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1 Combination of MAP6 deficit, maternal separation and MK801 in
2 female mice: a 3-hit animal model of neurodevelopmental disorder
3 with cognitive deficits

4

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15

16 **Abstract:**

17 Schizophrenia is a major psychiatric disease still lacking efficient treatment,
18 particularly for cognitive deficits. To go further in research of new treatments that
19 would encompass all the symptoms associated with this pathology, preclinical animal
20 models need to be improved. To date, the aetiology of schizophrenia is unknown, but
21 there is increasing evidence to highlight its multifactorial nature. We built a new
22 neurodevelopmental mouse model gathering a triple factor combination (3-M): a
23 genetic factor (partial deletion of MAP6 gene), an early stress (maternal separation)
24 and a late pharmacological factor (MK801 administration, 0.05 mg/kg, *i.p.*, daily for 5
25 days). The effects of each factor and of their combination were investigated on
26 several behaviours including cognitive functions.

27 While each individual factor induced slight deficits in one or another behavioural test, 3-M
28 conditioning induces a wider phenotype with hyperlocomotion and cognitive deficits
29 (working memory and social recognition). This study confirms the hypothesis that
30 genetic, environmental and pharmacological factors, even if not deleterious by
31 themselves, could act synergistically to induce a deleterious behavioural phenotype.
32 It moreover encourages the use of such combined models to improve translational
33 research on neurodevelopmental disorders.

34

35 **Keywords:** psychosis; glutamate; environment; genetics

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43 1. Introduction

44

45 Schizophrenia is a neurodevelopmental disorder affecting approximately 1% of the
46 population, with an unknown aetiology. Most symptoms can be distinguished in 3
47 categories: positive symptoms (hallucinations, delirium), negative symptoms (social
48 withdrawal, anhedonia), and cognitive deficits such as memory issues and altered
49 executive functions [1,2]. Antipsychotic drugs which are currently in use to treat
50 patients are only symptomatic and not curative. Besides, they do not cover the
51 entirety of symptoms, including cognitive deficits. Moreover, negative symptoms and
52 cognitive deficits are often not supported or sometimes even worsened by
53 antipsychotics [3,4]. There is, therefore, a crucial need to improve treatments'
54 efficiency, particularly for cognitive functions, which necessarily goes through the
55 refinement of animal models.

56 Even if few two-factor [5,6] and rare three-factor [7–11] animal models of
57 schizophrenia have been developed, most existing models are based on a single
58 intervention (genetic deletion, adverse environmental exposure, pharmacological
59 modulation etc.), suffering thus from a lack of construct validity given the commonly
60 admitted multifactorial aspect of schizophrenia aetiology [12–15]. Actually, the
61 combination of a genetic predisposition with early and late factor has been described
62 as a key requirement for an animal model with high construct validity [16].

63 A first 3-hit schizophrenia model have been developed in our laboratory by combining
64 a partial deletion of the MAP6 gene as the genetic vulnerability, a 24h maternal
65 separation of PND 9 pups as the early environmental factor, and chronic
66 adolescence administration of THC as the late pharmacological factor. This 3-hit
67 model displays several behavioural and electrophysiological alterations
68 corresponding to a schizophrenia-like phenotype, but no positive-like symptoms
69 [10,17]. We describe another 3-hit model varying only the last factor, to help
70 understanding its own impact. It is of importance to extend the number of available

71 models of schizophrenia spectrum disorders to model the symptomatology as large
72 as possible.

73 Herein, we designed and characterised a new mouse model of neurodevelopmental
74 disorder based on the combination of three factors (3-M). This 3-M model combined:
75 a partial deletion of the MAP6 gene (Microtubule-Associated Protein 6, also called
76 Stable-Tubule-Only-Polypeptide, STOP), a 24h maternal separation of 9-days old
77 pups as the early environmental factor, and then a sub-chronic intraperitoneal
78 administration of MK801 (dizocilpine, a non-competitive N-Methyl-D-Aspartate
79 receptor antagonist NMDAR) in early adulthood as the late pharmacological factor.
80 All these risks factors were selected for their potential to favour the development of a
81 psychosis-like phenotype. MAP6 alterations have been detected in schizophrenic
82 patients [18,19]. Further, in animals studies, unlike the strong behavioural deficits
83 displayed by full knock-out MAP6 mice [20–23], a partial deletion (heterozygous
84 mice) leads to a very mild phenotype of schizophrenia-like symptoms [24,25]. Finally,
85 this partial deletion has been described to increase susceptibility to stress factors,
86 such as maternal separation [10,25]. Childhood trauma is known to be a major risk
87 factor of psychiatric disorders, including schizophrenia [26,27]. In rodents, maternal
88 separation conducts to the emergence of some behaviours related to schizophrenia-
89 like phenotype [28–30]. Moreover, maternal separation induces long-term alteration
90 of glutamatergic and dopaminergic neurotransmission [31]. Finally, as the
91 pharmacological factor, repetitive administration of the NMDAR antagonist MK801
92 was chosen. Indeed, glutamatergic neurotransmission and more particularly that
93 mediated by NMDAR, is disturbed in schizophrenia [32–35]. Therefore, sub-
94 anaesthetic doses of NMDAR antagonists, such as ketamine [36], have been largely
95 employed as experimental models of psychosis in humans [37–39], as well as in

96 rodents (see per reviews [12,40]). Herein, we chose a low dose (0.05mg/kg) of
97 MK801 with sub-chronic administration (5 days) [41–44] (see per review [45]).
98 The effects of the three factors, taken apart or in combination, were assessed
99 through a set of behavioural tests, covering some aspects of schizophrenia-like
100 symptoms (positive-like - locomotion, negative-like - sociability and cognitive deficits
101 – recognition and working memories) [46].

102 The choice for female over male mice in this first single-sex study of a new 3-M
103 model is supported by several arguments concerning interindividual variability and
104 possible biases. Firstly, it has been showed that the higher social hierarchy in males
105 increases behavioural variability in a quite similar amplitude than oestrus cycles of
106 females, decreasing any particular advantage to use males [43,47]. Secondly, the
107 sociability assessment used herein is based on social interaction and is consequently
108 more appropriate, and less biased, in female mice considering that males display a
109 high territoriality [48], and could possibly display aggressive-related behaviour. Lastly,
110 Andiné and collaborators have reported a higher behavioural sensitivity of female
111 rodents to MK-801 [49] , allowing the use of a sub active dose with possible
112 decreased side effects.

113 **In the present study**, while each individual factor induced slight deficits in one or
114 another test, none of them was able to mimic all behavioural aspects of
115 schizophrenia related symptoms. Conversely, through a potential synergistic effect,
116 3-M conditioning induces deficits, representative of phenotypes of
117 neurodevelopmental disorder. Indeed, 3-M mice displayed an increased locomotor
118 activity (positive-like phenotype), a deficit in social recognition task and in working
119 memory task (cognitive-like phenotype).

120 Material and methods

121 Animal experiments were performed in accordance with the ARRIVE guidelines and
122 the EU Directive 2010/63/EU for animal experiments (project n° 016120817103795).
123 All efforts were made to minimise the number of animals used as well as their
124 suffering.

125 2.1 Animals

126 Heterozygous MAP6 (MAP6^{+/-}) female mice and their Wild Type (WT) littermates
127 were obtained from crossing C57Bl6/129SvPas F1 males [23] (bred in Centre
128 Universitaire de Ressources Biologiques, Unicaen, France) with C57Bl/6 females
129 (Janvier Labs, France). After weaning, mice were shortly anaesthetised (5%
130 isoflurane in an O₂/N₂O mixture (0.3/0.7) for identification procedures with a
131 subcutaneous chip and tail sampling for genotyping [25]. Animals were kept in
132 standard laboratory cages in groups of 3 to 5 individuals, with mixed genotypes and
133 treatments per cage, in a reversed 12h/12h light/dark cycle (light on at 19:00 p.m., off
134 at 7:00 a.m.), with regulated temperature (21±1°C) and humidity (55±10%). Food and
135 water were given ad libitum. Enrichment consisted of craft paper and a small scrap
136 paper igloo.

137 *Maternal separation (MS)*

138 When aged 9 days (post-natal day PND9), half of the litters was separated from their
139 mother for 24h. Mothers were removed and individually placed in a cage close to the
140 pups' cage, from 9:00a.m. to 9:00a.m., the day after. Then, the mothers were
141 returned to their litters and left undisturbed until weaning. Control mice were
142 submitted to the same protocol, with only 20s of separation [28,29].

143 *MK801 treatment*

144 MK801 (Sigma-Aldrich®, Ref#M107) was dissolved in a vehicle saline solution
145 (0.9%) for a final intraperitoneal administration dose of 0.05mg/kg [43,44] in a volume

146 of 10mL/kg. Administrations were performed daily for five consecutive days. Control
147 animals received the vehicle saline solution.

148 *Animal groups*

149 Forty-eight female mice, aged two-month, were allocated to the animal groups
150 according to the experimental design depicted on Figure 1. **Behavioural assessment**
151 **was performed during business days. Open field test was assessed at PND 66;**
152 **spontaneous alternation test at PND 71; sociability and social recognition tests at**
153 **PND 73; object recognition test, including habituation phase, between PND 85 and**
154 **PND 89.**

155

156 2.2 Behavioural assessment

157 Animals were handled daily for 5min during the week preceding the experiments.

158 Behaviour was assessed through several tests, from the day after the last MK801
159 injection and performed in the following order: open field, spontaneous alternation,
160 sociability, social recognition and object recognition. For all behavioural tests, animal
161 cages were placed in the experimental room 30min before the test.

162

163 *Open field test*

164 Locomotor activity was assessed in a squared (50x50x50cm) open field arena for
165 30min, through video tracking (NOLDUS®, Ethovision). The parameters collected
166 were the total distance travelled and the time spent in the centre of open field [50].

167

168 *Spontaneous alternation test*

169 Spatial working memory performances were assessed by spontaneous alternation
170 behaviour in a single-session Y-maze test [51,52]. The maze consisted of three

171 equally spaced arms (22x6.5x10cm) made of black painted wood. Each mouse was
172 individually placed at the end of one arm, facing the ending wall, and allowed to
173 freely explore the maze during a 5min session. The number of rearings as well as the
174 number and the sequence of arm entries were recorded. An arm entry was scored
175 when all four feet crossed into the arm. Alternation behaviour was defined as
176 consecutive entries into three different arms. The percentage was calculated by the
177 formula: (number of alternations/maximum theoretical number of alternations) ×100.

178

179 *Sociability and social recognition tests*

180 Briefly, the apparatus consisted of three interconnected compartments
181 (27x18x21cm), with two lateral side chambers (6.5x18x21cm) [28]. Those two
182 chambers were separated from the compartments by transparent perforated walls to
183 ensure visual, auditory, and olfactory contact. Two successive trials were performed
184 to assess sociability behaviour [28], and social recognition performance]. For the first
185 trial, the tested mouse was individually placed in the central compartment of the
186 apparatus. A 45-day-old female mouse (stimulus mouse unknown from the tested
187 mouse) was randomly placed in one of the two lateral chambers. Time spent in each
188 compartment (close and far from the stimulus mouse) was collected for 5min, before
189 replacing the mouse in its home cage [53,54].]. A sociability index was calculated as
190 follows: [(time spent close to the stimulus mouse)–(time spent far from the stimulus
191 mouse)]/[(time spent close to the stimulus mouse)+(time spent far from the stimulus
192 mouse)]x100 [28]. After 30min elapsed, the tested mouse was placed again in same
193 conditions, while a novel stimulus mouse placed in the opposed lateral chamber.
194 Time spent in each compartment (close to the previously encountered mouse and
195 close to the novel stimulus mouse) was collected for 5min and a social recognition

196 index was calculated as follows: $[(\text{time spent close to the novel stimulus mouse}) -$
197 $(\text{time spent far from the novel stimulus mouse})] / [(\text{time spent close to the novel}$
198 $\text{stimulus mouse}) + (\text{time spent far from the novel stimulus mouse})] \times 100$ [55].

199

200 *Object recognition test*

201 For four consecutive days, animals were habituated to the arena (30x30x20cm) and
202 to the presence of an object as follows: the first day, all mice from the same cage
203 were placed together in the arena for 10min. The 2nd, 3rd and 4th day, each mouse
204 was placed alone for 5min. The 4th day, an object, different from those used in the
205 test, was placed in the centre of the arena. For the test sessions, towers of Lego®
206 bricks and Falcon® tissue culture flasks filled with sand were used. The 5th day, the
207 object recognition test was performed in two sessions (10min each, inter-session
208 interval ITI: 1h) [56,57] (see per review [58]). For the acquisition session (1st session),
209 the mouse was free to explore two identical objects placed in the apparatus (tower or
210 flask, random), before being replaced in its home cage. For the retention session (2nd
211 session), the mouse was placed with an object identical to one of the objects used
212 during acquisition session («familiar object»), the other being new to the mouse
213 («novel object»). Object exploration time was instantly collected manually when the
214 mouse was oriented toward the object at a distance of 1cm or less, touching or
215 sniffing the object (exclusion criteria: total exploration < 5s). Objects were cleaned with
216 70% ethanol after each session. A discrimination index was calculated as follows:
217 $(\text{exploration time of the novel object} - \text{exploration time of the familiar object}) / (\text{total}$
218 $\text{exploration time})$. Due to exclusion criteria, one mouse from the MS group was
219 excluded from the analysis.

220

221 2.3 Statistical analyses

222 Data analyses were conducted using R software 3.4.2 (Free Software Foundation,
223 Vienna, Austria; R Development Core team 2009). Data normality was assessed
224 using the Shapiro test. When data were normally distributed, intergroup comparisons
225 were performed with ANOVA and Tukey method for post hoc analysis. Comparison
226 to a reference value was performed with two-sample t tests. When data were not
227 normally distributed, intergroup comparisons were performed using ANOVA with
228 permutation test and t test with permutation for post hoc analysis with the FDR (False
229 Discovery Rate) adjustment method. Comparison to a reference value was
230 performed with Wilcoxon tests. Concerning open field test, statistical analysis was
231 carried out using the nparLD package for non-parametric analysis of group and time
232 effects. Group comparisons within each 5-min interval were analysed using
233 previously described method. The significance level was set as 0.05 for all statistical
234 analyses.

235

236 2. Results

237

238 *Open field test*

239 In the open field test, an increased locomotor activity could reflect positive-like
240 symptom and a decrease time spend in centre part is currently considered as an
241 indicator of an anxiogenic effect [50].

242 Distance travelled in the open field was significantly different between groups
243 (ANOVA $F_{4, 43}=2.638$, $P=0.037$), and there was a significant time effect (ANOVA $F_{5, 42}=6.177$, $P<0.001$, no group x time interaction). Post-hoc analysis revealed that the
244 3-M group travelled more distance than the control group and the MS group (pairwise
245 test, respectively: $P=0.02$ and 0.01) (Figure 2.A). Looking at time course of activity for
246

247 each 5min interval, a statistical group difference was observed at the first two 5min
248 (ANOVA, $F_{4, 43}=4.560, 2.764, 1.471, 1.812, 1.787, \text{ and } 1.581$; $P=0.004, 0.039, 0.228,$
249 $0.144, 0.149 \text{ and } 0.197$, for 0-5min, 5-10min, 10-15min, 15-20min, 20-25min and 25-
250 30min, respectively). For the first 5min interval, the 3-M group travelled a longer
251 distance than the control, MS and MK801 groups (Tukey's method, respectively:
252 $P=0.004, 0.005 \text{ and } 0.022$). During the second 5min interval, 3-M locomotor activity
253 was only higher than the control group (Tukey's method, $P=0.026$) (Figure 2.B).
254 Time spent in the centre of the open field did not globally differ between groups
255 (ANOVA $F_{4, 43}= 1.701, P=0.15$), a time effect appears (ANOVA $F_{5, 42}=11.876, P<0.01,$
256 no group x time interaction) (Figure 2.C). Interestingly, a focus on the first 5-min
257 interval revealed significant difference between groups (ANOVA $F_{4, 43}=5.537,$
258 $P=0.001$). Indeed, the 3-M group spent more time in the centre of the open field than
259 both the control and the MS group (Tukey's method, respectively: $P=0.001 \text{ and}$
260 0.005). A trend towards significant differences was also noticed between 3-M and
261 MAP6 $^{+/-}$ and between control and MK801 groups (Tukey's method, respectively:
262 $P=0.062 \text{ and } 0.051$) (Figure 2. D). There was no difference between groups
263 concerning the latency to visit the centre of the open field (ANOVA with permutations,
264 $P= 0.934$).

265

266 *Spontaneous alternation test*

267 In the Y maze apparatus, normal working memory performances are attested by an
268 alternation percentage significantly higher than 50% [10].

269 The number of arm entries in the Y-maze was significantly different between groups
270 (ANOVA, $F_{4, 43}=2.734, P=0.041$). Post-hoc revealed a trend towards an increased
271 number of arm entries for the 3-M group compared to the MS group (Tukey's method,

272 P=0.066) (Figure 3.A). There was no difference between groups for the number of
273 rearings (ANOVA $F_{4, 43}=0.783$, $P=0.543$). Concerning spontaneous alternation
274 behaviour, there was no difference in alternation percentages between groups
275 (ANOVA $F_{4, 43}=0.469$, $P=0.758$). However, only the control and MS groups displayed
276 a percentage significantly above chance value, 50% (t test, $P=0.018$ and 0.002 , for
277 control and MS respectively). While the MK801 group was close to the significant
278 value (t test, $P=0.061$), MAP6^{+/-} and 3-M groups did not display an alternation
279 percentage above 50% (t test, $P>0.05$) (Figure 3.B).

280

281 *Sociability test*

282 This test measures the sociability performances of mice through their natural
283 behaviour to prefer staying close to than far from a congener [28].

284 The sociability index was not different between groups (ANOVA $F_{4, 43}=0.229$,
285 $P=0.921$), and none of the groups displayed a sociability index above 0 (t test,
286 $p>0.05$) (Figure 3.C).

287

288 *Social recognition test*

289 This test is used to measure the social memory performance of mice through their
290 natural preference to investigate a new than a previously encountered congener
291 [55,56].

292 The social recognition index significantly differed between groups (ANOVA with
293 permutations, $P=0.014$). Although there was no significant difference by inter-group
294 comparisons, a tendency for a higher social recognition for control group compared
295 to the MAP6^{+/-}, MK801 and 3-M groups was observed (permutation t test, $P=0.053$,
296 0.053 and 0.053 , respectively). Further, only the control group showed a social

297 recognition index significantly above the reference value of 0 (t test, $P=0.001$) (Figure
298 3.D).

299

300 *Object recognition test*

301 The exploration time of the novel object during the retention session of the object
302 recognition test reflects significant long-term memory performances if the index is
303 higher than the reference value of zero [57].

304 There was no global difference in the object recognition index between groups
305 (ANOVA $F_{4, 42}=1.190$, $P=0.329$). However, except the MK801 group (t test, $P=0.148$),
306 all other groups displayed an object recognition index significantly above 0 (t test,
307 respectively: $P=0.003$, 0.009 , 0.001 , 0.015 for control, MAP6+/-, MS and 3-M groups,
308 respectively) (Figure 3.E). With regard to exploration times, while no group difference
309 was observed during the acquisition session (ANOVA $F_{4, 42}=1.897$, $P=0.129$), the
310 control group explored more than the MS group during the retention session
311 (ANOVA, $F_{4, 42}=3.260$, $P=0.02$; followed by Tukey's method, $P=0.012$) (Figure 3.F).

312

313 3. Discussion

314 We assessed herein the behavioural phenotype of a new mouse model of
315 neurodegenerative disorder displaying a high construct validity based on complex
316 interactions between genetic, environmental, and pharmacological factors (3-M). The
317 3-M mice displayed an increased locomotor activity considered reflective of positive-
318 like phenotype, a deficit in social recognition and in working memory arguing for
319 cognitive-like deficits. The behavioural phenotype displayed shows promising face
320 validity of the 3-M murine model, supporting arguments for its use as a model of
321 neurodevelopmental disorders. While submitted to only one factor, i.e. 1-hit, animals

322 were not severely affected showing thus an incomplete phenotype reflecting only
323 some aspects of the pathology. Indeed, whatever the factor considered, mice
324 submitted to only one of them did not show any increase in locomotor activity,
325 indicating no positive-like symptoms. However, each 1-hit group displayed a less
326 specifically altered behavioural profile than 3-M group. Thus, while both MAP6+/- and
327 MS groups showed working memory deficits, only the MK801 group showed a deficit
328 in the object recognition task. Similarly, only the MS group spent less time than the
329 control group exploring objects during the object recognition task. Each factor alone
330 induces specific phenotype, suggesting that some deficits observed in the 3-M group
331 could be more related to one or the other factors. For instance, deficits in working
332 memory could be due to the genetic deletion or to the MK801 administration given
333 that they are observed in the MAP6+/- and MK801 groups. Conversely, the increased
334 locomotor activity was observed only in the 3-M group, and not in any of the 1-hit
335 groups, which indicates a synergistic effect (possibly between 2 or 3 factors) for this
336 positive-like symptom to occur.

337 To our knowledge, only four others animal models using 3 hits have been
338 reported in the literature as schizophrenia models, two in rats [8,9] and two in mice
339 [7,10]. All factors combinations have been studied in two of them and synergistic
340 effects between factors appeared systematically [7,8]. Interestingly, all these
341 multifactorial models studies investigated cognition, which is a key point in current
342 neuropsychiatric disorders research.

343 Another schizophrenia model of Buki and collaborators (2019), describe as a
344 “two-hit” model, can be considered as three-factor in consideration with the fact that
345 the model was designed in selected rats with isolation rearing and sub-chronic
346 ketamine administration [11].

347 In 3-hit models of neuropsychiatric diseases, genetic modifications are not
348 systematically used. In the present study, and that of Bouet and collaborators, MAP6
349 partial deletion was used [10]; Sultana and collaborators used DISC KO mice [7]
350 which is considered as a schizophrenia model per se [59]. These two studies in mice
351 are constructed on the same design than the 3-M model, with a genetic factor
352 associated with early and late environmental factors; a construct based on the “3-hit”
353 hypothesis of Ellenbroek [16] considering gene-environment interactions in the
354 etiology of neurological and psychiatric diseases.

355 The environmental factors chosen by authors are diverse; moreover, even if
356 maternal separation is frequent, the protocols differ from each other. Chen and
357 collaborators and Sultana and Lee studies used chronic protocols [7,8], whereas
358 acute separation was used by Bouet and collaborators [10] and by ours in the
359 present study. Concerning the pharmacological factor, glutamate antagonists have
360 been widely used, as in the studies of others 3-hit schizophrenia models which
361 concerned active doses [7–9], and herein with a sub active dose. On the contrary,
362 here we showed only few effects of the sub active dose of MK801. **The 3-hit model**
363 **previously developed in our laboratory (partial deletion of MAP6 gene as the genetic**
364 **vulnerability, 24h maternal separation of PND 9 pups as the early environmental**
365 **factor, and chronic adolescence administration of THC as the late pharmacological**
366 **factor), , displays several behavioural and electrophysiological alterations**
367 **corresponding to a schizophrenia-like phenotype and a strong construct validity**
368 **[10,17]. However, the altered phenotype mostly concerned negative-like symptoms**
369 **and cognitive deficits, which is per se, highly relevant to improve current treatment**
370 **that do not act on these categories of symptoms. In the present study, we modified**
371 **the late pharmacological factor by replacing THC exposure by MK801**

372 administrations. Several arguments determined this choice. Firstly, the 3-hit model
373 displays no positive-like symptoms, and the use of MK801 in place of THC was
374 useful to induce positive-like symptom, by a synergistic effect, giving an increased
375 potency to test new treatments.

376 Secondly, schizophrenia is a complex pathology and our opinion is that the
377 development and characterisation of several multiple-hit models is one of the keys to
378 address the multiple facets of the disease and to improve treatments. In the end,
379 varying only one factor leads to a better understanding of its respective impact.
380 Therefore, 3-hit and 3-M models have to be considered as complementary because
381 they display different schizophrenia-like phenotypes and could be used to mimic
382 different categories of schizophrenia spectrum, fitting to recent theories suggesting
383 that schizophrenia has to be considered as a multitude of syndromes that vary
384 between patients [60].

385 Although historically designed to assess emotionality in rodents [61], the open field
386 test is now also widely used to measure spontaneous locomotor activity (distance
387 travelled). Herein, the 3-M group displayed an increase in locomotor activity.

388 Additionally, during the first five minutes, 3-M mice displayed an increase in time
389 spent in the centre part of the open field. This last measurement is considered to
390 reflect anxiety level [50,62–64]. Of note, mice were tested in a darkened room that
391 could decrease the anxiety effect of the centre part in comparison with a classical
392 lighted room [50]. Besides, the time spent in centre part of the open field can also be
393 considered to be related to behavioural inhibition. Indeed, a longer time spent in the
394 centre part is reflective of a decrease in behavioural inhibition [65]. For example, an
395 increased locomotion and longer time spent in the centre zone of the open field has
396 been observed in mice submitted to a chronic stress [66]. Thence, instead of

397 concluding to a decrease of anxiety level, a potential alteration of behavioural
398 inhibition could underlie the increased time spent in the centre of the open field. The
399 novelty effect present in the open field, which measures anxiety/inhibition, may be
400 more relevant during the first part of the test [67]. That could explain the progressive
401 disappearance of significant differences in the time spent in centre of the open field
402 between groups according to time in the open field. However, time spent in centre
403 zone of the open field is usually measured for 5min [50,64]. Using the 30min test for
404 the assessment of distance travelled (spontaneous activity) and only the first 5min for
405 the time spent in centre zone (anxiety-related behaviour) remains consistent.

406 An increased anxiety level has been reported, through open field parameters, in
407 Chen and collaborators and Sultana and Lee models [7,8], but Kekesi and
408 collaborators model fail to observe this aspect in the hole-board task [9].

409 Unexpectedly, we did not observe a social preference in the sociability test whatever
410 the group considered, and neither differences between groups. This was not
411 expected for the control group given that C57Bl/6 mice are supposed to be able to
412 discriminate the side containing the congener from the empty one with a similar
413 protocol [68]. Our results can be explained by our testing apparatus that differed
414 slightly from that used by Moy and collaborators [68] (a compartment separated by a
415 drilled transparent wall versus small grid cages containing the stimulus mouse).

416 Moreover, the stimulus mice used came from the same animal facility, possibly
417 rendering the olfactory discrimination not obvious or attractive enough.

418 Negative-like symptoms, assessed via social measurements, are not systematically
419 observed in 3-factor schizophrenia models [8,9]. Sultana and Lee, and Bouet and
420 collaborators showed, as in the 3-M model, a decreased sociability in animal
421 submitted to the three factors [7,10]. Kekesi and collaborators showed decreased

422 motor dexterity and exploration during hole-board test, considered by the authors to
423 reflect negative-like symptoms [9].

424 To determine the cognitive status of animals, working and recognition memories
425 capacities were appraised through spontaneous alternation, novel object recognition
426 and social recognition tests. In the spontaneous alternation test, although no group
427 difference was evidenced, the 3-M group showed working memory impairments, in
428 comparison with a random level (as well as the MAP6+/- group, the MK801 group
429 only shows a trend). Working memory deficit is amongst the core cognitive symptoms
430 in schizophrenia patients [69,70] and thus necessarily sought in animal models of this
431 disease [6,7,71]. We did observe deficits in social but not in object recognition
432 memory in the 3-M group. Long-term memory impairments are less frequently
433 reported in schizophrenic patients than working memory deficits [72,73] and in animal
434 models as well [71]. Therefore, deficits in working memory (rather than in long-term
435 memory) appear as a stronger indicator of the face validity of an animal model of
436 schizophrenia. Quite interestingly, MK801 animals did display a deficit in the novel
437 object recognition test. Thus, it could be that either MAP6 partial deletion, maternal
438 separation or both, have afforded 3-M mice to present a kind of resilience that
439 preserves recognition memory performances. At least for maternal separation, such
440 beneficial effect has already been reported, with notably a positive impact on the
441 long-term object location test [74,75].

442 As noted above, all 3-hit schizophrenia models tested until now displayed cognitive
443 deficits assessed with different tests, although the spontaneous alternation for
444 measuring working memory performances was largely used [7,9,10].

445 Surprisingly, sociability of the MS group did not differ from the control group. Several
446 arguments can be proposed to explain this absence of difference. Thus, while we

447 used female C57Bl/6 mice, the experiment from Bouet and colleagues was
448 performed on NMRI male mice [28]. Further, Bondar and colleagues showed an
449 increase sociability in female C57Bl/6 submitted to a maternal separation protocol
450 [76]. However, the experimental protocol of maternal separation reported by Bondar,
451 was quite different. Indeed, contrarily to the 24h single separation protocol we used,
452 Bondar and colleagues performed repeated and short (3h) daily separations (PND2
453 to PND14) [76]. Regarding the cognitive assessment, we reported no deficit either in
454 recognition or working memory, as observed for spontaneous alternation by Bouet
455 and colleagues with the same protocol [28]. A deficit in recognition memory has been
456 previously described using the novel object recognition test in rats submitted to a 24h
457 maternal separation protocol at PND9 [30,77] as well as in C57Bl/6 male mice after a
458 chronic maternal separation protocol (3h per day, from PND1 to PND21) [78].
459 Differences in animal model, separation protocol and/or sex could account for this
460 discrepancy.

461 The late factor was a chronic administration of MK801 ranging from 0.2 to 0.3mg/kg
462 in rodents, described as a pharmacological way to induce schizophrenia-like
463 phenotype [79–81] (see per reviews [40,82]). Such administrations induce several
464 behavioural modifications in rodents, mimicking positive-like phenotype with
465 hyperlocomotion and stereotypies [83,84], negative-like phenotype with decreased
466 social interactions [42,85,86] and cognitive deficits with impairments of working, long-
467 term, and spatial memory [42,85,87] (see per review [88]). Herein, a low, sub
468 efficient, dose of MK801 (0.05mg/kg) was chosen to avoid a direct effect, but to allow
469 possible synergistic effects with other factors. Thonnard and colleagues used
470 0.1mg/kg (i.p. acute administration), considered as a “relatively low-dose” and
471 demonstrated weak effects on locomotion and cognition [43]. Besides, using the

472 same strain, sex and administration protocol, Wu and colleagues reported that a
473 dose above 0.1mg/kg induced an increased locomotor activity [44]. In a chronic
474 protocol similar to ours, five days of 0.3 or 0.6mg/kg i.p., MK801 administration did
475 not disturb locomotion or cognition in male mice except for some specific variables
476 (decrease number of rearings and time exploring displaced objects in object
477 recognition task) with the highest dose [89]. Surprisingly, a low dose of MK801
478 chronic administration at 0.05mg/kg induced object and social recognition deficits in
479 our experiment, as well as working memory deficits (in the spontaneous alternation
480 task). Deficits observed after MK801 administration with such a low dose, not
481 reported elsewhere, could be explained by the sex of mice. Indeed, an increased
482 sensitivity to MK801 administration has been showed in female rats compared to
483 male in a locomotor test [49]. However, no sex differences were found in mice
484 concerning cognitive performances after MK801 administration [90]. Whatever the
485 sex of mice, acute i.p. administration of 0.05mg/kg MK801 did not modify locomotion
486 [91–96]. Moreover, increased locomotion and stereotypies appears in C57Bl/6 male
487 mice only from 0.3mg/kg after i.p. acute administration [44]. While acute 0.05mg/kg of
488 MK801 is enough to induce memory deficits in the novel object recognition test in rats
489 [97–99], a minimal dose of 0.2mg/kg seems necessary to induce such deficits in mice
490 [41,99]. However, lack of effect of chronic i.p. administration of 0.25mg/kg of MK801
491 (7 days) has been shown by Rodrigues Da Silva in C57Bl/6 mice in social interaction
492 and novel object recognition tests [42]. Even if we did not expect any change in
493 locomotor activity, the effect of MK801 chronic administration could be explained by
494 the experimental design of our study. Indeed, in da Silva and colleagues study, mice
495 were tested in the novel object recognition test 14 days after the last injection [42]. In
496 the present study, we started to test mice the day after the last MK801 injection and

497 deficits appeared in social novelty preference and novel object recognition tests. The
498 behavioural effects of chronic MK801 administration seem therefore to be time-
499 dependent. Indeed, while a low dose (0.05mg/kg) induced deficits the day after the
500 last administration in the present study, and a stronger dose (0.25mg/kg) did not
501 induce a deficit 14 days after the last administration [42]. It might be excluded that the
502 hyperlocomotion displayed by 3-M mice in this test is related to a direct effect of
503 MK801 because the MK801 group, that only received MK801, did not display such an
504 effect on locomotion.

505 However, repeating behavioural assessment 2 weeks after the end of treatment
506 could be interesting to understand how the duration of the washout period affects
507 behavioural results.

508 This single-sex study would be strengthened by a sex comparison, as suggested by
509 several authors for research on animal models of schizophrenia [100–105].

510 Moreover, male mice could display a different phenotype, as regard to the gender
511 differences in schizophrenic patients and in other animal models of schizophrenia
512 [100,106]. For example, Kekesi and collaborators showed a sexual dimorphism in
513 PPI with stronger deficits in males [9]. As suggested here, PPI seems to be a cue
514 measurement to highlight gender differences, and it would be interesting to extend
515 the behavioural characterisation of the 3-M model with this test. Sexual dimorphism
516 showed by Kekesi and collaborators [9] highlights the importance of completing the
517 phenotyping with male mice.

518 4. Conclusion

519 Behavioural data revealed a synergistic effect of the three factors, which is consistent
520 with the multifactorial aetiology of schizophrenia and brings a strong construct validity

521 to the 3-M model. 3-M mice showed positive-like phenotype (hyperlocomotion) and
522 cognitive deficits (working memory and social recognition).

523 Further behavioural investigations, particularly sensorimotor gating, a key component
524 of schizophrenia-like phenotype need to be performed. Moreover, the study of 2-hit
525 groups will bring supplementary information about factors interact to induce the
526 synergistic effect. Our study therefore suggests that the 3-M model is powerful as a
527 new model of neurodevelopmental disorder with a strong construct validity, according
528 to the multifactorial nature of the schizophrenia pathology, and a promising face
529 validity.

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534 CRediT author statement:

535 **Solenn Percelay**: Writing- Original draft preparation, Formal analysis, Methodology

536 **Nicole Turnbull**: Data Curation, Writing - review & editing

537 **Thomas Freret**: Conceptualization, Writing - review & editing

538 **Valentine Bouet**: Conceptualization, Writing - review & editing, Methodology,

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962 Figure 1: Animal groups and experimental design.

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966 Figure 2: Open field parameters in control, 1-hit (MAP6^{+/-}: partial deletion of MAP6

967 gene; MS: maternal separation; 3-M (MAP6^{+/-} x MS x MK801:) mice. (A) Total

968 distance travelled in the open field during 30 min. (B) Distance travelled during each

969 5min interval of the open field session. (C) Total time spent in the centre of the open

970 field over 30min. (D) Time spent in the centre of the open field during each 5min

971 interval. For A and C, data are boxplots with horizontal black lines as the median, first

972 and third quartiles; the outer lines represent the range of scores or 1.5 box-lengths

973 from the median; white diamonds indicate the means, vertical black lines indicate the

974 standard deviation. Intergroup comparisons use non-parametric ANOVA-type test

975 statistic with Box approximation followed by tests for pairwise comparisons; *:

976 $p < 0.05$. For B and D, data are Mean \pm SEM. Intergroup comparisons use ANOVA or

977 ANOVA with permutation followed by Tukey's method or permutation t test: *: $p < 0.05$,

978 **: $p < 0.01$, ***: $p < 0.001$.






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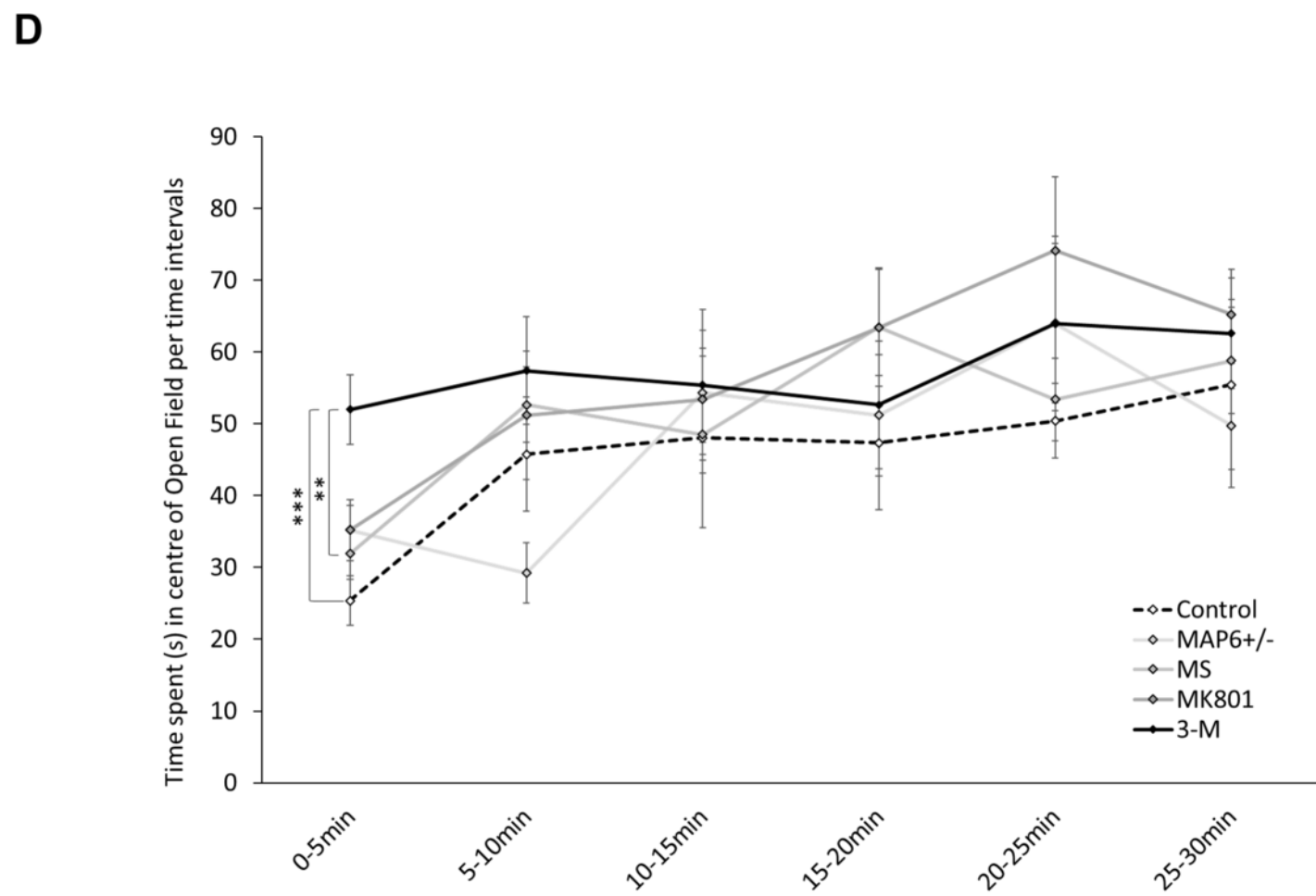
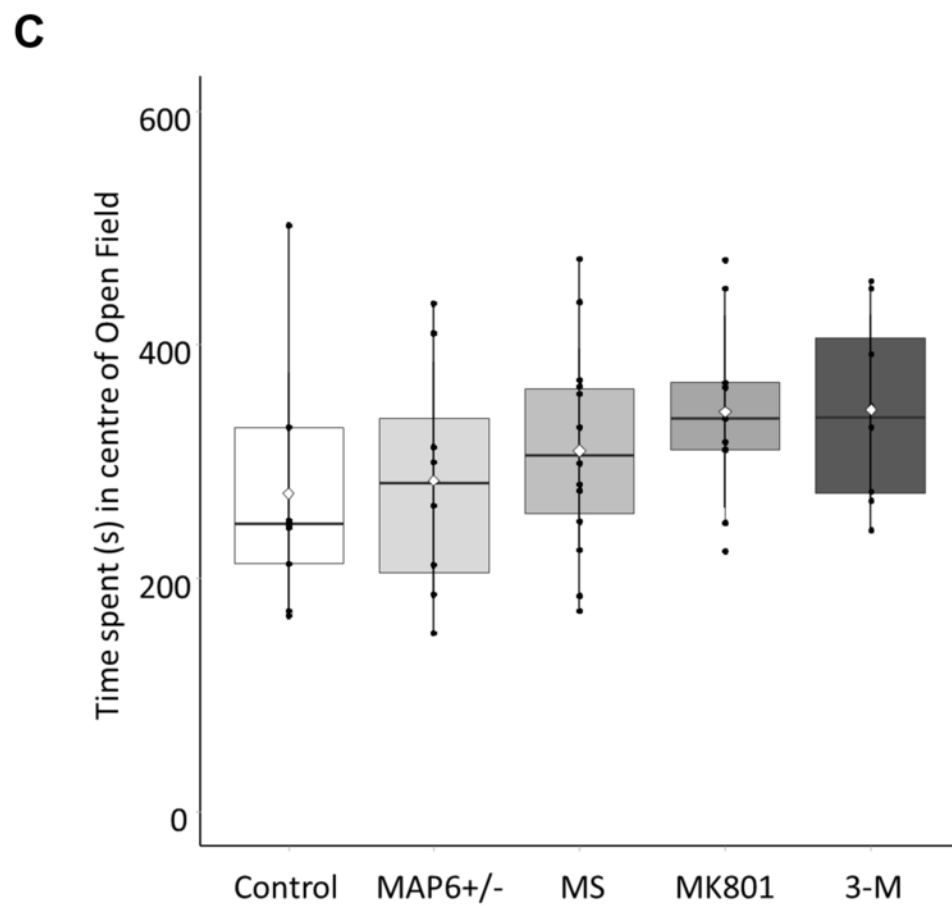
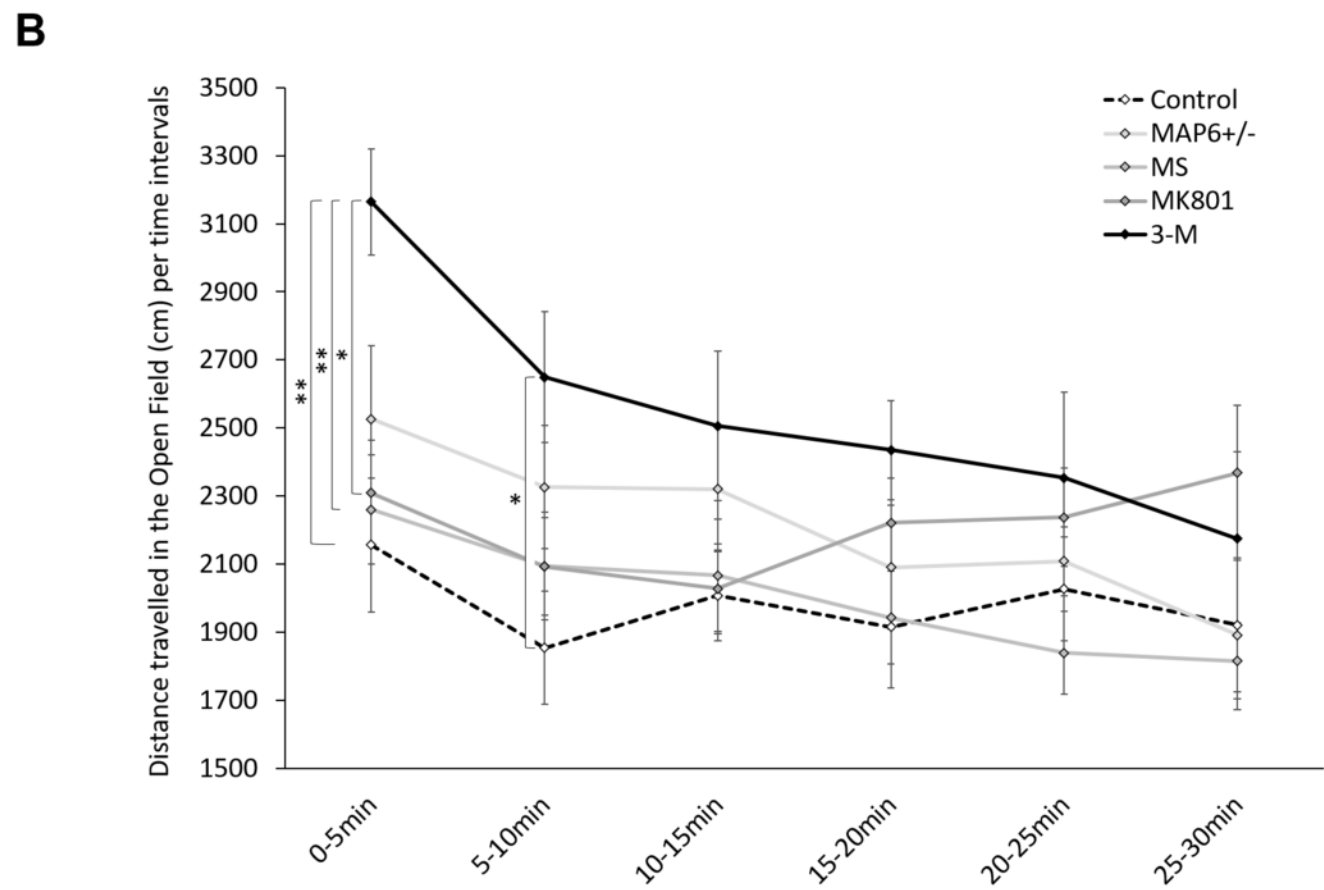
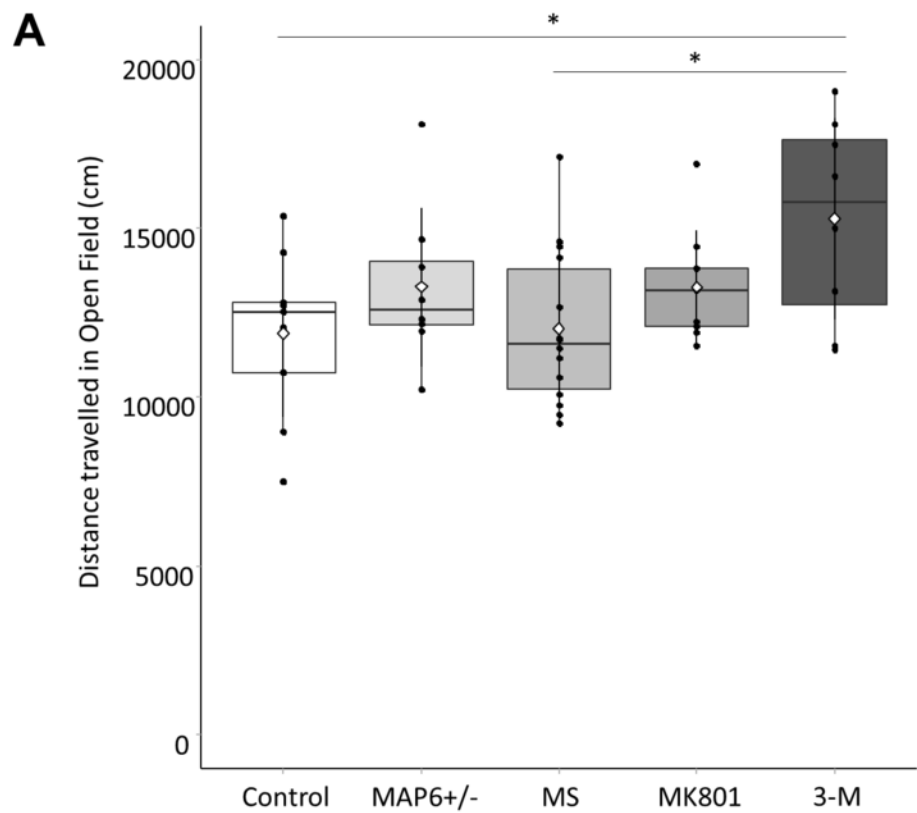
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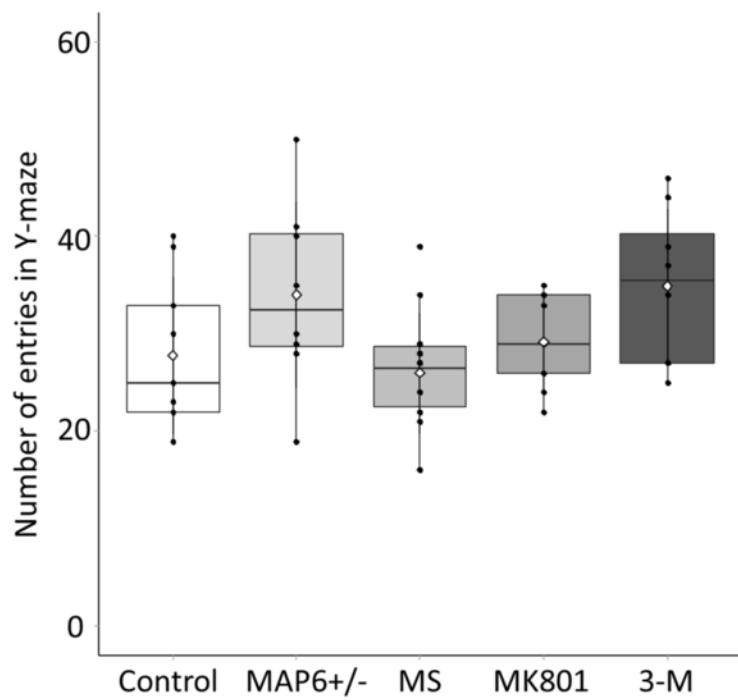
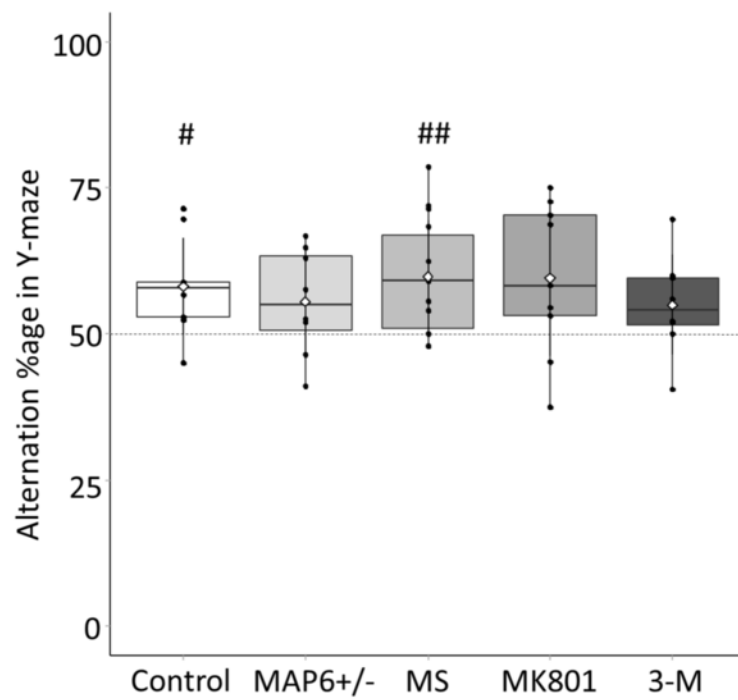
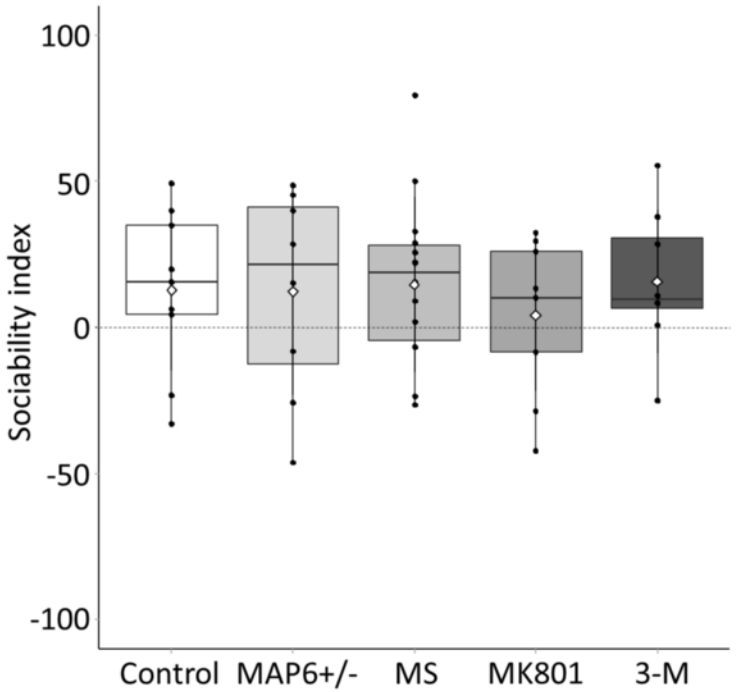
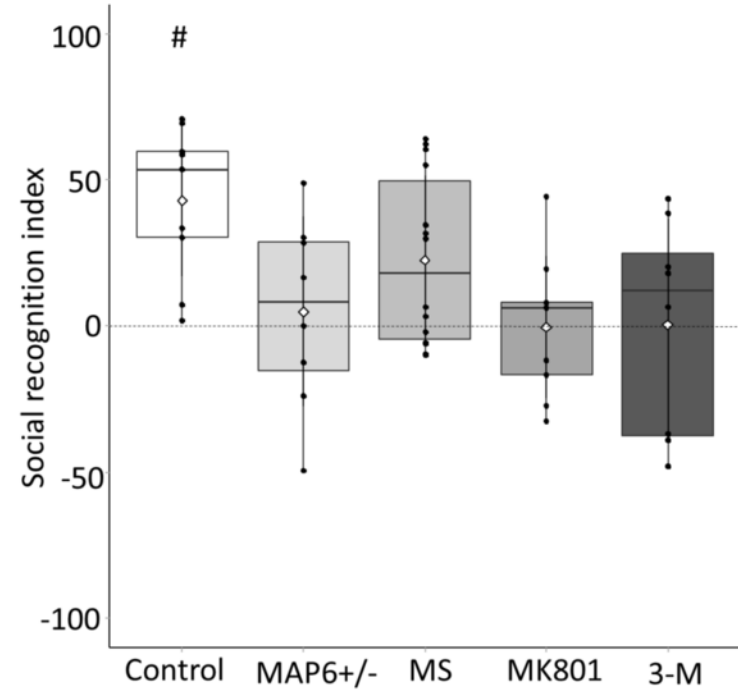
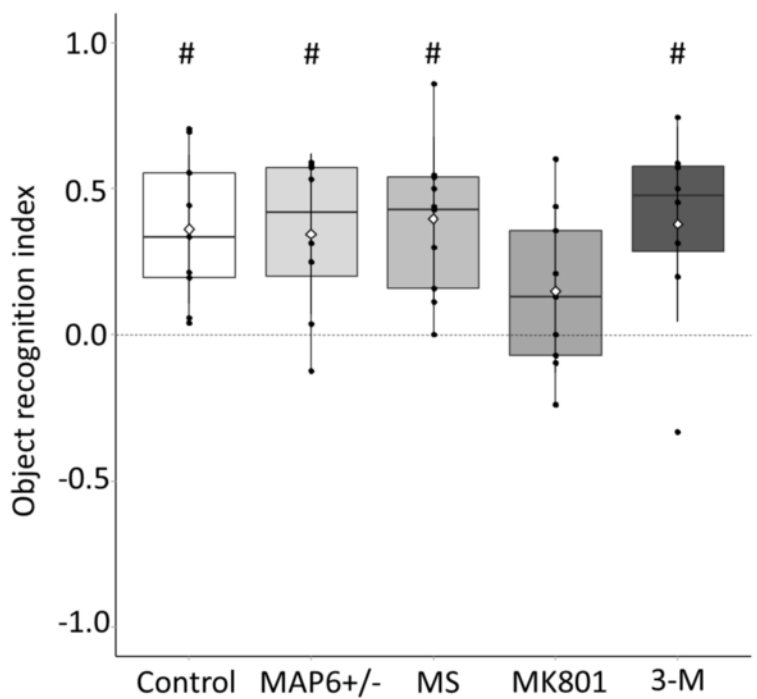
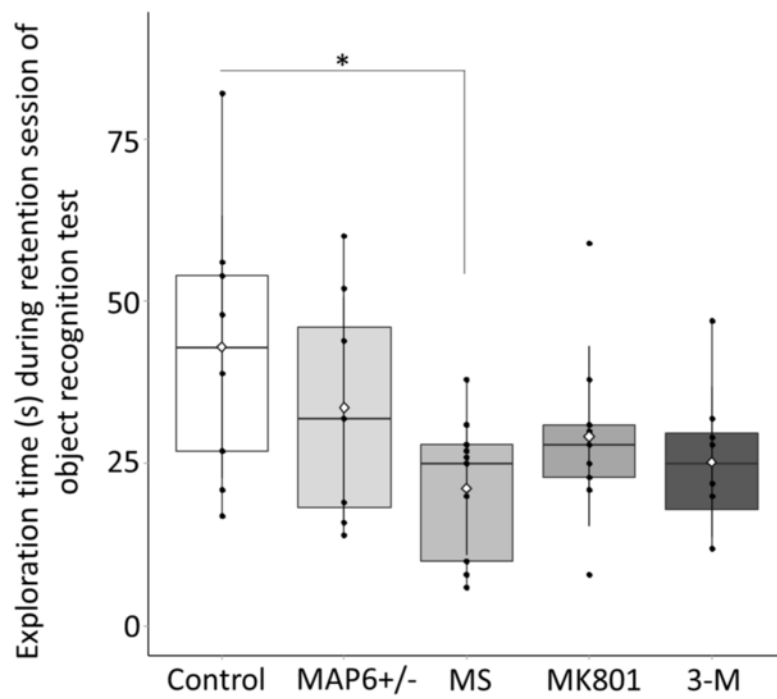
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982 Figure 3: Behavioural phenotype assessed in control, 1-hit (partial deletion of MAP6
983 gene: MAP6^{+/-}; maternal separation: MS; MK801 administration: MK801) and 3-hit
984 (partial deletion of MAP6 gene, maternal separation MK801 administration: 3-M)
985 mice. (A) Number of entries in the Y-maze. (B) Alternation percentage in the Y-maze.
986 (C) Sociability index. (D) Social recognition index. (E) Object recognition index. (F)
987 Exploration time during the retention session of the object recognition test. Data are
988 boxplots with horizontal black lines as the median, first and third quartiles; the outer
989 lines represent the range of scores or 1.5 box-lengths from the median. White
990 diamonds indicate the means, vertical black lines indicate the standard deviation.
991 Intergroup comparison uses ANOVA or ANOVA with permutation followed by Tukey's
992 method or permutation t test: *: $p < 0.05$. Comparison to a reference value using a t
993 test or Wilcoxon test: #: $p < 0.05$; ##: $p < 0.01$.

994

	1 st M Gene susceptibility		2 nd M Early stress	3 rd M Environmental exposure	Behaviour
Age (days)		0	9-10	60-65	66-89
		Birth	Maternal separation		
Control <i>n</i> =9	Wild Type		20s	Vehicle	Open field Spontaneous alternation Approach-avoidance Social novelty preference Object recognition
MAP6+/- <i>n</i> =8	MAP6+/-		20s	Vehicle	
MS <i>n</i> =14	Wild Type		24h	Vehicle	
MK801 <i>n</i> =9	Wild Type		20s	MK801 treatment 0.05mg/ kg/day	
3-M <i>n</i> =8	MAP6+/-		24h	MK801 treatment 0.05mg/ kg/day	



A**B****C****D****E****F**



Neurodevelopmental disorder

Genetic factor



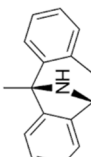
Early environmental factor



Maternal separation

PND 9 24h

Late environmental factor



Sub-chronic MK801
0.05mg/kg

Multifactorial model

3-M

Synergic effect

*

