Accepted Manuscript

Cannabidiol (CBD) reduces anxiety-related behavior in mice via an FMRP1-independent mechanism

Jerzy Zieba, Duncan Sinclair, Terri Sebree, Marcel Bonn-Miller, Donna Gutterman, Steven Siegel, Tim Karl

PII: S0091-3057(18)30646-4
DOI: https://doi.org/10.1016/j.pbb.2019.05.002
Reference: PBB 72720
To appear in: Pharmacology, Biochemistry and Behavior
Received date: 21 December 2018
Revised date: 21 April 2019
Accepted date: 1 May 2019

Please cite this article as: J. Zieba, D. Sinclair, T. Sebree, et al., Cannabidiol (CBD) reduces anxiety-related behavior in mice via an FMRP1-independent mechanism, Pharmacology, Biochemistry and Behavior, https://doi.org/10.1016/j.pbb.2019.05.002

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Cannabidiol (CBD) reduces anxiety-related behavior in mice via an FMRP1-independent mechanism

Jerzy Zieba\(^a\)*, Duncan Sinclair\(^a,b,#\), Terri Sebree\(^c\), Marcel Bonn-Miller\(^c\), Donna Gutterman\(^c\), Steven Siegel\(^d\), and Tim Karl\(^a,e\)

\(^a\)Neuroscience Research Australia (NeuRA), NSW 2031, Australia
\(^b\)Wicking Centre, University of Tasmania, TAS 7001
\(^c\)Zynerba Pharmaceuticals Inc. Devon 19333, USA
\(^d\)Department of Psychiatry and Behavioral Sciences, Keck School of Medicine, University of Southern California, Los Angeles 90033, USA
\(^e\)School of Medicine, Western Sydney University, NSW 2560, Australia

* Authors contributed equally to the work

**Short title:** Acute CBD in a Mouse Model of Fragile X Syndrome

# To whom correspondence should be addressed:
Dr Duncan Sinclair
University of Tasmania
Wicking Centre
Hobart, TAS 7001, Australia
Email: duncan.sinclair@utas.edu.au
Abstract

Fragile X Syndrome is a neurodevelopmental disorder which affects intellectual, social and physical development due to mutation of the Fragile X mental retardation 1 (FMR1) gene. The resultant loss of Fragile X mental retardation protein can be modelled by Fmr1 gene knockout (KO) in mice. The current study investigated the behavioural effects of cannabidiol (CBD; a non-psychoactive phytocannabinoid) in male Fmr1 KO mice as a preclinical model for therapeutic discovery. Vehicle or CBD (5 or 20 mg/kg body weight) was administered to adult Fmr1 KO and wild type-like (WT) mice before they were tested in behavioural tasks including: open field (OF), elevated plus maze (EPM), spontaneous alternation, social preference, and passive avoidance tasks. Fmr1 KO mice were hyperlocomotive and hyperexplorative and habituated more slowly to a novel environment compared to control animals. Furthermore, Fmr1 KO mice showed fewer anxiety-related behaviours across tests. Effects of CBD were subtle and limited to the EPM, where CBD decreased the anxiety response of all mice tested. Acute CBD had no impact on locomotion or anxiety-related parameters in the OF. Cognitive performance of Fmr1 KO mice was equivalent to controls and not affected by CBD treatment. Brain concentrations of CBD were equivalent between genotypes, but in animals sacrificed 90 min post-administration, decreased plasma CBD in Fmr1 KO mice compared to WT suggested more rapid clearance of CBD by transgenic animals. Overall, acute CBD at the doses chosen did not selectively normalize behavioural abnormalities in Fmr1 KO mice, but reduced anxiety-like behaviour in both Fmr1 KO and WT mice.

Keywords: Cannabidiol (CBD); Fragile X Syndrome; Mouse Model; Locomotion; Anxiety; Cognition; Social Behaviours
1. Introduction

Fragile X Syndrome (FXS) is a neurodevelopmental disorder which affects intellectual, social and physical development of both men (1 in 4000) and women (1 in 8000) \(^1\). Individuals with FXS experience intellectual disability and symptoms of autism \(^2,3\) as well as altered sensory sensitivity \(^4-6\), repetitive behaviours \(^7\), social communication deficits \(^8\), and increased anxiety \(^2\). FXS is caused by CGG repeat expansion in the 5’ untranslated region of the Fragile X mental retardation 1 gene \((FMR1)\) \(^9\). Repeat expansion in excess of 200 copies results in epigenetic silencing of \(FMR1\) and loss of Fragile X mental retardation protein (FMRP). FMRP is an RNA-binding protein \(^10\) which negatively regulates protein translation and is required for normal neural development, as it binds to transcripts of proteins involved in synaptic function \(^11\). Other molecular signaling pathways are also affected in FXS, such as mTOR signalling \(^12\), a crucial factor for protein synthesis and cellular growth \(^13\). Treatment options in FXS are currently limited, and have until recently focused on specific domains of symptom relief (predominantly anxiety, attention deficit, and hyperactivity) \(^14,15\). Thus, new treatment alternatives informed by increased understanding of FXS neurobiology are urgently required.

As a tool for understanding FXS, a germline \(Fmr1\) knockout (KO) mouse model was developed \(^16\). This rodent model mimics the complete loss of FMRP which occurs in individuals with the full mutation (>200 repeats), and can be used for evaluation of novel therapeutic strategies. \(Fmr1\) KO mice display a range of altered behaviours compared to control mice, some of which are consistent with the clinical picture for FXS or autism (for review see \(^17\) and \(^18\)). \(Fmr1\) KO mice display less preference for social novelty than wild type-like mice \(^19\), consistent with social deficits in FXS \(^8\) and are consistently hyperactive \(^20-24\). Spatial working memory abnormalities have been described \(^16,25-27\), but not consistently \(^28,29\). Similarly, studies on the anxiety-related phenotype of this mouse model report both
decreased as well as increased anxiety-like behaviours compared to control animals. Humans with FXS often exhibit increased anxiety. \textit{Fmr1} KO mouse model phenotypes can be utilised pre-clinically to evaluate some efficacy parameters of new treatment candidates.

Recently, the endocannabinoid system has become a target of preclinical research into FXS as FMRP, which is diminished in FXS, facilitates the production of the endocannabinoid 2-arachidonoylglycerol (2-AG). In line with this, \textit{Fmr1} KO mice exhibit lower levels of 2-AG than control mice and diminished retrograde 2-AG signaling in the hippocampus. The phosphatidylinositol-3-kinase (PI3K)-protein kinase B (Akt)-mTOR-p70S6 kinase (p70S6K) signalling pathway is a downstream target of the endocannabinoid system and is dysregulated in \textit{Fmr1} KO mice. The non-psychoactive phytocannabinoid cannabidiol (CBD) may have benefits for FXS patients based on its capacity to modulate FXS-compromised endocannabinoid signalling. CBD may attenuate the pathophysiology of the disease by indirectly increasing the concentration of the two main endocannabinoids, 2-AG and \textit{N}-arachidonoylethanolamine (AEA, anandamide). In the mouse hippocampus, levels of anandamide but not 2-AG increase after 14 day treatment with 30mg/kg CBD. Furthermore, CBD has neuroprotective effects and can increase adult hippocampal neurogenesis. On a behavioural level, CBD has been found to carry anti-anxiety and anti-psychotic-like properties and improve social impairments, suggesting therapeutic potential for FXS. Indeed, CBD is a promising new therapy for Dravet and Lennox-Gastaut syndromes, which cause seizures and developmental delay. Importantly, the ability of CBD to reverse FXS-related behavioural abnormalities of \textit{Fmr1} KO mice has not previously been evaluated.

The current study assessed the ability of CBD treatment to rescue behavioural deficits of male \textit{Fmr1} KO mice. The open field test, elevated plus maze, passive avoidance test,
continuous Y maze, and the social preference test were performed to index locomotion, anxiety-related behaviour, social behaviours and working memory respectively, and to evaluate the potentially therapeutic-like effects of CBD on Fmr1 KO-related deficits. Plasma and brain concentrations of CBD were also analysed to check for potential differences in CBD pharmacokinetics across genotypes.

2. Materials and methods

2.1 Animals

Fmr1 knockout mice \(^{16}\) were sourced from the Jackson Laboratory [Bar Harbor, Maine, USA; strain name B6.129P2-Fmr1\(^{tm1Cgr}\)/J, Stock No. 003025)]. Male Fmr1 knockout mice (Fmr1 KO: \(n = 36\)) and C57BL/6J controls (WT: \(n = 36\)) were sent from The Jackson Laboratory to the Animal BioResources (Moss Vale, Australia) post-weaning and group-housed in independently ventilated cages (Airlaw, Smithfield, Australia) for around two weeks of habituation. At 10 weeks of age (±1 week), test and control mice were transported to Neuroscience Research Australia (NeuRA), where they were group-housed in Polysulfone cages (1144B: Techniplast, Rydalmere, Australia) equipped with nesting material. Mice were kept under a 12:12 h light:dark schedule [light phase: white light (illumination: 124 lx) – dark phase: red light (illumination: < 2 lx)]. Food and water were provided \textit{ad libitum}. Mice were habituated to the NeuRA facilities for three weeks before behavioural testing commenced. Adult, male A/JArc mice from Animal Resources Centre (Canning Vale, Australia) were used as standard opponent for the social preference test (see more information below). Research and animal care procedures were approved by the University of New South Wales Animal Care and Ethics Committee in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.
2.2 Acute Cannabidiol (CBD) Treatment

*Drug preparation and administration:* Powdered cannabidiol (National Measurement Institute, NSW, Australia) was dissolved in equal amounts of Tween 80 (Sigma-Aldrich Co., St Louis, USA) and 100% ethanol and diluted with 0.9% sodium chloride to the appropriate concentration to a final ratio of 1:1:18 as published previously.\(^5\) Ethanol and Tween 80 comprised 10% of the total volume. A vehicle control treatment was set up similarly without the addition of CBD. *Fmr1* KO and WT mice (*n* = 12 per CBD dose) were administered either vehicle, 5 mg CBD /kg body weight, or 20 mg CBD /kg body weight, one dose before each behavioural test (see below). These doses were chosen based on previous studies which identified benefits of acute or chronic CBD treatment at 5 and 20mg/kg.\(^5\) Mice received intraperitoneal (i.p.) injection (injection volume of 10 ml/kg body weight) 30 min prior to the start of behavioural testing, with an inter-test interval of at least three days between tests. For the passive avoidance task CBD was administered 30 min before the start of the training session.

2.3 Behavioural Phenotyping

Starting at 5 months of age (±1 week; after 3 weeks of habituation to the facilities), mice were tested in a battery of behavioural tests. All tests were conducted during the first 5 h of the light phase to minimise effects of the circadian rhythm on the performance of test mice. The test order was as follows: open field, elevated plus maze, spontaneous alternation in the Y maze, social preference, and passive avoidance tasks. Equipment and apparatus were cleaned between trials using 70% ethanol except where specified (i.e. open field testing).

2.3.1 Open field (OF): The OF mimics the natural conflict in mice between the tendency to
explore a novel environment and to avoid an exposed open area \(^{57, 58}\). Mice were placed into an infrared photobeam controlled open field activity test chamber (MED Associates Inc., USA, Vermont) for 30 min. The arena (43.2 cm x 43.2 cm) was divided into a central and a peripheral zone (MED Associates Inc. software coordinates for central zone: 3/3, 3/13, 13/3, 13/13). The animal’s horizontal activity (i.e. distance travelled), vertical activity (i.e. rearing), small motor movements (i.e. movements below the ambulation threshold, e.g. grooming), and resting behaviour (no infrared photobeam-detectable movements), were recorded automatically for the different zones (software settings for ambulation threshold: box size: 3; ambulatory trigger: 2; resting delay: 1000 ms; resolution: 100 ms). The ratio of central to total distance travelled and time spent in the central zone were taken as measures of anxiety \(^{59}\). Equipment was cleaned between experiments using detergent.

2.3.2 Elevated plus maze (EPM): The EPM assesses the natural conflict between the tendency of mice to explore a novel environment and avoidance of a brightly lit, elevated and open area \(^{60}\). The grey plus maze was “+” shaped [for details of apparatus see \(^{61}\). Mice were placed at the centre of the + (faced towards an enclosed arm) and were allowed to explore the maze for 5 min. The percentage time spent in the open arms and total distance travelled in the open arms were recorded as anxiety measures using Any-Maze\(^\text{TM}\) (Stoelting, Wood Dale, USA) tracking software.

2.3.3 Continuous spontaneous alternation in the Y-maze (SA): The Y-maze SA test measures the willingness of mice to explore novel environments. Rodents typically prefer to investigate a new arm of a maze rather than returning to one that was previously visited \(^{62}\). The Y-maze used in our laboratory consisted of three grey acrylic arms (10 cm x 30 cm x 17 cm) placed at 120° with respect to each other. Around the arms were distal cues. Animals were placed into
the centre of the Y-maze and allowed to freely explore the environment for 10 min. Order of entries into the three different arms (A, B, or C) was recorded and successful arm entry triplets (i.e. ABC, ACB, BCA, BAC, CAB, CBA) calculated (maximal number of correct triplets = total number of arm entries – 2)\(^2\). Percentage of correct arm entries was calculated. AnyMaze\(^\text{TM}\) software was used to track the animal’s movement in the maze: an arm entry was scored whenever an animal entered the 2\(^\text{nd}\) half of the arm with more than 60\% of its body length.

2.3.4 Passive avoidance: In this basic hippocampus-dependent learning test, the avoidance of a naturally less aversive dark compartment after it is paired with an electrical foot shock indicates the retention of this memory\(^63\). In the training session, CBD was administered then 30 min later mice were placed in an illuminated compartment (illumination: 25 lx; 25 x 15 x 25 cm; model H10-11R-TC, Coulbourn Instruments, USA). After 10 s, the door to a dark chamber was opened and the latency to enter was measured manually. Once the mouse had entered the dark chamber (illumination: 1 lx; 25 x 12 x 25 cm), the door was closed and a single foot shock (0.4 mA for 2 s) was delivered. Mice were kept in the dark chamber for another 60 s to facilitate the formation of an association between the dark chamber and the foot shock. In the retention session 24 h later, mice were placed in the light compartment and the latency to enter the dark chamber was measured manually (cut-off time: 300 s) as published previously from our laboratory\(^64\). Latency was compared between training and test sessions- increased entry latency on the second day indicates memory of the aversive stimulus.

2.3.5 Social preference test (SPT): The SPT was used to assess sociability and social recognition memory\(^65\) and performed as described in our earlier studies\(^66,67\). Test animals
were isolated for an hour prior to the start of testing. During the habituation trial, mice were allowed to explore a three-chamber apparatus, consisting of a centre chamber (9 x 18 cm; height: 20 cm) and two outer chambers (16 × 18 cm; height: 20 cm), freely for 5 min. For the following sociability test an unfamiliar (male A/J) mouse was placed in a small enclosure in one of the outer chambers, which allowed nose contact between A/J and test mice. The test mouse was returned to the apparatus and allowed to explore all three chambers and the animal enclosures for 10 min. Following the sociability test, test mice were observed in the social recognition test. For this, a second, unfamiliar A/J standard mouse was placed in the previously empty chamber so that the test mouse had the choice to explore either the familiar mouse (from the previous trial) or the novel, unfamiliar mouse in the following 10 min. The inter-trial interval (ITI) was 5 min. The chambers and enclosures were cleaned with 70% ethanol in-between trials and fresh corn cob bedding was added to the chambers prior to each test trial. AnyMaze™ software was used to determine the time spent in the different chambers, number of entries and distance travelled by the test mice in each trial. Primary measures of interest were the time spent with a mouse (i.e. in the sociability trial) or a novel mouse (i.e. in the social recognition trial) as a percentage of total time in both chambers.

2.4 Analysis of CBD Concentrations in Plasma and Brain

At least one week after the completion of the behavioural testing (see 2.4), mice were treated one more time with vehicle or CBD (5 mg or 20 mg i.p.) and blood and brain tissue were collected. One half of the cohort was sacrificed 30 min post CBD administration (N=6 per genotype and dose) and the other half, 90 min post CBD administration. Analysis and quantification of plasma and brain concentrations of CBD were conducted by XenoBiotic Laboratories, Inc. (New York, USA) using liquid chromatography tandem mass spectrometry (LC-MS/MS) with positive electrospray ionization – multiple reaction monitoring mode to
quantify CBD. For plasma sample preparation, 50 μl of plasma sample was mixed with internal standard working solution and water, then loaded to a preconditioned solid phase extraction plate. After washing with water and a mixture of water and methanol, CBD and internal standard were eluted with acetonitrile and reconstituted. For brain sample preparation, each sample was individually weighed and the volume of control mouse plasma was adjusted for each sample to achieve 1:4 ratio of brain:plasma (i.e., 200 mg of brain tissue mixed with 800 μl of plasma). This mixture was then homogenized. Next, 50 μl of the processed mouse brain sample was used for the extraction procedure and extracted the same as mouse plasma samples.

2.5 Statistical Analysis

Two-way analysis of variance (ANOVA) was performed to investigate main effects of ‘genotype’ and ‘CBD dose’ and possible interactions. Repeated measures (RM) three-way ANOVAs were used to investigate total distance travelled across time, i.e. ‘5-min block’ (OF), ‘latency’ (training session vs test session; PA), and ‘chamber’ (SPT) and ‘time’ (CBD concentrations) as published previously. In line with Rothman and Perneger, the data were not adjusted for multiple comparisons and were interpreted as such in the discussion. Paired t-tests were performed to investigate the preference of mice in the SPT test against chance levels (i.e. 50%). Finally, for analysis of CBD levels, missing values (i.e. samples below the limit of detection of 0.5ng/ml) were assigned a value of 0.49. Three-way ANOVAs were then employed to determine relationships between ‘genotype’, ‘CBD dose’ and ‘time’ (between CBD administration and sample collection) and Pearson’s correlations to determine the linear relation between plasma and brain concentrations of CBD. Differences were regarded as significant if $p < .05$. F-values and degrees of freedom are presented for ANOVAs. Data are shown as means ± standard error of means (SEM). Analyses were
conducted using SPSS 20.0 for Windows.

3. Results

3.1 Locomotion and exploration

*Fmr1* KO mice exhibited a hyperlocomotive and hyperexplorative phenotype in the 30 min OF test (Fig. 1A-B). *Fmr1* KO mice travelled further throughout the test [main effect of ‘genotype’ on total distance travelled: F(1,66) = 14.9, p < .0001] (Fig. 1A) and exhibited increased vertical activity [F(1,66) = 28.1, p < .0001] (Table 2) compared to WT mice. These parameters were not influenced by CBD treatment (‘genotype’ x ‘CBD’ interactions: p’s > .05). Mice of both genotypes habituated to the novel OF environment [main effect of ‘5 min block’ on distance travelled: F(5, 330) = 240.1, p < .0001] (Fig. 1B). However, knockout mice displayed a slower locomoter habituation to the novel environment than control mice, as evidenced by interaction of ‘genotype’ and ‘5-min block’ [F(5,330) = 3.1, p = .009; Fig. 1B]. This was not affected by CBD treatment (no ‘CBD’ x ‘genotype’ x ‘5-min block’ interaction; p > .05).

Figure 1A-B: Overall locomotion and habituation of locomotive response to novelty in the open field (OF): A) Total distance travelled [cm] and B) distance travelled [cm] across
5-min blocks. Data for control (WT) and Fmr1 knockout mice (Fmr1 KO) after acute treatment with vehicle (VEH), 5 mg/kg bodyweight of CBD (CBD5) or 20 mg/kg of CBD (CBD20) are shown as means + SEM. There was a significant main effect of ‘genotype’ for total distance travelled ($p = .0003$; Fig. 1A) and a significant ‘5 min block’ x ‘genotype’ interaction ($p = .009$; Fig. 1B). *** $p<0.0005$

### 3.2 Anxiety

The time spent in the central zone of the OF was greater in Fmr1 KO mice than control mice [$F(1,66) = 58.3, p < .0001$] (Fig. 2A). Similarly, the percentage distance travelled in the central zone of the OF was increased in Fmr1 KO mice [$F(1,66) = 30.7, p < .0001$] (Fig. 2B). Acute CBD treatment had no impact on anxiety-related parameters of the OF test (no ‘CBD’ main effects and no ‘genotype’ by ‘CBD’ interactions, all $p$'s > .05).

In the EPM, Fmr1 KO mice spent more time in the open arms [$F(1,63) = 42.3, p < .0001$] (Fig. 2C) and also travelled further in the open arms compared to control mice [i.e. as a percentage of total distance: $F(1,63) = 23.9, p < .0001$] (Fig. 2D). CBD increased time in the open arms regardless of genotype [main effect: $F(2,63) = 4.3, p < .05$, no ‘genotype’ by ‘CBD’ interaction, $p > .05$] (Fig. 2C). Collapsed across genotype, animals treated with 20mg/kg CBD spent longer in the open arm than those treated with vehicle or 5mg/kg ($p<0.005$ and $p<0.05$ respectively, Fig 2C). There was also a strong trend for CBD treatment to increase the percentage of total distance travelled on open arms in all mice [$F(2,63) = 2.9, p = .06$, no ‘genotype’ by ‘CBD’ interaction, $p > .05$] (Fig. 2D). Collapsed across genotype, animals treated with 20mg/kg CBD performed a greater percentage of their locomotion in the open arm than those treated with vehicle or 5mg/kg (both $p<0.05$, Fig 2D).
<table>
<thead>
<tr>
<th></th>
<th>OF Vertical activity [n]</th>
<th>OF Small motor movements [n]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT VEH</strong></td>
<td>283.7 ± 16.5</td>
<td>2283.5 ± 52.8</td>
</tr>
<tr>
<td><strong>Fmr1 VEH</strong></td>
<td>364.8 ± 29.5</td>
<td>2295.9 ± 34.5</td>
</tr>
<tr>
<td><strong>WT CBD5</strong></td>
<td>226.8 ± 24.0</td>
<td>2168.4 ± 57.4</td>
</tr>
<tr>
<td><strong>Fmr1 CBD5</strong></td>
<td>380.4 ± 27.9</td>
<td>2320.9 ± 45.7</td>
</tr>
<tr>
<td><strong>WT CBD20</strong></td>
<td>249.0 ± 21.3</td>
<td>2277.5 ± 62.9</td>
</tr>
<tr>
<td><strong>Fmr1 CBD20</strong></td>
<td>326.5 ± 22.8</td>
<td>2226.0 ± 31.5</td>
</tr>
</tbody>
</table>

**Table 1:** Open field (OF) behaviours of wild type-like control (WT) and *Fmr1* knockout mice (*Fmr1*) after acute treatment with vehicle (VEH), 5 mg/kg bodyweight of CBD (CBD5) or 20 mg/kg of CBD (CBD20). N = 12 for each dose and genotype. Data are shown as means ± SEM. There was a main effect of ‘genotype’ on vertical activity (rearing; *p* < .0001).
Figure 2A-D: Anxiety-related behaviours in the open field test (OF) and the elevated plus maze (EPM): A) time spent in the central zone of the OF [s], B) ratio of total distance travelled in the central zone of the OF, C) time spent on open arms [s], and D) percentage distance travelled on open arms [%]. Data for control (WT) and Fmr1 knockout mice (Fmr1 KO) after acute treatment with vehicle (VEH), 5 mg/kg bodyweight of CBD (CBD5) or 20 mg/kg of CBD (CBD20) are shown as means + SEM. There were significant main effects of ‘genotype’ for OF centre time and centre distance ratio (both $p < .0001$). In the EPM, time spent as well as distance ratio on open arms was influenced by both ‘genotype’ (both $p < .0001$) and ‘CBD’ ($p < .05$ and $p = .06$, respectively). *** $p<0.0005$

3.3 Spatial memory

There was no difference between Fmr1 KO and WT mice in spontaneous alternation (% correct entries) in the Y maze [(F(1,66) = 2.7, $p > .05$], nor an effect of CBD [(F(2,66) = .1, $p$]
Paired t-tests showed that only control mice treated with 20 mg CBD displayed levels of spontaneous alternation significantly above the chance level of 50% (Table 2). In the passive avoidance task, there were no baseline differences in the latency to enter the dark compartment during training between genotypes [F(1,66) = 0.8, p > .05] nor between CBD treatment groups [F(2,66) = 1.6, p > .05]. Latency to enter the dark compartment increased between training and testing in all mice equally [three-way RM ANOVA for ‘latency’: F(1,66) = 18.5, p < .0001; no interaction of ‘latency’ with ‘genotype’ or ‘CBD’ was detected; both p > .05], suggesting that all mice had learned equally the association between the foot shock and the dark compartment and CBD treatment did not alter this association (Fig. 3).

Figure 3: Fear-associated memory in the passive avoidance test (PA): Latency [s] to enter a dark compartment on training day and again, 24 h later on test day. Data for control (WT) and Fmr1 knockout mice (Fmr1 KO) after acute treatment with vehicle (VEH), 5 mg/kg bodyweight of CBD (CBD5) or 20 mg/kg of CBD (CBD20) are shown as means + SEM. *** p<0.0005

3.4 Social Behaviours

Mice across all experimental groups demonstrated a significant preference for exploring a
mouse over an empty chamber [three-way RM ANOVA for ‘chamber’: F(1,66) = 13.8, \( p = .0004 \)]. There was no difference in sociability (i.e. preference for mouse chamber) between \textit{Fmr1} KO and WT mice (‘chamber’ x ‘genotype’ interaction: \( p > .05 \)) and CBD did not influence sociability (‘chamber’ x ‘CBD’ interaction: \( p > .05 \)). Paired t-test against chance level mouse chamber exploration showed that all groups except control mice treated with CBD developed a trend or significant preference for the chamber containing the mouse (Table 2).

There was also a significant effect of ‘chamber’ across experimental groups for the social recognition session (i.e. exploring novel and familiar mice) [‘chamber’: F(1,66) = 6.2, \( p < .05 \)]. This phenomenon was not affected by genotype (‘chamber’ x ‘genotype’ interaction \( p > .05 \)) nor CBD treatment (‘chamber’ x ‘CBD’ interaction \( p > .05 \)). However, detailed paired t-test analyses revealed that only \textit{Fmr1} KO mice treated with 5 mg of CBD showed a strong trend for an aversion of the novel mouse (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>YM Correct entries [%]</th>
<th>SPT Time in mouse chamber [%]</th>
<th>SPT Time with novel mouse [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT VEH</td>
<td>55.7 ± 3.5</td>
<td>57.4 ± 3.5 ( p = .06 )</td>
<td>44.8 ± 4.3</td>
</tr>
<tr>
<td>\textit{Fmr1} VEH</td>
<td>55.5 ± 2.6 ( p = .07 )</td>
<td>56.7 ± 3.4 ( p = .07 )</td>
<td>49.8 ± 2.7</td>
</tr>
<tr>
<td>WT CBD5</td>
<td>54.8 ± 3.2</td>
<td>52.7 ± 3.7</td>
<td>45.3 ± 6.2</td>
</tr>
<tr>
<td>\textit{Fmr1} CBD5</td>
<td>53.7 ± 2.2 ( p = .004 )</td>
<td>58.1 ± 2.2 ( p = .05 )</td>
<td>40.2 ± 4.4</td>
</tr>
<tr>
<td>WT CBD20</td>
<td>57.0 ± 2.2 ( p = .01 )</td>
<td>49.4 ± 5.2</td>
<td>45.4 ± 5.2</td>
</tr>
<tr>
<td>\textit{Fmr1} CBD20</td>
<td>51.8 ± 3.4 ( p = .08 )</td>
<td>53.6 ± 1.9</td>
<td>47.0 ± 3.2</td>
</tr>
</tbody>
</table>
Table 2: Y maze (YM), and social preference test (SPT) behaviours of wild type-like control (WT) and Fmr1 knockout mice (Fmr1 KO). Data are shown as means ± SEM for WT and Fmr1 KO mice after acute treatment with vehicle (VEH), 5 mg/kg bodyweight of CBD (CBD5) or 20 mg/kg of CBD (CBD20). N = 12 per genotype and dose. For Y maze and SPT testing, paired sample t-test results against chance levels (i.e. 50%) are shown.

3.5 CBD concentrations

In both Fmr1 KO and WT mice, CBD concentrations in the brain and plasma were highly correlated (Fmr1 KO: r = .985, p < .001; WT: r = .980, p < .001). In brain, CBD levels did not differ between genotypes [F(1,60) = 1.9, p > .05], regardless of CBD dose (vehicle, 5mg or 20mg) or time (collection of tissue 30 min or 90 min post CBD administration) (interactions- all p’s > .05, Table 3). Across genotypes, CBD levels differed significantly according to CBD dose [F(2,60) = 105.2, p < .001] and in brains collected 30 min post CBD administration compared to those collected 90 min post administration [‘time’: F(2,60) = 17.0, p < .001]. Across genotypes there was also a significant ‘CBD’ x ‘time’ interaction (p < .001), with CBD levels decreasing more acutely between 30 and 90 min for the 20 mg CBD dose than the 5 mg dose (Table 3).

In plasma, CBD levels did differ subtly between genotypes [F(1,60) = 4.7, p = .03]. Interestingly, this effect was driven by differences at 20 mg CBD in brains collected 90 min post administration (three-way ‘genotype’ x ‘CBD’ x ‘time’ interaction, p < .005) where CBD levels were lower in Fmr1 KO mice than WT mice (Table 3). As in brain, CBD levels differed significantly according to CBD dose [F(2,60) = 109.5, p < .001] and time of blood collection [F(2,60) = 18.0, p < .001] for both genotypes, with a significant ‘CBD’ x ‘time’ interaction (p < .001; Table 3). Collapsed across genotypes, a strong correlation between plasma and brain concentrations of CBD was observed for both 5 mg CBD (r = .870, p <
.001) and 20 mg CBD ($r = .956, p < .001$).

<table>
<thead>
<tr>
<th></th>
<th>Brain (30 min)</th>
<th>Brain (90 min)</th>
<th>Plasma (30 min)</th>
<th>Plasma (90 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmr1 VEH</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>WT CBD5</td>
<td>229.3 ± 56.3</td>
<td>95.7 ± 24.6</td>
<td>123.3 ± 22.9</td>
<td>75.5 ± 18.2</td>
</tr>
<tr>
<td>Fmr1 CBD5</td>
<td>201.8 ± 47.5</td>
<td>198.2 ± 41.6</td>
<td>114.4 ± 26.3</td>
<td>100.7 ± 16.5</td>
</tr>
<tr>
<td>WT CBD20</td>
<td>1580.0 ± 79.4</td>
<td>820.8 ± 122.3</td>
<td>1000.3 ± 64.6</td>
<td>550.5 ± 101.6</td>
</tr>
<tr>
<td>Fmr1 CBD20</td>
<td>1209.0 ± 305.2</td>
<td>588.2 ± 147.2</td>
<td>737.4 ± 179.7</td>
<td>301.9 ± 68.3</td>
</tr>
</tbody>
</table>

**Table 3:** Concentration of CBD [ng/ml] in blood plasma and brain of WT and Fmr1 KO mice, 30 min and 90 min after an acute injection with vehicle, 5 mg of CBD or 20 mg of CBD. N = 6 per timepoint, for each dose and genotype. Data are shown as means ± SEM. WT = wild type-like, KO = knockout, CBD = cannabidiol, n.d. = not detectable.

### 4. Discussion

Here we present the effects of acute CBD treatment on an established genetic mouse model for FXS. The Fmr1 KO mice in our study displayed a phenotype which was broadly consistent with the literature. Compared to WT mice, Fmr1 KO mice were hyperactive, hyperexplorative and displayed fewer anxiety-like behaviours. This is consistent with the majority of studies using these tests $^{20-22, 26, 30}$. As in this study, other studies have failed to observe social behaviour deficits $^{30, 70}$ or spatial memory deficits $^{28, 29}$. However it is important to note that the Fmr1 KO behavioural phenotype is variable. There have been differing findings from those reported here for tasks which assess locomotor activity $^{26, 71}$.
anxiety, social behaviour and spatial memory. This includes work by a subset of the current authors in a different facility.

In this context, acute CBD had no impact on locomotion or anxiety-related parameters of the OF. However, in the EPM test, 20mg/kg CBD (but not 5mg/kg CBD) decreased the anxiety response of all mice tested. CBD treatment did not affect cognitive performance of animals in the spontaneous alternation task and the passive avoidance task.

To assess pharmacokinetics we administered CBD (5 or 20mg/kg) to Fmr1 KO and WT mice, then sacrificed them 30 or 90 min post-administration and measured CBD in brain and plasma of each animal. Although CBD levels in brain were equivalent in both genotypes at both timepoints, and in plasma were equivalent at 30 min post-administration, CBD levels were lower in plasma in Fmr1 KO mice at 90 min post-administration. This suggests that Fmr1 KO mice may clear CBD more rapidly than WT mice. However, there was no evidence from our behavioural tests that CBD had differential pharmacodynamic effects in Fmr1 KO and WT mice (all genotype x CBD interactions were non-significant).

Male mice only were used for initial characterisation of effects of CBD, because males with FXS typically have greater severity of symptoms and male Fmr1 KO mice have been more exhaustively characterised in the literature. However, use of homozygous female Fmr1 KO mice in future experiments would be beneficial.

Individuals affected by FXS have been found to be hyperactive. Similarly, Fmr1 KO tested in our study exhibited a hyper-locomotive and hyper-explorative phenotype in the OF, consistent with previous studies. This OF phenotype was evident in both the vehicle-treated as well as the CBD-treated cohorts. The lack of locomotor effects of CBD in the current study are consistent with previous data from our lab indicating that neither acute nor chronic CBD treatment (ranging from 1-50 mg/kg bodyweight) induces sedative-like effects in male C57BL/6J mice or reverses the hyperactive phenotype of an established genetic
mouse model for schizophrenia. However, it is important to note the discrepant findings in this research area, as CBD has been shown to be effective in ameliorating the ‘psychotic-like’ stimulating effect of acute amphetamine on locomotion. Furthermore, some human studies have observed that high dose CBD can produce sedative-like effects [e.g. at 600 mg].

Analysis of anxiety-related open field and the elevated plus maze behaviours in Fmr1 deficient mice revealed a pronounced and consistent (i.e. task-independent) decrease in anxiety-like phenotypes in the Fmr1 KO mice. Previous studies using this Fmr1 mouse model revealed varying anxiety phenotypes for Fmr1 KO mice. While some find no genotype effect, others report fewer anxiety-related behaviours in Fmr1 knockout models as seen in our study. These inconsistencies across research studies appear to be independent of the genetic background of the mouse model in question [reviewed in] and could be related to the nature of the test protocol (e.g. nature of apparatus, time and duration of testing, level of illumination), housing conditions or other factors. We found that acute CBD (in particular at the dose of 20 mg/kg) decreased the anxiety-like responses of all mice in the EPM. The task-specific characteristics of the anxiolytic-like effect of CBD treatment have been found in other studies including humans as well as mice [e.g.]. The effect of acute CBD treatment was similar for all mice and the genotype differences were not affected by the treatment, suggesting that FMRP is not required for anxiolytic-like effects of acute CBD.

Pharmacological blockade of the cannabinoid receptor 2 (CB₂) (but not the CB₁ receptor) has been effective in normalizing the anxiety behaviour of Fmr1-deficient mice. However, since CBD has low affinity and/or weak indirect action at CB₁ and CB₂ receptors, it is more likely that CBD decreases anxiety via another mechanism, such as binding to the 5-HT₁A receptor. Given that individuals with FXS experience increased anxiety but we (and others) find decreased anxiety in Fmr1 KO mice, the therapeutic value of CBD’s anti-anxiolytic effect in FXS requires clarification in future studies.
Most people with FXS are affected by mild to severe intellectual impairments. We did not identify a deficit of fear-associated memory (i.e. passive avoidance) in Fmr1 KO mice in this study - all mice displayed increased latencies to enter the dark chamber after receiving a foot shock. We also did not observe a difference between Fmr1 KO mice and controls in spatial working memory (i.e. spontaneous alternation). This result should be interpreted with caution since only WT mice treated with 20mg/kg CBD showed levels of alternation significantly above chance (50%). Similar to the anxiety phenotype, the literature provides an inconsistent picture of the cognitive phenotype of Fmr1 KO mice although knockout mice can show impaired spatial memory when tested in the passive avoidance and Y maze paradigms. In our study, CBD had no impact on spatial working memory (spontaneous alternation) and fear-associated spatial memory (passive avoidance). Other studies in our laboratory have found beneficial effects of CBD in cognitive domains, as deficits in recognition memory of a transgenic mouse model for Alzheimer’s disease were rescued and prevented after chronic CBD treatment. In line with the latter finding, two studies explored the effects of modulating the endocannabinoid system on passive avoidance behaviour in Fmr1 KO mice. Blocking the CB1 (but not the CB2) receptor pharmacologically (acutely as well as chronically) using rimonabant ameliorated impairments in recognition memory. In another study, increasing the endocannabinoid tone using propofol or URB-597, both inhibitors of fatty acid amide hydrolase activity (FAAH: catabolic enzyme for endocannabinoids), post training resulted in an improved passive avoidance performance of Fmr1 KO mice without any effect on control animals. However, the experimental protocol of Qin and colleagues (2015) was substantially different from our study, as they used habituation trials, two training days, and a post-training administration regime. Although all mice (regardless of genotype and treatment) developed the preference to explore a mouse over an empty chamber, a significant preference for social novelty was absent in
these mice. This lack of social novelty preference in control mice in the vehicle condition was unexpected, as previous studies suggest the test protocol is valid in other mouse models. FXS patients are often diagnosed with social withdrawal or social phobias, suggesting that Fmr1-deficient animals would demonstrate similar reductions in the social preference test. However, findings regarding the social behaviour of Fmr1-deficient mice are inconsistent across previous studies with some studies reporting intact preference for social novelty or even increased social interaction in free-running social tests whereas others report deficient social preference (reviewed in 18). Furthermore, studies have found that testing mice in the 3-chamber preference test can result in different results dependent on which parameters have been evaluated. CBD did not substantially impact the social behaviours of test mice in this 2-test trial paradigm despite having been found to improve social preference and interaction deficits in mouse models for schizophrenia and Alzheimer’s disease. Interestingly, by post-hoc analysis Fmr1 KO mice displayed significant preference for the familiar mouse when treated with 5mg/kg CBD but not under other conditions. This may suggest that CBD may have benefit for increasing social affiliation and/or decreasing social anxiety in the context of repeated exposures but future research will have to clarify this in more detail.

The current study investigated the effects of acute CBD across multiple behavioural domains in Fmr1 KO mice as a preclinical model for therapeutic discovery. Data indicate that Fmr1-deficient male mice displayed behaviours consistent with increased activity, reduced anxiety, and preserved social and cognitive performance. CBD administration resulted in a further reduction in anxiety-like behaviour in both Fmr1-deficient and WT mice, without concomitant effects on locomotor activity, social or cognitive performance. Data suggest that CBD may have anxiolytic effects, which are not dependent on Fmr1, and thus may be considered for use in individuals with FXS. Future studies could evaluate the chronic effects of CBD on FXS-related mouse phenotypes.
5. References


11. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE et al. FMRP
stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell

Dysregulation of mTOR signaling in fragile X syndrome. J Neurosci 2010; 30(2):
694-702.

149(2): 274-293.

Fragile X Syndrome: From Bench to Bedside and Back. Neurotherapeutics 2015;
12(3): 584-608.

15. Hagerman RJ, Polussa J. Treatment of the psychiatric problems associated with

Fmr1 knockout mice: a model to study fragile X mental retardation. Cell 1994; 78(1):
23-33.

17. Bernardet M, Crusio WE. Fmr1 KO Mice as a Possible Model of Autistic Features.

18. Kazdoba TM, Leach PT, Silverman JL, Crawley JN. Modeling fragile X syndrome in
the Fmr1 knockout mouse. Intractable Rare Dis Res 2014; 3(4): 118-133.

Social Behavior Deficit and Elevated Protein Synthesis in a Mouse Model of Fragile
X Syndrome. The international journal of neuropsychopharmacology / official
scientific journal of the Collegium Internationale Neuropsychopharmacologicum
(CINP) 2015; 18(9).

20. Ding Q, Sethna F, Wang H. Behavioral analysis of male and female Fmr1 knockout
mice on C57BL/6 background. Behav Brain Res 2014; 271: 72-78.

social enrichment rescues adult behavioral and brain abnormalities in a mouse model


42. Campos AC, Ortega Z, Palazuelos J, Fogaca MV, Aguiar DC, Diaz-Alonso J *et al.* The anxiolytic effect of cannabidiol on chronically stressed mice depends on hippocampal neurogenesis: involvement of the endocannabinoid system. *The international journal of neuropsychopharmacology / official scientific journal of the*


52. Long LE, Chesworth R, Huang XF, McGregor IS, Arnold JC, Karl T. A behavioural comparison of acute and chronic Delta9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. The international journal of neuropsychopharmacology / official


6. Acknowledgements and Conflict of Interest Disclosure

This work was funded by Zynerba Pharmaceuticals. TK was supported by a Career Development Fellowship (Level 2) from the National Health and Medical Research Council (NHMRC: #1045643), and currently receives funding from two NHMRC project grants (#1102012 and #1141789), and the NHMRC dementia research team initiative (#1095215). JZ is supported by an A.M. Wood Scholarship from the Schizophrenia Research Institute. DS was supported by a NHMRC CJ Martin Fellowship (#1072878).

SJS is a consultant to, and receives grant support from, Zynerba Pharmaceuticals Inc., Astellas Pharma Inc. and Bohringer Ingleheim. TS, MB-M and DG are full-time employees of Zynerba Pharmaceuticals Inc. All other authors declare no competing financial interests.

We would like to thank Jerry Tanda for critical comments on the manuscript and Adam Bryans for taking care of our test mice at NeuRA.
Highlights

- Acute cannabidiol (CBD) decreased anxiety-related behaviours in both Fmr1 knockout mice and wildtype controls in the elevated plus maze
- Fmr1 KO mice were hyperlocomotive, hyperexplorative, showed fewer anxiety-related behaviours and habituated more slowly to a novel environment than controls
- Acute CBD had no impact on locomotion, spatial working memory or fear-associated memory in Fmr1 knockout mice or controls.