Regional vulnerability of the hippocampus to repeated motor activity deprivation

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HIGHLIGHTS

- Rearing-deprived rats indicated levels of emotional disturbances after rearing deprivation.
- HPA system was significantly affected by chronic rearing deprivation.
- Spatial search strategies were considerably influenced by the deprivation protocol.
- Hippocampal CA2-specific vulnerability was observed in relation to rearing deprivation.

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ABSTRACT

Spontaneous vertical and horizontal exploratory movements are integral components of rodent behavior. Little is known, however, about the structural and functional consequences of restricted spontaneous exploration. Here, we report two experiments to probe whether restriction in vertical activity (rearing) in rats could induce neuro-hormonal and behavioral disturbances. Rearing movements in rats were deprived for 3 h/day for 30 consecutive days by placing the animal into a circular tunnel task. Rats temporarily deprived of rearing behavior showed elevated plasma corticosterone levels but no detectable psychological distress and/or anxiety-related behavior within an elevated plus maze. However, rats emitted a greater number of 22-kHz ultrasonic vocalizations and spent significantly more time vocalizing than controls when deprived of their rearing behavior. Despite intact spatial performance within wet- and dry-land spatial tasks, rearing-deprived rats also exhibited a significant alteration in search strategies within both spatial tasks along with reduced volume and neuron number in the hippocampal subregion CA2. These data suggest a new approach to test the importance of free exploratory behavior in endocrine and structural manifestations. The results support a central role of the CA2 in spontaneous exploratory behavior and vulnerability to psychological stress.

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1. Introduction

From an evolutionary viewpoint, animals' exploratory behavior including horizontal and vertical movements [36] serves the purpose to determine benefits (e.g., food and partners) and recognize potential dangers (e.g., predators) in a novel environment [2,23]. Specifically in rats, this behavior not only represents an organized sequence of trips and progressions with variable direction and speed [67,70] but also includes an unpredictable number of stops each of which is mostly linked to vertical or rearing behavior. Rearing in rats during exploration can be observed when the animal intermittently discontinues its horizontal activity and rear up by lifting its forelimbs from the ground. In this position, the animal

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usually seems stationary and appears to be visually inspecting the environment [3]. Thus, in conjunction with horizontal movements, rearing plays a key role in the integrity of exploratory behavior.

Vertical activity along with its potential cognitive importance in exploration also is a manifestation of emotional state. Rearing during open-field exploration was previously regarded as reflecting emotional components of behavior [27]. If this is the case, it seems justified to hypothesize that rearing deprivation could impair emotional behavior along with the integrity of free and goal-directed exploration.

Importantly, the neuroanatomical correlates of vertical activity indicate a close correlation between the hippocampal morphology and the occurrence of rearing in rats and mice [13,54,12,29,62,1]. For example, cholinergic responses in the hippocampus seem stronger in animals which show more frequent rearing behaviors during open-field exploration [61]. Also, animals with a higher rate of rears show larger intra- and infra-pyramidal mossy fiber projections in hippocampus compared to their less frequently-rearing counterparts [31]. However, the hippocampal regional involvement in vertical activity has not yet been directly investigated.

The present experiments were designed to examine the neuro-hormonal, emotional and behavioral consequences of reduced exploratory movements, with particular focus on vertical activity (rearing). Rats were deprived of rearing for a limited time each day by placing animals into a circular tunnel task that resembled features of a rat’s natural environment. The tunnel segment in a rat burrow allows only one rat to pass at a time without space for rearing. The burrow usually ends in a cup-shaped nest or dead end where the rat establishes a colony; spends extended periods of time and, more importantly, is free to show vertical activity. Here, we examined whether restricted rearing influences the activity of the hypothalamic-pituitary-adrenal (HPA) axis, a system that is intimately linked to psychological distress and hippocampal function [55]. The experiments investigated established endocrine (corticosterone and glucose), affective (anxiety-like behavior and ultrasonic vocalizations) and cognitive (spatial learning and memory) manifestations of altered HPA axis activity [19,23]. Furthermore, we asked whether rearing deprivation induces regional alterations in the hippocampus based on evidence that changes in rearing frequency are reflected in altered hippocampal morphology and function [1]. Results of the present study indicate neuro-hormonal disturbances and regional hippocampal vulnerability to restricted exploratory activity. The results support a central role of the CA2 in exploratory hippocampus-dependent behaviors and vulnerability to psychological stress.

2. Materials and methods

This study involved 29 male Wistar rats (8–9 weeks old). The animals were housed in pairs under a 12:12 h light/dark cycle with light starting at 07:30 h. Animals were provided with water and food ad libitum. The room temperature was set at 22 °C, and experimental procedures were conducted during the light phase of the cycle at the same time of day. All procedures were approved by the Avicenna Institute of Neuroscience (AIN) Animal Care Committee and were carried out in accordance with NIH guidelines.

3. Experimental procedures

3.1. Experiment 1 Vertical activity deprivation and spatial performance in wet- and dry-land tasks

Fourteen rats (control, N=6; deprived, N=8) in this experiment were trained and tested before and after vertical activity deprivation for spatial performance in two tasks, the Morris water task (MWT; [59,49]) and the ziggurat task (ZT; [20]. All behavioral analyses were performed by an experimenter blind to the group identities.

3.1.1. Assessment of vertical activity

Preliminary assessment (pre-test) of vertical activity (number of rears) during free navigation was performed before rearing deprivation using a square black open-field arena (70 × 70 × 35 cm) under dim illumination. Rats were individually placed in the middle of the field, and the number of rears was counted for 10 min regardless of whether rears occurred on or off the walls [61]. The same procedure for counting the number of rears was used for the post-test session. Vertical activity in this experiment was scored when rats reared on their hind limbs with their forelimbs unsupported. During a rearing movement, the rat appears stationary with slow or absent whisker movement [3].

3.1.2. Morris water task (MWT)

A hidden platform version of the MWT was employed to assess spatial performance [19]. Briefly, animals were taught to escape from the water (22 ± 1 °C) by climbing onto the hidden platform. Each trial began with the rat being placed in the pool at one of the four cardinal compass positions around the perimeter of the pool according to a pseudo-random sequence. The location of the hidden platform remained constant from trial to trial. Thus, we were able to assess trial-independent spatial learning. The maximum duration of each swim trial was 60 s. Animals in this experiment were tested in 12 trials for one day before (pre-test) and after (post-test) vertical activity deprivation. Latency and path speed were recorded and analyzed by an image-computerized tracking system (HVS Image, UK). A no-platform probe trial was also performed two hours after the completion of the single session hidden platform testing as an additional measure for reference memory. The platform was removed from the pool and the rats were allowed to swim freely for 30 s. Because in one of our pilot studies both aged (N=7) and young (N=5) rearing-deprived rats showed (data not presented) more thigmotaxis (wall hugging behavior) and circling (swimming in tight circles; [7] within the MWT, the number of circling during spatial navigation in the task was manually calculated later via the path graphics for each animal generated by the tracking system.

3.1.3. Ziggurat task (ZT)

The procedures for ZT testing were previously reported [21]. Briefly, animals were food-restricted one week prior to habituation sessions and spatial testing in the ZT, and maintained at about 90–95% of their initial body weight throughout the experiment. To maintain body weight, rats were given an additional amount of food in their home cage at least 2–3 h after completion of the behavioral training and testing. Animals were weighed daily throughout the experiment in order to monitor food consumption.

Rats were habituated to the ZT environment for four days. After habituation, the testing sessions were conducted over 12 trials per day within the standard version of the ZT, and began the day immediately following the last session of habituation. Two sets of ziggurats were defined in the arena. First, “start” ziggurats, located in each corner, and second, the rest of ziggurats or “goal” ziggurats [21]. On the testing day, rats were released from each starting point and allowed to explore the environment. One central goal ziggurat was baited with spaghetti for each trial. During each testing trial, rats started from one of four different starting points in a randomized sequence. Across trials, the starting location varied among the four corners of the apparatus, and on each trial, animals navigated in the environment for 80 s or until they found the central goal ziggurat. It should be pointed out that the location of the goal ziggurat remained constant from trial to trial.
All animals were tested in the ZT before and after vertical activity deprivation. Moreover, post-training probe trial-dependent behaviors in the ZT were measured two hours after the completion of the 12-trials spatial testing as an additional measure for spatial performance. Each rat was given one 60-s probe trial, released from one starting point to reach the central goal ziggurat. Rats were allowed to navigate freely in the environment during the specified time. The movements of the animals were recorded and analyzed by a HVS Image tracking system and a Sony digital videocassette recorder (GV-D 900 NTSC, Sony Inc.) for further examination. The number of rears produced by rats during the spatial navigation was manually calculated for both groups using the path graphics and videocassette tapes. It should also be noted that because latency and path length consistently reveal similar profiles of spatial navigation within the MWT and ZT [65,39,21] we have considered and reported only latency and speed for spatial performance.

3.1.4. Circular tunnel task for vertical activity deprivation

For vertical activity deprivation, rats were transferred to a dark room. Each animal in the deprived group (N = 8) was placed in a gray transparent Plexiglas circular tunnel (9 cm inner diameter; Taj-Abzar Co., Tabriz; Fig. 1A) from 8:30 am to 11:30 am for 30 consecutive days. Only one rat was placed in each tunnel at a time. The tunnel allowed animals to freely move and turn around in the tunnel but prevented rearing on their hind limbs. Windows inserted in the wall of the apparatus supported air ventilation. For the same period of time, control animals (N = 6) were individually placed in a round Plexiglas cylinder (35 cm diameter; 45 cm high; Fig. 1B) with no overhead cover located in a dark room near the testing room. They were allowed to freely explore the chamber.

3.2. Experiment 2 vertical activity deprivation, emotionality and ultrasonic vocalization

3.2.1. Glucose and corticosterone measurements

All animals (control, N = 7; deprived, N = 8) in this experiment underwent blood sampling for glucose (GLU) and corticosterone (CORT) measurements as two main physiological indicators of HPA axis activation [40]. Blood samples were taken 5 days prior to rearing deprivation (baseline). Blood sample were also taken after the first session of deprivation (acute time point), on the fifteenth day (chronic time-1) and the last day (chronic time-2) of deprivation. Rats were placed in a restraint tube and blood samples were obtained by tail notch with a scalpel blade [19]. Blood samples were collected from all rats within the first 1–2 min of being placed in the tube to ensure that circulating CORT levels did not change in response to the brief restraint procedure during collection. Blood GLU was measured using an HNC Blood Glucose Monitoring System (HNC, CDC series, China). All blood samples (0.5–0.6 mL) placed in heparinized tubes were then transferred to centrifuge tubes and plasma was obtained by centrifugation at 6000 rpm for 7 min. The plasma samples were stored at −20 °C until analyzed by enzyme-linked immunosorbent assays (ELISA).

3.2.2. Elevated plus maze (EPM)

As previously described [66,72], anxiety-related behavior of all animals before and after deprivation was assessed in the EPM under dim ambient illumination. The wooden apparatus consisted of two open and two closed arms (each 50 × 10 cm) and was elevated 100 cm above the floor. The open arms had no side or end walls, and the closed arms had side and end walls (40 cm high). The rats were placed individually in the central square (10 cm × 10 cm) facing either the left or right open arm, and were allowed to explore the apparatus for 5 min. Each animal was tested only once, and the behavior of the animals in the EPM was video recorded (Sony HDR-PJ220B, Japan). Anxiety-related behavior was measured by an experimenter blind to the animals’ experimental condition through analysis of the traditional measures (time spent in the open and closed arms, time spent in the end of the open arms, time spent in the central square).

3.2.3. Circular tunnel task for vertical activity deprivation

The vertical activity deprivation procedure used in the present experiment was identical to Experiment 1, with the exception that rats’ ultrasonic vocalizations were recorded while they were exploring the task. Vocalizations from control animals were also recorded while they explored the cylinder.

3.2.4. Ultrasonic vocalization recording (USV)

An ultrasound microphone was set at a distance of 3–4 cm from one of the side windows of the deprivation apparatus. The microphone was connected to a recording interface (PooyaSound-PS2 Ltd., Tabriz, Iran). The microphone was sensitive to frequencies of 10–120 kHz. Data acquisition hardware and recording software (PooyaSoft-2012; Version 2.0; Yazd) on an LG Desktop PC computer L7047-C9463 were used in the present experiment and acoustic data files were converted into spectrograms. Only 22-kHz calls (either single pulses or short bouts) were determined automati-
ugically for each recording and animal. It should be noted that calls were considered in the 22-kHz category if they fell between 18 and 32 kHz with a duration of 300–3,000 ms, as previously reported [71]. Because of some technical and procedural limitations, only one rat in each group was chosen daily in a randomized order for USV recording. The tunnel selected for USV recording was placed in a different room, and no other deprived rats were present in the room during the deprivation period and USV recording session. The same procedure for USV recording was also utilized for an isolated control rat in a different room, with the exception that rat’s USVs were recorded by the ultrasound microphone positioned about 30 cm above the base of a chamber in which the rat was placed.

USVs wave generation by each control and deprived rat was recorded four times per session: (i) after the first 5 min, (ii) after the first 60 min, (iii) after the second 60 min, and (iv) final phase, each for 5 min (total 20 min). The main parameters used for analysis of USV calls in the present experiment were the average number of 22-kHz USVs and average time spent calling [71,33,53]. Furthermore, for rats that vocalized, the mean call length [75] per individual animal in each session was calculated.

3.3. Histological procedures

Rats were euthanized with an overdose of sodium pentobarbita-
tal (300 mg/kg i.p.) and intracardially perfused with saline (0.9%; 200 mL/rat) followed by 4% paraformaldehyde (PFA; 200 mL/rat). Brains were removed and treated as described previously [22]. To determine the hippocampal volume a series of tissue (control, N = 5; deprived, N = 6) was stained with cresyl violet. The hippocampal volume in each rat was estimated according to the Cavalieri method [57] using a set of 10–11 cross sections of the hippocampal area, from −2.30 mm to −6.04 mm relative to bregma. In the case of missing or damaged sections (less than 10 sections for each rat) data was calculated as average area values from preceding and following sections. A second series of tissue in a subset of control (N = 7) and deprived (N = 9) rats was labeled with 4', 6-diamidino-2-phenylindole (DAPI, Sigma) and total cell number estimates in all hippocampal subregions (CA1, CA2, CA3, DG) were recorded utilizing the optical fractionator technique [69,38]. One animal from the deprived group had to be excluded from the volumetric analysis because of missing and/or damaged sections. It should be noted that all counting was performed by the same person, who was blinded to the groups’ assignment.

3.4. Statistical analyses

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., USA), Behavioral data were normally distributed (Shapiro–Wilk test), and statistical comparisons were performed using the repeated-measure and one-way analysis of variance (ANOVA) followed by the post hoc Tukey-HSD test. To test for correlations between the level of circulating CORT and USVs Spearman’s rank correlation coefficient was applied. Comparison means between groups were also performed using dependent and independent samples t-tests for within-subject comparison. Familiarly error was considered prior to the multiple post hoc analyses if necessary. In order to evaluate magnitudes of effects of experimental manipulation (here, vertical activity deprivation) on spatial performance and emission of 22-kHz calls, effect sizes ($\eta^2$ for ANOVA and Cohen’s d for the post hoc test) were calculated. Values of $\eta^2 = 0.14$, 0.06 and 0.01, and Cohen’s $d = 0.80$, 0.50 and 0.20 were considered for large, medium and small effects, respectively [33]. All data are displayed as the mean ± standarderror, and the criterion for statistical significance was $p < 0.05$ for all comparisons.

4. Results

4.1. Behavioral analysis

4.1.1. Experiment 1

4.1.1.1. No group difference in the number of rears during free exploration. During a 10-minute period of assessment within the open field task at both pre- and post-test time points, DEPRIVED animals showed lower numbers of rears than CONTROL rats (Pre-test: 48.78 ± 6.01 vs. 54.3 ± 6.74; p ≤ 0.77; Post-test: 44.39 ± 4.66 vs. 51.30 ± 5.2; p > 0.096, independent samples t-test), but the observed differences were not significant. Also, there were no significant differences between the pre- and post-test number of rears in each group (both $p > 0.05$; dependent samples t-test).

4.1.1.2. Rearing deprivation altered spatial search strategies within wet- and dry-land tasks without altering spatial learning.

4.1.1.2.1. Spatial performance in the MWT. The latencies to reach the hidden platform and swim speed for both CONTROL and DEPRIVED rats in the MWT before and after rearing deprivation are depicted in Fig. 2, Panels A–F. The categorization for search strategies in MWT employed in the present experiment was similar to those reported previously [7]. The latency by the rats in both groups decreased over 12 trials of testing within the MWT in both time points suggesting that all rats, regardless of their experimental positions, were able to acquire and retrieve the spatial information at a similar rate. A significant effect of Trial (Pre-test: $F_{11,12} = 18.64, p < 0.029, \eta^2 = 0.23$; Post-test: $F_{11,12} = 11.08, p < 0.033, \eta^2 = 0.26$; repeated-measures ANOVA), but no effect of Group (Pre-test: $p > 0.411$; Post-test: $p > 0.674$) was observed at any time point. Group by Trial effects for both time points were significant (All $p < 0.05$).

Furthermore, examination of swim speed during the 12 tri-
als of acquisition showed a relatively flat speed profile across 12 testing trials for both groups at both time points. No significant difference was observed between DEPRIVED and CONTROL rats (Pre-test: $p > 0.760$; Post-test: $p > 0.109$). Also, rats in both groups showed similar preferences to spend time in the target quadrant (Pre-test: quadrant three; SW; Post-test: quadrant one; NE) within the MWT. No significant group difference was found in the percentage time spent in the target quadrant (Pre-test: $p > 0.088$; Post-test: $p > 0.141$).

Pre-test examination of the number of circling produced by the rats during spatial navigation also indicated no differences between DEPRIVED and CONTROL groups (22.73 ± 4.66 vs. 27.19 ± 4.81; $p = 0.069$, ANOVA). Further, no difference was observed between groups in the probe trial in terms of the number of circling behaviors ($p = 0.251$; independent samples t-test) in the pre-test session. While both groups showed similar spatial path trajectories across 12 trials and probe performance prior to rearing deprivation, DEPRIVED animals showed more circling than CON-
TROL rats when they were searching for the hidden platform in the post-test session. The observed differences were confirmed by statistical analysis conducted on the 12-trial session (CONTROL: 19.66 ± 3.41 vs. DEPRIVED: 52.07 ± 4.18; $F_{11,12} = 8.33, p < 0.041, \eta^2 = 0.17$; repeated-measures ANOVA) and probe trial ($F_{11,3} = 3.58, p < 0.03, d = 0.84$, independent samples t-test). In summary, perform-
ance in the MWT showed that rats that were repeatedly deprived of vertical activity exhibited significant alterations in their spatial search strategies in the absence of spatial learning and memory deficits.

4.1.1.2.2. Spatial performance in the ZT. Fig. 2, Panels A–F shows a summary of latency and path speed in the ZT during the pre-
test (Panels A and B) and post-test (Panels D and E) sessions. Like MWT, all rats regardless of their experimental condition were able to locate the spatial target within the ZT in both test sessions. An
Fig. 2. Spatial performance measured by latency and speed within the wet- and dry-land tasks before and after rearing deprivation. Morris Water Task (MWT). A–F. Spatial memory assessment within the MWT using a one-day testing protocol. Latency and swim speed to locate the hidden platform during 12 trials of testing before (A and B) and after (D and E) rearing deprivation indicated no significant difference between groups in terms of spatial performance. Zigzag Task (ZT). A–F. Single-day testing in the standard version of the ZT for spatial performance. Latency and path speed to find the goal ziggurat during 12 trials of testing confirmed that all animals were able to locate the spatial goal before (A and B) and after (D and E) rearing deprivation. Note the occupancy of target area locations (light quadrant) within the MWT and ZT during the pre- and post-test probe trials (C and F) that indicate no differential search strategy between groups.

ANOVA conducted for the latencies over the 12 trials of the ZT indicated no significant differences among groups (Pre-test: $p \geq 0.061$; Post-test: $p \geq 0.104$) suggesting that vertical activity deprivation had no impact on the time to locate the goal ziggurat. A significant main effect of Trial, however, was found for both time points (Pre-test: $F_{11,12} = 11.84, p \leq 0.03, \eta^2 = 0.23$; Post-test: $F_{11,12} = 17.22, p \leq 0.037, \eta^2 = 0.19$; repeated-measures ANOVA).

Examination of path speed during acquisition showed a similar profile of spatial performance in the ZT and the MWT. ANOVA did not show a significant effect of Group (Pre-test: $p \geq 0.110$; Post-test: $p \geq 0.093$) and Trial (Pre-test: $p \geq 0.47$; Post-test: $p \geq 0.946$).
Fig. 3. Spatial navigation within the wet- and dry-land tasks before and after rearing deprivation. A. Representative swim paths of control and rearing deprived rats across testing trials (trials 1, 3, 6, 9, 12) within the MWT before and after deprivation. Red stripes indicate starting points and small white circles indicate the location of the hidden platform. Despite intact spatial memory in the wet land to locate the hidden platform, rearing deprived rats displayed different search strategies even during the last trials by showing more “circling” than controls. Occupancy plots (probe-right row) of paths taken by rats during 30s probe trial within the MWT. Each plot that is compiled from individual tracks shows a spatial bias toward the former training quadrant. Again, deprived animals showed enhanced circling. B. Examples of search paths in control and rearing deprived rats in the standard version of the ZT before and after deprivation. Red arrows indicate starting point. Like MWT, frequency density analysis of accumulated trajectories (probe-right row) reveals paths focused on the target quadrant where the non-baited goal ziggurat was presented. Note black arrows that represent points where rearing occurred during spatial navigation to the central goal ziggurat. C. The number of rears after rearing deprivation was higher than in the control condition. Panels D and E depict samples of error and rearing that occurred during spatial navigation in the ZT. Deprived animals also showed more rears than control rats in post-test probe trial (F). However, the observed difference was not significant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
MWT, both groups exhibited a focused pattern of spatial searching in the probe trial within the ZT, with clear spatial bias toward the former training quadrant (Pre-test: quadrant four; NW: Post-test: quadrant one; NE: Fig. 2C and F). There was no significant difference between groups in terms of the search time in training compared to the opposite quadrant of the task (Pre-test: \( p = 0.67 \); Post-test: \( p ≥ 0.083 \); ANOVA) suggesting that rats in both groups spent a considerable portion of their time searching in the quadrant of the ZT in which the goal ziggurat had previously been baited.

Despite their intact spatial performance in the ZT, DEPRIVED rats reared more than CONTROL animals in the post-test phase of spatial performance assessment. These results were confirmed by statistical analysis for Group (Pre-test: 96.04 ± 12.19 vs. 87.44 ± 13.19, \( p ≥ 0.088 \); Post-test: 128.46 ± 12.47 vs. 66.81 ± 11.46; \( F_{1,12} = 7.24, p ≤ 0.047, n^2 = 0.16 \)) and Trial (Pre-test: \( F_{1,12} = 6.82, p ≤ 0.036, n^2 = 0.19 \); Post-test: \( F_{1,12} = 10.33, p ≤ 0.042, n^2 = 0.15 \); ANOVA) effects with no significant interaction effect. DEPRIVED animals also showed more rears than CONTROL rats in the post-test probe trial (6.22 ± 1.31 vs. 4.77 ± 1.14), but the observed difference was not significant (\( p ≥ 0.058 \)). Overall, our results (Fig. 3) revealed that vertical activity deprivation had no significant impact on spatial performance in the ZT. However, DEPRIVED rats reared significantly more than CONTROL rats when they were searching for the spatial goal ziggurat in the task (\( p ≤ 0.041 \)).

4.2. Experiment 2

4.2.1. Rearing deprivation differentially affected corticosterone and glucose levels

CORT measurements (Fig. 4) revealed a significant effect of Group (\( F_{1,12} = 3.57, p ≤ 0.041, n^2 = 0.21 \)) and Time (\( F_{1,11} = 8.14, p ≤ 0.046, n^2 = 0.21 \)), but no significant interaction between Group and Time (\( p ≥ 0.170 \)). Both CONTROL and DEPRIVED groups showed approximately the same levels of CORT at baseline (129.57 ± 39.71 ng/ml and 117 ± 42.13 ng/ml, respectively: \( p = 0.570 \)). The acute-time CORT levels for CONTROL and DEPRIVED rats indicated that DEPRIVED animals showed slightly higher CORT levels than CONTROL rats (209.01 ± 53.19 ng/ml and 133.86 ± 61.55 ng/ml: \( p ≥ 0.063 \)). However, blood samples assayed for plasma CORT levels at chronic time-1 (day 15th) and 2 (day 30th) showed that HPA activity was affected by the rearing deprivation protocol as indicated by significantly enhanced CORT levels in DEPRIVED animals (chronic time-1: 346.72 ± 67.39 ng/ml vs. 113.07 ± 78.52 ng/ml; \( p ≤ 0.0313 \); chronic time-2: 306.20 ± 58.11 ng/ml vs. 139.68 ± 57.02 ng/ml; \( p ≤ 0.042 \)).

GLU measurements, however, revealed a different profile of rearing deprivation-induced alterations (Fig. 4). There were no differences between groups in blood GLU levels at any time point. Average baseline blood glucose levels were 5.17 ± 0.41 mmol/L for the CONTROL group and 4.66 ± 0.38 mmol/L for the DEPRIVED group (\( p ≥ 0.068 \); ANOVA). Although DEPRIVED animals indicated higher levels of GLU than CONTROL rats at both acute time and chronic time-1 (acute time: 5.03 ± 0.38 mmol/L vs. 4.81 ± 0.46 mmol/L; chronic time-1: 5.69 ± 0.48 mmol/L vs. 4.90 ± 0.42 mmol/L), the observed differences were not significant (\( p ≥ 0.337 \) and \( p ≥ 0.072 \), respectively). There was also no significant difference between groups in terms of the GLU levels at the chronic time-2 (CONTROL: 4.76 ± 0.39 mmol/L, DEPRIVED: 5.11 ± 0.44 mmol/L; \( p ≥ 0.066 \)) suggesting that repeated rearing deprivation had no significant impact on blood GLU at any time point of assessment. Overall, our analysis indicate that blood GLU was not significantly affected by rearing deprivation, whereas rearing deprivation resulted in elevated CORT levels at chronic time points-1 and 2 compared to CONTROL rats.

4.2.1.1. Rearing deprivation does not alter anxiety-like behavior in the EPM. One DEPRIVED rat was excluded from scoring and analysis for anxiety-related behavior in the EPM due to accidental loss of information. Both CONTROL (\( N = 7 \)) and DEPRIVED (\( N = 7 \)) animals spent most of the time in the closed arms before and after deprivation. No significant between-group difference was observed in the time spent in the open arms, time spent in the end of the open arms, and time spent in the central square of the EPM (all \( p ≥ 0.05 \)). Thus, rearing deprivation does not affect anxiety-like behaviors in the EPM.

4.2.1.2. Rearing-deprived rats emitted a greater number of 22-kHz USV calls. The 22-kHz USVs were observed in 6 out of 8 animals. The DEPRIVED rats emitted a significantly greater total number of USVs than CONTROL rats (main effect of Group: \( F_{1,10} = 14.70, p ≤ 0.026, n^2 = 0.24 \); repeated-measures ANOVA; Fig. 5A and B). Also, DEPRIVED rats emitted significantly more calls than CONTROL animals at the second (minutes 61–65; \( p ≤ 0.008 \)), third (minutes 121–125; \( p ≤ 0.036 \)) and fourth (minutes 176–180; \( p ≤ 0.041 \)) time points. No interaction between Group and Time, however, was observed. Further analysis of the duration of calls indicated that DEPRIVED rats not only spent significantly longer time for producing distress calls than CONTROL animals (main effect of Group: \( F_{1,10} = 19.13, p ≤ 0.04, n^2 = 0.22 \); main effect of Time: \( F_{3,10} = 8.06, p ≤ 0.041, n^2 = 0.19 \), repeated-measures ANOVA; Fig. 5C), they also emitted shorter USVs at the second time points when compared to the third and fourth recording sessions (\( F_{3,5} = 5.78, p ≤ 0.046, n^2 = 0.18 \); one-way ANOVA; Fig. 5D). The latter observation indicates that early times of deprivation imposed different emotional loads to the DEPRIVED rats than the later times. In summary, the 22-kHz USV parameters (number and duration of the calls) in the present experiment indicated that rearing deprived rats emit more distress calls compared to controls.

4.3. Structural assessment

4.3.1. Rearing deprivation does not alter total hippocampal volume

Despite slightly reduced hippocampal volume in DEPRIVED rats, no significant difference was observed between groups in terms of the total hippocampal volume (effect of Group: \( p ≥ 0.116 \), effect of Hemisphere: \( p ≥ 0.082 \)) indicating that rearing deprivation had no significant impact on the volume of the left and right hippocampus.
4.3.2. Rearing deprivation reduces CA2 volume and neuronal quantity

Volumetric analysis of hippocampal regions (granular layer of the DG, and pyramidal layers of the CA1, CA2 and CA3) revealed that DEPRIVED rats had significantly reduced volume in only CA2 when compared to the CONTROL group (0.19 ± 0.04 vs. 0.28 ± 0.04; F1,8 = 5.18, p ≤ 0.04, η² = 0.17, repeated-measures ANOVA; Fig. 6). No interaction between Group and Region, and no effect of Hemisphere, and interaction between Hemisphere and Region was observed (all p ≥ 0.05).

The number of neurons only in the CA2 was significantly reduced by rearing deprivation (F1,13 = 10.04, p ≤ 0.039, η² = 0.18, repeated–measures ANOVA; Fig. 7) suggesting that not only the CA2 volume, but also the number of neurons in this region of the hippocampus was influenced by the rearing deprivation (Control: 37 ± 1.24, Deprived: 30.1 ± 1.28 [×10³]). No significant differences, however, were found in neuronal quantity between DEPRIVED and CONTROL groups in the DG, CA1, and CA3 regions (all p ≥ 0.05). An additional analysis conducted for dorsal-ventral neuronal quantity in the rearing-deprived rats indicated lesser number of neurons in dorsal CA2 compared to the ventral CA2 (t2 = 11.61, p ≤ 0.039, d = 0.71, dependent samples t-test) suggesting that the dorsal CA2 was more susceptible to structural changes induced by rearing deprivation than ventral CA2 (Fig. 8).

5. Discussion

The present study examined the neuro-hormonal, behavioral and neuromorphological consequences in rats that were restricted in their vertical exploratory activity by temporary housing in a circular tunnel. The major findings of this study include following: (1) rats that were temporarily deprived of their rearing movements showed emotional and HPA axis disturbances; (2) spatial search strategies within wet- and dry-land spatial navigation tasks were considerably influenced by rearing deprivation, although there was no discernible effect on spatial performance; and (3) deprived animals exhibited hippocampal regional vulnerability involving the CA2 subregion.

Rearing represents a vital component of the normal exploratory behavioral repertoire of most rodents when introduced to novelty, such as an open field. Because three-dimensional exploratory behavior of rats is highly organized [70], the study of rearing during free and goal-directed exploration provides important insights underlying physiological, emotional and motoric disturbances. Both neurochemical and behavioral assessments indicate that changes in vertical activity are associated with altered brain morphology and behavior [61,29,62,2,1]. From a neuropsychological point of view, rearing also reflects emotional responses to a changing environment, such as novelty and increased stimulus intensity [27]. These findings predominantly emphasize on the dynamic role of vertical activity spatial and motor behaviors in the rat.

Using three standard measurements of emotionality in rats, including motor activity in elevated plus maze, plasma CORT and 22-kHz USVs, the present study characterized the emotional consequences of rearing deprivation. Despite the lack of the anxiety-related behavior within the elevated plus maze, elevated CORT levels and vocalization patterns indicate that the lack of rearing in combination with exposure to the circular tunnel task activated the HPA axis. Interestingly, the deprived group showed no habituation to the repeated exposure to the restrictive circular tunnel task. Habituation is a characteristic aspect of neurohormonal responses to repeated stressful experiences [25,18], which is usually accompanied by reduced CORT levels. However, chronic
measurements of plasma CORT in the present experiment showed a persistent physiological vulnerability to rearing deprivation. This lack of physiological habituation confirms the need of rats to participate in free exploration. In addition, this observation suggests that involuntary tunnel exposure, although it mimics the natural burrow environment, induces persistently high emotionality and is perceived as an enduring challenge that prompts a lasting stress response.

Signals of emotional disturbance related to rearing restraint were also supported by changes in USV patterns. In contrast to 50-kHz USVs or happy calls, the 22-kHz USVs, termed distress calls, have been found to be a valid measure of stress-induced emotionality in rats. These calls are mainly emitted in negative situations or during presentation of aversive stimuli such as stress and aggression. One of the main findings of the present study was that 22-kHz calls were emitted at consistent and relatively high rates throughout the rearing deprivation period in the tunnel. The line histogram of USV calls during the deprivation indicates that there was a warm-up period (the first 5 min) in which both number of USVs and the time spent calling gradually rose and then are found in higher rates during the second to fourth periods of deprivation. Rearing deprivation exposure also induced an evident profile of changes in the pattern of distress calls in which the duration of the 22-kHz USVs in the second 5-min interval (minutes 61–65) of the deprivation period.
were significantly shorter than the third 5-min interval (minutes 121–125). It is not obvious whether the short or long USVs represent more severe distress responses to the deprivation periods. Both, however, reveal a significant emotional sensitivity to acute and chronic associated with the tunnel and limited freedom of exploration. More importantly, in conjunction with the interpretation of a definite behavioral relevance of USVs to the deprivation exposure, the 22-kHz calls may also be construed as a predictive emotional index for learned helplessness in animal models of stress employing inescapable, aversive stimuli [68]. Aside from emotionality, our data indicate that repeated periods of rearing deprivation may challenge other aspects of behavioral capabilities such as spatial performance.

Despite intact spatial performance, the present study revealed a unique profile of changes in search strategies within both wet- and dry-land testing arenas. Search strategies have been shown to be affected by brain lesions [17,7]. In our data, however, search strategies were influenced by repeated rearing deprivation. In particular, stress associated with rearing deprivation turned the formerly spatial search strategies into mainly non-spatial strategies, especially inducing “circling” (swimming in close circles; [7] within the Morris water task and “rearing-dependent navigation” in the ziggurat task. Changes in search strategies may not necessarily reveal a direct effect of rearing deprivation when other aspects of spatial performance (e.g., path latency, length and speed) remained unaffected. However, these changes may confirm the hypothesis [26] that spatial performance, at least under certain experimental conditions, needs to be assessed through alternative hippocampus-specific parameters.

It remains to be determined whether circling – or rearing – based search strategies in deprived animals originate from a failure to utilize egocentric and/or allocentric route-knowledge, or they simply serve as a set of compensatory behaviors secondary to a primary structural deficit. Both increased circling and rearing during spatial navigation can arguably be attributed to a lower contribution of allocentric comprehensions. Interestingly, hippocampal lesions have been shown to prevent the occurrence of allocentric search strategies [17]. Furthermore, an egocentric route-knowledge in spatial tasks like Morris water task or ziggurat task does not necessarily result in longer latency or path length, although it may be associated with less efficient strategies combined with, for instance, “scanning” and “local search”. Therefore, the enhanced number of circling in the Morris water task and rearing in the ziggurat task after rearing deprivation may be considered a subtype of a compensatory and/or integrating behavior. Specifically, we hypothesize that these behaviors promote the animals’ capabilities to compensate a transient central processing deficit and, in the context of spatial function, they increase the integration of egocentric route-knowledge into an allocentric representation [26]. Such a process is the most important feature of a successful spatial navigation with which an animal is able to increase search accuracy for a hidden or baited goal, and it critically depends upon hippocampal regional integrity.

The present findings demonstrate striking regional differences in hippocampal volume emphasizing changes in the CA2 inter-

![Fig. 7. DAPI-stained coronal sections of the dorsal hippocampus. Atlas plates of the HPC shown in the top row depict the corresponding CA2 subregions on the sections. Note the reduced neuronal quantity in the CA2 subregion of the HPC in the deprived (D–F) compared to control (A–C) rats. Subpanel f shows that deprivation reduced neuron numbers in right and left hippocampus when compared with controls. *p < 0.05, ANOVA. Error bars show ±SEM. Atlas plates are from [51] approximately equal to −3.24, −3.48 and −4.20 mm relative to bregma. Magnification 10×.](image)

![Fig. 8. Changes in neuronal quantity in dorsal versus ventral CA2 caused by rearing deprivation. Panel shows brain sections labeled with DAPI (N = 8). Each dark and light green square represents the corresponding section (dorsal or ventral) in which rearing deprivation caused a significant difference. Neuronal quantity in both left (A) and right (B) hemispheres indicated that dorsal CA2 had significantly lower numbers of neurons than ventral CA2 in rearing-deprived animals. No significant difference was found between left and right CA2 in terms of the neuronal quantity. B: brain; L: left; R: right. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
estingly, dorsal CA2 cells were more affected by the deprivation protocol than ventral CA2 cells. The anatomical characterization [46,10,9,14,41] of the hippocampal cornu ammonis (CA2) and its contribution to hippocampus-dependent performance [37,32] still continue to be important questions.

First introduced by [44], the hippocampal CA2 is a relatively small region with a small population of large pyramidal cells [9]. Perhaps because of the difficulty in determining its anatomical borders (between the hippocampal CA1 and CA3 regions) and narrow regional extent that CA2 has been much less investigated than other hippocampal CA regions. However, the most recent studies of hippocampal neural circuits linked to CA2 indicate that dentate gyrus (DG) cells, the highly specified hippocampal granule neurons for spatial behavior send unique functional monosynaptic inputs to CA2 pyramidal cells [41]. CA2 also plays a key role in coordinating the hippocampal circuitry [47,10] via its extended axons and broad dendritic arbors with all CA regions, indicating that the CA2 represents more than just a CA1–CA3 passive transient zone. Beyond the previous and current challenges over the anatomical correlates of the hippocampal function (e.g., the classical trisynaptic circuit of the entorhinal–hippocampal connectivity; [4,63,10,41]), the modulatory role of the CA2 region in the hippocampal circuitry and function is increasingly well documented.

In the present study, despite a pronounced loss of the CA2 pyramidal cells, we failed to observe a significant deficit in rats’ spatial performance, although there was a trend for deprived rats, for e.g., to learn the ziggurat task more slowly. These behavioral results support the latest findings in which functional inactivation of dorsal CA2 caused no deficit in hippocampus-dependent spatial memory [52]. However, given the previously reported involvement of the hippocampus and its general modulatory role in rearing behavior [13,54,31,12,29,62] regional structural alterations, especially in dorsal CA2, highlight the pivotal role of the CA2 region in such behaviors that commonly appear independent of the hippocampus. Whether this unique profile of changes induced by deprivation in CA2 affects the hippocampal capacity for encoding, processing, storage and/or recall still require further evidence at the subcellular level. Furthermore, a limitation of the present results is the use of different animals for Experiments 1 and 2. This experimental design does not allow correlations between the individual measurements of the two experiments and limits further interpretation of the data with respect to the aversive structural and functional consequences of vertical activity. Nevertheless, for the first time our data decipher CA2 specific function in exploratory behavior, and that rearing probably is a key behavioral component linked to hippocampal function.

The current study suggests that hippocampal dorsal CA2 is particularly susceptible to the stress caused by exploratory deprivation. The presence of mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs) renders the hippocampal formation sensitive to CORT effects and thus modulates spatial navigation [15,50,74]. Because the hippocampus is a major component for negative feedback regulation of the HPA axis, chronic stress may produce structural changes in hippocampal subregions, such as the CA2, that in turn makes the hippocampus more vulnerable to neurotoxic challenges [24]. In the present study, rats with elevated circulating CORT levels showed reduced volume and neuronal numbers in CA2. The CA2 in particular is characterized by high MR density [30], which is reduced by chronic stress [42]. Repeated rearing deprivation may disrupt the balance of MR and GR in the CA2 thus altering spatial navigation strategies and exploratory movements [35], which may be further aggravated by its reduction in volume. It is also well-documented that CA2 receives direct inputs from amygdala [4], a brain structure intimately involved in the formation and storage of memories associated with emotional events. Furthermore, CA2 is the only hippocampal CA subfield to be innervated by the supramammillary (SuM) body [47], a hypothalamic nucleus thought to be actively involved during stressful experiences [11]. Therefore, the CA2 regional susceptibility to psychological stress offers a new direction for future investigations of hippocampus-dependent behavioral dynamics underlying free and goal-directed navigation.

Conflict of interest
None to declare.

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