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# Identifying therapeutic targets for the treatment of Fragile X Syndrome: Implications for Autism Spectrum Disorders

William Fyke

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DOCTORAL SCHOOL OF HEALTH AND LIFE SCIENCES AT THE UNIVERSITY OF  
BORDEAUX

DOCTORAL SCHOOL OF GRADUATE STUDIES AT SUNY DOWNSTATE  
NEUROSCIENCES

By William Fyke

**IDENTIFYING THERAPEUTIC TARGETS FOR THE  
TREATMENT OF FRAGILE X SYNDROME:  
IMPLICATIONS FOR AUTISM SPECTRUM DISORDERS**

Under the supervision of Susanna Pietropaolo, Ph.D.  
and Juan Marcos Alarcon Ph.D.

Viva on 17, March 2021

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**Title:** Identifying Therapeutic Targets for the Treatment of Fragile X Syndrome: Implications for Autism Spectrum Disorder

**Abstract:** Fragile X syndrome (FXS) is a neurodevelopmental disorder due to an X-linked mutation in the *FMR1* gene that results in intellectual disability (ID), autism spectrum disorder (ASD), anxiety, attention deficit hyperactivity disorder (ADHD) and sensory processing deficits. There is substantial overlap between FXS and ASD as approximately 30 to 50% of individuals diagnosed with FXS also meet the diagnostic criteria for ASD. Furthermore FXS-ASD patients represent approximately 5% of all cases of ASD. Since there is currently no targeted therapeutic approach, novel pharmacological agents addressing the neurobiological underpinnings of these disorders are crucially needed. Due to the overlap between the two conditions, systems which are disrupted in FXS and ASD patients may provide targets for treating the ASD symptoms observed in FXS-ASD patients and some non-syndromic ASD patients. FMRP, the protein lost by the *FMR1* mutation, is a potent regulator of the endocannabinoid system (ECS) and BKCa channels. These function to regulate presynaptic excitability. Dysfunction in these systems is found in FXS patients and some ASD patients. The presynaptic role of these agents conceptualizes the “presynaptic hypothesis of FXS-ASD”. This project used genetic and pharmacological approaches which target FMRP, the ECS or BK<sub>Ca</sub> channels in combination with an extensive behavioral characterization of FXS and ASD-relevant phenotypes, in order to assess the therapeutic value of these targets. This work demonstrates that the ECS and BKCa channels contribute to behavioral domains affected in neurodevelopmental disorders and offer several targets for therapeutics which should be explored.

**Keywords:** neurodevelopmental disorders, animal models, fragile x syndrome, autism spectrum disorder

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**Titre :** Identification de cibles thérapeutiques pour le traitement du syndrome de l'X fragile : conséquences pour le spectre de l'autisme

**Abstract :** Le Syndrome de l'X fragile (FXS) est un trouble du neurodéveloppement causé par la mutation du chromosome X dans le gène *FMR1*. Les manifestations de cette mutation sont un déficit intellectuel, le trouble du spectre de l'autisme (TSA), trouble d'hyperactivité avec ou sans déficit de l'attention (TDHA), et les défauts du traitement de l'information sensorielle. Il existe un lien entre FXS et TSA. Environ 30 à 50% des individus qui sont diagnostiqués avec FXS aussi remplissent le critère pour TSA. En plus, les patients qui ont FXS-TSA comptent pour environ 5% de toutes les personnes qui ont TSA. Étant donné qu'il n'existe actuellement aucune approche thérapeutique ciblée, de nouveaux agents pharmacologiques visant les bases neurobiologiques de ce trouble sont indispensables. Parce qu'il y a un lien entre ces conditions, les systèmes qui sont perturbés pour les patients qui ont FXS ou TSA pourraient fournir des cibles pour le traitement des symptômes de TSA observés en les patients FXS-TSA et certains patients TSA non-syndromiques. FMRP, la protéine perdue par la mutation du gène *FMR1*, est un régulateur puissant du système endocannabinoïde (ECS) et les canaux de conductance potassique qui sont calciques et voltage dépendants (BKCa). Ces canaux travaillent pour réguler l'excitabilité du neurone présynaptique. Le dysfonctionnement de ces systèmes est observé avec les patients de FXS et quelques patients de TSA. Le rôle présynaptique de ces agents conceptualise « l'hypothèse présynaptique de FXS-TSA ». Ce projet a utilisé des approches génétiques et pharmacologiques qui ciblent FMRP, l'ECS, ou les canaux BKCa en combinaison avec une caractérisation comportementale des phénotypes FXS et TSA pertinents, afin d'évaluer la valeur thérapeutique de ces cibles. Ces travaux démontrent que les canaux ECS et BKCa contribuent aux domaines comportementaux affectés dans les troubles neurodéveloppementaux et offrent plusieurs cibles thérapeutiques qui devraient être explorées.

**Keywords :** trouble du neurodéveloppement, modèles animaux, syndrome de l'X fragile, le trouble du spectre de l'autisme

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## **FOREWORD**

The work presented in this thesis has been carried out for its first part at the INCIA (Bordeaux) between September 2017 and May 2019, and for the remaining part at the SUNY (NY, USA). The experiments described in Chapters 3.1 and Chapter 4 were designed and performed under the supervision of Dr. S. Pietropaolo at the INCIA, while the experiments presented in chapters 3.2 and 3.3 were designed and performed under the supervision of Dr. J.M. Alarcon at SUNY.

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## ACCOMPLISHMENTS

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### **Activities**

COVID Task Force (March 2020 – present), Department of Pathology, SUNY Downstate Medical Center, Brooklyn, NY. Laboratory of Dr. Jenny Libien. Chair of the Department of Pathology.

- Developed and validated SARS-Covid19 RT-PCR for use at SUNY Downstate
- Designed (validation ongoing) study for SARS-Covid19 detection in saliva and novel nasopharyngeal swab

### **Awards and Distinctions**

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## **CHAPTER 1 – INTRODUCTION**

### **1.1 Neurodevelopmental disorders**

Neurodevelopmental disorders are a broad group of neurological and psychiatric conditions wherein the development of the central nervous system is disrupted. These disorders are often recognized early in life, follow a steady progression, and persist into adulthood. The most notable manifestation is the failure to achieve age appropriate developmental milestones in one or more of the following domains: social/emotional, language/communication, cognitive, and motor. Importantly, neurodevelopmental disorders affect approximately 15% of the population and thus place an immense clinical and economic weight on the healthcare system (Boyle et al., 2011).

Clinically, neurodevelopment disorders are diverse. High levels of heterogeneity, in patient presentations, is found in most, if not all, of the disorders. Additionally, the degree of impairment can span from mild to profound which, in many cases, requires patients to have substantial supports for daily functioning. The variability in symptoms and the level of impairment has resulted in many neurodevelopmental disorders being conceptualized as occurring along a continuum or spectrum. Moreover, a notably high degree of symptom overlap occurs and adds more complexity to the clinical picture. A poignant example is the impairments in social communication, as this is a diagnostic criterion for intellectual disability, communication disorders, and also Autism Spectrum Disorder (ASD) (American Psychiatric Association, 2013).

In addition to an overlap in symptoms, a high rate of comorbidity is the rule, rather than the exception (Gilger & Kaplan, 2001). This is apparent with ASD, as approximately up



to 79% of patients have motor delays, 70% depression, 45% intellectual disability (ID), 56% anxiety disorder, 70% gastrointestinal (GI) disturbances, 44% Attention Deficit Hyperactivity Disorder (ADHD), and 30% seizure disorder (M.-C. Lai et al., 2014).

It is not surprising that given the diverse clinical presentations, the degree of symptom overlap, and the high rate of co-morbidity, that the underlying neuropathology is complex. In the case of neurodevelopmental disorders, such as ASD or ADHD, hundreds of genetic variants have significant associations with these disorders (De Rubeis & Buxbaum, 2015; Velinov, 2019). In addition to genetic links, exposures while in utero or during early development, such as toxins (e.g. lead, alcohol), medications (e.g. valproate, thalidomide), or stress, are causal for many neurodevelopmental disorders (Christensen et al., 2013; Nanson et al., 1995; Strömmland et al., 1994). Risk increasing genetic variants and early life exposures occur within unique heterogeneous genetic backgrounds and environmental contexts (Folstein & Rutter, 1977). These factors combine to create an intricate etiological landscape, which presents unique challenges in comparison to other areas of medicine. This is particularly evident in regard to etiology, classification, and the development of therapeutics.

## **1.2 Origins and Categories**

Neurodevelopmental disorders are categorized into discrete entities on the basis of clinical observations or patient/caregiver report, as no universally agreed upon biomarkers for these disorders has been found. The poorly understood biological basis and overlapping symptoms have made classification a difficult process (Lyll et al., 2017; Thapar et al., 2017). Despite the heterogeneity among patients, extensive work

has produced functional definitions and diagnostic criteria that enable researchers, clinicians and patients to conceptualize and communicate about these disorders (American Psychiatric Association, 2013).

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) is considered the guide for diagnosis and researching neuropsychiatric disorders (American Psychiatric Association, 2013). This classification system is based on clusters of frequently co-occurring symptoms and the point in the lifespan where they predominantly manifest themselves. Thus, disorders which occur due to an alteration in developmental processes (e.g. neurodevelopmental) have been grouped together, while acquired (e.g. neurocognitive) disorders or those which typically manifest in adolescence or adulthood (e.g. mood disorders, personality disorders) fall into a separate cohort. Accordingly, the DSM-5 provides criteria for the following neurodevelopmental disorders: Intellectual Development Disorder, Communication Disorders, ASD, ADHD, Specific Learning Disorder, Motor Disorders, and other specified or unspecified neurodevelopmental disorders.

While the DSM-5 is the principal diagnostic guide, the preface emphasizes that its use should heavily rely on experience based clinical reasoning and additional diagnostic tools (American Psychiatric Association, 2013). For example, clinical observations, a detailed history and physical, should be combined with diagnostic tools such as the Wechsler Intelligence Scale for Children (WISC<sup>®</sup>-5) for ID, Connors scales for ADHD or the Autism Diagnostic Observation Schedule (ADOS<sup>™</sup>-2) and Autism Diagnostic

Inventory (ADI<sup>®</sup>-R) for ASD to obtain a detailed clinical picture that can best guide diagnosis and management (Hessl et al., 2009). To emphasize the importance of thorough clinical work as a requirement for diagnosis, specifiers for symptom severity are included for each disorder. This provides a way to communicate the clinical significance of a patient's symptoms within the context of the diagnostic criteria and allows for the inclusion of descriptors, such as genetic conditions (Harris, 2014).

Since a consensus on the pathophysiology and biological markers does not exist, diagnostic criteria are clinical, however putative pathophysiological mechanisms have been elucidated. A general overview of the pathobiology of neurodevelopmental disorders posits that at the molecular level, genetic variants or environmental insults cause altered cellular physiology and result in computational errors at the synaptic level, thus disturbing key neural mechanisms such as plasticity or excitation/inhibition ratio (Ethridge et al., 2016; Ethridge et al., 2017; Kamionowska et al., 1985). This underlies local circuit and global network alterations that ultimately manifest clinically during development (Krol et al., 2018).

A major hurdle in defining the underlying neuropathology is the overlap of biological associations. A true biological definition of these disorders would provide distinct and consistent correlations among genomic variants, pathophysiological processes and clinical phenotypes. Furthermore, this would aid the development of novel therapeutics. However, any of the identified risk genes are typically only found in a subset of patients and often share correlations with multiple disorders (De Rubeis & Buxbaum, 2015;

Gilissen et al., 2014; Iossifov et al., 2014). A similar situation is encountered with pathology at the cellular, circuit and network levels (Sahin & Sur, 2015).

Monogenetic neurodevelopmental disorders, such as Fragile X Syndrome (FXS) and Rett syndrome, can trace the root of their pathology to loss of a single gene (Cassidy & Allanson, 2010). Initially, one would anticipate that discrete genetic causes of neurodevelopmental disorders would provide relatively straightforward answers to questions about the underlying neuropathology. To the contrary, even in case of monogenetic disorders, complex processes govern the downstream effects. FXS and Rett syndrome exemplify this, as the loss of a single gene results in drastic effects on the activity of a multitude of other cellular processes (Faundez et al., 2019; Salcedo-Arellano et al., 2020).

### **1.3 Modeling neurodevelopmental disorders**

Animal models are powerful tools in assessing the relationship between causative or risk factors and neurodevelopmental disorders (Chadman et al., 2009; Crawley, 2012). Of the animal models available, mouse models provide an extremely valuable resource for these investigations due to close genetic and physiological homology to humans (DeBry & Seldin, 1996).

The degree to which a mouse model recapitulates a human disorder is assessed by three types of validity (Chadman et al., 2009). **(1) Construct validity** is the degree to which the etiology of a human disease is captured. A one to one translation with neurodevelopmental disorders is nearly impossible due to the polygenic nature of these

disorders (Pietropaolo, Crusio, & Feldon, 2017). However, monogenetic (syndromic) neurodevelopmental disorders are amenable to the development of models with high construct validity. Construct validity is a necessary foundation for elucidating etiology and drawing strong connections to human pathology. **(2)** *Face validity* describes the degree to which a model recapitulates human phenotypes. These can be assessed by measures of behavioral and biological abnormalities. A model with high face validity would demonstrate core and co-morbid phenotypes across a developmental time frame. This highlights the need for longitudinal studies which investigate developmental pathology and effectively model this across development. **(3)** *Predictive validity* describes the degree of equivalency in treatment response between the mouse model and humans (Crawley, 2012). This has been a hurdle for many neurodevelopmental disorders, such as ASD, however there have been successes. Preclinical models have resulted in the development of numerous medications which treat epilepsy, hyperactivity, anxiety, behavioral disturbances and depression (Crawley, 2012). These have been used extensively and, while they are far from perfect in their efficacy or safety, provide therapeutic benefits to many patients and for some forms of epilepsy are lifesaving.

It is important to recognize that neurodevelopmental disorders are uniquely human and therefore, difficult to model in animals. This highlights the need for a variety of animal models which can fill gaps where other models lack a high degree of validity. For example, the genetic background of the mouse strain can heavily influence phenotypes (Crusio, 1998; Moy et al., 2007; Pietropaolo et al., 2011). While this may appear as a

confounding factor, it has provided the researchers with an added tool, as the choice in genetic background can accentuate a desired phenotype or drug response. This may provide an opportunity to model the variability found in neurodevelopmental disorders and provide insight into the heterogeneity found from patient to patient (Pietropaolo et al., 2011).

Even though monogenetic neurodevelopmental disorders are not fully understood, they have provided insight about common pathophysiology (Bernardet & Crusio, 2006). Of the neurodevelopmental disorders, FXS and ASD provide an avenue to investigate common pathophysiology. The association between these two conditions is significant, and evidenced by the importance of FXS in clinical efforts to identify a distinct etiology for idiopathic ASD patients (Cassidy & Allanson, 2010). When ASD with an unknown etiology is present, testing for the *fragile X mental retardation 1 (FMR1)* gene mutation, the mutation responsible for FXS, is one of the first genetic tests performed.

#### **1.4 Fragile X Syndrome (FXS)**

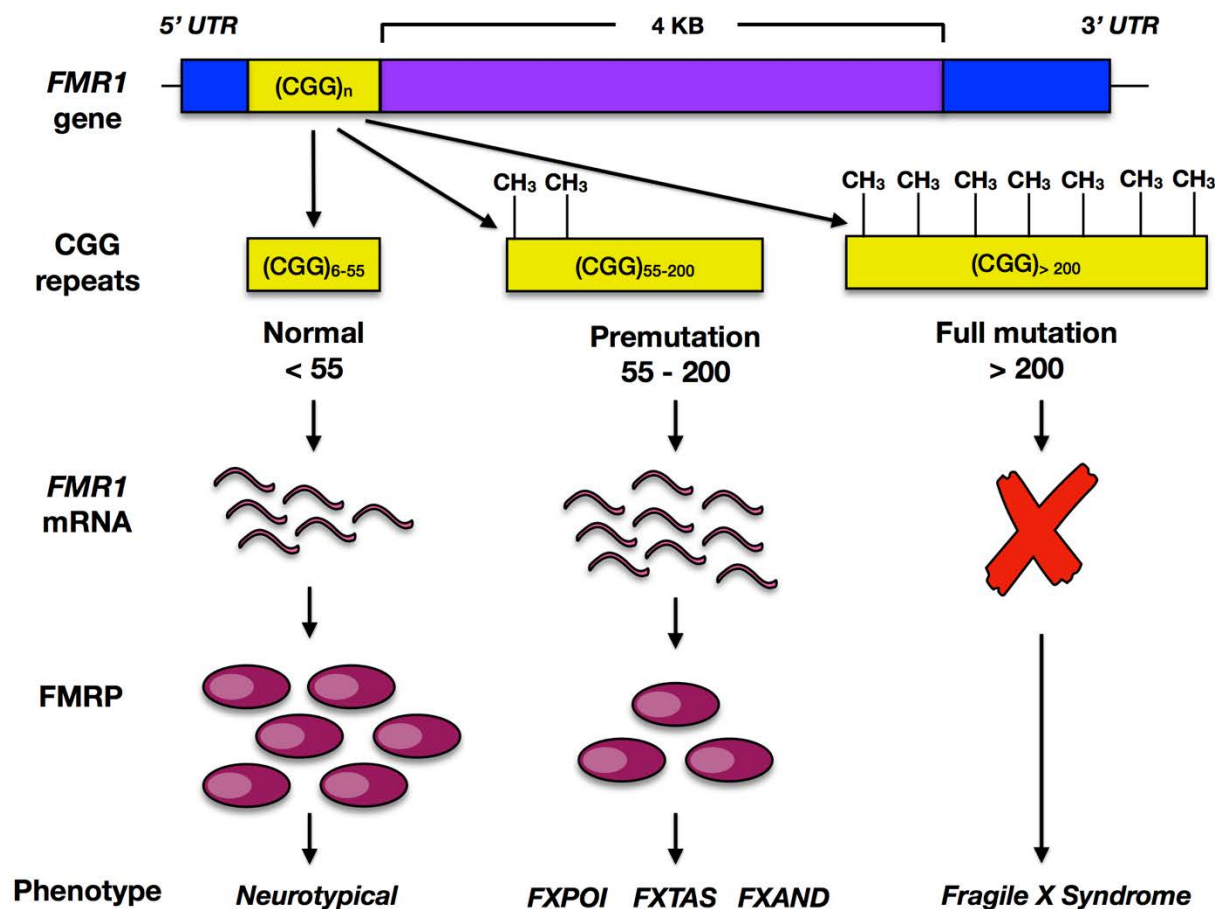
Fragile X syndrome (FXS) is a neurodevelopmental disorder due to an X-linked mutation in the *FMR1* gene. The overall prevalence of FXS is approximately 1 in 2000 to 3000 (Crawford et al., 2002; Murray et al., 1996). In 95% of known cases, the FXS phenotype is due to an expansion of more than 200 repeats and subsequent methylation of CGG triplets in the 5' untranslated promoter region of the *FMR1* gene (Cassidy & Allanson, 2010). The remaining 5% of FXS cases, in which triplet repeat expansions are not found, are often due to point mutations or deletions in the *FMR1* gene which, as in the other 95% of cases, result in absent or markedly decreased

production of the fragile X mental retardation protein (FMRP). FMRP regulates the translation of approximately 4% of fetal brain mRNA and directly regulates several classes of ion channels (Brown et al., 2010; Brown et al., 2001; Deng et al., 2013; Ferron et al., 2014). Clinically, FXS patients present with distinct physical and behavioral features. Physically, individuals have characteristic facial abnormalities (e.g. elongated face, large ears), macroorchidism, hyperlaxity of joints and hypotonia. Behaviorally, FXS patients often have intellectual disability (ID), autism spectrum disorder (ASD), anxiety, attention deficit hyperactivity disorder (ADHD) and sensory processing deficits (Cassidy & Allanson, 2010).

#### **1.4.1 Fragile X Syndrome – Genetics**

Studies from the 1980s identified nonpenetrant male carriers in families with FXS, indicating a unique pattern of inheritance for the syndrome (Sherman et al., 1985). This peculiar pattern of inheritance remained unresolved until the *FMR1* gene was identified in 1991 by positional cloning (Annemieke JMH Verkerk et al., 1991). The 5'-untranslated promoter region for the *FMR1* gene typically contains less than fifty-five CGG trinucleotide repeats and is located at Xq27.3 (Fig. 1). Individuals with the full *FMR1* mutation typically have more than 200 repeats which results in heavy methylation and subsequent gene silencing. Intermediate repeat lengths of 55-200 are defined as premutation (Cassidy & Allanson, 2010). The presence or absence of FXS phenotypes from generation to generation is caused by a mechanism called anticipation, wherein an expansion of the premutation occurs during meiosis and produces the full mutation. Mothers with a premutation of greater than 90 to 100 repeats, have an approximately

50% risk of passing on the full mutation to their children (Nolin et al., 2003). Individuals with the premutation do not express the severe phenotypes seen with the full mutation, however several disorders are associated with premutation carriers, namely, fragile X-associated tremor and ataxia syndrome (FXTAS), fragile X-associated primary ovarian insufficiency (FXPOI), and fragile X-associated neuropsychiatric disorder (FXAND) (Salcedo-Arellano et al., 2020).



**Figure 1. *FMR1* gene and fragile X pathology.** CGG repeats (yellow) in promoter region. < 55 repeats are typical. Repeat expansion resulting in the premutation (55-200) is found in 1/130-250 females and 1/260-800 males. The premutation expansion increases mRNA transcription and is associated with fragile x primary ovarian insufficiency (FXPOI), fragile X-associated tremor and ataxia syndrome (FXTAS), and fragile X associated neuropsychiatric disorder (FXAND). Repeats greater than 200 result in methylation of the promoter region and gene silencing.



Due to the X linked nature of FXS, a more variable and less severe phenotype is often found in female carriers of the full mutation. This phenotypic variation among females with FXS is due to presence of a functional *FMR1* allele on a second X chromosome. (Tassone et al., 1999). This provides some protection, however the degree of protection and thus the severity of the phenotype, is dependent on the X inactivation ratio. A linear relationship between higher levels of FMRP and outcomes on measures of fluid intelligence and behavioral skills in FXS females has been detected (Tassone et al., 1999). Furthermore, less than half of females diagnosed with FXS meet the criteria for ID, which stands in contrast to FXS males, who nearly all meet the diagnostic criteria for ID (Baker et al., 2019).

Historically, FXS research has played an important role in understanding neurogenetic disorders, as it was the first of the trinucleotide repeat disorders identified (Fu et al., 1991). Additionally, the monogenetic nature of FXS provides an opportunity for hypothesis testing of co-morbid FXS pathologies. For example, FXS is recognized as the most common monogenetic cause of ID and ASD. (Schaefer & Mendelsohn, 2008). Approximately 30 to 50% of individuals diagnosed with FXS meet the criteria for a diagnosis of ASD with FXS-ASD patients composing approximately 3% of all cases of ASD (Mendelsohn & Schaefer, 2008; Schaefer & Mendelsohn, 2008). Due to this notable overlap, studies of the *FMR1* mutation may reveal shared pathophysiological mechanisms with non-syndromic forms of neurodevelopmental disorders. Indeed, studies of specific mutations in the *FMR1* gene have been identified and linked with

clinical pathology. For example, Myrick et al. (2015) identified a patient that did not have the pathognomic repeat expansion but had a missense mutation (R138Q) in the *FMR1* gene was detected. This resulted in loss of function in FMRP to regulate large conductance voltage and calcium sensitive potassium (BKCa) channels. The patient who carried this mutation only exhibited a partial FXS phenotype, primarily ID, epilepsy and a history of developmental motor and speech delays. This mutation was also found in his mother, who also had a history significant for developmental delays. His maternal grandfather (already deceased at the time of the study) had a history of developmental delays, however he could not be tested for the mutation.

#### **1.4.2 Modeling Fragile X Syndrome**

The monogenetic nature of FXS make it a pathology which is amenable to modeling. In fact, a number of models have been developed (e.g. drosophila, cell cultures, rats, mice). Of the existing models, the majority of studies have modeled FXS with the original *Fmr1*-KO (*Fmr1<sup>tm1Cgr</sup>*) mouse, wherein FMRP expression was inactivated by mutation of exon 5 of the *Fmr1* gene (The Dutch-Belgian Fragile X Consortium et al., 1994). In this model, despite the absence of FMRP, the *Fmr1* promoter region remains intact, and thus residual *Fmr1* RNA transcript expression occurs (Tassone et al., 2000). A second-generation mutant, *Fmr1*-KO2, that lacks residual *Fmr1* mRNA transcript expression, has been developed and is used for both brain and behavioral studies (Gaudissard et al., 2017; Mientjes et al., 2006; Zhang et al., 2014).

The *Fmr1*-KO mouse approximates many clinical FXS phenotypes, however these are often nuanced and lack exact symmetry to their clinical correlates (Bernardet & Crusio, 2006). For example, the *Fmr1*-KO mouse demonstrates cognitive impairments only on some measures of cognitive ability (Bernardet & Crusio, 2006; Spencer et al., 2011). This is concerning as intellectual disability (ID) is one of the most consistent symptoms found in FXS (> 85%) (Hagerman, 2002). Modeling anxiety-like behavior has also presented challenges. *Fmr1*-KO mice demonstrate an inverse anxiety phenotype on behavioral assays that is inconsistent between laboratories and is modulated by genetic background (Hebert et al., 2014; Mineur et al., 2002; Nielsen et al., 2002). Altered prepulse inhibition responses are found in the *Fmr1*-KO mouse, however these phenotypes also demonstrate an inverse phenotype to those seen in patients (Frankland et al., 2004; Pietropaolo et al., 2011).

The presence of ASD symptoms in the majority of FXS patients make this an area of focus in *Fmr1*-KO mouse studies. Social behavior and communication deficits have been detected (Belagodu et al., 2016; J. K. Lai et al., 2014; Nolan et al., 2020; Pietropaolo et al., 2011). Notably, both social behavior and communication phenotypes have been rescued by potential therapeutics (Hebert et al., 2014; Rotschafer et al., 2012; Toledo et al., 2019). Increased repetitive behaviors (e.g. self-grooming, marble burying) are found in the *Fmr1*-KO mouse and, emerging data showed a positive response to potential therapeutics (Carreno-Munoz et al., 2018).

The *Fmr1*-KO mouse presents with neurological phenotypes that appear to approximate those seen clinically and, as with behavioral phenotypes, demonstrate asymmetries with respect to human studies. Seizures show an age dependent phenotype (> 10 weeks) sensitivity to audiogenic seizure inducing stimuli which differs from those seen in FXS patients, seizures are typically not audiogenic in origin and are much milder in severity (i.e. decrease in seizures with age) (Cassidy & Allanson, 2010; Chen & Toth, 2001). Despite these phenotype differences, cortical hyperexcitability, which likely plays a role in some forms of epilepsy, is found in FXS patients and the *Fmr1*-KO mouse (Bianchi et al., 2009; Ethridge et al., 2016; Ethridge et al., 2017). This model provides some insights about developmental mechanisms which may underlie cortical hyperexcitability, as delays during critical periods for synaptic plasticity and the GABA excitatory to inhibitory shift are evident during early life (Bureau et al., 2008; Gibson et al., 2008; Harlow et al., 2010; He et al., 2014; Kim et al., 2013). Studies of post-mortem brains from FXS patients have revealed morphological dendritic defects which are recapitulated in in the mouse model (Hebert et al., 2014; Kamionowska et al., 1985; Rudelli et al., 1985). Additionally, the *Fmr1*-KO demonstrates hyper-connectivity within and hypo-connectivity between cortical regions (Bureau et al., 2008; Haberl et al., 2015). This bears similarities to neurological phenotypes found in some patients with *FMR1* mutations (Hall et al., 2013; van der Molen et al., 2014; Wang et al., 2012)

Comparing neurobiological phenotypes found in the *Fmr1*-KO model (and all mouse models) to those seen in humans must be done with caution. In addition to the obvious limitation of modeling a strictly human disorder in a non-primate mammal, there are

technological limitations. Current human brain imaging techniques do not provide a resolution as detailed as those which can be acquired with mouse models. Therefore, mouse models provide an opportunity for insight into the underpinnings of neurodevelopmental disorders that are unable to be acquired directly from humans at this time.

There are some challenges that have been encountered in modeling the human pathology with *Fmr1*-KO mouse. These can be summarized in four points: (1) Some, but not all, of the FXS phenotypes are recapitulated, (2) some phenotypes are inconsistent, (3) the presence or absence of FXS phenotypes is dependent on multiple factors (e.g. genetic background, protocol, etc.) and (4) the developmental trajectory has asymmetries with humans (Bernardet & Crusio, 2006; Pietropaolo et al., 2011).

While these aforementioned statements must be acknowledged, patients with FXS also demonstrate a large degree of phenotypic variation that ranges from mild to severe pervasive dysfunction (Cassidy & Allanson, 2010). Therefore, it is reasonable to anticipate a degree of variation in this model. Indeed, phenotypes that vary in response to genetic background and environmental conditions are more reflective of what is seen clinically. Moreover, the *Fmr1*-KO mouse continues to generate insightful data regarding behavior and biology of FXS and other neurodevelopmental disorders, such as ASD.

This knowledge has laid the groundwork for clinical trials, most notably, arbaclofen and mavoglurant (Berry-Kravis et al., 2017; Veenstra-VanderWeele et al., 2017).

## **1.5 Autism Spectrum Disorder**

Autism spectrum disorder (ASD) is characterized by persistent deficits in social communication and social interaction with restricted, repetitive patterns of behavior, interests or activities (American Psychiatric Association, 2013). The most recent estimates place the prevalence of ASD at 1 in 60 children, and in some communities a rate as high as 1 in 34 is reported (Baio, 2018). Diagnosis of ASD is made through clinical and behavioral assessments with diagnostic tools (ADI<sup>®</sup>-R and ADOS<sup>™</sup>-2) as there are no diagnostic biomarkers (Lord et al., 2000; Rutter et al., 2003). In terms of clinical presentation, ASD is heterogenous, however 78% of individuals have severe behavioral impairments, requiring permanent full time care, while only 12% live independent lives (Billstedt et al., 2005). The best predictors for long term prognosis are IQ and early language ability (Magiati et al., 2014).

### **1.5.1 ASD – Genetics**

The co-occurrence of syndromic disorders, such as FXS, and the high level of heritability seen in twin studies were among the first findings to make clear the importance of genes in the etiology of ASD (Blomquist et al., 1985; Folstein & Rutter, 1977). Twin studies have indicated that monozygotic twins have a 60-90% concordance rate for ASD (Hallmayer et al., 2011; Tick et al., 2016). Many copy number variants (CNV), chromosome duplications/deletions, point, de novo, and monogenetic mutations are associated with ASD pathology (Bitar et al., 2019; Sebat et al., 2007; Velinov, 2019). In fact, hundreds of genes have been linked to ASD however many of these genes are involved in only a small percentage of cases (Abrahams & Geschwind, 2008; State & Levitt, 2011). A comprehensive genomic database which indexes and evaluates

genetic ASD associations has been developed by the Simons Foundation Autism Research Initiative (SFARI), (<https://gene.sfari.org/>).

### **1.5.2 Models of ASD**

An ideal animal model of ASD would recapitulate the core criteria, some of the comorbid and biological pathology across a developmental timeline (Bernardet & Crusio, 2006; Chadman et al., 2009; Crawley, 2004, 2012). Since ASD is a disorder that is distinctly human, not occurring naturally in other species, it may be unreasonable to demand that an animal model perfectly replicate the symptomatology. In regard to behavior, a reasonable expectation is that any proposed model would demonstrate relevant phenotypes on at least two assays within a given behavioral domain (Chadman et al., 2009; Crawley, 2004, 2012).

ASD behaviors in mouse models are evaluated with experimental paradigms which approximate the deficits found in patients (Table 1). Impairments in social behavior are assessed by measures of approach, novelty, and direct interactions (Crawley, 2004). Social communication deficits can be detected through measurements of ultrasonic vocalization production (USV) (Crawley, 2004). USV production is particularly of interest since this represents one of the few phenotypes available for examination throughout the life span of the mouse, thus providing a method for modeling the developmental and context specific nature of communication delays seen in ASD (Wöhr & Krach, 2017). Repetitive behaviors are assessed in various paradigms by measurements of spontaneous motor behaviors, such as self-grooming and digging (Crawley, 2012). The

behavioral rigidity and restricted interests that are characteristic of many ASD patients are more difficult to model in mice, however some tests, such as tests of reversal.

<b>ASD Symptom</b>	<b>Examples of tests and behavioral measures</b>
<b>Core symptoms</b> Inappropriate social interactions	Three chamber test sociability (novel mouse vs object) social novelty (familiar vs novel mouse)  Direct social interaction <i>Affiliation behaviors</i> Sniffing Allogrooming Mounting Ano-genital sniffing  <i>Non-social behaviors</i> Self grooming Digging Rearing
Impairments in social communication	Maternal separation induced USVs USVs during direct social interaction
Repetitive, ritualistic, behaviors, resistance to change, and restricted activities	Assessments of: Self grooming Digging
<b>Secondary symptoms</b> Hyperactivity (ADHD) Anxiety Sensory processing hyper/hypo sensitivity Intellectual Disability	Open field Elevated plus maze Acoustic startle Novel Object Recognition

**Table 1. ASD core or secondary symptoms and selected tests of mouse behavior which recapitulate these symptoms.**

ASD models are generated via manipulations which replicate the biological associations found in ASD patients (Table 2). Monogenetic models of ASD such as *Fmr1*-KO, *Shank3*, and chromosome 15q11-13 have elucidated molecular pathways and neurobiological phenotypes that are likely causal for symptoms in some patients with ASD (Berg et al., 2018; Nakatani et al., 2009; Peixoto et al., 2019; The Dutch-Belgian Fragile X Consortium et al., 1994; Wang et al., 2011; Yoo et al., 2013; Yoo et al., 2019). While these have been useful, the majority of patients have multiple genetic



variants of unknown significance and therefore these models only capture a small percentage of the ASD population. A model of the polygenic component seen in many ASD patients has been established with the BTBR T+tf/J (BTBR) mouse strain (Chadman, 2012; Moy et al., 2007).

Models of gestational exposures linked to ASD, such as valproate or the presence of maternal autoantibodies have been developed (Bromley et al., 2013; Christensen et al., 2013; Nicolini & Fahnstock, 2018; Quadros et al., 2018). Studies with the anti-folate receptor antibody model have demonstrated translational importance of animal models, as these laid the groundwork for clinical studies which indicate that treatment of ASD children with folinic acid, improves verbal communication (Desai et al., 2017; Frye et al., 2018).

To better address both overlap and heterogeneity, multiple models should be used to validate positive findings (Pietropaolo, Crusio, & D'Amato F, 2017). In the context of this research, models that would best identify treatment targets for a specific ASD (e.g. FXS, Rett syndrome, autoimmune) may produce results which could generalize to a larger cohort of ASD patients. This highlights the critical need for new models of ASD. This work proposes that targeted models which recapitulate part of the pathology will improve our understanding of mechanisms in both syndromic and non-syndromic ASD patients.

Model of ASD	Phenotype	Selected references
<b>Fragile X syndrome</b>	Fragile X syndrome is the most common genetic cause of intellectual disability and ASD. <i>Fmr1</i> -KO mutant mice display phenotypes which recapitulate those seen in FXS patients. Phenotypes include hyperactivity, anxiety-like behavior, altered social and communication behaviors, increased self-grooming, audiogenic seizures, and impairments on learning and memory tasks. Dendritic morphology defects are reported. Long-term potentiation is impaired, and long-term depression enhanced, in <i>Fmr1</i> -KO mice.	<i>Cassidy et al 2010</i> <i>Pietropaolo et al 2011</i> <i>Jung et al 2012</i> <i>Spencer et al 2011</i> <i>Hebert et al 2014</i> <i>Belagodu et al., 2016</i> <i>Huber et al 2002</i> <i>Bureau et al 2008</i>
<b>Chr 15q11–13</b>	Deletions and duplications in the 15q11–13 chromosomal region are associated with Angelman’s syndrome, Prader–Willi syndrome, and autism. Mice with the homologous deletion displayed impaired sociability and communication, anxiety-like behavior, impaired learning and memory	<i>Nakatani et al 2009</i>
<b>BTBR</b>	BTBR T+ tfJ (BTBR) is a genetically homogenous inbred strain of mice. BTBR mice display decreased sociability, altered communication and high levels of spontaneous self-grooming. Assessment of general health, motor and sensorimotor abilities are normal in BTBR. This supports the interpretation that abnormalities in the BTBR mouse are selective for phenotypes relevant to ASD	<i>Moy et al 2007</i> <i>Chadman et al 2012</i> <i>Scattoni et al 2008</i> <i>Crusio et al 2008</i> <i>Martin et al 2014</i>
<b>Prenatal Valproate</b>	Exposure to the anti-epileptic valproate (VPA) during pregnancy increases the likelihood of ASD in the child. Rats of mother treated with VPA during pregnancy demonstrate impaired social behaviors, repetitive behaviors, and impaired performance on learning and memory tasks	<i>Bromley et al 2013</i> <i>Christensen et al 2013</i> <i>Nicolini et al 2018</i> <i>Hirsch et al 2018</i>
<b>22q13 deletion syndrome</b>	22q13 deletion syndrome results from a loss of a segment of the distal long arm of chromosome 22. ~25% of patients have ASD. The major neurological features are due haploinsufficiency of the <i>SHANK3/PROSAP2</i> gene. Mutations in this gene are also associated with ASD. Rats and mice null for the <i>Shank3</i> demonstrated impaired social behavior, communication, and enhanced self-grooming behavior. Mice null for <i>Shank3</i> have neurobiological phenotypes associated with ASD.	<i>Cassidy et al 2010</i> <i>Yoo et al 2013</i> <i>Berg et al 2018</i> <i>Yoo et al 2019</i> <i>Peixoto et al 2019</i>

**Table 2. Descriptions of five mouse models of ASD.** Representative syndromic (FXS, Chr 15q11-13, 22q13 deletion syndrome), non-syndromic (BTBR), and prenatal toxin exposure (valproate) models of ASD are listed. *Adapted from Rouillet and Crawley (2011).*

## 1.6 Fragile X Syndrome and Autism Spectrum Disorder: Shared pathology?

The overlap between FXS and ASD extends beyond the clinical presentation and into the molecular pathology. Our knowledge about the underpinnings of these disorders and where they may overlap has increased over the past decades. It has been well demonstrated that loss of FMRP results in excess and dysregulated mRNA translation, delocalization of FMRP regulated proteins, and thus profound changes in the structure and physiology of the synapse (Brown et al., 2001; Schutt et al., 2009). From this foundation emerged the “mGluR5 theory” of FXS (Bear et al., 2004). Type 1

metabotropic glutamate receptors (mGluR1 or 5) are G coupled protein receptors (GCPR) located post-synaptically which regulate multiple cellular signaling pathways (Abe et al., 1992). Stimulation of mGluR5 receptors induces FMRP translation at the synapse and FMRP functions as a repressor of protein synthesis (Antar et al., 2004). In the *Fmr1*-KO mouse an mGluR5 regulated form of synaptic plasticity, long term depression (LTD) is exaggerated (Huber, 2002). The pathology seen in *Fmr1*-KO mice is reflected in both FXS and non-syndromic ASD patients, as alterations in mGluR5 expression are seen in post-mortem ASD brains (Fatemi & Folsom, 2015; Fatemi et al., 2011). Furthermore, high-throughput sequencing of mGluR signaling pathway genes reveals enrichment of rare variants in ASD (Kelleher et al., 2012).

Since dysfunctional mGluR activity is present in FXS and some ASD patients, this has prompted detailed investigations into downstream components of mGluR5 signaling. Targeted mutations of mGluR5 scaffolding proteins such as *Homer1a*, *Shank3*, *Ngln3* produced phenotypes that approximate those seen in FXS and ASD (Foldy et al., 2013; Guo et al., 2016; Sledziowska et al., 2019). Many of the functional defects uncovered are in mechanisms of plasticity, such as  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor expression, N-methyl-D-aspartate (NMDA) receptor localization, and endocannabinoid (eCB) signaling (Aloisi et al., 2017; Foldy et al., 2013; Jung et al., 2012; Tang & Alger, 2015; Yang et al., 2012). Notably, mutations in many of these components, particularly FMRP, mGluR and NMDAR, disrupted endocannabinoid mediated regulation of presynaptic activity (Foldy et al., 2013; Jung et al., 2012; Krueger & Brose, 2013; Tang & Alger, 2015). These deficits are also found in

the *Fmr1*-KO model and importantly, genetic variants for these proteins are found in non-syndromic ASD patients and are predictive of an ASD diagnosis (Durand et al., 2007; Jamain et al., 2003; Pinto et al., 2014; Skafidas et al., 2014).

FMRP also functions as a direct regulator of voltage gated ion channels, and thus loss of FMRP results in physiological deficits which are independent of protein synthesis (Deng et al., 2013). Importantly to this work, genetic variants for these FMRP targets have been detected in the ASD population and, in some cases, linked directly with pathology. For example, whole-exome sequencing study and subsequent transmission and de novo association (TADA) analysis detected genetic variants which strongly increase the risk of ASD in the regulatory subunit of P/Q ( $Ca_v2.1$ ) and N ( $Ca_v2.2$ ) type calcium channels (De Rubeis & Buxbaum, 2015). The surface expression of these channels is directly regulated by FMRP, the loss of which increases  $Ca_v$  expression and results in increased neurotransmitter release (Ferron et al., 2014; Ferron et al., 2020).

FXS and ASD pathologies overlap at presynaptic  $Ca_v2.1$  and  $Ca_v2.2$  channels. These represent a key dysfunctional regulatory point for both disorders. Large conductance voltage and calcium sensitive potassium channels (BKCa) regulate action potential duration and neurotransmitter release by directly interacting with  $Ca_v2.1$  and  $Ca_v2.2$  (Berkefeld et al., 2006; Salkoff et al., 2006). The  $\beta_4$  regulatory subunit specific for BKCa channels located in central neurons (Petrik & Brenner, 2007; Weiger, 2000). Data from several genomic databases were analyzed for diagnostic predictors of ASD, single nucleotide polymorphisms (SNPs) in the *KCNMB4* gene were found to be predictive for

a diagnosis of non-syndromic ASD (Skafidas et al., 2014). Importantly, BKCa channel activity is directly regulated by FMRP at the  $\beta 4$  unit and loss of FMRP results in physiological deficits in voltage and calcium regulation by BKCa that increases excitability and neurotransmitter release (Deng et al., 2013). A separate study identified mutations in the *KCNMA1* gene were identified in two patients with ASD and intellectual disability (Laumonnier, 2006). A patient with intellectual dysfunction who had a mutation only in  $\beta 4$  BKCa regulatory domain of FMRP was identified (Myrick et al., 2015).

### **1.7 Therapeutic Targets for FXS and ASD**

Despite decades of extensive research an FDA approved treatment for the core ASD deficits has yet to be obtained, however there have been encouraging advances in recent year (Heussler et al., 2019; Tartaglia et al., 2019; Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019). One systematic approach toward the identification of treatment targets has been categorizing FXS and ASD associations into smaller cohorts of related or overlapping pathobiology. Logically, a commonly shared point of dysfunction between causal candidate mechanisms would represent a point of interest for the development of therapeutics.

An approach toward identifying therapeutic targets for FXS and ASD, would consider targets which (1) have clinical or population-based risk alleles, (2) which modulate a common mechanism found in syndromic and non-syndromic ASD, (3) can be manipulated without deleterious effect, and (4) produce significant functional improvements when acted upon by therapeutics.

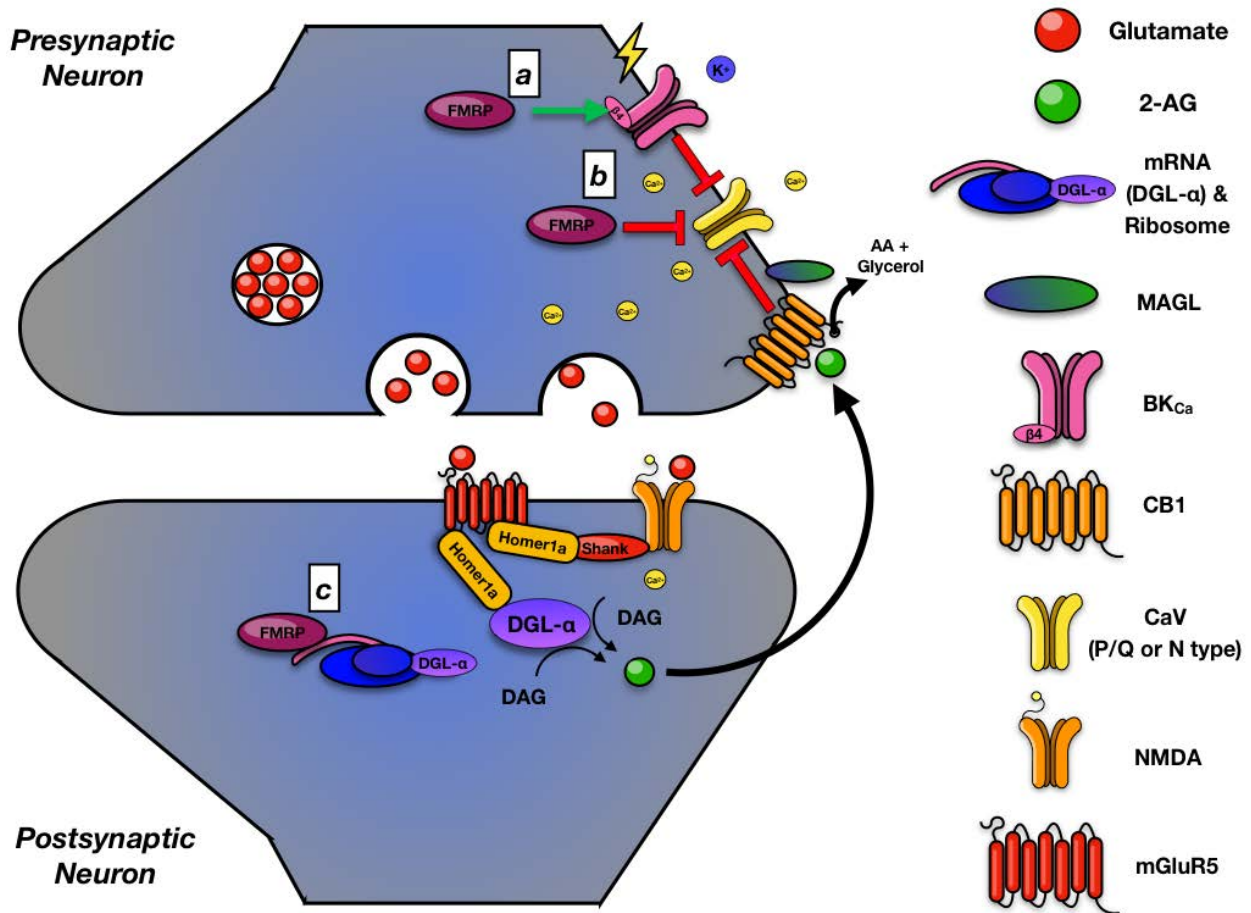
## 1.8 The Presynaptic Hypothesis

A commonality found in many etiological ASD studies is the presence of risk variants or environmental insults which are causal or strongly correlated with synaptic dysfunction (Bagni & Zukin, 2019). This has led to the proposal that ASD be conceptualized as a “synaptic disease” (Auerbach et al., 2011; Chen et al., 2014). Synaptic dysfunction can be subdivided into various subcategorizations (e.g. channelopathies) (Mullin et al., 2013; Zhang et al., 2014). Each of these subcategorizations provides a framework for generating hypotheses and may be a useful for identifying treatment targets.

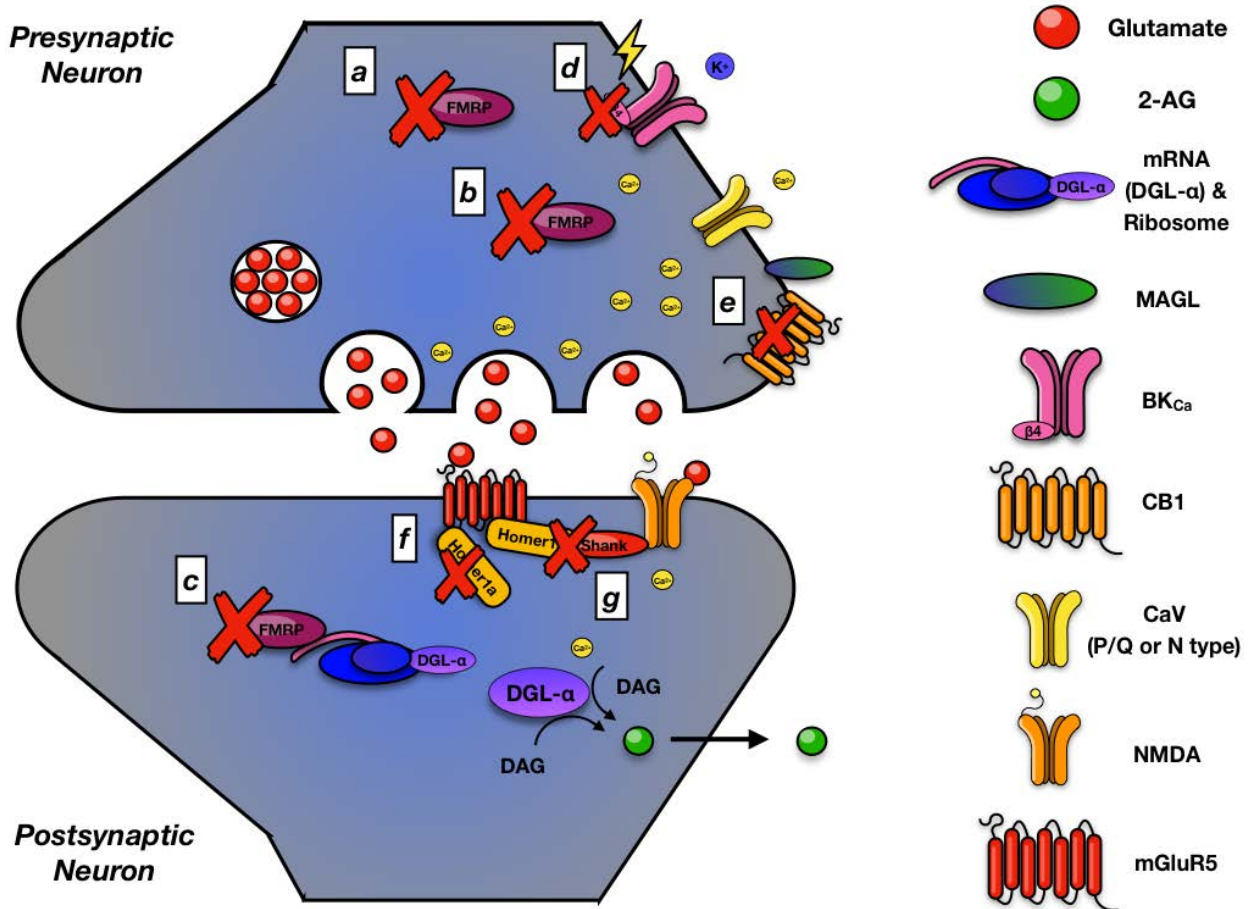
Of the many pathophysiological processes associated with FXS and ASD, those which are imperative for appropriate presynaptic activity have a substantial body of clinical and preclinical evidence implicating them in the pathology both disorders. At the synaptic level, presynaptic dysregulation results in aberrant neurotransmitter release and altered synaptic plasticity, which underlies the hyperexcitability seen at the circuit level (Ferron et al., 2014; Ferron et al., 2020; Tang & Alger, 2015; Zhang et al., 2014; Zhang et al., 2012). Dysfunctional local circuits may underlie larger scale brain network dysfunction (e.g. connectopathy) often detected in ASD patients (Assaf et al., 2010; Cardon et al., 2017; Just et al., 2012).

To summarize, *the presynaptic hypothesis posits that presynaptic dysregulation causes a computational problem at the synaptic level, resulting in circuit level and systems level brain network dysfunction that manifests as neurodevelopmental pathology.*

More specifically, three of these presynaptic regulatory mechanisms, FMRP, the endocannabinoid system (ECS), and BKCa channels, converge on the same regulatory targets, presynaptic P/Q and N type  $Ca^{2+}$  channels (Ferron et al., 2014; Ferron et al., 2020; Twitchell et al., 1997). These channels are also linked to FXS and ASD pathology (Damaj et al., 2015; Gargus, 2009; Li et al., 2015) (Fig. 2 and 3).



**Figure 2a. Regulation of presynaptic Cav (P/Q or N type) channels by FMRP.** FMRP contributes to presynaptic regulation by (a) stimulating BKCa channel to inhibit CaV channels, (b) directly inhibiting Cav channels, or (c) controlling the translation and localization of the eCB producing enzyme DGL- $\alpha$  in the post-synaptic density. DGL- $\alpha$  exists in a complex (synaptosome) with the scaffolding protein Homer1a and mGluR5. mGluR5 activity stimulates DGL- $\alpha$  production of the CB1 ligand 2-AG. CB1 responds to 2-AG stimulation by inhibiting P/Q and N type Cav channels.



**Figure 2b. Dysregulation of presynaptic Cav (P/Q or N type) channels.** Absence of FMRP due to the FMR1 mutation results in a loss of presynaptic Cav channels by (a) BKCa channels and by (b) direct FMRP interactions; (c) absence of post-synaptic FMRP results in delocalized DGL-α and 2-AG production. Mutations in (d) the β4 regulatory unit of BKCa channels, (e) CB1, (f) Homer1a, and (g) Shank3 have associations with syndromic and non-syndromic ASD. Each of these defects causes increased Ca<sup>2+</sup> entry and neurotransmitter release (computational dysfunction).



While other targets may also have therapeutic potential, the ECS and BKCa channels and FMRP regulate presynaptic neurotransmitter release at presynaptic Cav2.1 and Cav2.2 channels. Furthermore, each of these components has pre-clinical and clinical evidence linking them to the pathology of FXS, ASD and other neurodevelopmental disorders. Importantly, both can be manipulated pharmacologically to rescue some neurodevelopmental phenotypes (Hebert et al., 2014; Jung et al., 2012; Wei et al., 2016). Therefore, the ECS and BKCa channels make attractive targets for testing the presynaptic hypothesis.

To begin testing the role of the ECS and BKCa channels presynaptic hypothesis, three questions must be addressed:

1. *What is the function of these systems?*
2. *What is their relationship to FXS and ASD with respect to clinical correlations and data from preclinical studies?*
3. *What is known (and unknown) about their potential as therapeutic targets?*

### **1.9 The Endocannabinoid System**

The endocannabinoid system (ECS) is composed of two primary cannabinoid receptors, cannabinoid type 1 receptor (CB1) and cannabinoid type 2 receptor (CB2), and two primary ligands, arachidonoyglycerol (2-AG) and N-arachidonylethanolamine (AEA) (Devane et al., 1992; Matsuda et al., 1990; Munro et al., 1993). CB1, a G-coupled protein receptor (GPCR), is expressed extensively in the central nervous system, with higher levels of expression found in the hippocampus, amygdala, striatum, and cortex (Kano et al., 2009). CB2, also a GPCR, is expressed at low levels in the CNS and

largely on microglial cells where they mediate immune responses (Munro et al., 1993; Núñez et al., 2004). Endocannabinoids are hydrophobic lipids which are biosynthesized and released on demand, unlike the majority of neurotransmitters, which are water soluble, synthesized in advance, and stored in vesicles (Makriyannis et al., 2005).

Of the two endocannabinoid ligands, 2-AG is the most abundant found in the mammalian CNS and is a full agonist at CB1 (Stella et al., 1997; Sugiura et al., 1995; Suhara et al., 2000). 2-AG synthesis has two distinct mechanisms by which it occurs: First (eCB<sub>mGluR</sub>), activation of group I mGluR which activates phospholipase C  $\beta$  (PLC- $\beta$ ) to cleave phosphatidylinositol-4,5-bisphosphate (PIP<sub>2</sub>) to produce the 2-AG precursor, 1,2-diacylglycerol (DAG). This is hydrolyzed by the serine lipase, diacylglycerol lipase alpha (DGL- $\alpha$ ) in central neurons and diacylglycerol lipase beta (DGL- $\beta$ ) in immune cells (e.g. microglia, macrophages), to form 2-AG (Bisogno et al., 2003; Jung et al., 2005). The second mechanism for 2-AG synthesis is dependent on rapid increases of intracellular Ca<sup>2+</sup> via NMDA receptors (eCB<sub>NMDA</sub>) (Ohno-Shosaku et al., 2007; Zhang et al., 2011). PLC- $\beta$  is activated in a Ca<sup>2+</sup> dependent manner and produces the precursor, DAG, needed for production of 2-AG by DGL- $\alpha$  (Brenowitz & Regehr, 2003). The synthesis of 2-AG in post-synaptic neurons occurs within a supramolecular complex wherein mGluR5 receptors are bound to Homer1a scaffolding proteins which also bind PLC- $\beta$  and DLG- $\alpha$  resulting in rapid and spatially localized 2-AG synthesis (Jung et al., 2012). Approximately 85% of 2-AG is hydrolyzed into arachidonic acid (AA) and glycerol the presynaptic enzyme monoacylglycerol lipase (MAGL), with the remaining 15% metabolized by the enzymes  $\alpha/\beta$ -hydrolase-6 (ABHD6)

and  $\alpha/\beta$ -hydrolase-12 (ABHD12) (Dinh et al., 2002; Gulyas et al., 2004). The second endocannabinoid, AEA, is a partial agonist at CB1 (Felder et al., 1993; Sugiura et al., 2002). AEA synthesis occurs in a  $\text{Ca}^{2+}$  dependent manner, in response to an influx on intracellular  $\text{Ca}^{2+}$  causes cleavage of phosphatidylethanolamine (PE) by N-acetyltransferase into the AEA precursor, N-arachidonoyl-PE (NAPE), which is then cleaved by the NAPE-hydrolyzing phospholipase D (NAPE-PLD) into AEA. Metabolism of AEA is carried out by fatty acid amide hydrolase (FAAH) which hydrolyzes AEA into AA and ethanolamine (EA). Once synthesized, 2-AG and AEA diffuse retrosynaptically and interact with CB1 receptors located on the presynaptic neuron (Sugiura et al., 2002).

CB1 signaling by 2-AG or AEA can result in the activation of multiple signaling pathways mediated by the  $G_{i/o}$  protein subunits of CB1. CB1 activation inhibits adenylyl cyclase and reduces cAMP production (Felder et al., 1995). Activation by CB1 agonists also induces mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways which controls gene transcription and cellular activity (Bouaboula et al., 1995). Crucially, CB1 inhibition of neurotransmitter release, responsible for synaptic plasticity, is mediated by  $G_{i/o}$  protein inhibition of presynaptic  $\text{Ca}_v2.1$  and  $\text{Ca}_v2.2$  channels (Twitchell et al., 1997).

The retrograde nature of the ECS provides a unique method of modulating synaptic plasticity called depolarization-induced suppression of inhibition (DSI) at inhibitory GABAergic synapse and depolarization-induced suppression of excitation (DSE) at excitatory synapses (Pitler & Alger, 1992; Wilson & Nicoll, 2001). Briefly, depolarization

at the post-synaptic neuron induces the production of eCBs which act retrosynaptically to inhibit neurotransmitter release from the presynaptic neuron.

Endocannabinoids also exhibit activity at transient receptor potential cation channel subfamily V member 1 (TRPV1), G protein-coupled receptor 18, 55, and 119 (GPR 18; GPR55; GPR119) (Lauckner et al., 2008; Maccarrone et al., 2008). While the activity of these ligand-receptor interaction is not yet fully understood, it has been shown that signaling at these receptors with exogenous cannabinoids may mediate some of the anxiolytic and anti-epileptic properties of these molecules (Hill et al., 2012; Hill et al., 2013). The ECS also has a critical developmental role, as during gestation, DGL- $\alpha$  mediated 2-AG-CB1 signaling is necessary for appropriate neurogenesis, neuronal migration and axonal targeting (Berghuis et al., 2007; Keimpema et al., 2013).

### **1.9.1 Clinical Data**

A growing body of clinical evidence associates the ECS with FXS and ASD phenotypes. Post-mortem studies of brain tissue from ASD patients indicated reduced expression of *CNR1*, the gene for CB1R (Purcell et al., 2001). Additionally higher expression of CB2R has been found to be upregulated in some children with ASDs (Siniscalco et al., 2013). Analysis of multiple genomic databases found variants in *CNR1* and *DAGLA*, the gene for DGL- $\alpha$ , were significantly associated with autism (Smith et al., 2017). A series of studies investigated gaze to facial stimuli, a behavior frequently altered in FXS and ASD patients, and found that polymorphisms in the *CNR1* gene modulate striatal responses and gaze duration to happy faces (Chakrabarti & Baron-Cohen, 2011; Chakrabarti et al.,

2006). Two recent studies detected lower levels of circulating endocannabinoids in ASD patients (Aran et al., 2019; Karhson et al., 2018). Risk increasing variants have been detected in synaptic proteins important for ECS function such as *GRM5*, *NGLN3*, *HOMER1A*, and *SHANK3*, (Kelleher et al., 2012; Moessner et al., 2007; Pinto et al., 2014; Quartier et al., 2019). Given the known role that mGluR5 dysfunction plays in FXS pathology and ECS activity, it is important to note that mutations in *GRM5*, the gene for mGluR5, are risk variants for ASD (Kelleher et al., 2012).

### **1.9.2 Preclinical Studies**

Pharmacological and genetic manipulations have demonstrated a pathophysiological link between the ECS and neurodevelopmental pathology. Behavioral phenotyping of mice null for CB1 expression revealed deficits in social behavior, cognition, and repetitive behaviors (Haller et al., 2002; Haller et al., 2004; Litvin et al., 2013). Selective deletion of CB1 revealed that a loss of CB1 in glutamatergic, but not GABAergic, cortical neurons resulted in a reduction of social interest (Terzian et al., 2014). Mice with a targeted DGL- $\alpha$  deletion from direct pathway medium spiny neurons (dMSNs) of the striatum had impaired social interest and increased repetitive behaviors (Shonesy et al., 2018). Mice with global DGL- $\alpha$  deletion showed increased anxiety, stress and fear responses (Jenniches et al., 2016; Shonesy et al., 2014).

Importantly, the ECS demonstrates functional redundancy in the modulation behavioral phenotypes, as both major eCBs appear to exhibit brain region specific effects that overlap in some regions and have a reciprocal relationship in others (Bedse et al., 2017;

J. Z. Long et al., 2009). This has been studied mostly in anxiety behaviors, as inhibition of 2-AG or AEA signaling in wild type mice induced anxiety and impaired stress responses (Bedse et al., 2017; Imperatore et al., 2015). This also showed that both major eCBs display functional redundancy in the regulation of stress and anxiety responses. Molecular and physiological studies demonstrated that the two primary eCBs enable the ECS to function as a polymodal integration system, allowing for multiple forms of synaptic plasticity (e.g. short-term depression, long-term depression) within a single neuron (Puente et al., 2011).

Studies with the *Fmr1*-KO mice consistently show evidence of ECS dysfunction (Jung et al., 2012; Maccarrone et al., 2010; Straiker et al., 2013; Tang & Alger, 2015). FMRP binds the mRNA of DGL- $\alpha$  and controls its appropriate translation and localization at the post synaptic density (PSD) (Jung et al., 2012). Loss of FMRP expression resulted in delocalization of DGL- $\alpha$  and dysfunctional 2-AG mediated plasticity. It was demonstrated that, in the absence of FMRP, mGluR5 stimulation fails to induce 2-AG production in the prefrontal cortex (PFC), and thus the mGluR hypothesis of FXS is tied to dysfunctional eCB activity. Molecular and physiological studies indicated that appropriate eCB<sub>mGluR</sub> production requires a scaffolding protein called Homer1a, which complexes mGluR5 and DGL- $\alpha$  (Jung et al., 2007; Tang & Alger, 2015). In *Fmr1*-KO mice, interactions between mGluR5 and Homer1a are reduced and this is causal for hyperexcitability of cortical neurons and seizures (Ronesi & Huber, 2008). Homer1a proteins also mediate mGluR5 and NMDA interactions, likely coordinating eCB<sub>mGluR</sub> and eCB<sub>NMDA</sub> forms of 2-AG synthesis, although this coordination has not been directly

demonstrated at this time (Aloisi et al., 2017). These interactions are disrupted in *Fmr1*-KO mice and upregulation of Homer1a expression rescued cognitive deficits.

Importantly, increasing the bioavailability of 2-AG normalized plasticity deficits and rescued the hyperactive, anxiety, and cognitive impairments phenotypes of the *Fmr1*-KO mouse (Jung et al., 2012; Wang et al., 2017). Furthermore, pharmacological enhancement of AEA availability rescued deficits in social approach, memory and deficit frequently found in *Fmr1*-KO mice (Busquets-Garcia et al., 2014; Gomis-Gonzalez et al., 2016; Qin et al., 2015; Wei et al., 2016).

Developmental studies show that a temporally orchestrated pattern of ECS expression and activity is imperative for appropriate brain connectivity (Berghuis et al., 2007; Heng et al., 2011; Keimpema et al., 2013; Mulder et al., 2008; Oudin et al., 2011). The results of a postmortem study of brain tissue from various developmental times points revealed that CB1 and the enzymes responsible for endocannabinoid synthesis and breakdown (e.g. DGL- $\alpha$ , MAGL, FAAH) have distinct patterns of expression across development, particularly during neonatal and infancy age ranges (Long et al., 2012). This is further demonstrated by mouse studies where in mice null for the CB1R have altered brain connectivity (Abbas Farishta et al., 2015; Hill et al., 2011). This appears to approximate a neurobiological phenotype frequently seen in patients with neurodevelopmental disorders (Just et al., 2012; Keown et al., 2017; Keown et al., 2013).

### 1.9.3 Therapeutics

Previous studies support the ECS as a promising target for therapeutics.

Phytocannabinoids (pCBs) are exogenous cannabinoids derived from the plant *Cannabis Sativa* and have been used by humans for millennia (Adams & Martin, 1996). Clinical studies with the pCBs cannabidiol (CBD) showed an improvement in aggression, hyperactivity, sleep problems, speech impairment, seizures, and anxiety in ASD patients (Bar-Lev Schleider et al., 2019; Barchel et al., 2018). Phase 1 and 2 trials of CBD treatment with FXS patients produced substantial reductions in hyperactivity, social avoidance, anxiety, and compulsive behavior after 12 weeks of treatment (Heussler et al., 2019). Importantly the frequency of adverse events was low, and no serious adverse events were reported. Additionally, several case reports of FXS patients and CBD treatment report improvement of symptoms (Tartaglia et al., 2019). Moreover these molecules typically avoid the undesired psychotropic side effects that result from CB1 activation, strengthening their appeal as potential treatments for neurodevelopmental disorders (Morales et al., 2017).

Cannabidivarin (CBDV), a propyl analog of CBD, has emerged as another candidate pCB for the treatment of neurodevelopmental disorders. Preclinical evidence supports this, as a promising treatment for Rett Syndrome and epilepsy (Hill et al., 2012; Hill et al., 2013; Vigli et al., 2018; Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019).

Currently a clinical trial, funded by the United States Department of Defense, is underway for CBDV treatment in ASD patients (Clinicaltrial.gov; NCT03202303).

Imaging studies with both CBD and CBDV demonstrate that these pCBs have distinct



effects on brain function in ASD patients, however further studies are needed to define if this underlies a therapeutic effect (C. M. Pretzsch, J. Freyberg, et al., 2019; C. M. Pretzsch, B. Voinescu, et al., 2019).

Currently, there are high hopes that pCBs will be useful in the treatment of FXS, ASD and other neurodevelopmental disorders. This is not without a solid foundation. CBD has received FDA approval for the treatment of two forms of epileptic neurodevelopmental disorders: Dravet and Lennox-Gastaut syndromes (Devinsky, Nabbout, et al., 2018; Devinsky, Patel, et al., 2018).

### **1.10 Large Conductance Voltage and Ca<sup>2+</sup> Sensitive K<sup>+</sup> (BKCa) Channels**

Large conductance voltage and calcium sensitive potassium (BKCa) channels are expressed ubiquitously throughout the body, however regulatory subunits of these channels are tissue specific (Tseng-Crank, 1994). In the central nervous system, the  $\beta 4$  regulatory subunit is referred to as the neuronal auxiliary subunit and is the most abundant of the subunits expressed with BKCa channels in central neurons (Petrik & Brenner, 2007; Weiger, 2000). In the CNS, BKCa channels are expressed in most brain regions at presynaptic terminals, however higher levels of expression are found in the cortex, basal ganglia, hippocampus and cerebellum (Hu, 2001; Petrik & Brenner, 2007).

Functionally, the  $\alpha$  subunit of the BKCa channel opens in response to membrane depolarization and intracellular increases in Ca<sup>2+</sup> (Wallner et al., 1996). It has a bimodal

response to these events; opening to allow a large efflux of  $K^+$  ions (thus hyperpolarizing the membrane) and complexing with P/Q and N type  $Ca^{2+}$  channels to inhibit  $Ca^{2+}$  entry and control neurotransmitter release (Berkefeld et al., 2006; Salkoff et al., 2006). Of these two stimuli,  $Ca^{2+}$  entry is the rate limiting step for BKCa activation (Berkefeld & Fakler, 2013). FMRP regulates the  $Ca^{2+}$  sensitivity of BKCa channels through direct interactions with the  $\beta 4$  subunit (Deng et al., 2013). This reduces action potential duration, controlling neurotransmitter release and repetitive neuronal activity.

### **1.10.1 Clinical Data**

Genetic studies have uncovered a relationship between genetic variants for BKCa genes and ASD. Skafidas et al. (2014) examined the occurrence of specific single nucleotide polymorphisms (SNPs) and a diagnosis of ASD. A genetic diagnostic classifier of 237 SNPs in 146 genes was used to with 85.6% accuracy in predicting a diagnosis of ASD in a cohort of central European individuals gathered from two different databases: SFARI and Wellcome Trust 1958 Normal Birth Cohort (WTBC) databases. Two of the SNPs determined to be most effective at determining a classification of non-syndromic ASD vs non-ASD were found in the *KCNMB4* gene, ( $\beta 4$  BKCa subunit), and *GRM5* gene, (mGluR5) were two of the three identified genes. This is particularly important in regard to the overlap between FXS and ASD since BKCa channel activity is directly regulated by FMRP at the  $\beta 4$  unit and mGluR5 dysfunction in FXS has been well established (Bear et al., 2004; Deng et al., 2013; Hebert et al., 2014).

Two studies which investigated chromosomal abnormalities in ASD patients discovered a mild to moderate association between mutations in *KCNMA1* and a diagnosis of autism (Alarcon et al., 2002; International Molecular Genetic Study of Autism, 2001). Additionally, mutations in the *KCNMA1* gene were identified in two patients with ASD and intellectual disability (Laumonnier, 2006). Genome analysis of the first patient discovered a balanced de novo translocation (9q23/10q22) resulting in haploinsufficiency for the  $\alpha$  subunit, while the second patient revealed a single point mutation in the *KCNMA1* gene which resulted in an ALA138VAL substitution.

BKCa dysfunction is also associated with other neurodevelopmental disorders. A patient with moderate to mild intellectual disability and febrile seizures was identified as having a mutation only in  $\beta 4$  BKCa regulatory domain of FMRP (Myrick et al., 2015). Analysis of the family found a maternal and paternal history of learning problems, however this specific mutation, being X linked, was found only in the maternal genome. Additionally, the *CRBN* gene, which codes for the protein necessary for appropriate assembly and surface expression of BKCa channel in neurons, was found to be associated with autosomal recessive non syndromic mental retardation (ARNSMR) (Higgins et al., 2008).

### **1.10.2 Preclinical Studies**

Studies with the *Fmr1*-KO mouse demonstrated that loss of FMRP regulation of BKCa channels increased action potential duration (Deng & Klyachko, 2016; Deng et al., 2013). Specifically, loss of FMRP increased the after-hyperpolarization phase (AHP) of

the action potential, increasing neuronal excitability, presynaptic Ca<sup>2+</sup> influx, and neurotransmitter release. Zhang et al. (2014) showed that loss of FMRP was also responsible for downregulation of BKCa channel expression in the *Fmr1*-KO mice. These factors were determined to be contributory for the sensorimotor hypersensitivity phenotype in the *Fmr1*-KO mouse. A genetic mouse model null for the BKCa  $\alpha$  subunit gene (*Slo1*) was developed to explore the role of BKCa channels in neurodevelopmental disorders (Typlt et al., 2013). This study found that mice null for BKCa  $\alpha$  expression had impaired sensorimotor and spatial memory, with normal locomotor activity. Currently, phenotyping of the social behaviors of the BKCa<sup>-/-</sup> mouse has not been performed.

### **1.10.3 Therapeutics**

A BKCa channel agonist, BMS-204532 (BMS), was developed in 2002 for the treatment of ischemia stroke, however it failed to demonstrate clinically significant therapeutic effects in phase III trials (Jensen, 2002). Since BMS has a favorable safety profile it is currently under investigation as a treatment of BKCa channelopathies. Detailed analyses of cells cultured from patients with ASD and BKCa mutations demonstrated that channel function could be rescued by BMS (Laumonnier, 2006).

Studies with the *Fmr1*-KO mouse have demonstrated promise for BMS as a therapeutic for FXS. In an initial study, BMS treatment rescued social, cognitive and anxiety phenotypes and normalized dendritic morphology in the *Fmr1*-KO mouse (Hebert et al., 2014). Two subsequent studies have demonstrated that BMS can rescue dendritic

hyperexcitability and the increased self-grooming and sensorimotor hypersensitivity phenotypes of the *Fmr1*-KO mouse (Carreno-Munoz et al., 2018; Zhang et al., 2014). One of the challenges in using BMS clinically, is the short half-life in brain tissue ( $t_{1/2}=1.9$ ) (Jensen, 2002). This would result in a difficult dosing schedule and therefore additional development is needed for molecule refinement. Despite these challenges, these preclinical studies strongly suggest that BMS or a next generation BMS derived molecule could provide a pharmacological intervention for these neurodevelopmental disorders.

## CHAPTER 2 - GOAL OF THESIS AND GENERAL HYPOTHESIS

The goal of this research is the identification and characterization of therapeutic targets for the treatment of FXS. Due to the large overlap of FXS and ASD, targets which are identified for the treatment of FXS, particularly the treatment of the FXS-ASD symptoms, may have efficacy in treatment of non-syndromic ASD patients. The work presented in this thesis operates under the organizational paradigm *of the presynaptic hypothesis of FXS and ASD*, wherein dysfunctional regulatory mechanisms of presynaptic Ca<sub>v</sub> channel activity, namely the ECS and BKCa channels are proposed to be causal for FXS and ASD phenotypes. These mechanisms are amenable to pharmacological manipulation, and thus, present a crucial opportunity to assess their potential as therapeutic targets. This work predicts that inhibition of the ECS or BKCa is inductive for FXS and ASD phenotypes. Conversely, activation of these systems in a preclinical model of FXS will rescue behavioral and biological phenotypes. The specific goal of this research is to evaluate the potential of ECS and BKCa channels, as treatment targets for FXS with implications for the core deficits seen in ASD.

## **SPECIFIC AIMS**

### **1. Evaluate the inhibition of CB1R and BKCa channels as causative for FXS and ASD relevant phenotypes**

This aim, which will be expanded in chapter three of this thesis document, tested the hypothesis that inhibition of either CB1R or BKCa function will induce behavioral alterations similar to FXS and ASD. To do so, we utilized genetic and pharmacological methods to interfere with CB1R or BKCa function.

**SA1.1** will use mice which are a full or heterozygous genetic knockout for the *CB1* gene (*CB1*-KO; *CB1*-HET) to assess the role of CB1R social communication and behavior during post-natal development (*SA1.1a*) and at adulthood (*SA1.1b*). An additional cohort will be assessed for the effect of the *CB1* deletion on anxiety-like behavior and sensorimotor responses in adult mice (*SA1.1c*). These studies will, *for the first time*, assess the *CB1*-KO mouse as a model for developmental disorders.

**SA1.2** will use wild type mice (B6) with pharmacological methods to inhibit CB1R function via selective CB1R blockage with Rimonabant or downregulation of 2-AG production, a CB1R endogenous agonist, via DGL- $\alpha$  selective inhibition with DO34; as well as BKCa function with the specific inhibitor Paxilline in C57BL/6J mice. Evaluations of the effects of treatment will be assessed using a battery of behavioral paradigms for FXS and ASD behaviors.

The main outcome of specific aim 1 is the addition of genetic and pharmacological models of FXS and ASD phenotypes.

## **2. Evaluate the therapeutic efficacy of increasing CB1R and BKCa activity in *Fmr1*-KO mice.**

This aim, which will be expanded in chapter two of this thesis document, will test the hypothesis that activation of either CB1R or BKCa channels will rescue behavioral and neurobiological phenotypes in the *Fmr1*-KO mouse model. To do so, we will utilize pharmacological manipulations which increase CB1R or BKCa activity in *Fmr1*-KO mice.

**SA2.1** will use a sub-chronic (10 day) dosage schedule with the phytocannabinoid (pCB), cannabidivarin (CBDV) in adult or juvenile *Fmr1*-KO mice followed by the battery of behavioral paradigms to assess FXS and ASD relevant behaviors (SA 2.1a).

Additionally, the effects of CBDV treatment on neurobiological markers of inflammation (cytokines) and plasticity (BDNF), which are known to be altered in the FXS mouse model, will be assessed with qt-PCR (SA 2.1b).

**SA2.2** will increase 2-AG levels using JZL-184, a highly selective inhibitor of the metabolic enzyme for 2-AG, monoacylglycerol lipase (MAGL) in *Fmr1*-KO mice. This study will include a group treated with a direct CB1R agonist (CP-55940) in order to contrast the effects of increasing 2-AG-CB1 signaling with direct CB1R activation. This study will be composed of three sub-studies: The first will investigate the behavioral effects of acute administration (SA2.2a), the second will follow a sub-chronic 10-day dosing paradigm (SA2.2b) evaluating the effects of repeated administration, and the third will assess the effects of these manipulations on mRNA and protein expression for key ECS and BKCa components (SA2.2c).



The main outcomes of this aim are the evaluation of the ECS and BKCa channels as therapeutic targets and the identification of temporal windows for treatment efficacy.

## **CHAPTER 3 – INDUCTION OF FXS AND ASD PHENOTYPES BY DIMINISHING CB1R AND BKCa ACTIONS**

### **General Introduction**

Preclinical studies demonstrate that dysfunction in the ECS or BKCa channels are contributory for FXS and ASD patho-phenotypes. These studies are supported by clinical evidence which links dysfunction of these systems in FXS and ASD patients.

The function of these systems, particularly with regard to behavior, has only been partially elucidated. Importantly, manipulations which increased the activity of CB1 or BKCa channels rescued some of the pathological phenotypes seen in preclinical models (Hebert et al., 2014; Jung et al., 2012; Wei et al., 2016; Zhang et al., 2014).

Despite the evidence linking these systems with neurodevelopmental disorders, their contribution, particularly to social behavior and communication, is largely unknown. This represents a critical gap in our knowledge. Furthermore, it is reasonable to suspect that manipulations which interfere with ECS or BKCa activity would be inductive for pathological phenotypes. This would provide insight on their contributory role to neurodevelopmental disorders. To this end, this chapter contains three manuscripts which utilized genetic or pharmacological manipulations to induce neurodevelopmental phenotypes. These studies aimed to 1) provide further insight on the relationship of the FXS and ASD phenotypes and 2) provide novel genetic and pharmacological models for core and co-morbid symptoms of neurodevelopmental disorders, particularly as these relate to FXS and ASD.

### 3.1 Study 1

#### **Communication and social interaction in the Cannabinoid-type 1 Receptor (CB1R) null mouse: Implications for Autism Spectrum Disorder**

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## **Abstract**

Clinical and pre-clinical findings have suggested a role of the endocannabinoid system (ECS) in the etiopathology of Autism Spectrum Disorder (ASD). Previous mouse studies have investigated the role of ECS in several behavioral domains; however, none of them have performed an extensive assessment of social and communication behaviors, i.e., the main core features of ASD. This study employed a mouse line lacking the primary endocannabinoid receptor (*CB1r*) and characterized ultrasonic communication and social interaction in *CB1<sup>-/-</sup>*, *CB1<sup>+/-</sup>* and *CB1<sup>+/+</sup>* males and females. Quantitative and qualitative alterations in ultrasonic vocalizations (USVs) were observed in *CB1* null mice both during early development (i.e., between post-natal days 4 and 10), and at adulthood (i.e., at 3 months of age). Adult mutants also showed marked deficits in social interest the three-chamber test and social investigation in the direct social interaction test. These behavioral alterations were mostly observed in both sexes and appeared more marked in *CB1<sup>-/-</sup>* than *CB1<sup>+/-</sup>* mutant mice. Importantly, the adult USV alterations could not be attributed to differences in anxiety or sensorimotor abilities, as assessed by the elevated plus maze and acoustic startle tests. Our findings demonstrate the role of *CB1r* in social communication and behavior, supporting the use of the *CB1* full knock-out mouse in preclinical research on these ASD-relevant core domains.

## **Lay Summary**

The endocannabinoid system (ECS) is important for brain development and neural function and is therefore likely to be involved in neurodevelopmental disorders such as Autism Spectrum Disorder (ASD). Here we investigated changes in social behavior and communication, which are core features of ASD, in male and female mice lacking the chief receptor of this system. Our results show that loss of this receptor results in several changes in social behavior and

communication both during early development and in adulthood, thus supporting the role of the ECS in these ASD-core behavioral domains.

## Introduction

Autism Spectrum Disorder (ASD) is a highly heterogeneous group of neurodevelopmental disorders characterized by a broad range of behavioral deficits (Harris, Hessler et al. 2008, Shubrata, Sinha et al. 2015); among these, social alterations, especially in communication and social interest, are a major core component of ASD symptoms and provide with a diagnostic criterion, together with the presence of repetitive/inflexible behaviors (Association 2013). In the last years, an impressive impetus has been given to research on this neurodevelopmental disorder, with a special emphasis on preclinical animal models.

Despite the substantial and varied recent research efforts, the pathological mechanisms underlying ASD are far from being understood and therapeutic targets still need to be identified. It is known that ASD has a strong, though complex, genetic component, as it has been associated with a diverse array of copy number variants (CNV), chromosome duplications/deletions and point mutations (Folstein and Rutter 1977, Sebat, Lakshmi et al. 2007, De Rubeis and Buxbaum 2015, Velinov 2019). With regard to brain function, a view of ASD as a connectivity disorder has emerged (Keown, Datko et al. 2017), since patients often present with heterogeneous patterns of functional and structural connectivity between and within brain regions which differ significantly from neurotypical individuals (Assaf, Jagannathan et al. 2010, Nair, Treiber et al. 2013).

The endocannabinoid system (ECS) is a promising candidate to understand the etiopathology of ASD and to provide novel therapeutic targets. It is primarily a retrograde inhibitory signaling pathway that includes the primary cannabinoid type-1 (*CB1r*) receptors and the endogenous ligands, the endocannabinoids (Devane, Hanus

et al. 1992, Sugiura, Kondo et al. 1995). It is a modulator of neuronal functions, as demonstrated by the abundance of *CB1* receptors in the brain (Mackie 2005), and regulates synaptogenesis and neuronal interconnectivity during development (Berghuis, Rajniecek et al. 2007, Mulder, Aguado et al. 2008), all of these processes are altered in ASD pathology (Pardo and Eberhart 2007). Also, the brain expression levels of *CNR1*, the gene coding for *CB1r*, increase during the late embryonic stage and peak during post-natal development (Marsicano and Lutz 1999).

Alterations in the expression of *CB1r* and other ECS components, as well as in their functionality, have been reported in ASD patients (Smith, Stanley et al. 2017, Karhson, Krasinska et al. 2018) and in several animal models of ASD [reviewed in (Zamberletti, Gabaglio et al. 2017)]. Furthermore, recent clinical and preclinical studies support the efficacy of modulators of ECS in treating ASD symptoms (Jung, Sepers et al. 2012, Bar-Lev Schleider, Mechoulam et al. 2019, Pretzsch, Freyberg et al. 2019). Studies with null mice (*CB1*<sup>-/-</sup>) have demonstrated that *CB1r* plays a key role in the regulation of several behavioral responses (Haller, Varga et al. 2004, Litvin, Phan et al. 2013, Shonesy, Parrish et al. 2018), including ASD-relevant social behaviors (Haller, Varga et al. 2004, Haring, Kaiser et al. 2011, Litvin, Phan et al. 2013, Terzian, Micale et al. 2014). Mice lacking *CB1r* also have brain connectivity alterations, a neurological phenotype of ASD (Hill, Hillard et al. 2011, Abbas Farishta, Robert et al. 2015). However, to the best of our knowledge, no studies have been performed specifically on the role of *CB1r* and the ECS in modulating social communication, i.e., one of the major domains altered in ASD.

The major form of social communication in mice is based on ultrasonic vocalizations (USVs), produced in the range above human hearing (> 20 kHz). USVs are produced

throughout the life of the animal (Wohr and Scattoni 2013). Shortly after birth, mouse pups vocalize in response to separation from the mother to induce maternal retrieval (Brudzynski 2009, Scattoni, Crawley et al. 2009). These isolation-induced USVs represent one of the earliest social behaviors that can be analyzed quantitatively (Ricceri, Moles et al. 2007), and have functional similarities to cries produced by human infants, namely the induction of maternal care. During the first post-natal week, mouse pups increase their number of vocalizations, while a subsequent decrease follows during the second postnatal week, i.e., through an inverted U-shaped typical developmental pattern (Branchi, Santucci, & Alleva, 2001; Sungur, Schwarting, & Wohr, 2016). At adulthood, male mice emit USVs during courtship interactions with females (White, Prasad et al. 1998, Hammerschmidt, Radyushkin et al. 2009). USVs are also produced in female to female interactions in a resident-intruder setting, possibly as a strategy of the resident to reduce the aggressiveness of the intruder (Maggio and Whitney 1985, Moles, Costantini et al. 2007). Therefore, USVs may provide researchers with an accessible tool for modeling ASD-like communication deficits throughout development and adulthood (Crawley 2004), as demonstrated in several studies with mouse models of ASD (Scattoni, Gandhy et al. 2008, Spencer, Alekseyenko et al. 2011, Schmeisser, Ey et al. 2012, Wohr, Silverman et al. 2013, Belagodu, Johnson et al. 2016, Gaudissard, Ginger et al. 2017).

Surprisingly, to our knowledge, ultrasonic communication in mice lacking *CB1r* has not been investigated yet, as only one USV study has been conducted in *CB1<sup>-/-</sup>* pups, but within a chronic stress paradigm (Fride, Suris et al. 2005). Also, most behavioral studies - including those investigating social interest and interaction (Haller, Varga et al. 2004,



Haring, Kaiser et al. 2011, Litvin, Phan et al. 2013, Terzian, Micale et al. 2014) - have so far focused exclusively on homozygous  $CB1^{-/-}$  male mice. Hence, little is known about potential “dosage” and sex-dependent effects of the  $CB1$  mutation on behavior, especially in relation to ASD-relevant phenotypes. Here we therefore examined ultrasonic communication in male and female  $CB1$  null mutants, homo ( $CB1^{-/-}$ ) or heterozygous ( $CB1^{+/-}$ ) for the mutation, during development (i.e., between post-natal days 4 and 10) and at adulthood (3 months). Both quantitative and qualitative analyses of spectrographic measurements were performed in order to provide with an extensive characterization of USVs in  $CB1$  null mutants and their WT littermates. To complete the assessment of ASD-relevant social phenotypes, social interest in the 3-compartment test and social investigation toward a conspecific were also evaluated at adulthood. As confounding differences in anxiety (Vivian and Miczek 1993, Fish, Faccidomo et al. 2004, Veronesi, Batista et al. 2017, Simola and Granon 2019) or acoustic responsiveness (Webber, Mankin et al. 2013, Wada 2017) may influence ultrasonic communication and social behavior, adult mice were also assessed in the elevated plus maze and acoustic startle tests.

## **Material and Methods**

### ***Ethics Approval***

All experimental procedures were in accordance with the European Communities Council Directive 2010/63/EEC, as well with local French and Spanish legislation.

### ***Animals***

All experiments were performed in homozygous *CB1* null mutant (*CB1*<sup>-/-</sup>) mice with a targeted deletion of *CNR1* gene and their heterozygous (*CB1*<sup>+/-</sup>) and wildtype (*CB1*<sup>+/+</sup>) littermates. Mice were obtained from breeders on a C57BL/6N congenic background, generated as previously reported (Marsicano, Wotjak et al. 2002). *CB1*<sup>+/-</sup> males and females were paired for breeding in a temperature- (21±1°C) and humidity- (40%) controlled animal facility (lights on at 07:00 am); approximately two weeks afterwards, pregnant females were individually housed and left undisturbed. The day of birth was considered as postnatal day (PND) 0.

Three batches of mice were used, as described in detail in Table 1: one batch (36 males and 36 females) was tested for USVs during development between PND 4 and 10 (Experiment 1a); a subgroup of the same batch (24 males and 25 females) was tested again for USVs at adulthood (Experiment 1b). A second batch of adult mice (23 males and 25 females) underwent the tests of social interest in the 3 compartment apparatus and of direct social interaction with an adult female. A third batch of mice (36 males and 27 females) was tested at adulthood in the elevated plus maze followed by the acoustic startle test.

Experiment 1a used male and female pups obtained from 11 litters, including all three genotypes (*CB1*<sup>-/-</sup>, *CB1*<sup>+/-</sup>, and *CB1*<sup>+/+</sup>). On PND4 pups were marked after testing by paw tattoo, using a non-toxic tattoo ink (Ketchum permanent Tattoo Inks green paste, Ketchum MFG. Co, NY), as previously described (Wohr, Rouillet et al. 2011, Yang, Bozdagi et al. 2012). On the same day tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes as previously described (Marsicano, Wotjak et al. 2002). Mice were weaned at 3 weeks of age (PND21), housed

in same-sex cages in groups of 3–5 mice/cage in polycarbonate standard cages (33 × 15 × 14 cm in size; Tecniplast, Limonest, France). Mice were left undisturbed until experiment 1b began, i.e., at 3 months of age. Animals for experiments 2 and 3 were bred and housed as described from experiment 1, but they were left undisturbed until PND21, when they were weaned, identified and genotyped and they were all tested at 3 months of age.

Stimulus mice used for the adult assessment of USVs (Experiment 1b) and of social interest and investigation (Experiment 2) were adult (10 weeks of age) female NMRI mice (Janvier, Le Genest-Saint-Isle, France), as this strain is commonly employed in social studies (Moles and D'Amato F 2000, Moles, Costantini et al. 2007). They were housed in groups of 3-4 per cage in the same conditions used for test subjects and left undisturbed for 2 weeks before being used in behavioral tests. Since the estrous phase of the stimulus female does not influence ultrasonic communication in male (Pomerantz, Nunez et al. 1983, Kim, Son et al. 2016) or female (Moles and D'Amato F 2000, Moles, Costantini et al. 2007) mice, but may instead markedly affect their social interest and investigation (Baudoin, Feron et al. 1991, Liu, Erkkila et al. 2010), we employed intact and ovariectomized NMRI stimulus females, for the adult assessment of USVs (Experiment 1b) and social interest/investigation (Experiment 2), respectively.

All animal cages were covered by a stainless metal wired lid, provided with sawdust (SAFE, Augy, France) and ad libitum food and water; they were provided with nesting material as environmental enrichment (Cotton Nestlets).

Experiment	Testing Age/cohort	N (Male)	N (Female)	Behavioral test	Behaviors Analyzed
1a	PND 4, 6,8,10/ cohort 1	9 WT 14 HET 13 KO	13 WT 17 HET 6 KO	Maternal separation	Ultrasonic vocalizations
1b	Adult (3m)/ cohort 1	6 WT 11 HET 7 KO	8 WT 11 HET 5 KO	Direct social interaction with an intact adult NMRI female	Ultrasonic vocalizations
2	Adult (3m)/ cohort 2	8 WT 10 HET 5 KO	11 WT 8 HET 6 KO	3 compartment-test, direct social interaction with an ovx adult NMRI female	Social interest, social investigation
3	Adult (3m)/ cohort 3	9 WT 16 HET 9 KO	7 WT 9 HET 11 KO	Elevated plus maze, acoustic startle	Anxiety-like behavior, startle reactivity

**Table 1. Experimental plan of the study:** A subgroup of the cohort used for experiment 1a was re-assessed in experiment 1b, while separate cohorts of adult mice underwent experiments 2 and 3. Ovx= ovariectomized female. PND= post-natal day.

### ***Behavioral Testing***

As mentioned above, three behavioral experiments were carried out, using 3 independent cohorts of mice (Table 1). First, ultrasonic communication was evaluated on PND 4, 6, 8 and 10 in response to maternal separation (Experiment 1a), and again

at adulthood in response to an adult female intruder (Experiment 1b). Second, adult social interest and investigation were assessed respectively in the 3 compartment and direct social interaction tests (Experiment 2). Finally, adult mice were tested for anxiety-like behavior in the elevated plus maze and for acoustic startle response, i.e., two behavioral confounding variables potentially acting on ultrasonic communication and social behavior [e.g., (Webber, Mankin et al. 2013, Simola and Granon 2019)]. All behavioral procedures were based on experimental protocols used in our previous studies on genetic mouse models of ASD (Pietropaolo, Guillemot et al. 2011, Hebert, Pietropaolo et al. 2014, Pietropaolo, Goubran et al. 2014, Zhang, Bonnan et al. 2014, Oddi, Subashi et al. 2015, Gaudissard, Ginger et al. 2017, Gauducheau, Lemaire-Mayo et al. 2017). Behavioral tests were performed in adult mice with a 48h-interval between subsequent tests, and they were carried out by experimenters blind to animals' genotypes. Except for pups' assessment, male and female mice were tested on separate days, in order to avoid olfactory interference in the testing environment.

*Experiment 1a: assessment of isolation-induced ultrasonic vocalizations in pups*

USVs of  $CB1^{-/-}$ ,  $CB1^{+/-}$ , and  $CB1^{+/+}$  littermates were repeatedly assessed on PND 4, 6, 8, and 10, during a 3-min daily session at room temperature (22-24°C). Pups were taken individually from the nest in a random sequence and placed into a glass container (10x8x7 cm; open surface), containing clean bedding material (3 cm). USVs were captured by an UltraSoundGate Condenser Microphone CM 16 (Avisoft Bioacoustics, Berlin, Germany) placed 20 cm above the the bedding. The microphone used is sensitive to frequencies of 15 to 180 kHz with a flat frequency response ( $\pm 6$  dB) between 25 and 140 kHz. It was connected via an UltraSoundGate 116 USB audio

device (Avisoft Bioacoustics) to a personal computer, where acoustic data were recorded with a sampling rate of 250 kHz in 16-bit format by Avisoft RECORDER (version 2.97; Avisoft Bioacoustics). At the end of the 3 minute session, each pup was weighed and identified, while the container was cleansed with 70% EtOH solution and filled with clean bedding.

For acoustic analyses, recordings were transferred to Avisoft SASLab Pro (Version 5.20; Avisoft, Berlin, Germany) and a Fast Fourier transformation was applied (512 FFT length, 100% frame, Hamming window, and 75% time window overlap). Call detection was provided by an automatic threshold-based algorithm and a hold time (0.004 s) mechanism. The accuracy of call detection by the software was verified manually by an experienced user. Based on previous studies (Wohr, Rouillet et al. 2011), the number of USVs was computed, as well as their mean duration, peak frequency and peak amplitude. In addition call subtypes were determined by density plots depicting the distribution of total calls for each genotype at peak frequency versus peak amplitude, peak frequency versus duration, and peak amplitude versus duration, as described in details elsewhere (Wohr 2014, Mosienko, Beis et al. 2015).

*Experiment 1b: assessment of interaction-induced ultrasonic vocalizations in adults*

$CB1^{-/-}$ ,  $CB1^{+/-}$ , and  $CB1^{+/+}$  male and female littermates were then tested at adulthood in a 33x15x14 cm plastic cage with 3 cm of sawdust and a metal flat cover. Male experimental subjects were habituated to this apparatus for 15 min prior to testing, while female subjects were isolated in the testing cage for 72hs, in order to induce a status of resident in adult females and therefore promote the emission of USVs towards an adult female intruder (Moles, Costantini et al. 2007). An unfamiliar stimulus mouse (an adult

intact NMRI female) was then introduced into the testing cage of either male or female subjects and left there for 3 minutes. Previous studies have shown that in these experimental settings USVs are mainly emitted by the male mouse in the male-female interaction (Whitney, Coble et al. 1973, Warburton, Sales et al. 1989, Wang, Liang et al. 2008), and by the female resident in the female-female interaction (Maggio and Whitney 1985, Moles, Costantini et al. 2007). The ultrasonic microphone previously described was mounted 2 cm above the cover of the testing cage; subsequent scoring of USV parameters was performed following the same procedures described for experiment 1a. The estrus phase of adult females was assessed by analysis of vaginal smears (Caligioni 2009) performed on the testing day in both the experimental subjects and NMRI stimulus mice. The evaluation of  $CB1^{-/-}$ ,  $CB1^{+/-}$ , and  $CB1^{+/+}$  females was conducted after their testing, in order to minimize the potential stress effects of the manipulation necessary for determining the estrous phase. Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was equally distributed between genotypes and sexes, although the estrus phase of the stimulus female is known to have no influence on ultrasonic communication in mice of both sexes (Pomerantz, Nunez et al. 1983, Moles, Costantini et al. 2007, Kim, Son et al. 2016). The estrus phase of experimental female subjects included pro-estrus, estrus and diestrus, following a distribution that was balanced across genotypes.

#### *Experiment 2: assessment of social interest and social investigation in adult mice*

Mice of a second cohort were assessed first in the 3-compartment test for social interest and 48hs later in the direct social interaction; both tests used an ovariectomized NMRI

adult female as the social stimulus, since the estrous phase of the stimulus animal is known to affect social interest and investigation (Baudoin, Feron et al. 1991, Liu, Erkkila et al. 2010). The estrus phase of experimental female subjects was assessed as described in Experiment 1b, and no differences in the distribution of estrous phases were found between genotypes.

*Three compartment test for social interest:* The 3-compartment apparatus was made of transparent Plexiglas (its detailed description was provided elsewhere (Gauducheau, Lemaire-Mayo et al. 2017). Each side compartment contained a perforated stimulus cage (8 x 8 x 15cm) placed at a distance of 5.5cm from the side walls.

Each experimental animal was placed in the middle of the central compartment and allowed to explore the whole apparatus for 2 trials of 5 minutes each (Pietropaolo, Guillemint et al. 2011). On the first trial the stimulus cages were empty and the experimental mouse was left undisturbed to explore the apparatus and habituate to the testing environment. At the end of this trial, the experimental mouse was confined in the central compartment using two transparent Plexiglas magnetic doors for 40 seconds. On the second trial, a stimulus mouse (an ovariectomized NMRI adult female) was introduced in one of the stimulus cages, while a novel object (a glass red cylinder) was introduced in the other one. The position of the social stimulus and of the object was counterbalanced between genotypes. The apparatus and the stimulus cages were cleansed with 70% EtOH solution at the end of the second testing trial.

The time spent in each of the side compartments containing the stimulus cages was computed from the videofiles obtained from a camera placed above the center of the



apparatus. An experimenter blind to stimulus position and animals' sex and genotypes performed the analysis using Observer XT (version 7, Noldus, The Netherlands).

*Direct social interaction with an adult female:* Each experimental animal was confined in one of the side compartments of the 3-chambered apparatus and an unfamiliar stimulus NMRI female was introduced and left for 10 min. Testing sessions were recorded by a camera placed on the side of the compartment and videos analyzed with Observer XT. One observer who was unaware of the genotype and sex of the animals scored the behavior of the test mice, quantifying the time spent performing affiliative behaviors, i.e., sniffing the head and the snout of the partner, its anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming. Nonsocial activities were also evaluated: rearing (standing on the hind limbs sometimes with the forelimbs against the walls of the cage); digging; self-grooming (the animal licks and mouths its own fur).

*Experiment 3: Assessment of potential confounding non-social behavioral alterations in adult mice*

Mice of a third cohort were assessed first for anxiety-like behavior in the elevated plus maze and 48hs later in for acoustic startle response. The estrus phase of female subjects was assessed as described in Experiment 1b at the end of each behavioral test, and no differences in the distribution of estrous phases were found between genotypes. Prior to each test, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried with paper towels.

*Elevated Plus Maze* The maze described in details elsewhere (Pietropaolo and Crusio 2009, Pietropaolo, Guilleminot et al. 2011) was placed 55cm above floor level, in a quiet

testing room with diffuse dim lighting. A digital camera was mounted above the maze, and images were transmitted to a PC running the Ethovision (Verision 11, Noldus Technology, the Netherlands) tracking system. To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms and allowed to explore freely for 5 minutes. We measured the percent time in open arms as  $(\text{time}_{(\text{open arms})} / \text{time}_{(\text{open} + \text{closed arms})}) \times 100$ . Total distance moved was also assessed.

*Auditory Startle Response* The whole-body startle response to low intensity auditory stimuli was measured using startle response boxes (SR-LAB, San Diego Instruments), as described in details elsewhere (Gaudissard, Ginger et al. 2017). Briefly, mice were habituated to the boxes for 24 hr prior to testing for 5 min to reduce stress. On the days of testing, mice were presented with pulses of 20-ms duration and varying intensity: +6, +12, +18 and +24 dB over a white background noise at 66 dB (namely 72, 78, 84 and 90 dB). Startle reactivity was assessed by the scores obtained for the mean of trials for each stimulus level presented.

### ***Statistical Analysis***

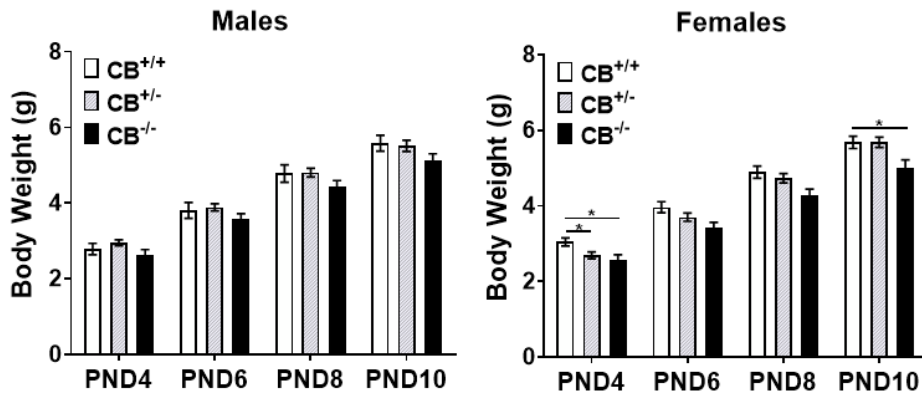
Data from experiment 1a were analyzed using an ANOVA with genotype and sex as between-subject factors, and day as within-subject factor. For all other experiments, data from males and females were analyzed separately using an ANOVA with only genotype as between-subject factor. These separate analyses were necessary as male and female mice had to be tested (i) on different days to avoid odor interference in all experiments, and (ii) using a different experimental protocol to allow USV detection in Experiment 1b.

Within-subject factors, i.e., stimulus compartment, 5-min-bins, stimulus intensities, were added to the ANOVAs of the data of social interest, social interaction and acoustic startle. Post-hoc comparisons using Fisher's PLSD test were performed when appropriate. To better conform to the assumptions of parametric ANOVA, a natural logarithmic transformation was applied to the startle reactivity scores (Experiment 3). All statistical analyses were performed using SPSS Statistics Version 25 (Tokyo, Japan) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA).

## Results

### ***Experiment 1a: assessment of isolation-induced ultrasonic vocalizations in mouse pups***

*CB1* mutation affected the body weight of mouse pups and this effect was detected only in females, where it differed across post-natal days [interaction sex x genotype x day:  $F_{6, 198} = 3.58$ ,  $p < 0.01$ , and interaction genotype x day in females:  $F_{6, 33} = 3.596$ ,  $p < 0.05$ ; Fig. 1]. On PND 4, both *CB1*<sup>+/-</sup> and *CB1*<sup>-/-</sup> females weighted less than their *CB1*<sup>+/+</sup> littermates, and this difference was still found on PND 10, but for *CB1*<sup>-/-</sup> pups only [post-hoc,  $p < 0.05$ ]. No significant genotype difference in body weight gain emerged in male pups [genotype and interaction genotype x day, all n.s].



**Figure 1. Effects of the *CB1* null mutation on body weight during development (Experiment 1a).** \* =  $p < 0.05$ . N (males)= 9 (*CB1*<sup>+/+</sup>), 14 (*CB1*<sup>+/-</sup>), 13 (*CB1*<sup>-/-</sup>). N (females)= 13 (*CB1*<sup>+/+</sup>), 17 (*CB1*<sup>+/-</sup>), 6 (*CB1*<sup>-/-</sup>). Data are mean $\pm$ SEM.

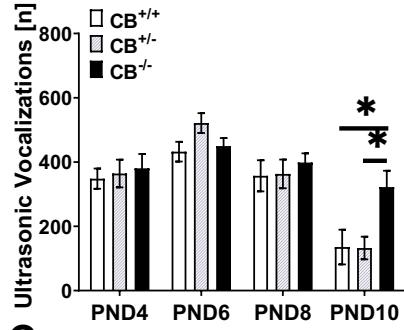
All USV parameters followed a developmental pattern, with changes across PNDs. As expected, the number of USVs emitted by pups of both sexes showed a peak occurring on PND 4 and 6 followed by a decrease on PND 8 and PND 10 [day effect:  $F_{3, 198} = 44.272$ ,  $p < 0.0001$ ; Fig. 2-A and B ). This pattern was altered in *CB1* mutants, with slight differences between sexes [genotype x day:  $F_{6, 198} = 2.645$ ,  $p < 0.05$ ; sex x genotype x day:  $F_{6, 198} = 2.309$ ,  $p < 0.05$ ]. The most prominent decrease was observed in males on PND 10, and it was less marked in *CB1*<sup>-/-</sup> littermates only [genotype x day in males  $F_{6,99} = 2.674$ ,  $p < 0.05$ ; post-hoc:  $p < 0.05$ ; Fig. 2-A], while in females it was observed on PND 8 and it was attenuated in both *CB1*<sup>+/-</sup> and *CB1*<sup>-/-</sup> animals [genotype x day in females  $F_{6,99} = 2.95$ ,  $p < 0.05$ ; post-hoc:  $p < 0.05$ ; Fig. 2-B].

A similar pattern was detected also on USV duration, with a peak occurring on PND 4-6 and a reduction afterwards [day effect:  $F_{3,198} = 19.13$ ,  $p < 0.0001$ ; Fig. 2-C and D]. This pattern was more marked in male than in female mice, with a more dramatic decrease

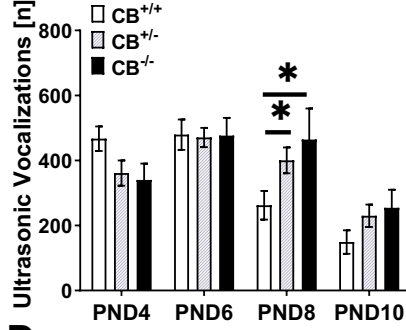
in call duration on the last days in males [interaction sex x day:  $F_{3,18} = 5.67$ ,  $p < 0.01$ ], where it tended to be attenuated in  $CB1^{-/-}$  mice [interaction genotype x day in males:  $F_{6,99} = 2.75$ ,  $p < 0.05$ ; post-hoc:  $p < 0.05$ , Fig. 2-C; in females, genotype effects or its interactions: all n.s., Fig. 2-D].

The peak frequency of the calls increased on PND 8 and 10, and this pattern differed between sexes and genotypes [genotype x sex:  $F_{2,66} = 3.07$ ,  $p = 0.05$ ; Fig. 2-E and F]. The highest increase in peak frequencies was observed in males on PND10 and this was markedly reduced in  $CB1^{-/-}$  pups only [interaction genotype x day in males:  $F_{6,33} = 10.463$ ,  $p < 0.0001$ ; Fig. 2-E], while in females it was detected already on PND 8, and it was almost absent in both  $CB1^{-/-}$  and  $CB1^{+/+}$  pups [interaction genotype x day in females:  $F_{6,33} = 4.989$ ,  $p < 0.05$ ; Fig. 2-F]. The peak amplitude of USVs tended instead to decrease (softer calls) on PND8 and PND10 [day effect:  $F_{3,198} = 155.959$ ,  $p < 0.0001$ ; Fig. 2-G and -H], with no differences between sexes and genotypes [all effects and interactions, n.s.].

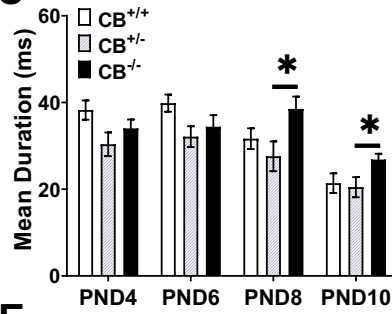
### A Males



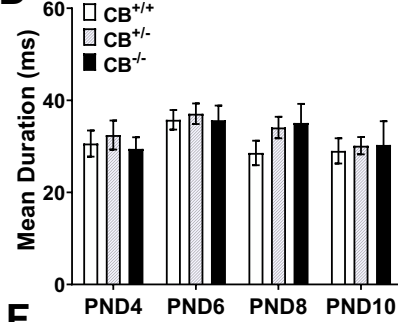
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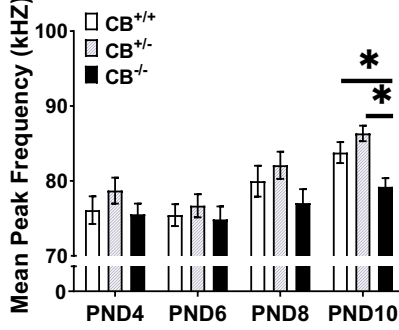
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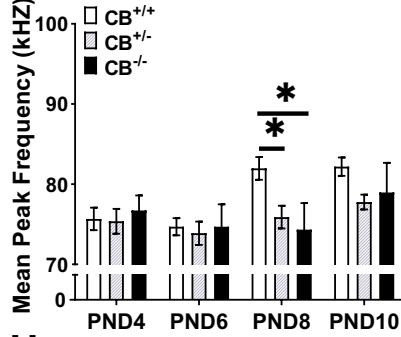
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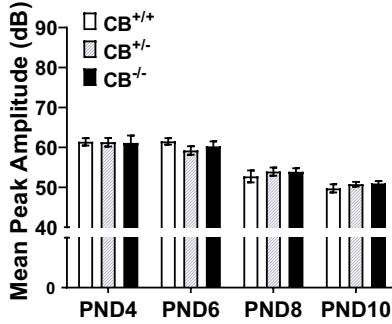
### E



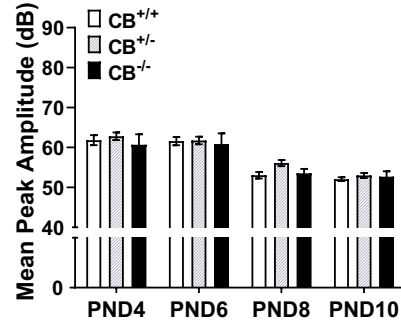
### F



### G

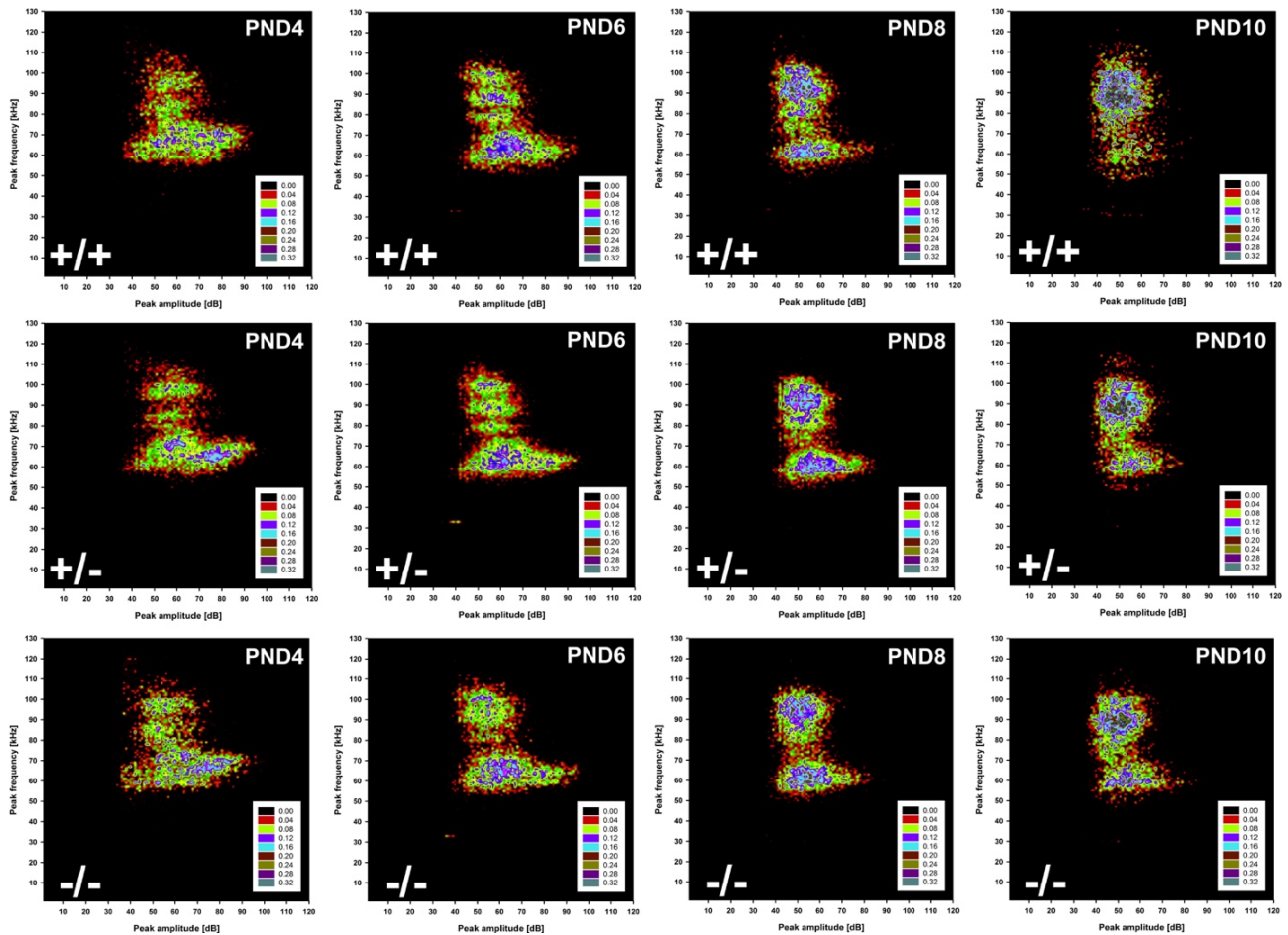


### H



**Figure 2. Effects of the *CB1* null mutation on ultrasonic vocalization (USV) during development in mouse pups.** Isolation-induced USVs were analyzed in terms of quantitative (A, B) and qualitative (C to H) parameters. \* =  $p < 0.05$ . N (males)= 9 (*CB1<sup>+/+</sup>*), 14 (*CB1<sup>+/-</sup>*), 13 (*CB1<sup>-/-</sup>*). N (females)= 13 (*CB1<sup>+/+</sup>*), 17 (*CB1<sup>+/-</sup>*), 6 (*CB1<sup>-/-</sup>*). Data are mean $\pm$ SEM.

In a subsequent detailed analysis based on 28 756 calls emitted by *CB1<sup>+/+</sup>* pups, 44 724 calls by *CB1<sup>+/-</sup>* pups, and 31 452 calls by *CB1<sup>-/-</sup>* pups, different clusters of isolation-induced USVs were revealed by density plots (Fig. 3). In *CB1<sup>+/+</sup>* mice a single cluster was identified on PND 4, most USVs being characterized by peak frequencies between 60 and 70kHz. On PND 6, a second cluster between 80 and 100kHz appeared, became more prominent on PND 8, and included most USVs as a single cluster on PND10. A similar pattern was observed in *CB1<sup>+/-</sup>* and *CB1<sup>-/-</sup>* pups except on PND10, when both mutants continued to produce the majority of their USVs in two distinguishable clusters. This effect was found in both male and female mice

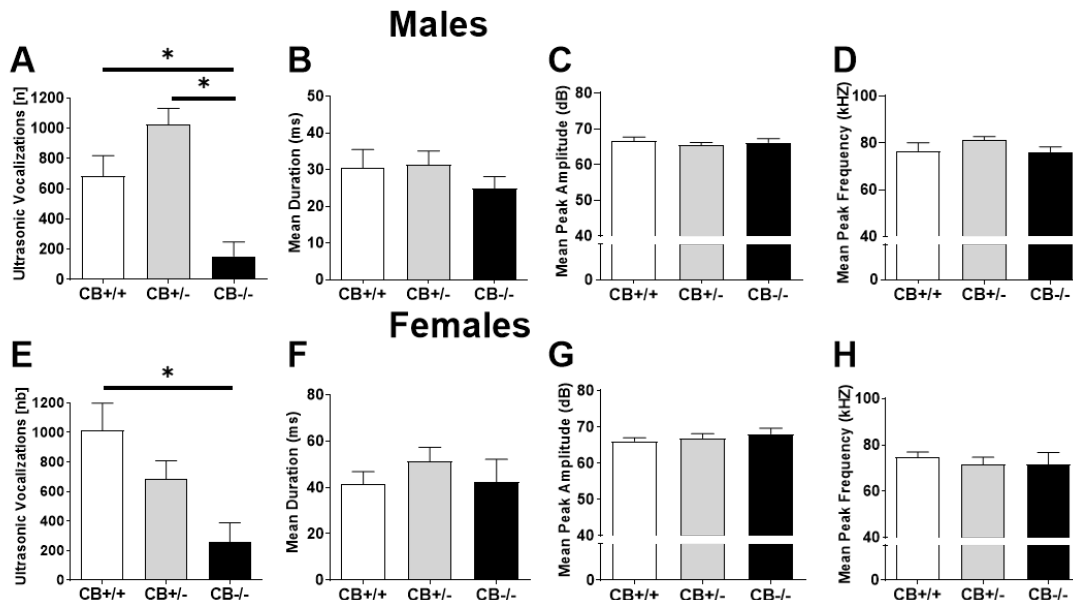


**Figure 3. Density plots depicting the distribution of individual isolation-induced ultrasonic vocalizations by postnatal day (PND) in mouse pups. Distribution of individual USVs depending on peak frequency in kHz and amplitude in dB in  $CB1^{+/+}$ ,  $CB1^{+/-}$  and  $CB1^{-/-}$  littermates. Color coding reflects frequency in percentages. Pooled data for both sexes are represented, as no difference between males and females was detected.**



**Experiment 1b: assessment of interaction-induced ultrasonic vocalizations in adult mice**

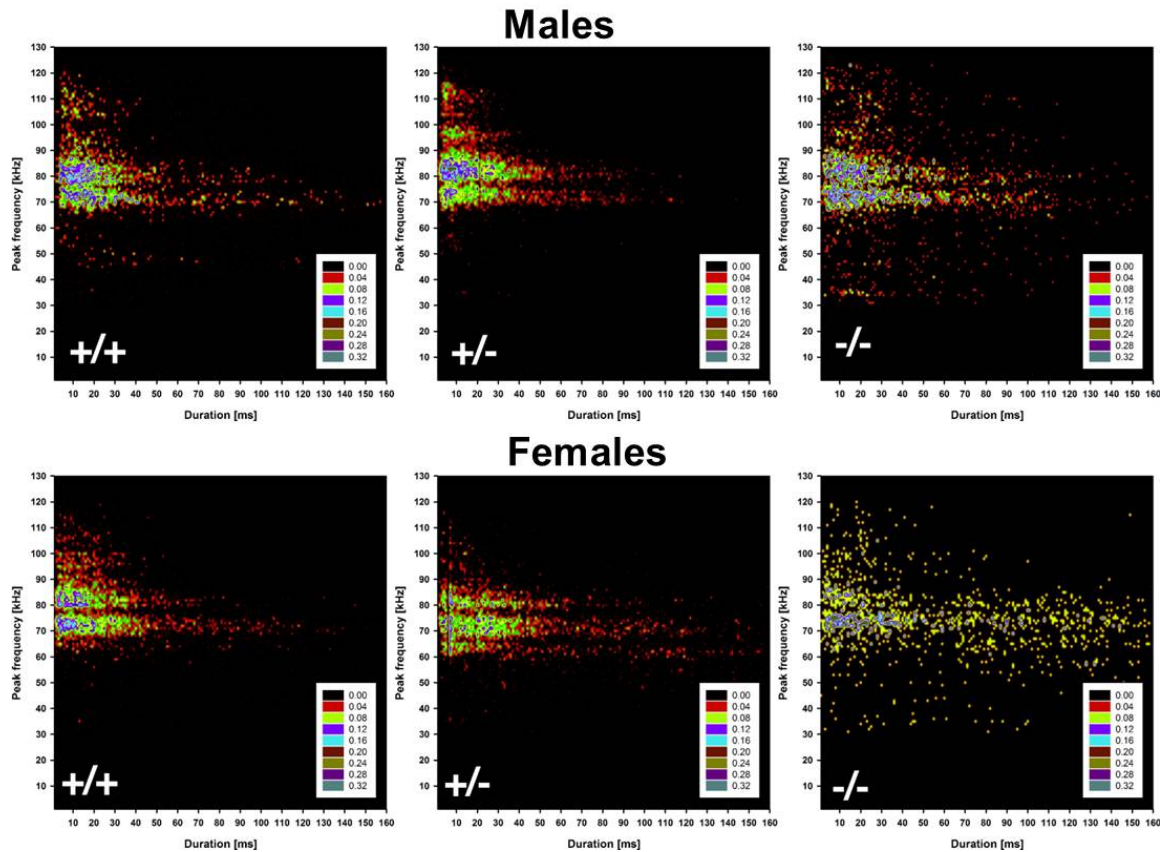
The *CB1* mutation affected the number of USVs produced at adulthood by both males and females [genotype effect, respectively:  $F_{2,21} = 15.89, 4.06$ , and  $p < 0.05$ ; Fig. 4-A and -E], with *CB1*<sup>-/-</sup> mice emitting less USVs than their *CB1*<sup>+/+</sup> and *CB1*<sup>+/-</sup> littermates [post-hoc:  $p < 0.05$ ]. No differences in other parameters, including duration, peak frequency and peak amplitude were detected in either sex [all genotype effects, n.s.; Fig. 4-B, C, D, F, G, H].



**Figure 4. Effects of the *CB1* null mutation on interaction-induced ultrasonic vocalization (USV) in adult mice.** USVs were analyzed in male and female mice during a 3-min session of direct social interaction with an intact NMRI stimulus female. Female experimental subjects were single caged for 72hs prior testing, in order to promote their USV production towards the NMRI intruder. USVs were analyzed in terms of quantitative (A, B) and qualitative (C to H) parameters. \* =  $p < 0.05$ . N (males)= 6 (*CB1*<sup>+/+</sup>), 11 (*CB1*<sup>+/-</sup>), 7 (*CB1*<sup>-/-</sup>). N (females)= 8 (*CB1*<sup>+/+</sup>), 11 (*CB1*<sup>+/-</sup>), 5 (*CB1*<sup>-/-</sup>). Data are mean±SEM.

Stimulus NMRI females were approximately half in diestrus and half in estrus phases, and their assignment to social encounters was balanced between genotypes and sexes. In males, no significant main effect of the estrous phase of the stimulus females ( $F_{1,18} = 1.24, 0.51, 0.03, 1.17$ , for number, mean duration, peak amplitude and peak frequency, all n.s.) or its interaction with genotype ( $F_{2,18} = 0.09, 1.02, 0.79, 0.73$ , for number, mean duration, peak amplitude and peak frequency, all n.s.) was detected on any USV parameter. In females, similar results on the impact of the estrous phase of the stimulus animals were obtained, with no main effect ( $F_{1,18} = 0.03, 0.01, 0.62, 0.05$ , for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ( $F_{2,18} = 0.03, 1.13, 1.4, 0.83$ , for number, mean duration, peak amplitude and peak frequency, all n.s.). The estrus phase of experimental female subjects included pro-estrus, estrus and diestrus, following a distribution that was mostly balanced across genotypes. The estrous phase of the experimental subjects did not induce any significant main effect ( $F_{2,15} = 0.29, 0.94, 1.14, 1.12$ , for number, mean duration, peak amplitude and peak frequency, all n.s.) or interaction with genotype ( $F_{4,15} = 0.07, 0.69, 0.93, 1.89$ , for number, mean duration, peak amplitude and peak frequency, all n.s.) on all considered USV parameters.

As in experiment 1a, a detailed analysis (Fig. 5) was performed in males based on 4106 calls for *CB1<sup>+/+</sup>*, 11289 calls for *CB1<sup>+/-</sup>*, 1082 calls for *CB1<sup>-/-</sup>* mice, and in females based on 9237 calls for *CB1<sup>+/+</sup>*, 8600 calls for *CB1<sup>+/-</sup>*, and 1283 calls for *CB1<sup>-/-</sup>* mice. In *CB1<sup>+/+</sup>* males the majority of calls were clustered between 70 to 85kHz for peak frequency and 5 to 25ms for the mean duration; while *CB1<sup>+/-</sup>* littermates exhibited a similar pattern, *CB1<sup>-/-</sup>* males showed substantially more variation in their calls in both mean peak frequency and duration, with the majority of calls occurring in clusters between 65 to 90kHz and durations between 5 to 50ms. In *CB<sup>+/+</sup>* females most USVs were distributed in two clusters, one between 70-75 kHz and another between 80- 85 kHz, both with durations between 5 and 40ms. These two clusters were less distinguishable in *CB<sup>+/-</sup>* females, and tended to disappear in *CB1<sup>-/-</sup>* mice, emitting USVs with a wider variation in both mean peak frequency and duration.



**Figure 5. Distribution of individual interaction-induced ultrasonic vocalizations (USV) in adult males and females.** Density plots depict the distribution of individual USV emitted during 3-min social interaction with an intact NMRI adult female, plotted by frequency in kHz and duration in ms. Color coding reflects frequency in percentages.

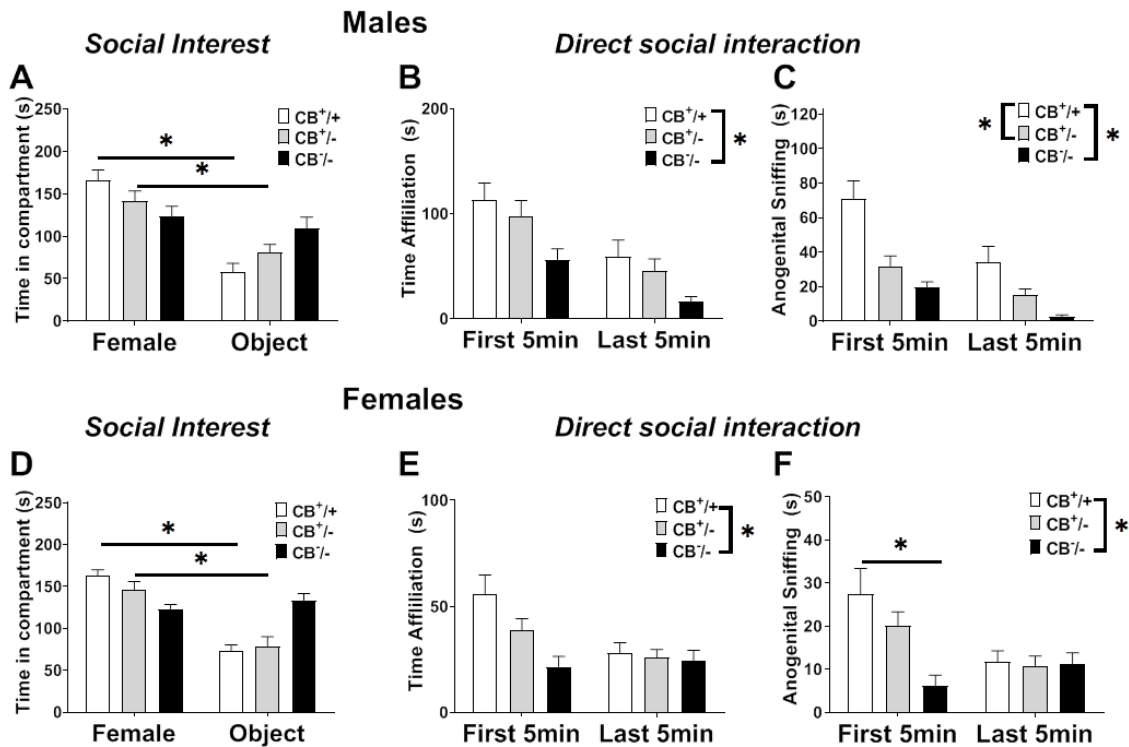
### ***Experiment 2: assessment of social interest and social investigation in adult mice***

In the 3 compartment test, CB1 mutation affected social interest in both sexes

One  $CB1^{+/-}$  and a  $CB1^{+/+}$  mouse, both females, were excluded respectively from the analysis of the data of the 3 chamber and direct social interaction tests because of problems in video recording (the precise n for each test is described in the legend of [Fig.6](#)). In the 3--chamber test, CB1 mutation affected social interest in both sexes [interaction genotype x compartment:  $F_{2,20} = 5.32$  and  $F_{2,22} = 12.04$ ,  $p < 0.05$ , respectively in males and females; Fig. 6-A and -D].  $CB1^{+/+}$  and  $CB1^{+/-}$  mice showed a clear preference for the compartment containing the

social stimulus the [compartment effect:  $F_{1,7} = 46.91$  and  $F_{1,9} = 10.97$ ,  $p < 0.01$ , respectively in  $CB1^{+/+}$  and  $CB1+/-$  males, and  $F_{1,11} = 43.66$  and  $F_{1,6} = 16.73$ ,  $p < 0.01$ , respectively in  $CB1^{+/+}$  and  $CB1^{-/-}$  females], while social interest was absent in  $CB1^{-/-}$  adult males and females [compartment effect:  $F_{1,4} = 0.49$  and  $F_{1,5} = 3.09$ , n.s., respectively in male and female mutants].

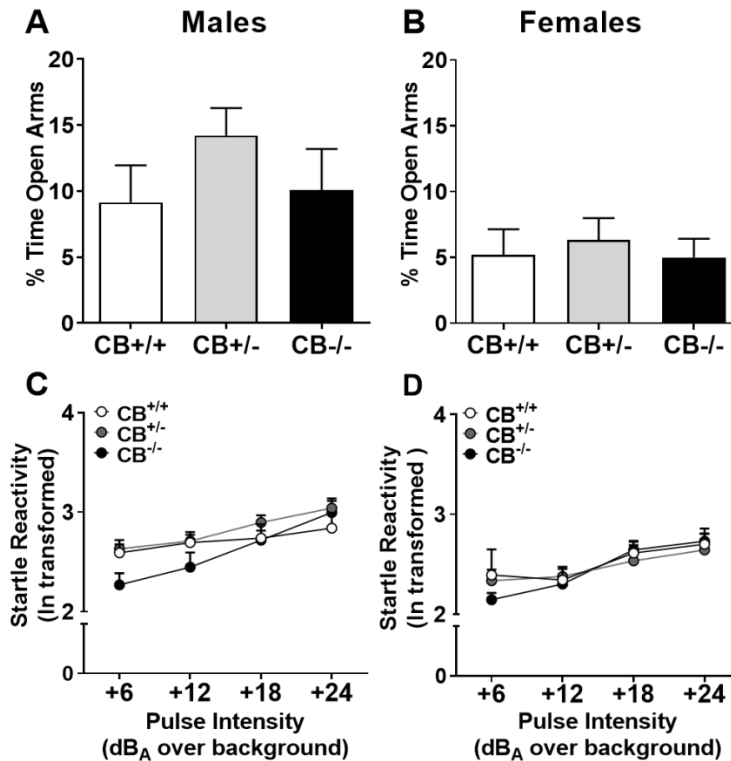
In the direct social interaction test, all mice displayed social habituation, as demonstrated by the reduced time spent in affiliation from the first to the last 5-min of the testing session [effect of 5-min bins,  $F_{1,19} = 28.19$ ,  $F_{1,22} = 4.51$ ,  $p < 0.05$ , respectively, in males and females; Fig. 6-B and E].  $CB1$  mutation reduced social investigation, as demonstrated by the reduced time spent in affiliation by  $CB1^{-/-}$  males and females compared to their littermates [genotype effect, respectively:  $F_{2,19} = 3.61$  and  $F_{2,22} = 4.42$ ,  $p < 0.05$ ; post-hoc:  $p < 0.05$ ; Fig. 6-B and E]. In mice of both sexes, this effect was mainly due to a reduction in the time spent performing anogenital sniffing (Fig. 6-C and -F); in males, this reduction was observed in both  $CB1^{+/+}$  and  $CB1^{-/-}$  mice during the entire duration of the test [genotype effect:  $F_{2,19} = 11.62$ ,  $p < 0.001$ ; post-hoc:  $p < 0.05$ ; Fig. 6-C]. In females, reduced anogenital sniffing was detected only in  $CB1^{-/-}$  mice and during the first 5 min of the test [genotype effect:  $F_{2,22} = 4.45$ ,  $p < 0.05$ ; interaction genotype x 5-min bin:  $F_{2,22} = 4.39$ ,  $p < 0.05$ ; post-hoc:  $p < 0.05$ ; Fig. 6-F]. No difference among experimental groups was found on non-social behaviors (data not shown).



**Figure 6. Effects of the *CB1* null mutation on social interest and investigation in adult mice.** Social interest (A, D) was assessed in the 3-compartment test. Social investigation was evaluated 48hrs afterwards during a 10-min session of direct social interaction. Both tests used an unfamiliar ovariectomized NMRI stimulus female. \* =  $p < 0.05$ . N (males)= 8 (*CB1*<sup>+/+</sup>), 10 (*CB1*<sup>+/-</sup>), 5 (*CB1*<sup>-/-</sup>). N (females)= 11 (*CB1*<sup>+/+</sup>), 8 (*CB1*<sup>+/-</sup>), 6 (*CB1*<sup>-/-</sup>). Data are mean±SEM.

### **Experiment 3: Adult assessment of potentially confounding non-social alterations**

In the elevated plus maze, the *CB1* mutation in males or females did not result in behavioral differences either in the percent time in the open arms [all genotype effects, n.s.; Fig.7-A and B], or in the total distance traveled (data not shown). Similarly, no genotype differences were detected in acoustic startle response in both sexes, with only an overall expected effect of pulse intensity [ $F_{3, 72} = 12.529$ ,  $F_{3, 93} = 16.49$ ,  $p < 0.0001$  in males and females, respectively; all genotype effects, n.s.; Fig.7-C and D].



**Figure 7. Effects of the *CB1* null mutation on confounding non-social behaviors in adult mice.** Anxiety-like behavior (A, B) in the elevated plus maze and acoustic startle response (C, D) were assessed in adult (3-months old) mice. N (males)= 9 (*CB1*<sup>+/+</sup>), 16 (*CB1*<sup>+/-</sup>), 9 (*CB1*<sup>-/-</sup>). N (females)= 7 (*CB1*<sup>+/+</sup>), 9 (*CB1*<sup>+/-</sup>), 11 (*CB1*<sup>-/-</sup>). Data are mean±SEM.

## Discussion

Our data demonstrate that the *CB1* mutation affects ultrasonic communication, both during development and at adulthood, as well as social interest/investigation at adult age. These ASD-relevant behavioral alterations were observed in both male and female mice, and overall seemed more marked in *CB1*<sup>-/-</sup> than *CB1*<sup>+/-</sup> mutants. Importantly, the adult USV alterations were not confounded by differences in anxiety or acoustic response, as assessed by the elevated plus maze and acoustic startle tests. These findings provide further evidence to support the role of the ECS in the etiopathology of

ASD and its relevance as a therapeutic target for autistic pathologies. Our data also suggest the validity of the *CB1* null mouse line for preclinical studies on ASD, but also on the neurobiological mechanisms involved in the general control of social behaviors and communication.

The present study performed for the first time a comprehensive analysis of ultrasonic communication in *CB1* mutants during early post-natal development (between PND 4 and 10) and at adulthood (at 3 months of age). The alterations in USVs observed in *CB1* mutants were both quantitative and qualitative at both time points. During the postnatal period both male and female *CB1*<sup>-/-</sup> pups showed altered day-dependent patterns of expression of multiple USV parameters. These patterns included the typical inverted U-shaped profile (Branchi, Santucci et al. 2001) characterizing the number and mean duration of calls produced by *CB1*<sup>+/+</sup> mouse pups, with a peak around PND 6, followed by a reduction on PNDs 8 and 10 (Fig. 2-A, B, C and D). While *CB1*<sup>-/-</sup> pups demonstrated a similar peak in USV rate and duration, they did not show a comparable reduction on the following days. This finding may suggest a delay in the development of communication abilities in *CB1*<sup>-/-</sup> pups, a finding supported by the analysis of other parameters of pups' USVs. Indeed, USV mean peak frequencies also followed a clear developmental pattern, increasing from PND 4 to PND 10 (Fig. 2-E and F), but this linear increase was markedly reduced in *CB1*<sup>-/-</sup> male and female pups. Furthermore, density plots revealed in *CB1*<sup>+/+</sup> pups the presence at PND 4 of a single cluster of lower mean peak frequency calls (50-70kHz), associated at PND 6 and 8 with a second cluster of higher frequency calls (80-100kHz), and disappearing on PND 10, when only the higher frequency cluster remained (Fig. 3). In *CB1*<sup>+/+</sup> and *CB1*<sup>-/-</sup> pups both the high



and low frequency clusters were instead still evident at PND 10; this finding resembles the pattern observed in the *Shank* mouse model of ASD (Sungur, Schwarting et al. 2016), and further supports a delay in the communication abilities of *CB1* mutants.

The hypothesis of a general developmental delay in *CB1* mutants is further supported by their reduced body weight gain during the first ten post-natal days (Fig. 1); nonetheless, this reduced body growth, that was previously described (Fride, Ginzburg et al. 2001, Fride, Suris et al. 2005), is unlikely to directly explain the alterations in USV emission rates of *CB1* mutant pups. First, body weight differences were mostly observed in female mutant pups and already at PND 4, while USV alterations were detected in both sexes and mostly starting at PND 8. Second, reductions in body weight are thought to lead to reduced emission rates of USVs because of the decreased pulmonary-thoracic size (Scattoni, Gandhi et al. 2008), while here an increase in USV number was observed on the last post-natal days (Fig. 2-A and -B). It is instead possible that an overall developmental delay in terms of reflexes and neuro-physiological development may be associated with the USV quantitative and qualitative alterations found in *CB1* mutant pups; future studies evaluating in depth developmental milestones (Branchi, Santucci et al. 2004) are needed to assess this issue that is, to our knowledge, still unknown in these mouse mutants.

An alternative explanation for USV alterations in *CB1* pups may involve their altered response to stress, as USVs were assessed following a short maternal separation; several studies demonstrated an abnormal behavioral response of *CB1* null mice to

stressors in general (Miller, Ward et al. 2008, Busquets-Garcia, Gomis-Gonzalez et al. 2016), often accompanied by enhanced neuro-endocrine reactivity (Barna, Zelena et al. 2004). However, previous data demonstrated a reduced response to acute stress in *CB1*<sup>-/-</sup> pups during early development (Fride, Suris et al. 2005). Moreover, the persistence of USV alterations into adulthood in our *CB1* mutants strongly supports the presence of a genuine deficit in communication abilities in these mutants, which was indeed confirmed in the non-stressful context of direct social interaction with an adult female.

Male and female *CB1*<sup>-/-</sup> mice showed in fact again at adulthood quantitative and qualitative USV alterations, including a reduction in the call rate (Fig. 4-A and -E), and higher variations in the peak frequency and mean duration of the calls (i.e. reduced clustering), as revealed by the analysis of the density plots (Fig. 5). The USV reduced rate is in line with what observed in other ASD mouse models, e.g., the BTBR (Scattoni, Ricceri et al. , McFarlane, Kusek et al. 2008), *Shank* (Ey, Leblond et al. 2011, Schmeisser, Ey et al. 2012) or *Fmr1*-KO mouse lines (Rotschafer, Trujillo et al. 2012), thus supporting the relevance of this quantitative USV alteration as an ASD-like phenotype. Interestingly, the USV alterations of *CB1* mutants were not accompanied by altered anxiety or reduced startle response, as found in other mouse models for ASD (Pietropaolo and Subashi 2014, Yang, Mahrt et al. 2015). In fact here *CB1* mutants did not differ from their WT littermates in the elevated plus maze and acoustic startle tests (Fig. 7), in line with previous studies showing that behaviors in these tests were not consistently and robustly affected by *CB1* homozygous deletion (Haller, Bakos et al. 2002, Haller, Varga et al. 2004, Marongiu, Poddie et al. 2012). While the USV

alterations observed in *CB1* male and female mutants at adulthood were not linked to emotional or acoustic abnormalities, they were instead associated with deficits in social interest in the three compartment test and in social investigation (Fig. 6); interestingly, the genotype differences were more marked on anogenital sniffing, a behavior that has been shown to positively correlate with USVs rate in adult mice (Nyby 1983, Moles, Costantini et al. 2007). This finding, together with the presence of social and USV alterations in both sexes, suggests that *CB1* mutation may affect social interactions and communication by specifically acting on the general sociability of mice.

The deficits showed in social interest and behavior in adult *CB1* mutants are in agreement with previous reports on *CB1*<sup>-/-</sup> male mice (Haller, Varga et al. 2004, Haring, Kaiser et al. 2011, Litvin, Phan et al. 2013, Terzian, Micale et al. 2014), even with other type of stimuli and genetic backgrounds, thus confirming these as a robust behavioral phenotype of *CB1* null mice. Also, similar deficits in USVs rate and social investigation (again, especially on anogenital sniffing) were observed following pharmacological *CB1* inhibition in male mice, in a dose-dependent manner. To our knowledge, this is the first time that USV and social alterations are described also in *CB1*<sup>+/-</sup> mice, also including female subjects; here, females seem more sensitive to the early effects of *CB1* mutation, since during development *CB1*<sup>+/-</sup> females (but not males) differed in body weight and USVs from WT littermates similarly to *CB1*<sup>-/-</sup>. At adulthood, “a dosage” effect of *CB1* mutation seemed evident in males and females, with one allele somehow protecting from the effects of *CB1* deletion, and *CB1*<sup>+/-</sup> positioning between *CB1*<sup>-/-</sup> and *CB1*<sup>+/+</sup>. Despite a traditional focus on the male sex, ASD-research is indeed increasingly interested in evaluating pathological behavioral phenotypes also in females subjects, as

ASD female patients may have unique clinical presentations relative to their male counterparts, a factor that may have led to under diagnosis of ASD in the female sex (Loomes, Hull et al. 2017). Hence, the presence of communication and social phenotypes in *CB1* mutant females add to the value of the *CB1* null mouse to study ASD, an issue that is receiving increasing attention in preclinical research on this pathology (e.g., studies in female *Fmr1*-KO mice modelling ASD, (Gauducheau, Lemaire-Mayo et al. 2017)).

In conclusion, our data support the use of the *CB1* null mouse in pre-clinical research on ASD. The lack of non-social alterations, i.e., emotional or acoustic abnormalities, does not undermine the validity of *CB1* mutants to study ASD, although they may be considered ASD-like phenotypes; first, because recapitulating the full ASD-like phenotypes is increasingly considered an unrealistic and unnecessary goal of mouse models (Crawley 2004, Moy, Nadler et al. 2006, Crawley 2007), second because it allows to rule out important confoundings potentially acting on on social and communication behaviors. Thus, the *CB1* null mouse may be instrumental in specifically investigating the neurobiology of social behaviors and communication, i.e., the core ASD symptoms, without including other non-social symptoms. This approach is particularly suitable to the *CB1* null model, because of the availability of mutant *CB1* mouse lines with region- and cell-specific deletions (Marsicano and Lutz 1999, Bellocchio, Lafenetre et al. 2010, Hebert-Chatelain, Reguero et al. 2014, Busquets-Garcia, Gomis-Gonzalez et al. 2016, Oliveira da Cruz, Robin et al. 2016, Robin, Oliveira da Cruz et al. 2018), allowing dissecting the behavioral role of *CB1* according to its expression site (e.g., glutamatergic, gabaergic, dopaminergic neurons, in the whole cell

or mitochondria only). Hence, future studies combining region-and cell-specific deletions of *CB1* will be able to identify the structures and circuits responsible for the social communication deficits, thus providing novel avenues for research on ASD.

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### **3.2 Study 2**

**Pharmacological inhibition of the primary endocannabinoid producing enzyme, DGL- $\alpha$ , induces ASD-like and co-morbid ASD phenotypes in adult C57BL/J mice**

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The authors have declared no conflict of interests exists

## **Abstract:**

Accumulating evidence links dysfunction in the endocannabinoid system (ECS) with the pathology of neurodevelopmental disorders, particularly Autism Spectrum Disorder (ASD). Variants in endocannabinoid system genes *CNR1* and *DAGLA* have been associated with neurological phenotypes in humans. The endocannabinoids (eCBs), 2-AG and AEA, which act at the primary cannabinoid receptor (CB1), mediate behaviors relevant to neurodevelopmental disorders. The overlap between these two eCBs is poorly understood. Most ECS studies have focused on stress responses, anxiety, and epilepsy, however its role in social behavior and communication has only recently come under investigation. This represents a critical gap in our understanding of the ECS and its relationship to ASD. Furthermore, the increasing prevalence of ASD and a lack of therapeutics emphasize a crucial need for novel therapeutic targets. To this aim, we used a highly selective inhibitor of the eCB producing enzyme DGL- $\alpha$ , DO34, and the CB1 inverse agonist, rimonabant, to evaluate the role of the primary eCB, 2-AG, in ASD. Adult male C57BL/6J mice were used in a series of behavioral paradigms which assessed social behavior, social communication, repetitive behaviors, anxiety and locomotor activity. Both DO34 and rimonabant increased anxiety-like behavior, while only DO34 induced hyperactivity, social deficits, and repetitive self-grooming behavior. These data indicate that reduced 2-AG bioavailability, but not CB1 inhibition, play a role in behavioral phenotypes relevant to neurodevelopmental disorders, particularly ASD. This suggests there are fundamental differences in CB1 signaling, particularly for social behaviors, and that 2-AG signaling may represent a target for the development of novel therapeutics.

**Lay Summary:** Endocannabinoids play a critical role in the developing nervous system. Alterations in the endocannabinoid system are linked to neurodevelopmental disorders. Studies suggest these variants may play a critical role in the core symptoms of autism spectrum disorder. In this study pharmacological inhibition of the primary endocannabinoid producing enzyme, DGL- $\alpha$ , induced a constellation of deficits in behavioral domains associated with autism.

**Keywords:** DGL- $\alpha$ , endocannabinoids, autism spectrum disorders, mouse models, neurodevelopmental disorders

## **Introduction**

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in social behavior and communication which co-occur with restricted, repetitive patterns of behavior, interests or activities (American Psychiatric Association, 2013). Epidemiological data indicates a notable increase in the prevalence of ASD from 1 in 150 children between 2002-2010 to 1 in 59 children in 2014 (Baio, 2018). This dramatic increase has placed a large clinical and financial demand on the public healthcare system (Leigh & Du, 2015). This underscores that identification of causes and therapeutic targets are a public health priority.

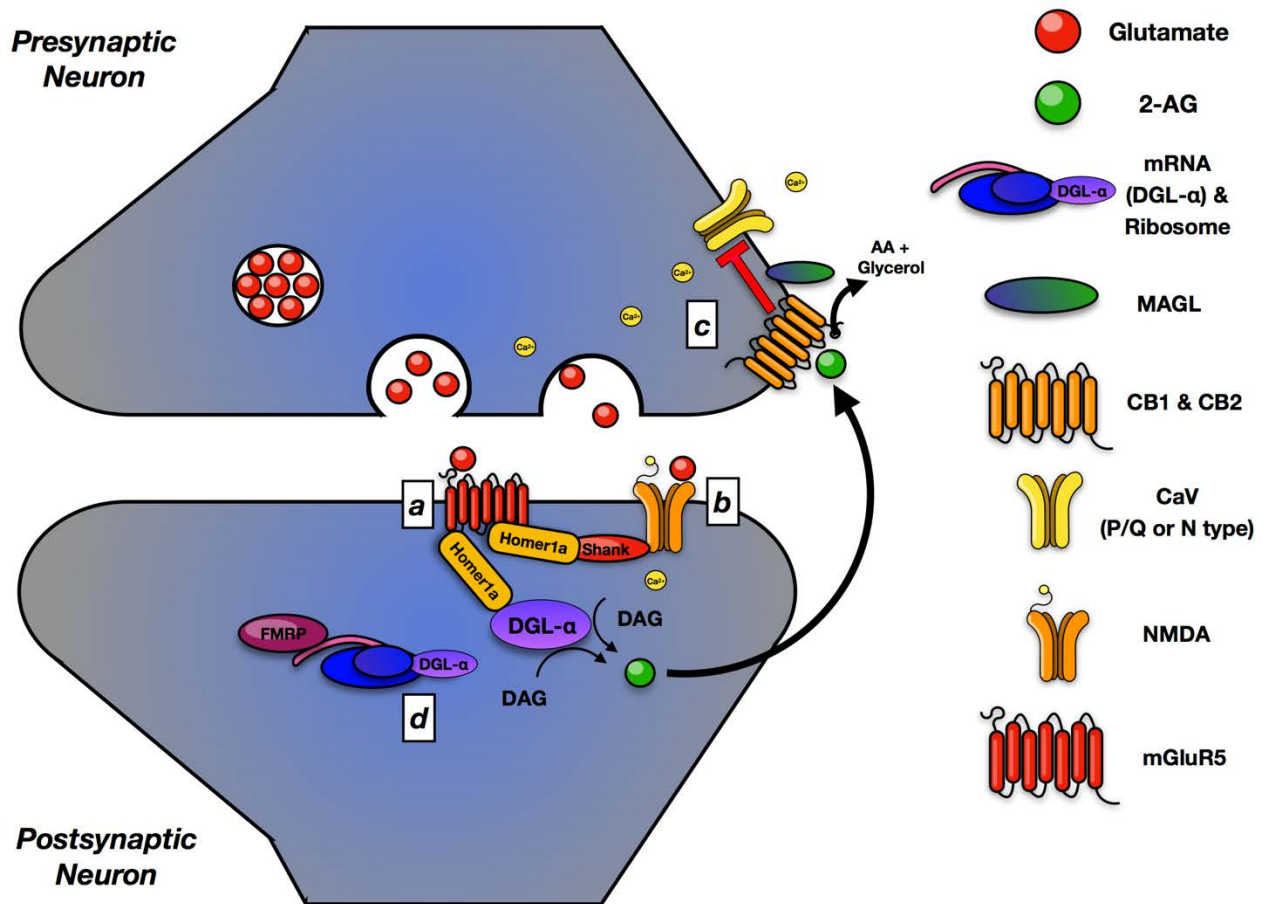


Recent studies have revealed a relationship between alterations in the endocannabinoid system (ECS) and patients with non-syndromic ASD or syndromic ASD, such as Fragile X Syndrome (FXS) (Aran et al., 2019; Karhson et al., 2018; Smith et al., 2017). Clinical studies have found lower levels of circulating endocannabinoids in ASD patients and post-mortem studies found lower expression levels of the primary cannabinoid receptor (CB1) (Aran et al., 2019; Karhson et al., 2018). Genetic studies detected associations between neurodevelopmental disorders and genetic variants in the genes for CB1 (*CNR1*) and the primary endocannabinoid (eCB) producing enzyme diacylglycerol lipase alpha (*DAGLA:DGL- $\alpha$* ) (Miller et al., 2010; Prasad et al., 2012; Smith et al., 2017). Furthermore, variants in genes which are critical for intact eCB signaling, but not considered part of the ECS itself, such as Fragile X Mental Retardation Protein (*FMR1*), metabotropic glutamate receptors (*GRM5*), Neuroligin (*NLGN3*, *NLGN4*), Shank (*SHANK3*), and Homer (*H1a*, *H2a*) are associated with ASD (Foldy et al., 2013; Laumonnier et al., 2004; Wang et al., 2016; Wenger et al., 2016; Yan et al., 2005).

Preclinical studies with mouse models of ASD have partially identified the role ECS dysfunction may play in ASD pathology. Studies with mouse models of the *NLGN3* mutation detected altered eCB activity, deficits in social behavior, ultrasonic vocalization (USV) production, sensorimotor responses, reversal learning, and increased locomotor behavior (Chadman et al., 2008; Foldy et al., 2013; Jaramillo et al., 2014). Importantly, Chadman et al. (2008) detected delayed neurological reflex and growth development in this model. Studies with the fragile X syndrome (FXS) mouse model (*fmr1*-KO) revealed that FMRP, the protein lost due to the fragile X mutation, regulates the translation and

localization of DGL- $\alpha$  at the post synaptic density (Jung et al., 2012; Maccarrone et al., 2010). Loss of FMRP resulted in delocalization of DGL- $\alpha$  and 2-AG, the eCB produced by DGL- $\alpha$  (Jung et al., 2012). This study found that increasing the bioavailability of 2-arachidonoylglycerol (2-AG) with an inhibitor of the 2-AG deactivating enzyme, monoacylglycerol lipase (MAGL), rescued the hyperactivity and reduced anxiety-like behavior seen in the *Fmr1*-KO mouse. Subsequent studies demonstrated that the scaffolding protein Homer, a protein correlated with ASD, is integral for appropriate 2-AG production (Ronesi et al., 2012; Ronesi & Huber, 2008).

DGL- $\alpha$  forms 2-AG through the hydrolysis of 1,2-diacylglycerol (DAG). 2-AG production occurs on demand through two mechanisms: activation of group I metabotropic glutamate receptors (mGluR5) (eCB<sub>mGluR</sub>) or via N-methyl-D-aspartate (NMDA) receptors (eCB<sub>NMDA</sub>) (Fig. 1). 2-AG, produced at post-synaptic neurons, is a retrograde messenger which acts on presynaptic CB1 and CB2 receptors to suppress neurotransmitter release via P/Q and N-type Ca<sup>2+</sup> channel inhibition (Sugiura et al., 2002; Sugiura et al., 1995; Suhara et al., 2000; Tanimura et al., 2010). Studies with *DAGL $\alpha$* <sup>-/-</sup> mice or the highly selective DGL- $\alpha$  inhibitor DO34, showed that interfering with DGL- $\alpha$  activity eliminated the two major forms of eCB mediated synaptic plasticity, depolarization induced suppression of excitation (DSE) and inhibition (DSI), in prefrontal cortex, hippocampus, striatum, and cerebellum (Gao et al., 2010; Ogasawara et al., 2016; Tanimura et al., 2010; Yoshino et al., 2011)



**Figure 1. 2-AG synthesis by DGL-α.** Glutamate released from presynaptic vesicles can stimulate DGL-α to produce 2-AG from DAG via **a)** mGluR5 activation or **b)** NMDA mediated  $\text{Ca}^{2+}$  entry into the post-synaptic neuron. Newly synthesized 2-AG moves retrogradely to pre-synaptic CB1 and CB2 receptors which subsequently

inhibit P/Q or N type  $Ca_v$  channels to suppress  $Ca^{2+}$  entry **c)** and neurotransmitter release. FMRP, the protein lost due to the fragile X mutation, controls the appropriate **d)** translation and localization of DGL- $\alpha$  within post-synaptic density.

In regard to behavior, studies which interfered with 2-AG production detected phenotypes relevant to neurodevelopmental disorders. Schurman et al. (2019) compared *DAGL $\alpha$ <sup>-/-</sup>* mice to DO34 in C57BL/6J mice in assays of learning and memory. DO34 treatment induced deficits in acquisition and reversal learning without deficits in expression, extinction, forgetting, perseveration or object location. In contrast *DAGL $\alpha$ <sup>-/-</sup>* mice displayed profound deficits in all of these domains. Highly specific genetic deletion of DGL- $\alpha$  in direct pathway striatal medium spiny neurons (dMSNs) induced deficits in synaptic plasticity (DSE and DSI), social behavior, and increased repetitive self-grooming behavior (Shonesy et al., 2018; Shonesy et al., 2013). Studies that used either genetic or pharmacological disruption of DGL- $\alpha$  induced anxiety-like behavior (Bedse et al., 2017; Shonesy et al., 2014). These studies highlight the important contribution of 2-AG to behavioral domains impaired in neurodevelopmental disorders.

To our knowledge, no study has directly tested the effect of pharmacological interference with DGL- $\alpha$  activity on measures of social behavior or communication. Accumulating preclinical and clinical evidence shows that pharmaco-therapeutics which target ECS dysfunction are promising treatments for neurodevelopmental disorders

(Bar-Lev Schleider et al., 2019; Jung et al., 2012; Pretzsch et al., 2019; Wei et al., 2016). Therefore, this represents a critical gap in our knowledge regarding the ECS and neurodevelopmental disorders, particularly with respect to DGL- $\alpha$  and 2-AG.

To address this need, we used the highly selective DGL- $\alpha$  inhibitor DO34, and an inverse agonist of CB1, rimonabant, to evaluate pharmacological 2-AG depletion and CB1 inhibition on measures of social behavior and communication. Due to their relevance to ASD and neurodevelopmental disorders, assays of anxiety-like behavior and locomotor activity were also included.

## **Material and Methods**

### *Animals*

Adult male C57BL6/J (B6) mice (n = 51) treated with DO34 50 mg/kg (n = 14), DO34 10 mg/kg (n = 13), rimonabant 2 mg/kg (n = 13); or Vehicle ([Ethanol:Kolliphor:Saline]; n = 14), aged 3 to 6 months were bred in house (from breeders obtained from Jackson Labs) and used for all experiments. 129S1/SvImJ mice (n = 6) were used as the stranger mice because they have very low levels of activity so that all interactions were initiated by the subject mice (Moy et al., 2007). Adult female C57B6/L mice (n = 6) were used in the direct social interaction test. Because ASD affects a higher percentage of males than females, only male mice were used in the current study. Mice were housed 3 to 5 per cage with ad lib food and water and 12-h light/dark cycle. All experiments

were conducted during the light phase between 9 am and 5 pm. All procedures were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the New York State Institute for Basic Research in Developmental Disabilities' Institutional Animal Care and Use Committee.

### *Drug treatment*

The drugs used were the DGL- $\alpha$  inhibitor DO34 (10 or 50 mg/kg) (AOBIOUS INC, Hopkinton, MA) and the CB1R antagonist rimonabant (2 mg/kg) (Sigma-Aldrich, St. Louis, MO). Animals were assigned to one of four experimental treatment conditions and administered DO34, rimonabant or vehicle by intraperitoneal (i.p.) injection at a volume of 10 mL/kg in a formulation containing ethanol:Kolliphor:saline (1:1:18; Sigma-Aldrich, St. Louis, MO). Drug pretreatment time was 2 hours before behavioral testing. Two doses of DO34 were selected, one for full inhibition (50 mg/kg) and a second at the 50% inhibitory concentration ( $IC_{50}$ ) (10 mg/kg) as established by Ogasawara et al. (2016) to evaluate the a possibility of dose dependent effects on behavior. Rimonabant dosage was selected based on dosage used by Bedse et al. (2017).

### *Behavioral testing*

#### *Order of testing*

Subjects were run in five cohorts of nine mice, each with 2 to 3 mice per drug/dose. The order of tests was based on the need of performing first those tests that are more influenced by previous testing experience (such as the elevated plus maze), while leaving last tests involving a certain degree of stressful experience (such as those requiring social interactions). Therefore, tests were conducted in the following order: Day 1: EPM, Day 2: Open Field and Social Approach, Day 3: Direct Social Interaction. Tests were conducted > 24 hours apart to allow for drug washout. Mice received the same drug dose for each experiment.

#### *Elevated plus maze (EPM)*

Anxiety-like behavior was tested in the elevated plus maze as previously described (Chadman, 2011) The elevated (95 cm) plus maze consists of 2 open arms (30 X 5 cm) and 2 closed arms (30 X 5 X 15 cm) extending from a central (5 X 5 cm) area. A raised lip (0.25 cm) around the open arms minimized falling off the edges of the open arms. Mice were placed in the central area facing an open arm and allowed to traverse the maze freely for 5 min. Arm entries (70% of mouse in the arm) and time spent in the open and closed arms were tracked and scored using ANY-maze software (Stoelting, Inc., Wood Dale, IL). The center of the maze was lighted at 200 lux. This lighting condition was chosen based on Haller et al. (2004), where *CB1*-KO animals demonstrated anxiety-like behavior under high (198 lx), but not low (red) light conditions. Prior to each and all tests, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried.

### *Open field*

The open field test can be used to measure general exploration, anxiety, and locomotor activity in a novel environment. Mice were placed in a 40 x 19 x 22 cm<sup>3</sup> transparent plexiglass apparatus for 10 minutes. The center of the open field was defined as 7.5 x 7.5 cm square. Distance travelled, average speed, and center duration were scored using ANY-maze, while grooming and rearing were hand scored.

### *Social approach test*

This experiment has two habituation phases (center and all 3 chambers) followed by two testing phases (sociability and novelty). The first test compares the preference for a social stimulus versus an inanimate object. The second test, or social novelty phase of the test, compares the preference for a now familiar social stimulus to a novel social stimulus. Social approach behaviors were tested in an apparatus with 3 chambers in a single 40-min session, divided into 4 phases. The subject mouse was acclimated to the apparatus for 10 min in the center chamber (phase 1), and then for an additional 10 min with access to all 3 empty chambers (phase 2). The subject was then confined to the middle chamber, while the novel object (an inverted wire cup, Galaxy Cup, Kitchen Plus, Streetsboro, OH) was placed into one of the side chambers, and the stranger mouse (stranger 1), inside an identical inverted wire cup, was placed in the opposite side chamber. The location (left or right) of the novel object and stranger mouse counterbalanced across subjects. The chamber doors were opened simultaneously, and



the subject had access to all 3 chambers for 10 min (phase 3). After this, the fourth 10-min session provided a measure of preference for social novelty (phase 4). The subject mouse was gently guided to the center chamber, the doors closed, and the novel object removed, and a second novel mouse (stranger 2) was placed in the side chamber. The chamber doors were opened simultaneously, and the subject again had access to all 3 chambers for 10 min. The fourth 10-min phase provided a measure recognition and discrimination and is used to confirm olfactory abilities for detection and discrimination of social odors. Video tracking with ANY-maze (Stoelting, Inc.; Wood Dale, IL) automatically scored the time spent in each of the 3 chambers, time spent sniffing, and number of entries into each chamber during each 10-min phase of the test. Animals used as strangers were male 129S1/SvImJ mice habituated to the testing chamber for 30-min sessions on 3 consecutive days and were enclosed in the wire cup to ensure that all social approach was initiated by the subject mouse. Both end chambers maintained a lighting level of 26-27 lux with 2 desk lamps angled away from the maze.

#### *Direct social interaction*

Direct social interaction was assessed in 33 x 15 x 14 cm cage plastic cage with 3 cm of sawdust and a metal flat cage roof. Male test mice were isolated for two hours in this cage prior to testing. An unfamiliar stimulus mouse (a 16-week year old C57BL/6 female) was then introduced into the testing cage and left there for 6 minutes. The

ultrasonic microphone was mounted 2 cm above the top of the testing cage to record the session for subsequent scoring of USV parameters (see methods. Testing sessions were recorded, and videos were analyzed, with ANY-maze software (Stoelting, Inc., Wood Dale, IL). One observer who was unaware of the drug treatment of the animals scored the behavior of the test mice, quantifying the time spent performing each of the following behavioral categories and elements:

*Affiliative behaviors:* sniffing the head and the snout of the partner, anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming (grooming the partner).

*Nonsocial activities:* rearing (standing on the hind limbs sometimes with the forelimbs against the walls of the cage); digging; self-grooming (the animal licks and mouths its own fur).

#### *Assessment of estrus cycle*

The estrous phase was assessed by analysis of vaginal smears performed on the day of the direct social interaction test in the female C57BL/6J stimulus mice. The evaluation of the test subjects was conducted after testing, in order to minimize the potential stress effects of the manipulation on direct social interaction. Cell types were identified in

unstained wet preparations, and estrus stages categorized (Caligioni, 2009). Stimulus females were all in metestrus or proestrus.

### *Ultrasonic Vocalization Analysis*

Ultrasonic vocalizations were captured by a Noldus ultrasonic microphone (Noldus Information Technology Inc., Leesburg, VA) and rendered into audio files (.wav) by UltraVox XT (3.0.80) software (Noldus Information Technology, The Netherlands). Wav files were converted and spectrograms were generated by DeepSqueak (Coffey et al., 2019). Sonogram parameters for short duration vocalization are: nfft = 0.0032s, overlap = 0.0028 s, window = 0.0032s. Sonogram parameters for long duration vocalizations are: nfft = 0.01s, overlap = 0.005s, window = 0.01s.

### *Statistical Analysis*

EPM, open field, direct social interaction and USV data were analyzed using a one-way analysis of variance (ANOVA). To evaluate habituation to the three-chamber maze for chamber bias (left vs right) a repeated measures ANOVA was used. For the social approach task, a repeated measures ANOVA was used to compare time spent in the chamber and sniff time for trial 3 (sociability) and trial 4 (novelty). However, the time spent in each of the three chambers was not independent; for the analysis, only times spent in the side chambers (containing the stranger mouse and novel object) were compared. Time spent in the center chamber is shown in the graphs to illustrate where

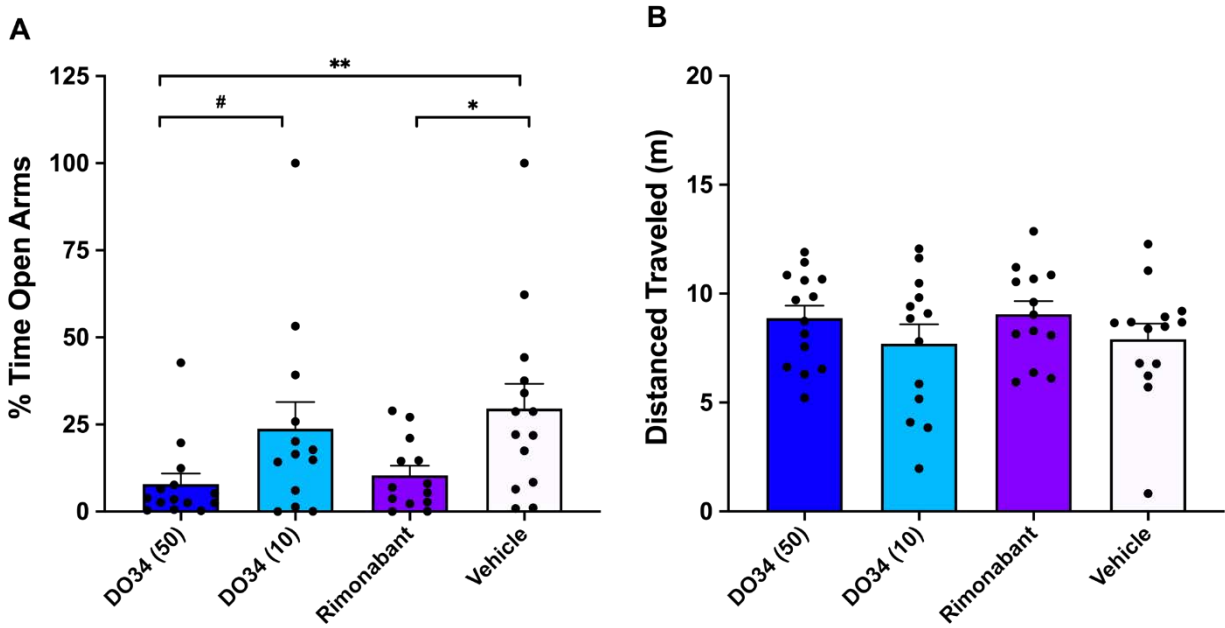
the subject mouse spent time during the entire 10-min phase. Chamber time, time spent sniffing the novel object versus the stranger mouse, and number of entries to the side chambers in the social approach test were analyzed. For USV data, the number of USV vocalizations failed the Shapiro-Wilks normality test, therefore outliers were removed using the ROUT method with  $Q = 10\%$ , and data were analyzed using a one-way ANOVA. Statistical analyses were performed using IBM® SPSS Statistics Version 25 (IBM SPSS Japan, Tokyo, Japan) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Fisher's LSD post-hoc analysis was run when a main effect or when the repeated measure (stranger mouse or novel object) was significant to determine the group differences. Density plots were calculated and generated using IBM® SPSS Statistics Version 25 (IBM SPSS Japan, Tokyo, Japan) Sigma Plot 14 (Jandel Scientific).

## **Results**

### **Elevated Plus Maze**

An effect of treatment on percentage of time spent in the open arms was detected ( $F_{3,50} = 3.52$ ,  $p < 0.05$ ). Animals treated with 50 mg/kg of DO34 or rimonabant spent less time in the open arms ( $p < 0.01$  and  $p < 0.05$  respectively; Fig. 1A), with this effect being more pronounced in animals injected with high dose 50 mg/kg.

Total distance traveled was not affected, ( $F_{3,50} = 0.93$ ,  $p > 0.05$ ; NS), suggesting that locomotor activity in this test was not affected by inhibition of DGL- $\alpha$  or direct inhibition of CB1.

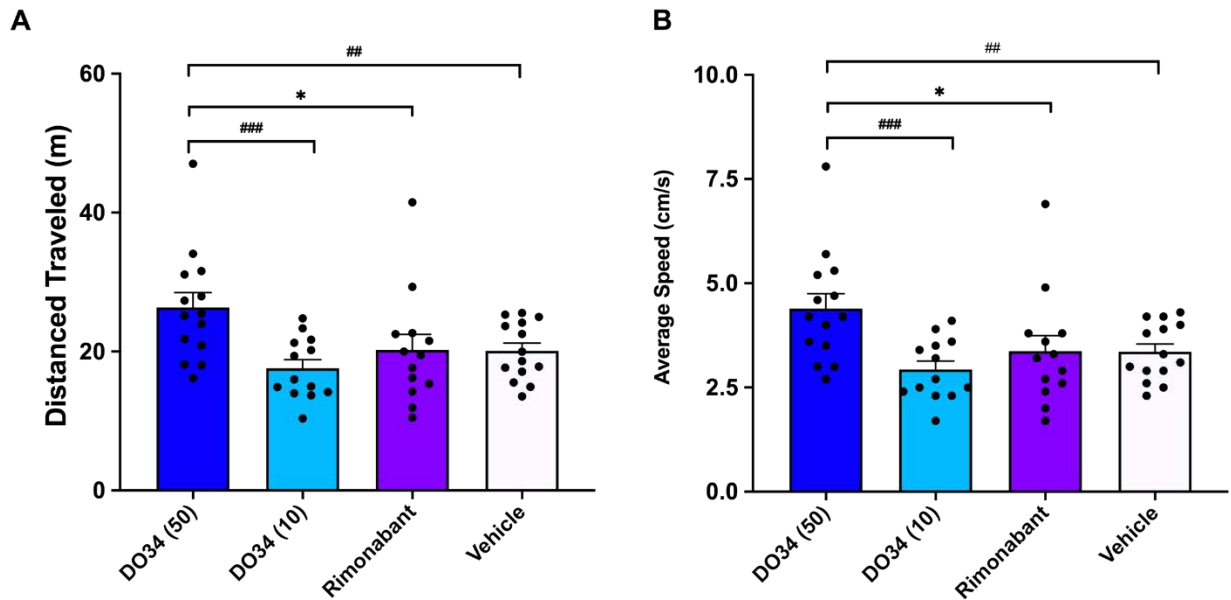


**Figure 1. Elevated Plus Maze.** DO34 (50 mg/kg) and rimonabant induced anxiety-like behavior in C57BL/6 mice. **(A)** DO34 (50 mg/kg) induced more anxiety-like behavior ( $p < 0.01$ ) in comparison to vehicle treated mice than rimonabant ( $p < 0.05$  in comparison to vehicle). A trend toward significance was detected for DO34 (50 mg/kg) in comparison to DO34 (10 mg/kg) mice. **(B)** No significant difference was found for the distance traveled. #  $p = 0.05$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ . All data are presented as mean and  $\pm$  SEM. DO34 (50 mg/kg):  $n = 13$ ; DO34 (10 mg/kg):  $n = 14$ ; rimonabant  $n = 13$ ; vehicle:  $n = 14$ .

### Three Chambered Maze

#### *Open Field (Center Habituation)*

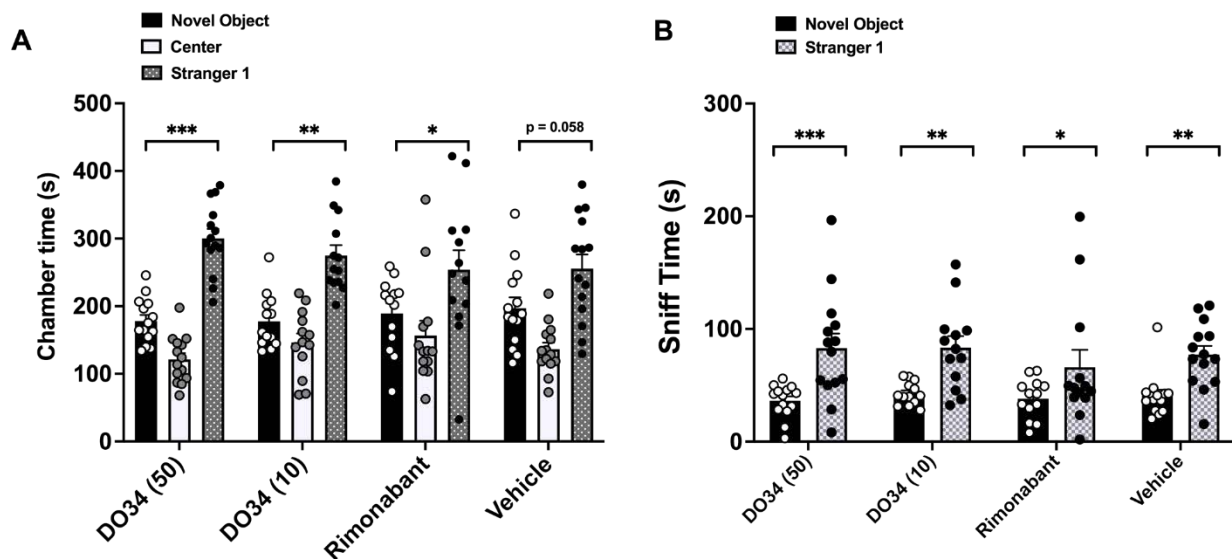
An effect of treatment on distance traveled during the center habituation trial was detected ( $F_{3,50} = 4.50$ ,  $p < 0.01$ ). DO34 (50 mg/kg) increased activity relative to all treatments ( $p = 0.001$  vs DO34 (10 mg/kg),  $p < 0.05$  vs rimonabant;  $p < 0.01$  vs vehicle; Fig. 2A). DO34 (50 mg/kg) mice also demonstrated increased average speed during this trial ( $F_{3,50} = 4.55$ ,  $p < 0.01$ ; Fig. 2B).



**Figure 2. Open Field. (A)** DO34 (50 mg/kg) treatment increased locomotion relative to DO34 (10 mg/kg) ( $p = 0.001$ ), rimonabant ( $p < 0.05$ ), and vehicle ( $p = 0.01$ ). **(B)** DO34 (50 mg/kg) treatment increased the average speed (cm/s) relative to DO34 (10 mg/kg) ( $p = 0.001$ ), rimonabant ( $p < 0.05$ ), and vehicle ( $p < 0.05$ ). \*  $p < 0.05$ ; ##  $p = 0.01$ ; ###  $p < 0.001$ . All data are presented as mean and +/- SEM. DO34 (50 mg/kg):  $n = 13$ ; DO34 (10 mg/kg):  $n = 14$ ; rimonabant  $n = 13$ ; vehicle:  $n = 14$ .

## Sociability trial

Fig. 3 A-B illustrates social approach behaviors in B6 mice treated with DO34 (10 or 50 mg/kg), rimonabant, or vehicle. An effect of chamber was detected but not of treatment (chamber,  $F_{1,50} = 33.16$ ,  $p < 0.0001$ ; treatment x chamber,  $F_{3,50} = 0.3587$ ,  $p > 0.05$ ; Fig. 3A). Mice treated with DO34, both 10 mg/kg ( $p < 0.01$ ) and 50 mg/kg ( $p < 0.0001$ ), or rimonabant ( $p < 0.05$ ) showed a preference for the chamber containing the stranger mouse relative to the chamber with the novel object. Vehicle treated mice showed a similar trend that did not achieve statistical significance (vehicle  $p = 0.058$ ). When sniffing behavior was evaluated, each group, regardless of treatment spent more time sniffing the stranger mouse than the novel object (chamber,  $F_{1,50} = 33.161$ ,  $p < 0.0001$ ; treatment x chamber,  $F_{1,50} = 0.359$ ,  $p > 0.05$ ; Fig. 3B) DO34 (50 mg/kg)  $p < 0.0001$ ; DO34 (10 mg/kg)  $p < 0.01$ ; rimonabant  $p < 0.05$ ; vehicle  $p < 0.01$ ; Fig. 3B).



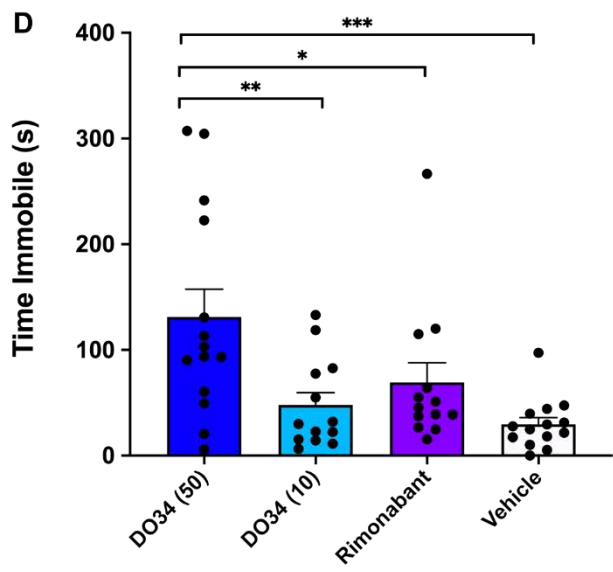
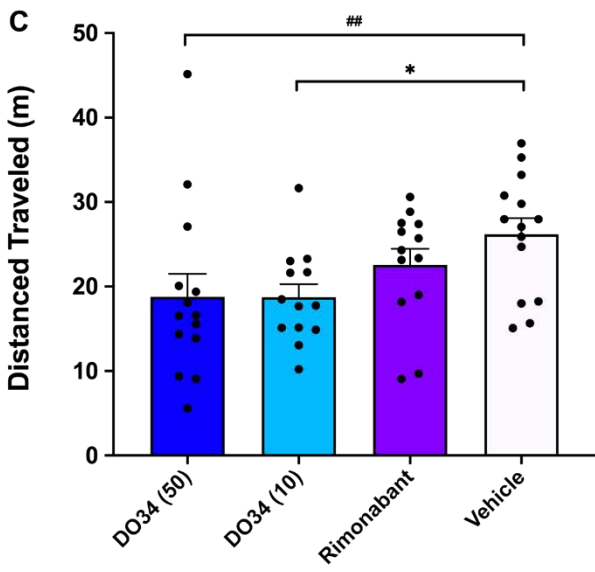
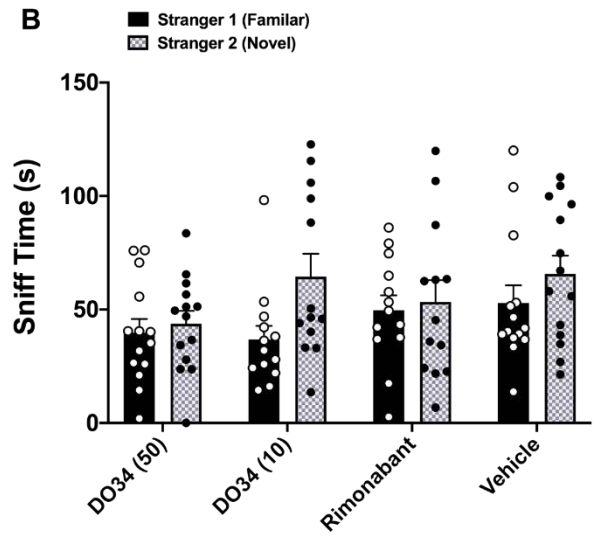
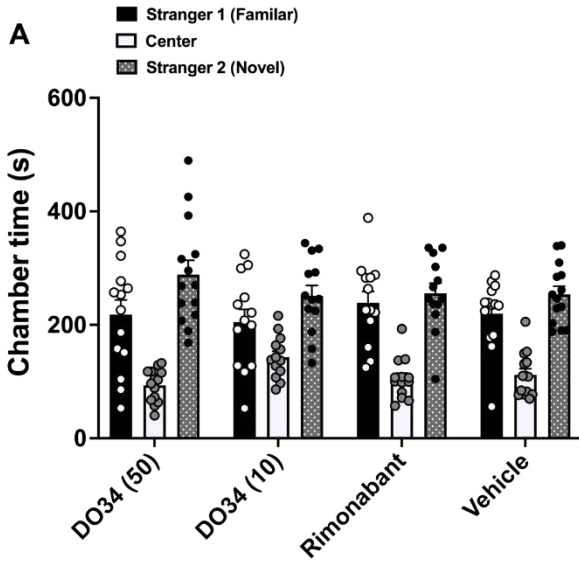
**Figure 3. Sociability trial. (A) Chamber time:** Mice treated with DO34 (50 or 10 mg/kg) or rimonabant demonstrated preference for the stranger mouse relative to the novel object. Vehicle treated mice showed a similar trend that did not reach statistical significance. **(B) Sniff time:** All groups spent more time sniffing the stranger mouse relative to the novel object. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ;  $p < 0.001$ . All data are presented as mean and +/- SEM. DO34 (50 mg/kg):  $n = 13$ ; DO34 (10 mg/kg):  $n = 14$ ; rimonabant  $n = 13$ ; vehicle:  $n = 14$ .

#### **Novelty (Trial 4)**

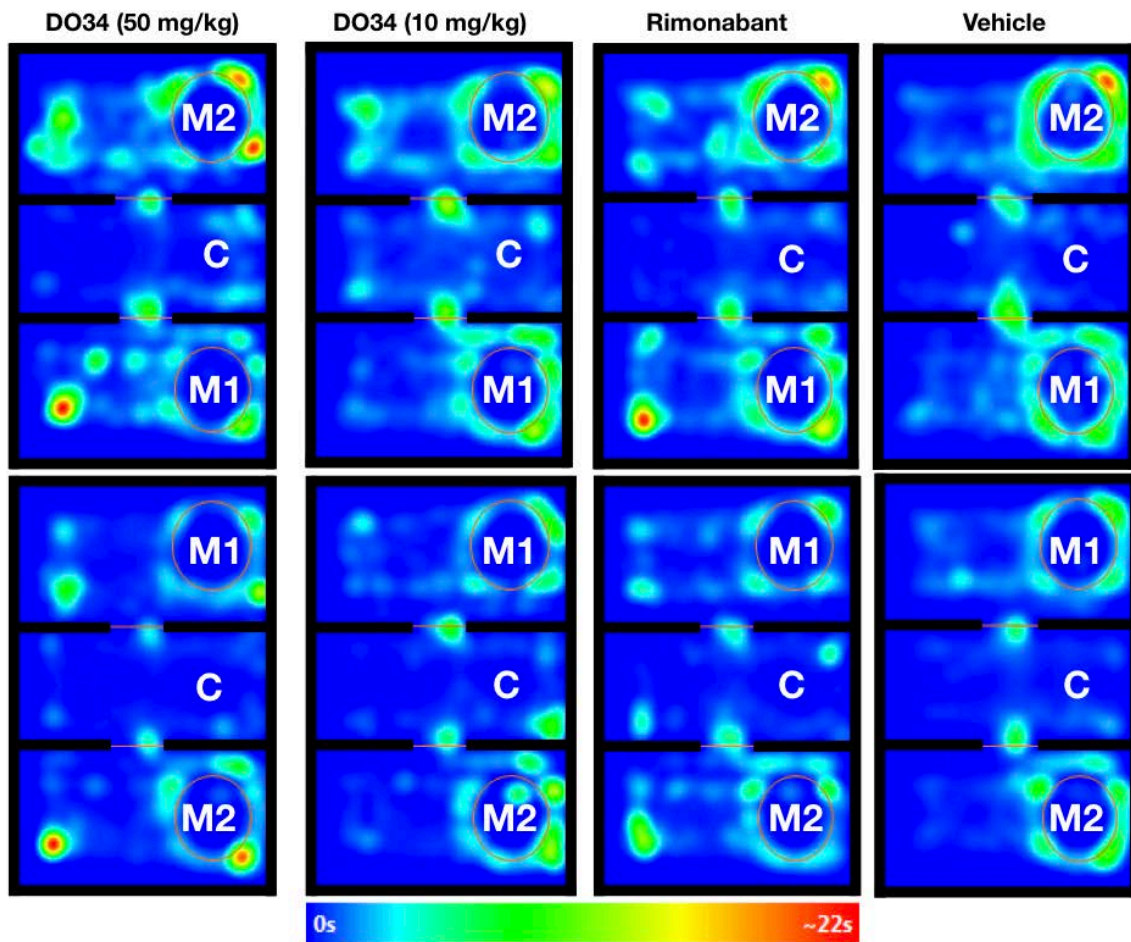
Fig. 4A-D illustrate the preference for social novelty in each of the groups when the novel object has been substituted with a second stranger mouse. An effect of chamber was detected but not of treatment (chamber,  $F_{1,50} = 4.51$ ,  $p < 0.05$ ; treatment x chamber,  $F_{3,50} = 0.332$ ,  $p > 0.05$ ). When sniffing time was analyzed, an effect of chamber but not treatment was found (chamber,  $F_{1,50} = 5.364$ ,  $p < 0.05$ ; treatment x chamber,  $F_{3,50} = 1.144$ ,  $p > 0.05$ ; Fig. 4B). Treatment altered locomotor behavior during this trial ( $F_{3,50} = 2.959$ ,  $p < 0.05$ ). DO34 (50 mg/kg) and DO34 (10 mg/kg) decreased locomotor behavior ( $p = 0.01$  and  $p < 0.05$  respectively; Fig. 4C). No significant effects for number of entries between chambers were detected (chamber,  $F_{1,50} = 7.068$ ,  $p = 0.01$ ; treatment x chamber,  $F_{3,50} = 1.082$ ;  $p > 0.05$ ; not shown). Treatment groups demonstrated differences in the time spent immobile during this trial ( $F_{3,50} = 6.472$ ,  $p < 0.001$ ; Fig. 4D). Pairwise comparisons revealed that DO34 (50 mg/kg) treated mice



spent more time immobile than DO34 (10 mg/kg) ( $p < 0.01$ ), rimonabant ( $p < 0.05$ ), and vehicle treated mice ( $p = 0.0001$ ). Heat maps revealed that DO34 (50 mg/kg) treated mice spent increased amounts of time in one spot in the right chamber of the maze, regardless of whether this chamber contained the stranger mouse or the novel object (Fig. 5). Evaluation of the habituation phase to the empty maze showed no preference for either the left or right side of the chamber, nor an effect of treatment (chamber,  $F_{1,50} = 0.177$ ,  $p > 0.05$ ; treatment x chamber,  $F_{3,50} = 2.242$ ,  $p > 0.05$ ).



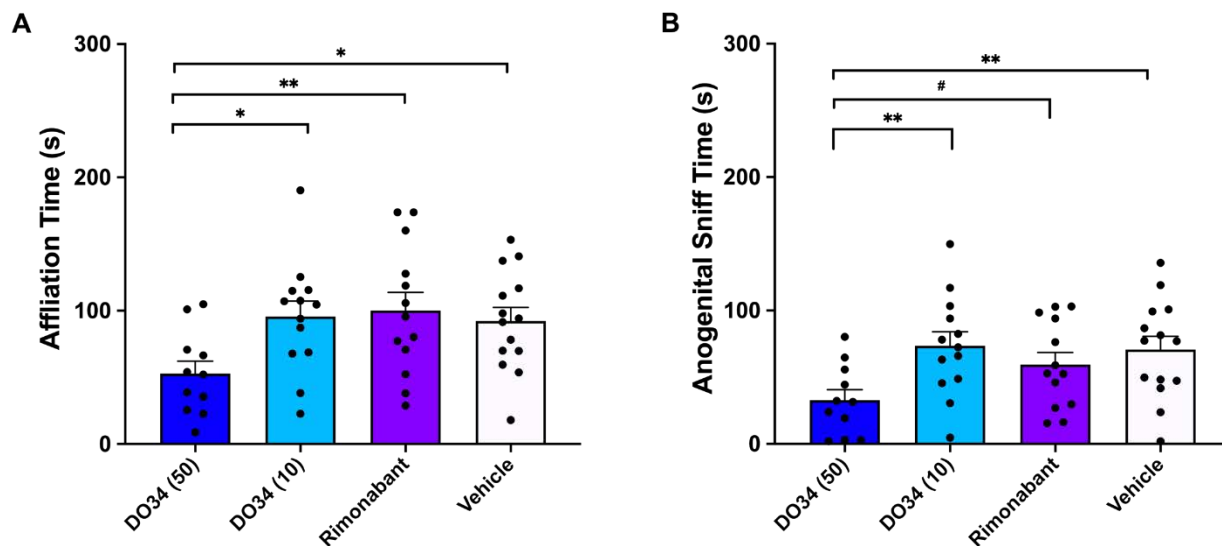
**Figure 4. Novelty trial. (A) Chamber time:** DO34 (50 mg/kg) treated mice spent more time in the side chamber containing stranger mouse 2 (Novel) than the chamber containing stranger mouse 1 (Familiar). **(B) Sniff time:** DO34 (50 mg/kg) and rimonabant treated mice appeared to have similar average times spent sniffing the stranger and novel object in comparison to DO34 (10 mg/kg) and Vehicle treated mice however no statistically significant differences were detected. **(C) Distance traveled:** DO34 (50 mg/kg) and DO34 (10 mg/kg) demonstrated increased levels of locomotor behavior during the novelty trial ( $p = 0.01$  and  $p < 0.05$  respectively). **(D)** DO34 (50 mg/kg) treatment increased the time spent immobile relative to all other treatment groups ( $p < 0.01$  vs DO34 (10 mg/kg);  $p < 0.05$  vs rimonabant;  $p < 0.0001$  vs Vehicle). \*  $p < 0.05$ ; ##  $p = 0.01$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ . All data are presented as mean and +/- SEM. DO34 (50 mg/kg):  $n = 13$ ; DO34 (10 mg/kg):  $n = 14$ ; rimonabant  $n = 13$ ; Vehicle:  $n = 14$ .



**Figure 5. Time immobile during the sociability trial.** Heat maps illustrate that DO34 (50 mg/kg) treated mice remained immobile in the right chamber of the maze regardless of whether the chamber contained the stranger mouse or novel object. M1 = familiar mouse, M2 = novel mouse, C = center chamber of maze.

### Direct Social Interaction

An effect of treatment was detected for time engaged in affiliation behaviors during the direct social interaction ( $F_{3,47} = 3.33$ ,  $p < 0.05$ ). DO34 (50 mg/kg) treated mice engaged in less affiliative behaviors than DO34 (10 mg/kg) ( $p < 0.05$ ), rimonabant ( $p < 0.01$ ) and vehicle injected mice ( $p < 0.05$ ; Fig 6A). When individual affiliative behaviors (sniffing: anogenital or rest of body, mounting, allogrooming) were analyzed, an effect for anogenital sniffing was detected ( $F_{3,47} = 3.47$ ,  $p < 0.05$ ). DO34 (50 mg/kg) engaged in less anogenital sniffing than DO34 (10 mg/kg) ( $p < 0.01$ ), and vehicle injected mice ( $p < 0.01$ ). DO34 (50 mg/kg) compared to rimonabant treatment trended toward but did not achieve significance ( $p = 0.06$ ; Fig. 6B).



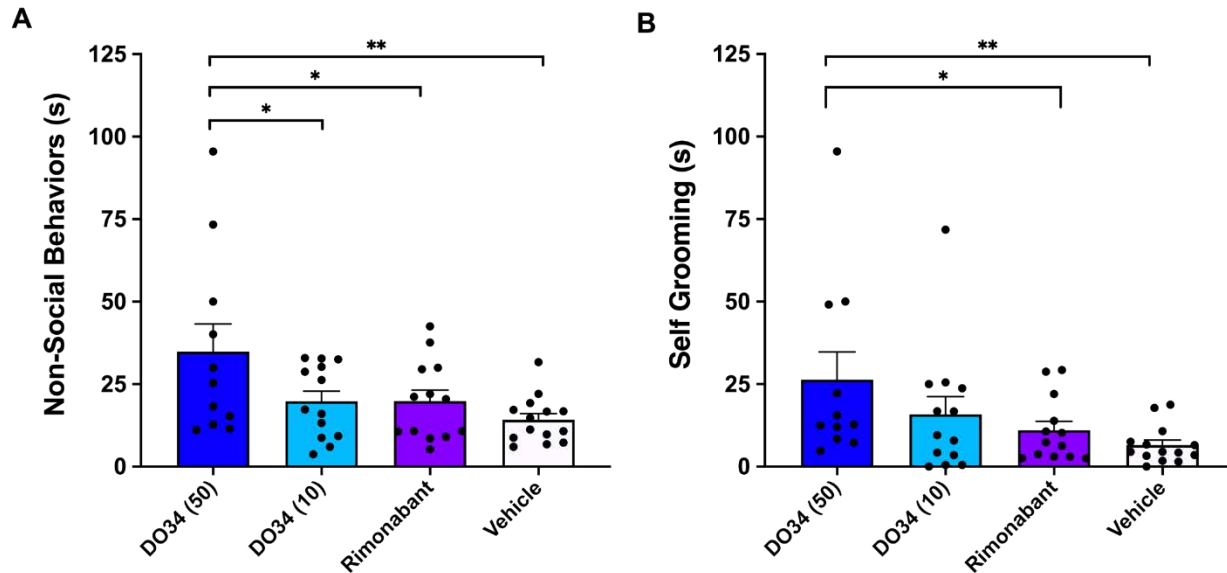
**Figure 6. Affiliation behaviors during a direct social interaction with a novel**

**female. (A)** DO34 (50 mg/kg) treatment decreased affiliative behaviors in comparison to DO34 (10 mg/kg) ( $p < 0.05$ ), rimonabant ( $p < 0.01$ ), and vehicle ( $p < 0.05$ ) treated mice.

**(B)** Of the affiliation behaviors analyzed, DO34 (50 mg/kg) treatment reduced

anogenital sniffing in comparison to DO34 (10 mg/kg) treatment and Vehicle ( $p < 0.01$ ); when compared to rimonabant treatment a trend was detected that failed to achieve statistical significance ( $p = 0.06$ ). All data are presented as mean and +/- SEM. #  $p = 0.06$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ . DO34 (50 mg/kg):  $n = 11$ ; DO34 (10 mg/kg):  $n = 13$ ; rimonabant  $n = 13$ ; Vehicle:  $n = 14$ .

An effect of treatment was detected for time engaged in non-social behaviors ( $F_{3,47} = 3.72$ ,  $p < 0.05$ ; Fig. 7). DO34 (50 mg/kg) treated mice engaged in more non-social behaviors than DO34 (10 mg/kg;  $p < 0.05$ ), rimonabant ( $p < 0.05$ ) and vehicle ( $p < 0.01$ ) injected animals. When individual non-social behaviors were analyzed (e.g. digging, rearing, self-grooming), an effect of treatment was again detected for self-grooming behaviors ( $F_{3,47} = 2.98$ ,  $p < 0.05$ ). DO34 (50 mg/kg) treated mice engaged in more self-grooming than rimonabant and vehicle treated mice ( $p < 0.05$  and  $p < 0.01$  respectively) but not DO34 (10 mg/kg) ( $p > 0.05$ ).



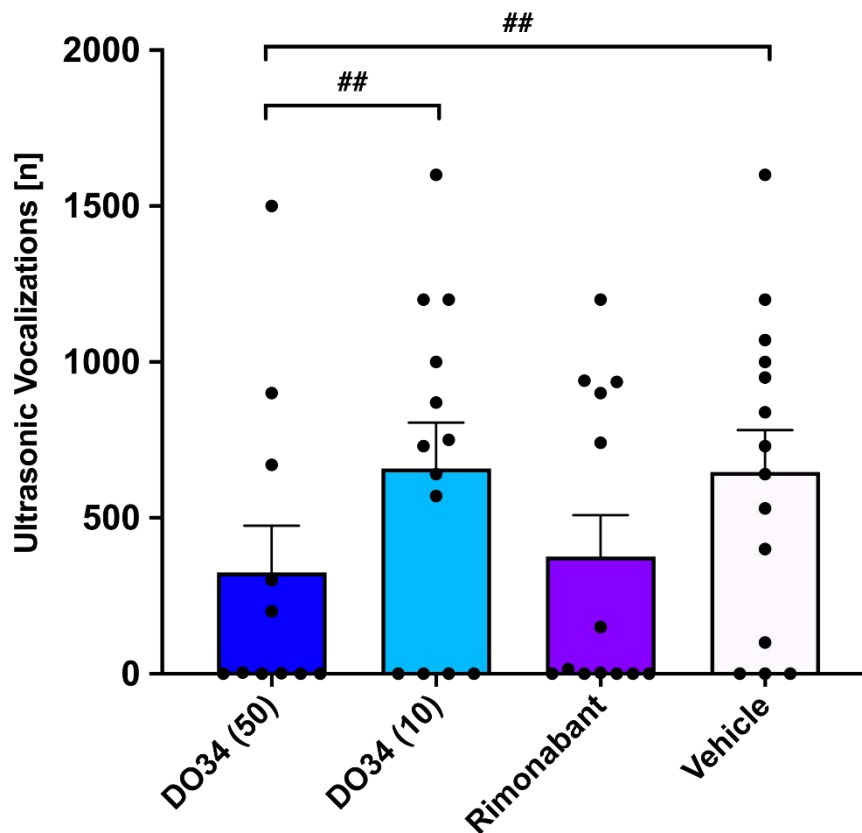
**Figure 7. Non-social behaviors during a direct social interaction with a novel**

**female. (A)** DO34 (50 mg/kg) treatment increased non-social behaviors in comparison to DO34 (10 mg/kg) ( $p < 0.05$ ), rimonabant ( $p < 0.05$ ), and vehicle ( $p < 0.01$ ) treated mice. **(B)** DO34 (50 mg/kg) treatment increased self-grooming behavior in comparison to rimonabant ( $p < 0.05$ ), and vehicle ( $p < 0.01$ ) treated mice, but not DO34 (10 mg/kg) ( $p < 0.05$ ) mice. All data are presented as mean and +/- SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$

DO34 (50 mg/kg):  $n = 11$ ; DO34 (10 mg/kg):  $n = 13$ ; rimonabant  $n = 13$ ; Vehicle:  $n = 14$ .

### Ultrasonic Vocalizations (USVs)

USVs produced by the male during the 6-minute direct social interaction with a novel female were recorded and analyzed. A significant effect of treatment was found for the number of USVs produced (treatment,  $F_{3,45} = 3.114$ ,  $p < 0.05$ ). DO34 (50 mg/kg) treated mice produced significantly fewer vocalizations than DO34 (10 mg/kg) and Vehicle



treated mice (both,  $p = 0.01$ ), but not rimonabant treated mice ( $p > 0.05$ ; Figure 8).

**Figure 8. Number of ultrasonic vocalizations (USVs) produced during an interaction with a novel female.** DO34 (50 mg/kg) reduced the number of USVs relative to DO34 (10 mg/kg) and Vehicle treated animals but not rimonabant treated



animals. ##  $p = 0.01$ .  $n = 11$ ; DO34 (10 mg/kg):  $n = 13$ ; rimonabant  $n = 13$ ; Vehicle:  $n = 14$ .

## Discussion

To investigate the contribution of 2-AG-CB1 signaling in behaviors relevant in ASD, we utilized two pharmacological approaches. First, a reduction in DGL- $\alpha$  synthesis of the primary CB1 ligand, 2-AG (Ogasawara et al., 2016), and second, antagonism of CB1 with the inverse agonist, rimonabant (Rinaldi-Carmona et al., 1995). Our main findings are as follows: 1) inhibition of DGL- $\alpha$  with DO34 induced anxiety-like behavior, hyperactivity, social behavior deficits, communication deficits, and increased self-grooming behavior; and 2) CB1 inverse agonism induced anxiety-like behavior and communication deficits but not hyperlocomotion, increased self-grooming behaviors, and only partially influenced social behavior.

Consistent with previous reports, inhibition of DGL- $\alpha$  or antagonism of CB1 induced anxiety-like behavior in C57BL/6 mice (Bedse et al., 2017; Bluett et al., 2017). In agreement with these findings, mice that are null for CB1 (CB1<sup>-/-</sup>) show anxiety-like phenotypes in the elevated plus maze, light-dark box, and open field (Haller et al., 2002; Haller et al., 2004; Uriguen et al., 2004). Our findings with rimonabant are consistent with those reported clinically. Rimonabant was approved for the treatment of obesity in

the European Union (E.U.), however reports of neuropsychiatric side effects, such as anxiety, depression, and suicidal ideation, resulted in its removal from the market (Christensen et al., 2007; Moreira & Crippa, 2009). Interestingly, acute inhibition of 2-AG had a more pronounced effect for anxiety-like behavior (% time in the open arms) on the elevated plus maze than CB1 inhibition by rimonabant. The results we obtained may be due to contributions of 2-AG signaling at cannabinoid receptor 2 (CB2). 2-AG acts as a full agonist at both CB1 and CB2 (Sugiura et al., 2002). Therefore, reduction in 2-AG production would reduce signaling at both CB1 and CB2, whereas rimonabant, which is highly specific for CB1, does not affect 2-AG-CB2 activity (Ogasawara et al., 2016). Our studies are consistent with previous pharmacological studies which indicate that 2-AG-CB2 signaling contributes to anxiolysis (Almeida-Santos et al., 2013; Busquets-Garcia et al., 2011).

Previous studies have found that inhibition of CB1 with rimonabant does not induce a hyperactive phenotype (Long, Li, et al., 2009; Long, Nomura, et al., 2009; Marinho et al., 2015). Consistent with these studies CB1 inhibition with rimonabant did not induce a hyperactive phenotype, in the open field test but reduction of 2-AG did. Interestingly, hyperactivity did not contribute to our findings on the EPM or on the social approach test. This strongly suggests that effects on locomotor behavior due to a reduction in 2-AG are highly context dependent.

Our results on the social approach test indicate that DGL- $\alpha$  inhibition did not affect social behavior or locomotor activity when one stranger mouse was present in the apparatus (sociability), however when a second stranger mouse was introduced (novelty trial) DO34 treatment increased immobility time. These results are intriguing, since DO34 treatment increased locomotor activity in the open field test. It is possible that our findings on the social approach test reflect a context specific social anxiety phenotype and that the presence of two mice stimulus mice in the apparatus is necessary to elicit this phenotype. This effect was only found with DO34 and not with rimonabant. These data suggest the possibility that this behavior is mediated by 2-AG signaling at CB1 and CB2. Indeed, CB2 signaling contributes to social behavior and anxiety in mice (Almeida-Santos et al., 2013; Argue et al., 2017; Rodríguez-Arias et al., 2015). Our results are congruent with those of Folkes et al. (2020) that showed treatment with DO34 (50 mg/kg) increased time immobile during a social behavior test. Interestingly, Fragile X syndrome (FXS) and prenatal exposure to valproate are linked to DGL- $\alpha$  dysfunction (Jung et al., 2012; Kerr et al., 2013; Tang & Alger, 2015). Persons with these syndromes have unique social phenotypes, one of which is social anxiety (Budimirovic et al., 2006; Cassidy & Allanson, 2010; Christensen et al., 2013; Harris et al., 2008; Hong et al., 2019; Kaufmann et al., 2004). Our results here suggest the connection between DGL- $\alpha$  activity and social anxiety needs to be investigated further.

We employed a second test of social behavior, direct social interaction with a novel female, to examine the effects of our manipulations under a different context. Inhibition

of DGL-  $\alpha$  at 50 mg/kg reduced affiliation behaviors and increased non-social behaviors, specifically self-grooming behavior. These effects were not found with CB1 antagonism.

Recent studies using genetic methods to knock out DGL- $\alpha$  function in dorsal striatum detected reduced social interest and increased repetitive self-grooming behavior (Shonesy et al., 2018; Shonesy et al., 2013). Folkes et al. (2020) demonstrated that inhibiting the action of 2-AG on the basolateral amygdala– nucleus accumbens (BLA-NA) circuit induced social deficits in B6 mice, while pharmacological augmentation of 2-AG activity in this circuit rescued social behavioral impairments in *SHANK3B*<sup>-/-</sup> mice. Our manipulations appeared to show dose dependent effects with regard to self-grooming. Rimonabant produced a non-significant increase in self-grooming behavior while DO34 10 mg/kg, and 50 mg/kg produced larger increases. These findings with regard to rimonabant are consistent with those found by Terzian et al. (2014) in a similar direct social interaction paradigm with wildtype C57BL/6N mice. Overall our results suggest that reduction in 2-AG production results in larger insults to social behavior and repetitive self-grooming than inverse agonism at CB1 with rimonabant.

When we analyzed the USVs produced during this interaction, a different pattern was detected. Rimonabant treatment reduced the number of vocalizations males produced, however, this effect was more pronounced in the case of selective 2-AG reduction. These data suggest that the 2-AG contributions to communication behavior during a courtship interaction are mostly mediated by interactions with CB1, while the social deficits we detected appear to be mediated through a CB1 independent mechanism.

DO34 does not augment levels of the second eCB, AEA, and thus it unlikely that changes in AEA signaling occurred in these manipulations. (Ogasawara et al., 2016). It is important to note that the relationship between AEA signaling and social behavior is largely unexplored. A single study with Male Sprague–Dawley rats showed that 2-AG and AEA have overlapping developmental roles in social play behavior (Manduca et al., 2015). In regard to neurodevelopmental disorders, a single study found that increasing AEA levels rescued social deficits in two mouse models of ASD, namely *Fmr1*-KO and BTBR mice (Wei et al., 2016). It is possible that 2-AG and AEA exhibit functional redundancy with social behavior, as is the case with anxiety behavior (Bedse et al., 2017). Therefore, we cannot exclude the possibility that inhibition of AEA-CB1 signaling plays a role in our results.

The bimodal nature of CB1 signaling may explain some of the results obtained here. With respect to anxiety-like behavior, CB1 signaling exhibits anxiolytic properties at moderate levels of stimulation and anxiogenic effects at high levels of stimulation (Bhattacharyya et al., 2017; Ruehle et al., 2012). Whether or not CB1 exhibits bimodal properties with regard to social behavior has not been directly investigated to the best of our knowledge.

On the surface our data appear somewhat at odds with CB1<sup>-/-</sup> mice studies which indicate a strong role for CB1 in social behavior (Haller et al., 2004; Litvin et al., 2013;

Terzian et al., 2014). We do not view the results obtained in our study as contradictory, but complementary. The CB1 mutation used in those studies were bred on a CD1 background, which may contribute to the phenotypic differences we detected. Furthermore, CB1<sup>-/-</sup> mice, relative to wild-type mice, have an altered developmental trajectory, and therefore phenotypic differences compared to pharmacological studies are expected. The CB1<sup>-/-</sup> mouse continues to help unravel the importance of the ECS in ASD relevant behaviors, however a complete genetic knockout of CB1 does not closely recapitulate human pathology, as genetic variants for *CNR1* and *DAGLA*, not complete loss of the gene, are associated with ASDs. Indeed, ASD is a uniquely human disorder. Mouse models are only rough approximations which provide insight, by proxy, into selective pathology related to ASD. Therefore, the development of novel mouse models is critical for improving our understanding of this complex and diverse disorder.

Our manipulations of the ECS illustrate a crucial point: neurodevelopmental disorders are a complex mix of alterations due to different mechanisms, these data, and those from previous studies, suggest that the ECS supports a cluster of mechanisms responsible for certain wavelength ranges of behavior. This is particularly evident in regard to social behavior and communication. Here we supply a novel pharmacological mouse model for exploring behaviors which are relevant to neurodevelopmental disorders, particularly ASD. Our findings illustrate the need for exhaustive studies regarding imbalances in 2-AG signaling and neuropathology. Furthermore, these findings support and extend the accumulating body of evidence that the ECS,

particularly with respect to 2-AG, is a target of therapeutic interest for neurodevelopmental disorders.

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### **Conflict of Interest**

The author(s) have no potential conflicts of interest to declare with respect to the research, authorship, and/or publication of this article.

### **3.3 Study 3**

#### **Pharmacological inhibition of BKCa channels induces a specific social deficit in adult C57BL6/J mice**

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## **Abstract**

Genetic variants in large conductance voltage and calcium sensitive potassium (BKCa) channels have associations with neurodevelopmental disorders such as autism spectrum disorder, fragile X syndrome, and intellectual disability. In the case of fragile X syndrome, early pre-clinical studies suggest that BKCa channels may be a promising treatment target for neurodevelopmental disorders. While BKCa channel dysfunction has been investigated within the context of fragile X syndrome, it is unknown whether interference with BKCa channel function is inductive for deficits in behavioral domains relevant to neurodevelopmental disorders. This represent a critical gap in our knowledge regarding the relationship between BKCa dysfunction and neurodevelopmental disorders. To this aim we used the BKCa channel antagonist paxilline to evaluate the role of BKCa channel function in phenotypes of neurodevelopmental disorders. Here we used adult male C57BL/6J mice and a series of behavioral paradigms which assessed anxiety-like behavior, locomotor activity, social behavior, and repetitive self-grooming. We found that acute inhibition with paxilline induced a specific social deficit, but not anxiety-like behavior, hyperactivity . These findings support the relationship between BKCa channel impairment and social behavior. This demonstrates a need for future studies which further examine the contribution of BKCa channels to social behavior, particularly during critical periods of development.

Keywords: BKCa channel, behavior, developmental disorders, channel dysfunction

Genetic variants for large conductance voltage and calcium sensitive potassium (BKCa) channels have associations with neurodevelopmental disorders, such as autism spectrum disorder (ASD), fragile X syndrome (FXS), intellectual disability, epilepsy, and motor disorders (Cavalleri et al., 2007; Laumonnier et al., 2006; Myrick et al., 2015; Skafidas et al., 2014). In regard to ASD, an association has been identified between chromosomal abnormalities in *KCNMA1*, the gene for the  $\alpha$  unit of BKCa channels, or *KCNMB4*, the gene for the  $\beta 4$  subunit of BKCa channels, and a diagnosis of autism (Alarcon et al., 2002; International Molecular Genetic Study of Autism, 2001; Laumonnier et al., 2006; Skafidas et al., 2014). In the case of FXS, the silenced protein responsible for the syndrome, the fragile X mental retardation protein (FMRP), directly regulates the activity of BKCa channels (Deng et al., 2013). In accord with this, mutations which are specific for functional deficits in FMRP-BKCa interactions, have been strongly linked to the neurodevelopmental deficits of some patients (Laumonnier et al., 2006; Myrick et al., 2015).

BKCa channels are located at presynaptic terminals and respond to voltage and intracellular  $\text{Ca}^{2+}$  influx by complexing with P/Q and N type  $\text{Ca}^{2+}$  channels to inhibit  $\text{Ca}^{2+}$  entry and control neurotransmitter release (Berkefeld et al., 2006; Salkoff et al., 2006; Tseng-Crank, 1994). FMRP regulates the  $\text{Ca}^{2+}$  sensitivity of BKCa channels through

direct interactions with the  $\beta 4$  subunit (Deng et al., 2013). Via these mechanism BKCa channels play a pivotal role in regulating synaptic activity.

Several preclinical studies with the *fmr1*-KO mouse demonstrated that increasing BKCa activity can rescue neuronal and behavioral deficits in this mutant mouse model (Deng & Klyachko, 2016; Hebert et al., 2014; Zhang et al., 2014). Additionally, mice with genetic deletion of the  $\alpha$  unit of BKCa channels were found to have learning deficits in reversal and sensory learning (Typlt et al., 2013).

These studies suggest that BKCa channels may be a viable target for pharmacological treatments for neurodevelopmental disorders. While an association between BKCa channel dysfunction and neurodevelopmental disorders exists, its role in these disorders is far from understood. To the best of our knowledge impairments in BKCa function and their direct relationship to social behavioral deficits, a prevalent feature of many neurodevelopmental disorders, has not been investigated. Therefore, we hypothesized that pharmacological inhibition of these channels may induce behavioral deficits in wildtype mice. To this aim, we used the highly specific BKCa channel blocker paxilline in combination with behavioral tests for anxiety-like, locomotion, and social behaviors to investigate if acute pharmacological inhibition of these channels would produce phenotypes relevant to neurodevelopmental disorders (Choi, Lee, Kim, Jo, et al., 2018; Choi, Lee, Kim, Bae, et al., 2018; Imlach et al., 2008; Sanchez & McManus, 1996; Sheehan et al., 2009; Strøbaek et al., 1996; Wang et al., 2016; Zhou & Lingle, 2014). Due to the relationship to both non-syndromic and syndromic ASD (e.g. FXS) we

used two different social behavioral assays to investigate for unique social deficits related to acute paxilline treatment.

## **Material and Methods**

### *Animals*

Adult male C57BL6/J (B6) mice (n = 22; n = 12 [paxilline], n = 10 [vehicle]) aged 3 to 6 months were bred in house and used for all experiments. Male 129S1/SvImJ male mice (n = 6) were used as the stranger mice for the social approach test because they have very low levels of activity so that all interactions were initiated by subject mice (Moy et al 2007). Since the direct social interaction test is a measure of courtship behavior, female C57BL6 (n = 6) were used as stranger mice for this test. Because ASD affects a higher percentage of males than females, only male mice were used in the current study. Mice were housed 3 to 5 per cage with ad lib food and water and 12-h light/dark cycle. All experiments were conducted during the light phase between 9 am and 5 pm. All procedures were conducted in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals and approved by the New York State Institute for Basic Research in Developmental Disabilities' Institutional Animal Care and Use Committee (IACUC).

### *Drug treatment*

Paxilline (Sigma-Aldrich, St. Louis, MO) was dissolved to 10 mM in DMSO and then diluted further to 1:2000 (also in DMSO) (3 µg/kg). This dosage and pretreatment time were chosen based on previous studies which examined physiological and neurological

effects of paxilline on behavior (Choi, Lee, Kim, Jo, et al., 2018; Choi, Lee, Kim, Bae, et al., 2018; Knaus et al., 1994; Sanchez & McManus, 1996; Zhou & Lingle, 2014).

Animals were assigned to one of two experimental treatment conditions and administered paxilline (3 µg/kg) or vehicle (0.05% DMSO) by intraperitoneal (i.p.) injection at a volume of 10 mL/kg. Drug pretreatment times were three hours before behavioral testing.

### *Behavioral testing*

#### *Order of testing*

Subjects were run in five cohorts of nine mice each with 2 to 3 mice for each treatment. The order of tests was based on the need of performing first those tests that are more influenced by previous testing experience (such as the elevated plus maze), while leaving last, tests involving a certain degree of stressful experience (such as those requiring social interactions). Therefore, tests were conducted in the following order: Day 1: Elevated plus maze, Day 2: Open Field and Social Approach, Day 3: Direct Social Interaction. Tests were conducted > 24 hours apart.

#### *Elevated plus maze (EPM)*

Anxiety-like behavior was tested in the elevated plus maze as previously described (Chadman, 2011). The elevated (95 cm) plus maze consists of 2 open arms (30 X 5 cm) and 2 closed arms (30 X 5 X 15 cm) extending from a central (5 X 5 cm) area. A raised lip (0.25 cm) around the open arms minimized falling off the edges of the open arms. Mice were placed in the central area facing an open arm and allowed to traverse the

maze freely for 5 min. Arm entries (70% of mouse in the arm) and time spent in the open and closed arms were tracked and scored using ANY-maze software (Stoelting, Inc., Wood Dale, IL). The center of the maze was lighted at 200 lx. This lighting condition was chosen based on Haller et al. (2004), where *CB1*-KO animals demonstrated anxiety-like behavior under high (198 lx), but not low (red) light conditions. Prior to each and all tests, behavioral equipment was cleaned using a 70% ethanol solution, followed by water, and dried with paper towels.

### *Open field*

The open field test can be used to measure general exploration, anxiety, and locomotor activity in a novel environment. Mice were placed in a 20 x 40 x 22 cm<sup>3</sup> transparent plexiglass open field apparatus for 10 minutes. Distance travelled, average speed, and center duration, were scored using ANY-maze.

### *Social approach test*

This experiment has two habituation phases (center and all 3 chambers) followed by two testing phases (sociability and novelty). The first test compares the preference for a social stimulus versus an inanimate object. The second test, or social novelty phase of the test, compares the preference for a now familiar social stimulus to a novel social stimulus. Social approach behaviors were tested in an apparatus with 3 chambers in a single 40-min session, divided into 4 phases. The subject mouse was acclimated to the apparatus for 10 min in the center chamber (phase 1), and then for an additional 10 min with access to all 3 empty chambers (phase 2). The subject was then confined to the

middle chamber, while the novel object (an inverted wire cup, Galaxy Cup, Kitchen Plus, Streetsboro, OH) was placed into one of the side chambers, and the stranger mouse (stranger 1), inside an identical inverted wire cup, was placed in the opposite side chamber. Male 129S1/SvlmJ mice were used as the stranger mice. The location (left or right) of the novel object and stranger mouse alternated across subjects. The chamber doors were opened simultaneously, and the subject had access to all 3 chambers for 10 min (phase 3). After this, the fourth 10-min session provided a measure of preference for social novelty (phase 4). The subject mouse was gently guided to the center chamber, the doors closed, and the novel object removed, and a second novel mouse (stranger 2) was placed in the side chamber. The chamber doors were opened simultaneously, and the subject again had access to all 3 chambers for 10 min. The fourth 10-min phase provided a measure recognition and discrimination and is used to confirm olfactory abilities for detection and discrimination of social odors. Video tracking with ANY-maze (Stoelting, Inc.; Wood Dale, IL) automatically scored the time spent in each of the 3 chambers, time spent sniffing, and number of entries into each chamber during each 10-min phase of the test. Animals used as strangers were male 129S1/SvlmJ mice habituated to the testing chamber for 30-min sessions on 3 consecutive days and were enclosed in the wire cup to ensure that all social approach was initiated by the subject mouse. Both end chambers maintained a lighting level of 26-27 lux with 2 desk lamps angled away from the maze.

#### *Direct social interaction*

Direct social interaction was assessed in 33 x 15 x 14 cm cage plastic cage with 3 cm of sawdust and a metal flat cage roof. Male test mice were isolated for two hours in this cage prior to testing. An unfamiliar stimulus mouse (a 16-week old C57BL/6J female) was then introduced into the testing cage and left there for 6 minutes. Testing sessions were recorded, and videos were analyzed, with ANY-maze software (Stoelting, Inc., Wood Dale, IL). One observer who was unaware of the genotype of the animals scored the behavior of the test mice, quantifying the time spent performing each of the following behavioral categories and elements:

*Affiliative behaviors:* sniffing the head and the snout of the partner, its anogenital region, or any other part of the body; contact with partner through traversing the partner's body by crawling over/under from one side to the other or allogrooming (grooming the partner).

*Nonsocial activities:* rearing (standing on the hind limbs sometimes with the forelimbs against the walls of the cage); digging; self-grooming (the animal licks and mouths its own fur).

#### *Assessment of estrus cycle*

The estrous phase was assessed by analysis of vaginal smears performed on the day of the direct social interaction test in the female C57BL/6J stimulus mice. The evaluation of the test subjects was conducted after testing, in order to minimize the potential stress effects of the manipulation on direct social interaction. Cell types were identified in



unstained wet preparations, and estrus stages categorized (Caligioni, 2009). Stimulus females were all in metestrus or proestrus.

### *Statistical Analysis*

EPM, open field, direct social interaction and USV data were analyzed using an independent samples t-test. For the social approach task, a repeated measures ANOVA was used to compare time spent in the chamber. However, the time spent in each of the three chambers was not independent; for the analysis, only times spent in the side chambers (containing the stranger mouse and novel object) were compared. Time spent in the center chamber is shown in the graphs to illustrate where the subject mouse spent time during the entire 10-min phase. Chamber time, time spent sniffing the novel object versus the stranger mouse, and number of entries to the side chambers in the social approach test were analyzed. Statistical analyses were performed using IBM® SPSS Statistics Version 25 (IBM SPSS Japan, Tokyo, Japan) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA). Fisher's LSD post-hoc analysis was run when a main effect or when the repeated measure (stranger mouse or novel object) was significant to determine the group differences.

## **Results**

### **Anxiety-like and locomotor behavior**

Statistically significant differences between animals treated with paxilline were not found for the percentage of time spent in the open arms ( $t_{(20)} = -0.6723$ ,  $p > 0.05$ ; Fig. 1A),

total distance traveled ( $t_{(20)} = -0.8333$ ,  $p > 0.05$ ; Fig. 1B). Similarly in the open field test, paxilline treatment did not significantly change locomotor behavior in either the distanced traveled ( $t_{(20)} = -0.1043$ ,  $p > 0.05$ ; Fig. 1C) or the average speed ( $t_{(20)} = -0.0382$ ,  $p > 0.05$ ; Fig. 1D) on the open field test relative to vehicle treated animals.

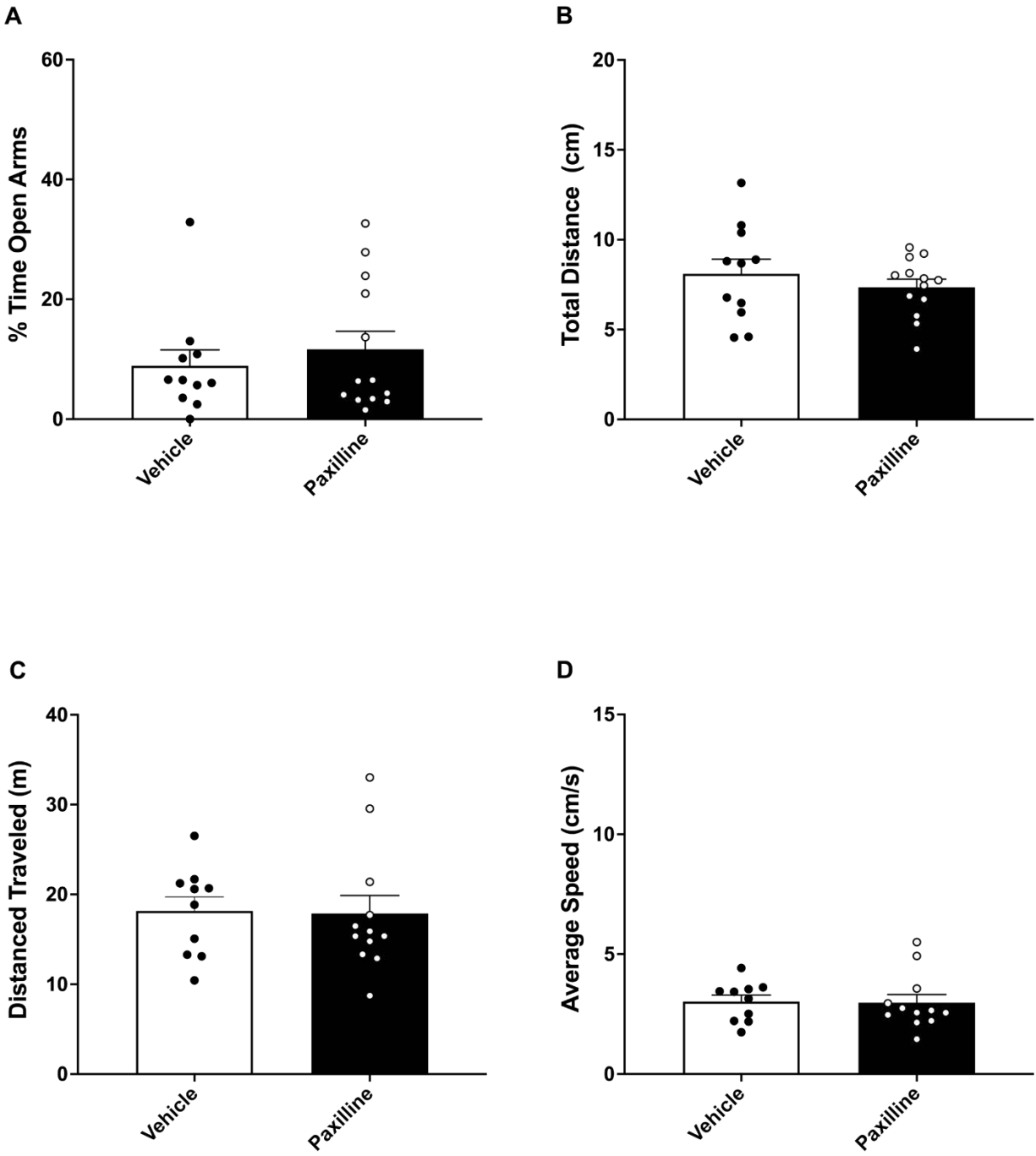


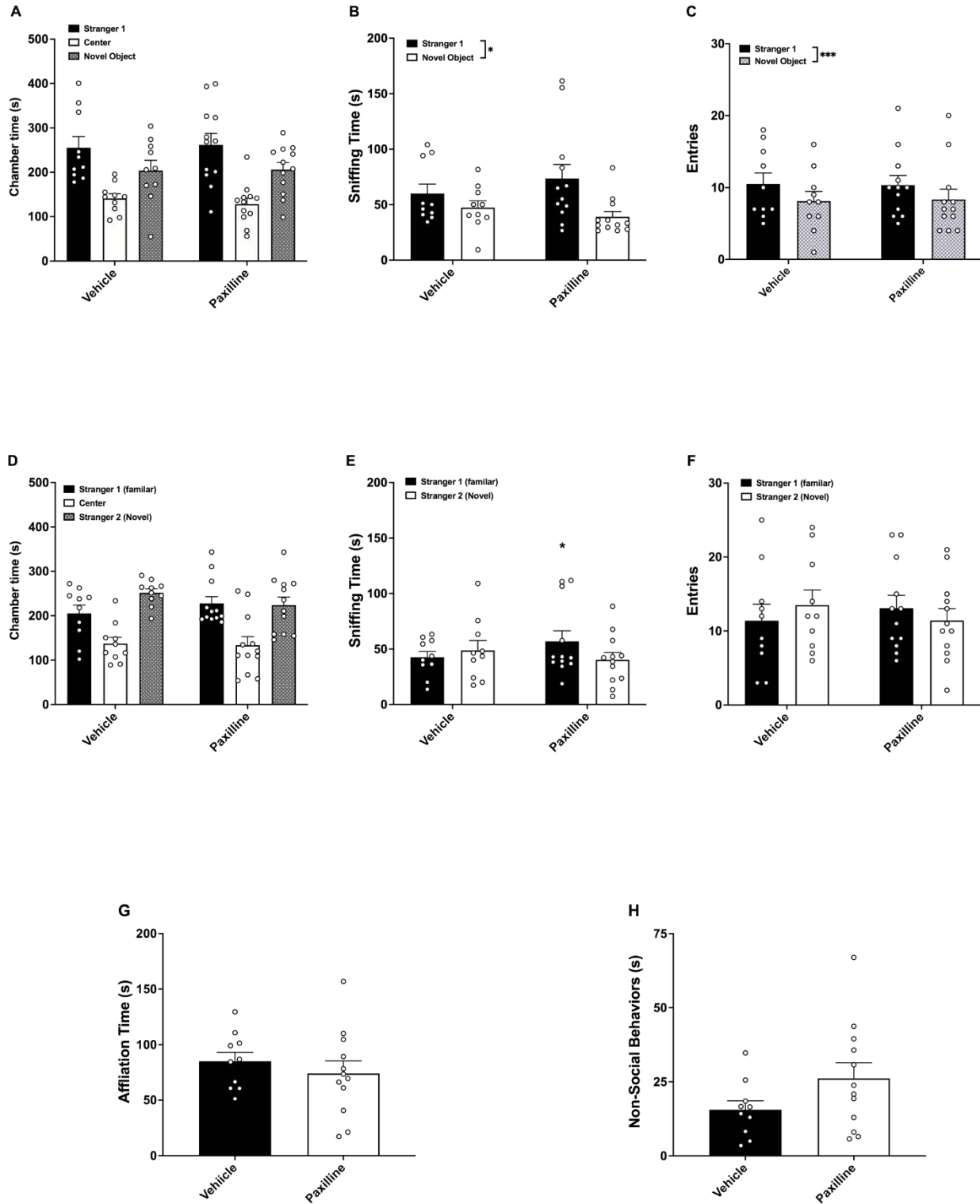
Figure 1. Tests for anxiety-like and locomotor behavior. Elevated plus maze test of anxiety-like behavior (panel A-B). Percentage time spent in the open arms (panel A) and the total distance traveled (panel B) in the elevated plus maze apparatus. Open field test of locomotive behavior (panel C-D). The total distance traveled (panel C) and the average speed (panel D). All data are presented as mean and  $\pm$  SEM. Paxilline n = 12; Vehicle: n = 10.

**Social Behavior**

During the sociability trial a statistically significant effect of chamber was not obtained (chamber,  $F_{1,20} = 2.943$ ,  $p > 0.05$ ; Fig. 2A). No effect of treatment or interaction of chamber and treatment was detected (treatment,  $F_{1,20} = 0.283$ ,  $p > 0.05$ ; chamber x treatment,  $F_{1,20} = 0.005$ ,  $p > 0.05$ ). When sniffing time was evaluated, a significant effect was found, as mice spent more time sniffing the stranger mouse than the novel object (sniff,  $F_{1,20} = 6.235$ ,  $p < 0.05$ ; Fig. 2B). No effect of treatment or interaction of chamber and treatment was detected (treatment,  $F_{1,20} = 0.098$ ,  $p > 0.05$ ; chamber x treatment,  $F_{1,20} = 1.333$ ,  $p > 0.05$ ). An effect of chamber was detected for the number of entries (chamber,  $F_{1,20} = 6.235$ ,  $p < 0.01$ ; Fig. 2C). Mice from both groups made more entries into the chamber containing the stranger mouse. No effect of treatment or interaction of chamber and treatment was detected (ns,  $p > 0.05$ ).

During the novelty trial mice did not show a preference for the chamber containing the familiar mouse (stranger 1) or the novel mouse (stranger 2) ( $F_{1,20} = 1.281$ ,  $p > 0.05$ ; Fig. 2D). Paxilline treatment did not affect the time spent in either chamber (treatment,  $F_{1,20} = 0.040$ ,  $p > 0.05$ ; treatment x chamber,  $F_{1,20} = 1.798$ ,  $p > 0.05$ ). An overall effect of chamber was not detected for sniffing time or treatment (sniff,  $F_{1,20} = 1.087$ ,  $p > 0.05$ ; treatment,  $F_{1,20} = 1.087$ ,  $p > 0.05$ ). An interaction between treatment and sniffing time was detected (treatment x sniff,  $F_{1,20} = 5.170$ ,  $p < 0.05$ ; Fig 2E). Unlike vehicle treated mice that spent more time sniffing the novel mouse (stranger 2), paxilline treated mice spent significantly more time sniffing the familiar mouse (stranger 1). Mice did not show significant differences for the overall number of entries made into either chamber or an overall effect of treatment (ns; Fig. 2F).

Paxilline treatment did not induce significant differences in the amount of time male mice spent in affiliative behaviors with a novel stimulus female mouse ( $t_{20} = 0.7678$ ,  $p > 0.05$ ; Fig. 2G), nor was a statistically significant effect of treatment found for non-social behavior ( $t_{20} = 1.653$ ,  $p > 0.05$ ; Fig. 2H). When individual affiliative (e.g. anogenital sniffing, mounting, allogrooming, contact) or non-social (e.g. self-grooming, rearing, digging) behaviors were analyzed no significant differences were detected (all  $p > 0.05$ ).



*Figure 2. Social behaviors. Social approach test (panel A-F). Sociability trial (panel A-C). Chamber time:* Vehicle and paxilline treated mice did not show significant preference for either the chamber containing the stranger mouse versus the novel object (panel A). *Sniff time:* All mice, regardless of treatment, showed a significant preference for time spent sniffing the stranger mouse relative to the novel object (panel B). *Entries:* Mice of both groups made more entries into the chamber containing the stranger mouse relative to the novel object (panel C). *Novelty trial (panel D-F). Chamber time:* Vehicle and paxilline treated mice did not show significant preference for either chamber (panel D). *Sniff time:* Paxilline treated mice spent significantly more time sniffing the familiar mouse than the vehicle treated group (panel E). *Entries:* Paxilline and vehicle treated mice did not differ significantly in the number of entries they made into the chamber containing the familiar or novel mouse (panel F). *Direct social interaction with novel female (panel G-H).* Affiliation behavior was not affected by paxilline treatment (panel G). Non-social behaviors were not significantly affected by paxilline treatment (panel H). \*  $p < 0.05$ ; \*\*\*  $p = 0.001$ . All data are presented as mean and +/- SEM. Paxilline:  $n = 12$ ; Vehicle:  $n = 10$ .

## Discussion

The current study is the first to directly investigate if pharmacological BKCa inhibition would induce behavioral phenotypes associated with neurodevelopmental disorders. We found that acute treatment with paxilline induced a specific social deficit in one, but not both, of our social behavior paradigms. This deficit was found only during the social novelty trial of the social approach test, and not during the sociability trial or during a direct social interaction. Paxilline treatment did not induce anxiety-like behavior or hyperactivity.

The results of this study suggest that BKCa channels, at least at adulthood, may contribute to specific social behaviors, namely social novelty. Social novelty has not been well examined in the context of BKCa function and the data for B6 mice is not consistent, therefore it is difficult to elucidate why this measure was affected by paxilline (Brigman et al., 2009; Keum et al., 2016; Langguth et al., 2018; Martin et al., 2014; Shoji

& Miyakawa, 2019). One possibility is that paxilline's inhibition of BKCa channels induced a social anxiety-like phenotype. Reduced activity of BKCa channels is most closely associated with FXS (Laumonnier et al., 2004; Laumonnier et al., 2006). Patients with FXS often present with unique social phenotypes compared to ASD, one of which is social anxiety (Cassidy & Allanson, 2010). This phenotype is recapitulated by the *Fmr1*-KO mouse (McNaughton et al., 2008).

One may ask why a more robust phenotype, or impairments in other behavioral domains, were not induced. BKCa channel expression in the central nervous system follows a specific developmental time course, peaking in the late embryonic and early post-natal period (Higgins et al., 2010). Therefore, it is likely that more robust deficits require BKCa disruption during critical periods of development and at adulthood. If this is the case, it is not surprising that inhibition of BKCa channel activity at adulthood in wild-type mice only induced a specific social deficit. It is also possible that BKCa channels may only contribute to a subset of behavioral phenotypes, such as was seen in this study with regard to social novelty.

Currently, the mechanistic links between BKCa channel activity and behavioral deficits are largely derived from studies with the *fmr1*-KO mouse (Carreno-Munoz et al., 2018; Hebert et al., 2014; Zhang et al., 2014). It is likely that disruption of BKCa channels activity due to a syndromic mutation, which occurs in parallel with dysregulation of numerous physiological processes, results in more robust impairments. In this context,



impaired BKCa activity likely makes contributions to, but is certainly not the sole player in these deficits.

It must be considered that the effects obtained in our study could be due to a possible off target effect of paxilline treatment or be mediated by BKCa channels outside of the CNS. While this possibility must be acknowledged, biochemical studies have demonstrated that paxilline is highly specific for BKCa channels (Sanchez & McManus, 1996; Zhou & Lingle, 2014). However, one may ask if this effect is due to BKCa channels in central neurons or those found in other regions of the body. Paxilline is an indole alkaloid, a class of molecules whose ability to cross the blood brain barrier has been well demonstrated (Porter, 1995; Sanchez & McManus, 1996; Shruti et al., 2008). Additionally, a studies with neuron specific  $BKC_{(\beta 4)}$ -KO mice or evaluation of early gene expression with immunohistochemistry following paxilline treat have demonstrated that the behavioral results obtained were indeed due to paxilline's action on BKCa channels in the CNS (Imlach et al., 2008; Sheehan et al., 2009). In consideration of these findings, it is likely that the effects obtained in this study were due to the action of paxilline at BKCa channels in central neurons.

This study supports a link between BKCa activity and a specific type of social behavior. Here we chose the dosage defined in the literature which is known to have physiological and behavioral effects in order to demonstrate “proof of concept” regarding BKCa channels, paxilline, and social behavior (Choi, Lee, Kim, Jo, et al., 2018; Choi, Lee, Kim, Bae, et al., 2018; Knaus et al., 1994; Sanchez & McManus, 1996). Paxilline has

been considered as a possible therapeutic agent for epilepsy caused by a BKCa gain of function mutation (Du et al., 2005; Sheehan et al., 2009; Shruti et al., 2008). Paxilline has also demonstrated the ability to reverse cognitive deficits in certain mouse models (Choi, Lee, Kim, Jo, et al., 2018; Choi, Lee, Kim, Bae, et al., 2018). These studies, in parallel with ours, highlight the potential of paxilline to investigate both causative mechanisms and therapeutic applications for neurodevelopmental disorders. Follow up studies which investigate dose-dependent effects in combination with comprehensive assays of motivated social and learning behaviors within a neurodevelopmental context are critically needed. These will further our understanding of the relationship between BKCa channels, neurodevelopmental disorders, and the potential of paxilline or other indole alkaloids as therapeutics.

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## CHAPTER 4 – TREATMENT OF FXS AND ASD PHENOTYPES BY ENHANCING THE ACTIVITY OF THE ECS

### General Introduction

There are currently no pharmaco-therapeutics which treat FXS or ASD. The rapid increase in prevalence of ASD has brought the lack of therapeutics to the attention of the public. In fact, the available pharmaco-therapeutics for neurodevelopmental disabilities are limited in number and carry risks (Silverman & Crawley, 2014). Current pharmacological interventions used with FXS and ASD patients often target symptoms of aggressive behavior, anxiety, and attention deficits. The core symptoms of social behavior/communication deficits and restrictive rigid repetitive behaviors/interests, currently have no FDA approved therapy (American Psychiatric Association, 2013).

The chapter examined the ECS and BKCa channels as therapeutic targets. This chapter contains one manuscript and two appendices. The *Fmr1*-KO mouse model of FXS was used to examine their efficacy in rescuing behavioral and/or neurobiological patho-phenotypes. Chapter one examined the hypothesis that interference with ECS or BKCa channel function would induce phenotypes found in neurodevelopmental disorders. In the case of the ECS, genetic and pharmacological inhibition of CB1 or a reduction in the primary eCB, 2-AG, induced mouse phenotypes considered homologous to those seen in human FXS and ASD patients. For BKCa channels, inhibition at adulthood induced a mild, context specific, social deficit which suggests that inhibition of BKCa channels during critical periods of development may inform further on their contribution to neurodevelopmental disorders.

Preclinical studies with mouse models of FXS and ASD suggest that the ECS and BKCa channels are targets for the treatment of neurodevelopmental disorders. In regard to the ECS, manipulations which increased the activity of the primary eCBs have rescued some behavioral and neurobiological deficits in the mouse model of FXS and the BTBR mouse model of ASD (Jung et al., 2012; Wei et al., 2016).

Clinically, phytocannabinoids (pCBs), plant derived molecules with similar chemical structures as eCBs, have demonstrated success in treatment neurodevelopmental disorders. The pCB, cannabidiol (CBD) has FDA approval for the treatment of two forms of epilepsy: Dravet Syndrome and Lennox-Gastaut Syndrome (Devinsky, Nabbout, et al., 2018; Devinsky, Patel, et al., 2018). In regard to FXS, a phase 1/2 study with CBD and FXS patients found that 12 weeks of treatment significantly improved behavioral and emotional symptoms (Heussler et al., 2019). Additionally, several earlier case reports support these findings (Tartaglia et al., 2019). Emerging evidence indicates that CBD may be useful as a treatment for ASD (Barchel et al., 2018; Poleg et al., 2019; C. M. Pretzsch, J. Freyberg, et al., 2019; Charlotte M Pretzsch et al., 2019).

Cannabidivarin (CBDV), a propyl analog of CBD, is currently under investigation as treatment for neurodevelopmental disorders (C. M. Pretzsch, B. Voinescu, et al., 2019; Vigli et al., 2018; Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019). Studies indicate that these molecules act on the ECS, however the mechanism of action for pCBs is not well understood (Atwood et al., 2012; De Petrocellis et al., 2011; Hill et al., 2013; Iannotti et al., 2014; Rock et al., 2013; Rosenthaler et al., 2014).

In the case of BKCa channels, three studies have demonstrated that treatment with the BKCa agonist, BMS-204352, rescues neurobiological and behavioral phenotypes in the *Fmr1*-KO mouse (Carreno-Munoz et al., 2018; Hebert et al., 2014; Zhang et al., 2014). BMS-204352 was developed in 2002 for the treatment of ischemia stroke, while it failed to demonstrate therapeutic effects (Jensen, 2002). However, BMS treatment for FXS has yet to move to clinical trials. Since BMS has a favorable safety profile, and shows promising preclinical results, further studies into its efficacy may provide needed evidence to support clinical trials.

The ECS studies contained in this chapter investigate the hypothesis the pCB, CBDV, or increasing the availability of the primary eCB, 2-AG, can rescue behavioral and neurobiological phenotypes in the *Fmr1*-KO mouse. In the case of 2-AG, a single study demonstrated that increasing 2-AG rescued the anxiety and hyperactivity phenotype of the *Fmr1*-KO mouse (Jung et al., 2012). This study did not examine social or repetitive behaviors. This represents a critical gap in our knowledge regarding 2-AG as a therapeutic target for FXS and, perhaps, non-syndromic ASD. To address this aim, the behavioral studies in this chapter include two tests of social behavior, in addition to measures of anxiety, locomotion, and repetitive behaviors.

## 4.1 Study 4

### **Evaluation of the neurobehavioral effects of sub-chronic CBDV administration in the adult Fmr1-KO mouse model of Fragile X Syndrome**

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## Abstract

Non-psychoactive phytocannabinoids, such as Cannabidiol (CBD) and its analogues, are promising compounds for therapeutic applications in a variety of pathologies. For example, pre-clinical studies have shown that Cannabidivarin (CBDV), a propyl analogue of CBD, has anti-convulsant and anti-inflammatory properties and ameliorates behavioural abnormalities in models of Rett syndrome (RTT) and autism spectrum disorder (ASD). Fragile X syndrome (FXS), where there are mutations in Fragile X mental retardation protein (FMRP), is a leading monogenic cause of autism. The aim of this study was to provide an initial investigation of the potential therapeutic effects of CBDV treatment on the neurobehavioural abnormalities present in the fragile X mental retardation 1 (*Fmr1*)-Knock out (KO) mouse model of FXS. CBDV (20 and 100 mg/kg,daily, i.p, n=6-8 per group) was administered for 10 days to 12 week old *Fmr1* KO mice at an age when the pathology is at an advanced stage. Behavioural tests assessing anxiety (elevated plus maze (EPM)), hyperactivity (open field), cognitive (novel object recognition (NOR)), social (3-compartment test and direct social interaction) and sensory (acoustic startle) behavioural domains were performed. Of these, hyperactivity, hyper responsiveness in the acoustic startle test, social and cognitive deficits are consistently demonstrated as aberrant behaviours in *Fmr1* KO mice. Anxiety alterations are less consistent and were evaluated from an opportunistic perspective. Brain samples were also collected to evaluate inflammatory (interleukin-1beta (IL-1 $\beta$ ), interleukin-6 (IL-6), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF $\alpha$ ), cluster of differentiation 11b (CD11b), cluster of differentiation 45 (CD45) and plasticity (using brain derived neurotrophic factor (BDNF)) markers in cortical (prefrontal

cortex (PFC)) and hippocampal (CA1, CA3 and dentate gyrus (DG)) areas, i.e., those brain regions where FMRP normally is most abundant. Fmr1 KO mice were hyperactive compared with their WT littermates in the open field test. Fmr1 KO mice also showed a deficit in the social interaction test and were hyperresponsive to acoustic stimuli. Cognitive and social novelty deficits were present in Fmr1 KO mice in the NOR test and 3 compartment tests. There was no difference in sociability between groups. Although there was a treatment difference between groups on hyperactivity this effect was most evident in Fmr1 KO mice treated with 20 mg/kg CBDV compared to vehicle treated KO mice. There was a difference between Fmr1 KO mice treated with CBDV at 100mg/kg compared to their WT littermates suggesting an anxiolytic effect of CBDV at this dose in Fmr1 KO mice. CBDV treatment in Fmr1 KO mice had no effect on reduced social interaction compared with vehicle treatment. The hyper-responsiveness to an acoustic stimulus was not present in Fmr1 KO mice treated with CBDV 20 mg/kg compared with the same dose in WT mice. There were some changes in potential markers associated with the Fmr1 KO phenotype (decrease in TNF $\alpha$  in CA3, increase in BDNF in DG and differences between WT and Fmr1 KO in IL-1b in DG) but these were not affected by CBDV treatment. There were treatment effects present for IL-1b in CA3 of WT mice, which was associated with administration of 100 mg/kg CBDV as was an increase in CD45 in CA3 compared with vehicle treatment.



## **Conclusions**

In these adult Fmr1 KO mice a phenotype was demonstrated that was broadly consistent with that seen in previous studies from this laboratory, but it was not always possible to differentiate between vehicle and CBDV treatment. Differences between treatments were observed on some behaviours and a small number of brain markers, independently of genotype. The lack of an interaction between these factors suggests that evidence supporting the ability of sub-chronic administration of CBDV to modulate specific deficits present in adult Fmr1 KO mice is sparse.

## Introduction

Non-psychoactive phytocannabinoids such as Cannabidiol (CBD) and its analogues, are promising compounds for therapeutic applications in a variety of pathologies. For example, pre-clinical studies have shown that Cannabidivarin (CBDV), a propyl analogue of CBD, has anti-convulsant and anti-inflammatory properties and ameliorates behavioural abnormalities in models of neurodevelopmental disorder such as Rett syndrome (RTT) (Zamberletti, Gabaglio, Piscitelli, et al., 2019) and autism spectrum disorder (ASD) (Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019). Fragile X (FXS) is the principal monogenic cause of inherited intellectual disability and autism, and is characterized by significant anxiety, attention deficit hyperactivity disorder (ADHD), and hyperarousal to sensory stimuli (Hessl et al., 2001; McLennan et al., 2011; Miller et al., 1999). It is caused by an unstable expansion of CGG repeats in the 5'untranslated region of the *FMR1* gene, producing loss of expression of FMRP, a synaptically expressed RNA-binding protein regulating translation (Pieretti et al., 1991; A. J. Verkerk et al., 1991). Studies with the fragile X mental retardation 1 (*Fmr1*)-knockout (KO) mouse, the animal model of FXS, recapitulate several of the behavioural domains of FXS, including motor, sensory, cognitive, emotional and social behaviours (Hebert et al., 2014; Oddi et al., 2015; Pietropaolo, Goubran, et al., 2014; Pietropaolo et al., 2011; Zhang et al., 2014).

The aim of this study was to investigate potential therapeutic effects of CBDV treatment in adult *Fmr1* KO mice, at an advanced stage of the disease (Pietropaolo & Subashi, 2014) using tests where *Fmr1*-KO mice are known to exhibit a robust behavioural phenotype, for example, motor (open field), cognitive (object recognition), social (direct

social interaction and three-compartment test) and sensory (acoustic startle) domains, as have been previously demonstrated (Hebert et al., 2014; Oddi et al., 2015; Pietropaolo, Goubran, et al., 2014; Pietropaolo et al., 2011; Zhang et al., 2014). Although alterations in anxiety-like behaviour in the elevated plus maze are not consistently described in Fmr1-KO mice, this test was also performed in order to investigate potential anxiolytic effects induced by CBDV treatments and their confounding impact on the other behavioural tests. Brain samples were also collected from tested mice in order to evaluate inflammatory (IL-1 $\beta$ , IL-6, IL-10, TNF $\alpha$ , CD11b, CD45) and plasticity (BDNF) markers in cortical and hippocampal areas, i.e., those brain regions where FMRP normally is most abundant (Bakker et al., 2000; Khandjian, 1999). The same markers were assessed in a previous study, which demonstrated the therapeutic benefits of omega-3 dietary enrichment on aberrant behavior in the same Fmr1-KO model (Pietropaolo, Goubran, et al., 2014).

## Methods

### Animals

Subjects were adult ( $12\pm 1$  weeks old) male *Fmr1*-KO and their wild-type littermates, bred in our animal facility at Bordeaux University. C57BL/6J*Fmr1*<sup>tm1Cgr/Nwu</sup> (B6) breeders were originally obtained from Neuromice.org (Northwestern University). Breeding trios were formed by mating two heterozygous *Fmr1* females with a wild-type C57BL/6J male purchased from Janvier (Le Genest St Isle, France). After 2 weeks the sire was removed and the females were single caged and left undisturbed until weaning of the pups. Mice were weaned at 21 days of age and group-housed with their same-sex littermates (3–5/cage). On the same day, tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes as previously described (Dutch-Belgian Fragile X Consortium, 1994). Only male mice were used for the study, as they are the most commonly used in mouse studies on FXS, due to the higher prevalence of this syndrome in the male sex. Only litters including males of both genotypes (WT and KO) were used for experiments, for a total of 62 subjects (31 WT and 31 KO, i.e., 10-11 mice per experimental condition).

NMRI female mice ( $12\pm 2$  weeks old) and juvenile (4 weeks old) males purchased from Janvier (Le Genest St Isle, France) were used as social stimuli respectively during the direct social interaction and three-compartment tests. This strain has been selected for its high level of sociability (Moles & D'Amato F, 2000) and was previously employed in several social studies from our group on *Fmr1*-KO mice (Pietropaolo, Goubran, et al.,

2014; Pietropaolo et al., 2011; Zhang et al., 2014). Mice were group-housed (4/ cage) and left undisturbed upon arrival at least one week before the social interaction test. All animals were housed in polycarbonate standard cages (33x15x14 cm in size; Tecniplast, Limonest, France), provided with litter (SAFE, Augy, France) and a stainless steel wired lid. Food (SAFE, Augy, France) and water were provided ad libitum. The animals were maintained in a temperature (22°C) and humidity (55%) controlled vivarium, under a 12:12 hr light–dark cycle (lights on at 7 a.m.). All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and local French legislation (Authorization N° 2017073113175079).

### **Experimental procedures**

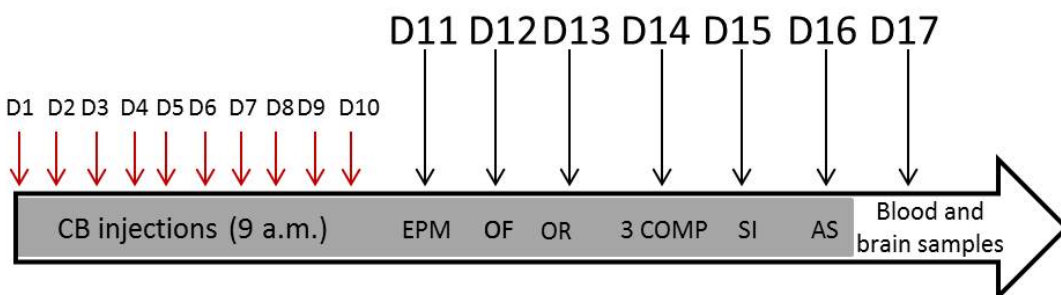
At adulthood (12±1 weeks of age) mice of both genotypes were assigned to one of the three experimental conditions, i.e., injected with vehicle alone (VEH: Cremophor® EL:Ethanol:saline in a ratio of 1:2:17), or with a dose of CBDV (GW Research Ltd., Cambridge, UK) either of 20mg/Kg (CBDV-20) or 100mg/Kg (CBDV-100).

*Fmr1*-KO mice and WT littermate controls were injected daily i.p. (around 9.00 a.m.) during the entire duration of the study, i.e., 17 consecutive days, as illustrated in Figure 1 below.

Behavioral tests began after 10 days of injections, in line with previous studies with CBDV in *Fmr1* KO mice, where effects on cognitive deficits were demonstrated after sub-chronic dosing (10 days) but not after acute dosing (1 day) (data not shown),

following the timing described in Figure 1. After between 3 and 7 days of injections, some animals (belonging to all experimental conditions, 2-3 mice/group) showed signs of distress, such as reduced locomotion in the home-cage, weight loss or hypothermia, and were therefore excluded from further injections and behavioural testing. A total of 46 mice were subjected to all behavioural tests and brain analysis (N=8 for WT-VEH, KO-VEH, WT-CBDV20 and KO-CBDV100; n=7 for KO-CBDV20 and WT-CBDV100). The order of the tests was based on the need of performing first those tests that are more influenced by previous testing experience (such as the elevated plus maze), while leaving until last tests involving a certain degree of stressful experience (such as the acoustic startle, requiring a short confinement in the startle box).

All the tests were performed during the light phase, between 10 a.m. and 5 p.m. by an experimenter who was blind to the genotype and treatment of the animals. Mice were injected one hour before the beginning of each testing procedure; after injection, each mouse was left undisturbed in a waiting cage containing sawdust bedding, food and water. Mice were habituated to the testing room at least one hour and half before the beginning of each behavioural test.



**Fig. 1: Schematic representation of experimental plan of the study:** Daily i.p. injections (indicated by arrows) were given during the entire experimental period, including the days of behavioural testing and brain sampling (one hour before their beginning). EPM= elevated plus maze, OF=open field, OR=object recognition, 3-COMP= three-compartment test for sociability, SI=social interaction, AS=acoustic startle.

### **Elevated plus maze**

The elevated plus maze consisted of grey acrylic glass with a removable plastic grey floor. It was elevated 55 cm above floor level and placed in a quiet testing room with diffuse dim lighting (30 lux in the centre of the maze). The maze consisted of four equally spaced arms (29.5 cm long and 8 cm wide) radiating from a central square measuring 8 × 8 cm. One pair of opposing arms was enclosed with opaque walls (16 cm high, 3 mm thick), except for the side adjoining the central square. The remaining two arms were exposed. A digital camera was mounted above the maze; recorded videos were analysed manually by an observer blind to the experimental condition of the animals using Observer XT (Version 7, Noldus Technology, The Netherlands). The position of each subject in the open or closed arms as well as in the centre was scored.

To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms and allowed to explore freely and undisturbed for 5 min. Anxiety-like behaviours were measured by percent time in open arms = time in open arms / time in all arms × 100%, while locomotor activity was assessed by the total number of entries into the maze arms.

### **Open field (habituation phase of object recognition test)**

The apparatus consisted of 2 identical plastic rectangular arenas, each measuring 24x30 cm in surface area and with 22-cm walls. The arenas were located in a testing room under diffused dim lighting (30 lux in the arena centre). A digital camera was mounted directly above the arenas, capturing images at 5 Hz that were transmitted to a PC running the Ethovision tracking system (version 11, Noldus, The Netherlands).

Each mouse was gently placed in the centre of the appropriate arena and allowed to explore undisturbed for 20 min. The choice of the arena was counterbalanced across experimental groups. Locomotor activity was indexed by distance travelled; the time spent in the central area was assessed as a measure of emotionality and anxiety-like behaviour. At the end of the third trial the maze was cleaned with a 30% ethanol solution and dried.

### **Object recognition**

The open field test served as habituation phase for the object recognition test. At the end of the open field session, two identical objects were placed in two opposite corners and the mouse introduced in the centre of the arena for a 5-min sample phase. Twenty-four hours later, the mouse was returned to the arena for a 5-min test phase, where one of the objects was replaced with a novel one of different shape and material. Both the type of object used for the sample phase and the position of the novel object during the test phase were counterbalanced across experimental groups. During the training and test phases, the time spent sniffing each object was manually scored by an observer unaware of the experimental conditions of the animals using Observer XT (version 7, Noldus, the Netherlands). During the test phase, a percent recognition index, was used to measure object recognition as follows:  $100 \times T_{\text{novel object}} / (T_{\text{novel object}} + T_{\text{familiar object}})$ . (lack of novel object recognition= 50%). At the end of the sample and test phases the apparatus as well as the objects were cleansed with a 30% ethanol solution and dried.



### Three compartment test

The testing apparatus (described in details elsewhere (Pietro Paolo et al., 2011)) consisted of a central chamber connected on each side to another compartment containing a perforated stimulus cage (8x8x15cm) to allow the test mouse to interact with the mouse or the object inside the stimulus cage. Each stimulus cage was placed at a distance of 5.5cm from the side walls and there was no space between the stimulus cage and the back wall. The object employed for the test was a plastic black cylinder and the stimulus mice were NMRI juvenile males, in order to minimize aggressive tendencies and exclude sexual interest.

Each experimental subject was introduced in the middle of the central compartment and allowed to explore the apparatus for 3 trials of 5 min each: in trial 1 habituation to the apparatus containing empty stimulus cages was evaluated, while in trial 2 the preferential exploration of the social (a juvenile male mouse) *versus* the non-social (an object) novel stimulus was measured, and in trial 3 the preferential exploration of a novel *versus* familiar social stimulus was assessed, by replacing the object with a novel stimulus mouse.

In all trials the total distance travelled as well as the time spent in each contact area (20 × 22 cm) containing the stimulus cages was computed using the Ethovision tracking system (version 11, Noldus, The Netherlands). A percentage score was also computed for the last two trials as follows:

- On trial 2: Sociability index =  $100 \times \frac{T_{\text{social stimulus}}}{(T_{\text{social stimulus}} + T_{\text{non-social stimulus}})}$ ,

- On trial 3: Social novelty preference index =  $100 \times \frac{T_{\text{novel social stimulus}}}{(T_{\text{novel social stimulus}} + T_{\text{familiar social stimulus}})}$ .

At the end of each trial the experimental animal was confined in the central compartment by means of two Plexiglas magnetic doors for 30sec. At the end of the third trial the apparatus as well as the object and the stimulus cages were cleansed with a 30% ethanol solution and dried.

### **Direct social interaction**

Direct social interaction was assessed as described in detail elsewhere (Pietropaolo et al., 2011). Briefly, an unfamiliar adult NMRI female mouse was introduced into a testing cage (32 x 14 x 12.5cm, with a flat metal grid as cover and approximately 3 cm of clean sawdust bedding) to which experimental subjects were habituated for one hour. Six min-testing sessions were recorded and videos analyzed with Observer XT (version 7, Noldus, The Netherlands). One observer who was unaware of the experimental conditions of the animals scores the time spent performing affiliative behaviors, i.e., social investigation (nose, body and anogenital sniffings) and contact. At the beginning of the testing day, the estrous cycle of the stimulus females was assessed through the analysis of the vaginal smear, so that only females in the non-estrous phase were used for social interaction sessions.

### **Sensory responsiveness (acoustic startle test)**

The apparatus consisted of four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA). Each startle chamber comprises a non-restrictive

cylindrical enclosure made of clear Plexiglas attached horizontally on a lightweight mobile platform, which was in turn resting on a solid base inside a sound-attenuated isolation cubicle. A high-frequency loudspeaker mounted directly above the animal enclosure inside each cubicle produced a continuous background noise of 66 dB and the various acoustic stimuli in the form of white noise. Twenty-four hours before testing, mice were placed in the recording chamber of a startle response box for 5 min without being exposed to any stimuli, in order to habituate them to the confinement and reduce the related stress.

On the test day, mice were presented with continuous white noise of 66 dB (background) and, after a 5 min habituation period, mice were presented with pulses of white sound of 20 ms duration and of varying intensity: +6, +12 +18 and +24 dB over background levels (namely 72, 78, 84 and 90 dB). Each intensity was presented 8 times, in a randomized order with variable intervals (10 sec to 20 sec) between the onset of each pulse. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animal were converted into analogue signals by a piezoelectric unit attached to the platform. These signals were digitised and stored by a computer. A total of 130 readings were taken at 0.5-ms intervals (i.e., spanning across 65 ms), starting at the onset of the pulse stimulus. The average amplitude (in mV) over the 65 ms was used to determine the stimulus reactivity and further averaged across trials of the same stimulus intensity.

## **Brain analyses**

### **Assessment of inflammatory and plasticity markers using reverse transcription and real-time RT-PCR**

Mice were sacrificed by cervical dislocation; brains were immediately extracted and cut in two hemispheres that were separately frozen using dry ice for the half that was used for RT-PCR analysis (while for the other half, that was stored for subsequent analysis, liquid nitrogen was used). Frozen brains were thawed to  $-20^{\circ}\text{C}$  in a cryostat chamber (CM3050 S, Leica Microsystems, Wetzlar, Germany).

Whole brain tissue was sectioned at  $50\ \mu\text{m}$  using a Leica cryostat and mounted in series with 8-10 sections per slide on polyethyl-ene-naphthalate membrane 1mm glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) that were pretreated to inactivate RNase. Serial sections were created from distinct coronal sections (bregma positions based on a reference brain atlas by Georges Paxinos and Keith B.J. Franklin) and individual regions were matched across section and harvested by LCM.

The pre-frontal cortex (PFC) (Infralimbic cortex and prelimbic cortex) series were collected from bregma 1.98 mm to 1.54 mm, cornu Ammonis 1 (CA1), cornu Ammonis 3 (CA3) and dentate gyrus (DG) of the hippocampus series were collected from bregma -1.22 mm to -2.80 mm. Subsequently, the sections were immediately fixed for 30 seconds with 95% ethanol, followed by 75% ethanol for 30 seconds and by 50% ethanol for 30 seconds to remove the OCT. Sections were stained with 1% cresyl violet in 50% ethanol for 30 seconds and dehydrated in 50%, 75% and 95% ethanol for 30 seconds each, 2x in 100% ethanol for 30 seconds.

Laser Pressure Catapulting microdissection (LPC) of samples was performed using a PALM MicroBeam microdissection system version 4.6 equipped with the P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). Laser power and duration were adjusted to optimize capture efficiency. Microdissection was performed at 5X magnification. The microdissection of pure brain structures were collected in adhesives caps and re-suspended in 250µl guanidine isothiocyanate-containing buffer (BL buffer from ReliaPrep™ RNA Cell Miniprep System, Promega, Wisconsin,USA) with 10 µl 1-Thioglycerol, and stored at -80°C until extraction was done. Total RNA was extracted from microdissected tissues using the ReliaPrep™ RNA Cell Miniprep System (Promega, Wisconsin,USA) according to the manufacturer's protocol. The integrity of the RNA was checked by capillary electrophoresis using the RNA 6000 Pico Labchip kit and the Bioanalyser 2100 (Agilent Technologies, Massy, France), and quantity was estimated using a Nanodrop 1000 (Thermo Scientific, Waltham, USA). The RNA integrity number (RIN) were above 7/8.

### **Quantitative Real-Time PCR (q-PCR)**

RNA was processed and analyzed according to an adaptation of published methods (Bustin et al., 2009). Briefly, cDNA was synthesized from 140 ng of total RNA for each structure by using qSript™ cDNA SuperMix (Quanta Biosciences). qPCR was performed with a LightCycler® 480 Real-Time PCR System (Roche, Meylan, France). qPCR reactions were done in duplicate for each sample by using LightCycler 480 SYBR Green I Master (Roche) in a final volume of 10 µl. The qPCR data were exported and analyzed in an informatics tool (Gene Expression Analysis Software Environment)

developed at the University of Bordeaux. The Genorm method was used to determine the reference gene (Bustin et al., 2009). Relative expression analysis was normalized against two reference genes. Succinate dehydrogenase complex subunit (Sdha) and tubulin alpha 4 a (Tuba4a) were used as reference genes for PFC. Succinate dehydrogenase complex subunit (Sdha) and tyrosine 3 mono oxygenase tryptophan 5 mono oxygenase (Ywhaz) were used as reference genes for CA1. Tubulin alpha 4 a (Tuba4a) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used as reference genes for CA3. Tubulin alpha 4 a (Tuba4a) and non-POU-domain-containing, octamer binding protein (Nono) were used as reference genes for DG. The relative level of expression was calculated with the comparative ( $2^{-\Delta\Delta CT}$ ) method (Livak & Schmittgen, 2001). Primer sequences are reported in Table 1.

Gene	GenBank ID	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Nono	NM_023144	CTGTCTGGTGCATTCCTGAACTAT	AGCTCTGAGTTCATTTTCCCATG
Sdha	NM_023281	TACAAAGTGCGGGTCGATGA	TGTTCCCAAACGGCTTCT
Ywhaz	NM_011740	CTTGTGAGGCTGTGACACAAACA	CAAGAGTGTGCACGCAGACA
Tuba4a	NM_009447	CCACTTCCCCTTGGCTACCTA	CCACTGACAGCTGCTCATGGT
Gapdh	NM_008084	TCAAGAAGGTGGTGAAGCAG	TGGGAGTTGCTGTTGAAGTC
IL-1b	NM_008361	TCGCTCAGGGTCACAAGAAA	TCAGAGGCAAGGAGGAAAACAC
IL-6	NM_031168	TACTCGGCAAACCTAGTGCGT	ATTTTCTGACCACAGTGAGGAATG
IL-10	NM_010548	AGTTGTGAAGAACTCATGGGTCT	TGCTGCAGGAATGATCATCAA
TNF $\alpha$	NM_013693	GGCACTCCCCCAAAGATG	GCCACAAGCAGGAATGAGAAG
ITGAM (CD11b)	NM_001082960	CTCATCACTGCTGGCCTATACAA	GCAGCTTCATTCATCATGTCTT
PTPRC (CD45)	NM_011210	TGGGACAACGCAGACTCTCA	CTGCACAGCCATGTTCTTTCAT
BDNF	NM_007540	CCCGTCTGTACTTTACCCTTTGG	TGACTAGGGAAATGGGCTTAACA

**Table 1: Primer Sequences.**

### **Drug preparation**

CBDV (synthetic; Batch N°: 10300001; purity by HPLC: 95.9%) was supplied by GW Research Limited (Cambridge, UK) and stored at approximately -20°C), protected from light. Injectable solutions were prepared fresh each day and were continuously stirred until injection.

### **Statistical Analyses**

Data were inspected for the identification of possible outliers, i.e., using Grubb's test. Outliers were excluded from statistical analysis of the specific dataset and variable. The number of outliers (1-2/group, if observed) is in line with that seen in other similar studies conducted by this laboratory. For each test, the exact N were the following:

<b>Test</b>	<b>Variable</b>	<b>WT - VE H</b>	<b>WT- CBD V20</b>	<b>WT- CBD V100</b>	<b>KO - VE H</b>	<b>KO- CBDV2 0</b>	<b>KO- CBDV10 0</b>
<b>EPM</b>	All	7	7	7	8	7	7
<b>OF</b>	Distance moved	8	8	7	8	7	7
	%Time centre	8	8	7	8	7	8
<b>OR: sample</b>	Time contact objects	8	8	7	8	7	8
<b>OR: test</b>	% recognition	8	8	7	8	7	8
<b>3-Comp: tr1</b>	Distance moved	8	8	7	8	6	8
	%Time contact	8	8	7	8	7	8
<b>3-Comp: tr2</b>	Distance moved	8	8	7	8	7	8
	%Time contact	8	8	6	7	7	8
<b>3-Comp: tr3</b>	Distance moved	8	8	6	8	7	8
	%Time contact	8	8	7	8	7	8
<b>SI</b>	Time in affiliation	8	8	7	6	7	7
<b>AS</b>	Ln (startle response)	8	8	7	8	7	8

**Table 2: Number of mice/group after exclusion of outliers (differing  $\geq 2SD$  from the mean).** EPM= elevated plus maze, OF=open field, OR=object recognition, 3COMP= three-compartment test for sociability, SI=social interaction, AS=acoustic startle.

Data from the outliers are included in the raw data files provided in the appendix (marked in yellow and named “outlier”). Normality was assessed through the Shapiro-Wilks test for each experimental group (genotype x dose) and each variable of interest. Data from startle reactivity did not show a normal distribution and were therefore subjected to natural logarithmic (ln) transformation in order to meet the normality requirements of ANOVA. For all other variables, data distribution was found to be normal and a parametric 2x3 ANOVA with genotype and treatment as the between-



subject factors was applied. Within-subject factors were included according to the specific test and used as repeated measures in the ANOVA; these included for example, 5-min bins for the total distance travelled in the open field, the stimulus area for the three-compartment test, the type of object for the object recognition test, 3-min-time bins for the social interaction test and the stimulus intensity for the acoustic startle assessment. Post-hoc comparisons were conducted, when a significant interaction was found, using Tukey's HSD (multiple comparisons) test where  $P < 0.05$  was taken as significant. Otherwise, separate one-way ANOVAs in each treatment group with genotype as the between subject factor were conducted, if appropriate. For the object recognition index, sociability and social novelty scores in the three compartment test, a one-sample t test was used for comparison with chance level/lack of preference (i.e., 50%), as done in previous behavioural studies (see for example, (Oddi et al., 2015; Vandesquille et al., 2013)). All analyses were carried out using PASW Statistics 18 and Statview. Data were expressed as mean  $\pm$  SEM.

## **RESULTS**

### **Behavioural tests**

#### **Elevated plus maze**

Anxiety measured in the elevated plus maze was an opportunistic endpoint and test, which has not been previously assessed in Fmr1 KO mice by this laboratory. Anxiety-like behaviour, assessed by the percent time spent in the open arms, was not affected by genotype or treatments [all effects, ns. Mean  $\pm$  standard error of the mean (SEM) for

WT were:  $7.40 \pm 1.34$ (VEH),  $9.64 \pm 1.51$  (CBDV-20),  $8.68 \pm 1.63$  (CBDV-100); for KOs were:  $9.50 \pm 3.15$ (VEH),  $14.81 \pm 3.91$  (CBDV-20),  $6.39 \pm 1.34$  (CBDV-100)].

Locomotor activity did not differ among experimental groups, as demonstrated by the number of total arm entries [all effects, ns. Mean $\pm$ SEM for WT were:  $22.14 \pm 1.71$ (VEH),  $23.86 \pm 2.56$  (CBDV-20),  $25 \pm 2.99$  (CBDV-100); for KOs were:  $22.25 \pm 2.55$  (VEH),  $27 \pm 1.7$  (CBDV-20),  $23.57 \pm 2.24$ (CBDV-100)].

## **Open field**

### **Object recognition test, habituation phase, total distance travelled**

The open field arena used for the object recognition test was used to assess hyperactivity, which is a robust end point for Fmr1 KO mice, and anxiety. The distance travelled during the 20-min session of the habituation to the open field was analysed in 5-min bins (using a 2x3x4 ANOVA with genotype and treatment as the between-subject factors and 5-min bins as the within subject factor), in order to assess locomotor habituation (Fig.2-A). Indeed, a time-dependent reduction in locomotion was observed in all experimental groups, independently of the genotype and the treatment [5 min-bin effect:  $F(3,117)=156.08$ ,  $p<0.0001$ ].

Fmr1 KO mice were more active than their WT littermates [genotype effect:  $F(1,39)=15.65$ ,  $p<0.001$ ; Fig.2-B] confirming this phenotype in Fmr1 KO mice. Although there was also a difference between treatments for hyperactivity [ $F(2,39)=11.29$ ,  $p<0.001$ ,], there was no interaction and when these individual factors were examined further within the 2-way ANOVA, hyperactivity was only present in Fmr1 KO mice

treated with 20 mg/kg CBDV compared to vehicle treated KO mice ( $p < 0.05$  Tukey's multiple comparisons test) [Fig.2B].

Consistent with previous data, anxiety levels appeared reduced in KO mice compared to their WT littermates as shown by the increased time spent in the centre of the open field [Genotype :  $F(1, 39) = 4.216, p < 0.05$ ]. On examination of the data this increase was seen only in Fmr1 KO mice treated with CBDV at 100 mg/kg compared to their WT littermates treated with 100mg/kg CBDV [interaction genotype x treatment:  $F(2,40)=3.54, p < 0.05$ , post-hoc Tukey's multiple comparisons test:  $p < 0.05$  KO-CBDV100 versus WT-100; Fig.2-C]. This suggests that the highest dose of CBDV may induce the appearance of a novel (anxiolytic) phenotype in Fmr1-KO mice.

### **Object recognition, sample phase, exploration time**

During the sample phase, all mice explored equally the two sample objects irrespective of their position (object position effect; ns, data not shown). Overall, there was no difference in the object exploration among experimental groups, although a tendency towards an increased exploration consistent with the hyperactive phenotype could be observed in KO-VEH mice, but it failed to reach statistical significance [genotype x treatment interaction:  $F(2,40)=3.21, p=0.05$ ; Fig. 2D].

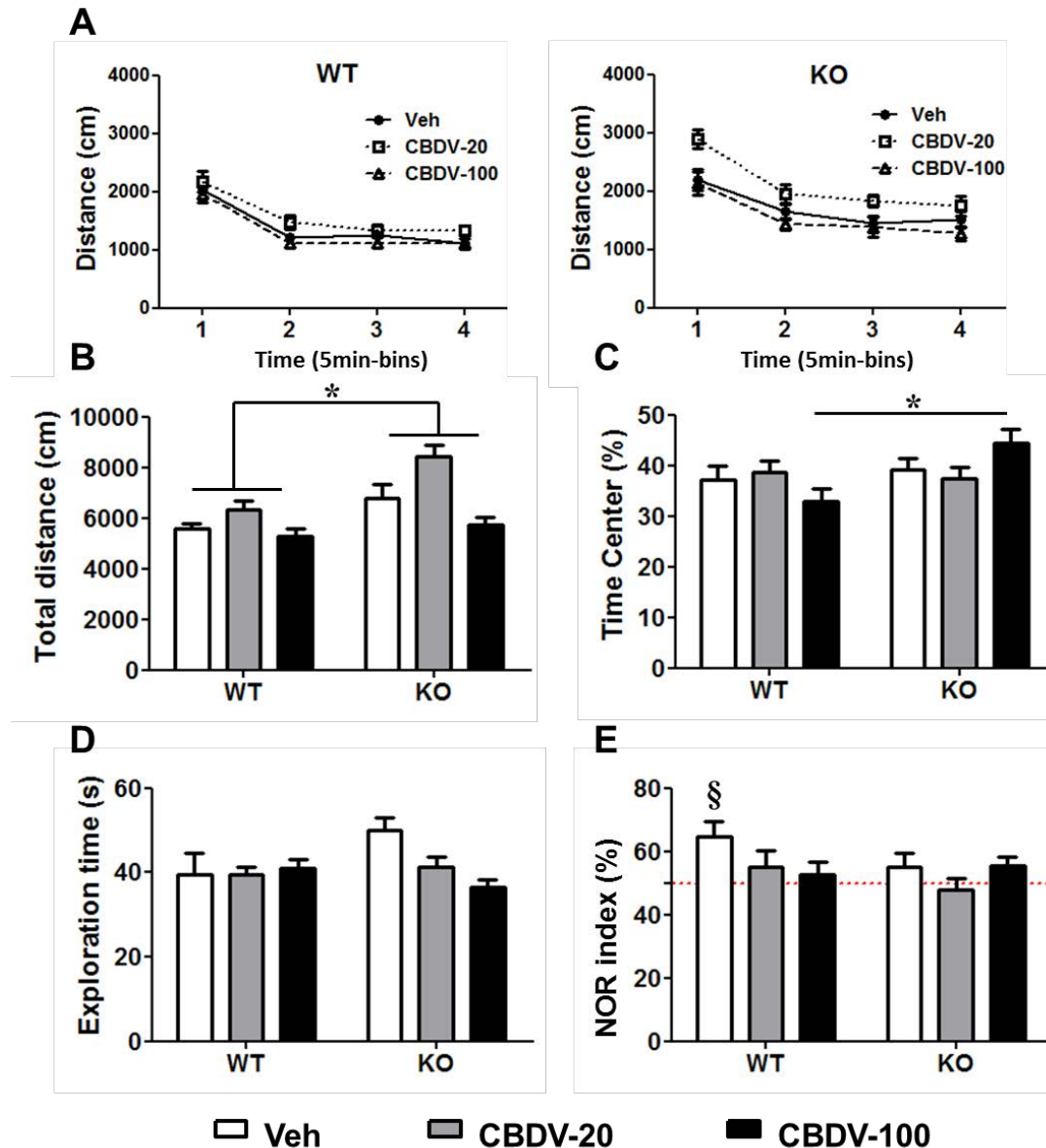
### **Object recognition, test phase**

During the test phase only the WT-VEH mice showed a recognition index that was significantly above the chance level [one sample t-test versus 50%:  $p < 0.05$  in WT-VEH;

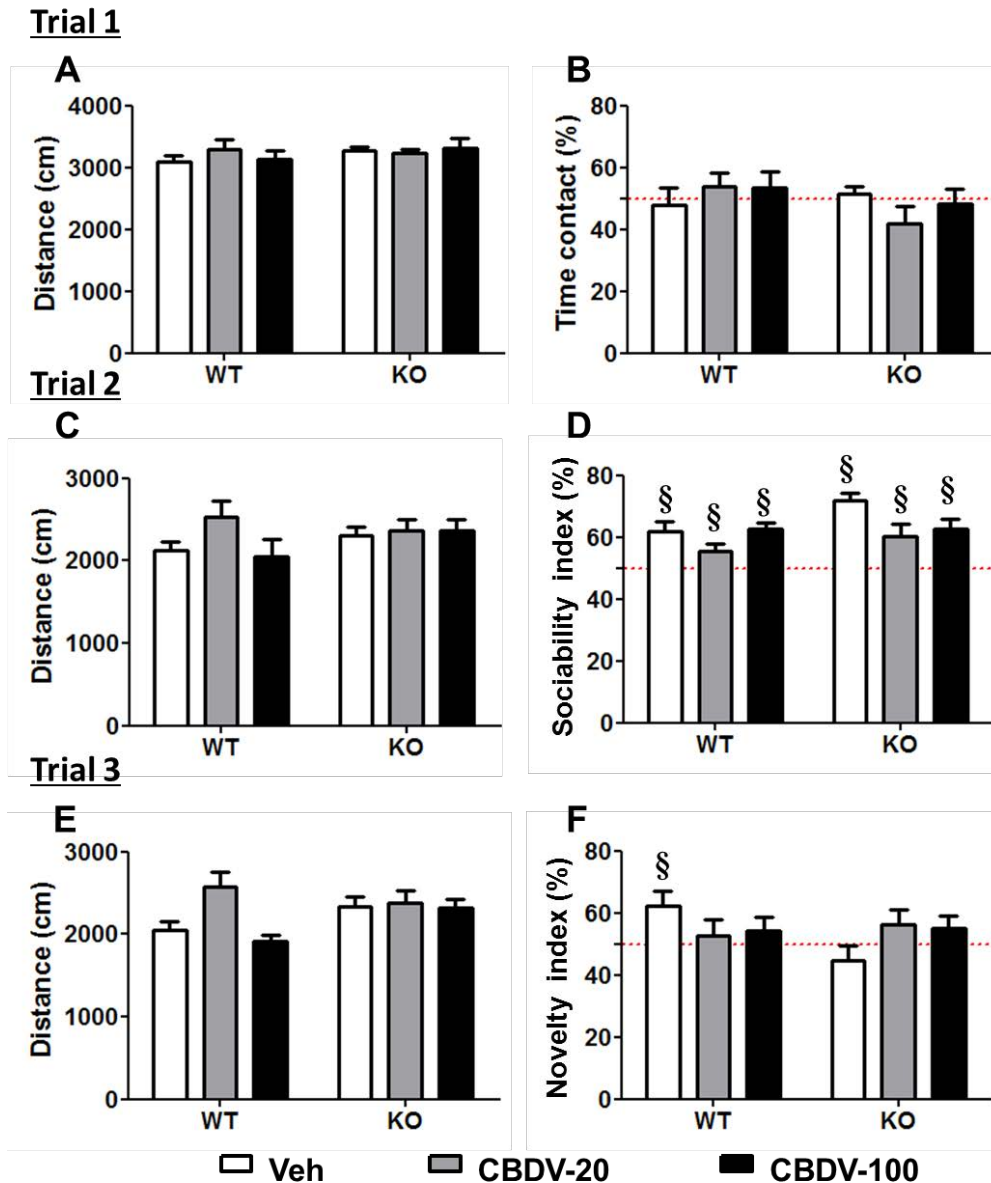
ns in all other groups, Fig. 2E] suggesting that a memory deficit was present in all other treated groups. Hence, an object recognition deficit was present in KO-VEH mice, as previously described, but was also evident following CBDV treatment in both WT and Fmr1 KO mice.

### **Three-compartment test for sociability and social novelty**

In trial 1 (habituation phase) no difference was observed among groups in the total distance moved in the apparatus [all effects, ns; Fig. 3-A], and mice equally explored the two side compartments containing the stimulus cages, showing no bias for any of the two [all effects ns, and the difference from chance level ns, Fig.3B ]. On trial 2 (sociability), again no difference was found on locomotion [all effects, ns; Fig. 3C]. All mice preferred to explore the social versus the inanimate stimulus, as demonstrated by a mean percentage time spent in the contact area containing the social stimulus significantly >50% in all experimental groups [t-test difference from chance level of 50%,  $p < 0.05$  in all genotype x treatment groups, Fig. 3D]. This lack of sociability deficit in KO-VEH mice was as expected, based on previous data from our and other studies (reviewed in (Pietropaolo & Subashi, 2014)). On trial 3, locomotor activity did not differ between experimental groups ([all effects, ns Fig.3E). In this trial, the WT-VEH group was the only one to show a preference for the novel social stimulus [One sample t-test versus 50% chance level:  $p < 0.05$ ; Fig. 3F] demonstrating a deficit in Fmr1-KO mice in this test, but also in CBDV treated mice.



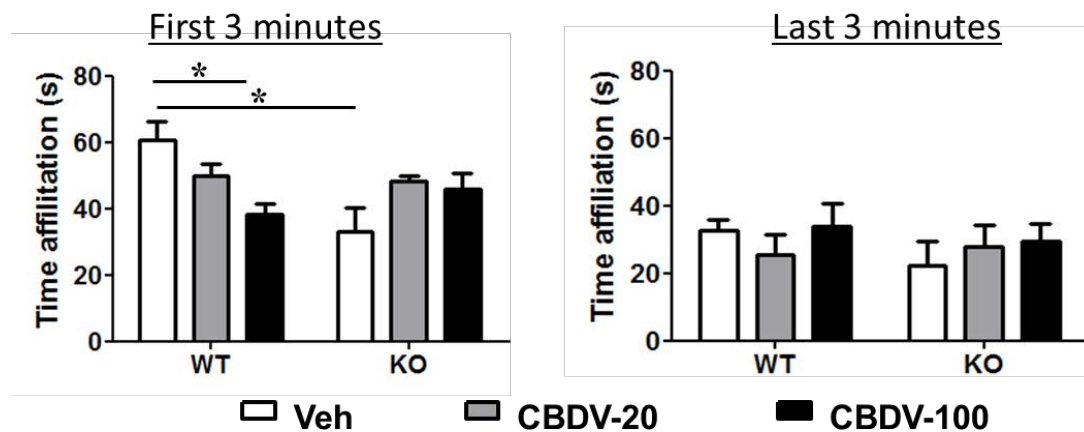
**Fig. 2: Object recognition:** Locomotor habituation (A), overall activity, Genotype:  $F(1,39)=15.65$ ,  $p<0.001$ , Treatment:  $F(2,39)=11.29$ ,  $p<0.001$ , Genotype x Treatment: NS (B) and anxiety-like behavior in the empty arena during the 20-min session of the open field used as the habituation phase., Genotype:  $F(1,39)=4.216$ ,  $p<0.05$ , Genotype x treatment:  $F(2,40)=3.54$ ,  $p<0.05$ , post-hoc Tukey's multiple comparisons test:  $p<0.05$  KO-CBDV100 versus WT100 (C) Exploration of the two identical objects introduced in the arena during the 5-min sample phase (D). Twenty-four hours later object recognition was measured during the 5-min test phase by the percent novel object recognition index (NOR)=%time spent exploring the novel object/the time spent exploring the novel+familiar objects (E). Data are expressed as mean±SEM. § versus lack of NOR (50%, red dotted line),  $p<0.05$  one sample t-test



**Fig. 3: Three compartment test:** Locomotion (A, C and E) and percent sociability (B, D) and social novelty recognition (F) scores during the 3 trials of the test (lasting 5 min each). These included a first trial of habituation to the apparatus containing the empty stimulus cages (A, B), a second trial of sociability (C,D), assessing the percent preference for a social versus a non-social novel stimulus (juvenile male mouse versus object), and a third trial of social novelty preference (E,F), assessing the percent preference for a novel versus a familiar stimulus mouse. Data are expressed as mean±SEM. § versus chance level (50%, red dotted line),  $p < 0.05$  one sample t-test

## Direct social interaction with an adult female

The 6-min interaction session was analyzed using 3-min bins as the within-subject factor, in order to assess habituation to the social stimuli. Indeed, the 2 x 3 x 2 ANOVA of the time spent performing affiliative behaviors led to a significant effect of 3-min bins [ $F(1,37)=29.91$ ,  $p<0.0001$ ; Fig. 4], independently of genotype and treatment, due to the fact that most of these social behaviors were displayed during the first 3 min and decreased afterwards.



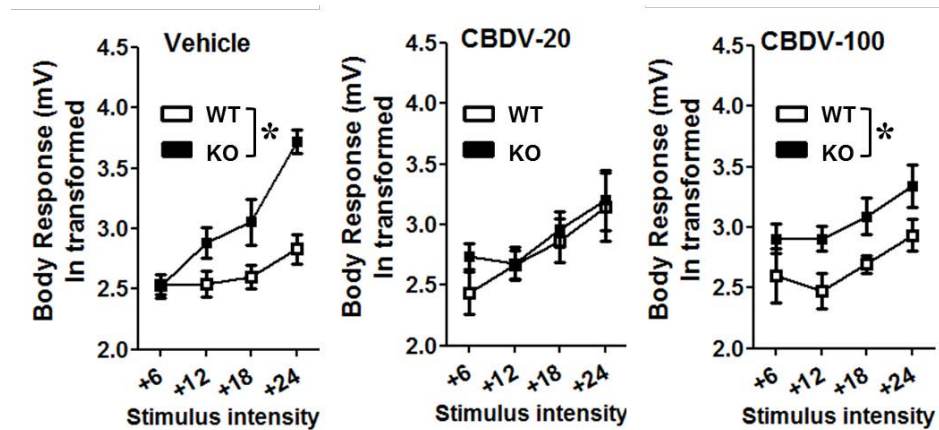
**Fig. 4: Social interaction:** Time spent performing affiliative behaviors (including sniffings and contact) towards a WT adult female during a 6-min session of direct social interaction test, genotype x treatment:  $F(2,37)=10.16$ ,  $p<0.001$ , WT-VEH versus KO-VEH,  $p<0.001$ , WT- VEH vs WT CBDV100,  $p<0.05$ , post-hoc Tukey's test. Data are expressed as mean $\pm$ SEM.

Analysis of the time spent in affiliative behaviors during the first 3 min alone showed that KO-VEH mice displayed a deficit compared to WT-VEH [genotype x treatment:  $F(2,37)=10.16$ ,  $p<0.001$ , WT-VEH versus KO-VEH,  $p<0.001$ , post-hoc Tukey's test, confirming the *Fmr1* KO mouse phenotype in this test. However, CBDV treated *Fmr1* KO mice were not different to *Fmr1* KO mice treated with vehicle. CBDV at the highest dose of 100 mg/kg reduced affiliation in WT mice, WT- VEH vs WT CBDV100,  $p<0.05$ , post-hoc Tukey's test; Fig. 4.

## Acoustic startle

Reactivity data did not follow a normal distribution for all experimental groups and stimulus intensities; therefore, a natural logarithmic transformation was applied, which allowed the normality criterion to be met before performing a 2 x 2 x 4 (genotype x treatment x stimulus intensity) ANOVA of the startle response (Fig.5).

As expected, body startle response increased with the stimulus intensity [intensity effect:  $F(3,120)=21.13$ ,  $p<0.0001$ ; Fig. 5A]. KO mice showed an overall startle hyper-responsiveness [genotype effect:  $F(1,40)=15.07$ ,  $p<0.001$ ], which was not present in *Fmr1* mice treated with CBDV 20 mg/kg treatment, as demonstrated by separate ANOVAs yielding genotype effects only in the VEH [ $F(1,62)=13.25$ ,  $p<0.001$ ] and in the CBDV-100 [ $F(1,58)=12.50$ ,  $p<0.001$ ] groups, Fig. 5A and B).



**Fig. 5: Acoustic startle:** the body startle response to acoustic stimuli of 6, 12, 18 and 24 dB over the background of 66 db (A) Startle reactivity was ln-transformed in order to meet the normality requirement for a parametric repeated measures ANOVA, genotype effect:  $F(1,40)=15.07$ ,  $p<0.001$ . Separate ANOVAs performed between genotypes for each treatment group yielded a genotype effect in the vehicle VEH [ $F(1,62)=13.25$ ,  $*p<0.001$ ] and CBDV 100 mg groups [ $F(1,58)=12.50$ ,  $*p<0.001$ ], but no difference between WT and *Fmr1* KO mice treated with 20 mg/kg CBDV groups. Data are expressed as mean $\pm$ SEM.

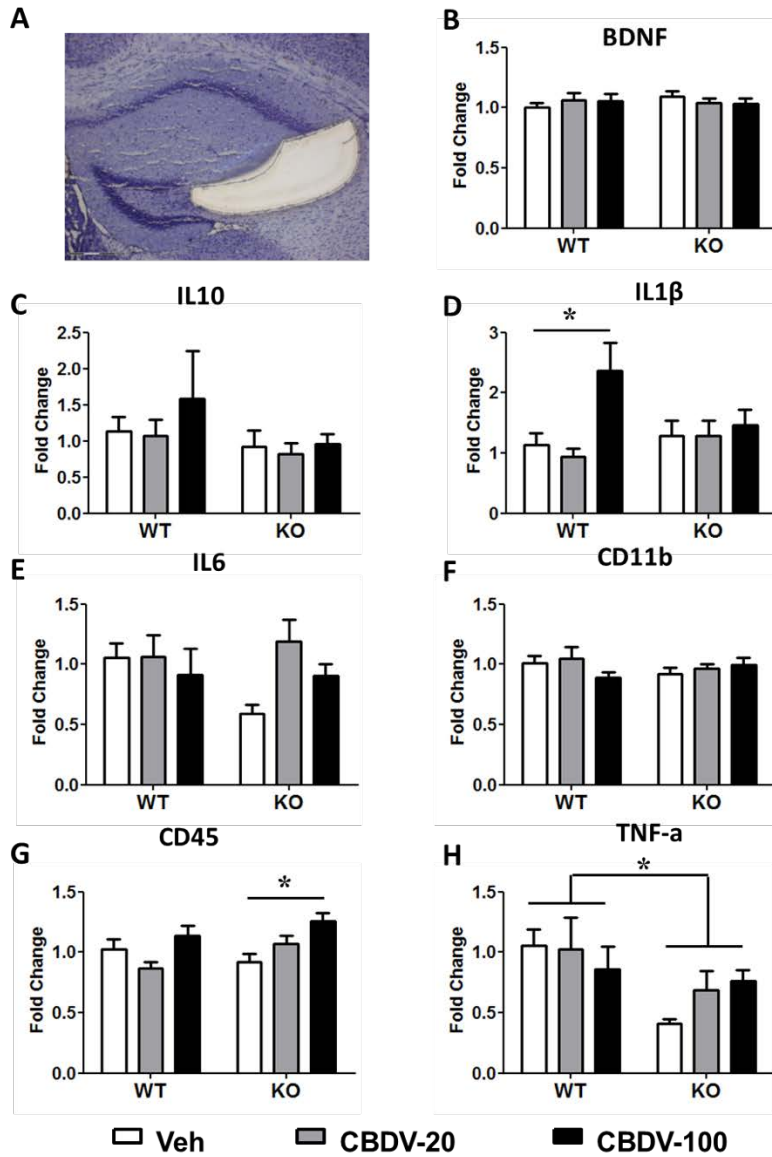


## Brain analysis

### Hippocampus, CA3

TNF $\alpha$  gene expression was decreased in KO mice compared to WT [genotype effect:  $F(1,38)=7.76$ ,  $p<0.01$ ; Fig. 6H], and this effect was not rescued by CBDV treatment. There was treatment effect for IL-1b gene expression [treatment:  $F(2,39)=5.13$ ,  $p<0.05$ ] and whilst there was no effect of genotype nor an interaction [genotype x treatment effect:  $F(2,39)=2.99$ ,  $p=0.06$ ], when the treatment effect was examined further, there was an increase in IL-1b expression in WT mice associated with 100 mg/kg CBDV compared to vehicle,  $p<0.01$ , Tukey's test (Fig.6D). There was also a difference between treatment groups for CD-45 gene expression [treatment effect:  $F(2,39)=7.0$ ,  $p<0.01$ ; Fig. 6F], but again no genotype effect or interaction [genotype x treatment effect:  $F(2,39)=2.5$ ,  $p=0.09$ ]. When this treatment effect was examined further, the increase in CD-45 was associated with Fmr1 KO mice treated with CBDV at 100mg/kg. There was no change in the expression of BDNF, IL-6, IL-10 or CD11b between groups.

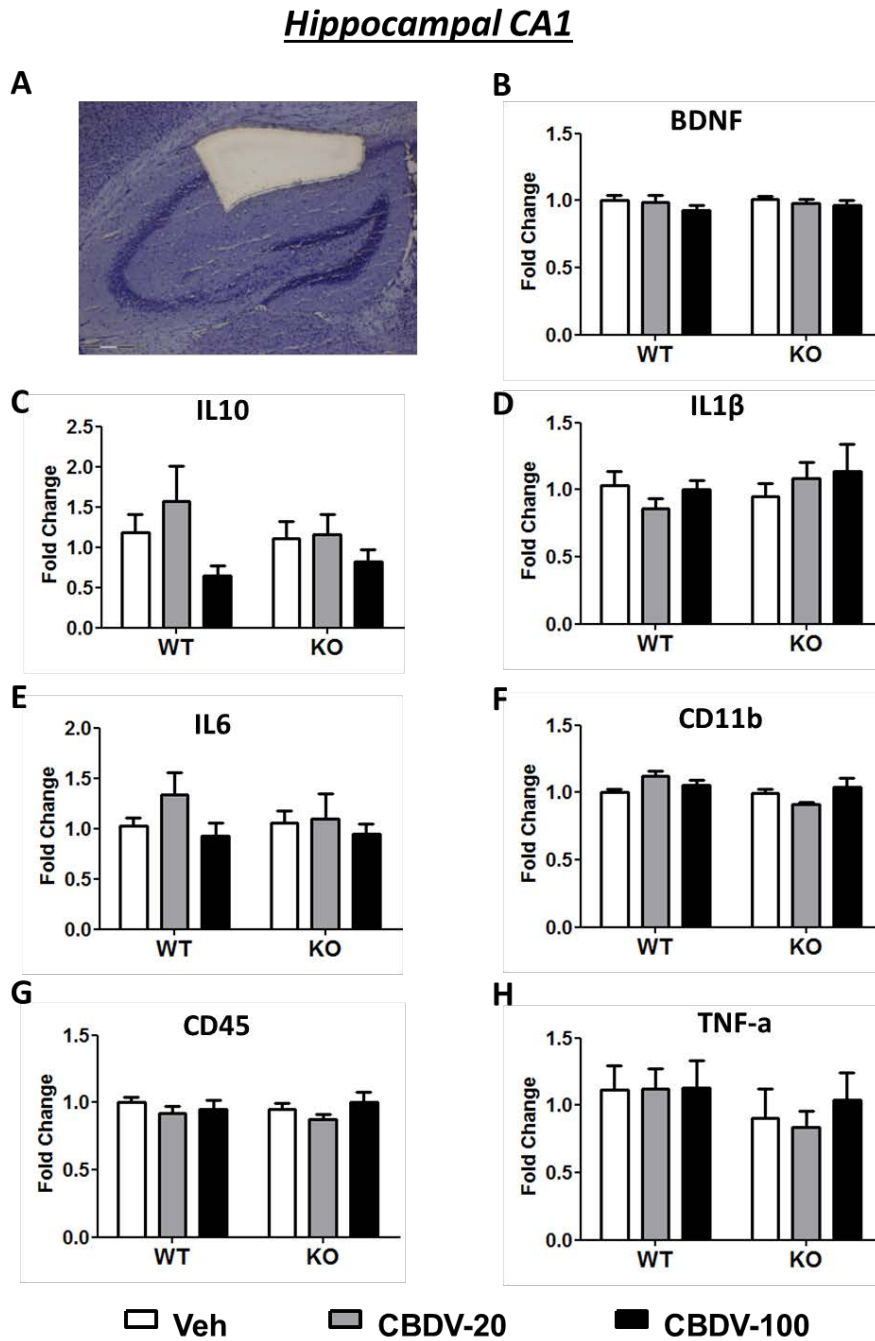
### Hippocampal CA3



**Fig. 6: BDNF and inflammatory markers in CA3:** Representative image of CA3 obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). TNF $\alpha$  was decreased in FMR1 KO mice compared with WT, genotype:  $F(1,38)=7.76$ ,  $p<0.01$ ; An increase in IL-1 $\beta$  was associated with CBDV at 100mg/kg in WT mice, treatment:  $F(2,39)=5.13$ ,  $p<0.05$ , #  $p<0.01$  Tukey's multiple comparison test; an increase in CD45 in Fmr1 KO mice was associated with treatment with 100 mg/kg CBDV, treatment:  $F(2,39)=7.0$ ,  $p<0.01$ , \*  $p<0.05$  Tukey's multiple comparison test. Data are expressed as mean $\pm$ SEM.

## Hippocampus, CA1

In CA1, no difference in any marker was found between genotypes and treatments (Fig.7).

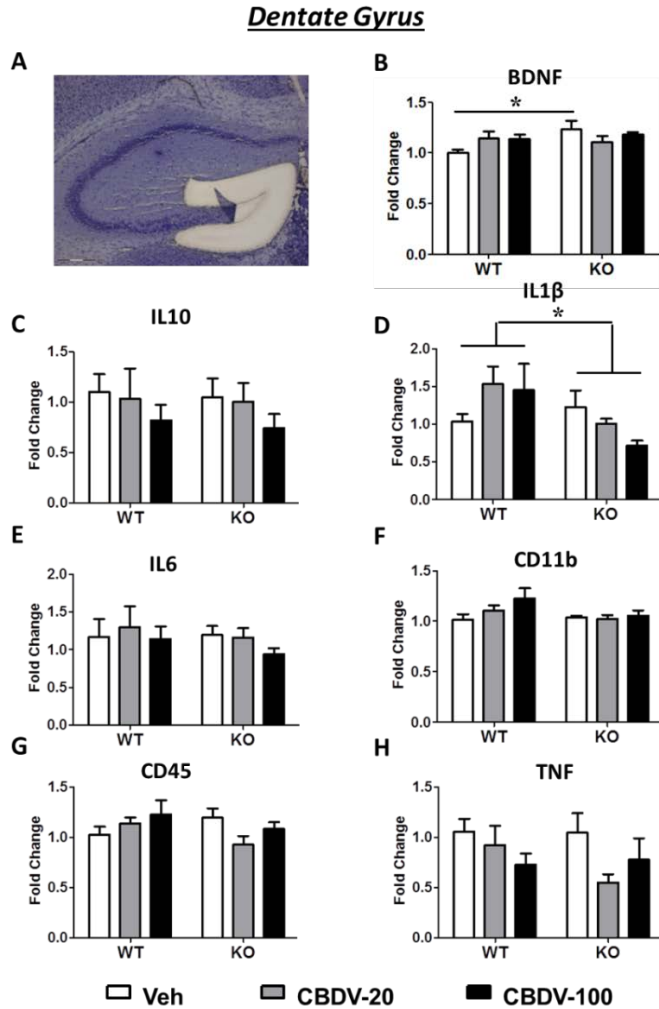


**Fig. 7: BDNF and inflammatory markers in CA1:** Representative image of CA1 obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). Data are expressed as mean $\pm$ SEM

## Hippocampus, dentate gyrus (DG)

In the dentate gyrus (DG, Fig.8), BDNF was increased in KO-VEH mice compared with WT-VEH mice, but there was no difference in BDNF levels between vehicle treated and CBDV-treated Fmr1 KO mice [genotype x treatment:  $F(2,40)=3.27$ ,  $p<0.05$ , Fig.8B; post-hoc Tukey's multiple comparisons test, \*  $p<0.05$ , WT-VEH versus KO-VEH].

There was a difference in IL-1 $\beta$  levels between Fmr1 KO mice and their WT littermates [genotype effect:  $F(1,38)=4.75$ ,  $p<0.05$ , Fig. 8D], but no effect of CBDV treatment. There was no change in expression of IL10, IL6, CD11b, CD45 or TNF $\alpha$ .

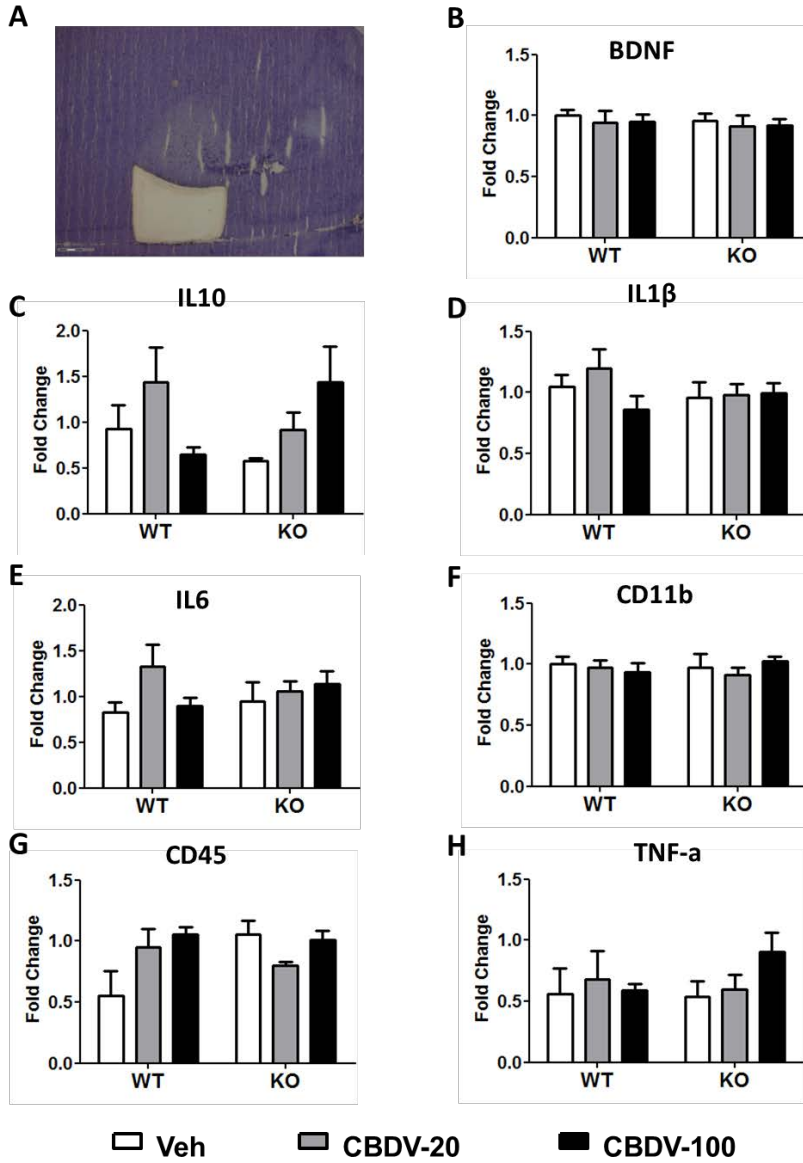


**Fig. 8: BDNF and inflammatory markers in DG:** Representative image of the dentate gyrus (DG) obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). BDNF was increased in KO-VEH mice compared with WT-VEH, genotype x treatment:  $F(2,40)=3.27$ ,  $p<0.05$ , post-hoc Tukey's multiple comparisons test, \*  $p<0.05$ , WT-VEH versus KO-VEH. There was a difference in IL-1b levels between Fmr1 KO mice and their WT littermates [genotype effect:  $F(1,38)=4.75$ ,  $p<0.05$ . Data are expressed as mean $\pm$ SEM. \*  $p<0.05$ .

### **Prefrontal cortex (PFC)**

In the prefrontal cortex (PFC), There was no effect of Fmr1 mutation or of treatment detected (Fig.9). Despite weak genotype x treatment interactions observed for IL-10 [ $F(2,35)=2.16$ ,  $p=0.05$ ; Fig.9C] and for CD45 [ $F(2,38)=3.73$ ,  $p<0.05$ ; Fig.9G], post hoc analysis revealed no differences between groups.

### Prefrontal Cortex



**Fig. 9: BDNF and inflammatory markers in PFC:** Representative image of the prefrontal cortex (PFC) obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). Data are expressed as mean±SEM.

## Discussion

This study provided an initial characterization of the neurobehavioural effects of CBDV in the adult *Fmr1*-KO mouse model of FXS using behavioural endpoints where a phenotype has been previously demonstrated by us and others (hyperactivity in the open field (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; de Diego-Otero et al., 2009; Eadie et al., 2009; Hayashi et al., 2007; Liu et al., 2011; Mineur et al., 2002; Oddi et al., 2015; Olmos-Serrano et al., 2011; Peier et al., 2000; Pietropaolo, Goubran, et al., 2014; Restivo et al., 2005; Spencer et al., 2011; Spencer et al., 2005; Thomas et al., 2011; Uutela et al., 2012), reduced direct social interaction (Dahlhaus & El-Husseini, 2010; Mineur et al., 2006; Oddi et al., 2015; Pietropaolo et al., 2011; Pietropaolo & Subashi, 2014; Spencer et al., 2011) and lack of preference for social novelty (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Hebert et al., 2014; Heitzer et al., 2012; Liu et al., 2011; Mines et al., 2010; Pietropaolo et al., 2011; Spencer et al., 2011), sensory hyper-responsiveness in the acoustic startle test (Michalon et al., 2012; Zhang et al., 2014) and deficit in novel object memory (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Pietropaolo, Goubran, et al., 2014; Ventura et al., 2004)). Other more opportunistic endpoints, , leading to inconsistent genotype differences in our (Hebert et al., 2014) and others' (Bilousova et al., 2009; de Diego-Otero et al., 2009; Eadie et al., 2009; Heulens et al., 2012; Jung et al., 2012; Mineur et al., 2002; Nielsen et al., 2002; Qin et al., 2011) studies in this model were also evaluated (anxiety in the elevated plus maze).

Although most aspects of the *Fmr1* mouse phenotype were confirmed, e.g., lack of social novelty preference and object memory, reduced social interaction and elevated



acoustic startle response, it was not always possible to demonstrate a specific difference between vehicle-treated WT and Fmr1 KO groups, rather an overall genotype effect was seen (e.g., hyperactivity in the open field). In the social novelty test (and the NOR test), it was not possible to differentiate between CBDV treatment effects and genotype effects and all groups, apart from vehicle treated WT mice, demonstrated a deficit in social novelty and were not different to a nominal 50% chance level. For sociability, the lack of a phenotype was confirmed, as previously demonstrated by us (Hebert et al., 2014; Pietropaolo et al., 2011) and others (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Heitzer et al., 2012; Liu et al., 2011; Mines et al., 2010; Spencer et al., 2011).

CBDV treatment had little effect on those behaviours where a phenotype could be demonstrated, apart from acoustic startle, where there was no difference between WT and Fmr1 KO mice treated with CBDV at 20 mg/kg, in contrast to the difference seen after vehicle treatment, which was still present after treatment with CBDV at 100 mg/kg. There are also some instances where treatment effects were demonstrated that were independent of genotype. For example, cognitive and social novelty deficits were present in WT mice treated with CBDV at 20 and 100 mg/kg in the NOR test and 3-chamber test, as well as at 100 mg/kg in the direct social interaction test. There was also an anxiolytic effect of CBDV at 100 mg/kg in Fmr1 KO mice in the absence of a phenotype.

Although there were some changes in potential markers associated with the Fmr1 phenotype (decrease in TNF $\alpha$  in CA3, increase in BDNF in DG and differences between WT and Fmr1 KO in IL-1b in DG) these were not consistent with changes in these markers observed in a previous study (Pietropaolo, Goubran, et al., 2014) and were not affected by CBDV treatment. As with the behavioural arm of the study, there was an instance where a treatment effect was present for IL-1b in CA3 of WT mice, which was associated with administration of 100 mg/kg CBDV as was an increase in CD45 in CA3 compared with vehicle treatment.

In these adult Fmr1 KO mice a phenotype was demonstrated that was broadly consistent with that seen in previous studies from this laboratory, but it was not always possible to differentiate between vehicle and CBDV treatment between genotypes. Differences between treatments were observed on some behaviours and a small number of brain markers, independently of genotype. The lack of an interaction between these factors suggests that evidence supporting the ability of sub-chronic administration of CBDV to modulate specific deficits present in adult Fmr1 KO mice is sparse.

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## 4.2 – Study 5

### **Evaluation of the neurobehavioural effects of chronic CBDV administration starting at weaning in the Fmr1-KO mouse model of Fragile X Syndrome**

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## **ABSTRACT**

Cannabidivarin (CBDV) has beneficial effects in pre-clinical studies of Rett syndrome and Autism Spectrum Disorder and has the potential to treat other developmental disorders like Fragile X syndrome (FXS). In our previous study we demonstrated that sub-chronic (10 days) administration of CBDV to adult Fmr1-KO mice, the main animal model of FXS, had limited behavioural effects. The aim of the present study was to investigate whether chronic CBDV treatment, when started at weaning (i.e., 3 weeks of age), could prevent the alteration of behaviour observed in adult male Fmr1-KO mice. Our hypothesis was that targeting the juvenile phase, which is characterized by high levels of neuronal plasticity, would maximize the therapeutic effects of the treatment.

Hence, chronic (5 weeks) administration of two doses (20 and 100 mg/kg IP) of CBDV was performed in juvenile subjects starting at weaning. Behavioural tests assessing emotional (anxiety, elevated plus maze), motor (open field), cognitive (object recognition), social (direct social interaction and three-compartment test) and sensory (acoustic startle) domains were then performed. Brain samples were collected to evaluate markers of inflammation (IL-1 $\beta$ , IL-6, IL-10, TNF $\alpha$ , CD11b, CD45) and plasticity (BDNF) in cortical (prefrontal cortex, PFC) and hippocampal (CA1, CA3 and dentate gyrus, DG) areas. Vehicle-treated Fmr1 KO animals displayed cognitive deficits, such as lack of novel object recognition and of preference for social novelty (in trial 3 of the 3-compartment test), as well as reduced social interaction and sensory hyper-responsiveness in the acoustic startle test. KO mice were also hyperactive compared with their WT littermates and less anxious in the elevated plus maze and open field test. Sociability was unaltered. Chronic CBDV administration at 20 and 100



mg/kg IP, started at weaning, was associated with improvements in cognitive and social deficits in adult mice. Sensory alterations of adult KO mice were also not present in the startle test after CBDV treatment as juveniles. No effect of CBDV treatments was detected on hyperactivity and anxiety-like phenotypes in Fmr1 KO mice. At the brain level, KO-VEH animals displayed little change in the markers examined. There was a slight increased expression of IL10 in the DG compared with WT-VEH mice. Treatment effects were observed in both WT and KO mice on some markers, i.e. both doses of CBDV increased CD11b levels in the CA3 compared to VEH, while only CBDV 100 treatment increased CD45 levels in the DG in mice of both genotypes. Overall, these data demonstrate that CBDV (20 and 100 mg/kg IP), when administered chronically (5 weeks), to juvenile male Fmr1 KO mice (3 weeks old), is associated with improvements in social interaction, cognitive deficits and normalisation of startle responsiveness in adult KO mice. As these behaviours are correlates of the most relevant symptoms in FXS, CBDV may have therapeutic benefit in this condition.

## INTRODUCTION

Non-psychoactive phytocannabinoids such as Cannabidiol (CBD) and its analogues, are promising compounds for therapeutic applications in a variety of pathologies. For example, pre-clinical studies have shown that Cannabidivarin (CBDV), a propyl analogue of CBD, has anti-convulsant and anti-inflammatory properties and ameliorates behavioural abnormalities in models of neurodevelopmental disorder such as Rett syndrome (RTT) (1) and autism spectrum disorder (ASD) (2). Fragile X (FXS) is the principal monogenic cause of inherited intellectual disability and autism, and is characterized by significant anxiety, attention deficit hyperactivity disorder (ADHD), and hyperarousal to sensory stimuli (3-5). It is caused by an unstable expansion of CGG repeats in the 5'untranslated region of the *FMR1* gene, producing loss of expression of FMRP, a synaptically expressed RNA-binding protein regulating translation (6, 7). Studies with the fragile X mental retardation 1 (*Fmr1*)-knockout (KO) mouse, the animal model of FXS, recapitulate several of the behavioural domains of FXS, including motor, sensory, cognitive, emotional and social behaviours (8-12).

This study has therefore evaluated the therapeutic impact of CBDV in the *Fmr1*-KO mouse model of FXS. Either 20 or 100 mg/Kg CBDV were given daily for 5 weeks to juvenile (3 weeks-old) *Fmr1*-KO mice and their WT littermates before behavioural testing. Tests where *Fmr1*-KO mice are known to exhibit a robust behavioural phenotype were chosen, affecting motor (open field), cognitive (object recognition), social (direct social interaction and three-compartment test for sociability and social novelty) and sensory (acoustic startle) domains, as we have previously demonstrated

(Hebert et al., 2014; Oddi et al., 2015; Pietropaolo, Goubran, et al., 2014; Pietropaolo et al., 2011; Zhang et al., 2014). Emotional alterations in the elevated plus maze are not consistently described in Fmr1-KO mice, but this test was included in this study in order to assess potential differences in anxiety-like behaviour induced by CBDV treatments and their confounding impact on the other behavioural tests. Brain samples were also collected from tested mice in order to evaluate markers of inflammation (IL-1 $\beta$ , IL-6, IL-10, TNF $\alpha$ , CD11b, CD45) and plasticity (BDNF) in cortical and hippocampal areas, i.e., those brain regions where FMRP normally is most abundant (Bakker et al., 2000; Khandjian, 1999) and where Fmr1-KO mice have previously shown altered expression of these markers (Pietropaolo, Goubran, et al., 2014). Also, the brain expression of some of these inflammatory markers (e.g., TNF $\alpha$ , CD11b) was previously shown to be modulated by CBDV, at least in other animal models of developmental pathologies (Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019).

The doses of CBDV used here were chosen as they have already shown efficacy in (i) recent studies using young and adult animal models of other developmental disorders, i.e., Rett syndrome (Vigli et al., 2018; Zamberletti, Gabaglio, Piscitelli, et al., 2019) and ASD (Zamberletti, Gabaglio, Woolley-Roberts, et al., 2019), (ii) our previous study in adult Fmr1-KO mice (Study 4). In this latter project, we demonstrated the partial rescue of some FXS-like phenotypes following sub-chronic (10 days) CBDV treatment when started at adulthood (i.e., 12 weeks of age), i.e., once the pathology is at the advanced stage in the Fmr1 mouse model (Pietropaolo & Subashi, 2014).

The aim of the present study was to investigate the therapeutic effects of CBDV with 5 weeks of treatment from weaning, as done previously in a Rett model (Zamberletti, Gabaglio, Piscitelli, et al., 2019) to coincide with an age where neuronal plasticity is expressed at high levels (Spear, 2000). Hence, our hypothesis was that chronic CBDV treatment started at weaning could prevent the neurobehavioural alterations displayed by adult male *Fmr1*-KO mice. A schematic representation of the experimental procedure of the study is provided in the method section, Fig.1).

## **Methods**

C57BL/6J*Fmr1*<sup>tm1Cgr/Nwu</sup> (B6) breeders were originally obtained from Neuromice.org (Northwestern University) and afterwards bred in our animal facility of Bordeaux University for 10 years. Breeding trios were formed by mating two heterozygous *Fmr1* females with a wild-type C57BL/6J male purchased from Janvier (Le Genest St Isle, France). After 2 weeks the stud was removed and the dams were singly caged and left undisturbed until weaning of the pups. Mice were weaned at 21 days of age and group-housed with their same-sex littermates (3–5/cage). On the same day, tail samples were collected for DNA extraction and subsequent PCR assessment of the genotypes as previously described (Dutch-Belgian Fragile X Consortium, 1994). Only male mice were used for the study, as they are the most commonly used in mouse studies on FXS, due to the higher prevalence of this syndrome in the male sex. Only litters including males of both genotypes (WT and KO) were used for experiments, for a total of 60 subjects (30 WT and 30 KO, i.e., 10 mice per experimental condition) at the start of the experiment.

NMRI female mice (12±2 weeks old) and juvenile (4 weeks old) males purchased from Janvier (Le Genest St Isle, France) were used as social stimuli respectively during the direct social interaction and three-compartment tests. This strain has been selected for its high level of sociability (Moles & D'Amato F, 2000) and was previously employed in several social studies from our group on *Fmr1*-KO mice (Pietropaolo, Goubran, et al., 2014; Pietropaolo et al., 2011; Zhang et al., 2014). Mice were group-housed (3/4 cage) and left undisturbed upon arrival at least one week before the social interaction test.

All animals were housed in polycarbonate standard cages (33x15x14 cm in size; Tecniplast, Limonest, France), provided with litter (SAFE, Augy, France) and a stainless steel wired lid. Food (SAFE, Augy, France) and water were provided ad libitum. The animals were maintained in a temperature (22°C) and humidity (55%) controlled vivarium, under a 12:12 hr light–dark cycle (lights on at 7 a.m.). All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and local French legislation (Authorization N° 2017073113175079).

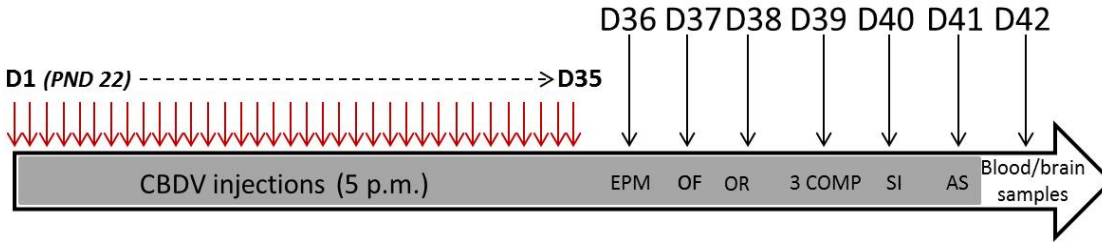
### **Experimental procedures**

The day after weaning, on PND 22, mice of both genotypes were assigned to one of the three experimental conditions, i.e., injected with vehicle alone (VEH: Cremophor® EL:EtOH:saline in a ratio of 1:2:17), or with a dose of CBDV (GW Research Ltd., Cambridge, UK) either of 20mg/kg (CBDV-20) or 100mg/kg (CBDV-100). *Fmr1*-KO mice

and WT littermate controls were injected once daily i.p. (around 5.00 p.m.) during the entire duration of the study as illustrated in Figure 1 below.

Behavioural tests began after 5 weeks of injections, following the timing described in Figure 1. After some days of injections, a few animals (1-2 per group, belonging to all experimental conditions) showed adverse effects, such as reduced locomotion in the home-cage, weight loss or hypothermia, as assessed by daily animal inspection by the experimenters and were therefore excluded from further injections and behavioural testing. A total of 47 mice were subjected to all behavioural tests and brain analysis (n=7 for WT-VEH and WT-CBDV100; n=8 for WT-CBDV20, KO- CBDV20 and for KO-CBDV100; n=9 for KO-VEH). The order of the tests was based on the need for performing first those tests that are more influenced by previous testing experience (such as the elevated plus maze), while leaving to last tests involving a certain degree of stressful experience (such as the acoustic startle, requiring a short confinement in the startle box).

All the tests were performed during the light phase, between 9 a.m. and 4 p.m. by an experimenter who was blind to the genotype and treatment of the animals. On testing days, the treatments were given after each behavioural test, to avoid evaluating acute effects of CBDV (Figure 1). The order of testing was counterbalanced across experimental groups. Mice were habituated to the testing room at least one hour before the beginning of each behavioural test.



**Fig. 1: Schematic representation of experimental plan of the study:** Daily i.p. injections (indicated by arrows) started at weaning (i.e., 3 weeks of age) and were given during the entire experimental period, including the days of behavioural testing when they were administered after completion of each testing procedure. EPM= elevated plus maze, OF=open field, OR=object recognition, 3COMP= three-compartment test for sociability, SI=social interaction, AS=acoustic startle. All tests were performed during the light phase, between 9 a.m. and 4 p.m. PND=post-natal day.

### Elevated plus maze

The elevated plus maze consisted of grey acrylic glass with a removable plastic grey floor. It was elevated 55 cm above floor level, and placed in a quiet testing room with diffuse dim lighting (30 lux in the center of the maze). The maze consisted of four equally spaced arms (29.5 cm long and 8 cm wide) radiating from a central square measuring 8 × 8 cm. One pair of opposing arms was enclosed with opaque walls (16 cm high, 3 mm thick), except for the side adjoining the central square. The remaining two arms were exposed. A digital camera was mounted above the maze; recorded videos were analysed manually by an observer blind to the experimental condition of the animals using Observer XT (Version 7, Noldus Technology, The Netherlands). The position of each subject in the open or closed arms as well as in the center was scored.

To begin a trial, the mouse was gently placed in the central square with its head facing one of the open arms and allowed to explore freely and undisturbed for 5 min. Anxiety-

like behaviours were measured by percent time in open arms = time in open arms / time in all arms × 100%, while locomotor activity was assessed by the total number of entries into the maze arms.

### **Open field (habituation phase of object recognition test)**

The apparatus consisted of 2 identical plastic rectangular arenas, each measuring 24x30 cm in surface area and with 22-cm walls. The arenas were located in a testing room under diffused dim lighting (30 lux in the arena center). A digital camera was mounted directly above the arenas, capturing images at 5 Hz that were transmitted to a PC running the Ethovision tracking system (version 11, Noldus, The Netherlands).

Each mouse was gently placed in the center of the appropriate arena and allowed to explore undisturbed for 20 min. The choice of the arena was counterbalanced across experimental groups. Locomotor activity was indexed by distance travelled and analyzed across 5-min bins in order to assess locomotor habituation (as commonly done in behavioural mouse studies, e.g. (Belzung, 1999; Pietropaolo et al., 2006; Pietropaolo, Feldon, et al., 2008, 2014; Pietropaolo et al., 2007; Pietropaolo, Singer, et al., 2008)); the time spent in the central area was assessed as a measure of emotionality and anxiety-like behaviour (Belzung, 1999; Prut & Belzung, 2003). At the end of the third trial the maze was cleansed with a 30% ethanol solution and dried with a paper towel.



## **Object recognition**

The open field test served as habituation phase for the object recognition test. At the end of the session, two identical objects of different shape and material were placed in two opposite corners and the mouse introduced in the center of the arena for a 5-min training phase. Twenty-four hours later, the mouse was returned to the arena for a 5-min test phase, where one of the objects was replaced with a novel one. Both the type of object used for the sample phase and the position of the novel object during the test phase were counterbalanced across experimental groups. During the training and test phases, the time spent at each object was manually scored by an observer unaware of the experimental conditions of the animals using Observer XT (version 7, Noldus, the Netherlands). During the test phase, a percent recognition index, was used to measure object recognition as follows:  $100 \times T_{\text{novel object}} / (T_{\text{novel object}} + T_{\text{familiar object}})$ ; lack of novel object recognition= 50%. At the end of the sample and test phases the apparatus as well as the objects were cleansed with a 30% ethanol solution and dried.

## **Three compartment test**

The testing apparatus (described in details elsewhere (Pietropaolo et al., 2011)) consisted of a central chamber connected on each side to another compartment containing a perforated stimulus cage (8x8x15cm) to allow the tested mouse to interact with the mouse or the object inside the stimulus cage. Each stimulus cage was placed at a distance of 5.5cm from the side walls and there was no space between the stimulus cage and the back wall. The object employed for the test was a plastic black cylinder and the stimulus mice were NMRI juvenile males, in order to minimize aggressive

tendencies and exclude sexual interest. Each experimental subject was introduced in the middle of the central compartment and allowed to explore the apparatus for 3 trials of 5 min each: in trial 1 habituation to the apparatus containing empty stimulus cages was evaluated, while in trial 2 the preferential exploration of the social (a juvenile male mouse) *versus* the non-social (an object) novel stimulus was measured, and in trial 3 the preferential exploration of a novel *versus* familiar social stimulus was assessed, by replacing the object with a novel stimulus mouse. In all trials the total distance travelled as well as the time spent in each contact area (20 × 22 cm) containing the stimulus cages was computed using the Ethovision tracking system (version 11, Noldus, The Netherlands). A percentage score was also computed for the last two trials as follows:

- On trial 2: Sociability score =  $100 \times T_{\text{social stimulus}} / (T_{\text{social stimulus}} + T_{\text{non-social stimulus}})$ ,
- On trial 3: Social novelty preference score =  $100 \times T_{\text{novel social stimulus}} / (T_{\text{novel social stimulus}} + T_{\text{familiar social stimulus}})$ .

At the end of each trial the experimental animal was confined in the central compartment by means of two Plexiglas magnetic doors for 30sec. At the end of the third trial the apparatus as well as the object and the stimulus cages were cleansed with a 30% ethanol solution and dried.

### **Direct social interaction**

Direct social interaction was assessed as described in details elsewhere (Pietropaolo et al., 2011). Briefly, an unfamiliar adult NMRI female mouse was introduced into a testing cage (32 x 14 x 12.5cm, with a flat metal grid as cover and approximately 3 cm of clean

sawdust bedding) to which experimental subjects were habituated for one hour. Six min-testing sessions were recorded and videos analyzed with Observer XT (version 7, Noldus, The Netherlands). One observer who was unaware of the experimental conditions of the animals scores the time spent performing affiliative behaviours, i.e., social investigation (nose, body and anogenital investigation) and contact. At the beginning of the testing day, the estrous cycle of the stimulus females was assessed through the analysis of the vaginal smear, so that only females in the non-estrous phase were used for social interaction sessions.

### **Sensory responsiveness (acoustic startle test)**

The apparatus consisted of four acoustic startle chambers for mice (SR-LAB, San Diego Instruments, San Diego, CA, USA). Each startle chamber comprises a non-restrictive cylindrical enclosure made of clear Plexiglas attached horizontally on a lightweight mobile platform, which was in turn resting on a solid base inside a sound-attenuated isolation cubicle. A high-frequency loudspeaker mounted directly above the animal enclosure inside each cubicle produced a continuous background noise of 66 dB and the various acoustic stimuli in the form of white noise. Twenty-four hours before testing, mice were placed in the recording chamber of a startle response box for 5 min without being exposed to any stimuli, in order to habituate them to the confinement and reduce the related stress. On the test day, mice were presented with continuous white noise of 66 dB (background) and, after a 5 min habituation period, mice were presented with pulses of white sound of 20 ms duration and of varying intensity: +6, +12 +18 and +24 dB over background levels (namely 72, 78, 84 and 90 dB). Each intensity was

presented 8 times, in a randomized order with variable intervals (10 sec to 20 sec) between the onset of each pulse. Vibrations of the Plexiglas enclosure caused by the whole-body startle response of the animal were converted into analogue signals by a piezoelectric unit attached to the platform. These signals were digitised and stored by a computer. A total of 130 readings were taken at 0.5-ms intervals (i.e., spanning across 65 ms), starting at the onset of the pulse stimulus. The average amplitude (in mV) over the 65 ms was used to determine the stimulus reactivity and further averaged across trials of the same stimulus intensity.

## **Brain analyses**

### **Assessment of inflammatory and plasticity markers using reverse transcription and real-time RT-PCR**

Mice were sacrificed by cervical dislocation; brains were immediately extracted and cut in two hemispheres that were separately frozen using dry ice for the half that was used for RT-PCR analysis (while for the other half, that was stored for subsequent analysis, liquid nitrogen was used). Frozen brains were thawed to  $-20^{\circ}\text{C}$  in a cryostat chamber (CM3050 S, Leica Microsystems, Wetzlar, Germany). Whole brain tissue was sectioned at  $50\ \mu\text{m}$  using a Leica cryostat and mounted in series with 8-10 sections per slide on polyethyl-ene-naphthalate membrane 1mm glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) that were pretreated to inactivate RNase. Serial sections were created from distinct coronal sections (bregma positions based on a reference brain atlas by Georges Paxinos and Keith B.J. Franklin) and individual regions were matched across section and harvested by LCM. The pre-frontal cortex (PFC) (Infralimbic cortex and prelimbic cortex) series were collected from bregma 1.98 mm to 1.54 mm, cornu Ammonis 1 (CA1), cornu Ammonis 3 (CA3) and dentate gyrus (DG) of the hippocampus series were collected from bregma -1.22 mm to -2.80 mm. Subsequently, the sections were immediately fixed for 30 seconds with 95% ethanol, followed by 75% ethanol for 30 seconds and by 50% ethanol for 30 seconds to remove the OCT. Sections were stained with 1% cresyl violet in 50% ethanol for 30 seconds and dehydrated in 50%, 75% and 95% ethanol for 30 seconds each, 2x in 100% ethanol for 30 seconds. Laser Pressure Captapulting microdissection (LPC) of samples was performed using a PALM MicroBeam microdissection system version 4.6 equipped with

the P.A.L.M. RoboSoftware (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). Laser power and duration were adjusted to optimize capture efficiency. Microdissection was performed at 5X magnification. The microdissection of pure brain structures were collected in adhesives caps and re-suspended in 250µl guanidine isothiocyanate-containing buffer (BL buffer from ReliaPrep™ RNA Cell Miniprep System, Promega, Wisconsin, USA) with 10 µl 1-Thioglycerol, and stored at -80°C until extraction was done. Total RNA was extracted from microdissected tissues using the ReliaPrep™ RNA Cell Miniprep System (Promega, Wisconsin,USA) according to the manufacturer's protocol. The integrity of the RNA was checked by capillary electrophoresis using the RNA 6000 Pico Labchip kit and the Bioanalyser 2100 (Agilent Technologies, Massy, France), and quantity was estimated using a Nanodrop 1000 (Thermo Scientific, Waltham, USA). The RNA integrity number (RIN) were above 7/8.

### **Quantitative Real-Time PCR (q-PCR)**

RNA was processed and analyzed according to an adaptation of published methods (Bustin et al., 2009). Briefly, cDNA was synthesized from 140 ng of total RNA for each structure by using qSript™ cDNA SuperMix (Quanta Biosciences). qPCR was performed with a LightCycler® 480 Real-Time PCR System (Roche, Meylan, France). qPCR reactions were done in duplicate for each sample by using LightCycler 480 SYBR Green I Master (Roche) in a final volume of 10 µl. The qPCR data were exported and analyzed in an informatics tool (Gene Expression Analysis Software Environment) developed at the University of Bordeaux. The Genorm method was used to determine the reference gene (Bustin et al., 2009). Relative expression analysis was normalized

against two reference genes. Succinate dehydrogenase complex subunit (Sdha) and tubulin alpha 4 a (Tuba4a) were used as reference genes for PFC. Succinate dehydrogenase complex subunit (Sdha) and tyrosine 3 mono oxygenase tryptophan 5 mono oxygenase (Ywhaz) were used as reference genes for CA1. Tubulin alpha 4 a (Tuba4a) and glycéraldéhyde-3-phosphate déshydrogénase (Gapdh) were used as reference genes for CA3. Tubulin alpha 4 a (Tuba4a) and non-POU-domain-containing, octamer binding protein (Nono) were used as reference genes for DG. The relative level of expression was calculated with the comparative ( $2^{-\Delta\Delta CT}$ ) method (Livak & Schmittgen, 2001). Primer sequences are reported in Table 1.

**Table 1. Primer Sequences.**

<b>Gene</b>	<b>GenBank ID</b>	<b>Forward Sequence (5'-3')</b>	<b>Reverse Sequence (5'-3')</b>
<b>Nono</b>	<b>NM_023144</b>	<b>CTGTCTGGTGCATTCCTGAA CTAT</b>	<b>AGCTCTGAGTTCATTTTCCC ATG</b>
<b>Sdha</b>	<b>NM_023281</b>	<b>TACAAAGTGCGGGTTCGATG A</b>	<b>TGTTCCCCAAACGGCTTCT</b>
<b>Ywha z</b>	<b>NM_011740</b>	<b>CTTGTGAGGCTGTGACACA AACA</b>	<b>CAAGAGTGTGCACGCAGAC A</b>
<b>Tuba4 a</b>	<b>NM_009447</b>	<b>CCACTTCCCCTTGGCTACCT A</b>	<b>CCACTGACAGCTGCTCATG GT</b>
<b>Gapd h</b>	<b>NM_008084</b>	<b>TCAAGAAGGTGGTGAAGCA G</b>	<b>TGGGAGTTGCTGTTGAAGT C</b>
<b>Il1b</b>	<b>NM_008361</b>	<b>TCGCTCAGGGTCACAAGAA A</b>	<b>TCAGAGGCAAGGAGGAAA ACAC</b>
<b>Il6</b>	<b>NM_031168</b>	<b>TACTCGGCAAACCTAGTGC GT</b>	<b>ATTTTCTGACCACAGTGAG GAATG</b>
<b>Il10</b>	<b>NM_010548</b>	<b>AGTTGTGAAGAACTCATG GGTCT</b>	<b>TGCTGCAGGAATGATCATC AA</b>
<b>Tnf-a</b>	<b>NM_013693</b>	<b>GGCACTCCCCCAAAGATG</b>	<b>GCCACAAGCAGGAATGAGA AG</b>
<b>Itgam (CD11 )</b>	<b>NM_001082 960</b>	<b>CTCATCACTGCTGGCCTATA CAA</b>	<b>GCAGCTTCATTCATCATGTC CTT</b>
<b>Ptprc (CD45 )</b>	<b>NM_011210</b>	<b>TGGGACAACGCAGACTCTC A</b>	<b>CTGCACAGCCATGTTCTTTC AT</b>
<b>BDNF</b>	<b>NM_007540</b>	<b>CCCGTCTGTACTTTACCCTT TGG</b>	<b>TGACTAGGGAAATGGGCTT AACA</b>



### **Drug preparation**

CBDV (synthetic; Batch N°: 10300003; purity by HPLC: 95.9%) was supplied by GW Research Limited (Cambridge, UK) and stored at approximately -20°C, protected from light. Injectable solutions were daily prepared and were continuously stirred until injection.

### **Statistical Analyses**

Data were inspected for the identification of possible outliers using Grubbs' test. The number of outliers (1-2/group, if any) is in line with that seen in other similar studies conducted by this laboratory. Outliers were excluded from statistical analysis of the specific dataset and variable only; this explains the slight differences that may occur among tests in the number of animals per group. For each test, the exact n were the following:

**Table 2. number of mice/group after exclusion of outliers (as detected by Grubb's test)**

<b>Test</b>	<b>Variable</b>	<b>WT-VEH</b>	<b>WT-CBDV2 0</b>	<b>WT-CBDV10 0</b>	<b>KO-VEH</b>	<b>KO-CBDV2 0</b>	<b>KO-CBDV10 0</b>
<b><i>EPM</i></b>	<b>%Time open arms</b>	6	8	6	9	8	8
	<b>Total arm entries</b>	7	7	7	9	7	8
<b><i>OF</i></b>	<b>Distance moved</b>	7	8	7	9	8	8
	<b>%Time center</b>	7	8	7	9	8	8
<b>OR: sample</b>	<b>Time contact objects</b>	7	8	7	9	8	8
<b>OR: test</b>	<b>%recognition</b>	6	8	7	9	8	8
<b>3-Comp: tr1</b>	<b>Distance moved</b>	7	7	7	9	8	7
	<b>%Time compartment</b>	7	8	7	9	8	8
<b>3-Comp: tr2</b>	<b>Distance moved</b>	7	8	6	9	7	8
	<b>%Time compartment social</b>	7	8	7	9	8	8

<b>3-Comp: tr3</b>	<b>Distance moved</b>	7	8	7	8	7	8
	<b>%Time compart novel</b>	7	8	7	9	8	8
<b>SI</b>	<b>Time in affiliation</b>	7	8	7	9	8	8
<b>AS</b>	<b>Ln(startle response)</b>	7	8	7	9	8	8

EPM= elevated plus maze, OF=open field, OR=object recognition, 3COMP= three-compartment test for sociability, SI=social interaction, AS=acoustic startle.

Data from the outliers are included in the raw data files provided in the appendix (marked in yellow and named “outlier”). Normality was assessed through the Shapiro-Wilks test for each experimental group (genotype x dose) and each variable of interest. Data from startle reactivity did not show a normal distribution at all stimulus intensities and were therefore subjected to natural logarithmic (ln) transformation in order to meet the normality requirements of ANOVA.

For all other variables, data distribution was found to be normal and a parametric 2x3 ANOVA with genotype and treatment as the between-subject factors was applied. Within-subject factors were included according to the specific test and used as repeated measures in the ANOVA; these included for example, 5-min bins for the total distance travelled in the open field, the stimulus area for the three-compartment test, the type of object for the object recognition test, 3-min-time bins for the social interaction test and the stimulus intensity for the acoustic startle assessment.

Post-hoc comparisons were conducted using the Tuckey's HSD test when a significant interaction was found. For the object recognition index, sociability and social novelty scores in the three compartment test, a one-sample t test was used for comparison with chance level/lack of preference (i.e., 50%), as done in previous behavioural studies (see for example, (Oddi et al., 2015; Vandesquille et al., 2013)). All analyses were carried out using Statview and PASW Statistics 18.

## Results

### Behavioral tests

#### Elevated plus maze

Anxiety-like behaviour, assessed by the percent time spent in the open arms, was overall reduced in Fmr1 KO mice independently of CBDV treatment [genotype effect:  $F(1,39)=13.1$ ,  $p<0.001$ ], although this effect seemed to be exaggerated by the high levels of anxiety shown by WT-100 mice, which showed low a percentage of time spent in the open arms compared with WT untreated mice from most previous studies (e.g.,(Holmes, 2013)): Mean±standard error of the mean (SEM) for WT were:  $6.98\pm 2.36$ (VEH),  $6.81\pm 1.96$  (CBDV-20),  $1.96\pm 1.14$  (CBDV-100); for KOs were:  $10.21\pm 2.44$ (VEH),  $11.03\pm 1.43$  (CBDV-20),  $12.24\pm 1.79$ (CBDV-100).

Locomotor activity, indexed by the number of total arm entries, was overall enhanced in KO animals [genotype effect:  $F(1,39)=14.9$ ,  $p<0.001$ ], although this effect seemed to be mainly due to the lower activity levels of both WT-20 and WT-100 mice. No significant difference between treatment groups was detected. Mean±SEM for WT were:  $26.57\pm 1.77$ (VEH),  $23\pm 1.36$  (CBDV-20),  $22.43\pm 1.86$  (CBDV-100); for KOs were:  $28.33\pm 1.25$  (VEH),  $29.57\pm 1.34$  (CBDV-20),  $28.5\pm 1.5$ (CBDV-100).

#### Open field (habituation phase of the object recognition test)

The habituation phase to the open field arena used for the object recognition test was used to assess hyperactivity, which is a robust end point for Fmr1 KO mice, and anxiety. The distance travelled during the 20-min session of the habituation to the open

field was analysed in 5-min bins (using a 2x3x4 ANOVA with genotype and treatment as the between-subject factors and 5-min bins as the within subject factor), in order to assess locomotor habituation (Fig.2-A). Indeed, as expected, all mice showed a time-dependent decrease in locomotion, independently of their genotype and treatment [5 min-bin effect:  $F(3,123)=117.44$ ,  $p<0.0001$ ]. Fmr1 KO mice were more active than their WT littermates [genotype effect:  $F(1,41)=17.65$ ,  $p<0.001$ ; Fig.2-B], confirming this phenotype in Fmr1 KO mice and no difference between treatments was found on hyperactivity [treatment effect and its interaction with genotype, ns].

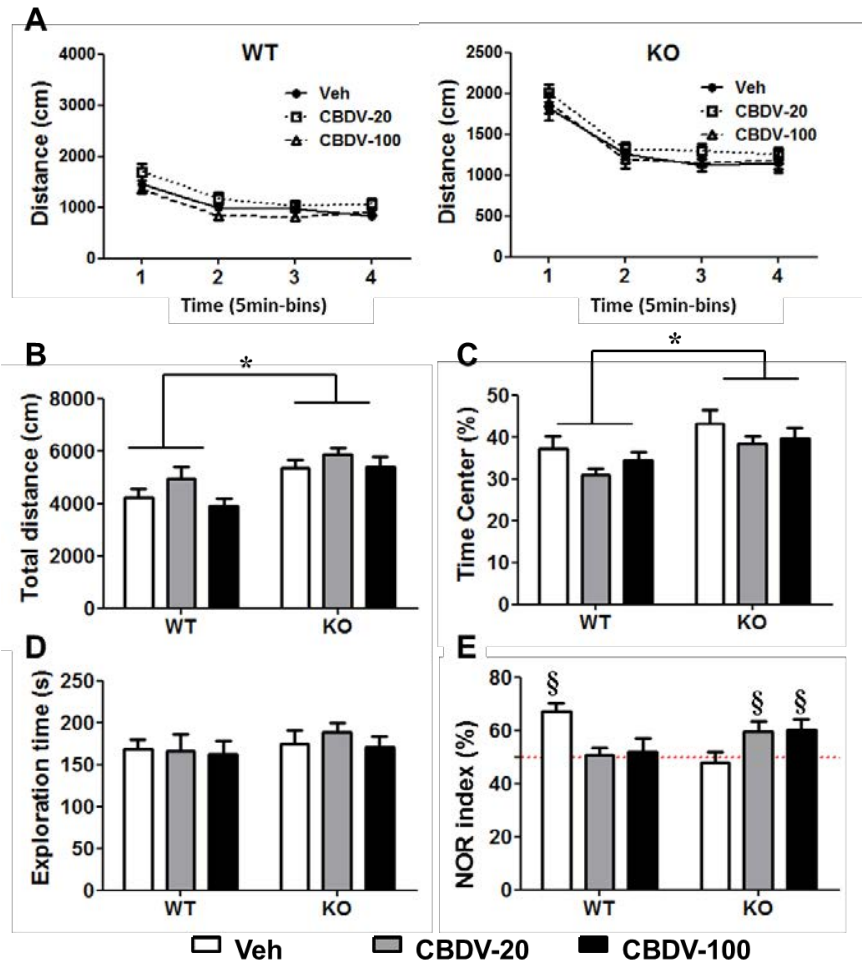
Consistent with previous data, anxiety levels appeared reduced in KO mice compared to their WT littermates as shown by the increased time spent in the centre of the open field [Genotype :  $F(1, 41) = 9.42$ ,  $p < 0.01$ ; Fig.2-C], and again this phenotype in the open field was not modified by treatment.

### **Object recognition**

During the sample phase, all mice explored equally the two sample objects irrespective of their position (object position effect; ns, data not shown). Overall, there was no difference in the object exploration among experimental groups [all effects, ns; Fig. 2D].

During the test phase a clear object recognition deficit was detected in KO-VEH mice, as shown by no difference in the NR index compared with a chance level of 50%, as previously described by us and others, and this deficit was not present following both CBDV treatments. In contrast, CBDV treatments were associated with an impairment in

the cognitive performance of WT mice, which was not significantly different from the chance level [one sample t-test versus 50%:  $p < 0.05$  in WT-VEH, KO-20 and KO-100; ns in all other groups, Fig. 2E].



**Fig. 2: Object recognition:** Locomotor habituation (A), overall activity [Genotype:  $F(1,41)=17.65$ ,  $p < 0.001$  (B)] and anxiety-like behaviour [ $F(1, 41) = 9.42$ ,  $p < 0.01$  (C)] in the empty arena during the 20-min session of the open field used as the habituation phase. Exploration of the two identical objects introduced in the arena during the 5-min sample phase (D). Twenty-four hours later object recognition was measured during the 5-min test phase by the percent novel object recognition index (NOR)=%time spent exploring the novel object/the time spent exploring the novel+familiar objects (E). Data are expressed as mean $\pm$ SEM. § versus lack of NOR (50%, red dotted line),  $p < 0.05$  one sample t-test.

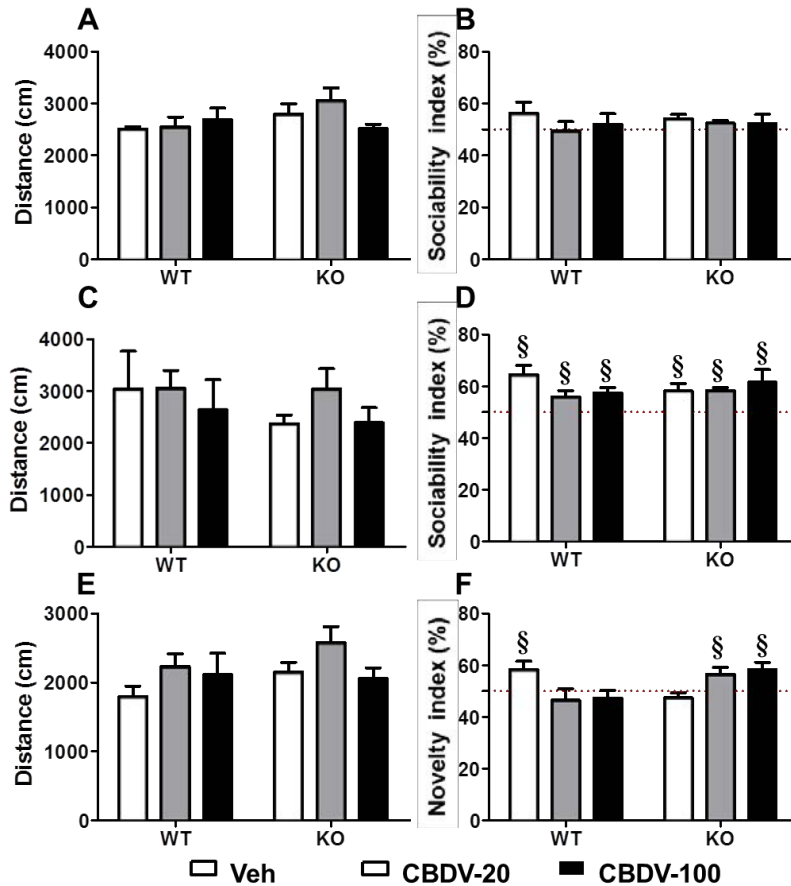
### **Three-compartment test for sociability and social novelty**

On trial 1 (habituation phase) no difference was observed among groups in the total distance moved in the apparatus [all effects, ns; Fig. 3-A], and mice equally explored the two side compartments containing the stimulus cages, showing no bias for any of the two [all effects ns, and the difference from chance level ns, Fig.3B ].

On trial 2 (sociability), no difference was found on locomotion [all effects, ns; Fig. 3C]. All mice preferred to explore the social versus the inanimate stimulus, as demonstrated by the mean percentage time sociability score that was significantly >50% in all experimental groups [t-test difference from chance level of 50%,  $p < 0.05$  in all genotype x treatment groups, Fig. 3D]. This lack of sociability deficit in KO-VEH mice was expected, based on previous data from our and other studies ((McNaughton et al., 2008; Mines et al., 2010; Pietropaolo, Goubran, et al., 2014; Pietropaolo et al., 2011)).

On trial 3, locomotor activity did not differ between experimental groups ([all effects, ns Fig.3E). In this trial, the KO-VEH group showed a clear lack of preference for social novelty, as expected by several previous reports, and this deficit was not present in CBDV treated animals, at both doses. CBDV treatments were also associated with an impairment in the performance of WT mice in this task, as neither WT-20 nor WT-100 treated mice showed a preference for the novel social stimulus [One sample t-test versus 50% chance level:  $p < 0.05$  in WT-VEH, KO-20 and KO-100; ns in other groups; Fig. 3F].



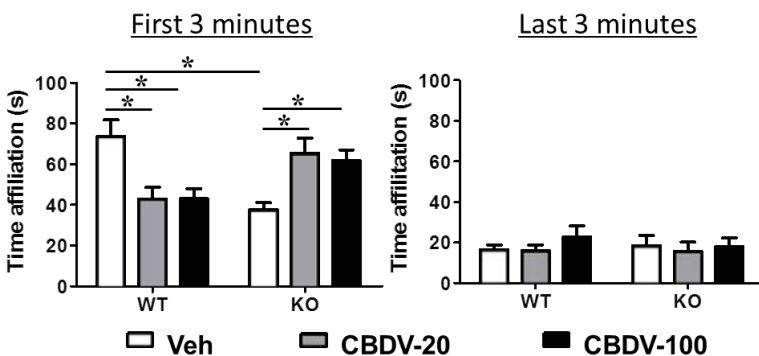


**Fig. 3: Three compartment test:** Locomotion (A, C and E), percent sociability (B, D) and social novelty recognition (F) scores during the 3 trials of the test (lasting 5 min each). These included a first trial of habituation to the apparatus containing the empty stimulus cages (A, B), a second trial of sociability (C,D), assessing the percent preference for a social versus a non-social novel stimulus (juvenile male mouse versus object), and a third trial of social novelty preference (E,F), assessing the percent preference for a novel versus a familiar stimulus mouse. Data are expressed as mean±SEM. § versus chance level (50%, red dotted line),  $p < 0.05$  one sample t-test

### Direct social interaction with an adult female

The 6-min interaction session was analysed using 3-min bins as the within-subject factor, as it is well known that the highest level of social affiliative behaviours are displayed during the first 3 min and decreased afterwards. Indeed, the 2 x 3 x 2 ANOVA of the time spent performing affiliative behaviours led to a significant effect of 3-min bins [ $F(1,41)=225.45$ ,  $p < 0.0001$ ; Fig. 4], confirming this social habituation. Furthermore, the

experimental groups differed in their affiliation time only during the first 3 min of the testing session [overall interaction genotype x treatment x 3-min bins [F(1,41)=225.45,  $p < 0.0001$ ; Fig. 4], when KO-VEH mice displayed a clear deficit compared to WT-VEH, which was attenuated following CBDV treatments at both doses. CBDV treatment was again associated with reduced affiliation in WT mice [genotype x treatment on the first 3 min: F(1,41)=15.95,  $p < 0.0001$ ; Fig. 4; post-hoc Tukey test: WT-VEH versus KO-VEH and versus WT- CBDV20 and WT-CBDV100, KO-VEH versus KO-CBDV20 and KO-CBDV100, all  $p < 0.05$ ].

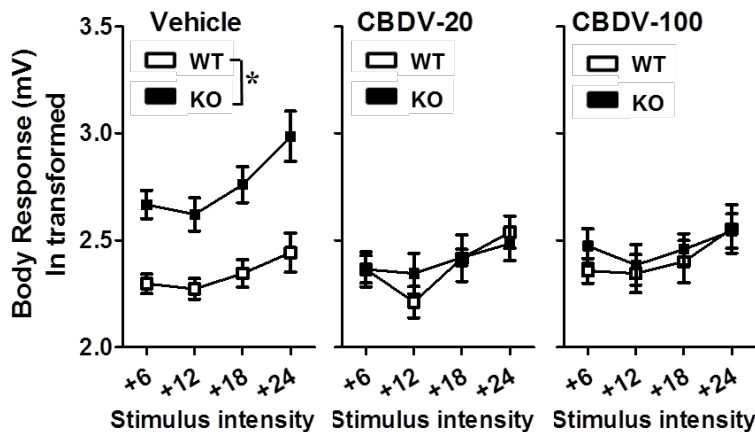


**Fig. 4: Social interaction:** Time spent performing affiliative behaviours (including sniffings and contact) towards a WT adult female during a 6-min session of direct social interaction test [genotype x treatment on the first 3 min: F(1,41)=15.95,  $p < 0.0001$ ; WT-VEH versus KO-VEH and versus WT- CBDV20 and WT-CBDV100, KO-VEH versus KO-CBDV20 and KO-CBDV100,  $p < 0.05$ , post-hoc Tukey's test]. Data are expressed as mean $\pm$ SEM.

### Acoustic startle

Reactivity data did not follow a normal distribution for stimulus intensities in all experimental groups and were therefore submitted to a natural logarithmic transformation before performing a 2 x 2 x 4 (genotype x treatment x stimulus intensity) ANOVA of the startle response (Fig.5). As expected, body startle response increased with the stimulus intensity [intensity effect: F(3,123)=11.65,  $p < 0.0001$ ; Fig. 5]. Also, KO

mice showed an overall startle hyper-responsiveness [genotype effect:  $F(1,41)=12.41$ ,  $p<0.01$ ], and this expected phenotype was not present following CBDV administration [genotype x treatment interaction:  $F(2,41)=7.68$ ,  $p<0.01$ ; post-hoc Tukey test: WT-VEH versus KO-VEH, KO-VEH versus KO-CBDV20 and KO-CBDV100,  $p<0.05$  Fig. 5].

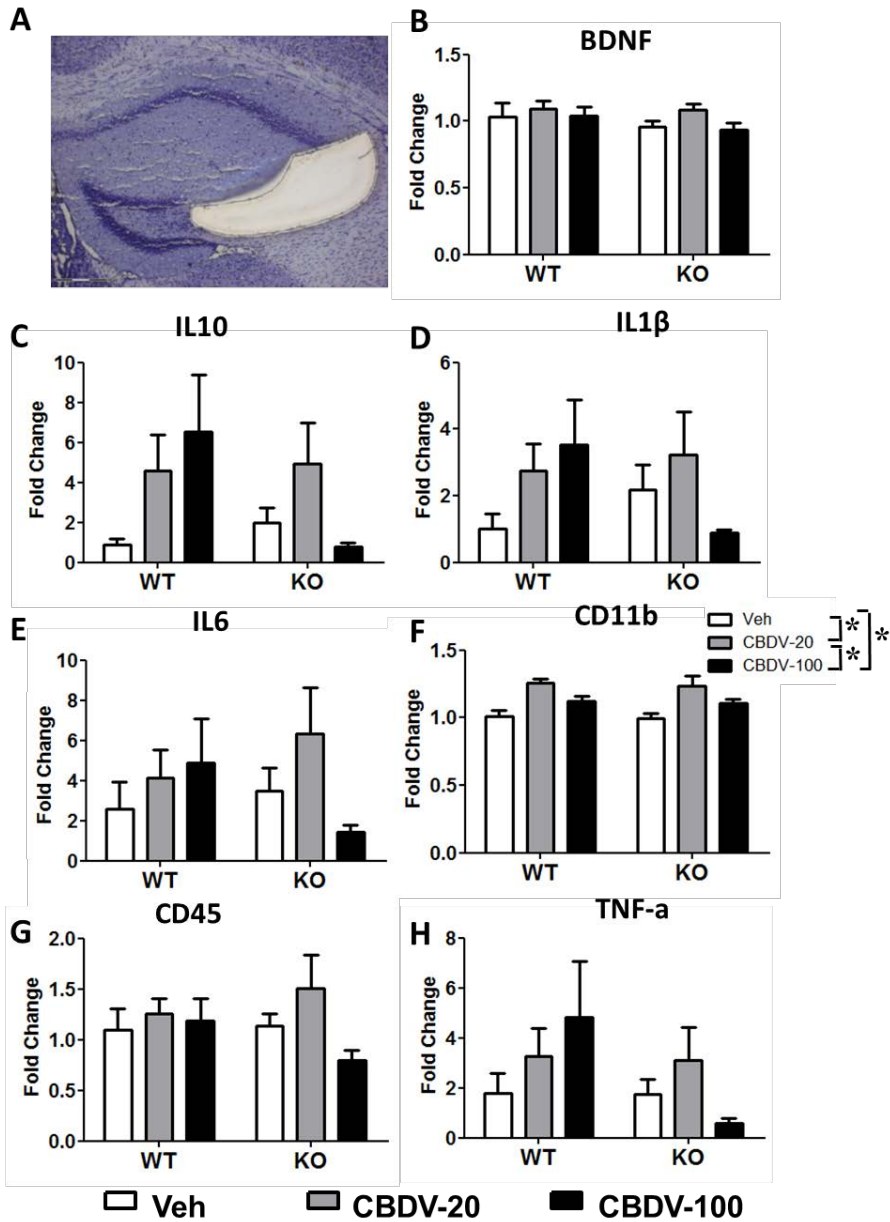


**Fig. 5: Acoustic startle:** the body startle response to acoustic stimuli of 6, 12, 18 and 24 dB over the background of 66 db (A) Startle reactivity was In-transformed in order to meet the normality requirement for a parametric repeated measures ANOVA; [genotype x treatment interaction:  $F(2,41)=7.68$ ,  $p<0.01$ ; WT-VEH versus KO-VEH, KO-VEH versus KO-CBDV20 and KO-CBDV100,  $p<0.05$ , post-hoc Tukey's test]. Data are expressed as mean±SEM.

## Brain analyses

### Hippocampus, CA3

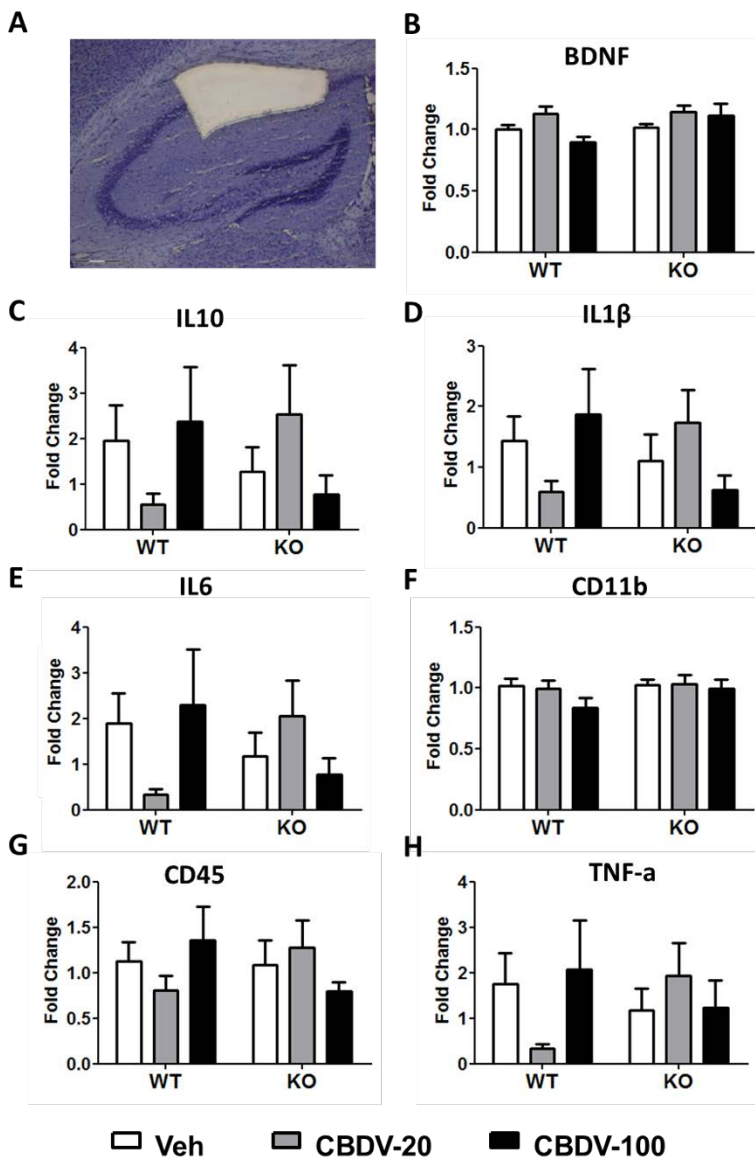
In CA3, there was no effect of genotype on any of the markers examined, suggesting no phenotype, although there was an effect of treatment for CD11b. This microglial marker was increased compared with vehicle by both doses of CBDV treatment in both WT and KO mice, but this effect seemed significantly stronger for CBDV 20 [treatment effect:  $F(2,41)=15.23$ ,  $p<0.0001$ ; Fig. 6-F; post-hoc: CBDV 20 versus CBDV 100 and VEH; CBDV 100 versus VEH] (Fig. 6).



**Fig. 6: BDNF and inflammatory markers in CA3:** Representative image of the CA3 region obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). CD11b was increased by both doses of CBDV treatment in both WT and KO mice [treatment effect in the genotype x treatment ANOVA:  $F(2,41)=15.23, p<0.0001$ ; Fig. 6-F; post-hoc Tukey's test: CBDV 20 versus CBDV 100 and VEH; CBDV 100 versus VEH, \*  $p<0.05$ ]. Data are expressed as mean±SEM..

### Hippocampus, CA1

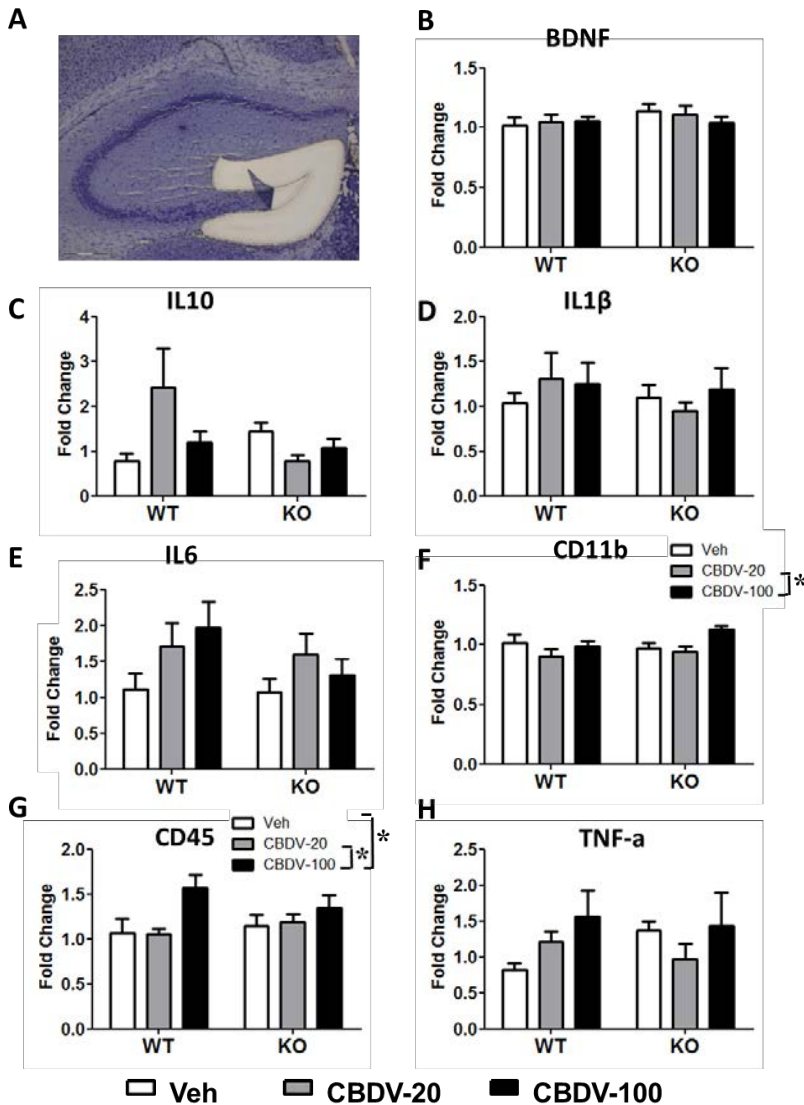
In CA1 with IL-6, although there was an interaction between genotype and treatment [interaction genotype x treatment  $F(2,36)= 2.27$ ,  $p<0.05$ ; Fig.7-E], there was no difference between WT and Fmr1 KO mice treated with vehicle suggesting no phenotype. Although CBDV 20 was associated with an increase in IL-6 in Fmr1 KO mice compared with WT mice, this was not seen for CBDV 100 and is not relevant without a phenotype. (Fig. 7).



**Fig. 7: BDNF and inflammatory markers in CA1:** Representative image of the CA1 region obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). CBDV 20 was associated with an increase in IL-6 in Fmr1 KO mice compared with WT mice [interaction genotype x treatment  $F(2,36)= 2.27$ ,  $p<0.05$ ; Fig.7-E], but this was not seen for CBDV 100 and is not relevant without a baseline phenotype ., although no baseline phenotype was observed ). Data are expressed as mean $\pm$ SEM. \*  $p<0.05$ .

### Dentate gyrus

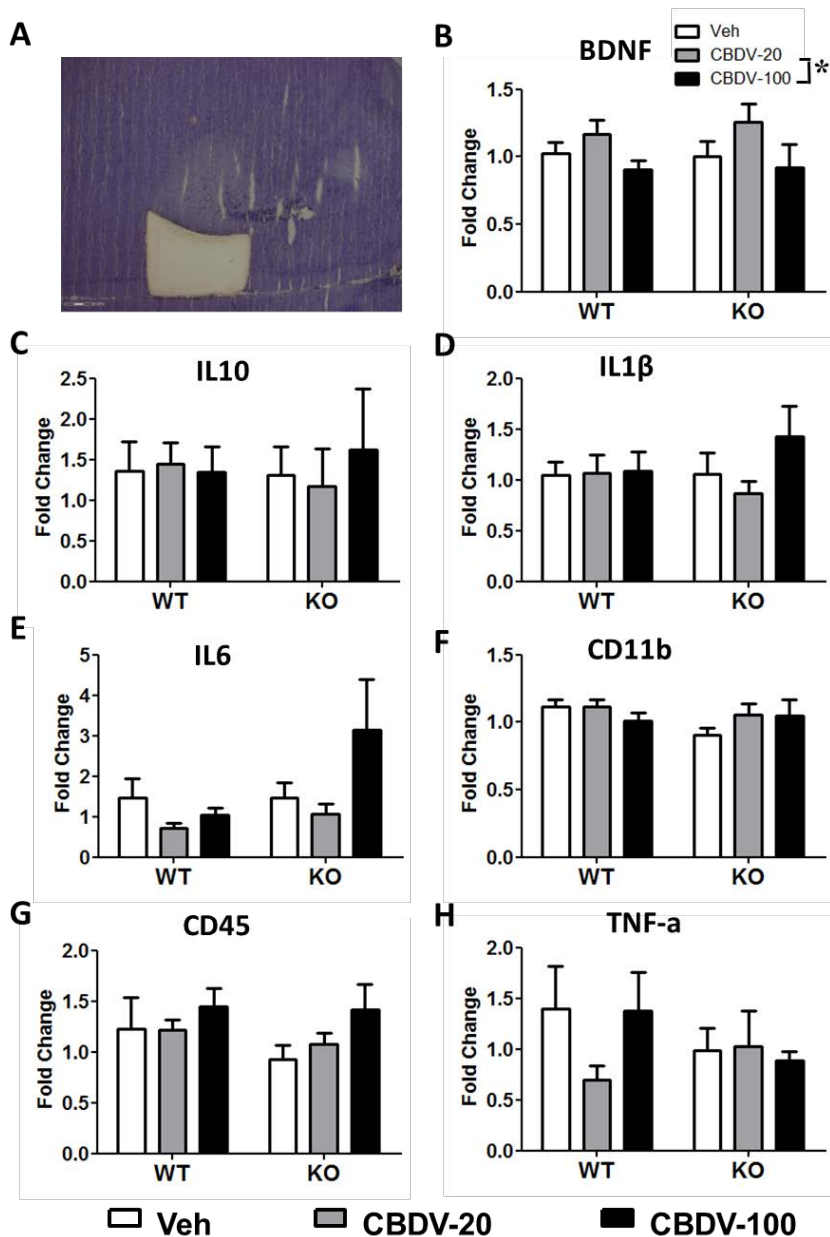
In DG, there was no effect of genotype on any of the markers examined, suggesting no phenotype for these, apart from IL-10, which was increased in KO mice compared to WT an effect that tended to be more evident under VEH treatment, [genotype x treatment  $F(2,37)= 3.67$ ,  $p<0.05$ ; Fig.8-C); Although there were treatment effects with CD11b and CD45 [respectively, treatment effect:  $F(2,40)=3.86$  and  $5.13$ ,  $p<0.05$ ), only the CBDV 100 showed an increase in CD45 compared with both Veh and CBDV 20 in mice of both genotypes whereas with CD11b, CBDV 100, was only different compared with CBDV 20, which is not relevant; ( $p<0.05$  post-hoc Tukey's test, Fig. 8-F and G]. (Fig. 8).



**Fig. 8: BDNF and inflammatory markers in DG:** Representative image of the dentate gyrus hippocampal (DG) region obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). IL-10 was increased KO mice, but only under VEH treatment [genotype x treatment  $F(2,37)=3.67$ ,  $p<0.05$ ; Fig.8-C; genotype effect in VEH:  $F(1,12)=5.32$ ,  $p<0.05$ ]. CD11b and CD45 were increased by CBDV in mice of both genotypes but only at CBDV 100 [respectively, treatment effect:  $F(2,40)=3.86$  and  $5.13$ ,  $p<0.05$ ; pst-hoc: CBDV 100 versus CBDV 20 for CD11b; CBDV 100 versus CBDV 20 and Veh for CD45; Fig. 8-F and G]. Data are expressed as mean $\pm$ SEM. \*  $p<0.05$ .

## Prefrontal cortex

In PFC, there was no effect of genotype on any of the markers examined, suggesting no phenotype for these. Although there was a treatment effect for BDNF, neither dose of CBDV was different to vehicle and CBDV 100 was only different to CBDV 20, which is not a relevant comparison. [treatment effect:  $F(2,38)=3.32$ ,  $p<0.05$ ; post-hoc: CBDV 100 versus CBDV 20; Fig. 9A]. (Fig. 9).





**Fig. 9: BDNF and inflammatory markers in PFC:** Representative image of the prefrontal cortex (PFC) obtained by laser microdissection (A) and levels of plasticity (B) and inflammatory markers (C-H). There was no effect of genotype on any of the markers examined, suggesting no phenotype for these. Although there was a treatment effect for BDNF, neither dose of CBDV was different to vehicle and CBDV 100 was only different to CBDV 20 [treatment effect:  $F(2,38)=3.32$ ,  $p<0.05$ ; post-hoc: CBDV 100 versus CBDV 20; Fig. 9A]. Data are expressed as mean $\pm$ SEM.

## Discussion and Overall Conclusions

This study provided a further characterisation of the behavioural effects of CBDV in the *Fmr1*-KO mouse model of FXS, following the initial investigation described in Study 4. In this previous study we administered the same doses of CBDV over 10 days, starting at 3 months of age, in adult mice and we detected a beneficial effect of CBDV (at CBDV 20) only on the startle hyper-responsiveness of KO mice, with little meaningful effect at the brain level on a range of plasticity and neuroinflammatory markers. In the current study, we instead demonstrated several beneficial behavioural effects of chronic CBDV treatment (5 weeks) starting at weaning (PND 22).

As in our previous study, we confirmed here most aspects of the *Fmr1* mouse phenotype, that were previously demonstrated by us and others (hyperactivity in the open field (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; de Diego-Otero et al., 2009; Eadie et al., 2009; Hayashi et al., 2007; Liu et al., 2011; Mineur et al., 2002; Oddi et al., 2015; Olmos-Serrano et al., 2011; Peier et al., 2000; Pietropaolo, Goubran, et al., 2014; Restivo et al., 2005; Spencer et al., 2011; Spencer et al., 2005; Thomas et al., 2011; Uutela et al., 2012), reduced direct social interaction (Dahlhaus & El-Husseini, 2010; Mineur et al., 2006; Oddi et al., 2015; Pietropaolo et al., 2011; Pietropaolo & Subashi, 2014; Spencer et al., 2011) and lack of preference for social novelty

(Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Hebert et al., 2014; Heitzer et al., 2012; Liu et al., 2011; Mines et al., 2010; Pietropaolo et al., 2011; Spencer et al., 2011), sensory hyper-responsiveness in the acoustic startle test (Michalon et al., 2012; Zhang et al., 2014) and deficits in novel object memory (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Pietropaolo, Goubran, et al., 2014; Ventura et al., 2004). Anxiety-like behaviour was also evaluated as a more opportunistic endpoint, as it was previously described as an inconsistent phenotype in our own (Hebert et al., 2014) and others' (Bilousova et al., 2009; de Diego-Otero et al., 2009; Eadie et al., 2009; Heulens et al., 2012; Jung et al., 2012; Mineur et al., 2002; Nielsen et al., 2002; Qin et al., 2011) studies on this model. Indeed, here we found reduced anxiety of KO mice in the elevated plus maze and open field test, as reported by others (Hayashi et al., 2007; Heulens et al., 2012; Jung et al., 2012; Spencer et al., 2011; Spencer et al., 2005), while no genotype difference was detected in our previous report and in previous work by us (Hebert et al., 2014) and others (Eadie et al., 2009; Mineur et al., 2002; Nielsen et al., 2002; Spencer et al., 2011; Veeraragavan et al., 2011). Our data therefore confirmed the inconsistency of the anxiety-like phenotype of the Fmr1-KO model, in contrast with that observed in FX patients where there is a reported increase in anxiety levels (Bagni et al., 2012; Hagerman et al., 1991; Hagerman et al., 1999; Paribello et al., 2010; Tartaglia et al., 2019). For sociability (on trial 2 of the 3-compartment test), the lack of a KO phenotype was confirmed, as previously demonstrated by us (Hebert et al., 2014; Pietropaolo et al., 2011) and others (Bhattacharya et al., 2012; Dahlhaus & El-Husseini, 2010; Heitzer et al., 2012; Liu et al., 2011; Mines et al., 2010; Spencer et al., 2011). It should be noted that the hyper-activity shown here by KO mice in the open

field test was less marked than what previously reported in the literature, also by us (for a review see (Pietropaolo & Subashi, 2014), as it emerged only as an overall genotype difference, but lost significance if WT-VEH and KO-VEH mice only were compared (see Fig. 2-B). The same result was found in our previous study and may be perhaps explained by the confounding effects of the repeated i.p. injections.

Chronic CBDV administration at 20 or 100 mg/kg started at weaning was associated with the absence of all behavioural alterations examined in Fmr1 KO mice, apart from hyperactivity and reduced anxiety, thus providing amelioration of the most relevant and marked Fragile X-like symptoms. In particular, the effects of CBDV on affiliative behaviours during the social interaction test, clearly showed a therapeutic impact, where the decreased interaction was improved in Fmr1 KO mice after administration of CBDV (20 and 100 mg/kg) as compared with vehicle. Although this was in contrast to the effects of CBDV in WT mice where affiliative behaviours were decreased compared with vehicle. Increased startle responsiveness in Fmr1 KO mice was also clearly eliminated after treatment with CBDV 20 and 100 mg/kg.

In addition to the effects of CBDV observed in WT mice in the direct social interaction test, CBDV administration also reduced their performance in the OF and 3-COMP tests, similar to what was described following adult treatment in study 4.

At the brain level, KO-VEH animals displayed very limited alterations compared with WT mice, with only increased expression of IL10 observed in the DG. CBDV treatment

exerted some overall effects on brain parameters in both WT and KO mice, for instance CBDV at both doses increased CD11b in CA3 while CBDV-100 increased CD45 levels in the DG compared with vehicle treated mice of both genotypes and CBDV 20 increased BDNF levels in PFC.

Overall, these data demonstrate that CBDV (20 and 100 mg/kg IP), if administered chronically (5 weeks), to juvenile male Fmr1 KO mice (3 weeks old), is associated with improvements in social interaction, cognitive deficits and normalisation of startle responsiveness in adult KO mice.

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## CHAPTER 5 - CONCLUSION OF THESIS WORK

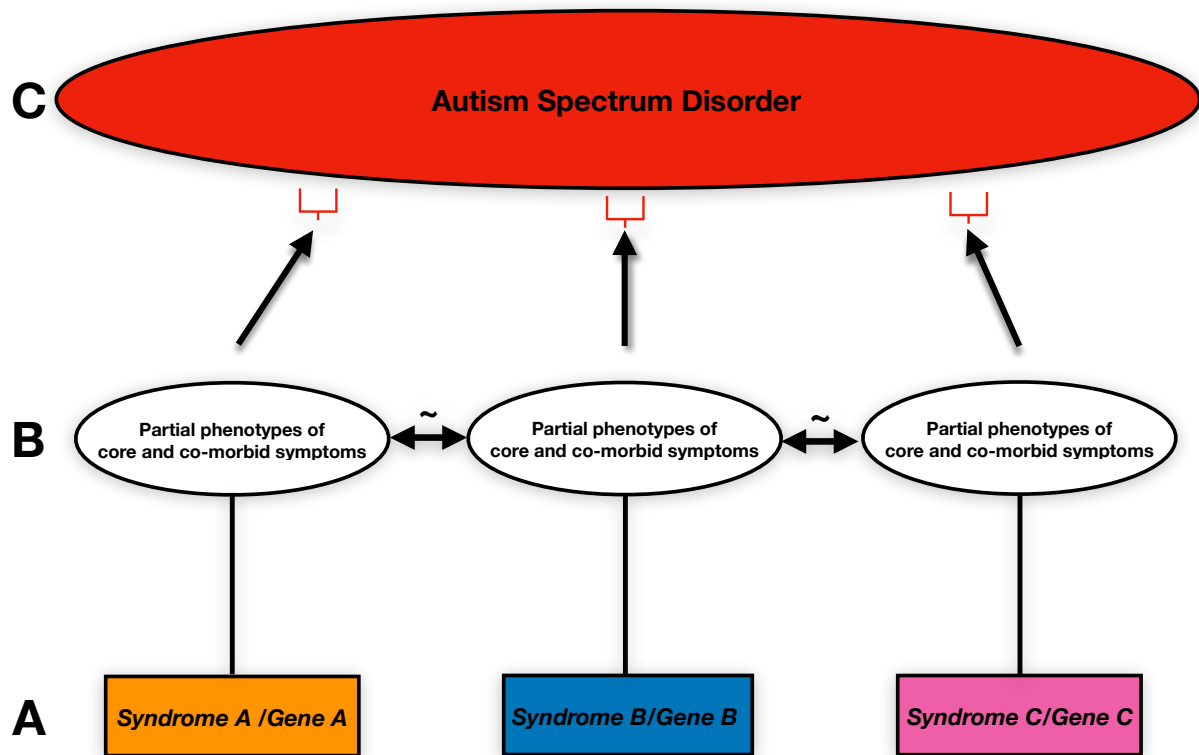
The goal of this research was the identification and characterization of therapeutic targets for the treatment of fragile X syndrome (FXS), with implications for autism spectrum disorders (ASD). This research explored two molecular systems, the ECS and BKCa channels, using pharmacological and genetic manipulations. A comprehensive battery of tests and analyses were used in order to examine the effect of genetic or pharmacological manipulations within a developmental timeline and under various contexts. Overall these studies demonstrated that:

- Genetic and pharmacological interference with ECS function induces a range of phenotypes of neurodevelopmental disorders. This is most apparent in the case of 2-AG, as reduction of this eCB induced robust phenotypes which recapitulated core and co-morbid ASD symptoms (*Chapter 3/SA1*).
- Acute pharmacological interference with BKCa activity at adulthood induces a mild social deficit (*Chapter 3/SA1.2*).
- Treatment with the pCB, CBDV, rescued most of the FXS-like phenotypes of Fmr1-KO mice, but the effects seem more evident following early (juvenile) administration (*Chapter 4/SA2.1*).

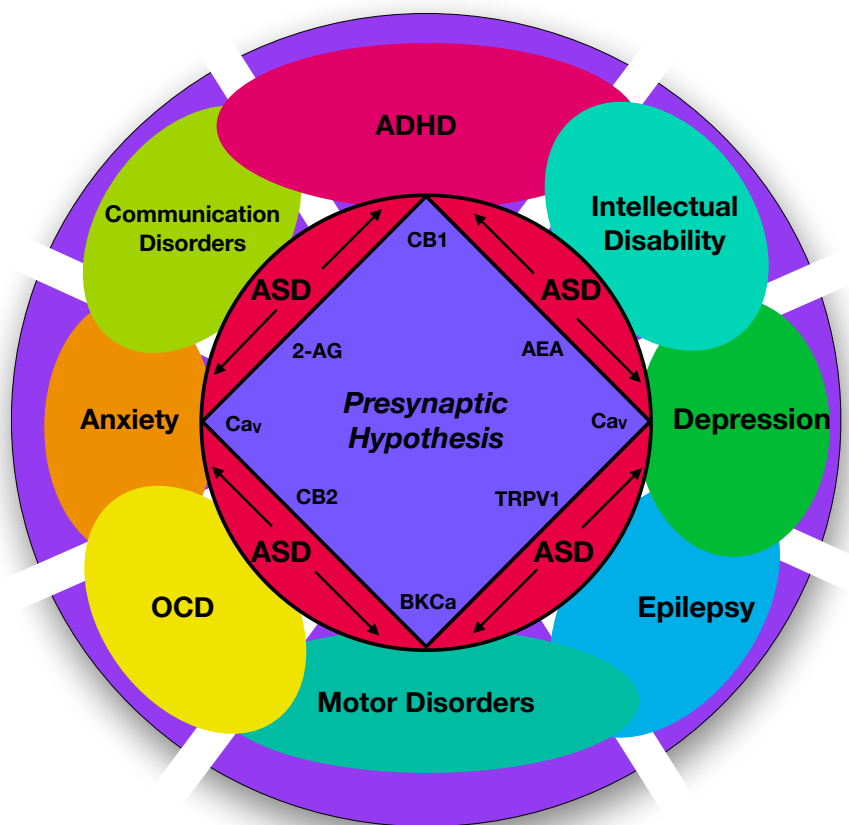
This work expands the current body of knowledge regarding the ECS, BKCa channels and neurodevelopmental disorders. The link between these systems and neurodevelopmental disorders, specifically FXS and ASD, is a relatively recent area of research (Gaffuri et al., 2012; Karhson et al., 2016; Zamberletti et al., 2017; Zou et al., 2019). This thesis work demonstrates that: (1) the primary eCB, 2-AG, makes substantial contributions to social behavior and communication in the C57BL/6 mouse, (2) loss of the CB1 expression is causal for developmental communication delays which are persistent across the lifespan and influenced by sex and context, (3) the pCB, CBDV, has therapeutic potential for behavioral deficits in FXS, and (4) interference with BKCa function induces a mild social deficit. Overall these results demonstrate that insults to the ECS and BKCa produces a range of neurodevelopmental phenotypes.

Since neurodevelopmental disorders often have a spectrum-like range of phenotypes, it is imperative that systems which are causally linked to these disorders and, shown to modulate a spectrum of behavior, be thoroughly studied. This is particularly crucial in regard to ASD. Novel methods for modeling this disorder are needed. Therefore, the development of new models is critical for improving our understanding of this complex and diverse disorder. This thesis work contributes three novel methods for modeling the phenotypes of neurodevelopmental disorders, particularly for, but not exclusive to, FXS and ASD.

This work proposes a system-based approach toward the identification and characterization of therapeutic targets for spectrum disorders. This approach posits that systems which can produce spectrum like phenotypes should be the subject of comprehensive and exhaustive investigations. This represents a new conceptual view of modeling ASD pathology that aims to produce models which better approximate the behavioral nuances observed clinically with ASD patients. Most efforts to generate animal models of ASD have focused on syndromic single gene mutations (e.g. *Fmr1*-KO, *Shank3*) or environmental insults (e.g. valproate). These models have provided a wealth of insight regarding the syndromes they model, such as FXS or Phelan-McDermid Syndrome, however the degree to which they inform about ASD is a matter of debate. These models incompletely recapitulate a small fraction of ASD phenotypes. Furthermore, the degree of overlap between models for ASD phenotypes is unclear (Fig. 3). A system-based approach toward modeling ASD will methodically investigate the capacity of a conceptual model, such as the presynaptic hypothesis, to produce a range of phenotypes (Fig. 4). The phenotypes produced by manipulations of these systems would ideally manifest (a) across a developmental timeline, and with (b) context and (c) sex specific phenotypes. This approach encourages new conceptualizations of ASD pathology which overlap and, in no way are mutually exclusive. Importantly, the characterization of systems which produce spectrum like phenotypes will identify multiple candidate targets for novel therapeutics. Indeed, in this thesis work, the critical findings are that CB1, CB2, 2-AG, TRPV1, and BKCa channels are targets with potential for the treatment of FXS and non-syndromic ASD (Table 4).



**Figure 3. Single gene (syndromic) modeling of ASD.** (A) A candidate gene is chosen as representative of a syndrome with strong associations to ASD (construct validity). (B) With this gene altered, a mouse partially recapitulates core and co-morbid phenotypes with varying degrees of overlap between syndromes (*double headed arrow*). (C) The ASD phenotypes recapitulated by syndromic mouse models are applicable to a small fraction of the ASD population.



**Figure 4. Hypothetical representation of a system-based approach toward modeling ASD.** Here the presynaptic hypothesis is used as an example. A conceptual framework for a group of pathological mechanisms is developed based on related molecular components which have associations to ASD (purple). These various combinations of specific insults in the system may demonstrate a spectrum like effect in pathophenotypes for ASD (red). These insults may also induce co-morbid disorders which have their own definitions (purple) and also overlap with each other and with ASD to various degrees (various colors).

System	Type of manipulation	Target	Behavioral Effect
ECS	Genetic knockout (Induce deficits)	CB1	<ul style="list-style-type: none"> <li>- social communication deficits post-natally and at adulthood</li> <li>- post-natal: communication delay is developmental</li> <li>- increased self-grooming in CB1<sup>-/-</sup> females at adulthood</li> </ul>
	Pharmacological inhibition (Induce deficits)	CB1	<ul style="list-style-type: none"> <li>- anxiety-like deficit</li> <li>- reduction vocalization behavior</li> <li>- mild increase in self-grooming behavior</li> </ul>
	Pharmacological antagonism (Induce deficits)	DGL- $\alpha$	<ul style="list-style-type: none"> <li>- anxiety-like deficit</li> <li>- social novelty deficit</li> <li>- decreased affiliation behavior</li> <li>- increased non-social behavior</li> <li>- increased self-grooming behavior</li> <li>- reduced vocalization behavior</li> </ul>
	Pharmacological modulation with pCB (Rescue deficits)	TRPV1 and/or DGL- $\alpha$ (?)	<ul style="list-style-type: none"> <li>- rescued most of FXS-like behavioral deficits following (juvenile)</li> <li>- rescued sensorimotor alterations following adult administration</li> </ul>
BKCa	Pharmacological antagonism (Induce deficits)	BKCa	<ul style="list-style-type: none"> <li>- social novelty deficit</li> </ul>

**Table 4. Summary of results**

Future studies should explore mechanistic questions surrounding the presynaptic hypothesis of FXS and ASD, such as: Do the ECS and BKCa channels interact? It is well established that they each regulate the same presynaptic Ca<sup>2+</sup> channels. It has not been explored if eCBs have activity at BKCa channels in central neurons, however eCBs can modulate BKCa channels in cell culture (Sade et al., 2006); Do mutations in P/Q and N-type channels Ca<sub>v</sub> channels, which interfere with either ECS or BKCa mediated regulation, produce models of ASD phenotypes? Can insults to 2-AG activity during critical periods produce developmental delays that are persistent across the life



span, and if so when are these critical periods? Does increasing 2-AG activity rescue social deficits across mouse models of ASD, and if so, are there critical periods of treatment which may produce better long-term treatment outcomes? Our data showing enhanced efficacy of pCBs following juvenile administration supports this hypothesis.

This thesis work has provided evidence which expands the understanding of the ECS and BKCa channels in FXS and ASD. This research has also provided a novel conceptual framework for future studies on the pathology and treatment of FXS and ASD. The results obtained here provide support that this approach can demonstrate causal pathology and potential targets for novel ASD therapeutics.

## FUTURE PERSPECTIVES

### **Pharmacological increasing 2-AG and social behavior and communication in the *Fmr1*-KO mouse model of Fragile X Syndrome**

#### **Summary Statement:**

The experiments conducted in the manuscript “Pharmacological inhibition of the primary endocannabinoid producing enzyme, DGL- $\alpha$ , induces ASD-like and co-morbid ASD phenotypes in adult C57BL/J mice” demonstrated that DGL- $\alpha$  production of the 2-AG contributes significantly to behavioral domains altered in FXS and ASD. The study “*Pharmacological increasing 2-AG and social behavior and communication in the Fmr1-KO mouse model of Fragile X Syndrome*” was planned to test the hypothesis that pharmacological increases in 2-AG signaling will rescue the pathophenotypes of the *Fmr1*-KO mouse, however the completed study could not be incorporated into the thesis project because of the COVID19 pandemic. The scientific premise for the study is as follows: Jung et al. (2012) demonstrated that JZL-184, an inhibitor of the 2-AG inactivating enzyme monoacylglycerol lipase (MAGL), rescued pathophysiological phenotypes in the ventral striatum and prefrontal cortex. Furthermore, this treatment rescued behavioral phenotypes of hyperactivity and altered anxiety like behavior. This study aimed to replicate and extend these findings. To this end, this study employed the same behavioral paradigms of the DGL- $\alpha$  study to examine inhibition of MAGL as a therapeutic target for FXS. As was done in the DGL- $\alpha$  study, two tests of social behavior and an assessment of social communication were planned since these have relevance to the broader category of autism spectrum disorders. Treatments for FXS or ASD will

most likely need to chronic treatment (10 days) with JZL-184 in order to examine the effects of chronic MAGL inhibition in comparison to acute treatment.

Electrophysiological and molecular studies were planned to test the hypothesis that it is loss of presynaptic regulation of neurotransmitter release which contributes to the pathophenotypes of the *Fmr1*-KO mouse. This study was expected to provide insights into both mechanisms of pathology and potential therapeutic targets for FXS. Recent studies by Folkes et al. (2020) demonstrated that inhibiting the action of 2-AG on the basolateral amygdala– nucleus accumbens (BLA-NA) circuit induced social deficits in B6 mice, while pharmacological augmentation of 2-AG activity in this circuit rescued social behavioral impairments in *SHANK3B*<sup>-/-</sup> mice. It is our hope that our planned studies will be able be conducted at a near point in the future when the Covid-19 pandemic is not an inhibiting factor.

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