Supplementary Material

Castration of adult male C57BL/6JRj mice allows for resocialization and social housing of previously single-housed males: a harm-benefit analysis

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Material and Methods (Supplementary)

Ethics statement

The study was performed according to the German Animal Welfare Act and the Directive 2010/63/EU for the protection of animals used for scientific purposes. Animal housing and husbandry were approved by the Berlin State Authority (*Landesamt für Gesundheit und Soziales*, permit number: G 0053/15). Mice had been previously used in an experiment classified as "mild" with regards to animal suffering as defined by current EU regulations on severity classification of animal studies. After castration and resocialization in groups of three to four, the mice were rehomed.

Animals and housing

19 male C57BL/6JRj mice from Janvier Labs (Saint-Berthevin Cedex, France) aged 18 to 42 weeks had previously been used in an experiment, which did not necessitate killing of the animals. Due to aggressive behaviour, the mice had been individually housed for 6 to 32 weeks. All 19 mice were castrated and subsequently resocialized in groups of three to four animals.

Since sample size depended on the number of male mice in our previous experiment, power analysis based on the faecal corticosterone metabolites (FCM) concentrations was performed after the study was carried out (Geisser-Greenhouse Corrected F Test; power = 0,9989).

All mice had been single-housed in Makrolon type III open top cages (42×26×15 cm) for 6 to 32 weeks. Their cages contained fine wooden bedding material (LIGNOCEL® 3-4 S, J. Rettenmaier & Söhne GmbH + Co. KG, Rosenberg, Germany), cotton nestlets (Ancare, UK agents, Lillico, United Kingdom), cocoons (ZOONLAB GmbH, Castrop-Rauxel, Germany), one red plastic house (length: 100 mm, width: 90 mm, height: 55 mm; ZOONLAB GmbH, Castrop-Rauxel, Germany), and two tunnels (metal; length: 125 mm, diameter: 50 mm).

Thirty-five to 36 days after castration, the mice were resocialized in groups of three to four in cages of the same type, also containing the cage enrichment mentioned above, except from a plastic house. The animals were maintained under standard conditions (room temperature 22±2 °C; relative humidity 55±10%) on a light:dark cycle of 12:12 hours of artificial light with a 5 min twilight transition phase (lights on from 6:00 a.m. to 6:00 p.m.). The mice were fed pelleted mouse diet *ad libitum* (Ssniff rat/mouse maintenance, Spezialdiäten GmbH, Soest, Germany) and had free access to tap water. After surgery, all mice received food pellets soaked in water for five days.

In order to prevent any stress-related influence caused by male persons (Sorge et al. 2014), both the technician and veterinarian were female. Mice were handled using a combination of tunnel and cup handling which is known to induce less stress than tail handling (Hurst and West 2010).

Surgery, anaesthesia, and analgesia

Anaesthesia was induced with 4% isoflurane in 100% oxygen in an induction chamber and maintained with 1.5–2.5% isoflurane via nose cone. During anaesthesia and surgery, mice were warmed with a heating pad. Eye ointment was used to prevent dehydration of the eyes. After induction of anaesthesia, systemic analgesia was provided by meloxicam (1 mg/kg body weight, s.c.; Metacam 2 mg/ml injection solution for cats, Boehringer Ingelheim Vetmedica GmbH, Ingelheim, Germany), a long-acting non-steroidal anti-inflammatory drug (NSAID) which requires a single dose per day (Tubbs et al. 2011). This pain management protocol was based on recommendations by the German Society for Laboratory Animal Science which suggests the use of NSAIDs for one day following castration if no complication or severe swelling occurs (Henke et al. 2015). Lidocaine/prilocaine was used as topical local anaesthetic for the scrotum (Emla Creme, AstraZeneca GmbH, Wedel, Germany: 1 g containing 25 mg lidocaine and 25 mg prilocaine). Surgical castration was performed according to Behringer et al. (2014) using the scrotal approach. Mice were placed in supine position and the scrotum was disinfected. Both testicles were pushed into the scrotal sacs by gently applying pressure to the abdomen. Then, an incision of approximately 1 cm was made through the skin at a right angle to the midline of the scrotal sac. The testes were removed one by one. The membrane covering the testicle was incised and the testicle was carefully pushed out. The Vas deferens including its blood vessels was clamped with two artery forceps. Ligation between the two forceps was performed using absorbable suture material (3–0). After removal of the forceps, the testicles were each dissected from the fat pad and removed. The skin was stitched with a single button suture.

Test schedule
The test schedule can be divided into two parts: well-being of mice was assessed 1) post-castration (until day 14 post-surgery) and 2) post-resocialization (day 35 until day 56 post-surgery). The test schedule is outlined in Figure 1 of the main article and all time points of testing are listed.

Post-castration
In order to recognize and alleviate post-operative pain, a clinical score sheet was used before surgery, at 30 min, 150 min, and 300 min after surgery as well as on days one to seven after surgery (Supplementary Table 1). The Mouse Grimace Scale (MGS) (Langford et al. 2010) was applied before surgery, 30 min, 150 min, and 300 min as well as on day one, two, and three after surgery. On day one after surgery, burrowing behaviour was monitored (Deacon 2006). On the following day, nesting (Gaskill et al. 2013) was evaluated and the time-to-integrate-to-nest test (TINT) (Rock et al. 2014) was conducted. Faecal samples for corticosterone metabolite analysis were collected over a period of 24 hours prior to surgery (i.e. between day 2 and day 1 prior to castration) as well as on days two and 14 after surgery. Mice were weighed regularly during the entire study period. In the event of any signs of pain, mice were treated with meloxicam (1 mg/kg body weight, s.c.) once daily.

Post-resocialization
Approximately a week post-castration and 28 days prior to resocialization, mice were habituated to the odour of their future group members by distributing nest material among all group members every three to four days. Nest material frequently contains pheromones originating from plantar and other body glands, which have been shown to exert an aggression-inhibiting effect (Mugford 1973, Van Loo et al. 2003). On day 35 or day 36 post-castration, mice were resocialized in groups of three or four. All mice of a group were transferred to a Makrolon type III cage with new bedding and nesting material of 20 cocoons, as well as two tunnels. Immediately after resocialization, group interaction was video-recorded and monitored live by the experimenter for three hours in order to terminate the experiment if an endpoint criterion was reached. Fights lasting longer than 10 min or the occurrence of open wounds were defined as endpoint criteria, i.e. mice had to be separated again. On day one and eight after resocialization, the group nests of the mice were scored and the TINT was carried out. All mice were examined daily for lesions resulting from fights. Mice were regularly weighed over the entire study period.

Clinical score sheet
A clinical score sheet (Supplementary Table 1) was applied prior to surgery, at 30 min, 150 min, and 300 min after surgery as well as on days one to seven after surgery. To examine the animals, they were observed in their home cage and then transferred to the hand using a tunnel. In addition, a separate score sheet was used covering the time period of three weeks after post-resocialization. Apart from the scores evaluated after castration, it also included scores for fight-associated lesions (instead of scores for the castration wound). During the post-resocialization phase, the animals were examined daily (Monday to Friday by a veterinarian using the clinical score sheet, Saturday and Sunday by an animal care technician, who was instructed to immediately call the veterinarian if the well-being of a mouse was compromised).

SUPPLEMENTARY TABLE 1: *Clinical Score Sheet*

Criteria	Action according to Ullmann et al. (2018)	Definition
Body posture		Sitting upright, uninhibited movement, rearing
	A	Unsteady, careful gait, and reduced rearing
	B	Reduced forward movement, hunched back, sunken flanks
Coat condition		Smooth, shiny
	A	Dull, slightly unkempt
	B	Dirty, unkempt, prolonged piloerection, sticky or wet
Audible vocalisation		None
	B	Present
Respiration		Smooth, regular
	B	Hypoventilation/hyperventilation
	C	Dyspnoea, breathing noises, oral respiration
Pain behaviour		None
	A	Staggering
	B	Flinching and/or abdominal press
Diarrhoea		None
	B	Present
Castration wound		Dry, not swollen, not reddened
	A	Exuding, swollen, reddened to a mild degree
	B	Exuding, swollen, reddened to a higher degree
Fight-associated lesions (resocialization process)		No presence of fight-associated wounds
	B	Fight-associated wounds are present
Eye closure		Open
	B	Eye lids partially closed with more than 50% of the eye visible
	B	Less than 50% of the eye visible up to eye lids being tightly closed; wrinkles or dirty/wet coat may be visible around the eye

Mouse Grimace Scale
MGS scores according to Langford et al. (2010) were obtained as described in our previous studies (Hohlbaum et al. 2017, 2018). Mice were transferred to photography cube (22×29×39 cm, with three white walls and one clear wall, and 0.5 cm of bedding material) and approximately 20–40 photographs from each mouse were taken for each time point using a high definition camera (Canon EOS 350D, Canon Inc., Tokyo, Japan). After selecting all sharp frontal or lateral photographs, one photograph was selected for each time point and for each mouse by simple randomization. Two blinded individuals independently analysed the photographs according to Langford et al. (2010): the five facial action units (i.e. orbital tightening, nose bulge, cheek bulge, ear position, and whisker

change) were scored on a scale from 0 to 2 (0 = not present, 1 = moderately present, 2 = obviously present). For each scorer, the mean of the five facial action units was calculated. Mean MGS scores of the two observers were averaged for each mouse for further statistical analysis (Hohlbaum et al. 2017, 2018).

Burrowing behaviour

For burrowing behaviour analysis (Deacon 2006, Deacon et al. 2001) we modified a protocol by Jirkof et al. (2010). A standard opaque plastic water bottle served as burrow (250 ml, 150 mm length, 55 mm diameter, 45 mm diameter of bottle neck). Three days prior to testing the burrow – filled with food pellets normally supplied as diet – was placed in the home cage for habituation (Jirkof et al. 2010). For the test it was filled with 140±2 g food pellets (Jirkof et al. 2010) and placed in parallel to the right long wall of the home cage. After 2 hours the weight of food pellets removed from the burrow relative to the initial weight was calculated (%). If the general condition of a mouse is impaired post-surgery, for instance due to post-operative pain, the mouse will remove less pellets from the burrow when compared to baseline (Arras et al. 2007, Jirkof et al. 2010).

Nest building

Ten cocoons (nest of a single mouse) or 20 cocoons (group nest) (ZOONLAB GmbH, Castrop-Rauxel, Germany) were provided as nest material. In the morning of the following day two individuals independently assessed the nests using the protocol by Hess et al. (2008). In short, if no nest site was to be identified, the nest score is 0 (undisturbed: mice did not move the material, interact with or manipulate it) or 1 (disturbed: mice interacted with the material but did not use it for nest building). If a nest site was present, the nest was considered to be composed of four parts which were scored individually (i.e. 2 = flat nest, 3 = cup, 4 = incomplete dome, 5 = dome) (Hess et al. 2008). For further statistical analysis the scores obtained by the two scorers were averaged for each mouse.

Time-to-integrate-to-nest test (TINT)

The time-to-integrate-to-nest test by Rock et al. (2014) was slightly modified. The home cage of the animals was removed from the rack and placed on a table. A cocoon piece of 2 cm in length was placed on the opposite side of the cage from the main nest site. The mice were monitored for 10 min and the latency to first interaction with novel material as well as the latency to integrate novel material into the present nest were manually recorded.

Behavioural analysis of group interaction

Group interaction was video-recorded for 3 hours (180 min). Minutes 1–10, 60–65, and 120–125 were manually analysed using the video material. For each mouse, the number of occurrences of following behavioural parameters was counted: aggressive behaviour (i.e. chasing, attacking, fighting, biting), body sniffing, ano-genital sniffing, social groom, social interaction in the nest site (i.e. nest-building while sitting in the nest, grooming, resting next to each other). An occurrence ended when the particular behaviour was interrupted by another behaviour.

Analysis of faecal corticosterone metabolites

FCMs were analysed as stress indicators (Palme 2019) by using an assay employing an antibody directed against 5α-pregnane-3β,11β,21-triol-20-one. Faecal samples were collected over a period of 24 hours, as described previously (Hohlbaum et al. 2017, 2018). Mice were moved to a new cage for the testing period. Distress caused by the new cage was minimized by scattering soiled bedding material without faeces from their home cage on top of the new bedding. At the end of the testing period, all dry faecal pellets were collected using forceps whilst wet pellets contaminated with urine were eliminated.

FCMs were extracted from the 24-h-bulk samples according to Palme et al. (2013). Briefly, faecal samples were dried at 60–70 °C and homogenized in a mortar. An aliquot of 0.05 g was suspended in 1 ml of 80% methanol for 30 min on a multi-vortex. After centrifugation (2500 g, 15 min), 0.5 ml of supernatant was pipetted in an Eppendorf tube. Before and after extraction, the samples were stored at -80 °C. The samples were analysed for corticosterone metabolites using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay as described and validated for mice by Touma et al. (2003, 2004).

Statistical analysis

Statistical analysis was performed with IBM SPSS Version 23 (IBM Corporation, Armonk, NY, USA). Explorative data analysis and tests for normality were performed for each parameter. Data were analysed using the respective test indicated in the results section (related samples Friedman's two-way analysis of variance by ranks, related samples Wilcoxon signed rank test, repeated measures ANOVA). Differences were considered significant at $p < 0.05$.

Since this case report did not include a control group, experimenters could not be blinded in the following examinations and tests: clinical score sheet, burrowing behaviour, nest building, time-to-integrate-to-nest test, and behavioural analysis of group interaction.

References

Arras M, Rettich A, Cinelli P, Kasermann HP, Burki K (2007): Assessment of post-laparotomy pain in laboratory mice by telemetric recording of heart rate and heart rate variability. BMC Vet Res 3: 16–16.

Behringer R, Gertsenstein M, Vintersten Nagy K, Nagy A (2014): Protocol 11: Castration of mice. In: Behringer R, Gertsenstein M, Vintersten Nagy K, Nagy A (eds.), Manipulating the mouse embryo: a laboratory manual. 4th edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 234–235.

Deacon RM (2006): Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. Nat Protoc 1(1): 118–121.

Deacon RM, Raley JM, Perry VH, Rawlins JN (2001): Burrowing into prion disease. Neuroreport 12(9): 2053–2057.

Gaskill BN, Karas AZ, Garner JP, Pritchett-Corning KR (2013): Nest building as an indicator of health and welfare in laboratory mice. J Vis Exp (82): 51012. DOI 10.3791/51012.

Henke J, Haberstroh J, Sager M, Becker K, Eberspächer E, Bergadano A, Zahner D, Arras M (2015): Fachinformation aus dem Ausschuss für Anästhesie der GV-SOLAS in Zusammenarbeit mit dem Arbeitskreis 4 in der TVT: Schmerztherapie

bei Versuchstieren. Available from: www.gv-solas.de/fileadmin/user_upload/pdf_publikation/Anaest._Analgesie/Schmerztherapie_Mai2015.pdf (last accessed on March 1, 2019).

Hess SE, Rohr S, Dufour BD, Gaskill BD, Pajor EA, Garner JP (2008): Home improvement: C57BL/6J mice given more naturalistic nesting materials build better nests. J Am Assoc Lab Anim Sci 47(6): 25–31.

Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C (2017): Severity classification of repeated isoflurane anesthesia in C57BL/6JRj mice-Assessing the degree of distress. PLoS One 12(6): e0179588.

Hohlbaum K, Bert B, Dietze S, Palme R, Fink H, Thöne-Reineke C (2018): Systematic assessment of well-being in mice for procedures using general anesthesia. J Vis Exp 133. DOI 10.3791/57046.

Hurst JL, West RS (2010): Taming anxiety in laboratory mice. Nat Methods 7(10): 825–826.

Jirkof P, Cesarovic N, Rettich A, Nicholls F, Seifert B, Arras M (2010): Burrowing behavior as an indicator of post-laparotomy pain in mice. Front Behav Neurosci 4: 165.

Langford DJ, Bailey AL, Chanda ML, Clarke SE, Drummond TE, Echols S, Glick S, Ingrao J, Klassen-Ross T, Lacroix-Fralish ML, Matsumiya L, Sorge RE, Sotocinal SG, Tabaka JM, Wong D, van den Maagdenberg AM, Ferrari MD, Craig KD, Mogil JS (2010): Coding of facial expressions of pain in the laboratory mouse. Nat Methods 7(6): 447–449.

Mugford RA (1973): Intermale fighting affected by home-cage odors of male and female mice. J Comp Physiol Psychol 84(2): 289–295.

Palme R (2019): Non-invasive measurement of glucocorticoids: advances and problems. Physiol Behav 199: 229–243.

Palme R, Touma C, Arias N, Dominchin MF, Lepschy M (2013): Steroid extraction: Get the best out of faecal samples. Wien Tierarztl Monatsschr 100(9–10): 238–246.

Rock ML, Karas AZ, Rodriguez KB, Gallo MS, Pritchett-Corning K, Karas RH, Aronovitz M, Gaskill BN (2014): The time-to-integrate-to-nest test as an indicator of wellbeing in laboratory mice. J Am Assoc Lab Anim Sci 53(1): 24–28.

Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, Wieskopf JS, Acland EL, Dokova A, Kadoura B, Leger P, Mapplebeck JC, McPhail M, Delaney A, Wigerblad G, Schumann AP, Quinn T, Frasnelli J, Svensson CI, Sternberg WF, Mogil JS (2014): Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods 11(6): 629–632.

Touma C, Palme R, Sachser N (2004): Analyzing corticosterone metabolites in fecal samples of mice: a noninvasive technique to monitor stress hormones. Horm Behav 45(1): 10–22.

Touma C, Sachser N, Mostl E, Palme R (2003): Effects of sex and time of day on metabolism and excretion of corticosterone in urine and feces of mice. Gen Comp Endocrinol 130(3): 267–278.

Tubbs JT, Kissling GE, Travlos GS, Goulding DR, Clark JA, King-Herbert AP, Blankenship-Paris TL (2011): Effects of buprenorphine, meloxicam, and flunixin meglumine as post-operative analgesia in mice. J Am Assoc Lab Anim Sci 50(2): 185–191.

Ullmann K, Jourdan T, Kock M, Unger J, Schulz A, Thöne-Reineke C, Abramjuk C (2018): Recommendations for the development and use of score sheets as a tool for applied refinement. Berl Münch Tierärztl Wochenschr 131(7–8): 292–298. DOI 10.2376/0005-9366-17095.

Van Loo PL, Van Zutphen LF, Baumans V (2003): Male management: coping with aggression problems in male laboratory mice. Lab Anim 37(4): 300–313.