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Marquinta Juvon Lee
mlee4@utk.edu

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I am submitting herewith a thesis written by Marquinta Juvon Lee entitled "The Role of 5-HT2A and 5-HT2C Receptors in Conditioned Defeat." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts, with a major in Psychology.

Matthew A. Cooper, Major Professor

We have read this thesis and recommend its acceptance:

Todd Freeberg, Rebecca Prosser

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

The Role of 5-HT2A and 5-HT2C Receptors in Conditioned Defeat

A Thesis Presented for the
Master of Arts
Degree
The University of Tennessee, Knoxville

Marquinta Juvon Lee
May 2011

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DEDICATION

To my husband
Brandon M. Harvey

my mother
Regina L. Dorsey

my father
Henry W. Lee

my step-father
Dwayne L. Dorsey

my grandparents
Jasper G. Hatcher
Thelma T. Hatcher
Josephine Lee

and cousins
Aundrea Anderson
Kamaria Anderson
Sharonda Anderson
Katrina Perry 'my ace'

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ABSTRACT

Previous research indicates that serotonin (5-HT) enhances the acquisition of stress-induced changes in behavior; although it is unclear which serotonin receptors mediate this enhancement. 5-HT2 receptors are potential candidates because activation at these receptors is associated with increased fear and anxiety. In this study we investigated whether pharmacological treatments targeting 5-HT2A and 5-HT2C receptors modulated the acquisition and expression of conditioned defeat. Conditioned defeat is a social defeat model in Syrian hamsters (*Mesocricetus auratus*) that is characterized by increased submissive and defensive behavior and a loss of territorial aggression following social defeat. In experiment 1, we injected the 5-HT2C receptor agonist mCPP (0.3, 1.0, or 3.0 mg/kg) or vehicle prior to social defeat and tested subjects for conditioned defeat behavior in a social interaction test 24 hours later. In experiment 2, subjects received a social defeat, and 24 hours later we injected mCPP (0.3, 1.0, or 3.0 mg/kg) or vehicle prior to a social interaction test. We found that injection of mCPP increased the expression, but not acquisition, of conditioned defeat. In experiment 3, we injected the 5-HT2A receptor antagonist MDL 11,939 (0.5 or 2.0 mg/kg) or vehicle prior to a social defeat and tested subjects for conditioned defeat behavior. In experiment 4, subjects received a social defeat, and 24 hours later we injected MDL 11,939 (0.5 or 2.0 mg/kg) or vehicle prior to a social interaction test. We found that injection of MDL 11,939 significantly decreased the acquisition, but not expression, of conditioned defeat. These data suggest that pharmacological activation of 5-HT2C receptors

enhances the expression of conditioned defeat, while pharmacological blockade of 5-HT2A receptors impairs the acquisition of conditioned defeat. These data extend other studies indicating that 5-HT signaling at 5-HT2A receptors facilitate memories for aversive events and 5-HT signaling at 5-HT2C receptors enhance stress-induced anxiety.

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INTRODUCTION

Psychosocial stress in humans can lead to a variety of psychiatric disorders, including major depression, acute stress disorder, and post-traumatic stress disorder (Ramboz et al., 1998, Naughton et al., 2000, Middlemiss et al., 2002, Davidson, 2003). The biological basis for stress-related mental illness remains poorly understood. Animal models of fear and anxiety have been used to understand the neural mechanisms underlying these psychiatric disorders. Because most of the stressors that are experienced by humans are social in nature (Brown and Prudo, 1981, Kessler, 1997, Bjorkqvist, 2001), ethologically relevant animal models that examine social conflict are particularly useful for determining how social experience alters the brain and subsequent behavior. Previous research has used physical stressors such as forced swim, foot shock and immobilization test. Although these are potent stressors that activate the stress response, physical stressors activate slightly different neural circuitry compared to psychosocial stressors (Canteras and Blanchard, 2008).

Social defeat is a robust stressor that activates the HPA-axis (Blanchard et al., 1995, Koolhaas et al., 1997). Social defeat also leads to several long-lasting behavioral and physiological changes, such as decreased locomotor activity /exploratory behavior (Meerlo et al., 1996a, Koolhaas et al., 1997, Rygula et al., 2005), changes in circadian rhythmicity (Meerlo et al., 1996a, Meerlo et al., 2002) and altered feeding and body weight (van de Poll et al., 1982, Meerlo et al., 1996b, Bartolomucci et al., 2004, Foster et al., 2006). The behavioral

effects produced by social defeat stress are noticeably similar to symptoms of depression, and many of these effects are reversed with antidepressant treatments (e.g., drugs or controlled sleep deprivation) (Fuchs et al., 1996, Meerlo et al., 1996a, Berton et al., 1999). Social defeat also produces changes in the serotonergic system. Serotonin's (5-HT's) specific role in fear and anxiety-like behavior is mixed. Conflicting data from human and animal research support both an anxiolytic and anxiogenic role for 5-HT (Gordon and Hen, 2004). More research is being conducted on various 5-HT receptors that may be mediating the changes in anxiety behavior.

Siberian and Syrian hamsters have been used as rodent models in circadian rhythms, obesity, and agonistic behavior (Wade and Bartness, 1984a, b). Syrian hamsters are especially useful for studying changes in agonistic behavior because they are solitary aggressive animals that will defend their territory from conspecifics (Nowack and Paradiso 1983). In a laboratory setting, singly housed hamsters defend their territory from intruders who are placed in their home cage (Albers 2002). However if a Syrian hamster loses an aggressive encounter, it will fail to display its' natural territorial aggression in future encounters and instead display submissive and defensive behavior towards a novel intruder (Huhman et al., 2003). This switch in agonistic behavior has been called conditioned defeat and has been used as a model for stress-induced anxiety disorders (Huhman, 2006).

Stressful events are known to increase fear and anxiety. Exposure to a predator is an ethologically relevant stressor that causes an increase in flight,

avoidance, and risk assessment in a mouse defensive test battery (Blanchard et al., 1990, Griebel et al., 1995). Predator odor increases different types of defensive behavior in both mice and rats depending on whether the threat is uncertain, distal, or proximal (Blanchard and Blanchard 2008). For instance, rats perform cautious exploration, such as risk assessment, in novel environments, when danger is certain. When a predator is perceived at a distance, tense and attentive immobility (freezing) ensues. Finally, when a predator is near or in actual contact with the rat, the animal flees whenever possible or otherwise threatens back or even attacks the predator defensively (Blanchard and Blanchard, 1988). Our lab has attempted to differentiate these defensive behaviors in hamsters by quantifying flight as a fear-like response, and stretch attends as an anxiety-like response.

Serotonin (5-HT) I a neurochemical increased during stressful events and known to modulate fear and anxiety. Previous research suggests that disruption of 5-HT is linked to anxiety disorders and serotonergic drugs are used as pharmacological treatment for many anxiety disorders (Owens and Nemeroff, 1998, Ballenger, 1999). The majority of 5-HT neurons that innervate stress-sensitive regions of the forebrain project from the dorsal raphe nucleus (DRN). Increases in 5-HT concentrations in the DRN are associated with exposure to stressful stimuli such as forced swim (Kirby et al., 1995) and foot shock (Yoshioka et al., 1995). 5-HT has been shown to increase anxiety in conflict tests in rats tested in an elevated t-maze (Graeff, 2002). Our lab has shown that social defeat activates 5-HT neurons in the DRN (Cooper et al., 2009). Additionally, we

have shown that blocking 5-HT activity by activating 5-HT1A autoreceptors in the DRN disrupts the acquisition and expression of conditioned defeat (Cooper et al., 2008).

It is unclear which 5-HT receptors in the forebrain facilitate stress-induced changes in fear and anxiety. 5-HT2 receptors are potential candidates for translating stress-induced increases in 5-HT into increased anxiety-like behavior. 5-HT2 receptors are postsynaptic, G-protein coupled receptors, that elevate cytosolic Ca++ (Conn and Sanders-Bush, 1986). The three subtypes of 5-HT2 receptors (2A, 2B, and 2C) have different distributions in the brain. While 5-HT2B receptors are found mainly in the periphery, 5-HT2A and 5-HT2C receptors are widely distributed throughout the brain. 5-HT2A receptors occur in high densities in the frontal cortex, piriform cortex, ventro-caudal part of the hippocampus (CA3), medial mammilary nucleus, the pontine nuclei, the motor cranial nerve nuclei in the brainstem, and the ventral horn of the spinal cord (Pompeiano et al., 1994). High densities of 5-HT2C receptors are found in retrosplenial, piriform and entorhinal cortex, anterior olfactory nucleus, lateral septal nucleus, subthalamic nucleus, amygdala, subiculum and ventral part of CA3, lateral habenula, substantia nigra pars compacta, several brainstem nuclei and the whole grey matter of the spinal cord (Pompeiano et al., 1994). 5-HT2C receptors contribute to the expression of fear and anxiety. Pharmacological activation of 5-HT2C receptors has induced panic attacks in humans (Kahn et al., 1988). Administration of a 5-HT2C receptor agonist before testing has been shown to increase the expression of learned helplessness behavior, such as reduced

social exploration, in rats (Strong et al., 2009). 5-HT2A receptors are important for the formation of emotional memories. Injection of 5-HT2A receptor agonists prior to training has been shown to facilitate eye blink conditioning in rabbits (Harvey, 2003).

The purpose of this study was to examine the role of 5-HT2A and 5-HT2C receptors in conditioned defeat. We chose the drug mCPP, a 5-HT2C receptor agonist, because previous research in animal and human studies has shown that mCPP increases anxiety. We choose MDL 11,939, a 5-HT2A receptor antagonist because of its' high affinity for 5-HT2A receptors over other 5-HT receptors. We hypothesized that 5-HT2C receptor activation prior to testing would increase the production of conditioned defeat behavior. Also we hypothesized that 5-HT2A receptor blockade prior to social defeat training would impair the formation of conditioned defeat.

METHODS

Animals

Subjects were adult male Syrian hamsters (*Mesocricetus auratus*) that weighed 130–190 g (3–4 months) at the start of the study, and were individually housed for 10–14 days prior to testing. Older hamsters that weighed 180–200 g (>6 months) were individually housed and used as resident aggressors for social defeat training. Immature hamsters that weighed 90–120 g (2 months) were group-housed (three or four animals per cage) and used as non-aggressive opponents for conditioned defeat testing. All animals were housed in polycarbonate cages (20 cm × 40 cm × 20 cm) with corncob bedding, cotton nesting materials, and wire mesh tops. Animal cages were not changed for at least 1 week prior to testing to allow individuals to scent mark their territory. Animals were housed in a temperature-controlled colony room (20 ± 2 °C) and maintained on a 14:10 h light-dark cycle with food and water available ad libitum. All procedures were approved by the University of Tennessee Animal Care and Use Committee and are in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Conditioned defeat protocol

During social defeat training subjects experienced either one 15-min or one sub-optimal 10-min aggressive encounter in a resident aggressor's home-cage. The 10-min aggressive encounters were used to avoid a ceiling effect when we expected drug treatment to increase conditioned defeat behavior.

During social defeat training subjects were reliably attacked and defeated by resident aggressors. To standardize the amount of aggression received and the duration of social defeat, timing of aggressive encounters began at the first attack by the resident aggressor, which usually occurred within the first 60 s. During social defeat training we recorded the total duration of aggression and the number of attacks displayed by resident aggressors. No-defeat control subjects did not receive a social defeat. To investigate whether drug treatments affected agonistic behavior in the absence of social defeat experience, we included no-defeat control groups that were exposed to a resident aggressor's empty cage. We performed all training and subsequent testing under red or dim light (< 40 lux) during the first 3 h of the dark phase of the light-dark cycle.

Behavioral testing occurred 24 h after training and consisted of one, 5-min encounter with a novel, non-aggressive opponent in the subject's home cage. Testing sessions were digitally recorded and later scored by researchers blind to the experimental conditions using an ethogram adapted from Albers et al.(2002). A second researcher scored a subset of testing sessions; inter-observer reliability was 91% with a kappa of coefficient of .292. We recorded the total duration of four classes of behavior during the 5-min tests: (a) non-agonistic social (approach, investigate, sniff, and nose touch); (b) nonsocial (locomotion, exploration, self-groom, nest build, and feed); (c) submissive and defensive (flight, avoid, tail up, upright and side defense, full submissive posture, stretch-attend, head flag, attempt to escape from cage); and (d) aggressive (upright and side offense, chase, and attack including bite). For a more detailed analysis of

the subject's agonistic behavior, we also quantified the frequency of flight, stretch-attend, and attack.

Drugs

We dissolved 1-(3-Chlorophenyl)piperazine (mCPP; Sigma-Aldrich) in sterile saline as per previous research (Fox at al. 2008). mCPP is a non-selective 5-HT2 receptor agonist which shows a preferential affinity at 5-HT2C receptors (Kimura et al., 2009). We dissolved α-phenyl-1-(2-phenylethyl)-4-piperidine methanol (MDL 11,939; Tocris) in sterile saline with 1% of acetic acid and adjusted the pH to 5.5 with NAOH as per previous research (Welsh et al., 1998). MDL 11,939 is a highly selective 5-HT2A receptor antagonist (Welsh et al., 1998). All drugs were administered in a 0.3 ml volume using an intraperitoneal injection (i.p.) with a 1ml syringe.

Experiments 1 and 2: 5-HT2C receptor agonist

We designed experiment 1 to test whether injection of a 5-HT2C receptor agonist would enhance the acquisition of conditioned defeat. We injected mCPP (0.3 mg/kg, N=11; 1.0 mg/kg, N=11; or 3.0 mg/kg, N=11) or vehicle (N = 11) 15 min prior to a 10-min social defeat. Animals were tested for conditioned defeat behavior 24 h later.

We designed experiment 2 to test whether injection of a 5-HT2C receptor agonist would enhance the expression of conditioned defeat. Hamsters received a 10-min social defeat, and 24 hours later we injected mCPP (0.3 mg/kg, N=10;

1.0 mg/kg, N=11; or 3.0 mg/kg, N=10) or vehicle (N=11) 15-minutes prior to conditioned defeat testing. No defeat controls received exposure to a resident aggressor's empty cage during training, and 24 h later we injected mCPP (1.0 mg/kg, N=8) or vehicle (0.0 mg/kg, N=8) 15-minutes prior to conditioned defeat testing.

Experiments 3 and 4: 5-HT2A receptor antagonist

We designed Experiment 3 to test whether injection of a selective 5-HT2A receptor antagonist would reduce the acquisition of conditioned defeat. We injected MDL 11,939 (0.5 mg/kg, N=11; or 2.0 mg/kg, N=10) or vehicle (N=10) 30 min prior to a 15-min social defeat. For no defeat controls, we injected MDL 11,939 (2.0 mg/kg, N=8) or vehicle (0.0 mg/kg, N=9) 30 min prior to exposure to a resident aggressor's empty cage. Animals were tested for conditioned defeat behavior 24 h later as described above.

We designed Experiment 4 to test whether injection of a selective 5-HT2A receptor antagonist would reduce the expression of conditioned defeat. Hamsters receive a 15-min social defeat, and 24 h later we injected MDL 11,939 (0.5 mg/kg, N=11; or 2.0 mg/kg, N=10) or vehicle (N=10) 30 min prior conditioned defeat testing. Likewise, no defeat controls received exposure to a resident aggressor's empty cage during training, and 24 h later we injected MDL 11,939 (2.0 mg/kg, N=8) or vehicle (0.0 mg/kg, N=8) 30 min prior to conditioned defeat testing.

Data analysis

Several subjects were not included in statistical analysis because of difficulties with the conditioned defeat protocol. Nine animals were excluded because they were attacked by intruders during testing, two were excluded because of insufficient defeats, and 25 animals were excluded because two cohorts of subjects failed to show conditioned defeat behavior. The 2 excluded cohorts were bred in the Walters Life Science building at the University of Tennessee, Knoxville, born in June and July, and had no obvious reason for not showing conditioned defeat. Our lab is currently investigating individual variation in our subjects to explain why Syrian hamsters can widely vary in their display of conditioned defeat behavior. We analyzed the data with both cohorts included, dropping all animals that did not shown conditioned defeat, and by dropping cohorts when vehicle control subjects did not show conditioned defeat and the statistical results were similar for all three types of analyses.

For social defeat training, we analyzed the total duration of aggression received by subjects and the frequency of attacks received by subjects. For conditioned defeat testing, we separately analyzed the total durations of submissive and defensive, non-agonistic social, nonsocial, and aggressive behavior, as well as the frequencies of attack, flight, and stretch-attend posture. Conditioned defeat data were analyzed using two-way ANOVAs with defeat experience (defeat; no defeat) and drug dose as independent variables. To investigate a dose-response relationship for drug treatments in defeated subjects we performed one-way ANOVAs with Tukey or LSD post-hoc tests. We used t-tests to further investigate the effect of drug treatment in no defeat controls. All

statistical tests were two-tailed and the alpha level was $p < 0.05$. Data are presented as mean ± S.E.

RESULTS

Experiment 1: mCPP and acquisition of conditioned defeat

Injection of mCPP prior to social defeat did not significantly alter the acquisition of conditioned defeat (Fig 1). mCPP treatment did not significantly alter the duration of submissive and defensive behavior ($F_{(3,43)}$ = 0.279, p = 0.840). Likewise, animals injected with 0.3, 1.0 or 3.0 mg/kg of mCPP did not show significant changes in frequency of flight ($F_{(3,43)}$ = 1.177, p = 0.330) or stretch-attend postures ($F_{(3,43)}$ = .320, p = 0.811) compared to vehicle controls (Table 1). Injection of mCPP prior to social defeat did not alter the duration of non-agonistic social behavior ($F_{(3,43)}$ = .390, p = 0.760), nonsocial behavior ($F_{(3.43)}$ = .346, p = 0.792), or aggressive behavior ($F_{(3,43)}$ = 1.306, p = 0.286).

Injection of mCPP prior to social defeat training did not alter the amount of aggression resident aggressors directed toward subjects. Vehicle controls received 200.5 s (±33.3) of aggression during social defeat and individuals injected with 0.3, 1.0, or 3.0 mg/kg of mCPP received 250.5 s (±28.9), 291.5 (±24.6) and 296.0 s (±28.4) of aggression, respectively ($F_{(3,43)}$ = 0.55, p = 0.567). Vehicle controls received 12.3 (±1.1) attacks during social defeat and individuals injected with 0.3, 1.0 and 3.0 mg/kg of mCPP received 10.8 (±1.6), 13.7 (±2.1) and 12.7 (±1.9), attacks, respectively ($F_{(3,43)}$ = 1.41, p = 0.796).

Experiment 2: mCPP and expression of conditioned defeat

Injection of mCPP prior to behavioral testing dose dependently increased the expression of conditioned defeat (Fig 2). A nearly significant drug by defeat

interaction ($F_{(1,36)}$ = 4.116, p = 0.051) was found for the duration of submissive and defensive behavior. This result indicates that 1.0 mg/kg of mCPP increased submissive and defensive behavior in defeated subjects, but not in no defeat controls. Also, a one-way ANOVA on defeated subjects showed a significant increase in submissive and defensive behavior at 1.0 mg/kg mCPP ($F_{(3,40)}$ = 4.204, p = 0.012, Tukey, p = .011) but not at 3.0 mg/kg (Tukey, p = ???). However, mCPP did not significantly change the frequency of flight ($F_{(1,36)}$ = .216, p = 0.645) or stretch-attend postures ($F_{(1,36)}$ = .430, p = 0.517) compared to vehicle controls (Table 2). To measure the selectivity of mCPP's effect on conditioned defeat behavior we also quantified three other classes of behavior, non-agonistic social, nonsocial, aggressive behavior. We expect a selective effect of mCPP on submissive/defensive behavior but not on the other classes of behavior. Our results were as expected, injection of mCPP prior to behavioral testing did not alter the duration of other classes of behavior such as non-agonistic social ($F_{(1,36)}$ = 0.081, p = 0.778), nonsocial ($F_{(1,36)}$ = 1.270, p = 0.268), or aggression ($F_{(1,36)}$ = 0.515, p = 0.478).

No defeat controls did not show greater aggression ($F_{(1,36)}$ = .515, p = 0.478) but did show less submissive and defensive behavior ($F_{(1,36)}$ = 17.382, p < 0.001) compared to defeated subjects (Fig. 2). Also, injection of mCPP in no defeat control animals did not alter the duration of submissive and defensive (t = -1.260, p = 0.248), aggressive (t = 1.000, p = .351), non-agonistic social (t = .476, p = .649), or nonsocial behavior (t = -.643, p = .541). Similarly, no defeat controls injected with mCPP or vehicle did not significantly differ in the number of

attacks initiated during conditioned defeat testing (t = 1.000, p = .351; Table 2).

Experiment 3: MDL 11,939 and acquisition of conditioned defeat

Injection of MDL 11,939 prior to social defeat dose dependently decreased the acquisition of conditioned defeat (Fig 3). We found significant drug by defeat interaction ($F_{(1,36)}$ = 4.793, p = 0.036) was found for the duration of submissive and defensive behavior. This result indicates that 2.0 mg/kg of MDL 11,939 reduced submissive and defensive behavior in defeated subjects, but not in no defeat controls. However, a one-way ANOVA on defeated subjects showed a marginally significant decrease in submissive and defensive behavior at 2.0 mg/kg ($F_{(1,30)}$ = 2.594, p = 0.093, LSD, p = .05). MDL 11,939 did not significantly change the frequency of flight ($F_{(1,36)}$ = .378, p = 0.543) or stretch-attend postures ($F_{(1,36)}$ = .757, p = 0.391) compared to vehicle controls (Table 3). Also, injection of MDL 11,939 prior to social defeat did not alter the duration of other classes of behavior such as non-agonistic social ($F_{(1,36)}$ = 0.204, p = 0.661), nonsocial ($F_{(1,36)}$ = 0.012, p = 0.912), or aggression ($F_{(1,36)}$ = 0.196, p = 0.661).

No defeat controls showed greater aggression ($F_{(1,36)}$ = 9.412, p = 0.004) and less submissive and defensive behavior at testing ($F_{(1,36)}$ = 17.945, p = 0.000) compared to defeated subjects (Fig. 3). However, injection of MDL 11,939 in no defeat control animals did not alter the duration of submissive and defensive ($t_{(1,36)}$ = 1.174, p = 0.279), aggressive ($t_{(1,36)}$ = .417, p = .689), non-agonistic social ($t_{(1,36)}$ = -.361, p = .729), or nonsocial behavior ($t_{(1,36)}$ = -.203, p = .845). Similarly, no defeat controls injected with MDL 11,939 or vehicle did not

significantly differ in the number of attacks displayed during conditioned defeat testing ($t_{(1,36)} = .403$, $p = .699$; Table 3).

Injection of MDL 11,939 did not alter the level of aggression subjects received during social defeat training. Vehicle controls received 307 s (±57.9) of aggression during social defeat and individuals injected with 0.5 or 2.0 mg/kg of MDL 11,939 received 303 s (±47.5) and 277.3 s (±53.3), respectively ($F_{(2,28)} = 0.095$, $p = 0.909$). Vehicle controls received 21.7 (±1.2) attacks during social defeat and individuals injected with 0.5 or 2.0 mg/kg of MDL 11,939 received 19.5 (±2.5)and 16.4 (±3.3), attacks, respectively ($F_{(2,28)} = 0.857$, $p = 0.436$).

Experiment 4: MDL 11,939 and expression of conditioned defeat

Injection of MDL 11,939 prior to behavioral testing did not significantly alter the expression of conditioned defeat (Fig 4). We did not find a significant drug by defeat interaction for the duration of submissive and defensive behavior ($F_{(1,37)} = 0.98$, $p = 0.757$). Also, a one-way ANOVA on defeated subjects did not reveal a significant difference in submissive and defensive behavior ($F_{(1,31)} = 0.248$, $p = 0.782$). Likewise, animals injected with 0.5 or 2.0 mg/kg of MDL 11,939 did not show significant changes in frequency of flight ($F_{(1,37)} = 0.287$, $p = 0.837$) or stretch-attend postures ($F_{(1,37)} = 1.400$, $p = 0.245$) compared to vehicle controls (Table 4). Injection of MDL 11,939 prior to conditioned defeat testing did not produce changes in the duration of non-agonistic social behavior ($F_{(1,37)} = 1.227$, $p = 0.276$) or aggressive behavior

($F_{(1,37)}$ = 0.314, p = 0.579). However we found a significant drug by defeat interaction for nonsocial behavior ($F_{(1,37)}$ = 4.672, p = 0.038), but a one-way ANOVA on defeated subjects did not show a significant effect of drug treatment on nonsocial behavior ($F_{(2,31)}$ = 2.619, p = 0.090). The modest increase in nonsocial behavior appears related to increased cage climbing, nest building, and self-grooming and is not directly attributed to drug-induced hyper-locomotion.

No defeat controls showed elevated aggression ($F_{(1,37)}$ = 5.309, p = 0.027) and reduced submissive and defensive behavior ($F_{(1,37)}$ = 10.354, p = 0.003) compared to defeated subjects (Fig. 4). Also, injection of MDL 11,939 in no defeat control subjects did not alter the duration of aggression ($t_{(1,36)}$ = 1.052, p = .328), submission ($t_{(1,36)}$ = .037, p = .971), non-agonistic social ($t_{(1,36)}$ = -.361, p = .729), or nonsocial behavior ($t_{(1,36)}$ = -.203, p = .845). Similarly, no defeat controls attacked non-aggressive opponents at testing more often than did defeat animals ($F_{(1,37)}$ = 5.309, p = 0.027), although MDL 11,939 injection did not alter frequency of attacks ($t_{(1,36)}$ = 1.055, p = .326; Table 4).

DISCUSSION

In each experiments we found an effect of social defeat on conditioned defeat behavior. Specifically, defeated animals show increased submissive and defensive behavior and decrease in aggressive behavior, when compared to no defeat control subjects. Also, in all experiments pharmacological manipulations did not produce a significant change in the behavior of no defeat subjects. Because the effects of drug treatment were limited to defeated subjects, we concluded the prior psychosocial stress is required for the 5-HT2 ligands used here to modulate agonistic behavior. Our study shows that administration of mCPP, a nonselective 5-HT2C receptor agonist, increases the expression but not acquisition of conditioned defeat behavior. These results suggest that activation of 5-HT2C receptors are important for the production of submissive and defensive behavior at testing but not the development of conditioned defeat behavior. We found that injection of MDL 11,939, a selective 5-HT2A receptor antagonist, reduces the acquisition but not expression of conditioned defeat behavior. These results suggest that 5-HT2A receptor blockade impairs the development of conditioned defeat but is not critical for the production of submissive and defensive behavior at testing. Together these data suggest that 5-HT may act at 5-HT2C and 5-HT2A receptors to facilitate the expression and acquisition of conditioned defeat, respectively. In sum, these results support our overarching hypothesis that defeat-induced increases in serotonin act at 5-HT2 receptors in the forebrain to promote conditioned defeat behavior.

Administration of mCPP exacerbates panic attacks in humans with panic disorder causing behavioral effects such as increased anxiety, depression and panic attacks (Kahn et al., 1988). Also, mCPP increases the expression of anxiety-like behavior in several animal models including the social interaction test (Bagdy et al., 2001), light/dark transition box test (Bilkei-Gorzo et al., 1998), and open field test (Campbell and Merchant, 2003). Although mCPP often is used as a 5-HT2C receptor agonist, the drug pharmacology is complex. mCPP activates several other receptors and binds with equal affinity to 5-HT2C and 5-HT2B receptors. It binds to 5-HT2C receptors with a ten-fold greater selectivity than 5-HT2A receptors and a two fold greater selectivity than 5-HT1A (Roth et al., 1995, Campbell and Merchant, 2003). The non-selective binding of mCPP at 5-HT receptors could explain the non-linear dose response curve in our results. We found that 1.0 mg/kg of mCPP increased conditioned defeat, whereas 3.0 mg/kg was less effective. Our data is consistent with other research showing inverted U-shaped dose response curves for mCPP effects. For example, mCPP treatment increases anxiety in an open field test at doses between 3 and 300 pmol but not at 3000 pmol (Campbell and Merchant, 2003). One, possibility is that mCPP fails to increase conditioned defeat at high doses because it binds to other receptors, such as the 5-HT1A receptor. This possibility would be consistent with our previous finding that activation of 5-HT1A receptors in the basolateral amygdala (BLA) decreases conditioned defeat (Morrison et al., 2011). In a learned helplessness model, another 5-HT2C receptor agonist, CP-809101, has been shown to impaire escape behavior in the absence of prior stress (Strong et al.,

2009). Unlike with Strong et al. (2009), activation of 5-HT2C receptors in our study did not create conditioned defeat behavior in our no defeat subjects. Thus, activation of 5-HT2C receptors appears to interact with social defeat to enhance the display of submissive and defensive behavior, however it does not produce conditioned defeat itself.

We also quantified flees and stretch attends in an attempt to differentiate fear and anxiety. Threat stimuli, like predator odor, increases different types of defensive behavior in both mice and rats. These defensive behaviors have been divided into fear-like responses, which include escape behavior, and anxiety-like behavior, which include risk assessment (Blanchard and Blanchard 2008). In our animals we used flees to represent escape behavior and stretch attends to represent risk assessment behavior. MDL and mCPP failed to significantly alter the frequency of flees or stretch attends. Because there were no significant changes in flee or stretch attend behavior our study was unable to differentiate the effect of 5-HT2 receptors on this aspect of fear and anxiety. Future research will require us to modify our ethogram to more carefully address fearful and anxious types of behavior.

Several brain regions may underlie the effect of mCPP on the expression of conditioned defeat. Brain regions such as the bed nucleus of the stria terminalis (BNST) and central nucleus of the amygdala (CeA), have been implicated in the link between 5-HT2C receptors and the expression of anxiety and fear-like responses. 5-HT2C receptor knock-out mice show reduced c-Fos immunoreactivity in the BNST and CeA following exposure to an anxiety-

provoking stimulus (Heisler et al., 2007). Systemic mCPP administration has been shown to increase the expression of c-Fos in the anterolateral BNST (Singewald et al., 2003). Also, the anxiogenic effects of mCPP have been linked to 5-HT2A/2C receptors expressed by BNST projection neurons (Hammack et al., 2009). The BLA is another key brain region because it contains 5-HT2C receptor protein (Pompeiano et al., 1994) and plays a critical role in the expression of conditioned defeat . Others have found that 5-HT2C receptor activation within the BLA causes acute fear-like responses in an open-field test (Campbell and Merchant, 2003). Similarly, 5-HT2C receptor activation in the BLA reduces social exploration in the learned helplessness model (Christianson et al., 2010).

Pharmacological treatments targeting 5-HT2A receptors have been shown to modulate several types of learning including spatial, emotional, and associative learning in several species (Harvey et al., 1982, Alhaider et al., 1993, Williams et al., 2002). Activation of 5-HT2A receptors by lysergic acid diethylamide, LSD (Gimpl et al., 1979, Siegel et al., 1996), 2,5-dimethoxy-4-methylamphetamine, DOM (Harvey et al., 1982), 3,4 methylenedioxyamphetamine, MDA (Romano et al., 1991), and methylenedioxymethamphetamine, MDMA (Romano and Harvey, 1994) enhances eye blink conditioning in rabbits. Also blockade of 5-HT2A receptors with ritanserin (Welsh et al., 1998), mianserin (Romano et al., 1991), MDL 11,939 (Welsh et al., 1998), and pizotifen (Ginn and Powell, 1986) has been shown to impair eye blink conditioning in rabbits. These studies suggest that 5-HT2A

receptor activation facilitates, while 5-HT2A receptor blockade disrupts eye blink conditioning. The 5-HT2A receptors' role in modulating aversive learning is not limited to eye blink conditioning in rabbits; other animal and human studies have shown that activation or blockade of 5-HT2A receptors modulates the formation of memories for aversive events. For example, the acquisition of active avoidance was enhanced in rats using quipazine, a 5-HT agonist, and was blocked by ketanserin, a 5-HT2A/2C antagonist, suggesting that the enhanced formation of active avoidance was facilitated by 5-HT2A receptors (Alhaider et al., 1993). Similarly, cyproheptadine, a 5-HT2A/2C receptor antagonist, impaired the acquisition of active aviodance (Titov et al., 1983, Ma and Yu, 1993). In humans, injection of ritanserin has been shown to impair learning in an aversive classical conditioning test (Hensman et al., 1991).

Consistent with the research on classical conditioning and active avoidance, our results support a role for 5-HT2A receptors in the acquisition of stress-related memories. Our results indicate that blockade of 5-HT2A receptors prior to social defeat impairs the acquisition of conditioned defeat behavior. MDL 11,939 may impair the acquisition of conditioned defeat by acting in several brain regions that have been implicated in emotional memories. 5-HT2A receptors in the hippocampus and frontal cortex have been implicated in eye blink conditioning (Takehara et al., 2003). Importantly, neural transmission in the hippocampus and prefrontal cortex are necessary for the development of conditioned defeat. Previous research has shown that inactivation of the hippocampus using musicmol disrupted the acquisition of conditioned defeat

(Markham et al., 2010) and inactivation of medial prefrontal cortex impairs conditioned defeat resistance in dominant hamsters (Morrison and Cooper, 2010. Online). The BLA is another candidate brain region for mediating the effect of MDL 11,939 on the development of conditioned defeat. We have previously shown that Syrian hamsters have 5-HT2A receptors in the BLA (Morrison et al., 2011), and neural plasticity in the BLA is critical for the development of conditioned defeat (Jasnow et al., 2005, Markham et al., 2010, Day et al., 2011). Also, 5-HT2A receptors are present on GABAergic interneurons and glutamatergic pyramidal cells in the BLA of rats (McDonald and Mascagni, 2007). One possible explanation for our results is that MDL 11,939 may preferentially block 5-HT2A receptors on BLA glutamatergic cells and thereby impair the development of conditioned defeat. Interesting, serotonergic input can desensitize 5-HT2A receptors in vitro (Roth et al., 1995). Thus, another possibility is that MDL 11,939 might prevent the desensitization at 5-HT2A receptors on GABAergic neurons in the BLA and thus enable serotonergic inhibition of the BLA pyramidal neurons at testing (Fig. 5).

These data extend our understanding the role of 5-HT in the acquisition and expression of conditioned defeat. We have previously shown that enhancing 5-HT signaling in the DRN increases conditioned defeat (Cooper et al., 2008). It was unclear which post-synaptic receptors mediated this increase in conditioned defeat. The current study indicates that the 5-HT2 receptors play a key role in facilitating conditioned defeat. Our data are consistent with previous research suggesting that activation of 5-HT2C receptors is important for the expression of

anxiety-like behavior. While the role of 5-HT2A receptors in the acquisition of anxiety-like behavior is unclear and our findings provide a novel example of the role of 5-HT2A receptors in the formation of anxiety-like behavior. This study builds upon our working model of mechanisms by which 5-HT can modulate the acquisition and expression of conditioned defeat (see Fig. 5). In sum, our results indicate that conditioned defeat is an elegant model for investigating 5-HT's role in anxiety disorders.

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APPENDICES

APPENDIX A

Table 1.

Behavior	0.0 mg/kg	0.3 mg/kg	1.0 mg/kg	3.0 mg/kg	p value
Flee	.455 ± .366	.091 ± .091	1.091 ± .732	.182 ± .122	> .05
Stretch Attend	.364 ± .203	.364 ± .152	.273 ± .141	.545 ± .287	> .05
Attack	.000 ± .000	.000 ± .000	.000 ± .000	.545 ± .455	> .05

The frequencies of flee, stretch attend, and attack (mean ± SE) during conditioned defeat testing are shown. All subjects received social defeat and were treated with 0.0 mg/kg, 0.3 mg/kg, 1.0 mg/kg, and 3.0 mg/kg of mCPP prior to social defeat.

Table 2.

Behavior	D 0.0 mg/kg	D 0.3 mg/kg	D 1.0 mg/kg	D 3.0 mg/kg	ND 0.0 mg/kg	ND 1.0 mg/kg	p value
Flee	1.800 ± .853	1.222± 1.102	1.091 ± .995	.182 ± .122	.000 ± .000	.000 ± .000	> .05
Stretch Attend	.000 ± .000	.111 ± .111	.091 ± .091	.167 ± .167	.000 ± .000	.250 ± .250	> .05
Attack	.500 ± .500	.000 ± .000	.000 ± .000	.455 ± .282	.500 ± .500	.000 ± .000	> .05

The frequencies of flee, stretch attend, and attack (mean ± SE) during conditioned defeat testing are shown. Defeated (D) animals were treated with 0.0 mg/kg, 0.3 mg/kg, 1.0 mg/kg, 3.0 mg/kg of mCPP and No Defeat (ND) animals treated with 0.0 mg/kg and 2.0 mg/kg of mCPP did not significantly differ in any category of behavior. Subjects received i.p. injection prior to conditioned defeat testing.

Table 3.

Behavior	D 0.0 mg/kg	D 0.5 mg/kg	D 2.0 mg/kg	ND 0.0 mg/kg	ND 2.0 mg/kg	p value
Flee	.400 ± .267	.091 ± .091	.200 ± .267	.200 ± .133	.000 ± .000	> .05
Stretch Attend	.400 ± .163	.182 ± .182	.200 ± .267	.000 ± .000	.000 ± .000	> .05
Attack	.000 ± .000	.273 ± .273	.200 ± .200	.556 ± .444	.375 ± .263	> .05

The frequencies of flee, stretch attend, and attack (mean ± SE) during conditioned defeat testing are shown. Defeated (D) animals were treated with 0.0 mg/kg 0.5 mg/kg, or 2.0 mg/kg of MDL 11,939 prior to social defeat. No Defeat (ND) animals were treated with 0.0 mg/kg or 2.0 mg/kg of MDL 11,939 before exposure to an aggressor's empty cage. Subjects did not significantly differ in any category of behavior.

Table 4.

Behavior	D 0.0 mg/kg	D 0.5 mg/kg	D 2.0 mg/kg	ND 0.0 mg/kg	ND 2.0 mg/kg	p value
Flee	1 .182 ± 1.086	1.900 ± .824	1.455 ± .824	.000 ± .000	.625 ± .625	> .05
Stretch Attend	.000 ± .000	.300 ± .300	.273 ± .195	.000 ± .000	.000 ± .000	> .05
Attack	.000 ± .000	.000 ± .000	.000 ± .000	2.125 ± 1.716	.250 ± .250	= .027

The frequencies of flee, stretch attend, and attack (mean ± SE) during conditioned defeat testing are shown. Defeated (D) animals were treated with 0.0 mg/kg, 0.5 mg/kg, or 2.0 mg/kg of MDL 11,939 prior to conditioned defeat testing. No Defeat (ND) animals were treated with 0.0 mg/kg or 2.0 mg/kg of MDL 11,939 before conditioned defeat testing. No defeat controls attacked more often than did defeat subjects.

APPENDIX B

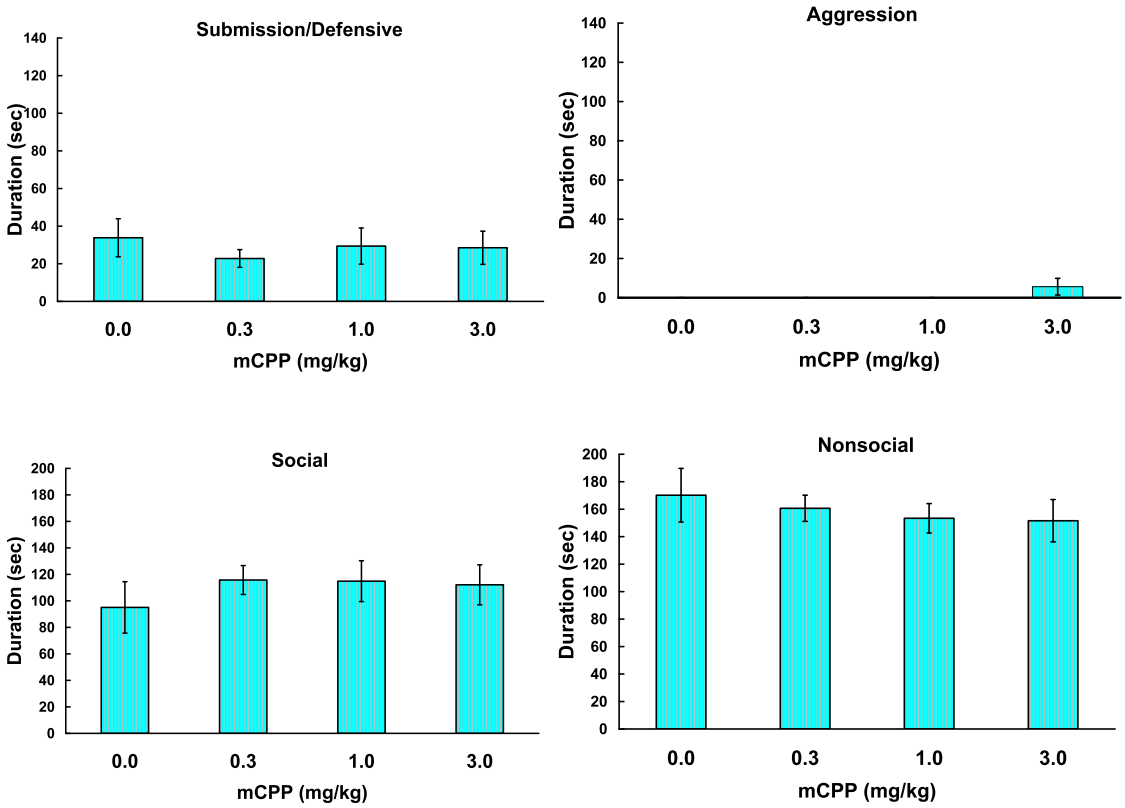

Figure 1. Durations (mean ± S.E.) of submissive and defensive, aggressive, non-agonistic social, and nonsocial behavior are shown for a 5-minute test with a novel, non-aggressive opponent. Subjects received an injection of mCPP (0.3 mg/kg, N=11; 1.0 mg/kg, N=11; or 3.0 mg/kg, N=11) or vehicle (N=11) 15 min before social defeat training.

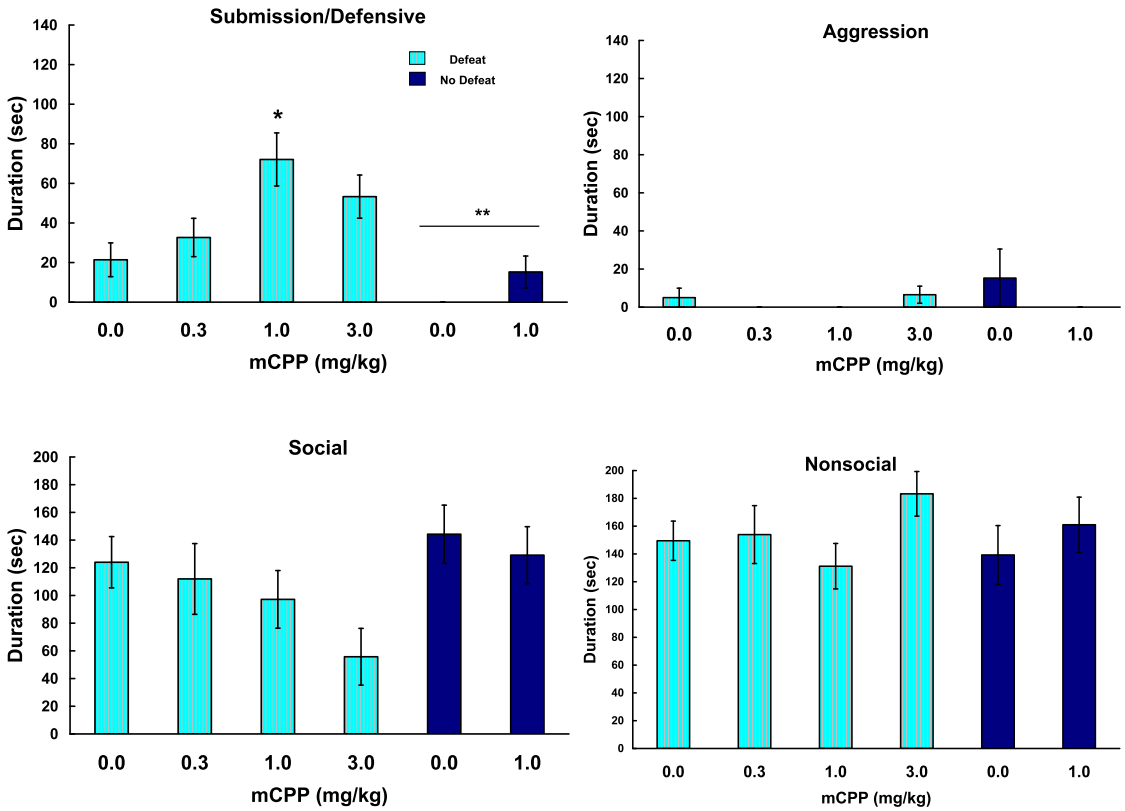

Figure 2. Durations (mean ± S.E.) of submissive and defensive, aggressive, non-agonistic social, and nonsocial behavior are shown for a 5-minute test with a novel, non-aggressive opponent. Defeated animals received an injection of mCPP (0.3 mg/kg, N=10; 1.0 mg/kg, N=11; or 3.0 mg/kg, N=10) or vehicle (N=11) 15 minutes before behavioral testing. Likewise controls received an injection of mCPP (1.0 mg/kg, N=8) or vehicle (N=8) 15 minutes before behavioral testing. * indicates significantly different than defeated, vehicle controls. ** indicates significantly different than defeated subjects.

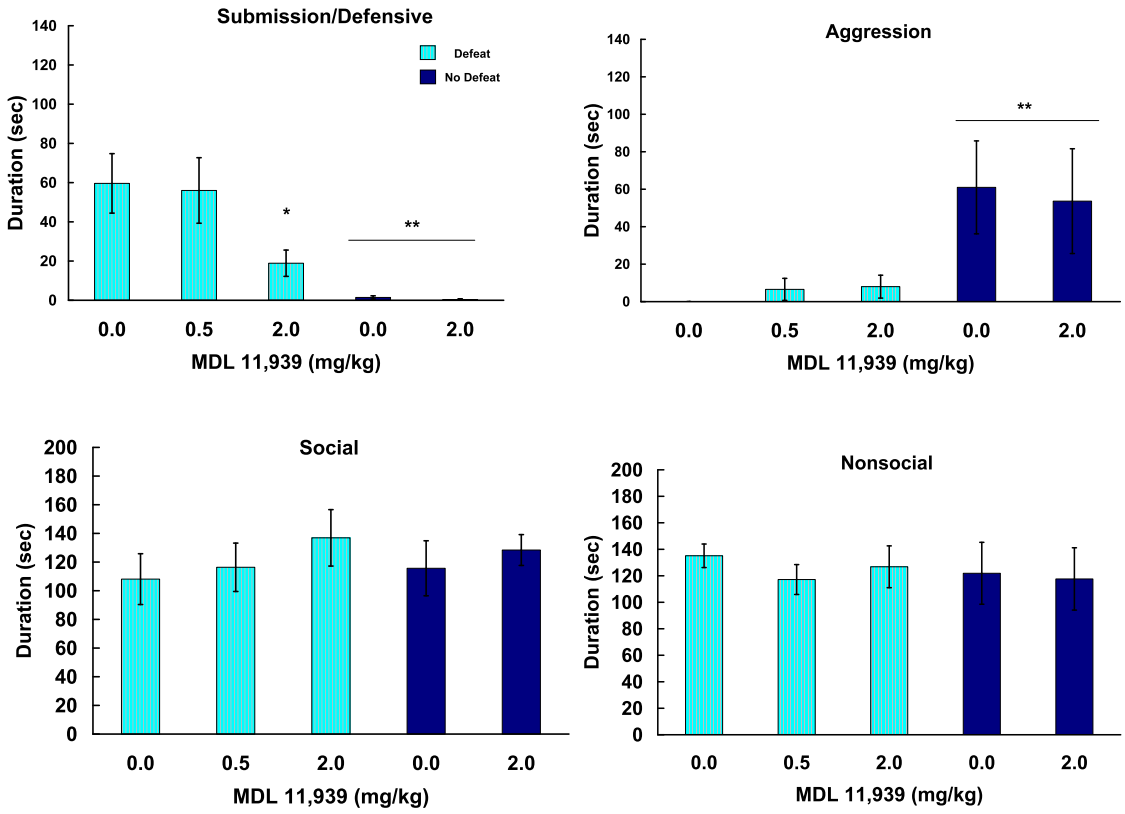

Figure 3. Durations (mean ± S.E.) of submissive and defensive, aggressive, non-agonistic social, and nonsocial behavior are shown for a 5-minute test with a novel, non-aggressive opponent. Defeated animals received an injection of MDL 11,939 (0.5 mg/kg, N=11 or 2.0 mg/kg, N=10) or vehicle 30 minutes before social defeat training. Likewise, controls received an injection of MDL 11,939 (2.0 mg/kg, N=8) or vehicle (N=9) 30 minutes before exposure to a resident aggressor's empty cage. * indicates significantly different than defeated, vehicle controls. ** indicates significantly different than defeated subjects.

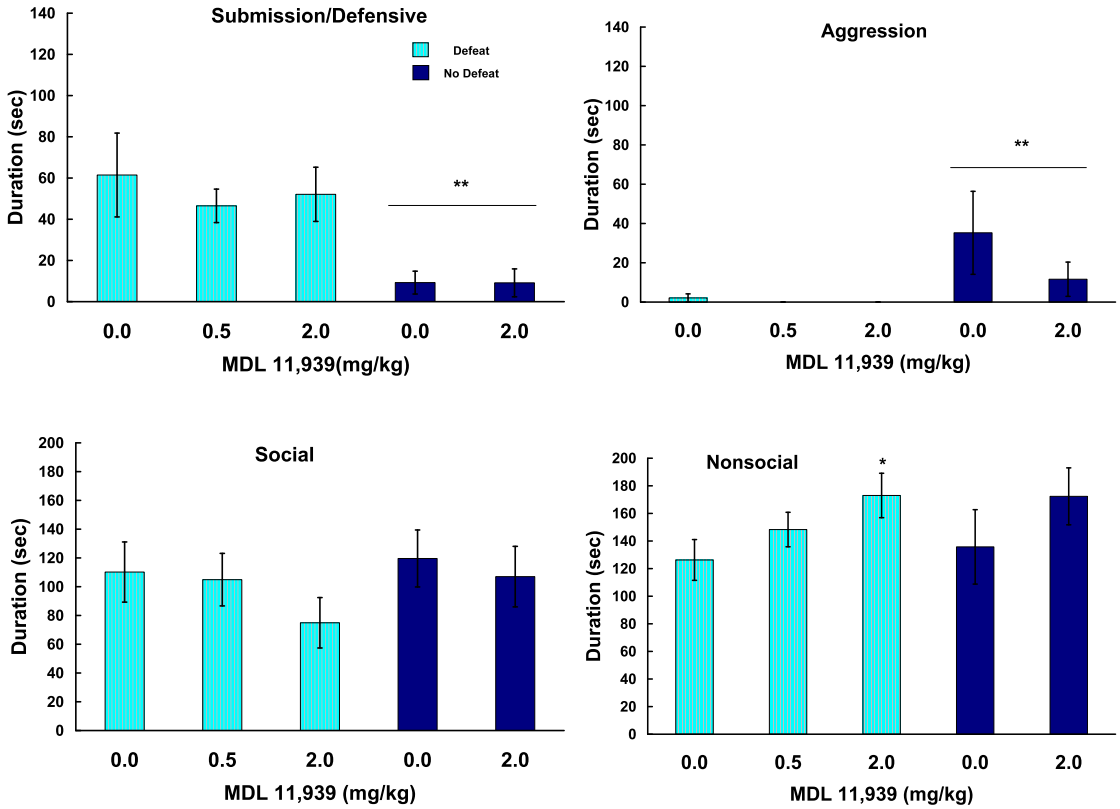

Figure 4. Durations (mean ± S.E.) of submissive and defensive, aggressive, non-agonistic social, and nonsocial behavior are shown for a 5-minute test with a novel, non-aggressive opponent. Defeated animals received an injection of MDL 11,939 (0.5 mg/kg, N=11 or 2.0 mg/kg, N=10) or vehicle (N=10) 30 minutes before behavioral testing. Likewise controls received an injection of MDL 11,939 (2.0 mg/kg, N=8) or vehicle (N=8) 30 minutes before behavioral testing. * indicates significantly different than defeated, vehicle controls. ** indicates significantly different than defeated subjects.

APPENDIX C

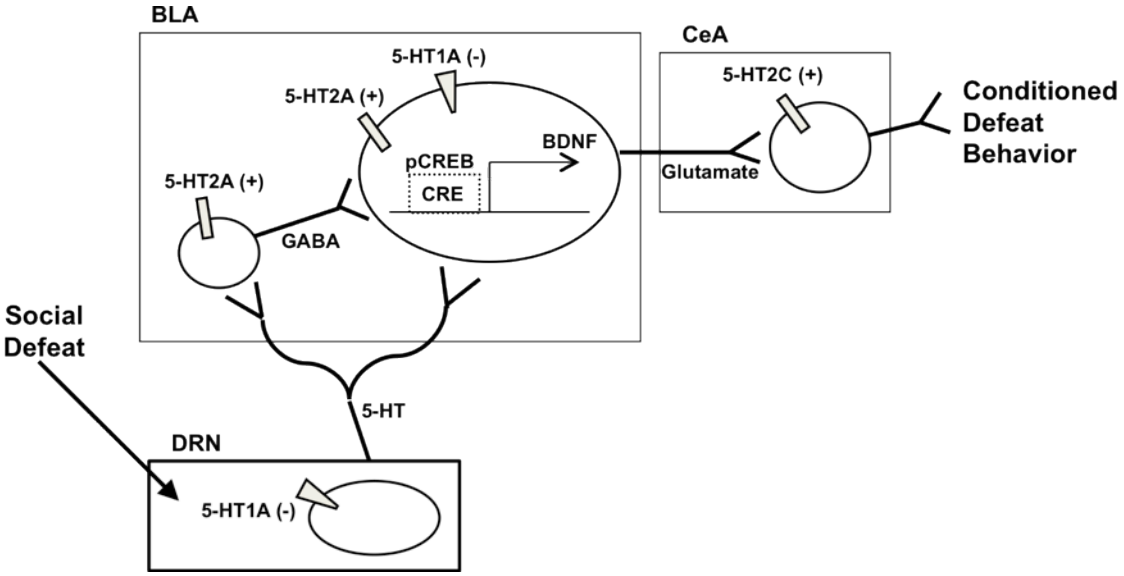

Figure 5. Proposed neural circuit underlying 5-HT1A, 5-HT2A, and 5-HT2C receptors role in conditioned defeat behavior. Social defeat activates 5-HT neurons in the dorsal raphe nucleus (DRN), in which in turn increases 5-HT release into the basolateral amygdala (BLA). 5-HT1A receptor activation in the BLA inhibits glutametergic projection cells causing a reduction in the acquisition of conditioned defeat behavior. 5-HT2A receptors may facilitate the acquisition of conditioned defeat in two separate ways. 5-HT2A receptor activation in the BLA may enhance activity of the glutametergic cells projecting to the central amygdala, causing an increase in the acquisition of conditioned defeat behavior. Also, 5-HT2A receptor activation in the BLA may cause desensitization of 5-HT2A receptors on GABAergic interneurons and disinhibit glutametergic projection cell causing a reduction in conditioned defeat behavior. 5-HT2C receptor activation in the central amygdala on glutametergic projection cell may an increase the expression of conditioned defeat behavior.

VITA

Marquinta Lee Harvey was born in Nashville, Tennessee on March 27, 1981. She obtained a B.S. degree in Microbiology at the University of Tennessee, Knoxville in May 2004. She entered the graduate program in the Psychology Department in August 2009. After obtaining a M.A. degree in Psychology in May 2011, she left to pursue a Doctorate of Philosophy with a focus in Behavioral Neuroscience. She plans to pursue a career in neuroscience research.