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Single cannabidiol administration affects anxiety-, obsessive compulsive-, object memory-, and attention-like behaviors in mice in a sex and concentration dependent manner

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ABSTRACT

Rationale: The behavioral effects of cannabidiol (CBD) are understudied, but are important, given its therapeutic potential and widespread use as a natural supplement.
Objective: The objective of this study was to test whether a single injection of CBD affected anxiety-like or attention-like behavior, or memory in wildtype mice or mice with reported trait anxiety due to a targeted gene-deletion in a voltage-dependent potassium channel, Kv1.3.
Methods: Wildtype C57BL/6 J and Kv1.3−/− mice of both sexes were reared to adulthood and then administered an intraperitoneal injection of 10 or 20 mg/kg CBD. Mice were behaviorally-phenotyped using the marble-burying test, the light-dark box (LDB), short (1 h) and long-term (24 h) object memory test, the elevated-plus maze (EPM), and the object-based attention task in order to assess obsessive compulsive-, anxiety-, and attention-like behaviors, and memory.
Results: We discovered that acute CBD treatment reduced marble burying in male, but not female mice. CBD was effective in lessening anxiety-like behaviors determined by the LDB test in both male and female wildtype mice, whereby the effective dose required to observe the effect in females was less. In Kv1.3−/− mice, CBD increased anxiety-like behaviors in the LDB in both sexes at the higher concentration of CBD and it similarly increased anxiety-like behavior in females in the EPM at the lower concentration of CBD. Long-term object memory was reduced in male wildtype mice at the lower concentration of CBD. Finally, ADHD- or attention-like behaviors were not altered by CBD in wildtype mice, but in Kv1.3−/− mice, females were observed to have a loss in attention while males demonstrated improved attention.
Conclusions: We conclude that administration of a single dose of CBD has immediate effects on mouse behavior that is dose, sex, and anxiety-state dependent – and that these behavioral outcomes are important to examine in parallel human trials.

Abbreviations: 2-w ANOVA, two-way analysis of variance; ADHD, attention deficit hyperactivity disorder; ARRIVE, Animal Research: Reporting of In Vivo Experiments; AVMA, American Veterinary Medicine Association; C57BL/6J, an inbred strain of laboratory mouse; Ca^{2+}, calcium ions; CBD, cannabidiol; CB1 (CB_1-R), cannabinoid-1 receptor; CB2 (CB_2-R), cannabinoid-2 receptor; dF, degrees of freedom; EPM, elevated plus maze; EtOH, ethanol; FO, familiar object; h, hour; HPLC, high-performance liquid chromatography; IACUC, Institutional Animal Care and Use Committee; IP, intraperitoneal; Kv1.3, voltage-dependent potassium channel 1.3; Kv1.3−/−, mice with targeted deletion of the Kv1.3 gene; LDB, light-dark box; MS, mass spectrometry; NIH, National Institute of Health; NMR, nuclear magnetic resonance; NO, novel object; OBS, object bias score; OCD, obsessive compulsive disorder; RI, recognition index; RM, repeated measure two-way analysis of variance; SD, standard deviation; THC, Δ9-tetrahydrocannabinol; UV, ultraviolet; WIN, WIN55,212–2, a synthetic agonist of the cannabinoid CB_1 receptor; WT, wildtype.

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1. Introduction

Olivetolic phytocannabinoids are the dominant and most widely studied compounds derived from the plant species *Cannabis*. Historically, the use of *Cannabis* originates 10,000 years ago at the end of the Ice Age in Central Asia (Pisanti and Bifulco, 2019). Derivatives of the plant were prescribed as medication in the United States until restrictions were placed on their use in the 1930s, and eventually components became classified as Schedule I substances in the 1970s (Bostwick, 2012). Since the recent legalization of *Cannabis* products in some states in 2018, there has been a growing demand for medical opinion for use of the components based upon scientific research (Hill and Palastro, 2017).

Cannabidiol (CBD) holds high therapeutic potential because it is a non-psychoactive ingredient of *Cannabis* and lacks addictive properties (Crippa et al., 2018; Calapai et al., 2020). CBD's behavioral effects are understudied, but important, given the public and commercial advertisement of unsubstantiated uses and applications. Because there is varied government- and state-regulated distribution of CBD, it is important to determine measurable behavior outcomes of CBD use. CBD treatment has demonstrated changes in anxiety, chronic pain, sleep, seizure, and prevention of substance abuse in mouse and human subjects (Blessing et al., 2015; Rosenberg et al., 2017; De Gregorio et al., 2019; Elsaid et al., 2019; Shannon et al., 2019; Calapai et al., 2020; Uberall, 2020). Among these, anxiety disorders are the most prevalent mental illness worldwide, and in the United States 15% of adults report symptoms of anxiety (Terlizzi, 2020). Anxiety is also well known to disrupt attention by narrowing an individual's focus (Derryberry and Reed, 1998; Najmi et al., 2012) and can also bias memory processing by the hippocampus (Kuga and Sasaki, 2022). Anxiety demonstrates comorbidity with other psychiatric disorders, especially that of depression (up to 60%) (Sartori et al., 2011).

There is a need to examine alternative compounds for treatment of anxiety disorders because up to 40% of patients seeking treatment for anxiety are refractory to traditional treatments such as serotonin reuptake inhibitors or behavioral therapy (Bandelow et al., 2017). Herein, we examined a newly found mouse model (Kv1.3−/−) of anxiety-like and attention deficit-like behaviors (Huang et al., 2018) to explore any reduction or elimination of these behaviors following CBD treatment. Kv1.3−/− mice that lack the voltage-dependent potassium channel Kv1.3 have been shown to exhibit trait anxiety and impaired attention that can be ameliorated by methylphenidate treatment (Huang et al., 2018). Mouse models of anxiety can be described as “state” anxiety where a stressor is induced and the experimenter can observe how the rodent perceives and responds to the stressor in time. Alternatively, mouse models can be described as “trait” anxiety in which it is a durable feature of the rodent whereby the experimenter can observe how the animal persistently responds to its environment because the anxiety trait does not vary over time (Bourin, 2015). Because our mouse model of anxiety was generated via genetics, it represents a beneficial manner to study innate elevated anxiety without the necessity of environmental manipulations (maternal separation, isolation housing) or pharmacological manipulations (corticosteroid treatment). Although CBD is thought to have over 70 targets, it is known that CBD is a negative allosteric inhibitor of the principle endocannabinoid receptors, CB1 and CB2 (Di Marzo and Piscitelli, 2015). The molecular mechanism of how targeted deletion of Kv1.3 channels in mice evokes trait anxiety is not known, but in general, activation of endocannabinoid signaling targets excitatory and inhibitory synaptic transmission in response to changes in intracellular Ca^{2+} concentration and modulation of neurotransmitter release (Zou and Kumar, 2018).

Measurable behavioral effects in response to CBD treatment are understudied but important given public access to the compound without a large number of studies comparing dose, delivery route, sex-dependent effects, purity and stability, and duration of treatment. Moreover, there is contradiction in behavioral outcomes of CBD treatment with some studies reporting anxiogenic-like effects in rodents (ElBatsh et al., 2012; Schleicher et al., 2019), while others report anxiolytic tendencies (Blessing et al., 2015; Zieba et al., 2019). Our current study compares two pharmacological doses using an intraperitoneal (IP) delivery route to ensure rapid bioavailability; tests both male and female animals; uses synthetic-derived compound to confirm activity, known purity, and stability; and uses an acute drug treatment that can be compared against chronic or one-month duration studies. Using Kv1.3−/− vs. wildtype mice, we compare trait vs. state anxiety across a battery of 6 different behavioral tasks (buried marble, light dark box, elevated plus maze, short-term object memory, long-term object memory, and object-based attention task) designed to examine obsessive-compulsive-like, anxiety-like, attention-deficit-like behaviors, and object memory in response to CBD.

2. Methods

2.1. Subjects

Experiments were performed on 3-month old male and female mice with C57BL6/J as the principle strain or strain background. A total of 116 mice were generated for our behavioral study, of which 98 mice were analyzed. Mice were either wildtype (Jackson Laboratories, stock number 000664, https://www.jax.org/strain/000664, RRID: IMSR_JAX:000664) or with a gene-targeted deletion of the voltage-dependent potassium channel, Kv1.3 (Kv1.3−/−) that have previously been reported to exhibit trait anxiety-like and attention deficit-like behaviors (Huang et al., 2018). Kv1.3−/− mice were generated via deletion of a large promoter region as well as the N-terminal third of the coding sequence for Kv1.3 (Koni et al., 2003; Xu et al., 2003). The mice were a generous gift of Drs. Leonard Kaczmarek and Richard Flavell (Yale University, New Haven, CT) and have now been deposited at Jackson Laboratories (Bar Harbor, ME; B6;129S1-Kcna3tm1Lys/J, stock number 027392, https://www.jax.org/strain/027392, RRID: MGI:2679442). All experiments in this study were approved, under protocol number #202000036, by the Florida State University (FSU) Institutional Animal Care and Use Committee (IACUC). These experiments were done in accordance with the guidelines set by the National Institutes of Health (NIH), the American Veterinary Medicine Association (AVMA), and the ARRIVE guidelines (du Sert et al., 2020). Upon weaning, all mice were singly housed with two sources of enrichment (house and bedding square) using open-style conventional cages. Mice were kept on a reverse 12-h/12-h light/dark cycle with lights off at 8:00 A.M. and lights on at 8:00 P.M. All mice were given a standard diet (LabDiet 5001 Rodent Chow; 13.5% kcal from fat, https://www.labdiet.com/Products/StandardDiets/Rodents/index.html), and had access to food and water *ad libitum* in their home cages. For the duration of the behavioral experiments, mice were temporarily not given access to food or water for up to 2 h.

2.2. Drug and solutions

Cannabidiol (CBD) was obtained as synthetically derived from Purisys, LLC (Athens, GA; Batch NQS1951; NDC# 516342155) as purchased from and analytically certified by Emerald Scientific using mass spectrometry and infrared spectrometry. CBD was also obtained from Sigma-Aldrich as used in one experiment, which was synthetically derived from PhytoLab (Vestenbergsgreuth, Germany; Batch 113,083,344, CAS #13956–29-1) who analytically certified it through NMR/MS Spectroscopy and HPLC using UV detection. All CBD was of Pharmaceutical Grade, GMP Certified, with 100% purity factor rating, and without detection of Δ9-tetrahydrocannabinol (THC). Drug was delivered by intraperitoneal (IP) injection at two different final concentrations (high – 20 mg/kg, low – 10 mg/kg). Animal delivery volumes ranged from 60 to 180 μl depending upon dose and weight of the subject. Drug was initially reconstituted in 100% ethanol and then

stored protected from light and at -20°C, before being diluted to a working concentration in a vehicle solution containing 0.9% Sodium Chloride: Ethanol: Tween 80 (90%:5%:5% final ratio). The control solution was the 90%:5%:5% Sodium Chloride:Ethanol:Tween 80 delivered at an equivalent volume as used for the two drug concentrations. The sodium chloride was purchased from Hospira, Inc. (Lake Forest, IL) as a Rx, preservative free sterile diluent (NDC 0409–4888-02, RL-7302), Tween 80 was from Sigma Chemical (St. Louis, MO; P1754-25ML), and ethyl alcohol was from Pharmco-aaper (Shelbyville, KY; CatE200).

2.3. Experimental design

2.3.1. Behavioral tasks

Behavioral tasks were conducted to test anxiety-, attention-, and obsessive compulsive-like behaviors and object memory in mice. All mice received all six tests sequentially in the same order as schematically presented in Fig. 1. None of the anxiety-based behavioral tests we employed required hippocampal-dependent learning (Sartori et al., 2011), and any experience acquired in a given task that might affect a subsequent task (novel object or attention tasks; (Antunes and Biala, 2012)) would have been equivalent for all mouse subjects due to the fixed order of the experiments. The sequential design also permitted us to reduce total number of mice in our study that otherwise would have been 6-fold. Only one behavioral test was performed per day, with the exception of day 2, when mice first completed their 24-h memory test and were then introduced to the light-dark box, and day 3, when mice were first examined through the attention-task followed by direct introduction to the elevated plus maze. Mice were randomly assigned to either drug or control vehicle, where animals performed each of the six behavioral tests once and then were euthanized. Mice were phenotyped in cohort sizes of 8 to 16 mice, ensuring that all treatment groups and sexes were represented within a cohort. Treatment group sizes were matched through staggered breeding as close as possible but 18 mice had to be removed from the study due to failure in the Dixon Q test ($n = 4$), failure in the object bias criteria ($n = 11$), accidental injury during injection ($n = 2$), or unexplained death prior to experimentation (n = 1). During all animal handling and behavioral testing, investigators wore gloves, coats, masks, and protective shoe covers, and spoke minimally to avoid animal stress. Regardless of behavioral task, mice were acclimated to the new experimental chamber or testing room prior to data collection for varying durations as described individually below. Experimental tests were carried out thirty minutes after injection of the drug. This timing matched previous pharmacokinetic results by other investigators that have demonstrated plasma levels of CBD following IP delivery methods (Xu et al., 2019; Ochiai et al., 2021). Based upon the reported half-life ($t_{1/2\alpha}$) for CBD by Ochiai et al. (2021), we conservatively estimated that 3% of our original drug injection would be available in the plasma following 24 h; making any cumulative effect of daily drug injections negligible. All behavioral experiments were conducted during the dark cycle (08:00–20:00) in a room with temperature at 22°C and with humidity between 50 and 60%. All objects, boxes, and mazes were cleaned with 70% ethanol and air dried between subjects. All behaviors were filmed using a 16.6-megapixel Sony 4 K Handycam with a 26.8 mm wide angle lens and still image recording (FDR-AX53; Best Buy, Tallahassee, FL) mounted to a Sony tripod using a 1 K Gorillapod (Joby Aviation, Santa Cruz, CA). Films were uploaded to private access YouTube (YouTube, San Bruno, CA) so that researchers could score activity blinded to the drug or genotype condition. Following download from YouTube, QuickTime Player (Apple, Inc., Cupertino, CA) and Movies and TV (Microsoft, Redman, WA) were used as the platforms to manually score respective behavior activities by Mac and IBM users, respectively. Films were stored on Passport External Drives (Western Digital, San Jose, CA) and are available through request.

2.3.1.1. Marble burying test. The marble burying test assesses anxiety-like and obsessive-compulsive-like behavior in mice (Nicolas et al., 2006; Dixit et al., 2020). We followed the procedure as previously used by Marks et al., 2009 and described by others (Marks et al., 2009; Dixit et al., 2020). Briefly, mice were taken from their home cage and placed in a standard rat cage/box (45 cm [L] × 23 cm [W] × 20 cm [H]) with 3 cm of bedding to acclimate for 30 min. The cage lid, water, and food were removed and replaced with a sheet of plexiglass (Amazon, Seattle, WA) to prevent distraction and allow video recording of behaviors. For testing, 18 black metallic marbles (Amazon) were placed in a 6-marble x 3-marble grid. To initiate a trial, mice were placed in the center of the test cage and were allowed to move freely for 30 min. A picture was taken of the cage before the marbles were touched and after the mouse was recovered to its home cage to ensure accuracy of the marbles-buried count. To be considered buried, the surface of the marble had to be covered $^2/_3$ of the way with bedding. Following the trial, marbles were recovered from the bedding and washed with Versa-Clean (VWR, Radnor, PA) diluted in water, sprayed with 70% EtOH, and air dried before

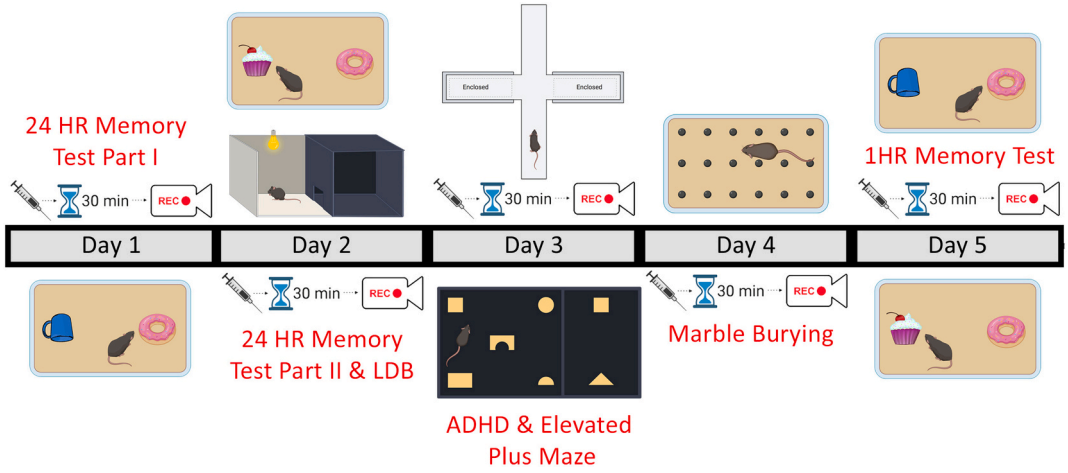

Fig. 1. Experimental timeline for behavioral testing following cannabidiol (CBD) treatment in mice.
Schematic demonstrating the sequential behavioral tasks that all mice underwent over a 5-day time interval that included: the light-dark box (LDB) and elevated plus maze (EPM) to evaluate anxiety-like behavior, the marble burying assay to evaluate obsessive compulsive-like behavior, the object-based attention test to evaluate attention-like deficit behavior, and object memory tests of 24- and 1-h duration to evaluate any deficits in long- or short-term memory, respectively. Male and female wildtype and Kv1.3−/− mice received a single, daily treatment with 10 mg/kg or 20 mg/kg CBD via IP injection (needle symbol) thirty minutes (hour glass symbol) prior to completing the behavioral task that was video recorded (camera symbol) and manually scored by an observer that was blinded to the experimental condition.

being used again.

2.3.1.2. Light-dark box. The light-dark box (LDB) assesses anxiety-like behavior in rodents that avoid time spent in the illuminated side (Hascoët et al., 2001; Bourin and Hascoët, 2003; Takao and Miyakawa, 2006; Bourin, 2015). In addition to time spent in each compartment, the number of transitions between the two compartments can be scored as an index of general locomotor activity. Latency of first movement to the opposite compartment than initially placed is also an index of anxiety. The LDB testing chamber was a standard rat cage (45 cm [L] × 23 cm [W] × 20 cm [H]) painted black on one side and painted white on the other side. A black divider was positioned in the center with a small entry door (7 cm [L] x 7 cm [H]) to allow ease of mouse movement and transition between the created two compartments. A 60 W light bulb was hung 70 cm directly over the center of the white chamber and a sheet of plexiglass again served as the cage lid to prevent distraction and allow video recording of behaviors. The LDB protocol was as previously performed (Marks et al., 2009). Briefly, mice were acclimated to the testing room 30 min prior to the experiment. Mice were then transferred from their home cage into the LDB apparatus by placing the mouse in the light compartment. The trial duration was 5 min, where the time to first latency to the dark, number of total transitions, and time spent in each compartment were scored. Entrance into a compartment was defined as all four paws of the mouse crossing into that compartment. The LDB was cleaned using Kimwipes (VWR) and 70% ethanol between each mouse trial.

2.3.1.3. Elevated plus maze. The elevated plus maze (EPM) assesses anxiety-like behavior in rodents due to their proclivity for movement to enclosed spaces and their unconditioned fear of heights (Montgomery, 1955; Pellow et al., 1985; Walf and Frye, 2007). The EPM can be used to evaluate potential changes in anxious behavior based upon changed distribution of time spent in the maze compartments (Komada et al., 2008). Our apparatus consisted of four arms (35 cm length x 5 cm width each) raised to a height of 45 cm from the ground. Two arms were completely flat and lacked barriers (open arms) and two arms had 15 cm tall barriers (closed arms). The procedure was as performed previously (Huang et al., 2018), whereby mice were acclimated to the experiment room for 30 min prior to experimentation. Mice were then introduced to the middle of the plus maze facing the open arm, and allowed to freely explore the apparatus for a 5-min duration. The time spent in the open and closed arms and the total number of transitions were scored. Entrance into an arm was defined as all four paws of the mouse crossing into that arm. The EPM was cleaned with Kimwipes and 70% ethanol between mouse subjects.

2.3.1.4. Short- and long-term memory testing. Mice were tested for short- (1 h) and long-term (24 h) object recognition that are tests for memory in rodents as previously performed (Marks et al., 2009; Antunes and Biala, 2012; Tucker et al., 2012; Huang et al., 2018). Briefly, mice were acclimated for 30 min in a standard rat cage (45 cm [L] × 23 cm [W] × 20 cm [H]) filled with a layer of bedding and with the cage lid and food/water removed and replaced with a sheet of plexiglass as described above. Following acclimation, two unfamiliar objects (object 1, object 2) were placed at opposite ends of the cage and mice were given the opportunity to explore for a duration of 5 min. Then, either 1 h (short-term memory) or 24 h (long-term memory) later, one familiar object (object 1) and one novel object (object 3) were alternatively placed in the cage and the mice were given opportunity to explore for a second 5-min duration. The mouse was considered to be exploring the object when all of the following criteria were met: the mouse was oriented toward the object, the nose was within 2 cm of the object, and both of these criteria were met for at least 1 s. Chewing or standing on an object did not count toward exploration time. A recognition index (RI) was calculated based upon time spent with the novel object, or RI = object 3/(object 1 + object 3), whereby a higher RI was associated with better memory retention. In order to assure equal time exploration of the initial object 1 and object 2, an object bias score was calculated, or OBS = object 1/(object 1 + object 2). Mice exhibiting an OBS below 0.20 or above 0.80 (i.e. signifying avoidance or attraction of object 1, respectively) were noted and excluded from the subsequent 1 h or 24 h object memory test. The objects used were plastic toys of similar size but different shapes (McDonald's, Chicago, IL), and they were cleaned using Kimwipes and 70% ethanol between mouse subjects.

2.3.1.5. Object-based attention testing. Attention deficit-like behavior (ADHD) in rodents can be assessed with an object-based attention test as originally designed (Alkam et al., 2011; Ishisaka et al., 2012) and as previously performed (Huang et al., 2018). The object-based attention test is similar in design as the object memory paradigm, but it is of shorter duration and more objects are presented. The testing chamber is larger and has two unequal compartments separated with a removable divider: a larger chamber (40 cm [L] x 40 cm [W] x 22 cm [H]) and a smaller (20 cm [L] x 40 cm [W] x 22 cm [H])) chamber. The testing chamber was filled with a layer of bedding, and the cage lid and food/water were removed and replaced with plexiglass as described above. Mice were first acclimated to the full chamber (no divider) for 10 min. Mice were gently coaxed into the larger compartment, the divider was inserted, and mice were allowed to explore for 10 min. Mice were then coaxed into the smaller compartment, and allowed to explore for an additional 10 min. Following this full acclimation period of 30 min, five wooden shapes (Walmart, Bentonville, AR) were placed in the larger compartment and mice were allowed to explore the objects for 3 min. Mice were then gently coaxed into the smaller compartment where they were presented with two objects, one was a novel object (NO) and the second was a familiar object (FO) matching one of the five shapes from the larger compartment, whereby mice were allowed to explore for 3 min. Criteria for object exploration were the same as described previously for the short- and long-term memory testing. RI was calculated as NO/(NO + FO), based upon time spent exploring the objects. The mouse was considered to be exploring the object when all of the following criteria were met: the mouse was oriented toward the object, the nose was within 2 cm of the object, and both of these criteria were met for at least 1 s. A lower RI indicated increased attention-like deficit behavior. In the object-based attention test, the box and shapes were cleaned with 70% ethanol and new bedding was added for each mouse subject.

2.4. Statistical analysis

Data scored from video playback were organized in Excel (Microsoft Office 365 Suite), then analyzed and graphed in Prism v9.0 (GraphPad Software, Inc.), and finally compiled to Photoshop CS4 (Adobe, San Jose, CA) to create figure layouts. Prior to performing any statistical comparisons, data were first analyzed with the Dixon's Q test to identify any outliers. Data were then checked for normal distribution and homogeneity of variance using the F_{max} test. Analysis of data collected across the behavioral phenotyping experiments infrequently identified outliers (data from one mouse in the EPM was discarded, one in the LDB, and two mice in the first latency test) nor did any collected data violate homogeneity of variance (fail the F_{max} test). The number of buried marbles, number of transitions and time to first latency in the LDB, and number of transitions in the EPM were compared between vehicle and drug treated animals using a two-tailed Student's *t*-test ($\alpha \leq 0.05$). Mouse behavior in the EPM and LDB was analyzed using a mixed repeated measure (RM) 2-way analysis of variance (2-w ANOVA) with drug and location (RM) as factors, and also drug x location interactions at the 95% confidence level ($\alpha \leq 0.05$). The Bonferroni method for multiple comparison testing was used as the *post-hoc* analysis to make mean-wise comparisons between treatments. Recognition Indexes (RI) computed in the short- and long-term object memory and object-based

attention tasks were compared between vehicle and drug treated animals using a Mann Whitney U (non-parametric equivalent to the *t*-test) appropriate for percentage data. All data were analyzed within drug concentration, genotype and sex as independent cohorts. All reported values in the text and figures are mean ± standard deviation (SD). Sample sizes are reported as individual data points in the graphs and represent number of mice. Individual *F* statistic and/or *p* values are reported for each experiment within the corresponding graph, as described in the results section, and as noted in Supplementary Table 1.

3. Results

3.1. Obsessive compulsive- and anxiety-like behaviors

Obsessive compulsive- and anxiety-like behaviors were measured in male and female wildtype and Kv1.3−/− mice that had been treated with a high dose (20 mg/kg) or low dose (10 mg/kg) of CBD using three standard assays: the marble burying test, the elevated plus maze, and the light-dark box.

3.1.1. Marble-burying test

When wildtype male mice were treated acutely with 10 mg/kg CBD, they buried significantly fewer marbles in comparison to their vehicle-treated counterparts, as determined by Student's *t*-test ($p = 0.0364$; Fig. 2B). Wildtype male mice treated with the higher dose of CBD (20 mg/kg) did not follow a similar trend. There was no significant difference in number of marbles buried between the drug-treated and vehicle-treated groups (Student's *t*-test; $p = 0.8376$, Fig. 2B). Additionally, there was no reduction in number of marbles buried between female wildtype mice treated with either concentration of CBD and their vehicle-treated counterparts (Student's *t*-test; $p > 0.05$, Fig. 2A). Kv1.3−/− male mice treated with the higher concentration of CBD (20 mg/kg) buried significantly fewer marbles than those treated with vehicle alone (Student's *t*-test; $p = 0.0156$; Fig. 2D). Male mice of the same genotype trended to bury less marbles in response to the lower concentration of CBD but the behavior did not reach statistical significance (Student's *t*-test; $p = 0.1078$, Fig. 2D). Female Kv1.3−/− mice, in contrast to their male counterparts, did not respond to either concentration of CBD (Student's *t*-test; $p > 0.05$, Fig. 2C).

3.1.2. Elevated plus maze

All mice spent more time in the closed arms of the maze compared with that of the open arms or the center compartment, regardless of sex or genotype. This was determined using a mixed RM 2-w ANOVA using arm location as the factor (all tests were highly significant $p < 0.0001$ and the individual Bonferroni post-hoc *p* values are reported on the graphs (*), see main effect – arm, Figs. 3 and 4). Because location was highly significant in all tests, and we wanted to focus on drug x location interactions, the significance of the latter was also denoted on the EPM graphs (#). In both male and female wildtype mice, no significant drug treatment x location interaction was observed (female 10 mg/kg - $F(2,30) = 1.353$, female 20 mg/kg - $F(2, 24) = 1.390$, male 10 mg/kg - $F(2, 24) = 1.864$, male 20 mg/kg - $F(2,25) = 0.2323$; Fig. 3A-D). Therefore, acute treatment with either 10 mg/kg CBD or 20 mg/kg CBD failed to change the distribution of time spent in the arms of the maze for wildtype mice. In female Kv1.3−/− mice, treatment with 10 mg/kg CBD was anxiogenic and showed a significant drug treatment x location interaction, while treatment with 20 mg/kg CBD had no significant effect (female 10 mg/kg - $F(2,21) = 6.380$, $\#p = 0.0251$ closed arms, $\#p = 0.0357$ open arms; female 20 mg/kg - $F(2,24) = 1.390$; Fig. 4A,B). In male Kv1.3−/− mice, there was no deviation in behavior for the distribution of time spent in the arms of the maze between the 10 mg/kg or 20 mg/kg CBD-treated groups and the vehicle-treated groups (male 10 mg/kg - $F(2,24) = 1.864$, male 20 mg/kg - $F(2,32) = 0.4156$; Fig. 4C, D).

In order to confirm that the CBD treatments did not cause malaise or change in general locomotor activity, the total number of elevated plus maze transitions was quantified. There was no significant difference in the number of transitions made by the majority of all groups of mice for the sum of all movement across all five compartments of the elevated plus maze (two open compartments, two closed compartments, and the center area) (Student's *t*-test; $p > 0.05$, Supplemental Fig. 1). The only exception was for Kv1.3−/− female mice that had a significant decrease in the number of transitions when treated with 20 mg/kg CBD (Student's *t*-test, $p = 0.0058$, Supplemental Fig. 1C).

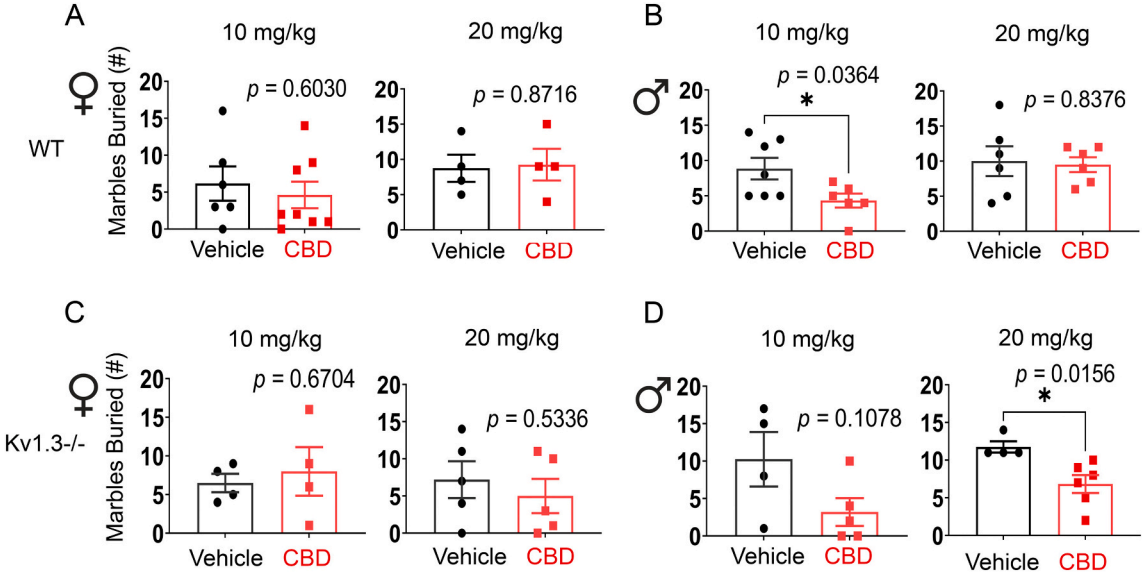

Fig. 2. Male mice treated acutely with CBD bury fewer marbles in a dose-dependent manner according to genotype.
Bar graphs plotting the mean number (±s.d.) of marbles buried by mice following an intraperitoneal (IP) injection of CBD (■) vs. vehicle (●). Data points represent individual mice (sample size), wildtype mice (WT; top row), Kv1.3−/− mice (bottom row), female (left), and male (right). Concentration received as 10 mg/kg or 20 mg/kg as noted, in this and subsequent figures, with vehicle being volume matched. See female and male symbols in this and subsequent figs. A, Wildtype females, B, Wildtype males, C, Kv1.3−/− females, and D, Kv1.3−/− males. Student's *t*-test, significantly-different means, $*p < 0.05$.

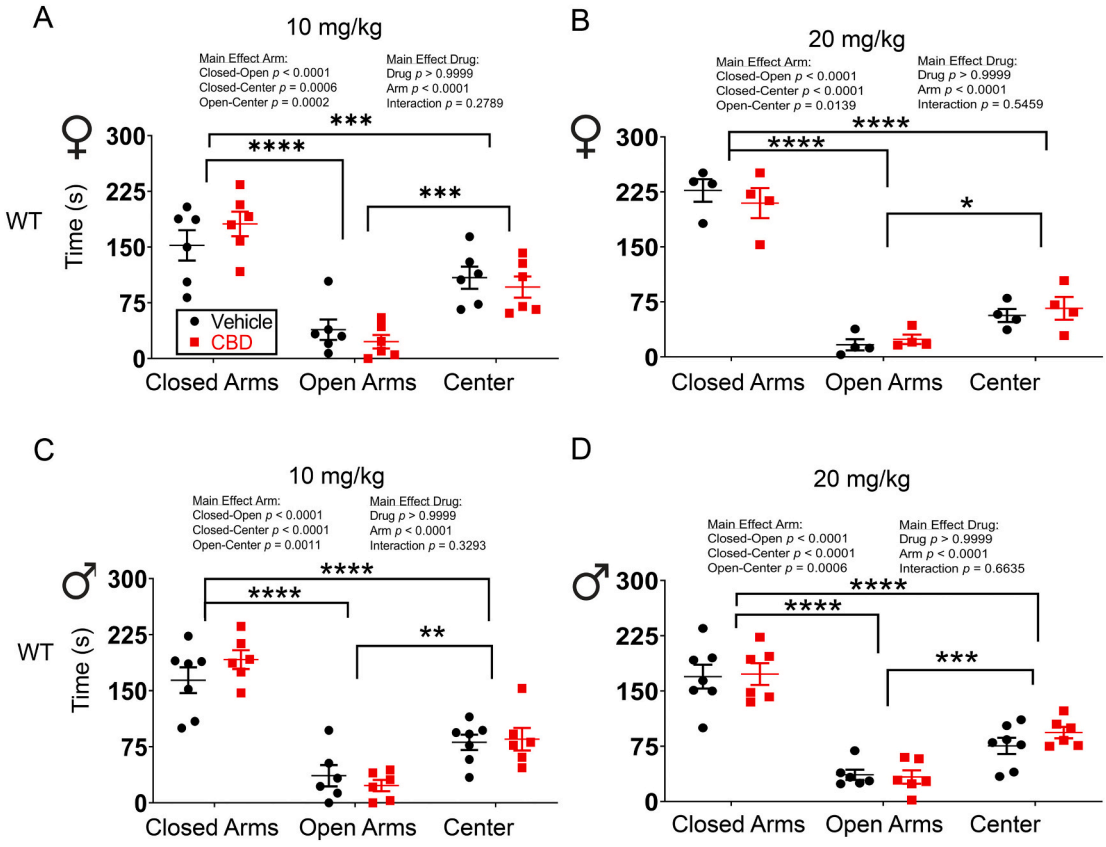

Fig. 3. Wildtype mice spend more time in the closed arms of an elevated plus maze (EPM), which is not altered when treated acutely with CBD. Scatter plot of the time a mouse spends in the closed arms, open arms, or center area of an EPM following an intraperitoneal (IP) injection of CBD (■) vs. vehicle (●). In this and subsequent scatter plots, data points represent individual mice (sample size), with mean denoted as a long horizontal bar (± s.d. brackets); A, B female mice (top row) and C, D male mice (bottom row). Mixed two-way repeated measure analysis of variance (2-w RM ANOVA) using arm location and drug as factors. The F values and degrees of freedom (dF) for the ANOVA are reported in text for this and subsequent figures. Significantly different main effect arm location, Bonferroni *post-hoc* test, ***$p < 0.001$, ****$p < 0.0001$. Not significantly different main effect drug.

3.1.3. *Light-dark box*

All mice spent more time in the dark compartment compared with the light compartment, regardless of sex or genotype. This was determined using a mixed RM 2-w ANOVA using compartment location as the factor (all tests were highly significant ranging from *$p = 0.0026$ to ****$p < 0.0001$; individual p values are reported on the graphs (*), see main effect location, Figs. 5 and 6). Because our objective was focused specifically on location x drug interactions to determine if drug treatment affected distribution of time spent in a compartment, we noted significant interactions on the LDB graphs using red font for clarity (see Interaction). The associated Bonferroni's *post-hoc* p values (#) are noted when significant for drug treatment multiple comparisons.

In wildtype mice of both sexes, CBD lessened anxiety-like behaviors in the LDB apparatus in comparison to the vehicle treated group. We observed a significant drug x location interaction ($F(1,20) = 5.507$, $p = 0.0294$) in which wildtype female mice receiving a 10 mg/kg dose of CBD no longer spent significantly greater time in the dark compartment compared with those receiving vehicle (female 10 mg/kg - #$p < 0.001$ vehicle, $p > 0.05$ CBD; Fig. 5A). When the CBD dose was increased to 20 mg/kg, there was no longer a significant drug x location interaction in the wildtype female mice ($F(1,10) = 0.1573$, $p = 0.7000$; Fig. 5B). In comparison to the female mice, wildtype male mice did not exhibit a significant drug x location interaction with the lower dose of 10 mg/kg ($F(1,22) = 3.000$, $p = 0.0973$; Fig. 5C), but did exhibit such at the higher dose of 20 mg/kg ($F(1,18) = 8.237$, $p = 0.0102$; Fig. 5D). Here, as with females treated with the lower dose of CBD, males no longer spent a significantly greater time in the dark compartment compared with those receiving vehicle (male 20 mg/kg - #$p < 0.0001$ vehicle, $p > 0.05$ CBD; Fig. 5D). Therefore, in the wildtype mice, treatment with CBD disrupts the tendency of mice to prefer the dark compartment over that of the light (reduces anxiety), and does so differentially across sex with different dose sensitivity. The female effective dose is lower than that of males.

In contrast to what was observed for wildtype mice, Kv1.3 −/− mice of both sexes appeared to exhibit increased anxiety-like behaviors in response to the higher CBD dose. At the 10 mg/kg CBD concentration, there was not a significant drug x location interaction for either male or female Kv1.3−/− mice (female, $F(1,16) = 0.0009$, $p = 0.9763$; male, $F(1,16) = 0.0089$, $p = 0.9257$; Fig. 6A,C). However, at the 20 mg/kg CBD concentration, there was a significant drug x location interaction for both sexes (female, $F(1,14) = 16.40$, $p = 0.0012$; male, $F(1,18) = 5.531$, $p = 0.0303$; Fig. 6B,D). Here, unlike what was observed for wildtype mice, both male and female Kv1.3−/− mice, treated with the higher dose of CBD, spent a significantly greater time in the dark compartment over that of the light compartment compared with those receiving vehicle (female 20 mg/kg - $p > 0.05$ vehicle, ##$p < 0.01$ CBD; male 20 mg/kg - $p > 0.05$ vehicle, ##$p < 0.01$ CBD; Fig. 6B,D). Consistent with locomotor activity observed for the EPM, there was also no significant difference in the number of light-dark transitions made by any group of CBD-treated mice in comparison to their vehicle-treated counterparts (Student's *t*-test, $p > 0.05$; Supplemental Fig. 2A-D) in the LDB test. Interestingly, female wildtype mice initially moved more quickly to the dark compartment (First Latency measurement) when treated with 20 mg/kg CBD, indicating heightened initial anxiety at this dose (Student's *t*-test; $p = 0.0212$; Supplemental Fig. 3A). CBD did not affect the first latency to the dark compartment in male wildtype mice, or for either sex

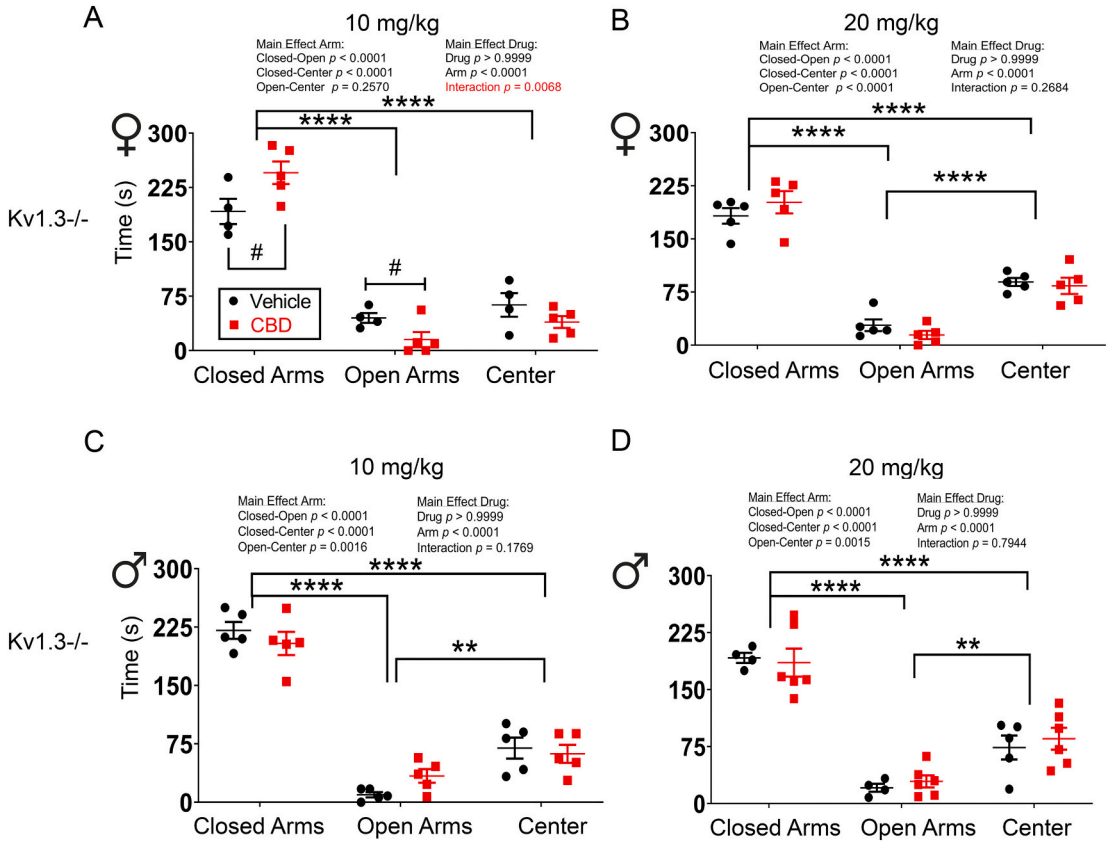

Fig. 4. Kv1.3−/− mice spend more time in the closed arms of an elevated plus maze (EPM), which is increased in females when treated acutely with 10 mg/kg CBD. Same as Fig. 3, but for Kv1.3−/− mice. Mixed 2-w RM ANOVA, significantly different main effect arm location, Bonferroni *post-hoc* test, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Significantly different main effect drug, Bonferroni *post-hoc* test, #$p < 0.05$.

in Kv1.3−/− mice (Student's *t*-test; $p > 0.05$: Supplemental Fig. 3B-D).

3.2. Object memory and object-based attention testing

3.2.1. 1-hour and 24-hour object memory

Mice were required to spend >20% of their observation time, but not >80%, with each of the two objects during the familiarization phase to ensure they had no initial bias for an object. Using this definition, 6 mice had an initial object bias during the short-term memory test and 5 mice had an initial object bias during the long-term memory test. The mice were therefore eliminated from analysis in those respective tests. Mice treated with CBD demonstrated no significant change in the recognition of a novel object following familiarization with two objects and a subsequent wait interval of 1 h prior to novel object presentation. This was true regardless of genotype, sex, or concentration of CBD (Fig. 7, Mann-Whitney *U* tests, all $p > 0.05$). Mice treated with CBD, familiarized with two objects, and then presented with a novel object 24 h later also showed no change in exploration of the novel object over the familiar - with one exception (Fig. 8, Mann-Whitney U tests, all $p > 0.05$). Wildtype male mice exhibited a significantly reduced recognition index (Fig. 8B) compared with mice receiving vehicle (Mann-Whitney U test, $p = 0.0484$) for animals receiving the 10 mg/kg CBD dose and asked to explore a novel object 24 h later. Therefore, a low concentration of CBD reduced long-term memory in male wildtype mice.

3.2.2. Object-based attention test

Object-based attention tests were used to determine if CBD affected immediate changes in the recognition of a novel object after a brief presentation of five dissimilar objects. A lower recognition index is associated with poorer attention or ADHD-like behavior. Wildtype mice were observed to have no significant change in their recognition index when comparing CBD vs. vehicle treated groups. This was true regardless of sex or concentration of CBD (Fig. 9A,B; Mann-Whitney U tests, all $p > 0.05$). However, in Kv1.3−/− mice, the observed recognition index was significantly different in a sex and concentration manner. Female Kv1.3−/− mice exhibited a reduced recognition index when treated with 10 mg/kg CBD (Fig. 9C; Mann-Whitney U, $p = 0.0079$) that was not observed in males (Fig. 9D; Mann-Whitney U, $p = 0.6623$). When treated with the higher dose of CBD (20 mg/kg), the opposite occurred – males exhibited an increased recognition index (Fig. 9D; Mann-Whitney U, $p = 0.0370$) and females were unaffected (Fig. 9C; Mann-Whitney U, $p = 0.3874$). Therefore, female Kv1.3−/− exhibit a decreased attention when treated with a low dose of CBD, whereas male Kv1.3−/− exhibit an improved attention when treated with a high dose of CBD.

A descriptive summary of all behavioral phenotyping results (Figs. 1 to 9, Supplementary Figs. 1-3) can be found in Table 1.

4. Discussion

This study tested whether an injection of CBD acutely affected anxiety-like or attention-like behavior, or memory in wildtype and Kv1.3−/− mice. We discovered that acute CBD treatment reduced marble burying in male, but not female mice, inferring that CBD decreased obsessive-compulsive-like behaviors in male subjects. CBD was effective in lessening anxiety-like behaviors determined by the LDB test in both male and female wildtype mice, whereby the effective dose required to observe the same response in females was less than that required to observe the effect in males. In Kv1.3−/− mice, CBD was anxiogenic in that the drug increased anxiety-like behaviors in the LDB in both sexes at the higher concentration of CBD and it similarly increased anxiety-like behavior in females in the EPM at the lower concentration of CBD. Treatment with CBD did not affect short-term

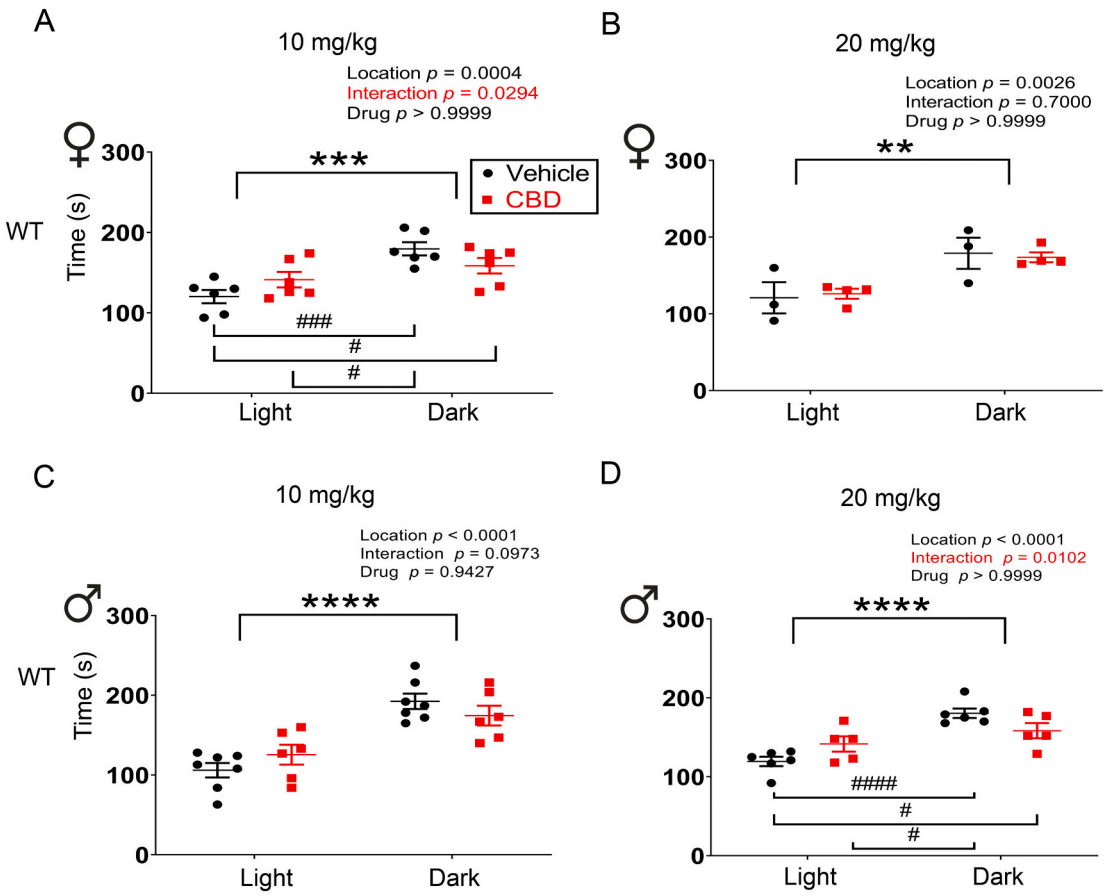

Fig. 5. Wildtype mice spend less time in the lighted, over that of the darkened, compartment of a Light Dark Box (LDB), which is increased when treated acutely with CBD. The response to increase time in the light with drug is dose-dependent according to sex.
Scatter plot of the time a mouse spends in the light vs. dark compartment of a LDB following an intraperitoneal (IP) injection of CBD (■) vs. vehicle (●). Data points represent individual mice (sample size), A, B female mice (top row) and C, D male mice (bottom row). Mixed two-way repeated measure analysis of variance (2-w RM ANOVA) using location and drug as factors. Significantly different effect of location ($p = 0.0004$), Bonferroni *post-hoc* test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Significantly different effect of drug x location interaction ($p = 0.0294$), Bonferroni *post-hoc* test, $\#p < 0.05$, $\#\#\# p < 0.001$.

object memory in mice of either genotype or sex, but long-term object memory was reduced in male wildtype mice at the lower concentration of CBD. Finally, ADHD- or attention-like behaviors were not altered by CBD in wildtype mice, but in Kv1.3−/− mice, females were observed to have a loss in attention while male mice demonstrated improved attention.

Our study increases the understanding of the effect of a single treatment of CBD on a spectrum of associated behaviors ranging from anxiety, obsessive compulsive, attention, and memory. Unlike previous studies that were limited to only the male sex, or one to two different behavioral tests, or a single drug dose, we attempted to design a battery of 6 behavioral tests that would compare across both sexes and also at two drug concentrations. Moreover, we incorporated mice that had a gene-targeted deletion of the Kv1.3 gene that are known to have trait anxiety and attention-like deficiencies as a potential model for ADHD-like behaviors (Huang et al., 2018). By doing such, we discovered that CBD affects behavior in a dose-, sex-, and state-dependent manner. It is interesting that CDB effectively reduced obsessive-compulsive behaviors in male subjects without having any significant effect in females. Obsessive compulsive behaviors in humans are thought to present earlier in childhood for males, but are more common in females in adolescence and adulthood (Mathes et al., 2019). Treatment outcome, however, is judged to be sex independent, which is counter to what our results in mice would predict given that CBD was only effective in male mice. Nardo et al., 2014 previously reported a similar anti-compulsive effect of CBD administration in mice, which was additionally able to block obsessive-compulsive behavior (enhanced marble burying) produced by the serotonin agonist meta-chloro-phenyl-piperazine (mCPP) (Nardo et al., 2014). While they only examined male mice, they also reported that doses lower than 30 mg/kg were subthreshold and had no effect on marble burying. This is in contrast with our study in that only 10 mg/kg was significantly able to reduce marble burying. Murphy et al., 2017 also only studied male mice and the effect of CBD on obsessive compulsive, object memory, and anxiety outcomes (Murphy et al., 2017). These investigators did not examine single use exposure, but rather following 20 days of daily injections in either early postnatal or adult mice. Nonetheless, chronic administration of 3 mg/kg CBD did act as an anti-compulsive agent and decreased number of marbles buried.

While both wildtype and Kv1.3−/− control mice treated with vehicle exhibited the expected anxiety-like behaviors in both the EPM and the LDB, the two test apparatuses did not yield similar outcomes for a main effect of the drug in the wildtype mice. It is not known why CBD was clearly anxiolytic for the wildtype mice in the LDB – both males and females demonstrated a reduction of time spent in the dark compartment and increased time in the light compartment – but then in the EPM, there was no change in the distribution of time spent in the open or closed arms following CBD treatment. Both the LDB and the EPM represent unconditioned anxiety tests that examine ethologically relevant stresses for rodents as defined by Bourin and collaborators (Bourin et al., 2007). While other unconditioned tests, such as the open field test (OFT), have been criticized for the inability to discern locomotion or

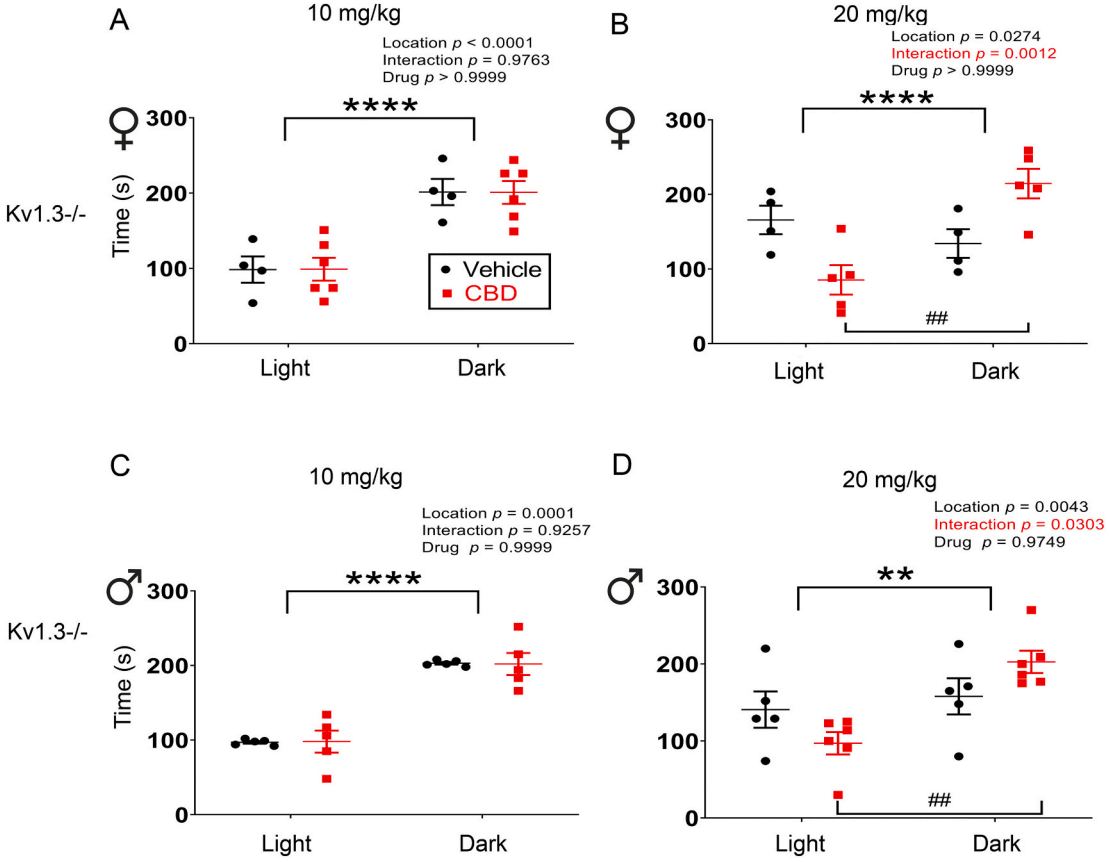

Fig. 6. Kv1.3−/− mice spend less time in the lighted, over that of the darkened, compartment of a Light Dark Box (LDB), which is further decreased for both sexes when treated acutely with 20 mg/kg CBD.
Same as Fig. 5, but for Kv1.3−/− mice. Mixed 2-w RM ANOVA, significantly different effect of location, Bonferroni *post-hoc* test, **p < 0.01, ****p < 0.0001. Significantly different effect of drug x location interaction ($p = 0.0303$), Bonferroni *post-hoc* test, ## $p < 0.01$.

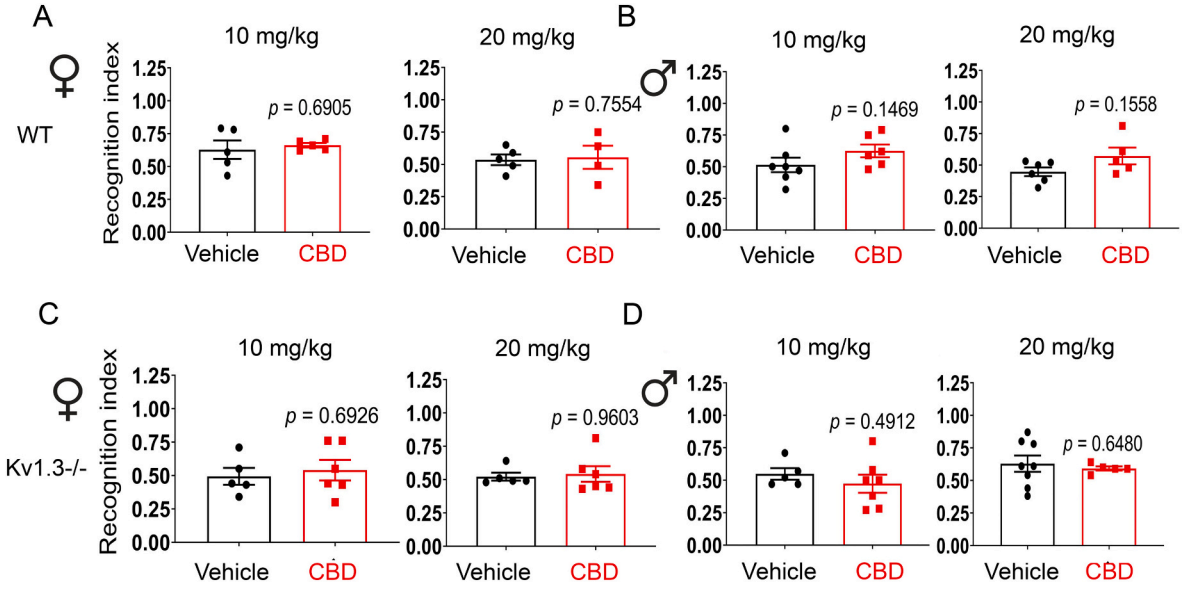

Fig. 7. Mice have a defined recognition index for a novel vs. familiar object that is calculated following presentation of the two objects across a 1-h interval (short-term memory), which is not changed when treated acutely with CBD.
Bar graphs plotting the recognition index for mice following an intraperitoneal (IP) injection of CBD (■) vs. vehicle (●). Data points represent individual mice (sample size), wildtype mice (WT; top row), Kv1.3−/− mice (bottom row), female (left), and male (right). A, Wildtype females, B, Wildtype males, C, Kv1.3−/− females, and D, Kv1.3−/− males. Mann-Whitney U, not significantly different, $p > 0.05$.

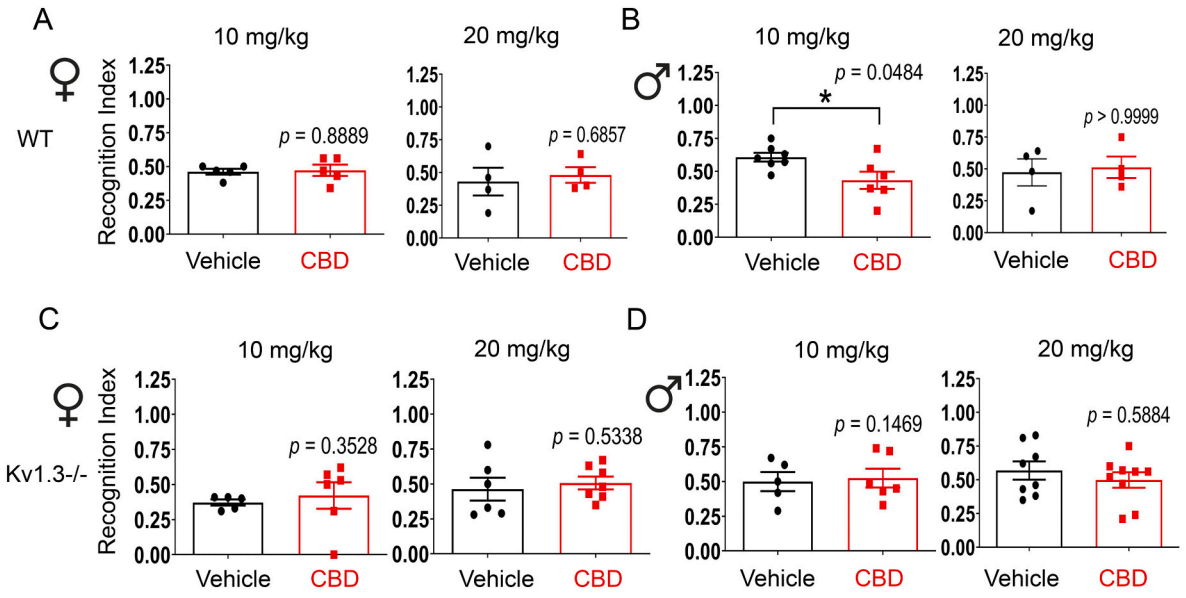

Fig. 8. Mice have a defined recognition index for a novel vs. familiar object that is calculated following presentation of the two objects across a 24-h interval (long-term memory), which is significantly reduced for male mice when treated acutely with 10 mg/kg CBD.
Same as Fig. 7, but for long-term memory.

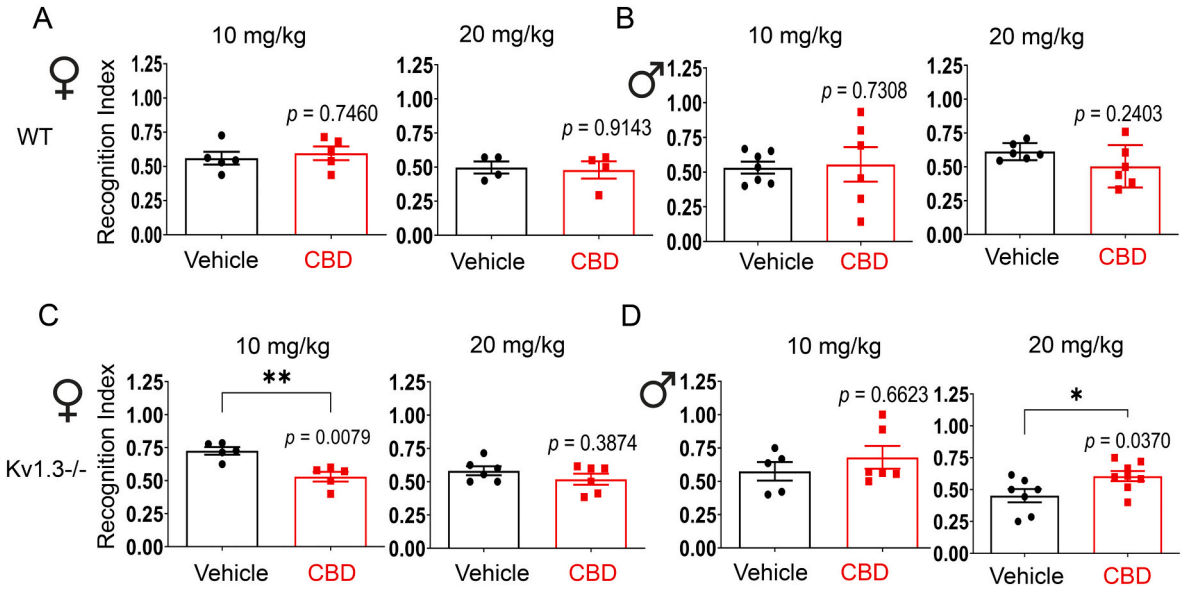

Fig. 9. Mice have a defined recognition index for a novel vs. familiar object that is calculated following presentation of the two objects after brief exposure to 5 different objects in an attention task. Female Kv1.3−/− mice have a reduced attention ability and male Kv1.3−/− mice have an opposite, increased attention ability when treated acutely with 10 and 20 mg/kg CBD, respectively.
Bar graphs plotting the recognition index for mice following an intraperitoneal (IP) injection of CBD (■) vs. vehicle (●). Data points represent individual mice (sample size), wildtype mice (WT; top row), Kv1.3−/− mice (bottom row), female (left), and male (right). A, Wildtype females, B, Wildtype males, C, Kv1.3−/− females, and D, Kv1.3−/− males. Mann-Whitney U, significantly different, $*p < 0.05$, $**p < 0.01$.

exploration from anxiety (File, 2001; Tucker and McCabe, 2021), within the LDB, the most reliable measure for assessing anxiolytic drugs has been time spent in the light chamber (Hascoët and Bourin, 1998; Tucker and McCabe, 2021) as opposed to number of transitions. The EPM is the mostly widely applied anxiety test, however, it is sensitive to some anxiolytic drugs and not others (Pellow et al., 1985; Lister, 1987). Here, both the number of entries into open arms and total time spent in the open arms are equivalent metrics for identifying reduction in anxiety (Tucker and McCabe, 2021). Interestingly for the EPM, there are additional behaviors that have been observed such as stretch attenuated postures (more anxious) and head dipping (directed exploration, less anxious) that have been reported (Cryan and Holmes, 2005; Tucker and McCabe, 2021). In the future it would be advantageous to score these selective approach/avoidance behaviors alongside the classical measures of occupancy within the EPM. It may be possible that sequential behavioral testing over the course of the week could have lessened the basal anxiety of the mice through repeat handling (Gouveia and Hurst, 2019), however, the vehicle control animals responded as anticipated, demonstrating a significant location effect and avoidance of the open arm compartment. In performing analysis of anxiety-like behaviors, it is always important to apply more than one type of anxiety measuring test. In fact, some investigators have even integrated the OFT/EPM/LDB into

Table 1
Summary results of behavioral phenotyping tests in wildtype and Kv1.3−/− mice following IP administration of CBD at 10 mg/kg or 20 mg/kg dosage. *$p < 0.05$ by Student's *t*-test, %$p < 0.05$ by Mann Whitney *U* test, #$p < 0.05$, ##$p < 0.01$ by mixed 2-way RM ANOVA, NS = Not significantly different. Increased = significantly elevated anxiety-like, obsessive-compulsive-like, attention-like behaviors or memory. Decreased = significantly lessened anxiety-like, obsessive-compulsive-like, attention-like behaviors or memory. For full statistical summary see Supplementary Table 1.

Behavioral Test	Female		Male	
	10 mg/kg	20 mg/kg	10 mg/kg	20 mg/kg
Marble Burying				
Wildtype	NS	NS	Decreased*	NS
Kv1.3−/−	NS	NS	NS	Decreased*
Elevated Plus Maze (EPM)				
Wildtype	NS	NS	NS	NS
Kv1.3−/−	Increased##	NS	NS	NS
Light Dark Box (LDB)				
Wildtype	Decreased#	NS	NS	Decreased##
Kv1.3−/−	NS	Increased##	NS	Increased#
Short-term Memory (1 h)				
Wildtype	NS	NS	NS	NS
Kv1.3−/−	NS	NS	NS	NS
Long-term Memory (24 h)				
Wildtype	NS	NS	Decreased %	NS
Kv1.3−/−	NS	NS	NS	NS
Object-based Attention				
Wildtype	NS	NS	NS	NS
Kv1.3−/−	Decreased% %	NS	NS	Increased%

a single trial by connecting the apparatus (Ramos et al., 2008). Although it is not certain what relationship the inter-test differences have across the OFT/EPM/LBD, with the various forms of human anxiety, it is acknowledged that the three tests measure different types of anxiety-like behavior (Cryan and Holmes, 2005). Another alternative interpretation rests upon the report of Rogers and colleagues who report that exposure of mice to the EPM in their studies prior to that of the LDB, eliminated the observed anxiolytic properties of diazepam (Rodgers and Shepherd, 1993). In the future, it would be interesting to determine if we observed anxiolytic properties from CBD if mice were challenged with the EPM prior to that of the LDB, inferring that CBD's anxiolytic properties can be lost with repeat anxiety testing.

It is interesting, nonetheless, that the anxiolytic effect of CBD was sex-dependent for these mice observed in the LDB – female mice showed reduced anxiety-like behavior at 10 mg/kg, but for males, 20 mg/kg was required to observe such an anxiolytic effect of CBD. These data underscore a potential need to investigate sex differences in CBD use when cannabinoids are prescribed in human subjects. Moreover, in the Kv1.3−/− mice, the results of the EPM and LDB were more congruent in terms of similar drug-induced outcomes, yet unexpectedly CBD was anxiogenic rather than anxiolytic. These results might infer that administration of CBD may be state dependent – in that CBD decreased anxiety behaviors for wildtype mice that were placed in a temporary anxiety producing environment (LDB/EPM; (Bourin et al., 2007; Tucker and McCabe, 2021)), but CBD increased anxiety behaviors for Kv1.3−/− mice have trait anxiety that is more chronic. When considering the possible implications of our findings for human subjects, it would be important to determine if CBD might exasperate anxiety behaviors in individuals with heightened anxiety or a diagnosed anxiety disorder. This is also an idea presented by Sartori and colleagues that discuss the need to reproduce the pathophysiology of the human anxiety disorder so that the mouse model of heightened anxiety is predictive for a clinical test (Sartori et al., 2011). Our data in the Kv1.3−/− model might be particularly relevant for female subjects given that female Kv1.3−/− exhibit increased anxiety-like behavior in response to CBD in both the EPM and the LDB. Females are known to have increased diagnosis of generalized anxiety disorder (GAD) at a rate 2:1 compared to that of males (Bahrami and Yousefi, 2011), and thus there is a need to tailor their mental health care needs differently than that of males historically speaking. With these comparisons between mouse and human subjects, it is important to note that there is no single mouse model that encompasses the complexity of human anxiety/depression, however, rodents can be used for predictive values.

It is also well-known that high levels of anxiety or generalized anxiety disorder can negatively affect cognition or memory (Lukasik et al., 2019). Despite this, we did not observe any changed attention or short-term memory in our wildtype mice treated with CBD, even though the drug clearly decreased anxiety-like behavior. The wildtype male mice did have a reduction in long-term memory, which is opposite as to what would be anticipated since the CBD was anxiolytic, not anxiogenic. Interestingly, the Kv1.3−/− mice that exhibited enhanced anxiety with CBD treatment (Figs. 4 and 6), did have changes in attention that were sex dependent (Fig. 9), but the drug did not change short- or long-term memory (Figs. 7–8). The female Kv1.3−/− mice did follow the anticipated negative association between anxiety and memory, but the male Kv1.3−/− had a positive association that is not understood. It is beneficial that CBD lacked any effect on attention or short-term object memory in the wildtype mice, thereby retaining its potential beneficial therapeutic use as a non-psychoactive chemical, however, it did affect long-term object memory in male subjects and this should be considered. Because CBD was strongly anxiogenic in Kv1.3−/− mice and there was both a negative and positive association with attention that was sex dependent, this potential relationship should be further examined in clinical trials when considering individuals with trait anxiety or generalized anxiety disorder, for example.

We observed behavioral responses to CBD that were not linearly dose dependent, and in some instances these responses were significant at low rather than high dose (see Table 1). In fact, this is congruent with other investigations that report a biphasic effect of cannabinoids in terms of anxiety, in particular (reviewed by Blessing et al., 2015; Sharpe et al., 2020; Petrie et al., 2021), but also concerning the biphasic effect of the drug on inflammation, tumor growth, motor activity, or neurotransmitter release (Tzavara et al., 2003; Rey et al., 2012; Katsidoni et al., 2013; Huestis et al., 2019; Griffiths et al., 2021). Because there are so many targets for CBD signaling (Griffiths et al., 2021), combined with the fact that there is a diversity of expression for any one receptor (i.e. CB1) throughout the brain, and also modulation of endocannabinoid signaling by exogenous CBD, this may lead to a varied concentration effectiveness of CBD dose.

It is important to note that there was no apparent change in generalized locomotor activity associated with single CBD treatment regardless of genotype, sex, or drug concentration. This conclusion was drawn following quantification of the number of EPM or LDB transitions comparing vehicle vs. CBD treated mice. A significant change in the number of compartment transitions in a test could indicate hyperactivity, malaise, or lack of sensation for the animal, which could affect proper interpretation of the anxiety behavior. Prior to the accessibility of CBD with known synthetic purity, and due to the regulation of THC being classified as a Substance I chemical, many investigators alternatively designed behavioral assays using orthosteric antagonists or agonists of the cannabinoid CB_1 receptor such as AM-251 and WIN 55,212–2 (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate, respectively (Hájos and Freund, 2002). Because CBD is a known antagonist and negative allosteric modulator of the CB_1-receptor (Laprairie et al., 2015), we initially examined if we could observe the opposite behavioral phenotype through injection of 1 mM WIN 212–2.

Unfortunately, this drug evoked catalepsy in the mice (demonstrating zero compartment transitions and failure to position on a ring-stand; see (Pertwee, 1972; Banafshe et al., 2005)), and subsequently we could not examine any behavioral effects of CB_1 receptor activation by WIN. Moreover, behavioral experiments comparing agonists and antagonists to only the CB_1 receptor with that of global CBD action may be simplistic in design given that there are >70 receptor targets for CBD (Ibeas Bih et al., 2015).

5. Conclusions

In conclusion, single treatment with CBD exhibits changes in obsessive compulsive and anxiety-like behaviors in mice, which could be therapeutically beneficial if further confirmed in human subjects. CBD appears to be useful in decreasing obsessive compulsive-like behaviors in male mice, and is ineffective in modifying this behavior in females. The fact that CBD lessens long-term object memory, which appears to be uncoupled from anxiety changes, is a potential negative side effect that should be followed up in human clinical trials. Our data observed for Kv1.3−/− mice suggest that CBD effects might be state dependent, thereby having implications for the utility of CBD use by individuals with anxiety or generalized anxiety disorders. Our data examining anxiety-like behaviors in wildtype mice emphasize that CBD-induced behavioral changes are sex dependent in terms of effective dose, and those examining attention deficit-behavior in Kv1.3−/− mice also underscore the sex-dependent effect of whether CDB treatment is positively correlated (female) or negatively correlated (male) with anxiety-like behavior.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pbb.2022.173498.

Declaration of competing interest

The authors declare no competing interests financial or scientific.

Data availability

All video recordings acquired during our behavioral experiments can be made available through contact of the communicating author.

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