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CRE mice exhibit hyperactive and impulsive behavior affecting their learning and retention performances

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Abstract: CRE recombinase is a protein that recognizes and mediates site-specific recombination between loxP site sequences. The *Cre/loxP* recombination system has become a useful tool for genetic manipulation. Spatial regulation of recombination can be achieved by using cell type-specific promoters that drive expression of CRE in the tissue of interest. The temporal regulation can be obtained with CreER recombinase, which consists of Cre fused to mutated hormone-binding domain of the estrogen receptor (ER). In the more improved versions of the construct, the CRE-mediated gene regulation can be controlled both spatially and temporally, by combining tissue-specific expression of a CreER recombinase with its tamoxifen-dependent activity. We recently generated and characterized an astrocyte specific mutant of the lipolysis-stimulated lipoprotein receptor *lsr* gene by crossing *Glast* ERT2 mice with floxed *lsr* mice (El Hajj et al., 2022). During the behavioral analysis of generated mice, we identified specific hyperactive traits in the *Glast* ERT2 mice (CRE mice) that prevented them from being used as a control group. Here we further assessed the hyperactive trait of those CRE mice using a battery of behavioral tests. We showed that CRE mice exhibited hyperactive behavior combined with attention-deficit, sleep disturbance and impulsivity that affect their learning and memorization performances. These mice may therefore serve as a model to study attention deficit / hyperactivity disorder. Our work also pointed out the need for proper behavioral analysis of control groups in transgenic animal generation to avoid misinterpretation and misattribution of behavioral traits.

48 **Keywords:** behavioral phenotyping, hyperactivity, attention deficit / hyperactivity disorder
49 (ADHD), Cre recombinase, astrocyte
50

51 **1. Introduction**

52 Vectors expressing CRE recombinase have been utilized to turn gene
53 expression on or off. The CRE recombinase is a protein that recognizes and
54 mediates site-specific recombination between *loxP* site sequences in
55 bacteriophage P1 [1]. CRE-mediated recombination between two *loxP* sites
56 can result in gene deletion, insertion, translocation, and inversion
57 depending on the location and orientation of the *loxP* sites. The *Cre/loxP*
58 recombination system has turned into a useful tool for directed genetic
59 manipulation *in vitro* [2] and *in vivo* [3]. The *Cre/loxP* site-specific
60 recombination system is particularly useful for conditional somatic
61 mutation in mice. Spatial regulation of recombination can be achieved by
62 using cell type-specific promoters that drive expression of CRE in the tissue
63 of interest, such as using the *Glast* promoter to drive CRE expression in
64 astroglial cells that specifically express this glutamate transporter [4].
65 Temporal regulation can be obtained with CreER recombinase, which
66 consists of Cre fused to the mutated hormone-binding domain of the
67 estrogen receptor (ER). The CreER recombinase is inactive but can be
68 activated by the synthetic estrogen receptor ligand tamoxifen (TAM). The
69 more improved versions of the chimeric CRE recombinase have been
70 developed, including CreER^{T2} [5]. By combining tissue-specific expression
71 of a CreER recombinase with its TAM-dependent activity, the CRE-
72 mediated gene regulation can be controlled both spatially and temporally
73 [6]. For instance, induction of *GLASTCreER^{T2}* mice with TAM drives, in a
74 temporally-dependent manner, the glia-specific expression of CRE enzyme
75 [4].

76 At the time we established the behavioral phenotyping of a new glia-
77 specific conditional knockout mouse (cKO) using the
78 *GLASTCreER^{T2}* construct [7], we chose to include CRE littermates as a
79 control group in the behavioral studies. The only difference between them
80 and the group of interest (cKO) was, supposedly, the absence of deletion of
81 the target gene in the CRE group. Despite this, CRE mice exhibited
82 abnormal behavior and low results in all tests requiring attention. They
83 showed signs of hyperactivity that prevented us from using them as a
84 proper control group. However, this hyperactive trait could turn the CRE
85 mice into an interesting model to study attention deficit / hyperactivity
86 disorder (ADHD). This disorder is characterized by a persistent pattern of
87 inattention and/or hyperactivity-impulsivity that has a direct negative
88 impact on academic, occupational, or social functioning [8]. In children,
89 attention deficit may result in poor school performance. In addition,
90 ADHD is associated with mental disorders and substance abuse in
91 adulthood [9]. Since CRE mice exhibited excessive motor activity that could
92 account for hyperactive trait and performed poorly in attention-requiring

93 tasks that could reflect distraction and inattention, we performed an
94 extensive behavioral analysis of the CRE mice by comparing their score to
95 control wild type in specific attention- and activity- related tests. Results
96 obtained confirmed their hyperactive behavior, specific memory and
97 learning deficits, but ruled out anxiety trait or sensory deficits. Altogether
98 the profile of CRE mice resembled ADHD, highlighting their potential
99 usefulness as model of this cognitive disorder, in addition to existing
100 animal models [10]. It also pointed out possible bias introduced in
101 behavioral studies where abnormal behavior in generated cKO was linked
102 to CRE and not to the excision of the target gene.

104 2. Materials and Methods

105 2.1. *Animals*

106 Initial C57Bl6J genitors of Glia-specific CRE/+ transgenic mice (Tg(Slc1a3-
107 cre/ERT2)^{45-72Fwp}) [4] were a kind gift from Dr FW Pfrieder (INCI,
108 Strasbourg). C57Bl6J WT control mice of the same genetic background
109 were purchased from Charles River Laboratories (Saint Germain Nuelles,
110 France). Male CRE and WT mice were housed in certified animal facilities
111 (#B54-547-24), under controlled environmental conditions including a
112 constant temperature (22 ± 2 °C), and a relative humidity of 50 ± 5 % with
113 *ad libitum* access to food (standard chow diet, Envigo Teklad, Gannat,
114 France) and water. Animals were maintained on a standard 12-h light/dark
115 cycle: dark cycle starting at 12:00 P.M. (noon) and ending at 12:00 A.M.
116 (midnight). Animal studies were conducted in accordance with the
117 European Communities Council Directive (EU 2010/63) for the use and care
118 of laboratory animals. All experimental procedures were approved by the
119 institutional ethical board (authorization number APAFIS #12079-
120 201711081110404).

121 2.2 *Tamoxifen (TAM) injection*

122 TAM (Sigma, St Louis, MO) was dissolved in a 9:1 (v/v) sunflower oil
123 (Sigma, St Louis, MO) and ethanol (Carlo Erba Reagents, Val de Reuil,
124 France) mixture at a concentration of 15 mg/mL at 37 °C, sterile-filtered
125 (0.22 µm), and stored at 4 °C for up to 7 days in the dark. A 23G needle
126 tuberculin syringe (Henke Sass Wolf, Tuttlingen, Germany) was used for
127 intraperitoneal injections. At the age of 8 weeks, all mice were injected for
128 5 consecutive days (every 24 h) with 150 µg of TAM per g of body weight
129 [4]. Each mouse was randomly chosen from a different litter to avoid any
130 litter-specific bias (litter effects).

131 2.3. *Behavioral tests*

132 Two weeks after TAM induction, behavioral tests detailed in Table 1 were
133 performed over an 8-month period using the same mice during the entire

134 study (15 WT and 14 CRE mice). All behavioral tests were performed 1 h
135 after the beginning of the dark cycle using only red light.

136 **Table 1. Timeline of behavioral tests**

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Behavioral test	Age of animals
Open field test (OFT)	3 months
Y-maze	4 months
Object recognition test	4.5 months
3-chamber sociability and social novelty test	5 months
Home cage activity	6 months
Barnes maze	11 months

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139 *2.3.1 Open field*

140 The general locomotor activity was assessed on 3-month old animals (15
141 WT and 14 CRE mice) in a white circular open field (80 cm
142 diameter × 50 cm high walls) coupled with a Smart video tracking system
143 (Bioseb, Vitrolles, France). Using this software, the radius of the circular
144 area was virtually divided into three sections to establish three circular
145 areas: peripheral (Z1), median (Z2) and central areas (Z3). Four adjustable
146 lamps, arranged on the ceiling allowed indirect illumination of the area.
147 Luminous intensities were 20, 35 and 80 lux in Z1, Z2 and Z3 respectively.
148 Testing was conducted between 2:00 P.M. and 5:00 P.M. Animals were
149 placed in the peripheral zone (Z1) of the device, head facing the wall, and
150 ambulation was monitored during 3 min. Parameters recorded for the
151 locomotor activity assessment were: total traveled distance, cumulated
152 duration in each area, as well as latency of the first entry and number of
153 entrances in Z3. A moving fast threshold was established for speed above
154 14 cm/s, allowing the measure of the total time moving at high speed.

155 *2.3.2 Y maze*

156 Immediate spatial working memory performance was evaluated on 4-
157 month old animals (15 WT and 14 CRE mice) using a classic Y maze. The
158 device was composed of three opaque black plexiglass arms (40 L × 9 W ×
159 16 H cm, and positioned at equal angles) with a floor made of transparent
160 plexiglass, allowing the animal to see geometric patterns depending on the
161 arm, as proximal clues. The Y-maze task was conducted between 2:00 P.M.
162 and 5:00 P.M., in a dimly illuminated room (80 lux in maze arms). Mice
163 were placed at the extremity of one arm, head facing the wall of the maze,
164 and allowed to move freely through the apparatus during a 5-min session.
165 The percentage of spontaneous alternation was calculated as the ratio of
166 successful overlapping alternations by the total possible triplets (defined as
167 the total number of arm entries minus 2) multiplied by 100. An arm entry
168 was considered to be completed when the four paws of the mouse were all
169 placed in the arm.

170 *2.3.3. Object recognition test*

171 The object recognition test was performed on 4.5-month old animals (15
172 WT and 14 CRE mice) according to the procedure of Leger *et al.* (2013) [11].
173 Briefly, each mouse was first habituated to the device (acclimation) before
174 being subjected to an acquisition phase during which two similar objects
175 were presented to it, then an hour later, had to discriminate a new object
176 from the one already acquired (phase of recognition memory). The
177 procedure was conducted from 1:00 P.M. to 5:00 P.M. in an empty squared
178 opaque plastic box (30 L x 30 W x 26 H cm) placed in a dimly illuminated
179 room (25 lux in the center of the arena). During the acclimation session, the
180 arena was left empty and the mouse was allowed to freely investigate its
181 new environment for 3 min in order to avoid any later potential neophobic
182 response. Monitoring of the mice placement was carried out using the
183 video tracking system to highlight, if existing, any initial place preference
184 in the device especially in the 2 opposite corners of the arena (namely
185 position A and B) where objects would be placed in the following steps of
186 the test. Fifteen minutes after the acclimation phase, an acquisition session
187 was conducted in which the mouse was presented two similar objects
188 (either two Lego blocks or two litter-filled tissue culture flasks, 15 cm high
189 to prevent the mouse from climbing on it) positioned in positions A and B.
190 The animal was allowed to explore the objects for 3 min. During this
191 session, the monitoring of both similar objects was performed to enlighten
192 any initial object preference that would induce an experimental bias in the
193 later recognition memory test. One hour later, one of the known objects
194 was randomly removed and a new one (either Lego or Falcon) was placed
195 at the same position. The novel object recognition session was then
196 performed, in which exploratory behaviors toward objects were recorded
197 via the Smart video tracking system. To this end, we used the TriWise
198 option, which allowed tracking the position of the mouse's head relatively
199 to the animal's center. According to Leger *et al.*, the mouse was considered
200 as exploring the object when its head was directed toward the object at a
201 distance less than or equal to 2 cm. Mice memory was assessed by
202 calculating the ratio of "time spend exploring the novel object" to "total
203 time of objects exploration" (recognition index) obtained during the
204 discrimination task. We also focused on mice interest toward their
205 environment during both acquisition and discrimination phases by adding
206 times of exploration toward both objects, with no interest for a possible
207 object preference.

208 2.3.4. *Social interaction / memory test*

209 The social memory assessment was performed on 5-month old animals
210 (15 WT and 14 CRE mice) using a device made of an open-top transparent
211 plastic box (63 L x 42 W x 22 H cm), divided into three successive chambers
212 by two opaque partition walls (Imetronic, Reganeau, France). Small
213 openings (5 x 5 cm) in these partitions allowed mice to access each
214 compartment of the apparatus. Both opposite chambers were equipped
215 with a cylindrical wire cup made of chrome bars spaced 1 cm apart (11 cm
216 H; 11 cm bottom diameter). We followed the Kaidanovich-Beilin's
217 procedure [12], consisting of three phases, *i.e.*, a first phase of

218 familiarization to the device, a phase of sociability assessment and a phase
219 of social memory testing. During the familiarization phase, the mouse was
220 placed in the middle chamber and was allowed to freely explore the whole
221 device for 5 min to reduce anxiogenic response to novelty. Again, we used
222 video tracking to highlight, if existing, a place preference (cumulated time
223 of presence in each of the opposite chambers) which could potentially
224 induce bias in the socialization and/or social memory results. Following
225 this familiarization phase step, the mouse was replaced in its home cage for
226 5 min before sociability assessment phase. Once again, the tested animal
227 was placed in the middle chamber of the apparatus, the device now
228 containing an unknown congener previously positioned under one of the
229 wire cups. The TriWise option from Smart video tracking system software
230 was used to record the sniffing time around both wire cups in a range of 2
231 cm and for a 5 min duration. Thus, the social motivation was assessed via
232 the time spent sniffing the cup containing congener versus the empty cup.
233 At the end of the socialization phase, the mouse was replaced in its home
234 cage for one hour before the social discrimination test was performed. In
235 this aim, the now-known congener was placed under the same wire cup
236 used in the socialization step, while an unknown congener was placed in
237 the second cup of the opposite chamber. Again, the tested animal was
238 placed in the device's central chamber and allowed to explore the device
239 for 5 min during which sniffing time around the wire cups (2 cm) was
240 recorded and considered as exploration time. Total investigation time and
241 the ratio of social discrimination (time spent sniffing unfamiliar mice
242 relative to total investigation time) were calculated.

243 2.3.5. *Spontaneous home cage activity*

244 The assessment of the spontaneous home-cage activity was performed on
245 6-month old animals (15 WT and 14 CRE mice) using four beam-break
246 activity monitors (Promethion, Sable Systems International, North Las
247 Vegas, USA). The array of each monitor was composed of three
248 independent infrared (wavelength 900 nm) beams (X + Y + Z axes), whose
249 captors were spaced 0.25 cm on each axis. The recording sessions took
250 place in the animal room to avoid any disruptive or stress effect that could
251 occur if moving the mice to another room. At 11:00 A.M., cages were
252 placed in the center of each beam-break monitor. The duration of
253 monitoring was set at 24 h, beginning at 12:00 A.M. (light "off"). The
254 subsampling factor was set at 1 s and raw data were processed using
255 ExpeData software (Sable Systems International). According to the
256 manufacturer's instructions, ambulation exceeding 1 cm/s was considered
257 as directed locomotion (ped-meters), while values below this threshold
258 were considered as non-ambulatory movements, *i.e.*, fine activity
259 (grooming, scratching or feeding). The variable "all meters" was
260 established as the sum of all distances traveled within the beam-break
261 system including fine movement as well as direct locomotion. Moreover,
262 using guidelines from Pack *et al.* (2007) [13], any episode of continuous
263 inactivity for ≥ 40 s was considered as sleep and expressed as percentage of
264 the cycle. Given the wide difference of activity in mice between the light

265 and dark phases, data were initially examined according to each phase of
266 the cycle, then to the total circadian cycle. Also, in order to allow genotype
267 comparisons for each activity-related variable, the 12 values within a
268 cycle's phase were summed up. Thus, the level of significance of the
269 subsequent unpaired *t*-test (CRE vs WT) was equal to the main factor
270 "genotype" from the two-way ANOVA with repeated measures of the
271 activity-related variable, and thus, not mentioned.

272 2.3.6. Barnes maze

273 Eleven-month old animals (15 WT and 14 CRE mice) were tested on a
274 modified Barnes maze, made according to the work of Youn *et al.* (2012)
275 [14]. The maze consists of a white circular area (radius of 56 cm),
276 positioned in height (40 cm), and pierced with 44 openings (5 cm diameter)
277 placed according to 3 circumferences (peripheral, median and central), and
278 following a similar pattern for its four quadrants. Only one escape hole
279 was provided with a small mesh staircase leading to the escape chamber, a
280 dark and restricted space in which the mice were left for one minute after
281 having found the target hole. According to Youn's procedure, the escape
282 hole was never placed on the peripheral circumference to avoid
283 experimental bias induced by serial thigmotaxic explorations. The
284 apparatus was mildly illuminated (100 lux at the center), and 3 rotating
285 fans placed equilaterally around the device induced aversive conditions to
286 obtain sufficient motivation in the mice to seek for the escape hole during
287 the trial sessions. Four distant clues (black geometric shapes on white
288 panels) were placed on the walls around the device at the cardinal points,
289 30 cm higher from its surface to allow mice's allocentric navigation. The
290 escape hole was set under one of the clues and its location never changed
291 during all the test sessions. The mouse was placed on the modified Barnes
292 maze device inside a small opaque plastic cylinder (10 cm diameter /
293 height) to avoid any pre-trials spatial recognition. The location of the trial's
294 starting point was set at the center of the quadrant opposite to the target
295 quadrant. The test started with the withdrawal of the cylinder, which was
296 performed when the mouse had its head placed in the opposite direction to
297 the exit hole. In this way, the animal was required to perform an initial
298 half-turn to move to the escape hole. Mice were given 3 min to find the
299 escape hole. When an animal did not succeed in the imparted time, it was
300 gently positioned and maintained by the tail for 10 s in front of the escape
301 hole, *i.e.*, facing its specific distant clue, before being allowed to enter the
302 hole. Familiarization and acquisition sessions took place for three
303 consecutive days (D1, D2 & D3), in which mice were given three trials a
304 day to acquire the location of the escape hole. Time to find the escape hole
305 were recorded for each trial. Two probe tests were performed on the fifth
306 (D5) and eighth days (D8) consisting of only one trial/day to evaluate
307 memory retention. The latter is expressed as the percentage of the time
308 spent in the target quadrant compared to untargeted quadrants according
309 to the total time needed for the trial.

310 2.4. Statistical analyses

311 All variables were tested for its distribution by the Kolmogorov-Smirnov
312 test. Since all variables followed a normal distribution, parametric statistics
313 were used for the analyses of this work and results are presented as
314 mean \pm SEM. Data obtained from the spontaneous home cage activity and
315 Barnes maze acquisition sessions were analyzed using the two-way
316 repeated measures analysis of variance, considering genotype and time as
317 main factors. Intra-group performances in the Barnes maze were analyzed
318 using a 1-way ANOVA with repeated measure, with time as unique main
319 factor. If significant, a post-hoc Tuckey test was used to enlighten
320 significant differences of the variable between specific times. Data resulting
321 from non-time-dependent comparisons were performed via unpaired
322 Student *t* test for strain comparisons, while intra-group performances were
323 achieved using paired Student *t* test. Statistics were performed using
324 StatView software (version 5.01) and in all analyses, $p < 0.05$ was
325 considered statistically significant.

326 3. Results

327 3.1. Open field

328 When comparing the total distances traveled in the open field during the
329 3 min of the test, CRE mice traveled a greater distance when compared to
330 controls ($p = 0.027$, **Table 2**). By examining the distribution of the distances
331 traveled according to the area of the device, it appeared that compared to
332 controls, CRE mice moved significantly more in the peripheral Z1 area
333 ($p < 0.0029$) and less in the central Z3 area ($p = 0.0074$). However, no
334 difference was found regarding the distance travelled in the middle area
335 (Z2) of the open field ($p = 0.76$). This thigmotaxic behavior was still found
336 when examining the times of presence in each zone according to the mouse
337 strain. CRE mice also exhibited a significantly longer time in Z1 ($p = 0.0031$),
338 with shorter times in Z2 ($p = 0.06$) and Z3 ($p = 0.0016$) when compared to
339 controls (Table 1). The analysis of the two variables "latency of the first
340 entry", as well as "total number of entries" in the central zone, as
341 representative of the level of anxiety in rodents, revealed no
342 difference between strains (latency of the first entrance in Z3, $p = 0.982$; total
343 number of entries in Z3 during the 3-min test, $p = 0.095$). Finally, the analysis
344 of the total time spent by animals moving at high speed, i.e., > 14 cm/s
345 threshold, was found to be significantly different ($p = 0.0038$), with the CRE
346 animals spending a total of 57 s at high speed while control animals
347 exhibited a duration of 33 s. This most likely explains the difference
348 observed between the two groups concerning the distance travelled in the
349 open field.

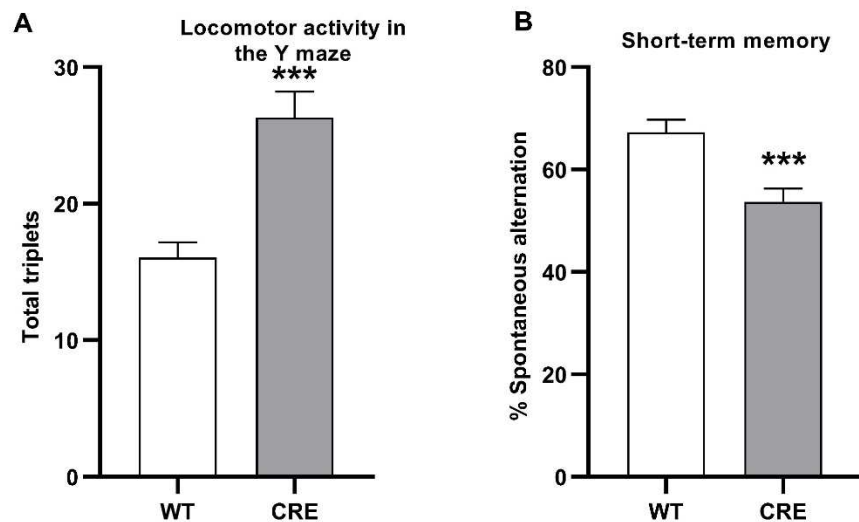
350 **Table 2.** Behavior of CRE and WT in open-field test (CRE: $n = 14$; WT: $n = 15$). Data are
351 reported as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, significantly different from control group
352 (unpaired *t* test).

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	WT (n=15)	CRE (n=14)
Total distance (m)	9.81 ± 0.3	11.58 ± 0.7 *
Distance in Z1 (m)	7.38 ± 0.3	9.56 ± 0.6 **
Distance in Z2 (m)	1.81 ± 0.3	1.72 ± 0.3
Distance in Z3 (cm)	61.4 ± 8	29.8 ± 6 **
Time in Z1 (s)	135.4 ± 4	152.0 ± 3 **
Time in Z2 (s)	25.6 ± 2	20.1 ± 2
Time in Z3 (s)	18.9 ± 3	7.0 ± 1 **
Latency of the first entrance in Z3	34.2 ± 8	34.5 ± 7
Number of entries in Z3	5.3 ± 0.7	3.5 ± 0.7
Time moving at high speed (s)	33.1 ± 3.1	57.1 ± 7.1 **

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The total number of triplets performed by mice in the Y-maze device revealed a higher ambulatory activity ($p < 0.0001$), where CRE mice completed up to 26.3 triplets (± 1.9) and WT control mice up to 18 triplets (± 1.1) (**Figure 1A**). Nevertheless, this performance of CRE mice was also accompanied by a poor immediate spatial memory, since the percentage of spontaneous alternation was found close to the chance threshold for CRE mice ($X=53.7 \pm 2.6$ %), while WT mice exhibited higher performances of 67.2 ± 2.5 %, $p = 0.0008$, **Figure 1B**).



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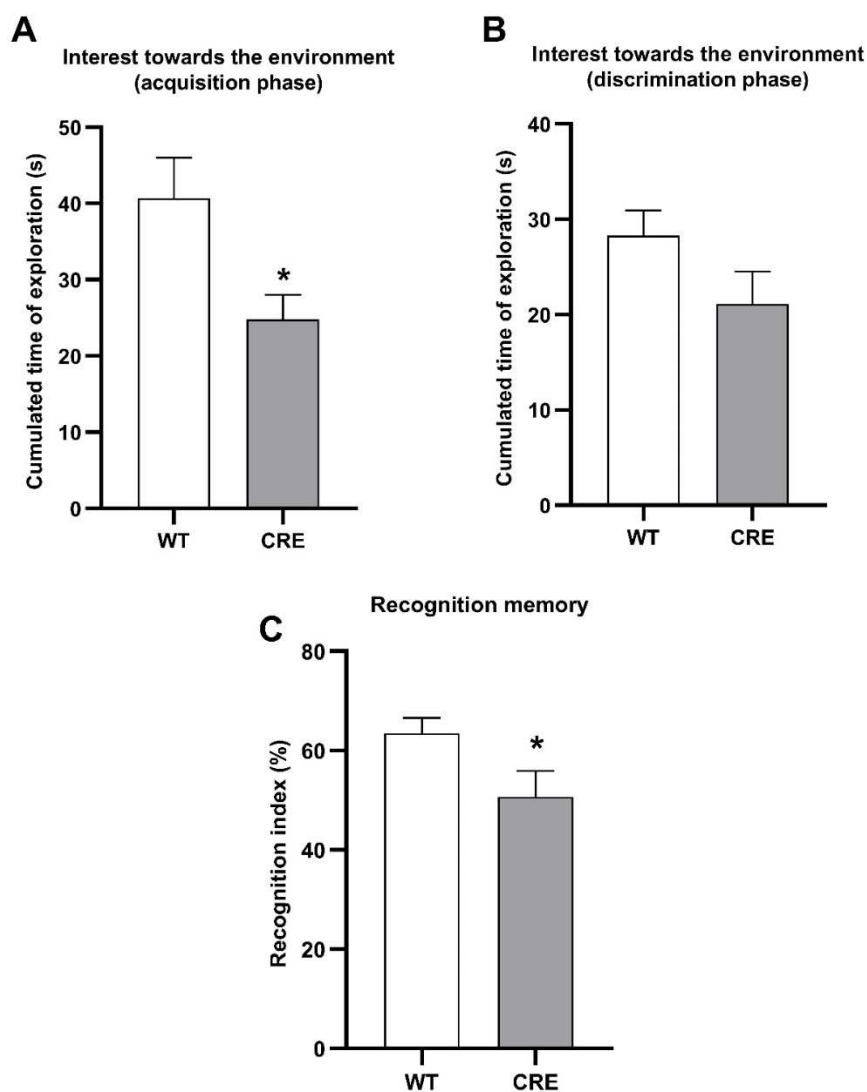
Figure 1. Effect of *Cre* genotype compared to WT on the behavior in the Y maze (CRE n=14, WT n=15). Data are reported as mean \pm SEM. *** $p < 0.001$ significantly different from control (Fisher *post hoc* test).

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3.3. Object recognition test

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No place preference in the device was observed during the acclimation step, neither in WT control mice ($p = 0.17$) nor in CRE mice ($p = 0.23$). Also, no object preference was revealed during the acquisition session neither in control mice ($p = 0.30$) nor in CRE mice ($p = 0.15$). Concerning their interest towards their environment, control mice showed a longer exploration time ($X = 40.7 \pm 5$ s) when compared to CRE mice ($X = 24.8 \pm 3$ s, $p = 0.019$, **Figure 2A**). A difference was still found during the discrimination task, but did not reach the level of significance ($p = 0.10$, **Figure 2B**). The discrimination task, performed 1 h after the acquisition phase, revealed that control mice spent 63.5 ± 3 % of their exploration time towards the novel object, while CRE mice recognition index was found close to the chance threshold (50.7 ± 5 %, $p = 0.04$, **Figure 2C**).



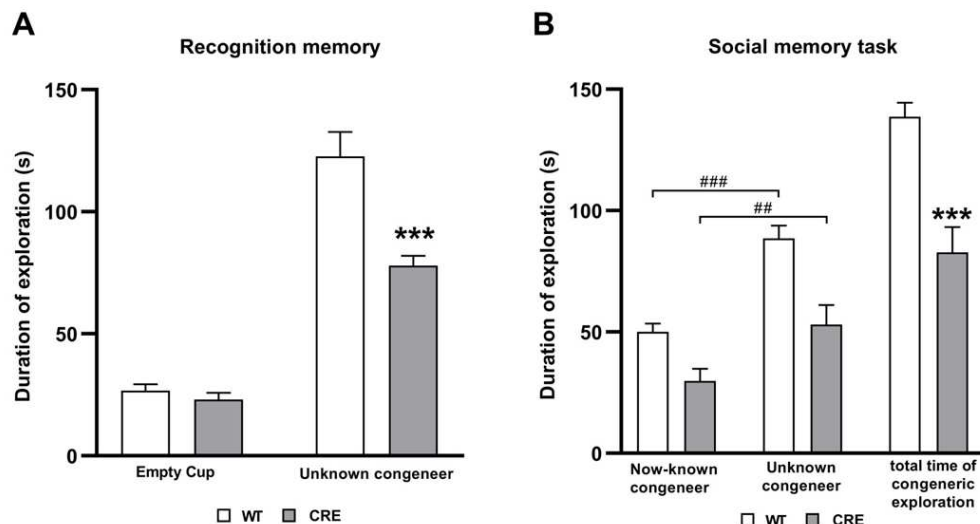
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Figure 2. Effect of *Cre* genotype compared to WT on the interest towards the environment (A & B) and recognition memory (C) in the object recognition test (CRE n=14, WT n=15). Data are reported as mean ± SEM. * $p < 0.05$ significantly different from control (Fisher *post hoc* test)

3. 4. Social discrimination test

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Since CRE mice exhibited a lack of interest toward the inanimate environment of the object recognition test, we performed a two-trial social memory test to assess the possible influence of the type of stimuli on CRE mice attention (**Figure 3**).



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Figure 3. Effect of *Cre* genotype compared to WT on times of congener exploration during sociabilisation and discrimination phases (CRE n=14, WT n=15). Data are reported as mean \pm SEM. *** $p < 0.001$ significantly different from control (Fisher *post-hoc* test). ** $p < 0.01$; ### $p < 0.0001$, intra-group significant difference (paired t-test).

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Initial comparison of mice movements in the empty apparatus during the familiarization phase did not reveal any place preference for a specific compartment (data not shown). Evaluation of mice sociability showed that CRE mice spent more time exploring the wire cup containing an unknown congener than the empty one ($p < 0.0001$). This same discriminative behavior was also found in controls ($p < 0.0001$). Nevertheless, it was observed that the CRE mice spent significantly less time ($p = 0.0004$) to explore the congener (78 ± 4 s) than the controls (123 ± 10 s). However, no difference was found between the groups with regard to the time spent in exploring the empty wire cup ($p = 0.33$, **Figure 3A**). Social memory task revealed that despite a lower time of congener exploration during the social motivation assessment, CRE mice were nonetheless able to discriminate the congeners ($p = 0.0038$). Not surprisingly, this result was also found in WT mice ($p = 0.0004$, **Figure 3B**). The ratios of exploration between unknown and now-known congener were similar in both groups, revealing no deleterious effect of social memory ($p = 0.99$) in CRE mice ($63.4 \pm 3\%$) when compared to WT mice ($63.5 \pm 3\%$). Nevertheless, the total time of congener explorations was still found significantly lower ($p < 0.0001$) in CRE animals (83 ± 6 s) than in controls (139 ± 10 s, **Figure 3B**).

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3.5. Home cage activity

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Data collected from the actimeter revealed different patterns of circadian activity according to mice genotype (Figure 4). Total activity is expressed

430 as the variable "All meters", which represents the sum of all distances
431 traveled within the beam break system (**Figure 4A**). The total mouse
432 activity recorded during the dark phase was significantly higher
433 ($p = 0.032$) in CRE mice ($X = 125.4 \pm 18.7$ m) as compared to WT
434 ($X = 77.4 \pm 10.8$ m). Regarding the light cycle, CRE mice displaying higher
435 activity levels ($X = 26.1 \pm 4.4$ m) than controls ($X = 16.6 \pm 3.2$ m), but the
436 difference between the two strains did not reach statistical significance
437 ($p = 0.088$). On a complete circadian cycle, CRE mice exhibited a
438 significantly higher ($p = 0.034$) home cage activity ($X = 161.7 \pm 24.7$ m) as
439 compared to controls ($X = 100.8 \pm 12.9$ m).

440 **Figure 4.** Effect of *Cre* genotype compared to WT on the behavior in home cage activity (CRE
441 $n=14$, WT $n=15$). A) Measurement of all activities over a 24-h period covering an activity
442 phase (dark phase) of 12 h and a resting phase (light phase) of 12 h. B) Displacement over the
443 24-h period. C) Fine movement (immobile activities like scratching and grooming) over the
444 24-h period. D) Sleeping percentage over the 24-h period. Data are shown as mean \pm SEM.
445 * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, significantly different from control (Fisher *post hoc* test)
446

447 As seen in **Figure 4B**, locomotor activity ("Ped meters") counted for the
448 major part of the "All meters" variable measured in **Figure 4A**, which
449 reflected the total activity. The sum of direct locomotions recorded during
450 the dark phase was found to be significantly higher ($p = 0.043$) in CRE mice ($X = 120.3 \pm 19.8$ m) when compared to controls ($X = 72.7 \pm 11.2$ m). The sum of
451 direct locomotions performed during the light cycle revealed CRE mice
452 displaying higher levels of locomotor activity ($X = 21.3 \pm 3.7$ m) than controls
453 ($X = 13.6 \pm 2.8$ m) but the difference did not reach statistical significance
454 ($p = 0.115$). Over the entire circadian cycle, CRE mice performed significantly
455 more ($p = 0.039$) ambulatory activity ($X = 141.5 \pm 23.1$ m) than WT ($X = 86.4 \pm$
456 11.9 m).

458 The sum of all the displacements recorded due to fine activity was measured
459 over the 24 h period (**Figure 4C**). During the dark phase, fine activity was
460 found significantly higher ($p = 0.039$) in CRE mice ($X = 15.3 \pm 1.4$ m) when
461 compared to controls ($X = 11.5 \pm 1.1$ m). Interestingly, CRE mice showed two
462 peaks of immobile activity 3 h after the beginning of the dark phase and 2 h
463 before the light phase (beginning of the sleeping phase). As in the dark
464 phase, CRE mice exhibited more fine activity ($X = 4.81 \pm 0.7$ m) than controls
465 ($X = 3 \pm 0.4$ m) during the light phase ($p = 0.023$). Another increase of
466 immobile activity was found in CRE mice when compared to controls
467 during the light phase at a specific time interval (from 8:00 P.M. to 9:00
468 P.M.). This phenomenon is similar to the peak observed during the dark
469 phase which was also detected 2 to 3 h before the phase switch. Regarding
470 the whole circadian cycle, CRE mice were found to perform significantly
471 more ($p = 0.022$) fine activity ($X = 20.1 \pm 2$ m) than controls ($X = 14.4 \pm 1.3$ m)
472 over a complete circadian cycle.

473 As reported in **Figure 4D**, the sleep sequences in CRE mice were greatly
474 reduced during the dark phase when compared to those of control mice
475 ($p = 0.0005$). It should be noted that two particular periods of arousal were

concomitantly found to the two peaks of locomotor and fine activity previously highlighted during this dark phase, and thereby could contribute to the differences observed between the both strains. The sleep sequences in CRE mice were also reduced during the light phase when compared to those of control mice: the average sleep percentage was found at $95 \pm 0.6\%$ in WT mice, while it was found at $91.1 \pm 1\%$ in CRE mice ($p = 0.0037$). This was also the case for the whole circadian cycle: the average sleep percentage was found at $87.1 \pm 1\%$ in WT mice, while it was found at $79.0 \pm 1.7\%$ in CRE mice ($p = 0.0002$), which clearly shows different patterns of sleep during the whole circadian cycle between both CRE and WT.

3.5. Barnes maze

Mice spatial learning performances were assessed using a modified Barnes maze. Animals were given three trials a day for three consecutive days to learn the location of the escape hole. Learning performances and retention memory of both strains are shown in **Figure 5**. Concerning the acquisition period (Day 1 to Day 3, T1 to T9), the two-way ANOVA with repeated measures revealed at first a strong time effect ($p < 0.0001$), indicating that both strains were able to learn the location of the escape hole as shown by reduction of time to perform the task. Nevertheless, looking to the second main factor, a clear genotype effect ($p = 0.0049$) was found, CRE mice spending significantly more time to find the escape than WT. Finally, no interaction between both factors was found ($p = 0.23$).

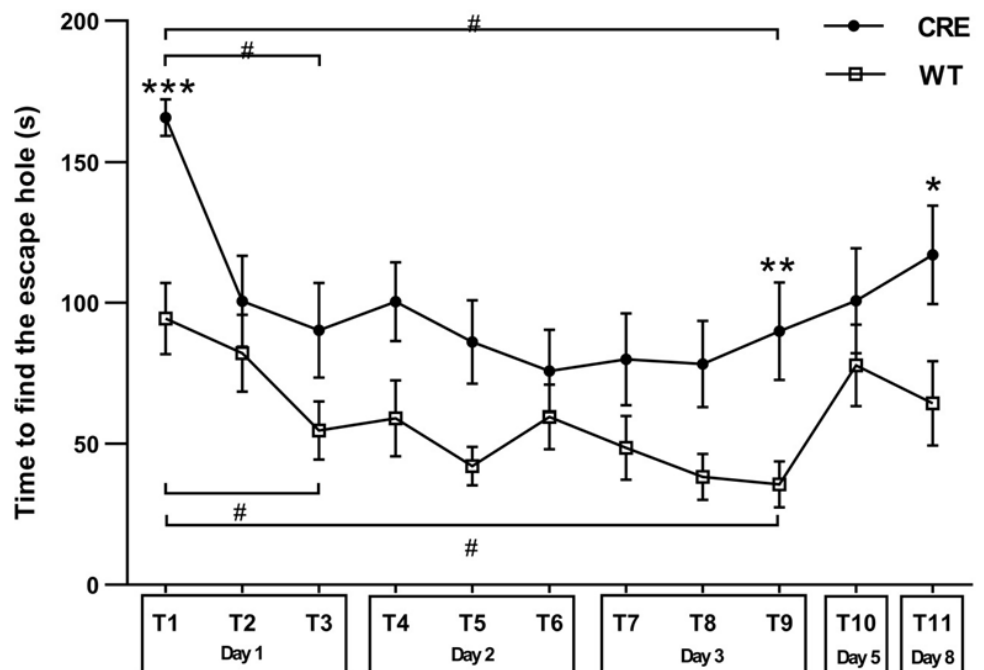


Figure 5. Effect of *Cre* genotype compared to WT on the latency to find the escape hole in the modified Barnes maze (CRE n=14, WT n=15). Data are reported as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significantly different from control (unpaired *t* test post-hoc test). # $p < 0.05$ intra-group significant difference (Tuckey test)

For short-term learning, *post-hoc* Tuckey's *t*-tests applied at 5% probability performed on trial 1 *vs.* trial 3 revealed a significant decrease in time to locate the escape hole in both groups. This indicated that short-term spatial learning took place since the first day of testing (mean difference of 39.7 with a critical difference of 37.2 for WT; mean difference of 75.4 with a critical difference of 49.3 for CRE; $p < 0.05$). *Post-hoc* Tuckey's *t* tests also reached significance for both groups when comparing trial 1 *vs.* trial 9 (last trial of the acquisition period), suggesting continued learning over time (mean difference of 58.9 with a critical difference of 37.2 for WT; mean difference of 75.8 with a critical difference of 49.3 for CRE; $p < 0.05$). Thus, both groups evolved in time according to the same pattern. Both CRE and WT demonstrated short and long-term learning, but not at the same rate, with CRE mice exhibiting systematically longer durations to find the escape hole. Trial 1 has an intrinsic specificity, since none of the animals had ever been exposed to the maze before, which allowed to compare the performance of both groups at this particular time. Here, the unpaired *t* test showed a clear genotype effect ($p < 0.0001$), where CRE mice required up to 165 ± 6 s before finding the escape hole, as compared to a mean time of 94 ± 12 s for control mice to perform the same task. Finally, when looking for exploratory behavior in the last trial of the acquisition period (T9: third trial of the third day), a significant difference was still found. CRE mice required up to 90 ± 17 s when control mice showed a duration of 35 ± 8 s ($p < 0.0073$) to escape the maze's aversive conditions. Two probe tests were performed on Day 5 and Day 8. No difference between both groups was found on Day 5 ($p = 0.33$). However, the Day 8 probe test revealed a deleterious effect of long-term memory in CRE mice when compared to controls ($p = 0.03$), since the time needed to find the escape hole was approximatively doubled in CRE mice (117 ± 18 s) in comparison with controls (64 ± 15 s).

4. Discussion

4.1. Hyperactivity in CRE mice

All performed tests pointed towards the hyperactive trait of CRE mice. This was especially noticeable for home cage activity where CRE mice walked longer distances than WT mice and had significantly more stationary activity including grooming and scratching than WT mice (**Figure 4**). In addition, CRE mice exhibited state anxiety behavior (**Table 2**) rather than trait anxiety behavior. The hyperactive trait of CRE mice most likely influenced their performance in all tests requiring attention and retention. CRE mice showed short-term memory problems (**Figure 1**), learning ability deficits and long-term memory problems (**Figure 5**). In addition, this disruption of information acquisition inevitably led to an impact on their performance in the object recognition test (**Figure 3**). Since the mice explored the objects significantly less as compared to WT, they had difficulties memorizing the familiar object and thus discriminating it from the newly introduced one.

547 CRE mice performed poorly in Barnes maze since their hyperactivity trait
548 apparently blocked them from learning and retaining information (**Figure**
549 **5**). There is evidence in the literature that hyperactivity can interfere with
550 escape latency, distance and speed in behavioral tests [15]. However, since
551 CRE mice also performed badly in object recognition test, it is possible that
552 they also have a visual deficit in addition to their hyperactive trait. Further
553 tests that assess vision could be needed in order to rule out low vision
554 ability in CRE mice. However, the retinal structure and organization of
555 GLAST-CreERT2 mice appeared fully normal [4, 16], and routine
556 morphological observation of the anterior chamber did not reveal corneal
557 or lens opacification in CRE mice. In addition, Barnes maze can
558 discriminate learning and retention abilities independently of visual abilities
559 [17]. Thus, it is more likely that the hyperactive trait of CRE mice led to the
560 low scores in the Barnes maze and object recognition tests, rather than
561 global visual deficiency.

562 At the olfactory level, CRE mice were able to smell, as they performed well
563 in buried cookie test (**Supplementary Figure S1**) and showed high interest
564 in female urine odor in odor discrimination test (**Supplementary Figure**
565 **S2**). They performed well in sociability and social novelty test (**Figure 3**),
566 most probably due to a well-functioning olfactory system. However, they
567 weren't as interested in rose odor as in female urine and were unable to
568 discriminate 1 % lemon additive (**Supplementary Figure S2**). This is most
569 probably due to their hyperactivity trait rather than a deficient sensory
570 entry. This was clearly visible in the habituation phase of the buried cookie
571 test, when the cookie is not buried, but placed in a visible location in the
572 cage. CRE mice took 4-times longer time to find the cookie compared to
573 control. This was very likely not due to impaired sensory entry but rather
574 inattention since the CRE mice were able to find the buried cookie. This
575 hyperactive trait rather than olfactory deficits might also be linked to the
576 lower interest for congeners identified in sociability tests (**Figure 3**).

577 578 *4.2. Uncertainties about the mechanism of action*

579 Noxious CRE effects have been documented over the past decade. First,
580 CRE integration in mouse genome is random and can lead to multiple
581 copies on single-chromosomal loci, as well as potentially disrupting
582 endogenous gene expression [18]. Our CRE mice were generated using the
583 bacterial artificial chromosome technology that are thought to minimize
584 position effects on variegation and random recombination [4]. Even if we
585 cannot fully rule out this possibility, it is unlikely that our CRE mice exhibit
586 hyperactivity due to random gene disruption. *Cre* expression has been
587 shown to induce DNA damage and apoptosis in the absence of genuine
588 *loxP* sites [19, 20]. Toxic effects associated with *Cre* expression have been
589 observed in various systems including the nervous system [21-23].
590 Moreover, TAM-inducible CRE expression has also been demonstrated to
591 induce DNA damage in absence of valid *loxP* site [24]. However, the

592 behavioral profile of our CRE mice is independent of the TAM treatment as
593 both uninduced and induced CRE mice exhibited the same hyperactive
594 trait (**Supplementary Figure S3**). This is in line with previous work in the
595 field [25], where authors observed that short TAM treatments used to
596 activate the CRE do not have a measurable impact on adult neurogenesis
597 or on their performance in behavioral tests. In addition, in the original
598 publication, no CRE recombinase activity was detected after vehicle
599 injection (see Figure 3 in [4]), indicating absence of TAM-independent
600 CreER^{T2} activity. Hyperactive trait is therefore neither linked to estrogen
601 treatment, nor to detectable CRE recombinase activity that could have
602 targeted invalid and degenerated *loxP* sites that are abundant in mouse
603 genome [23].

604 The question whether accumulation of *cre* mRNA or inactive CRE
605 recombinase protein could exert extra-genomic activity is still unanswered
606 and the mechanism underlying the hyperactive behavior of our CRE mice
607 is still to be deciphered. Interestingly, when we generated specific glia-
608 specific conditional knockout mice based on this CRE recombinase system,
609 we did not observe any hyperactive traits [7]. This would support the
610 hypothesis of low CRE recombinase activity or that *cre* RNA accumulation
611 might impair attention and activity in this CRE mice.

612 613 *4.3. Importance of behavioral analysis of CRE transgenic mice*

614 The hyperactivity trait observed in our CRE mice prevents it's use as a
615 negative control group in behavioral phenotyping of previously published
616 conditional mutant mice [7]. In addition, the abnormal behavioral profile
617 reported in this strain of CRE mice could indicate that CRE mice in general
618 suffer from overlooked cognitive deficits. Indeed, there is little to no
619 information on the specific behavior of CRE mice in the literature. In most
620 behavioral studies, the group of interest (cKO) is usually compared to WT
621 littermates, rather than CRE. Some of these studies showed that their group
622 of interest was hyperactive when compared to *WT* controls [26-29].
623 Ignoring the behavioral status of CRE mice may generate a bias in the
624 behavioral analysis of cKO mice by identifying a specific trait as a
625 consequence of the targeted gene deletion while it might be linked in fact
626 to the CRE recombinase activity. CRE group should therefore be analyzed
627 in parallel to WT and cKO group in behavioral phenotyping experiments
628 to avoid misinterpretation of gene deletion consequences. Moreover,
629 similar results of specific tests might be due to different behavioral traits.
630 For instance, CRE mice and previously characterized cKO mice [7]
631 exhibited similar behavioral patterns in some of the tests, such as equal
632 exploration time of objects and subjects, delay before departure in Barnes
633 maze, and low proper alternation rates. However, these results observed
634 were not caused by the same reason and were rather linked to
635 hyperactivity and attention errors in CRE mice, as opposed to anxiety in

636 the cKO mice that did not exhibit any hyperactive behavior [7]. Home cage
637 activity and open field tests appear therefore as essential tests to easily
638 discriminate between hyperactivity and anxiety and to avoid
639 misinterpretation of behavioral profile linked to gene deletion
640 consequences.

641 642 4.4. CRE mice as a model of attention-deficit hyperactivity disorder?

643 The hyperactive trait of CRE mice might serve as a model for attention
644 deficit and hyperactivity disorder (ADHD). Here we showed that CRE
645 mice were hyperactive, impulsive, and lacked motivation and attention,
646 coinciding with ADHD which is generally characterized by inattention,
647 hyperactivity and impulsivity, and deficits in motivation [30]. Under
648 normal circumstances, the prefrontal cortex regulates attention, behavior,
649 and emotion. ADHD is characterized by poor impulse control, weak
650 sustained attention, and heightened distractibility, and has been linked to
651 deficits in prefrontal cortex functioning [31]. In a stressed state, there is
652 excessive dopamine and accompanied norepinephrine release which
653 impairs prefrontal cortex abilities [32]. The pharmacological treatment for
654 ADHD is based on methylphenidate that blocks the activity of dopamine
655 transporter and norepinephrine transporter, leading to increased
656 availability of catecholamines in the synaptic cleft and to improved
657 dopaminergic synapse functioning thereby restoring attention. Unlike
658 amphetamine, methylphenidate does not induce dopamine release from
659 presynaptic vesicles [33]. Animal models are critical to understand the
660 pathological mechanisms involved in ADHD and to allow assessment and
661 development of new therapeutics. The currently existing models are
662 heterogeneous regarding physiopathological alterations, behavioral
663 symptoms and response to medication [10, 34-36]. Indeed, no existing
664 rodent model captures all aspects of ADHD. We demonstrated here that
665 CRE mice exhibited impulsive and hyperactive traits. Their poor
666 performances in attention-based tests were exacerbated when visual entry
667 was required for a specific task (*i.e.*, finding visible cookie in habituation
668 phase). Those poor performances were linked to attention deficit rather
669 than functional visual problems as they were able to oriente themselves
670 based on clues in the Barne's maze or discriminate illumination changes in
671 the open field test. As we did not observe any other visual deficit in CRE
672 mice, vision-based attention was therefore especially affected in CRE mice.
673 In another study, authors identified ADHD-like behavior using vision-
674 based attention tests in mutant mice exhibiting a duplicated retino-
675 collicular map [37]. As those mice did not exhibit other visual impairment,
676 it underlies the importance of superior colliculus organisation in vision-
677 based attention. In addition, we pointed out a significant reduction of sleep
678 periods for CRE mice (**Figure 4D**). Sleep disturbance is associated with
679 ADHD in patients [38] and could also have affected CRE mice
680 performances requiring attention and memorization. Interestingly, it was
681 recently shown that sleep-deprived mice could serve as models of ADHD

682 [39]. Altogether, CRE mice exhibit specific vision-based attention deficits
683 associated with sleep disturbance, hyperactivity and impulsivity traits that
684 affect their exploration, learning and retention abilities. CRE mice could
685 therefore be proposed as an ADHD model in view of their behavioral
686 response. Further experiments and studies would be needed to assess their
687 adequation level with ADHD condition regarding physiopathological and
688 pharmacological aspects to fullfull the list of criteria for an optimal animal
689 model of ADHD as suggested previously [34].

690 **Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1,
691 Figure S1: Buried cookie test; Figure S2: Habituation/Cross habituation test for odor
692 discrimination; Figure S3: Home cage activity in absence of tamoxifen treatment

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694 F.D., A.E.H., A.H., F.Dj. V.B., I.D.; validation, F.D., A.E.H., A.H., F.T.Y., M.C.L., T.C.; formal
695 analysis, F.D., A.E.H., A.H., investigation, F.D., A.E.H., A.H.; ressources, F.Dj., T.O., C.M.,
696 F.T.Y., M.C.L., T.C.; data curation, F.D., A.E.H., A.H., F.T.Y., M.C.L., T.C.; writing—original
697 draft preparation, F.D., A.E.H., A.H.; writing—review and editing, F.Dj., V.B., I.D., T.O.,
698 C.M., M.V., F.T.Y., M.C.L., F.T.Y., T.C.; visualization, F.D., A.E.H., A.H.; supervision,
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