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Developmental intervention with CBD on BDNF levels and prosocial behavior in a mouse model of autism spectrum disorder

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Chapter 1: Expanding pharmacological therapies for autism spectrum disorder with cannabis-based medicines

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Introduction

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder characterized by core symptoms of impaired social interaction, communication deficits, and repetitive behaviors. Also referred to as autism, ASD is categorized under 'pervasive developmental disorders' (PDD) to account for the broad spectrum of social communication deficits that occur, while additional clinical modifiers may be used to differentiate individuals for diagnosis (Lord et al., 2020). ASD affects 1% of the population worldwide and frequently occurs with additional health conditions that lead to overlapping symptoms (American Psychiatric Association, 2013). Common medical comorbidities include epilepsy, anxiety, sleep and attention disorders, along with gastrointestinal and immune abnormalities (Al-Begati, 2021). As such, ASD may share pathological mechanisms with a variety of neurological disorders. Signs of autism begin to emerge during early development, and screening might start as early as 18 months (Lord et al., 2018). Diagnosis must be based on behavioral presentation due to a lack of reliable biomarkers, though this can introduce bias into autism prevalence ratios. Females often show less severe behavioral symptoms than their male counterparts and are much less likely to be diagnosed as a result (Halladay et al., 2015). This sex bias is exacerbated because research studies often include low sample sizes of females while diagnostic tools are developed in males. As research progresses, it is especially important to focus attention on core symptoms of ASD for optimizing treatment strategies and improving quality of life for affected individuals in all stages of development.

Etiology and Current Therapeutic Approach

The etiology of ASD is multifactorial with both genetic and environmental factors known to play a role. Over 100 risk genes have been recognized (Forrest and Penzes, 2020), and genetic testing is now recommended to individuals with ASD to identify potential causes and co-occurring medical conditions. A meta-analysis of twin studies in 2016 estimated 64-91% heritability, though this does not discount the possibility for nongenetic influence (Tick et al., 2016). A study published in 2019 estimated the heritability of ASD to be approximately 80% with no contribution from maternal effects (Bai et al., 2019). Additionally, they found that nonshared environment, but not shared environment, contributed to risk variability. Still, several environmental factors have been associated with ASD, including advanced parental age, maternal infection, and preterm birth (Modabbernia et al., 2017). It is likely that gene-environment interactions contribute to ASD risk, adding to the complex neurochemical mechanisms that may be involved in its pathology. Sex-specific factors may also influence liability with autism risk for males being three times higher than that of females (Loomes et al., 2017). One theory suggests a female protective effect in which increased etiological load is required for the manifestation of autism in girls. Indeed, Robinson et al. (2013) found that fewer familial risk factors were necessary for males to reach equivalent threshold of autistic impairment. The interplay of genetic, environmental, and sex-specific characteristics are all important aspects of autism etiology that should be considered in order to move toward early diagnosis and intervention.

Beyond behavioral intervention, treatment for core ASD symptoms remains extremely limited. Currently, risperidone and aripiprazole are the only two FDA-approved drugs that exist in the United States to treat "irritability" associated with ASD. Antipsychotics remain the most frequently prescribed medication in the US, followed by attention deficit hyperactivity disorder (ADHD) medications and antidepressants (Jobski et al, 2016). These psychotropic drugs target other comorbid conditions such as inattention/hyperactivity, anxiety, and aggression. Despite widespread use for treating ASD, many of

these drugs have limited efficacy for autism symptoms and introduce undesirable side effects. Antipsychotic treatment is associated with significant weight gain in youth (Yoon et al., 2016), and there is limited evidence for serotonin reuptake inhibitors (SSRIs) in successful treatment of autism symptoms (Lamy et al., 2020). Methylphenidate improved ADHD-related symptoms in children with PDD, but also caused several adverse events including insomnia, decreased appetite, and social withdrawal (Reichow et al., 2013). Developing additional pharmacological therapies capable of alleviating core symptoms of autism is a primary goal as research into ASD pathophysiology continues to grow. Novel treatments may not only more effectively target relevant symptoms but could offer wider accessibility to medications for affected individuals and their families.

Cannabis-based Intervention for ASD

Recent evidence suggests strong involvement of the endocannabinoid system in the pathophysiology of ASD. The endocannabinoid system comprises a vast network of lipid signaling molecules (endocannabinoids), their receptors, and enzymes in the central nervous system (CNS). The main endogenous endocannabinoids include anandamide and 2-arachnidonoyl glycerol that act primarily on cannabinoid receptors type 1 (CB1) and type 2 (CB2). As a modulator of neurotransmission and neuronal plasticity (Zou et al., 2021), the endocannabinoid system plays a fundamental role across health and disease conditions. Dysregulation of this network is observed in ASD with evidence from both human and animal studies revealing altered signaling in relation to social reward responsivity, neural development, circadian rhythm, and anxiety-related symptoms (Chakrabarti et al., 2015). The endocannabinoid system contributes to social functioning by controlling neurotransmitter release at glutamate and γ-aminobutyric acid (GABA) synapses through CB1 receptors (Karhson et al., 2016). This mediates the level of excitation/inhibition (E/I) in the brain, a balance that is essential for establishing normal circuit function and neuronal connections early in life. An elevated E/I ratio is implicated in autism (Rubenstein & Merzenich, 2003), with several studies identifying reduced GABAergic neurotransmission as a common neurological substrate (Fatemi et al., 2009; Han et al., 2014; Sgadò et al., 2013). Furthermore, anandamide is found to be lower in children with ASD (Aran et al., 2019; Karhson et al., 2018). These findings suggest this system may contribute to key phenotypic features of autism, imparting new cellular and molecular targets for drug development. Additionally, an imbalance in E/I signaling may disrupt neural circuit development and drive the core social deficits in ASD.

Cannabis-based medicines provide an unconventional opportunity to modulate affected neurochemical systems and target core symptoms of ASD. With more studies looking into medical cannabis over the recent years, cannabidiol (CBD) has emerged as a promising therapeutic agent for ASD as reviewed by Loss et al. (2021). CBD is an abundant phytocannabinoid produced by *Cannabis sativa L* and demonstrates anti-inflammatory and neuroprotective properties (Bergamaschi et al., 2011). In addition to modulating the endocannabinoid system, CBD exerts its effects through a range of mechanisms in the CNS (Loss et al., 2021). Its high safety profile and nonintoxicating features, as compared to the other major intoxicating phytocannabinoid, Δ9-tetrahydrocannabinol (THC) (Cifelli et al., 2019), have supported those investigating administration of CBD to adolescent populations. Evidently, a study investigating the efficacy of CBD treatment in children with ASD demonstrated improved behavioral symptoms with minor side effects (Schleider et al., 2018). Another study treated patients with a CBD-enriched *Cannabis sativa* extract and showed strong symptom improvements related to seizures, ADHD, communication and social interaction deficits (Fleury-Teixeira et al., 2019). Out of 18 patients, only one exhibited no symptom improvement and three withdrew from treatment due to adverse events. Bilge and Ekici (2021) found that a lower dose of CBD-enriched cannabis improved social interaction and cognition and decreased behavioral problems and stereotypes in autism patients, though responses to these outcomes varied among participants. The genetic heterogeneity of ASD and the fact that most individuals take additional neuropsychiatric medications for comorbid conditions highlight that not all patients will benefit equally when receiving the same medication. Therefore, more

personalized treatment of CBD such as adjusting medication regimen on a case-by-case basis may be required.

Research in preclinical models of ASD is necessary to provide robust evidence for cannabis-based intervention and elucidate the mechanisms by which CBD attenuates core symptoms. A review by Zamberletti et al. (2017) discusses alterations in the endocannabinoid system in several rodent models of ASD. However, there is a lack of research on the effects of CBD in these animal models, particularly with studies that include female subjects (Loss et al., 2021). At present, it has been shown that CBD can improve autism-like social deficits and restore an elevated E/I imbalance in a mouse model of Dravet syndrome (Kaplan et al., 2017; Patra et al., 2020). In the valproic acid model of ASD, cannabidivarin, an analog of CBD, recovered social impairments and repetitive behaviors in addition to restoring hippocampal endocannabinoid signaling (Zamberletti et al., 2019). Pharmacological enhancement of anandamide signaling in the BTBR model of ASD also improved social behavior (Wei et al., 2016). Further research in relevant ASD models is necessary to provide empirical information for CBD treatment of core autism symptoms. Additionally, these models may reveal the involvement of specific cellular and molecular processes and efficacy of therapeutic doses that will aid in translation to clinical study.

Conclusions

The prevalence of autism is rising globally, yet much of its pathophysiology remains elusive. The complex etiology and interplay of comorbid diseases presents challenges for diagnosis and treatment. There is a critical need for pharmacological therapies that can effectively treat the core symptoms of autism. This requires an understanding of the complex cellular, molecular, and behavioral changes that occur through the course of development. Amassing evidence for cannabis-based intervention for neurodevelopmental disorders offers a promising route for therapeutic development. Current literature suggests that CBD can be used to effectively manage autism-related symptoms, including some of the core deficits. Preclinical research should continue to evaluate the efficacy of CBD in validated models of autism to expand available strategies for affected individuals.

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Chapter 2: Cannabidiol and Cannabis-Inspired Terpene Blends Have Acute Prosocial Effects in the BTBR Mouse Model of Autism Spectrum Disorder

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Abstract

Cannabidiol (CBD) is a non-intoxicating phytocannabinoid with increasing popularity due to its purported therapeutic efficacy for numerous off-label conditions including anxiety and autism spectrum disorder (ASD). Those with ASD are commonly deficient in endogenous cannabinoid signaling and GABAergic tone. CBD has a complex pharmacodynamic profile that includes enhancing GABA and endocannabinoid signaling. Thus, there is mechanistic justification for investigating CBD's potential to improve social interaction and related symptoms in ASD. Recent clinical trials in children with ASD support CBD's beneficial effects in numerous comorbid symptoms, but its impact on social behavior is understudied. Here, we tested the prosocial and general anxiolytic efficacy of a commercially available CBD-rich broad spectrum hemp oil delivered by repeated puff vaporization and consumed via passive inhalation in the female cohort of the BTBR strain, a common inbred mouse line for preclinical assessment of ASD-like behaviors. We observed that CBD enhanced prosocial behaviors using the 3-Chamber Test with a different dose-response relationship between prosocial behavior and anxiety-related behavior on the elevated plus maze. We also identified that inhalation of a vaporized terpene blend from the popular OG Kush cannabis strain increased prosocial behavior independently of CBD and acted together with CBD to promote a robust prosocial effect. We observed similar prosocial effects with two additional cannabis terpene blends from the Do-Si-Dos and Blue Dream strains, and further reveal that these prosocial benefits rely on the combination of multiple terpenes that comprise the blends. Together, our results illustrate the added benefit of cannabis terpene blends for CBD-based treatment of ASD.

Introduction

Cannabis sativa L synthesizes hundreds of distinct chemicals (ElSohly and Slade, 2005). Differences in this composition across genetic strains and products confer unique psychopharmacological effects and impacts the purported therapeutic effects (Sholler et al., 2020). Although cannabis has been used medicinally for millennia (Russo, 2007), the individual chemical or combination of chemicals responsible for symptomatic relief across numerous clinical indications are just starting to be understood. Δ^9-tetrahydrocannabinol (Δ^9-THC), which is responsible for the euphoric and intoxicating nature of cannabis, has historically drawn the bulk of research attention (Liu et al., 2020). However, the shared and complementary pharmacodynamic mechanisms across numerous phytocannabinoids has stoked research interest into the impact that non-intoxicating phytocannabinoids, like cannabidiol (CBD), may have across different therapeutic domains (Russo, 2011, 2019; Mandolino et al., 2019). Furthermore, volatile organic compounds, although not unique to *Cannabis sativa L*, are synthesized by the plant in unique "blends" and confer its unique odor and flavor (Sommano et al., 2020). Terpenes are a category of volatile organic compounds that are abundantly produced and may interact with the phytocannabinoids themselves or act as cannabimimetics to confer their own therapeutic properties (LaVigne et al., 2021).

Together, this vast diversity of phytocannabinoids and cannabis terpene blends exposes the immense complexity of the pharmacodynamic interactions in whole-plant cannabis extracts that may impact its medicinal characteristics. It also reveals optimization potential for developing cannabis-based medicines with improved efficacy or extended effective dose ranges (Ferber et al., 2020).

Cannabis use has increased along with legalized access to medicinal and recreational products (Han et al., 2018; Lapham et al., 2022). Currently, the only approved cannabis-derived medicine by the United States Food and Drug Administration (FDA) is a CBD extract, in the form of Epidolex, for intractable pediatric epilepsies. However, anxiety, sleep problems, and stress are among the most common off-label uses of CBD (Moltke and Hindocha, 2021). CBD is non-intoxicating and abundantly produced in the hemp variety of *Cannabis sativa L*, which is typically classified as having less than 0.3% Δ^9-THC (Nahler, 2019). The perception that CBD is safe (Kaur Bhamra et al., 2021) has even led to the off-label administration of CBD to children for treating numerous conditions including anxiety, hyperactivity, and autism spectrum disorder (ASD) (Poleg et al., 2019). These symptoms may derive from similar etiologies that could be targeted by a single treatment approach. Indeed, 30% of patients with ASD also have epilepsy which increases severity of additional comorbid symptoms that involve anxiety, sleep, and locomotor disturbances (Gillberg and Billstedt, 2000). Therefore, CBD may reduce symptoms associated with ASD (da Silva Junior et al., 2021), not to reduce neurodiversity, but to improve daily functioning and quality of life.

ASD is a complex neurodevelopmental disorder defined by core deficits with ranging severities in social, locomotor, and communicative behaviors (Fusar-Poli et al., 2020). Reduced GABAergic signaling (Coghlan et al., 2012; Cellot and Cherubini, 2014) and low levels of the endocannabinoid, anandamide (Karhson et al., 2018), have been implicated in the etiology of ASD symptoms. Boosting GABAergic signaling in preclinical mouse models of ASD (Yizhar et al., 2011; Han et al., 2012; Kaplan et al., 2017) or elevating anandamide signaling through inhibition of its degrading enzyme, FAAH (Kerr et al., 2016; Wei et al., 2016), rescues core social deficits. Initial clinical studies of CBD-rich cannabis treatment in human patients with ASD focused exclusively on comorbid symptoms but demonstrated promising effects (Aran et al., 2018; Barchel et al., 2019). Sublingual consumption of a 20:1 CBD: Δ^9-THC oil led to considerable behavioral improvement on the Clinical Global Impression of Change scale in 61% of patients (Aran et al., 2018). These improvements were accompanied by reduced anxiety levels, less frequent disruptive behavior, and improved communication. In a separate study, CBD-rich cannabis improved symptoms relating to hyperactivity, rage attacks, self-injurious behavior, sleep impairment, and anxiety (Barchel et al., 2019). Notably, 75% of patients reported improvement following treatment whereas symptoms only worsened in 4%. The most recent clinical study of CBD-rich cannabis was conducted in 60 5-11 year old children and found substantial benefits on social interaction (Silva Junior et al., 2022) making it the first clinical investigation to assess CBD's effect on a core ASD symptom. Additional improvements were observed in psychomotor agitation and food intake. The rates of symptom improvement reported in these studies are consistent with traditional prescription medications used in ASD therapy (McCracken et al., 2002; Marcus et al., 2009; Nadeau et al., 2011; Rossignol and Frye, 2014; Sturman et al., 2017), highlighting the potential of a cannabis-based treatment approach as a monotherapy.

Despite the promising outcomes emerging from the clinical trials, there are several limitations to which preclinical investigation will be able to inform clinical use of CBD-based treatment strategies in ASD. First, current clinical investigations have used cannabinoid formulations either not currently commercially available or prohibitively expensive (Elliott et al., 2020). Understanding the efficacy of commercially available products may help make cannabis-based treatment approaches more financially

attainable for some families. Second, while CBD may be efficacious in the current trials' formulations, its efficacy and effective dose range may be enhanced through the additive or synergistic actions of cannabis-derived volatile organic compounds such as terpenes (Russo, 2011, 2019).

The recognition that volatile organic compounds can have therapeutic properties stems back to around 3000 BC with the first recorded description of aromatherapy (Hedaoo et al., 2021). Essential oils used in aromatherapy may have calming, anxiolytic, and even pain-reducing properties under certain conditions (e.g., (Navarra et al., 2015)). These essential oils are usually comprised of multiple volatile compounds, similar to the terpene blends tested in this study, and it's unclear whether their benefits derive from the action of a single compound in the oil or the coordinated action of many. Preclinical models provide a platform for systematically examining how phytocannabinoids (e.g., CBD) and cannabis-inspired terpenes (e.g., formulated blends found in the OG Kush variety containing myrcene, limonene, and β-caryophyllene among other volatile organic compounds) impact behavior alone or in combination. Here, we tested the efficacy of vaporized CBD isolate in commercially available hemp oil as well as common cannabis-influenced terpene blends, either alone or in combination, on core social deficits and anxiety-related behavior in the well-defined BTBR mouse model of ASD (McFarlane et al., 2008). We hypothesized that passive inhalation of CBD rich vapor would induce prosocial effects and these effects would be enhanced by the addition of cannabis inspired terpenes. Our results reveal that both CBD and terpene blends inspired by popular cannabis strains have prosocial effects, and together, lead to improved symptom management in BTBR mice.

Materials and Methods

Animals

BTBR *T+ Itpr3^{tf}*/J (BTBR; Jackson Laboratories, Bar Harbor, ME) litters were bred in-house at Western Washington University. A total of 150 mice (18 males, 132 females) were used in these experiments. Mice were raised in standard laboratory housing in groups of 3-5 mice per cage on a 12-hour light/dark cycle (lights on at 0700). Food and water were provided *ad libitum*. Mice were handled and habituated to the experimenter for a minimum of 5 minutes/day for 3 days prior to experimental assessment. All drug exposures and behavioral testing were conducted during the light cycle. All procedures conform to the regulations detailed in the National Institutes of Health *Guide for the care and use of laboratory animals* and were approved by the Institutional Animal Care and Use Committee at Western Washington University.

Terpenes and CBD vape oils

Three commercially available unflavored CBD isolate-containing hemp oils were initially tested for CBD content (see details below): Savage Vape Shot (Savage Enterprises, Irvine, CA), Koi (Koi CBD, Norwalk, CA), and Blue Moon (Blue Moon Hemp, Pompano Beach, FL), each with a declared 1000 mg of CBD isolate per 30 ml bottle. Terpene blends (i.e., OG Kush, Blue Dream, and Do-Si-Dos) and monoterpenes (i.e., β-caryophyllene, myrcene, and D-limonene) were gifted from Abstrax Tech (Tustin, CA). See Table 1 for composition details. Savage Vape Shot was used for all experiments when CBD oil was indicated (actual CBD concentration: 24.26 mg CBD/ml). CBD in Savage Vape Shot was CBD isolate with a 70/30 vegetable glycerin/propylene glycol base. When indicated, Savage Vape Shot or the terpenes were diluted in a vehicle solution comprised of 70% vegetable glycerin, 30% propylene glycol purchased from La Jolla Alcohol Research, Inc. (La Jolla, CA). The terpene blend concentration in vape oil was diluted to 5%. Terpene concentration was determined from the concentration found in each blend: D-limonene makes up 20% of the OG Kush blend, and therefore, was diluted to 1%; myrcene makes up 35% of the Blue Dream

blend, and therefore, was diluted to 1.75%; β-caryophyllene makes up 24% of Do-Si-Dos, and therefore, was diluted to 1.2% with vehicle. Vape oil dilutions were prepared on the day of experiments.

Drug administration

Four 36 cm x 27 cm x 23 cm (L x W x H) ~17 L passive vapor inhalation chambers (La Jolla Alcohol Research, Inc) were programed to deliver precise vapor pulls for 6 seconds every 5 minutes for 30 minutes (starting at time point 0 for a total of 6 pulls per session; see Supplementary Figure 1). 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. Each 6 second vapor pull draws 83.3 microliters of vape oil and leads to an approximately 2-minute exposure (120.25 ± 4.55 seconds) to the vapor as it gets pulled through the chamber. 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 vapor pull. Since the vapor gets evenly distributed across 4 chambers, each 6 second vapor pull leads to the delivery of 0.51 mg of CBD in the undiluted CBD product.

Behavioral Assessment

Behavioral assessments began between postnatal day 80 and 200. All experiments were run as a repeated measures design, except for the elevated plus maze for which ages were counterbalanced across conditions. Exposure conditions were counterbalanced for all experiments. Each experiment included subjects from a minimum of two litters. Animals are removed from the chambers 5 minutes after the last vapor exposure and are then moved to the behavioral room. Animal behavior was tested approximately 20 minutes following the last vapor exposure. Animal movement was recorded in the presence of overhead fluorescent light using a digital camera (Microsoft LifeCam) mounted above the behavioral apparatus. Behavior was analyzed using ezTrack open-source animal tracking software (Pennington et al., 2019). Each video was checked for accurate assessment by visually inspecting output bokeh plots and calculating total ratios to ensure that 100% of their behavior was captured in analysis. At the end of each trial, the behavioral apparatus was cleaned with 70% ethanol and wiped with paper towels.

Three Chamber Test of Social Interaction

Experiments were conducted as a within-subjects design and exposure conditions were counterbalanced between subjects. 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. For each experiment, mice did not show a side preference during the habituation period (all p > .05). Locomotor activity was also measured during the habituation phase and compared across conditions in a between-subjects manner for only the first round of each experiment to eliminate any practice effects on exploratory locomotor activity. After habituation, the test mouse was then returned briefly to its home cage. For the test phase, a stranger age- and sex-

matched C57BL/6J mouse (Jackson Laboratories, Bar Harbor, ME) 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 trials. Time spent within a 5-cm radius proximal to each wire cage was measured and recorded as time interacting with the "social" or "object" stimulus. A social preference was defined as a statistically significant preference for engaging in social interaction as a function of the total interaction (time spent interacting with the social stimulus and non-social, "object", stimulus): 0.5 > (interaction with social stimulus / total stimulus interaction).

Elevated Plus Maze

These experiments were conducted as a between-subjects design to prevent practice effects. Ages were counterbalanced across conditions. Subjects were placed in the center of the white plus-shaped maze and allowed to explore for 5 minutes. Each of the 4 maze arms is 60 cm x 6 cm connected in the middle at a 6 x 6 cm open center (total 126 cm in length). Two "closed" arms are surrounded by 21 cm opaque plexiglass walls on 3 sides while the other two "open arms" are open on all sides. The maze is elevated 93 cm above the floor. The ratio of time spent in the open arms/closed arms was assessed using ezTrack. Head dip frequency, grooming frequency, and grooming duration were assessed over video by an observer blind to the experimental condition. Experimenters left the behavioral room during the experiment and monitored behavior on a computer monitor through a narrow window. The EPM test was conducted with full overhead lighting.

Quantification of CBD in commercial products by LC/Q-TOF

We used an LC-QTOF-MS system to quantify CBD in our samples, specifically an Agilent 1290 UHPLC with an AdvanceBio 6545 XT Q-TOF. Separation was attained with an Agilent Eclipse+ C18 RRHD column, a 0.2 ml/min flow rate, and a 10-minute gradient transitioning between water with 0.1% formic acid (solvent A) and acetonitrile (solvent B), see Table 2. The system was fitted with an electrospray source with the capillary voltage and nozzle voltage set at 3500 and 2000 V respectively. Within the mass spectrometer the fragmentor voltage was set at 175 V while the skimmer was at 60 V. Analyte confirmation and peak integration was completed with Agilent MassHunter software.

Quantification of CBD in plasma by LC/Q-TOF

Male and female BTBR mice (n = 4 male and n = 4 female) were exposed to undiluted Savage Vape Shot for the same 30-minute protocol as used throughout the experiments (i.e., 6 second vapor pulls every 5 minutes for 30 minutes). Immediately after the 30-minute exposure period, blood was collected by cardiac puncture and placed in lithium heparin BD Mircotainer tubes (Becton Dickson, NJ, USA). The blood sample was then centrifuged at 2,000 rcf (g) for 10 minutes at 4°C. Serum plasma was then transferred to a separate tube for the liquid-liquid extraction procedure. Here 100 uL of plasma was added to 200 uL of acetonitrile and vortexed for 60 seconds. 50 mg salt mixture containing a 4:1:1 ratio of magnesium sulfate, sodium chloride, and sodium citrate was added and vortexed for another 60 seconds. The samples were then centrifuged at 10,000 rcf (g) for 10 minutes at 4°C. The plasma was then extracted and stored at -80°C until testing.

For the quantification of CBD in mouse plasma, the same instrument was used as was detailed previously for the CBD oil concentration verification in commercial products. The system, column, solvents and flow rate remained the same. To achieve separation of this more complex sample matrix an extended gradient was used, see Table 3. The system was fitted with an electrospray source with the capillary voltage and nozzle voltage set at 3000 and 1500 V respectively. Within the mass spectrometer the

fragmentor voltage was set at 100 V while the skimmer was at 60 V. Analyte confirmation and peak integration was completed with Agilent MassHunter software. It was known that the sample concentrations would be low and near the detection limit, so a larger injection volume was used to concentrate the sample within the instrument. This concentration factor as well as the dilution done during the liquid-liquid extraction was used to calculate the final dilution factor of 0.2, which was used calculate the final sample concentration.

Statistical Analysis

All data are shown as mean ± S.E.M. and analyzed by either one-way ANOVA, repeated measures ANOVA, or paired t-tests where appropriate using Sigma Plot software (SPSS Inc) with an alpha set at 0.05, all tests two-tailed. For analyzing the elevated plus maze data, we used a Kruskil-Wallis ANOVA on ranks due to a statistically significant Shapiro-Wilk test suggesting we violated the assumption of a normal distribution of data ($P < .05$). Tukey's HSD post hoc comparisons were used to analyze main effects and interactions. Descriptive statistics (mean ± S.E.M.), as well as the number of subjects and litters used in each experiment are included in Table 4. For all figures, * indicates $P < .05$; ** indicates $P < .01$; *** indicates $P < .001$.

Results

Because of the increasing popularity of commercially available CBD-rich hemp products (Leas et al., 2019; Williamson et al., 2020), we assessed the efficacy of a commercially available CBDrich hemp extract (see methods for product details). Since the declared CBD content on the product label of commercial CBD hemp products is often inaccurate(Johnson et al., 2022; Miller et al., 2022), we first sought to identify the CBD concentration in three hemp samples that all claimed a concentration of 33.3 mg/ml. Analysis of CBD concentration using LC/Q-TOF revealed that CBD concentrations were lower than reported in all three samples and ranged from 21.32 mg/ml to 24.26 mg/ml (Supplemental Figure 2). For our behavioral assessment, we chose to use the Savage Vape Shot product because it had the highest CBD concentration.

To test CBD's efficacy in treating core ASD-like social deficits in our BTBR mice, we used the 3-Chamber Test of Social Interaction(Crawley, 2012) and measured the effect of vaporized CBD oil at four different concentrations (vehicle, 1 part CBD oil to 2 parts vehicle [1:2], 1 part CBD oil to 1 part vehicle [1:1], and undiluted CBD oil [1:0]) on the ratio of time spent interacting with a novel stranger mouse compared to the total time in interaction with either the mouse or an inanimate object in male and female mice. Each six second vapor pull of the undiluted CBD oil delivered 0.51 mg of CBD into each chamber (see method section for details). By the end of the 30-minute exposure session, in which mice were exposed to six, six second vapor pulls, plasma CBD concentrations were $0.13 ± 0.02$ ng/ml (range: 0.08 ng/ml to 0.19 ng/ml; Supplemental Figure 3). A two-way repeated measures ANOVA revealed a main effect of exposure condition, $F_{(3,42)} = 17.57$, $P < .001$, and an interaction between mouse sex and CBD concentration, $F_{(3,42)} = 5.03$, $P = .005$, on the social interaction ratio (Figure 1; Table 3). In male mice, both the diluted 1:2 ratio and undiluted 1:0 ratio hemp oil increased the social interaction ratio, whereas the 1:1 ratio reduced the social interaction ratio compared to vehicle treatment (all $P < .05$, Figure 1a). In female mice, all 3 CBD concentrations increased social interaction, all $P < .01$, Figure 1b) without affecting locomotor activity, $P = 78$ (Supplemental Figure 4). Notably, males and females differed in their social interaction ratios in the vehicle condition, $P = .001$. In contrast to what we predicted based on previous reports (Yang et al., 2012), one-sample t-tests revealed that male BTBR mice showed a significant social interaction preference, $t(7) = 4.54$, $P = .003$, whereas female mice did not, $P = .4$. We replicated this prominent social preference in a separate cohort of male mice, $t(5) = 4.36$, $P = .004$.

Therefore, we focused the rest of our behavioral assessment to female mice to further investigate pharmacological strategies that impact social deficits and related comorbidities.

While CBD oil caused prosocial effects in our BTBR strain, it's unclear if cannabis terpenes also contribute therapeutic benefits on their own and may be responsible for the high degree of efficacy reported anecdotally from whole-plant preparations. We started by testing the effect of vapor delivery of a blend of terpenes from the OG Kush cannabis strain (5% OG Kush terpenes, 95% vegetable glycerin/propylene glycol vehicle) on social behavior in the 3-Chamber Test. Like CBD, a paired t-test revealed that OG Kush terpenes caused a robust increase in the social interaction ratio compared to vehicle, $t(8) = 3.69$, $P = .006$ (Figure 1c). Interestingly, a KruskalWallis one-way ANOVA on ranks identified that only the undiluted CBD oil, but not other concentrations, nor OG Kush terpenes, reduced general anxiety on the elevated plus maze, $H(4) = 12.25$, $P = .02$ (Figure 1d). There were no impacts of any of the CBD concentrations nor OG Kush on head dips, grooming frequency, or time spent grooming (all $P > .05$; Supplemental Figure 5). This suggests that some of CBD and OG Kush's prosocial benefits are independent of reducing general anxiety since changes in social interaction behavior were observed without changes in general anxiety.

We next tested the hypothesis that a combination of CBD oil with added terpenes would be more efficacious than the two components on their own. This is often referred to as the "Entourage Effect", and although it has strong theoretical basis (Russo, 2011, 2019), the impact that innovative combinations of terpenes and cannabinoids have on various conditions, including social behavior in ASD, are largely understudied (Ferber et al., 2020). We therefore retested the effect of a 1:2 CBD oil:vehicle solution and 5% OG Kush terpenes, alone and in combination on prosocial behavior in the 3-Chamber Test. A priori pairwise comparisons supported the replication of our earlier results that both a 1:2 CBD oil:vehicle solution and 5% OG Kush terpenes, independently increased the ratio of time spent in social interaction compared to vehicle (all $P < .05$). A one-way repeated measures ANOVA with Tukey's posthoc comparisons found a main effect of exposure condition on social behavior, $F(3,36) = 7.66$, $P < .001$, and the combination of 1:2 CBD oil combined with 5% OG Kush terpenes had the most robust prosocial effect ($P < .001$; Figure 2a). These findings suggest that a combination of CBD oil and OG Kush terpenes leads to stronger and more robust prosocial benefits. These prosocial effects were independent of changes to general anxiety as the combination of OG Kush and 1:2 CBD oil had no impact elevated plus maze performance, $P = .58$ (Figure 2b).

The prosocial effects we observed with fresh OG Kush terpenes prompted investigation of the potential prosocial effects of other cannabis terpene blends from common strains such as Do-SiDos and Blue Dream. Each blend's composition of volatile organic compounds is listed in Table 2. Similar to OG Kush, a repeated measures ANOVA found that both Blue Dream and Do-SiDos terpene blends (5% terpenes, 95% vegetable glycerin/propylene glycol vehicle) increased the social interaction ratio, $F(2,14) = 4.56$, $P = .03$ (Figure 3a). Together, these findings demonstrate that cannabis terpene blends can contribute to the prosocial benefits in ASD and highlight the benefits of a commercially available hemp oil containing CBD isolate. We hypothesized that it was the unique blends of volatile organic compounds, and not a single terpene within the blend, that conveyed the prosocial effects we observed. To test this hypothesis, we assessed each terpene blend's most abundant terpene alone and at the concentration found in each blend on social interaction behavior. Since each terpene blend was tested at a concentration of 5%, the following terpene concentrations were tested to match the individual terpene concentration from each blend: 1% D-Limonene (most abundant in OG Kush), 1.5% β-caryophyllene (most abundant in Do-Si-Dos), and 1.75% myrcene (most abundant in Blue Dream). Consistent with our hypothesis, a one-way repeated measures ANOVA did not reveal any effect of the individual terpenes on the social interaction

ratio, $F_{(3,18)} = 1.20$, $P = .34$ (Figure 3b). Therefore, our results suggest that the most abundant terpene in each blend is not solely responsible for the prosocial benefits we observed from the complete blends. Instead, the unique combination of volatile organic compounds in each blend are important in promoting prosocial behavior.

Discussion

Numerous anecdotal cases and accumulated caregiver reports suggest that CBD can reduce core symptoms of ASD and improve quality of life (Bar-Lev Schleider et al., 2019; Barchel et al., 2019). Several early clinical trials have found that CBD can improve comorbid symptoms of ASD (Aran et al., 2018; Barchel et al., 2019), but only one assessed and found benefits on core social interaction behaviors (Silva Junior et al., 2022). These studies used CBD-rich cannabis oils that contain a 1:20 ratio of $\Delta 9$ - THC to CBD, more than the 0.3% limitation of $\Delta 9$ -THC to legally classify as "hemp", and therefore, may make it increasingly difficult for patients to access because of legal restrictions or prohibitive cost. Safety concerns among caregivers with administering $\Delta 9$ -THC to children may also limit this formulation's utility. This and other medicinal cannabis formulations tend to focus exclusively on phytocannabinoids (e.g., CBD and $\Delta 9$ -THC) and fail to consider the potential therapeutic benefits conferred by terpenes. An empirical understanding of the effects that common terpene blends have on ASD symptoms could lead to the development of safer, more effective, and more accessible treatment options. Our findings suggest that combining cannabis-inspired terpene blends with CBD may be an efficacious strategy for improving social behavior that avoids legal restrictions on THC levels and mitigates concern over administering THC to children and adolescents.

We tested the effect of a commercially available CBD-rich hemp oil along with several terpene blends from common cannabis strains on social interaction behavior in BTBR mice. Our results add further support to preclinical findings of CBD's prosocial effects in ASD models (Kaplan et al., 2017; Mastinu et al., 2022) and the latest clinical trial (Silva Junior et al., 2022). We also provide the first known evidence for the prosocial effects of terpene blends from popular cannabis strains. Our findings support four general conclusions: 1) a commercial hemp oil can have prosocial effects, 2) cannabis terpene blends confer their own prosocial effects and can lead to more robust prosocial effects when combined with CBD, and 3) the prosocial effects of cannabis terpene blends derive from the combination of multiple independent terpenes, and 4) the prosocial effects can be achieved independent of reductions in general anxiety. These findings should inform the development of novel phytocannabinoid and terpene compositions for treating symptoms of ASD, not to reduce neurodiversity, but to improve the quality of life for patients.

We observed prosocial effects from the inhalation of vaporized blends of terpenes found in popular cannabis strains, OG Kush, Do-Si-Dos, and Blue Dream. We tested if the single most abundant terpene in each blend was sufficient to have prosocial effects, or whether the combination of terpenes was needed. Since the most abundant terpenes, at the concentration and delivery dose administered for the blends, did not meet the threshold for increasing social interaction, we conclude that the combination of multiple volatile compounds found in the OG Kush, Do-Si-Dos, and Blue Dream terpene blends is important for reliably conferring the prosocial effects we observed. However, since we only tested a single terpene from each blend, it is possible that other constituents in the blends that were expressed at lower concentrations may have conferred its prosocial effects. Other benefits have been observed with individual terpenes such as reduced anxiety (Malcolm and Tallian, 2017), dampened pain (Klauke et al., 2014), and improved mood (Ferber et al., 2020), but their effects on prosocial behavior in ASD hadn't been assessed until this study. Our findings suggest that, at least in the BTBR mouse model of ASD,

unique combinations of these terpenes lead to more robust prosocial effects than individual terpenes alone.

Another main finding is that the combination of diluted CBD oil and OG Kush terpenes had robust prosocial effects in the Three Chamber Test. We planned this experiment to be a test of the Entourage Effect Hypothesis which posits that the combination of multiple phytochemicals can improve the efficacy of a single one (Ben-Shabat et al., 1998). Our observation that the combination of CBD and OG Kush terpenes led to more robust prosocial effect than either component on their own supports this hypothesis. Based on the prosocial effects of both CBD and OG Kush terpenes, independently, the robust prosocial benefit of the combination is consistent with additive effects, as opposed to synergistic potentiation of two sub-therapeutic doses. The prosocial benefits stemming from the combination of CBD and terpenes supports the hypothetical, but previously untested, assertion that prosocial efficacy can be enhanced by the combination of terpenes and phytocannabinoids, which may be a safer effective alternative than adding THC. Like combinatorial benefits of adding OG Kush to CBD, we found that two additional cannabis-inspired terpene blends, Do-Si-Dos and Blue Dream, had prosocial effects, but the three most abundant terpenes in each did not improve sociability more than vehicle. Therefore, the combination and presumably the relative ratio of these volatile organic compounds is important for their prosocial effects. Together, these findings highlight that combinations of phytochemicals can lead to enhanced therapeutic benefits than individual chemicals.

At this point, our conclusions only pertain to female mice. Although we observed that CBD had prosocial effects in both males and females, we failed to observe a baseline social interaction deficit in males in two separate cohorts of male BTBR mice. This was surprising given that BTBR mice are a commonly used mouse model of ASD (McFarlane et al., 2008) and male mice are more commonly tested than females. While male subjects have historically dominated preclinical research, it's especially prevalent in ASD research (e.g.,(Pearson et al., 2011)), which is often justified by the higher prevalence of males than females with ASD. However, given the 1:3 ratio of females to males with ASD (Loomes et al., 2017), females certainly warrant investigation as well. Most reports describe a robust social interaction deficit among male BTBR mice in the Three Chamber Test of Social Interaction, whereas female BTBR mice are less consistent in their social deficit phenotype because of their enhanced sensitivity to different characteristics of the stimulus mice (Meyza et al., 2012). In our hands, female BTBR mice displayed a consistent lack of social preference in the Three Chamber Test whereas males initially did not. The exact reasons for this are unclear. BTBR mice have an exaggerated response to stress (Benno et al., 2009), especially for novel social situations (Pobbe et al., 2011), and so it's possible that the male mice experienced some unique interaction of these factors during their testing period that promoted a social preference. Another possibility is that the repeated periods of discrete inhalation periods of the vehicle vapor affected their olfactory processing that disrupted their social sensory cues in a way that facilitated more interaction time, perhaps by requiring longer sniff durations which are normally shorter in male BTBRs (Yang et al., 2012). Future experiments should seek to understand the impacts of terpene blends in males to identify if the therapeutic utility in females can extend to both sexes since sex differences in the response to volatile organic compounds have been reported across a number of phenotypes including anxiety(Bradley et al., 2007), pain(Ceccarelli et al., 2004), and neurotransmitter release(Ceccarelli et al., 2002).

One of this study's limitations is that estrus cycle was not controlled for in female subjects. Recent evidence highlights the importance of estrus cycle for interpreting female mouse social behavior (Chari et al., 2020). Social behavior may be particularly elevated during estrus due to enhanced excitability of midbrain dopamine neurons from increased estradiol levels (Shanley et al., 2023).

However, these assessments are commonly conducted in C57BL/6 mice who typically display high social preference. The impact of estrus cycle on female social behavior in BTBR mice is not well-documented. Given the importance of estrus cycle on social behavior in other mouse strains, future investigations assessing the interaction between estrus cycle phase and the prosocial efficacy of CBD and terpenes are warranted.

One of this study's strengths is that we administered CBD and terpenes via discrete pulls of vaporized oils. Vapor inhalation better models human consumption patterns of cannabis (Aston et al., 2019; Lim et al., 2022) and more closely matches the pharmacokinetic parameters of sublingual/oromucousal absorption (Millar et al., 2018) used in human studies of CBD's effect on ASD symptoms (Aran et al., 2018; Barchel et al., 2019). However, given the notable variability in blood drug levels following passive drug inhalation (MacLean et al., 2017), we sacrificed precise dose control obtained with injection methods to better model the use and pharmacokinetic parameters relevant for ASD treatment. This lack of precision is illustrated in the fair amount of variability in plasma CBD concentrations we measured. Notably, our observed plasma CBD concentration range is quite low compared to those achieved for treating other disorders. This highlights that small amounts of CBD may be effective for some behavioral symptoms but not others. For instance, on the lower end, plasma CBD concentrations of 4.7 to 17 ng/ml were associated with reduced neural responses to threatening faces in humans (FusarPoli et al., 2009). On the higher end, antiepileptic CBD plasma concentration often build to several hundred ng/ml following several weeks of daily dosing to achieve maximal clinical efficacy (Szaflarski et al., 2019). These differences in the therapeutic plasma levels for epilepsy and other disorders can be quite drastic, especially in the case of comparing CBD's effects in ASD compared to epilepsy where effective doses for treating ASD can be 71 times lower (Bilge and Ekici, 2021) than for epilepsy (Szaflarski et al., 2019). This has been confirmed in the Scn1a+/- mouse model that shares both epilepsy and ASD-like social impairment phenotypes where the prosocial benefits were found at 1/10th that of the antiepileptic dose (Kaplan et al., 2017). Unfortunately, the minimal prosocial dose threshold was never determined that may otherwise have corresponded to the prosocial benefits associated with low plasma levels observed in this study, which we reveal here to be relatively low. The prosocial benefits of lowdose CBD disappear with higher doses (Kaplan et al., 2017), consistent with the common inverted-U dose-response curve of CBD's therapeutic efficacy (Guimarães et al., 1990; Zuardi et al., 2017). This highlights the dosing challenge when trying to treat multiple symptoms simultaneously, such as social behavioral in ASD and seizures in epilepsy which may be comorbid in approximately 30% of cases (Tye et al., 2018). Integrating additional chemicals to CBD, such as the terpene blends studied here, may extend this therapeutic dose range to achieve symptom control across several conditions.

There is debate over the necessity of olfactory stimulation to experience the therapeutic benefits of volatile compounds, such as cannabis terpenes, as several have been shown to act directly on neurotransmitter systems. For instance, β-caryophyllene activation of cannabinoid type II receptors (Gertsch et al., 2008) contributes to its anti-inflammatory and pain-relieving properties (Klauke et al., 2014). Additionally, linalool and some of its metabolic products enhance GABAergic currents, in vitro (Milanos et al., 2017). However, linalool's direct targeting of limbic GABAergic signaling may not be its therapeutic mechanism since ablating the olfactory epithelium blocked its anxiolytic action in mice, thereby suggesting that olfactory stimulation is necessary to achieve its anxiolytic effects, at least (Harada et al., 2018). Whether olfactory stimulation also mediates the prosocial effects of the terpene blends or if they work directly on central signaling mechanisms downstream of the olfactory epithelium remains to be tested. Yet, this may be important for therapeutic efficacy of the terpene blends since the reliance on repeated bouts of olfactory stimulation, as achieved by our discrete vapor puff protocol, may not transfer

to non-vaporization consumption methods (e.g., oral capsules) or experimental protocols (e.g., injection methods).

The reliance on olfactory stimulation for the terpene's prosocial effects may also be impacted by one's olfactory sensitivity. There is great olfactory heterogeneity among those with ASD for odor detection thresholds, identification (Larsson et al., 2017), and neural responses to odorant presentation (Xu et al., 2020). Those with ASD may show extreme effect sizes for hyposensitivity or hypersensitivity (Larsson et al., 2017). BTBR mice effectively discriminate both social and non-social odors but display lower-than average sniff times (Moy et al., 2007; Yang et al., 2012), thereby suggesting intact but somewhat abnormal olfactory processing. If olfactory stimulation by the terpenes is necessary for their prosocial effect, then terpene blend concentrations may need to be modulated depending on the individual's olfactory sensitivity phenotype.

In conclusion, we present the first known evidence for the prosocial effects of cannabis terpene blends in a preclinical ASD model. Further, combining terpenes with CBD promotes more robust therapeutic benefits. These findings highlight the value of including cannabis terpenes in formulations being tested in human ASD clinical trials. Future studies should seek to validate these findings in males showing social deficits and continue to optimize CBD and terpene blends for improved efficacy.
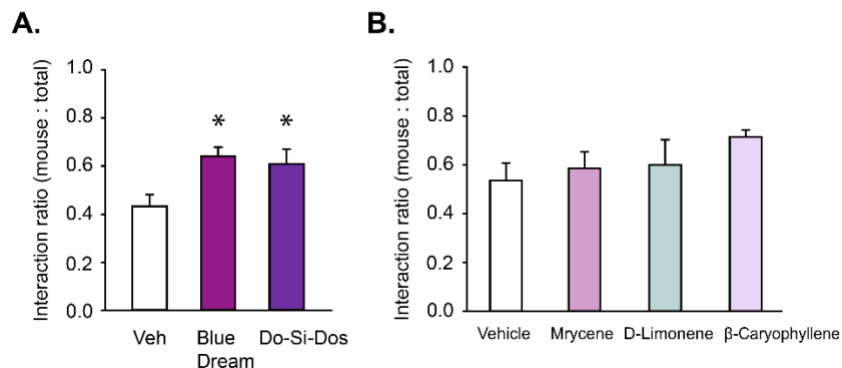
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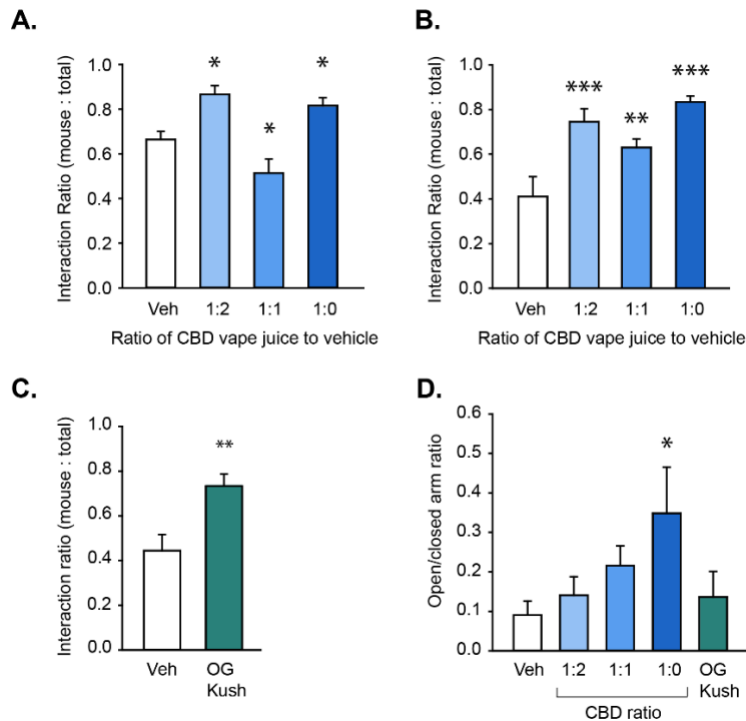
Figure 1: CBD and OG Kush terpenes have prosocial effects. A. Bar chart showing the effect of vehicle and four different CBD oil concentrations on the social interaction ratio in the Three Chamber Test of Social Interaction in male mice. **B**. Bar chart showing the effect of vehicle and four different CBD oil concentrations on the social interaction ratio in female mice. **C.** Bar chart showing the effect of the OG Kush terpene blend on the social interaction ratio in female mice. **D.** The effect of different CBD oil ratios and OG Kush terpenes on the ratio of time spent in the open versus closed arms of the elevated plus maze in female mice. * Indicate differences compared to the vehicle condition.
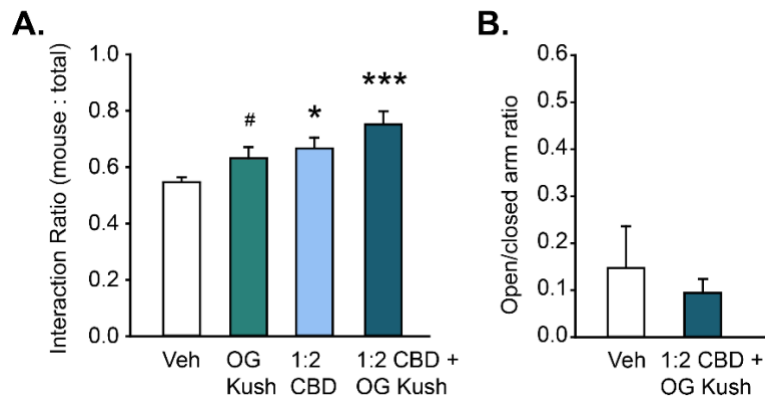
Figure 2. Combination of CBD oil and OG Kush terpenes have robust prosocial effects. **A.** Bar chart showing the effect of OG Kush terpenes, CBD oil, or the combination on the social interaction ratio in the Three Chamber Test of Social Interaction in female mice. the combination and presumably the relative ratio of these volatile organic compounds is important for their prosocial effects. **B.** Bar chart showing the effect of a ratio of 1:2 CBD to vehicle plus OG Kush terpenes on the ratio of time spent in the open versus closed arms of the elevated plus maze in female mice. * Indicates difference compared to the vehicle condition following Tukey's posthoc comparisons; # indicates difference compared to vehicle from a priori paired contrasts, $P < .05$.

Figure 3. Cannabis terpene blends confer prosocial effects. A. Bar chart showing the effect of Blue Moon and Do-Si-Dos terpene blends have on the social interaction ratio in the Three Chamber Test of Social Interaction in female mice. **B.** Bar chart showing the effect of individual terpenes on the social interaction ratio in female mice. * Indicates differences compared to the vehicle condition.

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Chapter 3: Exploring the developmental role of BDNF and interventional cannabidiol in the BTBR mouse model of autism spectrum disorder

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Abstract

The current understanding of molecular processes contributing to autism pathogenesis during development is limited. As a result, available therapeutic strategies able to effectively treat this disorder in youth are scarce. Dynamic changes in brain-derived neurotrophic factor (BDNF) may be involved in ASD progression by exerting changes in synaptic function and neuronal development. Additionally, CBD may interact with BDNF as a therapeutic agent to alter disease states. This preliminary work found that BTBR mice had higher levels of BDNF than wildtype controls in the hippocampus. Chronic CBD treatment appears to lower BDNF levels in BTBR mice but does not improve social behavior assessed in adulthood. Future work in preclinical studies is necessary to understand the developmental neuropathology of ASD. Identifying drug-induced pathological changes remains important for knowing how certain therapeutics bring about phenotypic changes. It is worth investigating different approaches using cannabis-based medicines in younger populations based on the beneficial effects that have been exemplified in adults.

Introduction

Investigating abnormalities in the developing brain is a necessary step to identify markers of autism onset and develop early therapeutic strategies for improved quality of life. Autism spectrum disorder affects one in 44 children in the United States (Maenner et al., 2021), but there is a surprising lack of therapeutic strategies for youth. Total yearly costs for children with ASD are estimated between $61-66 billion for the United States and medical expenditures are 4.1-6.2 times greater for these individuals than those without autism (Buescher et al., 2014; Shimabukuro et al., 2007). Since the publication of these studies, expenditure rates have steadily increased; one model predicts the total cost of ASD in the U.S. to be $5.54 trillion/year by 2060 after accounting for the rise in autism prevalence over time (Blaxill et al., 2022). The substantial healthcare costs combined with a lack of available medications places a significant burden on families. However, it may be possible to mitigate some of this impact through early intervention approaches with the development of new and efficacious therapies.

ASD typically emerges during the first few years of life followed by phenotypic consolidation shortly thereafter (Piven et al., 2017). Targeting this period could be key for improving behavioral deficits in youth and functional outcomes into adulthood. Evidently, this requires the ability to diagnose autism as early as infancy with clear neurobiological evidence prior to full symptomatic onset. The complex etiology of ASD encompasses a wide range of pathological processes that could be involved during early development, challenging efforts for accurate diagnosis; it is apparent that autism involves atypical trajectories of neuronal maturation and synaptic differentiation, but the exact mechanisms by which this occurs remains unclear. Neurotrophins are a family of proteins that play a central role in regulating neuronal survival and function during development (Huang & Reichardt, 2001). Brain-derived neurotrophic factor (BDNF) is one molecule from this family that has gained particular interest in ASD research. BDNF is known to be involved in the maturation of glutamatergic and GABAergic synapses (Qi et al., 2022). It exerts modulatory actions by binding tropomyosin-related kinase B receptors, activating signaling cascades that regulate protein synthesis and synaptic transmission for development and cognitive function (Scattoni et al., 2013). Changes in BDNF expression have been implicated in pathological brain conditions, including several neurodevelopmental disorders that may be caused, in

part, by neurotrophic dysregulation. Investigating the scope of BDNF through cellular and molecular processes in autism contributes to a deeper understanding of one aspect of the disorder's pathology.

Along with genetic testing for mutations in candidate genes, plasma levels of BDNF have been suggested as a potential diagnostic marker for autism due to its ability to cross the blood-brain barrier (Bryn et al., 2015; Barbosa et al., 2020). There is robust evidence for abnormalities in BDNF homeostasis in ASD, though these changes are not unidirectional. A meta-analysis of 20 studies reported significantly higher blood BDNF levels in ASD patients compared to controls (Saghazedah & Razaei et al., 2017). A few studies have reported lower levels of BDNF in newborns and adults (Hashimoto et al., 2006; Skogstrand et al., 2019). One study found that children ages 0-9 years old have significantly lower serum levels of BDNF, while children ages 10-19 have higher levels of BDNF compared to age-matched controls (Katoh-Semba et al., 2007). Together, this suggests age-related changes in BDNF levels as a critical component of autism progression. ASD is a complex disorder with a high rate of co-occurring disease, so it is possible for many confounding factors to influence serum BDNF levels. While most studies point to an increase in plasma BDNF during development, sensitivity in measuring blood concentration may explain differences across some studies (Barbos et al., 2020). Additionally, changes in BDNF are involved in many neuropsychiatric disorders, limiting the discriminatory power for diagnosis (Miranda et al., 2019). While plasma BDNF may serve as a more relative marker for autism diagnosis, it nevertheless remains an important etiological component of the disorder. Research focused on specific developmental stages of autism may reveal key pathological mechanisms that occur during ASD progression.

Animal models are a valuable tool for identifying pathological mechanisms with less confounding biological or environmental variables that influence disease states in humans. BTBR mice are commonly used to model ASD and exhibit aberrant social behavior. A study by Jasien et al. (2014) reported the presence of social deficits, a characteristic phenotype of this strain, along with a significant reduction of BDNF in the hippocampus of aged BTBR mice. At the beginning of development, however, an upregulation of BDNF expression has been identified in fetal brains of BTBR mice (Hwang et a., 2015). Similar findings were reported in the valproic acid (VPA) animal model of ASD. An increase in BDNF expression in fetal brains of VPA mice identified in one study did not continue past late adolescence (Almeida et al., 2014; Chau et al., 2017). This stark contrast is in line with clinical studies that show increased plasma levels of BDNF in children with ASD, followed by decreased levels in adulthood (Katoh-Semba et al., 2007). While there seems to be an apparent shift in BDNF expression during the span of development, when this shift occurs and whether it induces phenotypic changes is unclear. Moreover, no studies have looked at changes in BDNF levels in relation to core autism phenotypes in adolescent BTBR mice. Here, we chose to investigate BDNF protein levels during the adolescent period in BTBR mice with the hypothesis that hippocampal BDNF levels would be increased in the middle of the murine developmental period.

In addition to researching distinct molecular mechanisms of disease, it is important to look at how interventional strategies might influence these pathological states. BDNF has been suggested as a common mediator of the therapeutic actions of neuropsychiatric drugs (Bazzari & Bazzari, 2022). Drug-induced BDNF changes may influence the therapeutic efficacy of pharmacological agents. Functional crosstalk between BDNF and endocannabinoid signaling has been reported in a variety of brain regions (Gangarossa et al., 2020). Furthermore, the endocannabinoid system plays an integral role in neurodevelopmental processes (Meyer et al., 2018). Thus, it is worth exploring the effects of cannabis-based treatments on BDNF levels and phenotypic expression of ASD models. To achieve this, we tested the effects of developmental CBD exposure on BDNF protein levels and social behavior in BTBR mice.

Previous research has shown that chronic CBD exposure during adolescence is without harmful effects (Kaplan et al., 2021). In humans, a recent open label study reported improvements in social symptoms in children with ASD following 6 months of CBD-rich cannabis treatment (Hacohen et al., 2022). Chronic developmental exposure to CBD as an interventional strategy may produce longer-lasting behavioral changes into adulthood. Interestingly, ElBatsh et al. (2012) previously reported that chronic CBD administration caused a significant reduction of BDNF expression in the hippocampus of rats. The exact mechanistic action by which CBD interacts with BDNF is unknown. It is possible that prolonged CBD treatment reduces the atypical elevation in BDNF expression during adolescence by acting on developing neural circuits to confer the beneficial effects reported in open label studies. Therefore, we hypothesized that chronic CBD administration would reduce BDNF levels in the hippocampus of adolescent BTBR mice. For behavior, we hypothesized that chronic CBD treatment to adolescent BTBRs would improve social deficits into adulthood. For both experiments, we chose to look at the hippocampus which is particularly altered during the adolescent period. BDNF is highly abundant in this brain region affecting synaptic plasticity, neurogenesis, and neuroprotection during development (Miranda et al., 2019). The hippocampus is also involved in the underlying processes of flexible cognition and social behavior (Rubin et al., 2014). Additionally, CBD influences endocannabinoid signaling via proneurogenic action here (Campos et al., 2013). Therefore, the aim of this work was to investigate BDNF levels in the BTBR mouse model and whether effects of chronic CBD would involve changes in BDNF signaling and social behavior.

Materials and Methods

Animals

BTBR T+ Itpr3tf/J (BTBR; Jackson Laboratories, Bar Harbor, ME) and C57BL/6J (B6; Jackson Laboratories, Bar Harbor, ME) litters were bred in-house at Western Washington University. A total of 32 female mice were used in these experiments. C57BL/6J mice served as wildtype controls (WT) for these experiments. Mice were raised in standard laboratory housing in groups of 3-5 mice per cage on a 12-hour light/dark cycle (lights on at 0700). Food and water were provided *ad libitum*. All drug exposures and behavioral testing were conducted during the light cycle. All procedures follow regulations detailed in the National Institutes of Health Guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Western Washington University.

Drugs

A CBD isolate solution of 30mg/mL was prepared by diluting CBD isolate in a vehicle solution comprised of 70% vegetable glycerin, 30% propylene glycol purchased from La Jolla Alcohol Research, Inc. (La Jolla, CA). The CBD isolate solution was heated and vortexed prior to exposure. The vehicle solution for control mice were 70/30 vegetable glycerin/propylene glycol. Drug administration occurred through four 36 cm x 27 cm x 23 cm (L x W x H) ~17 L passive vapor inhalation chambers (La Jolla Alcohol Research, Inc). These chambers were computer controlled to deliver precise vapor pulls for 3 seconds every 5 minutes for 30 minutes, for a total of 6 pulls per session. The air in the chambers appeared visibly clear of vapor prior to subsequent vapor pull.

Western Blot Analysis

For Western blot analysis, mice were anesthetized using 1.5-2mL isoflurane in a holding cage for two minutes and sacrificed by decapitation at P35. Brains were quickly removed, and the hippocampus was rapidly dissected on an ice-cold glass petri dish. Samples were immediately frozen and stored at -80º until use.

Frozen hippocampi were individually homogenized in RIPA lysis buffer (200µL RIPA + 1% protease inhibitor cocktail) and centrifuged at 16000xg for 20 minutes at 4ºC. Supernatants were collected and protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific #23225). Samples were incubated at 70ºC for 10 minutes and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 10% NuPAGE Bis-Tris gels followed by protein transfer to a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated in blocking buffer (5% nonfat dry milk in 20mL Tris-buffered saline) for 1 hour at room temperature and then incubated with mouse anti-BDNF (1:1000; ABclonal #A18129) overnight at 4ºC in blocking buffer with 0.2% Tween-20. After washing, the membranes were incubated with goat-anti mouse IgG (1:20,000) for 1 hour at room temperature. Images were captured with the Odyssey Fc Imaging System and bands were quantified with Image Studio Lite software (LI-COR Biosciences). For all three western blots, samples were loaded in a random order to reduce the impact of position effects and a control sample was loaded for comparison across blots.

Three Chamber Test of Social Interaction

The three-chamber 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. A BTBR test mouse was placed in the center of the empty apparatus for a 10-minute habituation period and then returned to its home cage. For the test phase, a female, age matched WT mouse was placed under one of the wire cups to serve as a novel stimulus. The BTBR mouse was returned to the center of the apparatus to explore for another 10-minute period. Time spent within a 5-cm radius proximal to each wire cage was measured and recorded as time interacting with the "social" or "object" stimulus. A social preference was defined as a statistically significant preference for engaging in social interaction as a function of the total interaction (time spent interacting with the social stimulus and non-social, "object", stimulus): $0.5 >$ (interaction with social stimulus / total stimulus interaction).

Experimental Design

Experiment 1 – Evaluation of BDNF Levels in BTBR and Control Mice

To evaluate differential regulation of BDNF in BTBR mice during development, hippocampal tissue was collected from BTBR and WT mice at postnatal day (P) 35. The adolescent period in rodents is generally considered to be from P21-60, and puberty typically occurs in females between P28-42 (Hueston et al., 2017). We only used females in these experiments because we failed to observe baseline social interaction deficit in males from previous work. BDNF protein levels were compared between strains using western blot analysis. A total of 8 female BTBR mice and 8 female WT mice were used for this experiment, distributed across two blots. Mice were taken from various litters to account for litter effects.

Experiment 2 – Developmental CBD Effects on Prosocial Behavior and BDNF Levels in BTBR Mice

To investigate CBD effects on prosocial behavior and drug-induced changes in BDNF levels, BTBR mice were subjected to a chronic developmental exposure period. A total of 15 female BTBR mice were used for this between-subjects experimental design, separated into a CBD-exposed group and a vehicle-exposed group. Mice were weaned at P21 and began a two-week long exposure period, receiving twice daily (0800 and 1600) exposures to CBD or a vehicle solution. On day fourteen, only the morning

exposure was administered. Half the mice from each group were sacrificed for western blot at P35 and the rest were saved for behavioral testing at P80. Behavioral testing consisted of the three-chambered test to examine social behavior by observing the experimental mouse's affinity for social novelty (Moy et al., 2007).

Statistical Analysis

An independent samples t-test was used for analyzing BDNF protein expression levels in experiments 1 and 2. A 2x2 factorial ANOVA was used for analyzing the three chamber sociability test results. $p < 0.05$ is regarded as statistically significant.

Results

Experiment 1

An independent samples t-test did not find a difference between genotype on BDNF expression, t(13) = 1.26, $p = .23$; our power for this test was .24. Based on descriptive statistics, BTBR mice exhibited higher average levels of BDNF protein in the hippocampus compared to wildtype mice (Figure 1b, $n = 7$ mice of WT group, 8 mice of BTBR group). One WT sample was omitted from analysis as an outlier due to relative expression levels being three-fold higher than the rest and a disruption to the BDNF band on the blot image. An increase in power is required to reduce the high type II error risk for proper statistical analysis.

Experiment 2

An independent samples t-test did not find an effect of CBD on BDNF expression, t(6) = 1.08, $p = .32$; our power for this test was .18. Based on descriptive statistics, chronic CBD administration lowered BDNF protein levels in the hippocampus of BTBR mice compared to vehicle treated BTBR mice (Figure 1c, $n = 4$ mice of vehicle group, 4 mice of CBD group). The same control was used across experiments 1 and 2, enabling comparison across all western blots; CBD appears to lower BDNF expression close to levels in WT mice. An increase in power is required to reduce the high type II error risk for proper statistical analysis.

A 2x2 factorial ANOVA found an effect of treatment condition on ratio of time spent in social interaction, F(1,10) = 9.77, $p = .01$. 3CT analysis revealed an increased social preference in the vehicle treated group (Figure 2, $n = 4$ mice of vehicle group, 3 mice of CBD group). This experiment was even more limited by the low sample size and should be considered when interpreting these results.
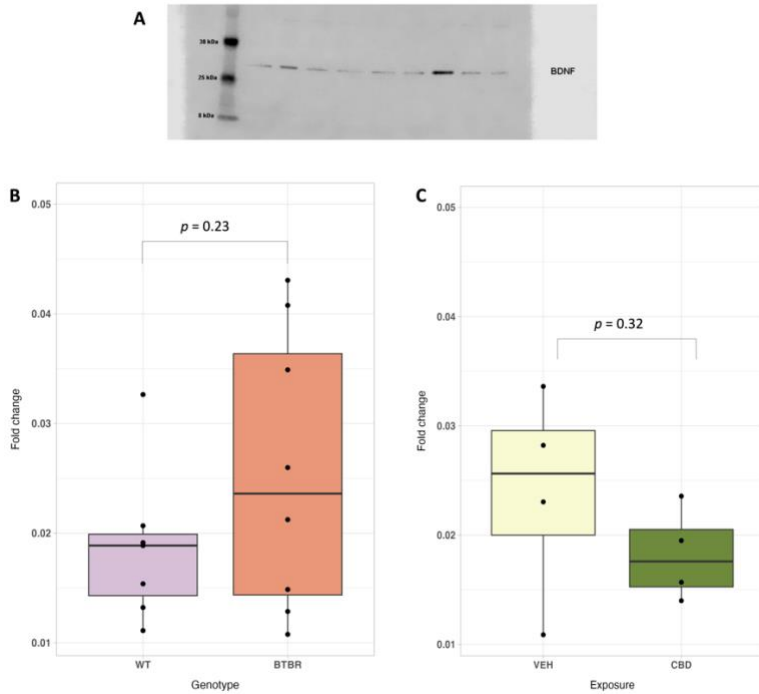
Figure 1. Elevated BDNF levels during development are reduced by chronic CBD treatment. A. Western blot image showing bands of BDNF at 28kDa. **B.** Boxplot showing relative fold change levels in WT and BTBR mice normalized to total protein expression, $p = 0.23$. **C.** Boxplot showing relative fold change levels in WT and BTBR mice normalized to total protein expression, $p = 0.32$.
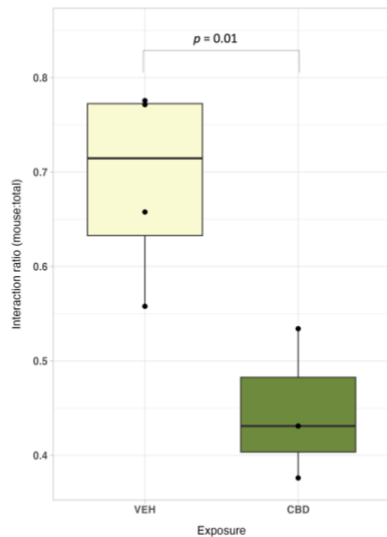
Figure 2. Vehicle-exposed mice exhibit increased social interaction in adulthood. Boxplot showing the social interaction ratio of BTBR mice on the 3CT. Vehicle-exposed mice exhibited improved social behavior in adulthood as compared to CBD-exposed mice, $p = 0.01$.

Discussion

Given the profound developmental role of neurotrophins in healthy and pathological brain states, further research is needed to evaluate the impact of BDNF levels in the progression of ASD. In this study, hippocampal tissue was collected to determine whether BDNF levels were differentially regulated in BTBR as compared to WT mice; cannabis-based treatment was assessed for drug-induced pathological changes in BDNF expression and social behavior. Consistent with our hypothesis, we found that BDNF levels in the hippocampal region of pubescent female BTBR mice are higher as compared to WT controls. Additionally, chronic CBD exposure during development appears to reduce hippocampal BDNF levels to near wildtype expression. Elevated expression of BDNF has been reported in fetal brains of BTBR mice, and this data indicates higher levels are maintained into the adolescent period (P35). A confounding factor in this data is the biological variability present in our BTBR group.

Although this preliminary evidence suggests that chronic CBD treatment restores atypical molecular mechanisms of BDNF during development, it did not improve social behavior in adulthood as expected. On the contrary, the observed treatment effect was driven by an increased social interaction in the vehicle-treated group. It is possible that vapor administration functions as an early life stressor to adolescent mice, potentially inducing lasting changes in synaptic plasticity. BTBR mice exhibit enhanced stress reactivity and may be more sensitive to environmental disturbances (Ayriyants et al., 2023). However, this explanation does not account for the differential impact on social behavior between treatment groups. Additionally, it has been shown that early life stress does not worsen autism phenotypes in BTBR mice (Reshetnikov et al., 2021). Oral administration of CBD may present a better option because it eliminates the potential stressor of vapor exposure.

BDNF is suggested to play a role in modulating social behavior. Higher levels of neurotrophic factors are associated with increased social interaction in adulthood and indicative of social status in adult mice (Branchi et al., 2006). This is consistent with the fact that adult BTBR mice with impaired social behavior also show a reduction in BDNF levels. To the contrary, elevated levels of BDNF are present during adolescence when social impairments have already emerged. These social deficits may begin to consolidate independently from BDNF, but deficient levels could play a contributory role to impaired social behavior in adulthood. Additional behavioral testing directly following the treatment period could assess changes in sociability from adolescence into adulthood.

It is also interesting to consider the effects of CBD as a therapeutic agent at different developmental periods. Our findings are consistent with a previous study reporting hippocampal BDNF-lowering after chronic CBD exposure in rats (ElBatsh et al., 2012). Other studies indicate that treatment induces BDNF in the opposite direction, with acute CBD administration increasing BDNF in the hippocampus of adult rodents (Sales et al., 2018). The duration of treatment and the age of the animals used in these studies could influence the effects of CBD on BDNF. Indeed, developmental changes in the endocannabinoid system may alter the mechanism of action of CBD. Heng et al. (2011) demonstrated an age-dependent downregulation of CB1 receptor expression in rats. Dose-dependent effects have also been identified in the medial prefrontal cortex: single exposure of CBD upregulated BDNF, but repeated exposures slightly decreased levels (Mottarlini et al., 2022). The ways in which these plastic changes impact social behavior in autism is currently unknown. It is possible that chronic CBD exposure during adolescence may alter neural circuits leading to reduced sociability later in life. Evidently, the modulatory effects of CBD on BDNF should be an additional consideration for evaluating treatment regimens for different age groups. This requires knowledge of BDNF expression across all age groups and further investigation into the behavioral effects of CBD from different dosing schedules.

Some other considerations to this pilot study include the fact that we did not control for the estrus cycle in these female mice. Chari et al. (2020) found a significant differences in 3CT social interaction between female mice at different stages of their estrus cycle. In future studies, we plan to monitor this to

control variability this can introduce in behavioral tests. There is also a possibility that changes in BDNF are not related to social interaction but may correspond to other behavioral changes. The study by ElBatsh et al. (2012) reported that chronic CBD exposure had anxiogenic-like effects in healthy rats. It would be worth assessing the effects of the developmental exposure in BTBR mice on general anxiety using the elevated plus maze. Incorporating a control strain receiving developmental CBD and vehicle treatment would be useful for comparison. Given the role of the hippocampus in learning and memory, relevant behavioral testing for these functions should be considered in future experiments as well.

There are several important limitations to these studies. First, there are no male subjects in these experiments due to a lack of social impairments in males of our autism model. The potential sex-differences in autism pathology and responses to therapies are still important to consider and this research should be replicated in male subjects. Preliminary studies show that cannabis may produce sex-specific alterations leading to different neurodevelopmental trajectories (Crane et al., 2013). Sex-dependent BDNF levels may also be an important factor; male mice show higher expression of BDNF in the hippocampus, though there is no difference in hippocampal BDNF expression in humans (Sardar et al., 2021). Second, the small sample size of our data limits interpretation of these results. The low statistical power (i.e., 0.2) reveals the high type II error rate risk in our conclusion. Third, much is still unknown regarding autism pathology at all stages of ASD. The developmental changes to BDNF and the endocannabinoid system presents complex mechanisms that may be sensitive to specific periods. While this research attempts to investigate one piece of the puzzle, several other factors are likely at play. Further behavioral assessments may help identify the impact of CBD-induced changes in developmental BDNF expression beyond social behavior and potentially inform management or prevention of secondary symptoms in ASD.

Conclusions

This pilot study identified the potential for developmental exposure to CBD to normalize levels of BDNF in female BTBR mice. These findings are consistent with the current literature indicating higher BDNF expression prior to a developmental shift toward deficient levels in adulthood and highlight the ability of CBD to restore them back to levels detected in a healthy control strain. Although it was predicted that this BDNF restoration would correspond to improved social behavior, likely through the modification of relevant social circuits, our pilot assessment did not show this to be the case. We found that vehicle-exposed mice, but not CBD-exposed mice, exhibited improved social behavior in adulthood. Additionally, the adolescent period is characterized by complex cellular processes with neuroplastic changes influenced by the endocannabinoid system and neurotrophic signaling. Several factors could be at play including the timing and duration of treatment, which can differentially affect BDNF signaling and prosocial behavior.

Given that this study confers only preliminary findings, future work should continue to elucidate the role of the BDNF and cannabis-based treatments in ASD progression. Changes in BDNF signaling occur in the BTBR mouse model from the adolescent period into adulthood, consistent with observations in patients with ASD. CBD has proven beneficial effects on social behavior in the BTBR model and remains a promising therapeutic to treat core symptoms. Identifying novel interventional strategies for youth will be optimal for improving quality of life for affected individuals. Optimizing this strategy for youth could include different treatment regimens or other cannabis-based medicines. It is also possible that CBD exerts prosocial effects only at certain developmental stages. Additional research focused on understanding the cellular and molecular processes of autism will also benefit our progress in developing appropriate therapies for this population.

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