Behavioral assessment of mice with cell-type specific deletion of FMRP in parvalbumin and somatostatin interneurons

REPORT RESEARCH INTERNSHIP
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ABSTRACT

Fragile X syndrome (FXS) is a genetic cause of autism spectrum disorder and intellectual disability. The mutational basis of this disorder is an abnormal trinucleotide CGG repeat expansion in the 5' untranslated region of the fragile X mental retardation 1 (FMR1) gene, which results in silencing of this gene and lack of transcription and translation of fragile X mental retardation protein (FMRP). Lack of FMRP expression leads to dysregulation of mRNA translation, increased protein synthesis and disturbances in synaptic plasticity. To understand how FMRP contributes to FXS pathophysiology, the *Fmr1* knockout (*Fmr1* KO) mouse model was generated. *Fmr1* KO mice showed alterations in excitatory glutamatergic signaling and inhibitory gamma-aminobutyric acid (GABA) signaling, which may result in an imbalance between excitatory and inhibitory signaling in the brain that underlies autism spectrum disorders.

Despite these observations, is the role of FMRP in the GABAergic system less characterized. Alterations in GABAergic inhibitory neurotransmission were found in several brain regions including amygdala, hippocampus and cortex of *Fmr1* KO mice. However, the consequence of FMRP deletion in specific GABAergic interneuron subtypes, parvalbumin (PV) and somatostatin (SOM), which are the main inhibitory neurons of the brain, and their specific contribution to behavioral deficits observed in *Fmr1* KO mice have never been examined. Therefore, we used Cre/Lox recombinase technology to generate mice with cell type-specific deletion of the FMRP in PV or SOM interneurons (PV-$Fmr1^{-/y}$ and SOM-$Fmr1^{-/y}$), to assess whether this deletion in specific interneurons results in behavioral impairments that are observed in *Fmr1* KO mouse models

A battery of behavioral tests for anxiety and repetitive behavior (open field, elevated plus maze, marble burying and self-grooming), locomotor function and learning (rotarod), cognition (novel object recognition, T-maze, Morris water maze and Y-maze) and social interaction (three chamber social interaction test) were performed in this study. Our results demonstrate that SOM-$Fmr1^{-/y}$ mice showed impairments in repetitive behavior (stereotypic counts) and sociability. On the other hand, PV-$Fmr1^{-/y}$ mice showed mild anxiety, impairments in social novelty, spatial memory and cognitive flexibility. Based on these observations, we conclude that deletion of FMRP in specific classes of interneurons results in different types of behavioral phenotypes observed in *Fmr1* KO mouse models.

Nevertheless, the possible underlying mechanisms remain partly unclear. Although, SOM interneurons are known for their important role in regulating the balance of motor activity and other studies found loss of social preference after specific deletion of FMRP in SOM interneurons. Finally, GABAergic SOM an PV interneurons seem to have different distinct underlying mechanisms that modulate motor activity, social behavior and spatial memory. More research is necessary to investigate the effects of FMRP deletion in specific interneuron classes, whereby future studies with optogenetics and electrophysiological techniques could give more insight in underlying mechanisms.

1. INTRODUCTION

1.1 Fragile X syndrome

Fragile X syndrome (FXS) is the most common genetic cause of intellectual disability and autism spectrum disorders (ASD), with a prevalence of approximately 1 in 7,000 males and 1 in 11,000 females (Verkerk et al, 1991; Coffee et al, 2009; Hagerman et al, 2017). FXS is characterized by severe behavioral alterations, including hyperactivity, attention deficit, shyness, anxiety and autism, in combination with physical features, such as prominent ears, long face and flat feet (Crawford et al, 2001; Hagerman et al, 2017). The mutational basis of FXS is the abnormal expansion of a CGG repeat in the 5'-untranslated region of the fragile X mental retardation 1 (*FMR1*) gene (Fu et al, 1991). Full mutations have over 200 copies of the repeat and lead to hypermethylation and silencing of *FMR1* expression (Sutcliffe et al, 1992).

Due to the silencing of *FMR1*, there is a lack of transcription and translation of fragile X mental retardation protein (FMRP). FMRP is an mRNA binding protein with an important role in the translational control of ~800 brain mRNAs as well as mRNA localization and transport (Garber et al, 2008; Darnell et al, 2011; Ascano et al, 2012; Hagerman et al, 2017). Lack of FMRP expression leads to dysregulation of mRNA translation, increased protein synthesis and disturbances in synaptic plasticity (Darnell & Klann, 2013; Richter 2015). Furthermore, other cellular functions of FMRP have been proposed, including a role in the activation of potassium channels, DNA damage response, RNA editing, hippocampal dependent learning and regulation of the endocannabinoid system (Hagerman et al, 2017). Thus, FMRP is a crucial protein with many important cellular functions in the human body, and lack of FMRP expression as result of *FMR1* silencing underlies the pathophysiology of FXS.

The *Fmr1* knockout (*Fmr1* KO) mouse model was generated to understand how the loss of FMRP contributes to neurological deficits and FXS pathophysiology (Bakker et al, 1994; Bakker et al, 2003; Dahlhaus 2018). This mouse model showed autistic-like phenotypes, including cognitive deficits, hyperactivity and social deficits (Hagerman et al, 2017). In addition, increased protein synthesis and alterations in neuronal plasticity in specific brain regions were found (Hagerman et al, 2017). Altogether, the use of *Fmr1* KO mouse models is well accepted nowadays and has revealed several alterations and disturbances in neuroplasticity and signaling. However, a treatment for FXS is still lacking, because FMRP affects multiple cellular functions and molecular pathways in the brain. It remains unknown how alterations in brain connectivity, caused by the lack of FMRP, are responsible for the specific behavioral phenotypes of FXS, whereby certain findings and phenotypes observed in FXS mouse models may not be relevant for FXS patients (Berry-Kravis et al, 2018).

1.2 Excitatory / inhibitory imbalance underlies autism spectrum disorders

The observed behavioral phenotypes in *Fmr1* KO mouse models suggested a possible imbalance between excitatory glutamatergic signaling and inhibitory gamma-aminobutyric acid (GABA) signaling. It all started with the observations that FXS neuronal networks are hyperexcitable. The metabotropic glutamate receptor (mGluR) theory of FXS, as proposed by Bear and co-workers, suggested that excessive glutamate signaling in the absence of FMRP leads to increased protein synthesis and deficits in synaptic plasticity, including enhanced long-term depression in response to activation of group I mGluRs (Bear et al, 2004; Pop et al, 2014).

Additional studies revealed indeed that genetic reduction or pharmacological blockage of mGluR5 receptors rescues several phenotypes observed in *Fmr1* KO mice (Santoro et al, 2012). Thus, FXS neuronal networks are hyperexcitable, whereby glutamate receptor antagonists could be used as potential treatment.

Despite promising results in animal models, most clinical trials using mGluR5 antagonists in FXS have not been successful (Erickson et al, 2017). Nevertheless, these studies showed alterations in downstream effectors of glutamate signaling in the absence of FMRP. Increased phosphorylation of eukaryotic translation initiation factor 4E (eIF4E) and ribosomal p70 S6 kinase 1 (S6K1), the downstream targets of mammalian target of rapamycin complex 1 (mTORC1) signaling, have been observed (Sharma et al, 2010; Hoeffer et al, 2012; Gkogkas et al, 2014). All in all, are the effects of the absence of FMRP in the excitatory glutamate signaling pathway widely studied and well-established in *Fmr1* KO mouse models.

Impaired inhibition and altered neural synchrony are a possible cause for the excessive glutamate signaling in the absence of FMRP (Gonçalves et al, 2013). Recent studies found that lack of FMRP is responsible for dysregulation of the GABAergic system, which is the main inhibitory signaling system in our brain (Paluszkiewicz et al, 2011). This inhibitory neurotransmission is mediated by presynaptic and postsynaptic ionotropic GABAA and G-protein-coupled GABAB receptors (Farrant & Nusser, 2005; Padgett & Slesinger et al, 2010; Paluszkiewicz et al, 2011). In addition, recent molecular and electrophysiological studies showed that specific components of the GABAergic system are regulated by FMRP expression. Several brain regions in *Fmr1* KO mice, including hippocampus, cortex and amygdala showed alterations in GABAergic signaling, with or without decreased expression of multiple GABA receptor subunits and enzymes (Gantois et al, 2006; D'Hulst et al, 2006; Selby et al, 2007; Penagarikano et al, 2007; Centonze et al, 2008; Gibson et al, 2008; Curia et al, 2009; Olmos-Serrano et al, 2010; Paluszkiewicz et al, 2011; Braat & Kooy, 2015; Hagerman et al, 2017).

The observed inhibitory deficits in *Fmr1* KO mouse models are brain region specific, whereby reductions in inhibitory synapse numbers and GABA release were found in the amygdala, meanwhile increased GABA release and inhibitory synapses were found in was found in the striatum and hippocampus (Dahlhaus & El-Husseini et al, 2010; Maccarrone et al, 2010; Olmos-Serrano et al, 2010; Paluszkiewicz et al, 2011). Thus, studies in *Fmr1* KO mice showed specific alterations in GABAergic inhibitory transmission that differs between brain regions.

Altogether, *Fmr1* KO mouse models showed alterations in the excitatory glutamatergic signaling and inhibitory GABAergic signaling, which are brain region specific and may result in an imbalance between excitatory and inhibitory signaling (Rubenstein & Merzenich, 2003). The disrupted ratio of excitation and inhibition (E/I imbalance) is thought to underlie ASD and has been observed in several other mouse models of ASD (Yizhar et al, 2011; Lee et al, 2017). Although promising effects of glutamatergic antagonists and GABA modulators were found in *Fmr1* KO mouse, translation to clinical setting remains problematic (Hagerman et al, 2014; Ligsay et al, 2017; Lee et al, 2018). None of the trials has been able to demonstrate efficacy, which highlights knowledge gaps in drug development for FXS, as reviewed by Berry-Kravis and co-workers (Berry-Kravis et al, 2018). Therefore, a better understanding of the mechanisms

underlying the alterations in glutamatergic and GABAergic transmission and how this is related to specific FXS behavioral phenotypes is necessary.

1.3 Inhibitory interneurons: parvalbumin (PV) and somatostatin (SOM)

Despite the knowledge about disturbances of the inhibitory system in *Fmr1* KO mouse models, the role of FMRP in GABAergic neurons itself is still less characterized. Recent studies showed evidence that FMRP affects GABAergic signaling, but only a few studies address the specific role of GABAergic interneurons in FXS. Interneurons are the main inhibitory neurons in the brain and form 20-30% of the total neuron population (Hu et al, 2014; Riedemann, 2019). They control hyperexcitability by mediating the precise gathering of information by controlling the amounts of excitatory and inhibitory input neurons receive (Markram et al 2004; Marin, 2012; Hu et al, 2014).

In addition, GABAergic interneurons are known for their morphological, biochemical and physiological diversity (Markram et al, 2004; Hu et al, 2014; Kepecs & Fishell, 2014; Riedemann, 2019). Interneurons vary in their patterns of functional connectivity by making local connections in different cortical layers. Although a few GABAergic interneurons showed axonal projections across their home brain region, these interneurons are called long-range projection interneurons (Riedemann, 2019). In recent years, classification of interneurons according to their neurochemical properties became increasingly popular, because it makes genetic manipulation in a subset of cells that are target by their cell-type-specific genes possible (Riedemann, 2019). In this way are three non-overlapping subtypes of GABAergic interneuron classes found, parvalbumin (PV), somatostatin (SOM) and serotonin 5-HT3a-receptor expressing interneurons (Freund & Buzsaki et al, 1996; Rudy et al, 2010; Urban-Ciecko & Barth, 2016; Riedemann, 2019). Of note, in this study were only PV and SOM interneurons investigated.

PV interneurons account for approximately 40-50% of the GABAergic interneurons and include fast spiking basket cells and chandelier cells, which are found throughout cortical layers 2-6 (Paluszkiewicz et al, 2011; Rudy et al, 2011). Basket cells generate synapses at the soma and chandelier cells are known to target axon initial segments of pyramidal cells (Markram et al, 2004; Rudy et al, 2011; Riedemann, 2019). Moreover, PV interneurons express FMRP and *Fmr1* KO mouse models showed significantly reduced PV density in cortical regions in addition to larger soma size (Selby et al, 2007; Patel et al, 2013; Yavorska & Wehr, 2016). However, no differences in density or size of PV interneurons were found in hippocampal DG and CA1 regions of *Fmr1* KO mice (Selby et al, 2007). Removal of PV interneurons from hippocampal CA1 regions in mice causes problems in spatial working memory, indicating that PV interneurons could control cognitive function (Murray et al, 2011). Impaired visual discrimination and orientation were also found in *Fmr1* KO mice, which could be rescued by restoring PV interneuron activity (Goel et al, 2018).

On the other hand, SOM interneurons form approximately 30% of total population of GABAergic interneurons. Except for cortical layer 1, SOM interneurons are present in all cortical layers (brain (Markram et al, 2004; Paluszkiewicz et al, 2011; Rudy et al, 2011; Yavorska & Wehr, 2016; Riedemann, 2019). SOM interneurons can be divided in non-Martinotti cells and Martinotti cells. Non-Martinotti cells comprise, amongst others, basket cells with axonal projections that remain in each cortical layer and long-range neurons with axons that

project to other brain regions (Markram et al, 2004; Riedemann, 2019). Meanwhile low threshold spiking Martinotti cells are known for modulating the activity of excitatory pyramidal neurons (Riedemann, 2019). SOM interneurons express FMRP and *Fmr1* KO mouse models showed reduced activation of low-threshold spiking SOM interneurons in response to glutamate receptor agonist 3,5-dihydroxyphenylglycine (DHPG), resulting in impaired synaptic inhibition (Paluszkiewicz et al, 2011). SOM receptor knockout mice showed also motor and spatial learning deficits, whereby hippocampal and ventricular injections of SOM facilitate spatial learning in a variety of tasks (Lamirault et al, 2001; Zeyda et al, 2001; Tuboly & Vecsei, 2012).

Thus, fast spiking PV and low spiking SOM interneurons are the main inhibitory interneurons of our brain. Both types of interneurons express FMRP and *Fmr1* KO mouse models showed deficits in PV and SOM interneuron functioning, which indicates deficits that range from a reduction in GABAergic receptors and enzymes, to a reduction in the number of GABAergic interneurons after FMRP deletion.

However, previous studies found also differences in key features of PV and SOM interneurons. SOM interneurons are low threshold spiking, target dendrites, have a more depolarized potential, higher input resistance and shorter membrane time compared to fast-spiking PV interneurons (Rudy et al, 2011). This makes SOM interneurons more excitable. PV interneurons are fast spiking and mediate precise and powerful inhibition of pyramidal excitatory neurons (Rudy et al, 2011). Furthermore, excitatory synapses onto FS cells (which are PV interneurons) are typically strongly depressing, meanwhile excitatory inputs onto Martinotti cells (which are SOM interneurons) are generally strongly facilitating (Rudy et al, 2011). Nevertheless, the underlying mechanisms of deletion of FMRP leads to altered interneuron function or expression, as well as the effects of interneurons on cellular and circuit properties in relation to behavioral phenotypes in *Fmr1* KO mouse models, remain unclear.

1.4 Contribution of specific FMRP deletion in interneurons to behavioral deficits

PV and SOM interneuron functioning are altered in *Fmr1* KO mouse models. However, the consequences of FMRP deletion in specific PV and SOM interneurons and their contribution to behavioral deficits observed in *Fmr1* KO mice have never been examined. Therefore, we used Cre/Lox recombinase technology to generate mice with cell type-specific deletion of the FMRP in PV or SOM interneurons.

The aim of this study is to assess whether FMRP deletion in PV or SOM interneurons results in behavioral impairments that are observed in *Fmr1* KO mouse models. To this end, a battery of behavioral tests for anxiety, repetitive, locomotor, cognition and social behavior were performed. Moreover, because FMRP is a mRNA binding protein with an important role in translational control and protein synthesis, *de novo* protein synthesis was assessed in PV and SOM *Fmr1* KO mice and wildtype (WT) control mice. Because our study is the first to assess the specific effects of FMRP deletion in PV and SOM interneurons and the consequences on behavioral phenotypes of *Fmr1* KO mice, it is hard to hypothesize any possible outcomes. Conflicting results of previous studies in tests for anxiety, cognition and social phenotype in *Fmr1* KO mouse models, as reviewed by Kazdoba and co-workers, makes it even harder to determine the outcome of our study (Kazdoba et al, 2014).

Finally, PV and SOM interneurons together comprise approximately 70% of the total GABAergic interneurons, but both types of interneurons are independent from each other (Kelsom & Lu, 2013). As mentioned before, PV interneurons are fast spiking, meanwhile SOM interneurons are low spiking. Therefore, our study could give new insights in the role of specific classes of interneurons and their effects on behavioral phenotypes observed in *Fmr1* KO mice. Altogether, the effects of FMRP deletion in PV and SOM interneurons, and their consequences on behavioral phenotypes, could give a novel and cell specific understanding of the function of FMRP in FXS.

2. MATERIALS & METHODS

2.1 Animals

Floxed *Fmr1* mice were bred to PV or SOM *Cre* knock-in mice, which express *Cre* recombinase in PV or SOM expressing neurons only. In this way, mice with FMRP deletion specific in PV (PV-*Fmr1*$^{-/y}$ mice) or SOM interneurons (SOM-*Fmr1*$^{-/y}$ mice) were generated. These mice and their WT littermates were used in a battery of behavioral tests. A total of 16 mice (n=8 SOM-*Fmr1*$^{-/y}$ mice and n=8 WT) were used in this experiment. The mice were divided over two cohorts with an age between 3-6 months.

All mice were housed in the New York University animal facility and were complaint with the NIH Guide for Care and Use of Laboratory Animals. Mice were housed individually or with their littermates in groups of 2-3 animals per cage under a 12 hours regular light/dark cycle. Ad libitum access to food and water was provided to the mice. The cages contained sawdust bedding and cage enrichment in the form of nesting material. All experimental procedures involving mice were performed in accordance with protocols approved by the New York university Animal Welfare Committee and followed the NIH Guidelines for the use of animals in research.

2.2 Behavioral tests

Prior to behavioral testing, mice were weighted and handled for 1 minute a day for 2 days. Mice were allowed to habituate for 30 minutes before every behavioral test and all tests were performed in a sequence from least stressful to most stressful. The behavioral tests can be divided in tests for anxiety and repetitive behavior (open field, elevated plus maze, marble burying and self-grooming), locomotor function and learning (rotarod), cognition (novel object recognition, T-maze, Morris water maze and Y-maze) and the three chamber social interaction test (sociability and social novelty). Objects and test apparatus were rinsed with 30% ethanol between each trial and mice. The researcher was blind to the genotype of the animals and EthoVision XT 13 video tracking software (Noldus Information Technologies, Inc) was used for recordings of different behavioral tests. In the following sections each of the behavioral tests will be shortly mentioned and explained.

2.3 Anxiety / repetitive / locomotor behavioral tests

Open field

Anxiety-related behavior and repetitive behavior was tested in the open field test. After the mice were placed in the open field box, their movements were tracked for 15 minutes. Data about the preference for being in the center or close to the walls (periphery) was used to

determine anxiety related behavior. Furthermore, average velocity and total distance traveled were measured to give insight in locomotor behavior and repetitive behavior. Meanwhile stereotypic counts and jump counts were also measured as indication for repetitive behavior. The measurements for repetitive behavior were also measured in time blocks of 5 minutes, in addition to the total time.

Elevated Plus Maze (EPM)

The elevated plus maze (EPM) is a simple method for assessing anxiety responses of mice (Walf & Frye et al, 2007). The EPM apparatus consists of two open arms and two enclosed arms, which extend from a central platform. Mice were placed at the junction of the open and closed arms, facing the open arm opposite to where the experimenter was. The explorative behavior of each mouse was tracked for 5 minutes. The total time spent in center, open versus closed arms, and the number of entries in the open and closed arms were measured.

Marble burying

Marble burying is a test used for measurement of compulsive-like or repetitive behavior. The test takes advantage of the fact that burying is a behavior that occurs spontaneously in mice and is therefore of great value and easy to perform (Perez et al, 2013). In our study we added 8-10 centimeter of fresh bedding to each cage. The bedding material was distributed evenly and patted down to become perfectly flat. After this, 20 black marbles were placed in each cage (5 columns with 4 rows of 4 marbles) and the mice were recorded for 30 minutes. The average number of marbles buried, the number of marbles buried after 10, 20 and 30 minutes and the total digging time was scored for each mouse.

Grooming

Repetitive behavior and anxiety can be measured with the grooming test, whereby abnormal or excessive self-grooming is considered as ASD-like behavior (Kalueff et al, 2016). Each mouse was placed in a separate box and recorded for 30 minutes during the grooming test. The total time each mouse spent grooming was measured, even as the time spent grooming in three intervals of 10 minutes (0-10 minutes, 10-20 minutes and 20-30 minutes).

Rotarod

The rotarod test was used to evaluate the motor coordination and learning of rodents and could give insight of the maximal motor performance in mice, because of the accelerating speed of the rotarod (Shiotsuki et al, 2010). In our experiment the rotarod test took two consecutive days and consisted of 4 trials for maximal 300 seconds each. At least 15-20 minutes was in between each trial to prevent for excessive fatigue. Mice were placed on the rotarod facing away from the experimenter and the baseline speed of the rotarod was 3 rotations per minute and was gradually increased to 40 rotations per minute after 300 seconds. Failure was characterized as falling off or slipping and making a swing around the rotarod. The total time mice spent on the rotarod before failure was recorded. Mice were picked up from the rotarod if they reached the maximum of 300 seconds for each trial without failure.

2.4 Cognitive behavioral tests

Novel object recognition

The novel object recognition test evaluates the differences in exploration time of novel and familiar objects. This enables to study learning and memory, including preferences for novelty and recognition (Antunes & Biala et al, 2012). In our experiment the novel object test took 4 consecutive days. On the first two days, mice were placed in the apparatus in the presence of two identical objects and allowed to explore for 10 minutes. During these days the interaction time with both objects was measured.

On day 3, mice were placed back in the same apparatus, with the same identical objects and allowed to explore for 10 minutes. However, after a period of 1.5 hours of recovery, one familiar object was replaced with a novel object and placed in the apparatus on the side the mice did not prefer. Mice could explore both objects for 5 minutes, whereby the interaction time with each object was scored for determining the short-term memory performance. On day 4 (24 hours later) the long-term memory performance was determined by replacing the short-term memory object for a different long-term memory object. Mice were once again allowed to explore the objects for 5 minutes and the interaction time with each object was measured. Preference index (PI) for short term and long-term memory object was calculated by dividing the total time spent with the novel object by the time spent with the novel and familiar object.

T-maze

The T-maze spontaneous alternation test measures exploratory behavior in mice and is based on their tendency to explore a novel environment. In addition, this test is used to asses if mice prefer to visit a new arm of the maze rather than the familiar arm, whereby mice normally show less tendency to enter a previously visited arm (Deacon & Rawlins, 2006). At the start of this test, each mouse was placed in the home arm starting box with the gate down. Then the gate was opened, and the mouse started exploring. Each mouse could choose between two arms (left or right) during exploration. As soon as the mouse made a choice, the gate to the unchosen arm was closed, which forced the mouse to go back to the home arm. Once all the 4 paws of the mouse were back in the home arm, the gate of the unchosen arm was re-opened, and the mouse could once again choose between both arms (left or right). The preference of each mouse was examined for 15 trials and the percentage of correct alterations was measured.

Morris Water Maze

Spatial memory and reference memory were determined in the Morris water maze (MWM) test (Vorhees & Williams et al, 2006). Before starting the MWM test, white paint was added to the pool and a hidden platform was placed about 1-1.5 cm below the water surface. The MWM pool was divided in four quadrants with each their own visual clue attached to the wall of the pool. The MWM took place on 8 consecutive days, whereby the first day consisted of 4 trials, meanwhile the second, third and fourth day consisted of 3 trials each day. Each trial took a maximum of 60 seconds and every mouse started each trial from a different position.

On the first day of the test, the mouse was guided to the platform after 60 seconds. After guiding, each mouse was hold for 10-15 seconds on the platform, to associate it with safety. If the mouse didn’t find the platform within 60 seconds on the second, third or fourth day it was removed from the pool and put back in their drying cage. When all mice reached the hidden

platform within +- 20 seconds (after 4 days of training) the probe trial was performed. On this day, the swim latency, swim distance, velocity, duration and frequency of thigmotaxic behavior, swim path, proximity to platform and number of platform crossing were assessed.

Reversal learning was measured on day 6, 7 and 8, whereby the hidden platform was placed in the opposite quadrant. On each day, 3 trials of maximal 60 seconds were performed for each mouse. During reversal learning there was no guiding of the mouse into the direction of the platform, and again every mouse started each trial from a different position. To rule out any visual deficits, a flag was placed on the hidden platform on day 8.

Y-maze

The Y-maze apparatus consists of three arms which are joined in the middle to form a Y shape. This test is based on the innate curiosity of mice to explore novel areas and all arms were identical without any clues. At the first day a hidden platform was placed ~0.5 cm under the water surface in one of the arms of the maze. After this each mouse was placed in the start arm and allowed to explore the maze and find the hidden platform within 60 seconds. If the mouse found the platform within 60 seconds, it was picked up from the platform and placed back in their drying cage. However, if the mouse failed to find the platform within 60 seconds, it was guided to the platform and only rescued from the platform. To determine the learning capacity of each mouse, trials (60 seconds) were given until each mouse reached a score of 4 out of 5 consecutive successful trials. Success was determined if the first decision of the mouse was to go from the home arm to the arm containing the platform and failure was determined as the first choice to the arm without platform.

The LTM and reversal learning capacity of each mouse was determined on the second day. Again, each mouse was given a 60 second trial in the same way as described on day 1, whereby the hidden platform was still in the same arm. For the LTM test the criteria of success and failure were similar as described on day 1. After the LTM test, mice were placed back for 1.5 hours in their home cage. During this interval the hidden platform was switched to the opposing arm. For the reversal test each mouse was given again trials of 60 seconds, whereby the mouse was blocked for 20 seconds by a barrier (arm of the researcher), when they entered the wrong arm. In this manner we ensured aversive association with the previously learned hidden platform location. The trials (with the 20 seconds block by a barrier) were repeated until each mouse first decision was to go from the home arm to the correct arm. The criteria for success was no determined by reaching 9 out of 10 consecutive successful trials.

2.7 Social behavioral test

Three chamber social interaction test

The main principle of the three-chamber social interaction test is that mice normally prefer spending time with another mouse over that of a novel object (sociability). In addition, mice will normally spend more time exploring a novel intruder compared with a familiar one (social novelty). Thus, the experimental design of this test evaluates two important, but distinguishable aspects of social behavior (Kaidanovich-Beilin et al, 2011).

The test consists of 3 trials of 5 minutes for each mouse. At the start of each trail, the mouse was placed in the center quadrant, and the gates were removed so that the mouse could

choose between the left or right chamber. After each trial the mouse was guided back to the center quadrant of the box after which the gates were closed. In the first trial there was no mouse or object placed in the left and right chambers. In the second trial a stranger mouse and a novel object were placed in one of the chambers. Finally, in the third trail the object from the second trial was replaced by a new stranger mouse, meanwhile the stranger mouse of trial 2 remained in the same chamber. Time spent in each chamber and interaction time were measured, even as the preference index. The preference index was calculated by dividing the total time spent with the novel object or mouse by the time spent with the novel and familiar object or mouse.

2.9 Statistical analysis

Statistical analyses were performed using Graphpad Prism Version 8.1.2 (GraphPad Software, La Jolla California USA). All data was assumed to be normally distributed and tested by a two-way ANOVA, with a Tukey test as post hoc test. Furthermore, for comparison between SOM-*Fmr1*$^{-/y}$ and WT mice only, an unpaired T-test was used. P-values ≤ 0.05 (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****) were considered to indicate statistical significance. P values of $p \leq 0.10$ (#) were considered to indicate a trend for a difference. In the graphs data are expressed as the mean ± standard error of mean (SEM).

3. RESULTS

In the following sections, results of PV-*Fmr1*$^{-/y}$ and WT mice will be shorty mentioned. These results were already available while writing this report (data graphs are not shown). After this, the results of the different behavioral tests performed in SOM-*Fmr1*$^{-/y}$ and WT mice will be described. These results will follow the same structure as described in the materials & methods section, which means that the outcomes of the anxiety / repetitive and locomotor behavior tests will be described first, followed by the outcomes of the cognitive behavior tests and in the end by the results of the social behavior test in in SOM-*Fmr1*$^{-/y}$ and WT mice.

3.1 FMRP deletion in PV interneurons led to behavioral phenotypes observed in *Fmr1* KO mice

PV-*Fmr1*$^{-/y}$ mice showed a specific set of autistic-like behaviors, such as mild anxiety, impaired social interactions with deficits in behavioral flexibility and mild impairments in spatial memory (data graphs not shown). Thus, PV interneurons contribute to specific aspects of behavioral phenotype observed in *Fmr1* KO mice, but most of the behavioral test results of PV-*Fmr1*$^{-/y}$ mice were not significant and only slightly different compared to WT mice.

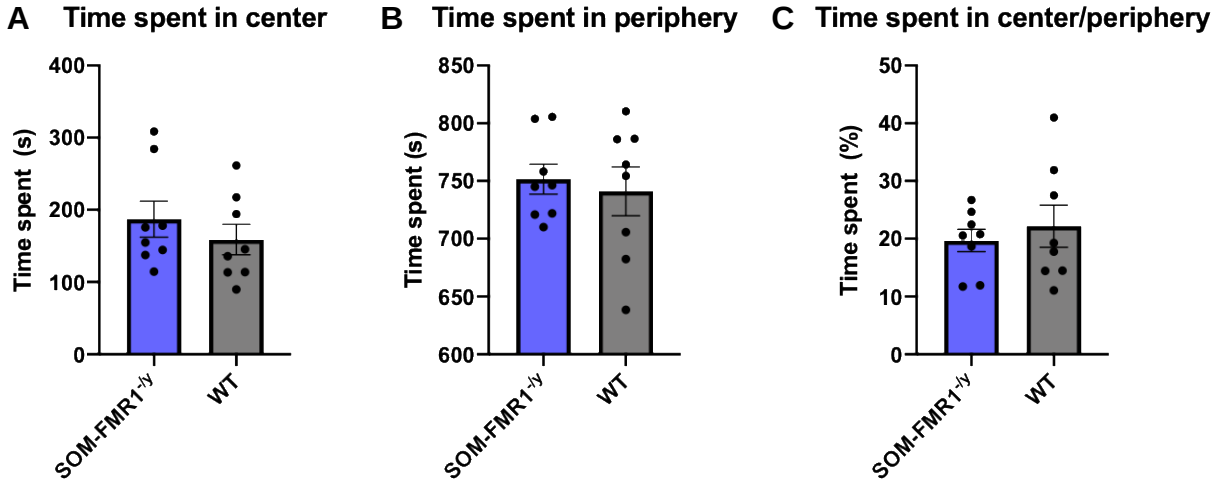
3.2 SOM-*Fmr1*$^{-/y}$ mice showed significant increased repetitive behavior in open field test

The results of the open field test can be divided in anxiety, expressed in time spent in the center or periphery and the percentage of time spent in the center relative to periphery, locomotor behavior, expressed in distance travelled and velocity and repetitive behavior, expressed in the number of stereotypic counts and jumps. This study found no significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice in time spent in center or periphery, and the percentage of time spent in center compared to periphery (figure 1 A, B & C). Despite this, the results showed that SOM-*Fmr1*$^{-/y}$ mice spent on average slightly more time in the center and periphery.

In addition, the total distance traveled is higher in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice, although these results are not significant (figure 1D). If we look at the results of distance traveled in time blocks, we see as expected based on the total distance traveled, that SOM-*Fmr1*$^{-/y}$ mice traveled more than WT mice in each of the 5-minute time blocks. Moreover, the distance traveled in WT mice was significantly lower between time block 1 and time block 2 (p=0.0239), and between time block 1 and time block 3 (p=0.0015), as shown in figure 1E. These significant differences between distance traveled in the different time blocks was not observed in SOM-*Fmr1*$^{-/y}$ mice (figure 1E). Results of velocity showed a higher average velocity in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice, although these results were not significant (figure 1F & 1G).

Lastly, this study found a significant higher number of total stereotypic counts in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice (p=0.0155, figure 1H). When divided in time blocks of 5 minutes, SOM-*Fmr1*$^{-/y}$ mice showed a higher amount of stereotypic counts in each of the time blocks compared to WT mice, as shown in figure 1I. The stereotypic counts of the WT mice were also significantly lower in the third time block compared to the first-time block (p=0.0214, figure 1I), meanwhile the amount of stereotypic counts remained more or less the same in the different time blocks for SOM-*Fmr1*$^{-/y}$ mice.

The results of number of stereotypic counts were comparable to the results of the number of jump counts, whereby SOM-*Fmr1*$^{-/y}$ mice showed a higher number of total jump counts compared to WT mice. However, these results were no longer significant (figure 1J). The number of jump counts divided in time blocks of 5 minutes, showed also a higher number of jumps for the SOM-*Fmr1*$^{-/y}$ mice compared to WT mice, as shown in figure 1K. The number of jumps in time block 3 was lower than in time block 1 for WT mice (figure 1K), but no longer significant as it was by the number of stereotypic jumps.



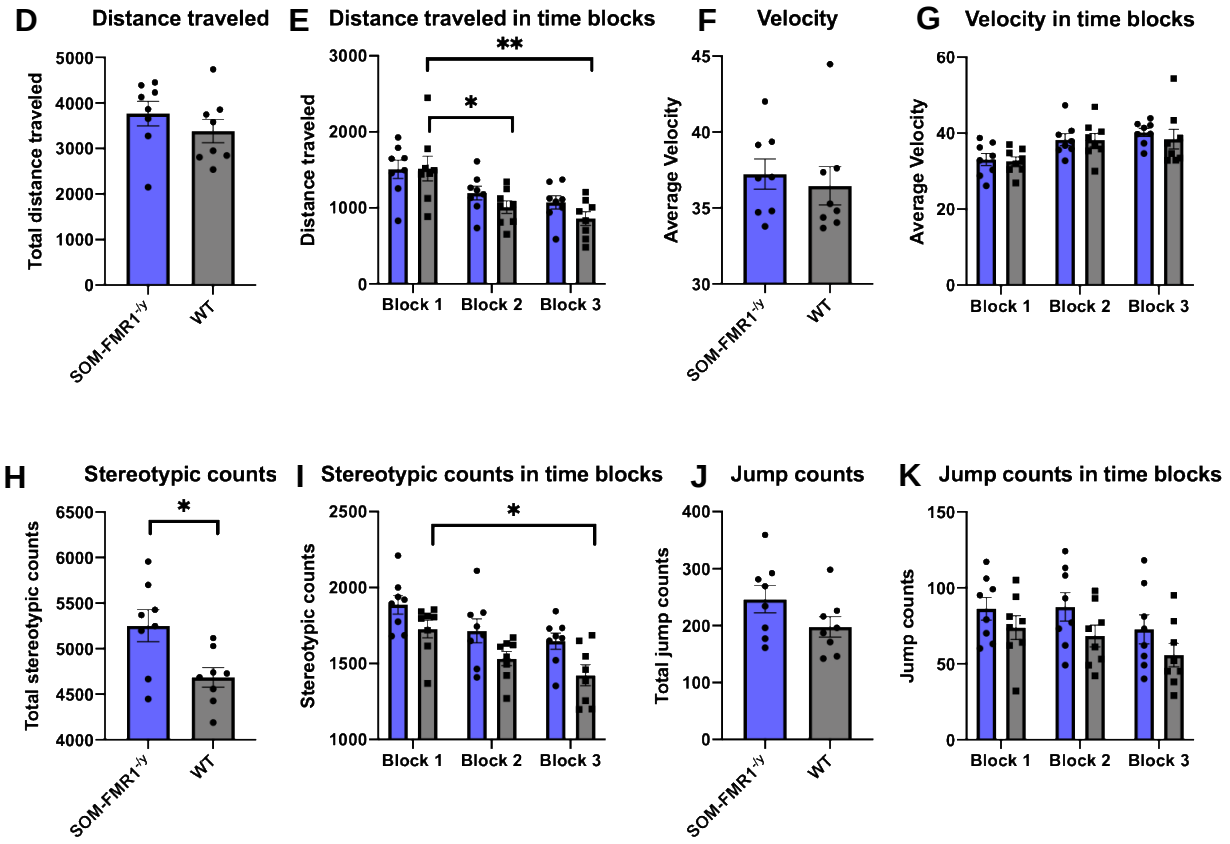


Figure 1: Open field test. *No significant differences were found between SOM-Fmr1$^{-/y}$ and WT mice in time spent in center (1A) or periphery (1B) and the percentage of time spent in center relative to periphery (1C). The total distance traveled was also not significant between both groups (1D), meanwhile the distance traveled in time blocks was significant lower in block 2 and 3 compared to block 1 for WT mice only, whereby every time block indicates 5 minutes (1E). Results of average velocity (1F) and velocity in time blocks (1G) showed no significant differences between SOM-Fmr1$^{-/y}$ and WT mice. Furthermore, a significant higher number of total stereotypic counts was found in SOM-Fmr1$^{-/y}$ mice compared to WT mice (1H) and the number of stereotypic counts was significantly lower in time block 3 compared to time block 1 in WT mice (1I). Finally, SOM-Fmr1$^{-/y}$ mice showed a higher number of total jumps compared to WT mice, although these results were not significant (1J). Between the different time blocks for the number of jumps were no significant differences found in SOM-Fmr1$^{-/y}$ and WT mice as well (1K).*

3.3 SOM-*Fmr1*$^{-/y}$ and WT mice showed no significant differences in elevated plus maze, marble burying, grooming and rotarod behavioral tests

The results of the elevated plus maze showed no significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice, as shown in figure 2. Nevertheless, the results showed that SOM-*Fmr1*$^{-/y}$ mice spent slightly less time in the open arms and slightly more time in the closed arms and center area compared to WT mice (figure 2 A, B & C). In addition, SOM-*Fmr1*$^{-/y}$ mice showed slightly lower number of entries in the open and closed arms compared to WT mice (figure 2 D & E).

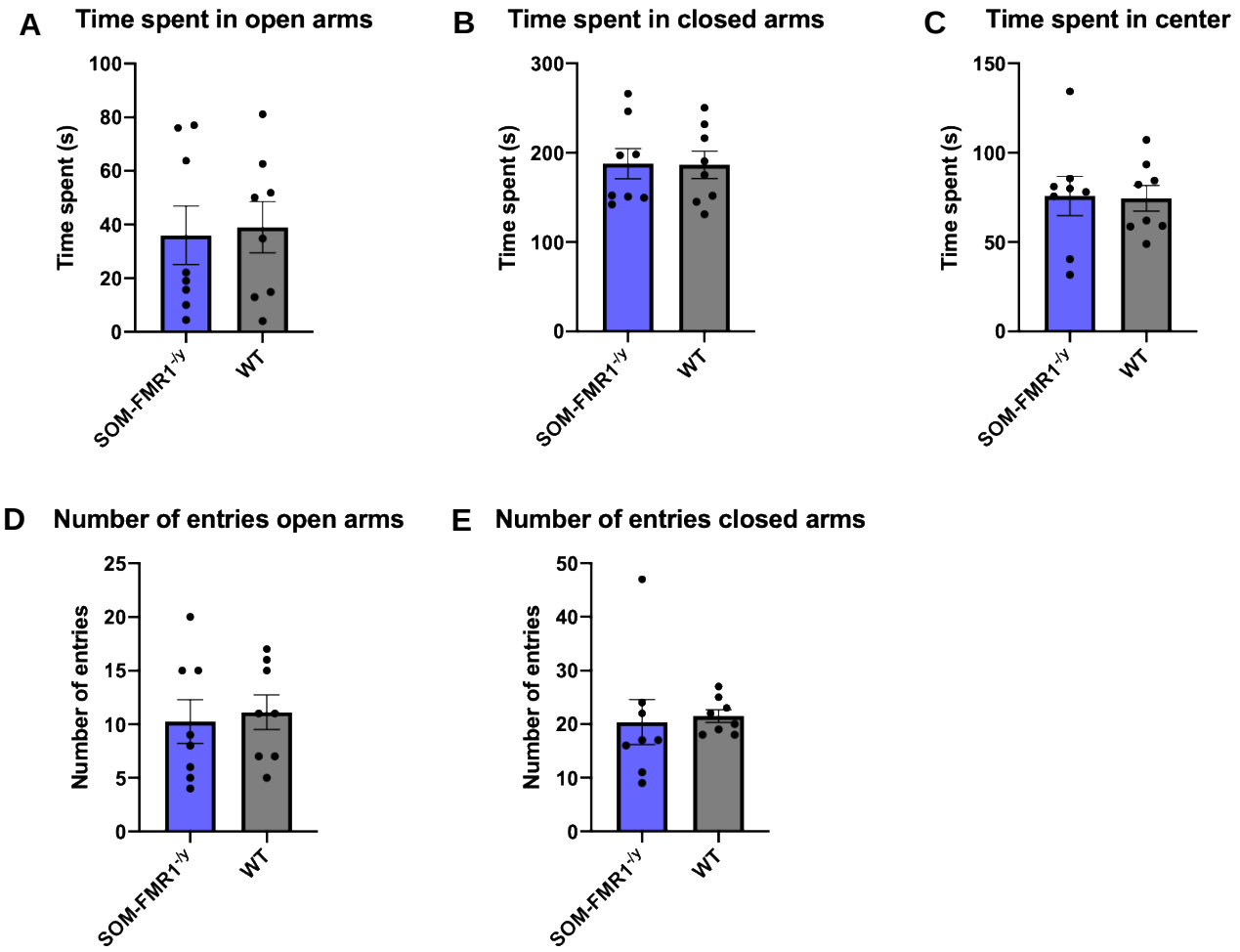


Figure 2: Elevated plus maze test. *No significant differences were found between SOM-Fmr1$^{-/y}$ and WT mice in time spent in open arms (2A), closed arms (2B) or center (2C). The number of entries in open arms (2D) and closed arms (2E) showed also no significant differences between both groups of mice.*

Results of marble burying showed an average lower number of marbles buried and less time spent digging in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice (figure 3 A & C), although these results were not significant. Moreover, the number of marbles buried after 10, 20 and 30 minutes showed no major differences between SOM-*Fmr1*$^{-/y}$ and WT mice. After 10 minutes SOM-*Fmr1*$^{-/y}$ mice buried more marbles compared to WT mice, meanwhile the number of marbles buried after 20 and 30 minutes was lower in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice (figure 3B).

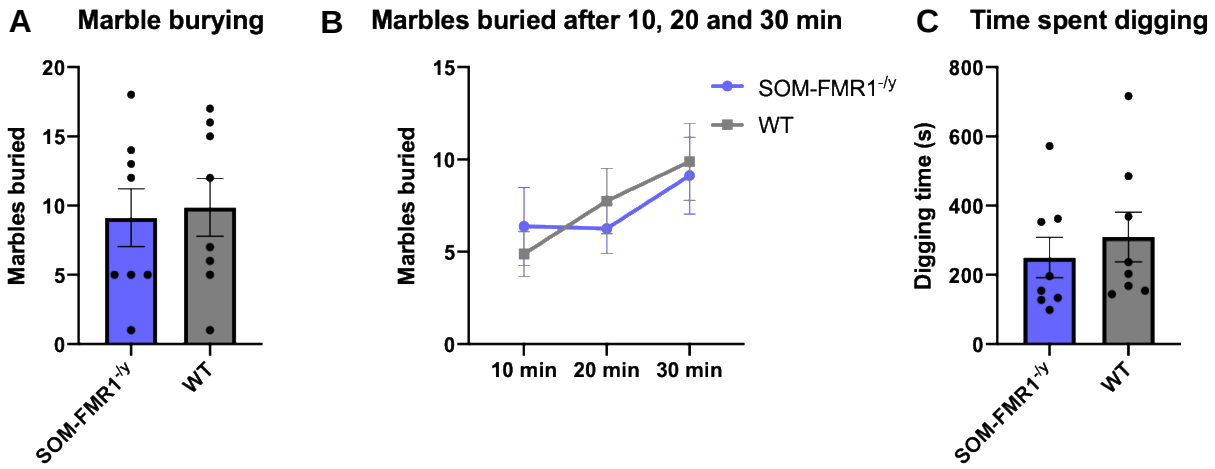


Figure 3: Marble burying test. *The results of number of marbles buried after 30 minutes (3A), the number of marbles buried after 10, 20 and 30 minutes (3B) and the digging time (3C) showed no significant differences between SOM-Fmr1$^{-/y}$ and WT mice. Nevertheless, the WT mice seem to burry slightly more marbles and spent more time digging on average.*

The results of the grooming test showed also no significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice. In the time spent grooming after 10, 20 and 30 minutes (figure 4A) and in the average total time spent grooming after 30 minutes (figure 4B) were no significant differences observed between SOM-*Fmr1*$^{-/y}$ and WT mice.

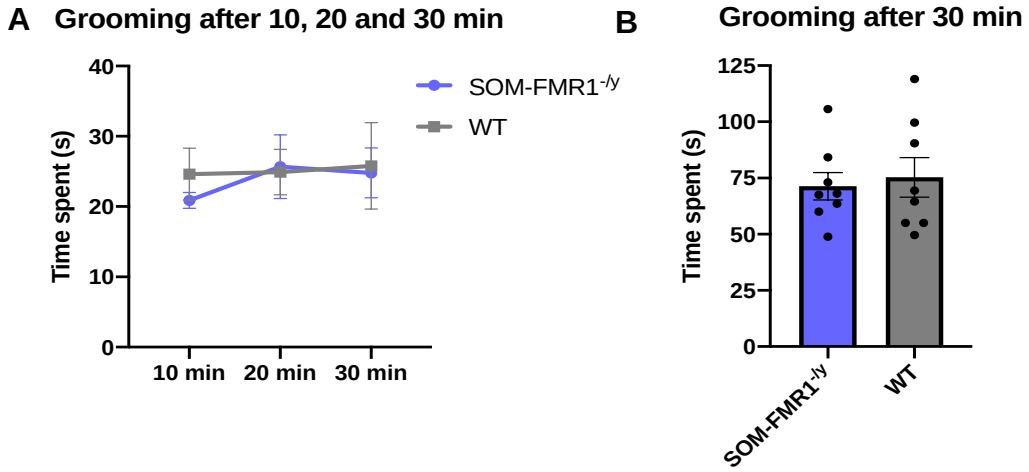


Figure 4: Grooming test. *The results of time spent grooming after 10, 20 and 30 minutes (4A) and the time spent grooming after 30 minutes (4B) showed no significant differences between SOM-Fmr1$^{-/y}$ and WT mice.*

Finally, no major differences were found in time spent on the rotarod during day 1 and day 2 (figure 5 A & B). However, SOM-*Fmr1*$^{-/y}$ mice showed a non-significant shorter latency to fall, on especially day 2 of testing, compared to WT mice. SOM-*Fmr1*$^{-/y}$ mice spent in the first 3 trials of day 2 less time on the rotarod compared to WT mice (figure 5B).

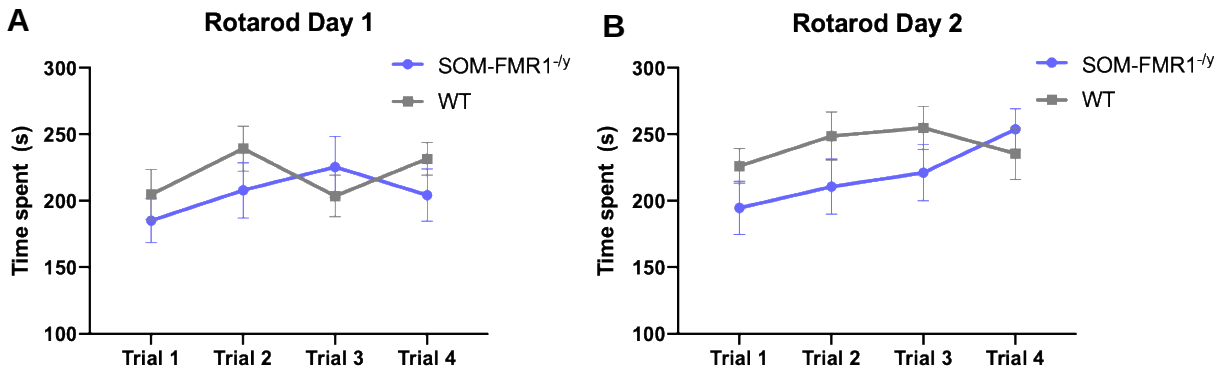


Figure 5: Rotarod test. *No significant differences were found between SOM-Fmr1$^{-/y}$ and WT mice in any of the different trials on both the first (5A) and second day (5B) of the rotarod test. Although, SOM-Fmr1$^{-/y}$ mice spent on average less time on the rotarod in the first three trials of the second day compared to WT mice.*

3.4 SOM-*Fmr1*$^{-/y}$ mice showed normal performance in learning, memory and behavioral flexibility in cognitive behavioral tests

The results of the different cognitive behavioral tests showed no major differences between SOM-*Fmr1*$^{-/y}$ and WT mice. The novel object recognition test showed in the STM memory test for both groups of mice a preference for the novel object compared to the familiar object, as shown in figure 6A. SOM-*Fmr1*$^{-/y}$ mice showed even a trend for a higher interaction time for the novel object compared to the familiar object (p=0.0839). These outcomes are also expressed in figure 6B, where the preference index for the novel object compared to the familiar object was measured. SOM-*Fmr1*$^{-/y}$ and WT mice showed both a preference index of around 0.7 for the novel object versus the familiar object.

In addition, the results of the LTM test showed similar results, whereby SOM-*Fmr1*$^{-/y}$ and WT mice showed both a higher interaction time for the novel object compared to the familiar object (figure 6C). These results were significant for the SOM-*Fmr1*$^{-/y}$ mice (p=0.0117) and a trend for a higher interaction time was found for WT mice (p=0.0661), as shown in figure 6C. The preference index for the long-term memory test was also similar in both groups of mice, with a score around 0.75 for the novel object compared to the familiar object (figure 6D).

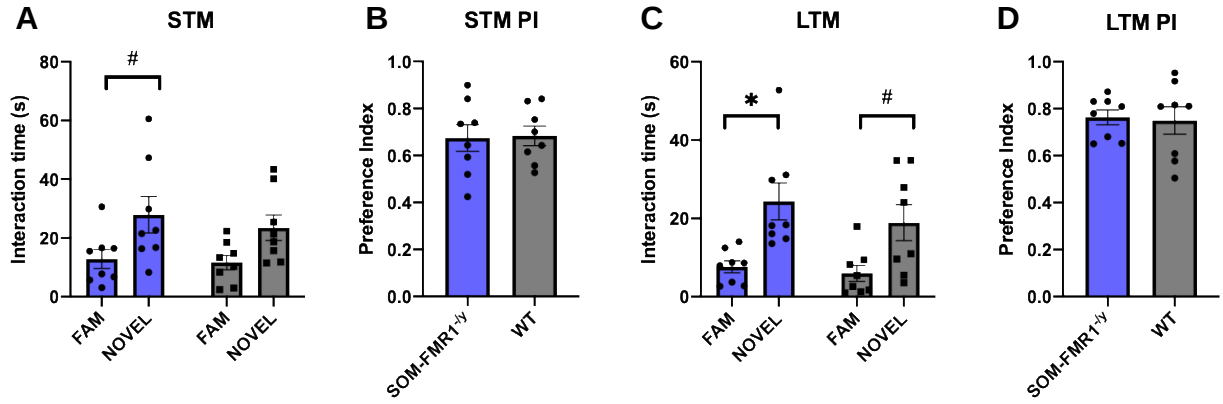


Figure 6: Novel object recognition test. *SOM-Fmr1$^{-/y}$ and WT mice showed in the short-term memory test both a higher interaction time for the novel object compared to the familiar object, whereby SOM-Fmr1$^{-/y}$ mice showed even a trend for a higher interaction time for the novel object compared to the familiar object (6A). The preference index in the short-term memory test was in both groups of mice the same (6B). The long-term memory test showed similar results, with a clear preference for the novel object compared to the familiar object (expressed in interaction time) in SOM-Fmr1$^{-/y}$ and WT mice (6C). These preferences for the novel object were significant for SOM-Fmr1$^{-/y}$ mice and a trend was found in WT mice. Finally, both groups of mice showed a similar clear preference index for the novel object compared to the familiar object (6D).*

The second test performed for cognitive capacity was the T-maze test, which tests working memory. This test showed no significant differences between SOM-*Fmr1$^{-/}$* and WT mice, as shown in figure 7. Nevertheless, the percentage of correct alterations was slightly lower in SOM-*Fmr1$^{-/y}$* mice compared to WT mice

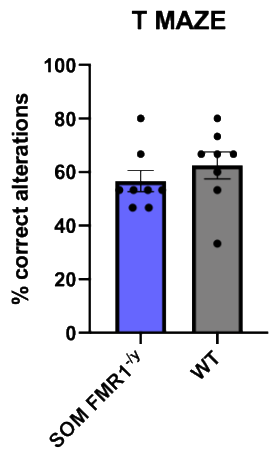


Figure 7: T-maze test. *The percentage of correct alterations was not significantly different between SOM-Fmr1$^{-/y}$ and WT mice. Although the percentage of correct alterations was slightly lower in SOM-Fmr1$^{-/y}$ mice compared to WT mice.*

The next test was the MWM, which consisted of 8 consecutive days divided in a training phase (day 1- day 4), probe trial (day 5) and reversal phase (day 6 – day 8). Results of the MWM training phase showed no significant differences in escape latency between SOM-*Fmr1$^{-/y}$* and WT mice (figure 8A). The escape latency is the time it took for the mice to find the hidden platform. Both groups of mice showed a decreased escape latency as a function of training time, compared to the first day of training (figure 8A). Furthermore, the probe trial showed no significant differences in the amount of target crossings between SOM-*Fmr1$^{-/y}$* and WT mice (figure 8B).

In addition, both groups of mice showed a similar score for the percentage of time spent in the different quadrants, as shown in figure 8C and 8D. SOM-*Fmr1$^{-/y}$* and WT mice spent more time in the target quadrant, which is the quadrant where the hidden platform was placed during the training phase (figure 8C). SOM-*Fmr1$^{-/y}$* mice showed a significant lower percentage time spent in the left quadrant compared to target quadrant (p=0.0237), in the right quadrant compared to the target quadrant (p=0.0058) and in the opposite quadrant compared to the target quadrant

(p=0.0072), as shown in figure 8D. Similar results were found for the WT mice, whereby a significant lower percentage of time was spent in the left quadrant compared to the target quadrant (p=0.0002), the right quadrant compared to the target quadrant (p=0.0011) and for the opposite quadrant compared to the target quadrant (p=0.0001), which is also shown in figure 8D.

Lastly, the MWM reversal phase, whereby the hidden platform was placed in the opposite quadrant compared to the training phase, showed no significant differences in escape latency between SOM-*Fmr1*$^{-/y}$ and WT mice, as shown in figure 8E. Both groups of mice were learning the task, indicated by a decrease of the escape latency in the third day compared to first day (figure 8E). No significant differences were also found in the escape latency between SOM-*Fmr1*$^{-/y}$ and WT mice in the flag test, which was used to measure visibility in mice (figure 8F).

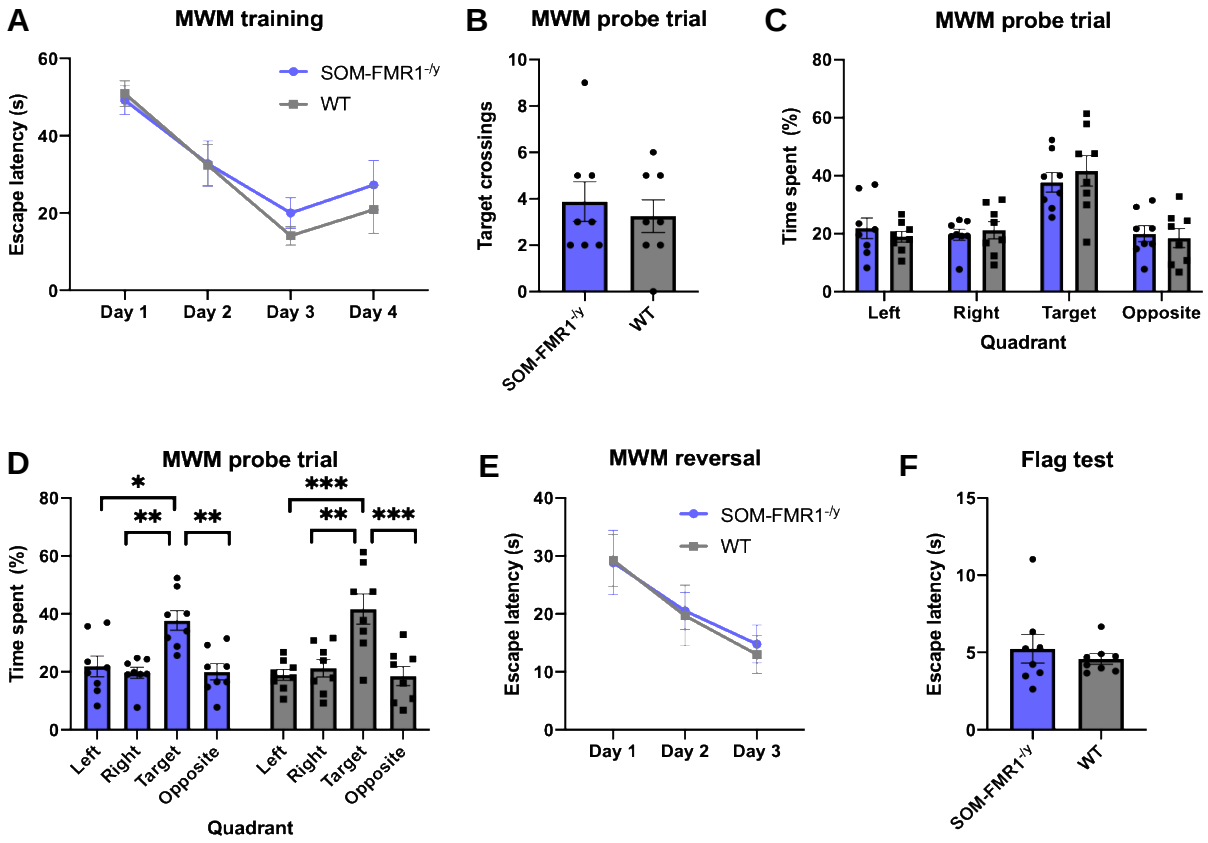


Figure 8: MWM test. *The MWM training phase showed no significant differences between SOM-Fmr1*$^{-/y}$ *and WT mice. Both groups of mice learned the task and showed a decreased escape latency in the last days of the training phase compared to the first day (8A). The probe trial showed no significant differences between the amount of target crossings (8B) and both groups of mice spent significant more time in the target quadrant compared to the left, right or opposite quadrant (8C & 8D). The MWM reversal phase showed also no significant differences between SOM-Fmr1*$^{-/y}$ *and WT mice, whereby both groups of mice learned the task and showed a decreased escape latency at the last day of the test compared to the first days (8E). At the end, the flag test was performed and showed no significant differences in escape latency between SOM-Fmr1*$^{-/y}$ *and WT mice, indicating no problems in visibility in both groups of mice (8F).*

The last test performed in the set of cognitive behavioral tests was the Y-maze. This test is made up of a training phase, LTM measurement and reversal phase. The results are depicted in trial blocks, whereby every trial block consisted of three different trials. Correct arm choices were measured and showed no significant differences in the training phase between SOM-*Fmr1*$^{-/y}$ and WT mice, as shown in figure 9A. Both groups of mice learned the task in the same way, whereby after 4 trial blocks the 100% score of correct arms was reached.

Results of the LTM test were similar, both SOM-*Fmr1*$^{-/y}$ and WT mice reached the 100% score of correct arm choices after a total of 5 trials (figure 9A). The reversal phase of the Y-maze test showed also similar cognitive performance in both groups of mice, whereby after 5 trial blocks the 100% score of correct arm choices was reached. These results indicate that SOM-*Fmr1*$^{-/y}$ and WT mice learned the task in a similar way (figure 9A). Finally, the number of reversal trials was measured, but showed no significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice. However, SOM-*Fmr1*$^{-/y}$ mice needed on average slightly more reversal trials to reach the 100% score of correct arm choices, as shown in figure 9B.

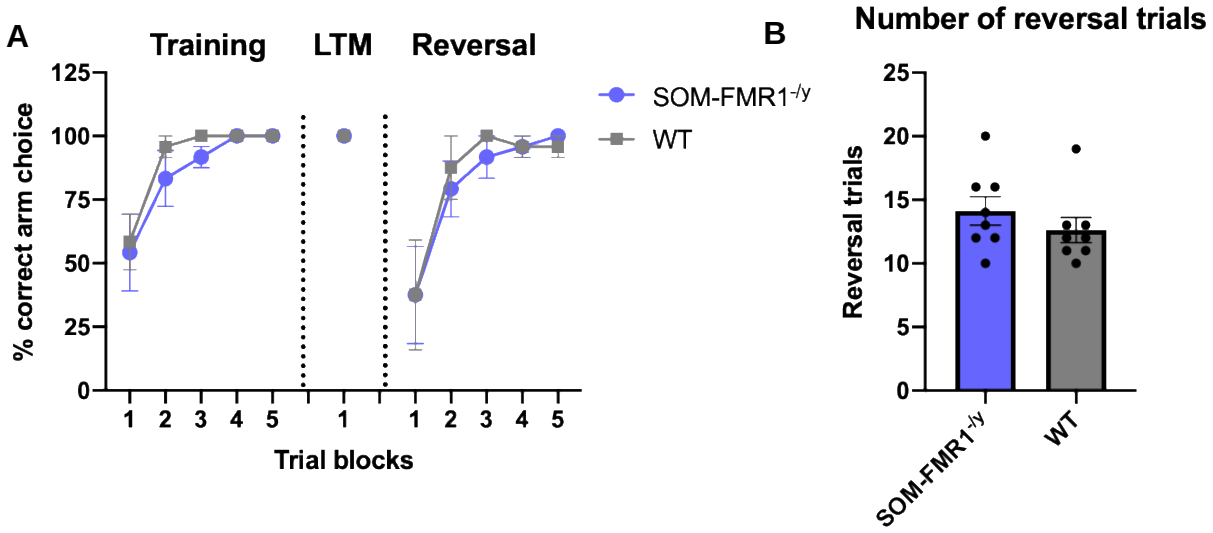


Figure 9: Y-maze test. *No significant differences in percentage of correct arm choices were found between SOM-Fmr1*$^{-/y}$ *and WT mice in the training, LTM and reversal phase of the Y-maze test (9A). Both groups of mice learned the task after a similar amount of trial blocks, whereby every trial block consisted of 3 trials. The number of reversal trials showed no significant differences between SOM-Fmr1*$^{-/y}$ *and WT mice (9B).*

3.5 SOM-*Fmr1*$^{-/y}$ showed significant impairments in sociability, but not in social novelty

The three-chamber social interaction test was used to test sociability and social novelty. During the habituation phase SOM-*Fmr1*$^{-/y}$ and WT mice spent more time in the left and right chamber compared to the starting chamber in the center, as shown in figure 10A. SOM-*Fmr1*$^{-/y}$ mice showed a trend for more time spent in the left chamber compared to the center chamber (p=0.0584) and a significant more time was spent in the right chamber compared to the center chamber (p=0.0012). Similar results were found for WT mice, whereby a trend was observed for significant more time spent in the right chamber compared to the center chamber (p=0.0612). These results showed the normal tendency of mice to explore novel areas.

However, results of the sociability test were more interesting, during this test mice had the choice between an object or a novel stranger mouse 1, as described in the experimental setup in the materials & methods section. SOM-*Fmr1*$^{-/y}$ mice showed no significant preference for spending time in the chamber of the object or stranger mouse 1(figure 10B), meanwhile WT mice spent significantly more time in the chamber with the stranger mouse 1 compared to the chamber with the object (p= < 0.0001, figure 10B).

Additionally, the interaction time for object or stranger mouse 1 was different between both groups of mice. SOM-*Fmr1*$^{-/y}$ mice showed only a trend for more time spent in the interaction zone with stranger mouse 1 compared to the object (p=0.0723, figure 10C). On the other hand, WT mice spent significantly more time in the interaction zone of the stranger mouse 1 compared to the object (p= < 0.0001), which is also shown in figure 10C. Furthermore, the preference index between stranger mouse 1 and the object was measured, whereby SOM-*Fmr1*$^{-/y}$ mice showed a lower preference index for the stranger mouse compared to the object than WT mice (figure 10D). Although these results were no longer significant.

Finally, the three-chamber social interaction test was used to assess preference for social novelty in SOM-*Fmr1*$^{-/y}$ and WT mice. These results showed no significant differences, but SOM-*Fmr1*$^{-/y}$ mice spent more time in the chamber with novel stranger mouse (stranger 2) compared to stranger mouse 1 (figure 10E), meanwhile WT mice spent more time in the chamber of stranger mouse 1 compared to novel stranger mouse 2, also depicted in figure 10E. Despite these results, both SOM-*Fmr1*$^{-/y}$ and WT mice spent more time in the interaction zone of the new stranger mouse 2 compared to stranger mouse 1, as shown in figure 10F, although the results for both groups were not significant. The preference index for novel stranger mouse 2 compared to stranger mouse 1 showed also no significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice. However, the preference index for novel stranger mouse 2 compared to stranger mouse 1 was slightly higher in the SOM-*Fmr1*$^{-/y}$ mice compared to the WT mice (figure 10G).

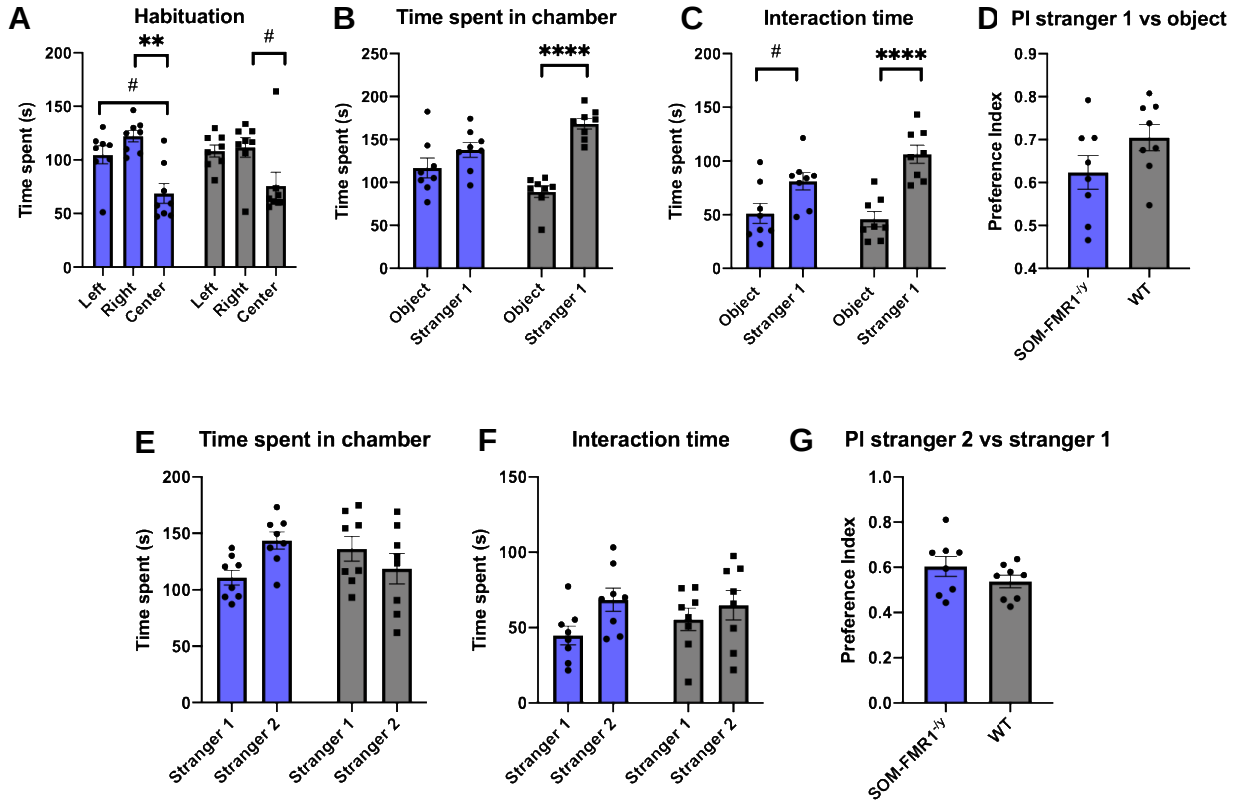


Figure 10: Three chamber social interaction test. *During the habituation phase SOM-Fmr1$^{-/y}$ and WT mice spent more time in the left and right chamber compared to the center chamber (10A). The time spent in the chamber and interaction zone of the object and stranger mouse 1 was not significantly different in SOM-Fmr1$^{-/y}$ mice, meanwhile WT mice showed a significant preference for spending time with stranger mouse 1 compared to the object (10B & 10C). This was also translated in a higher preference index for stranger mouse 1 in WT mice compared SOM-Fmr1$^{-/y}$ mice (10D). The time spent in chamber and interaction time of stranger mouse 1 and 2 showed no significant differences between SOM-Fmr1$^{-/y}$ and WT mice (10E & 10 F), whereby both groups of mice seemed to have a minor preference in interaction time for novel stranger mouse 2 compared to stranger mouse 1. This is also expressed in the preference index for stranger mouse 2 compared to stranger mouse 1 (10G).*

3.6 Summary SOM-*Fmr1*$^{-/y}$ mice behavioral test results

In summary, results of the different behavioral tests performed in this study showed a significant difference in repetitive behavior, as shown in significant higher number of stereotypic counts for SOM-*Fmr1*$^{-/y}$ mice compared to WT mice. Moreover, another measurement of repetitive behavior, the number of jumps was also higher in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice, but no longer significant. However, the other tests for anxiety, repetitive and locomotor behavior showed only minor or slight impairments in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice. Results of the cognitive behavioral tests showed no major differences between SOM-*Fmr1*$^{-/y}$ and WT mice. The novel object recognition, T-maze, Morris water maze and Y maze showed all normal cognitive learning and performance in SOM-*Fmr1*$^{-/y}$ mice comparable to WT mice.

Nevertheless, the results of the three-chamber social interaction tests were more striking. SOM-*Fmr1*$^{-/y}$ mice showed in our study major impairments in sociability, whereby no clear preference was found in time spent in the chamber or interaction zone of the object compared to

the stranger mouse 1. In contrast to WT mice, which showed a significant preference for spending more time in the chamber and interaction zone of the stranger mouse 1 compared to the object. However, the results of social novelty (stranger mouse 1 versus stranger mouse 2) showed no major differences between SOM-*Fmr1*$^{-/y}$ and WT mice. Table 1 shows also an overview of the behavioral test results found in our study between SOM-*Fmr1*$^{-/y}$ and WT mice.

Table 1: Overview of behavioral test results of SOM-*Fmr1*$^{-/y}$ and WT mice.

	SOM-*Fmr1*$^{-/y}$	WT
EPM	✓	✓
Open field	✗ significantly increased number of stereotypic counts	✓
Marble burying	✓	✓
Grooming	✓	✓
Rotarod	✓	✓
NOR	✓	✓
T-maze	✓	✓
MWM	✓	✓
Y-maze	✓	✓
Three-chamber social	✗ impaired sociability	✓

Note: SOM-Fmr1$^{-/y}$ *mice showed significant increased numbers of stereotypic counts in the open field test and significantly impaired sociability in the three-chamber social interaction test. The other behavioral test results showed no significant differences between SOM-Fmr1*$^{-/y}$ *and WT mice.*

4. DISCUSSION

In this study a battery of behavioral tests for anxiety, repetitive, locomotor, cognition and social behavior were performed, with as consequence a lot of data. The discussion section starts with discussing the results of SOM-*Fmr1*$^{-/y}$ mice. Especially the repetitive and social behavior tests will be discussed, because these tests showed some significant differences between SOM-*Fmr1*$^{-/y}$ and WT mice. After this, the behavioral test results of SOM-*Fmr1*$^{-/y}$ and PV-*Fmr1*$^{-/y}$ mice will be compared and possible differences in behavioral phenotypes will be discussed in depth in the last sections of the discussion.

4.1 SOM interneuron inhibitory projections from cortex to striatum could be disturbed, which lead to repetitive behavior observed in SOM-*Fmr1*$^{-/y}$ mice

One of the key findings of this study is the significantly increased number of stereotypic counts in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice. Furthermore, SOM-*Fmr1*$^{-/y}$ mice showed an increase in the number of jumps compared to WT mice. Both the number of stereotypic counts and the number of jumps are measurements of repetitive behavior. Nowadays several studies studied repetitive behavior and concluded that various brain regions and pathways are involved, whereby the cortico-basal ganglia-thalamo cortical loop (CBGTC loop) is the most studied one (McGeorge & Faull, 1989; Parent & Hazrati, 1995; Bolam et al, 2000; Kim et al, 2016).

This loop starts with excitatory input from the cortex to the striatum. The striatum consists for 95% out of GABAergic spiny projection neurons (SPNs) and for 5% out of interneurons (5%) (Bolam et al, 2000). After receiving the excitatory input out of the cortex,

SPNs in the striatum projects into the direct pathway and indirect pathway that regulate motor function. Whereby the direct pathway is involved in activation of movements and the indirect pathway is involved in the inhibition of movements. Furthermore, dopaminergic receptors in the substantia nigra can activate and inactivate the direct and indirect pathways by expressing GABAergic inhibitory or glutamatergic excitatory neurons (Parent & Hazrati 1995; Balom et al, 2000; Kim et al, 2016). Thus, a balanced excitation and inhibition via the direct and indirect pathway of the CBGTC loop is crucial for motor output and this balance seems to be disturbed in FXS and could lead to repetitive behavior.

Interneurons play and important role in regulating the balance of motor activity. SPNs from the striatum and different classes of interneurons are interconnected in highly organized synaptic micro circuitry and communicate using numerous neurotransmitters and neuromodulators to modulate striatal excitability (Burke et al, 2017). But what is the specific role of SOM interneurons in this process? First, a study with *Fmr1* KO mice showed increased GABAergic neurotransmission in the striatum (Centonze et al, 2008). This could indicate that in the direct pathway, which is involved in the activation of movements, now more GABAergic neurotransmission takes place from the striatum to other brain regions as consequence of FMRP deletion in SOM interneurons. The consequence of increased GABAergic neurotransmission results in more excitatory projections to the motor cortex, resulting in an abnormal activation of movements and possible repetitive behavior. This overactivation of the direct pathway must be stronger and counteract the increased inhibition of movements as consequence of increased inhibition of GABAergic neurotransmission in the indirect pathway. Only in this manner is the balance between excitation and inhibition disturbed, which underlies the pathophysiology of FXS.

Secondly, several anatomical and physiological studies have showed that the striatum receives excitatory glutamatergic projections from the cortex (McGeorge & Faull, 1989). However, the study of Rock et al, showed with the help of optogenetics, that GABAergic neurons also project directly to the striatum from the motor cortex (Rock et al, 2016). Direct inhibitory influence from the cortex, mostly via corticocstriatal SOM interneurons, on the output of SPNs in the striatum was found. These results describe a corticostriatal long-range inhibitory circuit underlying the control of both SPNs of the direct and indirect pathway (Rock et al, 2016). Thus, disturbances in SOM interneuron functioning, as consequence of FMRP deletion, could lead to less direct inhibitory long-range projections directly from the cortex to striatum, whereby the striatum is now more active and responsible for increased motor function and repetitive behavior.

Nevertheless, our study found only significant increased number of stereotypic counts and a higher number of jumps in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice. Other tests for repetitive behavior, such as grooming and marble burying showed no significant or major differences between SOM-*Fmr1*$^{-/y}$ and WT mice. Therefore, more research is necessary into the effects of FMRP deletion in interneurons and neuronal circuits underlying repetitive behaviors observed in *Fmr1* KO mouse models.

4.2 The specific role of FMRP deletion in SOM interneurons and the effects on social behavior remain unclear

This study showed impairments in sociability in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice. Social impairments are one of the main symptoms of ASD, including FXS. We found no significant differences in SOM-*Fmr1*$^{-/y}$ mice between time spent in the chamber of the object and in the chamber of the novel stranger mouse, meanwhile WT mice spent significant more time in the chamber of the novel stranger mouse compared to the object. These observations indicate impairments in sociability, whereby sociability is defined as mice spending more time in the chamber containing the novel target mouse than in the chamber containing the inanimate novel object (Silverman et al, 2010). However, other studies with *Fmr1* KO mouse models found contradictory results of the three-chamber social interaction test. A few studies found normal sociability with no genotype differences, meanwhile other studies showed abnormal sociability in *Fmr1* KO mice with no preference for the novel mouse (Kazdoba et al, 2014). Therefore, the effects of *Fmr1* silencing and FMRP deletion in regulating social behavior remains unclear.

The main brain circuit involved in regulating social behavior is the corticolimbic circuitry, which includes the prefrontal cortex (PFC) (Riga et al, 2014). The PFC mainly consists of excitatory cells, which form 80-90% of total population. GABAergic interneurons form the rest of the population of neurons in the PFC (Riga et al, 2014). Direct alterations in the excitatory/inhibitory balance within in the PFC has a strong effect on social behavior as described in the study of Yizhar et al. In this study was showed, with the help of optogenetic techniques, that activation of PFC excitatory pyramidal neurons leads to disrupted social interactions and preference in the three-chamber social interaction test (Yizhar et al, 2011). On the other hand, the activation of inhibitory interneurons showed no effect on social behavior (Yizhar et al, 2011). Thus, only elevation and not reduction of the excitatory/inhibitory balance in the PFC seems to play an important role in the regulation of social behavior.

Despite these observations, is the role of FMRP deletion in specific populations of interneurons (also in the PFC) and corresponding effects on social behavior not yet completely understood. This makes it hard to find any evidence for the role of SOM interneurons in social behavior. Our study is the first that found impairments in sociability after specific deletion of FMRP in SOM interneurons. Interestingly, our results are comparable with another study involved in investigating Rett syndrome, which is also a neurodevelopmental disorder with an ASD component. In this study resulted deletion of *MeCP2* (which is the gene responsible for Rett syndrome) in SOM interneurons in a loss of social preference in mice (Mossner et al, 2017). Nevertheless, more research is necessary to investigate how FMRP deletion in specific interneuron populations and microcircuits in PFC and other brain regions is related to impairments in social behavior observed in *Fmr1* KO mice.

4.3. SOM-*Fmr1*$^{-/y}$ mice showed impairments in repetitive behavior and sociability, meanwhile PV-*Fmr1*$^{-/y}$ mice showed impairments in spatial memory, behavioral flexibility and social novelty

As previously mentioned, this study found significantly increased numbers of stereotypic counts in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice, which is used as measurement of repetitive behavior. Furthermore, SOM-*Fmr1*$^{-/y}$ mice showed impairments in sociability, meanwhile WT mice showed normal social behavior. In contrast, PV-*Fmr1*$^{-/y}$ mice showed mild anxiety and

impaired social novelty compared to WT mice. Furthermore, we noticed more target crossings during the probe trial of the MWM and deficits during the training phase of the Y-maze in PV-*Fmr1*$^{-/y}$ mice compared to WT mice. These results indicate impairments in spatial memory and cognitive flexibility in PV-*Fmr1*$^{-/y}$ mice, that were not observed in SOM-*Fmr1*$^{-/y}$ mice. Thus, SOM-*Fmr1*$^{-/y}$ mice showed only impairments in repetitive and social behavior, meanwhile PV-*Fmr1*$^{-/y}$ mainly showed impairments in spatial memory, behavioral flexibility and social novelty. An overview of the behavioral test results is also shown in table 2.

Table 2: Overview of behavioral test results of SOM-Fmr1$^{-/y}$, PV-Fmr1$^{-/y}$ and WT mice

	SOM-*Fmr1*$^{-/y}$	WT	PV-*Fmr1*$^{-/y}$
EPM	✓	✓	✓
Open field	✗ significant increased number of stereotypic counts	✓	✓
Marble burying	✓	✓	✓
Grooming	✓	✓	✓
Rotarod	✓	✓	✓
NOR	✓	✓	✓
T-maze	✓	✓	✓
MWM	✓	✓	✗ significantly decreased number of target crossings in probe trial
Y-maze	✓	✓	✗ significantly decreased correct arm choices and significantly increased number of reversal trials
Three-chamber social	✗ impaired sociability	✓	✗ impaired social novelty

Note: SOM-Fmr1$^{-/y}$ mice showed significant increased numbers of stereotypic counts in the open field test and significantly impaired sociability in the three-chamber social interaction test. In contrast, PV-Fmr1$^{-/y}$ mice showed significant decreased numbers of target crossings in the probe trial of the MWM, decreased correct arm choices and increased numbers of reversal trials in the Y-maze and impaired social novelty in the three-chamber social interaction test. The other behavioral test results showed no significant differences between SOM-Fmr1$^{-/y}$, PV-Fmr1$^{-/y}$ and WT mice.

4.4 Explanations why FMRP deletion in SOM and PV interneurons could lead to different impairments in behavior

Firstly, the differences in repetitive and social behavior between SOM-*Fmr1*$^{-/y}$ and PV-*Fmr1*$^{-/y}$ mice could be partly explained. Melzer et al. identified long-range projecting GABAergic neurons in the primary (M1) and secondary (M2) motor cortex that target the dorsal striatum. These long-range projecting GABAergic neurons comprises both SOM and PV interneurons, which target the direct and indirect pathway of the CBGTC loop (Melzer et al, 2017). Optogenetic stimulation of M1 PV+ and M2 SOM+ projecting neurons reduced locomotion, whereas stimulation of mainly M1 SOM+ projecting neurons enhanced locomotion and thus repetitive behavior (Melzer et al, 2017). Thus, GABAergic SOM and PV interneurons seem to modulate striatal output and motor activity in distinct ways. Perhaps both classes of GABAergic interneurons independently modulate the CBGTC loop and motor function.

Furthermore, PV and SOM interneurons regulate different regions of the striatum. PV interneurons control the dorsolateral striatum, meanwhile SOM interneurons control the dorsomedial striatum (Fino et al, 2018). These regions of the striatum are not directly involved in

motor output and repetitive behavior but are another indicator for distinct pathways and functioning of SOM and PV GABAergic interneurons.

Secondly, our study showed impairments in sociability in SOM-*Fmr1*$^{-/y}$ mice, meanwhile PV-*Fmr1*$^{-/y}$ mice showed only impairments in social novelty. These results correspond to the study of Mossner et al. as mentioned before. This study showed loss of social preference after SOM-specific deletion of *MeCP2*, meanwhile PV-specific deletion of *MeCP2* had no effects on social preference (Mossner et al, 2017). However, in our study PV-*Fmr1*$^{-/y}$ mice showed impairments in social novelty. A possible explanation was found in a recent study, where PV interneurons in the ventral hippocampus are functioning as a discriminator in social memory (Deng et al, 2019). In this study was found that PV interneurons are essential for identifying and distinguishing between familiar and novel mice (Deng et al, 2019). Interestingly, SOM interneuron inactivation in the ventral hippocampus had no impact on social memory, which corresponds with the results of our study where no impairments in social novelty were found in SOM-*Fmr1*$^{-/y}$ mice (Deng et al, 2019). Although, in our study WT mice spent for an unknown reason more time in the chamber of stranger mouse 1 compared to novel stranger mouse 2, as shown in figure 10E. However, the interaction time of both SOM-*Fmr1*$^{-/y}$ and WT mice was higher for the (novel) stranger mouse 2 compared to stranger mouse 1, as shown in figure 10F.

In summary, PV and SOM interneurons seem to have different distinct underlying mechanisms for social behavior, whereby PV interneurons in the ventral hippocampus are specifically involved in social memory. Nevertheless, further insights into the role of FMRP deletion in different classes of interneurons and their influence on social behavior is lacking. Therefore, more research is necessary for finding out how FMRP deletion leads to social impairments observed in *Fmr1* KO mouse models and FXS patients.

Thirdly, we found impaired memory in PV-*Fmr1*$^{-/y}$ mice, meanwhile SOM-*Fmr1*$^{-/y}$ mice showed no differences in memory compared to WT mice. Until now, we know that several studies with *Fmr1* KO mouse models found impairments in training and reversal phase of the MWM, and during tests involved in maze learning (Kazdoba et al, 2014). Meanwhile other studies showed no genotype differences in these tests between *Fmr1* KO and WT mice, as reviewed by Kazdoba et al (Kazdoba et al, 2014). The main brain region involved in cognition, learning and memory is the hippocampus (Keefe & Nadel, 1978; Burgess et al, 2002). The hippocampus showed abnormal synaptic plasticity in *Fmr1* KO mice, with alterations in spine density, length and morphology (Bostrom et al, 2016) Moreover, the absence of *Fmr1* is responsible for increased protein synthesis, which contributes among other things to excessive glutamatergic excitatory signaling and long-term depression in the hippocampus. At last, *in vivo* studies showed impairments in stability and reduced specificity in spatial representations in *Fmr1* KO mice compared to WT mice (Arbab et al, 2018). Thus, deletion of FMRP causes impairments in hippocampal (spatial) functioning in *Fmr1* KO mice.

But how different classes of GABAergic interneurons in the hippocampus influence (spatial) memory is partially unknown. Under normal circumstances PV interneurons drive hippocampal CA1 oscillations and reactivation of CA1, which directly promotes network plasticity and memory formation (Ognjanovski et al, 2017). Removal of PV interneurons from the hippocampal CA1 area in mice led indeed to impairments in spatial memory (Murray et al,

2011). In addition, PV and SOM interneurons control different space coding networks and have distinct activity patterns and stimulation effects in the medial entorhinal cortex, which is the main brain area involved in spatial memory (Kim et al, 2016; Miao et al, 2017). Input into hippocampal CA1 from the entorhinal cortex is associated with fast gamma oscillations (similar like PV), whereas input into other hippocampal brain regions form the entorhinal cortex is associated with slow gamma oscillations and different interneurons subtypes (Colgin et al, 2009; Keeley et al, 2017).

Finally, long-range projecting GABAergic neurons modulate inhibition of the hippocampus and entorhinal cortex (Melzer et al, 2012). Perhaps there is a difference between these long-range projections in PV and SOM interneurons, which is comparable to the situation between the striatum and motor cortex as described earlier in this report. However, despite observations of different oscillatory regimes are neuronal mechanism about why and how specific FMRP deletion in PV interneurons lead to impairments in spatial memory, meanwhile deletion of FMRP in SOM interneurons didn't influence spatial memory, not yet completely understood.

5. CONCLUSION

The aim of this study was to assess whether FMRP deletion in PV and SOM interneurons resulted in behavioral phenotypes observed in *Fmr1* KO mouse models. So far, we know that lack of FMRP transcription underlies the pathophysiology of FXS and that GABAergic interneurons, such as PV and SOM form the main inhibitory system in our brain and control the excitatory/inhibitory balance. *Fmr1* KO mouse models showed deficits in PV and SOM interneuron functioning as result of FMRP deletion, which may lead to hyperexcitability. However, our study is the first that assess whether FMRP deletion in PV or SOM interneurons results in behavioral impairments that are observed in *Fmr1* KO mouse models.

Our results showed significantly increased number of stereotypic counts in SOM-$Fmr1^{-/y}$ mice compared to WT, which is used as measurement of repetitive behavior. Furthermore, SOM-$Fmr1^{-/y}$ mice showed impairments in sociability, meanwhile WT mice showed normal levels of social behavior. In contrast, PV-$Fmr1^{-/y}$ mice showed mild anxiety and impairments in social novelty compared to WT mice. In addition, results of the MWM and Y-maze test showed impairments in spatial memory and cognitive flexibility in PV-$Fmr1^{-/y}$ mice compared to WT mice. Based on these results we conclude that SOM-$Fmr1^{-/y}$ mice show increased repetitive behaviors and impairments in sociability, meanwhile PV-$Fmr1^{-/y}$ mice show impairments in social novelty, spatial memory and cognitive flexibility in spatial memory. Thus, deletion of FMRP in specific classes of interneurons lead to specific impairments of different types of behavioral phenotypes observed in *Fmr1* KO mouse models.

At last, previous studies showed that GABAergic interneurons exhibit morphological, biochemical and electrophysiological diversity, which make interneurons enormously heterogenous. Therefore, further studies (see also the next section about future perspectives) that use optogenetics or electrophysiological techniques are needed to give more insight in the role of specific interneuron classes, responsible mechanisms and cellular and neural circuit properties in relation to behavioral phenotypes observed in *Fmr1* KO mice. Despite this, our study was the

first that showed differential effects on behavioral phenotypes in *Frm1* KO mice, after FMRP deletion in specific classes of interneurons. Our study can be used as starting point for further research and gives novel and cell-type specific insight in the understanding of FMRP in FXS.

6. FUTURE PERSPECTIVES

Limited time was the reason that only two cohorts of SOM-*Fmr1*$^{-/y}$ mice were analyzed and results from the fear conditioning test are missing in this report. Furthermore, other suggestions for future studies are an extra behavioral test for spatial memory for PV-*Fmr1*$^{-/y}$ mice, measurements of *de novo* protein synthesis in SOM-*Fmr1*$^{-/y}$ mice, examination of PV and SOM interneuron density, optogenetic and electrophysiological studies and additional FXS could be used. All of this will be shortly mentioned and explained in the following sections.

6.1 Results of only two cohorts of SOM-*Fmr1*$^{-/y}$ mice were analyzed

While writing this report, there was only time to analyze data from two cohorts of mice. Therefore, the data of 8 SOM-*Fmr1*$^{-/y}$ and 8 WT mice was available for the analysis of the different behavioral tests. Obviously, a sample size that is representative for a given population is necessary. To reach a representative sample size, we used instead of two, three cohorts of mice for this study. The data from the three cohorts of mice together, will give a sample size of about 12 mice for the SOM-*Fmr1*$^{-/y}$ and WT group as well, which is considered as a representative sample size. A final note is that sample sizes should not be too small or too excessive. Small sample sizes undermine the internal and external validity of a study, meanwhile very large sample sizes tend to transform small differences into statistically significant differences (Faber & Fonseca, 2014).

6.2 PV and SOM interneurons have a different role in amygdala and fear conditioning

Due to a lack of time, the results of fear conditioning tests of SOM-*Fmr1*$^{-/y}$ and WT mice were not analyzed. One of the key brain regions involved in fear behavior is the amygdala, which consists for 80% out of glutamatergic neurons and for 20% out of GABAergic interneurons (Krabbe et al, 2018). Processing in the amygdala depends heavily on inhibitory circuit functioning and FXS patients and mouse models showed amygdala dysfunctions (Paluszkiewicz et al, 2011). The amygdala in *Fmr1* KO mouse models is for example characterized by inhibitory deficits, including reduction in frequencies and amplitude of spontaneous inhibitory postsynaptic currents, decreased expression of vesicular GABA, impaired GABA release and reductions in inhibitory synapse numbers (Olmos-Serrano et al, 2010).

Despite these indications for dysfunction of the GABAergic system in the amygdala, results of fear conditioning tests in *Fmr1* KO mice showed various outcomes. Some studies found deficits in delay-cued and contextual fear conditioning, meanwhile other studies found no genotype differences between *Fmr1 KO* mice and WT mice (Kazdoba et al, 2016). In line with these observations, we found no significant differences between PV-*Fmr1*$^{-/y}$ and WT mice in the fear conditioning test. In addition, another study showed that PV and SOM interneurons in the amygdala control the acquisition of fear conditioning through two distinct disinhibitory mechanism (Wolff et al, 2014). This study found that PV and SOM interneurons exhibit different responses to the conditioned and unconditioned stimulus during fear conditioning learning. Exposure to the auditory conditioned stimulus increased firing of PV interneurons in the

amygdala, meanwhile it resulted in damping of SOM interneuron activity (Wolff et al, 2014). Therefore, it could be interesting for our study to compare the results of the fear conditioning test of SOM-*Fmr1*$^{-/y}$ mice with PV-*Fmr1*$^{-/y}$ mice.

6.3 Object-place recognition as extra test for spatial memory in PV-*Fmr1*$^{-/y}$ mice

PV-*Fmr1*$^{-/y}$ mice showed impairments in spatial memory compared to WT mice, although these results were not significant. Performing another extra behavioral test, which is more specific for spatial memory could be interesting for future studies. For example, the object-place recognition task, which exploits the natural exploratory activity of rodents toward spatial novelty to assess the detection of spatial relocation of a known object and is critically dependent on the hippocampus (Oliveira et al, 2010). In this test is each mouse placed in a box with three distinct objects in the training phase. After 24h, each mouse is placed back in a box with the same three objects, whereby one of the three objects are displaced to a novel spatial location. Time spent exploring the displaced and non-displaced objects could be measured (Oliveira et al, 2010). Thus, the object-place test is specific for measuring spatial performance and could be interesting for PV-*Fmr1*$^{-/y}$ mice, who tend to show impairments in spatial memory compared to WT mice.

6.4 *De novo* protein synthesis levels are not yet measured for SOM-*Fmr1*$^{-/y}$

In general, *de novo* protein synthesis is necessary for long-lasting modifications in synaptic strength and dendritic spine dynamics that underlie cognition. FXS patients and *Fmr1* KO mouse models showed increased levels of *de novo* protein synthesis, resulting in deficits in synaptic plasticity and cognition (Darnell & Klann, 2013; Jacquemont et al, 2018). In our study we found increased levels of *de novo* protein synthesis in the hippocampus of PV-*Fmr1*$^{-/y}$ mice, which could explain impairments in (spatial) memory observed in the behavioral tests. However, due to a lack of time are measurements for *de novo* protein synthesis not yet performed in SOM-*Fmr1*$^{-/y}$ mice. Although, the results of the behavioral tests for cognition showed no impairments in SOM-*Fmr1*$^{-/y}$ mice compared to WT mice.

Furthermore, a proportion of FXS patients and *Fmr1* KO mice showed no increase in levels of protein synthesis, meanwhile specific behavioral phenotypes of FXS were observed (Jacquemont et al, 2018). These observations indicate that not every individual with FXS has increased levels of protein synthesis and could explain why treatment focused on the reduction of increased levels of protein synthesis is not yet successful. Perhaps many other factors independent of FMRP are involved in the pathophysiology of FXS.

6.5 Measurements of PV and SOM interneuron density under specific FMRP deletion

FMRP targets over 800 mRNAs according to genome-wide microarrays and sequencing studies. Most of these targets of FMRP are involved in neurodevelopmental processes, such as neurite growth, spine development, neuronal signaling and synaptic functioning. (Lee et al, 2019). Several studies in post-mortem human FXS patients and *Fmr1* KO mouse models found defects in synaptic plasticity and increased numbers of long thin dendritic spines, which shows immature morphology (Darnell & Klann, 2013; Lee et al, 2019). Normal plasticity and spine dynamics are essential for normal synaptic communication and functioning of neuronal brain circuits, whereby FMRP deletion showed to cause impairments in functioning of these circuits (Darnell & Klann, 2013).

Nevertheless, the functional role and via which mechanisms FMRP regulates dendritic spine development in *Fmr1* KO mice is still unknown. The histological study of Lee et al found significantly fewer PV interneurons in combination with altered cortical lamination patterns in *Fmr1* KO mice (Lee et al, 2019). Thus, after FMRP deletion a fewer number of PV interneurons were found, and they did not reach their correct location for projection. This is an indication for a lower density and morphological differences in PV and SOM interneurons after FMRP deletion.

6.6 Interneurons are heterogeneous and hard to characterize

In our study we classified interneurons according to their neurochemical properties, because it makes genetic manipulation in a subset of cells that is targeted by cell-type specific genes easy (Riedemann, 2019). However, interneurons are enormously heterogeneous and could also be characterized on morphology or electrophysiology. Classification studies on morphology revealed seven different classes interneuron classes and classification of interneurons on electrophysiology divided interneurons in six different classes (Riedemann, 2019). Therefore, it could be hard to analyze all features of interneurons, especially when the experimental setup of an experiment is limited to analyze only a limited number of features.

In addition, detailed classification of interneurons is essential to understand why alterations in a certain type of interneuron are responsible for specific impairments in behavior or neuropathological conditions, meanwhile alterations in another interneuron class showed none or only minor effects. For example, this study found after removal of FMRP in SOM interneurons impairments in sociability. However, SOM interneurons consist of Martinotti cells and non-Martinotti cells with different morphological and electrophysiological projections. This makes it hard to distinguish which type of the general SOM interneuron class is responsible for the impairments in sociability after FMRP deletion. Thus, it is necessary to unravel specific cellular and neural circuit properties within specific interneurons classes to understand why a certain type of interneuron is responsible for a specific condition. Therefore, instead of focusing on only neurochemical properties, future studies could focus on other classifications methods of interneurons and the role of FMRP.

6.7 Optogenetics and electrophysiological techniques could give more insight in specific interneuron classes

One technique that received a lot of attention in the past years is optogenetics, which refers to the integration of optics and genetics to achieve gain of function or loss of function within specific cells or living tissue (Yizhar et al, 2011). In future studies, distinct classes of interneurons could be silenced or activated with the help of optogenetics, which could give more insight about the effects of specific interneuron classes on brain function and eventually behavior. For example, during a behavioral test, a class of interneurons could be silenced and compared to another group of mice with normal functioning of this class of interneurons. After the behavioural test, results between both groups could be compared and the role of a specific class of interneurons determined.

However, silencing of interneurons requires a constant function of the silencing tool, whereby prolonged optogenetic inhibition can lead to tissue heating. Hyperthermia is known for increasing excitability in the hippocampus, whereby the intrinsic membrane properties of excitatory pyramidal cells and inhibitory interneurons are altered (Kim et al, 2012). Therefore,

the choice of an appropriate silencing strategy of optogenetics should be considered, also because the number of optogenetic tools for inhibition of neural activity is large nowadays and they all have their own technical challenges (Wiegert et al, 2017).

Finally, electrophysiological studies could be performed in the future. Electrophysiology makes it possible to investigate the electrical activity of living neurons, by measuring the electrical activity in electrical signals that neurons use to communicate with each other. This makes it possible to determine the role of specific interneurons in a neural circuit or during behavioural tests (Booker et al, 2014). To determine the effects of FMRP deletion in interneurons, action potential duration and firing frequency of specific classes of interneurons could be measured. Patch-clamp recording from acute brain slices of PV-*Fmr1*$^{-/y}$, SOM-*Fmr1*$^{-/y}$ and WT mice could be compared, to target and electrophysiological characterize synaptic functions of specific classes of interneurons and the influence of FMRP deletion on these processes.

6.8 Additional FXS animal models: Drosophila Melanogaster *Fmr1* mutants or *Fmr1* KO rat models

In general, *Fmr1* KO mouse models are considered as a good model for FXS, because it shows most FXS behavioral phenotypes and allows genetic experimentation. However, different FXS animal models could be used for future research, such as Drosophila Melanogaster *Fmr1* mutants or *Fmr1* KO rat models (Drozd et al, 2018). Working with Drosophila Melanogaster *Fmr1* mutants models has several benefits compared to vertebrate animal models. They show deficits in the same behavioural phenotypes as human FXS patients and share many pathways that are altered in FXS. Furthermore, Drosophila Melanogaster models are less expensive, easier to maintain, raise less ethical questions, have a shorter lifespan and lays many eggs that are available for genetic screens (Drozd et al, 2018). But Drosophila Melanogaster models shows also disadvantages, such as that fact that complex human behaviors not always correspond to those of fruit flies, potential toxicity of drugs tested in fruit flies are hard to predict in humans because the metabolic differences and complexities and only few interneurons are characterized (Drozd et al, 2018).

Another option are *Fmr1* KO rat models, which also showed behavioral phenotypes often observed in FXS. Additional advantages of rats compared to mice are their bigger brain size, they are easier to train, can learn more complicated behaviors and have an elaborated social repertoire (Drozd et al, 2018). This elaborated social behavior repertoire could be interesting because in our study SOM-*Fmr1*$^{-/y}$ mice showed impairments in sociability and PV-*Fmr1*$^{-/y}$ mice in social novelty. However, rats are in general more expensive and less genetically amenable (Drozd et al, 2018). Thus, there are several accepted and well-established animal models for FXS. Each model has his own advantages and disadvantages and the purpose of the study determines which model is most suitable. If behaviour is not a purpose of the study, human induced pluripotent stem cells derived neurons are nowadays also available for FXS research.

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