Effects of preimmunization with a heat-killed preparation of *Mycobacterium vaccae* on serotonin receptor subtype 1A expression: implications for stress resilience

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Abstract

Disorders associated with inflammation, including allergy and allergic asthma, are increasing in modern urban societies. The hygiene, or "Old Friends," hypothesis attributes this escalation to the decreased exposure to immunoregulatory microorganisms. Evidence suggests that inflammation is also a risk factor for trauma- and stressor-related disorders including posttraumatic stress disorder (PTSD). PTSD can be characterized by exaggerated fear and startle and the inability to extinguish fear. *Mycobacterium vaccae* is a microorganism found in the soil that has immunoregulatory and anti-inflammatory properties; recent studies suggest that *M. vaccae* enhances fear extinction in rodent models, potentially through actions on brain serotonergic systems. The current study determined the effects of preimmunization with *M. vaccae*, or vehicle, on rats subsequently exposed to the fear-potentiated startle paradigm, when compared to home cage control rats. Immunization with *M. vaccae* enhanced between-session and within-session fear extinction. Changes in expression of *htr1a* mRNA were observed in this study, namely in the dorsomedial, rostral, and caudal parts of the dorsal raphe nucleus (DRN). These data are consistent with the hypothesis that *M. vaccae* may play a useful role in the prevention of disorders related to fear and serotonergic dysregulation.

Introduction

Posttraumatic stress disorder (PTSD) was redefined in the fifth version of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-5) as a Trauma- and Stressor-Related Disorder (American Psychiatric Association, 2013). With a lifetime prevalence of 6.8%, PTSD affects about 5.2 million adults in the United States (Kessler et al., 2005; Merikangas et al., 2011; WebMD, 2018). PTSD has a disproportionately high prevalence among those returning from combat deployment, though it is not limited to combat-related experiences (Kessler et al.,

1995; Tanielian et al., 2008). Distinguishable features of PTSD are exaggerated fear and an impaired ability to extinguish fear (Norrholm et al., 2011). Using the translational model of fear-potentiated startle (FPS), studies have found enhanced levels of startle and FPS in PTSD sufferers, along with an inability to suppress fear when safety cues are present (Grillon et al., 1998; Jovanovic et al., 2009). Recent studies have found that chronic low-grade inflammation may be another risk factor for developing PTSD (Gola et al., 2013). It has also been found that individuals with PTSD have increased serum levels of interleukin-1β, a proinflammatory cytokine (Spivak et al., 1997).This inappropriate inflammation and many inflammatory diseases are a growing problem in modern urban societies partially due to the decreased exposure of humans to beneficial microorganisms that support immune function (Rook and Lowry, 2008). This was termed the "hygiene hypothesis" by Strachan, who suggested that a clean household is strongly correlated with prevalence of atopic hay fever (Strachan, 1989). This suggests the possibility that reintroducing the "good" bacteria could be a potential preventative mechanism against excessive inflammation.

One such "good" bacteria is *Mycobacterium vaccae*, a soil-derived bacterium with anti-inflammatory and immunoregulatory properties. Evidence suggests that it may be a potential treatment or preventative strategy for ailments involving chronic low-grade inflammation, including anxiety-related disorders or trauma-, or stressor-related psychiatric disorders, including PTSD (Reber et al., 2016; Fox et al., 2017). *M. vaccae* is a saprophytic, non-pathogenic microorganism found in abundance in the soil (Zuany-Amorim et al., 2002; Hoisington, et al., 2015; Reber et al., 2016). This environmental organism increases induction of regulatory T cells (Treg) and anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor beta (TGF-β) (Zuany-Amorim et al., 2002). Furthermore, evidence was found to support the

hypothesis that immunization with heat-killed *M. vaccae* may decrease anxiety and fear-like behaviors in mice (Reber et al., 2016). Reber et al. (2016) also found that *M. vaccae* may prevent exaggeration of stressed-induced colitis and stress-induced exaggeration of chemically-induced colitis in a model of inflammatory bowel disease (IBD) (Reber et al., 2016). Data also support that *M. vaccae* may enhance fear extinction learning in rodents by altering the brain's serotonergic circuits (Fox et al., 2017).

Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter associated with the regulation of fear and anxiety states (Lowry et al., 2005; Johnson et al., 2015; Spiacci et al., 2016). A main source of serotonergic neurons lies within the dorsal raphe nucleus (DRN), a region that has direct anatomical connections with forebrain limbic structures (Parent et al., 1981; LaBar et al., 1998; Fanselow and LeDoux, 1999). Deakin and Graeff hypothesized that the DRN is split into separate subsections due to differing topography and functionality of each region (Deakin and Graeff, 1991). These regions are also thought to differ in neurochemistry, afferents, and efferents (Deakin and Graeff, 1991). Rodent studies have found that stress and anxiety-related stimuli may affect serotonin activity, specifically in the neurons located in the caudal and dorsal regions of the DRN, the dorsal raphe nucleus, caudal part (DRC) and dorsal raphe nucleus, dorsal part (DRD), respectively, which have direct anatomical connections with forebrain limbic structures, such as the amygdala (Grahn et al., 1999; Abrams et al., 2005; Spannuth et al., 2011; Paul et al., 2011; Fernandez et al., 2016; Hassell Jr. et al., 2017). Within the DRN, many presynaptic serotonin receptor subtype 1A (5-HT_{1A}) receptors are found on the serotonergic neurons (Weissmann-Nanopoulos et al., 1985). These presynaptic receptors are G protein-coupled receptors and act in an auto-inhibitory fashion to decrease serotonergic neuronal firing rates and serotonin release (Hoyer and Schoeffter, 1991; Julius, 1991; Blier et al., 1987;

Kennett et al., 1987; Sprouse and Aghajanian, 1988; Bohmaker et al., 1993; Jolas et al., 1993; Toth, 2003).

A number of lines of evidence suggest that serotonergic projections from the DRN to the amygdala play an important role in the regulation of fear. Consistent with activation of these pathways during fear conditioning and fear expression, Spannuth et al., (2011) found increased expression of the immediate-early gene, c-Fos, an indication of neuronal activity, in serotonergic neurons within the DRN after fear conditioning and fear-potentiated startle (FPS) in rats, when compared to home cage controls (Spannuth et al., 2011). Johnson et al. (2015) found that when serotonergic terminals within the amygdala were destroyed, freezing behavior, a fear response in rodents, decreased, demonstrating that serotonergic neurons projecting from the brainstem raphe nuclei to the amygdala play a role in control of fear states (Johnson et al., 2015).

Serotonin may also play a role in fear memory consolidation processes. Baratta et al. (2016) tested short-term, related to acquisition, and long-term, related to consolidation, memory of rats either exposed to immobilization stress or not exposed to immobilization stress prior to fear conditioning and discovered that freezing behavior was only increased in long-term testing in animals exposed to stress (Baratta et al., 2016). A second experiment found that when excitatory serotonin subtype 2C (5-HT_{2C}) receptors were antagonistically blocked in the lateral/basolateral amygdala, rats previously exposed to immobilization stress, but not home cage controls, exhibited less freezing behavior than rats given vehicle (Baratta et al., 2016). These findings suggest serotoninergic pathways may play an important role in fear memory consolidation enhanced by previous stress exposure.

An uncontrollable stressor, such as inescapable shock (IS), in which an animal has no control over the stressor leads to a number of negative behavioral outcomes in rats and humans

(Maier and Watkins, 1998; Maier and Seligman, 2016). These behaviors include, but are not limited to, exaggerated anxiety, impaired escape learning, exaggerated fear and fear conditioning, and reduced social interaction (Maier and Watkins, 2005). This phenomenon, coined "learned helplessness," is in contrast to the effects of controllable stress, such as escapable shock (ES) (Maier and Seligman, 1976; Maier and Watkins, 2005). Interestingly, IS, but not ES, activates and sensitizes DRN serotonergic neurons resulting in an increase in extracellular serotonin within the DRN when measured with *in vivo* microdialysis and c-Fos expression in serotonin neurons (Maswood et al., 1998; Grahn et al., 1999). One hypothesis is that the 5-HT_{1A} receptors in the DRN become desensitized, which consequently leads to an excessive amount of serotonin release in the DRN and projection regions, such as the amygdala, for prolonged periods of time (Bland et al., 2003; Christianson et al., 2010; Richardson-Jones et al., 2010; Rozeske et al., 2011). Norepinephrine release from the locus coeruleus projecting to the DRN's alpha1 adrenergic receptors is suggested to be a critical input for serotonin neuron depolarization, and consequently, serotonin release (Trulson and Crisp, 1984; Aghajanian, 1985; Grahn et al., 2002). The increase in serotonin is suggested to be partly responsible for the behavioral consequences of IS described above and the production of learned helplessness (Maier et al., 1995; Christianson et al., 2010).

The lateral habenula (LHb) is another region of the brain that provides excitatory input to the DRN (Lee et al., 2003; Aizawa et al., 2012; Sego et al., 2014). Dolzani et al. (2016) found that the increased serotonin production that IS creates could be eliminated by optogenetically silencing the LHb (Dolzani et al., 2016). This also eliminated the associated IS behaviors (Dolzani et al., 2016). Interestingly, Dolzani et al. (2016) discovered that both ES and IS led to the same magnitude of increase of Fos in LHb neurons projecting to the DRN (Dolzani et al.,

2016). This suggests that there may be other factors, such as stressor controllability, influencing the consequences of IS, rather than just afferent excitatory inputs (Dolzani et al., 2016).

The ventral medial prefrontal cortex (mPFCv) has been hypothesized to be a brain region that provides input about controllability to the DRN (Amat et al., 2005). The mPFCv contains efferent excitatory glutamatergic neurons that synapse with inhibitory GABAergic neurons in the DRN, providing the DRN with information from the mPFCv's infralimbic and prelimbic regions (Peyron et al., 1998; Jankowski and Sesack, 2004; Vertes, 2004). When stimulated, the mPFCv is expected to inhibit serotonergic neurons, and therefore, result in a decreased serotonin release (Hajos et al., 1998). Amat et al. (2005) locally injected rats with a $GABA_A$ receptor agonist, muscimol, in order to inactivate the mPFCv, and then exposed them to either uncontrollable stress or controllable stress. Under these conditions, even controllable stress resulted in exaggerated c-Fos production in serotonin neurons in the DRN and behavioral responses 24 hours later, therefore, controllable stress functioned as uncontrollable stress (Amat et al., 2005). In contrast, Amat et al. (2008) instead activated the mPFCv using local injections of the $GABA_A$ receptor antagonist, picrotoxin, and discovered that this made IS function as ES (Amat et al., 2008). Rats injected with picrotoxin and then exposed to IS did not experience the typical increase in serotonin in the DRN or the consequential IS behaviors, like impaired escape learning (Amat et al., 2008). Overall, it is reasonable to suggest that the mPFCv has critical influence on determining controllability, and thus altering neural and behavioral outcomes of stress.

Another study suggests that the stress related hormone, corticotropin-releasing hormone (CRH), has a major role in mediating IS behaviors through the corticotropin-releasing hormone receptor 2 (CRFR2) in the DRN (Koob et al., 1993; Ronan et al., 2000; Hammack et al., 2002; Hammack et al., 2003). Hammack et al., (2003) injected the CRFR2 agonist, urocortin II, into

the DRN and discovered that the rats displayed IS behavior, such as poor escape learning (Hammack et al., 2003). Furthermore, Hammack et al. (2003) injected the CRFR2 antagonist, anti-sauvagine-30, into the DRN and found that it blocked IS behaviors from occurring (Hammack et al., 2003). These findings suggest that CRFR2 may have implications in the production of learned helplessness (Hammack et al., 2003).

Another mechanism to produce learned helplessness is supported by receptor binding studies which have found that 48-72 hours following IS there is decreased 5-HT_{1A} receptor density in the DRN (Short et al., 2000). Rozeske et al., (2011) also suggests that IS leaves the serotonin neurons sensitized to further input, and indeed a 200% increase in serotonin was found in the amygdala, a terminal region of output from the DRN, after a footshock was given to rats that were exposed to IS 24 hours previously (Amat et al., 1998; Maier and Watkins, 2005). Similarly, Maier et al. (1993) found that rats previously exposed to IS demonstrated freezing behavior more than rats not exposed to IS after one or two shocks were administered, indicating an enhanced conditioned fear response 24 hours after IS (Maier et al., 1993). Furthermore, Maier et al. (1993) found that when the DRN was lesioned, it almost completely eliminated freezing behavior of IS-exposed rats before and after footshock (Maier et al., 1993). When the DRN was inactivated via the 5-HT_{1A} receptor agonist, 8-OH-DPAT, potentiation of fear conditioning and impaired escape response was also eliminated (Watkins et al., 1995). A similar result was found when the bed nucleus of the stria terminalis (BNST), part of the extended amygdala that projects to the DRN, which has been shown to release CRH under stress conditions, was lesioned (Hammack et al., 2004). Impaired escape learning and potentiated fear conditioning 24 hours after IS was blocked by the lesion (Hammack et al., 2004). Ultimately, it can be hypothesized

that the increased serotonin concentration in the DRN is a proximal cause for the IS behaviors, such as freezing, potentiated fear conditioning, and poor escape learning.

The increasing incidences of many chronic inflammatory disorders calls attention to a need for novel therapeutic approaches to treatment of anxiety and affective disorders. In the present study, we tested the effects of *M. vaccae* on fear reduction and extinction in rats using the FPS paradigm. Home cage controls were also used to compare the effects of *M. vaccae* on expression of the serotonergic gene that encodes for the 5-HT_{1A} receptor, *htr1a*, when rats were either exposed or not exposed to FPS. We predicted that we would see a change in *htr1a* mRNA expression in regions of the DRN related to fear-potentiated startle and stressor controllability.

Materials & Methods

Animals

Methods used in this study are also described in Fox et al., 2017 briefly, adult male Sprague Dawley® rats (Strain code 400, Crl:SD; Charles River, Wilmington, MA, USA) weighing 72-111 g (27-32 days old upon arrival) were pair-housed in standard polycarbonate rat cages (26 cm width x 47.6 cm length x 20.3 cm height; Alternative Design, Siloam Springs, AR, USA) containing an approximately 2.5 cm-deep layer of bedding (Cat. No. 7090; Teklad Sani-Chips; Harlan Laboratories, Indianapolis, IN, USA). This species, strain, and supplier were chosen for studies of FPS due to the extensive previous research conducted with these animals using this model (Davis, 1986). All rats were kept under standard laboratory conditions (12-h light/dark cycle, lights on at 0600 h, 22 °C) and had free access to tap water and standard rat diet (8640 Teklad 22/5 Rodent Diet, Harlan Laboratories, Indianapolis, IN, USA). Cages were changed once per week. All studies 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 the Institutional Animal Care and Use Committee at the University of Colorado Boulder approved all procedures. All possible efforts were made to minimize the number of animals used and their suffering.

Reagents. These studies used whole heat-killed *M. vaccae* NCTC 11659 suspension [10 mg/ml solution; strain National Collection of Type Cultures 11659, batch ENG#1, provided by BioElpida (Lyon, France), diluted to 1 mg/ml in 100 µl sterile borate buffered saline (BBS)].

***M. vaccae* and vehicle immunization**. Experimental rats received either subcutaneous immunization with 0.1 mg whole heat-killed *M. vaccae* suspension or received injections of 100 µl of the vehicle, sterile borate buffered saline (BBS), using 21 gauge needles and injection sites between the scapula. The dose used in these experiments (0.1 mg) was 1/10 of the dose used in human studies (1 mg) (O'Brien et al., 2004; Reber et al., 2016) and identical to the dose used in previous studies in mice (Lowry et al., 2007).

General experimental procedures

After arrival (Day –42) experimental rats (N = 47) were housed in pairs assigned to either the FPS paradigm (n = 24) or home cage controls (n = 23). Half of the rats from each group then received subcutaneous injections of *M. vaccae* while the other half received subcutaneous injections of BBS vehicle on days –35, –28, and –21. Sample sizes were 1) BBS vehicle/home cage control (n = 11), 2) BBS vehicle/FPS (n = 12), 3) *M. vaccae*/home cage control (n = 12); 4) *M. vaccae*/FPS (n = 12).

Startle apparatus

The apparatus used consisted of a darkened sound-attenuated chamber (35.6 cm width x 27.6 cm depth x 49.5 cm height; SM100SP StartleMonitor Cabinet/Service Pack; Kinder Scientific, Poway, CA, USA). Inside the chamber was an aluminum base plate (Kinder Scientific) connected to a grounding cable to reduce electrical noise. An animal sensing plate for rats (SM2001; Kinder Scientific) was mounted onto the base plate, and contained a piezoelectric transducer. The transducer measured the startle response by converting the mechanical displacement of the animal sensing plate caused by the rat's startle response into a voltage output, which was then amplified, 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 first 250 msec following each noise burst or during the first 500 msec after the onset of noise burst during a CS+ trial. The transducer was calibrated prior to baseline and once again before the first FPS testing session using a Newton Impulse Calibrator (SMCAL; Kinder Scientific). The full-scale setting was determined experimentally so that the maximum startle response reached no more than 75-80% of the full-scale setting. An animal restrainer (9.5 cm width x 17.8 cm length x 17.8 cm height) was placed on top of the animal sensing plate. The animal restrainer had an adjustable ceiling that was positioned so the rat had adequate headroom, but was unable to rear. This offered the added benefits of reducing the aversiveness of testing, while minimizing excessive movement. The ceiling level remained at the same height for the duration of FPS training and testing. The rat restrainer also contained 4 stainless steel bars (21.6 cm length; 0.6 cm diameter; 1.3 cm between each bar) used to deliver scrambled foot shocks (0.6 mA, 0.5 sec) that were generated by a Dual Programmable Shocker (scrambled output; DSCK-D; Kinder Scientific). The sound-attenuated chamber also contained a light bulb located 10 cm above the

rat restrainer that was used to deliver the conditioned stimulus (CS) and two speakers (each 10 cm above the rat restrainer) to administer the startle stimulus and background white noise (60 dB, 3 – 32 kHz). Noise bursts and background white noise were delivered using a computer-generated sound file that was amplified through an auxiliary amplifier (AUXAMP-B; Kinder Scientific) and was administered to the rats through the two speakers in the sound-attenuated chamber. The Startle Monitor software (Build 06262-18; Kinder Scientific) was used to program the presentation, timing and sequencing of all auditory, tactile and visual stimuli. A control chassis (SM100CC Control Chassis; Kinder Scientific) with an independent microprocessor controller provided a stable interface between the data collection effort and the host personal computer. After each baseline startle testing, fear conditioning training and FPS testing session, rats were returned to their respective home cages in the vivarium and, prior to subsequent testing and/or training sessions, the apparatus was thoroughly cleaned with a water and soap-wetted paper towel, or paper towel with 95% alcohol for FPS startle so as to not interfere with electrical circuits, and the floor bars were cleaned free of dried urine/feces with medium-grit sandpaper to ensure that the current flow was not obstructed.

Baseline acoustic startle matching and testing

Home cage control rats remained undisturbed in their home cages throughout the entire research protocol. For FPS, following four days of acclimation to the colony, on Day –38, (for experimental timeline, see Fig 1.) each experimental rat was placed into the animal restrainer inside the sound-attenuated chamber. After 5 min of acclimation to the restrainer, the rat was exposed to the first in a series of 30 noise stimuli (10 trials each of 90, 95, and 105 dB noise stimuli) presented quasi-randomly (each intensity only occurred once in a block of 3 noise

stimuli) with a 30-sec intertrial interval (ITI). The total session duration was 20 min. After the baseline startle test for matching, the subjects were matched into groups of 12 such that each group had equivalent baseline mean startle amplitudes. Rats were tested for baseline startle again on days 1 and 2, 36 days after the initial immunization with *M. vaccae* or vehicle, to determine if there was a difference in the baseline acoustic startle response between *M. vaccae*- and vehicle-immunized rats. The baseline startle testing also served to habituate the rats to the testing procedure and chambers in order to minimize the effects of contextual conditioning.

Figure 1

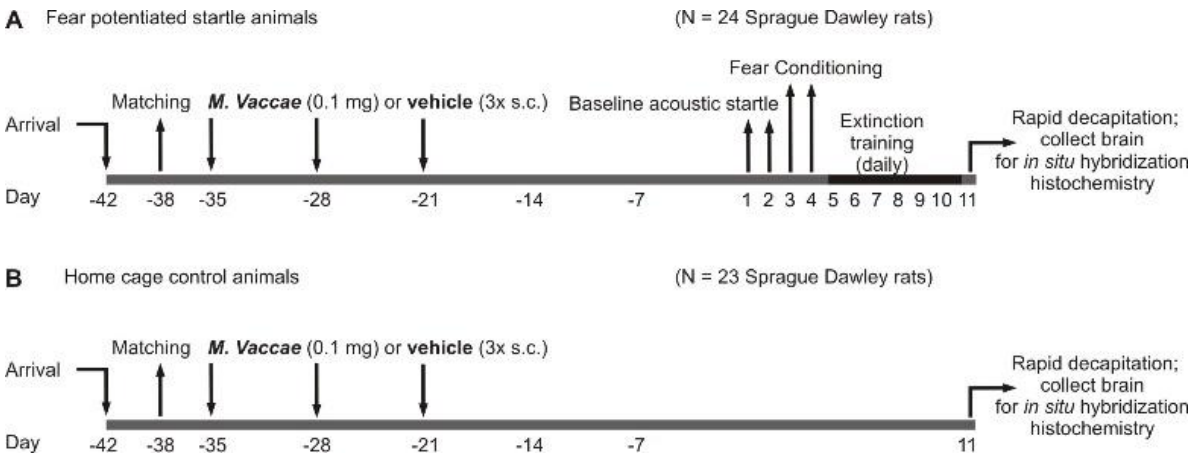

Figure 1. Diagrammatic illustration of experimental design and timeline. Day –1 is defined as the day before baseline acoustic startle testing and Day 1 is defined as the first day of baseline acoustic startle testing. One-half of the rats were assigned to the FPS protocol (**A**), whereas the remaining rats were assigned to a home cage control condition (**B**). Sample sizes (N = 47, vehicle/home cage control, n = 11; vehicle/FPS, n = 12; *M. vaccae*/home cage control, n = 12; *M. vaccae*/FPS, n = 12). All animals (N = 47) were matched for assignment of treatment groups on day –38. Abbreviations: FPS, fear-potentiated startle; s.c., subcutaneous.

Fear conditioning

Starting twenty-four hours after the second day of baseline acoustic startle testing, i.e., on days 3 and 4, rats were each placed back into the same animal restrainer/chamber on each of 2 consecutive days (separated by 24 h) for fear conditioning (or training for FPS), which consisted of 5 min of acclimation to the restrainer followed by the presentation of the first of 10 conditioned (CS, light; 115 lux) - unconditioned stimuli (US, footshock) pairings (variable ITI = 4 min; 3-5 min range). The light lasted for 3.7 sec and co-terminated with the footshock (0.6 mA, 0.5 sec). The total session duration was about 45 min.

Fear-potentiated startle (FPS) testing

On each of days 5-10, starting 24 h following the final day of two days of fear conditioning, rats were each returned to the same animal restrainer/chamber daily. After 5 min of acclimation to the restrainer, the rats were exposed to the first of 15 noise bursts (CS–/AS+ trials; 5 trials each at 90, 95 and 105 dB; presented quasi-randomly with each intensity only occurring once in a block of 3 trials; ITI = 30 sec), which were called “Leaders”. Thirty seconds after the last “Leader” trial, the rats were exposed to the first of 60 test trials (ITI = 30 sec). Half of the trials involved a 0.5 sec noise burst being administered 3.2 sec after the onset of the light (conditioned stimulus, CS+), which had been previously paired with shock, and co-terminated with the light (CS+/acoustic startle (AS)+ trials). For the other half of the trials, rats were presented with the startle-eliciting noise burst (0.5 sec) in the dark, in the absence of the light (CS–/AS+ trials). There were 60 trials for each of the CS+/AS+ and CS–/AS+ trials with 9 – 11 trials at each intensity of 90, 95 and 105 dB noise stimuli (9-11 CS+/AS+ trial and 9-11 CS–/AS+ trials) and presented quasi-randomly with the restriction that each trial type only occurred once within each

successive block of 6 trials. Overall, each session lasted around 42.5 min. The daily assessment for FPS over the 6-day period served to determine the rate of extinction learning to the CS+. Within-session extinction was determined based on measurement of FPS response during the average of the first block of 90 dB, 95 dB, 105 dB FPS trials (3 CS+/AS+ trials and 3 CS–/AS+ trials per block) after Leaders and the average of the last block of 90 dB, 95 dB, and 105 dB FPS trials (3 CS+/AS+ trials and 3 CS–/AS+ trials per block) on each day.

Euthanasia and blood collection

Rats were euthanized 24 h following the last testing session using an overdose of sodium pentobarbital (Fatal Plus®, Vortech Pharmaceuticals Ltd., Dearborn, MI, USA; 200 mg/kg, i.p.) after which rats were decapitated and trunk blood was collected and brains were frozen on dry ice immediately after decapitation.

***In situ* hybridization histochemistry**

Previously published methods were used for *in situ* hybridization histochemistry (Day and Akil, 1996). Briefly, brains were sectioned into 12 µm sections on a cryostat (Leica CM 1950, Leica Biosystems, Buffalo Grove, IL, USA), in a series of 7 sets of sections, thaw-mounted on Histobond® slides (Cat. No. 16,004-406; VWR, West Chester, PA, USA) and stored at −80 °C. [^{35}S]-UTP-labeled riboprobes directed against *htr1a* mRNA (provided by Dr. Heidi Day, Department of Psychology, University of Colorado Boulder, Boulder, CO, USA) were generated using standard transcription methods, as described previously (Day and Akil, 1996). Sections were fixed in 4% paraformaldehyde for 1 h, acetylated in 0.1 M triethanolamine hydrochloride with 0.25% acetic anhydride for 10 min, and dehydrated through graded alcohols. Sections were

hybridized overnight at 55 °C with a [^{35}S]-UTP-labeled riboprobe diluted in hybridization buffer containing 50% formamide, 10% dextran sulfate, 2× saline sodium citrate (SSC), 50 mM PBS, pH 7.4, 1× Denhardt's solution, and 0.1 mg/ml yeast tRNA. The following day, sections were treated with RNase A, 200 µg/ml at 37 °C for 1 h, and washed to a final stringency of 0.1× SSC at 65 °C (1 h). Dehydrated sections were exposed to x-ray film (BioMax MR; Eastman Kodak, Rochester, NY, USA) for region- and probe-appropriate times (1–3 weeks) prior to film development.

Autoradiographic images of the probe bound to *htr1a* mRNA, encoding the 5-HT$_{1A}$ inhibitory autoreceptor, together with ^{14}C-labeled standards were measured using a computer-assisted image analysis system. For this gene, all slides from the study were apposed to the same film, allowing us to use a single set of ^{14}C-labeled standards for reference per gene. Analysis was performed on a PC using the publicly available NIH-developed image analysis software ImageJ (https://imagej.nih.gov/ij/). Virtual matrices in the shape of the respective dorsal raphe nucleus (DRN) subregions were created, overlaid with the image, and the "mean gray value x area", taking into account the area of only the above threshold signal, within each matrix measured. All measurements were taken while blinded to the treatment groups. During the entire analysis a constant threshold function was applied, which determined the area that was actually measured within each matrix. Thus, all pixels with a gray density below threshold were automatically excluded. In addition, the individual background of each image was measured and subtracted from each value. A rostrocaudal analysis atlas for *htr1a* expression in the DRN was created by comparing the image of the tissue sections with a stereotaxic rat brain atlas (Paxinos and Watson, 1998) and with *tph2* mRNA expression topography as reported by Gardner et al. (Gardner et al., 2009b). According to Gardner et al. (Gardner et al., 2009b) and Tph immunostaining (Abrams et

al., 2004), each rostrocaudal level was further divided into respective subregions of the DRN. Throughout all rostrocaudal levels, the values for each subdivision were then averaged, and all values per treatment group also summarized to display the overall expression in the DRN. A total of 18 rostrocaudal levels were studied throughout the brainstem (see Fig. 2). The subdivisions studied were summarized into the following functional subregions of the DRN: dorsal raphe nucleus, caudal part (DRC), –8.336 mm to –8.672 mm from bregma; dorsal raphe nucleus, dorsal part (DRD), –7.244 mm to –8.252 mm from bregma; dorsal raphe nucleus, interfascicular part (DRI), –8.252 mm to –8.672 mm from bregma; dorsal raphe nucleus, ventral part (DRV), –7.244 mm to –8.504 mm from bregma; left and right dorsal raphe nucleus, ventrolateral part/ventrolateral periaqueductal gray region (left and right DRVL/VLPAG), –7.496 mm to –8.336 mm from bregma; left and right supralemniscal nucleus (B9), –7.244 mm to –7.580 mm from bregma; median raphe nucleus, rostral part (rMnR), –7.244 mm to –7.496 mm from bregma; mid-rostrocaudal median raphe nucleus, dorsal part (MnRd), –7.580 mm to –8.168 mm from bregma; mid-rostrocaudal median raphe nucleus, ventral part (MnRv) –7.580 mm to –8.168 mm from bregma; median raphe nucleus, caudal part (cMnR) – 8.252 mm to – 8.672 mm from bregma; pontomesencephalic reticular formation (PMRF) – 7.244 mm to – 7.496 mm from bregma.

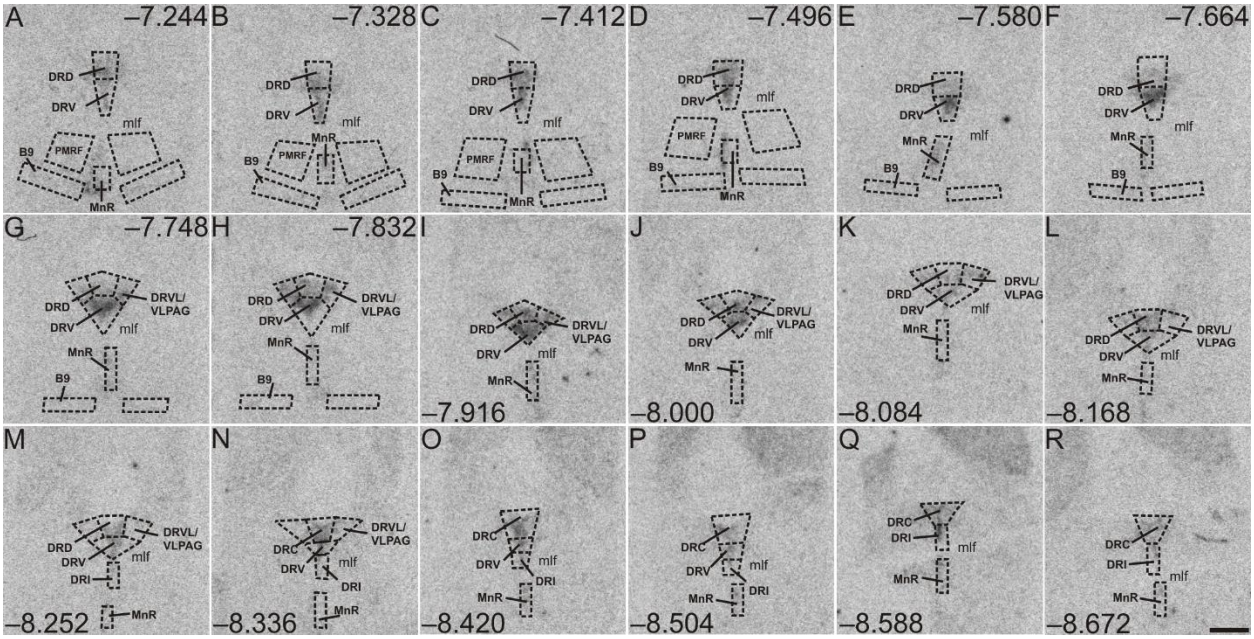

Figure 2. Atlas of serotonin receptor subtype 1a (*htr1a*) mRNA expression in the midbrain and pontine raphe complex (84 µm intervals) used for analysis of subregions of the dorsal raphe nucleus (DRN) and median raphe nucleus (MnR) with a high level of neuroanatomical resolution. Photographs are autoradiographic images of *htr1a* mRNA expression. The levels chosen for analysis ranged from (**A**) –7.244 mm bregma through (**R**) –8.672 mm bregma. Dotted lines delineate different subdivisions of the DRN analyzed in this study, based on a stereotaxic atlas of the rat brain (Paxinos and Watson, 1998). Abbreviations: B9, supralemniscal serotonergic cell group; cMnR, median raphe nucleus, caudal part; DRC, dorsal raphe nucleus, caudal part; DRD, dorsal raphe nucleus, dorsal part; DRI, dorsal raphe nucleus, interfascicular part; DRV, dorsal raphe nucleus, ventral part; DRVL, dorsal raphe nucleus, ventrolateral part; MnRd, mid-rostrocaudal median raphe nucleus, dorsal part; MnRv, mid-rostrocaudal median raphe nucleus, ventral part; PMRF, pontomesencephalic reticular formation; scp, superior cerebellar peduncle; VLPAG, ventrolateral periaqueductal gray; rMnR, median raphe nucleus, rostral part; xscp, decussation of the superior cerebellar peduncle. Numbers in the lower left of

each panel indicate the rostrocaudal coordinates relative to bregma (in mm). Scale bar, 1 mm. The rostrocaudal levels and matrices defining subregions of the DRN that were defined using *tph2* autoradiograms were also used in the analysis of *htr1a* to ensure sampling of the same anatomical regions for each gene.

Statistical analysis

For statistical comparisons, the software package IBM SPSS Statistics (versions 22.0 and 24.0, SPSS Inc., Chicago, IL, USA) was used. Extreme statistical outliers were identified using Grubbs' test for single outliers using two-sided $\alpha = 0.05$ (Grubbs, 1969) and were removed from the analysis. For baseline acoustic startle response, comparisons of two independent samples were made using Student's *t*-tests. For analysis of repeated measures data, a survey of linear mixed models with different covariance structures was performed and the model with the best -2 log-likelihood value, an information criterion function used for goodness of fit, was selected. For FPS analysis *M. vaccae,* decibel intensity, and day were used as fixed effects; post hoc between-subjects comparisons were made using Fisher's least significant difference (LSD) test and post hoc within-subjects comparisons were made using paired *t*-tests. For analysis of *in situ* hybridization histochemistry data, mean gray values x area for each DRN subdivision at each rostrocaudal level of the DRN in each treatment group were generated. Extreme outliers were identified using Grubbs' test for single outliers using two-sided $\alpha = 0.05$ (Grubbs, 1969) similar to what was performed with behavior. A survey of linear mixed models with difference covariance structures was performed and the model with the best −2 log-likelihood value was selected for *htr1a* with *M. vaccae,* FPS, rostrocaudal level, and

DRN subregion as fixed effects, and rostrocaudal level as the repeated effect. Additional linear mixed models were run and the best covariance structure was selected using the best −2 log-likelihood value for each individual subregion of the DRN, with *M. vaccae,* FPS, and rostrocaudal level, as fixed effects and rostrocaudal level as a repeated effect. Pairwise comparisons were made with Fisher's least significant difference (LSD) test. Furthermore, in the *in situ* hybridization histochemistry analysis no *posthoc* analyses were conducted at specific points in the rostrocaudal extent of the DRN, MnR, PMRF, or B9 if one of the group sample sizes was below 50% of the full sample size for that treatment group. Additionally, post hoc analyses were conducted only when overall and secondary linear mixed models yielded significant effects of *M. vaccae*, FPS, *M. vaccae* x FPS interactions, or interactions among these factors and rostrocaudal level within raphe subregion or rostrocaudal level. Data are presented as means ± standard error of means (SEM). Two-tailed significance was set at $p < 0.05$.

Results

The average body weights prior to treatment (day –42) and on the day of euthanasia (day 11) were as follows: vehicle/home cage control group, 93.6 g and 345.3 g; vehicle/FPS group, 95.6 g and 332.6 g; *M. vaccae*/home cage control group = 98.5 g and 352.1 g; *M. vaccae*/FPS group, 97.1 g and 330.2 g. Using a 2 x 2 ANOVA, a significant main effect of FPS was found on weight at euthanasia ($F_{(1,43)} = 6.37$, $p = 0.015$), with rats who underwent FPS weighing less than those that remained in their home cages while there was no effect *of M. vaccae*, or interaction between *M. vaccae* and FPS.

There were no significant differences in baseline acoustic startle between *M. vaccae*-immunized animals and vehicle-treated animals on either day of baseline testing (Fig 3A; days 1 and 2). In contrast, for between-session FPS data (Fig 3B), based on linear mixed model analysis, there were overall significant effects of *M. vaccae* ($F_{(1,397)}$ = 10.37, $p < 0.01$), decibel intensity ($F_{(2,397)} = 20.23$, $p < 0.001$), and day ($F_{(5,397)} = 5.22$, $p < 0.001$). Paired *t*-tests revealed a significant difference between day 5 and day 10 in vehicle-treated animals ($t_{(9)} = 2.98$, $p < 0.05$; Fig 3B). Fisher's least significant difference tests found animals immunized with *M. vaccae* expressed a lower FPS response compared to vehicle-treated animals on day 6 ($p < 0.001$; Fig 3B). Analysis using linear mixed model analysis of within-session extinction of FPS across days 5-10 revealed overall effects of *M. vaccae* ($F_{(1,620)} = 30.22$, $p < 0.001$), and time (comparing the first FPS trial block versus the last FPS trial block ($F_{(9,620)} = 2.52$, $p < 0.01$; Fig 3C)). On day 6, the second day of fear extinction training, paired *t*-tests revealed within-session extinction in *M. vaccae*-, but not vehicle-treated rats ($t_{(10)} = 3.39$, $p < 0.007$; Fig 3C). Additionally, *M. vaccae*-immunized animals also had a lower FPS compared to vehicle-treated animals ($p < 0.009$) at the end of the session on day 6. *M. vaccae*-immunized animals also showed within-session extinction on day 7 ($t_{(11)} = 7.01$, $p < 0.001$), day 8 ($t_{(10)} = 3.73$, $p < 0.01$), and day 9 ($t_{(11)} = 3.45$, $p < 0.01$), while vehicle-immunized animals showed within-session extinction on day 7 (Vehicle; $t_{(11)} = 3.36$, $p < 0.007$) and day 10 (Vehicle; $t_{(10)} = 4.20$, $p < 0.002$; Fig 3C).

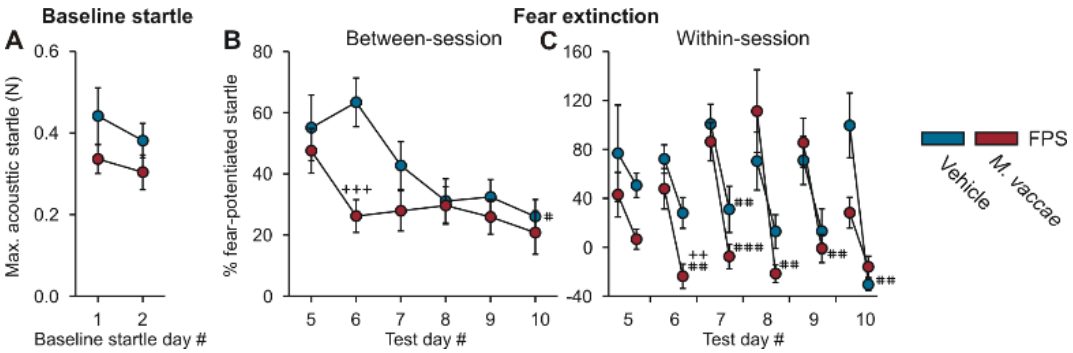

Figure 3. *M. vaccae* prevents fear extinction resistance and enhances extinction of conditioned fear in male rats. (**A**) Baseline acoustic startle, a measure of generalized anxiety, measured on days 1 and 2. (**B**) Between-session fear-potentiated startle (FPS) extinction; measured on days 5-10, starting 24 h after fear conditioning (FPS extinction training). (**C**) Within-session extinction (measured on days 5-10). Data are presented as means ± standard error of means (SEMs). Data were analyzed using (**A)** linear mixed model (LMM) with fixed effects of *M. vaccae* and day, (**B)** LMM with fixed effects of treatment, day, and decibel intensity, (**C**) LMM with fixed effects of *M. vaccae* and day, with block nested within day; post hoc comparisons were made using Fisher's least significant difference (LSD) tests for between-subjects comparisons and paired *t*-tests for within-subjects comparisons. $^{++}p < 0.01$, $^{+++}p < 0.001$, between-subjects effects; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ within-subjects effects, comparing the first and last 6 (CS–/AS+) trial blocks within the session. Sample sizes ($N = 47$, vehicle/home cage control, $n = 11$; vehicle/FPS, $n = 12$; *M. vaccae*/home cage control, $n = 12$; *M. vaccae*/FPS, $n = 12$).

In situ hybridization histochemistry

In situ hybridization histochemistry was used to evaluate the effects of *M. vaccae* immunization, with or without subsequent exposure to the FPS protocol, on expression of a gene thought to be important for control of serotonergic neurotransmission, *htr1a*.

Htr1a mRNA expression

Analysis of *htr1a* mRNA expression using linear mixed model analysis revealed an *M. vaccae* x FPS x rostrocaudal level within subregion interaction ($F_{(86,382.3)} = 2.3$, $p < 0.01$; Fig 4; Table 1. Based on this finding, secondary linear mixed models were used to determine effects of treatment within each subregion of the DRN, MnR, PMRF, and B9 serotonergic cell group. Linear mixed model analysis of specific subregions highlighted *M. vaccae* x FPS x rostrocaudal level interactions within both the DRD ($F_{(12,40.9)} = 2.1$, $p < 0.05$; Fig 4A; Table 1) and DRV ($F_{(15,40.7)} = 5.8$, $p < 0.01$; Fig 4B; Table 1). Among *M. vaccae*-immunized rats, those exposed to FPS had decreased *htr1a* mRNA expression levels compared to home cage controls within the rostral part of the DRD, whereas, more caudally in the DRD, this effect was reversed, with increased *htr1a* mRNA expression levels in rats exposed to FPS, compared to home cage controls. Immunization with *M. vaccae* also increased *htr1a* mRNA expression in home cage control rats, but this effect was only observed at –7.328 mm bregma.

Meanwhile, linear mixed model analysis revealed that *M. vaccae* had a main effect on *htr1a* mRNA expression in the DRVL/VLPAG ($F_{(1,62.7)} = 4.3$, $p < 0.05$; Fig 4C; Table 1.) Specifically, among rats exposed to FPS, immunization with *M. vaccae* increased *htr1a* mRNA expression. Similar effects of *M. vaccae*, increasing *htr1a* mRNA expression among rats exposed to FPS, were observed within the DRC (*M. vaccae* main effect, $F_{(1,42.9)} = 4.4$, $p < 0.05$; Fig 4E; Table 1), PMRF (*M. vaccae* main effect, $F_{(1,37.6)} = 5.0$, $p < 0.05$; Fig 4F; Table 1), and rMnR (*M. vaccae* x FPS x rostrocaudal level interaction, $F_{(3,33.0)} = 4.3$, $p < 0.05$; Fig 4G; Table 1.)

An *M. vaccae* x rostrocaudal level interaction was identified within the DRI ($F_{(4,56.5)} = 2.7$, $p < 0.05$; Fig 4D, Table 1); specifically, among rats exposed to FPS, *M. vaccae* decreased *htr1a* mRNA expression, but only at −8.672 mm from bregma.

Finally, linear mixed model analysis revealed an overall FPS effect within the B9 subregion ($F_{(1,41.0)} = 5.0$, $p < 0.05$, Fig 4K, Table 1. Among *M. vaccae* immunized rats, those exposed to FPS had increased *htr1a* mRNA expression at –7.748 mm and –7.916 mm from bregma.

There were no effects of FPS, *M. vaccae*, or interactions in the MnRv or cMnR.

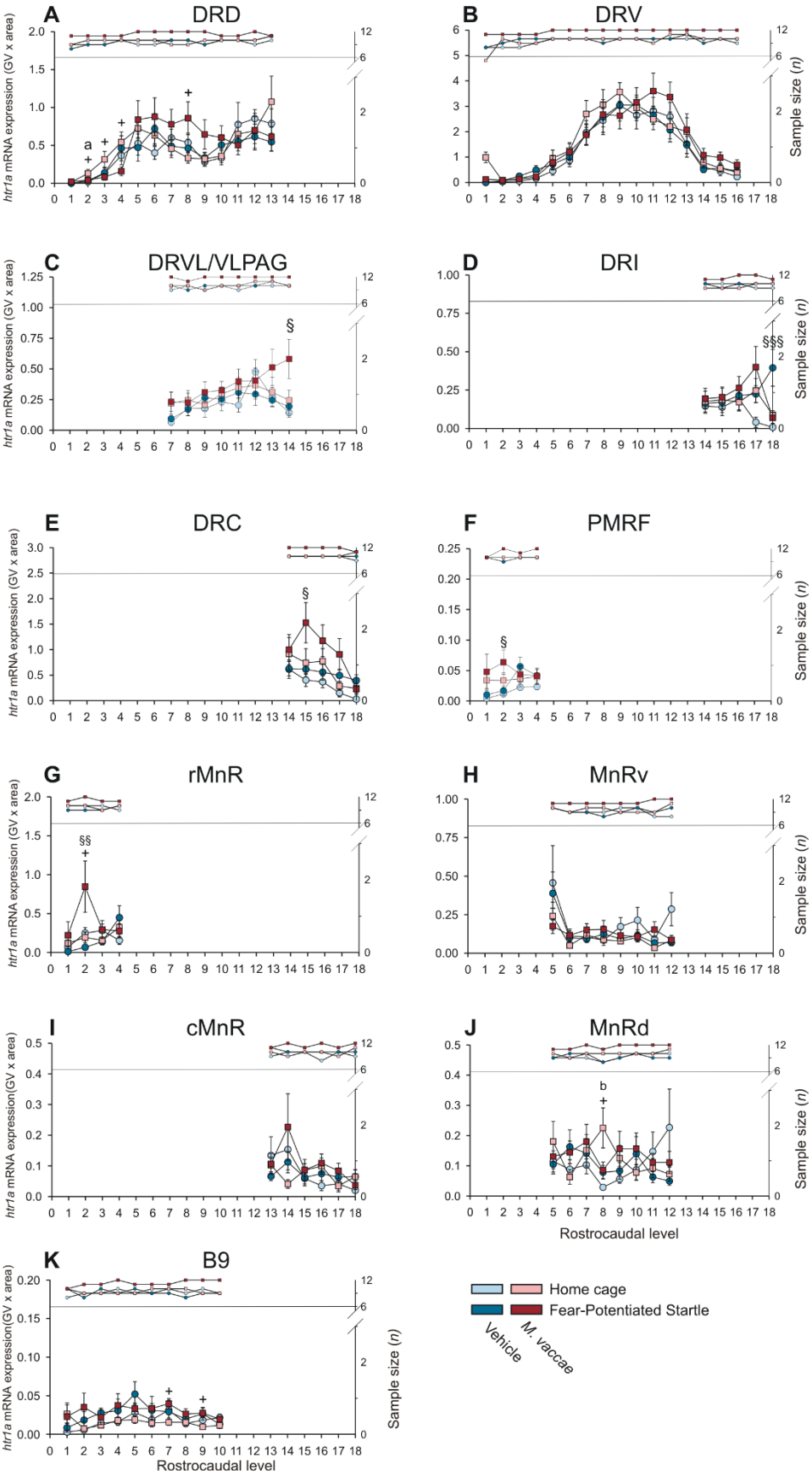
A
DRD
B
DRV
C
DRVL/VLPAG
D
DRI
E
DRC
F
PMRF
G
rMnR
H
MnRv
I
cMnR
J
MnRd
K
B9
htr1a mRNA expression (GV x area)
Sample size (n)
Rostrocaudal level
Home cage
Fear-Potentiated Startle
Vehicle
M. vaccae

Figure 4. Effects of repeated immunizations with a heat-killed preparation of *M. vaccae* and acoustic startle testing, fear conditioning, and fear extinction on *htr1a* mRNA expression in subdivisions of the dorsal raphe nucleus (DRN), median raphe nucleus (MnR), pontomesencephalic reticular formation (PMRF), and B9 serotonergic cell group. Each graph represents the mean ± SEM of *htr1a* mRNA expression levels throughout the rostrocaudal extent of different subdivisions of the DRN, MnR, PMRF, and B9. Rats received immunizations with a heat-killed preparation of *M. vaccae* or borate buffered saline vehicle (Vehicle) on days –35, –28, and –21, followed by baseline acoustic startle testing on days 1 and 2, fear conditioning on days 3 and 4, and fear extinction training on days 5-10, and were euthanized on day 11. Control rats were maintained under home cage control conditions. Graphs illustrate *htr1a* mRNA expression in the (**A**) dorsal raphe nucleus, dorsal part (DRD), (**B**) dorsal raphe nucleus, ventral part (DRV), (**C**) dorsal raphe nucleus, ventrolateral part (DRVL)/ventrolateral periaqueductal gray (VLPAG), (**D**) dorsal raphe nucleus, interfascicular part (DRI), (**E**) dorsal raphe nucleus, caudal part (DRC), (**F**) pontomesencephalic reticular formation (PMRF), (**G**) median raphe nucleus, rostral part, (rMnR), (**H**) median raphe nucleus, mid-rostrocaudal ventral part (MnRv), (**I**) median raphe nucleus, caudal part (cMnR), (**J**) median raphe nucleus, mid-rostrocaudal dorsal part (MnRd), (**K**) B9 supralemniscal serotonergic cell group (B9); Circles, vehicle-treated controls; squares, *M. vaccae*-immunized rats. $^{+}p < 0.05$, FPS versus home cage controls among rats treated with *M. vaccae*. $^{a}p < 0.05$. $^{b}p < 0.01$, *M. vaccae* versus vehicle among rats in the home cage condition, $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$, *M. vaccae* versus vehicle among rats exposed to FPS. Rostrocaudal levels relative to bregma: 1 = –7.244 mm, 2 = –7.328 mm, 3 = –7.412 mm, 4 = –7.496 mm, 5 = –7.580 mm, 6 = –7.664 mm, 7 = –7.748 mm, 8 = –7.832 mm, 9 = –7.916 mm, 10 = –8.000 mm, 11 = –8.084 mm, 12 = –8.168 mm, 13 = –-8.252 mm, 14 = –8.336

mm, 15 = –8.420 mm, 16 = –8.504 mm, 17 = –8.588 mm, 18 = –8.672 mm. Sample sizes for each treatment group at each rostrocaudal level of analysis are shown (upper section of each panel).

Table 1. Linear mixed model analysis results for *htr1a*

Model	Source	Test statistic	*p*-value
Overall Analysis			
Entire dataset (First-Order Ante-Dependence Covariance Structure)			
	Treatment (Mv vs. Veh)	$F_{(1, 555.2)} = 8.2$	0.004
	Fear training (FPS vs. HC)	$F_{(1, 555.2)} = 2.8$	0.093
	Rostrocaudal level	$F_{(17,462.0)} = 37.2$	0.001
	Raphe subregion	$F_{(10,319.8)} = 81.8$	0.001
	Treatment *Fear training	$F_{(1,457.8)} = 0.4$	0.544
	Treatment *Raphe subregion	$F_{(10,351.8)} = 1.8$	0.063
	Fear training *Raphe subregion	$F_{(10,351.8)} = 1.0$	0.447
	Fear training *Rostrocaudal level(Raphe subregion)	$F_{(76,362.9)} = 2.0$	0.001
	Treatment *Rostrocaudal level(Raphe subregion)	$F_{(76,362.9)} = 1.8$	0.001
	Treatment *Fear training *Rostrocaudal level(Raphe subregion)	$F_{(86,382.3)} = 2.3$	0.001
Subregional Analyses			
DRD (Unstructured Covariance Structure)			
	Treatment (Mv vs. Veh)	$F_{(1,43.3)} = 1.3$	0.268
	Fear training (FPS vs. HC)	$F_{(1,43.3)} = 0.06$	0.804
	Rostrocaudal level	$F_{(12,40.9)} = 18.2$	0.001
	Treatment *Fear training	$F_{(1,43.3)} = 0.2$	0.652
	Treatment *Rostrocaudal Level	$F_{(12,40.9)} = 1.7$	0.100
	Fear training *Rostrocaudal Level	$F_{(12,40.9)} = 3.0$	0.004
	Treatment * Fear training *Rostrocaudal Level	$F_{(12,40.9)} = 2.1$	0.036
DRV (Unstructured Covariance Structure)			
	Treatment (Mv vs. Veh)	$F_{(1,43.4)} = 2.8$	0.104
	Fear training (FPS vs. HC)	$F_{(1,43.4)} = 0.02$	0.888
	Rostrocaudal level	$F_{(15,40.7)} = 27.0$	0.001
	Treatment *Fear training	$F_{(1,43.4)} = 0.03$	0.876
	Treatment *Rostrocaudal Level	$F_{(15,40.7)} = 5.3$	0.001
	Fear training *Rostrocaudal Level	$F_{(15,40.7)} = 4.3$	0.001
	Treatment * Fear training *Rostrocaudal Level	$F_{(15,40.7)} = 5.8$	0.001
DRVL/VLPAG (Unstructured Covariance Structure)			
	Treatment (Mv vs. Veh)	$F_{(1,62.7)} = 4.3$	0.043
	Fear training (FPS vs. HC)	$F_{(1,62.7)} = 1.0$	0.333
	Rostrocaudal level	$F_{(7,83.1)} = 5.8$	0.001
	Treatment *Fear training	$F_{(1,62.7)} = 0.8$	0.374
	Treatment *Rostrocaudal Level	$F_{(7,83.1)} = 1.1$	0.405
	Fear training *Rostrocaudal Level	$F_{(7,83.1)} = 1.3$	0.259
	Treatment * Fear training *Rostrocaudal Level	$F_{(7,83.1)} = 0.8$	0.562

DRI (Unstructured Covariance)			
	Treatment (Mv vs. Veh)	$F_{(1,42.0)} = 0.6$	0.454
	Fear training (FPS vs. HC)	$F_{(1,42.0)} = 1.4$	0.251
	Rostrocaudal level	$F_{(4,56.5)} = 1.2$	0.335
	Treatment *Fear training	$F_{(1,42.0)} = 0.3$	0.590
	Treatment *Rostrocaudal Level	$F_{(4,56.5)} = 2.7$	0.043
	Fear training *Rostrocaudal Level	$F_{(4,56.5)} = 0.6$	0.696
	Treatment * Fear training *Rostrocaudal Level	$F_{(4,56.5)} = 1.7$	0.166
DRC (Unstructured Covariance Structure)			
	Treatment (Mv vs. Veh)	$F_{(1,42.9)} = 4.4$	0.041
	Fear training (FPS vs. HC)	$F_{(1,42.9)} = 3.7$	0.061
	Rostrocaudal level	$F_{(4,41.4)} = 10.4$	0.001
	Treatment *Fear training	$F_{(1,42.9)} = 0.4$	0.549
	Treatment *Rostrocaudal Level	$F_{(4,41.4)} = 2.0$	0.110
	Fear training *Rostrocaudal Level	$F_{(4,41.4)} = 2.2$	0.090
	Treatment * Fear training *Rostrocaudal Level	$F_{(4,41.4)} = 1.9$	0.136
PMRF	**(Unstructured Covariance)**		
	Treatment (Mv vs. Veh)	$F_{(1,37.6)} = 5.0$	0.031
	Fear training (FPS vs. HC)	$F_{(1,37.6)} = 3.0$	0.092
	Rostrocaudal level	$F_{(3,36.6)} = 1.6$	0.214
	Treatment *Fear training	$F_{(1,37.6)} = 0.004$	0.951
	Treatment *Rostrocaudal Level	$F_{(3,36.6)} = 1.8$	0.174
	Fear training *Rostrocaudal Level	$F_{(3,36.6)} = 0.5$	0.659
	Treatment * Fear training *Rostrocaudal Level	$F_{(3,36.6)} = 0.8$	0.502
rMnR	**(Unstructured Covariance)**		
	Treatment (Mv vs. Veh)	$F_{(1,38.0)} = 2.4$	0.130
	Fear training (FPS vs. HC)	$F_{(1,38.0)} = 1.5$	0.222
	Rostrocaudal level	$F_{(3,33.0)} = 4.1$	0.014
	Treatment *Fear training	$F_{(1,38.0)} = 2.2$	0.146
	Treatment *Rostrocaudal Level	$F_{(3,33.0)} = 2.4$	0.087
	Fear training *Rostrocaudal Level	$F_{(3,33.0)} = 1.3$	0.296
	Treatment * Fear training *Rostrocaudal Level	$F_{(3,33.0)} = 4.3$	0.012
MnRv (Unstructured Covariance)			
	Treatment (Mv vs. Veh)	$F_{(1,41.3)} = 1.8$	0.182
	Fear training (FPS vs. HC)	$F_{(1,41.3)} = 0.1$	0.739
	Rostrocaudal level	$F_{(7,35.8)} = 2.4$	0.044
	Treatment *Fear training	$F_{(1,41.3)} = 1.8$	0.184
	Treatment *Rostrocaudal Level	$F_{(7,35.8)} = 1.2$	0.348
	Fear training *Rostrocaudal Level	$F_{(7,35.8)} = 2.1$	0.068
	Treatment * Fear training *Rostrocaudal Level	$F_{(7,35.8)} = 1.1$	0.402
cMnR	**(Unstructured Covariance)**		
	Treatment (Mv vs. Veh)	$F_{(1,41.4)} = 0.7$	0.398
	Fear training (FPS vs. HC)	$F_{(1,41.4)} = 0.5$	0.496
	Rostrocaudal level	$F_{(5,32.7)} = 4.1$	0.005
	Treatment *Fear training	$F_{(1,41.4)} = 0.9$	0.358
	Treatment *Rostrocaudal Level	$F_{(5,32.7)} = 1.2$	0.346
	Fear training *Rostrocaudal Level	$F_{(5,32.7)} = 2.1$	0.091
	Treatment * Fear training *Rostrocaudal Level	$F_{(5,32.7)} = 2.0$	0.102
MnRd (Unstructured Covariance Structure)			

	Treatment (Mv vs. Veh)	$F_{(1,39.7)} = 1.0$	0.330
	Fear training (FPS vs. HC)	$F_{(1,39.7)} = 0.1$	0.722
	Rostrocaudal level	$F_{(7,38.0)} = 1.0$	0.422
	Treatment *Fear training	$F_{(1,39.7)} = 0.007$	0.935
	Treatment*Rostrocaudal Level	$F_{(7,38.0)} = 1.8$	0.119
	Fear training *Rostrocaudal Level	$F_{(7,38.0)} = 2.3$	0.044
	Treatment * Fear training *Rostrocaudal Level	$F_{(7,38.0)} = 2.3$	0.045
B9	**(Unstructured Covariance)**		
	Treatment (Mv vs. Veh)	$F_{(1,41.0)} = 0.08$	0.776
	Fear training (FPS vs. HC)	$F_{(1,41.0)} = 5.0$	0.031
	Rostrocaudal level	$F_{(9,36.1)} = 3.0$	0.009
	Treatment *Fear training	$F_{(1,41.0)} = 0.5$	0.478
	Treatment *Rostrocaudal Level	$F_{(9,36.1)} = 1.2$	0.340
	Fear training *Rostrocaudal Level	$F_{(9,36.1)} = 1.9$	0.089
	Treatment * Fear training *Rostrocaudal Level	$F_{(9,36.1)} = 0.7$	0.728

Discussion

Immunization with *M. vaccae* had no effect on acoustic startle or initial fear expression, but enhanced both between-session and within-session fear extinction, in association with changes in *htr1a* mRNA expression in the anxiety- and fear-related dorsomedial part of the DRN. Specifically, among rats that received immunization with *M. vaccae*, exposure to FPS increased *hrtr1a* mRNA expression in the dorsomedial DRN, an effect that has also been described in association with other interventions that promote stress resilience. Also among rats that received immunization with *M. vaccae*, exposure to FPS decreased *htr1a* expression in the rostral DRN. Also of interest, among rats exposed to FPS, those that were immunized with *M. vaccae* responded with increased *htr1a* mRNA expression in the DRC.

Immunization with *M. vaccae* had no effect on acoustic startle or initial fear expression, but enhanced between-session fear extinction. Between-session fear extinction has been shown to require input from the medial prefrontal cortex (mPFC), namely the infralimbic (IL) portion (Morawska and Fendt, 2012). When $GABA_A$ receptor agonist, muscimol, was injected into the mPFC, temporarily inactivating it, between-session extinction was impaired (Morawska and Fendt, 2012). In a study by Yang et al. (2012), administration of the nonclassical antidepressant

drug, venlafaxine (VEN), demonstrated facilitation of between-session fear extinction (Yang et al., 2012). VEN has also displayed some efficacy for treatment in individuals with PTSD, shown in two randomized, double-blinded, placebo-controlled clinical trials (Pae et al., 2007).

In addition to its effects on between-session fear extinction, immunization with *M. vaccae* enhanced *within*-session fear extinction. Brain-derived neurotrophic factor (BDNF) signaling within the basolateral nucleus of the amygdala (BLA) is suggested to have effects on fear extinction. In a study by Chhatwal et al. (2006), rats injected with lentivirus, known to induce neuroinflammation (Zhu et al., 2005), containing green fluorescent protein (GFP) or TrkB.t1, a genetically modified tyrosine kinase B receptor (TrkB) that cannot bind BDNF effectively, into the BLA produced within-session extinction solely within the GFP control rats (Chhatwal et al., 2006). This suggests TrkB receptor activation in the BLA may only be imperative for within-session fear extinction (Chhatwal et al., 2006). T cell activation and inflammation may also be associated with within-session fear extinction. Injections of a calcineurin (CaN) inhibitor or immunosuppressant cyclosporine A (CsA), was shown to block within-session fear extinction (Almeida-Corrêa et al., 2015). CaN is involved with T cell activation and CsA is involved in inhibiting T cell activation, so it is reasonable to suggest that there is a link between within-session fear extinction and T cell activation (Schreiber and Crabtree, 1992; Dutta et al., 2017).

Fear extinction is a form of associative learning and can be divided into separate mechanistic events, including encoding and consolidation (Toth et al., 2012). Between-session fear extinction is most closely related to consolidation and retention, while within-session fear extinction is most closely related to encoding (Toth et al., 2012). The endocannabinoid system may have important implications for both encoding and consolidation of fear memories. Pickens

and Theberge (2014) found that blocking cannabinoid receptor type 1 (CB1, with a CB1 receptor antagonist prevented between-session extinction (Pickens and Theberge, 2014). Conversely, Plendl and Wotjak (2010) found CB1 signaling to be imperative to within-session extinction in rats (Plendl and Wotjak, 2010). When the CB1 receptor was antagonistically blocked or altogether genetically deleted, within-session fear extinction was prevented (Plendl and Wotjak, 2010). These findings suggest convergence between the two mechanisms of fear extinction.

The effects of *M. vaccae* on between-session and within-session fear extinction were associated with increased *htr1a* mRNA expression in the anxiety- and fear-related dorsomedial part of the DRN. This result suggests a greater number of 5-HT_{1A} receptors in rats that experienced FPS rather than the home cage control environment, of which both groups received *M. vaccae*. Similarly, Greenwood et al., (2003) found increased 5-HT_{1A} receptor mRNA in the rostral-mid part of the dorsal DRN in physically active rats, who were allowed to voluntarily freewheel run for six weeks, when compared to sedentary rats (Greenwood et al., 2003). Greenwood et al. (2003) suggested that the physical activity-induced increase of 5-HT_{1A} receptor mRNA attenuated serotonergic neuronal activity in the DRN, and possibly led to the prevention of learned helplessness, a result that was also observed in these animals (Greenwood et al., 2003). The current study's findings that *M. vaccae*-immunized rats had the same 5-HT_{1A}-related response in the same region of the DRN suggests possible convergence between *M. vaccae* and the protective effects of exercise demonstrated by Greenwood et al. (2003).

The effects of *M. vaccae* on between-session and within-session fear extinction were associated with a decrease in *htr1a* mRNA expression in the rostral DRN, as well. The rostral DRN has been shown to project greatly to the striatum, an area associated with motor function (Imai et al., 1986). Strong et al. (2011) found that an increase in extracellular serotonin in the

dorsal striatum impaired escape behavior; an effect that can be eliminated by blocking excitatory 5-HT_{2c} receptors in the dorsal striatum (Strong et al., 2011). The decrease in *htr1a* mRNA expression may have implications in the increased serotonin noted here, due to the inhibitory actions of these receptors. Of note, *M. vaccae* has been observed to increase locomotion (Lowry et al., personal communication) and may be efficient in facilitating escape behavior, though this has not been studied.

Among rats exposed to FPS, those that were immunized with *M. vaccae* responded with increased *htr1a* mRNA expression in the DRC. Anxiogenic drugs, including caffeine, FG-7142, and the neuropeptide urocortin 2 (Ucn 2) activate serotonergic neurons in the DRC, shown by increases in c-Fos (Abrams et al., 2004; Table 2). In addition, Donner et al. (2012) found increased tryptophan hydroxylase 2 (*tph2*) mRNA expression, the rate-limiting enzyme involved in serotonin synthesis, in the DRC after injections of urocortin 1, a CRH-related neuropeptide, into the BLA (Donner et al., 2012). Given these findings, the current study's increased *htr1a* mRNA expression in the DRC in rats given *M. vaccae* and exposed to FPS, compared to rats given vehicle and exposed to FPS, may be a possible protective response of *M. vaccae* to stress.

The peroxisome proliferator-activated receptor (PPAR)-α is known to be involved in anti-inflammatory processes (Devchand et al., 1996). When endocannabinoid palmitoylethanolamide (PEA) binds to the PPAR-α antidepressant effects are reported (Yu et al., 2011). Additionally, PEA binding has been found to increase allopregnanolone in the hippocampus and amygdala which has been shown to be associated with faster fear extinction (Locci and Pinna, 2017b). This is of interest because allopregnanolone levels are reportedly low in individuals with PTSD (Rasmusson et al., 2006). Lowry et al. (personal communication) has identified an anti-inflammatory lipid portion within *M. vaccae* that binds to PPAR-α and may act in the same way

as an endocannabinoid. Therefore, immunization of *M. vaccae* may provide useful in individuals with PTSD.

Table 2. Functional associations of DRD/DRC serotonergic neurons

(Conditions associated with activation of DRD serotonergic neurons)

Stress/arousal/anxiogenic stimulus	*Effect on DRD/DRC*	*Dependent variable*	*Citation*
Anxiety due to intimate partner violence	↑ DRD/DRC	c-Fos	Cordero et al., 2012
Anxiogenic drug, caffeine	↑ DRD/DRC	c-Fos	Abrams et al., 2005
Anxiogenic drug, FG-7142	↑ DRD/DRC	c-Fos	Abrams et al., 2005
Avoidance task on elevated T-maze	↑ DRD/DRC	c-Fos	Spiacci et al., 2012
Diurnal variation in Tph activity	↑ DRD/DRC	Tph activity	Donner et al., 2012
Open-field	↑ DRD/DRC	c-Fos	Bouwknecht et al., 2007
Social defeat	↑ DRD/DRC	c-Fos	Gardner et al., 2005
Ucn 2, i.c.v.	↑ DRD/DRC	c-Fos	Hale et al., 2010; Staub et al., 2005; Staub et al., 2006
Ucn 3 overexpression (OE), 24 h post-restraint	↑ DRD/DRC	5-HT, 5-HIAA	Neufeld-Cohen et al., 2012
Ucn 2/Ucn 3 female KO	↑ DRC	5-HIAA	Neufeld-Cohen et al., 2010

Conclusion

Preimmunization with *M. vaccae* in a FPS paradigm enhanced both between-session and within-session fear extinction in rats. *M. vaccae* also produced changes in *htr1a* mRNA expression, namely in the dorsomedial DRN, rostral DRN, and caudal DRN in rats. These results

suggest that *M. vaccae* may be a novel preventative intervention for facilitating fear extinction and serotonergic regulation for individuals with PTSD.

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