunization with heat-killed *M. vaccae*

improves fear-potentiated startle responses and extinction learning in rats

By:

Emma J. Spratt

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Thesis Advisor:

Dr. Christopher A. Lowry, Dept. of Integrative Physiology & Center for Neuroscience

Defense Committee:

Dr. Christopher A. Lowry, Dept. of Integrative Physiology

Dr. David E. Sherwood, Dept. of Integrative Physiology

Dr. Steven K. Schmidt, Dept. of Ecology and Evolutionary Biology

## Introduction

*Mycobacterium vaccae (M. vaccae)* is a nonpathogenic bacterium that has come under focus by the scientific research community. *M. vaccae* is a rapidly growing mycobacterium that is normally encountered in the wild as a saprophyte, or a microorganism that lives on dead or decaying organic matter. *M. vaccae* has immunomodulatory properties (O'Brien et al. 2004, Zuaney-Amorim et al. 2002, Bazzi et al. 2015), and one study explored the possibility of synergy between chemotherapy and *M. vaccae* in the treatment of unresectable non-small-cell lung cancer (NSCLC) patients (O'Brien et al. 2015). In the study, NSCLC patients received a total of 5 intradermal injections of approximately $10^9$ heat-killed (HK) *M. vaccae* over the deltoid muscle every 4 weeks in addition to chemotherapy over the course of 16 weeks. It was anticipated that there may be a synergy between *M. vaccae* and chemotherapy. Ultimately, *M. vaccae* treatment did not impact survival times in this study. However, results surprisingly found marked increases in functioning and vitality, as well as a reduction in treatment-related adverse effects (nausea, vomiting and peripheral neuropathy) and relief of cancer-related symptoms (bodily pain and dyspnea). These unexpected findings catalyzed a new phase of exploration of *M. vaccae*, exploring its psychological effects.

Before psychological effects of *M. vaccae* were discovered, this strain of bacteria was widely known for its immunomodulatory properties (Bazzi et al. 2015, Smit et al. 2003, Zuany-Amorim et al. 2002). One explanation for the increase in some inflammatory disorders throughout the world is that urban living no longer exposes humans to nonpathogenic bacteria that humans evolved with, like *M. vaccae* (reviewed by Rook, Raison, & Lowry, 2012). Mycobacteria are characterized by a thick and complex cell wall structure, lending to its ability to modulate immune responses. *Mycobacterium bovis,* a bacterium similar in structure to *M. vaccae*, is used in its live and attenuated form as a prophylactic vaccine against human tuberculosis (TB), a globally prevalent disease caused by *Mycobacterium tuberculosis* (Romano et al. 2011). Further investigation determined *M. vaccae*'s role as a therapeutic agent for various conditions and diseases, including TB (Romano et al. 2011, Skinner et al. 1997), allergic reactions (Zuany-Amorim et al. 2002), asthma (Smit et al. 2003; Camporota et al., 2003; Yazi et al., 2008), psoriasis (Lehrer et al. 1998), and dermatitis (Arkwright et al., 2001). Studies have shown that immunization with HK *M. vaccae* increases levels of $CD8^+$ T cells, killing macrophages infected with *M. tuberculosis* (Skinner et al., 1997). Other studies show that immunization with *M. vaccae* reduced both airway hyperreactivity and bronchoalveolar fluid eosinophil count, which are white blood cells that primarily respond to parasites and allergens (Zuany-Amorim et al., 2002). The various immunomodulatory properties of *M. vaccae* have been studied in human blood from Caucasian donors (Bazzi et al., 2015). The exact mechanism *M. vaccae* uses to regulate the immune system is not clear, but this bacterium has displayed a rich variety of immunomodulatory effects in the last two decades of research.

The challenge of understanding the connection between immunization with *M. vaccae* and inflammation reduction with associated psychological effects lies in understanding the microbiota-gut-brain axis. The relationship between the brain and the health of the digestive system has been understood for many centuries, but microbial communication with the 'second brain' otherwise known as the enteric nervous system (ENS) is a relatively recent scientific breakthrough (reviewed by Mayer et al., 2011). The ENS has been likened to a peripheral extension of the limbic system into the gut due to its close connections with limbic and autonomic regions of the brain (MacLean 1993). The signaling between the

gastrointestinal tract and the brain is bi-directional (reviewed by Mayer et al., 2011), and the bodily structures involved in this line of communication include the ENS, the central nervous system (CNS), and the sympathetic and parasympathetic arms of the autonomic nervous system. The microbiota-gut-brain axis can be visualized as a hierarchy of reflexes, from the ENS reflex circuits to the highest reflex loop involving the insular cortex and anterior cingulate cortex of the CNS (Furness 2006). This reflex network with the gut microbiota includes afferent fibers projecting into the CNS and efferent fibers projecting into smooth muscle (Moser et al., 2014). These areas communicate not only through immune interactions like proinflammatory cytokines, but also via neural and hormonal avenues. The microbiota-gut-brain axis offers an explanation for how gut microbes influence mood, but its mechanism would be better understood with more research. Not only must we consider how bacteria stimulate the ENS on a mechanical level, but knowing where and how these signals influence the CNS to alter mood and behavior is crucial to understanding why the microbiota-gut-brain axis matters.

In terms of the microbiota-gut-brain axis and mood, it is worth noting the connection between the body's immune response and stress. A proinflammatory response was seen in one study following induction of a stressful situation in patients with coronary artery disease (Nijm et al., 2007). In this study, patients were exposed to a physical stress test on an electrically braked bicycle as well as two psychological stressors, an 'anger recall' and a mental arithmetic test. Urinary and evening salivary bioassays indicated a significant increase in C-reactive protein levels, which is indicative of a proinflammatory response. This immune response may induce changes in the central serotonin system (Zhu et al., 2006), which could explain the association between stress and neuropsychiatric disorders like anxiety and depression (Charney et al., 1993). Specifically, in one study, p38 mitogen-activated protein kinase, a protein that is responsive to stress stimuli like cytokines interleukin-1 beta and tumor necrosis factor alpha, activated the 5-hydroxytryptamine (5-HT) transporter SERT. 5-HT is a neurotransmitter that is intimately involved in depression, appetite, and sleep, along with many other behaviors (reviewed by Nestler et al., 2002). Further evidence of a link between depression and stress-induced proinflammatory cytokines like interleukin-6 is mounting (Pace et al., 2006; Maes et al., 1992; reviewed by Schiepers et al., 2005). Along with having immunomodulatory properties, *M. vaccae* also seems to have a protective role in depressive-like behavioral responses in the forced swim test (Lowry et al., 2007). In this study, *M. vaccae* activated a subset of serotonergic neurons in the interfascicular part of the dorsal raphe nucleus implicated in mood modulation (Lowry et al., 2007). Another study found that ingestion of *M. vaccae* decreases anxiety-related behavior and improves learning in mice (Matthews et al., 2013). All of these things considered, treating rats with this bacterium may have a mitigating effect on stress-induced inflammation and its presumed psychological effects, which may have implications of a new treatment for neuropsychiatric disorders.

Fear and anxiety are both aspects of the brain's defense circuitry, and variations in this circuitry and its outputs separate cue-specific fear and more generalized anxiety (reviewed by Lang et al., 2000). Many studies have implicated a small structure within the temporal lobe – the amygdala – to be involved in conditioned fear development and responses (Klinger et al., 1960, reviewed by Sarter et al., 1985, reviewed by Davis 1992). The amygdala receives sensory information through its lateral nuclei (LA) and basolateral nuclei (BLA), which both project into the central nucleus (CE) of the amygdala, which finally projects to the hypothalamus, the central gray and brainstem areas that mediate signals of fear and anxiety (reviewed by Davis 1992). Behaviors used to define a state of fear can be broadly organized into two classes, which include defense immobility ('freezing', 'fear bradycardia', and 'hyper-attentiveness', Kapp et al., 1992, Campbell 1997) and defensive action (variations in fight/flight). The

lateral, basal, and central amygdala are relevant in testing fear-potentiated startle (FPS), a particular fear state measured in this experiment. The central amygdala reaches into the nucleus reticularis pontis caudalis, which is directly involved in the startle pathway that is responsive to sound. The full neural pathway studied and proposed by Davis (1992), is visualized in Figure 1.

There are some important distinctions to note in terms of the proposed neural pathway involved specifically in FPS (Figure 1). This particular fear state is distinctively associated with vigilance and immobility, when the organism is reflexively mobilized, primed but still inactive. Examining this fear state involves activating a reflex, and this can be beneficial for a variety of reasons. The FPS reflex is a simple reflex with a non-zero baseline, meaning it allows separation of the effects of treatment on fear state from the effect of treatment on the response used to measure this fear state (Lang et al., 2000). Also, use of this reflex produces different response levels, so an experimenter can adjust the startle stimulus to match control condition, thus allowing fear assessment at equal parts of the measurement scale (Lang et al., 2000). In terms of neurobiology, although previous studies already show that *M. vaccae* played a role in the activation of serotonergic neurons in the DRN, it seems that FPS response is not impacted by treatments involving this area of the brain. One study showed that lesions in the serotonergic neurons in the DRN that alter 5-HT transmission did not affect potentiated startle (Davis et al., 1988). Lesions in this area have an anxiolytic profile in several other animal tests of fear and anxiety (i.e. operant-conflict test, lick-suppression test, social interaction test).
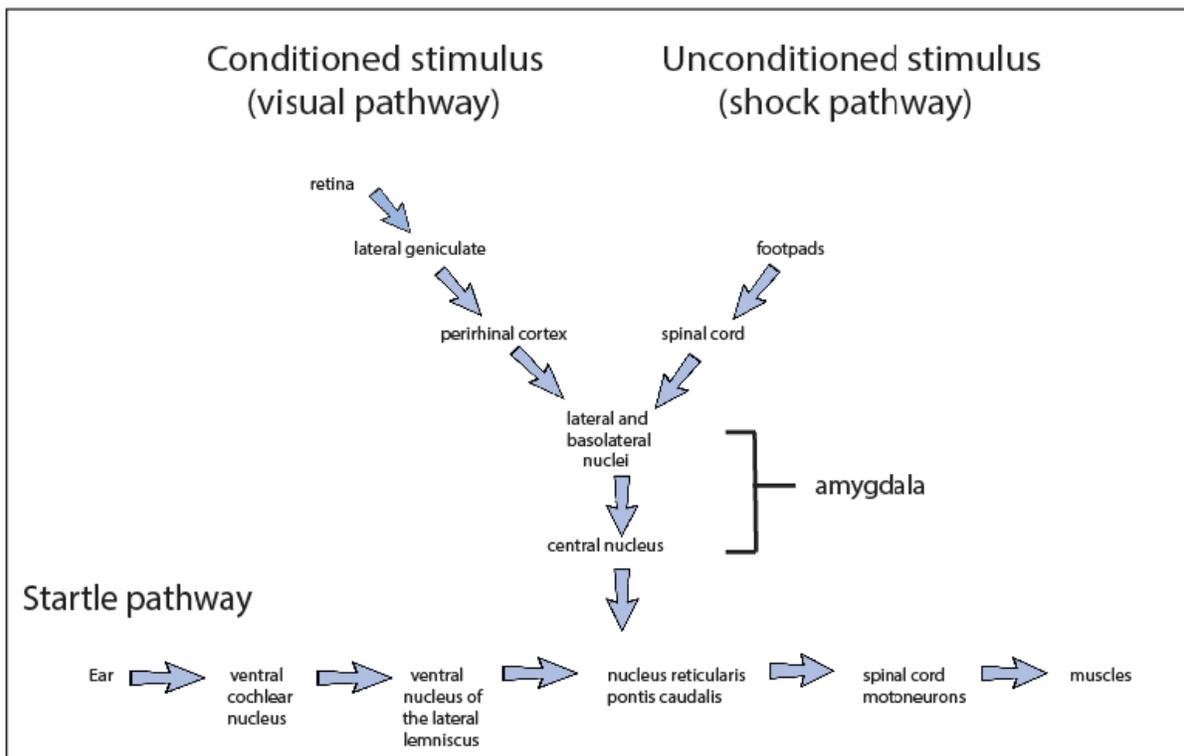


*Figure 1.* A hypothetical neural pathway outlining FPS. Retinal input and pain afferents join together at the lateral and basal nuclei of the amygdala, which project to the central nucleus of the amygdala. After being paired with a shock, the light may activate the lateral and basal nuclei of the amygdala and this

may be the catalyst for activating the acoustic startle pathway through the nucleus reticularis pontis caudalis. This proposed pathway was originally mapped by Davis (1992).

*M. vaccae*'s possible psychological impact has an array of implications. To narrow the scope, the main goal of the current research involves determining whether immunization with *M. vaccae* impacts FPS, or fear increasing startle response elicited by a sudden sound; the conditioned stimulus (CS) is light, and this CS is paired with an unconditioned stimulus (US). In this experiment, the CS is light and the US is footshock. In the test, fear is operationally defined by elevated startle amplitude, or the increase in the startle reflex, elicited by sudden noise in the presence of the light previously paired with footshock (Davis et al., 2001). The light is not responsible for the startle, but instead activates the retinal pathway that leads to a state of fear and in turn activates the startle pathway. This startle reflex effect has been shown to decrease with anxiolytic neurochemical agents such as morphine, diazepam, buspirone, gepirone, and high doses of ipsapirone (Davis et al., 1988; reviewed by Davis et al., 1986, Kehne et al., 1988). Some preliminary data found in the Behavioral Neuroendocrinology Laboratory at CU-Boulder suggests that administering *M. vaccae* as a preimmunization prior to the light and shock pairing reduces FPS in male rats, but no studies have tested whether immunization of rats with *M. vaccae* following the light and shock pairing could reduce conditioned fear responses, or alter fear extinction. Fear conditioning involves learning association between certain environmental cues with adverse events, while extinction learning involves the disappearance of the previously learned behavior when the behavior is not reinforced (Maren et al., 2001). It is important to note that conditioned fear and fear extinction are inherently separate (Pavlov 1927; Quirk et al., 2002; Konorski et al., 1967; Rescorla et al., 1975; Bouton et al., 1983; Herry et al. 2002). This distinction will be important in how the results are interpreted. A previous experiment displayed *M. vaccae*'s ability to act preventatively as an 'anxiety vaccine', whereas this experiment will determine if *M. vaccae* could be used to treat the conditioned fear by reducing fear conditioning or enhancing extinction learning.

**Methods**

*Subjects.* 24 adult male Sprague Dawley rats (weighed approximately 76-100g) were obtained from Charles River (Kingston, NY, USA) and arrived when 27-32 days old (Cat. No. SAS SD, Strain code: 400). Animals were acclimated for one week in the animal facility prior to experimental use. Animals were pair-housed in standard polycarbonate cages (26.0 cm (w) x 47.6 cm (l) x 20.3 cm (h)) containing a layer of bedding (approximately 300 g of bedding, 2 cm (h)). Stainless steel wire lids covered the cages and had compartments for Rodent diet food from Harlan Laboratories (Cat. No. WL88R, Alternative Designs). Tap water was also accessible, stored in 16 oz. reduced-height water bottles with screw lids. Food and water were accessible ad libitum. The vivarium in Ramaley Biology building (room N1B68F) was maintained on a 12-hour light-dark cycle, with lights on between 6am and 6pm. The room temperature was kept at 22°C, and room conditions were maintained and recorded daily. Cages were changed and the vivarium was cleaned weekly. All procedural elements were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Eighth Edition (The National Academies Press, 2011) and approved by the Institutional Animal Care and Use Committee at the University of Colorado Boulder. Rats were divided into two treatment groups.

*Treatments.* Vials of heat-killed (HK) *M. vaccae* (strain NCTC 11659, batch ENG #1) were provided by Immodulon Therapeutics, London, UK, manufactured by Eden Biodesign (Liverpool, UK), and shipped

by BioElpida (Lyon, France). 10 mg/ml HK *M. vaccae* was diluted to 1 mg/ml, and 0.1 mg was suspended in 100 µl sterile borate buffered saline. This mixture was packed into a single siliconized vessel for each day of injections, considering how *M. vaccae* antigen is a particulate suspension. The two treatment groups included n=12 vehicle immunized rats (vehicle group) and n=12 *M. vaccae* immunized rats (*M. vaccae* group). The *M. vaccae* group was immunized subcutaneously between the scapulae with the HK *M. vaccae* on day –35, day -28, and day –21 at 9:00 am. The vehicle group was treated with 100 µl sterile borate buffered saline. These methods and doses are in agreement with previous studies (Lowry et al., 2007). Injections were performed with 21 gauge needles. To ensure that each rat received an equal dose, the diluted suspension in the single vessel was gently swirled to avoid *M. vaccae* sticking to the sides and cap of the vessel. The suspension was then withdrawn directly into a syringe with no needle attached. The needle was then attached and each rat received 100 µl of the suspension, using a new needle for each animal and inverting the syringe several times after each new needle is placed.

*Apparatus.* The apparatus used has been described previously (Spannuth et al., 2011) and all parts were obtained through Kinder Scientific (Poway, CA, USA). The apparatus consisted of a darkened sound-attenuated chamber (49.35 cm (h) x 35.56 cm (w) x 27.62 cm (d)) (SM100SP StartleMonitor Cabinet/ Service Pack). Inside the chamber, an aluminum base plate was connected to a grounding cable which was meant to reduce any electrical noise. An animal sensing plate for rats (SM2001) containing a piezoelectric transducer was mounted on the base plate. The transducer measured startle response by converting mechanical displacement of the animal sensing plate due to the rat's startle response into a voltage output. This voltage output was amplified and presented to a 12-bit analog to digital (A/D) converter and recorded in newtons (N). Startle amplitude was defined as the maximum voltage output during the initial 200 msec following a noise burst. The transducer was calibrated prior to each baseline/FPS testing session using a Newton Impulse Calibrator (SMCAL; Kinder Scientific, Poway, CA, USA). The full-scale setting was determined experimentally so that the maximum startle responses did not exceed 75-80% of the full-scale setting. On top of the animal sensing plate, an animal restrainer (9.5 cm (w) x 17.75 cm (h) x 17.75 cm (l)) was placed and was equipped with an adjustable ceiling so the rat had sufficient head room but was unable to rear. This setup was also helpful in reducing stress on the rat while minimizing excessive movement. The height of the ceiling was kept consistent through the testing. The animal restrainer also contained 4 stainless steel bars (21.6 cm (l); 0.64 cm diameter; 1.3 cm between each bar) which were used to deliver scrambled foot shocks (0.6 mA, 0.5 sec). The shocks were generated by a Dual Programmable Shocker (scrambled output; DSCK-D; Kinder Scientific, Poway, CA, USA). The chamber also contained lights 10 cm above the animal restrainer for delivering the conditioned stimulus (CS) and two speakers (10 cm above animal restrainer) for startle stimulus and background white noise (60 dB, 3-32 kHz). Noise bursts and background noise were delivered using a computer-generated sound file that is amplified through and auxiliary amplifier (AUXAMP-B; Kinder Scientific, Poway, CA, USA) and delivered through the speakers in the chamber. The Startle Monitor software (Build 06262-18; Kinder Scientific, Poway, CA, USA) was used to program the presentation, timing and sequencing of all auditory, tactile and visual stimuli. A control chassis (SM100CC Control Chassis; Kinder Scientific, Poway, CA, USA) with a microprocessor controller provided an interface between the data collection effort and the PC.

*Baseline: startle testing and matching.* Following one week of acclimation to the colony, rats were placed into animal restrainer inside the sound-attenuated chamber for 20 minutes starting on day -35. After 5 minutes, rats were exposed to 30 noise stimuli (10 trials each of 90, 95 and 105 dB) presented quasi-randomly between every 30 second inter-trial interval (ITI), each intensity only occurring once in a block of 3 noise stimuli. Once the baseline startle tests were complete, the subjects were matched into 2 groups (12 subjects each) so each group had equivalent baseline mean startle amplitudes. This process was repeated again 29 days after the initial immunization to establish whether there was a difference in acoustic startle attributable to *M. vaccae* immunization. This baseline startle measurement acclimates animals to the procedure and chamber, avoiding additional contextual conditioning (Davis 2001). The experimental timeline can be visualized below in Figure 2.

**Experiment timeline**
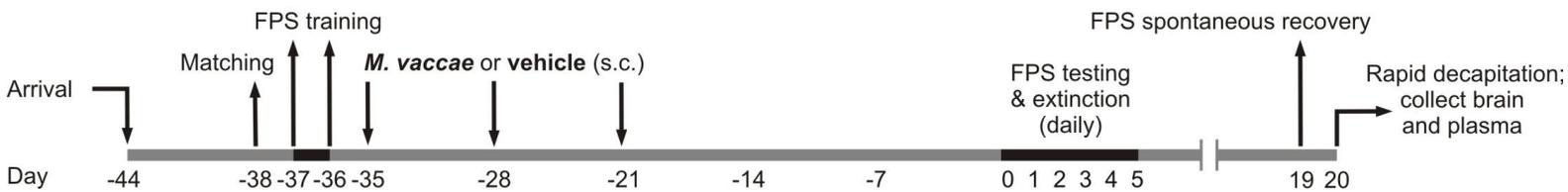(N = 24 Sprague Dawley rats)



*Figure 2.* The experimental timeline. Rats arrived on day -45. Starting on day –38, rats were placed in sound-attenuated chamber and tested for baseline startle responses. Based on these responses, rats were assigned to either the *M. vaccae* group or the Vehicle group, with each group having a roughly equal average baseline startle. On days –37 and –36, rats were fear conditioned. Starting on day –35, rats were immunized with *M. vaccae* or vehicle and again on day –28 and –21. On days 1-6, all rats were tested for FPS. On day 20, rats were again tested for FPS to investigate spontaneous recovery of fear. On day 21, rats were euthanized.

*Training: fear conditioning.* 24 hours and 48 hours following baseline testing (days 2 and 3), rats were returned to the animal restrainer and chamber for fear-conditioning training. This phase involved 5 minutes of acclimation followed by presentation with 10 conditioned (CS, Light; 115 lux) – unconditioned (UVS, foot shock; 0.6 mA) stimuli pairings. The ITI between stimuli varied between 3 and 5 minutes. The light (3.7 seconds) co-terminated with the foot shock (0.5 seconds). This procedural element can be visualized in Figure 3c.

*Testing: Fear-potentiated startle and extinction.* 6 weeks after the fear-conditioning training, rats were returned to animal restrainer and chamber for a 42.5-minute testing session. After 5 minutes of acclimation, "Leaders" were presented as 15 noise bursts (5 trials each at 90, 95 and 105 dB) with a 30-second ITI. Following the "Leader" trials, rats were exposed to test trials with a 30-second ITI. In the "light-noise" half of the test trials (CS+/AS+ trials), a 0.5 sec noise burst was administered 3.2 seconds after light onset (CS) and these were co-terminated. In the other "noise-alone" half of the test trials (CS-/AS+ trials), a startle-eliciting burst (0.5 seconds) occurred in the absence of the light (3). In both the CS+/AS+ and the CS-/AS+ trials, the sound intensity was divided equally among 90, 95 and 105 dB. Each trial was presented quasi-randomly with each trial type occurring only once within each successive block of 6 trials. Following test trials, rats were exposed to test trials with a 30-second ITI, called

"Trailers", which were identical to "Leaders" except exposure occurred following testing. These same conditions were repeated each day over 6 consecutive days (days 1-6) and on a recovery day (day 20) to assess any changes in the acoustic startle response in the presence of the CS+ to determine the rate of extinction learning to the CS+. Rats were euthanized 24 hours after the final FPS exposure with administration of 100 mg/kg sodium pentobarbital intraperitoneally followed by rapid decapitation.
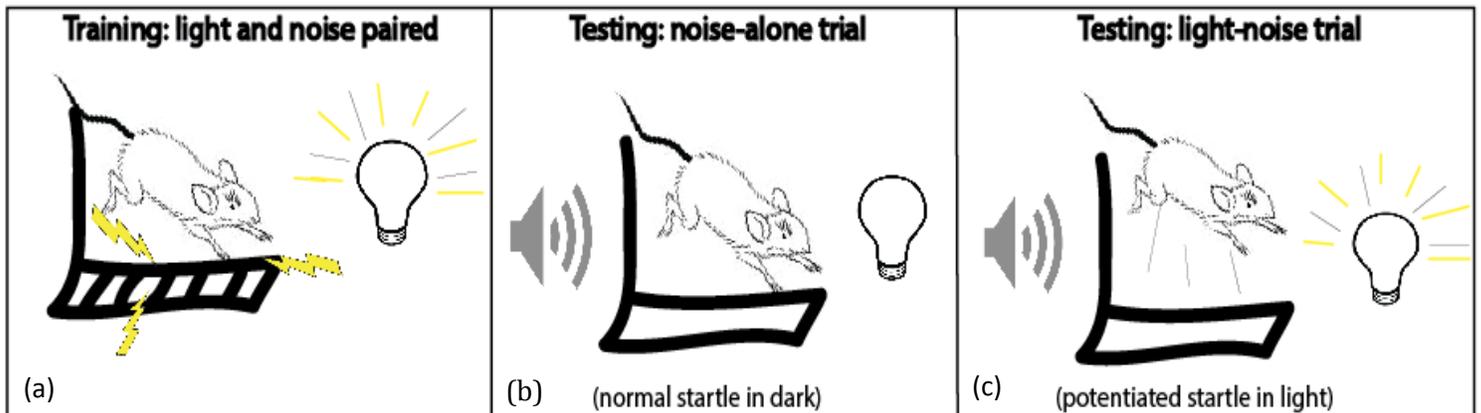


*Figure 3 (a-c).* The FPS model. A conditioned stimulus (light) is presented in training (a) and paired with a foot shock. During testing, startle is elicited through an auditory stimulus at three volumes, 90 dB, 95 dB, and 105 dB (b) in the dark for one group (b) and in the light (conditioned stimulus present) in the other group (c). This experimental design was originally tested by Brown et al., 1951.
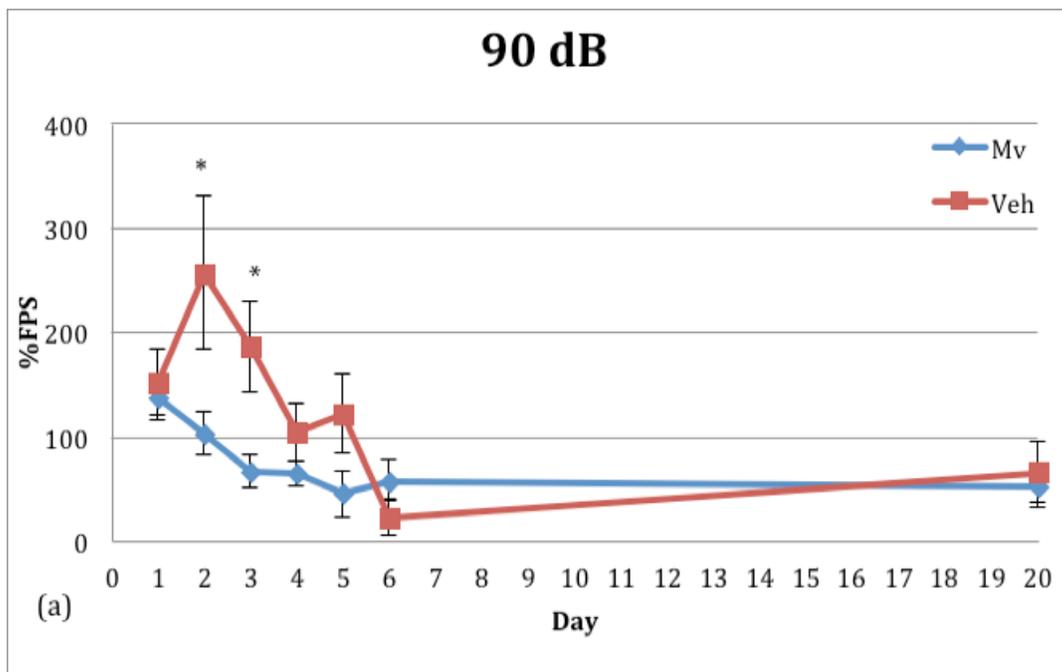
*Statistical analysis.* At each volume, fear potentiation was determined by measuring the increase in startle with the light on as compared to the light off, divided by the light off, multiplied by 100 (CS+ - CS-/CS-) x100. For the Davis analysis, the mean startle amplitude was calculated across 15 Leaders, 15 Trailers, 15 Leaders + 15 trailers, 30 Noise-alone test trials and 30 Light-noise test trials. The mean startle amplitude of Leaders + Trailers was subtracted from the mean startle amplitude of the 30 Light-noise test trials, and this result was divided by the mean startle amplitude of the Leaders + Trailers and multiplied by 100 to determine the percent potentiated startle relative to Leaders and Trailers. Next, the mean startle amplitude of Noise-alone test trials was subtracted from the mean startle amplitude of the Light-noise test trials. This result was divided by the mean startle amplitude on Noise-alone test trials and multiplied by 100 to yield the percent FPS relative to the Noise-alone test trials. Finally, the mean startle amplitude on the matching day was subtracted from the mean startle amplitude of the Leaders, then divided by the mean startle amplitude during matching (groups with the most similar mean startle amplitudes) and multiplied by 100 to yield the percent context conditioning from matching to testing relative to the Leaders. Prior to analysis, outliers were removed from the dataset using statistical tests for single outliers (Grubbs,1969); a maximum of one outlier was removed from a single group on any given day. These outliers were not included in the analysis and were not represented in the graphs. Data for FPS was analyzed two ways-- by a linear mixed model and a Fisher's Least Significant Difference (LSD) test. The linear mixed model was used to determine if any factors had any significant effects on the data results, and the Fisher's LSD test determined whether the two treatment groups differed from each other at any particular day. The linear mixed model is a model that is advantageous in a variety of ways. Mainly, this model allows the ability to accommodate missing data points as well as take into

consideration random experimental factors that may not have otherwise been considered (Krueger et al., 2004). These analysis methods were applied to all three volumes (90 dB, 95 dB, 105 dB) as well as the data analyzed using the Davis analysis (2001).

**Results**

The percent FPS calculated in the statistical analysis using the Fisher's LSD test and a linear mixed model was calculated, accounting for 6 consecutive days of FPS testing, as well as on the recovery day. The linear mixed model and Fisher's LSD tests were performed for 90 dB (Figure 4a), 95 dB (Figure 4b), 105 dB (Figure 4c), FPS relative to Leaders and Trailers (Figure 5a), and FPS relative to Noise-alone trials (Figure 5b). In the linear mixed model, treatment (*M. vaccae* or vehicle) was the fixed factor and time (days) were considered the repeated measure. A survey of the possible covariance structures was performed by assessing which -2 Log Likelihood value was closest to 0, indicating the most ideal structure. Structures compared were the Autoregressive, Compound Symmetry, Diagonal, Toeplitz, and Unstructured structures. The dependent variable used in the test was FPS, the fixed factors included were treatment, day, and a treatment*day nested factor. Random factors that may have been influencing the results were included, and both day and enclosure number were considered. The estimation method used was Maximum Likelihood, and the model statistics included parameter estimates.

The repeated covariance structure type used for 90 dB was a Diagonal structure, as the -2 Log Likelihood value (1908.478) was closest to zero compared to all other structures assessed. At the 90 dB intensity, day was found to be a significant factor ($p=.000$), as well as treatment ($p=.002$), and treatment*day ($p=.019$). The Fisher's LSD test performed for 90 dB showed significant differences between the *M. vaccae* and vehicle groups on day 2 and day 3 (Figure 4a).
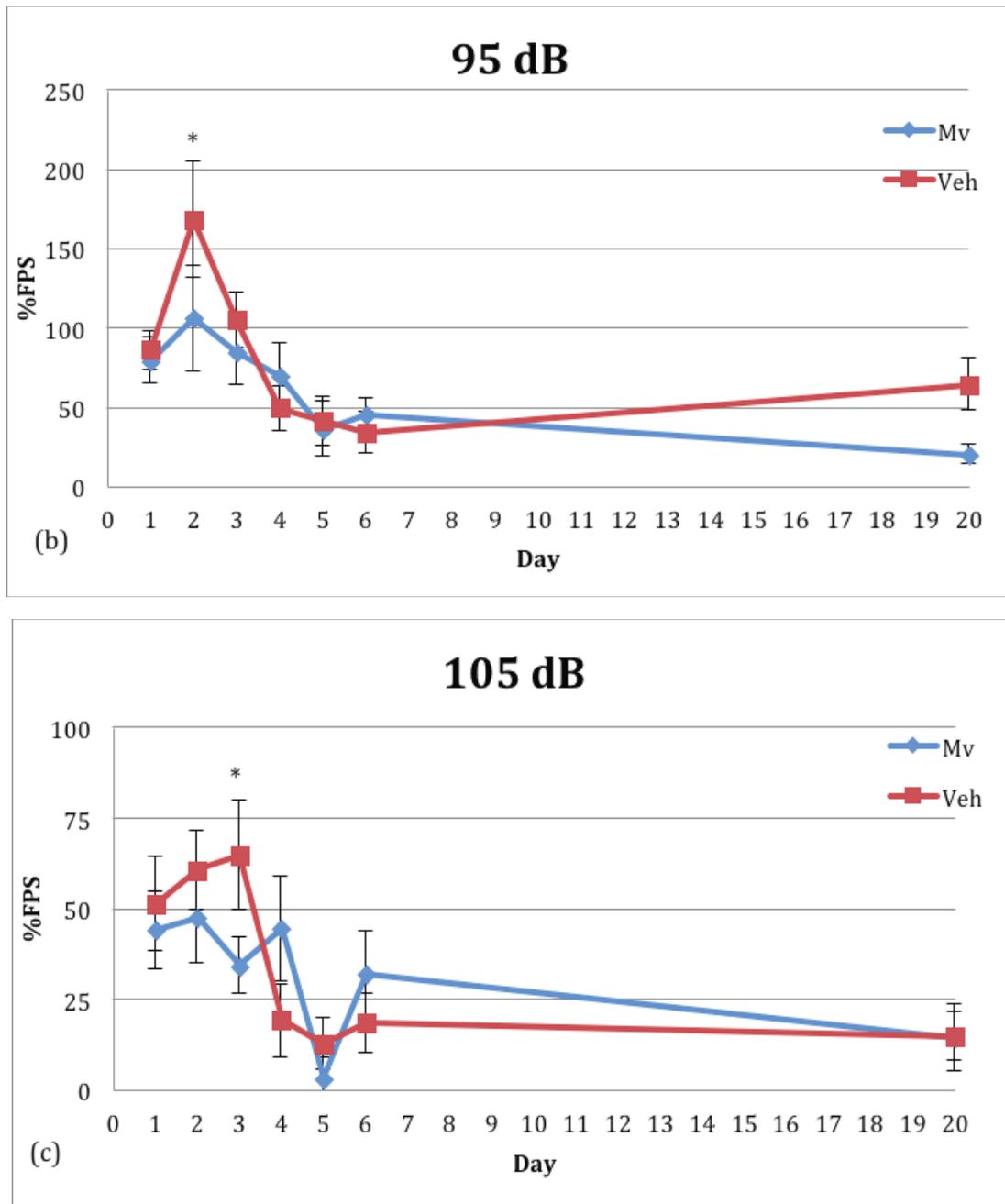

(a)

*Figure 4 (a-c).* Percent FPS was measured for all 6 consecutive days and the spontaneous recovery day at the 90 dB (a), 95 dB (b), and 105 dB dB (c) noise intensity levels. Percent fear potentiation was determined by measuring the ratio between startle with the light on as compared to the light off. Both *M. vaccae* (Mv) and vehicle (Veh) groups were included in the percent FPS analysis and compared. Sample included n=24 rats. Data represents mean +/- standard error. A Fisher's LSD test was used to compare means between groups on each day, for all intensities. FPS was significantly different between the *M. vaccae* and control group on days 2 and 3 (90 dB), day 2 (95 dB), and day 3 (105 dB). (* significant at p ≤ .05)

At the 95 dB intensity, an Unstructured repeated covariance structure type was chosen because the -2 Log Likelihood value reported (1754.618) was closest to 0 of the structures compared. At 95 dB, day was a significant factor ($p=.001$), but treatment was not significant ($p=.283$) and treatment*day was not significant ($p= .199$). For the 95 dB Fisher's LSD test, day 2 was found to be significantly different between the treatment groups (Figure 4b).

At the 105 dB intensity, an Unstructured covariance structure was used, with a -2 Log Likelihood score of 1590.217 closest to 0 as compared to other covariance structures assessed. At 105 dB, day was found to be a significant factor ($p=.000$), but treatment*day was not significant ($p=.071$), and treatment alone was not significant ($p=.757$). For the Fisher's LSD test conducted on each day at 105 dB, percent FPS was significantly different between the *M. vaccae* group and the control group on day 3 (Figure 4c).

In the Davis analysis, a Diagonal repeated covariance structure type was selected to be the best model for FPS relative to Leaders and Trailers, as the -2 Log Likelihood value was 1714.571, the closest value to 0 of any structure tested. For this analysis, it was found that day was a significant factor ($p<.000$), but treatment was not significant ($p=.862$) and neither was treatment*day ($p=.116$). For Fisher's LSD test, there were no specific days in the FPS relative to Leaders and Trailers analysis where the percent FPS for the two treatment groups were different from each other (Figure 5a). For the FPS relative to Noise-alone trials, an Unstructured repeated covariance structure type was selected to be the best model for FPS relative to Noise-alone test trials, as the -2 Log Likelihood value was 1628.010, closest to 0 of all structures considered. It was found that day was a significant factor ($p<.000$) as well as treatment*day ($p=.018$), but treatment alone was not significant ($p=.184$). For the Fisher's LSD test, percent FPS was significantly different between the *M. vaccae* group and the control group on day 2 (Figure 5b).
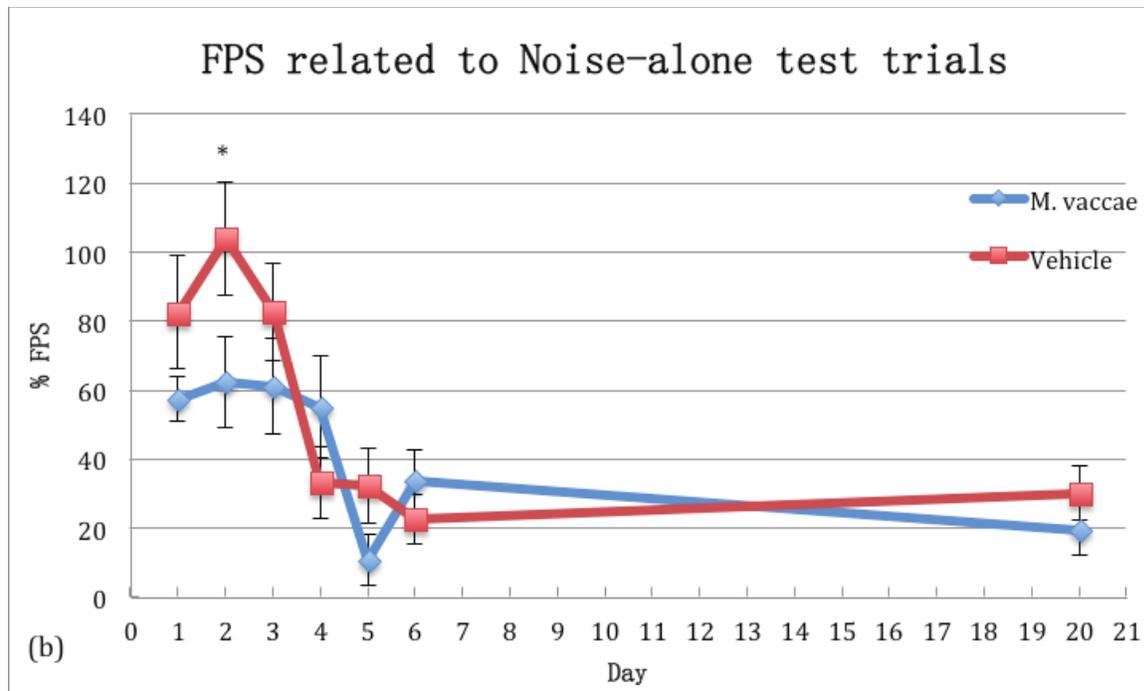


(a)
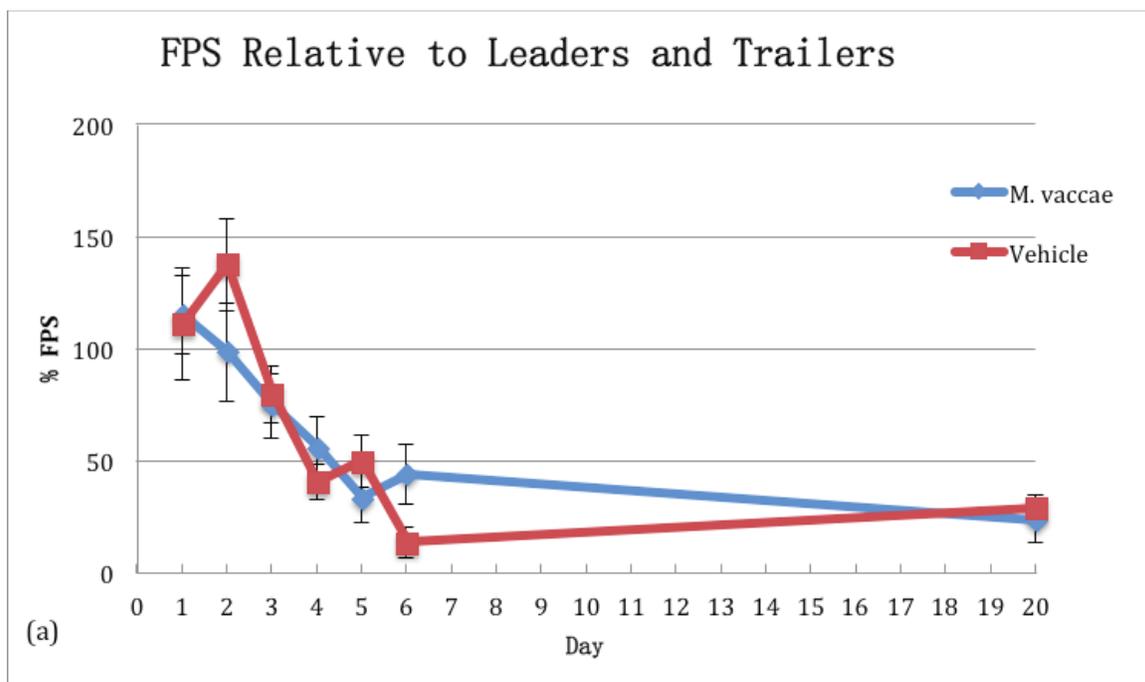
*Figure 5 (a and b).* Percent FPS at all noise intensities was measured relative to Leaders + Trailers (2a) and Noise-alone trials (2b). Results were plotted for all 6 consecutive testing days and the spontaneous recovery day 7. *M. vaccae* and vehicle groups were both considered. Percent fear potentiation was determined by measuring the ratio between startle with the light on as compared to the light off and following the steps in the Davis analysis (2001). Sample included n=24 rats. Data represents mean +/- standard error. A Fisher's LSD test was used to compare means between groups on each day, for all intensities. For the Fisher LSD test, there was a significant difference between percent FPS in the *M. vaccae* group as compared to the vehicle group on day 2. (p ≤ .05)

**Discussion**

Based on the results, percent FPS in the *M. vaccae* group was overall significantly different from percent FPS in the vehicle group at 90 dB. The treatment*day nested factor was found to be a significant factor at 90 dB and in the Davis analysis (FPS relative to Noise-alone trials). This finding may implicate *M. vaccae* as a viable treatment route for neuropsychiatric disorders associated with FPS. In the Behavioral Neuroendocrinology Laboratory at CU-Boulder, some preliminary results display that *M. vaccae* in the form of a preimmunization seems to act preventatively to reduce acquisition of fear due to conditioning. On the other hand, results from this experiment suggest *M. vaccae*'s ability to extinguish existing conditioned fear through extinction learning faster, thus reducing percent FPS more overall. Fear conditioning is a product of evolution, promoting survival and essential to defensive behaviors in mammals (reviewed by Fanselow 1994). Disturbances in this type of learning may contribute to neuropsychiatric disorders like PTSD, panic disorder and specific phobias (Rosen et al., 1998; reviewed by Wolpe et al., 1988). A difference in fear conditioning would have been apparent with significant differences towards the beginning of the experiment, when fear conditioning was recent. The reductions in FPS seen in the *M. vaccae* treatment group implicate it as a possible treatment for disorders involved with extinction learning as opposed to fear conditioning.

In addition to an overall reduction of FPS responses at 90 dB, since there were notable differences between the two groups tending to appear earlier in the testing on days 2 and 3, this indicates faster extinction learning with *M. vaccae* treatment. Fear extinction and conditioned fear responses are interwoven, but a body of research dating back nearly a century points to how the two are inherently separate (Pavlov 1927; Quirk et al., 2002; Konorski et al., 1967; Rescorla et al., 1975; Bouton et al., 1983; Herry et al. 2002). In his studies of appetitive conditioning, Pavlov showed that conditioned fear associations become extinguished when the conditioned stimulus (CS) is presented repetitively in the absence of the unconditioned stimulus (US), but the conditioned responses that had become extinguished would spontaneously recover over time (Pavlov 1927). His study showed that extinction did not erase the conditioned association, but rather inhibited the conditioned response. This discovery is what led to the concept that conditioning memory and extinction memory are inherently separate. The decrease in percent FPS seen throughout the experiment suggests that *M. vaccae* acted as a therapeutic agent resulting in the enhancement of extinction learning. Also, the significant effect of day on percent FPS throughout the experiment point to highlight use of extinction learning, considering the startle responses changes over time as the conditioned fear was extinguished.

In terms of neurobiology, the effectiveness of *M. vaccae* in reducing percent FPS brings into consideration the proposed association between the neural pathway (Figure 1) and experimental model. Assuming the validity of the suggested pathway, the convergence of the LA, BLA, and CE of the amygdala with the startle pathway must be a critical region in the acquisition of fear (reviewed Lang et al., 2000, Hitchcock et al., 1986, Davis et al., 1995, Sananes et al., 1992). Studies have shown that destruction of neurons in the BLA or CE is detrimental to the acquisition of fear (Davis et al., 1995; Maren et al., 1996). Another brain region of interest in terms of fear conditioning is the hippocampus. Using functional magnetic response imagery, one study found that hippocampal activity appears to develop during fear acquisition (Knight et al., 2004). Both the amygdala and the hippocampus, particularly the amygdala, are crucial in the process of forming new associations as environmental stimuli change.

The existing research on fear extinction primarily focuses on two interconnected brain regions: the medial prefrontal cortex (mPFC) and the amygdala. The ventral medial prefrontal cortex (vmPFC) was first connected to fear extinction in one study where vmPFC lesions impaired extinction (Morgan et al., 1993), but further investigation demonstrated solely long-term extinction learning impairment (Quirk et al., 2000). Some data suggests that extinction learning is impacted by changes in mPFC synaptic plasticity, or the ability of synapses to strengthen or weaken over time (Herry et al., 1999; Herry et al., 2002). In this study, depressing synaptic efficacy in the mPFC following extinction impaired the long-term memory of extinction learning. It has been shown that normal functioning of this brain region is essential to the benefits of cognitive therapy, and that up to 40% of patients with PTSD do not maintain these benefits in clinical follow-up studies (Tarrier et al., 1999). Stimulation of the mPFC has been shown to alter the response rate between the BLA, LA and CE of the amygdala (Quirk et al., 2003). The involvement of the amygdala displayed in studies of fear extinction validates the neural pathway proposed in Figure 1. Recent investigation into the amygdala's role in fear extinction focuses on N-methyl D-aspartate (NMDA) receptors, a subtype of glutamate-gated ion channel important in memory and learning (VanDongen 2008). One study found that injecting an NMDA agonist into the amygdala enhanced extinction learning (Walker et al., 2002). Both the acquisition and extinction of fear appear to

involve this critical brain region. Together, this body of evidence highlights the importance of pursuing a better understanding of the relationship between the mPFC and the amygdala in order to further understand the extinction learning effects seen in this experiment.

The results obtained in this study have a variety of applications, particularly in the realm of neuropsychiatric disorders. Exaggerated startle is known to be one of the cardinal symptoms of PTSD (Grinker 1945; Morgan III et al., 1995; Kardiner 1941). There is physiological evidence of an exaggerated startle response in veterans with combat-related PTSD (Butler et al., 1990). This disorder has been referenced in medical literature for hundreds of years, but it was not classified as a clinical diagnosis within the category of anxiety disorders in the DSM-III until 1980 (Kolb et al., 1984). This disorder can come about for a variety of catastrophic events falling outside the range of typical human experience. Although progress has been made in understanding and characterizing this disorder, most diagnostic criteria rely on patient self-reporting. Because exaggerated startle is listed as a symptom of PTSD, acoustic startle could represent an index of CNS dysregulation in this disorder. The startle reflex is applicable to both animals and humans, which makes the effectiveness of *M. vaccae* treatment in reducing percent FPS compelling. Although the results may offer a compelling treatment option for PTSD, some studies argue against the idea that startle responses are exaggerated in PTSD. One study found that a significant percentage of patients with PTSD lack exaggerated startle (Ross et al., 1989). In another study, war veterans with PTSD had a history of comorbid alcohol dependence (Morgan III et al., 1995). Alcohol withdrawal has been shown to increase startle and may have been a confounding variable in the study (Krystal et al., 1997). The lack of consensus about the relationship between PTSD and the startle response in the research community highlights a need for additional testing.

There is a list of current treatment options for PTSD, and with more research, *M. vaccae* may become a valuable addition to this list. According to one metaanalysis, one of the most effective PTSD treatments that exists currently is behavior therapy and Eye Movement Desensitization and Reprocessing (Van Etten et al., 1998). One class of drug therapy that has proven effective is selective serotonin reuptake inhibitors (SSRIs), which work to increase 5-HT levels in the brain. Fluoxetine is an example of an SSRI which has shown efficacy in the treatment of PTSD (Van Etten et al., 1998). Another interesting treatment option is cortisol. The logic behind this treatment is that patients with PTSD have been found to have lower levels of urinary cortisol (Yehuda et al., 1990). Since elevated cortisol levels inhibit memory retrieval, perhaps administration of this hormone may reduce excessive revisiting of traumatic memories in PTSD patients. One metaanalysis, however, argues that low cortisol levels in PTSD is only found under certain conditions, like physical abuse or afternoon samples (Meewisse et al., 2007). In one study, low-dose cortisol (10 mg/day) was administered orally for 1 month, and cortisol-related reductions of 38% in daily traumatic memory recall was recorded (Dominique et al., 2007). With further research on current PTSD treatment options and continuing *M. vaccae* animal trials, *M. vaccae* may someday reach human trials where its efficacy can be directly compared to current treatments.

## References

Arkwright, P. D., and Timothy J. David. "Intradermal administration of a killed Mycobacterium vaccae suspension (SRL 172) is associated with improvement in atopic dermatitis in children with moderate-to-severe disease." *Journal of allergy and clinical immunology* 107.3 (2001): 531-534.

Bazzi, S., et al. "Analysis of the immunomodulatory properties of two heat-killed mycobacterial preparations in a human whole blood model." *Immunobiology* 220.12 (2015): 1293-1304.

Bouton, M., et al. "Contextual control of the extinction of conditioned fear: tests for the associative value of the context." *Journal of Experimental Psychology: Animal Behavior Processes* 9.3 (1983): 248.

Brown, J. S., et al. "Conditioned fear as revealed by magnitude of startle response to an auditory stimulus." *Journal of experimental psychology* 41.5 (1951): 317.

Butler, R. W., et al. "Physiological evidence of exaggerated startle response in a subgroup of Vietnam veterans with combat-related PTSD." *Am J Psychiatry* 147.10 (1990): 1308-1312.

Campbell, B. A., et al. "Origins of orienting and defensive responses: An evolutionary perspective." *Attention and orienting: Sensory and motivational processes* (1997): 41-67.

Camporota, L., et al. "The effects of Mycobacterium vaccae on allergen-induced airway responses in atopic asthma." *European Respiratory Journal* 21.2 (2003): 287-293.

Charney, D. S., et al. "Psychobiologic mechanisms of posttraumatic stress disorder." *Archives of General Psychiatry* 50.4 (1993): 294-305.

Davis, M., et al. "Pharmacological and anatomical analysis of fear conditioning using the fear-potentiated startle paradigm." *Behavioral neuroscience* 100.6 (1986): 814.

Davis, Michael. "The role of the amygdala in fear-potentiated startle: implications for animal models of anxiety." *Trends in pharmacological sciences* 13 (1992): 35-41.

Davis, M., et al. "Involvement of the central nucleus and basolateral complex of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli." *The Journal of Neuroscience* 15.3 (1995): 2301-2311.

Davis, M., et al. "Fear-potentiated startle in rats." *Current Protocols in Neuroscience* (2001): 8-11.

Davis, M., et al. "Serotonin does not mediate anxiolytic effects of buspirone in the fear-potentiated startle paradigm: comparison with 8-OH-DPAT and ipsapirone." *Psychopharmacology* 94.1 (1988): 14.

Dominique, J. F. et al. "Glucocorticoid-induced reduction of traumatic memories: implications for the treatment of PTSD." *Progress in brain research* 167 (2007): 239-247.

Fanselow, M. S., et al. "Neural organization of the defensive behavior system responsible for fear." *Psychonomic Bulletin & Review* 1.4 (1994): 429-438.

Furness, J. B. *The Enteric Nervous System* (Blackwell, Oxford, 2006).

Gabriel, M. V., et al. *Neurobiology of cingulate cortex and limbic thalamus: a comprehnsive handbook*. No. 577.25 NEU. 1993.

Grinker, R. R. "War neuroses." (1945).

Grubbs, F. E. "Procedures for detecting outlying observations in samples." *Technometrics* 11.1 (1969): 1-21.

Herry, C., et al. "Plasticity in the mediodorsal thalamo-prefrontal cortical transmission in behaving mice." *Journal of Neurophysiology* 82.5 (1999): 2827-2832.

Herry, C., et al. "Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice." *The Journal of neuroscience* 22.2 (2002): 577-583.

Hitchcock, J., et al. "Lesions of the amygdala, but not of the cerebellum or red nucleus, block conditioned fear as measured with the potentiated startle paradigm." *Behavioral neuroscience* 100.1 (1986): 11.

Kapp, B. S., et al. "Amygdaloid contributions to conditioned arousal and sensory information processing." (1992).

Kardiner, A. *The traumatic neuroses of war*. National Academies, 1941.

Kehne, J. H., et al. "Anxiolytic effects of buspirone and gepirone in the fear-potentiated startle paradigm." *Psychopharmacology* 94.1 (1988): 8-13.

Klingler, J., et al. "The connections of the amygdala and of the anterior temporal cortex in the human brain." *Journal of Comparative Neurology* 115.3 (1960): 333-369.

Knight, D. C., et al. "Amygdala and hippocampal activity during acquisition and extinction of human fear conditioning." *Cognitive, Affective, & Behavioral Neuroscience* 4.3 (2004): 317-325.

Kolb, L. C. "The post-traumatic stress disorders of combat: a subgroup with a conditioned emotional response." *Military Medicine* (1984).

Konorski, J. "Qualitative versus directional cues in differential conditioning. I." *Left leg-right leg differentiation to cues of a mixed character* 27 (1967): 163-168.

Krueger, C., et al. "A comparison of the general linear mixed model and repeated measures ANOVA using a dataset with multiple missing data points." *Biological research for nursing* 6.2 (2004): 151-157.

Krystal, J. H., et al. "Evidence of acoustic startle hyperreflexia in recently detoxified early onset male alcoholics: modulation by yohimbine and m-chlorophenylpiperazine (mCPP)." *Psychopharmacology* 131.3 (1997): 207-215.

Lang, P. J., et al. "Fear and anxiety: animal models and human cognitive psychophysiology." *Journal of affective disorders* 61.3 (2000): 137-159.

Lehrer, A., et al. "Immunotherapy with Mycobacterium vaccae in the treatment of psoriasis." *FEMS Immunology & Medical Microbiology* 21.1 (1998): 71-77.

Lowry, C. A., et al. "Identification of an immune-responsive mesolimbocortical serotonergic system: potential role in regulation of emotional behavior." *Neuroscience* 146.2 (2007): 756-772.

MacLean, P. D., et al. *Neurobiology of Cingulate Cortex and Limbic Thalamus: A Comprehensive Handbook* 1-15 (Birkhauser, Boston, 1993).

Maes, M. "Major depression and activation of the inflammatory response system." *Cytokines, stress, and depression*. Springer US, 1999. 25-46.

Maren, S. "The amygdala and fear conditioning: has the nut been cracked?." *Neuron* 16.2 (1996): 237.

Maren, S. "Neurobiology of Pavlovian fear conditioning." *Annual review of neuroscience* 24.1 (2001): 897-931.

Margot A., et al. "Immunization with heat-killed Mycobacterium vaccae stimulates CD8+ cytotoxic T cells specific for macrophages infected with Mycobacterium tuberculosis." *Infection and immunity* 65.11 (1997): 4525-4530.

Matthews, D. M., et al. "Ingestion of Mycobacterium vaccae decreases anxiety-related behavior and improves learning in mice." *Behavioural processes* 96 (2013): 27-35.

Mayer, E. A. "Gut feelings: the emerging biology of gut–brain communication." *Nature Reviews Neuroscience* 12.8 (2011): 453-466.

Meewisse, M., et al. "Cortisol and post-traumatic stress disorder in adults." *The British Journal of Psychiatry* 191.5 (2007): 387-392.

Morgan, M. A., et al. "Extinction of emotional learning: contribution of medial prefrontal cortex." *Neuroscience letters* 163.1 (1993): 109-113.

Morgan III, et al. "Fear-potentiated startle in posttraumatic stress disorder." *Biological psychiatry* 38.6 (1995): 378-385.

Moser, V. "The Human Microbiome: The Brain-Gut Axis and its Role in Immunity." (2014).

Nestler, E. J., et al. "Neurobiology of depression." *Neuron* 34.1 (2002): 13-25.

Nijm, J., et al. "Impaired cortisol response to acute stressors in patients with coronary disease. Implications for inflammatory activity." *Journal of internal medicine* 262.3 (2007): 375-384.

O'Brien, M. E., et al. "SRL172 (killed Mycobacterium vaccae) in addition to standard chemotherapy improves quality of life without affecting survival, in patients with advanced non-small-cell lung cancer: phase III results." *Annals of Oncology* 15.6 (2004): 906-914.

Pace, T. W., et al. "Increased stress-induced inflammatory responses in male patients with major depression and increased early life stress." *American Journal of Psychiatry* (2006).

Pavlov, I. P. *Conditioned reflexes: An investigation of the physiological activities of the cerebral cortex.* 1927.

Quirk, G. J., et al. "The role of ventromedial prefrontal cortex in the recovery of extinguished fear." *The Journal of Neuroscience* 20.16 (2000): 6225-6231.

Quirk, G. J., et al. "Memory for extinction of conditioned fear is long-lasting and persists following spontaneous recovery." *Learning & Memory* 9.6 (2002): 402-407.

Quirk, G. J., et al. "Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons." *The Journal of Neuroscience* 23.25 (2003): 8800-8807.

Rescorla, R. A., et al. "Reinstatement of fear to an extinguished conditioned stimulus." *Journal of Experimental Psychology: Animal Behavior Processes* 1.1 (1975): 88.

Romano, M., et al. "Potential of Mycobacterium tuberculosis resuscitation-promoting factors as antigens in novel tuberculosis sub-unit vaccines." *Microbes and Infection* 14.1 (2011): 86-95.

Rook, G. A., Raison, and Lowry, C. A. "Can we vaccinate against depression?." *Drug discovery today* 17.9 (2012): 451-458.

Rosen, J. B., et al. "Immediate-early gene expression in the amygdala following footshock stress and contextual fear conditioning." *Brain research*796.1 (1998): 132-142.

Ross, R. J., et al. "Habituation of the startle reflex in posttraumatic stress disorder." *The Journal of neuropsychiatry and clinical neurosciences* (1989).

Sananes, C. B., et al. "N-methyl-D-aspartate lesions of the lateral and basolateral nuclei of the amygdaloid block fear-potentiated startle and shock sensitization of startle." *Behavioral neuroscience* 106.1 (1992): 72.

Sarter, M., et al. "Involvement of the amygdala in learning and memory: a critical review, with emphasis on anatomical relations." *Behavioral neuroscience* 99.2 (1985): 342.

Schiepers, O. J., et al. "Cytokines and major depression." *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 29.2 (2005): 201-217.Skinner,

Smit, J. J., et al. "Mycobacterium vaccae administration during allergen sensitization or challenge suppresses asthmatic features." *Clinical & Experimental Allergy* 33.8 (2003): 1083-1089.

Spannuth, B. M., et al. "Investigation of a central nucleus of the amygdala/dorsal raphe nucleus serotonergic circuit implicated in fear-potentiated startle." *Neuroscience* 179 (2011): 104-119.

Tarrier, N., et al. "Relatives' expressed emotion (EE) and PTSD treatment outcome." *Psychological Medicine* 29.04 (1999): 801-811.

VanDongen, A. M. *Biology of the NMDA receptor*. CRC Press, 2008.

Van Etten, M. L., et al. "Comparative efficacy of treatments for post-traumatic stress disorder: a meta-analysis." (1998).

Walker, D. L., et al. "Facilitation of conditioned fear extinction by systemic administration or intra-amygdala infusions of D-cycloserine as assessed with fear-potentiated startle in rats." *The Journal of Neuroscience* 22.6 (2002): 2343-2351.

Wolpe, Joseph, et al. "Panic disorder: A product of classical conditioning." *Behaviour Research and Therapy* 26.6 (1988): 441-450.

Yazi, D., et al. "Treatment with Mycobacterium vaccae ameliorates airway histopathology in a murine model of asthma." *Allergy and Asthma Proceedings*. Vol. 29. No. 1. OceanSide Publications, Inc, 2008.

Yehuda, R., et al. "Low urinary cortisol excretion in patients with posttraumatic stress disorder." *The Journal of nervous and mental disease* 178.6 (1990): 366-369.

Zhu, C., et al. "The proinflammatory cytokines interleukin-1beta and tumor necrosis factor-alpha activate serotonin transporters." *Neuropsychopharmacology* 31.10 (2006): 2121-2131.

Zuany-Amorim, C., et al. "Suppression of airway eosinophilia by killed Mycobacterium vaccae-induced allergen-specific regulatory T-cells." *Nature medicine* 8.6 (2002): 625-62.