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Gravity change differentially affects male and female cognition and anxiety-related parameters in mice

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Abstract

Variations in gravity affect multiple physiological and cognitive functions, therefore understanding how the body adapts to such changes is crucial, notably for space exploration. Astronauts exposed to altered gravity experience disturbances in sensory-motor functions, which are partly linked to vestibular system adaptations. Cognition and emotional regulation have been less studied and represent one of the most important challenges for long-term space missions. In this study, we examined the impact of exposure to 2G for 24 hours, 48 hours, or 15 days on locomotor activity, circadian rhythms, anxiety, blood corticosterone, sociability, short (spontaneous alternation) and long-term (object location memory OLM) memories in adult male and female C57BL/6JRj mice.

While horizontal locomotion remains unchanged, vertical activity strongly decreases after short duration exposure (24h and 48h), but less after a longer duration (15d). Furthermore, exposure to 2G affects circadian rhythm synchronization during the first 24h after return to 1G, with males showing a time lag in activity cycles after 24h (peak activity occurs 5 hours later than in control mice) and females demonstrating a more rapid adaptation. Anxiety-like behavior increases for short duration exposure (24h) particularly in females, while blood corticosterone concentrations remain unchanged in all groups. Besides, sociability is affected in both sexes only after 48h of exposure. Short-term memory is altered after 24h exposure, but only in females whereas long-term memory is impaired in both sexes whatever the duration of 2G exposure.

These findings provide new insights in the effects of HG exposure and its duration on behavioral abilities by 2G, with a particular sensitivity of recognition memory with a spatial component. They also highlight the importance of considering sex differences in gravitational adaptation and underline the need for targeted interventions to mitigate cognitive and physiological challenges for future space travels.

INTRODUCTION

Environmental factors exert a substantial influence on physiological and behavioral responses, thus impacting a wide range of biological functions, from fundamental homeostasis to complex cognitive processes[1,2]. In the context of space exploration, environmental modifications are multiple, and uncertainties persist regarding the potential health impact of changes in gravity, prolonged confinement, isolation, space radiation, and increased bacterial virulence[3,4].

Among these different stressors, gravity variations have attracted increasing interest in recent decades due to the necessity for adequate adaptation to long spatial missions[5]. Indeed, astronauts are subjected to significant and prolonged gravitational shifts during space travel, including alternating phases of microgravity (MG) and hypergravity (HG), which could affect not only motor and sensory control but also possibly cognitive performances[6–9]. Indeed, the vestibular system which is directly influenced by gravity, not only plays a central role in postural control, head position, movement perception, and gaze stability control but is also connected to brain structures involved in locomotion, emotional and cognitive

processing, such as cerebellum, amygdala, hippocampus and prefrontal cortex[10]. Disruptions of vestibular information due to gravitational changes could therefore have wide reaching consequences, such as altered locomotion, stress responses but also cognitive impairments[11]. However, when cognitive functions are essential in astronaut daily activities, how they are impacted by gravitational shifts still remains poorly documented, even in animal researches[12]. In fact, most information available so far are provided by studies using centrifugation-related strategies, enabling to investigate the effects of increased gravity on brain function and behavior[13]. Accordingly, it has been shown that HG exposure induces modifications in stress hormone levels, altered locomotor activity, and cognitive impairments in rodents[14–16]. In particular, learning and spatial memory is altered in spatial memory tasks involving object place recognition or orientation in mice following short exposure (1h per day during 5 days) and in rats following 15 days to 2G[17,18].

However, according to species, exposure duration, and timing of behavioral assessments, contradictory results are observed, highlighting the need for further researches. In addition, a difference between males and females has not yet been deeply considered, only one study showing sex-dependent reactivity in terms of cognitive performances after 2G exposure, with females showing better spatial flexibility than males[19].However this point is of importance if we refer to studies on astronauts showing that physiological and behavioral effects of spaceflight related to altered gravity may differ between men and women[20–24].

In order to address these different gaps, the present study aimed to characterize adaptations of physiological, emotional, and cognitive functions in male and female C57BL/6JRj mice in response to increasing durations of 2G exposure (24 hours, 48 hours, and 15 days). By examining multiple functional domains, this research seeks to provide a more comprehensive understanding of the biological consequences and the potential sex-specific reactivity to a moderate increase in gravity.

RESULTS

Spontaneous activity

Global analysis over 24 hours (through the number of crossed infrared beams) revealed a significant effect of 2G exposure on mice's vertical but not horizontal activity (see **Fig S.1**). Also analyses by 30mins increments confirmed that the horizontal activity profile which is similar in males and females, was not affected by 2G exposure regardless its duration during the following 24 hours after exit from the centrifuge *(*Fig. 1.**A and B***)*. Contrarily, the vertical activity was strongly reduced in both males and females, particularly for the two shortest durations of HG exposure. Indeed, male mice of the 24h and 48h groups performed fewer rearing than control mice, but this was not observed in 15 days exposed group (*ATS, p = 0.003, ctrl vs 24h p = 0.04, ctrl vs 48h p = 0.0001*) (Fig. 1.**C and D**). Similar results were found in females (*ATS, p = 0.0003, ctrl vs 24h p = 0.007, ctrl vs 48h p = 0.0001)*. It is worth notice that in females, the 15-day group not significantly different from the control one, was however significantly

elevated compared to the 24-hour and 48-hour groups (*ATS, 15d vs 24h p = 0.001, 15d vs 48h p = 0.0004*) suggesting some level of adaptation.

Based on cosinor analysis of vertical activity, the mesor, which reflects total activity over 24h, was significantly reduced in males after 24h and 48h of HG compared to the control group (*ANOVA, p = 0.0004, F3, 24 = 8.79, ctrl vs 24h p = 0.01, ctrl vs 48h p = 0.001*) **(**Fig. 2.**A)**. This hypoactivity is a direct consequence of the reduced amplitude of the numbers of rearing in both exposed groups, given that the amplitude is the difference between the maximum activity and the mesor (*ANOVA, p = 0.001, F3, 24 = 7.15, ctrl vs 24h p = 0.003, ctrl vs 48h p = 0.0004*) **(**Fig. 2.**B)**. This difference in mesor between the control group and the 24h and 48h group was also found in females (*Kruskal-Wallis, p = 0.0002, ctrl vs 24h p = 0.003, ctrl vs 48h p = 0.0004*) **(**Fig. 2.**C)**. Again, this vertical hypoactivity is indicated by the reduced amplitude of the numbers of rearing (*ANOVA, p = 0.0001, F3, 24 = 10.50, ctrl vs 24h p = 0.002, ctrl vs 48h p = 0.0004*) **(**Fig. 2.**D)**. Concerning the acrophase, which corresponds to the peak of rearing activity over 24h, the peak of vertical activity after only 24h of exposure to HG occurred in male mice on average 5h after that of control mice (*ANOVA, p = 0.0001, F3, 24 = 14.56, ctrl vs 24h p = 0.0001*) **(**Fig. 2.**E) while** no difference was found in females **(**Fig. 2.**F)**.

These results therefore indicate that exposure to different durations of 2G does not modify the horizontal displacements, but strongly affect circadian rhythm and the vertical component of the locomotor behavior (rearing). These effects are particularly observed in mice exposed to the shortest HG duration (24h and 48h) and recovered after 15 days of exposure.

Anxiety-like behavior

The latency of first entry into the dark box was not significantly different between regardless the sex or time of 2G exposure (data not illustrated).

In males, there was a global significant difference between groups in the percentage of time spent in the light box (Fig. 3.**A**) (*ANOVA, p = 0.022, F3, 36 = 3.59)*. However, multiple comparisons between the different 2G and the control groups did not reach any significant level. The number of transitions between the two compartments follows the same trend, with a global difference in the *non-parametric ANOVA* (*Krusal-Wallis test, p = 0.0021*) but no significance following multiple comparisons (Fig. 3.**B**).

In females, there was also a global significant difference between groups in the percentage of time spent in the light box (*Krusal-Wallis test, p = 0.0017*). Multiple comparisons revealed that the 24h exposed group spent significantly less time than the control group (*Kruskal-Wallis, p = 0.001, ctrl vs 24h p = 0.01*) (Fig. 4.**C**). A similar trend was observed for the number of transitions between the two compartments (Fig. 3.**D**), although the difference did not reach statistical significance (*ANOVA, p = 0.06*).

Plasma Corticosterone

Immediately after the 2G exposure, there was no significant difference in plasma corticosterone concentrations between groups in both sexes, even some trend to increase was found in female mice (*Krusal-Wallis test, p = 0,09)* (Fig. 3.**E and F**).

Short term memory

The number of arms entries in the Y-maze was not significantly different in either males or females, attesting for a similar level of exploration during the test (data not shown). In males, the alternation percentage of each group was significantly above the theoretical value of 50% (*t.test: ctrl p = 0.0003, 24h p = 0.006, 48h p = 0.001, 15d p = 0.001*) while a comparison between the different experimental groups did not reveal statistical differences (Fig. 4.**A)**. These results indicate that short-term memory is not impaired by 2G in male mice, regardless the duration of exposure.

In females, although there was no inter-group differences in spontaneous alternation percentages as in males (Fig. 4.**B**), comparison to 50% showed that when control, 24h and 15d exposed mice had robust performances (*t.test: ctrl p = 0.0002, 24h p = 0.02, 15d p = 0.01*), the percentage of the group exposed for 48h was not statistically different from the theoretical value of 50% (57 ± 4.9%, *t.test: p = 0.1*), indicating that short term memory is specifically impaired for this level of 2G exposure.

Place recognition

An overall analysis revealed significant differences between groups in male mice (Fig. 4.**C**). The 48h and 15d exposed mice exhibited a significant decrease in the discrimination index compared to the control group (*ANOVA, p = 0.03, F3, 33 = 3.11, ctrl vs 48h p = 0.04, ctrl vs 15d p = 0.03*). Additionally, only the 15d group did not differ to the theoretical value of 0 (*t.test: p = 0.06*) contrary to the other groups (*t.test: ctrl p = 0.0009, 24h p = 0.03, 48h p = 0.02*). These data suggest long-term spatial memory deficits that are related to the duration of 2G exposure, with the earliest manifestation occurring after 48h and increasing with longer exposition.

A similar pattern but even more severe was observed in female mice since a pronounced deficit was found already after 24h exposure (Fig. 4.**D**). *ANOVA* revealed that each group subjected to 2G exhibited a significant decrease in alternation percentage compared to the control group (*ANOVA, p = 0.0008, F3, 29 = 7.33, ctrl vs 24h p = 0.002, ctrl vs 48h p = 0.001, ctrl vs 15d p = 0.002*). Moreover, only the control group displayed a percentage statistically above the theoretical value of 0 (*t.test: ctrl p = 0.0003, 24h p = 0.9, 48h p = 0.5, 15d p = 0.9*). These results indicate a rapid and consistent impairment of long-term spatial memory in females induced by the 2G exposure.

Social behavior

Firstly, it appeared that the distance travelled in the device by the mice was not different between the groups, neither in males nor in females. Concerning the sociability index, *ANOVA* showed differences

between groups in males. Indeed, multiple comparisons revealed a significant difference between the 15d group and the control group, with the 15d group exhibiting a higher index value (*ANOVA, p = 0.005, F3, 36 = 4.91, ctrl vs 15d p = 0.03*) (Fig. 4.**E**). Comparison of the performance of each group to the value of 50% indicated that only the 48h exposed group was not significantly different (*t.test: ctrl p = 0.006, 24h p = 0.001, 48h p = 0.1, 15d p = 0.0001*) suggesting sociability deficits specifically associated with this amount of 2G exposure.

Also in female mice, there was no global statistical differences between the groups (*ANOVA*) (Fig. 4.**F**) and similarly to males, only the 48h exposed group showed deficits in sociability attested by an absence difference of its sociability index to 50% (*t.test: ctrl p = 0.02, 24h p = 0.005, 48h p = 0.09, 15d p = 0.01).*

DISCUSSION

This study aimed to explore the impact of prolonged exposures to 2G gravity on physiological, emotional, and cognitive abilities with a special emphasis on possible differences between male and female mice. The results show that after return to 1G, specific behaviors are differentially altered depending on time exposure but also on the gender of the animals. Thus, vertical activity is strongly decreased particularly in females whereas horizontal spontaneous activity is not modified, indicating sex and specific effects on the integration of the direction of gravity by the brain. Additionally, certain functions such as sociability, anxiety, and short-term memory are also altered in a sex-dependent manner, but only for short periods of exposure to 2G. In contrast, long-term memory is affected not only by short but also prolonged time exposure, suggesting a long-lasting sensitivity of this cognitive function to a moderate change in gravity. Intriguingly, it is worth notice that most changes we observed in the present study with 2G delivery in mice resemble to those described after 30 days spaceflight exposure to microgravity in the same species[12].

Our findings therefore provide new insights into sex-related differences in adaptation to prolonged gravitational changes, highlighting specific physiological and cognitive adjustment dynamics.

Locomotion/spontaneous activity

A first observation derived from either the actimeter or other behavioral tests, is that regardless the mice gender, no change in spontaneous horizontal activity occur after stopping the 2G exposure, as previously reported in mice in an endurance test on a treadmill after 21 days also at 2G[16]. Interestingly, this is in contrast with the lower spontaneous activity reported after very short application (1h or 2h) of the same level of HG[19,25]. It is acknowledged that during the first hours of the exposure, mice exhibit elevated levels of stress that could potentially account for their reduced mobility[26]. On the other hand, it is interesting to note that animals born and raised on HG are much more active than control animals[15,27,28]. Also, the way to assess locomotor activity has to be considered. In our study for example, the use of an

actimeter containing small cages does not allow the mice to perform prolonged, rapid movements, as described in the aforementioned articles which used open-field or mazes apparatus.

Besides, vertical displacements are drastically decreased by 24h and 48h exposure to 2G, in both males and females as previously reported also in rats[19,29]. This may be due to the fact that during the first few days of exposure, the mice feel twice their weight making rearing much more difficult to perform and also because they can suffer from motion sickness[25]. Interestingly, we show here that a robust vertical activity is restored after a 15d exposure suggesting that mice have adapted to the 2G gravity environment. However, it is important to note in males that if the 15-day group does not differ significantly from the control group, it is comparable to the 24h or 48h groups, thus suggesting a partial recovery of the rearing behavior. This is in contrast with the observation in females in which the 15d exposed group is similar to the controls and remains significantly different from the 24h and 48h groups, indicating a better recovery for this gender.

Regarding these results, it is interesting to see that only the vertical component is altered in mice exposed to 2G, as if the specific integration of this component of space and of vestibular encoding is affected. Interestingly, this observation has also been reported in astronauts requested to perform a geometric drawing task[30].

The cosinor analysis enabled to determine the acrophase, which provides the time of peak activity during the day. Our results show a 5h delay in acrophase after 24h of 2G in male mice consistent with the literature suggesting a disruption of the circadian cycle within the first few days of exposure to 2G in mice and rats[31,32]. This time lag in the circadian cycle is described as being associated with an abrupt drop in body temperature and food and water intake in the first few days of exposure[26,33]. We could hypothesize that the circadian rhythm alteration may be linked to the initial deregulation of the vestibular system, as a role of this system in synchronizing circadian cycles has been characterized[34]. Interestingly, mice exposed to HG in the absence of vestibular afferents do not display any decrease in body temperature, in food and water intake and do no show increase in blood corticosterone levels and modification of the circadian cycle[31,32,35–38]. Investigations on het-mice, a genetic model lacking otoconia on the macular membranes (the sensory vestibular organ sensitive to gravitational field), also concludes to a strong link between the vestibular system and the rhythmicity of the circadian cycle[39].

Our results show a more rapid adaptation of females than males to the modified gravity environment, as well as undisturbed circadian cycle during the first 24h. These findings offer novel insights into the study of the circadian cycle in females, particularly in light of the numerous differences that have been observed between the two sexes in the functioning and adaptation of circadian cycles[40].

Cognitive abilities

In terms of cognitive performances, our results show a specific impairment in spatial memory performance. This is in line with the pioneer study reporting spatial disorientation in hamsters after 4

months exposure to 2,5G[41] and with later studies in rats and mice exposed for 15 days and 1 or 2h respectively to 2G[19,42]. Interestingly, this impairment resembles to the disorientation experienced by the astronauts of the Apollo 11 mission who were unable to reach a previously known crater[43]. Like for the astronauts, the memory deficits observed in animals appear to be linked to impaired spatial learning[7,44]. Indeed, learning impairments have been reported in adult rodents after centrifugation for 4 weeks at 4G[45], 3 weeks at 3G and 4G[16], 15 days at 2G[18] and after several episodes of 1.85G lasting 1h for 5 days[17]. Similar deficits are also found after a very short exposure of 3min to high levels of 6G[29,46]. It should be noted that the protocols used in those studies differ both in the species, intensities, durations of exposure to HG, and in the time elapsed between ending of centrifugation and behavioral assessment. Nevertheless, all the related results argue the idea that hypergravity impairs learning and spatial memory.

Finally, while our study shows impairment of spatial recognition memory after 15d of exposure to 2G in both males and females, the deficits appeared earlier in females (after 24h of application) than in males for which 48h of exposure is required. These findings therefore indicate a temporal discrepancy in the deterioration of spatial memory functions between the male and female mice.

One question now is to ask for the possible mechanisms underlying the 2G-dependent learning deficit. A change in gravity stimulates the utricular and saccular membranes of the vestibular system thus altering both peripheral and central vestibular neurotransmission[47–52]. Considering the anatomical and functional links between the vestibular system and the hippocampal formation[45,53,54], it may be postulated that changes in vestibular activation could potentially impact hippocampal activity which is critical for memory trace formation. This hypothesis is further substantiated by the fact that a loss of vestibular function affects spatial cognition[55–57] as well as hippocampal functionality[53,54,58–60]. Alternately, evidence has also been provided that over-stimulation of the vestibular system such as exposure to HG alters synaptic activity within hippocampal neuronal networks[45,61]. In that case, a change in hippocampal genes expression could be involved since among 200 genes which are up-regulated by 2G exposure[62], 6 genes are specifically associated with synaptic transmission and functional plasticity (e.g. proSAAS, Ngfi-A binding protein 2, syndet). Also rats exposed to 2G for 14 days show an increased expression of the Igfbp2 gene known to be positively involved in hippocampal neuronal plasticity[42].

On the other hand, the memory deficits observed in the initial hours after HG exposure could also be attributed, at least partly, to the stress generated during the centrifugation. This could also explain why, unlike males, females show earlier deficits in our study. Indeed, the black-and-white box test reveals a higher level of anxiety in females exposed to 24h of HG compared to males. This hypothesis is supported by previous corticosterone assays carried out after or during exposure to 2G[14,36], but which are unfortunately not confirmed in our assays, possibly due to high inter-individual variability.

Our study also indicates that working memory seems to be less sensitive to changes in gravity than other cognitive functions, as we did not find any deficits in male mice regardless of time exposure to 2G and a deficit only for the 48h exposed group in females. This apparent resistance of working memory differs to the pronounced deficits reported in animals with vestibular lesions induced either chemically or mechanically (Besnard et al., 2012; Machado et al., 2012). This apparent contradiction may simply reflect the extent of the vestibular disruption between the respective studies with transient overstimulation of the system in one case vs complete or permanent deafferentation in the other condition. Additionally, the spontaneous alternation test is highly dependent on inter-individual variability, which may hide subtle deficits in our experiments[63].

Anxiety and stress-related response

Our results indicate that 2G affects anxiety-related behavior with a higher effect for short duration exposure and a more salient effect in females. These results provide additional indications in the contradictory debate related to the effects of time-related HG exposure on anxiolytic reactivity. In fact, we agree with the increase in anxiety reported in the EPM and black and white box tests in mice exposed to 2G for 21 days[16] but contradicts the decrease in the time spent in the white box found in the same species exposed for 3 weeks[64]. It is worth notice that in these two studies and in contrast to our investigation, behavioral tests were carried out 15 days after the exposure to HG suggesting that the observed response may reflect not only the effect of exposure but also the readaptation to the return to normal gravity. Furthermore, acute exposure (2h) does not impact anxiety responses in peri-adolescent CD1 male and female mice in the elevated plus maze[19], raising the question of the exact contribution of the centrifuge-induced stress in very short periods of HG exposure.

Stress-related environments induce long-term neuroendocrinal and behavioral effects related to the hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis influencing levels of corticosteron[63]e (Petrak et al., 2008; Review in Armorio et al., 2008). The higher stress reactivity in females observed herein may be attributed to sex-specific differences in the HPA function as previously suggested[65]. In fact, estrogens stimulate the activity of this axis[66], leading to greater corticosterone production in females. However, we did not find significant difference between the 2G-exposed groups in the blood concentration of corticosterone neither in males nor in females, though it tends to increase in the latter. The absence of change in corticosterone levels after at least 24h exposure to 2G agrees with previous assays obtained in the same HG conditions[67], as well as after exposures elapsing between 12 and 21 days[14,16,59,68–70]. This temporal pattern associated with an increase reported in the first few hours after the centrifugation[14] thus suggests an adaptive process to the new environment.

Sociability

Our study shows that social behavior is reduced both in male and female mice after 48 h exposure to 2G though a large variability between individuals is noted. The deficit may be related to a decrease in social

motivation induced by stress considering that stress-induced conditions such as a restrain for 6 hours a day during 21 days specifically alters sociability without affecting social recognition[71].

It is interesting to note that in our study, the sociability index increases in males exposed for 15 days is associated with a reduced anxiety and increased locomotor activity. This agrees with a greater sociability previously reported in rats born and raised for 2 months in 1.8G that is also linked to a reduction in anxiety[27]. Taken together, these data are rather reassuring in the context of long-duration space missions.

Our findings demonstrate significant sex-dependent differences in the adaptive responses to altered gravity. While long-term exposure to 2G impacts spatial memory in both sexes, short-term exposure results in decreased sociability, increased anxiety levels and short-term memory deficits more pronounced in females. We also provide cues that cognitive functions likely dependent on vestibular modulation such spatial memory, are particularly vulnerable to HG variations. Finally, this work contributes to new insights into changes in locomotion and circadian rhythms after 2G exposure, also revealing differences in activity dynamics between males and females.

Given the sex-related variability observed in behavioral responses, future researches are required to clarify the underlying neural mechanisms. In particular, electrophysiological recordings from the hippocampus would offer valuable insights into how HG affects neural activity and memory formation at the synaptic level. Furthermore, investigating the duration of deficits after the return to 1G conditions, could provide a better understanding of the reversibility of the cognitive and emotional impairments driven by gravitational shifts.

METHODS

Animals and hypergravity exposure

Experiments were performed in accordance with the French and European Community guidelines for the care and use of laboratory animals (2010/63/EU) and approved under the project number 2023041911573213 (approval date 2023/09/10). For these experiments, a total of 172 adult (10–14 weeks old) C57BL/6JRj mice were purchased from Janvier Labs (Le Genest-Saint-Isle, 53940, France) including 89 males and 83 females. Animals were kept in groups of 5 individuals in standard cages (42cm × 26cm × 19cm) with cardboard hut and nesting material, in a reversed 12h/12h light/dark cycle (light on at 7 pm), with regulated temperature (22 ± 1◦C) and humidity (55 ± 10%). Food and water were available ad libitum.

After a week of habituation period after arrival, the mice were handled by the experimenter at least 5-min every day for one week to reduce the stress associated with further handling. Then mice were transferred into the centrifuge specially designed for small animals (CNES, French national center for space studies, France) which consists in four arms (length: 0.73m), joined in a central rotating axis[13].

Each arm was equipped of a free-swinging gondola (55.5cm × 38.5cm × 31 cm) hung at its extremity, and each mouse cage was placed inside a gondola.

The rotation speed was set to 45 r.p.m to obtain a gravito-inertial acceleration of 2G on the floor of the gondolas, meaning that the mice were exposed to a doubling of their normal weight. Three different durations of 2G exposure were applied: 24h (n = 43), 48h (n = 43) and 15days (n = 43). Control (n = 43) (1G) animals were housed in the same room, to be subjected to the same olfactory and auditive stimuli than 2G animals, and in similar gondolas as the centrifuged mice. The centrifuge was stopped every day for less than 10-min in order to check and weigh the mice and to clean the cages when necessary. Mice behavior in the gondolas was observed and controlled by cameras (Axis M1031-W, Axis communications©) during the rotation. The device did not allow precise analysis of the behavior but allows a minimal visual check of animal welfare.

Behavioral assessment

The behavior of the mice was assessed in different ways. Firstly, the locomotor activity of the mice was recorded for 24 hours from 8.30 am to 8.30 am after 2G exposure (Fig. 5.**A)**. Then, on another batch of mice, behavior was assessed through a battery of tests performed on a single day between 9 am and 4 pm in the order depicted in Fig. 5.**B**. The mice were removed from the centrifuge in groups of five and transferred to the behavioral room for a 30-min acclimatization period to the environment and to reduce the stress associated with centrifugation, before starting the behavioral tests[14]. Throughout all tests, the room was illuminated at 30 lux. After each animal, the experimental apparatus was cleaned with 70% ethanol. Each test (except the light and dark box) was videotaped for *a posteriori* analysis.

- Spontaneous activity

To investigate the early and late effects of exposure to 2G and transfer to normal gravity, a batch of mice (n = 7 per group) was specifically used to assess spontaneous locomotor activity and chronobiological cycle in an actimeter (Fig. 1A.; Imetronic©, Pessac, France)[72]. Each mouse was individually placed in a cage of the actimeter (19cm × 11cm × 14cm) at 8:00 am, immediately after exit of the centrifuge, and observed for 24 hours. Food and water were provided ad libitum and horizontal and vertical activity was automatically counted. The raw data were thereafter transformed using cosinor method for analysis and meaningful graphical representation[73].

Waveform resolution attenuates outliers considered to be biological noise, and provides informative data related to circadian rhythm such as mesor (midline-estimating statistic of rhythm, the mesor is the rhythm adjusted mean that approximates the arithmetical mean of the data), amplitude (measure of one-half the extent of the rhythmic variation in a cycle estimated by the sinusoidal used to approximate the rhythm, e.g., the difference between maximum and mesor of a cosine) and acrophase (time of the maximal activity).

- Anxiety-like behavior

The light and dark box test was used to assess anxiety-like behavior in mice (Fig. 1B; Costall et al., 2004). The apparatus (LE810, Letica) was made of opaque black and white Plexiglas and consisted in two compartments: a large illuminated one (28cm × 27cm × 27cm, 900 lux) and a small dark one (17cm × 27cm × 27cm, 100 lux, red light), connected by a small opening (10cm × 10cm).

The protocol was that described by Lelong-Boulouard et al[74]. Each mouse was individually placed on the center of the light chamber. The time for first entry into the dark chamber was measured. Once the mouse has entered the dark chamber, a 5-min timer was started. A tilted mirror above the apparatus allowed the experimenter to collect the time spent in each chamber and the number of transitions, for each mouse. The percentage of time spent in the light box was then calculated and used as a parameter anxiety-related behavior (n = 10 for each group).

- Short term memory

Short term memory was assessed by evaluating spontaneous alternation behavior in a Y-maze. The apparatus was made in grey plastic with three arms (21cm × 7.5cm ×15cm), as previously described[75].

Each mouse was individually placed in one of the arms, head facing the wall, and allowed to freely explore the maze for 5-min. The number of entries into the different arms was collected (four-paw criterion) to define exploratory abilities of mice. The number of alternations, defined as 3 consecutive entries in the 3 different arms, was collected. The percentage of alternation was calculated as follows: number of alternation/(total number of entries-2)x100. The percentage of alternations for each group (n = 10) was compared to the reference value of 50%, which corresponds to random exploration of the maze.

- Object place recognition

Place recognition was carried out using Object Location Memory test (OLM). This test was assessed in a grey-painted wooden open-field (32cm × 32cm × 20cm), with visual cues on the walls of the room[76]. A period of habituation to the apparatus was first carried out in which each mouse was individually allowed to freely explore the device for 10-min. Then, two identical objects were placed in two consecutive corners (spaced 6 cm from the walls) for the encoding phase. The objects used were towers of LEGO®[77]. The mouse was free to explore the objects for 10-min before being replaced in its home cage. Two hours after the encoding phase, one of the two objects was moved to the opposite corner, and the mouse was again allowed to explore for 10-min (the moved object changed between each mouse). During the two sessions, mice were videotaped for further analysis. Exploration was considered when the mouse approached the objects with its nose to less than 1cm with the head being directed toward the objects. Touching the object with the nose or forepaws was also defined as an exploration. The following events were not considered as exploration: the mouse passed by the objects, climbed on the objects or groomed itself near the objects. During the encoding phase, an exclusion criterion based on total exploration of the objects was used: if the mouse explored the two objects for less than 20sec, it was excluded from the test (number of male mice excluded: ctrl = 0, 24h = 1, 48h = 1, 15d = 1; number of

female mice excluded: ctrl = 0, 24h = 2, 48h = 3, 15d = 2). A discrimination index was calculated as follows: ((exploration time of the displaced object – exploration time of the non-displaced object) / total exploration time) x 100. The discrimination indices were compared to the reference value of 50%, which corresponds to an equal exploration of both objects.

- Social behavior

The sociability apparatus was made of transparent Plexiglas and consisted of 3 equally sized chambers (40cm × 20cm × 40cm) interconnected by openings[72]. The mouse was first placed in the apparatus for 5-min familiarization. Thereafter, in one of the two lateral chambers, a 30-day-old mouse of the same sex (stimulus mouse) was placed in a small cage with bars, and in the other chamber an object was placed in another similar cage with bars (the object was a clear plastic bottle filled with clean sawdust). Five stimuli mice were used for each sex, so each stimulus mouse was used for two tests. To avoid stress, the stimuli mice were previously accustomed to the bar cages for 15 min/day for 5 days before the test. The location of the object and mouse (right or left) was randomized between each mouse. Animals were video-recorded and thereafter videotracked with EthoVision XT v.17.0 (Noldus©, The Netherlands). The sociability behavior (n = 10 for each group) was assessed during 10-min exploration and calculated with the following formula: ((time spent in the chamber containing the stimulus mouse - time spent in the chamber containing the object) / (total time spent in both lateral chambers) x 100. The sociability indices were compared to the reference value of 50%, which corresponds to an equal exploration of both cages.

Plasma Corticosterone

Blood corticosterone concentration was measured as an indicator of stress[78]. Blood samples collected immediately after centrifugation were obtained from another batch of mice after 2G exposure of 24h, 48h or 15 days (Fig. 1C.). To collect blood, mice were rapidly anesthetized with 5% isoflurane and decapitated. Trunk blood was collected in 2 ml sodium heparinized tubes and immediately centrifuged (3 000 g, 2 min, room temperature). Plasma was collected and stored at -20°C until assays. Analyses were performed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). Chromatography was performed on an ABSciex® API 5500 QTRAP triple quadrupole mass spectrometer (Framingham, MA USA) equipped with an electrospray ionization source.

Data analysis

Statistical analyses were performed with GraphPad® Software (San Diego, CA, USA 8.0) and Rstudio® software 4.2.0 (Free software foundation, Vienna, Austria, development core team 2009). P-value lower than 0,05 was considered significant. Normality of data distribution and variance homogeneity were assessed using Shapiro and Levene tests respectively, to select the statistical test to be used. If data followed a normal distribution and variances were homogeneous, intergroup comparisons were performed with *ANOVA* and Dunnett's method for post hoc analysis (comparing HG groups with the control group). Comparisons between two groups were made using paired or unpaired sample t-tests. When data were not normally distributed, intergroup comparisons were performed using Kruskal-Wallis

test, followed by Bonferroni-Dunn post hoc test for multiple comparisons. To compare the mean of a group with a reference value, a one sample t-test was used.

Two-way non-parametric statistic measures (*ATS: ANOVA-type statistic*) were used to analyse actimetry data for both males and females. The cosinor.online application was used to calculate the cosinor of the actimetry data and analyses biological cycles [73].

DATA AVAILABILITY

Data are available by request from the author for correspondence

Declarations

COMPETING INTERESTS

All authors declare no financial or non-financial competing interests.

Author Contribution

MW and VB carried out the design of the study. MW performed the experiments and wrote the manuscript. JMB and VB contributed to writing of the manuscript. All authors read and approved the final manuscript.

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Figures

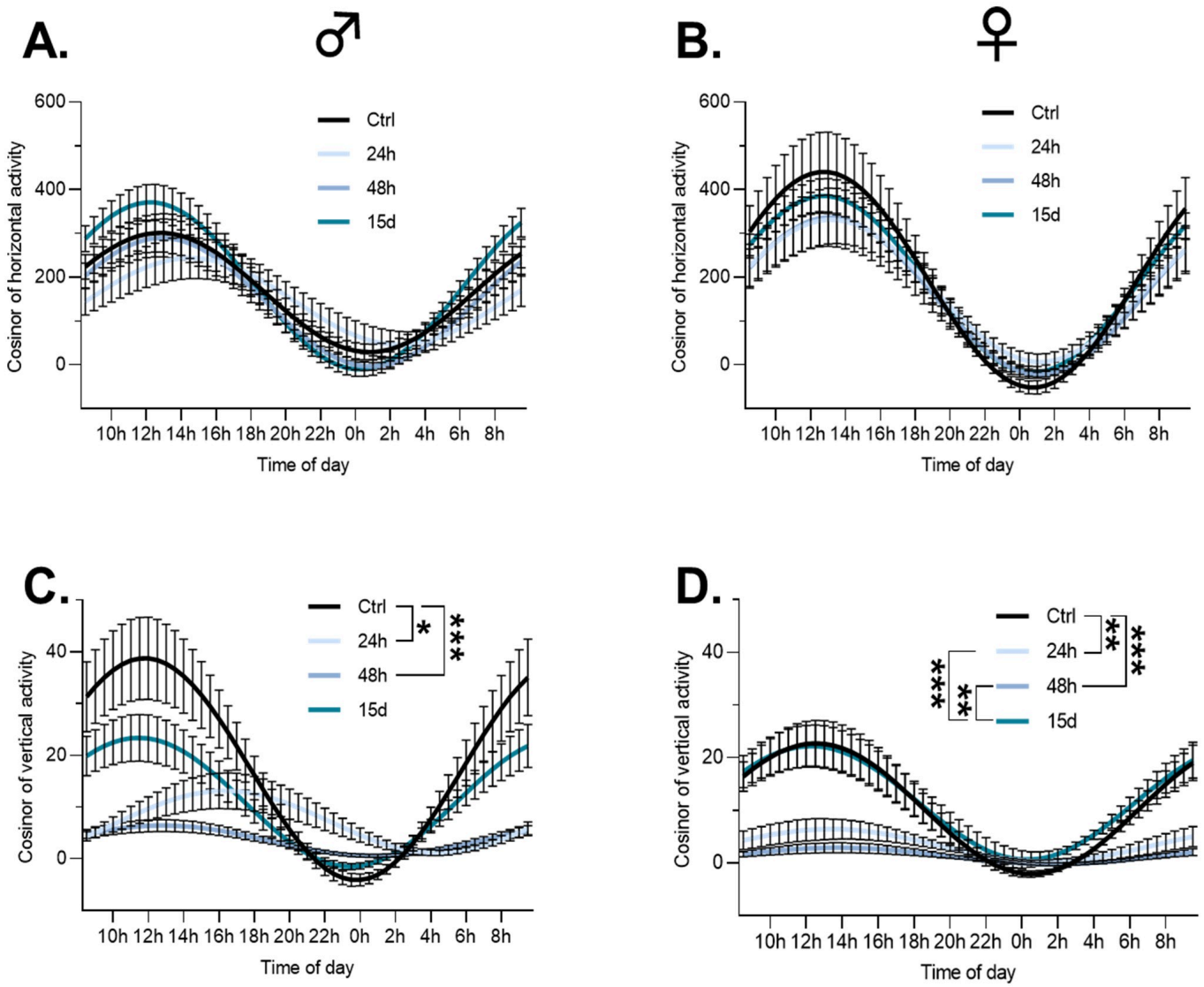


***Figure 1**: Sex-dependent actimetry activity of mice (obtained by number of cross of infrared beams by periods of 30min). **A**. Graph with mean ± SEM comparing the cosinor of the horizontally split infrared beams, during 24h after centrifugation, from control (n=7), 24h (n=7), 48h (n=7) and 15d (n=7) 2G-exposed male mice (* P<0.05, ATS). **B**. Graph with mean ± SEM comparing the cosinor of the horizontally split infrared beams, during 24h after centrifugation, from control (n=7), 24h (n=7), 48h (n=7) and 15d (n=7) 2G-exposed female mice (* P<0.05, ATS). **C**. Graph with mean ± SEM comparing the cosinor of the vertically split infrared beams, during 24h after centrifugation, from control (n=7), 24h (n=7), 48h (n=7) and 15d 2G-exposed (n=7) male mice (* P<0.05, ATS). **D**. Graph with mean ± SEM comparing the cosinor of the vertically split infrared beams, during 24h after centrifugation, from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) female mice (* P<0.05, ATS).*

Figure 1

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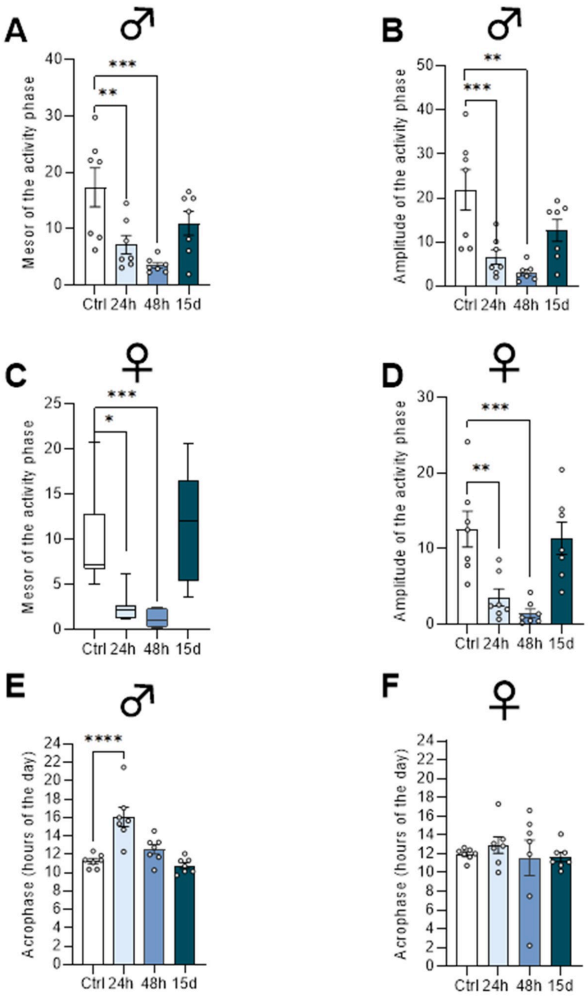


***Figure 2**: Sex-dependent effect of HG exposure on Cosinor analyses of vertical behavior. **A.** Bar graphs with mean ± SEM comparing the mesor value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) male mice (* P<0.05, One-way ANOVA with Tukey's post hoc tests). **B.** Bar graphs with mean ± SEM comparing the mesor value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) female mice (* P<0.05, Kruskal-wallis test with Bonferroni–Dunn post hoc tests). **C.** Bar graphs with mean ± SEM comparing the amplitude value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) male mice (* P<0.05, One-way ANOVA with Tukey's post hoc tests). **D.** Bar graphs with mean ± SEM comparing the amplitude value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) female mice (* P<0.05, One-way ANOVA with Tukey's post hoc tests). **E.** Bar graphs with mean ± SEM comparing the acrophase value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) male mice (* P<0.05, One-way ANOVA with Tukey's post hoc tests). **F.** Bar graphs with mean ± SEM comparing the acrophase value from control (n=7), 24h (n=7), 48h (n=7) and 15d HG exposed (n=7) female mice (* P<0.05, One-way ANOVA with Tukey's*

Figure 2

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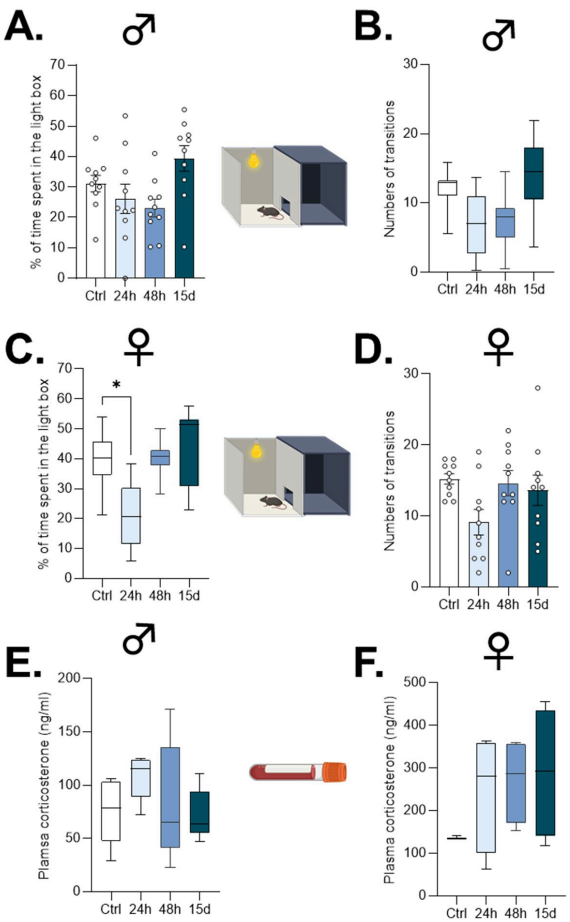


***Figure 3**: Sex-dependent effects of 2G exposure on anxiety like behavior and corticosterone plasma levels in male and female mice. **A.** Bar graphs with mean ± SEM comparing the percentage of time spent in the white box during 5min test, from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) male mice (* P<0.05). **B**. Bar graphs with median ± 5-95 percentile comparing the percentage of time spent in the white box during 5min test, from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) female mice (* P<0.05, Kruskal-wallis test with Bonferroni–Dunn post hoc tests). **C**. Bar graphs with median ± 5-95 percentile comparing the numbers of transitions between white and dark compartments during 5min test, from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) male mice (* P<0.05, Kruskal-wallis test with Bonferroni–Dunn post hoc tests). **D**. Bar graphs with mean ± SEM comparing the numbers of transitions between white and dark compartments during 5min test, from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) female mice (* P<0.05, One-way ANOVA with Dunnett's post hoc tests). **E**. Bar graphs with mean ± SEM comparing the plasma corticosterone concentration (ng/ml) at the end of centrifugation from control (n=6), 24h (n=5), 48h (n=5) and 15d HG exposed (n=5) male mice. **F**. Bar graphs with mean ± SEM comparing the plasma corticosterone concentration (ng/ml) at the end of centrifugation from control (n=3), 24h (n=4), 48h (n=4) and 15d*

Figure 3

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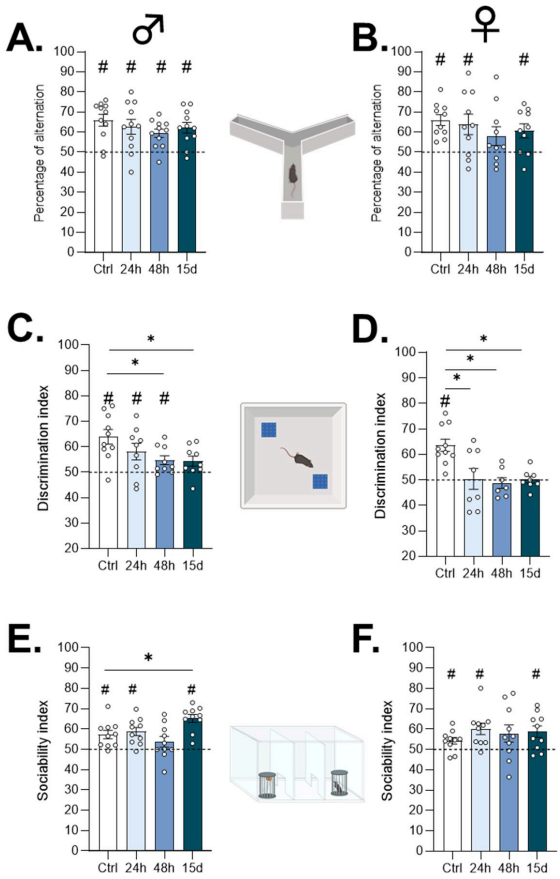


***Figure 4**: Sex-dependent effects of HG exposure on short term memory, spatial memory and sociability behavior in male and female mice. **A**. Bar graphs with mean ± SEM comparing the percentage of alternation between the 3 arms of the maze from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) male mice. # indicates a significant difference from 50%. **B**. Bar graphs with mean ± SEM comparing the percentage of alternation between the 3 arms of the maze from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) female mice. # indicates a significant difference from 50%. **C**. Bar graphs with mean ± SEM comparing the discrimination index obtained from the interaction time with the displaced object and the non-displaced object during the retention phase from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) male mice (* P<0.05). $ indicates a significant difference from 0. **D**. Bar graphs with mean ± SEM comparing the discrimination index obtained from the interaction time with the displaced object and the non-displaced object during the retention phase from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) female mice (* P<0.05). # indicates a significant difference from 0. **E.** Bar graphs with mean ± SEM comparing the sociability index obtained from time spent in the mice and object compartments from control (n=10), 24h (n=10), 48h (n=10) and 15d HG exposed (n=10) male mice (* P<0.05). # indicates a significant difference from 0. **F.** Bar graphs with mean ± SEM comparing the sociability index obtained from time spent in the mice and object compartments from control (n=10), 24h (n=10),*

Figure 4

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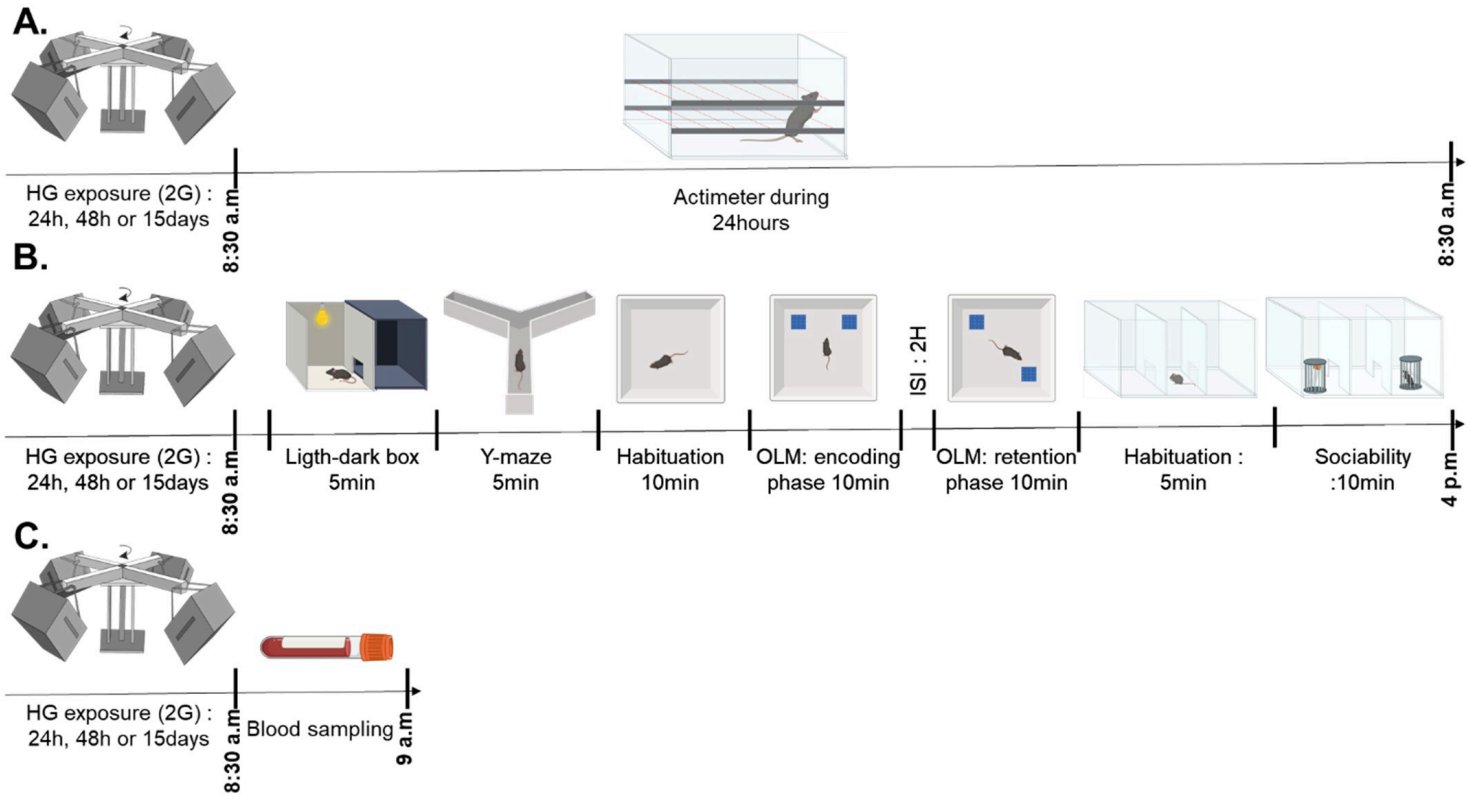


***Figure 5**: Experimental designs of behavioral assessments and blood samplings. **A**. Actimetry protocol (7 mice per group). **B.** Time-related sequences of the different behavioral tests (10 mice per group). **C.** Blood sampling protocol (5mice per group). (HG = Hypergravity).*

Figure 5

See image above for figure legend.

Supplementary Files

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