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The endocannabinoid system and autistic behavior in the Fmr1- KO mouse

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“ Confidence is what you have before you understand the problem” – **Woody Allen**

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Abbreviations

(m)RNA	(messenger) Ribonucleic acid
2- AG	2- Arachidonoylglycerol
AEA	Anandamide
ASD	Autism Spectrum Disorder
BSA	Bovine serum albumin
BW	Body weight
CA1- 3	Cornu amonis region 1- 3
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2
CNS	Central nervous system
COX- 2	Cyclooxygenase 2
DAG	Diacylglycerol
DAPI	4,6- diamidino- 2- phenylindole
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
DSM- IV	Diagnostic and Statistical Manual of Mental Disorders 4th Edition
DSM- V	Diagnostic and Statistical Manual of Mental Disorders 5th Edition
ECS	Endocannabinoid system
FISH	Fluorescent in situ hybridization
Fmr1- gene	Fragile X mental Retardation gene
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X syndrome
GIRKs	G protein coupled inwardly rectifying potassium channels
HRP	Horseradish peroxidase
i.p.	Intraperitoneal
kDa	Kilodalton
KO	Knock- out
MAPK	Mitogen- activated protein kinase
mGluR	Metabotropic glutamate receptor
mGluR5	Metabotropic glutamate receptor 5
PBS	Phosphate buffered saline

PFA	Paraformaldehyde
SDS	Sodium dodecyl sulfat
SDS- page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSC	Saline sodium citrate buffer
TBS	Tris buffered saline
TBS- T	Tris buffered saline plus Tween20
THC	Tetrahydrocannabinol
TNT	2,4,6- trinitrotoluene
TRPV2	Transient receptor potential V2
TSA	Tyramide signal amplification
Veh	Vehicle solution
WT	Wild- type

1 Introduction

In this chapter autism spectrum disorder, fragile X syndrome, mouse models for fragile X syndrome and the endocannabinoid system will be described.

1.1 Autism Spectrum Disorder

The medical term “autism” derives from the Greek word “autos” for “self” and was used in the 19th century to describe a pathological condition of egocentricity and social withdrawal in schizophrenic patients (Möller, 2011). Nowadays autism is perceived as a mental congenital and incurable developmental disorder associated with genetic and environmental factors and a highly complex symptomatology. The designation “autism spectrum disorder” (ASD) includes different forms, symptoms and degrees of severity to live up to the complex manifestation of the disease. The “Diagnostic and Statistical Manual of Mental Disorders” (DSM)- V from 2013 incorporates several previously separated diagnoses including autistic disorder, Asperger’s disorder, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (American Psychiatric Association, 2013). According to the DSM- V ASD is a pervasive developmental disorder mainly diagnosed in children before their third year of age. 2-5 of 10.000 children are suffering from ASD and boys are affected four times more often than girls (Baio, 2012). Diagnostic criteria presented by the DSM- V include a triad of core symptoms: 1. qualitative impairment in social interaction, 2. qualitative impairment in communication, and 3. restricted, repetitive and stereotype patterns of behavior, interests and activities. Further diagnostic criteria with onset prior to the 3rd year of age are delays or abnormal functioning within at least one of the areas: social interaction, language use in social communication and symbolic or imaginative play, and the disturbance is not better accounted for by Rett’s Disorder or Childhood Disintegrative Disorder (American Psychiatric Association, 2013). Besides these criteria, several secondary symptoms that are variable in occurrence and severity can be present in people suffering from ASD. They include cognitive impairment in about 75% of the patients (Tsai, 1999), which probably comes with long-term memory impairment and alteration in synaptic plasticity (Minshew and Goldstein, 2001; Pardo and Eberhart, 2007). In this regard a recent study could show that in ASD- affected humans as well as in animal models for ASD, some neurons build connections back to themselves in ladder-like patterns (Chung et al., 2013). Further symptoms include increased sensory reactivity (Baranek, 2002), a reduced prepulse inhibition of the acoustic startle response

(McAlonan et al., 2002; Perry et al., 2007; Yuhas et al., 2011), hyperactivity and sleep-pattern alterations (Gail Williams et al., 2004; Polimeni et al., 2005), an increased anxiety level (Muris et al., 1998), epileptic seizures (Tuchman and Rapin, 2002) and an altered stress response (Spratt et al., 2012).

The etiopathology of ASD is mostly unknown. Neuropsychology explains the development of ASD by a disturbed development of neuronal systems that are involved in forming central coherence (Frith, 1989), executive function (Ozonoff et al., 1991), theory of mind (Baron-Cohen et al., 1985) and empathy (Baron-Cohen, 2002). It suggests that inter- neuronal information transfer is reduced or otherwise modified due to local overconnectivity and long-rage underconnectivity between associated brain regions, i.a. frontal and parietal cortex, basal ganglia, thalamus and the limbic system including hippocampus and amygdala (Belmonte et al., 2004). Further the cerebellum seems to be particularly implicated in the anatomic variances with deficits of long- range connectivity and altered coordination of cognitive functions (Courchesne, 1997; Courchesne et al., 2007). Neuroscience includes genetic factors with the development of ASD since twin studies indicate concordance rates for monozygotic twins at 70- 90% and corresponding values for dizygotic twins at 0-10% (Steffenburg et al., 1989; Bailey et al., 1995; Ronald and Hoekstra, 2011). Currently scientists proceed on the assumption that polygenic transmission with multiple gene loci interacting as susceptibility factors are responsible for the development of ASD (Belzung et al., 2005; Betancur, 2011). Also epigenetic modifications and pre- and perinatal exposures to environmental factors are discussed to play a role (Kolevzon et al., 2007; Miyake et al., 2012).

Until today defined mutations, genetic syndromes and de novo copy number variation of genes, which account for about 10-20% of ASD cases, have been identified (Abrahams and Geschwind, 2008). Contributing to the strong genetic influence is the observation, that in families in which a sibling is affected, the relative risk for a child being diagnosed with ASD is increased 25- fold over the population prevalence (Jorde et al., 1991). Furthermore, siblings and parents of affected children are more likely than controls to show subtle cognitive or behavioral patterns, that are qualitatively similar to those observed in patients (Bolton et al., 1994; Bishop et al., 2004).

ASD treatment includes behavioral, educational and pharmacological components that have been shown to have positive effects on some dysfunctional autistic behavior in human (Bodfish, 2004; Benvenuto et al., 2013), especially when applied in early childhood and as intense behavioral intervention (Howlin et al., 2009).

Still there is little evidence for the relative effectiveness of these treatment options with highly individual responsiveness and a positive outcome for many behavioral and

pharmacological interventions in the sense that some form of treatment is favorable over no treatment (Leskovec et al., 2008; Seida et al., 2009). A general accepted therapeutic approach to ASD as well as proof for preconditions, effectiveness and efficiency in a best practice model is missing.

1.2 Animal models for Autism Spectrum Disorder

Important instruments to research and to assess psychiatric conditions in a complex organism instead of the human body are animal models, if information cannot be obtained through human studies. In order to make animal models transferable to humans, requirements of validity must be met. Therefore it is favorable if the cause for the animal model's phenotype and its development is similar to the one observed in human. Further treatment applied to the animal model or the affected human should reduce symptoms equally (McKinney and Bunney, 1969; Belzung et al., 2005).

Concerning the origin or cause of the disease, animal models can mimic ASD only to a certain extend since interaction of polygenetic susceptibility factors and epigenetic modifications are highly complex and not entirely known. Nevertheless, there are different approaches to employ genetically modified mouse models to ASD research given the genetic cause of the disorder and the high homology between human and mouse genomes (Oddi et al., 2013). One approach consists in observing autistic behavior in a model and searching for the possible target gene. Here, animals with altered expression of vasopressin, oxytocin and dysfunctional opioid receptors have been observed. They display amongst others impaired or decreased social recognition and modified social preferences (Engelmann and Landgraf, 1994; Wersinger et al., 2002; Bielsky et al., 2004; Wersinger et al., 2004). Another approach consists in modifying target genes and evaluating possible autistic behavior. A variant of this strategy is the employment of a mouse model of another, better defined human genetic disorder that presents some of the relevant autistic core symptoms such as Fragile X Syndrome (Mineur et al., 2002; Belzung et al., 2005). It represents a monogenic approach to autism and must be considered as reductionist and of limited validity (Belzung et al., 2005; Oddi et al., 2013). Still the advantage of using an intelligible monogenic mouse model is the ability to use a well- explored genetic modified model to identify and reproduce pathological mechanisms possibly underlying the development of ASD as a predominantly genetic disease (Gould and Gottesman, 2006). Apart from genetic approaches ASD research also exposes animal models to factors that are thought to increase the risk for autism in humans, such as perinatal exposure to anticonvulsants, thalidomide or developmental deficits in serotonin synthesis (Kahne et al., 2002; Moles

et al., 2004; Schneider and Przewłocki, 2005). Also neonatal lesions of brain areas that are assumed to be connected to abnormal behavior in autistic patients have been carried out, such as lesions in the cerebellum, the amygdala or the medial prefrontal cortex (Vicedomini et al., 1982; Bobée et al., 2000; Wolterink et al., 2001; Diergaarde et al., 2005).

To observe similarity in the development of ASD between animal models and human several tests assessing relevant autistic behavior have been designed, especially concentrating on the core symptoms of ASD. These tests can assess early social deficits (Ricceri et al., 2007), deficits in social interest and social novelty (Nadler et al., 2004) as well as deficits in direct social interaction like affiliation, grooming, sexual and aggressive behavior (Terranova et al., 1993; McFarlane et al., 2008). Further, there are attempts to unmask qualitative impairment of communication in mice by recording ultrasonic vocalization at different developmental stages and contexts (Oddi et al., 2013). Also restricted, repetitive and stereotype behavior can be assessed by measuring behavioral inflexibility, impairment in reversal learning and time spend engaging in non-social activities like self- grooming, digging or rearing while in social encounters (Moy et al., 2008; Blundell et al., 2010; Mines et al., 2010).

Concerning the requirement of a treatment that equally reduces autistic symptoms in animal models as well as in humans, behavioral and educational interventions are mainly unexplored and obviously limited. In terms of drug treatment there are different approaches to employ known drugs like antidepressants, antipsychotics or amphetamines. The employed treatment aims to decrease comorbidities in autistic patients but has poorly been tested on animal models and sometimes even exhibited an opposite effect (Belzung et al., 2005). The absence of an appropriate drug treatment is likely due to the missing evidence for a molecular drug target that can be associated with ASD. Still, there are some new insights into cellular and molecular mechanisms possibly underlying the development of an autistic phenotype in animal models, which will be introduced in the chapter “biochemical mechanisms underlying the Fmr1- KO phenotype”.

1.2.1 A mouse model for Fragile X Syndrome

The human genetic disorder Fragile X Syndrome (FXS) presents some of the relevant autistic core symptoms and is a well-described disorder which is caused by expansion of over 230 CGG repeats in the promoter region of the fragile X mental retardation (Fmr1)- gene, located on the X chromosome (Pieretti et al., 1991). The triplet repeat triggers the methylation of a regulatory site near the Fmr1- gene, which results in epi-

genetic silencing of the gene transcription and subsequent deficiency of the encoded protein (Oberlé et al., 1991), known as Fragile X Mental Retardation Protein (FMRP). This protein plays an important role in mRNA- transport along the dendrites and the control of mRNA translation in neurons, both processes that are indispensably involved in synaptic plasticity (Martin and Huntsman, 2012). Females with the full mutation tend to be less severely affected than males because generally only one of their X chromosomes carries the mutation and random X-inactivation results in some cells being able to produce FMRP. Males with the full mutation are amongst others invariably mentally challenged and exhibit a range of cognitive and affective impairments (Tsiouris and Brown, 2004). Autistic features are also common: 2% of all patients with ASD have FXS, whereas 25% of patients with FXS meet DSM-IV criteria for ASD before their third year of age (Philofsky et al., 2004; Tsiouris and Brown, 2004). Further 90% of all patients with FXS display abnormal behavior that is also common in patients with ASD such as social anxiety, gaze avoidance, sensory hypersensitivity, stereotypic movements, delayed speech development, poor motor coordination and echolalia (Belmonte and Bourgeron, 2006; Hatton et al., 2006; Geschwind and Levitt, 2007; Abrahams and Geschwind, 2008; Hernandez et al., 2009). Taken together, the FXS is the leading monogenic cause for ASD (Boyle and Kaufmann, 2010). In 1994, scientists designed a mouse model to study the pathophysiological mechanisms underlying mental retardation and behavioral abnormalities in humans with FXS (The Dutch-Belgian Fragile X Consortium, 1994). Later this mouse line became relevant to autism research. The so called Fmr1- knock- out (KO) mouse is a genetically modified model that has a neomycin resistance cassette replacing exon 5 of the Fmr1- gene, hence it lacks Fmr1- RNA and FMRP and resembles FXS. It was generated by homologous recombination of a targeting vector into the mouse germ line using the embryonic stem cell technology (Mansour et al., 1988; The Dutch-Belgian Fragile X Consortium, 1994).

1.2.2 Autistic behavior in the Fmr1- KO mouse

The Fmr1- KO mouse line displays autistic core symptoms such as impaired social interaction and interest as well as signs of repetitive behavior. This has been shown in social interaction tasks, in which mice present deficits in social recognition, reduced social investigation and reduced preferences for social novelty. Further, mice exhibit signs of repetitive behavior being persistently engaged in self- grooming during social interaction (Mineur et al., 2006; Mines et al., 2010; Liu et al., 2011; Pietropaolo et al., 2011). Mild deficits in communication in this mouse line, measured by analyzing ultrasonic vocalization, has been reported and is object of current research (Oddi et al., 2013). As secondary symptoms of ASD mice show cognitive impairment and reduced behavioral flexibility in learning tasks such as deficiencies in object recognition and spatial, reversal or associative learning. They further display inappropriate aggressive behavior in social situations as well as signs of social anxiety (The Dutch-Belgian Fragile X Consortium, 1994; D'Hooge et al., 1997; Paradee et al., 1999; Van Dam et al., 2000; Busquets-Garcia et al., 2014; Lim et al., 2014). On the other hand, mice show partially no or even the opposite of the expected abnormal behavior including a reduced sensory reactivity, an enhanced prepulse inhibition, no tendencies to epileptic seizures and no signs of hyperactivity or sleep- pattern alteration (Pietropaolo et al., 2011). Concerning treatment that equally reduces the autistic phenotype in Fmr1- KO mice and human suffering from ASD, a lot of research was done but no substance has been licensed yet and the existence of a potent molecular drug target is not sufficiently proven (Belzung et al., 2005). In conclusion, the Fmr1- KO mouse displays some of the main core and secondary symptoms of an autistic phenotype, including deficits in social interaction and repetitive behavior, cognitive impairment as well as signs of increased anxiety levels. Despite its reductionist origin and its limited validity, the Fmr1-KO mouse is a relevant and useful tool used in the experiments of this work to elucidate specific neurobiological mechanisms underlying the presented autistic behavior as well as to explore treatment options (Bernardet and Crusio, 2006; Pietropaolo et al., 2011; Oddi et al., 2013).

1.2.3 Biochemical mechanisms underlying the Fmr1- KO phenotype

Alterations in different neurobiochemical mechanisms have been proposed to explain the Fmr1- KO phenotype. For one, there is the “mGluR theory of fragile X”: FMRP controls the synthesis and cellular integration of metabotropic glutamate receptors (mGluR). As FMRP is missing in the Fmr1- KO mouse, phenotypical characteristics are explained by an over- supply of active mGluRs, leading to an excessive downstream protein synthesis (Bear et al., 2004). Several inhibitors of glutamate receptors were shown to be effective in the normalization of the Fmr1- KO phenotype (Michalon et al., 2012; Michalon et al., 2014). Further, there are findings of altered inhibitory gamma-aminobutyric acid (GABA)- receptor signaling and altered alpha- amino- 3- hydroxy- 5-methyl- 4- isoxazolepropionic-acid (AMPA)- receptor dependent synaptic plasticity. GABA- B receptor agonist STX209 (arbaclofen) as well as a combined treatment of serotonin 5HT2B- and dopamine D1- receptor agonists or 5HT2A- and D2- receptor antagonists improve neuronal protein synthesis, AMPA- dependent synaptic plasticity and associative learning in the Fmr1- KO mouse (Henderson et al., 2012; Lim et al., 2014). Other studies could show, that intracellular signaling pathways including extracellular- signal regulated kinase (ERK)-, protein kinase B (AKT)- and mammalian target of rapamycin (mTOR)- pathways are altered in neurons of the Fmr1- KO mouse (Weng et al., 2008; Gross et al., 2010; Sharma et al., 2010). More precisely the early- phase phosphorylation of ERK is delayed and activities of AKT- and mTOR- pathways are increased, also found post mortem in human brain tissue of patients with FXS (Hoeffer et al., 2012). It has been shown that genetic reduction and pharmacological inhibition of mTOR- components can partially restore the Fmr1- KO phenotype (Bhattacharya et al., 2012; Busquets-Garcia et al., 2013).

The indicated biochemical mechanisms are pointing to an unbalance between synaptic formation and excitatory and inhibitory synaptic processes in neuronal networks what possibly supports the development of autistic behavior in the Fmr1- KO mouse (Belmonte and Bourgeron, 2006). An important modulator of excitatory and inhibitory synaptic processes that is also involved in synaptic formation is the endocannabinoid system (Kano et al., 2009). The exploration of the endocannabinoid system (ECS) in connection with the development and treatment of autistic behavior in the Fmr1- KO mouse opens some new perspectives into autism research.

1.3 Role of the Endocannabinoid System in the development of autistic behavior in the Fmr1- KO mouse

The ECS is an important neuromodulatory system that became known in 1964 by the discovery of Tetrahydrocannabinol (Gaoni and Mechoulam, 1971). Components of the ECS include neurotransmitters, so called endocannabinoids named Anandamide and 2- Arachidonylglycerol, and their receptors, cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2). The ECS is operating in physiological processes like motor learning, pain sensation, appetite and body temperature regulation (Kano et al., 2009) as well as in neurophysiological processes like synaptogenesis, synaptic plasticity and neuronal interconnectivity during development (Freund et al., 2003). On a neuronal level, endocannabinoids are synthesized as a response to mGluR activation during synaptic activity in the postsynapse (Varma et al., 2001). Once released into the synaptic cleft, endocannabinoids travel retrograde to the presynapse where they bind and activate G- protein coupled CB1- receptors, illustrated in figure 1 (Kano et al., 2009). Due to suppression of calcium influx, the activation of CB1, the main cannabinoid receptor in the brain, leads to direct inhibition of vesicle- mediated neurotransmitter release from the presynapse and to unexcitability of the neuron through additional potassium efflux. Activation of CB1 also affects the regulation of cell functions by ERK-, AKT-, and mTOR- protein kinases signaling pathways, illustrated in figure 2 (André and Gonthier, 2010). CB1 receptors are mainly expressed at presynaptic GABAergic contacts and, to a lesser extend on glutamergic and serotonergic terminals (Marsicano and Lutz, 1999) CB1 is highly expressed in the frontal cortex, basal ganglia, hippocampus, amygdala, the olfactory bulb and cerebellum, modestly in the remaining cortical structures and diencephalon and less in brain stem and spinal cord (Herkenham et al., 1991b; Mailleux and Vanderhaeghen 1992; Tsou et al., 1998).

It is possible that the autistic phenotype of Fmr1- KO mouse is related to an altered ECS. The ECS is mainly represented in brain areas that have been associated with the development of autistic symptoms, i.e. frontal cortex, basal ganglia, hippocampus, amygdala and cerebellum (Belmonte et al., 2004). The ECS is directly involved in the regulation of emotional behavior (Martin et al., 2002) and cannabinoid treatment is suggested to modulate social behavior as it can decrease time rodents spent in social interaction (Viveros et al., 2005). Also synaptic plasticity and the regulation of excitatory and inhibitory synaptic processes depend on a steadily operating ECS, mechanisms that have been suggested to be altered in neurons of the Fmr1- KO mouse (Freund et al., 2003; Lim et al., 2014). Furthermore, the synthesis of endocannabinoids is de-

pendent on mGluR signaling, a process that is over- activated in the Fmr1- KO mouse according to the “mGluR theory of fragile X” (Varma et al., 2001; Bear et al., 2004) and may therefore lead to an altered signaling process of the ECS. Additionally, stimulation of CB1 activates intracellular protein kinases including ERK-, AKT- and mTOR- pathways, which have been shown to be quantitatively and qualitatively altered in neurons of Fmr1- KO mice (Weng et al., 2008; Kano et al., 2009; Gross et al., 2010; Sharma et al., 2010). Whether this contributes to the development of autistic behavior in the Fmr1- KO mouse remains unclear. To reason that this behavior is in fact due to an altered ECS, the functionality of the ECS must be evaluated within the autistic behavior of the Fmr1- KO mouse. The following work engages in this evaluation, using pharmacological studies with CB1- agonists to investigate the functionality of the ECS in connection with specific autistic behavior and the effects of cannabinoid treatment in the Fmr1- KO mouse. It contributes to the validity criteria for the Fmr1- KO mouse model as a model for autism and to therapeutic approaches concerning its autistic behavior. First it was evaluated whether Fmr1- KO mice responded differently to acute pharmacological CB1 stimulation. Long-term effects of CB1 stimulation on impaired social interaction were evaluated as well as the influence of cannabinoid treatment on deficient associative learning. Besides, the influence of cannabinoid treatment on the activity of altered intracellular signaling protein kinases ERK, AKT and mTOR (respectively the ribosomal S6- kinase, that is part of the mTOR activation) were analyzed by western blot in brain structures with a high density of CB1 expression.

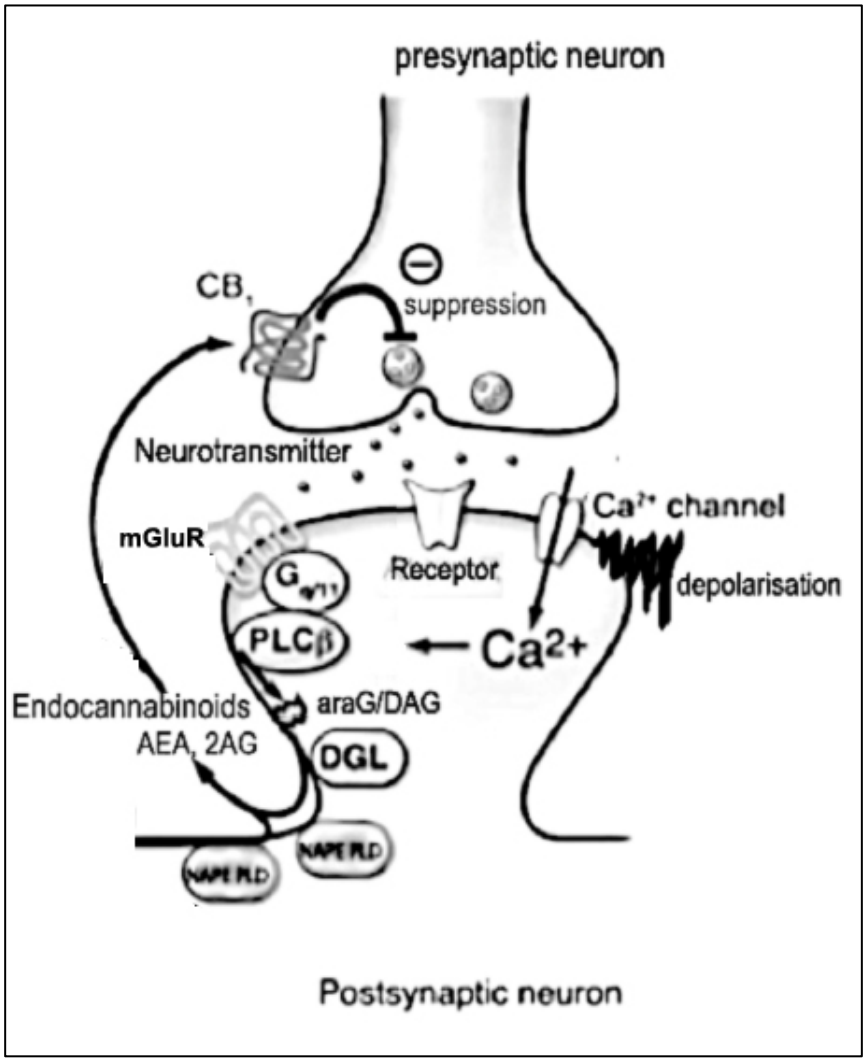


Figure 1: Endocannabinoid signaling, adapted from Kano et al., (2009). The figure shows endocannabinoid synthesis, release and action. mGluR= metabotropic glutamate receptor, PLC= phospholipase C, araG= arachidonate group, DAG= diacylglycerol, DGL= diacylglycerol lipase, NAPE PLD= phospholipid diesterase, AEA= Anandamide, 2AG= 2- Arachidonylglycerol).

Figure 1 shows a metabotropic glutamate receptor stimulated by neurotransmitter release from the inhibitory or excitatory presynapse. This leads to endocannabinoid synthesis and release from postsynapse and to retrograde messaging on CB1 at the presynapse. This again leads to suppression of inhibition or excitation.

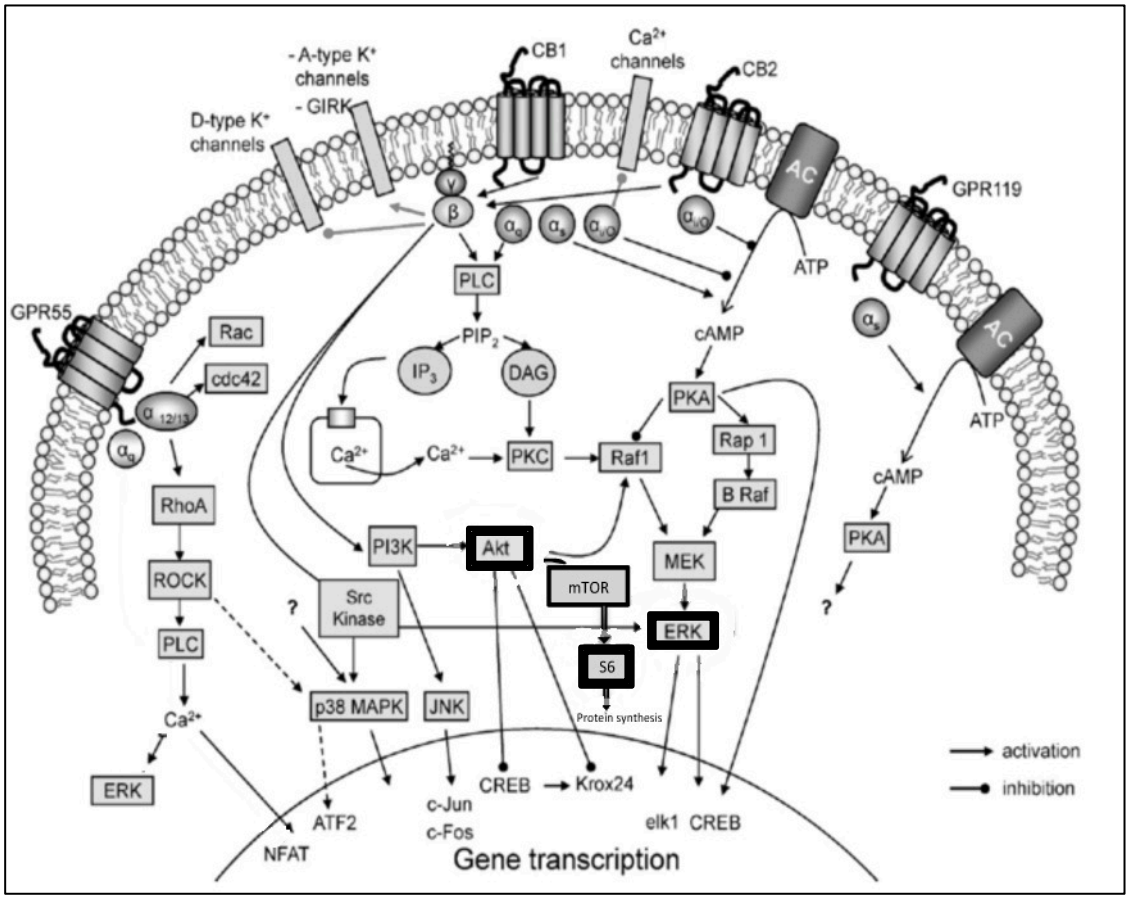


Figure 2: CB1 signaling (André and Gonthier 2010), modified. The figure shows G-protein-mediated signaling pathways of CB1. Protein kinases that are in focus of this study are highlighted in bold.

Figure 2 shows G-protein-mediated signaling pathways of CB1. Protein kinases that are in focus of this study are highlighted in bold. These are **ERK= extracellular- signal regulated kinase**, **mTOR= mammalian target of rapamycin/ respectively S6= ribosomal S6 kinase** and **AKT= protein kinase B**. Other pathway components shown are PKA= protein kinase A, AC= adenylate cyclase, ATF-2= activating transcription factor 2, CREB= catalytic response element binding, DAG= diacylglycerol, GPR55= G protein-coupled receptor 55, IP3= inositol triphosphate, NFAT= nuclear factor of activated T cells, PI3K= phosphatidylinositol 3 kinase, PIP2= phosphatidylinositol diphosphate, PKC= protein kinase C, PLC= phospholipase C, Raf1= rapidly accelerated fibrosarcoma kinase 1, Rap1= Ras- proximate-1, ROCK= Rho associated protein kinase, S6= ribosomal protein S6- kinase, c- Fos, c-Jun, elk1, Krox24= other transcription factors.

1.4 Aims of this work

ASD is a complex developmental psychiatric disorder. Aim of this work is to assess the hypothesis that an altered ECS mediates autistic behavior in a valid mouse model for autism. By measuring behavioral and molecular reactions to CB1 stimulation in the Fmr1- KO mouse, the involvement of the ECS in shaping autistic behavior can be explored as well as corresponding pharmaceutical possibilities can be elucidated. This leads to the following objectives:

1. Evaluation of typical cannabinoid- induced effects in the Fmr1- KO mouse compared to WT- mice
2. Investigation of the influenceability of autistic symptoms in the Fmr1- KO mouse with THC treatment
3. Analysis of the signalling cascade of the stimulated and unstimulated ECS in different brain regions of the Fmr1- KO mouse compared to WT- mice

2 Materials and Methods

Experiments consisted of behavioral tasks and western blots to investigate if and how CB1 stimulation influences the Fmr1- KO mouse and its autistic behavior as well as to determine intraneuronal mechanisms accounting for an alteration of the ECS in this mouse model. In the following chapters experiments and orders of experiments will be separated into a behavioral and an experimental part for reasons of simplification (figure 3).

As a preliminary point mice performed the so-called tetrad experiment to evaluate the basic functionality of the ECS in the Fmr1- KO mouse model. To assess typical and acute cannabinoid- induced effects in this experiment two different CB1- agonists, THC and WIN55,212 were tested exploratively. Since effects of both cannabinoids were comparable with a slightly greater effectivity in favour of THC all following experiments were carried out using THC. Subsequent to the tetrad experiment mice passed through a social interaction task 10 and 20 days later to assess long- term effects of THC treatment on deficits in social interaction, a core symptom of the autistic behavior presented by the Fmr1-KO mouse (Experiment 1). Because of a surprising and marked long- term effect on control WT- mice the experiment was repeated with WT- mice, which received a CB1- antagonist prior to cannabinoid treatment to validate the in-

volvement of CB1 (Experiment 2). This was not done in the context of ASD but as a contribution to the pharmacological study of long- term side effects of THC treatment. Further Fmr1- KO mice were tested on acute effects of THC treatment on deficits in associative learning, a secondary symptom of the autistic phenotype. Since the Fmr1-KO mouse model exhibits deficits in associative fear memory consolidation (Paradee et al., 1999), mice passed trough context- dependent fear conditioning task and subsequent THC injection to evaluate the effects of ECS- stimulation on context dependent learning (Experiment 3).

Experimental studies with western blot assessed the activity status of intraneuronal pathway components of the ECS in the Fmr1- KO mouse as well as its stimulatability with THC treatment. In brain tissue homogenates of cortex, hippocampus, striatum and cerebellum, regions that are high in CB1 expression (Herkenham et al., 1991b; Tsou et al., 1998) and partially addressed to be involved in the development of autistic behavior (Belmonte et al., 2004; Courchesne et al., 2007), quantities of activated protein- kinases ERK, AKT and S6 were measured using western blots (Experiment 4). All experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and local French legislation.

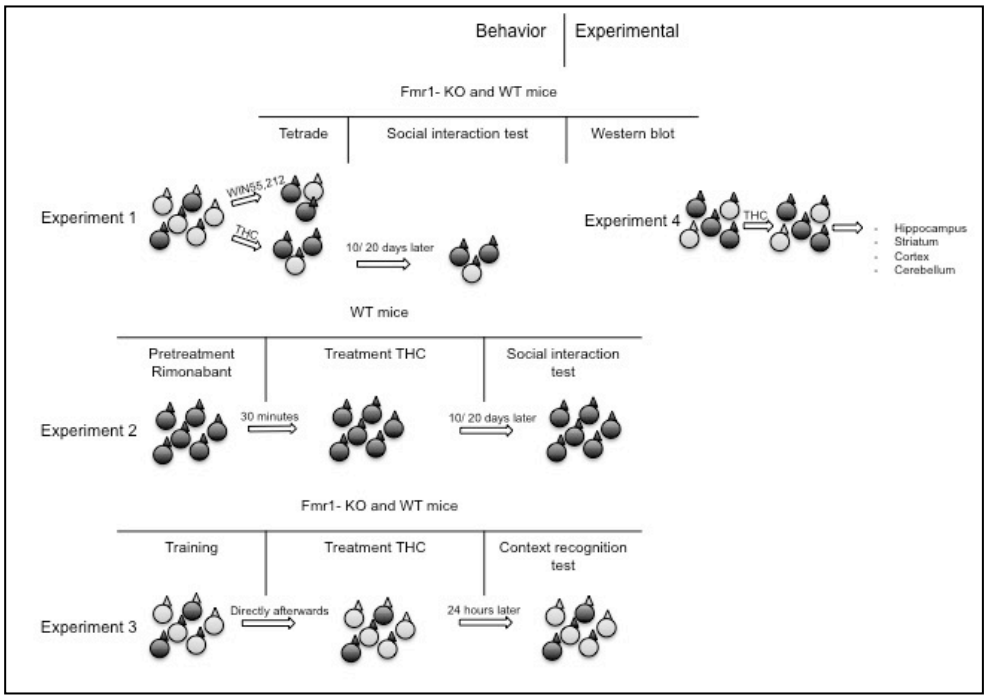


Figure 3: Synopsis of experiments in sequence and procedure. It shows Fmr1- KO (white) and WT (black) mice from the B6 background in order of experimentation. Animals of each experiment 1 to 4 were naive before testing.

2.1 Animals

Adult (12±1 weeks old) male Fmr1- KO and WT littermate mice from the C57BL/6J- (B6)- background were used for experimentation. B6 is the most widely used inbred strain and the first to have its genome sequenced. Animals from this background are as similar as possible, easily reproduced and validly used for comparing conclusions (Liu and Hewett, 2015). Fmr1- KO mice used for experiment 1 and 4 (see figure 3) were bred in the local institute. Female mice came from C57BL/6J- Fmr1$^{tm1Cgr/}$Nwu strains and were purchased from Neuromice.org (Northwestern University, IL 60208, USA; MGI ID: 1857169). Males came from the C57BL/6J.129P2-*Pde6b*$^{+}$*Tyr*$^{c\text{-}ch}$/AntJ strain, purchased from Charles River (L'Arbresle, France) and The Jackson Laboratory (Bar Harbor, ME 04609, USA; Stock number: 004828). By mating two heterozygous females with a wild- type male breeding trios were performed. After 2 weeks the sire was removed and the females were single caged and left alone in the cage 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 having the mice genotyped by PCR. Males with X- chromosomal knock- out for the Fmr1- gene (KO) or without (WT) were used for experiments. WT mice were employed as the control group. B6 mice used for experiment 2 (only WT) and experiment 3 (Fmr1- KO and WT, see figure 3) were purchased at Janvier (Le Genest-Saint-Isle, France). Adult (12 weeks old) virgin females of the NMRI strain were employed in addition in the social interaction task of experiment 1. Females of the NMRI strain were purchased from Janvier (Le Genest-Saint-Isle, France) and employed for testing after one week of customization to their new surroundings. NMRI mice are commonly employed in social behavior studies because of their high sociability (D'Amato and Pavone, 1996; Moles and D'Amato, 2000). All animals were housed in polycarbonate standard cages (33×15×14 cm in size; Tecniplast, Limonest, France), provided with sawdust bedding (SAFE, Augy, France) and a stainless steel wired lid. Food chow (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 12:12h light–dark cycle (lights on at 7 a.m.).

2.2 Pharmacological treatment

Table 1: Pharmacological treatment of mice

Drug	Form of administration	Dose mg/kg BW	Time of administration before testing/ sacrificing
Vehicle preparation for drugs/ control treatment	i.p.	5%ethanol (vol/vol), 5%cremophor-EL (vol/vol), 90%saline (vol/vol)	30 or 60min
CB1- agonist			
THC	i.p.	10	30min
WIN55,212	i.p.	3	30min
CB1- antagonist			
SR141716 (Rimonabant)	i.p.	3	60min

The applied doses are known to reliably induce or prevent neurocellular responses (Dewey 1986; Martin 1986; Little et al., 1988; Felder et al., 1995). THC was kindly provided by THC-Pharm-GmbH, WIN55,212 by Biomol-International and SR141716 by Sanofi-Aventis Recherche.

2.3 Behavioral tests

All behavioral tests were carried out during the light phase of the cycle (between 10 a.m. and 3 p.m.). Mice were excluded from testing in cases of aggressive behavior during the test or if accounting as statistical outliers. Test designs (see table 2 to 8) include treatment, genotype and number (N) of mice in each group.

2.3.1 Experiment 1: Tetrad experiment and social interaction task

Experiment 1 involved the measurement of acute cannabinoid induced effects on Fmr1- KO and WT mice, measured in the tetrad experiment and long- term cannabinoid induced effects on autistic behavior in this mouse model, measured in the social interaction task (SIT), 10 and 20 days after cannabinoid treatment.

2.3.1.1 Tetrad experiment

In the tetrad experiment mice were tested on typical acute cannabinoid-induced effects (figure 4). These effects include hypothermia, hypolocomotion, catalepsy and analgesia (Martin 1986; Howlett et al., 2002b; Varvel et al., 2005; Monory et al., 2007). A modified standard tetrad test protocol was used for testing (Martin, et al., 1991) and adopted with THC and WIN55,212.

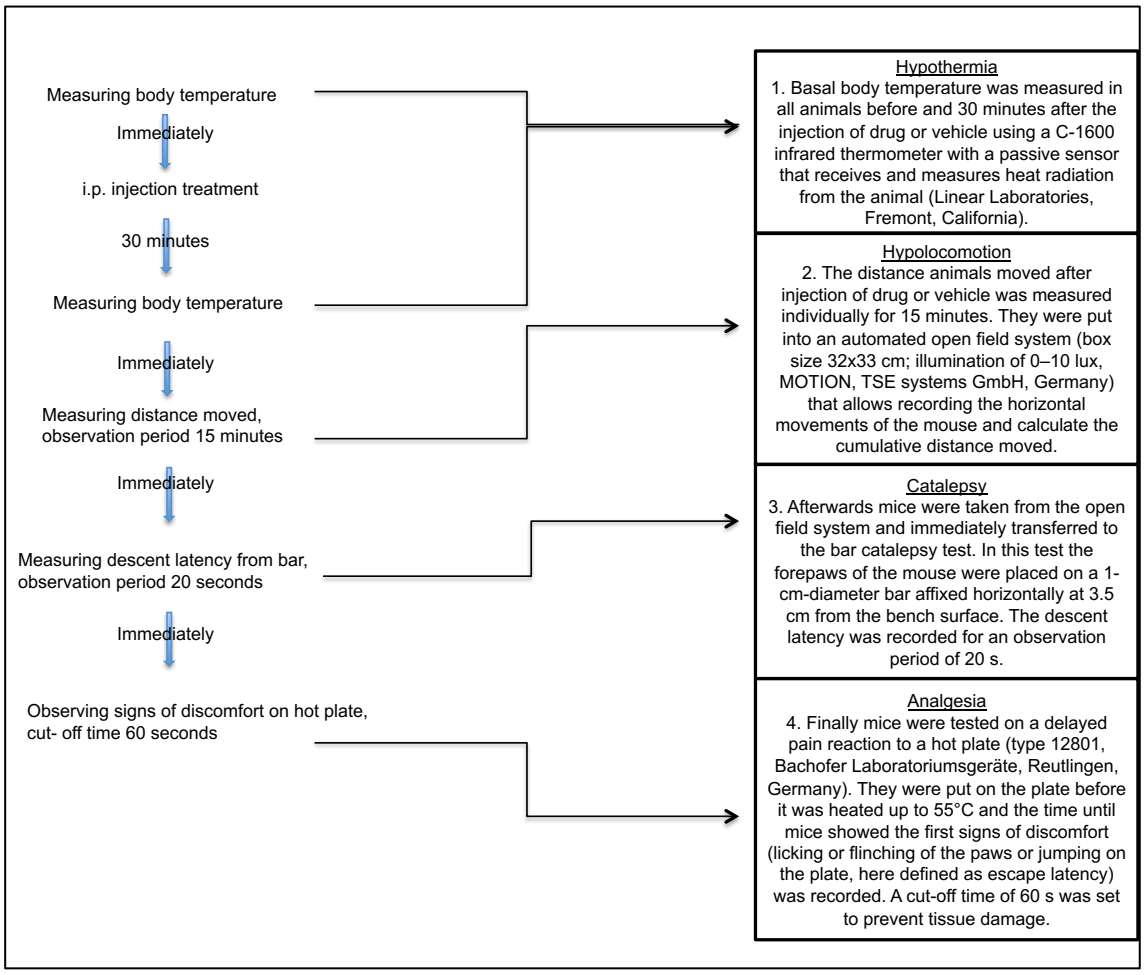


Figure 4: Test procedure of the tetrad experiment

2.3.1.2 Social interaction task

In the social interaction task (SIT) mice from the tetrad experiment were tested on long-term effects of THC treatment for deficits in social interaction. The time frame to analyze social encounter was chosen 10 and 20 days after cannabinoid treatment according to the half- life period of active THC metabolites. Since this period lasts up to 6 days (Law et al., 1984; Schwartz et al., 1985; Iten, 1994), an effect was still expected at 10. but not anymore at 20 days after treatment used as a control. Added test procedures for experiment 2 are put in brackets (figure 5).

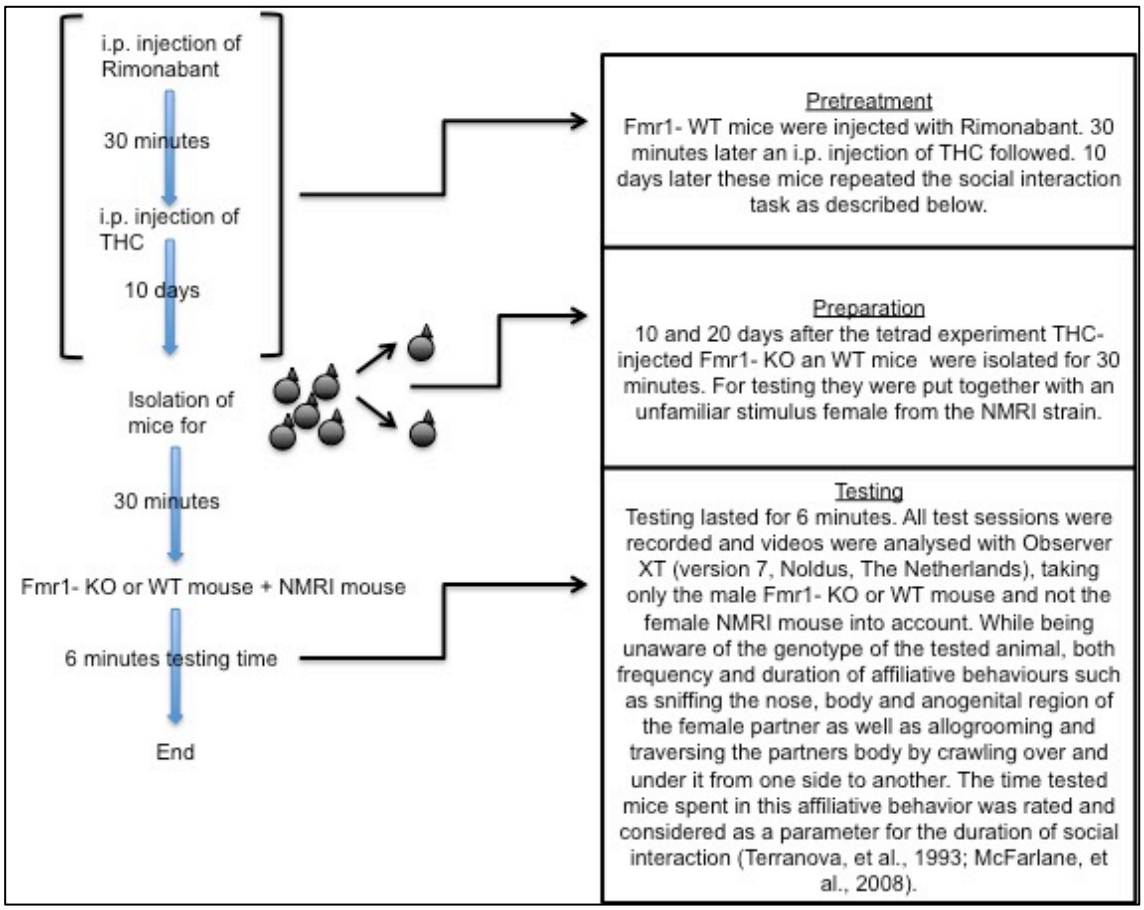


Figure 5: Test procedure of the social interaction tasks

Table 2: Number (N) of mice per genotype and treatment group, that took part in each of the three tasks of experiment 1, the tetrad experiment and the social interaction task (SIT) after 10 days and after 20 days

Test design of experiment 1						
Treatment	**WT**			**Fmr1- KO**		
	Tetrad	SIT 10 days	SIT 20 days	Tetrad	SIT 10 days	SIT 20 days
Veh	N= 8	N= 6 (8)	N= 6	N= 8	N=5 (8)	N=5
THC	N= 7	N=7	N= 7	N= 8	N=8	N= 8
Number (N) of mice vary between tasks. Five mice were excluded for aggression during testing from groups as indicated.						
Veh	N= 9	-		N= 6	-	
WIN55,212	N= 9	-		N= 7	-	

Apart from the number of mice shown in table 2 there were dropouts while measuring the catalepsy effect in the tetrad experiment. Six THC- treated mice (3 from WT and 3 from Fmr1- KO) presented the so called “popcorn- effect” (Adams and Martin, 1996) and were therefore not taken into account for statistical analyzes. This effect describes THC- sedated mice leaping up due to hypersensitivity to haptic or auditory stimuli. There were no dropouts measuring the other three effects in the tetrad experiment.

2.3.2 Experiment 2: Social interaction task with pre- treatment of a CB1- antagonist

Because of a strong effect on control WT mice, the social interaction task was repeated with naïve, purchased B6 WT mice from Janvier to verify the involvement of the CB1 receptor on the observed effect. Therefore mice were pre-treated with Rimonabant (SR141716), a CB1- antagonist, to impede THC induced deficits in social interaction by preventing THC- binding to CB1. Due to the novelty of the designed test protocol affiliative behavior was assessed in the first 3 and again in the whole 6 minutes of the experiment to find out whether differences will occur depending on half time and the amount of time analyzed. For test procedure see figure 4: Test procedure of the social interaction tasks. Table 3 shows number of mice used for testing. 3 mice were excluded for aggression during testing, one for accounting as statistical outlier.

Table 3: Number (N) of WT- mice per pre-treatment and treatment group that took part in the repetition of social interaction task 10 days after treatment. Numbers of mice that were used for statistical analyzes are put in brackets in case of drop outs.

Test design of experiment 2		
Pre- treatment with Rimonabant	**Treatment**	
	Veh	THC
No pre treatment (-)	N= 9	N= 9
Pre treatment (+)	N= 10 (9)	N= 9

2.3.3 Experiment 3: Context recognition task

In the context recognition task Fmr1- KO and WT mice were tested on acute effects of THC treatment on deficits in associative learning. In this experiment mice learn to fear a new environment because of its temporal association with an aversive stimulus. Observation of freezing 24 hours after conditioning in the conditioning chamber was regarded as contextual fear (Kim and Jung, 2006). Additionally, mice were analyzed on moving (all activities except grooming) to explore further behavioral aspects in this experiment. A modified test protocol was used for testing (Paradee et al., 1999) and adopted with THC (figure 6). Table 4 shows number of mice used for testing. One mouse was excluded for accounting as a statistical outlier.

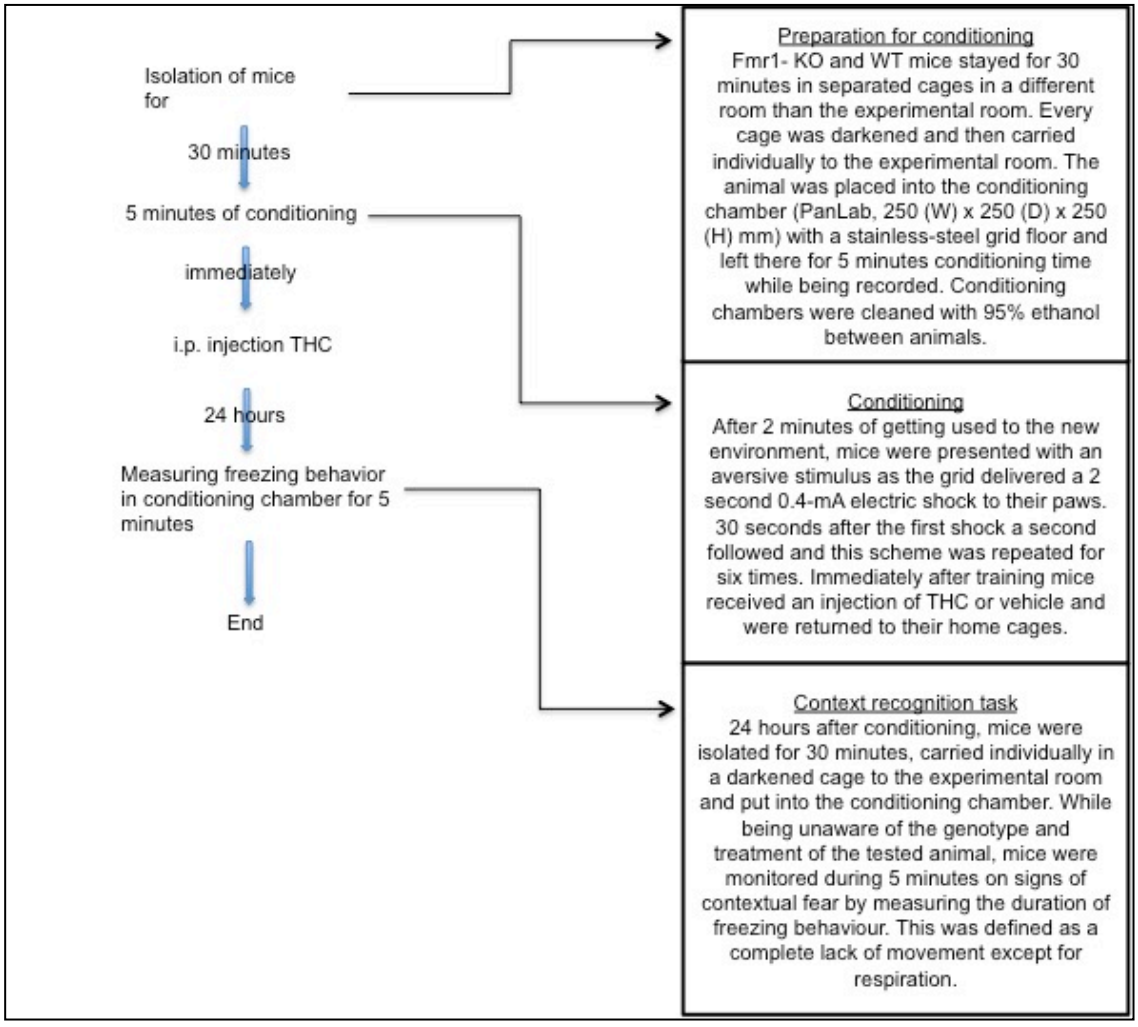


Figure 6: Test procedure of the context recognition task

Table 4: Number (N) of mice per genotype and treatment group that took part in context recognition task. Number of mice that were used for statistical analyzes is put in brackets in case of drop outs.

Test design of experiment 3		
Treatment	**WT**	**Fmr1- KO**
Veh	N= 5	N= 7 (6)
THC	N= 5	N= 8

2.4 Western blot

Western blotting was chronologically independent from behavioral testing. Mice used for experimental testing did not undergo any behavioral tasks and received pharmacological treatment as described above. Between injections a 5- minute gap left time for brain preparation. Mice were singly sacrificed by cervical dislocation, the head was directly divided from the body and opened for brain extraction. The whole brain was detached from the skull and immediately dissected in hippocampus, striatum, cortex and cerebellum according a modified dissection protocol (Spijker, 2011). Separated brain tissue was transferred to fresh sample tubes, directly frozen on dry ice and stored at -80°C until processing. Brain homogenates were prepared as described below (table 9) and employed for western blotting. Table 5 to 8 shows the test designs of western blots, based on N= 6 WT mice which were vehicle treated, N= 8 WT mice which were THC treated, N= 9 KO mice which were vehicle treated and N= 6 KO mice which were THC treated. The number of mice per genotype and treatment group differs, if mice were excluded from testing due to failure of the blot membrane (shown as "*", also in figure 7) or sample preparation errors (failure of protein assay, shown as "**"). If during western blot analyzes values for optical density differed extremely from others, they were accounted as statistical outliers (exemplary in figure 8).

Table 5: Number (N) of mice per genotype and treatment group, which were used for western blotting of hippocampus. In brackets number of mice used for statistical analyzes after exclusion of statistical outliers.

Test design of experiment 4 in hippocampus				
Kinase	**Treatment WT**		**Treatment KO**	
	Veh	THC	Veh	THC
(p)ERK	N= 6 (5)	N= 8 (7)	N= 9	N= 6
(p)AKT	N= 6	N= 8 (7)	N= 9	N= 6
(p)S6	N= 6 (5)	N= 8 (6)	N= 9 (7)	N= 6

Table 6: Number of mice per genotype and treatment group, which were used for western blotting of striatum. In brackets number of mice used for statistical analyzes after exclusion of statistical outliers.

Test design of experiment 4 in striatum				
Kinase	**Treatment WT**		**Treatment KO**	
	Veh	THC	Veh **	THC
(p)ERK	N= 6	N= 8 (7)	N= 8 (7)	N= 6 (5)
(p)AKT	N= 6 (5)	N= 8 (7)	N= 8	N= 6
(p)S6	N= 6 (4)	N= 8 (7)	N= 8	N= 6

Table 7: Number of mice per genotype and treatment group, which were used for western blotting of cortex. In brackets number of mice used for statistical analyzes after exclusion of statistical outliers.

Test design of experiment 4 in cortex				
Kinase	**Treatment WT**		**Treatment KO**	
	Veh	THC	Veh	THC
(p)ERK	N= 4 *	N= 6 * (5)	N= 6 * (5)	N= 6 (5)
(p)AKT	N= 6	N= 8 (7)	N= 9	N= 6
(p)S6	N= 6 (4)	N= 8 (6)	N= 9 (8)	N= 6

Table 8: Number of mice per genotype and treatment group, which were used for western blotting of cerebellum. In brackets number of mice used for statistical analyzes after exclusion of statistical outliers. (p)AKT detection in cerebellum is not presented due to experimentation errors.

Test design of experiment 4 in cerebellum				
Kinase	**Treatment WT**		**Treatmen KO**	
	Veh**	THC**	Veh**	THC
(p)ERK	N= 5	N= 7	N= 8	N= 6 (5)
(p)S6	N= 5	N= 7	N= 8	N= 6

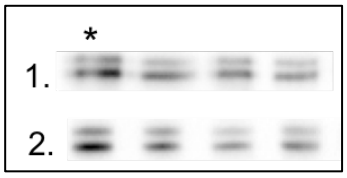


Figure 7: Exemplifies errors in western blot membrane (1., *) compared to an accurate one (2.) for detection of pERK in cortex. Errors of this kind result in deficient quantification of values for optical density and are therefore not evaluable.

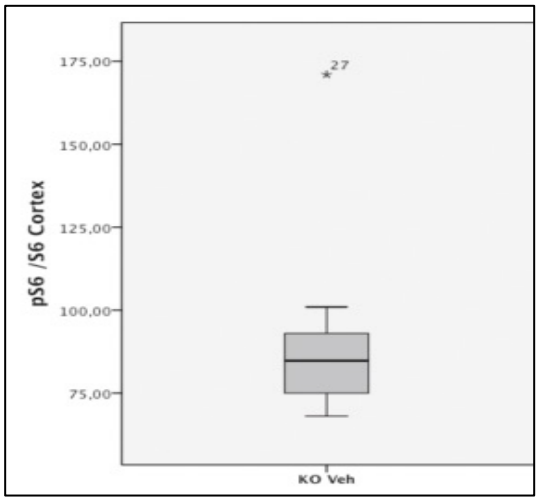


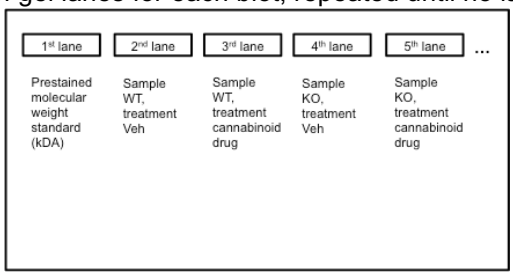
Figure 8: Exemplifies an outlier (* 27) of the western blot results, which was excluded from statistical analyzes.

2.4.1 Experiment 4: Western blot

Western blotting was used to visualize the activity status of mitogen- activated protein kinases Erk (ERK), proteinkinase B (AKT) and S6 ribosomal protein (S6) in the Fmr1-KO mouse with and without CB1 stimulation compared to their wild- type littermates. High levels of phosphorylated (p) targets were considered as activation of intraneuronal endocannabinoid signaling. The phosphorylation status of the selected CB1- targets was detected by quantification of optical density values of the emitted chemiluminescent signal by active phospho-specific and non phospho-specific antibodies. Values of phosphorylated targets were normalized to the values of unphosphorylated targets on the same blot.

Table 9: Procedure of western blotting

Preparation/ sample processing	- Defrosting samples on ice - Supplementing 500µl sample buffer (pH 7.6), protease (Roche, 1 pellet) and phosphatase inhibitors (NaF 2mM, against serine-threonine phosphatases) - Homogenizing with Precellys®24 homogenizer at 5000 rpm 2 times 30 seconds plus break of 10 seconds at 4°C - Centrifuging 10 min. at 10.000 rpm at 4°C (Eppendorf® centrifuge 5417 R) - Transfer supernatant into fresh tube - For protein assay: 50µl from each sample comparing to prepared albumin standard of 0µg/ml to 2µg/ml. 1ml bicinchoninic acid added as working reagent to each assay (chelating and coloring effects on sample proteins, induces a light absorption maximum at 562nm) Incubation in a water bath at 37°C for 30 min. Transfer to cuvettes for protein content determination with spectrophotometric analyzes (DU 700 Beckmann® Life Science UV/vis spectrophotometer) - Taking 300µg of protein from each sample and centrifuging for 10 minutes at 13.000rpm at 4°C (Eppendorf® centrifuge 5415 R) - Dropping supernatant, diluting pellet in 100µl laemmli sample buffer (removes secondary and tertiary structure of sample proteins and charges them negative with sodium dodecyl sulfate (SDS)) - Boiling samples at 95°C for 5 min - Cooling down and then freezing samples at minus 20°C until being used for western blotting
Western blotting	- Preparation of Tris- glycine 10% acrylamide gels (see addendum for protocol) in BioRad® Criterion midi format gel system. Components held clean with ethanol and distilled water - Transfere polymerized gel to a SDS-buffer (BioRad®)- filled running chamber - Carefully taking out spacers, washing wells with running buffer - Adding 10µl (equaling 30µg of protein) of each sample plus 5µl of prestained molecular weight standards for the first lane.

	- Order of gel lanes for each blot, repeated until no lane left:  - Running gel for about 1,5 hours at 125V. Negative charged SDS- covered proteins move to the cathode whereupon smaller proteins migrate faster and proteins are thus separated by size, usually measured in kilodaltons (kDa) - Assembling gel with ethanol activated PVDF membranes, Whatman- papers and sponges and submerging the assembly in cold transfer buffer containing Tris, glycine and ethanol - Transferring protein from gel to membrane for 2 hours at 250mA at 4°C in the blotting unit filled with transfer buffer - After blotting membranes were saturated for 30min in 5ml Tris buffered saline+ 0.1%Tween20 (TBS-T)+ 5% bovine serum albumin (BSA)
Immunodetection	- Proteins were immunodetected by incubating primary antibodies overnight at 4°C against - ERK (p44/42 MAPK, diluted 1:2000 in TBS-T 5% BSA) and pERK (phospho-p44/MAPK Thr202/Tyr204, diluted 1:1000 in TBS-T 5% BSA) - AKT (AKT, diluted 1:1000 in TBS-T 5% BSA) and pAKT (phospho-AKT Ser 473, diluted 1:2000 in TBS-T 5% BSA) - S6 (S6 ribosomal protein, diluted 1:1000 in TBS-T 5% BSA) and pS6 (phosphor-S6 ribosomal protein Ser240/244, diluted 1:1000 in TBS-T 5% BSA). All antibodies were obtained from Cell Signaling Technology - The next morning membranes were washed 6 times for 5 minutes in TBS-T and re-incubated for 60 minutes at room temperature with a secondary horseradish peroxidase (HRP)-conjugated antibody, diluted in 1:3000 in 5 ml TBS-T 5% BSA - Adding ultra-sensitive enhanced chemiluminescent agent as substrate for the HPR- signaling just before - visualization of relevant immunoreactive bands on a ChemiDoc XRS System (BioRad) controlled by The Quantity One software v4.6.3 (BioRad). - Relevant bands for (p)ERK were found at 44 and 42 kDa (two isoforms of ERK), for (p)AKT at 60 kDa and for (p)S6 at 32 kDa. - Immunoblots were processed with Adobe Photoshop 7.0. For quantitative purposes - Optical density values of active phospho-specific antibodies were normalized to the optical density of active nonphospho-specific antibodies. Normalized values were used for statistical analyzes.

2.5 Statistical methods

In experiments with two between subject factors, genotype (WT or KO) or pre- treatment (Veh or Rim) and drug treatment (cannabinoid drug or vehicle) two-way ANOVA was used to analyze all data. Results were considered to be significant at $p \leq 0.05$. A trend has been taken into account at $p \leq 0.06$. If results exhibited a significant interaction of the two between subject factors or were displaying a trend, Bonferroni's post hoc test was used and significance was pictured accordingly in all figures as "*" for $p<0.05$, main effects are pictured with brackets. Statistical analyzes were carried out using GraphPad Prism Version 5.04/d, data are presented as mean ± SEM (Bénard, 2012). If values deviated extremely from the rest of the samples, outliers were identified at arithmetic mean of all values in one group ± two times standard deviation and excluded.

3 Results

This chapter shows the statistical results of behavioral and experimental tests.

3.1 Behavioral tests

The statistical results of the behavior tests include the results of experiment 1 (tetrad and social interaction task), experiment 2 (Social interaction task with pre- treatment of a CB1- antagonist) and experiment 3 (context recognition task).

3.1.1 Experiment 1: Tetrad experiment and social interaction task

In the tetrad experiment mice were tested on the effectiveness of cannabinoid drugs on the Fmr1- KO mouse model by measuring typical acute cannabinoid- induced behavior using two different cannabinoid drugs. These effects include hypothermia, hypolocomotion, catalepsy and analgesia.

3.1.1.1 Influence of THC on Hypothermia

Treatment had a significant effect on body temperature change {F(1,27)= 17.72; p= 0.003}. Mice that were treated with THC exhibited a stronger decrease in body temperature compared to vehicle treated mice. Genotype did not have a significant effect on body temperature change {F(1,27)= 0.4; p= 0.53}. Interaction (genotype vs. treatment) did almost have a significant effect on body temperature change {F(1,27)= 4.02; p= 0.055}, figure 9.

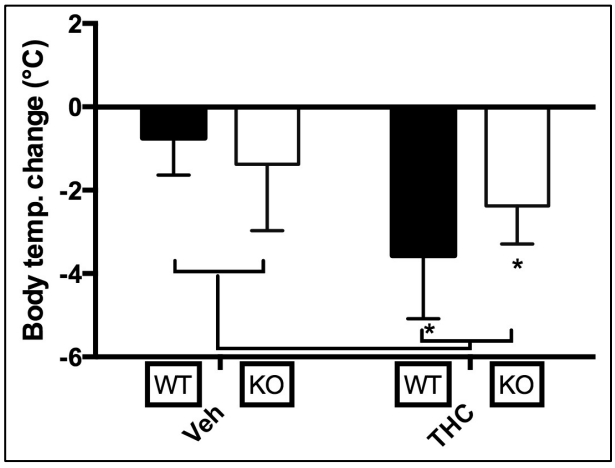


Figure 9: Effect of THC compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on body temperature decrease. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.2 Influence of WIN55,212 on Hypothermia

Treatment had a significant effect on body temperature change {F(1,27)= 53.55; p< 0.0001}. Mice that were treated with WIN55,212 exhibited a decrease in body temperature compared to vehicle treated mice. Genotype did not have a significant effect on body temperature change {F(1,27)= 2.34; p= 0.14}. Interaction (genotype vs. treatment) did not have a significant effect on body temperature change {F(1,27)= 1.91; p= 0.18}, figure 10.

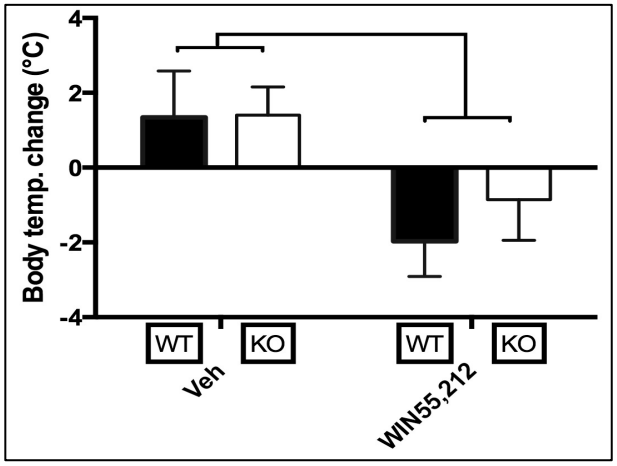


Figure 10: Effect of WIN55,212 compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on body temperature decrease. Significance for main effects are pictured with brackets.

3.1.1.3 Influence of THC on Hypolocomotion

Treatment had a significant effect on distance moved {F(1,27)= 114; p<0.0001}. Mice that were treated with THC moved less compared to vehicle treated mice. Genotype did not have a significant effect on distance moved {F(1,27)= 3.26; p= 0.08}. Interaction (genotype vs. treatment) had a significant effect on distance moved {F(1,27)= 4.97; p= 0.03 and subsequent post-hoc test}, figure 11.

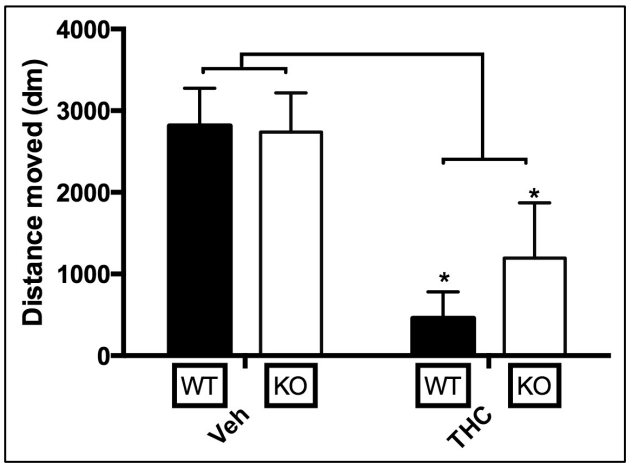


Figure 11: Effect of THC compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on the distance moved in an open field. Significance is shown as "*" for interaction effects and subsequent post-hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.4 Influence of WIN55,212 on Hypolocomotion

Treatment had a significant effect on distance moved {F(1,27)= 55.06; p< 0.0001}. Mice that were treated with WIN55,212 moved less compared to vehicle treated mice. Genotype did not have a significant effect on distance moved {F(1,27)= 2.07; p= 0.16}. Interaction (genotype vs. treatment) had a significant effect on distance moved {F(1,27)= 5.43; p= 0.03 and subsequent post-hoc test}, figure 12.

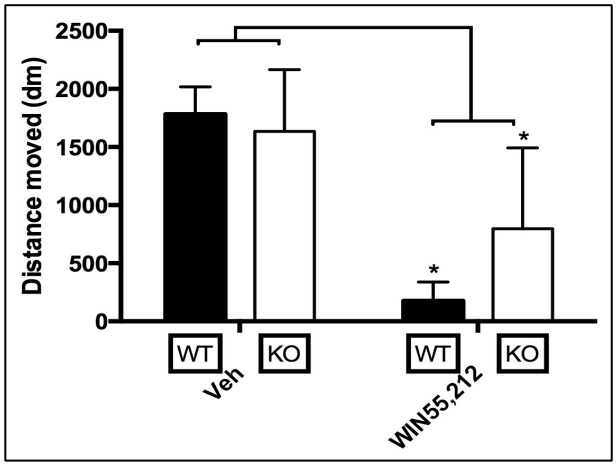


Figure 12: Effect of WIN55,212 compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on the distance moved in an open field. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.5 Influence of THC on Catalepsy

Treatment had a significant effect on catalepsy bar latency {F(1,21)= 56.24; p<0.0001}. Mice that were treated with THC stood longer on the catalepsy bar compared to vehicle treated mice. Genotype had a significant effect on catalepsy bar latency {F(1,21)= 6.82; p= 0.016}. WT mice stood longer on the catalepsy bar than KO mice. Interaction (genotype vs. treatment) had a significant effect on catalepsy bar latency {F(1,21)= 6.82; p= 0.016 and subsequent post-hoc test}, figure 13.

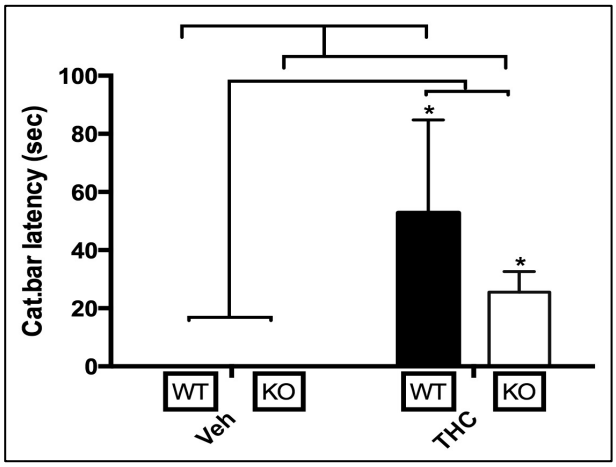


Figure 13: Effect of THC compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on the descent latency from a 1cm diameter bar. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.6 Influence of WIN55,212 on Catalepsy

Treatment had a significant effect on catalepsy bar latency {F(1,27)= 58.99; p< 0.0001}. Mice that were treated with WIN55,212 stood longer on the catalepsy bar compared to vehicle treated mice. Genotype had a significant effect on catalepsy bar latency {F(1,27)= 29.81; p< 0.0001}. WT mice stood longer on the catalepsy bar than KO mice. Interaction (genotype vs. treatment) had a significant effect on catalepsy bar latency {F(1,27)= 30.64; p< 0.0001 and subsequent post-hoc test}, figure 14.

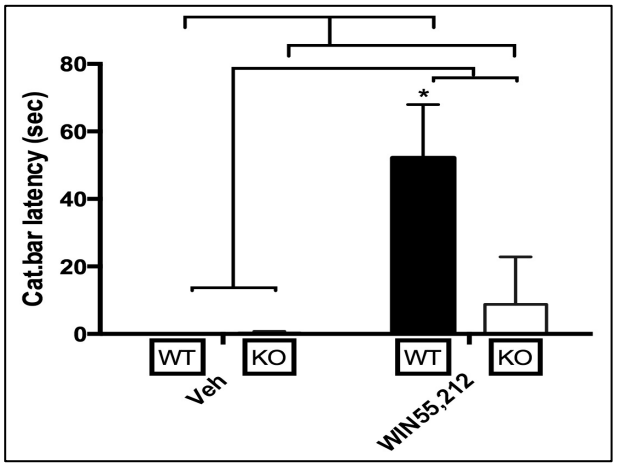


Figure 14: Effect of WIN55,212 compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on the descent latency from a 1cm diameter bar. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects is pictured with brackets.

3.1.1.7 Influence of THC on Analgesia

Treatment had a significant effect on latency to paw reaction {F(1,27)= 84.46; p<0.0001}. Mice that were treated with THC stood longer on the hot plate compared to vehicle treated mice. Genotype did not have a significant effect on latency to paw reaction {F(1,27)= 1.39; 0.25}. Interaction (genotype vs. treatment) had a significant effect on latency to paw reaction {F(1,27)= 5.95; p= 0.02 and subsequent post-hoc test}, figure 15.

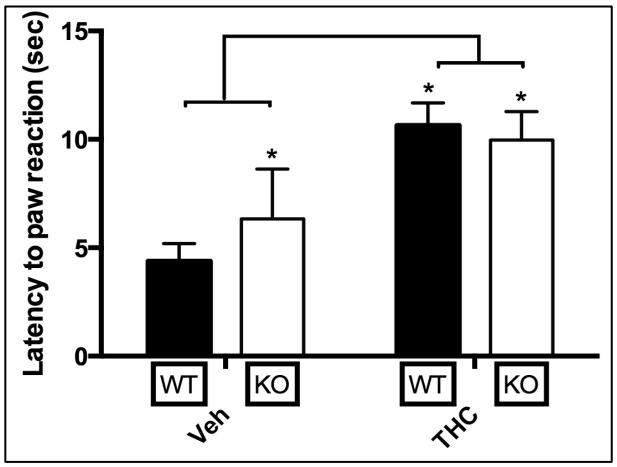


Figure 15: Effect of THC compared to vehicle on wild- type (WT) ■ and knock- out (KO)- □ mice on latency of expressing discomfort due to a painful stimulus. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.8 Influence of WIN55,212 on Analgesia

Treatment had a significant effect on latency to paw reaction {F(1,27)= 152.70; p< 0.0001}. Mice that were treated with WIN55,212 stood longer on the hot plate compared to vehicle treated mice. Genotype had a significant effect on latency to paw reaction {F(1,27)= 38.09; p< 0.0001}. WT mice stood longer on the hot plate than KO mice. Interaction (genotype vs. treatment) had a significant effect on latency to paw reaction {F(1,27)= 27.73; p< 0.0001 and subsequent post-hoc test}, figure 16.

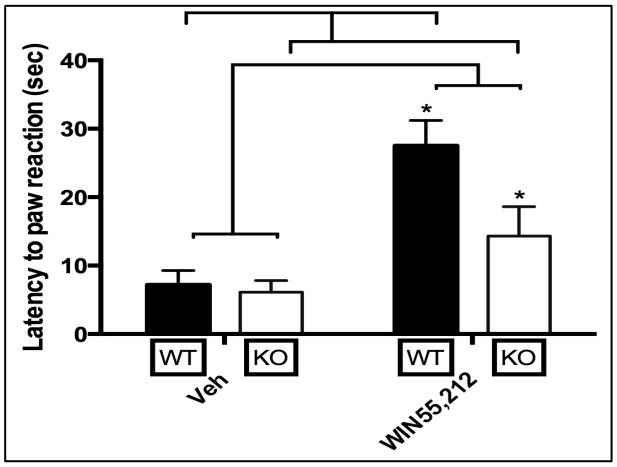


Figure 16: Effect of WIN55,212 compared to vehicle on wild- type (WT) ■ and knock- out (KO) □ - mice on latency of expressing discomfort due to a painful stimulus. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.1.1.9 Social interaction task 10 days after THC- injection

In the social interaction task mice from the tetrad experiment that received THC treatment were tested on long- term effects of THC treatment on deficits in social interaction 10 and 20 days after the tetrad experiment. Social interaction parameters were measured in time mice spent to nose-, body- and anogenital sniff the partner as well as allogrooming and traversing the partners body by crawling over and under it from one side to another. These parameters were observed in video recordings from the experiment and summarised as time mice spend in affiliative behavior.

Treatment did not have a significant effect on affiliative behavior {F(1,22)= 0.32; p= 0.58} Genotype did not have a significant effect on affiliative behavior {F(1,22)= 0.7; p= 0.42}. Interaction (genotype vs. treatment) had a significant effect on affiliative behavior {F(1,22)= 9.87; p= 0.005 and subsequent post-hoc test}, figure 17.

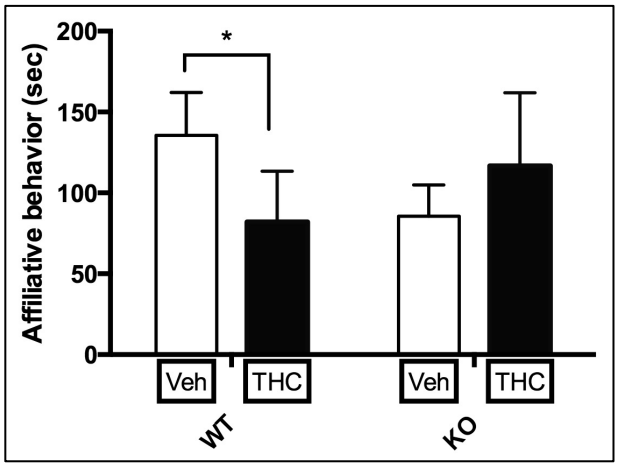


Figure 17: Effect of THC ■ compared to vehicle (Veh) □ on wild- type (WT) and knock- out (KO)- mice on the time spend in affiliative behavior with a female partner. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05.

3.1.1.10 Social interaction task 20 days after THC- injection

Treatment did not have a significant effect on affiliative behavior {F(1,22)= 0.56; p= 0.46}. Genotype did not have a significant effect on affiliative behavior {F(1,22)= 0.02; p= 0.88}. Interaction (genotype vs. treatment) did not have a significant effect on affiliative behavior {F(1,22)= 1,82; p= 0.19}, figure 18.

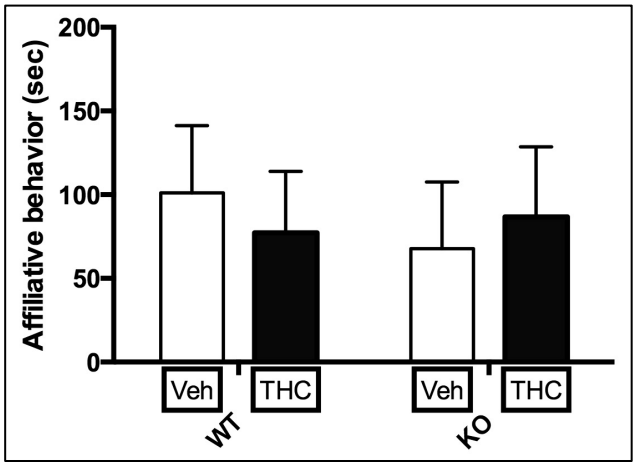


Figure 18: Effect of THC ■ compared to vehicle (Veh) □ on wild- type (WT) and knock- out (KO)- mice on the time spend sniffing the female.

3.1.2 Experiment 2: Social interaction task with pre- treatment of a CB1- antagonist

To reproduce the effect in WT mice from the social interaction task 10 days after THC treatment and to verify its dependency on CB1, purchased WT mice from the B6 background were pre- treated with either vehicle or Rimonabant and 30 minutes later additionally with THC or vehicle. Testing was done 10 days after injection, the same parameters as afore were analyzed for the first 3 and for the whole 6 minutes of social interaction testing.

3.1.2.1 First 3 minutes of experiment

Pre-treatment (Veh, Rim) did not have a significant effect on affiliative behavior in the first 3 minutes of experiment {F(1,32)= 1.418; p= 0.24}. Treatment (Veh, THC) did not have a significant effect on affiliative behavior in the first 3 minutes of experiment {F(1,32)= 0.35; p= 0.56}. Interaction (pre-treatment vs. treatment) had a significant effect on affiliative behavior in the first 3 minutes of experiment {F(1,32)= 9.70; p= 0.004 and subsequent post- hoc test}, figure 19.

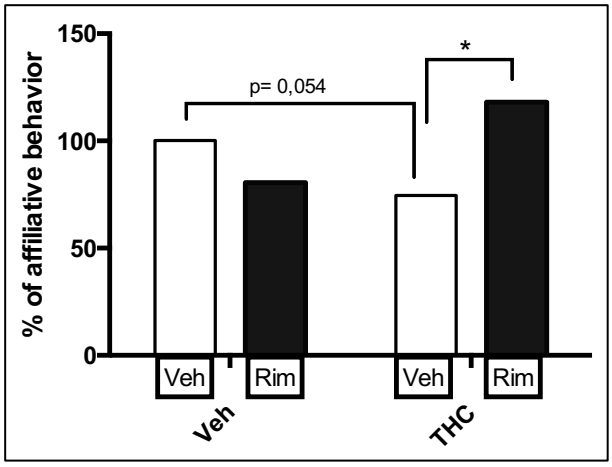


Figure 19: Effect of pre- treatment (Rim) ■ compared to pre- treatment of vehicle (Veh) □ on THC- or vehicle treated in wild- type mice on the percentage of affiliative behavior in the first 3 minutes of the social interaction task. The control group (Veh/ Veh) is shown as 100%. Significance is shown as "*" for p< 0.05. A trend has been taken into account at p≤ 0.06.

3.1.2.2 6 minutes of experiment

Pre-treatment (Veh, THC) did not have a significant effect on affiliative behavior {F(1,32)= 2.23; p= 0.15}. Treatment (Veh, RIM) did not have a significant effect on affiliative behavior {F(1,32)= 0.13; p= 0.72.}. Interaction (pre-treatment vs. treatment) did not have a significant effect on affiliative behavior {F(1,32)= 0.36; p= 0.55}, figure 20.

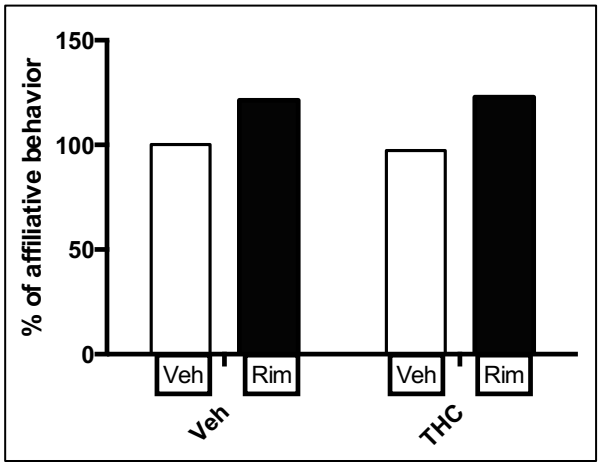


Figure 20: Effect of pre- treatment (Rim) ■ compared to pre- treatment of vehicle (Veh) □ on THC- or vehicle treated in wild- type mice on the percentage of affiliative behavior in the first 3 minutes of the social interaction task. The control group (Veh/ Veh) is shown as 100%.

3.1.3 Experiment 3: Context recognition task

In the context recognition task mice were tested on acute effects of THC treatment on deficits in associative learning in the Fmr1- KO mouse model. In this experiment mice learn to fear a new environment because of its temporal association with an aversive stimulus (foot shock). Directly after training mice received a single THC- injection that is known to have an amnesic- like effects in this context. 24 hours after conditioning mice were replaced into the conditioning chamber, the time of freezing (no movement apart from breathing) was measured and regarded as contextual fear (Kim and Jung 2006). Additionally, mice were analyzed for moving (all activities except grooming) to explore further behavioral aspects in this experiment.

3.1.3.1 Effect of THC on freezing

Treatment did not have a significant effect on freezing {F(1,20)= 2.32; p= 0.14}. Genotype did not have a significant effect on freezing {F(1,20)= 2.02; p= 0.17}. Interaction (treatment vs. genotype) had a significant effect on freezing {F(1,20)= 4.74; p= 0.04 and subsequent post- hoc test}, figure 21.

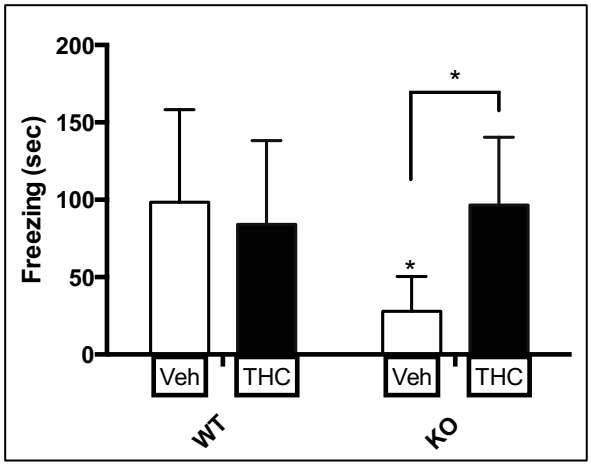


Figure 21: Effect of THC ■ compared to vehicle (Veh) □ on wild- type (WT) and knock- out (KO)- mice on contextual freezing behavior. Significance is shown as "*" for p< 0.05.

3.1.3.2 Effect of THC on moving

Treatment did almost have a significant effect on moving {F(1,21)= 3.92; p= 0.061}. Genotype did not have a significant effect on moving {F(1,21)= 1.49; p= 0.24}. Interaction (treatment vs. genotype) had a significant effect on moving {F(1,21)= 5.51; p= 0.029 and subsequent post- hoc test}, figure 22.

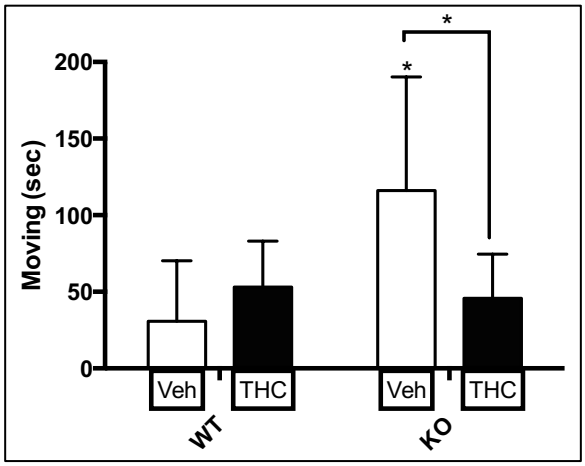


Figure 22: Effect of THC ■ compared to vehicle (Veh) □ on wild- type and knock- out mice on contextual moving behavior. Significance is shown as "*" for p< 0.05.

3.2 Experiment 4: Western blot

The effect of THC administration to Fmr1- KO an WT mice was analyzed through phosphorylation levels of MAP kinase Erk (ERK), proteinkinase B (AKT) and S6 ribosomal protein (S6) in hippocampus, striatum, cortex and cerebellum. The levels of these phosphorylated (p) forms of protein are known to correlate with their activation and the ECS signaling status. The statistical results of these experimental tests are shown below.

3.2.1 Effect of THC treatment on kinase- phosphorylation in hippocampus

THC treatment had a significant effect on pERK levels in hippocampus {F(1,23)= 6.44; p= 0.02}. Genotype did not have a significant effect on pERK levels {F(1,23)= 0.017; p= 0.9}. Interaction (genotype vs. treatment) did not have a significant effect on pERK levels {F(1,23)= 1.91; p= 0.18}, figure 23.

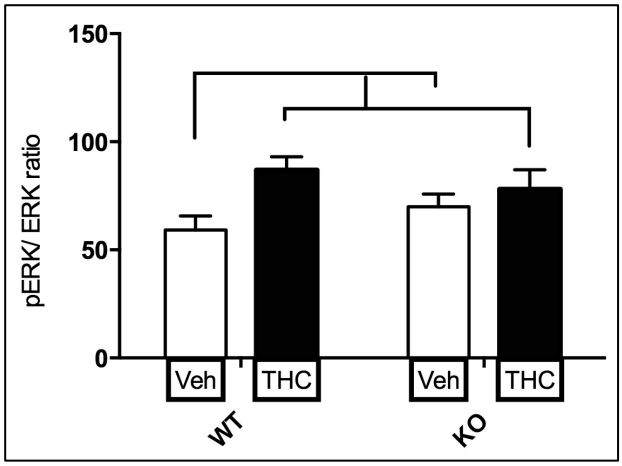


Figure 23: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of ERK- phosphorylation in hippocampus. Main effects are pictured with brackets.

THC treatment had a significant effect on pAKT levels in hippocampus {F(1,24)= 5.26; p= 0.03}. Genotype did not have a significant effect on pAKT levels {F(1,24)= 0.20; p= 0.7}. Interaction (genotype vs. treatment) did not have a significant effect on pAKT levels {F(1,24)= 0.86; p= 0.36}, figure 23.

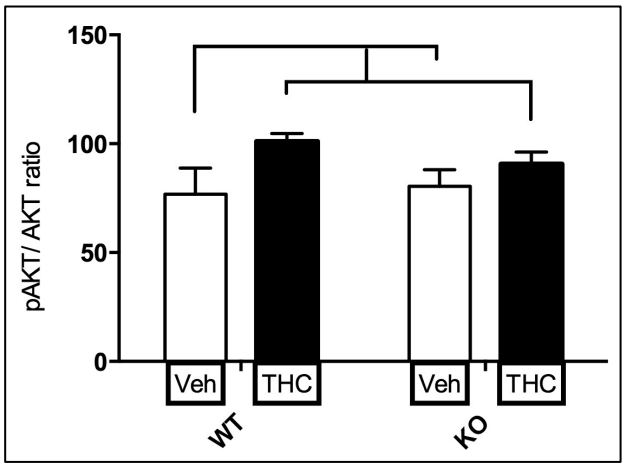


Figure 24: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of AKT- phosphorylation in hippocampus. Main effects are pictured with brackets.

THC treatment almost had a significant effect on pS6 levels in hippocampus {F(1,20)= 4.15; 0.055}. Genotype did not have a significant effect on pS6 levels {F(1,20)= 1.85; p= 0.19}. Interaction (genotype vs. treatment) did not have a significant effect on pS6 levels {F(1,20)= 1.58; p= 0.22}, figure 25.

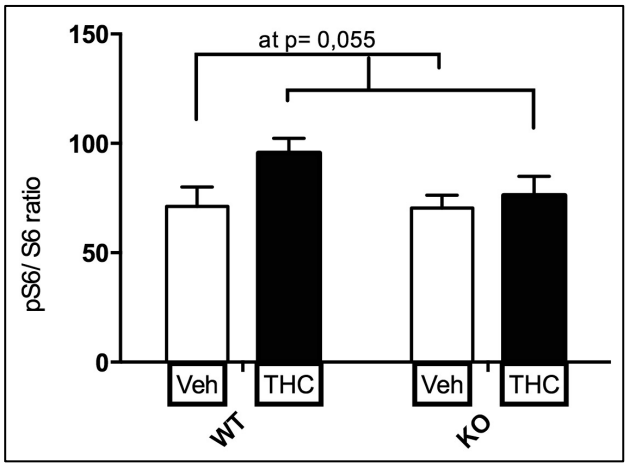


Figure 25: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of S6- phosphorylation in hippocampus. Trend of a main effect is pictured with brackets.

3.2.2 Effect of THC treatment on kinase- phosphorylation in striatum

In the striatum THC treatment had a significant effect on pERK levels {F(1,21)= 5.11; p= 0.04}. Genotype did not have a significant effect on pERK levels {F(1,21)= 1.77; p= 0.2}. Interaction (genotype vs. treatment) had a significant effect on pERK levels {F(1,21)= 6.99; p= 0.02 and subsequent post-hoc test}, figure 26.

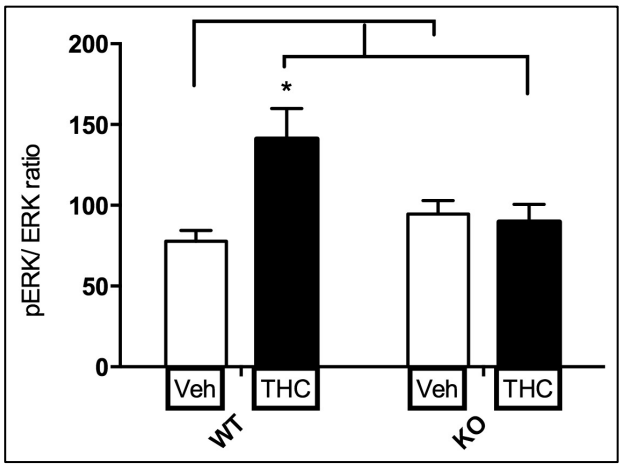


Figure 26: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of ERK- phosphorylation in striatum. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

In the striatum THC treatment did not have a significant effect on pAKT levels {F(1,22)= 0.34; p= 0.6}. Genotype did not have a significant effect on pAKT levels {F(1,22)= 1.31; p= 0.26}. Interaction (genotype vs. treatment) did not have a significant effect on pAKT levels but a trend is apparent {F(1,22)= 3.71; p= 0.06 }, figure 27.

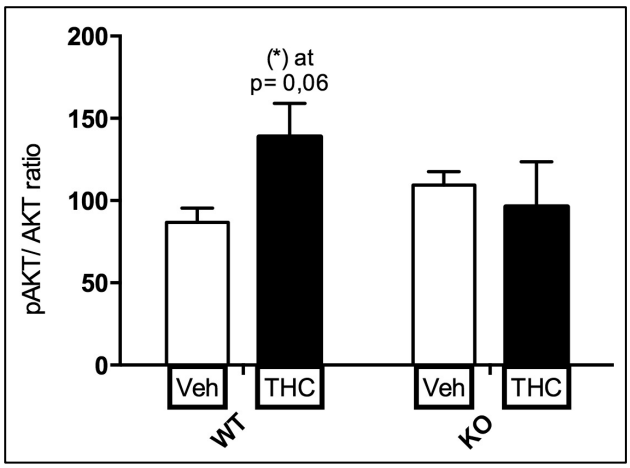


Figure 27: Effect of THC treatment ■ compared to vehicle treatment (Veh) ☐ on wild- type (WT) and knock- out (KO) mice on levels of AKT- phosphorylation in striatum. The trend of an interaction effect and subsequent post- hoc test at p= 0.06 is shown as (*).

In the striatum THC treatment had a significant effect on pS6 levels {F(1,21)= 6.76; p= 0.02}. Genotype did not have a significant effect on pS6 levels {F(1,21)= 3.26; p= 0.09}. Interaction (genotype vs. treatment) had a significant effect on pS6 levels {F(1,21)= 5.15; p= 0.03 and subsequent post-hoc test}, figure 28.

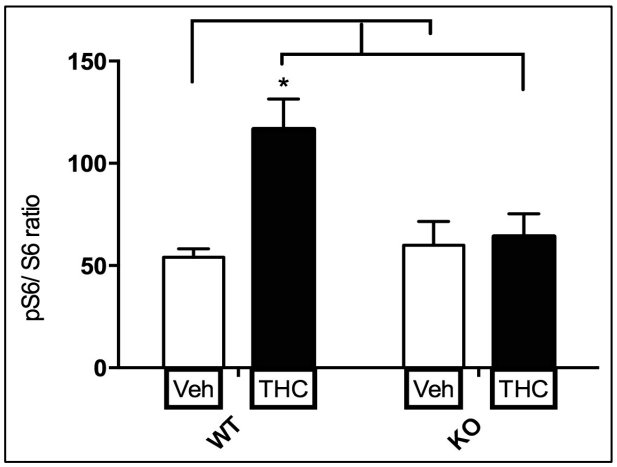


Figure 28: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of S6- phosphorylation in striatum. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

3.2.3 Effect of THC treatment on kinase- phosphorylation in cortex

In the cortex THC treatment did not have a significant effect on pERK levels {F(1,15)= 0.22; p= 0.64}. Genotype did not have a significant effect on pERK levels {F(1,15)= 0.33; p= 0.57}. Interaction (genotype vs. treatment) did not have a significant effect on pERK levels {F(1,15)= 1.09; p= 0.31}, figure 29.

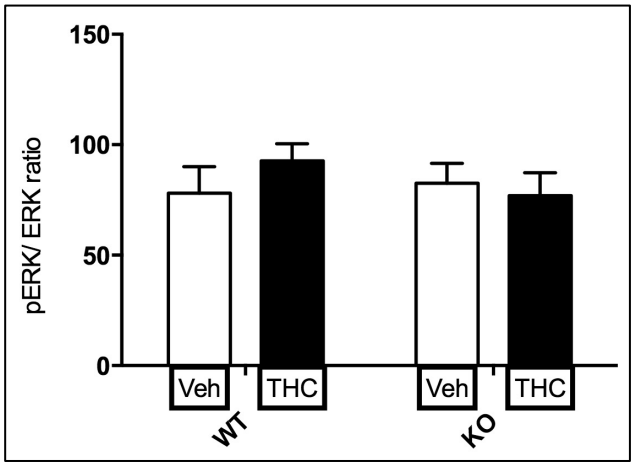


Figure 29: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of ERK phosphorylation in cortex.

In the cortex THC treatment did not have a significant effect on pAKT levels {F(1,24)= 0.60; p= 0.44}. Genotype did not have a significant effect on pAKT levels {F(1,24)= 0.49; p= 0.49}. Interaction (genotype vs. treatment) had a significant effect on pAKT levels {F(1,24)= 7.64, p= 0.01 and subsequent post- hoc test}, figure 30.

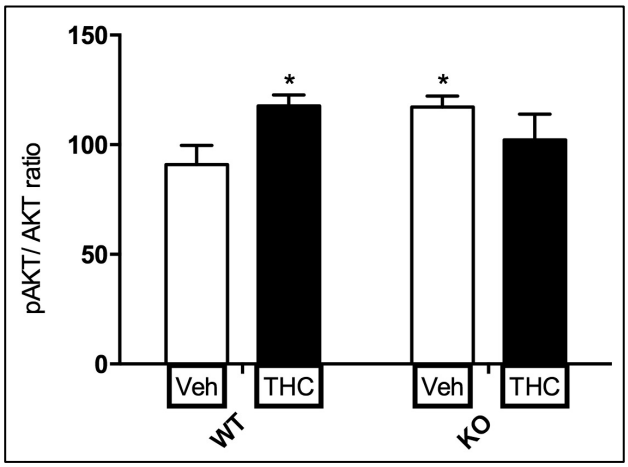


Figure 30: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on AKT phosphorylation in cortex. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05.

In the cortex THC treatment had a significant effect on pS6 levels {F(1,20)= 4.48; p= 0.05}. Genotype did not have a significant effect on pS6 levels {F(1,20)= 2.1; p= 0.16}. Interaction (genotype vs. treatment) did not have a significant effect on pS6 levels {F(1,20)= 1.48; p= 0.24}, figure 31

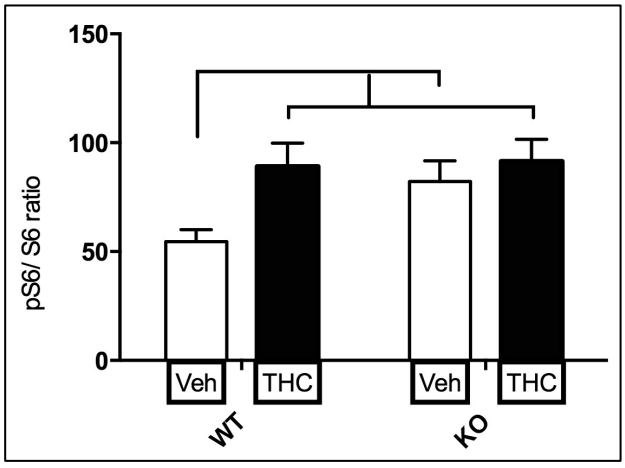


Figure 31: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of S6- phosphorylation in cortex. Main effects are pictured with brackets.

3.2.4 Effect of THC treatment on kinase- phosphorylation in cerebellum

In the cerebellum THC treatment had a significant effect on pERK levels {F(1,21)=18.15; p= 0.0003}. Genotype had a significant effect on pERK levels {F(1,21)= 12.18; p= 0.002}. Interaction (genotype vs. treatment) had a significant effect on pERK levels {F(1,21)= 27.62; p <0.0001 and subsequent post- hoc test}, figure 32.

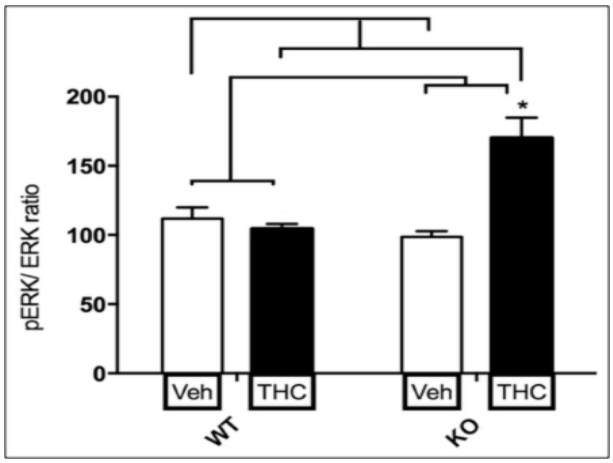


Figure 32: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of ERK- phosphorylation in the cerebellum. Significance is shown as "*" for interaction effects and subsequent post- hoc test at p< 0.05, main effects are pictured with brackets.

In the cerebellum THC treatment did not have a significant effect on pS6 levels {F(1,22)= 0.37; p= 1,49}. Genotype did not have a significant effect on pS6 levels {F(1,22)= 2.06; p= 0.16}. Interaction (genotype vs. treatment) did not have a significant effect on pS6 levels {F(1,22)= 1.11; p= 0.3}, figure 33.

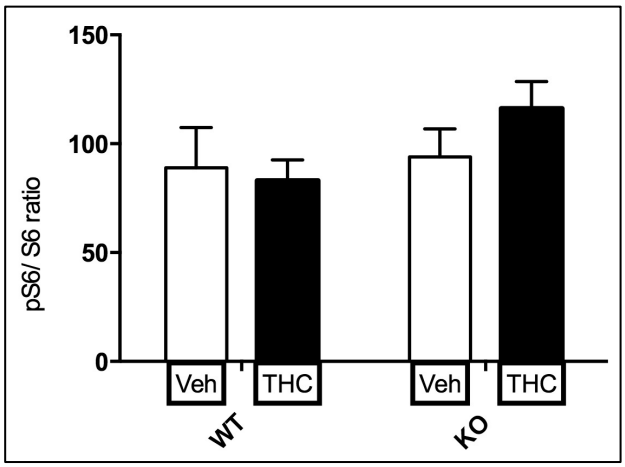


Figure 33: Effect of THC treatment ■ compared to vehicle treatment (Veh) □ on wild- type (WT) and knock- out (KO) mice on levels of S6- phosphorylation in the cerebellum.

4 Discussion

Aim of this study was to develop an understanding of the functionality of the endocannabinoid system in the Fmr1- KO mouse model as well as to represent the influence of cannabinoid treatment on its autistic behavior. This was accomplished using Fmr1- KO mice from the B6 background and testing them for immediate and long- term effects of cannabinoid treatment as well as for effects of THC treatment on specific autistic behavior. Finally the influence of THC treatment on kinase activation in different brain regions of the Fmr1- KO mouse, which are connected to autism and are high in CB1 expression was measured with western blot.

4.1 Behavioral tests

The discussion concerning results from behavioral tests include the results of experiment 1 (tetrad and social interaction task), experiment 2 (Social interaction task with pre- treatment of a CB1- antagonist) and experiment 3 (context recognition task).

4.1.1 Fmr1 KO mice display a desensitized endocannabinoid system

WIN55,212 or THC treated mice showed a significant decrease in their body temperatures compared to vehicle treated mice and the genotype had no significant influence on body temperature in either experiment (figures 9 and 10). THC treated WT and KO mice showed a significant decrease in body temperature compared to vehicle treated WT mice. This interaction effect was almost significant if mice were treated with WIN55,212. Body temperature loss after cannabinoid treatment could be due to peripheral cannabinoid receptors that have been shown to play a role in the regulation of energy homeostasis via lipogenic mechanisms (Cota et al., 2003) as well as to exhibit a normal responsiveness to pharmacological inhibition in Fmr1- KO mice (Pietropaolo, 2010).

Tested locomotion (figures 11 and 12) showed that mice move significantly less after THC or WIN55,212- injection compared to vehicle treated mice. Comparing WT and KO mice treated with THC or WIN55,212 revealed that hypolocomotion as a cannabimimetic effect is significantly less distinctive in Fmr1- KO mice.

The catalepsy part of the experiment (figures 13 and 14) revealed a significant cataleptic condition after THC or WIN55,212 treatment in mice. As for hypolocomotion, the cannabimimetic effect was significantly less distinctive in KO mice than in WT mice.

Measured analgesia (figures 15 and 16) exhibited a significant latency of moving the paws away from the hurting stimulus in THC or WIN55,212 treated mice compared to mice that received vehicle treatment. THC or WIN55,212 treated WT and KO mice mice stayed significantly longer on the hot plate than vehicle treated WT mice. Again the effect was less distinctive in KO mice, especially if mice were treated with WIN55,212.

Taken together the first results of the tetrad experiment revealed an impaired occurrence of typical immediate effects of cannabinoid treatment in the Fmr1- KO mouse. 30 minutes after cannabinoid treatment, KO mice showed significantly less signs of cannabinoid- induced behavior such as hypolocomotion, catalepsy and analgesia compared to their wild- type littermates. These results can be interpreted as desensitization to cannabinoids and it strongly suggests an alteration of the functionality of the ECS in the Fmr1- KO mouse model. In reference to the "mGluR theory of fragile X", a possible explanation for its altered functionality could be the neuronal increase of mGluR- activity that leads to an oversupply of endocannabinoids by augmenting their synthesis (Varma et al., 2001; Bear et al., 2004) and therefore to a general desensitization to cannabinoids. In fact, electrophysiological research could show that the ability of mGluR to mobilize endocannabinoids is heightened in the CA1 region of the hippocampus in Fmr1- KO animals from a B6 background (Zhang and Alger, 2010).

Further, it was shown that one of the affected glutamate receptors in the Fmr1- KO mouse is the metabotropic glutamate receptor 5, mGluR5 (Maccarrone et al., 2010). This receptor activates a diacylglycerol lipase (DGL/ DAGL), the main responsible enzyme for the synthesis of the endocannabinoid 2-AG (Varma et al., 2001; Maccarrone et al., 2008; Kano et al., 2009), illustrated in figures 1 and 34. The absence of the Fragile X Mental Retardation Protein (FMRP) results in heightened intracellular signaling of mGluR5 (Bear et al., 2004; Bear, 2005; Dölen et al., 2007; Michalon et al., 2012) and as a consequence into constant 2-AG production. Also, a local overproduction of 2- AG due to the loss of FMRP in hippocampal neurons cultured from Fmr1- KO mice has been suggested (Straiker et al., 2013).

A permanent oversupply of endocannabinoids could lead to an altered ECS signaling and a modified drug effect of cannabinoid drugs because of a diminishing supply of CB1 receptors in the brain of the Fmr1- KO mouse as a result of decreased receptor synthesis or receptor mediated endocytosis as recognized mechanisms in the down-regulation of transmembrane signal transduction (Tsao et al., 2001).

Further, a decreased CB1 synthesis might be due to the absence of FMRP, since FMRP is an important factor in protein synthesis and transport as well as cell mem-

brane assembly in neurons. Contradictory, different work found no decrease in protein levels of CB1 in western blots (Zhang and Alger, 2010).

Furthermore, a defective ECS signaling could come into being by a frequent and uncoordinated surplus of endocannabinoids in brains of Fmr1-KO mice that results in an uncoordinated activation of intracellular CB1 pathway components because of an uncoordinated CB1 stimulation. This could lead to modification of intracellular CB1- signaling as well as to an insensitivity to externally applied CB1 agonists like THC or WIN55,212. The hypothesis of an altered intracellular pathway component activation status in the Fmr1- KO mouse model and its stimulatability will be discussed in chapter 4.2.

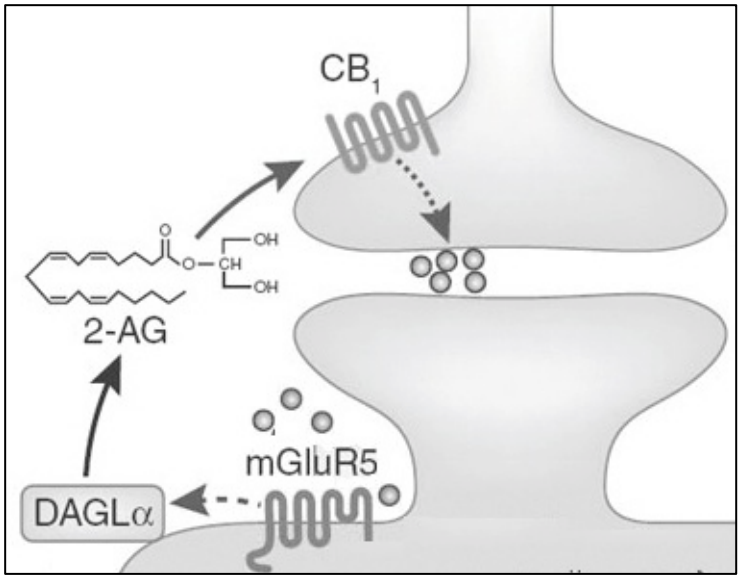


Figure 34: mGluR5 signaling, adapted from Di Marzo (2010). The figure shows 2-AG synthesis via mGluR5 activation followed by CB1 stimulation. mGluR5= metabotropic glutamate receptor 5, DAGLalpha= diacylglycerol lipase alpha, 2-AG= 2- Arachidonylglycerol, CB1= cannabinoid receptor 1.

4.1.2 THC possibly restores impairment in social interaction in Fmr1- KO mice

10 days after the tetrad experiment, THC or vehicle treated mice were used for social interaction testing. In this experiment, WT mice that had been treated with THC 10 days prior to testing spent significantly less time sniffing an unknown female partner than vehicle treated WT mice (figure 17). This effect has not been described in literature yet. It shows that THC treatment decreases the interest for social interaction in tested WT mice and possibly indicates a long lasting anxiogenic effect of THC. This could be due to still active metabolites of THC since it is known that a single dose of THC may result in detectable active metabolites in urine for up to 12 days (Law et al., 1984; Schwartz et al., 1985). Cannabinoid treatment has already been considered to possess some anxiogenic potency by other data that demonstrate conditioned anxiogenic effects in rats 24 hours after cannabinoid treatment (Genn et al., 2004).
However KO mice from this experiment show an inversed behavior. When treated with THC 10 days prior to testing, they spent more time sniffing an unknown female partner than mice that have been treated with vehicle. Although this effect was not significant it might suggest a positive effect of THC treatment on stimulating social interest in KO mice. This is especially noteworthy since this mouse model is amongst others characterized by a reduction in social interest (Pietropaolo et al., 2011). Treated with THC, KO mice almost behaved like vehicle treated WT mice and spent distinctly more time sniffing an unknown female partner than WT mice that had been treated with THC. The effect of THC on KO mice suggests, that THC possibly restores the phenotype in the Fmr1- KO mouse. The effect vanishes 20 days after the injection (figure 18).
In conclusion these results are in good agreement with the previous tetrad experiment as they also show an altered manner of functioning of the ECS in the Fmr1- KO mouse. Additionally, these results might exhibit a restoring character of cannabinoid treatment concerning the autistic core symptom of impaired social interaction. It is therefore also noteworthy that, by implication of the cannabinoid potency to restore a symptom of the autistic phenotype, an involvement of the ECS in the development of this phenotype is conceivable. Further it can be said that medical use of CB1- stimulation could modulate social behavior. In fact there are some studies suggesting cannabinoid treatment to help people overcome social anxiety (Rog et al., 2005; Frank et al., 2008; Narang et al., 2008; Skrabek et al., 2008; Bergamaschi et al., 2011). Still, these trials must be seen critically since some were judged as possibly highly biased in a systematic review and meta- analyzes of cannabinoids for medical use (Whiting et al., 2015). Further, cannabinoid treatment applied in these trials consisted mainly in the

use of THC plus cannabidiol (CBD) or only CBD. CBD is known for its anxiolytic, spasmolytic, antiemetic, anti-inflammatory and suggested antipsychotic effects (Mechoulam et al., 2007; Waldo Zuardi et al., 2012). Unlike THC, CBD has only a minor effect on CB1 (Pertwee, 2004), and shows a greater involvement in other neuronal mechanisms (Micale et al., 2013) including the activation of the vanilloid-receptor like 1, also known as transient receptor potential V2, TRPV2 (Pertwee, 2006; Qin et al., 2008). TRPV2 is further suggested to be associated with ASD as it mediates the release of oxytocin (Wainwright et al., 2004), a neuropeptide that has been found to have a restoring character on symptoms of ASD and their development in humans (Hammock and Young 2006; Bartz and Hollander 2008; Andari et al., 2010; Green and Hollander 2010). Also THC has been shown to act as an agonist on TRPV2, albeit less potent than CBD (Qin et al., 2008). Still, a synergy of both simulated receptors CB1 and TRPV2, might be responsible for the presented effects of THC treatment on social behavior in the Fmr1- KO mouse.

4.1.3 THC can reduce social interaction in wild- type mice 10 days after treatment

To further assess and verify the involvement of CB1 in reduced social interaction in WT mice 10 days after THC treatment as shown in chapter 4.1.2, the social interaction task was repeated with naïve WT mice purchased from Janvier. The test protocol was modified to include a pre- treatment with the CB1- antagonist Rimonabant 30 minutes before THC injection to impede the effect of THC by preventing it to bind to CB1.
The results of this experiment are shown in percentage of affiliative behavior to be able to compare them with the results from the previous social interaction task. The time the overall control group (Veh/ Veh- treatment, first bar in figures 19 and 20) engaged in affiliative behavior with a female partner is shown as 100%. Mice that had received only Rimonabant pre- treatment (Rim/ Veh) interacted not significantly less with the female partner compared to the control group (figure 19, first and second bar). This concludes that Rimonabant itself has no influence on the analyzed parameter. Mice that had received only THC treatment (Veh/ THC) interacted 25% less with the female partner than the control group (figure 19, first and third bar). This effect was not significant but a trend is apparent. THC- treated WT mice from the previous social interaction task interacted around 35% less with the female partner than the control group which was significant (figure 17). Accordingly, the results do not reproduce the long-term effects of THC treatment in WT mice but they still indicate the tendency of THC to decrease social interaction 10 days after treatment. The origin of mice used here (purchased at Janvier) differs from those of the experiment before (bred in the local institu-

tion) and might play a role in these results. More importantly the test history of the mice could have an influence on the different results. While mice from the Rimonabant experiment were naïve before testing, mice from the first social interaction task were not since they passed through the tetrad test battery beforehand. This can modulate behavior as it may decrease activity, exploratory sense, and emotionality in mice from the B6 background (McIlwain et al., 2001; Voikar et al., 2004) and thus influence behavior in subsequent experiments. Further, CB1- transmission has been shown to critically modulate the salience of rewarding and aversive emotional information (Tan et al., 2014). It is therefore possible that THC might have amplified the emotional impact of test history and moreover, that mice influenced by THC during previous testing experienced a lower salience for emotional information in following experiments, e.g. in the female partner mouse from the social interaction task. Mice that were treated with Rimonabant and THC (Rim/ THC) interacted not significantly more with the female partner compared to the control group (figure 19, first and fourth bar). Mice that had received only THC treatment (Veh/ THC) interacted 45% less with the female partner compared to mice that had first received Rimonabant before THC treatment (Rim/ THC, figure 19 third and fourth bar). This effect was significant and shows, that pretreatment with Rimonabant impedes the effect of THC to decrease social interaction 10 days after treatment and also verifies the involvement of CB1. However, this effect is only detectable in the first 3 minutes and not throughout the whole 6 minutes of the experiment (figure 20). This could indicate that neither THC, nor THC in combination with Rimonabant have a significant long term effect on social interaction behavior of the B6 mouse compared to the control group.

4.1.4 THC possibly restores impaired associative fear memory consolidation in Fmr1- KO mice

In the context recognition task, the influence of acute THC treatment on impaired associative fear memory consolidation in the Fmr1- KO mouse was examined in context-dependent fear conditioning with subsequent THC injection. Mice received an aversive stimulus (foot shock) while exploring a new environment (conditioning in the conditioning chamber). After 5 minutes of conditioning mice were immediately injected with THC. 24 hours later conditioned fear was measured as the time mice spent freezing (fear) or moving (no fear).

The results show that WT mice that had received THC treatment did not show a significant difference of freezing or moving compared to the control group (figures 21 and 22, first and second bar). Therefore this experiment did not reproduce the amnesic- like

effect of THC on aversive learning and memory in WT mice that had been demonstrated before (Puighermanal et al., 2009).

Vehicle treated KO mice freeze significantly less and move significantly more when returning to the conditioning chamber than the control group (WT vehicle treated), figures 21 and 22, comparing first and third bar. This confirms impaired associative learning in the Fmr1- KO mouse as reported before (Paradee et al., 1999; Lim et al., 2014). However KO mice, that had received a THC injection show no difference to the control group (figures 21 and 22, first and fourth bar). This suggests that they possibly recognized the context in which they had experienced an aversive stimulus as well as vehicle treated WT mice. Further, they freeze significantly longer and move significantly less than vehicle treated KO mice (figures 21 and 22, third and fourth bar). These results suggest that impaired associative learning can be influenced by THC treatment and, once again that CB1 stimulation possesses restoring potential on a symptom of the autistic phenotype of the Fmr1- KO mouse.

The mechanism of impaired associative fear memory consolidation in untreated Fmr1-KO mice could again be connected to an oversupply of endocannabinoids and thus to an increased extinction of the aversive experience from the training day. In fact, CB1 stimulation has been proposed to impact aversive memory consolidation trough selective inhibitory effects on local inhibitory networks in the amygdala and hippocampus and that endocannabinoids facilitate therefore the extinction of aversive memories (Marsicano et al., 2002; Azad et al., 2004).

That additional CB1- stimulation with THC in Fmr1- KO mice on the other hand leads to the opposite effect on aversive learning and memory consolidation seems contradictory. Yet, previous studies have shown that endocannabinoids and externally applied cannabinoids like THC provoke different behavioral responses (Long et al., 2009). THC is a partial agonist for CB1 (Shen and Thayer, 1999) unlike the endocannabinoid 2- AG that is a full agonist. If both are present at the same time, a partial agonist can compete with a full agonist at the receptor binding site, suppress its binding to the receptor and inhibit the effects of the full agonist (Calvey and Williams, 2009).

Combining this knowledge with the mGlur theory of FXS, which assumes an oversupply of endocannabinoids, THC could act as a partial antagonist in the Fmr1- KO mouse and thereby readjust the overstimulated endocannabinoid system. This might explain the restoring character of THC treatment on autistic behavior in the Fmr1- KO mouse as demonstrated in the social interaction task (chapter 4.1.2) and this chapter. However, it does not explain why KO mice exhibit less cannabimimetic effects shown in the tetrad experiment (chapter 4.1.1). Therefore the hypothesis of an altered intracellular

pathway component activation status in the Fmr1- KO mouse model and its stimulatability was examined using western blots.

4.2 Western blot reveals altered activity of CB1 downstream targets in Fmr1- KO mice

In Experiment 4 the activity status and stimulatability of intraneuronal CB1 pathway components ERK, AKT and S6 were assessed using western blots from brain tissue homogenates of hippocampus, striatum, cortex and cerebellum from cannabinoid treated and untreated Fmr1- KO an WT mice. The selected brain regions are high in CB1 expression (Herkenham et al., 1991b; Tsou et al., 1998) and suggested to be involved in the development of autistic behavior (Belmonte et al., 2004; Courchesne et al., 2007). Western blots were done to investigate the altered ECS signaling status in the Fmr1- KO mouse on a molecular level and to possibly determine its condition and responsiveness to THC treatment. Therefore activation levels of ERK, AKT and S6 were analyzed in the selected brain areas. The change of the ratio of phosphorylated kinases to not phosphorylated kinases in favor of the phosphorylated kinases indicates activation.

In the hippocampus THC treatment had a significant effect on ERK and AKT activation (figures 23 and 24) and no significant effect on S6 activation (figure 25) but a trend was detected ($p= 0.055$). No statement can be made about the effect of THC treatment on kinase activation in the different genotypes since the interaction (genotype vs. treatment) was not significant (figures 23, 24, 25).

In the striatum THC treatment had a significant effect on ERK and S6 activation (figures 26 and 28) and no significant effect on AKT activation (figure 27). The interaction (genotype vs. treatment) was significant for ERK and S6 activation, and a trend was detected for an interaction effect on AKT activation, respectively in THC treated WT mice (figures 26, 27, 28). This concludes that THC treatment increased ERK, S6 and possibly AKT activation in WT mice compared to vehicle treated WT mice. In KO mice THC treatment had no influence on kinase activation levels compared to vehicle treated KO mice.

In cortex THC treatment had a significant effect on S6- activation (figure 31) but no significant effect on ERK and AKT activation (figures 29 and 30). THC treatment significantly induced AKT activation in WT mice (figure 30) compared to vehicle treated WT mice. Vehicle treated KO mice exhibited significantly higher levels of activated AKT compared to vehicle treated WT mice.

In cerebellum THC treatment and mouse genotype had a significant effect on ERK activation (figure 32). THC treatment significantly induced ERK activation in KO mice (interaction, figure 32) compared to vehicle treated KO and WT mice, and THC treated WT mice. THC treatment had no effect on S6 activation in WT and KO mice (figure 33). In summary these results show an inhomogeneous distribution of kinase activation after THC treatment in different brain regions of WT and KO mice. In cortex and striatum of WT mice, THC treatment increased levels of activated kinases whereas THC treatment had no effect on levels of activated kinases in hippocampus and cerebellum. Therefore this experiment reproduced the effect of THC treatment on kinase activation in WT mice that had been demonstrated (Kano et al., 2009) but only in cortex and striatum. The strong activation of ERK in hippocampus (Derkinderen et al., 2003) was not reproducible.

The reason for a partially insufficient kinase activation after THC treatment could be the time frame in which the experiment was done. In behavioral experiments mice were tested 30 minutes after THC injection since this has been shown to be the best time to measure a response in the tetrad experiment (Martin et al., 1991). Accordingly the same time frame was applied in experiment 4. Yet, in N18TG2- cells maximal ERK phosphorylation has been suggested to occur already 5 minutes after CB1 stimulation (Dalton and Howlett, 2012). Therefore a shorter duration between THC treatment and western blot should be chosen to possibly allow for more valid results of kinase activation.

There was no activation of CB1 targets after THC treatment in the cerebellum of WT mice. It is possible that cerebellic CB1 activation in WT mice mostly results in adenylat cyclase inhibition that would lead to inhibition of kinase activation (Breivogel and Childers 2000; Tonini et al., 2006). Another explanation for a missing activation of CB1 targets in the cerebellum of WT mice would be the activation of GIRKs that are particularly enriched in the Purkinje cell bodies of the cerebellum (Mackie et al., 1995; Tsou et al., 1998). Yet unknown mechanisms of GIRKs could lead to inhibition of CB1 after cannabinoid binding (illustrated in figure 35).

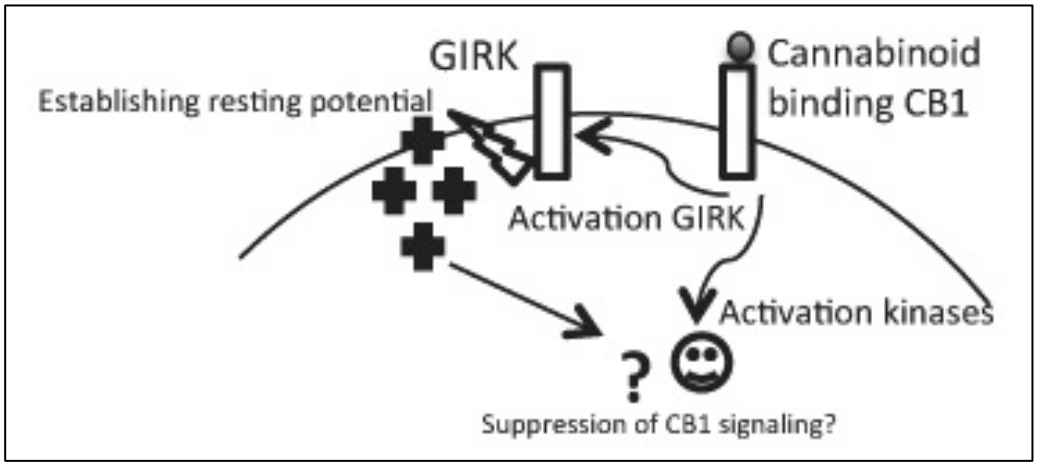


Figure 35: Possible mechanism of suppressing CB1 signaling after GIRK-activation in Purkinje cells.

In KO mice THC treatment induced no significant activation of ERK, AKT or S6 in the hippocampus (figures 23, 24, 25), striatum (figures 26, 27. 28) and cortex (figures 29, 30, 31). This is in accordance with findings from the tetrad experiment as it again suggests diminished responsiveness to cannabinoid treatment in the Fmr1- KO mouse.
As discussed in chapter 4.1.1, this might be due to a diminished supply of CB1 receptors or due to an uncoordinated activation of intracellular CB1 pathway components, leading to modifications of downstream CB1- signaling and to insensitivity to THC. Accordingly, cannabinoid drugs that bind to desensitized CB1 receptors would not increase the levels of activated kinases, similar to the demonstrated results. Further, the activation of AKT in the cortex of vehicle treated KO mice is significantly increased. This may suggest elevated kinase activation in the cortex as the regular condition in the Fmr1- KO mouse.
However, THC treatment causes a significant ERK- activation in the cerebellum of KO mice (figure 32). Even though THC treatment had no significant effect on S6 activation (figure 33), levels of pERK in the cerebellum of KO mice after THC administration suggest a strong activation of CB1 while there is no significant effect in WT mice in this brain area. It has been shown that the post-mortem cerebellum of humans suffering from a disease whose physiopathology is related to those of FXS exhibit overexpression of CB1, particularly in Purkinje cell bodies (Rodriguez-Cueto et al., 2013). Additionally cerebellar activation in autistic patients was shown to be abnormally high during simple tasks (Allen et al., 2004). Taken together, a cerebellar lowered neuronal sensitivity threshold for cannabinoids is thinkable and might have led to the strong kinase activation after THC treatment in Fmr1- KO mice. Also, Purkinje cell bodies have already been suggested to be responsible for suppression of activation of the analyzed CB1 targets in WT mice (Breivogel and Childers, 2000; Tonini et al., 2006). In the cer-

ebellum of KO mice the observed increase of pERK could be due to alteration or absence of GIRKs. It is likely that GIRK mRNA is altered in the absence of FMRP since mRNA of other voltage- gated potassium channels has been shown to be a target of FMRP (Darnell et al., 2011). If GIRK somehow suppresses CB1-signal transduction in WT mice the absence or alteration of GIRKs in KO mice would accordingly lead to an increase of CB1 signaling.

4.3 Conclusion

In conclusion the Fmr1- KO mouse displays a distinctly reduced common responsiveness to cannabinoid drugs in the tetrad experiment (chapter 4.1.1) and in western blots (chapter 4.2). This is likely due to an altered signaling status of the ECS in this mouse model. The exploration of the basal intraneuronal activity status of the ECS with western blots (chapter 4.2) did not reveal conclusively in which way the ECS might be altered in the Fmr1- KO mouse model. At the same time THC treatment influences the behavior of the Fmr1- KO mouse, as it may possess the potential to acutely (context recognition task, chapter 4.1.4) and in the long term (social interaction task, chapter 4.1.2) restore symptoms of the autistic phenotype of the Fmr1- KO mouse model.
Therefore this work suggests that the ECS plays a role in the development of the autistic phenotype of the Fmr1- KO mouse model and that it can be influenced by THC treatment.
The perspective of using cannabinoids such as THC as a therapeutic strategy to improve autistic symptoms is considerable and the objective of today's intense research (Busquets-Garcia, et al., 2013). Further, for the first time a single THC injection has been shown to notably reduce social interaction for up to 10 days in WT mice.

Abstract

Background:

Background of this work was the investigation of the endocannabinoid system (ECS) in the Fmr1 knock- out (KO) mouse. The Fmr1- KO mouse is a mouse model for fragile X syndrome (FXS). FXS is the leading monogenic cause for autism spectrum disorders (ASD) in humans. The Fmr1- KO mouse displays autistic behavior such as an impaired social interaction, repetitive behavior, cognitive deficits, increased anxiety and aggressiveness. Alterations of the ECS have been suggested to play a key role in the etiopathology of a variety of neuropsychiatric disorders. Until today, little has been described about the involvement of the ECS in ASD.

Interrogation:

1. Evaluating the manifestation of typical cannabinoid- induced effects in the Fmr1- KO mouse
2. Investigating the influenceability of autistic symptoms with THC treatment in the Fmr1- KO mouse
3. Analyzing the signaling cascade of the stimulated and unstimulated ECS in different brain regions of the Fmr1- KO mouse

Material and Methods:

Experiments were carried out on adult (12±1 weeks old) male Fmr1- KO and Fmr1- wild- type (WT) mice from the C57BL/6J- (B6)- background. N= 15 mice received THC (10mg/kg bodyweight) and N= 16 received WIN55,212 (3mg/kg bodyweight). 30min after injection, the body temperature was measured and the distance animals moved in an open field during 15min was recorded (locomotion). Then, animals were placed with their forepaws onto a horizontally fixed bar and the time remaining in this position (catalepsy) was measured. Finally animals were placed on a preheated plate and the temperature at which a pain stimulus occurred was determined (testing analgesia). All 4 experiments are called tetrad experiment. Afterwards changes in body temperature, locomotion, catalepsy and analgesia of the animals was evaluated.

To explore long-term effects of THC after the tetrad, N= 15 animals were tested in a social interaction test with a female contact mouse, 10 and 20 days after THC treatment. Therefore, the tested mouse and the contact mouse were placed together into a cage and the time mice spent in social interaction (nose, body and anogential sniffing, allogrooming and body contact) was manually quantified during 6min of recorded test-

ing time. Another group of N= 19 received a premedication of rimonabant (Cannabinoid-receptor 1 (CB1) antagonist, 3mg/kg bodyweight) 30min prior to THC treatment. Rimonabant prevents THC from binding to CB1 and therefore allows the assessment of the involvement of CB1 in mediating social behavior.

Furthermore the suggestibility of context-dependent fear conditioning with THC treatment has been tested on N= 13 mice. Animals were placed into a conditioning chamber that delivered 6 short electric shocks with a 30sec pause to their paws (conditioning phase). Immediately afterwards mice received THC or placebo. 24h later context-dependent fear was evaluated by quantification of the time mice spent freezing in the conditioning-chamber (fear) without receiving foot shocks.

Intraneuronal signaling of the ECS was analyzed with N= 29 animals using western blots. Quantities of phosphorylated ("activated") protein kinases (ERK, AKT and S6) from different brain homogenates (hippocampus, striatum, cortex and cerebellum) were therefore measured after THC or placebo injection (30 minutes prior to sacrificing).

Results:

Cannabinoids induced hypothermia, hypolocomotion, analgesia and catalepsy in WT-mice. These effects were significantly less detectable in Fmr1- KO mice. Effects of both cannabinoids, THC and WIN55,212, were comparable with a slightly greater but not significant efficiency of THC. THC treated WT- mice exhibited further reduced social interaction 10 days after treatment, an effect that was partially prevented by premedication with rimonabant. THC increased social interaction in Fmr1- KO mice comparable to the level of untreated WT- mice. THC had no effect on behavior of WT- mice in context-dependent fear conditioning. Fmr1- KO mice showed significant less context-dependent fear conditioning compared to WT- mice. THC facilitated the recognition of an anxiety-correlated context in Fmr1- KO mice comparable to untreated WT- mice.

In western blots significant changes in the THC- induced signaling cascade were detectable and depending on genotype, brain-region and analyzed protein-kinase.

In the hippocampus there were no changes in untreated Fmr1- KO mice compared to WT- mice. THC had no effect on activation of protein-kinases in WT- and Fmr1- KO mice.

In the striatum there were no changes in untreated Fmr1- KO mice compared to WT-mice. THC significantly increased activity of ERK, AKT and S6 in WT-mice and not in Fmr1- KO mice.

In the cortex of untreated Fmr1- KO mice AKT showed a significantly increased activity compared to WT- mice. THC significantly increased AKT activity in WT- mice without having an effect on KO- mice.
In the cerebellum there were no changes in untreated Fmr1- KO mice compared to WT- mice. THC significantly increased ERK- activity in Fmr1- KO mice but had no effect on protein kinase activity in WT- mice.

Conclusion:

We observed physiological cannabinoid effects in WT- mice after treatment with THC and WIN55,212. These effects are significantly attenuated in Fmr1- KO mice. This may be interpreted as a desensitization of the ECS in the Fmr1- KO mouse. At the same time it was demonstrated that THC has the potential to improve context dependent memory consolidation and to increase social interaction in the Fmr1- KO mouse. In particular the influence of THC on impaired social interaction should be a target of further investigations to find possible therapeutic options for this typical symptom of Autism. Underlying molecular mechanisms remain unclear and the analysis of THC stimulated intraneuronal signaling gave no clear indication of possible molecular alterations in the Fmr1- KO mouse.

Zusammenfassung

Hintergrund:

Ziel dieser Arbeit war die Untersuchung des Endocannabinoide Systems (ECS) in der Fmr1- knockout (KO) Maus. Die Fmr1- KO Maus ist ein Tiermodell für das Fragile X Syndrom (FXS). Das FXS ist die häufigste hereditäre Ursache für eine Autismus Spektrum Störung (ASD) beim Menschen. Auch in der Fmr1- KO Maus zeigen sich autistischer Verhaltensdefizite wie eine eingeschränkte sozialen Interaktion, repetitive Verhaltensweisen, kognitive Störungen, erhöhte Ängstlichkeit und Aggressivität. Veränderungen des ECS wurden mit verschiedenen neuropsychiatrischen Erkrankungen in Verbindung gebracht. Wenig ist bisher zum ECS im Rahmen der ASD bekannt.

Fragestellung:

1. Untersuchungen zur Ausprägung typischer, Cannabinoid- induzierter Mechanismen in der Fmr1- KO Maus im Vergleich zu Wildtyptieren
2. Untersuchung der Beeinflussbarkeit autistischer Verhaltensweisen in der Fmr1- KO Maus durch eine Behandlung mit THC
3. Analyse der intraneuronalen Signalkaskade des ECS in verschiedenen Gehirnregionen in der Fmr1- KO Maus im Vergleich zu Wildtyptieren

Material und Methoden:

Untersuchungen wurden an adulten (12±1 Wochen) männlichen Fmr1- KO und Wildtyp (WT)- Tieren (C57BL/6J-Hintergrund) durchgeführt. N= 15 Tiere erhielten THC (10mg/kg Körpergewicht) und N= 16 Tiere erhielten WIN55,212 (3mg/kg Körpergewicht). 30min nach Injektion wurde die Körpertemperatur gemessen und die Distanz aufgezeichnet, welche die Tiere innerhalb von 15min in einem offenen Feld zurücklegten (Lokomotion). Dann wurden die Tiere mit ihren Vorderpfoten auf eine fixierte Stange platziert und die Zeit gemessen, die sie in dieser Position verharrten (Katalepsie). Zum Schluss wurden die Tiere auf eine erhitzte Platte gesetzt und die Temperatur bestimmt ab welcher ein Schmerzreiz auftrat (Prüfung auf Analgesie). Diese 4 Experimente nennen sich Tetrade. Anschließend wurden sie hinsichtlich einer Veränderung von Körpertemperatur, Lokomotion, Katalepsie und Analgesie untersucht.

Um langfristige THC- Effekte nach der Tetrade zu überprüfen, wurde 10 bzw. 20 Tage nach THC- Injektion in N= 15 Tieren ein sozialer Interaktionstest mit weiblichen Kontaktmäusen durchgeführt. Dafür wurde je eine Testmaus mit einer Kontaktmaus in einen Käfig gesetzt und deren Interaktion (Schnüffeln an Nase, Körper, anogenitaler Re-

gion der Kontaktmaus, Allogrooming) für 6min aufgezeichnet und anschließend manuell quantifiziert.
Eine weitere Gruppe (N= 19) erhielt 30 min vor THC- oder Placeboinjektion eine Prämedikation mit Rimonabant (Cannabinoidrezeptor1 (CB1)-Antagonist; i.p., 3mg/kgKG). Rimonabant verhindert die Bindung von THC an CB1 und erlaubt so eine Untersuchung der Beteiligung des CB1- Rezeptors an der Vermittlung sozialer Interaktionsfähigkeit.
Im Weiteren wurde die Beeinflussbarkeit kontextabhängiger Angstkonditionierung durch THC an N= 13 Tieren getestet. Die Mäuse kamen in eine Konditionierungskammer in der 6 kurze Stromschläge im Abstand von 30 sec an die Pfoten abgesetzt wurden (Konditionierungsphase). Danach erhielten die Tiere THC oder Placebo. 24h später erfolgte die Prüfung auf kontextabhängig gelernte Angst durch Aufzeichnen der Zeit, in der sie regungslos in der Konditionierungskammer verharrten (Angst), ohne Stromschläge zu erhalten.
An N= 29 Tieren wurde die intraneuronale Signalkaskade des ECS mittels Western Blot- Analysen untersucht. Dafür wurde der Aktivitätsstatus anhand der Menge phosphorylierter (aktivierter) Proteinkinasen (ERK, AKT und S6) in Gehirnhomogenaten (Kortex, Hippocampus, Striatum und Zerebellum) bestimmt. Die Analyse erfolgte ohne und nach THC-Stimulierung (30 min vor Gehirnentnahme).

Ergebnisse:

Cannabinoide induzierten in Wildtyptieren eine Hypothermie, Hypolokomotion, Katalepsie und eine analgetische Wirkung. Diese Effekte waren in Fmr1- KO Mäusen signifikant weniger nachweisbar. Die Effekte beider Cannabinoiden THC und WIN55,212 waren vergleichbar wobei THC eine etwas stärkere Wirksamkeit als WIN55,212 zeigte (nicht signifikant). Wildtyptiere welche mit THC behandelt wurden zeigten darüber hinaus eine reduzierte soziale Interaktion 10 Tage nach Substanzapplikation, ein Effekt, der durch eine Vorbehandlung mit Rimonabant teilweise inhibiert werden konnte. In Fmr1- KO Mäusen erhöhte THC die soziale Interaktion auf das Niveau unbehandelter Wildtyptiere. Eine THC- Behandlung hatte keinen Effekt auf das Verhalten von Wildtyptieren in der kontext-abhängigen Angstkonditionierung. Fmr1- KO Mäuse zeigten eine signifikant schlechtere Angstkonditionierung im Vergleich zu Wildtyptieren. THC-Injektion in Fmr1- KO Mäusen erleichtere das Wiedererkennen des angstkorrelierten Kontextes, vergleichbar mit dem Niveau von Wildtyptieren. Auf neurobiologischer Ebene zeigten sich ebenfalls signifikante Veränderungen in der intrazellulären, durch Endocannabinoide stimulierten Signalkaskade in Anhängigkeit vom Genotyp, der Gehirn-

region und der spezifischen Proteinkinase. Unbehandelte Fmr1- KO Mäuse zeigten im Vergleich zu WT Mäusen eine unveränderte Aktivität von ERK, AKT und S6 im Hippocampus, Striatum und Cerebellum, während im Cortex die Aktivität von AKT in unbehandelten Fmr1- KO Mäusen im Vergleich zu WT Mäusen erhöht war. In WT Mäusen erhöhte THC im Striatum die Aktivität von ERK, AKT und S6 und im Kortex die von AKT. In Fmr1- KO Mäusen hatte THC keine Wirkung auf diese Regionen. THC hat keinen Effekt auf die Aktivierung der Proteinkinasen bei WT- und Fmr1- KO Mäusen im Hippocampus. THC erhöhte signifikant die Aktivität von ERK in Fmr1- KO Mäusen im Zerebellum, hatte aber keinen Effekt auf die Aktivität von Proteinkinasen in Wildtyptieren.

Schlussfolgerung:

Wir beobachteten physiologische cannabinoide Effekte in WT Mäusen nach Gabe von THC und WIN55,212. Diese Effekte sind in Fmr1- KO Mäusen abgeschwächt. Dies kann als eine Desensibilisierung des ECS auf Cannabinoide interpretiert werden. Gleichzeitig konnte gezeigt werden, dass die Applikation von THC sowohl das Potential besitzt, die kontextabhängige Konsolidierung von Gedächtnisinhalten bei Fmr1- KO Mäusen zu verbessern sowie die soziale Interaktion zu steigern. Insbesondere die genauere Analyse des THC Einflusses auf die soziale Interaktion sollte Ziel weiterer Untersuchungen sein, um eine mögliche therapeutische Option für dieses autische Symptom zu finden. Die molekularen Mechanismen sind unklar und die Analyse der Aktivität intraneuronaler, durch THC aktivierter Signalkaskaden ergab keinen eindeutigen Hinweis auf mögliche molekulare Veränderungen.

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Addendum

Protocol for western blot preparation

Laemmli sample buffer preparation 4X			
Product	Stock	Quantity	Final concentration
Tris	1M pH 6,8	2,5 ml	250 mM
Glycerol		4 ml	40%
SDS (pouder)		0.8g	8%
Bromphenolblue		3 tips of a tip	2%
MilliQ water		3,5 ml	
Betamercaptoethanol		1 ml	10%
1mm thick separating gel preparation (1 gel)			
Acrylamide (BioRad)			30%
Tris- HCL (BioRad)	1,5M pH 8,8	1,75 ml	373 mM
SDS (BioRad)	10%	70 µl	0.1%
Glycerol	66%	0.8 ml	7,5%
MilliQ water		2,7 ml	
APS (BioRad)	40%	11,6 µl	0.066%
Temed (BioRad)		4,7 µl	
Stacking gel preparation			
Acrylamide (BioRad)	30%	0.41 ml	5%
Tris- HCL (BioRad)	0.5M pH 6,8	0.63 ml	128 mM
SDS (BioRad)	10%	24,5 µl	0.1%
Glycerol	66%	0.56 ml	15%
MilliQ water		0.83 ml	
APS (BioRad)	40%	3,4 µl	0.055%
Temed (BioRad)		2,5 µl	