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1 Familiarization effects on the behavioral disinhibition of the cerebellar Lurcher mutant
2 mice: use of the innovative Dual Maze.

3

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19

1 **ABSTRACT**

2

3 Anxiety-related behaviors in mice are often assessed over short periods starting
4 immediately after introducing the animals in a dedicated apparatus. In these usual
5 conditions (5 to 10 minutes periods), the cerebellar Lurcher mutants showed
6 disinhibited behaviors characterized by abnormally high exploration of the aversive
7 areas in the elevated plus-maze test. We nevertheless observed that this disinhibition
8 sharply weakened after 10 minutes. We therefore decided to further investigate the
9 influence of the disinhibition on the intrinsic and anxiety-related exploratory behaviors
10 in Lurcher mice, with a special focus on familiarization effects. To this end, we used
11 an innovative apparatus, the Dual Maze, permitting to tune the familiarization level of
12 animals to the experimental context before they are faced with more (open
13 configuration of the device) or less (closed configuration of the device) aversive
14 areas. Chlordiazepoxide administration in BALB/c mice in a preliminary experiment
15 confirmed both the face and the predictive validity of our device as anxiety test and
16 its ability to measure exploratory motivation. The results obtained with the Lurcher
17 mice in the open configuration revealed that 20 minutes of familiarization to the
18 experimental context abolished the behavioral abnormalities they exhibited when not
19 familiarized with it. In addition, their exploratory motivation, as measured in the
20 closed configuration, was comparable to that of their non-mutant littermates,
21 whatever the level of familiarization applied. Exemplifying the interest of this
22 innovative device, the results we obtained in the Lurcher mutants permitted to
23 differentiate between the roles played by the cerebellum in exploratory motivation
24 and stress-related behaviors.

25

1. INTRODUCTION

The experimental procedures used in behavioral neuroscience always challenge a wide range of brain systems and functions including attention, motivation and emotions in addition to those they are primarily to be examined. These factors contribute to inconsistency and irreproducibility of results. Therefore, it is of importance to consider the general way the animals cope with the experimental environment as a whole before interpreting the specific behavioral aspect under investigation (as suggested, for instance, in [1]). Stress is at the forefront of the factors impeding the tests specificity [2]. Unfortunately, in spite of refined methods aiming at reducing suffering in laboratory animals, virtually all the experimental manipulations remain more or less stressful. The resulting stress-induced behaviors and hormone rise impact animal's performances in behavioral tests, regardless of the nature of the variable of interest: cognitive, emotional and even motor [3-7]. Such biases are particularly confounding when investigating emotional reaction with preset controlled anxiogenic factors (i.e. more or less open space, level of illumination or height) in standardized conditions. It obviously becomes of critical importance when testing animals having abnormal stress reactivity.

Novelty is a crucial component driving the rodents' behaviors in the classical exploration-based anxiety tests [8, 9]. Nevertheless, experimental procedure (manipulation) as well as novelty are also stressors [10], the effects of which depend on both the intrinsic features of the novel environment and the individual's sensitivity. The latter is largely determined by genetic factors and numerous mutant mice display specifically impaired reactivity to novelty [11-13], notably those modeling neuropsychiatric diseases such as autism spectrum disorders [14, 15]. In addition, reaction to novelty is a dynamic process influenced by habituation and familiarization mechanisms that can considerably change the coping strategies of animals over time. For instance, behaviors adopted by mice forcibly exposed to the elevated plus-maze first resulted from escape tendency, with long time spent in the aversive open arms. The approach-avoidance conflict, more in line with anxiety theory and characterized by a clear preference for the closed arm, progressively appeared only in a second phase [16].

The cerebellum plays a role in attention to novel stimuli [17]. As a consequence, cerebellar mutant mice could improperly manage novelty in the course

1 of tests evoking exploratory behaviors. Nevertheless, it would be hazardous to
2 attribute a unique cause to behavioral abnormalities observed in cerebellar mice in a
3 given test because of the various and deeply intertwined functions carried out by the
4 cerebellum [18, 19]. Although the precise mechanisms involved are still elusive, all
5 these functions, which range from motor coordination to cognitive processing and
6 from control of emotions to vegetative regulations, are sustained by complex,
7 bidirectional projections between the cerebellum and diverse brain structures [20-22].
8 So, the behavioral abnormalities of cerebellar mutants in any experimental situation
9 very likely result from dysfunctional interaction between several of these cerebello-
10 cerebral circuits. Considering such dysfunctions, the non-specific interfering effect of
11 stress could have a particular resonance in cerebellar mutants and even aggravate
12 their multiple deficits.

13 Lurcher mutant mice [23] are ones of the most commonly used cerebellar
14 mutants. Almost all of their Purkinje cells are extinct by the age of 3 months due to a
15 mutation in the delta2 glutamate receptor-encoding gene [24, 25]. The loss of
16 Purkinje cells is accompanied by massive secondary degeneration of cerebellar
17 cortex interneurons and inferior olive [24, 26] and by mild reduction of the cerebellar
18 nuclei [27]. These histological defects result in marked cerebellar ataxia and cognitive
19 impairments [17, 19, 28-32].

20 It was also extensively demonstrated that Lurcher mice exhibited profound
21 behavioral and physiological emotional-related disturbances [32-37]. For instance,
22 they did not avoid the aversive open arms of the elevated plus-maze test as much as
23 their littermate controls did [34]. Although such a behavior is classically interpreted as
24 attenuated anxiety, the paradoxical dramatic corticosterone rise observed in these
25 mutants in response to any manipulation (including the exposure to the elevated
26 plus-maze) led us to hypothesize that their functional cerebellar decortication actually
27 resulted in pathological behavioral disinhibition [34, 35, 38]. Further experiments
28 conducted in our laboratories revealed that the significant differences between the
29 behaviors of Lurcher and control mice noticed in the first 10 minutes of a 15-minutes
30 session in the elevated plus-maze test were no longer observed in the last 5 minutes
31 (see supplementary material). This clearly indicates that the conclusions of studies
32 comparing the Lurcher mutants and control mice behaviors strongly depend on the
33 duration of exposure to experimental conditions and the timing of data collection.

1 Moreover, it reinforces the idea that the Lurcher behavioral profile is shaped by the
2 dynamic of novelty-induced stress provoked by forcible exposure to an unknown
3 context. In line with these observations, we hypothesized that adding a resting period
4 in the experimental environment before starting the test itself could lead to the
5 normalization of the anxiety-related behaviors in Lurcher mice by neutralizing the
6 effects of handling- and novelty-induced stress.

7 The first aim of this study was to test this hypothesis by comparing the effects
8 of prior familiarization to the general experimental context on the behavioral reaction
9 of heterozygous Lurcher cerebellar mutants and their healthy littermates facing an
10 aversive and suddenly unveiled area they were kept to freely explore. We also
11 sought to investigate their behaviors in the same conditions but facing a much less
12 aversive environment in order to evaluate hypothetical genotype-dependent
13 differences in pure exploratory motivation, which could affect the anxiety variables.
14 Taking into consideration such a possible bias seemed to us particularly important
15 since Lurcher mice were previously described to suffer from exploratory impairments
16 in the hole board test [39]. Several well-documented tests based on exploratory
17 activity, such as the classical open field or elevated plus-maze or the more recent
18 zero maze, are available to investigate anxiety-related behaviors in mice.
19 Nevertheless, none of these apparatuses allows to properly carry out prolonged
20 familiarization of mice to experimental conditions or to separately evaluate the effects
21 of confrontation to aversive and less aversive places on their behaviors. We thus
22 designed an innovative apparatus coming in highly or lowly aversive versions: the
23 Dual-Maze. This new device permits 1/ to familiarize animals, for a controlled
24 duration, to experimental context before being tested and 2/ to investigate behavioral
25 reaction of animals confronted to more or less aversive environment. Before
26 submitting Lurcher mice and control littermates to this new apparatus, we first tested
27 its face validity (the mice would avoid open space) and predictive validity (well known
28 anxiolytic agent would reduce this avoidance) by measuring the chlordiazepoxide
29 effects on behaviors of BALB/c mice exposed to it.

30

31 **2. Experimental procedure**

32 **2.1. Experimental design**

1 The study was split into two distinct experiments: the first one consisted in
2 validating the Dual maze as a new apparatus for anxiety and exploratory behavior
3 assessment in mice. In this first experiment, we measured the effects of
4 chlordiazepoxide (CDP), an anxiolytic benzodiazepine-type substance, in
5 pathologically anxious BALB/c mice [40]. The second experiment evaluated the
6 impact of familiarization on anxiety- and spontaneous exploration-related behaviors in
7 the Lurcher mutant mice.

8 9 **2.1.1. Apparatus: The Dual Maze test (Figure 1)**

10 Two black-painted IffaCredo® individual cages 20 x 7 x 8 (length x width x
11 height in cm) were assembled to construct the apparatus. It consisted of a starting
12 closed arm facing a testing arm. The latter was either open (open configuration) or
13 closed (closed configuration: in this case, the testing arm was identical to the starting
14 arm). A guillotine door separated the starting and testing arms. Each of the arms was
15 virtually divided into two segments of the same surface. The segments adjacent to
16 the limit between the two arms of the device were called “proximal” segments, the
17 other ones “distal segments”. All the closed arms (e.g. the starting arm in both
18 configurations and the testing arm in the closed configuration) were covered with
19 plastic plates with ventilation holes. These plates were aimed at preventing the
20 escape of mice out of the arms. The whole apparatus was screwed to a pole and was
21 elevated to a height of 50 cm above the floor level.

22 23 **2.1.1.1. Closed configuration (named CC)**

24 In this configuration, the Dual maze is a parallelepiped box opened on its
25 upper face. This box is made of two contiguous individual Iffa Credo cages. Their
26 joined small sides were cut to allow the mice to stroll from one to another. Two
27 transverse partitions 1 cm long, located on both sides of the device, mark the limit
28 between these two cages.

29 **2.1.1.2. Open configuration (named OC)**

30 In this configuration, the testing arm is opened and has the same surface as in
31 the closed configuration. We added 3 mm high edges to this open arm to prevent the

1 fall of animals, especially that of ataxic Lurcher mice. In open configuration, the mice
2 are therefore confronted, from the familiar starting arm where they were deposited a
3 few seconds or minutes before, with an aversive open environment.

4 5 **2.1.2. Animals**

6 Adult male BALB/c mice, aged 3 months +/- 1 week, were used in the
7 experiment investigating the sensitivity of the Dual Maze to the anxiolytic effects of
8 CDP. We chose mice of this strain because they were described as a model of
9 pathological anxiety [40]. This first experiment was aimed at verifying the face validity
10 of the test (that is the occurrence of anxiety-related behaviors in it) and its predictive
11 validity (as indicated by the sensitivity of the variables recorded to the injection of an
12 anxiolytic agent). In the second experiment, adult male and female (approximately
13 1:1 ratio) heterozygous Lurcher mutant (+/Lc) and littermate wild type (+/+) mice from
14 B6CBA strain, aged 3 months +/- 10 days, were submitted either to the open or to the
15 closed configuration of the Dual Maze, after or not a 20 min familiarization period.
16 The detail of the number of mice per group (n) is given in figure 2.

17 All mice were obtained and bred in the lab under standard temperature- and
18 humidity-controlled conditions, with water and commercial pellet food available *ad*
19 *libitum* and 12:12 hours light:dark cycle. They were tested during the light period.

20 All the experimental procedures were performed in compliance with EU
21 guidelines about protection of animals used for scientific purposes and with the
22 permission of the Ethical Commission. All efforts were made to minimize the number
23 of animals used and their suffering.

24 25 **2.1.3. Drugs**

26 CDP hydrochloride was obtained from Sigma–Aldrich. It was dissolved in 0.9%
27 physiological saline, which, alone, served as vehicle control. CDP hydrochloride
28 (Sigma–Aldrich) dissolved in 0.9% saline was injected intraperitoneally in a dose of 5
29 mg/kg 20 min prior scoring the mouse behaviors. Control mice were treated by
30 injection of adequate volume (10 ml/kg) of saline (see Procedure – Experiment 1 for
31 details).

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2.2. Principle of the Dual Maze test

The test is based on the resolution of approach/avoid conflict which balance the innate motivation of rodents to explore their environment with their aversion for hazardous places. In line with what was repeatedly demonstrated in other devices [5, 41-43] we expected that our open testing arm was much more aversive for mice than closed one. In accordance with the classical meaning of this kind of variables in the literature, we therefore considered the number of entries and the time spent in the open testing arm as indices of anxiety. We also measured the number of protected stretched attend postures (pSAP: the body of the mouse is stretched from the starting arm toward the testing arm then retracted to the original position) and the number of protected head dips (pHD: downward visual screening over the edge of the open testing arm achieved from the testing arm) as additional markers indicating an elevated level of anxiety when they are high [44-46]. While rare pSAPs could be observed in the closed configuration of our device, we proposed the latter, considering its physical features not much aversive, was more suitable for isolating the motivational component of the mice exploratory activity. In both configurations, the total number of segments crossed was interpreted as an index of general activity.

Because both tests are based on the same principle, the variables measured in the Dual Maze test were similar to those usually measured in the plus-maze test. Nevertheless, the former allows to overcome some problems of interpretation encountered in the latter, especially those related to the center of the device [8, 47]. Incidentally, the zero maze was partly designed to answer this issue [41]. But the Dual Maze is even simpler than this alternative to the plus-maze and, as an important consequence for our purpose, it strongly limits the influence of Lurcher mouse motor disorders on their exploratory behaviors. So, the conformation of the device ensures that an entry into an aversive zone can hardly be accidental as it can happen in mice with motor disabilities in more complex devices. Indeed, ataxic mice have problems with straight trajectory maintenance and can enter initial part of an open arm of the elevated plus maze accidentally due to lateropulsions when just transiting from one closed arm to the opposite one. They also show typical backwards movements particularly confusing in the zero-maze, in which the closed arm is surrounded by two open arms.

1 The size of the starting arm, identical to that of an individual transport cage,
2 seemed to us ideal to carry out the familiarization of the animals. Lastly, the strict
3 segregation of the highly and lowly aversive testing arms in two separated
4 configurations facilitates the discrimination between pure exploratory motivation and
5 anxiety-related behaviors.

6 7 **2.3. Procedure**

8 **Common Protocol**

9 First, all animals were habituated for 30 minutes to the experimental room. At
10 the beginning of the experiment, each mouse was individually placed in the distal
11 segment of the starting arm. Whatever the level of prior familiarization (0 or 20 min.),
12 the following parameters were recorded for 5 min after removing the guillotine door:
13 the number of entries (four paws criterion) and the time spent in the different
14 segments of the apparatus and the number of pSAPs and pHD performed from the
15 starting arm. The number of entries and the time spent in the testing arm were
16 calculated from the data obtained for each segment. For more clarity, all the temporal
17 variables are expressed as relative values that is as a percentage of the total
18 experiment duration. Experimental sessions were recorded by a miniaturized camera
19 placed 1.5 m above the device and connected to a video recorder located in a room
20 next to that where the mice were tested. The experimenter (always the same) could
21 follow the experiment proceedings from this adjacent room by watching the screen
22 connected to the video recorder. All the behaviors of the mice were quantified later
23 from the camera footage using the ethological analysis software Etholog [48].

24 25 **2.3.1. Experiment 1 - The effects of CDP on BALB/c behaviors**

26 The mice were allocated to two different cohorts. The first one was submitted
27 to the closed configuration of the DualMaze and the second one to its open
28 configuration. Each mouse was intraperitoneally injected with saline or CDP (5mg/kg)
29 and was immediately placed in the starting arm of the device. Twenty minutes later,
30 the guillotine door was removed, and the mouse could freely access to the testing
31 arm. The dose of CDP chosen was determined using data from the literature [43, 44].
32 Moreover, in preliminary experiments, we observed that CDP was highly sedative at

1 a dose of 7.5 mg / kg (data not shown). Animals were randomly tested in a simple
2 blind procedure so that the experimenter did not know the compound administered to
3 the mice.

4 5 **2.3.2. Experiment 2 - Examination of Lurcher mice behaviors**

6 All the animals were tested randomly for the genotype and the configuration.
7 Due to the obvious ataxic gait of the Lurcher mice, the experimenter could not be
8 unaware of the genotype tested.

9 2.3.2.1. Without familiarization

10 In this procedure, animals were only habituated for 30 minutes to the
11 experimental room. Then, they were individually placed in the closed starting arm of
12 the device, either in its closed or open configuration, and the guillotine door was
13 immediately removed.

14 2.3.2.2. With familiarization

15 This procedure was similar to the previous one except that the mice were
16 familiarized to the starting arm of the device for 20 minutes before the removing of
17 the guillotine door.

18 2.4. Statistical analysis

19 The normality of residuals was assessed using normal probability plots.
20 Variances homogeneity was verified by comparing the externally studentized
21 residuals of groups to each other. For experiment 1, these assumptions were met
22 and the variables of interest were analyzed with a two-way ANOVA (treatment x
23 configuration) followed, where appropriate, by post-hoc t-test corrected for multiple
24 comparison with the Benjamini-Hochberg method. As most of the data of experiment
25 2 did not meet the parametric assumptions, we submitted them to a non-parametric
26 analysis. So, we first performed a Kruskal-Wallis test in order to detect any significant
27 heterogeneity in our results for each variable. When appropriate, multiple
28 comparisons were achieved with Fisher-Pitman permutation tests corrected with the
29 Benjamini-Hochberg method. The avoidance or preference for the testing arm was
30 assessed by comparing the relative time spent in it to the chance level with one-
31 sample t- (experiment 1) or Mann-Whitney (experiment 2) test. In all cases, the sex

1 factor was blocked (after checking it did not interact with any other factor). $P < 0.05$
2 was used as significance threshold in all analyses.

3

4 **3. RESULTS**

5 **3.1. Experiment 1 - Effects of CDP injection on BALB/c mice** 6 **behavior (table 1, figure 3)**

7

8 The statistical analysis presented in table 1 revealed that the mice behaviors
9 were dependent on the configuration they were submitted to. So, the configuration
10 factor exerted a significant effect upon the total number of segments crossed (fig.3A),
11 the number of entries in the testing arm (fig. 3B) and in its distal segment (fig. 3 C)
12 and the number of rears (fig. 3F). All these parameters were clearly decreased in the
13 open configuration when compared with what observed in the closed one,
14 demonstrating that both configurations had a very different impact on the exploratory
15 activity of animals. Interestingly, significant configuration x treatment interactions
16 were detected concerning the relative time spent in the testing arm (fig. 3D) and in its
17 distal segment (fig. 3E) (this segment being supposed to be the most aversive part of
18 the device in its open configuration).

19

20 Post hoc comparisons revealed that NaCl-injected mice spent more time in the
21 testing arm (fig. 3D) ($p < 0.001$) and in its distal segment ($p < 0.01$) (fig. 3E) when CC
22 rather OC was used, confirming that the closed testing arm is less aversive than the
23 open one. In accordance with this analysis, one-sample t-test showed the mice
24 tested in OC had a preference for the starting arm ($t = -3.0112$, $p < 0.05$, compared to
25 chance level). In CC, the preference for the testing arm (fig. 3D) probably reflected
26 the strong motivation of mice to explore new areas after prolonged familiarization to
27 the starting arm ($t = 5.1468$, $p < 0.05$, compare to chance level). In accordance with its
28 anxiolytic effects, CDP significantly increased the time spent in the distal segment of
29 the testing arm (fig. 3E) in OC ($p < 0.01$) while it did not significantly change it in CC
30 ($p > 0.05$). By the way, the responsivity of this parameter to pharmacological anxiolysis
31 was such that CDP suppressed the significant difference previously noticed between
32 both configurations in NaCl-mice ($p > 0.05$). Although the time spent in the open

1 testing arm as a whole (fig. 3D) did not sufficiently respond to the anxiolytic drug to
2 lead to a significant difference between NaCl and CDP-mice in OC ($p>0.05$), the
3 dramatic reduction of the number of pSAPs (fig.3G) by the treatment in this
4 configuration ($p<0.05$) and, at a lesser extent, that of the number of pHDs ($p>0.05$)
5 (fig. 3H), were another evidence of the potency of the anxiolysis induced by the
6 substance. It can be noted that pSAPs were only exceptionally observed in CC (for
7 this reason, they were not included in the statistical analysis), confirming the
8 extremely low aversiveness of this configuration.

9 As a whole, our results demonstrated that, at least after familiarization to the
10 starting arm: 1/ our device in OC was appropriate to precisely detect pharmacological
11 anxiolysis 2/ our device in CC, due to the virtually complete absence of any aversive
12 aspect (as indicated by the anxiety variables *per se* and the lack of CDP effect on
13 them), was appropriate to specifically investigate the intrinsic exploratory motivation
14 of mice.

15 **3.2. Experiment 2 -examination of Lurcher mice (tables 2-5,** 16 **figure 4)**

17
18 In accordance with the experiment 1 results, the B6CBA strain mice behaviors
19 strongly differed in CC and OC of the Dual Maze. In addition, our data patently
20 indicated that this configuration effect was influenced by both the genotype and the
21 level of familiarization. The data are shown on figure 4 and tables 2-5.

22
23 For more clarity, we decided to first present the results obtained in OC and CC
24 without any prior familiarization. The data collected after 20 minutes of familiarization
25 in the starting arm are described in the second paragraph.

26 27 **3.2.1. Without familiarization**

28 The analysis of the time spent in the testing arm clearly showed that mice of
29 both genotypes avoided this arm in OC ($U=0$; $p<0.01$, compared to chance level for
30 both genotypes) but not in CC where no significant preference for any arm was
31 observed (control mice: $U=35$; $p>0.05$ / Lurcher mice: $U=20$; $p>0.05$, compared to

1 chance level) (fig. 4D). Accordingly, the time spent in the open testing arm (fig. 4D)
2 was significantly shorter than that spent in the closed testing arm in control
3 ($p < 0.0001$) and Lurcher mice ($p < 0.05$). Nevertheless, the avoidance behavior
4 induced by the testing arm in OC was more marked in the former than in the latter
5 (number of entries in the open testing arm: $p < 0.005$, fig. 4B / time spent in the open
6 testing arm: $p < 0.05$, fig. 4D). In contrast, no significant difference between the
7 animals of both genotypes was noticed concerning the time spent in the closed
8 testing arm ($p > 0.05$) (fig. 4D), although the motor deficits of the mutants reduced the
9 number of entries they made in this arm ($p < 0.05$ when compared with the control
10 mice, fig. 4B) as well as the total number of segments they crossed ($p < 0.05$ when
11 compared with the control mice, fig. 4A). The results reported above, consistent with
12 the behavioral disinhibition previously reported in Lurcher mice, were confirmed by
13 the parameters regarding the distal segment of the open testing arm: the mutants
14 entered more often ($p < 0.01$) (fig. 4C) and spent more time ($p < 0.001$) (fig. 4E) in this
15 segment than their littermate controls. The disrupted aversion of the cerebellar
16 mutants for open spaces was particularly well illustrated by the fact they entered as
17 often in the testing arm ($p > 0.05$) (fig. 4B) and spent as much time in its distal
18 segment ($p > 0.05$) in OC as in CC (fig. 4E), contrary to wild type mice which entered
19 less this arm ($p < 0.0001$) (fig. 4B) and spent less time in its distal segment in OC
20 ($p < 0.0005$) (fig. 4E). In a more general way, the aversive configuration of our device
21 provoked a significant inhibition of locomotor activity only in wild type animals (control
22 mice: $p < 0.001$ / Lurcher mice: $p > 0.05$) strongly reflecting the impaired behavioral
23 reaction of the Lurcher mice to aversive environments (fig. 4A). Such an abnormal
24 reaction was also suggested by the higher proportion, albeit not significant ($p > 0.05$),
25 of mice performing some pSAPs (fig. 4G) in OC in the control group (8/12) than in the
26 Lurcher group (4/12). By contrast, the higher number of pHDs (fig. 4H) in the control
27 mice than in the mutant mice is only slight and very probably due to statistical noise
28 ($p > 0.05$). As expected, the number of rears (fig. 4F) were always significantly lower in
29 Lurcher mutants compared to control mice ($p < 0.05$ in OC and $p < 0.001$ in CC) and
30 decreased in both genotypes in open configuration (obviously due to the absence of
31 wall to support the rears; $p < 0.001$ for control mice and $p < 0.05$ for Lurcher mice).
32 Thus, in their all, the data we collected in the Dual Maze without prior familiarization
33 are consistent with those presented above regarding the BALB/c mice and those
34 previously obtained in the elevated plus-maze test with the Lurcher mutant mice.

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3.2.2. With familiarization

The familiarization considerably influenced the mice behaviors in the Dual Maze. While they still significantly avoided the open testing arm ($U=0$; $p<0.01$, compared to chance level for both genotypes), they displayed a clear preference for the closed testing arm where they spent about 75% of the total experiment time whatever their genotype (control mice: $U=91$; $p<0.01$ / Lurcher mice: $U=44$; $p<0.05$, compared to chance level) (fig. 4D). As a consequence, the control as well as the Lurcher mice spent about 8 times less time in the testing arm in OC than in CC ($p<0.0001$ for both genotypes) (fig. 4D). But the most relevant of our results was that the cerebellar mice were statistically indistinguishable from their littermate controls in terms of number of entries (fig. 4B) and time spent in the open testing arm (fig. 4D) and in its distal segment (fig. 4E) ($p>0.05$ for all the comparisons). These observations, contrasting with those made in unfamiliarized animals, suggested a kind of normalization of the anxiety-related behaviors in Lurcher mice by familiarization. Such an hypothesis was reinforced by the direct comparison of the data obtained with or without familiarization: while familiarized and unfamiliarized control mice did not behave differently in front of the open testing arm and its distal segment ($p>0.05$ for all comparisons), the 20-minutes period of familiarization was sufficient to lead to a drastic decrease in the number of entries and time spent in both areas (fig. 4B, 4C, 4D, 4E) in Lurcher mice (number of entries in the testing arm / its distal segment: $p=0.001$ / $p<0.0001$; time spent in the testing arm / its distal segment: $p<0.01$ / $p<0.001$). A hypothetical impairment of exploratory motivation could hardly account for this modification since the already evoked preference for the testing arm in CC had the same magnitude in mice of both genotypes ($p>0.05$ for the time spent in the closed testing arm between Lurcher and control mice). Furthermore, familiarization significantly decreased the number of segments crossed ($p<0.001$) (fig. 4A) and rears ($p<0.001$) (fig. 4F) in Lurcher mice only in OC ($p>0.05$ for all the comparisons in CC), suggesting an anxiety-induced inhibition of general activity. Although the mean number of pSAPs (fig. 4G) was not significantly changed by prior familiarization in Lurcher mutants ($p>0.05$), it is important to note that the proportion of the latter displaying this behavior in OC was much higher after familiarization than in the case of forced exposure without any prior familiarization (9/10 of the familiarized Lurcher

1 mice vs 4/12 of the unfamiliarized Lurcher mice). This tendency was also detectable
2 in control mice, but at a lesser extent (10/10 of the familiarized control mice vs 8/12 of
3 the unfamiliarized control mice). So, pSAPs, as well as the other variables
4 considered, indicated that 20-minutes familiarization prior testing mice in the aversive
5 version of the Dual Maze led to the convergence of the behavioral expression of
6 anxiety in Lurcher and control mice. The only exception to this picture is the number
7 of pHDs (fig. 4H) which is very higher in the mutants than in their control littermates
8 after familiarization ($p < 0.05$) while no significant difference was noticed in the
9 unfamiliarized animals ($p > 0.05$). The explanation for this exception is not obvious.
10 But we could hypothesize that the cerebellar deficiencies in Lurcher mice make the
11 information processing during their assessment of the height harder. This impairment
12 could be due to motor deficits (instability in head dipping and visual anchoring
13 resulting in interrupted information intake) as well as to specific cognitive difficulties.
14 Whatever the origin of the deficit, the mutants could have to repeat their scanning
15 behaviors to estimate the height of the open arm more often than control mice to
16 compensate it. Even so, the number of pHDs performed confirmed that the
17 familiarization to the experimental conditions radically changed the way the Lurcher
18 mice investigated the aversive environments (familiarized vs unfamiliarized mice:
19 Lurcher mice: $p < 0.05$ / control mice: $p > 0.05$).

20

21

22 4. DISCUSSION

23 In this work, we have 1) designed and tested an innovative apparatus to
24 investigate anxiety-related behavior, intrinsic exploratory behavior and the effect of
25 familiarization on them in mice, and 2) shown the impact of prior familiarization with
26 experimental apparatus on the behavioral disturbances expressed by cerebellar
27 Lurcher mutant mice.

28

29 4.1. Validation of apparatus and protocol

30 The types of behavior we took into consideration in the Dual Maze were
31 essentially the same as those measured in the elevated plus-maze test and are
32 thought to result from an approach/avoidance conflict [42]. Beyond this similarity, the

1 absence of a center in our device nevertheless eliminates an ambiguous area, often
2 discarded from the results analysis in the studies using the plus-maze [41, 45, 49]. It
3 permits to avoid any loss of information about the emotional status of animals or any
4 distortion in the appraisal of their real anxiety level.

5 The drop of horizontal and vertical exploratory activities observed in the open
6 configuration (figure 3) in both NaCl- and CDP-injected BALB/c mice obviously
7 resulted from their avoidance of the open testing arm (in which, moreover, they could
8 not lean on any wall). This avoidance was accompanied by numerous pSAPs from
9 the starting towards the testing arm, limiting the time the animals could allocate to
10 their locomotor movements. In closed configuration, pSAPs, which reflect the
11 approach-avoidance conflict evoked by an aversive space [50], were exceptional. As
12 expected, our results showed the open testing arm of our device was much more
13 aversive for mice than the closed one.

14 The profound modifications of mouse behaviors caused by CDP in the open
15 configuration of the apparatus further demonstrated the latter was particularly
16 valuable to measure anxiety level and detect anxiolysis. Thus, the drug specifically
17 elevated the time spent in the open testing arm (as denoted by the significant
18 configuration x treatment interaction). The time spent in the distal segment of this arm
19 seemed an especially reliable anxiety-related variable since CDP significantly
20 increased it. Although the lack of significant configuration x treatment interaction did
21 not permit us to statistically test it using post-hoc comparisons, it should be noted the
22 anxiolysis was also reflected by the dramatic increase in the number of entries in the
23 open testing arm as a whole and in its distal segment (+85% and +261%
24 respectively). The almost complete elimination of the pSAPs provoked by CDP
25 administration confirmed the open version of the Dual Maze was highly sensitive in
26 determining the level of anxiety in mice.

27 In the closed configuration, whatever the treatment administered, animals
28 exhibited a clear preference for the testing arm as indicated by the time they spent
29 (two thirds of the total time of the experiment) and the number of rears they achieved
30 into it (70% of the total number of rears). Such a preference for novelty has already
31 been observed in mice (especially in Swiss strain mice) which could freely explore an
32 environment after having been familiarized with a starting area [51]. In our
33 experimental conditions, 20 minutes of familiarization to a starting box was enough

1 for animals to mark a preference for an unfamiliar space. The procedure and the
2 device used in this study did not allow us to notice any significant influence of CDP
3 on the spontaneous exploratory activity of the mice suggesting that sedative effect of
4 CDP can be excluded in this study. To detect anxiolytic effects of a pharmacological
5 compound, it is needed that mice exhibit a sufficient baseline level of anxiety [8, 46].
6 So, the lack of behavioral modifications in BALB/c mice injected with CDP in the
7 closed configuration, confirmed that the latter induced only negligible anxiety in
8 animals.

9 Taken as a whole, the results we obtained in the BALB/c mice strongly
10 suggested that the Dual maze test was efficient at assessing the anxiety level of mice
11 in a clear-cut way (open configuration) and at specifically measuring their intrinsic
12 motivation to explore environment (closed configuration). A comprehensive
13 pharmacological study designed to test its bidirectional sensitivity (with anxiogenic
14 and anxiolytic drugs) in several strains of mice with more animals per group is
15 certainly needed to add value to it as an anxiety test. Nevertheless, our data are
16 sufficient to reasonably interpret the behaviors of Lurcher mice in the Dual maze in
17 terms of disinhibition. It is worthy to note that all the variables of anxiety we reported
18 here are influenced by CDP in the expected direction. In addition, the effect size, in
19 most cases, is particularly strong even when differences were not found significant
20 due to our limited sample sizes (that is the case for the number of entries in the
21 whole testing arm and in its distal part and for SAPs. The effect for pHD is a little bit
22 less strong). It is why we decided to make use of all the parameters measured in the
23 part of our work dedicated to the Lurcher mice.

24

25 **4.2. Examination of the Lurcher mutant mice**

26 The Lurcher mice display a very particular behavioral profile in experimental
27 devices, which can lead to bias and misinterpretation of classical test results. This
28 profile was proposed to be described by the concept of behavioral disinhibition. It
29 was defined as the paradoxical combination of inappropriate, risky behaviors with an
30 abnormal physiological reactivity to stressors [19, 34-37]. For instance, Lurcher mice
31 were consistently found to visit much more the aversive open arms of the elevated
32 plus-maze than their littermate controls and to realize fewer behaviors evocative of
33 anxiety-related inhibition like pSAPs, although being more physiologically stressed

1 [34, 38]. Such a disinhibition probably affects behaviors of the mutants in a wide
2 range of tests. So, their lack of immobility responses in the forced swimming test
3 could be a consequence of the impairment of the inhibition system rather than an
4 evidence of resistance to resignation [19, 52]. In the Morris water maze test, the
5 abnormally high levels of stress hormones reported in Lurcher mice [37] could denote
6 the disinhibition to which they are usually associated to. This disinhibition would
7 probably interfere with the selection of the most efficient strategy to complete the task
8 and could contribute to the poor performances of the mutants.

9 Whether it be the Lurcher or control mice, they all behave very differently in
10 the CC and OC of the Dual Maze when tested without any prior familiarization.
11 Nevertheless, the precise effect of the change of configuration depended on the
12 genotype of animals. In the CC, the Lurcher mice were distinguishable from their
13 littermate controls only by their lowest locomotor and rearing activity, likely due to
14 their motor deficits. On the other hand, the animals of both genotypes spent
15 equivalent time in the starting and closed testing arm, showing the device as a whole
16 appeared to them as a new, homogeneous space. The physical characteristic of the
17 testing arm in the OC deeply changed the exploratory strategy in control mice. The
18 sharp reduction of their locomotor activity coincides with a strong expression of
19 pSAPs, indicating the aversive valence of the open testing arm. Consistently, they
20 entered much less the latter and its distal segment than the control mice tested in the
21 CC. The same kind of changes were noticeable in the Lurcher mutants but at a much
22 lesser extent. Their preference for the starting arm in the OC clearly showed they
23 perceived the open arm as aversive. Nevertheless, compared to the control mice,
24 they exhibited reinforcement of the approach tendency (and / or attenuation of the
25 avoidance tendency) relative to the aversive area of the device as previously
26 observed in the plus-maze apparatus. This reinforcement (and / or this attenuation)
27 together with the fact that mutants very rarely used stretched postures, illustrate their
28 behavioral disinhibition in tests involving a forced confrontation to a novel
29 environment. Thus, in their all, the data we collected with the Lurcher mutant mice in
30 the Dual Maze without familiarization were in line with those previously obtained in
31 the elevated plus-maze test.

32

33

4.3. Effects of familiarization on the behavioral disinhibition of the Lurcher mice

In this study, we hypothesized that familiarization to the starting arm of the apparatus prior to testing phase could lower the novelty-induced stress and reduce the associated behavioral modifications in the animals. Although paradoxical behaviors caused by forcible exposure to novelty were observed in early periods of testing even in mice not suffering from any pathological disinhibition [16], it was expected the familiarization effects would be particularly strong in Lurcher mice.

20 minutes of familiarization in the starting arm were sufficient to strongly modify exploratory activity of mice of both genotypes into the Dual Maze in CC. Contrary to what observed without any prior familiarization, mutants and non-mutants showed a marked preference for the testing arm: they spent more than 2/3 of the total experiment time into it and reared much more in this area of the apparatus than in the starting arm. If such a preference was expected considering the data of the literature about the free exploration paradigm [51, 53], it is of particular interest regarding the Lurcher mutants. It has been previously shown that their exploratory activity was impaired in the hole board test [39, 54]. According to Caston and his collaborators [39], this deficit would primarily result from the alteration of the motivation to explore. Contrasting with these previous results, our data indicated that exploratory behavior of the Lurcher mutant mice was similar to that of their littermate controls. Such discrepancy could be explained by the simpler structure of the Dual Maze when compared to the hole-board apparatus. Consequently, the exploration of the latter could be more demanding and more sensitive to the visuospatial disturbances of the mutants [55, 56]. Be that as it may, our results strongly suggested there was actually not any significant impairment of the intrinsic exploratory motivation in the Lurcher mice.

As it was the case without any prior familiarization, open testing arm evoked passive avoidance behavior in all the familiarized mice, whatever their genotype. Nevertheless, the effect of familiarization in this configuration was very different in control and Lurcher mice. While this effect was moderate in the former, it led to a large reinforcement of the approach-avoidance conflict in the latter, as indicated by the (although non-significant) increase in the number of pSAPs they achieved. In addition, exploration of the open arm showed the resolution of the conflict resulted in

1 similar avoidance in both control and Lurcher mice. Actually, our results strongly
2 suggested that familiarization to the starting arm of the Dual Maze restored the
3 inhibition process in the mutants. The main consequence of familiarization is
4 probably the decline of the novelty-induced stress. Thus, it is reasonable to postulate
5 that the behavioral disinhibition only observed in non-familiarized Lurcher mice is
6 triggered by their intense stress in the experimental situation (as in our device without
7 familiarization or the elevated plus-maze in its classical version).

8 This means, *in fine*, that the cerebellum as a whole, and the cerebellar cortex
9 in particular, could participate in the modulation of coping strategies adopted by
10 animals confronted to a stressful experience. The multiple reciprocal connections the
11 cerebellum has with some limbic structures involved in the behavioral inhibition [57,
12 58], such as the septo-hippocampal system [59, 60], together with those it has with
13 the central noradrenergic [61-63] and serotonergic circuits [63-66] represent
14 candidates of choice as neurobiological substrates for such a function.

15 Using the Dual maze, an innovative device with face and predictive validity, we
16 could compare the impact of prior familiarization to the experimental context on both
17 anxiety-related behaviors and pure exploratory motivation in Lurcher mutant mice
18 and littermate controls. In its all, our experiment showed the behavioral disinhibition
19 of the Lurcher mice in a novel environment they were forcibly exposed to was
20 abolished by prior familiarization to the same environment. Such results support the
21 hypothesis that the cerebellum is involved in novelty-induced stress response and
22 contributes to the regulation of associated coping strategies. Beyond the
23 interpretation we proposed about the cause of the Lurcher disinhibition, our work
24 more generally underlines the fact that performance of laboratory mice, particularly
25 mice with abnormal stress reactivity, is a product of complex factors that must be
26 included in the interpretation of measured data in a given task.

27

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45

1 **Figure legends**

2 **Fig. 1**

3 Pictures of the Dual Maze: Picture A: open configuration / Picture B: closed
4 configuration. The floor of the arms was covered with sawdust. Each arm was
5 symbolically subdivided into distal and proximal segments of same dimensions
6 measuring 10 cm each, delimited here with dotted line. The closed arms were
7 covered with transparent plastic plates with ventilation holes. The open arm was
8 surrounded by 3 mm high plastic rods.

9

10 **Fig. 2**

11 Scheme of the experiment 1 and experiment 2: experimental groups and number of
12 mice per group (n) for BALB/c mice treated with chlordiazepoxide (CDP) or vehicle
13 tested in open or closed configuration, and for B6CBA Lurcher mutant (+/Lc) and wild
14 type (+/+) mice tested in open or closed configuration either with 20 min
15 familiarization (F) or with no familiarization (No F).

16

17 **Fig. 3**

18 Total number of crossed segments (A), number of testing arm entries (B), number of
19 entries into the distal segment of the testing arm (C), relative time in the testing arm
20 (D), relative time in the distal segment of the testing arm (E), number of rearings (F),
21 number of protected SAPs (stretched attend postures) in the open configuration (G),
22 number of protected head dips in the open configuration (H) in chlordiazepoxide- and
23 saline-treated BALB/c mice. Data are shown as means; error bars represent standard
24 error of the mean. Horizontal bars connect groups between which statistically
25 significant differences were found. For P values, see text.

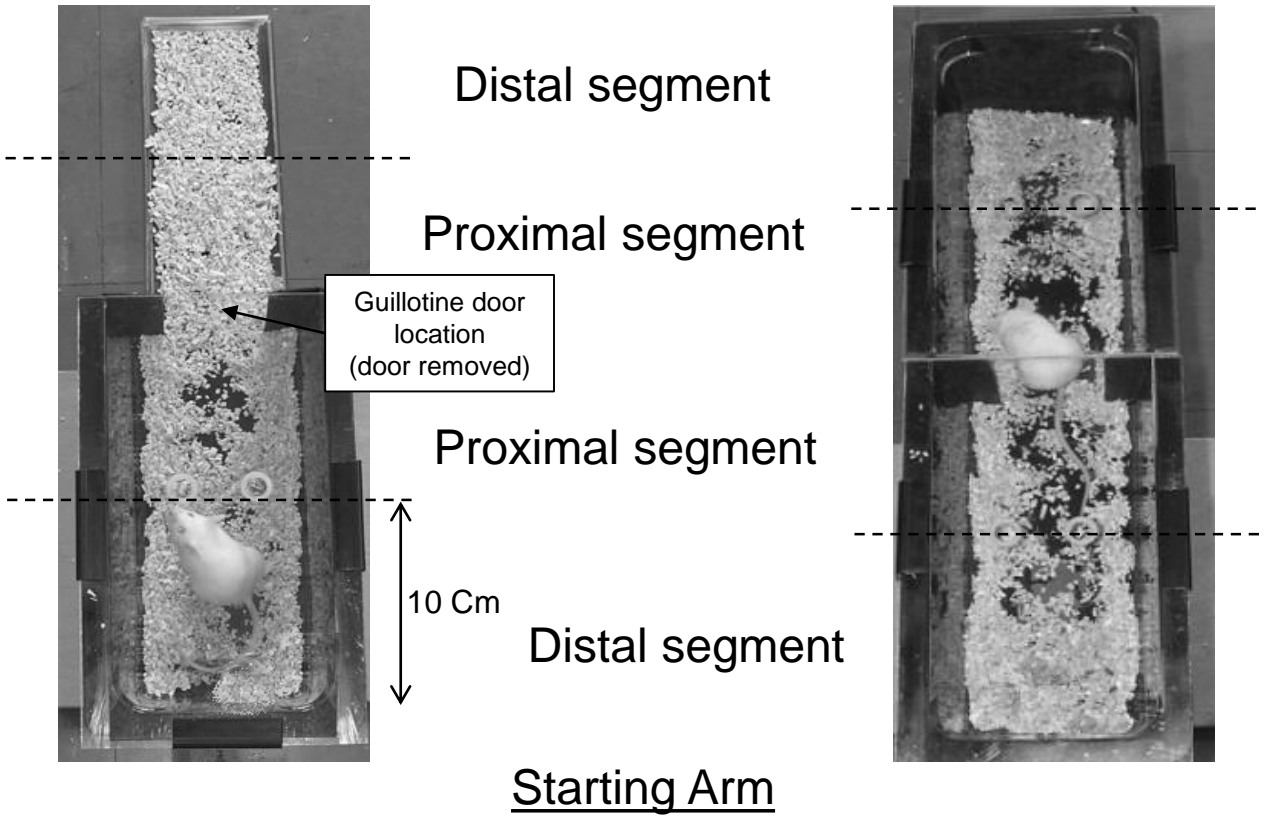
26

27 **Fig. 4**

28 Total number of crossed segments (A), number of testing arm entries (B), number of
29 entries into the distal segment of the testing arm (C), relative time in the testing arm
30 (D), relative time in the distal segment of the testing arm (E), number of rearings (F),

1 number of protected SAPs (stretched attend postures) in the open configuration (G),
2 number of protected head dips in the open configuration (H) in Lurcher mutant and
3 wild type B6CBA mice. Data are shown as means; error bars represent standard
4 error of the mean. Horizontal bars connect groups between which statistically
5 significant differences were found. For P values, see tables 2-5

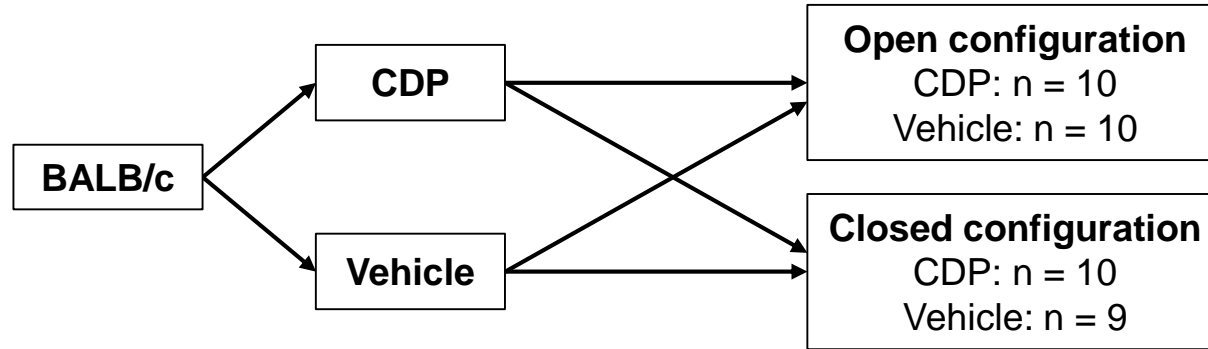
Testing Arm



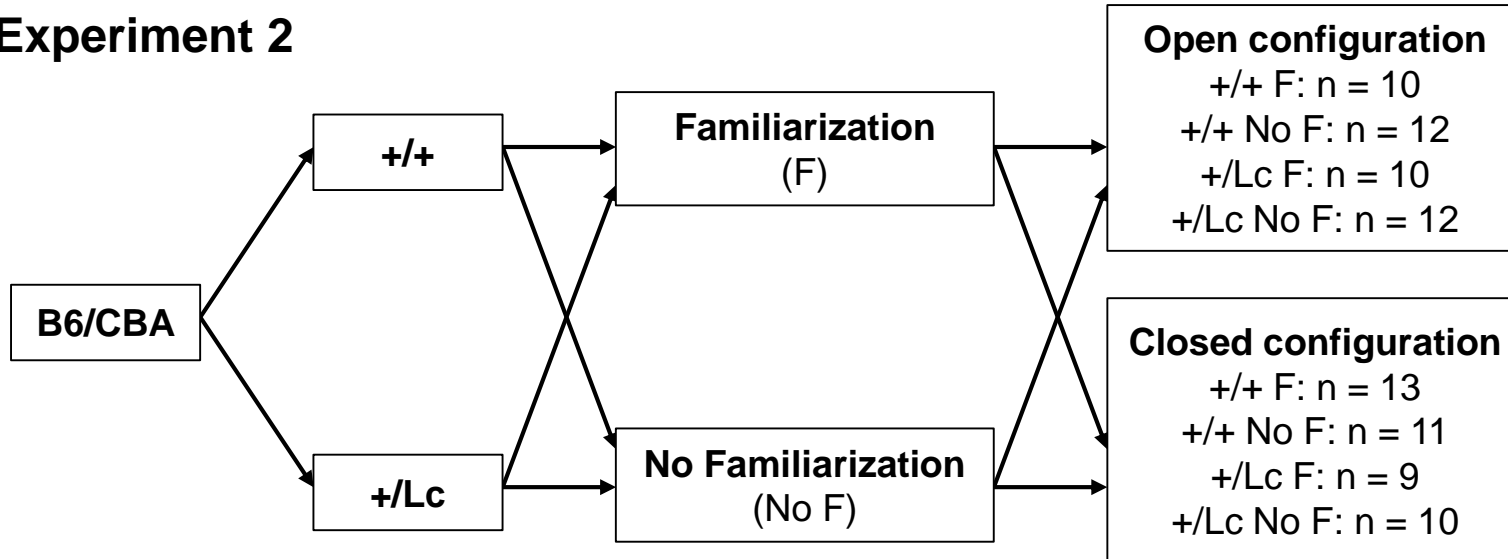
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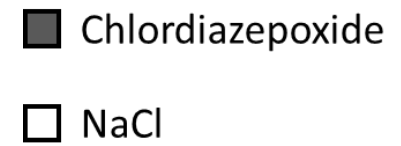
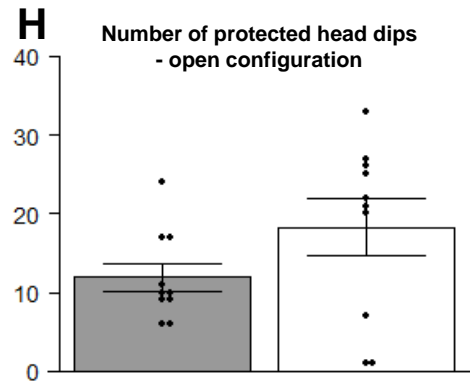
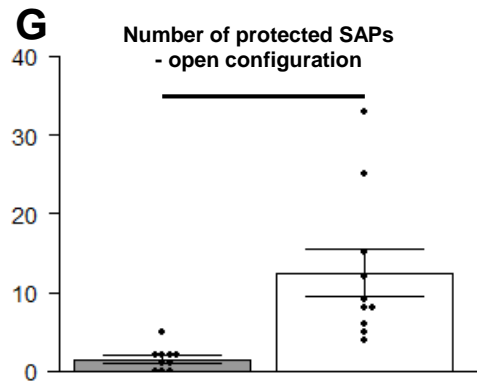
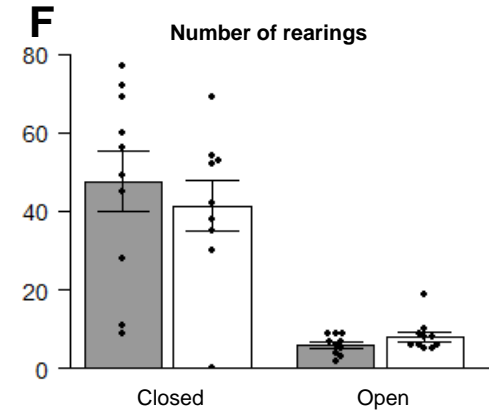
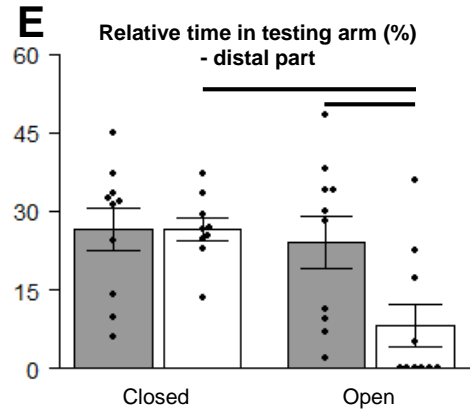
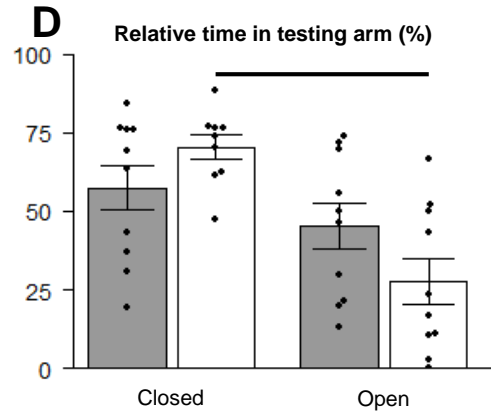
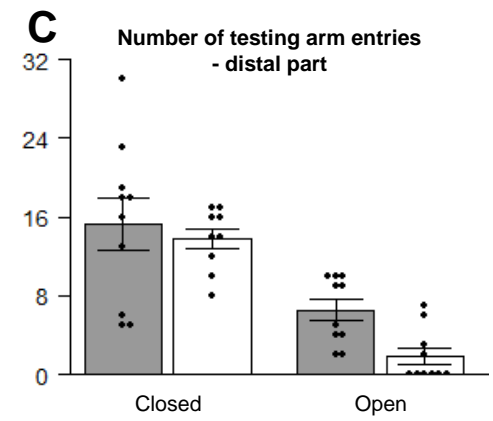
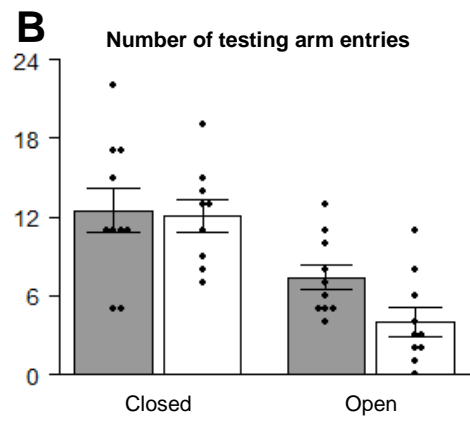
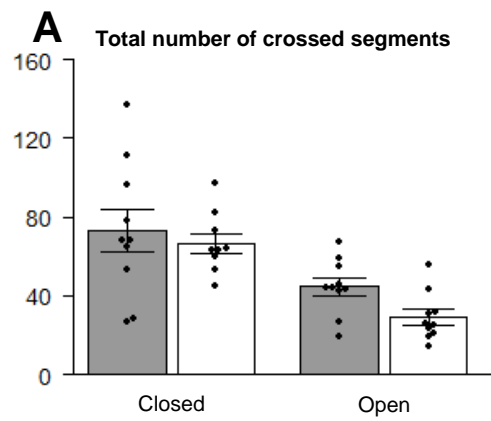
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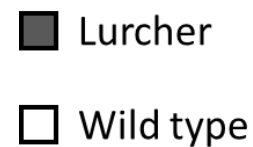
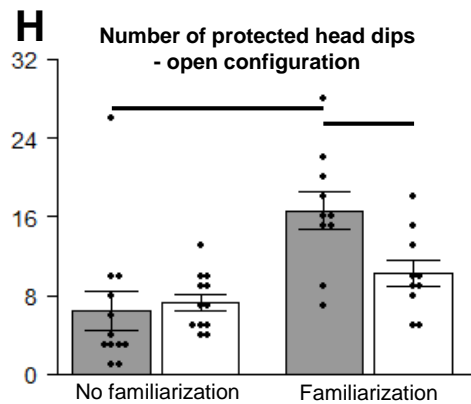
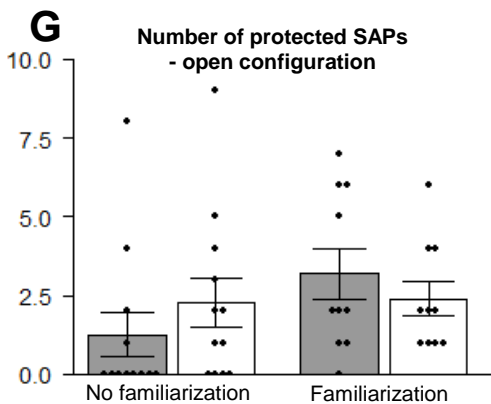
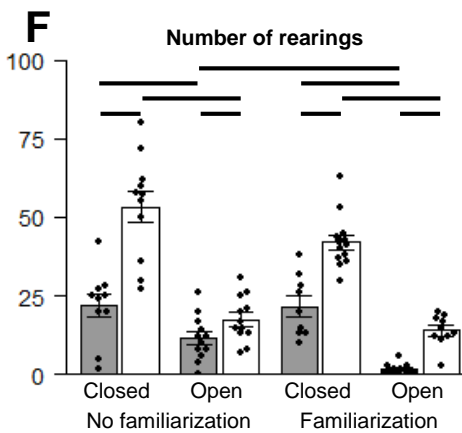
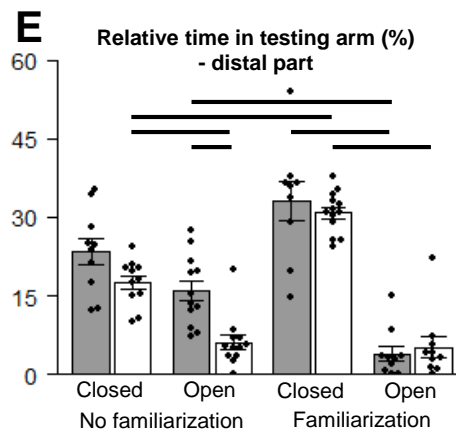
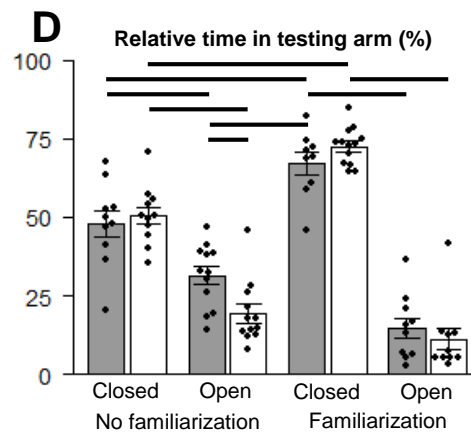
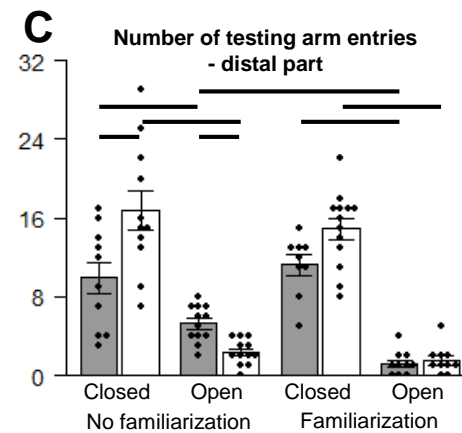
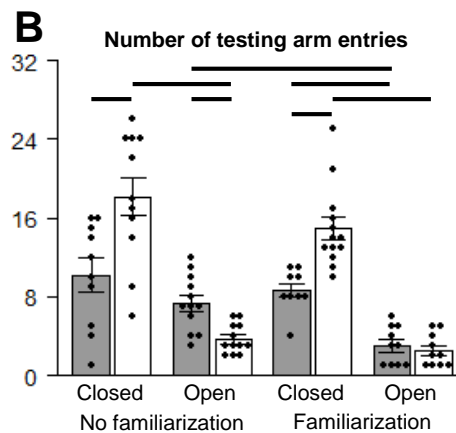
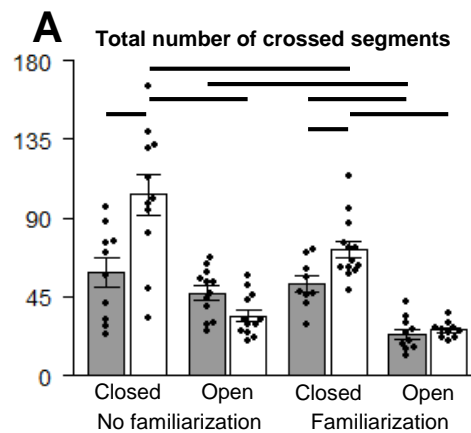
Experiment 1



Experiment 2







Parameter	Configuration effect		Treatment effect		Configuration by treatment interaction	
	$F_{(1,35)}$	P	$F_{(1,35)}$	P	$F_{(1,35)}$	P
Number of crossed segments	23.5	< 0.001	2.61	NS	0.45	NS
Number of entries into the testing arm	26.54	< 0.001	2.18	NS	1.38	NS
Number of entries into the distal part of the testing arm	42.75	< 0.001	3.83	NS	1	NS
Relative time in testing arm	16.65	< 0.001	0.12	NS	5.09	< 0.05
Relative time in the distal part of the testing arm	6.78	< 0.05	3.95	NS	4.12	< 0.05
Number of rearings	55.03	< 0.001	0.16	NS	0.67	NS
Number of pSAPs	-	-	Z = -2.83	< 0.001	-	-
Number of pHD	-	-	Z = -1,53	NS	-	-

Tab.1: Statistical significance of the effects of configuration (open/closed), treatment (chlordiazepoxide/saline) and their interaction on parameters measured in BALB/c mice in the Dual Maze. NS = statistically non-significant, - = not applicable (measured only in open configuration).

Relative time in testing arm ($\chi^2 = 69.822$; $p < 0.0001$)									
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F	
Lc OC F				NS		Z = 3.8228 P < 0.0001	Z = 2.8805 P < 0.01		Lc OC F
Lc OC N			Z = -2.463 P < 0.05		Z = 2.5616 P < 0.05			Z = 3.2984 P < 0.0005	Lc OC N
Lc CC F		NS			Z = -2.5767 P < 0.01			Z = 3.5569 P < 0.0005	Lc CC F
Lc CC N	NS					NS	NS		Lc CC N
WT OC F		Z = 4.4305 P < 0.0001	NS					NS	WT OC F
WT OC N	Z = 3.9414 P < 0.0001			NS			Z = -3.1121 P < 0.001		WT OC N
WT CC F	Z = -3.8985 P < 0.0001			Z = 4.2856 P < 0.0001		NS			WT CC F
WT CC N		Z = -4.0715 P < 0.0001	Z = 3.6065 P < 0.0005		NS				WT CC N
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F	
Relative time in the distal segment of the testing arm ($\chi^2 = 64.117$; $p < 0.0001$)									

Table 2

Statistical significance of differences in relative time spent in the testing arm (grey fields) and in relative time spent in the distal part of the testing arm (white fields) between individual experimental groups of mice (post hoc tests – planned comparison). χ^2 and p-value of the Kruskal-Wallis analysis is shown next to the parameter title.

WT = wild type mice, Lc = Lurcher mice, CC = closed configuration, OC = open configuration, F = with familiarization, N = no familiarization, NS = statistically non-significant

Number of entries into the testing arm ($\chi^2 = 61.336$; $p < 0.0001$)									
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F	
Lc OC F				NS		Z=3.7408 P < 0.0005	Z=3.1209 P < 0.005		Lc OC F
Lc OC N			Z = -2.932 P < 0.005		NS			Z = 3.5696 P < 0.001	Lc OC N
Lc CC F		Z = 3.0222 P < 0.0005			NS			Z = 3.8343 P < 0.0005	Lc CC F
Lc CC N	Z = 2.4306 P < 0.05						Z = 2.3688 P < 0.05		Lc CC N
WT OC F		Z = 4.1089 P < 0.0001	NS					NS	WT OC F
WT OC N	Z = 3.8794 P < 0.0001			NS			Z = -3.1762 P < 0.005		WT OC N
WT CC F	NS			Z = 4.2090 P < 0.0001		NS			WT CC F
WT CC N		NS	Z = 3.9164 P < 0.0001		Z = 2.2594 P < 0.05				WT CC N
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F	
Number of entries into the distal segment of the testing arm ($\chi^2 = 68.235$; $p < 0.0001$)									

Table 3

Statistical significance of differences in number of entries into the testing arm (grey fields) and in number of entries into the distal part of the testing arm (white fields) between individual experimental groups of mice (post hoc tests – planned comparison). χ^2 and p-value of the Kruskal-Wallis analysis is shown next to the parameter title.

WT = wild type mice, Lc = Lurcher mice, CC = closed configuration, OC = open configuration, F = with familiarization, N = no familiarization, NS = statistically non-significant

Number of crossed segments ($\chi^2 = 55.79$; $p < 0.0001$)								
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F
Lc OC F				NS		Z = 3.5420 P < 0.0005	Z = 3.266 P < 0.005	
Lc OC N			NS		NS			Z = 3.0463 P < 0.001
Lc CC F		Z = 2.2923 P < 0.05			NS			Z = 3.3929 P < 0.0001
Lc CC N	Z = 2.4826 P < 0.05					NS	Z = 2.1920 P < 0.05	
WT OC F		Z = 4.0010 P < 0.0001	NS					Z = 3.6521 P < 0.0001
WT OC N	Z = 3.6249 P < 0.0005			NS			Z = 2.3444 P < 0.05	
WT CC F	Z = 2.2772 P < 0.05			Z = 4.1492 P < 0.0001		Z = 3.4174 P < 0.0005		
WT CC N		NS	Z = 3.817 P < 0.0001		Z = 3.2867 P < 0.0005			
	WT CC N	WT CC F	WT OC N	WT OC F	Lc CC N	Lc CC F	Lc OC N	Lc OC F

Number of rearings ($\chi^2 = 64.481$; $p < 0.0001$)

Table 4

Statistical significance of differences in number of crossed segments (grey fields) and in number of rearings (white fields) between individual experimental groups of mice (post hoc tests – planned comparison). χ^2 and p-value of the Kruskal-Wallis analysis is shown next to the parameter title.

WT = wild type mice, Lc = Lurcher mice, CC = closed configuration, OC = open configuration, F = with familiarization, N = no familiarization, NS = statistically non-significant

Number of protected SAPs ($\chi^2 = 39.862$; $p < 0.0001$)					
	WT OC N	WT OC F	Lc OC N	Lc OC F	
Lc OC F		NS	NS		Lc OC F
Lc OC N	NS			$Z = -2.8272$ $P < 0.05$	Lc OC N
WT OC F	NS			$Z = -2.3059$ $P < 0.05$	WT OC F
WT OC N		NS	NS		WT OC N
	WT OC N	WT OC F	Lc OC N	Lc OC F	
Number of protected head dips ($\chi^2 = 16.83$; $p < 0.0001$)					

Table 5

Statistical significance of differences in number of protected SAPs (stretched attend postures; grey fields) and in number of protected head dips (white fields) between individual experimental groups of mice (post hoc tests – planned comparison). χ^2 and p-value of the Kruskal-Wallis analysis is shown next to the parameter title.

WT = wild type mice, Lc = Lurcher mice, OC = open configuration, F = with familiarization, N = no familiarization, NS = statistically non-significant