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

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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 behavioral and cognitive deficits associated with SCZ.
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Caen, 25th of March 2023,

Please find enclosed a manuscript entitled « **A new 2-hit model combining serine racemase deletion and maternal separation displays behavioral and cognitive deficits associated with schizophrenia** » to be considered for publication in *Schizophrenia Research*.

We describe here a new animal model of neurodevelopmental disorders that could substantially improve the research of new treatments. Our new multifactorial model presents both positive-like symptoms and cognitive deficits leading to an interesting phenotype for modelling paranoid schizophrenia. Because we believe that this model could be considered as a robust tool for testing new therapeutic strategies against cognitive deficits, we hope that this manuscript will attract your attention and will be considered for publication in *Schizophrenia Research*.

Sincerely Yours,

Caroline LAHOGUE and Valentine BOUET





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Declaration of competing interest: The authors declare no conflict of interest.

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1 Article

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

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12
13

14 **Abstract**

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

26 **Keywords:** multiple-hit; psychosis; stress; maternal separation; memory; serine racemase

27

28 **1. Introduction**

29 Schizophrenia (SCZ) is a psychotic disorder characterized by heterogeneous symptoms, including
30 positive (hallucinations, delusions), negative symptoms (avolition, anhedonia, social withdrawal) **and**
31 **cognitive impairments** (working memory deficits, attentional dysfunction) (Addington et al., 1991;
32 Tandon et al., 2013). The first psychosis episode usually occurs in the early adulthood (15 – 25 years)
33 and the course of the illness varies from one patient to another (Tandon et al., 2013). Although the
34 etiology of SCZ is still unknown, it is now well established that interactions between the genetic
35 background and environmental factors (gene x environment interactions) have a significant role in the
36 development of the disease (Moran et al., 2016; Guerrin et al., 2021; Wahbeh and Avramopoulos,
37 2021). Numerous genome-wide association studies reported genes polymorphism and copy number
38 variants in schizophrenic patients (Rees and Kirov, 2021). Several genes have been identified as risk
39 factors, including those related to neuronal communication involving glutamatergic neurotransmission

40 (Morita et al., 2007; Ripke et al., 2014; Nakazawa and Sapkota, 2020). The dual hit hypothesis of SCZ
41 suggests that the genetic vulnerability (first hit), in association with environmental factors (early stress
42 or late environmental factors; second hit) could favor its occurrence (Brown, 2011; Davis et al., 2016;
43 Millan et al., 2016; Stilo and Murray, 2019). Environmental factors include several complications during
44 pregnancy (such as mother infection, malnutrition, or psychosocial stress), childhood trauma (physical,
45 psychological, or sexual abuse, neglect, or exposure to physical and psychological abuse) and adversity,
46 bullying and discrimination or drug abuse during adulthood (Ellenbroek and Cools, 2002; Murthy and
47 Gould, 2018; Guerrin et al., 2021). Childhood trauma is one of the factors widely studied in the context
48 of SCZ (Larsson et al., 2013). The stress generated by a traumatic episode alters the hypothalamic-
49 pituitary-adrenal axis (HPA). Several paradigms have been developed to reproduce childhood trauma
50 in animal models of SCZ and one of the most frequent is maternal separation (MS) (reviewed in Nishi
51 et al., 2013; Marco et al., 2015; Čater and Majdič, 2022). MS for 24h at post-natal day (PND) 9 in rodent
52 disrupts the HPA axis (Tractenberg et al., 2016; Van Bodegom et al., 2017) and produces sensorimotor
53 gating deficit (Ellenbroek and Cools, 2002) while it increases anxiety-like behavior and alters sociability
54 in rodents (Bouet et al., 2011). These are all important hallmarks of SCZ.

55 Antipsychotic drugs are have been shown effective (even if not curative) to treat the positive
56 symptoms while the negative and cognitive symptoms remain resistant (Nucifora et al., 2019; Correll
57 and Howes, 2021). Among the possible explanations is the lack of reliability in the validity of animal
58 models of SCZ used in preclinical investigations (Sotiropoulos et al., 2021). Indeed, such animal models
59 are expected to mimic as much as possible the mechanisms underlying the disease (construct validity),
60 as well as to display behavioral abnormalities and cellular alterations similar to those observed in
61 patients (face validity). Finally, they have to respond to the existing treatments (predictive validity).
62 Although multiple animal models of SCZ have been developed, they mostly used a single risk factor
63 and therefore, do not accurately reflect the complexities of the disease (Jones et al., 2011). More
64 recently, some multifactorial models combining two factors (genetic x environment; genetic x genetic
65 or environment x environment factors) have been developed to better consider the face validity
66 (Moran et al., 2016; Guerrin et al., 2021; Sotiropoulos et al., 2021). Even for 2-hit animal models of
67 SCZ, it is complicated to observe all the symptoms of the disease, and each model presents a different
68 behavioral phenotype (for review see Guerrin et al., 2021). For example, alteration of the working
69 memory, which frequently occurs in SCZ patients (Conklin et al., 2005; Timofeeva and Levin, 2011a), is
70 not systematically reproduced in all 2-hit models. This could be due to the fact that few studies have
71 been conducted on this subject (Choy et al., 2009; Schroeder et al., 2015; Guerrin et al., 2021). In this
72 sense, the necessity to improve comprehension of the multifactorial models of the disease remains
73 therefore a major issue that prompted us to reconsider the development of a new 2-hits mouse model
74 of SCZ.

75 Among the genes associated with SCZ, serine racemase (SR) is now considered to be highly relevant
76 (Morita et al., 2007; Basu et al., 2009; Jacobi et al., 2019). SR is localized in the neuronal cells of the
77 forebrain and synthesizes D-serine, a co-agonist of the N-methyl-D-aspartate subtype of glutamate
78 receptors (NMDAR) (Wolosker et al., 1999b, 1999a). Some clinical studies have found a polymorphism
79 in the SR gene, associated with a reduction of D-serine levels in the cerebrospinal fluid and brain of
80 schizophrenic patients (Yamada et al., 2005; Goltsov et al., 2006; Morita et al., 2007; Jacobi et al., 2019;
81 Nakazawa and Sapkota, 2020). The total deletion of exon 1 of the SR gene (SR-KO) decreased D-serine
82 levels by at least 90 % in the brain of mice and is linked to behavioral alterations such as hyperactivity
83 and memory deficits (Basu et al., 2009; Ploux et al., 2020). Recent *ex vivo* electrophysiological studies

84 have reported an alteration of the excitatory/inhibitory balance in the CA1 hippocampal networks of
85 those mice (Ploux et al., 2020; Jami et al., 2021). Thus, the phenotype of the SR-KO mice, considered
86 as a model of NMDAR hypofunction, appeared as an interesting model to test the potential impact of
87 combination with an early stress exposure on cognitive abilities. Herein, we selected MS of 24 h at
88 post-natal day (PND9) as an early stress factor (Ellenbroek et al., 1998; Ellenbroek and Riva, 2003) since
89 it has been shown to impaired anxiety-like and social behavior in rodents (Fabricius et al., 2008; Bouet
90 et al., 2011). In the present study, four groups of male mice were constituted, including wild-type mice
91 (control group), 1-hit SR-KO mice (SR-KO group), 1-hit mice with maternal separation (MS group), and
92 SR-KO mice with MS (2-hit group). To characterize the behavioral deficits related to SCZ symptoms and
93 better highlight the impact of the interaction between the two risk factors, a large range of behavioral
94 assessments was performed. The 2-hit mice showed an increase in locomotor activity (assessed with
95 an open field test and actimeter) and an alteration in memory performances (including working
96 memory assessed with a spontaneous alternation test in the Y-maze and recognition memory assessed
97 with place preference in the Y-maze). These results indicate that this new 2-hit mouse model displays
98 an interesting phenotype, with both positive and cognitive symptoms of SCZ and could therefore be
99 considered as a robust tool for testing new therapeutic strategies.

100 2. Methods Materials and Methods

101 2.1. Animals

102 Male mice aged 2-4 months provided by our local facility (Centre Universitaire de Ressources
103 Biologiques, Université de Caen Normandie, France) were used including wild type (WT) C57BL/6J mice
104 and SR-KO mice originally developed in the Pr J.T. Coyle's laboratory (Belmont, MA, USA) (Basu et al.,
105 2009). Mice were housed in polycarbonate cages (n = 5 per cage) in standard controlled conditions,
106 including a reversed 12h-12h light-dark cycle (light on at 7:30 am), regulated temperature ($22 \pm 2^\circ\text{C}$),
107 humidity ($50 \pm 10\%$) and free access to food and water. Cage enrichment consisted in crinkle cut
108 shredded paper and a cardboard house. Experiments were performed in accordance with French and
109 European Economic Community guidelines for the care and use of laboratory animals (2010/63/UE,
110 project authorization n°2020121013375130 v2). Two cohorts were used to perform all behavioral
111 experiments. 23 WT and 25 SR-KO mice were used for the first experiment and 22 WT and 29 SR-KO
112 for the second one. Behavioral experiments are detailed on figure 1.

113

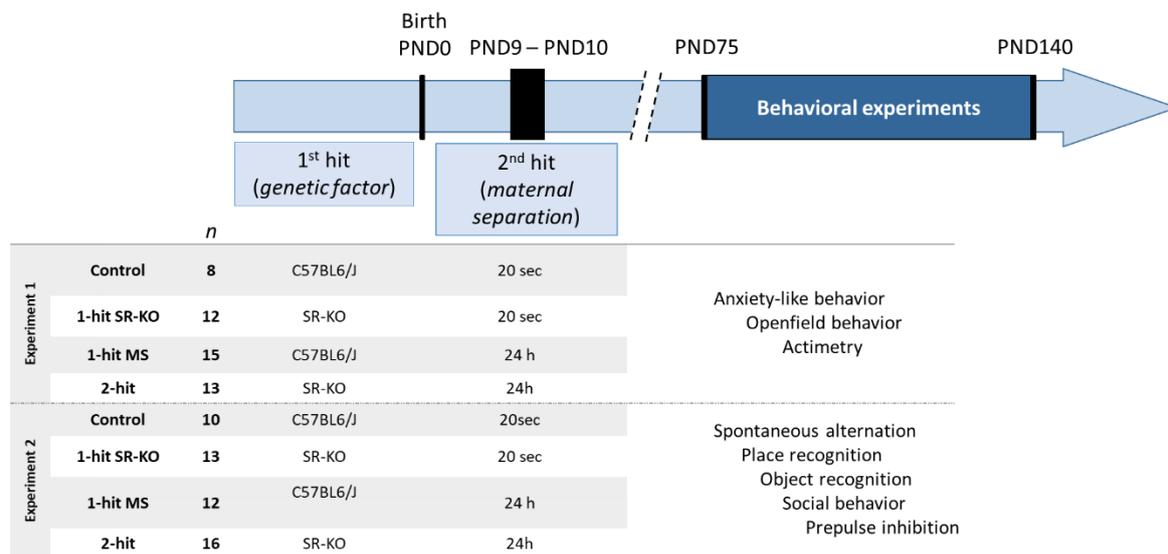
114 2.2. Maternal separation (MS)

115 At PND9, wild-type and SR-KO litters were separated from their mother for 24 h (early environmental
116 stress) (Ellenbroek and Cools, 2002; Bouet et al., 2011). Mothers were removed and placed in a cage
117 nearby (from 9:00 a.m. to 9:00 a.m., the day after). Then, the mothers were returned to their litters
118 and left undisturbed until weaning. Control mice were submitted to the same protocol with only 20
119 seconds of separation time.

120 2.3. Behavioral assessment

121 Animals were handled daily for 5 minutes during the week preceding the experiments. The
122 experiments were conducted during the active-dark phase of the cycle and were started at 8:00 am.
123 Each new behavioral test was carried out one week apart. Animal cages were placed in the
124 experimental room 30 min before the beginning of each experiment. Apparatuses were cleaned with

125 alcohol (70%) between each assessment. Two sets of experiments were performed for the behavioral
 126 assessment (Fig 1). Except for spontaneous locomotor activity (in actimeter), all behavioral tests were
 127 videotaped and scored using EthoVision XT v.16.0 (Noldus®, The Netherlands).



128

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

132

2.3.1. Anxiety-like behavior

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

138

2.3.2. Openfield behavior

139 Mice were individually placed for 30 min in a squared open arena (50 × 50 × 50 cm). The total distance
 140 and percentage of time spent in the center of the openfield (OF) were automatically collected (Prut
 141 and Belzung, 2003).

142

2.3.3. Actimetry

143 Motor activity was measured in Plexiglas cages (19 × 11 × 14cm) with an automated actimeter
 144 containing six lines of infrared beams (IMETRONIC®). Mice were individually placed for 30 min in cages
 145 (19 × 11 × 14 cm), and the number of beams crossing reflecting locomotor activity (horizontal and
 146 vertical movements) was automatically counted.

147

2.3.4. Spontaneous Alternation

148 Spatial working memory was assessed by spontaneous alternation in a single-session Y-maze test
 149 (Bazin et al., 2009). The maze consisted of three equally spaced plastic arms (22 × 6.5 × 10 cm). Each
 150 mouse was individually placed at the end of one arm, facing the front of the wall, and allowed to freely
 151 explore the maze during a 5-min session. The sequence of arm entries (four paws criterion) was

152 collected. An alternation was defined as consecutive entries into three different arms. The alternation
153 percentage was calculated using the formula: (number of alternations) / (total number of entries-
154 2)*100.

155 2.3.5. Place recognition

156 Place recognition was assessed in a Y-maze (Dellu et al., 2000; Asselot et al., 2017). The maze, different
157 from that used for the spontaneous alternation test, consisted of three equally spaced arms (gray
158 plastic; 34 × 8 × 16 cm). During the first training session, the mouse was placed in the starting arm,
159 facing the wall, and allowed to freely explore for 5 min the maze with one arm closed by a removable
160 door (left of right randomly), before being returned to its home cage. After a 60-min inter-trial interval,
161 the mouse was placed again in the maze for the test session and allowed to freely explore the three
162 arms for 2 min. The place recognition test is based on the preference of rodents for novelty, and the
163 remembrance of the previously visited arm. During each session (training and retrieval), the time spent
164 and the number of arm entries were recorded. A discrimination index was calculated the formula:
165 [(time in the novel arm) – (time in the familiar arm)] / [(time in the novel arm) + (time in familiar arm)]
166 *100.

167 2.3.6. Object recognition

168 The object recognition test was performed in a Y-maze (gray plastic; 34 × 8 × 16 cm for each arm) with
169 two different objects, available in four copies: lego® towers and sealed transparent bottles filled with
170 sand. The protocol used to evaluate object recognition memory was based on the study by Leger et
171 collaborators (Leger et al., 2013). The same maze as the one used for the place recognition test was
172 used for object recognition to benefit from the habituation induced by previous exposure. During the
173 acquisition session, two identical objects were placed in the maze in the distal parts of the arms. The
174 mouse was placed at the end of the arm containing no object, faced to the wall and was allowed to
175 freely explore the apparatus and the objects. Object exploration was considered when the nose of the
176 mouse was oriented towards the object at a distance lower than or equal to 2 cm. The criterion was
177 reached when the mouse had explored the objects for a total duration of 20 sec within a maximum of
178 10 min, and was returned to its respective home cage for 2h until the test session ended. During the
179 test phase, a copy of the familiar object and a novel object were placed in the maze. Again, the mouse
180 was allowed to freely explore the maze and the objects with the same criteria. The nature of the
181 new/familiar objects (Lego® vs bottle) and their position (in left/right arm) were randomized. The time
182 spent to fulfill the criterion was measured. The time of exploration of each object was manually
183 recorded and used as an index of memory performances, in comparison with the chance level (10 sec).

184 2.3.7. Social behavior

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

194 compartment)]/ [(time spent in the stimulus mouse compartment) + (time spent in the empty
195 compartment)] *100.

196 **2.3.8. Prepulse inhibition (PPI)**

197 PPI was measured with a startle reflex device (Imetronic®, France) (Valsamis and Schmid, 2011). Each
198 mouse was placed in a gray soundproof chamber, on a platform mounted on a weight-sensor and
199 surrounded with a transparent cylinder. Weight variation was used to calculate the amplitude of the
200 startle reflex. After 10 min of acclimatization to a background white noise (65 dB), 30 trials of pulse (85
201 dB) were performed to obtain startle reflex measurements, habituate the animal to the test, and
202 decrease intra-individual variability. During the test phase, the mouse was subjected to 5 types of trial
203 with an inter-trial interval of 10 to 20 s: no pulse (silence), pulse only (110 dB, 40 ms), pre-pulse (76,
204 80 or 84 dB, 20 ms presented 50 ms before the pulse) + pulse (110 dB, 40 ms). Mice were videotaped
205 throughout the protocol which lasted about 25 min. The percentage of prepulse inhibition was
206 calculated for each intensity as follows: $(1 - (\text{startle response magnitude of trials with prepulse} / \text{startle}$
207 $\text{response magnitude of startle-only trials})) * 100$.

208 **2.4. Statistics**

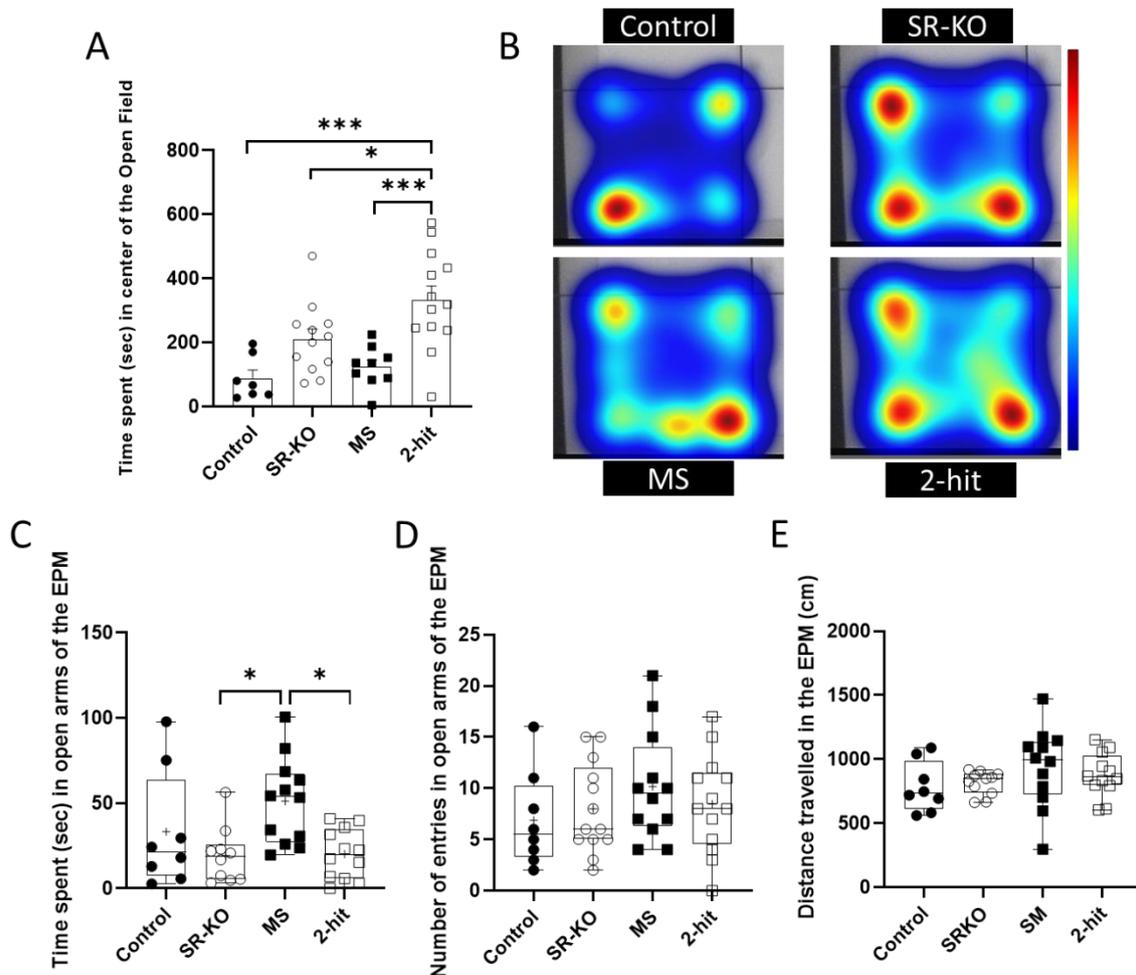
209 All analyses were performed with RStudio® software 4.2.0 and GraphPad® Prism 8.3. The normality of
210 the data was checked using the Shapiro-Wilk test, and the homogeneity of variances was assessed with
211 the Levene test. For data normally distributed, ANOVAs and a post-hoc Tukey honestly significant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

219 **3. Results**

220 **3.1. Experiment 1:**

221 **3.1.1. Anxiety-like behavior in OF and EPM**

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



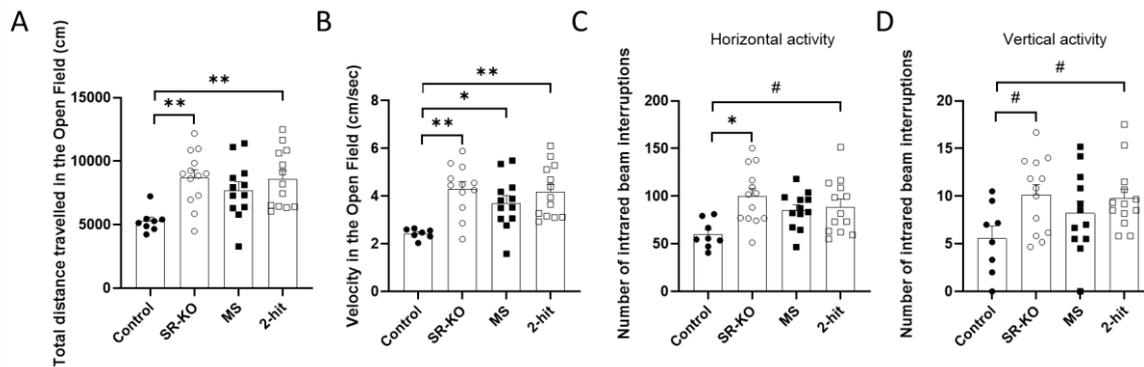
231

232 **Figure 2. The combination of SR-KO and maternal separation did not affect anxiety-like behavior.** (A) Total time spent in
 233 the center of the open field over 30 min. Data are presented as mean + SEM. ANOVA test with post-hoc tukeyHSD: * <0.05 ,
 234 *** <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
 235 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
 236 elevated-plus maze (EPM). (C) Total number of entries in the open arms of the EPM. (D) Total distance travelled for 5 min
 237 in the EPM. Data presented are boxplots, with horizontal black lines as the median, "+" as mean and vertical black lines as
 238 minimal and maximal. ANOVA with permutation and pairwise permutation test: * <0.05 .

239

3.1.2. Spontaneous activity

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



245

246 **Figure 3. Spontaneous locomotion is affected by SR deficits combined with maternal separation.** (A) Total distance travelled
 247 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)
 248 horizontal and (D) vertical activity. Data presented are individual values (squares and circles) and mean \pm SEM. ANOVA test
 249 with post-hoc tukeyHSD: #<0.1, *<0.05, **<0.01, ***<0.001.

250 3.2. Experiment 2:

251 3.2.1. Spontaneous alternation

252 Working memory was investigated in a Y-maze apparatus through spontaneous alternation. The
 253 number of arm entries in the Y-maze was significantly higher in the 2-hit groups than in the control
 254 groups. For the SR-KO and SM groups, the number of arms entries did not differ from control or 2-hit
 255 groups (Fig 4A). Concerning memory performances, there was no difference in the alternation
 256 percentage between groups. However, comparison to the chance value (50 %) showed that **while**
 257 **control, SR-KO and MS groups displayed an alternation percentage significantly above 50%, that of the**
 258 **2-hit group did not differ from 50%, suggesting a hazardous behavior in the Y-maze** induced by the
 259 combination of risk factors (Fig 4B).

260 3.2.2. Place recognition

261 Spatial recognition memory was assessed through place recognition in Y-maze. Unexpectedly, control,
 262 SR-KO and MS groups displayed a recognition index not different from 0, indicating no discrimination
 263 **compared with** the novel arm. The recognition index of the 2-hit group was significantly below the
 264 reference value of 0, suggesting that these mice had a preference **for remaining** in the familiar arm
 265 instead of exploring the new one (Fig 4C).

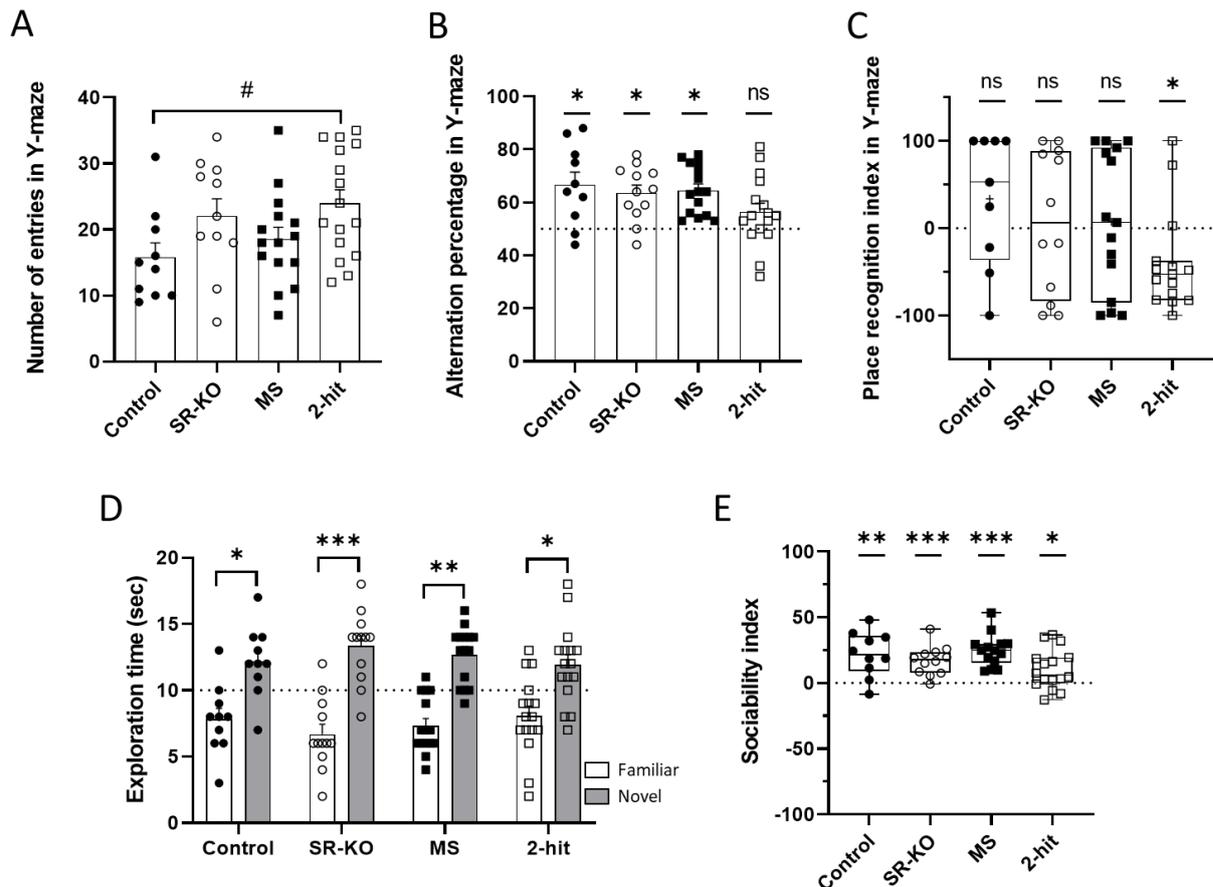
266 3.2.3. Object recognition

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

271

272 3.2.4. Social behavior

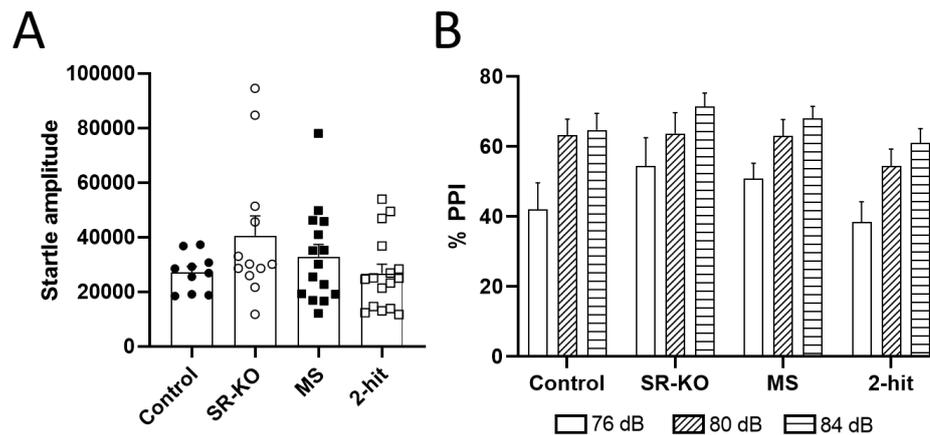
273 There was no difference between groups for the sociability index. All groups displayed a sociability
 274 index above 0, indicating a preference for the new congener (Fig 4E).



275
 276 **Figure 4. SR-KO combined with MS impairs working memory and recognition.** (A) Number of entries in the Y-maze. (B)
 277 Alternation percentage in the Y-maze. (C) Place recognition index in Y-maze. (D) Exploration time of the familiar (white
 278 histogram) and novel (gray histogram) object during the retention phase of an object recognition test. (E) Sociability index.
 279 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
 280 vertical black lines as minimum and maximum. Intergroup comparisons between groups were performed using ANOVA test
 281 with post-hoc tukeyHSD ($\# < 0.1$, $* < 0.05$, $** < 0.01$, $*** < 0.001$) and comparison to a reference value was performed with a
 282 one-sample t-test or one-sample Wilcoxon test: $* < 0.05$; $** < 0.01$, $*** < 0.001$.

283 3.2.5. Prepulse inhibition (PPI)

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



288
 289 **Figure 5. Sensorimotor gating is not altered in SR-KO, MS and 2-hit groups.** (A) Startle reflex amplitude triggered by a single
 290 85dB pulse, and (B) Prepulse inhibition (PPI) for increasing pre-pulse intensities (76 dB, 80 dB, 84 dB) in control, SR-KO, MS
 291 and 2-hit groups. ANOVA with two factors (intensity x group) revealed an intensity effect for all groups (p value < 0.0001).
 292 Data are presented as mean + SEM.

293 4. Discussion

294 This study aimed at characterizing the behavioral phenotype of a new 2-hit model **with behavioral and**
 295 **cognitive deficits** based on the combination of **two risk factors including** a deletion of serine racemase
 296 gene (SR-KO; 1st hit) that reproduces the SCZ-related glutamatergic hypofunction and a maternal
 297 separation (MS; 2nd hit) mimicking an early environmental stress. The hypothesis was that the genetic
 298 deletion would favor the vulnerability to the second risk factor (Guerrin et al., 2021). We showed here
 299 that the 2-hit mice displayed an increase in locomotor activity in three experimental protocols (open
 300 field, actimeter, and in Y-maze), as well as **limited** deficits in working memory (spontaneous alternation
 301 in the Y-maze) and in spatial recognition memory (place recognition test). On the contrary, the
 302 sociability-related behavior and the sensorimotor gating of 2-hit mice were not affected. This new
 303 animal model therefore presents a partial schizophrenic-like phenotype, with not only **positive**-like
 304 symptoms but also cognitive deficits, in contrast to most of the other 2-hit models **currently** available.

305 The genetic risk factor was selected to reproduce an altered activity of the NMDAR according to the
 306 glutamate hypofunction hypothesis of SCZ (Uno and Coyle, 2019). The NMDAR plays an essential role
 307 in cortical function but also in brain synaptic plasticity, underlying learning, memory, and other
 308 cognitive functions (Morris et al., 1990; Bliss and Collingridge, 1993; Izquierdo and Medina, 1995). SR-
 309 KO mice carry a deletion of the first exon of the SR gene, inducing 90% decrease in **brain levels of** the
 310 NMDA co-agonist D-serine (Basu et al., 2009; Ploux et al., 2020). **Current evidence indicates** that these
 311 mice display subtle but significant behavioral abnormalities (Basu et al., 2009; Labrie et al., 2009; Mori
 312 and Inoue, 2010; Sullivan et al., 2011; DeVito et al., 2011; Balu et al., 2013; Hagiwara et al., 2013; Jacobi
 313 et al., 2019; Ploux et al., 2020; Aguilar et al., 2021; Torres Jimenez et al., 2021; Jami et al., 2021) **which**
 314 **could be amplify by** additional **disruptors** such as those generated by environmental changes. Although
 315 many environmental risk factors have been pointed in SCZ, childhood trauma including physical or
 316 psychological abuse and neglect (Larsson et al., 2013; Misdrahi, 2016; Okkels et al., 2017) **are among**
 317 the most **predominantly studied** so far. It is reproduced in preclinical studies **through** a wide range of
 318 MS paradigms (Nishi et al., 2013; Marco et al., 2015). Among them, **we have previously shown** that MS
 319 **applied** for 24h at PND 9 induced behavioral disabilities in mice (increased anxiety levels and decreased

320 social behavior (Bouet et al., 2011)), which could possibly increase the genetic-related defects in a 2-
321 hit strategy.

322 The first major phenotype displayed by the 2-hit mice model is an increase in parameters related to
323 locomotion (velocity and total distance, horizontal and vertical activities) which have been considered
324 to mimic positive-like symptoms of SCZ (Van Den Buuse, 2010). The fact that the hyperlocomotion was
325 found in the 2-hit and SR-KO (Basu et al., 2009) mice but not in the MS group shows that a genetically-
326 induced glutamate hypofunction plays a major role in this deficit. Indeed, this behavioral impairment
327 is often observed in pharmacologically-induced SCZ models of dopaminergic hyperactivity but also of
328 glutamatergic hypofunction (Ogren and Goldstein, 1994; Gleason and Shannon, 1997; Carey et al.,
329 1998; Enkel et al., 2013; Akillioglu and Karadepe, 2021). For example, treatments with the
330 noncompetitive NMDAR antagonists phencyclidine (PCP) or MK-801 induce hyperlocomotion (Jentsch
331 et al., 1998; Enomoto et al., 2007; Kehrer, 2008). Besides, mice deprived of the NR1 subunit of the
332 NMDAR also display an increase in locomotor activity associated with stereotypies (Mohn et al., 1999;
333 Halene et al., 2009). This behavioral signature is also found in mice with genetic deletion of the
334 Disrupted in schizophrenia 1 gene (DISC1) (Ibi et al., 2010; Ma et al., 2013; Lipina and Roder, 2014),
335 one of the genes recognized to be associated with SCZ (Hennah et al., 2005), notably due to its
336 implication in glutamatergic neurotransmission and in the stabilization of SR (Ma et al., 2013; Jacobi et
337 al., 2019). The fact that the hyperlocomotion was not higher in 2-hit than in SR-KO mice suggests a
338 minor, if any, contribution of the MS to this particular phenotype.

339 In addition, other aspects of the locomotor activity more related to anxiety-like behavior, also suggest
340 a specific signature in the 2-hit mice. For example, this group is the one which spent the longer time in
341 the center of the OF (discrepancy with normal behavior), while the SR-KO and MS mice were not
342 different from controls, thus pointing to a synergetic effect of the risk factors. However, this synergy
343 remains to be fully established. Indeed, glutamatergic hypofunction has been rather linked to an
344 elevation in anxiety levels in other experimental conditions such as pharmacological NMDAR blockade
345 (McLean et al., 2010; Amani et al., 2013; Enkel et al., 2013; Akillioglu and Karadepe, 2021). In contrast,
346 increase in time spent in the center compared to the control has previously been reported in MS
347 models (Ito et al., 2010; Lussier and Stevens, 2016; Shin and Lee, 2023). This kind of behavior may refer
348 to reflect a lower anxiety-like level in the 2-hit mice than the other groups suggesting a behavioral
349 disinhibition process (or absence of inhibition). The behavioral disinhibition can be defined as a lack of
350 control of motor skills, attention and concentration, and can be similarly found in people with
351 impulsivity, lack of control, inappropriate euphoria or socially unacceptable behavior (Derryberry and
352 Rothbart, 1997; Kochanska et al., 1997). It is often studied in several comorbid diseases with SCZ, such
353 as bipolarity, Attention Deficit Disorder with or without Hyperactivity (ADHD) or autism (Stevens et al.,
354 2002; Hirshfeld-Becker et al., 2003; Kempf et al., 2005; Buckley et al., 2009; Arfaie et al., 2018; Popovic
355 et al., 2019; Hossain et al., 2020; Chien et al., 2021). This type of behavior, to our knowledge, is not
356 found in other 2-hit models, suggesting a characteristic behavior of our model (see Guerrin et al.,
357 2021).

358 Working memory impairment is an important feature of cognitive deficits observed in schizophrenic
359 patients (Conklin et al., 2005; Kebir and Tabbane, 2008; Timofeeva and Levin, 2011b). Paradoxically,
360 this aspect has been rather neglected in preclinical studies using pharmacologically-induced SCZ-
361 related deficits and only few studies using 2-hit models have assessed this parameter (Choy et al.,
362 2009; Schroeder et al., 2015; Guerrin et al., 2021). Indeed, another 2-hit model using 24h MS in PN9

363 rats combined with a treatment of 100 mg corticosterone (weeks 8 – 10) did not display synergistic
364 effects on working memory (Choy et al., 2008). On the contrary, a synergistic effect of 50 mg
365 corticosterone treatment (weeks 6 – 9) combined with genetic deletion of *Reelin* has been reported
366 (Schroeder et al., 2015). Interestingly, our results of the Y-maze test also revealed a synergy between
367 the two risk factors. Indeed, when SR-KO or MS groups had similar performances as control mice, a
368 deficit occurred when these two hits were combined. Once again, our new model confirms that
369 interactions between several contributing factors are essential for the development of the SCZ-related
370 phenotype and provide further evidence for a higher face validity compared to other available 2-hit
371 models.

372 The sensorimotor gating abnormality, which is observed in SCZ (San-Martin et al., 2020), is generally
373 assessed in preclinical studies through the prepulse inhibition (PPI) deficits test (Bullock et al., 1997;
374 Swerdlow and Light, 2018). In the present study, we did not observe any PPI changes restricting the
375 face validity of our 2-hit model, whatever the experimental condition. First, the absence of
376 sensorimotor gating deficit in SR-KO mice (see also Basu et al., 2009) indicates that the NMDAR
377 hypofunction generated by the D-serine depletion is not sufficient per se to affect this behavioral trait
378 in contrast to pharmacological treatments with the NMDAR antagonists PCP and MK-801 (Curzon and
379 Decker, 1998; Bubeníková et al., 2005; Rasmussen et al., 2007). Secondly, we did not reproduce PPI
380 disruption in the MS model compared to previous observations (see Ellenbroek et al., 1996, 1998;
381 Ellenbroek and Cools, 2002). Lastly, the absence of deficit in the 2-hit group suggests that a putative
382 synergy between SR-KO and MS-related factors is not enough to affect the sensorimotor gating of the
383 mice. The presence of PPI alteration is also more heterogeneous in other 2-hit models (Boucher et al.,
384 2007, 2011; Haque et al., 2012; Lim et al., 2012; Zamberletti et al., 2012; Long et al., 2013; Lukasz et
385 al., 2013; O'Leary et al., 2014; Howell and Pillai, 2016; Khan and Powell, 2018). For example, 2-hit
386 models combining MK-801 treatment with social isolation displays additive deleterious effects on PPI,
387 meaning that the deficit found in 2-hit group is similar to the one found in 1-hit groups (Lim et al.,
388 2012). Interestingly, 2-hit models combining the deletion of *Nurr1* with social isolation or maternal
389 immune activation present a synergistic effect on PPI deficit (Eells et al., 2006; Vuillermot et al., 2012).
390 It has been suggested that the disturbance induced in the dopaminergic system could be linked to the
391 disruption of PPI (Ellenbroek et al., 1996, 1998; Ellenbroek and Cools, 2000). Thus, in our model, it may
392 be necessary to verify the presence or absence of alterations in this neurotransmission system using
393 electrophysiological or immunological studies.

394 Finally, our study does not reveal any disruption in social behavior, neither in the 2-hit nor in the 1-hit
395 groups. Alteration of sociability has previously been reported in mice submitted to MS (Bouet et al.,
396 2011) whereas a defect in SR-KO mice is a matter of controversy (Labrie et al., 2009; Matveeva et al.,
397 2019; DeVito et al., 2011; Aguilar et al., 2021). Several explanations may account for these
398 discrepancies (Nishi et al., 2013) including differences in protocols and/or strains. On the one hand,
399 we used C57BL/6J mice in the present study whereas NMRI mice had been previously selected (Bouet
400 et al., 2011) which could be more sensitive to MS (Own and Patel, 2013; Powell and Swerdlow, 2023).
401 In the previous study, Bouet et al. (2011) used an approach-avoidance test instead. This test consists
402 of placing the stimulus mouse behind a wall with holes that may decrease the contact between
403 animals, lowering the social reward, and rendering the task more difficult. In two studies of
404 multifactorial models, social deficits were observed only if MS was combined with other genetic and/or
405 environmental factors (Sultana and Lee, 2020; Bouet et al., 2021). On the other hand, the parameter
406 that we used is maybe not sufficient to highlight the social deficit in the model. Indeed, it is found that

407 SR-KO mice displayed abnormal social investigation and vocalizations in the presence of a conspecific
408 (Matveeva et al., 2019). We can assume that our analysis of social behavioral is limited and that the
409 assessment of more subtle changes, such as those in vocalization or sniffing, grooming and licking
410 would be interesting to [study in further detail](#). One future direction [could](#) be to assess social behaviors
411 directly in home cages, in order to highlight specific social behaviors in a familiar environment (Fairless
412 et al., 2013; Kim et al., 2019).

413 To conclude, this model based on the “*two-hit hypothesis*” of SCZ (Davis et al., 2016; Guerrin et al.,
414 2021) contributes to better [understand](#) the interactions between two risk factors of SCZ, a genetic risk
415 factor (deletion of serine racemase) and an environmental risk factor (maternal separation). It is the
416 first time these risk factors have been combined in an animal model and their interaction results in a
417 partial phenotype of SCZ with the presence of both positive (hyperlocomotion) and cognitive
418 dysfunction (working memory deficit, disinhibition). The presence of cognitive deficits in this model
419 constitutes an attractive phenotype to develop new therapeutic drugs against these [types](#) of deficits
420 in SCZ. The lack of deficits in other parameters may be due to the behavioral tests used. Our tests are
421 brief (generally 5 to 10 min of testing) and need to place the animals in a new environment to which
422 they must adapt. Thus, these tests are highly sensitive to external environmental conditions, which
423 can disrupt the protocol (e.g. light, noise, smells or time of day). The “automated home cage
424 monitoring” [method](#) has been proposed to be more relevant to measure behavioral deficits and to test
425 the efficacy of new treatments in preclinical research (Jhuang et al., 2010; Robinson and Riedel, 2014;
426 Mingrone et al., 2020; Ho et al., 2023). The advantage of these cages is that they can monitor the
427 behavior of rodents to be recorded directly in their home cage over a long period with minimal human
428 contact (Bains et al., 2018; Ho et al., 2023). For example, this monitoring directly in the home cage has
429 made it possible to detect subtle and early phenotypes in the mouse model of Huntington's disease
430 compared to the control C57BL6/J mice (Bains et al., 2023). Thus, this could therefore be useful for
431 assessing more specifically the phenotype of the 2-hit [animal](#) model, which appears to display subtler,
432 but richer, behavioral deficits than the mono-factorial models. [It could also make it possible to observe
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Title: Gene-environment interactions in a new 2-hit mouse model of schizophrenia: behavioral phenotyping

Dear Matcheri S. Keshavan,
Editor in Chief
Schizophrenia Research

We thank you for the opportunity to revise our manuscript and the reviewers for their constructive comments. All the points raised have now been addressed, and our point-by point reply is presented below. According to the corrections, all changes in the manuscript are highlighted in blue.

Reviewer #1: Lahogue et al. make a convincing case for the involvement of serine racemase as a susceptibility factor for early life stress and demonstrate that the combination of these two factors results in behavioral deficits. Their data is presented clearly and transparently and I do not have concerns regarding the claims that are being made. However, I have two suggestions for improving this manuscript.

1) The writing style in the manuscript is very awkward at times. My suspicion is that the authors are not native English speakers and they directly translated from French without taking into account that sentence structure between the 2 languages are quite different. For example "Even if numerous animal models of SCZ have been developed, they are mostly based on a single risk factor (genetic, pharmacologic, environmental,...) which distanced them from the multifactorial aspect of the illness (Jones et al., 2011)." A better way to frame this is, "Although multiple animal models of SCZ have been developed, they mostly model a single risk factor and therefore, do not accurately reflect the complexities of the disease." In addition the statement in the abstract that, "Our study aimed to characterize an innovative mouse model of schizophrenia" is inaccurate. The study aims to develop a novel mouse model for schizophrenia and it should be stated as such. I also do not understand the term "The face validity." I recommend the authors get the manuscript edited/reviewed by a native English speaker.

We thank the referee for his very positive view of our study and we apologize if our writing style was sometimes awkward. Our manuscript has been proofread by a native English speaker and English professor, Sophie Pricot, who has been thanked in the Acknowledgements section. All changes are in blue in the text.

2) Not characterizing the molecular changes in the brain between the different animal paradigms (control, single hit and double hit) is a missed opportunity. Identifying the molecular changes in the brain between single and double hit animals where behavioral differences are being reported would be a fantastic addition to this paper. Although performing a genome wide characterization i.e. RNA seq would be ideal, I understand the authors need to publish their work. However, they should do immunohistochemistry (or other assays) to determine whether alterations in NMDA related activity is associated with behavioral changes as hypothesized.



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We agree that the characterization of the molecular changes would be an important step to identify the possible mechanisms underlying differences between 1-hit and 2-hit groups. In this direction, we have also performed in parallel an *ex vivo* electrophysiological study from hippocampal of the experimental groups. This was done using extracellular recordings in the CA1 area in which basal excitatory transmission and NMDA receptor-dependent synaptic plasticity were investigated. Although some changes have been recorded, they failed to reach statistical significance suggesting that behavioral alterations found in our study involve subtle changes in functionality or are generated outside the CA1 hippocampal area.

Minor comments

- Not clear why certain data are plotted as histograms and others as boxplots. It would be good to have a consistent style. This is a minor issue as the authors show individual data points.

The data are illustrated according to their distribution. Data normally distributed are expressed as mean + standard error of the mean while non-normally data distributed are expressed in median \pm interquartile. We clarified this in the Statistics section (lines 209 to 218).

- I would prefer if "Serine racemase" and "maternal separation" were included in the title which currently is vague.

We thank the referee for this remark. Accordingly, the title has been modified, and these aspects now appear in the following title:

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

- The animal model clearly has behavioral and cognitive deficits but I am not convinced that it is a model for schizophrenia. It may be wise to tone down these claims and state that this is an animal model with behavioral and cognitive deficits.

Regarding this concern, our enthusiasm is based on the construction of the model (gene x environment) which fits with the most recent view of schizophrenia etiology (see Guerrin et al., 2021 and Percelay et al, 2024) and on the behavioral and cognitive deficits we observed (hyperactivity, working and recognition memory) which mimic in animal models those found in the pathology. However, we agree that our model is incomplete. We have therefore reduced the emphasis on the aspect of schizophrenia modeling and replaced in several sentences "schizophrenia animal model" by "animal model with behavioral and cognitive deficits", as requested (lines 25 in the abstract, 294 in the text).



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Reviewers #2

In this manuscript, the authors advance a novel two-hit model of schizophrenia. While there are a number of publications describing a 2 hit model, this is to my knowledge the first to combine a specific genetic deletion with early maternal separation, particularly given that maternal separation is typically a model of affective disorders rather than schizophrenia. However, the data presented fail to support the hypothesis of a model of schizophrenia based on numerous inconsistencies in the results versus what would be predicted. There are a number of issues that need to be addressed:

Hyperlocomotion is not a marker of positive symptoms. Hyperlocomotion has been shown to be an exclusive domain of the ventromedial striatum, that is more involved in affect, as opposed to the associative striatum in which the hyperdopaminergic state has been repeatedly linked to psychosis. There are several review articles that highlight this discrepancy.

In his/her comment, the reviewer raises an interesting question. Indeed, it must be noted that hyperlocomotion is also considered as a nonverbal marker of positive symptoms that is supported in several articles (Daenen et al., 2003; Geyer et al., 2003; Powell & Miyakawa, 2006, Langova et al., 2020 for examples). Positive symptoms have been related to a dopaminergic increase in the mesolimbic pathway but, as mentioned by the reviewer, also to hyperdopaminergy in the associative striatum (for review: McCutcheon et al., 2019). Hyperlocomotion is relevant to assess an aspect of the positive-like symptoms in our model and suggest a possible deregulation of the mesolimbic pathway related to schizophrenia. However, we agree with the reviewer that our research should be extended to include tests also involving the associative striatum (as mentioned by Kesby et al (2018) in their recent review) which would give us further support for the presence of positive-like symptoms in the 2-hit model.

There are several models of adolescent stress leading to DA hyperactivity and hippocampal neuronal loss, but to my knowledge there are no papers showing that this occurs with trauma as early as maternal separation. This is an inaccurate citation.

We apologies for this inaccurate citation and removed it from the manuscript.

In the introduction, the pathologies associated with maternal separation are all affective disorder-related deficits, and it is not accurate to suggest that they are hallmarks of schizophrenia. Lines 49-56

We agree with the referee that maternal separation is linked to affective disorders and represents a form of early life adversity. However, schizophrenia is supposed to be due to several environmental adverse factors early in life, which could be modeled in animal model by maternal separation (Wang et al., 2020; Nishi, 2020, Guerrin et al., 2021). Thus, we, and others (Ellenbroek et al., 1998; Bouet et al., 2011; Guerrin et al., 2021) consider that maternal separation may be an interesting and useful tool, by its construct validity, to generate several disorders (affective, biological, behavioral, cognitive) associated with schizophrenia.

The use of a knockout of the serine racemase gene is not consistent with what is observed in schizophrenia, in which there is a polymorphism in the gene. The model used is an extreme and non-translatable aspect of genetic regulation.



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We agree with the referee that the deletion of the serine racemase gene could be seen an extreme aspect of genetic regulation, but it must be noted that most genetic procedures used so far to model schizophrenia are based, as ours, on complete single gene deletion (reelin, DISC, neuregulin, etc.) (for review: Winship et al., 2019). In our model, the serine racemase deletion is used as a way to mimic a glutamate hypofunction, described to be associated with schizophrenia (Uno and Coyle, 2019), and affecting more specifically NMDA receptor activation (Basu et al, 2009) The mutation of the SR-KO mice model used in our study is located on the exon 1 of the gene and induces a dysfunction in *serine racemase* promoter function. Interestingly, several gene polymorphisms found in schizophrenic patients are localized close to the 5'-promoter region and the exon 1 (1b notably) of the *serine racemase* gene, which induces a reduction in promoter function of at least 60 % (Yamada et al., 2005; Goltsov et al., 2006; Morita et al., 2006).

The authors need to justify the use of male mice exclusively.

This study was carried out only in male mice but, we are currently working on the behavioral assessment in female mice given the possible differences existing between the two genders in clinics (concerning in particular, the course of the pathology, the intensity of the symptoms, the sensitivity to antipsychotics and their adverse effects). Both genders have to be investigated, we started the study only in males on the basis of epidemiological data that show a sex ratio male/female (1.4), and because symptoms are described as more severe in men than women (Aleman et al., 2003; Lee et al., 2018).

Time spent in the center of the open field is a metric of low anxiety, not “disorganization.” The fact that the 2 hit models spend more time in the center area fails to support the hypothesis. This is also true of the elevated plus maze, which is a valid model of anxiety. In this model, the 2 hit also failed to show differences, and indeed had less “anxiety” than the maternal separation alone.

We agree with the referee regarding the “*less anxiety*” and complete our sentence line 366 for more precision. We also replace the “disorganization” term by “behavioral disinhibition” which seems to be more appropriate since it takes into account several locomotor abnormalities (as a lack of control of motor skills hyperactivity etc.) as we can find in our model.

In figure 4B, the authors argue that there is no difference in the 2 hit model in alternation performance above chance. However, saying there is no difference is NOT the same as saying they are the same; to argue they are the same one must show that they are within a 5% confidence interval of the 50% point; saying they are not significantly different in contrast has a 95% probability of being due to chance.

We thank the reviewer for this accurate comment. We changed the paragraph 3.2.1 (lines 252 to 259) and the discussion section (lines 300) regarding the spontaneous alternation test. Indeed, even if the spontaneous alternation percentage was not different between control, the fact that it did not differ from the chance value of 50% in the 2-hit group suggest a hazardous behavior in the Y-maze probably induced by working memory deficits in those mice.

The NOR test is a good cognitive performance metric. However, the authors failed to show that they 2 hit model had impairment in this domain. The failure to show deficits in social interaction is also not consistent with animal models of either affective or psychotic disorders. PPI is a deficit found



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across a number of disorders, not exclusively to schizophrenia. However, a failure to find any deficit across groups is a major concern, and is further evidence that this is not an effective model.

We agree with the referee deficits in NOR, PPI and social interaction would bring a more severe phenotype to our model. However, we believe that each animal model provides interesting and different characteristics, which are most of the times impossible to observe in each single model. Very few, if some exists, display all schizophrenia mimicking symptoms. The fact that we did not evidence deficits with all the tests used herein doesn't mean that the model is not effective. Instead, this model displays not only hyperactivity, but also lack of place recognition, and another aspect of cognitive deficit, such as working memory deficit, which is not common in multifactorial models and not often investigated. These aspects belong to the symptoms that are not yet reached by treatments, but are disabling for patients and for their daily living condition.



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