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Phenotyping spontaneous locomotor activity in inbred and outbred mouse strains by using Digital Ventilated Cages

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Mouse strains differ markedly in all behaviors, independently of their genetic background. We undertook this study to disentangle the diurnal activity and feature key aspects of three non-genetically altered mouse strains widely used in research, C57BL/6NCrl (inbred), BALB/cAnNCrl (inbred) and CRL:CD1(ICR) (outbred). With this aim, we conducted a longitudinal analysis of the spontaneous locomotor activity of the mice during a 24-h period for 2 months, in two different periods of the year to reduce the seasonality effect. Mice (males and females) were group-housed in Digital Ventilated Cages (Tecniplast), mimicking standard housing conditions in research settings and avoiding the potential bias provided in terms of locomotor activity by single housing. The recorded locomotor activity was analyzed by relying on different and commonly used circadian metrics (i.e., day and night activity, diurnal activity, responses to lights-on and lights-off phases, acrophase and activity onset and regularity disruption index) to capture key behavioral responses for each strain. Our results clearly demonstrate significant differences in the circadian activity of the three selected strains, when comparing inbred versus outbred as well as inbred strains (C57BL/6NCrl versus BALB/cAnNCrl). Conversely, males and females of the same strain displayed similar motor phenotypes; significant differences were recorded only for C57BL/6NCrl and CRL:CD1(ICR) females, which displayed higher average locomotor activity from prepuberty to adulthood. All strain-specific differences were further confirmed by an unsupervised machine learning approach. Altogether, our data corroborate the concept that each strain behaves under characteristic patterns, which needs to be taken into consideration in the study design to ensure experimental reproducibility and comply with essential animal welfare principles.

An accurate knowledge of the animal model is critical to ensure appropriate experimental design and subsequent reproducibility, reduce animal waste and comply with essential animal welfare principles. In the process of thoroughly comprehending and choosing a reliable animal model, phenotyping circadian rhythms and motor activity can provide information of paramount importance for the correct setting of—among other types of studies—behavioral, metabolic, neuroscience and cancer studies[1–4]. Circadian rhythmicity controls a wide variety of physiological events, including body temperature, activity, sleep, metabolism, heart rate, blood pressure and hormone and neurotransmitter secretion[5]. With the development and validation of multiple non-invasive recording instruments and variables of interest for different species, increasing numbers of research papers, ranging from assessment of focal behaviors mostly under experimental conditions (i.e., out of cage) to in-cage recording have been released, showing the scientific relevance of broadening the understanding of spontaneous behavior of undisturbed animals.

Despite the availability of several studies, to date, most focus on systematically reviewing C57BL/6J, C57BL/6J-related or genetically altered murine strains[2,6,7]. Little systematic data about characterization of spontaneous in-cage motor activity in inbred and outbred mouse stock strains are currently available from different vendors. Lack of such information can lead to inappropriate model choice, steering researchers to wrong experimental designs and confounding factors in experimental data analysis. On the other hand, a clear, unbiased characterization of spontaneous in-cage behaviors could improve comparability and reproducibility of models and data obtained apparently from similar strains but differently originated. Extensive literature documents strain-specific differences in circadian rhythms as well as remarkable differences in diurnal activity patterns[8] among inbred and hybrid strains[9]. Natural genetic polymorphisms manifested by inbred strains also indicate that background affects circadian rhythmicity[9]. The choice of mouse strain is thus the most important consideration for mouse circadian rhythm screen and ultimately dictates the ability to identify mutants. The implementation of large-scale phenotyping datasets may positively affect reduction measures, according to the 3Rs principles and policy[10], and accelerate global animal research.

On the basis of systematic observations made through extensive experience in the breeding of both outbred and inbred mouse strains, we decided to verify the existence of and eventually record relevant differences in circadian rhythms and spontaneous locomotor activity among different stock mouse strains. We focused on three non-genetically altered mouse strains widely used in research: C57BL/6NCrl (inbred), BALB/cAnNCrl (inbred) and CRL:CD1(ICR) (outbred). The choice of these strains was based on the following facts. First, although C57BL/6NCrl is commonly

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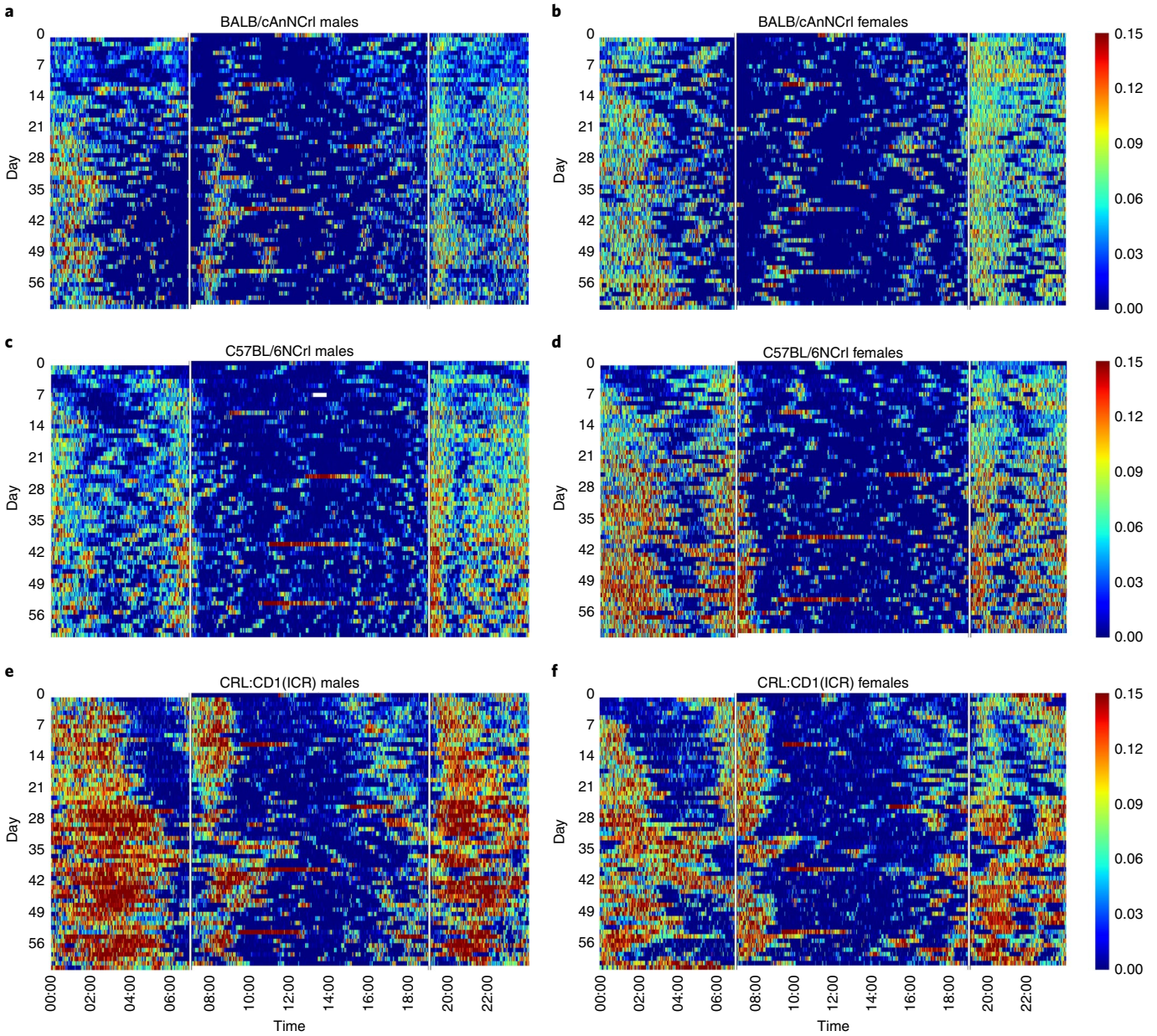

Fig. 1 | Heatmaps of spontaneous locomotor activity. a–f, Each panel shows 24-h activity during the experiment in an exemplifying cage of BALB/cAnNCrl males (**a**), BALB/cAnNCrl females (**b**), C57BL/6NCrl males (**c**), C57BL/6NCrl females (**d**), CRL:CD1(ICR) males (**e**) and CRL:CD1(ICR) females (**f**), with $n = 3$ mice per cage.

used for research purposes, it is less characterized than the substrain C57BL/6J. Remarkably, the two substrains, having clear phenotypic differences in various aspects, cannot be used interchangeably[11]. Second, because of the low genetic variability and phenotypic instability compared to the other substrains[12], BALB/cAnNCrl is frequently used in longitudinal neurobehavioral analyses. Third, among outbred strains, CRL:CD1(ICR) is the most commonly used in laboratories worldwide.

We screened our mice by using an automated recording home-cage device, the Digital Ventilated Cage (DVC by Tecniplast) to obtain an unbiased understanding of in-cage spontaneous mouse behavior and to track locomotor activity in the two sexes during a 24-h period. The DVC system, which relies on the detection of animal activity via the generation of tiny electromagnetic fields, has been proven to be safe for animals[13] and does not affect their behavior or welfare[14]. A previous study comparing C57BL/6NCrl and BALB/cAnNCrl mice housed in the DVC system has reported differences in measures such as bodyweight, water utilization and position within the cage, as well as a common test of anxiety-related behavior and cognition[14].

Here, we introduce new and different circadian metrics to analyze data obtained only from in-cage recording. We compared the 24-h spontaneous locomotor activity of the mice and extrapolated key aspects of the day and night activity patterns for each strain. All analyzed metrics clearly show significant differences in the circadian activity of the three selected strains, not only identifying differences when considering inbred versus outbred strains, but—consistent with available literature[2]—characterizing strain-specific spontaneous locomotor patterns during the 24-h period, proving once more that different strains have peculiar diurnal motor phenotypes. The strain-specific differences are further confirmed by an unsupervised machine learning approach.

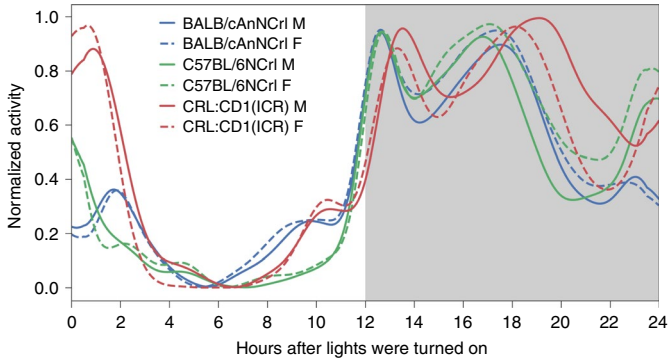

Fig. 2 | Activity pattern over 24 h of the three analyzed strains. The figure shows the average distribution of activity over the 24 h of a day (lights-on and lights-off periods). Each daily activity time series was normalized to its peak activity (=1.0), to compare groups by their relative 24-h pattern and not by the absolute level of activity. F, females; M, males.

Results

The DVC system allowed us to monitor the activity of three commonly used mouse strains (C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR)) from 4 to 12 weeks of age, covering the period between weaning, sexual maturity and early adulthood. Heat maps of representative cages of male and female C57BL/6NCrl mice, used as a reference strain, BALB/cAnNCrl mice and CRL:CD1(ICR) mice show how the activity was distributed across the 24 h of the experiment (Fig. 1). As expected, the overall highest recorded activity was concentrated during the night, although clear differences were observed between the three strains. To better disentangle the circadian phenotype of the three strains, we used the following metrics.

24-h locomotor activity pattern. We first qualitatively analyzed the average pattern of recorded locomotor activity for males and females of each strain, C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR), for 24 h and 7 days a week (24/7) for the two entire experimental periods. The activity of the three strains was not entirely confined to the dark phase, but cyclical patterns of increased and decreased activity over the light and dark phase were detected (Fig. 2). The locomotor activity of C57BL/6NCrl and CRL:CD1(ICR) mice began before dawn, and it lasted ~1 h. In contrast, BALB/cAnNCrl mice activated 2 h after lights were turned on. A clear pre–dark phase anticipatory activity (2 h before lights were turned off) was observed in the pattern of CRL:CD1(ICR) and BALB/cAnNCrl mice and progressively increased during the transition phase between light and dark. The peak of activity was recorded during the dark phase for the three strains, with a strain-specific pattern: C57BL/6NCrl mice showed remarkable peaks throughout the night, with extended activity for up to 1 h after lights were turned on; CRL:CD1(ICR) mice also displayed peaks of activity during the whole dark phase, but there was a gradual increase in activity at the start of the lights-off phase, reaching a peak 2 h later, and then alternated decreased and increased activity for up to 2 h after lights were turned on. In contrast, the recorded activity of BALB/cAnNCrl mice displayed bouts of intermediate activity beginning with clear anticipatory activity before the lights-off phase and continuing during the dark phase. BALB/cAnNCrl mice also showed a clear reduction in activity toward the end of the dark phase and had an additional short bout of activity 2 h after lights were turned on. No clear difference was observed between males' and females' cages, except for CRL:CD1(ICR) males revealing a more intense activity during the night compared to females.

Day and night activity. We then characterized more in depth the day and night level of activity for each strain. With this aim, we measured the average activity of each cage during 12 h of light and dark (Fig. 3). We used linear mixed models to question which of the following effects, including strain, sex, time and light, quantitatively correlates with the observed differences in the day and night activity levels. Because the mice are nocturnal, the average activity of the three strains in both males and females was much higher during the night than during light hours ($P_{light} < 0.001$). Indeed, the impact of light on average activity displayed a positive slope in all cages over time, with a shift depending on light and time-light interactions ($P_{time\text{-}light} < 0.001$). Although we did not observe significant differences in activity levels between BALB/cAnNCrl and C57BL/6NCrl mice ($P_{BALB/cAnNCrl} > 0.05$), CRL:CD1(ICR) mice displayed significantly more intense average activity during day and night compared to C57BL/6NCrl mice ($P_{CRL:CD1(ICR)} < 0.001$). The sex factor was not significant and was thus excluded from the model. Finally, we observed an increasing trend of activity over time ($P_{time} < 0.001$), with an estimated positive slope of 3.31×10^{-4}. We then compared the average activity during the second, fifth and eighth weeks of the experiment (i.e., 5, 8 and 12 weeks of age), probably corresponding to the pre-pubertal, post-pubertal and adulthood phases, respectively[15]. Our results confirmed that the activity significantly changed over these biological cornerstones ($P_{weeks} < 0.001$).

We observed that, overall, BALB/cAnNCrl and CRL:CD1(ICR) mice displayed a higher daylight activity than C57BL/6NCrl mice (Fig. 4). Specifically, CRL:CD1(ICR) mice had a significantly higher diurnality ($P_{CRL:CD1(ICR)} < 0.001$) than BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.05$), independently of sex differences.

Responses to lights being on and lights being off. Given that light deeply correlates with activity over 24 h, we decided to better analyze the locomotor activity in relation to light by identifying four critical moments over 24 h: (i) the first response during the lights-on phase, (ii) the last response during the lights-on phase, (iii) the first response during the lights-off phase and (iv) the last response during the lights-off phase.

The first response during the lights-on phase (Fig. 5a) occurred in a short time after lights were turned on for both C57BL/6NCrl and CRL:CD1(ICR) mice, whereas it was substantially delayed for BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.001$). In contrast, the last response during the lights-on phase (Fig. 5b) appeared earlier for CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$) and slightly later for BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.01$) compared to the reference strain. The effect of sex was excluded from both models, because no significant difference was observed.

The first response to the lights-off phase also suggested a different behavior between strains: whereas BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} > 0.05$) displayed an early response to lights being turned off, similarly to the reference strain, CRL:CD1(ICR) mice showed a clear delayed response to lights being turned off ($P_{CRL:CD1(ICR)} < 0.001$). This was particularly evident in males' cages ($P_{CRL:CD1(ICR):male} < 0.001$) (Fig. 6a). Conversely, in correspondence with the end of the dark phase, we observed a clear significant anticipation of the last peak of activity of BALB/cAnNCrl and CRL:CD1(ICR) mice, in either males or females, compared to C57BL/6NCrl mice ($P_{BALB/cAnNCrl} < 0.001$; $P_{CRL:CD1(ICR)} < 0.05$) (Fig. 6b).

Acrophase and activity onset. The acrophase and activity onset were evaluated in both males' and females' cages of the three strains to characterize the locomotor circadian rhythm. The acrophase was anticipated for BALB/cAnNCrl mice ($P_{BALB/cAnNCr} < 0.001$) compared to the reference strain (Fig. 7a) and clearly delayed in cages of CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$). Slight but significant differences were seen when measuring the activity onset (Fig. 7b): whereas the beginning of activity of C57BL/6NCrl mice probably corresponded to the transition from the lights-on phase to the lights-off phase, it was slightly anticipated in BALB/cAnNCrl mouse

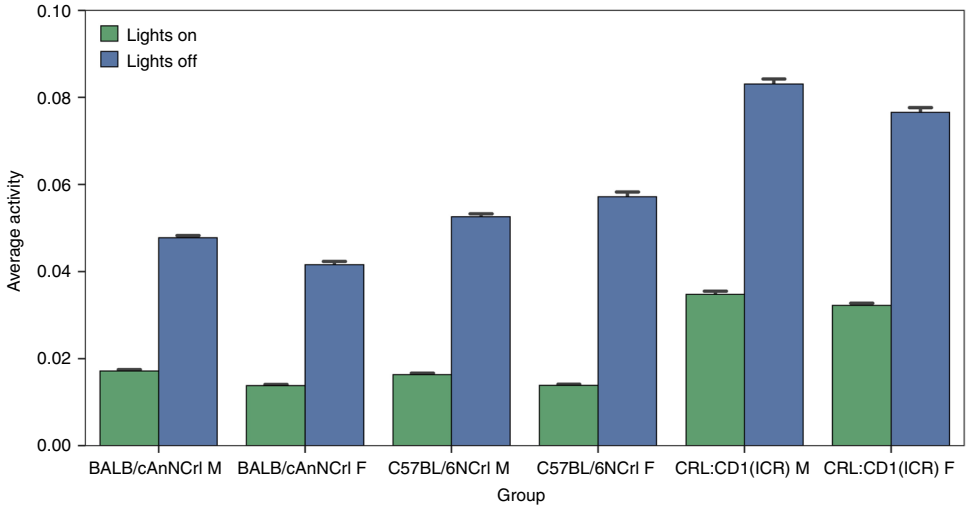

Fig. 3 | Day and night activity of male and female cages of each strain. Average (± s.e.m.) activity during lights-on and lights-off phases across multiple days and cages of each group.

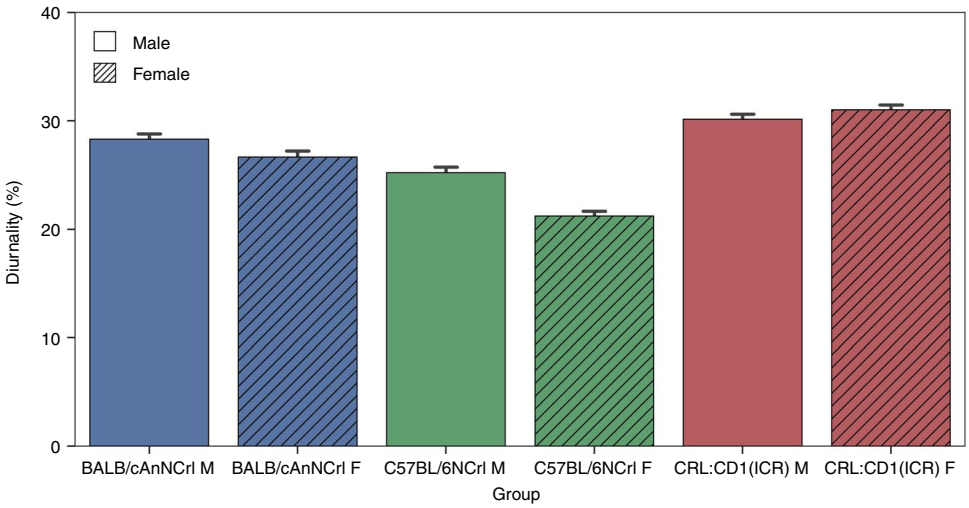

Fig. 4 | Average of diurnal activity (diurnality). The graph shows the percentage of recorded spontaneous locomotor activity during the lights-on phase with respect to the total activity recorded for 24 h. The percentage was measured in male and female cages of each strain, across multiple days and cages of each group.

cages ($P_{BALB/cAnNCrl} < 0.01$) and clearly delayed in CRL:CD1(ICR) mice ($P_{CRL:CD1(ICR)} < 0.001$).

Regularity disruption index (RDI). Finally, we calculated the RDI for females and males of each strain, to capture possible irregular mouse activity patterns during lights-on and lights-off phases over the entire experimental period. We observed that during the lights-on phase, C57BL/6NCrl and CRL:CD1(ICR) mice frequently changed their status, compared to BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.001$) (Fig. 8), which displayed the most stable locomotor behavior during the lights-on phase. As expected, RDI was much higher during the lights-off phase in all strains ($P_{light} < 0.001$). Remarkably, RDI was slightly higher in males of all strains than in females ($P_{female} < 0.05$).

Behavioral response to the cage change. We then decided to analyze and measure the locomotor activity within a range of 5 h after the cage change (Fig. 9a), to evaluate the response to such a stressful moment in the husbandry and management of mice[16,17]. The cage-change procedure was performed by trained animal care technicians under standardized practices: every 2 weeks, during the light phase of the light/dark cycle, from the dirty cage to the clean one and shortly restraining and moving the mice by tail grasping and suspension. We focused on two measurements: duration, as the average (± s.e.m.) estimate of the duration of the response to cage change, and average activity, as the average (± s.e.m.) activity recorded within the estimated response duration (Fig. 9b and c). BALB/cAnNCrl mice ($P_{BALB/cAnNCrl} < 0.05$) showed a significantly shorter response in terms of duration than did C57BL/6NCrl and CRL:CD1(ICR) mice (Fig. 9b). C57BL/6NCrl mice showed a longer duration of locomotor response to cage change, with slightly higher values in females than males, in contrast to BALB/cAnNCrl and CRL:CD1(ICR) mice (Fig. 9b). Slightly significant sex differences were observed in average activity, which increased in males—with the only exception being C57BL/6NCrl mice (Fig. 9c)—suggesting a potential correlation with strain and sex-related exploratory and marking behavior[18]. With the only exception of a clearly longer duration of locomotor response to cage change showed by C57BL/6NCrl

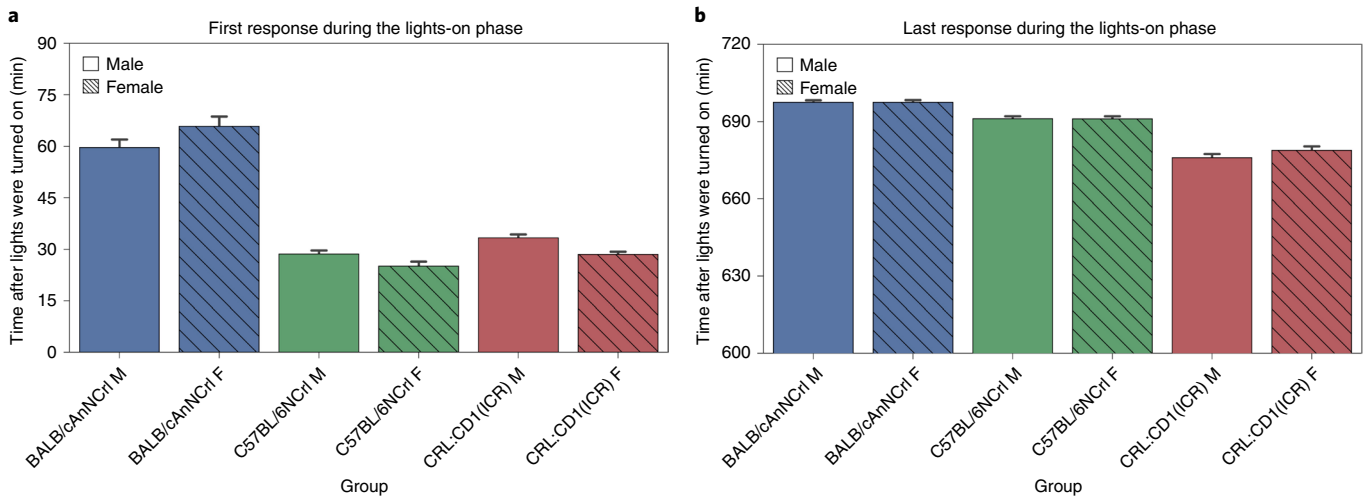

Fig. 5 | Behavioral responses during the lights-on phase. a, The graph shows the average (± s.e.m.) time of the first peak of activity during the lights-on phase across multiple days and cages of each group. **b**, Average (± s.e.m.) time of the last peak during the lights-on phase. The time is expressed as minutes after lights were turned on.

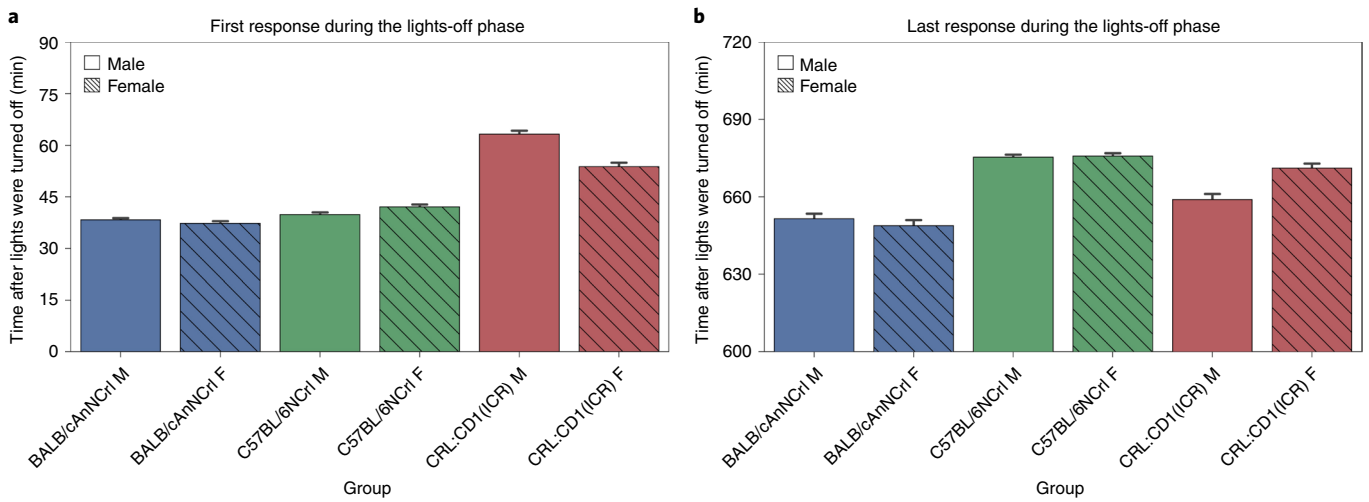

Fig. 6 | Behavioral responses during the lights-off phase. a, The graph shows the average (± s.e.m.) time of the first peak of recorded locomotor activity during the lights-off phase across multiple days and cages of each group. **b**, Average (± s.e.m.) time of the last peak of recorded locomotor activity during the lights-off phase. The time is expressed as minutes after lights were turned off.

mice (both males and females), other data on behavioral response to cage change should be investigated further to evaluate potential correlations between strain, handling and restraining techniques and sex-related exploratory and marking behavior.

K-means clustering. All analyzed metrics clearly highlighted differences in the circadian activity of the three selected strains (Table 1). To further confirm our results, we undertook the K-means clustering method and included each previously analyzed metric. To reduce the dimensionality, we first applied principal component analysis (PCA) and successive K-means clustering, aiming at separating only strains, and not sex. We were able to record ~600 measurements per strain (one measurement is one cage per day), obtained as 12 cages for 50 d per strain. We set K-means with three clusters. We observed that each cluster contains measurements mostly from a single strain, meaning that cages of the same strain are more similar to each other than to other strains. As represented in Fig. 10, BALB/cAnNCrl was assigned to cluster 1, C57BL/6NCrl to cluster 0 and CRL:CD1(ICR) to cluster 2. We further confirmed these results for each strain by calculating how many times each cage could be classified in the specific cluster over the two experimental periods. Our results show that each cage was classified according to the corresponding cluster of its strain, except for one C57BL/6NCrl cage that was classified in the BALB/cAnNCrl corresponding cluster (Table 2).

Discussion

The increasing number of available mouse strains and their genetically diverse background call for a need to identify strain-specific features to better guide the appropriate choice of models. This is even more relevant when conducting experiments to compare negative controls with the transgene, when modeling certain neurological conditions, as well as in the case of metabolic and cancer diseases, neurodegenerations and aging studies, among others. In parallel with the need for accurate phenotypic characterization, the scientific community is putting great effort into developing and validating continuous automated and non-intrusive home-cage analysis systems as unbiased approaches for behavioral evaluation[19–22],

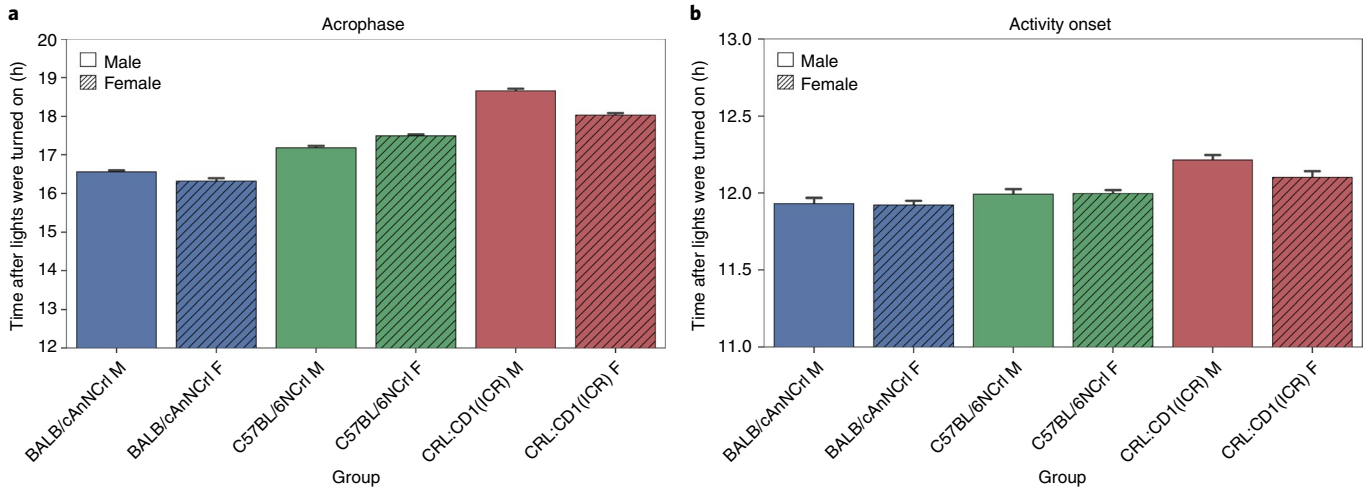

Fig. 7 | Acrophase and activity onset. a, The graph shows the average (± s.e.m.) of acrophase across multiple days and cages of each group, expressed as hours after the lights-on time. **b**, Average (± s.e.m.) of activity onset across multiple days and cages of each group, expressed as hours after the lights-on time.

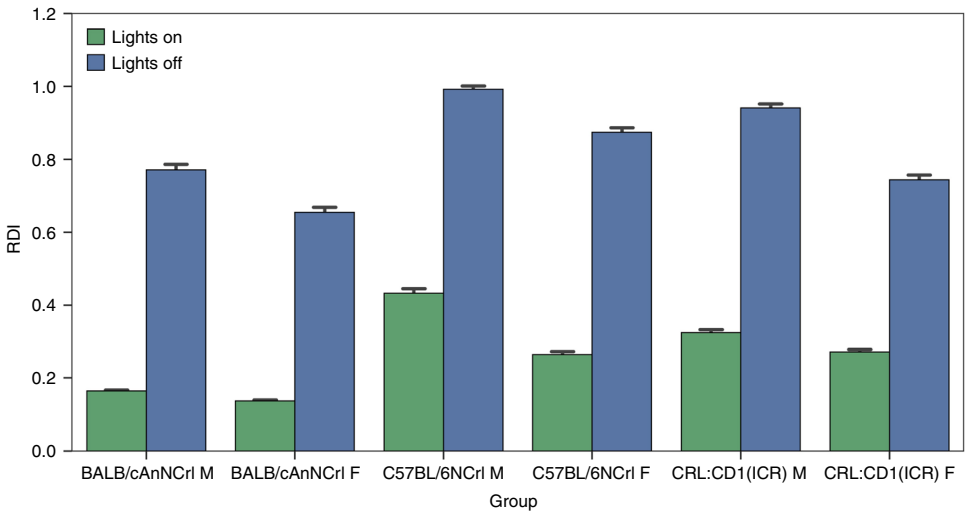

Fig. 8 | Day and night RDI. The graph shows the average (± s.e.m.) of RDI during lights-on and lights-off phases across multiple days and cages of each group.

with the advantage of reducing the effect of human handling and therefore improving animal welfare according to the 3Rs principles, without affecting experimental outcomes. Moreover, such technologies, allowing longitudinal observations, contribute to reducing the number of animals used per experiment or study, by enabling researchers to obtain either comparable levels of information from fewer animals or more information from the same number of animals, thereby avoiding further animal use.

The aim of this study was to characterize in depth and compare the spontaneous circadian rhythms of three commonly and widely used mouse strains, C57BL/6NCrl (inbred), BALB/cAnNCRL (inbred) and CRL:CD1(ICR) (outbred) in biomedical research. A longitudinal analysis of the circadian activity was conducted 24/7 in group-housed mice in the DVC system for 2 months in two cohorts in late summer and early spring, to avoid seasonal effects. To our knowledge, this is the first attempt to capture the diurnal phenotypic differences of the three selected strains, achieved by introducing new circadian metrics and confirming the results with a machine learning approach, which is a useful addition to the animal behaviorist's analytical toolkit[23].

As nocturnal animals, mice are active mainly during the dark phase, when the endogenous circadian clock dictates the behavior of the animal[5]. We observed that in all cages, the spontaneous locomotor activity revealed a clear rhythmicity, with the peak during the dark phase and the lowest activity during light hours[20,22,24]. C57BL/6NCrl and CRL:CD1(ICR) mice displayed an increased activity before the end of the dark phase, which lasted also during the first 1–2 h of the lights-on phase, confirming that circadian rhythms are internally generated patterns of activity[25] and function as an innate clock and that their development is genetically programmed independently of the environment[26]. The circadian phenotype of C57BL/6NCrl, herein used as a reference strain, matched with the description of C57BL/6J previously documented[19,20]. This represents a non-obvious observation, because several gene differences, some of which may regulate circadian clock function, including *Adcy5* (which influences locomotor activity levels), *Pmch* (which mediates sleep and arousal) and *Crb1* (which controls retina photoreceptor structure), have different regulation in the two substrains[27]. Furthermore, different behavioral and physiological responses to circadian disruption and wheel-running access have

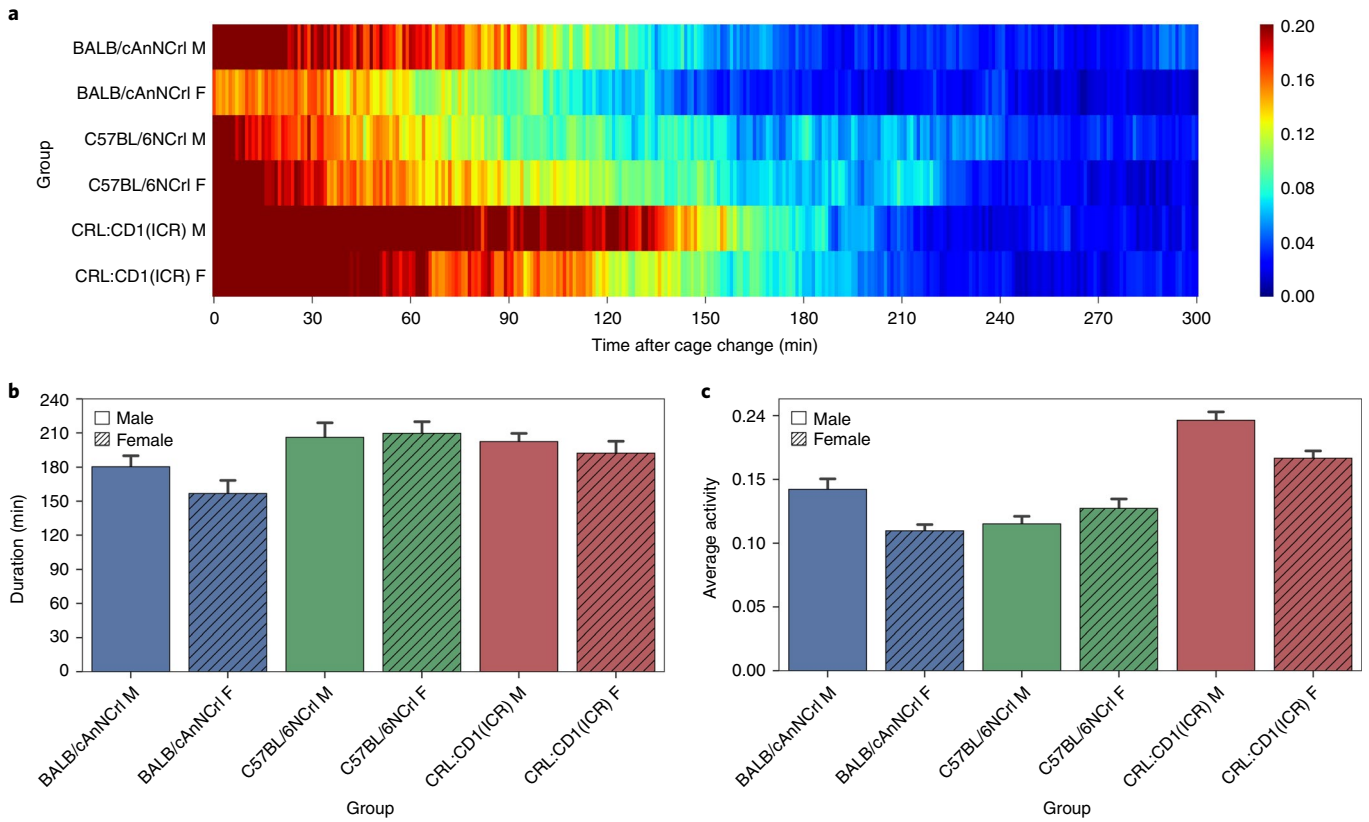

Fig. 9 | Response to cage change. a, The heatmap shows the minute-based activity recorded within 5 h after cage change, averaged across all days of cage change and all cages of the corresponding group (as rows). **b**, The graph shows the average (± s.e.m.) estimate of the duration of the response to cage change. **c**, The graph shows the average (± s.e.m.) activity recorded within the estimated response duration.

Table 1 | Key patterns of spontaneous locomotor activity recorded for each cage housing BALB/cAnNCrl, C57BL/6NCrl and CRL:CD1(ICR) mice of both sexes

Strain		Day and night activity	Diurnality	Response to the lights-on phase	Response to the lights-off phase	Activity from pre-puberty until adulthood	Acrophase	Activity onset	RDI
BALB/cAnNCrl	Males	++	++	Delayed	Early	+	Early	Early	++
	Females	++	++	Delayed	Early	+	Early	Early	+
C57BL/6NCrl	Males	++	+	Early	Early	++	Delayed	Concomitant	+++
	Females	++	+	Early	Early	+++	Delayed	Concomitant	++
CRL:CD1(ICR)	males	+++	+++	Early	Delayed	++	Delayed	Delayed	+++
	females	++	+++	Early	Delayed	+++	Delayed	Delayed	++

+++, ++, + indicate intense, medium and low average of locomotion, respectively.

been demonstrated in male C57BL/6NCrl and C57BL/6J mice[28]. However, against C57BL/6J, we were able to compare only the day and night activity pattern and the effect of cage change. Future experiments with the DVC system are necessary to dissect possible behavioral differences in the circadian activity of the two substrains.

C57BL/6NCrl and BALB/cAnNCrl mice showed both similarities (day and night activity levels, the first response to the lights-off phase and the last response to the lights-on phase) and differences (the first response to the lights-on phase, the last response to the lights-off phase, acrophase, RDI and the response to cage change) in their spontaneous locomotor activity, which supports previous studies comparing phenotypic characteristics of C57BL/6NCrl mice with BALB/cAnNCrl mice in different behavioral experimental settings[29,30]. Compared to C57BL/6NCrl mice, we observed that BALB/cAnNCRL mice showed a substantially delayed response to the lights-on phase and an anticipated peak toward the end of the night. Another evident difference was observed in the RDI, a digital biomarker used for phenotyping the onset and the evolution of neuromuscular diseases in murine models[6]. Notably, the recorded activity of BALB/cAnNCrl mice did not reveal significant irregularity and/or disturbances in the rest/sleep behavior during light hours compared to C57BL/6NCrl mice. The more stable locomotor activity in BALB/cAnNCrl mice, in either males or females, compared to the other two strains is in line with previous reports[31] and could be ascribed to the low sociability and conspecific interaction of this strain[12,32].

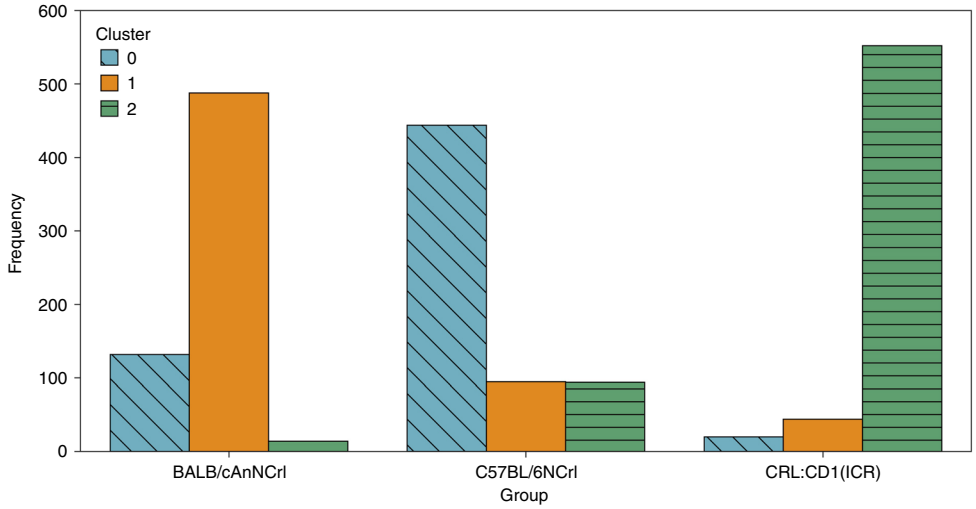

Fig. 10 | Cluster analysis. Each measurement (day per cage) was classified in a specific cluster by the K-means algorithm that took as input all the previously analyzed metrics (after a dimensionality reduction with PCA). The graph clearly shows three predominant clusters, each corresponding to a strain (cluster 1 to BALB/cANCrl, cluster 0 to C57BL/6NCrl and cluster 2 to CRL:CD1(ICR)). Notably, CRL:CD1(ICR) shows the highest percentage of measurements classified in a single cluster.

Table 2 | Relative frequencies of classification of cages in each cluster

Group	Cage	cl 0 (%)	cl 1 (%)	cl 2 (%)	Group	Cage	cl 0 (%)	cl 1 (%)	cl 2 (%)	Group	Cage	cl 0 (%)	cl 1 (%)	cl 2 (%)
BALB/cAnNCrl M	C_01	18.5	77.8	3.7	C57BL/6NCrl M	C_04	70.4	13.0	16.7	CRL:CD1(ICR) M	C_11	9.3	5.6	85.2
BALB/cAnNCrl M	C_03	9.3	83.3	7.4	C57BL/6NCrl M	C_06	53.7	3.7	42.6	CRL:CD1(ICR) M	C_13	1.9	0.0	98.2
BALB/cAnNCrl M	C_17	9.3	87.0	3.7	C57BL/6NCrl M	C_08	18.9	45.3	35.9	CRL:CD1(ICR) M	C_16	5.6	9.3	85.2
BALB/cAnNCrl M	C_20	46.2	53.9	0.0	C57BL/6NCrl M	C_19	90.4	9.6	0.0	CRL:CD1(ICR) M	C_21	0.0	0.0	100
BALB/cAnNCrl M	C_23	26.9	73.1	0.0	C57BL/6NCrl M	C_22	100	0.0	0.0	CRL:CD1(ICR) M	C_24	0.0	0.0	100
BALB/cAnNCrl M	C_26	36.5	61.5	1.9	C57BL/6NCrl M	C_25	92.0	4.0	4.0	CRL:CD1(ICR) M	C_27	0.0	0.0	100
BALB/cAnNCrl F	C_10	17.3	76.9	5.8	C57BL/6NCrl F	C_02	50.0	40.7	9.3	CRL:CD1(ICR) F	C_05	1.9	18.5	79.6
BALB/cAnNCrl F	C_12	11.1	87.0	1.9	C57BL/6NCrl F	C_15	44.4	13.0	42.6	CRL:CD1(ICR) F	C_07	0.0	6.4	93.6
BALB/cAnNCrl F	C_14	5.6	92.6	1.9	C57BL/6NCrl F	C_18	61.1	37.0	1.9	CRL:CD1(ICR) F	C_09	11.5	15.4	73.1
BALB/cAnNCrl F	C_29	9.6	90.4	0.0	C57BL/6NCrl F	C_28	78.9	7.7	13.5	CRL:CD1(ICR) F	C_30	0.0	6.3	93.8
BALB/cAnNCrl F	C_32	28.9	71.2	0.0	C57BL/6NCrl F	C_31	96.2	3.9	0.0	CRL:CD1(ICR) F	C_33	1.9	1.9	96.2
BALB/cAnNCrl F	C_35	32.7	67.3	0.0	C57BL/6NCrl F	C_34	90.4	0.0	9.6	CRL:CD1(ICR) F	C_36	5.8	21.2	73.1

The table shows how many times (as percentages) each cage was classified in the three clusters (cl 0, cl 1 and cl 2). On most of the days, each cage was classified to the corresponding cluster of its strain, except for cage C_08 of C57BL/6NCrl M group. CRL:CD1(ICR) cages show the overall highest percentages of being classified in their specific cluster (cl. 2).

CRL:CD1(ICR) mice exhibited the most clearly differentiated patterns in all metrics compared to the two inbred strains and had the highest average 24/7 activity recorded. Remarkably, only in this strain we observed sex differences, with males more active than females, although not statistically significant in all measurements. Our findings thus extend previously observed sex differences reported for this strain[33].

We also evaluated activity at three different time points, targeting cornerstones of mouse development from prepuberty to adulthood[15]. Previous studies involving wheel-running activity showed that daily activity reaches a peak and plateaus at 9–10 weeks of age in mice[34]. According to our data, free movement activity intensity significantly changes over the selected time points, confirming an increase in spontaneous activity and showing strain differences with a distribution pattern from CRL:CD1(ICR), the highest, to BALB/cAnNCrl, the lowest. Remarkably, BALB/cAnNCrl males and females and C57BL/6NCrl males showed a homogeneous activity pattern over the three time points, whereas C57BL/6NCrl and CRL:CD1(ICR) females showed a clear, progressive increased activity pattern. With wheel running, sex proved to be a significant factor in daily activity, with females showing higher intensity than male mice[34]. Conversely, our data show on average a higher activity intensity in male BALB/cAnNCrl and CRL:CD1(ICR) mice, and only C57BL/6NCrl females displayed higher activity intensity, suggesting that evaluation of spontaneous activity in cage locomotion provides a different perspective on activity intensity because it is a permanent and long-term parameter avoiding artefacts[35] and habituation bias[36].

Very interestingly, we confirmed our phenotypic analyses by an unsupervised machine learning approach. Each strain corresponded to a cluster, and notably the repeated and longitudinal measurements of all circadian metrics confirmed that data referring to cages housing each strain were included in the corresponding cluster, except for one C57BL/6NCrl cage that was classified in

the BALB/cAnNCrl corresponding cluster, corroborating the diversity of circadian phenotype of the three strains. Interestingly, all CRL:CD1(ICR) cages have been classified in the same cluster with very high rates, compared to the inbred strains. These observations further confirm a higher similarity in the diurnal locomotor activity between the two inbred strains and the phenotypic variability of outbred strains[37].

Finally, thanks to the automated home-cage 24/7 monitoring system, which allows researchers to longitudinally monitor individual group-housed cages without adverse behavioral and physiological effects, we were able to portray key features of C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR) mice, relying on their spontaneous locomotor activity, with CRL:CD1(ICR) mice more active and dynamic, C57BL/6NCrl mice more susceptible to environmental stimuli and BALB/c mice the least active strain. Overall, the systematic in-cage data recording potentially creates a large-scale and open behavioral database with a specific focus on spontaneous, unbiased locomotor activity patterns. The availability of such data from both non-genetically and genetically modified mice will allow precise comparison between strains and mutations[38], leading to more accurate understanding of deviations from baselines, pondered welfare assessment and phenotyping of genetically modified animals[39], with a further positive impact on implementation of refinements, including endpoints, increasing reproducibility and awareness in selecting appropriate models. We are confident that these phenotypic features will be helpful when selecting an appropriate model, independently also of the genetic variability of strains (inbred versus outbred), contributing thus to the effort to overcome the classical dichotomy of inbred versus outbred strains[40].

Online content

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41684-021-00793-0.

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Methods

Ethics statement. The study, data collection and analysis were approved by the Institutional Animal Care and Welfare Body of the CNR-IBBC/EMMA/Infrafrontier. Animal maintenance was performed in accordance with general guidelines regarding animal breeding and biotechnology, in compliance with the Italian Legislative Decree 26/2014.

Mice. The three strains here analyzed, C57BL/6NCrl, BALB/cAnNCrl and CRL:CD1(ICR), were obtained from Charles River Laboratories. The mice were bred under barriered specific pathogen free–condition facilities at the Charles River Laboratory facility in Calco, Italy according to internal breeding standard operating procedures, which include a genetic stability program and specific pathogen free conditions. At 3 weeks of age, after weaning, the mice were moved to the CNR-IBBC/EMMA-Infrafrontier-IMPC Core Structure (Monterotondo, Rome, Italy)—Consiglio Nazionale delle Ricerche (Rome, Italy) and housed in DVC racks for the whole duration of the study. After acclimatization, mice of each strain were housed in groups of three individuals per cage, fed ad libitum with standard diet (4RF21; Mucedola), under standard controlled environmental parameters (temperature = 21 ± 2 °C; relative humidity = 55% ± 15%), and mice were kept in a 12-h light/12-h dark cycle (7 AM—7 PM: lights on) with 12–15 air changes per hour and a 12:12 light cycle. Light intensity at room level was 230 lux, while cages were exposed to slight differences according to their position within the rack. Variations of light intensity at cage level were recorded, with lux levels ranging from 29 to 12 lux. Certified dust-free wood bedding (Scobis one; Mucedola) was provided in the cages. Mice were provided chlorinated, filtered water ad libitum. 2-week-interval cage changes were adopted with unaltered standard procedure and timing (Mondays at 10 AM). Differently from other studies[7], cage density was standardized to three mice per cage, with the intent to mimic possible standard housing conditions in research settings, avoiding the potential bias provided in terms of locomotor activity by single housing (i.e., absence of interaction with cage mates and altered (increased) time to integrate into the nest, leading to a prolonged activity time[41]).

Experimental groups were divided in two separate cohorts of mice in two different periods of the year (springtime and late summer/early autumn) to reduce the seasonality bias, as follows: C57BL/6NCrl mice, $n = 18$ males (6 cages); $n = 18$ females (6 cages); BALB/cAnNCrl mice, $n = 18$ males (6 cages); $n = 18$ females (6 cages); CRL:CD1(ICR) mice, $n = 18$ males (6 cages); $n = 18$ females (6 cages). Each cohort was thus composed of 54 individuals (27 females plus 27 males equally divided per strain).

Home-cage activity monitoring: DVC system and activity metrics. All cages were kept in a DVC rack, a home monitoring system that automatically measures animal activity 24/7[19]. An electronic capacitance sensing board is positioned below each cage and consists of 12 contactless electrodes that record the animal's presence in each electrode surrounding. We used the 'activation density' metric to capture mouse activity in the cage[19], aggregated in 1-min bins. We then analyzed lights-on activity (the average of all the 1-min bins within 12 h of daytime) and lights-off activity (the average of all the 1-min bins within 12 h of nighttime). On the basis of previous reports[42], we calculated 'diurnality', which is the (daily) fraction of activity performed during the lights-on phase with respect to the total activity performed in the whole day, measured as the sum of lights-on and lights-off activity. We also calculated the RDI during both lights-on and lights-off phases[6]. This metric captures the level of irregularity of the activity pattern: a time series in which all minutes have similar activity levels gives a low RDI, whereas a high RDI indicates that minutes of activity are very different from each other.

Responses to procedures estimated with Gaussian mixture models (GMMs). To determine the location of activity peaks during the 24 h, especially those related to responses to lights-on and lights-off phases, we used GMMs. We used the library scikit-learn (version 0.19.1) for Python[43] to fit each 720-min time series (12 h of the lights-on or lights-off phase) as a mixture of several normal-density components so that we could calculate their means, weights and standard deviations. We used a fixed number M of components for all the time series to homogeneously compare results between cages and groups, and we set $M = 7$ after empirically observing the model fitting and mean absolute error for different M's. Among these seven, we used the means of first and last components during both daytime and nighttime as estimates of the time of responses to lights-on and lights-off phases (Supplementary Fig. 1).

We used a similar approach to calculate the duration of the response to the cage change. We selected the 5-h time series after each cage change and fit a GMM with $M = 3$ components. We determined the duration as the interval between the time of cage change and the time at which the fitted curve goes below 10% of its peak (we reported some examples in Supplementary Fig. 2). We also made a comparison between the results with GMMs and those with the full-width half-maximum method already described[19] (Supplementary Figs. 2 and 3).

Analysis of circadian rhythmicity. Typical measures used in the analysis of circadian rhythmicity are 'acrophase' and 'activity onset'[44]. Acrophase is the time at which the peak of the circadian rhythm occurs, and thus it is an estimate of the centrality and concentration of the activity during a 24-h period[45]. Activity onset is the time that animals start being active, and in the case of rodents, it typically refers to the time around the lights-off phase. Conventional approaches to numerically determine these metrics are generally based on a clear separation between day (extremely low or zero activity) and night (very high activity). This is common when using running wheels, whereas with spontaneous locomotion, the separation is not always so clear, and conventional approaches possibly need to be modified[46].

We applied cosinor analysis[47] to fit a cosine wave with known period ($t = 24$ h) to each daily activity time series (1,440 min, i.e., 24 h). The acrophase is determined as the time at which the fitted curve reaches its maximum value (Supplementary Fig. 4).

We estimated activity onset time by the template-matching algorithm used by the ClockLab analysis package (Actimetrics Inc.), which we empirically adapted to spontaneous activity data, for which the separation between day and night is not always so sharp. We considered only data lying in an interval of 12 h centered on lights-off time, smoothed with a 30-min moving average. Each time series (12 h) was transformed to an array of 1's and −1's depending on whether each minute exceeded or fell below the 60th percentile of all non-zero activity data. We then computed the convolution (a mathematical operator that returns the product between one fixed sequence and another sequence that slides) between the transformed time series and a template of N hours of −1's followed by M hours of 1's ($M = 6$, $N = 6$; i.e., 720 min of −1's and 720 minutes of 1's). Finally, we weighted the convolution for the number of samples of the time series overlapping it and determined the onset time with the location of the maximum of this weighted convolution (Supplementary Fig. 5).

Cluster analysis. Machine learning can be a novel approach to model complex data in animal behavior studies[22]. Cluster analysis is one of the most common unsupervised learning techniques, aiming to find groups composed of units similar to each other and different from the units of other groups. Here, we decided to apply a K-means algorithm to cluster the daily data and see if strains do separate in an unsupervised and data-driven approach. All the previously described metrics (lights-on and lights-off activity, diurnality, lights-on and lights-off phases, RDI, acrophase, activity onset and all metrics relative to responses to lights-on and lights-off conditions) were used as input for the clustering algorithm. We applied PCA to reduce dimensionality and then applied K-means with K = 3 clusters, with the aim of separating strains and not sex (which was not always a significant factor in our analyses). Each day of each cage was therefore classified in a specific cluster.

Statistical tests. Because the same individuals were assessed over time and for a long period (60 d), we used general linear mixed models to quantitatively evaluate differences between strains, sexes and time and light conditions. We used lmerTest R software package to model data and test for fixed effects[48]. We resorted to a top-down approach and successive likelihood ratio tests to define the model best explaining the data[49]. All selected models and relative statistical results are reported (Supplementary Material and Supplementary Data 1).

We used Python to process and visualize data and R (version 3.4.3) to run all statistics, with significance level $\alpha = 0.05$. We excluded days of cage changing from the analysis, as well as days with missing values or with some technical issues. As a consequence of group housing, the statistical unit is the cage[50]: DVC measures the overall aggregated value of activity of the mice for each cage, with a reduction of statistical power that is not necessary scaled down exactly with the aggregation factor, because of probable intra-cage correlation[24].

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Datasets and codes used in the analyses are stored at the authors' home institution and will be provided upon request.

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Author contributions

S.F., L.D'A., P.d.G. and F.I. designed and supervised the research. M.Ra. and F.S. were responsible for mouse maintenance and prepared a first draft of Methods. M.Ri. and F.I. analyzed and generated the datasets. L.D'A., P.d.G. and S.F. provided a first draft of the manuscript. All the authors reviewed and approved the manuscript.

Competing interests

F.I. and M.Ri. were employed by Tecniplast SpA, which provided support in the form of salaries for authors F.I. and M.Ri. Tecniplast SpA did not have any additional role in the study design, data collection and analysis, decision to publish or preparation of the manuscript. S.F. was employed by Charles River Laboratories Italy, which provided support in terms of animal models, salary for the author and final review and approval of the manuscript. Charles River Laboratories did not have any additional role in the study design, data collection, analysis or interpretation.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41684-021-00793-0.

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Sample size	Samples size was made of 6 cages per group (the cage is the statistical unit of the analysis). Specifically: Males C57Bl6/NCrl, n=18 (6 cages); Females C57Bl6/NCrl, n = 18 (6 cages); Males BALB/cAnNCrl, n = 18 (6 cages); Females BALB/cAnNCrl, n = 18 (6 cages); Males CRL:CD1(ICR), n = 18 (6 cages); Females CRL:CD1(ICR), n = 18 (6 cages). This sample size was determined based on cage density (3 animals per cage) to mimic possible standard housing conditions in research settings.
Data exclusions	Data excluded from calculations were days with some missing values or some technical issues.
Replication	All data are replicable.
Randomization	Males and females of each murine strain were randomly subdivided in cages after acclimatation.
Blinding	Blinding was not relevant for this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☐ Antibodies
☐	☐ Eukaryotic cell lines
☐	☐ Palaeontology
☐	☒ Animals and other organisms
☐	☐ Human research participants
☐	☐ Clinical data

Methods

n/a	Involved in the study
☐	☐ ChIP-seq
☐	☐ Flow cytometry
☐	☐ MRI-based neuroimaging

Antibodies

Antibodies used	*Describe all antibodies used in the study; as applicable, provide supplier name, catalog number, clone name, and lot number.*
Validation	*Describe the validation of each primary antibody for the species and application, noting any validation statements on the manufacturer's website, relevant citations, antibody profiles in online databases, or data provided in the manuscript.*

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	*State the source of each cell line used.*
Authentication	*Describe the authentication procedures for each cell line used OR declare that none of the cell lines used were authenticated.*
Mycoplasma contamination	*Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.*
Commonly misidentified lines (See ICLAC register)	*Name any commonly misidentified cell lines used in the study and provide a rationale for their use.*

Palaeontology

Specimen provenance	*Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information).*
Specimen deposition	*Indicate where the specimens have been deposited to permit free access by other researchers.*
Dating methods	*If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement),*

Dating methods	*where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.*

☐ Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Mus musculus: C57Bl6/NCrl males and females from 5 to 12 weeks of age BALB/cAnNCrl males and females from 5 to 12 weeks of age CRL:CD1(ICR) males and females from 5 to 12 weeks of age
Wild animals	*Provide details on animals observed in or captured in the field; report species, sex and age where possible. Describe how animals were caught and transported and what happened to captive animals after the study (if killed, explain why and describe method; if released, say where and when) OR state that the study did not involve wild animals.*
Field-collected samples	*For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.*
Ethics oversight	The study, data collection and analysis were approved by the Institutional Animal Care and Welfare Body of the CNR-IBBC/ EMMA/Infrafrontier. Animal maintenance was performed in accordance with general guidelines regarding animal breeding and biotechnology, in compliance with the Italian Legislative Decree 26/2014.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics	*Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."*
Recruitment	*Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.*
Ethics oversight	*Identify the organization(s) that approved the study protocol.*

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration	*Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.*
Study protocol	*Note where the full trial protocol can be accessed OR if not available, explain why.*
Data collection	*Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.*
Outcomes	*Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.*

ChIP-seq

Data deposition

☐ Confirm that both raw and final processed data have been deposited in a public database such as GEO.

☐ Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links *May remain private before publication.*	*For "Initial submission" or "Revised version" documents, provide reviewer access links. For your "Final submission" document, provide a link to the deposited data.*
Files in database submission	*Provide a list of all files available in the database submission.*
Genome browser session (e.g. UCSC)	*Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.*

Methodology

Replicates	*Describe the experimental replicates, specifying number, type and replicate agreement.*
Sequencing depth	*Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.*
Antibodies	*Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.*
Peak calling parameters	*Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.*
Data quality	*Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.*
Software	*Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.*

Flow Cytometry

Plots

Confirm that:

☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☐ All plots are contour plots with outliers or pseudocolor plots.

☐ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	*Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.*
Instrument	*Identify the instrument used for data collection, specifying make and model number.*
Software	*Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.*
Cell population abundance	*Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.*
Gating strategy	*Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.*

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type	*Indicate task or resting state; event-related or block design.*
Design specifications	*Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.*
Behavioral performance measures	*State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).*

Acquisition

Imaging type(s) *Specify: functional, structural, diffusion, perfusion.*

Field strength *Specify in Tesla*

Sequence & imaging parameters *Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.*

Area of acquisition *State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.*

Diffusion MRI ☐ Used ☐ Not used

Preprocessing

Preprocessing software *Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).*

Normalization *If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.*

Normalization template *Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.*

Noise and artifact removal *Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).*

Volume censoring *Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.*

Statistical modeling & inference

Model type and settings *Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation).*

Effect(s) tested *Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.*

Specify type of analysis: ☐ Whole brain ☐ ROI-based ☐ Both

Statistic type for inference (See Eklund et al. 2016) *Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.*

Correction *Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).*

Models & analysis

n/a	Involved in the study
☐	☐ Functional and/or effective connectivity
☐	☐ Graph analysis
☐	☐ Multivariate modeling or predictive analysis

Functional and/or effective connectivity *Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).*

Graph analysis *Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).*

Multivariate modeling and predictive analysis *Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.*