Research Article

Vibrotactile Control of Spontaneous Gap Crossing in Premature Rat Pups

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Sensory feedback is critical for locomotor decisions, but its role in ethologically relevant behavior during early development remains poorly understood. Here, we investigated whether functionality of the vibrotactile system is necessary for spontaneous gap crossing in premature rat pups, and whether conventional open-field locomotion measures predicts such naturalistic behavior. Wistar rat pups received a bilateral vibrissaectomy (VE) on postnatal day (PND) 9, while sibling controls received sham treatment or left intact. Litters were reared in naturalistic home cages containing a dark nest area and equipped with a set of elevated platforms spaced 1-6 cm apart. Spontaneous gap-crossing activity was continuously videorecorded for 36 hours (PND 17-18) in darkness. On PND 18, a subset of pups underwent open-field testing followed by histological assessment of perineuronal nets (PNNs) in layer IV of the barrel cortex. Among pups that voluntarily attempted gap crossing, VE group showed impaired performance on larger gaps (5-6 cm) relative to controls. Notably, the degree of PNN maturation in layer IV of the barrel cortex correlated with the percentage of wide crossings, suggesting a link between cortical circuit maturation and sensorimotor competence. Gap-specific performance measures were independent of standard open-field behaviors, whereas aggregate locomotor metrics (total number of gaps crossed, platform-climbing attempts) correlated with locomotor vigor (mean speed and acceleration). Additionally, the trajectory compaction ratio (Cr) negatively correlated with these metrics of gap-crossing activity. These findings demonstrate that spontaneous dark-phase gap crossing in premature rat pups depends critically on the functionality of the vibrotactile system and correlates with structural maturation of the barrel cortex. The study underscores the need for naturalistic behavioral assays in developmental neurobiology.

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1. Introduction

1.1. General Introduction to Early Postnatal Development in Rats (Postnatal Weeks 2–3)

Rodent development is marked by a gradual transition from a predominantly nest-bound mode of life to active exploration of the external environment, a shift that coincides with eye opening, increasing locomotor competence, and growing independence from maternal support [1][2][3]. During this period, pups spend less time in the nest and progressively more time engaging in exploratory activity, reflecting a transition from passive dependence to more self-directed interaction with the environment [1][4][5]. This change is not purely behavioral, but also reflects the maturation of sensorimotor systems that support orienting, posture, and locomotion, as well as limbic and fronto-executive networks involved in novelty detection and motivational regulation [6][7]. In this sense, the emergence of exploratory behavior provides a functional readout of early postnatal brain development, a period characterized by rapid refinement of cortical circuits through activity-dependent mechanisms and experience-driven plasticity [8][9][10].

1.2. The Nesting Period and the Home Cage as a Behavioral Arena

Early behavioral organization in rodent litters is best understood in an ecological context that emphasizes the interdependence of maternal provisioning, thermoregulation through huddling, and rich social contact within the nest [11][12]. These factors jointly create a microenvironment in which pups spend the majority of their time during the first postnatal weeks, and in which their earliest motor behaviors—twitches, crawling, and short excursions—are embedded [12][13][14]. Within this setting, spontaneous locomotor activity recorded in the home cage provides a global measure of the pup's general level of activity, reflecting ongoing adaptations to the environment [6][15].

As development proceeds, pups progressively shift from remaining in the nest to exploring the surrounding cage space, indicating that sensorimotor circuits have matured sufficiently to support more sustained, coordinated movement [6][15]. Studying behavior in ecologically relevant contexts is essential to capture critical interactions between environmental variables in natural settings [16].

1.3. Two-dimensional and three-dimensional locomotion in rodent pups

Rodent locomotion can be usefully distinguished into 2-D and 3-D components. Two-dimensional locomotion refers to horizontal displacement on a flat surface, such as in the home cage or open field,

and is commonly quantified by measures of distance traveled, speed, and path organization [17][18][19][20]. By contrast, 3-D locomotion includes vertical exploration, rearing, jumping, climbing, and obstacle crossing, all of which place substantially greater demands on the nervous system [21][22]. In particular, the execution of 3-D movement requires continuous integration of visual cues, proprioceptive feedback, postural control, muscle strength, and motor planning, making it a more complex and ecologically relevant indicator of sensorimotor function than planar activity alone [23][24][25]. Accordingly, 3-D locomotor assays may reveal developmental or experimental phenotypes that are not detectable in conventional 2-D paradigms [26][27][28]. From an evolutionary perspective, the ability to move effectively in three dimensions is critical for survival in natural environments, where animals must negotiate uneven terrain, vertical surfaces, and discontinuous pathways in the context of foraging, predator avoidance, and shelter seeking.

1.4. Open Field Testing: Assessing Anxiety-Related and Exploratory Behavior

The open field paradigm remains a cornerstone for assessing anxiety-like behavior, exploratory activity, and locomotor patterns in rodents, capitalizing on the innate conflict between the drive to investigate a novel environment and the tendency to avoid exposed spaces. Behavioral readouts are typically framed in terms of the central versus peripheral sectors of the arena, with the periphery representing a relatively safe, wall-protected zone and the center an exposed, high-risk area; shifts in occupancy between these zones are therefore commonly interpreted as changes in anxiety level and exploratory motivation [29][30][31][32]. Standard metrics such as total distance traveled, movement speed, and freezing behavior allow decomposition of overall activity into components reflecting general arousal versus specific defensive strategies. Recently, we have shown that the spatial and locomotor parameters registered during open field test in rat pups might be differently sensitive to sensory deprivation [33]. A predominance of peripheral, wall-adjacent movement and compact trajectories is consistent with increased thigmotaxis, greater reliance on tactile and proprioceptive cues, and avoidance of open space, all of which are typically associated with elevated anxiety-like state [29][34][35].

1.5. The Barrel Cortex and Perineuronal Networks (PNNs)

The barrel cortex provides a model of precise topographic organization and experience-dependent development, making it highly relevant for studies of early sensory processing [36][37][38][39]. Single-barrel lesions permanently impair the discrimination of spatial object location [40]. In this region,

PNNs are closely associated with parvalbumin-positive interneurons and are thought to reflect the maturation of inhibitory circuits and the stabilization of critical-period plasticity [41][42]. Given their role in shaping sensory integration and motor performance, PNNs may contribute to the emergence of coordinated locomotion during [43][44][45]. Nevertheless, the relationship between cortical structural maturation and 3-D locomotor behavior in early development remains poorly understood.

The present work was designed to investigate the association between 2-D and 3-D locomotor patterns in rat pups at the third postnatal week and to evaluate the contribution of local PNN maturation to these behavioral outputs. We proposed two hypotheses: first, that PNN maturation is a prerequisite for successful 3-D locomotion, operationalized as a greater proportion of long gap jumps; and second, that parameters of induced locomotor activity in the open field would mirror the developmental status of spontaneous 3-D locomotion.

2. Materials and Methods

2.1. Animals

The heading Outbred Wistar rats sourced from the Stolbovaya breeding facility were paired for mating at the Animal Unit of the Institute of Higher Nervous Activity and Neurophysiology, Russian Academy of Sciences (IHNA&NPh, RAS). The animal study protocol was approved by the Ethics Committee of IHNA, the protocol N 04290520243. Pregnant females were housed individually in two-section polycarbonate cages (65 × 40 × 40 cm each) containing wood saw bedding and shredded paper towels as nesting material. The light and dark compartments were connected by a dark rectangular tunnel measuring 10 × 10 × 19 cm. In the dark compartment, nine plastic platforms (6 × 6 × 6 cm each) were placed at varying distances from each other (1-6 cm). The arrangement of the platforms is shown in Figure 1B. Video was recorded continuously in both compartments. For video recording in the dark compartment, a lamp emitting light in the red spectrum (606-656 nm), invisible to rats, was installed. The complete housing and continuous video-monitoring setup was provided by Open Science (Moscow, Russia). The animal rooms were kept under a 12:12 hour light-dark cycle (lights switched on at 8:00 AM), with temperature regulated at 22 ± 2 °C and relative humidity at 50 ± 10%. Standard pellet food and tap water were always available in the light compartment. Bedding was replaced once per week to maintain cleanliness and limit stress. Dams were checked daily for newborn pups. To minimize disturbances around the time of delivery, cages were not handled on the postnatal day (PND) 1. On PND 2, litter sizes were adjusted to nine

pups. The day of delivery was recorded as PND 0, and litters remained undisturbed until PND 9. Vibrissaectomy was performed on postnatal day 9 (PND 9; see below). Individual fur marks were renewed on PND 14.

Four litters were left undisturbed from the day of vibrissae trimming until postnatal day (PND) 18, at which point the pups were removed from the nest for behavioral testing (open field) and subsequent histological analysis. These litters are hereafter referred to as Experiment 1 (big litters). Nine additional litters were used in a combined experimental investigation. From each of these litters, three pups were allocated for behavioral testing and morphological assessment on PND 10 and PND 14 (results to be reported). Consequently, by PND 18, each litter contained only three pups. These litters are hereafter referred to as Experiment 2 (small litters).

2.2. Vibrissectomy

Prior to handling the pups, the mother was removed from the home cage. Right before the procedure, the pups were taken out and placed temporarily in a warm container with a homecage bedding. On postnatal day 9 (PND 9), pups from each litter were divided into an experimental group and 2 control groups. In the experimental group (VE group), all whiskers were cut down bilaterally to the fur level. In the control groups, a sham procedure was carried out over the same day, involving gentle contact of the scissors with the base of the whiskers without actually cutting them (Sham group), or the rat pups were left intact (Intact group). Throughout the procedure, each pup was gently grasped to restrict movement. Both the whisker trimming and the sham procedure took 60-120 seconds per pup. Following the procedure, the pups were marked for identification, and then placed back into their home cage. The mother was then returned to the cage.

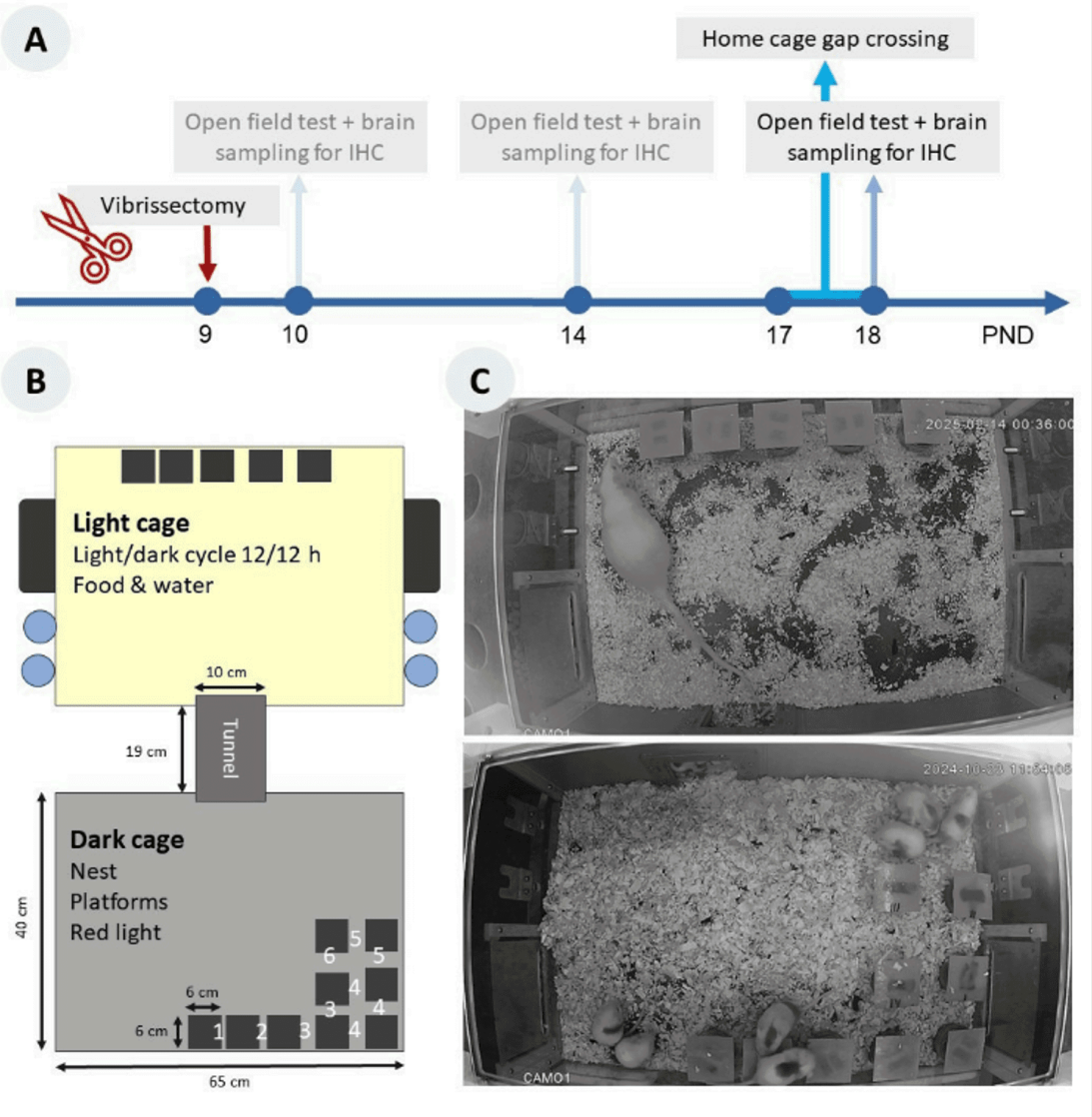


Figure 1. Experimental timeline and housing setup for home-cage gap crossing. (A) Timeline of experimental manipulations. (B) Schematic of the dual-compartment housing system, consisting of a light cage, a dark cage, and a connecting tunnel. (C) Representative images of the cage environment used during the experiment.

On postnatal day (PND) 2, litter sizes were adjusted to 9 pups. Two experimental protocols were followed. Experiment 1 (4 litters of 9 pups): Vibrissectomy was performed at PND 9, followed by home-cage gap crossing observation on PND 17-18 and brain collection for immunohistochemistry (IHC). Pups remained with their families until PND 18. Experiment 2 (9 litters): Vibrissectomy was performed at PND 9, followed

by the removal of 3 pups on PND 10 and 14, for behavioral testing and brain collection (shaded arrows; results to be reported). Home-cage gap crossing testing was observed on PND 17–18.

2.3. Assessment of Home Cage Activity

Under all experimental conditions, dams uniformly preferred to build their nests in the dark chamber, leaving it only to forage for food and water in the light chamber or to rest. Importantly, no gap crossing activity by pups was detected in the light compartment at any time.

Spontaneous locomotor activity in the home cages was assessed offline by tracking individual pups as they climbed onto, off of, and across elevated platforms. In recordings from the dark chamber, pups were identified by their fur markings. For each pup, every attempt to climb onto a platform (successful or not), and any subsequent horizontal locomotion on the platforms, was scored by observers blinded to experimental conditions. Attempts were classified as successful when all four limbs were placed on the platform. Attempts were classified as failed when the pup's forelimbs reached the platform but the pup did not fully climb onto it. The number of gap crossings was counted for each pup at each gap size (1-6 cm). Gap sizes were then grouped as small (1-2 cm), medium (3-4 cm), and wide (5-6 cm). A crossing was scored only if the pup traversed the gap without contacting the mother or littermates. Videos were analyzed over a 38-hour interval, from 00:00 on PND17 to 15:00 on PND18.

2.4. Open field

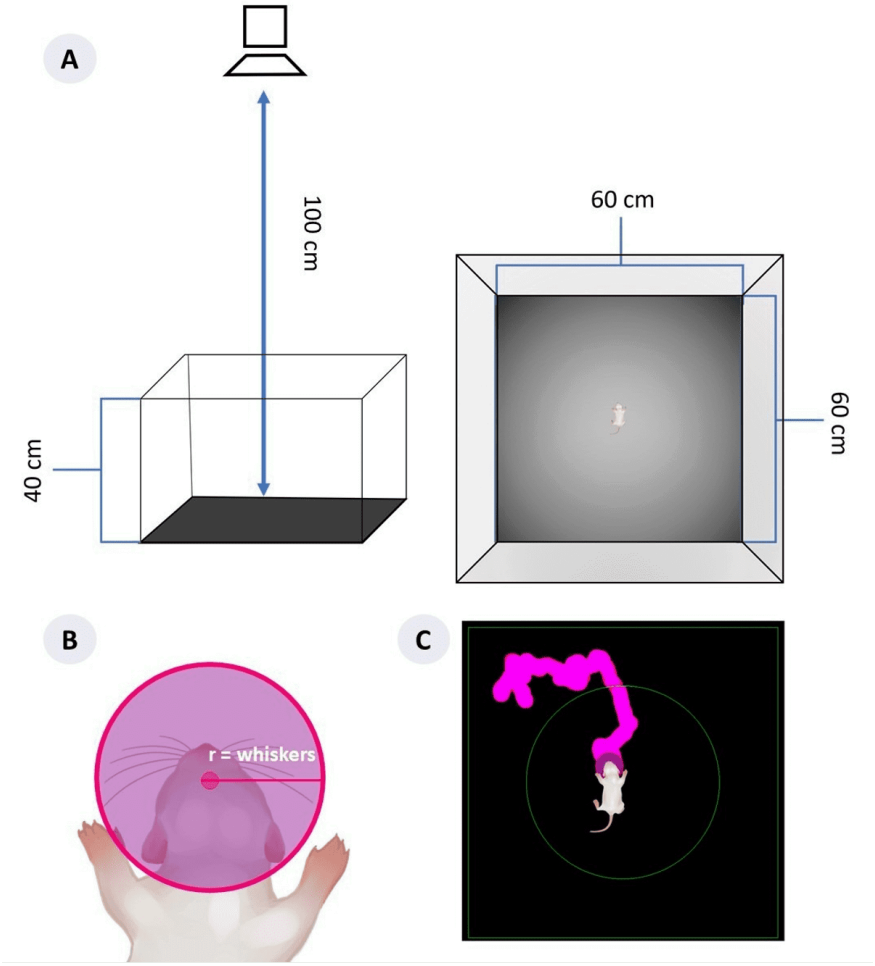


Figure 2. Experimental setup for open field test and trajectorial coating. (A) Overhead view of the open field apparatus. A digital camera was mounted 100 cm above the center of the arena to capture the full trajectory of pup locomotion. (B) Schematic representation of the whisking radius. The dashed circle indicates the approximate spatial range covered by vibrissae movements during active exploration, reflecting the vibrotactile sensing field available to the pup. (C) A schematic drawing of a rat pup on a real trajectory within the open field.

The behavioral testing procedure followed a previously described protocol [33]. To reduce stress, dams were separated from their litters only after they had spontaneously left their nests. The pups were taken out of the nest camera and then left undisturbed in a container with home beddings, allowing to stabilize in their huddles for 30 minutes. Individual pups were gently picked up from the edge of the huddle and

moved to an adjacent testing room. Every trial started immediately after placing the pup in the center of the arena. Each pup was tested once for 3 minutes, after which it was moved to another room and processed for morphological analysis (see the section 2.5.1.).

The open field apparatus was composed of two detachable components: vertical plywood walls (60 × 60 cm) painted black, and a replaceable plywood floor covered with black plastic (Figure 2A). This design prevented the accumulation of dust or olfactory cues in corners and gaps. Between trials, the arena was cleaned with a 50% ethanol solution and dried using lint-free wipes to remove any olfactory traces that might affect subsequent tests. A video camera was positioned 1 meter above the arena. The arena featured a brightly lit center (98-102 lux) and dimmer corners (32-38 lux). Pups were placed such that their heads faced away from the experimenter toward the opposite wall. Upon placement, video tracking commenced and lasted for 180 seconds.

2.4.1. Trajectory Analysis

Offline tracking was performed using ToxTrac (v 25.1.1) [46], which calculated the centroid of the white body against the black background at 400 ms intervals. Automated measurements included total distance traveled, average speed and acceleration calculations, and the duration of freezing episodes.

To quantify the spatial coverage of trajectories while reducing graphical noise, we calculated the trajectorial compaction ratio, as described recently [33]. Namely, each detected point was enclosed in a virtual circle whose radius matched the mean vibrissal length at PND 18 (Rv = 24 mm; Figure 2B). The total area of all superimposed circles was calculated in pixels. For each experimental trajectory, a linearized reference was generated by placing points along a straight line while preserving the subsequent inter-point distances. The track compaction ratio was defined as Cr = (coated linearized area) / (coated original area), with higher values indicating more spatially confined exploration. To isolate locomotor parameters independent of thigmotaxis, all analyses focused exclusively on a central zone (40 cm diameter), excluding the peripheral arena area [33].

2.5. Morphological Assessment

2.5.1. Tissue preparation

After the open field test, the rat pups were overdosed with chloral hydrate (450 mg/kg i.p.) and transcardially perfused with 200 ml of ice-cold 0.9% NaCl solution followed by 4% phosphate-buffered

paraformaldehyde (pH 7.4). After the end of perfusion, the brain was taken out of the scull and postfixed by immersion in 50 ml of 4% phosphate-buffered paraformaldehyde for a week with a change of the solution for fresh portion of the same fixative in 24 hours. After that sagittal 50-µm-thick sections were prepared using a Leica VT 1200 S vibrating microtome (Leica, Germany). The sections were collected in anti-freezing medium (PBS/glycerol/ethylene glycol) and stored at -20°C until processing.

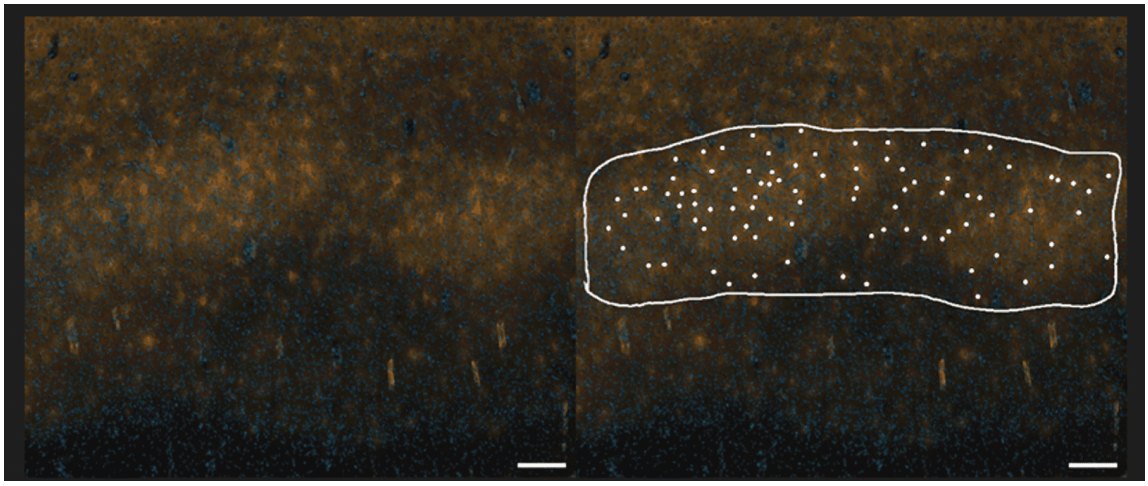


Figure 3. WFA staining of perineuronal nets in the barrel cortex and their quantification in layer IV. (A) Perineuronal nets (PNNs) labeled with WFA (Wisteria floribunda agglutinin) in the barrel cortex of a representative pup. PNNs appear as punctate or mesh-like structures surrounding neuronal cell bodies. (B) Identification of PNNs in cortical layer IV (the barrel field) for density counting. Only PNNs located within layer IV were included in the quantification to ensure consistency across animals. Scale bars = 100 µm WFA = Wisteria floribunda agglutinin; PNN = perineuronal net.

2.5.2. Histochemistry: staining for perineuronal nets markers

Perineuronal nets were stained using agglutinin of Wisteria Floribunda conjugated with biotin (WFA, L1516-2MG, Sigma-Aldrich, USA). Brain sections were washed in PBS 3 times for 5 minutes and then incubated with WFA diluted 1:1000 in 0.3% Triton X-100 (Serva, Germany) in PBS (PBST) at +4°C overnight. After that, the sections were three times for 5 minutes washed with PBST to clear off from the agglutinin and incubated with streptavidine conjugated with AlexaFluor546 fluorophores (# S11225, ThermoFisher, USA), diluted as 1:500 at the room temperature for 2 h. Then, the sections were washed with PBS, mounted onto glass slides and coverslipped using Fluroschield anti-fade reagent with DAPI (#F6057, Sigma-Aldrich, USA).

2.5.3. Microphotographs acquiring and processing

For quantitative analysis, we selected three neocortical areas: the barrel cortex and the secondary visual cortex. We used sagittal sections with coordinates 5.455 mm, -1.81 mm, 4.472 mm according to on-line rat brain atlas (https://atlases.ebrains.eu/). Microphotographs were acquired using Zeiss AxioImager.Z2 microscope, lens EC Plan-NEOFLUAR ×20, NA = 0.5, Axiocam 705 mono and software ZEN ver. 3.10 (Carl Zeiss, Germany). All the photos were taken with the same exposure which were equaled 300 msec for DAPI channel and 1500 msec for WFA channel. The size of images was fixed and equal to 1700.16×1418.64 µm (2464×2056 px), which included layers II-VI of neocortex. We took 1 image per neocortex area per section, and we used two sections per animal. Images saved as.szi files until further processing.

2.5.4. Estimation of perineuronal nets

Image processing was performed using ImageJ/Fiji software (version 1.53c; NIH, USA). The WFA channel was first extracted, and image contrast was enhanced using the Enhance Contrast function with histogram equalization (0.35%). To quantify the density of perineuronal nets (PNNs) in layer IV of the neocortex, PNNs clearly distinguishable from the background were counted within a defined region of interest. The contour of layer IV was manually outlined using the freehand selection tool, and the selected area was measured. PNNs located within this region and clearly distinguishable from the background were then counted (Fig. 3 A, B). The obtained counts were normalized to unit area (mm^2). All processing steps, from image opening to saving the results, were performed using a custom script.

2.6. Statistical analysis

Statistical processing was performed using a GLM ANOVA with type III sum of squares. The two control groups (Intact and Sham) were pooled for the analysis. The following predictors were entered into the model: litter size (big vs. small) and vibrissae status (trimmed vs. control) as fixed factors, and individual body weight at postnatal day (PND) 18 as a continuous covariate; the interaction between vibrissae status and body weight was also assessed. The sample comprised 4 litters of 9 pups each (Experiment 1) and 9 litters of 3 pups each (Experiment 2). For spontaneous gap crossing at the home cage, the following parameters were assessed: total number of gaps crossed, total number of trials to climb onto a platform, and total number of failed trials to climb onto a platform. Detailed analysis of gap crossing efficacy was subsequently restricted to pups that performed at least 20 gap crossings, regardless of gap width. Percentages of successful gap crossings for three gap sizes—small (1-2 cm), medium (3-4 cm), and large

(5-6 cm)—were analyzed as repeated measures, followed by Tukey's post-hoc tests. The minimum significance threshold was set at $p = 0.05$. Shapiro-Wilk test was used to assess the distributions' normality. For the open field test, the following parameters were assessed using a GLM ANOVA (as described above): average speed and acceleration, total distance traveled, total time spent freezing (obtained from automated analysis using ToxTrack software), and the compaction ratio for motion within the central and peripheral zones, as recently described, [33]. WFA staining in the barrel and visual cortices was analyzed in the same way, the vibrissae status (trimmed vs. control) was taken as fixed factors, and individual body weight at postnatal day (PND) 18 as a continuous covariate.

Additionally, Spearman's rank-order correlations with Benjamini–Hochberg correction were calculated for the full set of locomotor parameters, which included both the three-dimensional measures recorded under dark conditions and the two-dimensional open-field parameters obtained under light conditions (9 litters of 3 pups each). A separate correlation analysis was performed on a subset of data derived from the "behavior + morphology" experimental design, consisting of 5 litters with 3 pups each (5 experimental pups and 10 control pups).

3. Results

In total, we tested 63 pups from 13 litters across the two experimental series: 9 litters of 3 pups (as for PND 18), and 4 litters of 9 pups.

Experiment	Group	Sex	N	Body weight at PND 18	Weight gain from PND 9 to 18	Number of failed attempts	Total number of all attempts	All crossed gaps	Number of mother& sibling interactions
9-pups litters	Control	m	13	42.23 ± 1.61	20.3 ± 1.04	1.69 ± 0.38	20.46 ± 5.15	52.69 ± 15.96	6.54 ± 3.04
		f	11	37.91 ± 2.44	18.03 ± 1.2	2 ± 0.77	27.36 ± 7.55	52.73 ± 15.06	6.45 ± 1.84
	VE	m	7	41.86 ± 1.64	21.6 ± 1	3.57 ± 1.17	30.43 ± 7.78	60.57 ± 15.62	7.14 ± 3.77
		f	5	41.36 ± 2.91	19.15 ± 1.4	2 ± 0.45	26.6 ± 9.48	75.2 ± 35.74	11.2 ± 5.32
3-pups litters	Control	m	13	45.57 ± 2.52	27 ± 2.11	8.23 ± 2.79	30.69 ± 8.99	68.38 ± 21.49	3.23 ± 2.1
		f	5	43.63 ± 7.46	23.76 ± 5.83	3.2 ± 0.97	23.6 ± 11.61	38 ± 25.13	7.2 ± 4.45
	VE	m	5	48.21 ± 3.02	27.35 ± 3.93	12.2 ± 5.56	38.8 ± 16.79	65.8 ± 30.74	5.4 ± 4.65
		f	4	45.46 ± 2.59	28.31 ± 1.41	3.25 ± 1.25	12.25 ± 7.03	31.25 ± 27.4	3.25 ± 3.25
Litter size effect (p-value)				0.026	<0.01	<0.01	ns	ns	ns

Table 1. Experimental groups and summary of body weight, weight gain and home-cage gap crossing performance. Data are presented as mean ± SEM. Abbreviations: PND, postnatal day; N, number of animals per group. Number of failed attempts, Total number of all attempts, All crossed gaps, and Number of mother & sibling interactions refer to behaviors recorded during the spontaneous home-cage gap crossing task. Groups are defined according to vibrissae manipulation (trimmed vs. control), sex (female and male), and experiments (3-pups litters or 9-pups litters). Effects were estimated with ANOVA GLM. Only the effects of litter size were significant.

Body weights and sex ratios are presented in Table 1. Body weight differed between the two experimental series {F(1, 59)=5.23, p=0.03}, likely due to greater maternal milk availability for pups in small litters (3 pups) compared to big litters (9 pups). Sex and VE effects on body weights were insignificant.

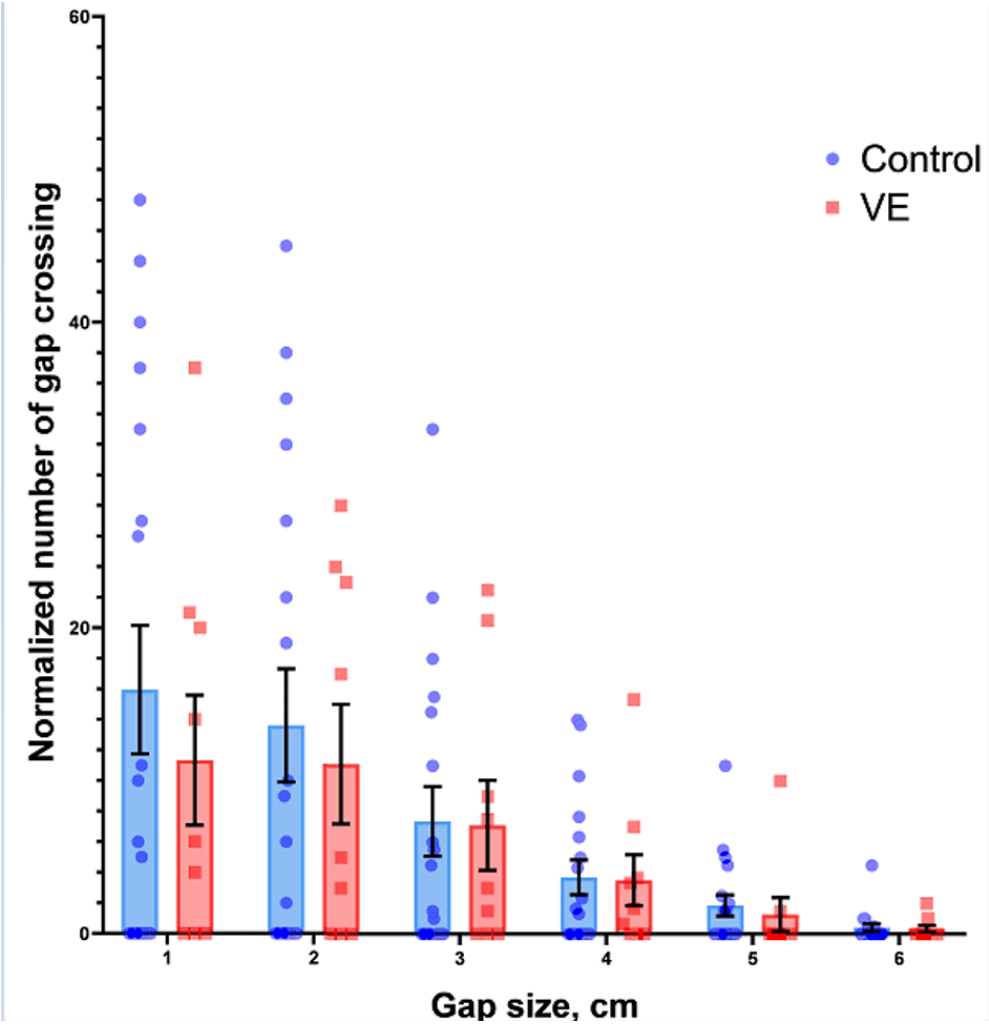


Figure 4. Normalized number of gap crossings as a function of gap size. The graph shows the normalized frequency of gap crossings for gap widths ranging from 1 to 6 cm in VE and control pups. Normalization was performed by dividing the actual number of crossings for each pup by the number of gaps of the same width available in the experimental setup. Data are presented as means ± SEM for each experimental group. VE = vibrissaectomized

All gap-crossing activity was observed exclusively in the dark compartment, with no such behavior occurring in the light chamber. To assess the frequency of crossings for each gap width, the data were

normalized: the actual number of crossings for each pup was divided by the number of gaps of the same width available in the experimental setup. In general, short gaps were crossed frequently, whereas large gaps posed the greatest challenge for the pups (Figure 4). Notably, three out of nine small litters did not initiate gap crossings. Despite having a greater body weight on PND 18 (Table 1), pups from small litters initiated gap crossing significantly less frequently than those from larger litters (17 out of 27 vs. 34 out of 36; χ^2 test with Yates' correction, p = 0.03). Also, the pups of small litters demonstrated a higher number of failed attempts to climb on a platform{F(1, 59)=9.44, p<0.01}, as compared to big litters. Together, these findings suggest that we are observing the earliest stage of three-dimensional locomotion, and that pups from small litters are likely to initiate this behavior later.

Given the assumption that vibrissectomy impairs three-dimensional locomotor performance in the dark compartment, we hypothesized that large gaps would pose greater difficulty for pups following VE relative to control pups. To evaluate this hypothesis, the percentage of successful large gap crossings was compared between the VE and control groups. The analysis was restricted to pups that commenced gap crossing and performed at least 20 spontaneous gap crossings (N=14 for VE group and N=22 for the controls). With this selection criteria, a repeated-measures ANOVA revealed a significant main effect of gap size (1-2, 3-4, or 5-6 cm) on gap crossing activity ($F(2, 62) = 154$, $p < 0.001$), indicating that activity changed across the gaps' widths. There was a significant gap size×body weight interaction ($F(2, 62) = 6.24$, $p = 0.003$), suggesting that the pattern of change in activity depended on body weight. The univariate analysis of the percentage of large gaps crossing (%5-6) showed significant main effects of experiment and body weight at PND18 (experiment: $F(1,31) = 17.22$, $p = 2.40 \times 10\text{^}-4$; body weight: $F(1,31) = 17.81$, $p = 1.97 \times 10\text{^}-4$). VE status also had a significant main effect on the percentage of large gap crossing ($F(1,31) = 4.98$, $p = 0.033$; partial $\eta 2 = 0.14$)). The VE status × body weight interaction approached significance ($F(1,31) = 3.57$, $p = 0.068$). This indicates that the percentage of large gap crossing is the most sensitive measure, being influenced by animal weight, VE status, and litter size. Post-hoc Tukey HSD test for the percentage large (5-6 cm) gap crossing was significant for VE status (p=0.046), supporting the presence of its effect on the large gap crossings.

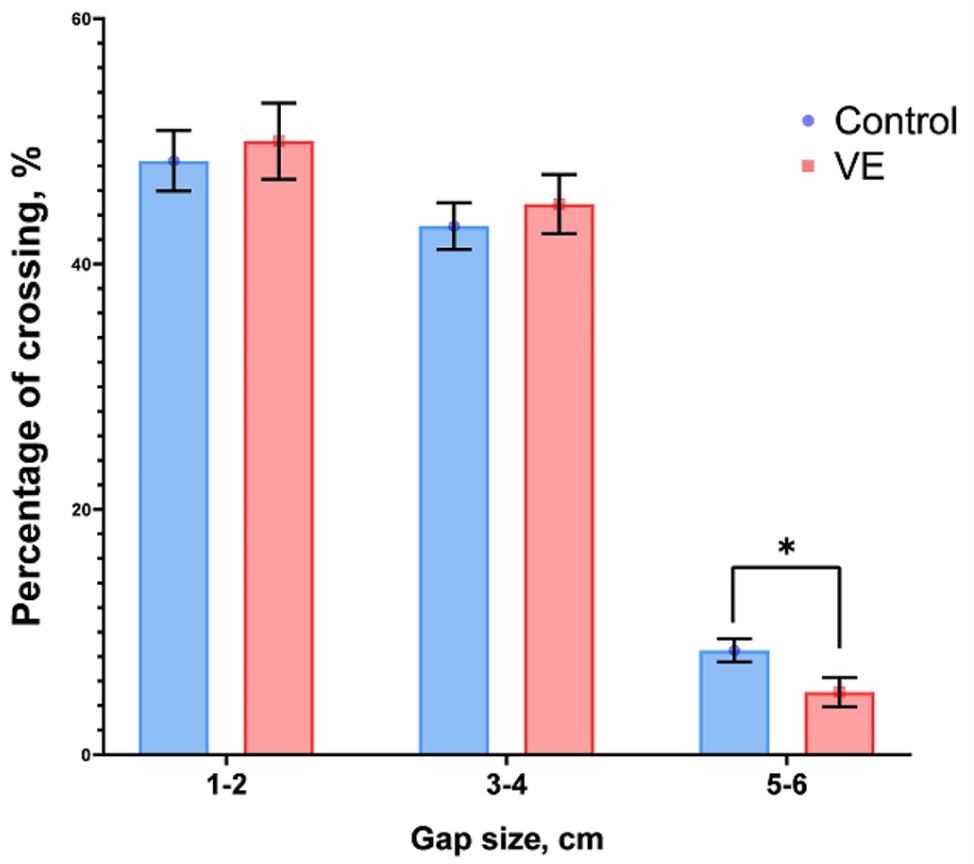


Figure 5. Percentage of large gap crossings is reduced in the vibrissae-removed (VE) cohort. Bar graph showing the percentage of successful crossings for large gaps (5-6 cm) in control and VE pups. Data are presented as LS means ± SEM. The VE cohort showed a significantly lower percentage of large gap crossings compared to controls; *p < 0.05, according to ANOVA GLM.

The morphological assay (5 litters of 3 pups) allowed us to assess putative correlations between perineuronal net (PNN) maturity and individual locomotor skills. First, no significant differences in local PNN density were observed between the two cortical areas studied (visual and barrel cortex), nor was the effect of vibrissaectomy significant. A multivariate analysis of variance (MANOVA) revealed no significant effect of vibrissaectomy status (Wilks' Λ = 0.836, F(2, 10) = 0.98, p = 0.407) or body weight (Wilks' Λ = 0.822, F(2, 10) = 1.08, p = 0.376) on the dependent variables (PNN measures and behavioral outcomes), indicating that neither predictor significantly influences the multivariate outcome in these groups. Univariate ANOVAs confirmed no significant effects of vibrissaectomy status on WFA-assessed PNN density per area in either the barrel cortex (F(1, 11) = 0.87, p = 0.372, partial η^2 = 0.07) or the visual

cortex ($F(1, 11) = 0.089$, $p = 0.771$, partial $\eta^2 = 0.01$). Similarly, body weight did not significantly predict PNN density in the barrel field (BF; $F(1, 11) = 1.76$, $p = 0.211$, partial $\eta^2 = 0.14$) or visual cortex (V2; $F(1, 11) = 0.052$, $p = 0.824$, partial $\eta^2 = 0.005$). Together, these results indicate that neither vibrissaectomy nor body weight significantly influences PNN density in the examined cortical regions.

However, correlation analysis revealed significant relationships between morphological parameters and the efficacy of wide gap crossings. Spearman rank-order correlations performed on all pups pooled ($N = 15$) showed two significant positive relationships of moderate strength. First, perineuronal net (PNN) density in the barrel cortex was significantly correlated with PNN density in the visual cortex ($\rho = 0.60$, $p < 0.05$), indicating coordinated PNN coverage across these cortical regions. Second, PNN density in the barrel cortex was significantly correlated with the percentage of 5-6 cm gaps crossed ($\rho = 0.57$, $p < 0.05$). In contrast, the correlation between visual cortex (V2) PNN density and the percentage of 5-6 cm gaps crossed was weak and non-significant ($\rho = 0.26$, $p \geq 0.05$). These results suggest that PNN density in the barrel field (BF) may be linked with the percentage of 5-6 cm gaps crossed, although it is coordinated with the neighboring visual cortex.

When analyzing only the subset of control animals (N=10), Spearman rank-order correlations revealed a positive relationship between the percentage of large gap crossings and PNN density in the barrel cortex ($\rho = 0.70$, $p < 0.05$) (Figure 6), which was stronger than in the full dataset (see above). This suggests that in the absence of vibrissaectomy, the ability to cross wide gaps and barrel cortex PNN density are tightly coupled. The correlation between BF and V2 PNN density was no significant in this subgroup ($\rho = 0.23$, $p \geq 0.05$).

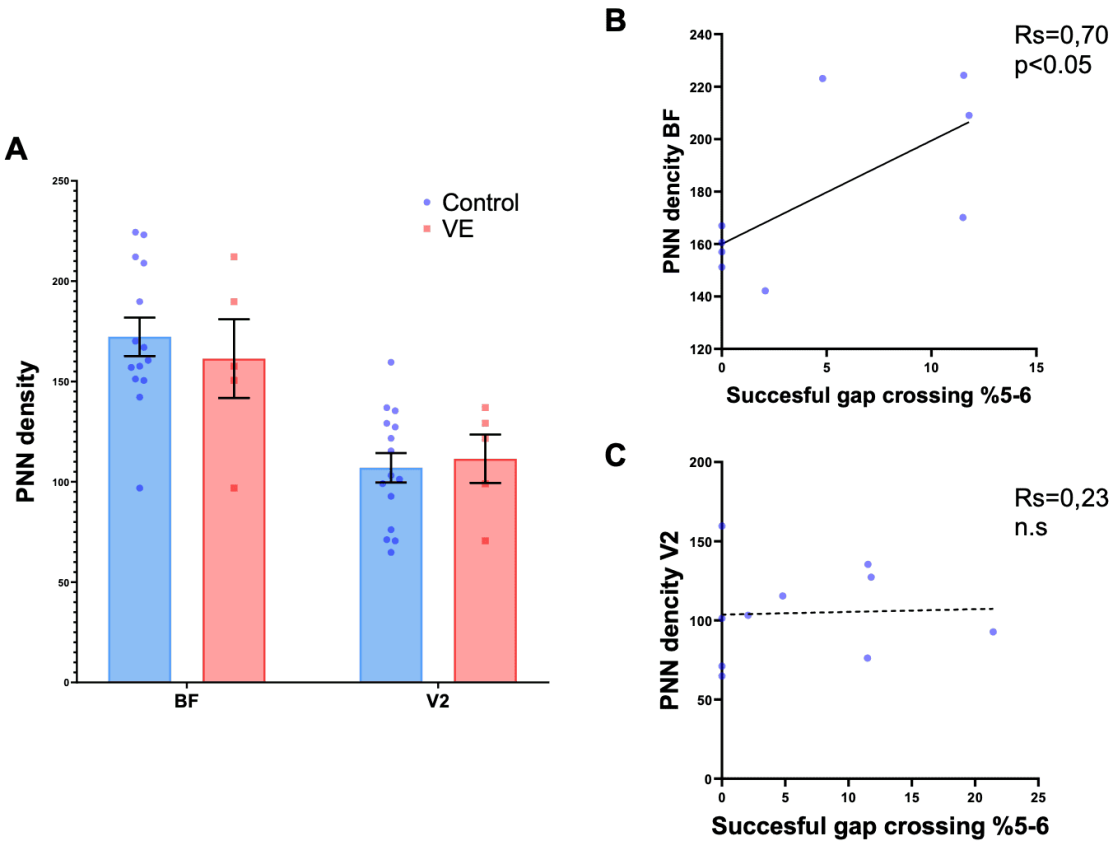


Figure 6 AB. Region-specific correlation between perineuronal net density and successful large-gap crossing. (A) PNN density in barrel and visual cortex in the control and VE pups. No significant group difference was seen. (B) PNN density in barrel, but not visual (C) cortex correlates with the percentage of large-gap crossing. Spearman rank-order correlation analyses revealed a significant positive relationship in the barrel cortex ($\rho = 0.70$, $p < 0.05$, $n = 9$), but a weak, non-significant relationship in the visual cortex ($\rho = 0.26$, $p \geq 0.05$, $n = 10$). Solid lines represent linear regression fits (shown for visualization only). PNN = perineuronal net.

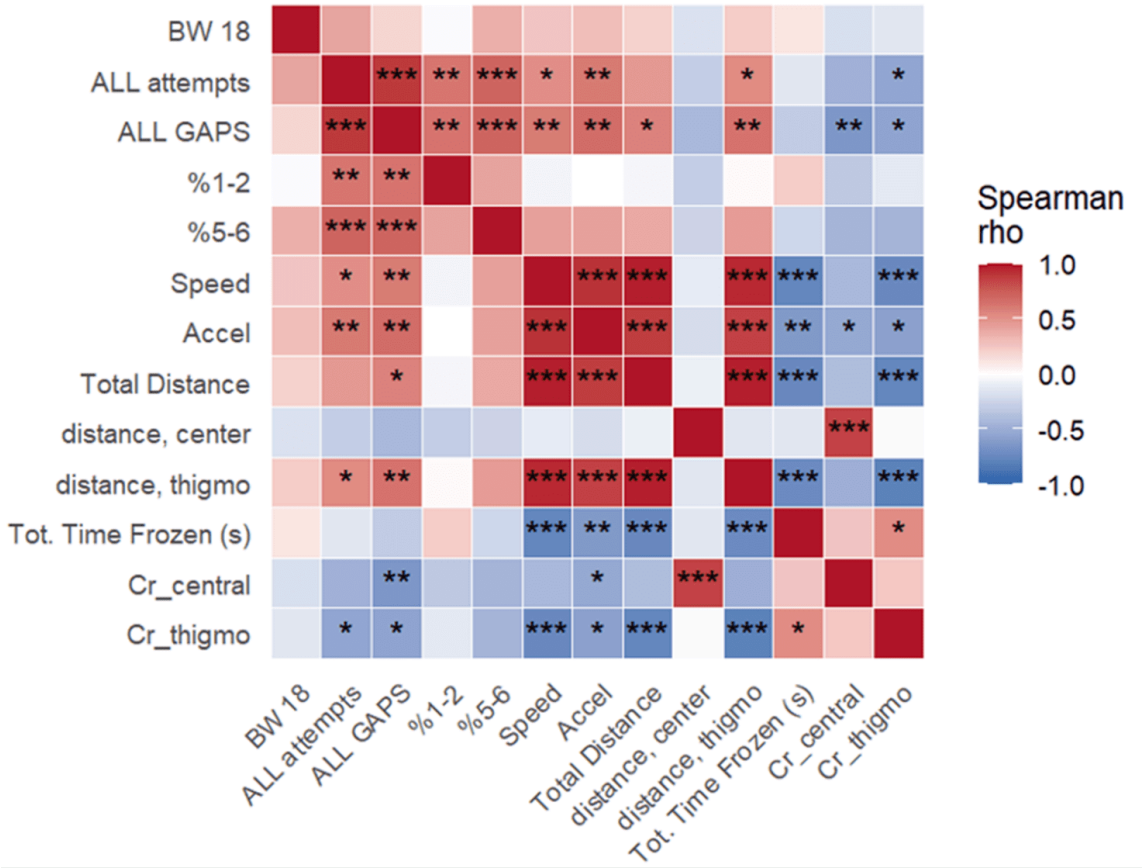


Figure 7. Correlation matrix of 2D open field and 3D home-cage locomotor parameters. Heatmap showing Spearman rank-order correlation coefficients (ρ) between 3D home-cage behaviors and 2D open field locomotion measures. Data were pooled from all control pups (N = 18). 3D parameters (rows/columns): platform climb attempts (total), total gaps crossed, percentage of short gaps (1-2 cm), percentage of large gaps (5-6 cm). 2D parameters (rows/columns): average speed, mean acceleration, total distance traveled, total freezing time, center compaction ratio, center distance traveled, thigmotaxis compaction ratio, thigmotaxis distance traveled. Correlation strength is color-coded: red indicates positive correlations (ρ close to +1), blue indicates negative correlations (ρ close to –1), and white indicates no correlation (ρ close to 0). *p < 0.05, **p < 0.01

Finally, we investigated whether spontaneous three-dimensional (3D) behavior in the dark nest chamber correlates with performance in the conventional open field test. No general effect of VE on the open field locomotor parameters was seen (Table 2). Correlation analysis integrating home cage spontaneous gap crossing and novelty-induced open field locomotion revealed unexpected patterns. Although we hypothesized that gap-crossing behavior would relate to conventional open field measures, neither small

nor large gap crossings showed such associations (Figure 7). Instead, overall 3D activity (sum of all gaps crossed and the number of all climbing attempts) displayed significant correlations with a locomotor vigor (average speed and acceleration in the open field), as well as with thigmotactic wall-following distance. Notably, compaction ratios (trajectory self-overlapping scaled to vibrissal length, [33]) were negatively correlated with overall 3D activity measures in both the central zone and during thigmotaxis (Figures 2, 7). This indicates that pups exhibiting less compact trajectories in the open field were more likely to display higher levels of spontaneous 3D activity.

Group	Sex	N	Average speed (mm/s)	Average acceleration (mm/s^2)	Total Distance (mm)	Total time frozen (s)	Compaction ratio
VE	m	3	31.56 ± 8.39	40.54 ± 9	5954.24 ± 1522.54	13.67 ± 6.33	5.21 ± 1.52
	f	4	14.64 ± 2.69	22.29 ± 4.63	2745.33 ± 487.12	41.75 ± 12.82	7.53 ± 2.16
Control	m	11	31.5 ± 5.91	44.17 ± 8.15	5693.3 ± 1052.72	21 ± 8.02	6.81 ± 1.17
	f	5	28.44 ± 5.85	37.39 ± 6.07	5040.48 ± 1090.59	30.8 ± 16.07	5.73 ± 0.42

Table 2. Open field locomotor and behavioral parameters across experimental groups. Data are presented as mean ± SEM. Abbreviations: N, number of animals per group; mm, millimeters; s, seconds. Average speed, average acceleration, total distance traveled, total time spent frozen, and compaction ratio (motion within central vs. peripheral zones) were derived from automated open field recordings. Compaction ratio was calculated as described earlier [33]. No significant group effect was seen.

4. Discussion

The present study yielded several findings. First, and surprisingly, pups from large litters achieved locomotor maturation more quickly than those from small litters (Table 1). This conclusion is supported by two observations: a lower proportion of pups from small (3-pups) litters initiated gap crossings compared to those from big (9-pups) litters, and a greater number of failed climbing attempts onto a platform occurred in small litters. Body weight and weight gain from postnatal day 9 to day 18 were

higher in small litters, suggesting that maternal care was at least sufficient under these conditions [47] [48]. Some delays in maturational milestone attainment with increasing litter size have been reported [49]. In contrast, early-life overnutrition in small litters decreases social play behavior [50]. The moderate acceleration of locomotor development in large litters is likely driven by social influences: pups in large litters must compete for optimal feeding positions, climb more frequently within the sibling huddle, and have more opportunities to observe and imitate motor behaviors. This topic is infrequently addressed in the literature [51], and few objective measures exist to quantify home cage effects of litter size on spontaneous locomotor maturation. Future studies will hopefully shed light on this important question, which holds high translational value.

We performed a minimal bilateral vibrissectomy on a single day only (PND 9). Few studies have described the effects of minimal whisker trimming (e.g., [52]); most experimental designs involve trimming lasting from 3 days to 4 weeks [53][54][55]. In our experiments, this single procedure produced a moderate but significant decrease in large gap crossing efficacy, as measured during spontaneous home cage behavior. The dark nest compartment, where spontaneous gap crossings occurred, ensures the absence of visual input, thereby rendering vibratactile, olfactory, and proprioceptive inputs more active [56]. Anticipatory whisker movements are required for tactile object identification [57]. The timing of the intervention (PND 9) coincides with the end of anatomical formation of the barrel somatosensory cortex, a developmental stage that also marks the beginning of a critical period for experience-dependent plasticity [58][59][60]. Importantly, this effect was observed in a naturalistic home cage environment under dark conditions, supporting the idea that tactile cues from the vibrissae play a functional role in guiding spatial navigation during spontaneous behavior.

Next, perineuronal net (PNN) maturity within the barrel cortex predicted successful long jumps in the control group. A significant positive correlation was found between PNN density in the barrel cortex and the percentage of large gaps crossed, whereas no such correlation was observed in the visual cortex. This region-specific relationship suggests that PNN maturation in the somatosensory barrel cortex may be functionally linked to neural circuits underlying 3D locomotor performance. Somatosensory deprivation via vibrissectomy was recently reported to cause some flattening of perineuronal nets [61]. In adult mice, perineuronal nets control visual input via thalamic recruitment of cortical parvalbumin-positive (PV+) interneurons [62]. In our study, the absence of correlation with visual cortex PNNs further supports the notion that tactile, rather than visual, processing dominates this behavioral pattern.

Our findings are consistent with previous literature demonstrating the role of PNNs in motor coordination and learning. In adult rodents, enzymatic degradation of PNNs restores plasticity but also impairs fine motor learning and coordinated movement, indicating that PNNs are necessary for maintaining stable motor performance [63][64]. Studies in developing animals have similarly shown that PNN formation precedes the emergence of complex motor behaviors, including skilled reaching and locomotor coordination. For example, PNN appearance in the cerebellum and its output regions is essential for the onset of coordinated limb movements and balance control [65][66]. Our results extend these findings by demonstrating that PNN maturation in the sensory barrel cortex, not just motor areas, predicts 3D locomotor success, highlighting the importance of sensorimotor integration in behavioral performance.

Finally, although two-dimensional and three-dimensional locomotion are interrelated, they represent non-identical markers of neurobiological maturation. Specific gap crossing activity (percentage of wide or narrow gaps crossed) did not correlate with conventional open field assays. This dissociation indicates that novelty-affected 2D locomotion (measured in the open field) and spontaneous 3D locomotion (measured by gap crossing) tap into different aspects of motor control and neural maturation. General activity markers—defined as the total numbers of gaps crossed and climbing attempts—showed positive correlations with measures of muscle power (average speed and acceleration) and negative correlations with spatial trajectory compaction. These findings suggest that pups must possess sufficient physical ability to move in 3D, and should not remain restricted to the small area surrounding their own trajectory.

Together, these findings suggest that 3D locomotor tasks in naturalistic settings provide unique insights into neurobiological development that cannot be adequately captured by traditional 2D open field tests alone.

4.1. Future directions

Future studies should address several critical questions to advance our understanding of PNNs and their behavioral relevance. Longitudinal monitoring of individual animals from the nestling period through adulthood will be essential to establish the developmental trajectory of PNN formation and its correlation with the emergence of 3-D locomotor capabilities. Causal manipulations of PNN integrity—using enzymatic degradation with chondroitinase ABC (ChABC) or pharmacological/genetic inhibition of PNN components—would directly test whether PNNs are necessary for normal 3-D locomotion. Expanding the anatomical scope to include PNN quantification in the motor cortex, hippocampus, and striatum will

determine whether the observed associations are localized solely to the barrel cortex. Additionally, integrating machine learning approaches to classify behavioral phenotypes from high-dimensional 3-D locomotion data could improve the sensitivity of behavioral assays.

5. Conclusions

The data presented here support the idea that the functional maturation of the barrel cortex critically gates the onset of dark-phase three-dimensional locomotor behavior, essentially relevant for nocturnal rodents. These findings suggest a temporal alignment between the consolidation of inhibitory microcircuits within the IV layer of barrel cortex and the emergence of exploratory and social play behaviors that require navigation through complex three-dimensional environments. Importantly, the analyses show that conventional two-dimensional open field measures, although widely used, provide limited insight into the emergence and refinement of 3D locomotor elements. Home cage-based assessments, which capture spontaneous behavior under minimally constrained conditions may be a more sensitive and ecologically valid approach for tracking neurobehavioral maturation during early life of laboratory rodents. When combined with quantitative histological characterization of perineuronal nets, this paradigm provides a toolset for investigating mechanisms of early neurodevelopment and for identifying developmental windows in which experience dependent interventions are likely to be most effective.

6. Limitations

This study has several important limitations that should inform both the interpretation of its findings and the design of future research. Although the sample sizes are consistent with standard practice in morphological and behavioral research, their relatively small magnitude reduces the reliability and generalizability of the results. Larger studies with greater statistical power will be needed to validate and refine the estimates reported here.

In addition, the reliance on a cross-sectional design precludes straightforward conclusions about causal relationships between neural measures and behavioral outputs. Longitudinal and, ideally, intervention-based designs will be required to address this issue.

The anatomical scope of the study is another constraint. By focusing solely on barrel and visual area of neocortex, the analyses do not account for potential contributions from motor cortex, hippocampus,

amygdala, and other nodes within the sensorimotor and limbic networks that are likely involved in the regulation of complex locomotor behavior.

Abbreviations

The following abbreviations are used in this manuscript:

- 2-D – two-dimensional
- 3-D – three-dimensional
- ANOVA – analysis of variance
- BF – barrel field (barrel cortex)
- Cr – compaction ratio (trajectory compaction ratio)
- DAPI – 4′,6-diamidino-2-phenylindole
- EPM – elevated plus maze
- GLM – general linear model
- IHC – immunohistochemistry
- MANOVA – multivariate analysis of variance
- OF – open field
- PBST – phosphate-buffered saline with Triton X-100
- PND – postnatal day (equivalent to P)
- PNN – perineuronal net
- PV – parvalbumin
- V2 – secondary visual cortex
- VE – vibrissectomy (whisker-trimmed group)
- WFA – Wisteria floribunda agglutinin

Statements and Declarations

Potential Competing Interests

The authors declare no conflict of interest.

Use of Generative AI

The authors confirm that artificial intelligence (AI) tools were used solely for language translation and editing of the manuscript. No AI tools were employed for data analysis, interpretation, or generation of scientific conclusions.

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Authors' contributions

Conceptualization, I.M. and A.M; investigation, M.O.; writing—I.M. and M.O.; visualization, M.O. and A.M.; supervision, I.M. All authors have read and agreed to the published version of the manuscript.

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