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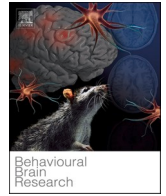
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Research article

A new 2-hit model combining serine racemase deletion and maternal separation displays behavioral and cognitive deficits associated with schizophrenia

Caroline Lahogue^{*}, Michel Boulouard, François Menager, Thomas Freret, Jean-Marie Billard, Valentine Bouet^{*}

Normandie Univ, UNICAEN, INSERM, FHU A2M2P, COMETE, Caen 14000, France

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ABSTRACT

Schizophrenia (SCZ) is a multifactorial psychotic disorder characterized by positive and negative symptoms as well as cognitive impairments. To advance the current treatments, it is important to improve animal models by considering the multifactorial etiology, thus by combining different risk factors. The objective of our study was to explore in a new mouse model, the impact of genetic deletion of *serine racemase* (genetic vulnerability) combined with an early stress factor induced by maternal separation (early environmental exposure) in the context of SCZ development. The face validity of the model was assessed through a wide range of behavioral experiments. The 2-hit mice displayed an increased locomotor activity mimicking positive symptoms, working memory impairment, cognitive deficits and recognition memory alterations, which could reflect neophobia. This new multifactorial model therefore presents an interesting phenotype for modelling animal model with partial behavioral and cognitive deficits associated with SCZ.

1. Introduction

Schizophrenia (SCZ) is a psychotic disorder characterized by heterogeneous symptoms, including positive (hallucinations, delusions), negative symptoms (avolition, anhedonia, social withdrawal) and cognitive impairments (working memory deficits, attentional dysfunction). The first psychosis episode usually occurs in the early adulthood (15 – 25 years) and the course of the illness varies from one patient to another [1]. Although the etiology of SCZ is still unknown, it is now well established that interactions between the genetic background and environmental factors (gene x environment interactions) have a significant role in the development of the disease [2,3]. Numerous genome-wide association studies reported genes polymorphism and copy number variants in schizophrenic patients [4]. Several genes have been identified as risk factors, including those related to neuronal communication involving glutamatergic neurotransmission [5,6]. The dual hit hypothesis of SCZ suggests that the genetic vulnerability (first hit), in association with environmental factors (early stress or late environmental factors; second hit) could favor its occurrence [7,8]. Environmental factors include several complications during pregnancy

(such as mother infection, malnutrition, or psychosocial stress), childhood trauma (physical, psychological, or sexual abuse, neglect, or exposure to physical and psychological abuse) and adversity, bullying and discrimination or drug abuse during adulthood [9,2,10]. Childhood trauma is one of the factors widely studied in the context of SCZ [11]. The stress generated by a traumatic episode alters the hypothalamic-pituitary-adrenal axis (HPA). Several paradigms have been developed to reproduce childhood trauma in animal models of SCZ and one of the most frequent is maternal separation (MS) (reviewed in [12]). MS for 24 h at post-natal day (PND) 9 in rodent disrupts the HPA axis [13,14] and produces sensorimotor gating deficit [9] while it increases anxiety-like behavior and alters sociability in rodents [15]. These are all important hallmarks of SCZ.

Antipsychotic drugs have been shown effective (even if not curative) to treat the positive symptoms while the negative and cognitive symptoms remain resistant [16,17]. Among the possible explanations is the lack of reliability in the validity of animal models of SCZ used in pre-clinical investigations [18]. Indeed, such animal models are expected to mimic as much as possible the mechanisms underlying the disease (construct validity), as well as to display behavioral abnormalities and

^{*} Correspondence to: UNICAEN COMETE, UMR S-1075, Bat GMPC, Campus Horowitz, Bd Henri Becquerel, Cedex 5, Cae CS14032, France.

E-mail addresses: caroline.lahogue@unicaen.fr (C. Lahogue), valentine.bouet@unicaen.fr (V. Bouet).

cellular alterations similar to those observed in patients (face validity). Finally, they have to respond to the existing treatments (predictive validity). Although multiple animal models of SCZ have been developed, they mostly used a single risk factor and therefore, do not accurately reflect the complexities of the disease [19]. More recently, some multifactorial models combining two factors (genetic x environment; genetic x genetic or environment x environment factors) have been developed to better consider the face validity [2,3,18]. Even for 2-hit animal models of SCZ, it is complicated to observe all the symptoms of the disease, and each model presents a different behavioral phenotype (for review see [2]). For example, alteration of the working memory, which frequently occurs in SCZ patients [20,21], is not systematically reproduced in all 2-hit models. This could be due to the fact that few studies have been conducted on this subject [22,2,23]. In this sense, the necessity to improve comprehension of the multifactorial models of the disease remains therefore a major issue that prompted us to reconsider the development of a new 2-hits mouse model of SCZ.

Among the genes associated with SCZ, serine racemase (SR) is now considered to be highly relevant [24,25,5]. SR is localized in the neuronal cells of the forebrain and synthesizes D-serine, a co-agonist of the N-methyl-D-aspartate subtype of glutamate receptors (NMDAR) [26, 27]. Some clinical studies have found a polymorphism in the SR gene (intron variant, single nucleotide polymorphism, [28]), associated with a reduction of D-serine levels in the cerebrospinal fluid and brain of schizophrenic patients [29,25,5,6,30]. The total deletion of exon 1 of the SR gene (SR-KO) decreased D-serine levels by at least 90 % in the brain of mice and is linked to behavioral alterations such as hyperactivity and memory deficits [24,31]. Recent *ex vivo* electrophysiological studies have reported an alteration of the excitatory/inhibitory balance in the CA1 hippocampal networks of those mice [31,32]. Thus, the phenotype of the SR-KO mice, considered as a model of NMDAR hypofunction, appeared as an interesting model to test the potential impact of combination with an early stress exposure on cognitive abilities. Herein, we selected MS of 24 h at post-natal day (PND9) as an early stress factor [33, 34] since it has been shown to impaired anxiety-like and social behavior in rodents [15,35]. The hypothesis is that the combination of two risk factors (SR-KO x MS) induces neurobehavioral alterations associated with SCZ. To characterize the behavioral deficits and to better highlight the impact of the interaction between the two risk factors, a large range of behavioral assessments was performed.

2. Materials and methods

2.1. Animals

Male mice aged 2–4 months provided by our local facility (Centre Universitaire de Ressources Biologiques, Université de Caen Normandie, France) were used including wild type (WT) C57BL/6 J mice and SR-KO mice originally developed in the Pr J.T. Coyle's laboratory (Belmont, MA, USA) [24]. Mice were housed in polycarbonate cages ($n = 5$ per cage) in standard controlled conditions, including a reversed 12h-12h light-dark cycle (light off at 7:30 am), regulated temperature ($22 \pm 2^\circ\text{C}$), humidity ($50 \pm 10\%$) and free access to food and water. Cage enrichment consisted in crinkle cut shredded paper and a cardboard house. Experiments were performed in accordance with French and European Economic Community guidelines for the care and use of laboratory animals (2010/63/UE, project authorization n°2020121013375130 v2). Two cohorts were used to perform all behavioral experiments. 23 WT and 25 SR-KO mice were used for the first experiment and 22 WT and 29 SR-KO for the second one. Behavioral experiments are detailed on Fig. 1.

2.2. Maternal separation (MS)

At PND9, wild-type and SR-KO litters were separated from their mother for 24 h (early environmental stress) [9,15]. Mothers were removed and placed in a cage nearby (from 9:00 a.m. to 9:00 a.m., the day after). Then, the mothers were returned to their litters and left undisturbed until weaning. Control mice were submitted to the same protocol with only 20 seconds of separation time.

2.3. Behavioral assessment

Animals were handled daily for 5 minutes during the week preceding the experiments. The experiments were conducted during the active-dark phase of the cycle and were started at 8:00 am. Each new behavioral test was carried out one week apart. Animal cages were placed in the experimental room 30 min before the beginning of each experiment. Apparatuses were cleaned with alcohol (70 %) between each assessment. Two sets of experiments were performed for the behavioral assessment (Fig. 1). Except for spontaneous locomotor activity (in

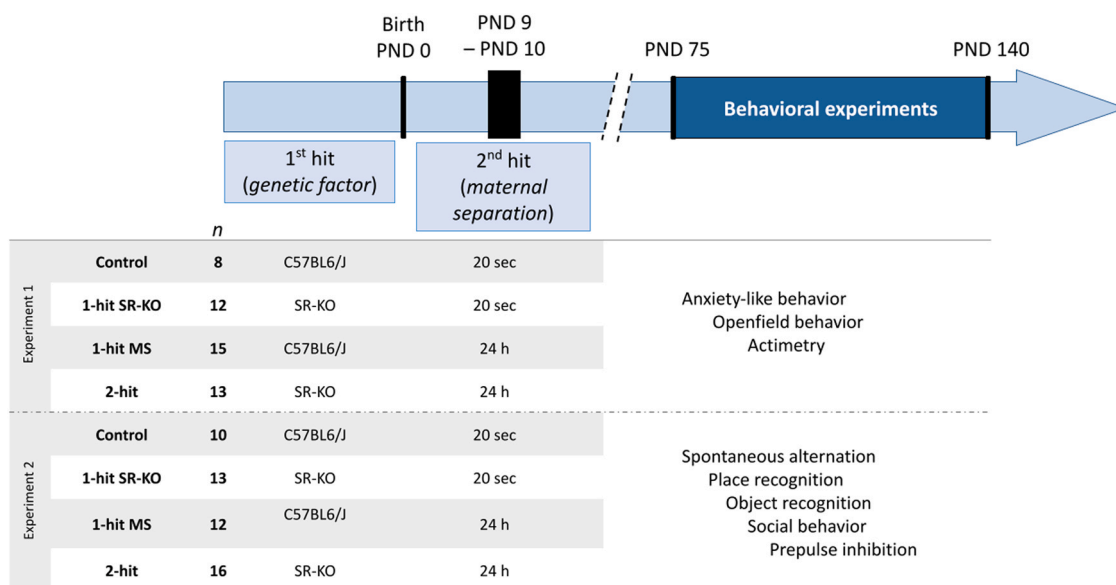


Fig. 1. Animal groups and experimental design. Two cohorts were used for all behavioral experiments. The first one was used for experiment 1 and the second one for the experiment 2. The number of animals used for each group appears in the "n" column. PND: post-natal day; SR-KO: serine racemase knock-out; MS: maternal separation.

actimeter), all behavioral tests were videotaped and scored using EthoVision XT v.16.0 (Noldus®, The Netherlands).

2.3.1. Anxiety-like behavior

The maze made of gray plastic consisted of two opposite open arms (30 × 5 cm) and two opposite closed arms (30 × 5 × 20 cm), elevated 75 cm above the floor. During 5-min free exploration (30 lux), the number of entries (four paws criterion) and the time spent in open and closed arms were recorded for each mouse, to obtain corresponding percentages. These parameters were used to assess anxiety-like behavior.

2.3.2. Openfield behavior

Mice were individually placed for 30 min (0 lux) in a squared open arena (50 × 50 × 50 cm). The total distance and percentage of time spent in the center of the openfield (OF) were automatically collected [36].

2.3.3. Actimetry

Motor activity was measured in Plexiglas cages (19 × 11 × 14 cm) with an automated actimeter containing six lines of infrared beams (IMETRONIC®). Mice were individually placed for 30 min in cages (0 lux), and the number of beams crossing reflecting locomotor activity (horizontal and vertical movements) was automatically counted.

2.3.4. Spontaneous alternation

Spatial working memory was assessed by spontaneous alternation in a single-session Y-maze test [37]. The maze consisted of three equally spaced plastic arms (22 × 6.5 × 10 cm). Each mouse was individually placed at the end of one arm, facing the front of the wall, and allowed to freely explore the maze during a 5-min session (30 lux). The sequence of arm entries (four paws criterion) was collected. An alternation was defined as consecutive entries into three different arms. The alternation percentage was calculated using the formula: (number of alternations) / (total number of entries-2)*100.

2.3.5. Place recognition

Place recognition was assessed in a Y-maze [38,39]. The maze, different from that used for the spontaneous alternation test, consisted of three equally spaced arms (gray plastic; 34 × 8 × 16 cm). During the first training session, the mouse was placed in the starting arm, facing the wall, and allowed to freely explore for 5 min (30 lux) the maze with one arm closed by a removable door (left of right randomly), before being returned to its home cage. After a 60-min inter-trial interval, the mouse was placed again in the maze for the test session and allowed to freely explore the three arms for 2 min. The place recognition test is based on the preference of rodents for novelty, and the remembrance of the previously visited arm. During each session (training and retrieval), the time spent and the number of arm entries were recorded. A discrimination index was calculated the formula: [(time in the novel arm) - (time in the familiar arm)] / [(time in the novel arm) + (time in familiar arm)] *100.

2.3.6. Object recognition

The object recognition test was performed in a Y-maze (gray plastic; 34 × 8 × 16 cm for each arm, 30 lux) with two different objects, available in four copies: lego® towers and sealed transparent bottles filled with sand. The protocol used to evaluate object recognition memory was based on the study by Leger and collaborators (2013) [40]. The same maze as the one used for the place recognition test was used for object recognition to benefit from the habituation induced by previous exposure. During the acquisition session, two identical objects were placed in the maze in the distal parts of the arms. The mouse was placed at the end of the arm containing no object, faced to the wall and was allowed to freely explore the apparatus and the objects. Object exploration was considered when the nose of the mouse was oriented

towards the object at a distance lower than or equal to 2 cm. The criterion was reached when the mouse had explored the objects for a total duration of 20 sec within a maximum of 10 min, and was returned to its respective home cage for 2 h until the test session ended. During the test phase, a copy of the familiar object and a novel object were placed in the maze. Again, the mouse was allowed to freely explore the maze and the objects with the same criteria. The nature of the new/familiar objects (Lego® vs bottle) and their position (in left/right arm) were randomized. The time spent to fulfill the criterion was measured. The time of exploration of each object was manually recorded and used as an index of memory performances, in comparison with the chance level (10 sec).

2.3.7. Social behavior

Sociability and social recognition tests were assessed with an apparatus consisting of three interconnected compartments (27 × 18 × 21 cm), with two lateral side chambers (6.5 × 18 × 21 cm). In the side compartments, two cages (8.5 cm × 16 cm in size) were positioned to ensure visual, auditory and olfactory contact between mice. To assess sociability behavior, the tested mouse was individually placed in the central compartment for 10-min free exploration (30 lux). A 45-day old C57BL/6 J male mouse (stimulus mouse, unknown from the tested mouse) was randomly placed in one of the two lateral chambers in the little circle cage. After 10 min, the mouse was returned to its home cage. Time spent in each compartment (i.e. close, middle, or far from the stimulus mouse) was recorded. A sociability index was calculated as follows: [(time spent close to the stimulus mouse) - (time spent in the empty compartment)] / [(time spent in the stimulus mouse compartment) + (time spent in the empty compartment)] *100.

2.3.8. Prepulse inhibition (PPI)

PPI was measured with a startle reflex device (Imetronic®, France) [41]. Each mouse was placed in a gray soundproof chamber, on a platform mounted on a weight-sensor and surrounded with a transparent cylinder (0 lux). Weight variation was used to calculate the amplitude of the startle reflex. After 10 min of acclimatization to a background white noise (65 dB), 30 trials of pulse (85 dB) were performed to obtain startle reflex measurements, habituate the animal to the test, and decrease intra-individual variability. During the test phase, the mouse was subjected to 5 types of trial with an inter-trial interval of 10–20 s: no pulse (silence), pulse only (110 dB, 40 ms), pre-pulse (76, 80 or 84 dB, 20 ms presented 50 ms before the pulse) + pulse (110 dB, 40 ms). Mice were videotaped throughout the protocol which lasted about 25 min. The percentage of prepulse inhibition was calculated for each intensity as follows: (1 - (startle response magnitude of trials with prepulse / startle response magnitude of startle-only trials)) * 100.

2.4. Statistics

All analyses were performed with RStudio® software 4.2.0 and GraphPad® Prism 8.3. The normality of the data was checked using the Shapiro-Wilk test, and the homogeneity of variances was assessed with the Levene test. For data normally distributed, ANOVAs and a post-hoc Tukey honestly significant difference (HSD) test were performed and data are expressed as mean + standard error of the mean (SEM). For data not normally distributed, ANOVA with permutation as well as the post-hoc pairwise permutation test were used and data are expressed in median ± interquartile. Statistical analysis of PPI data was carried out using the nparLD package for non-parametric analysis of group and intensity effects. The one-sample t-test (parametric data) or the one-sample Wilcoxon test (non-parametric data) was used for comparison to a reference value. The difference was considered as significant when *p* was below 0.05.

3. Results

3.1. Experiment 1

3.1.1. Anxiety-like behavior in OF and EPM

The time spent in the center of the open field did not differ between control and SR-KO or MS groups. On the contrary, the combination of factors disorganized the OF behavior, as highlighted by a significant increase in the time spent in the center of the OF in 2-hit mice, which was higher than all the three other groups (about 377 % that of controls and above the 159 % and 269 % for SR-KO and SM groups respectively; Fig. 2A). For the EPM test, SR-KO, SM and 2-hit groups showed no difference in the time spent in the open arms compared to the control groups. The MS group showed a significant increase compared to the SR-KO and 2-hit groups but not with the control group. Moreover, neither the number of entries in the open arms nor the distance travelled in the

EPM differed between groups (Fig. 2B and C).

3.1.2. Spontaneous activity

The total distance travelled in the open field was significantly higher in SR-KO and 2-hit mice than in controls (Fig. 3A). Interestingly, all the three experimental groups showed a higher velocity (walking speed) than the control group (Fig. 3B). In the actimeter box, a significant increase in infrared beam interruptions was also found in the SR-KO mice compared to control animals in both vertical (rearing) and horizontal activity, but only a slight increasing trend for the 2-hit mice (Fig. 3C and D).

3.2. Experiment 2

3.2.1. Spontaneous alternation

Working memory was investigated in a Y-maze apparatus through

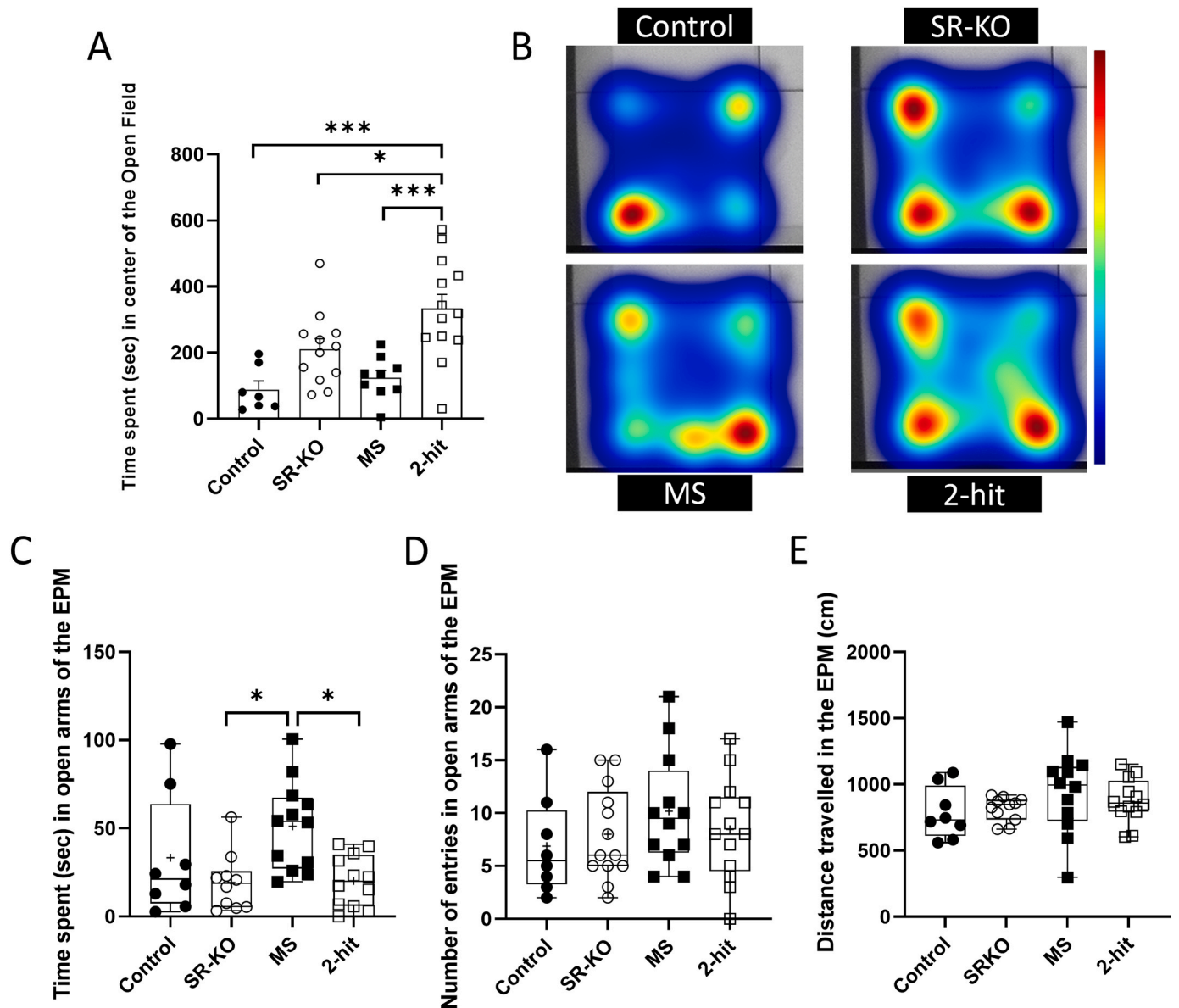


Fig. 2. The combination of SR-KO and maternal separation did not affect anxiety-like behavior. (A) Total time spent in the center of the open field over 30 min. Data are presented as mean + SEM. ANOVA test with post-hoc tukeyHSD: * <0.05 , *** <0.001 . (B) Example of heatmaps mean for each group (n=2 for control group, n=3 for MS et SR-KO groups and n=4 for 2-hit group). The longer the mouse stays in the zone, more the color tends towards red. (C) Time spent in the open arms of elevated-plus maze (EPM). (D) Total number of entries in the open arms of the EPM. Data presented as boxplots, with horizontal black lines as the median, “+” as mean and vertical black lines as minimal and maximal. ANOVA with permutation and pairwise permutation test: * <0.05 .

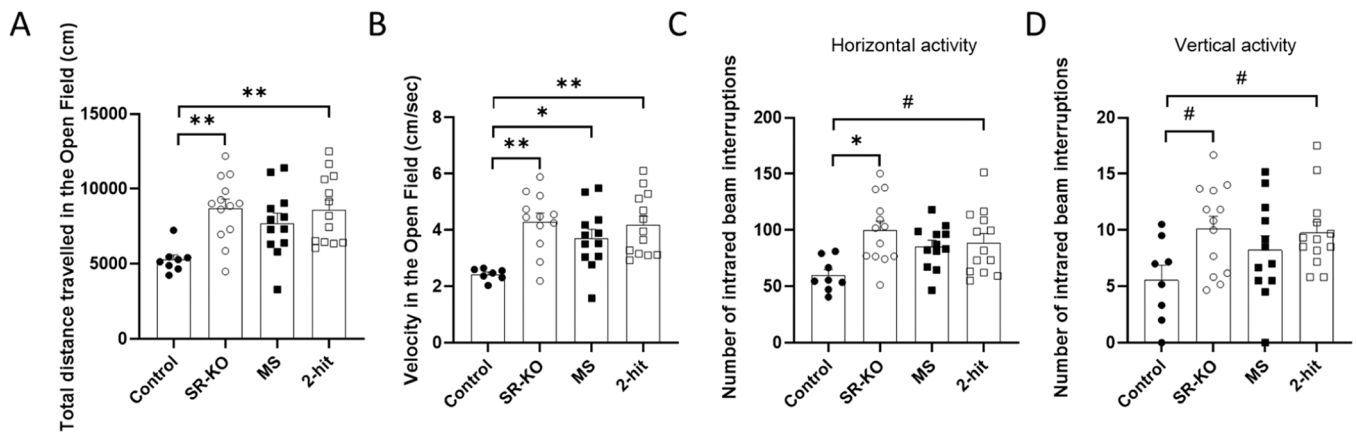


Fig. 3. Spontaneous locomotion is affected by SR deficits combined with maternal separation. (A) Total distance travelled in the open field for 30 min. (B) Mean of velocity in the open field for 30 min. Number of infrared beam interruptions for (C) horizontal and (D) vertical activity. Data presented are individual values (squares and circles) and mean \pm SEM. ANOVA test with post-hoc tukeyHSD: #<0.1, *<0.05, **<0.01, ***<0.001.

spontaneous alternation. The number of arm entries in the Y-maze was significantly higher in the 2-hit groups than in the control groups. For the SR-KO and SM groups, the number of arms entries did not differ from control or 2-hit groups (Fig. 4A). Concerning memory performances, there was no difference in the alternation percentage between groups. However, comparison to the chance value (50 %) showed that while control, SR-KO and MS groups displayed an alternation percentage

significantly above 50 %, that of the 2-hit group did not differ from 50 %, suggesting a hazardous behavior in the Y-maze induced by the combination of risk factors (Fig. 4B).

3.2.2. Place recognition

Spatial recognition memory was assessed through place recognition in Y-maze. Unexpectedly, control, SR-KO and MS groups displayed a

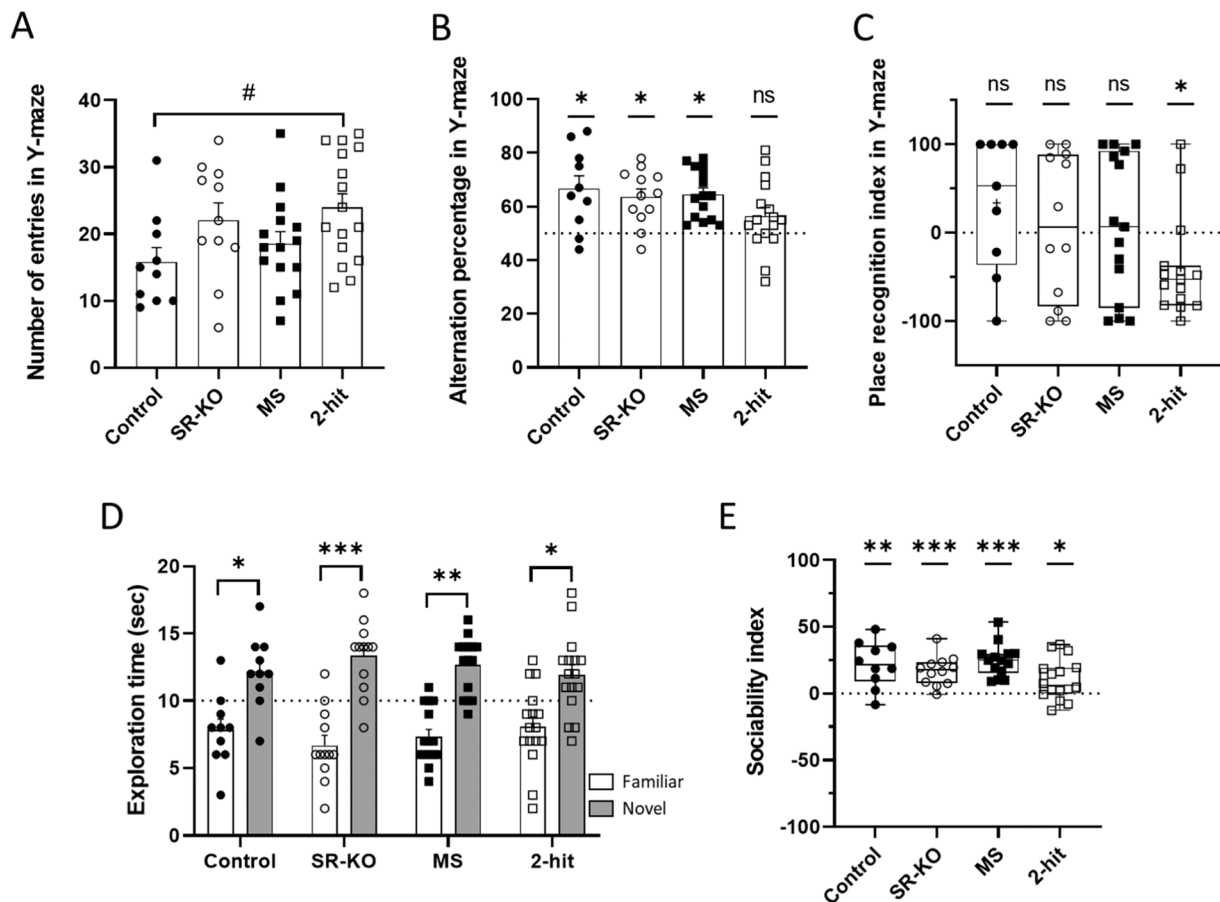


Fig. 4. SR-KO combined with MS impairs working memory and recognition. (A) Number of entries in the Y-maze. (B) Alternation percentage in the Y-maze. (C) Place recognition index in Y-maze. (D) Exploration time of the familiar (white histogram) and novel (gray histogram) object during the retention phase of an object recognition test. (E) Sociability index. For A, B and D, data are presented as mean \pm SEM. For C and E data are boxplots, with horizontal black lines as medians and vertical black lines as minimum and maximum. Intergroup comparisons between groups were performed using ANOVA test with post-hoc tukeyHSD (#<0.1, *<0.05, **<0.01, ***<0.001) and comparison to a reference value was performed with a one-sample t-test or one-sample Wilcoxon test: *<0.05; **<0.01, ***<0.001.

recognition index not different from 0, indicating no discrimination compared with the novel arm. The recognition index of the 2-hit group was significantly below the reference value of 0, suggesting that these mice had a preference for remaining in the familiar arm instead of exploring the new one (Fig. 4C).

3.2.3. Object recognition

There was no difference in the exploration time of novel object between groups. All groups displayed an exploration time of the novel object significantly above 10 and significantly different from the time exploration of the familiar one, indicating that all groups explored the new object significantly more than the familiar one (Fig. 4D).

3.2.4. Social behavior

There was no difference between groups for the sociability index. All groups displayed a sociability index above 0, indicating a preference for the new congener (Fig. 4E).

3.2.5. Prepulse inhibition (PPI)

The amplitude of the startle reflex triggered by an 85 dB stimulus was not different between groups, (Fig. 5A). Also, the percent of PPI was not affected and each group showed similar prepulse intensity effect (Fig. 5B). These results point to an absence of deficits in sensorimotor gating of the acoustic reflex in our experimental groups.

4. Discussion

This study aimed at characterizing the behavioral phenotype of a new 2-hit based on the combination of two risk factors including a deletion of serine racemase gene (SR-KO; 1st hit) that reproduces the SCZ-related glutamatergic hypofunction and a maternal separation (MS; 2nd hit) mimicking an early environmental stress. The hypothesis was that the genetic deletion would favor the vulnerability to the second risk factor [2] and that the combination of these two factors would mimic several symptoms of SCZ. We showed here that the 2-hit mice displayed an increase in locomotor activity in three experimental protocols (open field, actimeter, and in Y-maze), as well as limited deficits in working memory (spontaneous alternation in the Y-maze) and in spatial recognition memory (place recognition test). On the contrary, the sociability-related behavior and the sensorimotor gating of 2-hit mice were not affected. This new animal model therefore presents a partial schizophrenic-like phenotype, with not only positive-like symptoms but also cognitive deficits, in contrast to most of the other 2-hit models currently available.

The genetic risk factor was selected to reproduce an altered activity of the NMDAR according to the glutamate hypofunction hypothesis of SCZ [42]. The NMDAR plays an essential role in cortical function but also in brain synaptic plasticity, underlying learning, memory, and other cognitive functions [43-45]. SR-KO mice carry a deletion of the first exon of the SR gene, inducing 90 % decrease in brain levels of the NMDA co-agonist D-serine [24,31]. Current evidence indicates that these mice display subtle but significant behavioral abnormalities [46,24,47,48,31] which could be amplified by additional disruptors such as those generated by environmental changes. Although many environmental risk factors have been pointed in SCZ, childhood trauma including physical or psychological abuse and neglect [11,49,50] are among the most predominantly studied so far. It is reproduced in preclinical studies through a wide range of MS paradigms [12,51]. Among them, we have previously shown that MS applied for 24 h at PND 9 induced behavioral disabilities in mice (increased anxiety levels and decreased social behavior [15]), which could possibly increase the genetic-related defects in a 2-hit strategy.

The first major phenotype displayed by the 2-hit mice model is an increase in parameters related to locomotion (velocity and total distance, horizontal and vertical activities) which have been considered to mimic positive-like symptoms of SCZ [52]. The fact that the hyperlocomotion was found in the 2-hit and SR-KO [24] mice but not in the MS group shows that a genetically-induced glutamate hypofunction plays a major role in this deficit. Indeed, this behavioral impairment is often observed in pharmacologically-induced SCZ models of dopaminergic hyperactivity but also of glutamatergic hypofunction [53-55]. For example, treatments with the noncompetitive NMDAR antagonists phencyclidine (PCP) or MK-801 induce hyperlocomotion [56-58]. Besides, mice deprived of the NR1 subunit of the NMDAR also display an increase in locomotor activity associated with stereotypies [59,60]. This behavioral signature is also found in mice with genetic deletion of the Disrupted in schizophrenia 1 gene (DISC1) [61-63], one of the genes recognized to be associated with SCZ [64], notably due to its implication in glutamatergic neurotransmission and in the stabilization of SR [25, 63]. The fact that the hyperlocomotion was not higher in 2-hit than in SR-KO mice suggests a minor, if any, contribution of the MS to this particular phenotype.

In addition, other aspects of the locomotor activity more related to anxiety-like behavior, also suggest a specific signature in the 2-hit mice. For example, this group is the one which spent the longer time in the center of the OF (discrepancy with normal behavior), while the SR-KO and MS mice were not different from controls, thus pointing to a synergistic effect of the risk factors. However, this synergy remains to be

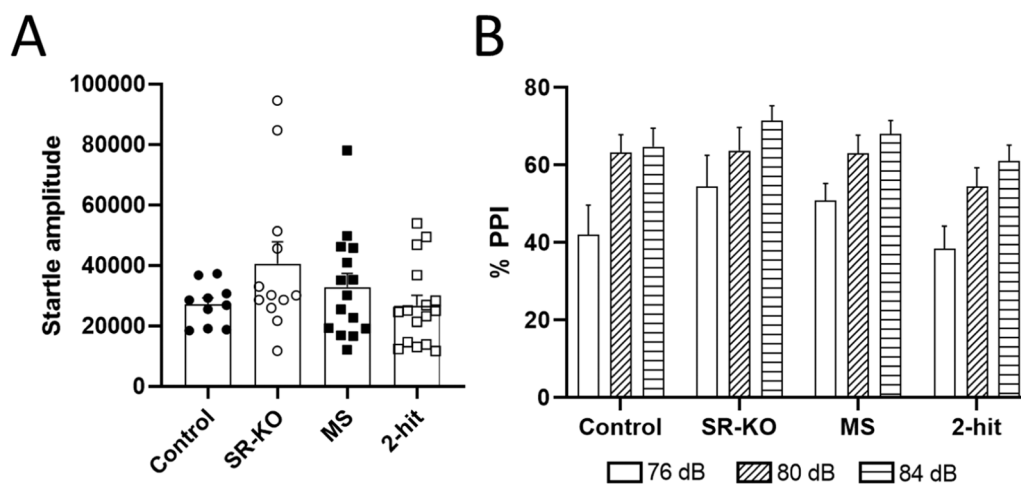


Fig. 5. Sensorimotor gating is not altered in SR-KO, MS and 2-hit groups. (A) Startle reflex amplitude triggered by a single 85 dB pulse, and (B) Prepulse inhibition (PPI) for increasing pre-pulse intensities (76 dB, 80 dB, 84 dB) in control, SR-KO, MS and 2-hit groups. ANOVA with two factors (intensity x group) revealed an intensity effect for all groups (p value < 0.0001). Data are presented as mean + SEM.

fully established. Indeed, glutamatergic hypofunction has been rather linked to an elevation in anxiety levels in other experimental conditions such as pharmacological NMDAR blockade [53,65,54,66]. In contrast, increase in time spent in the center compared to the control has previously been reported in MS models [67-69]. This kind of behavior may reflect a lower anxiety-like level in the 2-hit mice than the other groups suggesting a behavioral disinhibition process (or absence of inhibition). The behavioral disinhibition can be defined as a lack of control of motor skills, attention and concentration, and can be similarly found in people with impulsivity, lack of control, inappropriate euphoria or socially unacceptable behavior [70,71]. It is often studied in several comorbid diseases with SCZ, such as bipolarity, Attention Deficit Disorder with or without Hyperactivity (ADHD) or autism [72-74]. This type of behavior, to our knowledge, is not found in other 2-hit models, suggesting a characteristic behavior of our model (see [2]). Although the EPM and OF are two tests commonly used to assess the anxiety-like behavior, the anxiogenic environment for the animal is different between the two tests which may explain the differences obtained in anxiety-related parameters for the same animals (Bailey and Crawley, 2009; [75]). In the EPM test, MS mice showed a higher exploratory phenotype of the open arms compared to SR-KO and 2-hit mice suggesting a resistance effect in the 2-hit model (disappearance of the MS effect when cumulated with SRR partial deletion in 2-hit mice). Interestingly, an early maternal separation has been reported to decrease the anxiety-like behavior in the EPM test, confirming the results obtained in our study, and reinforcing the fact that an early stress could induce a resistance in case of exposure to an anxiogenic environment [76].

Working memory impairment is an important feature of cognitive deficits observed in schizophrenic patients [20,77,78], however only few studies using 2-hit models have assessed this parameter [22,2,23]. Indeed, another 2-hit model using 24 h MS in PND9 rats combined with a treatment of 100 mg corticosterone (weeks 8 – 10) did not display synergistic effects on working memory [79]. On the contrary, a synergistic effect of 50 mg corticosterone treatment (weeks 6 – 9) combined with genetic deletion of *Reelin* has been reported [23]. Interestingly, our results of the Y-maze test also revealed a synergy between the two risk factors. Indeed, when SR-KO or MS groups had similar performances as control mice, a deficit occurred when these two hits were combined. Once again, our new model confirms that interactions between several contributing factors are essential for the development of the SCZ-related phenotype and provide further evidence for a higher face validity compared to other available 2-hit models.

The sensorimotor gating abnormality, which is observed in SCZ [80], is generally assessed in preclinical studies through the prepulse inhibition (PPI) deficits test [81,82]. In the present study, we did not observe any PPI changes restricting the face validity of our 2-hit model, whatever the experimental condition. First, the absence of sensorimotor gating deficit in SR-KO mice (see also [24]) indicates that the NMDAR hypofunction generated by the D-serine depletion is not sufficient per se to affect this behavioral trait in contrast to pharmacological treatments with the NMDAR antagonists PCP and MK-801 [83,84]. Secondly, we did not reproduce PPI disruption in the MS model compared to previous observations (see [85,9,34]). Lastly, the absence of deficit in the 2-hit group suggests that a putative synergy between SR-KO and MS-related factors is not enough to affect the sensorimotor gating of the mice. The presence of PPI alteration is also more heterogeneous in other 2-hit models [86-89]. For example, 2-hit models combining MK-801 treatment with social isolation displays additive deleterious effects on PPI, meaning that the deficit found in 2-hit group is similar to the one found in 1-hit groups [90]. Interestingly, 2-hit models combining the deletion of *Nurr1* with social isolation or maternal immune activation present a synergistic effect on PPI deficit [91,92]. It has been suggested that the disturbance induced in the dopaminergic system could be linked to the disruption of PPI [85,93,34]. Thus, in our model, it may be necessary to verify the presence or absence of alterations in this neurotransmission system using electrophysiological or immunological studies.

Finally, our study does not reveal any disruption in social behavior, neither in the 2-hit nor in the 1-hit groups. Alteration of sociability has previously been reported in mice submitted to MS [15] whereas a defect in SR-KO mice is a matter of controversy [94-97]. Several explanations may account for these discrepancies [12] including differences in protocols and/or strains. On the one hand, we used C57BL/6 J mice in the present study whereas NMRI mice had been previously selected [15] which could be more sensitive to MS [98,99]. In the previous study, Bouet et al. [15] used an approach-avoidance test instead. This test consists of placing the stimulus mouse behind a wall with holes that may decrease the contact between animals, lowering the social reward, and rendering the task more difficult. In two studies of multifactorial models, social deficits were observed only if MS was combined with other genetic and/or environmental factors [100,101]. On the other hand, the parameter that we used is maybe not sufficient to highlight the social deficit in the model. Indeed, it is found that SR-KO mice displayed abnormal social investigation and vocalizations in the presence of a conspecific [97]. We can assume that our analysis of social behavioral is limited and that the assessment of more subtle changes, such as those in vocalization or sniffing, grooming and licking would be interesting to study in further detail. One future direction could be to assess social behaviors directly in home cages, in order to highlight specific social behaviors in a familiar environment [102,103].

To conclude, this model based on the “two-hit hypothesis” of SCZ [2,7] contributes to better understand the interactions between two risk factors of SCZ, a genetic risk factor (deletion of serine racemase) and an environmental risk factor (maternal separation). It is the first time these risk factors have been combined in an animal model and their interaction results in neurobehavioral alterations with the presence of working memory deficit, disinhibition and hyperlocomotion. These deficits referred to several symptoms observed in neurodevelopment disorders like SCZ (positive symptoms and cognitive deficits) however, the lack of other types of deficits (as negative symptoms and PPI deficit) is a missing point that requires an improvement of the model. One of the possibilities is the addition of a third factor (3-hit model) which may increase the severity of existing deficits and trigger other ones more related to SCZ symptoms (see [104] for more details about the 3-hit models).

The behavioral responses to the interaction between the risk factors depends on the parameters of the test used and are strongly influenced by the timing of factor occurrence and by interindividual responses. Each individual responds differently to the combination of the two factors. Thus, to better characterize the interaction effects, one other possibility would be to refine the tests and parameters collected. Indeed, our tests are brief (generally 5–10 min of testing) and need to place the animals in a new environment to which they must adapt. The “automated home cage monitoring” method has been proposed to be more relevant to measure behavioral deficits and to test the efficacy of new treatments in preclinical research [105-108]. The advantage of these cages is that they can monitor the behavior of rodents to be recorded directly in their home cage over a long period with minimal human contact [105,109]. For example, this monitoring directly in the home cage has made it possible to detect subtle and early phenotypes in the mouse model of Huntington’s disease compared to the control C57BL6/J mice [110]. Thus, this could therefore be useful for assessing more specifically the phenotype of the 2-hit animal model, which appears to display subtler, but richer, behavioral deficits than the mono-factorial models. It could also make it possible to observe the effects of subsequent pharmacological treatment.

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CRedit authorship contribution statement

Michel BOULOARD: Writing – review & editing. **François MENAGER:** Methodology, Funding acquisition. **Caroline LAHOGUE:** Writing – original draft, Visualization, Methodology, Funding acquisition, Formal analysis. **Valentine BOUET:** Writing – review & editing, Conceptualization. **Thomas FRERET:** Writing – review & editing. **Jean-Marie BILLARD:** Writing – review & editing, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest

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Institutional review board statement

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Data Availability

Data will be made available on request.

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