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Cannabidiol Administered via Vapor Inhalation Restores Social Interaction Deficits in a Mouse Model of Social Anxiety

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By

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Master's Thesis

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Brennen Risch

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A Thesis

Presented to

The Faulty of

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Abstract

Cannabidiol (CBD), the main non-intoxicating component of the plant cannabis, has shown various promising therapeutic effects in treatment of anxiety and depression in both humans and animals. One potential beneficial effect of CBD is restoration of social interaction deficits following chronic stress. Here I investigate the potential for CBD to be used as a treatment in animal models of Social Anxiety Disorder (SAD), as well as potential mechanisms of action by which CBD may produce these effects. Mice exposed to 10 days of chronic social-defeat stress were administered vaporized CBD in a single 30 minute session before being tested behaviorally. Mice exposed to CBD showed significantly higher levels of social interaction as measured by the three-chamber test compared to mice exposed to vehicle VG/PG vapor, and comparable levels of interaction to unstressed mice. Contrary to my hypothesis, restorative effects of CBD on social interaction were unrelated to levels of BDNF in the hippocampus. These results provide a foundation for the development of novel and improved treatments for chronic stress-related disorders, including social anxiety disorder. These studies also contribute to a growing body of literature on the potential therapeutic effects of CBD and its targeting of various neurobiological pathways in the treatment of chronic stress-related disorders.

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Introduction

Psychological disorders such as social anxiety disorder, generalized anxiety disorder, and major depressive disorder, are increasingly common in the United States, yet are poorly understood and variable in their response to treatment (Leichsenring et al., 2022). Identification of more effective treatments requires knowledge of the developmental processes and underlying neural perturbations that cause symptoms, as well as continuous experimentation with new medicines. This paper will examine the effects of cannabidiol, an abundantly produced phytocannabinoid by the cannabis plant purported to possess numerous medicinal properties, on a stress induced model of social anxiety disorder. Specifically, benefits to social interaction and social dominance deficits will be proposed, and potential mechanisms of both will be identified.

Stress and the Brain

Stress is a known risk factor for several negative physical and mental health outcomes, potentially contributing to abnormal brain development and functionality (Lupien et al., 2009; Nishi, 2020; Syed & Nemeroff, 2017). Stressors activate the hypothalamic-pituitary-adrenal axis (HPA), that primes the brain to handle stressful situations while also serving as a negative feedback mechanism, subsequently terminating responses to stressors. Activation of the HPA axis results in elevated levels of glucocorticoids (GC), stress hormones that bind to glucocorticoid receptors (GR) distributed throughout the brain. Chronic activation of the HPA axis results in HPA hyperactivity, and chronically elevated levels of GC's are thought to play a role in neurological impacts of stress (Tata & Anderson, 2010). Under normal circumstances, GC in the amygdala increase activity as well as levels of anxiety, while in the hippocampus and medial pre-frontal cortex (mPFC), they act to inhibit HPA axis activation decreasing further GC

release and attenuating the stress response (Myers et al, 2014; Mecholaum & Parker, 2013). Individuals who have experienced chronic or early life stress, or those who have been diagnosed with anxiety disorders such as generalized or social anxiety disorder, show altered HPA activation in the form of hypoactive or degenerated mPFC (Lupien et al., 2009), decreased inhibition of the HPA axis by the hippocampus , due to decreased GR expression (Herman et al., 2016; Syed & Nemeroff, 2017), and hyperactive limbic areas such as the amygdala (Linsambarth et al., 2017). It is thought that this altered HPA axis activation in response to stress in part underlies these and other psychological disorders. For instance, social anxiety, and many other anxiety disorders, are characterized by an overactive limbic system and an underactive inhibitory system (Minkova et al., 2017). Therefore, chronic stressors can promote neuropsychological disorders through an overly activated and dysfunctional HPA axis.

Social Anxiety Disorder

Individuals who experience chronic or early life stress are at a higher risk for development of social anxiety disorder (SAD; Miskovich & Schmidt, 2012). With lifetime prevalence rates in the United States ranging from 7-13%, SAD is one of the most common psychological disorders (Kessler et al., 2012), and is highly comorbid with other psychological disorders such as depression and/or generalized anxiety disorder (GAD; Lydiard, 2001; Erwin et al., 2002). SAD is primarily characterized by a fear or avoidance of social situations and is also associated with social subordinance (Weeks et al., 2011; Zimmerman et al., 2015).

Concerns over social dominance may contribute to behavioral manifestations of SAD (Maner et al., 2008; Weisman et al., 2011). Early theorists suggested that social fear may lead to submissive behaviors as an attempt to avoid and deescalate social conflict (Öhman, 1986). This theory of SAD became known as the psychobiological/ethological model (Trower & Gilbert,

1989). Indeed, prior research indicates that individuals with SAD are reported by peers as being more submissive (Walters & Inderbitzen, 1998) and show fewer dominance behaviors (Walters & Hope, 1998) than non-anxious individuals. Behaviors such as submissive posture and elevated vocal pitch have also been observed (Weeks et al., 2011). While properly navigating social hierarchies is essential for healthy functioning, social dominance effects are not always tested for in medications developed to target SAD. For instance, social dominance behaviors require a motivation to engage in social interaction, which is decreased in SAD. It may be of interest to examine neural mechanisms by which proposed SAD treatments might restore social dominance in animal models.

Current treatments for SAD include counseling therapy such as cognitive-behavioral therapy, and/or antidepressant or antianxiety medications, generally selective serotonin reuptake inhibitors (SSRIs) or benzodiazepines (Hjorth, 2020). SSRIs increase levels of serotonin (5-HT) by binding to 5-HT transporters (5-HTT) and inhibiting reuptake of 5-HT into the synapse, although increased BDNF in the dentate gyrus (DG) resulting from enhanced serotonin signaling may be responsible for the benefits of antidepressants (see Lee and Kim, 2010). While current medication is effective for some, between 30-40% of individuals fail to respond to SSRI treatment. Side effects such as nausea and impacted sex drive have been reported by some individuals. SSRI medications are also known for a delayed efficacy, where benefits are not felt for several weeks after beginning treatment, during which time symptoms may remain unchanged or even worsen (Rickels & Rynn, 2002). Benzodiazepines, the second class of common anxiety medications, are fast acting but possess habit-forming properties as well as produce significant cognitive deficits (Lader, 2011).

Mouse Models of Stress and SAD

It is common in behavioral and neurological research to use animal models of SAD to study effects and mechanisms of potential therapies. Most animal models of SAD employ some form of stress procedure to induce social avoidance in the animals, which is then treated with medication or other therapies (Toth & Neumann, 2013). Rodent models can be effectively utilized to study the effects of stress on psychological phenomenon because they are easily maintained, are genetically identical (in the case of inbred lab mice), and can be exposed to experimental conditions and methods of measurement not possible with human subjects. The most common mice used are C57BL/6, and the most common method of modelling early life stress is via maternal separation (Orso et al., 2019). Maternal separation is a paradigm used to induce stress by either acutely or repeatedly separating dams from litters for varying periods of time, anywhere from 15 minutes to 24 hours, and generally performed over the first two weeks of life (Récamier-Carballo, 2017; Orso et al., 2019). MS leads to various health consequences which mimic reports of consequences of severe early life stress in humans, such as increased levels of depression, anxiety, and impacted social behaviors (Orso et al., 2019; Niwa et al., 2011).

Maternal separation is a method of stress induction used to produce socially anxious mouse phenotypes in modelling SAD (Toth & Neumann, 2013). In mice, social anxiety is generally measured by contrasting time spent in social chambers of various behaviors paradigms, such as a three-chambered social interaction or social novelty test, to time spent in non-social chambers. Decreased time spent in the social relative to non-social chamber is indicative of impaired social functioning. Emmons and colleagues (2021) found significant decreases in time spent investigating a novel same-sex conspecific when comparing mice subjected to maternal separation combined with early weaning to controls. Similar results indicating decreased social

interaction as a function of early life stress have been found in rats subjected to maternal separation (Maciag et al., 2002) as well as mice subjected to extended (6 hour) maternal separation during the 3rd week of life (Niwa et al., 2011). Another stress procedure targeted specifically at inhibiting social interaction is the chronic social defeat stress paradigm (Golden et al., 2011). This procedure involves exposing experimental mice to larger and more aggressive mice to be attacked for 10 minutes daily over 10 days. In addition to the defeat sessions, experimental mice are housed opposite their attackers in a divided cage for the remainder of 24 hours before beginning again with a novel aggressor mouse. Repeated stress in the social domain appears to be sufficient to reliably induce a SAD phenotype.

CBD as an Anxiolytic Treatment

Cannabis use as a medicinal treatment for ailments ranging from pain to depression extends back centuries (Mechoulam & Parker, 2013). Today, SAD specifically is highly predictive of cannabis use and dependency, particularly as a form of coping (Buckner et al., 2007; Buckner et al., 2008; Buckner et al., 2014). Cannabidiol (CBD) is the major non-intoxicating component of the plant cannabis and is thought to be responsible for many of the plant's medicinal properties. Indeed, CBD is known to be anxiolytic in both animals and humans (Blessing et al., 2015; Crippa et al., 2011; Schier et al., 2012). CBD represents an attractive alternative to contemporary treatments of SAD, as it lacks many of the problematic properties of either SSRI's or benzodiazepines. CBD is effective in treatment of addiction (Hurd, 2017) and has little to no cognitive deficits associated with its use (Bergamaschi et al., 2011a. Bergamaschi et al. (2011b) found that in a sample of 24 treatment-naïve participants diagnosed with SAD, those administered a single dose (600 mg) of CBD showed significant reductions in reported anxiety during a simulated public speaking task. Chronic CBD administration (300mg/day for 4

weeks) has also been proven effective in treatment of SAD, as CBD was able to reduce self-reported anxiety in a group of treatment naïve Japanese adults diagnosed with either SAD or avoidant personality disorder (Masataka, 2019). While CBD has so far been effective in the limited number of human SAD trials in which it was used, its specific function in various disorders is unclear (Blessing et al., 2015; Schier et al., 2012).

The endocannabinoid system is proposed to be a key target of the phytocannabinoid's therapeutic effects, and CB1 receptors are thought to underly the anxiolytic effects from targeting the endocannabinoid system. CB1 receptor antagonists were found to be anxiogenic when rimonabant, a new drug targeting obesity, had to be recalled for severe side effects including anxiety, mood disorders, and risk of suicide (Christensen et al., 2007). CB1 receptors are generally located presynaptically, and function to inhibit neurotransmitter release. While CBD is not able to directly bind to CB1 receptors, it indirectly agonizes the receptor by boosting AEA levels through competitive inhibition of its hydrolytic enzyme, FAAH, as well as preventing its reuptake (Bisogno et al., 2009).

CB1 receptors are widely distributed throughout the CNS and can be found in several regions involved in anxiety, including the amygdala and hippocampus (Zou & Kumar, 2018). CB1 receptors in the amygdala can regulate either GABAergic (Katona et al., 2001) or glutamatergic (Kodirov et al., 2010) transmission, and are highly expressed in the basolateral amygdala (BLA). Stress is shown to decrease amygdalar AEA levels (Patel et al., 2005), and BLA infusion of an FAAH inhibitor reduces stress-induced corticosterone levels (Hill et al., 2009). However, Morena et al. (2018) found that BLA specific viral FAAH overexpression was associated with reductions in anxiety and fear, possibly from increased GABAergic signaling

from the BLA to the central nucleus. Therefore, CBD may moderate anxiety by increasing endocannabinoid signaling involving CB1 receptors

Importantly, activation of CB1 receptors in the hippocampus may contribute to the increased neurogenesis that is associated with CBD's anxiolytic effects. Administration of CBD elevates hippocampal AEA, and increases neurogenesis and spine density, an effect which was blocked by coadministration of a CB1 receptor antagonist (Campos et al., 2013; Fogaca et al., 2018). Coadministration of a CB1 antagonist also increased behavioral symptoms of anxiety in the EPM and light-dark box tests. This indicates that, at least in the hippocampus, activation of CB1 receptors is necessary for anxiolytic and neurogenerative effects of CBD. While CB1 receptors in the amygdala and hippocampus are known to be involved in CBD's anxiolytic action, there are several other mechanisms by which CBD produces its anxiolytic effects.

The serotonin 5-HT1a receptor is a receptor well known for its anxiolytic properties (Gross & Hen, 2004; Overstreet et al., 2003; Ramboz et al., 1998). Currently SSRI antidepressants are often the first medicine prescribed for anxiety disorders including SAD (Canton et al., 2012; Williams et al., 2017), and their benefits are thought partly to be due to activation of 5-HT1a receptors via increased serotonin availability. 5-HT1a receptors can be divided into presynaptic autoreceptors and postsynaptic heteroreceptors, with the former responsible for inhibitory feedback on 5-HT release, and the later responsible for reducing neuronal excitability (Altieri et al., 2013). 5-HT1a autoreceptors are located mainly in the dorsal raphe nucleus (DRN) of the brainstem, while 5-HT1a heteroreceptors are located throughout the brain. Thus, in this paper, "5-HT1a" receptors we will be used to refer to the postsynaptic heteroreceptors, unless otherwise specified. Like CB1 receptors, 5-HT1a receptors are found in many limbic regions associated with anxiety, including the amygdala, BNST, and hippocampus

(Altieri et al., 2013; Overstreet et al., 2003). Unlike CB1 receptors, CBD directly binds to and agonizes 5-HT1a receptors and may also desensitize 5-HT1a autoreceptors in the DRN (De Gregorio, 2018; Schier et al., 2012; Silvestro et al., 2020). The anxiolytic effect of CBD has been demonstrated to depend on 5-HT1a activation in numerous studies, where anxiolytic effects are blocked by coadministration of 5-HT1a antagonists (Resstel et al., 2009; for review see Blessing et al., 2015).

Gomes et al. (2012) showed that CBD acutely injected into the BNST prevents conditioned responses to contexts previously paired with footshock, and that this effect was dependent on activation of 5-HT1a receptors. 5-HT1a-mediated anxiolytic effects of CBD have also been found in the periaqueductal grey (PAG) where CBD administration results in improvement on EPM performance (Campos & Guimarães, 2008; Soares et al., 2010). Interestingly, 5-HT1a-mediated anxiolytic effects of CBD may be dependent on prior stress, as several studies have demonstrated anxiolytic effects of CBD after stress and no effect (Rock et al., 2017) or an anxiogenic effect (Fogaca et al., 2014) prior to stress.

Other receptors which may be involved in CBD's anxiolytic effect are the peroxisome proliferator-activated receptor gamma (PPAR-γ), and the adenosine A2a receptor (Campos et al., 2012; Silvestro et al., 2020). PPAR-γ is a receptor that regulates gene expression, and that can be found throughout the central nervous system. PPAR-γ is highly expressed in the amygdala and may regulate anxiety through various mechanisms, including modulating expression of BDNF or neuropeptide-y, or by preventing neuroinflammation (Rudko et al., 2020). In support of the role of PPAR-γ in anxiety, a selective PPAR-γ agonist, pioglitazone, was found to reduce anxiety in a mouse model of autism (Mirza & Sharm, 2019), and improve anxiety and depression measures in humans suffering from bipolar depression (Kemp et al., 2014; Zeinoddine et al., 2015). Further,

PPAR-γ knock-out mice have increased baseline levels of anxiety, and increased anxiety in response to stress, while in wild-type mice pioglitazone, a PPAR-γ agonist,was able to prevent anxiogenic effects of stress (Domi et al., 2016). CBD is a direct agonist of PPAR-γ (O'Sullivan, 2016), and action at PPAR-γ has been found to be partially responsible for anxiolytic effects of CBD following brain ischemia, which were attenuated by coadministration of a PPAR-γ antagonist (Mori et al., 2021).

The final mechanism to be reviewed by which CBD may affect anxiety is via interaction with the adenosinergic system. The adenosine receptors A1a and A2a are most widely known for being the targets of the drug caffeine, which is a nonselective and anxiogenic adenosine receptor antagonist (Fredholm et al., 1999). While CBD is unable to directly bind to adenosine receptors, like CB1 it can act as an indirect agonist via inhibition of adenosine reuptake. While both A1a and A2a tend to be anxiolytic when agonized, the A2a receptor has received more attention in anxiety literature (Calker et al., 2019). Indeed, A2a knockout mice show increased anxiety in both EPM and light-dark tests. However, on measures of social behavior, knockout mice surprisingly spent *more* time investigating novel conspecifics and socializing with familiar conspecifics (López-Cruz et al., 2017). Similarly, a selective A2a agonist was found to decrease social behavior as well as defensive behavior in a resident-intruder paradigm (Šulcová, 2001). This makes the A2a receptor a unique target for investigation of CBD's anxiolytic effects, for while enhancement of adenosine signaling may reduce general anxiety, it may also act to exacerbate social deficits seen in disorders such as SAD. While many A2a antagonists are anxiogenic, their effects are varied with some being anxiolytic, or having no effect on anxiety (Yamada et al., 2014). It may then be of benefit for individuals suffering from SAD, to combine

administration of CBD with a selective and non-anxiogenic A2a antagonist to capture both anxiolytic effects of CBD as well as prosocial effects of A2a antagonism.

With numerous neurological targets (Ibeas et al., 2015), several of which may be specifically involved in SAD, CBD may be useful as an anxiolytic compound in treatment of stress induced social avoidance. Below, region specific mechanisms by which CBD may affect social interaction and social dominance deficits in stress induced animal models of SAD will be proposed.

Social Dominance

Along with avoidance of and anxiety during socialization, social dominance deficits are also well documented in individuals with SAD (Walters & Inderbitzen, 1998; Weeks et al., 2011; Zimmerman et al., 2015). As social submissiveness can lead to a variety of negative consequences itself, such as impacted career advancement, potentially leading to depression and suicide, it should be considered as a behavioral target for treatments developed to combat SAD. While there is little to no research currently examining the effect of CBD on social dominance, a mechanism by which CBD might improve social dominance in a stress-induced model of SAD will be proposed.

Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is a protein belonging to the neurotrophin family of growth factors, so named because of their involvement in neuronal survival and growth. Indeed, BDNF action on its associated receptor TrkB leads to growth of new neurons, increased long-term potentiation, and increases in synaptic plasticity. BDNF is expressed throughout the brain, although the highest levels of BDNF are found in the dentate gyrus (DG)

region of the hippocampus (Miranda et al., 2019). Hippocampal BDNF can be upregulated via exercise (Erikson et al., 2011; Liu & Nusslock, 2018) and is downregulated by stress (Licinio & Wong, 2002; Marmigère et al., 2003). While hippocampal activity is involved in terminating the HPA axis in response to stressors, BDNF increases the number of excitatory postsynaptic currents (EPSC) and increases glutamatergic transmission (Binder & Scharfman, 2004; Rauti et al., 2020), potentially enabling the hippocampus to better inhibit responses to stress. Indeed, reducing BDNF in the hippocampus and hypothalamus increased cortisol levels, suggesting a critical role of BDNF-mediated signaling on HPA axis regulation. (Naert et al., 2015).

CBD and BDNF

Some of the antidepressant and anxiolytic properties of CBD administered after stress are thought to be due to increased hippocampal neurogenesis caused by elevated BDNF expression (Campos et al., 2013; Sales et al., 2019). The mechanism by which CBD elevates BDNF expression is not well understood, although it likely involves signaling at 5-HT1a and/or CB1 receptors. CBD is known to agonize 5-HT1a receptors, and 5-HT1a agonists are thought to increase BDNF via downstream CREB activation (the transcription factor that regulates BDNF; Jiang et al., 2016). This theory is supported by the numerous findings that increased BDNF signaling is necessary for the beneficial effects of antidepressants (D'Sa & Duman, 2002; Lee & Kim, 2010; Saarelainen et al., 2003). It is also possible that CBD regulates BDNF expression through interaction with hippocampal CB1 receptors. CB1 receptors are known to mediate the effect of CBD on hippocampal neurogenesis, as coadministration of a CB1 antagonist prevented increases in cell proliferation and spine density caused by CBD, as well as prevented anxiolytic effects in the EPM (Campos et al., 2013; Fogaca et al., 2018). Involvement of CB1 receptors in BDNF expression is supported by the findings that pharmacological inhibition of FAAH, and

subsequent increases in AEA and CB1 signaling, lead to increases in BDNF in a rodent model of depression (also likely via downstream CREB activation; Vinod et al., 2012), and that CB1 receptor knockout mice show deficiencies in hippocampal BDNF (Aso et al., 2008). Therefore, CBD may increase BDNF via serotonergic or endocannabinoid-mediated mechanisms.

BDNF and Social Dominance

Evidence that BDNF expression is associated with dominance ranking can be seen in various stress paradigms. While maternal deprivation resulted in decreased levels of hippocampal BDNF and a subordinate phenotype (Benner et al, 2014), environmental enrichment after weaning has been shown to elevate levels of hippocampal BDNF as well as tube-test rankings of social dominance in Swiss mice (Hoffman et al., 2020). Likewise, Schloesser et al. (2010) found that following social defeat stress transgenic mice adopted a chronically submissive phenotype, which was reversed by environmental enrichment only when accompanied by hippocampal neurogenesis. Hippocampal neurogenesis appears to be necessary for the beneficial effects of environmental enrichment, as Schloesser et al. (2010) found that transgenic mice subjected to social defeat stress developed a submissive phenotype, while mice in which neurogenesis was uninhibited did not show this phenotype. Similarly, bonnet macaques subjected to repeated social isolation stress show improvements in dominance behavior following antidepressant administration only when accompanied by hippocampal neurogenesis (Tarique et al., 2011).

Since BDNF is positively regulated by exercise (Erikson et al., 2011; Liu et al., 2018), elevated levels of BDNF in the hippocampi of dominant individuals have been suggested to arise from increased locomotor activity due to territoriality. It has also been suggested that increased levels of hippocampal BDNF may help dominant individuals assess their positions in a hierarchy

and climb the social ranks (So et al., 2015). The idea that BDNF might contribute to learning about dominance is supported by findings that winning, but not losing mice in resident-intruder paradigms show increased BDNF in the DG (Taylor et al., 2011). Similarly, agonistic encounters in aged male mice result in elevated hippocampal BDNF only in dominant individuals, while BDNF is decreased in submissive individuals (Fiore et al., 2003). Mice selectively bred to show a submissive phenotype begin to show dominance behavior after treatment with an antidepressant that elevated hippocampal BDNF levels (Moussaieff et al., 2012). Therefore, hippocampal BDNF levels appear to correlate with social dominance phenotype, and social dominance is negatively correlated with SAD.

Various forms of stress can impact BDNF levels, including early life stress (Park et al., 2018; Récamier-Carballo et al., 2017; Seo et al., 2016) and psychosocial stress (Jorgensen et al., 2019; Tse et al., 2014; Wu et al., 2014), as hippocampal BDNF expression is decreased following chronic GC exposure (Numakawa et al., 2017; Smith et al., 1995). Social dominance is both affected by stress as well as correlated with neurogenesis in the hippocampus, with dominant individuals showing increased neurogenesis and submissive animals showing decreases (Jorgensen et al., 2019). As CBD is known to encourage hippocampal neurogenesis and elevate BDNF expression in the DG, particularly after stress, CBD-mediated increases in BDNF may lead to reduced social submissiveness in stress-induced models of SAD. It was my hypothesis that CBD administered after chronic stress would restore social interaction by elevating levels of hippocampal BDNF. I tested this hypothesis by assessing the impact of acute CBD exposure on social interaction behavior and hippocampal BDNF following the induction of a social-anxiety phenotype. My results reveal that CBD may acutely rescue social anxiety caused by social defeat but the role of BDNF in its therapeutic action remains unclear.

Method

All procedures conform to the regulations detailed in the National Institute of Health's Guide for the Care and Use of Laboratory Animals(40) and were approved by the Institutional Animal Care and Use Committee at Western Washington University.

Animals

C57BL/6J

C57BL/6J mice bred at Western Washington University were used as target mice in these studies. Ages ranged from 7-12 weeks for behavioral studies and stress procedures excluding maternal separation and unpredictable maternal stress (MSUS). In the MSUS protocol, mice aged p3-p17 were deprived of maternal care daily, but were physically left undisturbed until weening, at which point they were split based on sex and housed in standard caging. For the following experiments only male mice were used. This is due to protocols for various stress procedures included in these studies pertaining to only male mice (Golden et al., 2011; Toth & Neumann, 2013). All mice were housed in standard caging with food and water ad-libitum.

Stress Procedures

Restraint Stress

Mice undergoing restraint stress were placed tail first into plexiglass perforated tubes measuring 3.1 inches long with an inner diameter of 1.0 inches and plugged on the opposite (nose) end with a rubber stopper. The rubber stoppers were perforated in the center enabling the mouse to place their nose through and breathe freely. Mice were left in the tubes for 30 minutes or 1 hour before being released and immediately tested behaviorally. Mice that were also

undergoing treatment exposure were administered treatment immediately following restraint, and behaviorally tested immediately following treatment.

Maternal Separation and Maternal Stress

From P3 – P17, a total of two weeks, mice were deprived of maternal care by removing the dam and placing her in a separate cage for two hours. During the maternal deprivation, the dam was placed in a cage directly next to her litter's to maintain olfactory contact. Maternal deprivation was performed at random times from 8am – 3pm which were changed daily. During the deprivation, the dam was provided with standard caging and free access to food and water. At a random time during the deprivation, the dam was restrained in a restraint tube for 30 minutes. Both the deprivation and restraint periods were fully supervised in an isolated room of the lab.

Chronic Social Defeat Stress

The chronic social-defeat stress procedure was performed as previously described (Golden et al., 2011). Male retired CD1 breeders were purchased from Jackson Labs, as CD1 retired breeder are known for their aggressive behavior. CD1 mice were pre-screened for aggressive behavior, defined as showing an attack latency of <30 s on 3 consecutive days using unique screener C57 mice on each day. Experimental (target) mice (C57BL/6J) were exposed to a novel CD1 aggressor mouse for 10 min each day on 10 consecutive days in a cage divided with plexiglass. After defeat, target mice were moved to the opposite side of the perforated divider and remained in olfactory contact with the aggressor for 24h. Both target and aggressor mice were provided access to food and water ad-libitum. Target mice were monitored for injury during defeat sessions and any mice exhibiting repeated injury or injury exceeding 1cm in size were removed from the study and euthanized immediately.

Behavioral Assays

Tube Test

To measure social dominance, the tube test was performed as described by (Fan et al., 2019). The tube was made of perforated plexiglass, 30 cm long tube with 3.5 cm diameter, and was ordered from and produced by the manufacturing department at Western Washington University. Mice were first trained to progress fully through the tube, before being placed with another trained mouse at one end each. The mice then meet in the middle of the tube and push each other in order to go through it. The mouse that pushed its opponent through to the far end was declared the victor for that round. In some instances mice from a single cage were tested against each other, whereas in others mice from opposite cages were tested against each other. Every mouse had an opportunity to interact in the tube with every other mouse, and following the final rounds, total victories were totaled to form a rank. The higher number of victories an individual mouse had, the higher that mouse's rank in the hierarchy. Only male mice were tested, as dominance hierarchies in female mice are less stable.

Three Chamber Test

The apparatus is a nontransparent Plexiglas box (58 × 30 cm) with two partitions that make left, center, and right chambers (30 × 19.3 cm). Each partition has a square opening (5 × 5 cm) in the bottom center. Inverted cylindrical wire cages (10.5-cm diameter; Galaxy Pencil Cup; Spectrum Diversified Designs) were placed in opposite corners of the chamber (top left and top right) and were used as an inanimate object or to cage the stranger mouse. Cylindrical bottles filled with water were placed on top of the wire cups to prevent the test mouse from climbing on top of the cups. The wire cups and chamber were cleaned with 70% ethanol and wiped with

paper towels between each test mouse. In the habituation phase, a test mouse was placed in the center of the chamber without wire cups and allowed to freely explore the three chambers for 10 min. The test mouse was then returned briefly to its home cage. For the test phase, a stranger age- and sex-matched C57BL/6J mouse was placed in one of the two wire cups; the opposite wire cup was empty. The test mouse was then returned to the center of the chamber and allowed to freely explore for 10 min. The side of the chamber with the stranger mouse was counterbalanced between cohorts. The movement of the mouse was recorded by a USB webcam and analyzed using EZTrack software. Time spent in each chamber and time spent within a 5-cm radius proximal to each wire cage were measured.

Elevated Plus Maze

The elevated plus maze (EPM) is a white plexiglass maze in the shape of a plus, with two of the four arms surrounded by 21cm walls on both sides. The other two arms of the maze are open platforms with no surrounding walls. The entire maze is raised above the floor by 93 cm. Each of the four maze arms is 60 cm × 6 cm connected in the middle at a 6 × 6 cm open center (total 126 cm in length). The maze is elevated 93 cm above the floor. Performance is measured by video recording each trial and comparing time spent in the open arms to that spent in the closed arms. Analysis was performed using EZTrack, an open-source animal tracking software. During EPM trials mice were left alone in the behavioral assessment room behind a closed door with a window for viewing. During restraint stress trials, mice were subjected to either 30 minutes or 1 hour of restraint stress immediately prior to testing. In experiments involving CBD administration, mice completed vaporization sessions (described below) immediately before testing. In CBD administration trials where the subjects were also stressed prior to testing, stressors occurred before CBD administration.

Treatment Administration

In experiments involving vaporized CBD administration, mice were placed for 30 minutes into 36 cm x 27 cm x 23 cm (L x W x H) ~17 L passive vapor inhalation chambers (La Jolla Alcohol Research, Inc) which were programed to deliver precise vapor pulls for 3 seconds every 5 minutes for 30 minutes (starting at time point 0 for a total of 7 pulls per session). Cannabidiol dosage was decided based on previous work by Javadi-Paydar et al. (2019), whose lab helped design the chambers used in these experiments. Dosages used for their experiments were higher than those used in the current experiments, however it was deemed appropriate as the plasma concentrations of CBD measured by Javadi-Paydar et al. (2019) are more appropriate for epilepsy related benefits instead of anxiety relief (Campos et al., 2012). The duration of vapor exposure was assessed by an experimenter who visually assessed the presence of the vehicle vapor and recorded the time on a stopwatch (n = 5 observations/pull duration). A consistent unidirectional airflow was created by a vacuum pump that pulled air and vapor through the chambers at a rate of 7.5 L/min. The air intake port in the front of each chamber was connected to an air flow meter and tubing connected to a commercial SMOK TFV8 Baby Beast Tank with a 0.4 Ω atomizer coil (40-60 W range) filled with the prepared vape oil. Vapor pulls were computer controlled, which would send an electrical current to the base of the atomizer and delivered through the air intake port. Chamber air was then pulled through the chamber and passed through an in-line Whatman HEPA-Cap filter (Millipore-Sigma, St. Louis, MI). The air in the chambers appeared visibly clear of vapor prior to subsequent pull. Mice undergoing vapor exposure were placed in small plexiglass cages with standard bedding and a wire top. The cages were then placed into the chambers and administered either CBD (1mg/ml) or vehicle (PG/VG).

After the final hit, mice remain in the chamber for a final 5 minutes to ensure the vapor is fully circulated.

BDNF Quantification

Western Blotting

Immediately following completion of behavioral testing, mice were euthanized and their hippocampi were collected for protein quantification. Hippocampi were collected by manually dissecting fresh brain tissue before immediately submerging in RIPA buffer for lysis. Tissue samples were homogenized in RIPA buffer containing 1% protease and phosphatase inhibitor cocktail and lysed using 1ml plastic syringe with a 23g needle. The homogenates were centrifuged for 20 min at 16,000 g at 4°C. Following centrifuging, the lysate was removed and the pellet discarded. 50ul aliquots of each lysate were collected and frozen at -80c. The lysates were then thawed to use for western blotting. Protein quantification was performed using BCA analysis, after which 90ug of protein were loaded into each well of a 10 well NuPage Bis-Tris gels. The far lane was used to load 10ul of Chameleon Duo ladder. The gel was then run at 200v for 60 minutes, or until the dye line reached the foot of the gel, after which the protein was transferred to a PVDF membrane. The membrane was scanned in the 700nm channel for normalization using Revert 700 Total Protein Stain and Empiria Studio scanning software. Following normalization, the membrane was blocked using a buffer of non-fat dried milk (NFDM 5%) in tris buffered saline, and incubated overnight at 4°C with monoclonal mouse primary BDNF antibody (ABClonal A18129I; diluted 1:10 000). Primary buffer was comprised of 5% NFDM in TBS-T (0.2% Tween-20). After primary incubation, the membrane was washed in blocking buffer and incubated in goat anti-mouse IRDye 800 secondary antibody (LICOR;

diluted 1:20,000), in 5% NFDM TBS-T (0.2% Tween-20) with 0.1% SDS. The blots were then scanned using a LICOR Odyssey imager and quantified using Empiria Studio software.

Series 1: Timeline of social defeat experiments leading to tissue collection. Mice were first exposed to 10 days of chronic social defeat, before being housed individually for 24 hours prior to behavioral testing. Vape administration was carried out prior to behavioral testing, after which mice were allowed to rest for 15 minutes prior to beginning behavior tests. A period of 3 days was included between behavioral tests, Immediately following the final behavioral test, mice were euthanized and their hippocampi collected and lysed.

Design

I first sought to identify a reliable procedure for inducing social avoidance and social dominance deficits in adult (8-20 weeks) C57BL/6J wildtype mice. I first decided to apply restraint stress to mice trained to complete the tube test measure of social dominance. While previously restraint stress has been shown to induce both social avoidance and social subordinance in mice (Park et al., 2018; Mei et al., 2020), I was unable to induce a socially subordinate phenotype using either 30 minute or 1 hour restraint sessions on dominant males, and ultimately decided to attempt a new stress induction procedure.

The stress procedure attempted following the failure of restraint stress to develop the desired phenotype was a modified version of a maternal separation and unpredictable maternal stress protocol developed by Franklin et al. (2010). In my procedure, I separated a newborn litter from its dam from days p3-p17 for two hours per day, during which the dam was subjected to a 30 minute restraint stress session intended to impair maternal behavior upon reintroduction to the

litter. Once the litter was weened and separated into male and female social groups, it was found that of the 6 pups, only 2 were male. As females have a less stable and more dynamic social structure than males, and as a social hierarchy is unable to be established with only 2 individuals, this litter was unable to be used for further study. At this point it was decided that the time requirements of this procedure combined with the unpredictability of litter sex distribution made the MSUS protocol an inefficient choice. Ultimately another stress procedure was selected instead.

This was also the point at which it was decided that I would focus this project solely on social avoidance and the mechanisms of action by which CBD may affect stress induced social avoidance, as I was unable to find other stress induction procedures that were reported to simultaneously induce subordinance. I therefore turned to an alternative approach to reliably induce a social avoidant phenotype: the chronic social defeat stress procedure described in Golden et al. (2011). After obtaining aggressive retired male CD-1 breeders from Jackson Laboratory, wildtype C57BL/6 mice were subjected to 10 consecutive days of 10 minute defeat sessions followed by 24 hours of olfactory contact, with a novel CD-1 each day. Using this method I was able to significantly reduce the amount of time spent in the social zone of the three chamber test of social interaction , without inducing generalized anxiety effects in the elevated plus maze.

Following successful induction of a SAD phenotype, the effect of cannabidiol vapor administration on stressed and unstressed mice was tested.

Results

Restraint Stress

Male C57 mice (n=12) were trained and tested on the tube-test of social dominance, with the top and bottom ranking mice exposed to either restraint stress or vaporized CBD exposure respectively. Statistical analysis revealed no effect of restraint stress at either 30 minutes or 1 hour on social dominance as measured by changes in hierarchical rank in the most dominant mouse. Similarly, CBD vapor in unstressed mice was unable to impact the lowest ranked mouse's place in the hierarchy.

Chronic Social Defeat Stress

A total of 17 mice were used in the CSDS paradigm to determine whether a socially avoidant phenotype could be reliably produced. N=9 mice were exposed daily to aggressive CD-1 while 8 were housed in control conditions as described by Golden et al. (2013). Behavioral data from defeated experimental mice were compared to control mice in a simple t-test to determine differences in both social interaction and generalized anxiety levels. As stated in the Golden protocol, only about 2/3 of C57 mice are susceptible to CSDS, with susceptibility being defined as a resulting social interaction ratio of <50% when measured in the three-chamber test. Of 9 defeated mice, 4 showed susceptibility to social defeat, with an average of 64% of interaction time spent interacting with the empty cup. No differences were found between control mice (.081) and susceptible mice exposed to CSDS (.097) in time spent in the open arms of the elevated plus maze ($df = 10$, $t = 0.393$, $p = .702$).

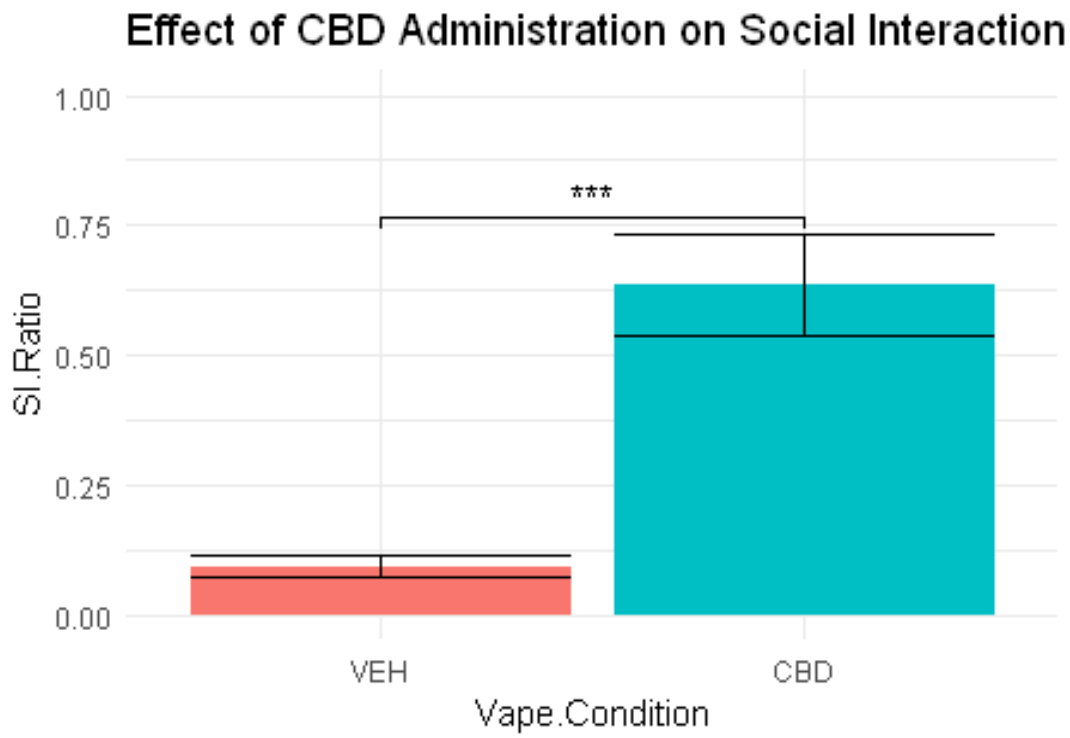

Figure 1: SI Ratio: Social Interaction Ratio. CBD or Vehicle VG/PG fluid vapor was administered in a single 30 minute session prior to completion of three-chamber test of social interaction. Stressed mice administered vehicle vapor spent significantly less time interacting with the social cup versus the empty cup compared to those administered CBD. Mice administered CBD vapor prior to behavioral measurement interacted at similar levels to unstressed mice.

Cannabidiol

After determining a reliable induction method for social avoidance, another 9 mice were subjected to daily social defeat before being administered either CBD vapor (n=4) or vehicle (n=5), after which behavioral data was collected and analyzed. It was found that stressed mice who were administered CBD prior to testing showed significantly higher levels of social interaction (63.27%) compared to mice administered VG/PG vehicle vapor (16.6%; Figure 1; df = 7, t = 3.81, p=.006). Mice administered CBD vapor also showed significantly reduced time

spent in open versus closed arms of the EPM (3.77%) when compared to mice administered vehicle vapor (17.42%; df = 7, t = 2.496, p = 0.04)

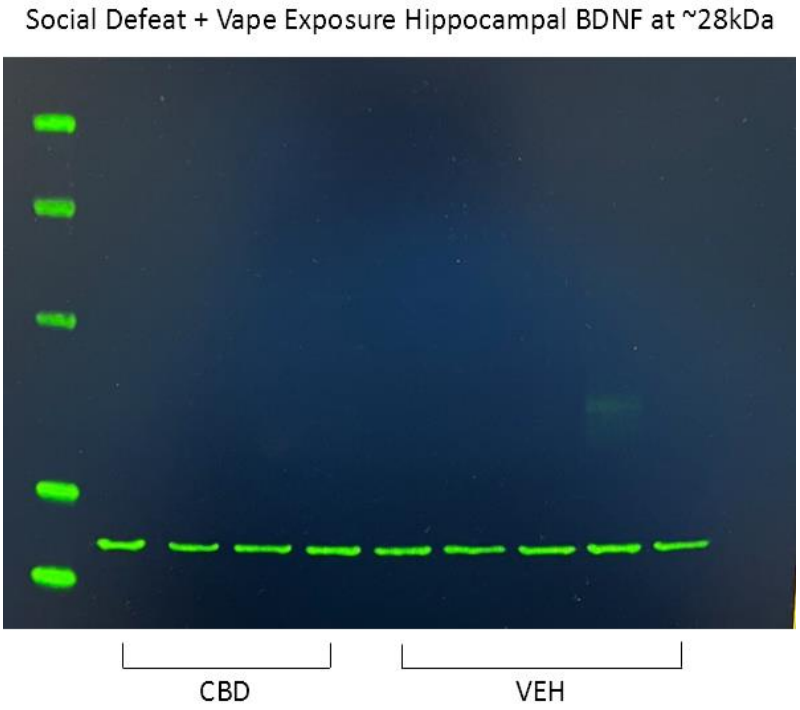

Figure 2: BDNF expression was quantified using western blotting and analyzing band signal with Empiria Studio software. Socially defeated mice who were administered either CBD or vehicle vapor prior to behavioral testing were euthanized immediately following completion and their hippocampi lysed and frozen for quantification. No significant differences were found between signal bands, indicating that there was no difference in relative BDNF expression between the samples.

BDNF Quantification

Protein quantification was achieved by performing western blotting on lysed hippocampal tissue, and the bands were analyzed using Empiria Studio software. Following normalization, BDNF signals were compared using standard t-tests. Mice administered either vehicle (n=4) or CBD (n=5) were compared in a single blot to determine relative concentration of BDNF. Contrary to hypothesis, no significant difference in levels of hippocampal BDNF

between mice administered CBD (0.0556) and vehicle (0.083) were found (Figure 2; df = 7, t = 1.795, p = 0.116).

Discussion

The aim of this study was to investigate the potential for vaporized CBD to be used as a medicinal treatment for stress-induced social anxiety disorder, and to test the relationship between its efficacy and expression of hippocampal BDNF. CBD is known to have various neurological targets on which it acts, many of which are involved in responses to stress and anxiety(Blessing et al., 2015; Schier et al., 2012). Due in part to action on these various targets, CBD has been found to be an effective treatment for various behavioral and physiological effects of animal models of stress. One theorized mechanism by which CBD may produce these therapeutic effects is by upregulation of the protein BDNF in the hippocampus (Campos et al., 2013). BDNF is known to both be affected by stress as well as upregulated after successful treatment with antidepressants, and numerous studies have shown upregulation of BDNF expression in conjunction with benefits seen from CBD administration (Mori et al., 2017;Reus et al., 2011; Sales et al., 2019). I therefore hypothesized that CBD would be an effective treatment for social interaction deficits in a model of SAD and that this effect would be related to an increase of BDNF in the hippocampus as measured by western blot protein quantification.

Through the chronic social defeat stress paradigm, I was able to successfully induce a socially avoidant phenotype while leaving generalized anxiety behaviors unaffected. This is important as while a number of stress-induction procedures are known to induce social avoidance, few have been shown to affect only social anxiety. Social and generalized anxiety are thought to have different underlying neurological pathways(Duval et al., 2015), and in clinical studies of social anxiety disorder, generalized anxiety does not necessarily manifest along with

social anxiety(Blair et al., 2008). The demonstration that mice in these studies showed only social deficits increases the generalizability of our findings to real-world applications, which is critical in animal studies of psychological disorders.

In these studies, I found that following induction of a socially avoidant phenotype using chronic social defeat stress, administration of vaporized CBD in a single 30-minute session was sufficient to restore social interaction ratios to that of control mice. This is consistent with my hypothesis as well as expectations from previous articles demonstrating therapeutic effects of CBD in chronically stressed mice (Campos et al., 2013; Fogaca et al., 2018). The finding that CBD works as expected in a vaporized state is particularly exciting, as increases in self-medication via vaporization by the public are likely to increase as laws regarding cannabis use continue to progress, yet few studies have utilized this method of administration.

While vaporized CBD was effective in restoring social interaction levels to that of controls, interestingly, defeated mice administered CBD actually showed higher levels of anxiety behavior in the elevated plus maze. While this is an unexpected result, it highlights the difference between generalized and social anxiety pathways on which CBD is likely acting. CBD has previously been shown to induce anxiogenic behaviors in certain doses and in unstressed animals (ElBatsh et al., 2012). It is possible that while our social stress procedure was effective in inducing social anxiety, that it was not sufficient to allow a beneficial effect of CBD to be seen on generalized anxiety behaviors.

Contrary to my hypothesis, I did not find a significant relationship between the effect of CBD on social behavior and the expression of BDNF in the hippocampus. This was surprising given that upregulation of BDNF after CBD administration has been shown in various studies (Campos et al., 2013; Mori et al., 2017), however there are various factors which may have

resulted in the contradictory findings of my data compared to that of others, including method of administration and dose. As stated previously few to no labs use vaporization administration methods as these studies did, and while many effects of CBD are dose dependent (Blessing et al., 2015), it is difficult to translate these dosages to that administered during vaporization.

SAD and the BNST

As CBD was unrelated to hippocampal BDNF expression, it is likely that the therapeutic effects on social interaction seen here are due to action on a different neurological target. Current studies are ongoing to determine what the mechanism of action may be, and are focused on potential action at the serotonin 5-HT1a receptor in the BNST.The bed nucleus of the stria terminalis (BNST) is a region considered to be part of the extended amygdala and is involved in sustained anxiety responses, particularly to unpredictable or uncertain stimuli (Walker et al., 2003). Recent research suggests that the BNST may be involved in the pathology of SAD, and a viable neuroanatomical target for treatment. BNST connectivity to the mPFC, a region implicated in BNST activation, was higher in individuals with SAD, and this may indicate hyperresponsivity of the BNST at rest (Clauss et al., 2019). The BNST particularly responds to aversive cues and this activation is greater for those with SAD, as illustrated by one study that showed that an unwanted camera facing the participants elicited a greater BNST response in SAD participants compared to healthy controls (Figel et al., 2019).

BNST functionality also shares several commonalities with SAD pathology, including behavioral inhibition (Clauss, 2019), unpredictability (Gorka et al., 2017), and prosocial interaction (Flannigan & Clash, 2020). Behavioral inhibition (BI) is a personality trait that can be measured in children through negative reactivity (e.g., fidgeting, crying) to novel situations (Kagan et al., 1998). BI is a potent predictor of SAD (Schwartz et al., 1999) with high-BI

children showing a 3.79 times greater likelihood of development of SAD over the lifetime. Clauss (2019) identified the BNST as a potential region involved in BI by relating BI expression to BNST function. High-BI children are known to have increased startle responsivity (Barker et al., 2014), and this startle responsivity is related to later development of SAD (Barker et al., 2015). The BNST mediates the startle response to certain cues, and BNST lesions affect startle responsivity to contextual fear stimuli (Sullivan et al., 2004). Although high-BI individuals tend to have increased responsivity to safe cues or intervals between cues as opposed to threat cues (Barker et al., 2015), it has been suggested that BNST functioning relative to startle response might be altered in BI (Clauss, 2019). Reactivity to novelty, another key component of BI (Kagen et al., 1998), may also involve the BNST.

As high-BI children tend to avoid novel situations, it was originally thought that the amygdala of these individuals would be hypersensitive to novelty (Kagen et al., 1998). Instead, research has shown sustained activation to recently introduced stimuli, suggesting that they still interpret these stimuli as novel. Blood-oxygenation levels measured using fMRI analysis revealed high-BI individuals did not show exacerbated amygdalar responses to novel faces but did show higher levels of activity during viewing of newly familiarized faces (Blackford et al., 2011). A similar pattern can be seen in the BNST of individuals with high trait anxiety[1], where activity was sustained during viewing of newly familiar neutral faces (Pedersen et al., 2017). This sustained BNST activation potentially contributes to the sustained vigilance that characterizes SAD (Rapee & Heimburg, 1997; Claus et al., 2019).

[1] Behavioral inhibition is highly associated with anxiety, and the measure used to determine trait anxiety scores in this study was highly correlated with measures social anxiety

BNST function is also involved in responsivity to uncertainty (Goode et al., 2019). Intolerance of uncertainty (IU) is a measure of an individual's reactivity to uncertainty in life and can be measured using the Intolerance of Uncertainty Scale (IUS), which includes items such as "I must get away from all uncertain situations" and "uncertainty keeps me from living a full life" (Freeston et al., 1994). Individuals with SAD show higher levels of IU, possibly due to ambiguity in social situations causing anxiety over potential for social evaluation (Boelen & Reijntjes, 2009; Rapee & Heimburg, 1997). While the BNST is known to play a role in stress responses to unpredictable threat, SAD is associated with an increased responsivity to unpredictable, but not predictable threat compared to individuals with generalized anxiety disorder (GAD) or major depressive disorder (Gorka et al., 2017). When compared to healthy individuals or individuals with other anxiety disorders, individuals with SAD also have altered BNST connectivity in response to unpredictable cues (Clauss et al., 2019). In social contexts, uncertainty and unpredictability can take the form of ambiguity regarding potential for social evaluation (Boelen & Reijntjes, 2009). Therefore, it is possible that BNST activity in response to uncertainty may contribute to worries regarding social evaluation during such settings, highlighting the importance of the BNST in SAD phenotypes.

Finally, prior research in animals implicates the BNST in social interaction and social anxiety after stress. BNST infusion of corticotrophin releasing factor (CRF), a key stress-hormone involved in activation of the HPA axis, increased social but not generalized anxiety as measured by social interaction and EPM tests (Lee et al., 2008), suggesting that CRF signaling to the BNST is specific for social anxiety. Likewise, administration of a CRF receptor antagonist increased social interaction in adult rats following maternal separation (Maciag et al., 2002), an effect which may likely be due to action in the BNST. The critical role of HPA axis signaling in

the BNST underlying SAD phenotype is highlighted by the finding that chemogenetic silencing of the BNST attenuates the impact of stress on social interaction in C57BL/6 mice (Emmons et al., 2021). Since silencing the BNST in unstressed mice did not further increase social interaction, and further activating the BNST in stressed mice had no further effect on social interaction, it suggests that the BNST's influence on social interaction is bounded by the unstressed and stressed states. Therefore, HPA axis dysregulation may contribute to SAD pathology and represent a targetable system for therapeutic intervention.

Taken together, there is strong support that the BNST is involved in the development and expression of SAD. The BNST is susceptible to stress, particularly early in life, and exposure to stress hormones such as CRF may contribute to deficits in social interaction behavior and aversion to uncertain contexts. Increasing BNST activity in unstressed animals lowers social interaction, whereas BNST inhibition in stressed animals increases social interaction. This is in line with SAD being characterized by an overactive limbic system and underactive inhibitory neurotransmitter systems (Miskovic & Schmidt, 2012). Therefore, treatments that reduce BNST output have therapeutic relevance in SAD.

As stated previously, 5-HT1a receptors' main function is inhibitory, and 5-HT1a receptors are distributed throughout the BNST. 5-HT1a activation in the BNST results in an anxiolytic effect in paradigms such as contextual fear conditioning (Gomes et al., 2012), acoustic startle (Levita et al., 2004), and OF/EPM tests (Alvaro et al., 2018), while 5-HT1a antagonization leads to a decrease in social interaction (Rhodes, 2008). 5-HT1a mediated anxiolytic effects in the BNST arise from inhibitory hyperpolarization of BNST neurons (Garcia-Garcia et al., 2019; Hammack et al., 2009; Rhodes, 2008). This is consistent with findings of anxiogenic effects BNST excitation via glutamate receptor activation (Molosh et al., 2012). CBD

has 5-HT1a receptor-dependent anxiolytic effects when administered into the BNST (Gomes et al., 2012), hence CBD-mediated BNST inhibition via 5-HT1a activation may also serve to attenuate social interaction deficits in stress-induced animal models of SAD.

Limitations

It would also be interesting to expand the current studies in a number of ways. For example, while the current studies only used male mice due to limitations in stress protocols, it may be possible to modify the social defeat protocol in order to effectively induce and manipulate a socially avoidant phenotype in females as well. Yin et al. (2019), using DREADD-based activation of the ventromedial hypothalamus, were able to induce male aggressors to attack female intruder mice. Using these modified aggressors they were able to successfully induce a socially avoidant phenotype in female C57 mice.

In addition to eliminating the sex split present in these studies, it would be interesting to repeat some of the studies involving socially defeated and control non-defeated animals. In the current studies, issues with western blotting protocol troubleshooting as well as ineffective antibodies made early quantification of BDNF levels difficult. It would be valuable to determine definitively whether social defeat in our lab was truly resulting in reduced hippocampal BDNF in order to make sense of the results shown here. If no reduction in BDNF was seen, no increase in BDNF could be expected from later CBD administration.

CBD administration and dose-response is also a potential area of interest for future studies. While the mice in these studies were chronically stressed for 10 days, they were only exposed to a single session of CBD vapor administration. It would be interesting to repeat these studies but manipulate the CBD administration protocol, either by extending the number of

sessions to examine effects of chronic administration, or to extend the range of dosages used in order to test what behaviors may be affected by what dosages. Effects of CBD are highly dose dependent (Campos et al., 2013) and dosages, particularly using CBD in its vaporized state, are far from well-defined. A valuable addition to this will be determining levels of CBD concentration in blood and plasma following various dosages and exposure durations.

In conclusion, our findings suggest that CBD may be a promising candidate for the treatment of social deficits associated with chronic stress, particularly in cases of stress-induced SAD. Although the exact mechanism of action of CBD in restoring social behavior in chronically stressed mice is still unclear, these results provide a foundation for the development of novel and improved treatments for chronic stress-related disorders, including social anxiety disorder. These studies also contribute to a growing body of literature on the potential therapeutic effects of CBD and its targeting of various neurobiological pathways in the treatment of chronic stress-related disorders.

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Appendix A

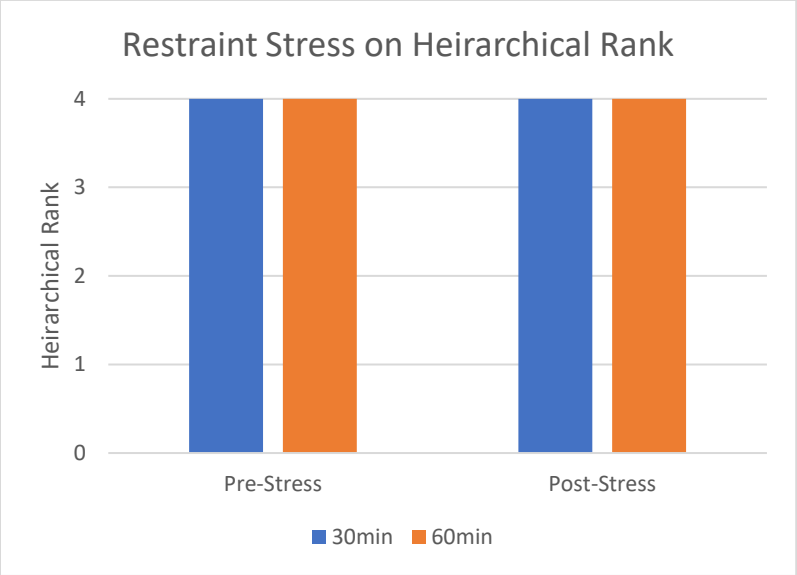

Figure 3A: Effect of restraint stress on hierarchical rank. 30 minutes (blue) and 60 minutes (orange) of restraint stress were performed on the most dominant mouse in an established hierarchy. In either duration, no downwards movement through the ranks was observed.

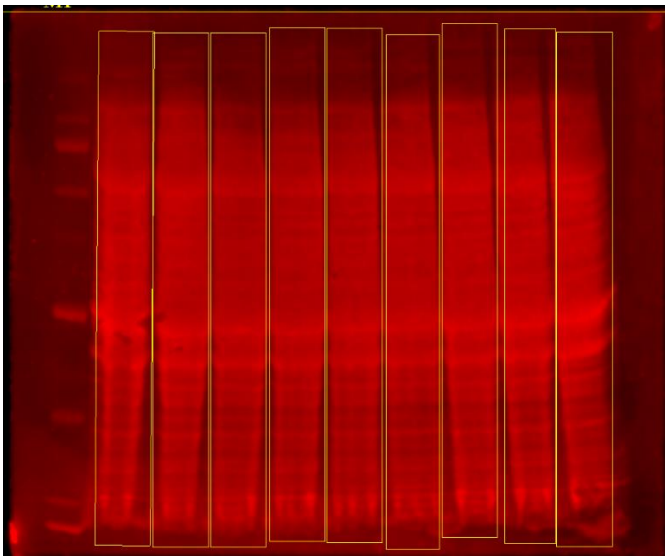

Figure 4A: Revert 700 total protein stain. Image used for normalization of 9 mice subjected to chronic social defeat stress and administered either CBD or VG/PG vapor. Normalization was performed using whole lane background to create a normalization factor, which was then applied to each of the bands in the final analysis.