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The effects of the rearing environment on measures of stress: a multi-laboratory study

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Summary

Lack of reproducibility is a prominent problem in biomedical research, with the neuroscience field being no exception. Here, we hypothesize that inconsistencies between replicate studies depend partly on idiosyncratic differences between rearing conditions of laboratory mice. Together with rigorous within-laboratory standardization, this may cause high between-study heterogeneity. To test this hypothesis, we will evaluate how differences in the environmental conditions (housing and husbandry) between different rearing laboratories affect the expression of stress responses in mice, from behaviour to the molecular level. We will rear genetically identical mice (C57BL/6 mice from a single breeding stock) in 5 different laboratories before testing them in our laboratory for phenotypic differences induced by the different rearing environments. We will focus specifically on environmentally induced variation in the hypothalamic-pituitary-adrenal (HPA) stress reactivity and anxiety-related behaviours. To assess the biological basis of the expected phenotypic differences, a genome-wide analysis of epigenetic changes in DNA methylation will be conducted. Our findings will reveal the range of phenotypic variation in stress response induced by the idiosyncratic differences between the rearing conditions of laboratory mice. Moreover, the associated epigenetic changes could reveal candidate genes that may uncover the molecular mechanisms of this variability. The results will contribute to a better understanding of between-study variability in laboratory animal research and the molecular mechanisms underlying such variability.

Registration details

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1. General Information

Keywords

Reproducibility, standardization, HPA, stress, rearing, mice, epigenetic

Funding sources

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International code of classification

NA

2. Study design

Introduction

Evidence over the last 10 years suggests that reproducibility in biomedical animal research is alarmingly low (1-5). Various potential causes of poor reproducibility have been identified, including inadequate animal study design, poor scientific rigour, low statistical power, analytical flexibility, and publication bias (1-9). As inadequate animal study design might be a major culprit, scientists and animal welfare regulators explicitly recommend environmental standardization as the best way to guarantee reproducible results in animal experiments (10). Even though studies with rodent models can be performed using genetically identical animals in highly controlled and standardized environments, reproducibility of results using such models has also emerged as a topic of concern (11-13). Therefore, contrary to the common belief that standardization guarantees reproducibility (10), it has been suggested that rigorous standardization (both genetic and environmental) may produce results that are idiosyncratic to the specific standardized conditions under which they were obtained, indicating that standardization might be an important factor of poor reproducibility (11-14).

The explanation for this phenomenon lies in the fact that an animal's phenotype (which represents the result of complex and dynamic interaction between its genotype and the environment in which it develops) significantly contributes to its response to an experimental treatment. Therefore, phenotypic plasticity caused by gene by environment interactions (G x E) determines the range of variation (reaction norm) of the animal's response and should be considered as an important mechanism of biological variation (14). Instead of incorporating biological variation in the experimental design, such variation is considered as a nuisance, which scientists aim to eliminate through rigorous standardization of both the genotype of the animals and the environmental conditions under which the animals are housed and tested (14,15).
However, despite efforts to standardize conditions even across laboratories, different laboratories always differ in many environmental factors that affect the animals' phenotype (e.g. noise, odours, microbiota, or personnel (16–19)). Therefore, different laboratories will inevitably standardize to different lab-specific conditions, potentially resulting in different animals with lab-specific phenotypes. Taken together, this suggests that a failure to replicate

the results of a study might indicate that the replication studies were testing animals of a different phenotype (11, 13).

To study this further, we designed a multi-lab study to investigate whether differences in housing and husbandry conditions between different rearing facilities induce variation in the hypothalamic-pituitary-adrenal (HPA) stress reactivity and anxiety-related behaviours. Since differences in stress responses could be mediated by epigenetic mechanisms (20), which act as molecular modulators between genetic make-up and environment, we will perform epigenomic analyses in the ventral hippocampus. The epigenetic analysis will be exploratory and restricted to mice from laboratories showing the greatest differences in HPA-axis reactivity. Thus, differences between the different cohorts of mice will reveal the range of phenotypic variation induced by common differences among laboratory conditions. Our findings will have implications for the reproducibility of results in animal research.

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Type of research

Confimatory

Hypothesis of your study

This experiment will test the hypothesis that differences in the environmental conditions (housing and husbandry) between different rearing laboratories cause substantial differences in the phenotype of mice, specifically in terms of HPA stress reactivity and anxiety.

Study design

We will use a multi-laboratory study design to model differences in environmental conditions between different rearing laboratories as realistically as possible. An overview of the study design is attached as Figure 1.

90 time-mated pregnant females C57BL/6JRj in the last third of pregnancy, all derived from the same breeding stock of a commercial breeder (Janvier Labs, Le Genest-Saint-Isle, France), will be randomly allocated to 5 different laboratories (n = 18 per lab). Ordering all

animals from the same breeder and deriving all pregnant females from the same breeding stock will guarantee that the cohorts of mice reared by the different laboratories will be as genetically similar as possible and that all differences between them can be attributed to the differential rearing environments. At weaning, in each rearing laboratory up to 12 litters with at least 3 pups of each sex will be selected randomly from all litters. If necessary, to achieve n=12, these will be complemented by litters with at least 2 pups of each sex. From each litter 3 (or 2) pups per sex will be selected randomly and reared together until the age of 8 weeks (PND 56) according to the specific protocols of housing and husbandry of each of the 5 animal facilities. At PND 57 one mouse per sex per cage of all cages with 3 mice will be sacrificed and various samples will be obtained for analysis of secondary outcome measures to control for changes induced by the transport to, and housing in, the test laboratory (See below list of study outcomes).

The remaining pairs of male and female offspring (n = 240) will be transported from the 5 rearing facilities to the testing facility at the University of Bern, where after an acclimation period of about 2.5 weeks, one mouse per sex per litter (n=120) will be tested for phenotypic differences in HPA stress reactivity (primary outcome variable) and anxiety-related behaviour (secondary outcome measures), while their cagemates (n = 120) will be sacrificed and the ventral hippocampus dissected and prepared for determination of epigenetic changes related to phenotypic differences between laboratories using genome-wide DNA methylation profiling. For this, test-naïve mice will be used to avoid the effects of testing on the epigenetic profile.

Two-established tests of anxiety-related behaviour, the open-field test and the light-dark box test, will be conducted in that order, with a break of 7 days in between, followed by assessing HPA-stress reactivity in response to 20 min physical restraint after another break of 7 days.

One week after the end of testing (at ~14.5 weeks of age), all mice will be euthanized by cervical dislocation followed by decapitation for post-mortem analysis.

The genome-wide DNA methylation analysis, as well as the molecular and histological analyses, will be restricted to the mice from the two laboratories showing the greatest differences in HPA stress reactivity. The final choice of the next-generation sequencing assays and the exact methods, including the bioinformatics pipeline, will be determined at a later stage.

The list of study outcomes

1) Primary outcome: HPA stress reactivity (measured by the area under the curve (AUC) of changes in corticosterone levels in the blood plasma in response to a standard stressor, acute restraint stress for 20 minutes).

2) Secondary outcomes:

a) measures to control for the effect of transport to and housing and testing in the test lab

* · Basal corticosterone levels in the blood plasma
* · Body weights

* · Weight of adrenal glands
* · Histological examination of the adrenal glands
* · Candidate gene expression analysis in the ventral hippocampus
* · Structural changes in the hypothalamus and ventral hippocampus

(b) secondary measures of HPA reactivity and of anxiety-related behaviour

* · Anxiety related behaviour (measured by light dark box test and open field test)
* · Weight of adrenal glands
* · Histological examination of the adrenal glands
* · Basal corticosterone levels in the blood plasma
* · Structural changes in the hypothalamus and ventral hippocampus
* · Genome-wide DNA methylation analysis in the ventral hippocampal neurons
* · Candidate gene expression analysis in the ventral hippocampus

(c) further secondary measures

* · Histological examination of the liver and thymus
* · Caecal and faecal samples to assess the gut microbiome
* · Histological examination of the germ cells development
* · Molecular analysis of epigenetic regulation in germ cells

Method of blinding

All experimenters performing the stress reactivity tests, behavioural tests, and post-mortem analyses will be blind to the "treatment", i.e. the rearing facility the animals were transferred from. Blinding will be done by two colleagues otherwise not involved in the execution of the experiments. Cages will be assigned new identification numbers and positions of cages within and between the cage racks will be randomly re-shuffled so that the experimenters cannot deduce the origin of the cages (i.e. treatment) from the ID number or the position of the cage. For assignment of ID numbers and cage positions a script will be written in the software Mathematica (version 11), using the inbuilt random number generator.

Method of randomization

The order of the cages during animal habituation and testing of all mice will be randomized using the random number generator of the software Mathematica (verion 11). Each of the 3 experimenters will get randomly assigned 20 male and 20 female mice which will be handled during the behavioural testing. Always two experimenters are testing animals in parallel (at the same time, but separated). Testing is done for both sexes by each experimenter in 3 blocks of 5 animals, each. The randomization and allocation procedure is restricted so that in each block for each experimenter there is exactly one from each lab in random order, with the addition that in no case animals tested at the same time are from the same lab. The allocation and randomization script is attached as supplementary file “Test Allocation.pdf”

Additional remarks

The list of methods is provided in Table 1. In brief, one mouse per cage (n=120) will be tested in the open-field and the light-dark box before being assessed for HPA-stress reactivity using blood corticosterone levels. All 3 tests will be performed during the animals’ light cycle (from 12:00h to 17:00h). In addition, the remaining one mouse per cage (n=120) will not be behaviourally tested and will be used for epigenetic analysis. These test-naïve mice will be used to avoid effects of testing on the epigenetic profile. One week after the HPA stress reactivity test, all mice (240) will be euthanised during the animals’ light cycle (from 12:00h to 17:00h) with cervical dislocation and decapitation for post-mortem analysis.

Uploads

Filename	Size (bytes)
Test Allocation.pdf	113355
Supplementary File 1.xlsx	22763
Figure 1.jpg	311123
Table 1.docx	14109

3. Methods

3. 1. Oestrous cycle determination

Description of the method

The oestrous cycle stage will be assessed by the cytological analysis of vaginal smears to account for the sex hormone status of the female mice. The vaginal smear will be taken immediately after behavioural testing and post-mortem. Briefly, after behavioural testing, the female will be placed on the cage lid with her hind end towards the experimenter. The rounded tip of a disposable pipette with 100 µl of sterile distilled water will be gently placed at the opening of the vaginal canal and vaginal smear cells will be collected by lavage. Smears will be placed on microscopic slides, allowed to dry, stained with 0.1% crystal violet solution, washed and then analysed using light microscopy. The stage of the oestrous cycle will be determined based on the relative ratio of nucleated epithelial cells, cornified squamous epithelial cells and leukocytes.

Narcotic/analgesic treatment

Not applicable

Drugs/substances

Not applicable

Antibodies

Not applicable

Cell lines, viruses, DNA or RNA constructs and bacteria

Not applicable

3. 2. Open-field test (OF)

Description of the method

The open-field test is a standard rodent test to assess anxiety-related behaviour (1).

The open-field apparatus to be used is a polycarbonate box (45 x 45 x 45 cm) with grey walls and a white base plate. OF testing will be run in batches during four consecutive days. On each trial, two animals will be tested simultaneously in two boxes by two experimenters.

Each mouse will be placed in one of the front (close to the experimenter) corners, facing the wall and allowed to freely explore the open field for 10 min. After the 10-min session, the mouse will be returned to its home cage. All testing will be video recorded using an infrared camera system and analysed using the Ethovision video tracking system.

Outcome measures will include: **1)** total distance travelled, **2)** time spent in the centre zone of the field, **3)** number of entries into the centre zone, **4)** number of rearings (supported and unsupported) and **5)** number of faecal boli produced.

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Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 3. Light-dark box test (LDB)

Description of the method

This is a well-established test to assess anxiety-related behaviour in rodents (1), in which the animal is exposed to a novel environment with a protected (dark compartment) and an unprotected (light compartment) area. Naturally, the rodents show a preference for the dark, protected compartment. The inherent conflict between risk avoidance and exploratory drive is thought to inhibit exploration. The key measure for assessing anxiety-related behaviour is the change in exploration of the illuminated (unprotected) area, as reflected in the time spent in each compartment, and the number of transitions between the two compartments. LDB testing will be run in batches during four consecutive days. All testing will be video recorded using an infrared camera system and analysed using the Ethovision video tracking system.

For the light–dark box test, the apparatus (37.5 x 21.5 x 15 cm) is partitioned into a brightly lit chamber (25 × 21.5 x 15 cm) and a closed, dark chamber (12.5 × 21.5 x 15 cm) connected

with a sliding door. Each mouse will be placed in the dark compartment and then the sliding door will be opened, and the mice will be allowed to move freely between the two chambers and explore the apparatus for 10 min. During the 10-min session, the time spent in each compartment and the number of transitions between the light and dark compartments will be recorded.

Outcome measures will include: 1) time spent in the light compartment, 2) time spent in the dark compartment, 3) number of transitions.

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Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 4. HPA stress reactivity

Description of the method

HPA stress reactivity test will be run in batches across four consecutive days, during the animals' light cycle (from 12:00h The blood samples will be taken at three timepoints: initial sample (as soon as the animal is taken from the home cage), reaction sample (after 20 minute restraint) and a recovery sample (90 minutes after the onset of stress).

Mice will be taken out of their home cages and a first blood sample will be taken by an incision of their tail veins using EDTA-coated capillary tubes (Microvette CB300, Sarstedt AG &Co., Germany). The procedure will be limited to 2 min to allow acquisition of basal (i.e., unstressed/initial) levels of CORT. Immediately after, the mice will be restrained for 20 min in 50 ml plastic tubes (with holes for breathing and the tail). After the 20-min stressor, a second blood sample will be taken from a fresh incision rostral to the first one followed by placing the mice back in their home cages. 90 minutes after the onset of stress, a third blood sample will be taken from a third incision rostral to the second one. Blood samples will be placed on ice and processed to obtain at least 20 µl of blood plasma, which will be stored at -80 until assayed. The plasma CORT levels will be quantified using the Enzyme-linked immunosorbent assay (ELISA).

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

3. 5. Tissue harvesting

Description of the method

All animals will be euthanised by cervical dislocation followed by decapitation for post-mortem analysis. Blood will be collected directly from the trunk into EDTA coated tubes. Plasma will then be separated by centrifugation and stored at −80 °C until assayed.

Immediately after decapitation, whole brains will be isolated, frozen, and then stored at −80°C. The brain region of interest, *i.e.* ventral hippocampus, will be dissected later.

The adrenal glands will be removed, dissected from the surrounding fat and weighed using an analytical scale. The thymus and liver will also be isolated. One of the adrenal glands, one half of the thymus and a piece of the liver will be immediately frozen using liquid nitrogen and then stored at −80°C for molecular analyses. The remaining adrenal gland, thymus and liver will be immediately fixed in a 4% buffered formaldehyde solution for histological analyses.

The mouse cecum and the distal colon together with its content will be isolated and frozen in liquid nitrogen and kept at -80°C for microbial DNA extraction and subsequent metagenome sequencing.

The mouse sperm will be collected post-mortem from the epididymis and frozen at -80°C for further molecular analyses. The ovaries and testicles will also be collected and frozen in liquid nitrogen (for the germ cell isolation) or fixed in the 4% buffered formaldehyde solution (for histological analysis of germ cell development).

Narcotic/analgesic treatment

Not provided

Drugs/substances

Not provided

Antibodies

Not provided

Cell lines, viruses, DNA or RNA constructs and bacteria

Not provided

4. Statistics

4. 1. Generalized linear mixed model (GLMM)

Assigned method(s)

Open-field test (OF)
Light-dark box test (LDB)
HPA stress reactivity

Main endpoints

Statistic: Body weight Name of statistical method: GLMM Main endpoint: Body weight (g) Secondary endpoint: none Sample size calculation: based on power analysis for corticosterone response Primary statistical analysis: GLMM (General Linear Mixed Effects Model), Response variable: body weight (g), explanatory variables: lab of origin, dam, batch. Sexes analysed in separate models. Diagnostics for assumptions: visual inspection of multi-panel scatter plots and Q-Q plots, VIF factor (following Zuur et al. (2010), Meth Ecol Evol, 1, 3–14). Statistic: Open-Field Test Name of statistical method: GLMM Main endpoint: Time spent in the centre (s) Secondary endpoint: Total distance travelled (cm) Sample size calculation: based on power analysis for corticosterone response Primary statistical analysis: GLMM, Response variable: total distance travelled (cm) and time spent in the centre (s), explanatory variables: lab of origin, dam, batch. Sexes analysed in separate models. Diagnostics for assumptions: visual inspection of multi-panel scatter plots and Q-Q plots, VIF factor. Statistic: Light-Dark Box Name of statistical method: GLMM Main endpoint: Time spent in the light area (s) Secondary endpoint: Total distance travelled in the light area (cm). Sample size calculation: based on power analysis for corticosterone response Primary statistical analysis: GLMM, Response variable: time spent in the light area (s), lab of origin, dam, batch. Sexes analysed in separate models. Diagnostics for assumptions: visual inspection of multi-panel scatter plots and Q-Q plots, VIF factor. Statistic: HPA stress reactivity Name of statistical method: GLMM Main endpoint: Restraint-stress induced change in Corticosterone concentration in blood plasma (area under the curve of all 3 samples) Secondary endpoints: corticosterone concentration in the blood plasma at the end of restraint stress (sample 2), restraint-stress induced increase in corticosterone concentration (difference between samples 1 and 2), recovery of the restraint-stress induced corticosterone response (difference between samples 2 and 3). Statistic: Absolute and relative organ weights Name of statistical method: GLMM Main endpoint: Absolute (mg) and relative organ weights (mg/kg body weight) Secondary endpoint: none Sample size calculation: based on power analysis for corticosterone response Primary statistical analysis: GLMM (General Linear Mixed Effects Model), Response variable: absolute and relative organ weights (mg or mg/kg body weight), explanatory variables: lab of origin, dam, batch. Sexes analysed in separate models. Diagnostics for assumptions: visual inspection of multi-panel scatter plots and Q-Q plots, VIF factor.

Secondary endpoints

Not provided

Sample size calculation

Sample size calculation: Appropriate sample sizes were determined a priori in a power analysis using simulated sampling for a two-way ANOVA design. The power analysis was done for the main outcome variable (area under the response curve for restraint-stress induced changes in CORT), aspired parameters of Alpha= 0.05, 1-Beta= 0.8 and a range of expected effect sizes from 10 to 30 per cent. Based on historical data (1,2), the effects to be observed are expected to be of medium size (i.e. means estimates for two randomly chosen groups (laboratory) are expected to be in the range of 20%, equivalent to a ratio of between-lab variation: within-lab variation of 1:2). This gives a required minimal sample size of n = 12 animals per sex and group (laboratory). Primary statistical analysis: GLMM, Response variable: corticosterone concentration in blood (ng/ml or mmol/ml), explanatory variables: time point (before restraint, 1st-time point after restraint) lab of origin, dam, batch. Sexes analysed in separate models. Diagnostics for assumptions: visual inspection of multi-panel scatter plots and Q-Q plots, VIF factor. 1. Herman JP, Cullinan WE, Morano MI, Akil H, Watson SJ. Contribution of the ventral subiculum to inhibitory regulation of the hypothalamo-pituitary-adrenocortical axis. J Neuroendocrinol. 7(6):475-82. 1995 2. Fediuc S, Campbell JE, Riddell MC. Effect of voluntary wheel running on circadian corticosterone release and on HPA axis responsiveness to restraint stress in Sprague-Dawley rats. Appl Physiol 100(6):1867-75. 2006

Primary statistical analysis

Primary statistical analysis: GLMM

Exclusion criteria

Not provided

5. Animals

5. 1. Mice (Mus musculus)

Animal strain/breed

C57BL/6JRj

Genetically modified

No

Sex

Female
Male

Further characteristics of the animals (e.g. age, body weight, size)

Each rearing facility will receive 18 time-mated pregnant, primipara, C57BL/6JRj females (gestational day 14 or 15) from the same breeding stock of a commercial breeder (Janvier Labs, Le Genest-Saint-Isle, France). Pregnant females will be 9 weeks old at the moment of arrival in the rearing facilities.

Housing conditions

Pregnant dams will be singly housed for approximately 5 days, from arrival to the rearing laboratory until parturition. Dams will be monitored daily for parturition and day of birth will be defined as postnatal day 0 (PND 0). Litters will not be culled during the lactation period and all healthy pups will be weaned at PND 22, into same-sex groups of 2 or 3 littermates. Both male and female offspring will be reared until the age of 8 weeks according to the specific protocols of housing and husbandry of each of the 5 animal facilities (e.g. type of cages, handling method, bedding, nesting material, diet, light regime). Detailed housing and husbandry conditions will be reported in the Excel file "Excel_questionnaire_Rearing Lab" (Supplementary 1).

At 8 weeks of age, the mice will be transferred from the 5 rearing facilities to the testing facility in Bern. Same-sex cage-mates will be placed into 4 compartment transport boxes (2 mice per compartment, 8 mice per box). Each compartment will contain 1 cm of bedding, nesting material from the home cage, food pellets and hydrogel. Mice will be shipped by a professional company using environmentally controlled vehicles.

Upon arrival, animals will be checked, and pair housed in freshly bedded Type 3 cages with nesting material and enrichment. Each cage will contain 3 cm of bedding (Lignocel® select), red mouse house (Tecniplast, Indulab, Gams, Switzerland), medium-size play cardboard tunnel (Play tunnel, #CPTUN00016P, Plexx B.V. Netherlands), and 10 g of nesting material (Sizzle Nest #SIZNEST00016P, Plexx B.V. Netherlands). Standard rodent chow (Kliba Nafag #3430, Switzerland) and tap water will be available at libitum. Females and males will be housed in separate rooms and all animals will be kept on a 12:12 light/dark cycle with lights on at 12:00 h. Two days after arrival in the housing facility, animals will be marked by ear tattoo in the testing room. Mice will be habituated to the new animal facility for 2.5 weeks after transport. Cages will be changed once a week in the testing room and animals will be weighed before moving to the new cage. During the cage change, the whole nest will be transferred with the nesting material to reduce the incidence of aggression. The females and males' cages will be changed on a different day. During habituation and behavioural testing, mice will be tail handled.

Refinement

Not provided

Uploads

Filename	Size (bytes)
Supplementary File 1.xlsx	22763