PLOS ONE

Genetic ablation of metabotropic glutamate receptor 5 in rats results in an autism-like behavioral phenotype

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Abstract

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in communication, and social skills, as well as repetitive and/or restrictive interests and behaviors. The severity of ASD varies from mild to severe, drastically interfering with the quality of life of affected individuals. The current occurrence of ASD in the United States is about 1 in 44 children. The precise pathophysiology of ASD is still unknown, but it is believed that ASD is heterogeneous and can arise due to genetic etiology. Although various genes have been implicated in predisposition to ASD, metabotropic glutamate receptor 5 (mGluR5) is one of the most common downstream targets, which may be involved in autism. mGluR5 signaling has been shown to play a crucial role in neurodevelopment and neural transmission making it a very attractive target for understanding the pathogenesis of ASD. In the present study, we determined the effect of genetic ablation of mGluR5 (*Grm5*) on an ASD-like phenotype using a rat model to better understand the role of mGluR5 signaling in behavior patterns and clinical manifestations of ASD. We observed that mGluR5 *Ko* rats exhibited exaggerated self-grooming and increased marble burying, as well as deficits in social novelty. Our results suggest that mGluR5 *Ko* rats demonstrate an ASD-like phenotype, specifically impaired social interaction as well as repetitive and anxiety-like behavior, which are correlates of behavior symptoms observed in individuals with ASD. The mGluR5 *Ko* rat model characterized in this study may be explored to understand the molecular mechanisms underlying ASD and for developing effective therapeutic modalities.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in behavior and communication [1–4]. The current occurrence of ASD in the United States is about 1 in 44 children [5]. There are three core symptoms of ASD: impaired social behavior,

stereotypic/repetitive behaviors, and sensory/communication deficits [1, 6–8]. The commonly observed behavioral disturbances also include aberrant sensitivity to sensory stimulations, hyperactivity, and possible self-injury [1].

ASD is a complex disorder, and a wide arsenal of factors have been involved in the pathophysiology of this neurodevelopmental disorder. Therefore, a multidisciplinary approach is the key to understand its etiology and for the design of rational interventions. Studies in animal models are aimed at simulating the core phenotypes associated with ASD to identify the mechanisms that underscore the entire spectrum of the disorder [9–15]. Due to the lack of suitable ASD models, the exact mechanisms by which ASD develops are still unknown. Rat models may be more appropriate than mice for understanding ASD pathogenesis, as rats exhibit complex social behavior especially during development [16–21]. Mouse play behavior during development is less conspicuous and comprises few interaction elements. In contrast, normal young rats are playfully aggressive creatures, wrestling, boxing, and pinning their siblings down by the neck unlike mice. This is important since ASD is a developmental disorder and modeling the impaired social behavior during development is critical in clinically relevant animal models of ASD. In addition, as compared to mice, rats use a rich acoustic communication system [22–27]. All these findings suggest that the rat may be a more suitable animal model as compared to mice for understanding the molecular mechanisms underlying ASD etiology.

Although the exact etiology of ASD remains an enigma, at least 30% of cases have an underlying genetic etiology [28–35]. The most common gene variants involved in ASD include *SHANK3*, *MECP2*, *NLGN3*, *NRXN1* and *FMR1* [36–50], some of which are regulated by the metabotropic glutamate receptor (mGluR) pathway, especially mGluR5, thus making it a very attractive target for understanding the pathogenesis of ASD [51]. mGluR5 is a seven-transmembrane spanning G-protein coupled receptor (GPCR) located in the postsynaptic membrane of excitatory synapses, neuronal nuclear membranes, glia, and oligodendrocytes [52–56]. mGluR5 is important for neuronal-glial communication and in neuronal homeostasis including the control of glutamate release and uptake by astrocytes [57]. mGluR5 signal transcription events occur either via phospholipase-C (PLC) to act ultimately on the mitogen activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), or via phosphoinositide-3-kinase (PI3K) and the mammalian target of rapamycin (mTOR) [58].

mGluR5 has been shown to play an important role in the pathophysiology of ASD by regulating the function of a number of proteins involved in synaptic transmission, including Shank3 [51]. As a proof of this concept, pharmacological enhancement of mGluR5 has been shown to rescue behavioral deficits in *SHANK3* knockout (*Ko*) mice [59]. mGluR5 and Shank3 interact with each other primarily through homer proteins [51]. In addition, the gene expression of *mGluR5* was significantly decreased in ASD patients versus control human subjects in a post-mortem brain stereological investigation [60]. The intensity of the staining of mGluR5-positive neurons was also significantly decreased in ASD versus control subjects [60]. The single nucleotide polymorphisms in *Grm5*, the gene that encodes mGluR5, have been found to be a predictive genetic classifier for ASD [61]. The mGluR5 antagonists administered to wild-type (WT) rats have been shown to impair social interaction, which is a core clinical deficit in ASD [62]. Further, reduced mGluR5 expression has been observed in *Mecp2 Ko* mice as well as in the motor cortex of autopsy samples from Rett syndrome (RS) patients [63]. RS is a neurodevelopmental disorder that results from *de novo* mutations in the *MECP2* gene and shares many symptomatic, as well as pathological commonalities with ASD [64, 65]. Additionally, treatment of *Mecp2*-deficient mice with mGluR5 positive allosteric modulator (PAM), VU0462807, improves behavior defects [63]. These basic science and clinical studies employing ASD individuals strongly lay the foundation for the usefulness and relevance of mGluR5

Ko rats as suitable models of ASD. The availability of a preclinical mGluR5 rat model that is a downstream target molecule for some of the genes implicated in ASD will provide a unique tool to understand the underlying molecular mechanisms behind the etiology of ASD. This model will help us to comprehend the neurodevelopmental changes that underlie the behavioral deficits observed in ASD and will open the doors for evaluating the efficacy of future therapeutic interventions.

Methods

Animals

Heterozygous breeders of mGluR5 *Ko* rats on Sprague Dawley background were obtained from the Envigo company (Indianapolis, IN, USA). The model was generated by a biallelic deletion of the metabotropic glutamate receptor 5 (mGluR5 or *Grm5*). All experimental animals were obtained from heterozygous crossings. Male and female rats were used in these experiments. In total, 36 rats [19 WT (9 males, 10 females) and 16 *Ko* (8 males and 8 females) animals] were subjected to a comprehensive battery of ASD-associated behavior tests at 12 weeks of age. Animals were group housed in a room with 12-hr light /12-hr dark Light/Dark cycle. Food and water were provided *ad libitum*. To control potential litter effect, one animal each of WT and *Ko* per litter was randomly selected for behavior phenotyping. This experimental design allows the use of standard statistical methods, such as *t* tests for analysis [66, 67]. The rest of the animals were used in additional different experiments. The animals for this study were derived from 19 pregnant dams as heterozygous breeding scheme was used providing both WT and *Ko* rats. Genotyping was performed by Transnetyx company (Cordova, TN, USA) using custom designed probes having primer sequence, mGluR5 F `CTTCATGAGGGTT GTACCTTCC`; mGluR5 R `GTGTGCACAGCTGAGACATAAG`. Behavioral analyses were conducted by the trained observers that were blinded to the rat genotype. The study protocol was approved by the Animal Care and Use Committee of the University of Miami and was in full compliance with the NIH guidelines for the care and use of laboratory animals.

Open field (OF)

Rats were brought to the testing room at least 30 minutes before the test. Each animal was videotaped for 10 minutes undisturbed after a 20-minute habituation time in their own cage (46 cm length × 23.5 cm wide × 20 cm high). The dimension of the open field (OF) chamber was 85 x 50 x 50 cm. Animals were placed in the middle of the arena at the start of the test. They remained in the arena for 10 minutes and their positions were tracked using Ethovision Version 11.5 (Noldus Information Technology, Netherlands). The center region was defined as an area that covered 42.35% of the total arena (artificial dimension: 60 x 30 cm inside vs. 85 x 50 cm total arena). Time spent in the center (center duration, in sec) and in the periphery of the arena (periphery duration, in sec) as well as the proportion of time spent in the perimeter of the box was used as an index of anxiety [68–70]. We also determined the total distance traveled (in cm) as an index of locomotor activity. In addition, the rearing frequency was used as a measure of exploratory behavior and anxiety [71, 72].

Self-grooming

After a 20-minute habituation period, animals were videotaped undisturbed for 10 minutes. The number of bouts of grooming sessions and the time spent in grooming was determined by two trained observers who were blinded to the experimental conditions. Self-grooming behaviors included wiping the nose, face, head, and ears with forepaws, as well as licking the body,

anogenital area and tail [68, 73, 74]. The influence of rat odors was prevented by thoroughly cleaning the cage at the beginning of each trial.

Social interaction test

The social interaction test was performed using a three-chamber compartment comprising of three phases. In the first phase, the test animal was introduced to the middle compartment of the three- chambered apparatus and was allowed 5 minutes to freely explore left and right chambers each containing an empty Plexiglas cage. In the second phase, a non-familiar wild-type rat matched for sex and age ("stranger rat") was placed in the cage in the right compartment. The test rat was placed in the middle chamber with closed connecting doors. The experiment started when the operator opened the doors, and the rat behavior was recorded over 10 minutes. Sociability was assessed by recording the time spent in the "stranger rat" chamber (in seconds), and in the empty cage chamber (in seconds) [70, 73, 75, 76].

Immediately following the second phase of the test, a novel wild-type rat matched for sex and age ("novel rat") was placed in the cage in the left chamber while the "stranger rat" (from the second phase of the test) became familiar ("familiar rat") to the test animal in this third phase. The search for social novelty was assessed by recording the time spent in the "familiar rat" chamber (in seconds) and in the "novel rat" chamber (in seconds) [70, 73, 75, 76].

Marble-burying test (MBT)

MBT was used to measure repetitive and anxiety-related behaviors [77, 78]. The test rat was left undisturbed for one hour in the testing room in its home-cage for acclimation. In five cm high fresh bedding, 20 marbles (previously washed with 90% alcohol) were placed equally distant in the testing area. After the acclimation period, the test animal was allowed to bury marbles freely for 30 minutes. At the end of the test, the number of marbles buried was counted. Marbles were considered buried if more than two-thirds of their height was covered with the bedding. The marbles were thoroughly cleaned after each experiment followed by replacement of new bedding in the testing chamber. Experiments were also video-recorded, and animal's cumulative time spent digging was manually scored with a stopwatch by two trained observers who were blinded to the experimental conditions.

Statistical analysis

Statistical analyses were conducted with XLSTAT and GraphPad Prism version 28. Quantitative variables were compared using a Student's t-test. A Mann-Whitney's test was performed for nonparametric samples. The threshold of statistical significance was set with a $p < 0.05$.

Results

Exaggerated self-grooming behavior in mGluR5 Ko rats

Excessive self-grooming has been observed in preclinical animal models of ASD representing repetitive behaviors and as expression of anxiety [79, 80]. We observed that the *Ko* rats self-groomed significantly more frequently and for longer durations compared to the WT animals ($p < 0.01$) (**Fig 1A and 1B**). The mean grooming frequency was 3.31 ± 1.35 for the *Ko* rats compared to 1.16 ± 0.89 for the WT rats. Average grooming time was 15.25 ± 8.37 seconds and 7.42 ± 2.25 seconds in the *Ko* and WT rats, respectively. However, there was no statistically significant difference in grooming frequency and grooming time between *Ko* males and *Ko* females ($p > 0.05$).
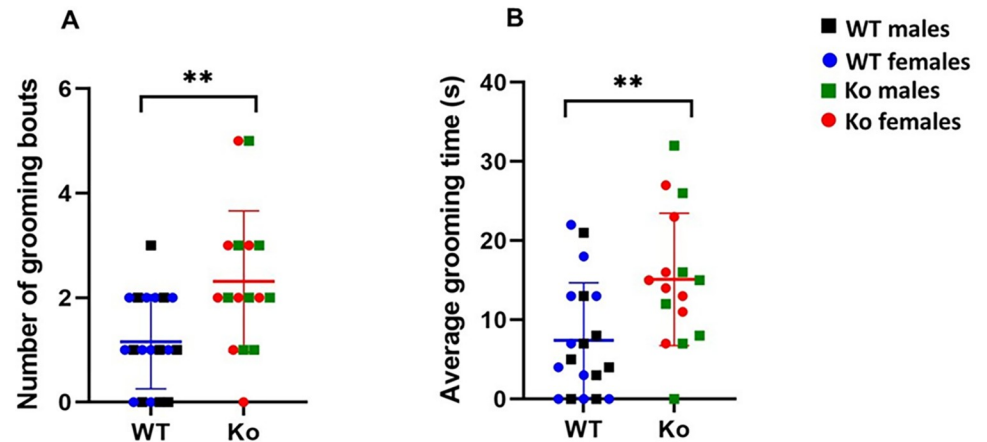
Fig 1. mGluR5 *Ko* rats show exaggerated self-grooming. The *Ko* rats groomed significantly more frequently (**A**) and for longer durations (**B**) compared to the WT animals. $^{**}p < 0.01$ *Ko* versus WT animals.

Increased rearing frequency in mGluR5 Ko rats

Rearing frequency is the frequency with which the rodent stands on its hind legs in the open field, which might be a direct measure of anxiety [71, 72, 81, 82]. We observed that the frequency of rearing was significantly higher in the *Ko* group than in the *WT* rats (*Ko*: *Mean* = 36.50, *SD* = 6.48; *WT*: *Mean* = 18.84, *SD* = 4.18; $p < 0.001$) (**Fig 2**). We did not find any statistically significant difference in rearing frequency between *Ko* males and *Ko* females ($p > 0.05$).
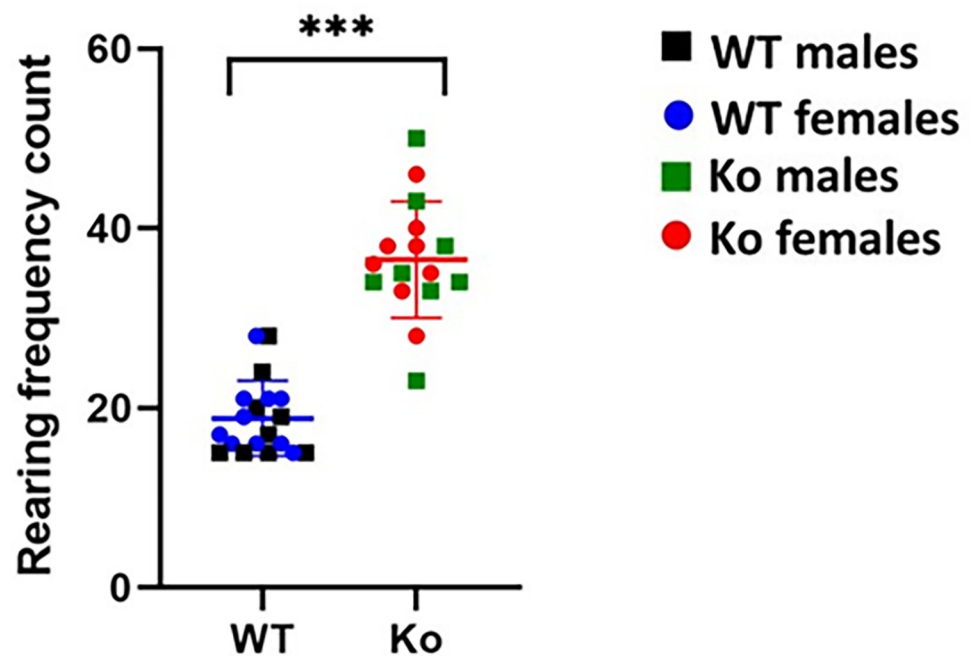

Fig 2. Rearing frequency is increased in mGluR5 *Ko* rats. The frequency of rearing was significantly higher in the *Ko* group than in the *WT* rats suggesting anxiety-like phenotype. $^{***}p < 0.001$ *Ko* versus WT animals.
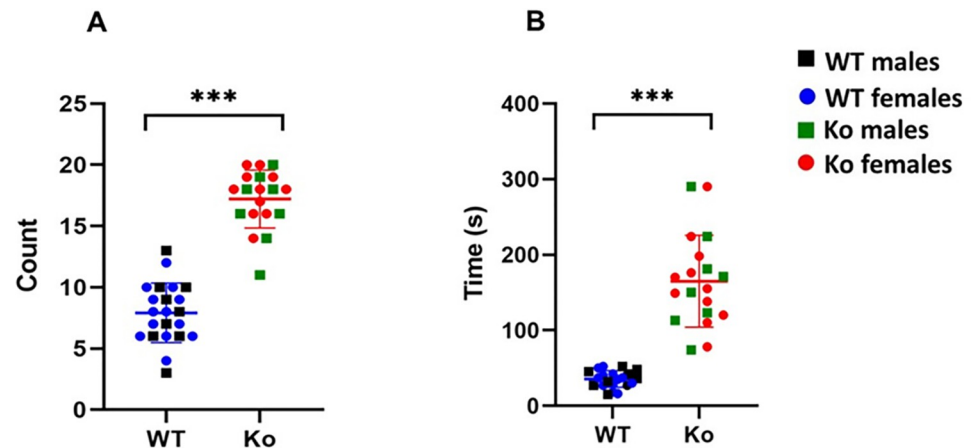
Fig 3. mGluR5 *Ko* rats show marble burying phenotype related with ASD-like phenotype. A. The *Ko* rats significantly buried more marbles compared to the WT animals. B. The total digging time was significantly higher in the *Ko* rats compared to the WT animals. ***$p < 0.001$ *Ko* versus WT animals.

Increased marble-burying behavior and digging time in mGluR5 Ko rats

The MBT allows in assessing anxiety-like, and repetitive behaviors. The effect of genetic ablation of mGluR5 (or *Grm5*) on MBT in a rat model is still not known. Therefore, we subjected WT and *Ko* rats to MBT. We determined the number of marbles buried as well as the total digging time in *Ko* and WT rats. We observed that the *Ko* rats significantly buried more marbles compared to the WT animals (*Ko*: *Mean* = 17.21, *SD* = 0.52; *WT*: *Mean* = 7.91, *SD* = 0.48; $p < 0.001$) (**Fig 3A**). In addition, the total digging time was significantly higher in *Ko* rats compared to WT animals (*Ko*: *Mean* = 169.95, *SD* = 9.48; *WT*: *Mean* = 35.41, *SD* = 8.33; $p < 0.001$) (**Fig 3B**). These results suggest that the *Ko* rats exhibit repetitive behavior and are more anxious than the WT animals, which are the hallmarks of ASD-like phenotype. However, there was no statistically significant difference between *Ko* males and *Ko* females ($p > 0.05$).

Open field test

The open-field test is used to measure locomotor activity and anxious behavior of animal models. We observed that there was no significant difference in the total distance travelled between the *Ko* and WT rats (*Ko*: *Mean* = 44.26, *SD* = 11.91; *WT*: *Mean* = 42.38, *SD* = 9.19; $p = 0.60$) (**Fig 4**). These results suggest that were no significant differences in the locomotor activity of *Ko* and WT rats. We then determined time spent in the center and periphery by the *Ko* and WT rats (**Fig 5A and 5B**). There was no statistical difference between the *Ko* and WT rats in time spent in periphery and center ($p > 0.05$).

mGluR5 Ko rats exhibit deficits in sociability and social novelty

Using the three-chamber test, we assessed the sociability and social novelty behaviors (**Figs 6A and 7A**). We observed that there was a statistically significant difference in sociability between *Ko* and WT rats. The WT rats spent significantly more time in the stranger rat chamber compared to the empty chamber (**Fig 6B**) ($p < 0.001$). On the contrary, the *Ko* rats showed no preference for stranger rat and empty ($p > 0.05$). These findings indicate that *Ko* rats show deficits in sociability. However, there was no statistically significant difference in sociability between *Ko* males and *Ko* females ($p > 0.05$).
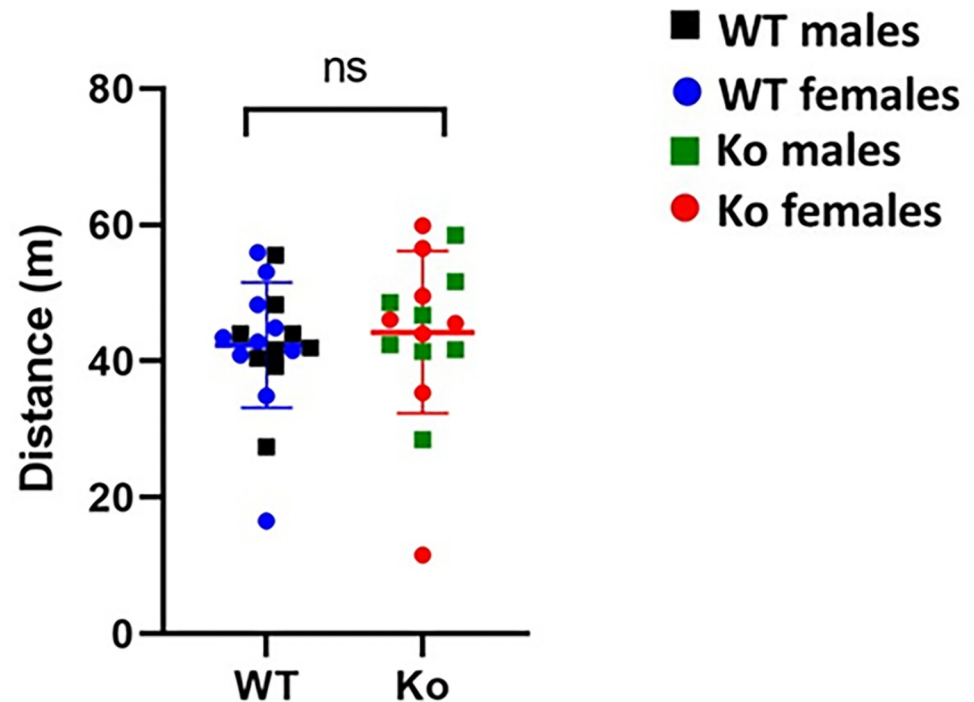
Fig 4. mGluR5 *Ko* rats do not show impaired locomotion. There was no significant difference in locomotor activity of the *Ko* rats compared to the WT animals. $p > 0.05$ *Ko versus* WT animals. ns: non-significant.

https://doi.org/10.1371/journal.pone.0275937.g004

During the third phase of the three-chambers test assessing the search for social novelty, a novel rat age and sex-matched ("novel rat") was inserted in the cage of the left chamber while the "familiar rat" remained in the right chamber (**Fig 7A**). The WT rats spent more time in the novel rat chamber compared to time spent in the familiar rat chamber ($p < 0.001$). On the other hand, *Ko* rats showed no preference and there was no statistically significant difference in time spent in familiar and novel rat chambers ($p > 0.05$) (**Fig 7B**). These results suggest the
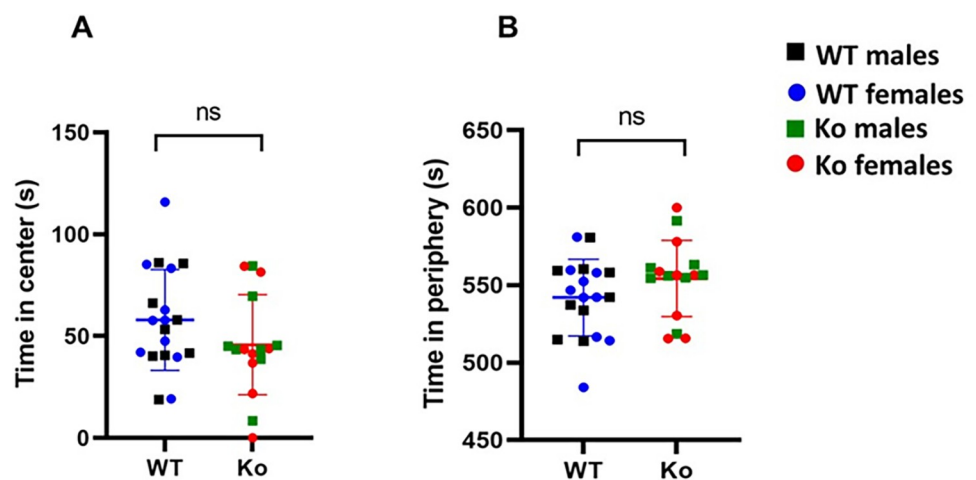

Fig 5. Perimeter preference in open-field test. The mGluR5 *Ko* rats do not show differences in time spent in center (**A**), and in perimeter (**B**) compared to WT animals. $p > 0.05$ *Ko* versus WT animals. ns: non-significant.

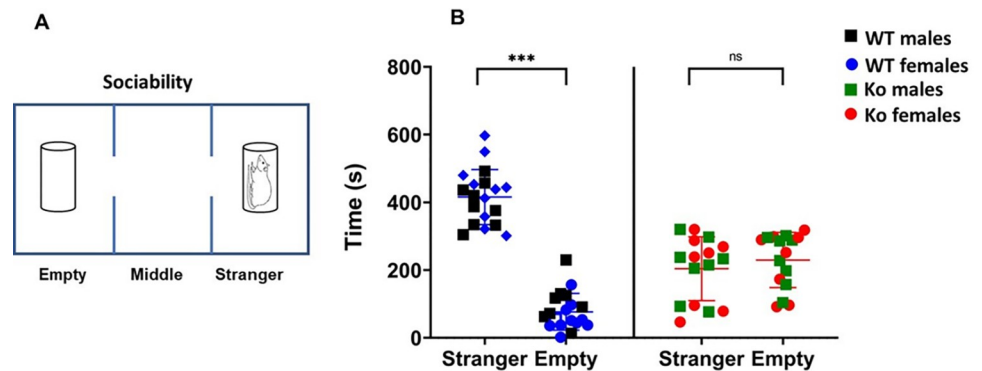https://doi.org/10.1371/journal.pone.0275937.g005

Fig 6. Three chamber sociability test. A) Schematic representation of three-chamber sociability test. **B)** The mGluR5 *Ko* rats do not exhibit sociability as there was no statistically significant difference between time spent with the stranger rat over the empty cage ($p > 0.05$). On the other hand, WT rats preferred spending time with the stranger rat compared to the empty cage (***$p < 0.001$). ns: non-significant.

reluctance to social novelty of *Ko* rats as compared to WT animals. However, there was no statistically significant difference in social reluctance between *Ko* males and *Ko* females ($p > 0.05$).

Discussion

Metabotropic glutamate receptor mediated signaling, especially through mGluR5, has been hypothesized to play a crucial role in the pathophysiology of ASD. In support of this concept, the pharmacological enhancement of mGluR5 ameliorates behavioral deficits in preclinical animal models of ASD [59]. As mGluR5 can play an important role in predisposition to ASD, it is worthwhile to examine the effects of genetic deletion of mGluR5 (or *Grm5*) on ASD-associated behavioral manifestations. In the present study, we subjected mGluR5 *Ko* rats to a battery of ASD-associated behavior phenotypes such as repetitive behavior, anxiety-like phenotype, social preference, locomotor activity and digging behavior. We observed that *Ko* rats several behavior deficits congruent with an ASD-like phenotype, suggesting the critical role of mGluR5 signaling in determining increased susceptibility to autism.
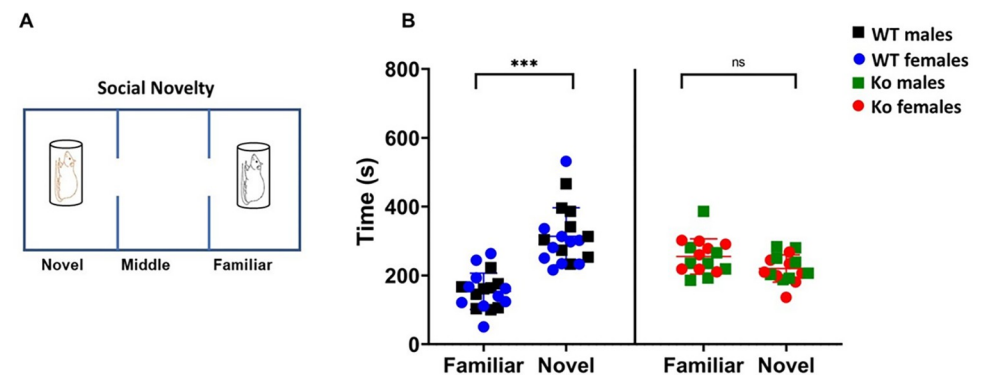
Fig 7. mGluR5 *Ko* rats exhibit no preference for social novelty. A) Schematic representation of social-novelty test. **B)** WT rats showed the preference for social novelty as they spent significantly more time with novel rat compared to the familiar rat (***$p < 0.001$). On the other hand, the mGluR5 *Ko* rats showed no preference and there was no statistically significant difference in total time spent with the novel rat over the familiar rat suggesting reluctance to social novelty ($p > 0.05$). ns: non-significant.

Aberrant self-grooming has been associated with ASD-like phenotype in animal models representing stereotyped repetitive behavior [74, 78, 83]. Self-grooming behaviors in rats often include licking the body, paws/legs, genital area, and tail, as well as using their paws to wipe the face, ears, and head [73, 84]. Although self-grooming is a typical animal or rodent behavior performed for seconds to minutes, it is often flagged as abnormal when done more frequently or for an extended amount of time [85]. A number of studies have associated exaggerated self-grooming with ASD-like phenotype. The valproic acid (VPA)-exposed rat offspring have been shown to spend significantly more time self-grooming than control rats, suggesting ASD-related stereotyped behavior [73, 85]. Furthermore, di-(2-ethylhexyl) phthalate (DEHP) exposure has been shown to result in autism-like behavior, illustrated in part by the increased self-grooming time, which is comparable to the elevated duration observed in VPA rats [86]. Our results are in agreement with these studies as we observed that the *Ko* rats self-groomed significantly more frequently and for longer durations than the WT animals, thus exhibiting ASD-like phenotype.

Marble burying test is commonly used to determine the repetitive and anxiety-like phenotype, which is one of the most important hallmarks of ASD. Both the number of the marbles buried, as well as the amount of time the animal spends exploring the marbles or digging are often studied, similar to the type of analyses performed in our study. We observed that *Ko* rats buried more marbles and spent more time in digging the marbles compared to WT animals showing repetitive and anxiety-like behavior. On par with these findings, increased marble burying has been observed in various ASD animal models. The loss of *Tsc2* in Purkinje cells and deficiency of cyclooxygenase-2 (COX2) have been associated with ASD-like phenotype in rodent models [76, 87, 88]. Similarly, in a VPA induced ASD model, increased marble burying was observed that correlated with repetitive and anxiety-like behavior, mimicking clinical manifestations of ASD in human subjects [89].

An important parameter in the open-field test is the total distance travelled. If there are no differences in total distance traversed between different strains, it facilitates making valid comparisons for various behavior tests, as locomotor activity is no longer a confounding variable in the data analysis. The significant differences in locomotor activity may skew the data by preventing analyses for time spent in certain designated zones of the maze as differences may be due to inactivity instead of due to the genotype effect. In the present study, we observed no statistically significant difference in the locomotor activity of the *Ko* and WT rats. Therefore, we determined whether there are differences in the time spent in the center and periphery zones between the Ko and WT rats in the open-field test. However, we observed no significant differences in the time spent in the different zones. These results are in agreement with previous findings where no perimeter preference was observed in other animal models of ASD [76]. It is possible that the brain regions involved in this perimeter preference are not affected following the genetic ablation of mGluR5 (*Grm5*), which needs to be explored in future studies.

Impairments in social behavior is one of the characteristic hallmarks that is observed in individuals with ASD. mGluR5 signaling has been shown to play an important role in social interaction. As a proof of this concept, the mGluR5 positive allosteric modulator (PAM) CDPPB has been demonstrated to ameliorate social interaction deficits in the *Shank2*, *Shank3*, and *Sarm1 Ko* mouse models of ASD [59, 90, 91]. In addition, mGluR5 PAM [3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl) benzamide] (CDPPB) attenuated social behavior deficits in *Sarm1* knock-out mouse model of ASD and prevented ASD-like alterations in rats exposed to cannabinoid during the prenatal period [91, 92]. These improvements in social behavior were attributed to the enhancement of mGluR5 levels. Our results are in agreement with these studies as mGluR5 *Ko* rats exhibited sociability deficits as well as impaired social behavior thus emphasizing the importance of mGluR5signaling in normal brain functioning.

In addition, our findings are in agreement with the data obtained from mGluR5 *Ko* mice. It has been shown that mGluR5 *ko* mice show impairments in social interaction and altered marble burying activity compared to WT controls [93]. However, no statistically significant differences were observed in self-grooming pattern and rotarod performance in mGluR5 *Ko* mice. Interestingly, mGluR5 *Ko* mice showed altered locomotor activity which may have confounded behavioral measurements. We do not observe such alteration in locomotor activity of *Ko* rats compared to WT animals thus suggesting this rat model can be used to understand the molecular underpinnings of ASD as well as testing the efficacy of future novel therapeutics for autism.

In summary, our findings suggest that mGluR5 *Ko* rats display ASD-like phenotype. Our results highlight the crucial role of mGluR5 signaling in ASD-associated behavior deficits. One of the limitations of our study is the constitutive deletion of mGluR5 expression. Future studies using conditional *Ko* rats with deletion of mGluR5 only in the brain or specific areas of the brain can shed further light on the role of mGluR5 signaling in the pathophysiology of ASD.

Acknowledgments

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Writing – review & editing: Adrien A. Eshraghi, Idil Memis, Florence Wang, Isaiah White, Emily Furar, Jeenu Mittal, Moeed Moosa, Coleen M. Atkins, Rahul Mittal.

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