Journal of the American Association for Laboratory Animal Science
Copyright 2022
by the American Association for Laboratory Animal Science

Vol 61 No 6
November 2022
Pages 615–623

Changes in the Behavior and Body Weight of Mature, Adult Male Wistar Han Rats after Reduced Social Grouping and Social Isolation

Milica S Potrebić,[1] Željko Z Pavković,[1] Maja M Srbovan,[1] Goran M Đmura,[2] and Vesna T Pešić[1,*]

Changes in housing density, including individual housing, are commonly necessary in animal research. Obtaining reproducibility and translational validity in biomedical research requires an understanding of how animals adapt to changes in housing density. Existing literature mainly addresses acclimatization after transportation. We used a within-subject design to examine changes in behavior and weight gain of 4-mo-old male Wistar Han rats after reduction of their social group (RSG; due to removal of one rat from a cage containing 3 rats) and social isolation (SI; the removed rat) for the subsequent 2 wk. Changes in weight gain and in exploratory and center-avoidance behavior in an inescapable open arena (OA) were measured before (D0) and on days 7 and 14 (D7 and D14, respectively) after social change. The motor response to d-amphetamine (1.5 mg/kg), which stimulates behavioral arousal in response to novelty, was assessed at D14. Within-subject design revealed that RSG rats in OA had less locomotion at D7 but not more center-avoidance behavior and had returned to the D0 activity level at D14; SI rats in OA had consistently less locomotion and more center-avoidance behavior. Rearing behavior during OA exposure did not change in either group. However, SI rats showed more center-avoidance behavior in OA, greater weight gain, and less amphetamine-induced rearing at D14 as compared with RSG rats. These data indicate that after RSG, mature adult male rats require 2 wk to return to their baseline level of OA-related behavior, while after SI they gain weight and acquire maladaptive exploratory and center-avoidance behavior. The finding that SI produces maladaptive behavioral and physiologic alterations in adult male rats deserves attention because these changes could have confounding effects on research findings.

Abbreviations: AMPH, d-amphetamine; OA, inescapable open arena; RSG, reduced social group; SI, social isolation.

DOI: 10.30802/AALAS-JAALAS-22-000032

Introduction

The social environment is an important health factor for all social species including rats, which are among the most used research subjects.[57] Compatible and stable social groups are crucial for animal welfare,[17] but changes in housing densities may be necessary due to aggression, sickness, or experimental requirements (to obtain accurate measurements, to avoid damage to monitoring apparatus). Given the importance of reproducibility and translational validity in biomedical research, we performed a systematic assessment of changes in social housing on experimental outcomes.[66] Our study examined the behavioral consequences of a reduced social group (RSG), how long such changes persisted and whether the behavioral effects of RSG differ from those of social isolation (SI). Existing literature mainly focuses on acclimatization after transportation and indicates that an acclimation period of at least 3 d is necessary for stabilization, based on physiologic parameters[10] while acclimation of home-cage behavioral parameters (a return to before-transportation levels) takes approximately 2 wk in Wistar rats.[4]

A bias toward using young animals has been reported in the literature.[33,42] However, the use of young animals can reduce the scientific validity of the findings, as in some cases the use of older animals may be more appropriate.[23,33] Brain maturation processes in rats suggest that they can be considered adult only after 3 mo of age.[45] Also, the level of testosterone, one of the major sex hormones involved in the regulation of socio-emotional behavior[15] stabilizes in rats at 3 mo of age.[7]

Grouping of rats before they reach puberty may avoid or minimize problems of aggression between unfamiliar individuals.[31] Moreover, a study on male Wistar rats showed that housing rats in groups of 3 or 4 per cage had the fewest physiologic effects.[34] Directive 2010/63/EU[17] recommended a floor area 350 cm^2 per rats weighing 300 to 400 g is and 450 cm^2 for rats weighing 400 to 600 g, which means that rats weighing around 400 g and sharing the same cage (enclosure size 800 cm^2) over a long period could be housed either in a stable group (to give priority to stable social structures, as indicated by the Directive 2010/63/EU[17]) or 2 per cage (especially if they are destined for use in a long-term experiment, which implies an additional increase in body weight). However, an important question is whether, and if so, when rats that experience RSG will be suitable for inclusion in experiments.

Clear recommendations are not available regarding minimal acclimatization periods after changing the number of animals per cage. Animals respond behaviorally and physiologically to adapt to changing environments and challenges, such as social interactions. Maintaining stability through change (allostasis) is a fundamental process through which organisms actively adjust to predictable and unpredictable events,[43] with the

Submitted: 07 Apr 2022. Revision requested: 26 May 2022. Accepted: 22 Jun 2022.
[1]Department of Neurobiology, Institute for Biological Research Siniša Stanković – National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia, [2]Animal Facility, Institute for Biological Research Siniša Stanković – National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia
[*]Corresponding author. Email: vesnav@ibiss.bg.ac.rs

emotional brain being essential in allostasis.[35] However, as already indicated,[4] applying the concept of allostasis would result in a different view on acclimatization periods, shifting anticipation from a return to baseline levels to stabilization of parameters at potentially different levels.

On the other hand, some recommendations are that rats should be housed individually only when approved by the Animal Ethics Committee of the institution based on compelling evidence for the need to house rats in this way.[2] Isolation studies using rodents are undoubtedly important in biomedical research,[19] as are studies that try to define the best conditions for the animal welfare.[6] In studies that intentionally use SI, the neurobehavioral outcomes must be closely evaluated because the type of housing is an important experimental variable. Actually, all studies should consider the effects of housing condition on neurobehavioral outcomes. Even if single housing is supported by a harm-benefit analyses,[40] the question is how housing conditions contribute to the complexity of the model and perhaps affect the external validity or generalization of data, such that the model is different than planned.[41] For example, studies in mice and rats reveal that isolation housing of juvenile animals may result in neurologic adaptations that promote locomotor sensitization and potentiate drug-taking,[13,20] while adult animals may be largely untested in this context. In rodents, increased locomotor activity in a novel environment reflects sensation seeking and is used in preclinical studies to examine the role of behavioral or personality traits in the acquisition of drug-taking behavior, specifically psychostimulants.[12,36] Behavior in a novel environment should predict responsiveness to d-amphetamine (AMPH) in rats.[18] However, differences in the behavioral response to AMPH can exist without measurable differences in the baseline activity; this outcome could be puzzling because it may give the impression that housing conditions do not interfere with the study outcomes. Moreover, one study suggests that SI for 1, 2, or 3 wk does not cause behavioral abnormalities in adult male rats, as none of the experimental groups showed statistically significant behavioral changes in comparison with a control group kept under standard conditions.[26] However, without considering the role of the animal personality in susceptibility to social changes, we cannot define the period of SI necessary to produce behavioral changes in an animal.

Considering all the above, the present study aimed to assess changes in behavior and body weight of mature (4 mo old) adult male Wistar Han rats, which are widely used in toxicology and carcinogenicity studies[29], as a result of RSG (due to removal of one rat from a cage with 3 rats) and SI (the removed rat), during the 2 wk after the social alterations. Changes in activity and center-avoidance behavior in an inescapable open arena (OA) and weight gain were measured on day 0, day 7, and day 14 (D0, D7, and D14) after social changes. Motor response to AMPH (1.5 mg/kg), which produces a behaviorally aroused state that can be predicted by an animal's responsivity to novelty,[18] was assessed on D14. A within-subject design was used to increase statistical power. Because the behavior of mature adult animals is not influenced by developmental events and senescence, weekly monitoring of the behavioral response of mature adult RSG and SI rats to the same environment should reflect alterations in animals' perception of and responsiveness to the testing environment after the changed housing conditions. We hypothesized that RSG rats would normalize exploratory behavior within 2 wk after the social alteration, while SI rats would show maladaptive characteristics due to the inability to overcome the new social environment.

Materials and Methods

Animals. The experiment used 4-mo-old and experimentally naïve male Wistar Han rats that had been bred and housed at the Institute for Biologic Research–Siniša Stanković. The rats were from 8 different litters, and the litter was an experimental unit. Three 7-wk-old males from the same litter were randomly chosen for housing in the same cage in which they lived until 4 mo of age. We applied the recommendation of the Directive 2010/63/EU[17] that, if in long-term studies the space available per animal falls below those indicated in the Directive 2010/63/EU,[17] priority should be given to maintaining a stable social structure. All animal procedures were in compliance with the Directive 2010/63/EU and were approved by the Ethical Committee of the Institute (Number 01 to 247) and by the National Ethics Research Committee (323-07-02030/2021-05).

The total number of rats used in this experiment was 24, of which 16 underwent behavioral testing ($n = 8$ per experimental group, 2 experimental groups). The sample size was chosen to obtain sufficiently informative results while also using the lowest number of animals based on previous experience with the proposed tests.[9,47,64]

The rats were housed under the following conditions: a room temperature of 22 ± 1 °C, relative humidity 50 ± 5%, 12:12-h light/dark cycle with lights (diffuse lighting, 20-50 lux light level along the cages on the shelves) on at 0700, cages 425 (L) × 265 (W) × 180 (H) mm with enclosure size of 800 cm^2 (European standard Type 3H, ZOONLAB, Castrop-Rauxel, Germany) made from transparent plastic, autoclaved wood shavings as bedding material (native spruce and fir; PREMIUMSPAN®, HVT Hobelspanverarbeitung GmbH, Dittersdorf/Thüringen, Germany) provided in sufficient quantity to cover the floor to a depth of 2 cm, chow (commercial pellets produced on demand, Gebi Doo, Cantavir, Serbia; protein 20%, fat 4%, fiber 5.5%, detailed content given in reference 50)and tap water provided ad libitum. Environmental enrichment was not provided as it could influence brain neurochemistry and behavior of the rats,[60] thus requiring the use of additional controls for the experimental paradigm and potentially providing a source of increased data variability.[63] The rats were free of all viral, bacterial, and parasitic pathogens listed in the Federation of European Laboratory Animal Science Associations (FELASA) guidelines. They were observed every day and were without observable signs of illness and distress. The study did not include humane endpoints.

Experimental procedure. A schematic of the experimental design is shown in Figure 1. At 4 mo of age (mature adult stage in rodents), rats originating from 8 different litters and housed in 8 cages (3 animals from the same litter per cage) were weighed and then evaluated for their spontaneous exploratory response in an inescapable open arena (OA) as a novel environment (D0). After this initial behavioral testing, 1 rat was randomly chosen and removed from each cage for single housing (that is social isolation, SI) while the other 2 remained in the reduced social group (RSG). In the RSG rats, 1 per cage was randomly selected for all individual behavioral tests; this rat was labeled using a nontoxic permanent marker on the tail. The cages were placed side by side and rats could smell, see, and hear rats in other cages, but could interact socially only in the group cages. On D7, rats were again weighed and tested in the OA. On D14 they were also weighed and tested in the OA; the latter test also provided the intrasession habituation for subsequent challenge with AMPH.

Rats were tested 3 times at 7-d intervals. The elapsed time between initial exposure and re-exposure gave the rats a greater opportunity to forget the experience of OA.[38] Rearing activity,

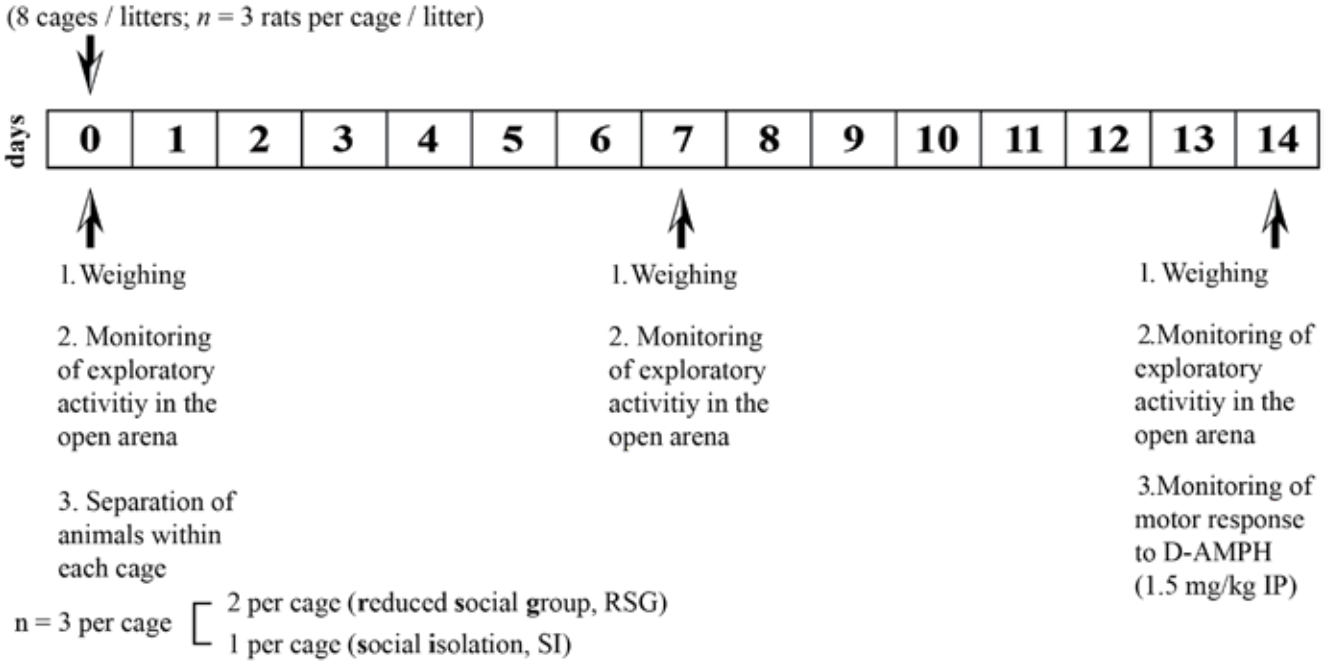

Figure 1. Schematic presentation of the experimental design.

which was our critical behavioral measure of habituation, should indicate whether a rat was habituated to the OA at the time of re-exposure.[56,62]

Before each testing, the rats were habituated (in home cages) to the testing room (sound isolated, temperature 22 ± 1 °C; light intensity averaged 150 lx) for at least 30 min. During the testing, the frequency and duration of human presence were equal for both experimental groups, with only one qualified person being in direct contact with the rats, thus preventing experimenter identity from being a source of data variability. Cage cleaning was never performed before behavioral testing.

Data collection in the OA test was automatic; blinding was applied to the analysis of the data. Outcome measures of the study were behavioral and body weight changes. No criteria were used to exclude rats, and all obtained data were included in the analysis (each analysis had n = 8 animals per group). Having all grouped rats coming from the same litter ensured that individual values in each experimental group were derived from independent samples. A within-subject design was used to allow each subject to be its own control. This type of design controls for extraneous participant variables, making it easier to detect the relationship between independent and dependent variables than with between-subject design.

Monitoring of behavioral response to the inescapable open arena. Exploratory activity was examined under the assumption that in satiated rats, it reflects the need to reduce environmental uncertainty[32] and can also indicate a positive emotional state with relevance to animal welfare[8]. All behavioral testing took place between 900 and 1400 to avoid influences of diurnal changes in plasma corticosterone levels.

The testing was done using Opto-Varimex cages (Columbus Instruments, Columbus, OH; 44.2 × 43.2 × 20 cm) that were linked online to an IBM-PC-compatible computer, as previously reported.[47] The equipment includes 2 experimental arenas, which allows parallel testing of rats from 2 experimental groups. Data were analyzed using Auto-Track software (Columbus Instruments). The Auto-Track interface collects data from the Opto-Varimex unit every $1/10^{th}$ of a second and categorizes the activity. The Auto-Track interface can detect movements in 16 (4 × 4) equal fictional squares, calculating the number of entries and time spent in the central zone (the 4 middle squares).

After termination of the 20-min exploratory period in the open arena, tested rats were returned to their home cages consistent with their previous housing condition, and arenas were cleaned and deodorized with a 20% ethanol cleaning solution to erase any smells that might interfere with the behavior of the next rat.

Monitoring of animals' body weight and weight gain. Body weight provided an objective physiologic and welfare indicator.[28] Rats were weighed on an electronic balance on D0, D7, and D14, one hour before behavioral testing. Each rat was confined in a perforated chamber that had been tared on the scale before placement of the rat. Weight gain was calculated by subtracting baseline weight (weight at D0) from the weights recorded on D7 and D14.

Monitoring of AMPH-induced behavioral response. The behaviorally activating effects of AMPH were examined because this drug produces an unconditional, behaviorally aroused state in mammals[1] and previous findings indicate that behavior in the novel environment predicts responsiveness to AMPH.[18]

After completing the 20-min exploratory period in the open arena at D14, rats were injected intraperitoneally with AMPH (Sigma-ALDRICH Chemie, Germany) at a dose of 1.5 mg/kg. This dose is within the dose range that produces a well-described behavioral pattern including hyperlocomotion and rearing.[49] The drug was dissolved in saline (0.9% NaCl) at a concentration of 1.5 mg/mL and was dosed at 1 mL/kg. Assessment of the behavioral response began immediately after the rats were returned to the arenas (Opto-Varimex cages) and lasted for 100 min. Data were classified and collected automatically, as described above.

Statistical analysis. The data were presented as means ± SD, with individual data plots along the column bars, and were statistically analyzed using Statistica 6.0 software (StatSoft). The normality of data sets was estimated by Shapiro-Wilk's test. The accepted level of significance was $P < 0.05$.

As some data sets did not have a normal distribution, the results obtained in behavioral tests were analyzed using nonparametric statistics: Friedman test was followed by pairwise Wilcoxon signed-rank tests (if appropriate) for repeated measures across time; independent groups were compared using Mann-Whitney *U* test. The results of the Wilcoxon test are subjected to Holm correction to determine which of the partial tests were effectively significant[24] and only those that met the criteria were considered in the results section.

The parameters analyzed in the OA were locomotor activity, vertical activity, time spent in the central zone, and the number of entries in the central zone. Changes in total counts were analyzed for each parameter. In addition, activity during the first and last 5 min of the test session was considered to indicate intra-session habituation, as an indication of adaptability (that is, the reduction in activity that occurred in a single exposure to the OA).[38]

Body weight data were analyzed using repeated measure ANOVA with the housing condition and the experimental day (repeated measure) as factors, followed by a post hoc Tukey test. Between-group differences in weight gain over particular time points were analyzed using a *t* test for independent samples, and within-group changes were assessed using a *t* test for dependent samples.

Results

Activity of mature adult male rats in the OA within 2 wk after social change. Locomotor activity counts in the OA showed significant changes across examined period (D0, D7, and D14) in both the RSG ($\chi 2_2 = 9.000$, $P < 0.011$) and SI ($\chi 2_2 = 9.250$, $P < 0.010$) groups (Figure 2A). For the RSG group, the Wilcoxon test revealed a significant difference between D0 and D7 (Figure 2A; * $P = 0.012$) that returned to baseline at D14 (* $P = 0.327$). For the SI rats, the Wilcoxon test revealed a significant difference between D0 and D7 (Figure 2A; * $P = 0.012$) and D0 and D14 (Figure 2A; * $P = 0.017$). No difference was detected between the RSG and SI groups either in the initial testing or at the end of the study (Figure 2A ; D0: $P = 0.208$; D14: $P = 0.115$, *U* test).

Vertical activity counts in the OA did not change significantly across the tests period in either the RSG or the SI groups (Figure 2B; Friedman test: $\chi 22 = 2.774$, $P < 0.250$ and $\chi 22 = 3.250$, $P < 0.197$, respectively). No difference was detected between the groups on either D0 or D14 (Figure 2B; D0: $P = 0.916$; D14: $P = 0.958$).

The small graphs embedded in Figures 2A and 2B show changes in locomotor and vertical activity, respectively, during the monitoring period (20 min). Comparison of activity during the first 5 min and last 5 min enables the assessment of within-session habituation (adaptive response). Regarding locomotor activity, both groups showed within-session habituation in all test sessions (the small graph embedded within Figure 2A; D0, D7, and D14, # $P = 0.012$, Wilcoxon test). Both groups of rats also showed within-session habituation of vertical activity in all test sessions (the small graph embedded within Figure 2B; D0, D7, and D14, # $P \leq 0.017$, Wilcoxon test).

Center avoidance behavior of mature adult male rats in the OA during the 2 wk period after social change. Total time spent in the central zone of the OA did not change significantly across test sessions in the RSG ($\chi 2_2 = 1.000$, $P < 0.606$) as compared with the SI rats ($\chi 2_2 = 12.452$, $P < 0.002$) (Figure 3A). For the SI group, the Wilcoxon test revealed a significant difference between D0 and D7 and D0 and D14 (Figure 3A; * $P = 0.012$ for both comparisons). No difference was found between the RSG and SI rats in total time spent in the central zone during the initial testing (Figure 3A; D0: $P = 0.916$) but a difference was evident at D14 (Figure 3A; $P =$ & 0.031).

The total number of entrances to the central zone of the OA did not change significantly across the monitoring period in

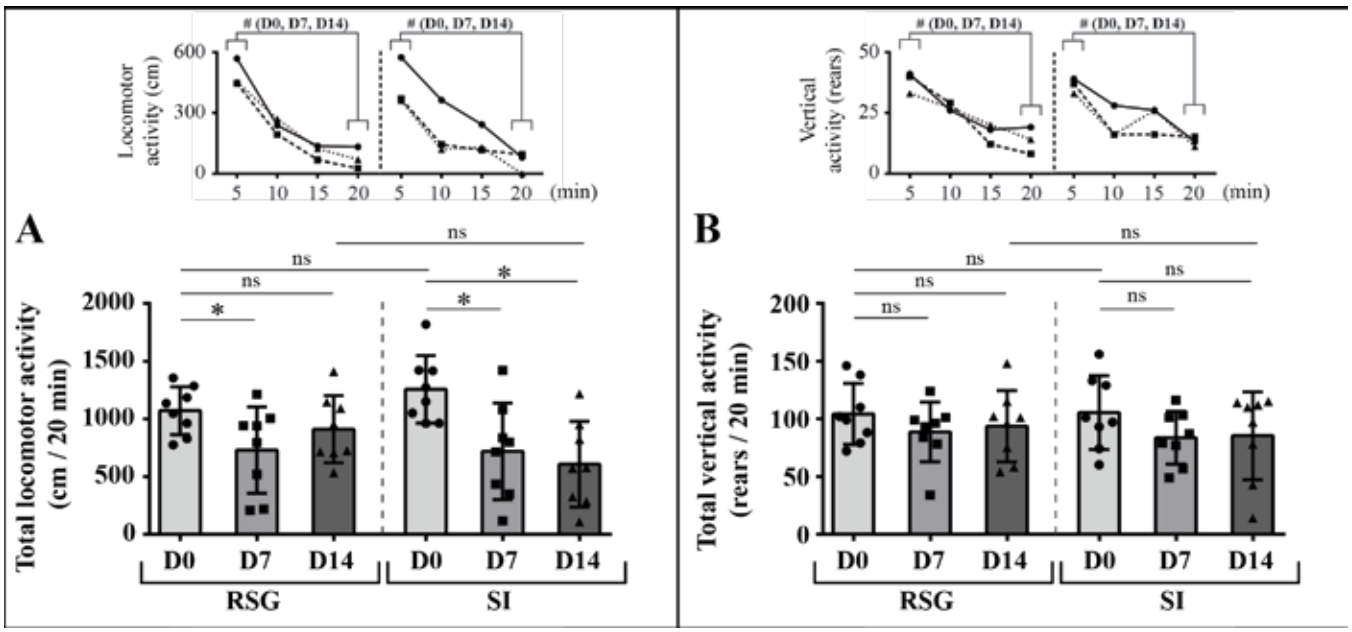

Figure 2. Exploratory behavior of mature adult male Wistar Han rats in the inescapable open arena on days 0, 7 and 14 after social change. Rats were tested before their social status changed (experimental day [D] 0), at D7 and at D14 after reduced social grouping (RSG) and social isolation (SI). The data are represented as mean ± SD, with individual data plotted along the column bars (8 rats per group). (A) Locomotor activity counts for a 20-min monitoring period. Inserted line graphs show changes in locomotor activity during this time. (B) Vertical activity counts for a 20-min monitoring period. Inserted line graphs show changes in vertical activity during this time. * $P < 0.05$ compared with D0 of the same group, Wilcoxon test; # $P < 0.05$ compared with the first 5 min of the observation period in the same group, Wilcoxon test.

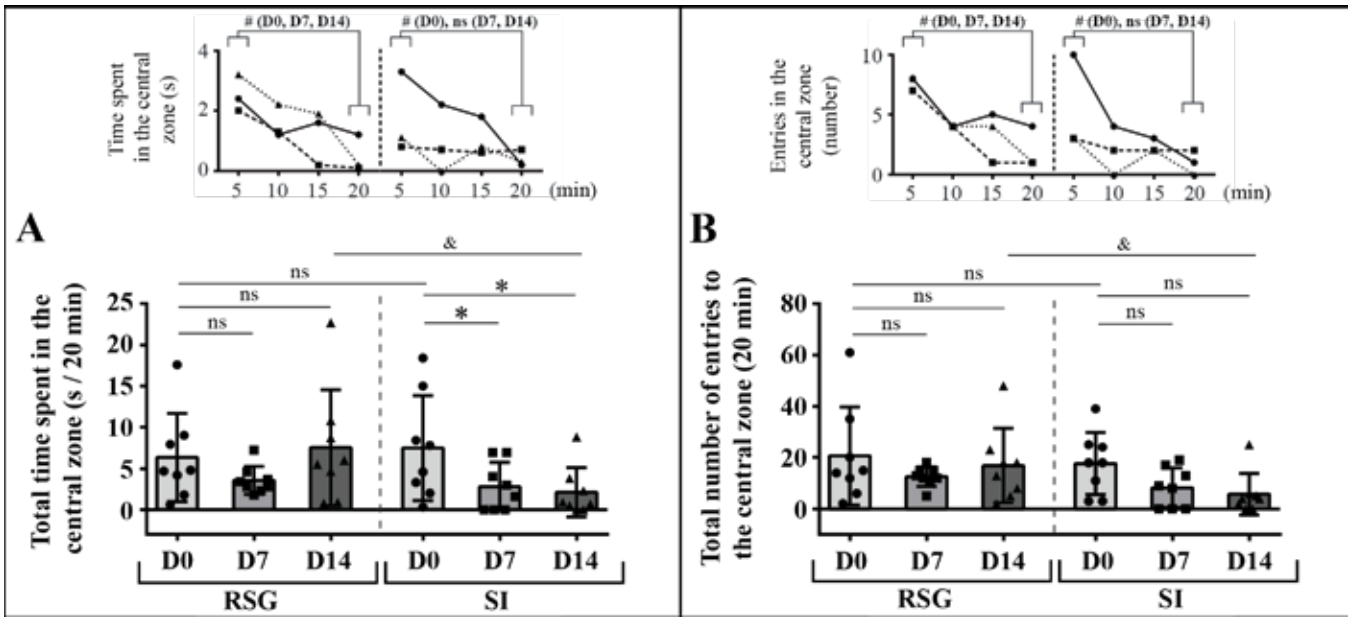

Figure 3. Center avoidance behavior of mature adult male Wistar Han rats in the inescapable open arena on days 0, 7 and 14 after social change. Testing was performed before the social status of the rats was changed (experimental day [D] 0), at D7 and at D14 after reduced social grouping (RSG) and in social isolation (SI). The data are represented as mean ± SD, with individual data plotted along the column bars (8 rats per group). (A) Total time spent in the central zone of the arena during a 20-min monitoring period. Inserted line graphs show changes in the parameter during this period. (B) Total number of entries in the central zone during a 20-min monitoring period. Inserted line graphs show changes in this parameter during this time. * $P < 0.05$ compared with D0 of the same group, Wilcoxon test; # $P < 0.05$ compared with the first 5 min of the observation period in the same group, Wilcoxon test; & $P < 0.05$ compared with the same day of the RSG group, U test.

the RSG ($\chi 2_2 = 1.000$, $P < 0.606$) but did change in the SI rats ($\chi 2_2 = 7.032$, $P < 0.029$) group (Figure 3B). However, the outcomes of the Wilcoxon test for the SI group did not pass the criteria of Holm correction and were considered insignificant. No difference was detected between the RSG and SI rats in the total number of entrances to the central zone during the initial testing (Figure 3B; D0: $P = 0.916$) but a difference was found at D14 (Figure 3B; & $P = 0.046$).

The small graphs embedded within Figures 3A and 3B show the dynamics of changes in the activities of the rats in the central zone during the 20 min monitoring period. Comparison of the activity of rats during the first 5 min and the last 5 min allows assessment of within-session habituation (adaptive response). In all test sessions (D0, D7, and D14), the RSG rats showed within-session habituation in the time spent in the central zone of the OA (the small graph embedded within Figure 3A; # $P \leq 0.028$, Wilcoxon test). However, although the SI rats had within-session habituation in time spent in the central zone of the OA at D0 (the small graph embedded within Figure 3A; # $P = 0.012$, Wilcoxon test), they lost this ability after isolation (D7: $P = 0.685$; D14: $P = 0.173$). In all test sessions (D0, D7, and D14), the RSG rats showed within-session habituation in the number of entrances to the central zone of the OA (the small graph embedded within Figure 3B; # $P \leq 0.042$, Wilcoxon test). However, although the SI rats had within-session habituation in the number of entrances to the central zone of the OA at D0 (the small graph embedded within Figure 3B; # $P = 0.012$, Wilcoxon test), they lost this ability after isolation (D7: $P = 0.589$; D14: $P = 0.075$).

Body weight and weight gain of mature adult male rats during 2 wk after social change. The ANOVA of body weight of both RSG and SI rats during 2 wk study (Figure 4A) revealed no significant influence of social change ($F_{1, 14} = 1.187$, $P = 0.294$) but a significant influence of time ($F_{2, 28} = 59.282$, $P < 0.001$) and social change x time ($F_{2, 28} = 6.534$, $P = 0.005$). Posthoc analysis (Tukey HSD test) revealed a difference between D0 and D7 (Figure 4A; * $P = 0.003$), D0 and D14 (Figure 4A; * $P < 0.001$) and D7 and D14 (Figure 4A; $ $P < 0.001$) for the SI group, while for the RSG group a difference was present only between D0 and D14 (Figure 4A; * $P < 0.001$; D0 *vs.* D7 $P = 0.112$; D7 *vs.* D14 $P = 0.156$). Between-groups differences (RSG *vs.* SI) were not obtained either in the initial time point (D0: $P = 0.999$) or at the end of the examined period (D14: $P = 0.927$).

A significant increase in weight gain during the 2 wk after social change occurred in both RSG and SI rats (Figure 4B; # $P < 0.001$, *t* test for dependent samples). At D14, the SI group gained more weight than the RSG group (Figure 4B; & $P = 0.017$; *t* test for independent samples).

AMPH-induced locomotor and rearing activity of mature adult male rats at 2 wk after the social change. AMPH-induced locomotor activity was not different between RSG and SI rats either at specific time points of the monitoring period (Figure 5A, left) or in the summary values (Figure 5A, right; $U = 25$, $P = 0.462$, *U* test). AMPH-induced rearing activity differed in RSG and SI animals periodically during the monitoring period (Figure 5B, left; $U \leq 13$, $P \leq 0.046$) and in the summary values (Figure 5B, right; $U = 9$, $P = 0.016$, *U* test).

Discussion

Understanding the impact of routine procedures on animal physiology and behavior is a prerequisite for improving data reliability and optimizing the number of animals used in experiments. The current study makes its contribution by emphasizing that RSG in mature adult male rats transiently reduces locomotor activity during OA exploration, which returns to a baseline level after 2 wk, while SI consistently affects locomotor activity, center-avoidance behavior, and AMPH-induced rearing behavior and weight gain after 2 wk of isolation. Although the lack of a socially intact group of rats limits the interpretation of this

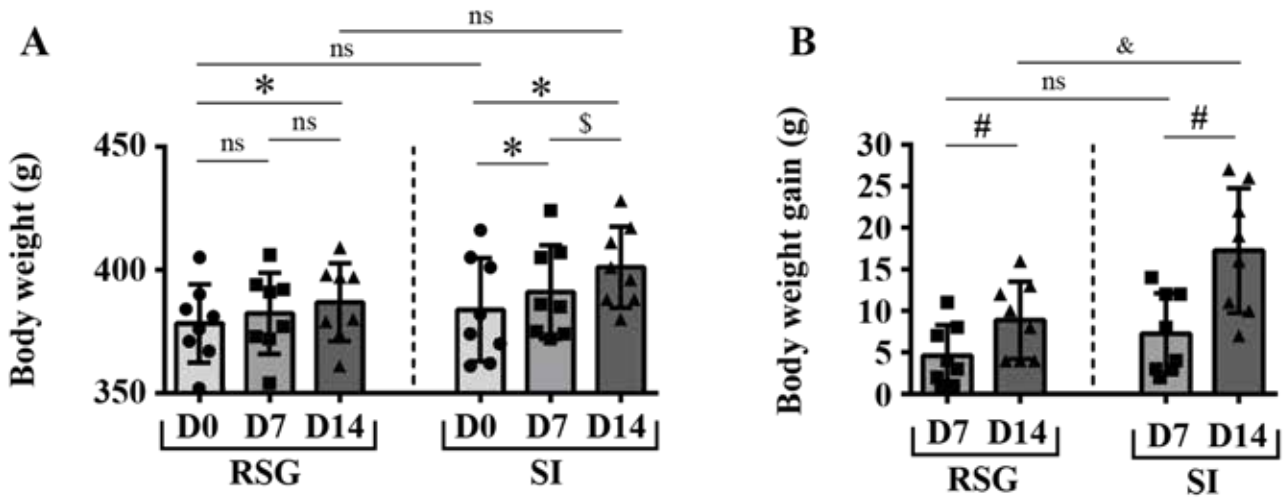

Figure 4. Body weight and weight gain in mature adult male Wistar Han rats on days 0, 7, and 14 after social change. Weights were measured before social change (experimental day [D] 0), at D7 and at D14 after reduced social grouping (RSG), and social isolation (SI). The data are shown as mean ± SD, with individual data plotted along the column bars (8 rats per group). (A) Body weight of rats during 2 wk after social changes. * $P < 0.05$ compared D0 of the same group, \$$P < 0.05$ compared with D7 of the same group, Tukey test. (B) Body weight gain, calculated by subtracting baseline weight (that is weight at the D0) from the weight recorded on D7and D14. # $P < 0.05$ compared with D7 of the same group, t test for dependent samples; & $P < 0.05$ compared with the same day of the RSG group, *t* test for independent samples.

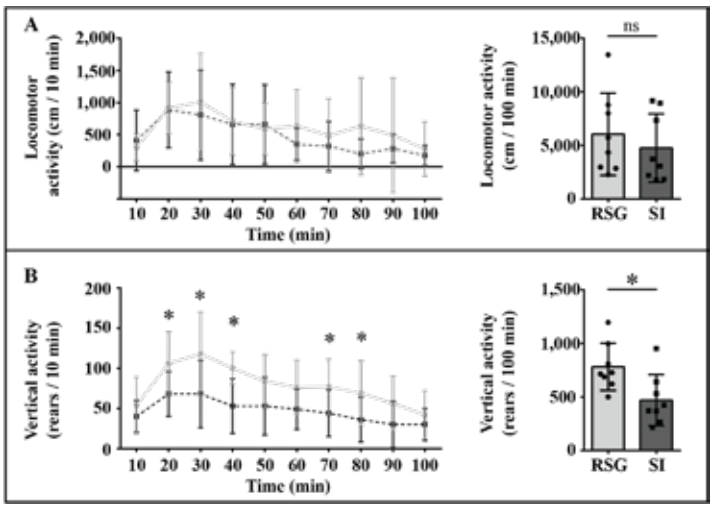

Figure 5. AMPH-induced locomotor (A) and vertical (B) activity of mature adult male Wistar Han rats on day 14 after social change. The data are shown as means ± SD of the examined parameters over a 100 min observation period after AMPH injection (left parts of the graphs), and as mean ± SD with individual data plotted along the column bars for activity counts (right parts of the graphs). AMPH was given at a dose of 1.5 mg/kg IP. Each group was comprised of 8 rats. RSG, reduced social group; SI, social isolation. * $P < 0.05$ compared with the RSG group, U test.

study, our obtained results indicate that using socially disrupted rats could interfere with research findings.

Exploration is a basic need and a potential positive emotional state indicator that is relevant to animal welfare.[8] Exploratory behavior in rats is characterized by increased locomotion and rearing. Characteristics of the individual animals can be assessed by examining differences in exploratory behavior.[44] In the present study, a detailed analysis of locomotor and vertical activity revealed that RSG and SI conditions influence these responses in a specific way. Precisely, neither the RSG nor SI affected rearing, which is a hippocampus-dependent[39] and stress-sensitive behavior.[61] Because rearing is a critical behavioral measure of habituation,[56,62] our findings suggest that the rats did not habituate to the OA as a result of repeated testing. On the other hand, locomotor activity in the OA was lower in both the RSG and SI rats as compared with their initial test in the same setting, with the proviso that this effect was temporary in the RSG rats and their locomotor response to OA returned to the baseline (D0 level) at D14. The current study also showed that, regardless of the reduction of total locomotor activity, neither RSG or SI affected the adaptability of either the horizontal or vertical responses to the OA as rats from both groups showed intrasession habituation (that is, significant decreases over the testing period in all test sessions[38]). Because locomotor activity in satiated rats represents information-gathering behavior directed toward reducing environmental uncertainty[32] and possibly a search for an escape,[18] our findings suggest that socially disrupted mature adult male rats have decreased interest in environmental uncertainty, while intra-session habituation, as an indication of adaptability,[38] is generally preserved.

A lower locomotor response to spatial novelty due to changed social conditions could be a consequence of 1) reduced motivation to explore, due to the less rewarding effect of novelty (a novelty signal from the hippocampus activates mesencephalic dopaminergic neurons in the substantia nigra/ventral tegmental area [SN/VTA] and enhances exploratory activity through the engagement of neural reward systems),[37,65] 2) increased anxiety in response to novelty (the interplay between brain circuits controlling the exploratory drive on one hand and the avoidance response on the other is crucial for the overall exploratory response) because activation of anxiety-associated regions may reduce exploratory drive and negatively affect exploratory activity.[44]

Previous findings have shown that locomotion alone is an unreliable indicator of emotionality, as both hypolocomotion and hyperlocomotion could express highly emotional behavior.[3,21] In the present study, we used a relatively small open arena to obtain a good measure of locomotor activity, but SI rats also showed changes in time spent in the central zone, which is typically found in large arenas (for a discussion please see references 59,64). Previous findings obtained using comparably sized fields highlighted thigmotaxic behavior as an indicator of emotionality in rats.[9,55,64] Although center avoidance in the open field is commonly considered an anxiety-like behavior, this simplified interpretation has been criticized as being insufficiently validated (for an extensive discussion on this topic please see reference 27). However, anxiety has only a moderate effect on the behavior of rodents in open areas, given that the effect of anxiolytic drugs on rodent thigmotactic behavior is limited.[52] Center-avoidance behavior is a defensive behavior that might occur with a certain degree of anxiety.[27] Therefore, based on the findings of the present study, we conclude that isolation housing in mature adult male rats promotes center-avoidance behavior and abolishes the adaptive profile of this response over test sessions (that is, intensifies avoidance behavior as an active choice).[5] Other assumptions and interpretations of the results could be unreliable given the limited amount of information available in this study.

Overall, the absence of a within-subject design and initial (D0) behavioral measurement may obscure the recognition that 1 wk of life in isolation can affect locomotor and center-avoidance behavior of mature adult male Wistar Han rats in OA. The RSG rats returned OA behavior to the baseline level 2 wk after social change, which means that they can then be validly used in experiments. Therefore, we compared responses of the RSG and SI rats at D14 time point. This comparison revealed that the SI rats, as compared with RSG rats, show increased OA-related center-avoidance behavior (i.e. less activity in the central zone), without significant differences in locomotor activity, suggesting that center-avoidance behavior is a more reliable indicator of maladaptive behavior promoted by isolation housing, at least in mature adult male rats. In addition to time point D14, responses of the RSG and SI rats were also compared at D0 time point to verify equivalent behavioral characteristics of both groups before social disruption.

The behaviorally activating effects of AMPH were examined in the present study because this drug produces an unconditional, behaviorally aroused state[1] and behavior in the OA should predict responsiveness to AMPH.[12,18] On D14 after social change, we saw less rearing activity without changes in locomotor activity in the SI as compared with RSG rats after AMPH challenge. However, locomotor and rearing activities in the OA did not show between-group differences immediately before the AMPH challenge, suggesting that nonpharmacological stimulation may not always be sufficient for correct assessment of the behavioral phenotype of rats. A reduction in AMPH-induced vertical activity without changes in locomotor activity, observed in the present study of SI compared with RSG rats 2 wk after the social change, has not been widely reported in the literature. A previous study[67] showed that a selective reduction in AMPH-induced vertical activity without alterations in total distance traveled is related to the stimulation of $GABA_B$ receptors in areas of the brain that mediate the rewarding and activating effects of psychostimulants.[67] The possibility that in mature adult male rats SI promotes selective activation of $GABA_B$ receptors in the motive circuit remains to be examined in further studies. Nevertheless, the results of the present study contrast previous findings that isolation housing brings the risk of neurologic adaptations that promote locomotor sensitization and potentiates drug-taking,[13,20] highlighting the importance of animals' age and, probably the duration of isolation housing when trying to define the consequences.

The present study showed a delicate relation between social change and body weight in mature adult male rats. Effects of separation were different in RSG and SI rats, as RSG rats did not show significant changes in body weight within a week after separation (D0 compared with D7), while SI animals showed an increase. Moreover, no significant difference in body weight was found in RSG rats between D7 and D14 (adaptation effect to the new housing condition) while SI rats showed an increase in body weight. Although a comparison of the body weights at given time points did not identify differences between groups, the analysis of weight gains showed that SI rats had gained more weight than RSG rats within 2 wk after social change. These findings emphasize that the analysis of the weight gain of RSG and SI rats during the period of interest can identify changes that cannot be observed by simply comparing the body weights at given time points. In social species, touch is a major rewarding sensory component of the composite social interaction stimulus.[16] An increase in weight gain of SI rats may be due to their inability to derive rewards from social contacts, so they may eat more to satisfy a need that can be fulfilled in other ways during group housing. Several studies have found that isolated animals eat more and gain more weight than do socially housed animals.[22,46,48,54]

Our study has several limitations. We did not measure corticosterone levels. The original stress concept[58] indicates that stress represents a nonspecific response to stressors and leads to the activation of the hypothalamo-pituitary-adrenocortical (HPA) axis and the release of glucocorticoids in the circulation, with serum corticosterone levels in stressed animals as a widely accepted neuroendocrine marker of a stress response. Further research has shown that stress reactions are specific and that each type of stressor has its own 'signature'[25] and distinct pathways and mechanisms of regulation. As a result, variation in HPA activity and thus serum corticosterone levels highly depend on the type of stress[14] and duration of exposure to stress.[53] Accurate measurement of changes in corticosterone levels would require measurements several times during the day. However, handling animals to obtain fecal samples[11,30,51] or taking blood or urine samples could affect the behavioral outcomes that were the primary purpose of the study and therefore we opted not to subject the rats to such analyses. The lack of the group of socially undisturbed adult male rats is also a limitation. For example, we cannot rule out the possibility that, given the increase in body weight, RSG rats may have gained too little weight. Our study also lacks behavioral measures from socially undisturbed adult male rats subjected to repeated testing, as well as the responses in other behavioral tests (for example, for anxiety and anhedonia). Therefore, the findings of this study are limited by the experimental design we used. Thanks to the constructive suggestions of an anonymous reviewer, we have tried to give a refined interpretation of our results.

In conclusion, understanding the effects of changes in social housing on the brain and behavior of rodents represents an important link between animal welfare science and the experiment itself, as it can potentially help to reduce data variability and the number of animals used. The present study revealed that mature adult male Wistar Han rats are sensitive to changes in social housing and require 2 wk to accommodate to a RSG (from 3 to 2). Therefore, they should be used during this pe-

riod if indicated by the experimental design. In contrast, SI rapidly and consistently influences exploratory and center avoidance behavior, weight gain, and AMPH-induced activity. These findings complicate the use of mature adult male SI rats in experiments that address behavioral, physiologic, and neuroactive compounds-induced dose-response psychomotor outcomes and do not otherwise consider the impact of housing conditions. The specificity of the current findings regarding strains and sex of laboratory rats remains to be assessed in further studies.

Acknowledgments

This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Contract Number 451-03-68/2022-14/200007).

References

1. **Alcaro A, Huber R, Panksepp J.** 2007. Behavioral functions of the mesolimbic dopaminergic system: an affective neuroethological perspective. Brain Res Rev **56:**283–321. https://doi.org/10.1016/j.brainresrev.2007.07.014.
2. **Animal Research Review Panel.** 2007. [Internet]. Guidelines for the Housing of Rats in Scientific Institutions. Orange, NSW, Australia: Animal Welfare Branch NSW Department of Primary Industries. [Cited 17 February 2021]. Available at: https://www.animalethics.org.au/__data/assets/pdf_file/0014/222512/housing-rats-scientific-institutions.pdf.
3. **Archer J.** 1973. Tests for emotionality in rats and mice: a review. Anim Behav **21:**205–235. https://doi.org/10.1016/S0003-3472(73)80065-X.
4. **Arts JW, Kramer K, Arndt SS, Ohl F.** 2012. The impact of transportation on physiological and behavioral parameters in Wistar rats: implications for acclimatization periods. ILAR J **53:**E82–E98. https://doi.org/10.1093/ilar.53.1.82.
5. **Aupperle RL, Paulus MP.** 2010. Neural systems underlying approach and avoidance in anxiety disorders. Dialogues Clin Neurosci **12:**517–531. https://doi.org/10.31887/DCNS.2010.12.4/raupperle.
6. **Begni V, Sanson A, Pfeiffer N, Brandwein C, Inta D, Talbot SR, Riva MA, Gass P, Mallien AS.** 2021. Correction: Social isolation in rats: Effects on animal welfare and molecular markers for neuroplasticity. PLoS One **16:**e0248070. https://doi.org/10.1371/journal.pone.0248070.
7. **Bell MR.** 2018. Comparing Postnatal Development of Gonadal Hormones and Associated Social Behaviors in Rats, Mice, and Humans. Endocrinology **159:**2596–2613. https://doi.org/10.1210/en.2018-00220.
8. **Boissy A, Manteuffel G, Jensen MB, Moe RO, Spruijt B, Keeling LJ, Winckler C, Forkman B, Dimitrov I, Langbein J, Bakken M, Veissier I, Aubert A.** 2007. Assessment of positive emotions in animals to improve their welfare. Physiol Behav **92:**375–397. https://doi.org/10.1016/j.physbeh.2007.02.003.
9. **Cameron LP, Benson CJ, Dunlap LE, Olson DE.** 2018. Effects of N, N-Dimethyltryptamine on Rat Behaviors Relevant to Anxiety and Depression. ACS Chem Neurosci **9:**1582–1590. https://doi.org/10.1021/acschemneuro.8b00134.
10. **Capdevila S, Giral M, Ruiz de la Torre JL, Russell RJ, Kramer K.** 2007. Acclimatization of rats after ground transportation to a new animal facility. Lab Anim **41:**255–261. https://doi.org/10.1258/002367707780378096.
11. **Cinque C, Zinni M, Zuena AR, Giuli C, Alema SG, Catalani A, Casolini P, Cozzolino R.** 2018. Faecal corticosterone metabolite assessment in socially housed male and female Wistar rats. Endocr Connect **7:**250–257. https://doi.org/10.1530/EC-17-0338.
12. **Dellu F, Piazza PV, Mayo W, Le Moal M, Simon H.** 1996. Novelty-seeking in rats–biobehavioral characteristics and possible relationship with the sensation-seeking trait in man. Neuropsychobiology **34:**136–145. https://doi.org/10.1159/000119305.
13. **Dickson PE, Mittleman G.** 2021. Environmental enrichment influences novelty reactivity, novelty preference, and anxiety via distinct genetic mechanisms in C57BL/6J and DBA/2J mice. Sci Rep **11:**3928. https://doi.org/10.1038/s41598-021-83574-6.
14. **Djordjevic J, Cvijic G, Davidovic V.** 2003. Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiol Res **52:**67–72.
15. **Eisenegger C, Haushofer J, Fehr E.** 2011. The role of testosterone in social interaction. Trends Cogn Sci **15:**263–271. https://doi.org/10.1016/j.tics.2011.04.008.
16. **El Rawas R, Saria A.** 2016. The Two Faces of Social Interaction Reward in Animal Models of Drug Dependence. Neurochem Res **41:**492–499. https://doi.org/10.1007/s11064-015-1637-7.
17. **European Union.** 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Off J Eur Union L **276/33**. https://doi.org/10.3000/17252555.L_2010.276.eng.
18. **Exner M, Clark D.** 1993. Behaviour in the novel environment predicts responsiveness to d-amphetamine in the rat: a multivariate approach. Behav Pharmacol **4:**47–56. https://doi.org/10.1097/00008877-199302000-00005.
19. **Fone KC, Porkess MV.** 2008. Behavioural and neurochemical effects of post-weaning social isolation in rodents-relevance to developmental neuropsychiatric disorders. Neurosci Biobehav Rev **32:**1087–1102. https://doi.org/10.1016/j.neubiorev.2008.03.003.
20. **Garcia EJ, Haddon TN, Saucier DA, Cain ME.** 2017. Differential housing and novelty response: Protection and risk from locomotor sensitization. Pharmacol Biochem Behav **154:**20–30. https://doi.org/10.1016/j.pbb.2017.01.004.
21. **Gentsch C, Lichtsteiner M, Feer H.** 1981. Locomotor activity, defecation score and corticosterone levels during an openfield exposure: a comparison among individually and group-housed rats, and genetically selected rat lines. Physiol Behav **27:**183–186. https://doi.org/10.1016/0031-9384(81)90320-6.
22. **Georgsson L, Barrett J, Gietzen D.** 2001. The effects of group-housing and relative weight on feeding behaviour in rats. Scand J Lab Anim Sci **28:**201–209. https://doi.org/10.23675/sjlas.v28i4.860.
23. **Ghasemi A, Jeddi S, Kashfi K.** 2021. The laboratory rat: Age and body weight matter. EXCLI J **20:**1431–1445. https://doi.org/10.17179/excli2021-4072.
24. **Giacalone M, Agata Z, Cozzucoli PC, Alibrandi A.** 2018. Bonferroni-Holm and permutation tests to compare health data: methodological and applicative issues. BMC Med Res Methodol **18:**81. https://doi.org/10.1186/s12874-018-0540-8.
25. **Goldstein DS, Pacak K, Kopin IJ.** 1996. Nonspecificity versus primitive specificity of stress response, p 3–20. In: McCarthy R, Aguilera G, Sabban E, Kvetnansk R, editors. Stress: Molecular Genetic and Neurobiological Advances. New York (NY): Gordon and Breach.
26. **Gorlova AV, Pavlov DA, Zubkov EA, Morozova AY, Inozemtsev AN, Chekhonin VP.** 2018. Three-Week Isolation Does Not Lead to Depressive-Like Disorders in Rats. Bull Exp Biol Med **165:**181–183. https://doi.org/10.1007/s10517-018-4125-7.
27. **Gromer D, Kiser DP, Pauli P.** 2021. Thigmotaxis in a virtual human open field test. Sci Rep **11:**6670. https://doi.org/10.1038/s41598-021-85678-5.
28. **Hawkins P, Morton DB, Burman O, Dennison N, Honess P, Jennings M, Lane S, Middleton V, Roughan JV, Wells S, Westwood K.** 2011. A guide to defining and implementing protocols for the welfare assessment of laboratory animals: eleventh report of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement. Lab Anim **45:**1–13. https://doi.org/10.1258/la.2010.010031.
29. **Hayakawa K, Mimura Y, Tachibana S, Furuya M, Kodama T, Aoki T, Hosokawa S, Fukui M, Shibata S, Yoshida M, Masuyama T, Narita T, Kuwagata M, Hisada S, Maki E.** 2013. Study for collecting background data on Wistar Hannover [Crl:WI(Han)] rats in general toxicity studies–comparative data to Sprague Dawley rats. J Toxicol Sci **38:**855–873. https://doi.org/10.2131/jts.38.855.
30. **Holson RR, Scallet AC, Ali SF, Turner BB.** 1991. "Isolation stress" revisited: isolation-rearing effects depend on animal care methods. Physiol Behav **49:**1107–1118. https://doi.org/10.1016/0031-9384(91)90338-O.

31. **Hurst JL, Barnard CJ, Tolladay U, Nevision CM, West CD.** 1999. Housing and welfare in laboratory rats: effects of cage stocking density and behavioural predictors of welfare. Anim Behav **58:**563–586. https://doi.org/10.1006/anbe.1999.1165.
32. **Inglis IR, Langton S, Forkman B, Lazarus J.** 2001. An information primacy model of exploratory and foraging behaviour. Anim Behav **62:**543–557. https://doi.org/10.1006/anbe.2001.1780.
33. **Jackson SJ, Andrews N, Ball D, Bellantuono I, Gray J, Hachoumi L, Holmes A, Latcham J, Petrie A, Potter P, Rice A, Ritchie A, Stewart M, Strepka C, Yeoman M, Chapman K.** 2017. Does age matter? The impact of rodent age on study outcomes. Lab Anim **51:**160–169. https://doi.org/10.1177/0023677216653984.
34. **Klír P, Bondy R, Lachout J, Hanis T.** 1984. Physiological changes in laboratory rats caused by different housing. Physiol Bohemoslov **33:**111–121.
35. **Korte SM, Olivier B, Koolhaas JM.** 2007. A new animal welfare concept based on allostasis. Physiol Behav **92:**422–428. https://doi.org/10.1016/j.physbeh.2006.10.018.
36. **Kuhn BN, Kalivas PW, Bobadilla AC.** 2019. Understanding Addiction Using Animal Models. Front Behav Neurosci **13:**262. https://doi.org/10.3389/fnbeh.2019.00262.
37. **Legault M, Wise RA.** 2001. Novelty-evoked elevations of nucleus accumbens dopamine: dependence on impulse flow from the ventral subiculum and glutamatergic neurotransmission in the ventral tegmental area. Eur J Neurosci **13:**819–828. https://doi.org/10.1046/j.0953-816x.2000.01448.x.
38. **Leussis MP, Bolivar VJ.** 2006. Habituation in rodents: a review of behavior, neurobiology, and genetics. Neurosci Biobehav Rev **30:**1045–1064. https://doi.org/10.1016/j.neubiorev.2006.03.006.
39. **Lever C, Burton S, O'Keefe J.** 2006. Rearing on hind legs, environmental novelty, and the hippocampal formation. Rev Neurosci **17:**111–133. https://doi.org/10.1515/REVNEURO.2006.17.1-2.111.
40. **Lidster K, Jefferys JG, Blumcke I, Crunelli V, Flecknell P, Frenguelli BG, Gray WP, Kaminski R, Pitkanen A, Ragan I, Shah M, Simonato M, Trevelyan A, Volk H, Walker M, Yates N, Prescott MJ.** 2016. Opportunities for improving animal welfare in rodent models of epilepsy and seizures. J Neurosci Methods **260:**2–25. https://doi.org/10.1016/j.jneumeth.2015.09.007.
41. **Manouze H, Ghestem A, Poillerat V, Bennis M, Ba-M'hamed S, Benoliel JJ, Becker C, Bernard C.** 2019. Effects of Single Cage Housing on Stress, Cognitive, and Seizure Parameters in the Rat and Mouse Pilocarpine Models of Epilepsy. eNeuro **6:**ENEURO.0179-18.2019. https://doi.org/10.1523/ENEURO.0179-18.2019.
42. **McCutcheon JE, Marinelli M.** 2009. Age matters. Eur J Neurosci **29:**997–1014. https://doi.org/10.1111/j.1460-9568.2009.06648.x.
43. **McEwen BS, Wingfield JC.** 2003. The concept of allostasis in biology and biomedicine. Horm Behav **43:**2–15. https://doi.org/10.1016/S0018-506X(02)00024-7.
44. **McGregor M, Richer K, Ananth M, Thanos PK.** 2020. The functional networks of a novel environment: Neural activity mapping in awake unrestrained rats using positron emission tomography. Brain Behav **10:**e01646. https://doi.org/10.1002/brb3.1646.
45. **Mengler L, Khmelinskii A, Diedenhofen M, Po C, Staring M, Lelieveldt BP, Hoehn M.** 2014. Brain maturation of the adolescent rat cortex and striatum: changes in volume and myelination. Neuroimage **84:**35–44. https://doi.org/10.1016/j.neuroimage.2013.08.034.
46. **Menich SR, Baron A.** 1984. Social housing of rats: life-span effects on reaction time, exploration, weight, and longevity. Exp Aging Res **10:**95–100. https://doi.org/10.1080/03610738408258550.
47. **Milanovic D, Pesic V, Loncarevic-Vasiljkovic N, Avramovic V, Tesic V, Jevtovic-Todorovic V, Kanazir S, Ruzdijic S.** 2017. Neonatal Propofol Anesthesia Changes Expression of Synaptic Plasticity Proteins and Increases Stereotypic and Anxyolitic Behavior in Adult Rats. Neurotox Res **32:**247–263. https://doi.org/10.1007/s12640-017-9730-0.
48. **Morgan M, Einon D.** 1975. Incentive motivation and behavioral inhibition in socially-isolated rats. Physiol Behav **15:**405–409. https://doi.org/10.1016/0031-9384(75)90205-X.
49. **O'Donnell KC, Gould TD.** 2007. The behavioral actions of lithium in rodent models: leads to develop novel therapeutics. Neurosci Biobehav Rev **31:**932–962. https://doi.org/10.1016/j.neubiorev.2007.04.002.
50. **Perović M, Jović M, Todorović S, Đorćević AM, Milanović D, Kanazir S, Lonćarević-Vasiljković N.** 2022. Neuroprotective effects of food restriction in a rat model of traumatic brain injury - the role of glucocorticoid signaling. Nutr Neurosci **25:**537–549. https://doi.org/10.1080/1028415X.2020.1769410.
51. **Pritchard LM, Van Kempen TA, Zimmerberg B.** 2013. Behavioral effects of repeated handling differ in rats reared in social isolation and environmental enrichment. Neurosci Lett **536:**47–51. https://doi.org/10.1016/j.neulet.2012.12.048.
52. **Prut L, Belzung C.** 2003. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. Eur J Pharmacol **463:**3–33. https://doi.org/10.1016/S0014-2999(03)01272-X.
53. **Retana-Márquez S, Bonilla-Jaime H, Vázquez-Palacios G, Domínguez-Salazar E, Martínez-García R, Velázquez-Moctezuma J.** 2003. Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats. Psychoneuroendocrinology **28:**207–227. https://doi.org/10.1016/S0306-4530(02)00017-3.
54. **Sahakian BJ, Burdess C, Luckhurst H, Trayhurn P.** 1982. Hyperactivity and obesity: the interaction of social isolation and cafeteria feeding. Physiol Behav **28:**117–124. https://doi.org/10.1016/0031-9384(82)90112-3.
55. **Sanberg PR, Zoloty SA, Willis R, Ticarich CD, Rhoads K, Nagy RP, Mitchell SG, Laforest AR, Jenks JA, Harkabus LJ, Gurson DB, Finnefrock JA, Bednarik EJ.** 1987. Digiscan activity: automated measurement of thigmotactic and stereotypic behavior in rats. Pharmacol Biochem Behav **27:**569–572. https://doi.org/10.1016/0091-3057(87)90369-8.
56. **Schildein S, Huston JP, Schwarting RK.** 2002. Open field habituation learning is improved by nicotine and attenuated by mecamylamine administered posttrial into the nucleus accumbens. Neurobiol Learn Mem **77:**277–290. https://doi.org/10.1006/nlme.2001.4017.
57. **Schweinfurth MK.** 2020. The social life of Norway rats (*Rattus norvegicus*). eLife **9:**e54020. https://doi.org/10.7554/eLife.54020.
58. **Selye H.** 1936. A Syndrome produced by Diverse Nocuous Agents. Nature **138:**32. https://doi.org/10.1038/138032a0.
59. **Sequeira-Cordero A, Salas-Bastos A, Fornaguera J, Brenes JC.** 2019. Behavioural characterisation of chronic unpredictable stress based on ethologically relevant paradigms in rats. Sci Rep **9:**17403. https://doi.org/10.1038/s41598-019-53624-1.
60. **Simpson J, Kelly JP.** 2011. The impact of environmental enrichment in laboratory rats–behavioural and neurochemical aspects. Behav Brain Res **222:**246–264. https://doi.org/10.1016/j.bbr.2011.04.002.
61. **Sturman O, Germain PL, Bohacek J.** 2018. Exploratory rearing: a context- and stress-sensitive behavior recorded in the open-field test. Stress **21:**443–452. https://doi.org/10.1080/10253890.2018.1438405.
62. **Thiel CM, Huston JP, Schwarting RK.** 1998. Hippocampal acetylcholine and habituation learning. Neuroscience **85:**1253–1262. https://doi.org/10.1016/S0306-4522(98)00030-X.
63. **Toth LA, Kregel K, Leon L, Musch TI.** 2011. Environmental enrichment of laboratory rodents: the answer depends on the question. Comp Med **61:**314–321.
64. **Verma P, Hellemans KG, Choi FY, Yu W, Weinberg J.** 2010. Circadian phase and sex effects on depressive/anxiety-like behaviors and HPA axis responses to acute stress. Physiol Behav **99:**276–285. https://doi.org/10.1016/j.physbeh.2009.11.002.
65. **Wittmann BC, Bunzeck N, Dolan RJ, Duzel E.** 2007. Anticipation of novelty recruits reward system and hippocampus while promoting recollection. Neuroimage **38:**194–202. https://doi.org/10.1016/j.neuroimage.2007.06.038.
66. **Würbel H.** 2017. More than 3Rs: the importance of scientific validity for harm-benefit analysis of animal research. Lab Anim (NY) **46:**164–166. https://doi.org/10.1038/laban.1220.
67. **Zhou W, Mailloux AW, McGinty JF.** 2005. Intracerebral baclofen administration decreases amphetamine-induced behavior and neuropeptide gene expression in the striatum. Neuropsychopharmacology **30:**880–890. https://doi.org/10.1038/sj.npp.1300635.