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Original scientific article

Failure to replicate? Exacerbated 8-OH-DPAT-induced hypothermia could not be established in single housed mice when tested as part of a battery of depression tests

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Summary

We have previously described a depressive phenotype in male mice stemming from three weeks' single housing, demonstrated as an exacerbated transient hypothermia following a challenge with the serotonin (5-HT) receptor agonist 8-OH-DPAT. In an attempt to flesh out the phenotype, we carried out a battery of tests used to assess depressive states on C57BL/6 mice of both sexes. When combining the 8-OH-DPAT challenge with the tail-suspension test, an open field test, a sucrose preference test, and blood samplings for measuring serum levels of oxytocin, we could find no clear evidence of depressive states in the single housed animals. The fact that we could not replicate our previous findings is puzzling; however, we suspect that the stressful nature of the test battery may have been detrimental to the model. These negative findings and their implications may prove of significant importance moving forward with studying natural models of depression.

Introduction

Despite being common practice in many laboratory animal facilities, the consequences of housing mice alone in shoebox cages is a divisive subject. Are there detrimental effects to a mouse's wellbeing from its lack of contact with conspecifics? Is its welfare compromised? Does research carried out in single housed mice produce significantly different results than the same research on group housed mice? The current EU directive (2010) dictates that social animals must be housed in groups; but is an adult mouse even to be considered a social animal? Is it not completely

unnatural to house adult male mice together? And so the debate rages on. A number of investigations have been carried out attempting to substantiate the arguments empirically, but the results, to date, have been as varied as the viewpoints. Whereas some studies find clear changes in behavior and physiology of single housed mice, other studies find no differences when comparing to group housed mice. An increased metabolic rate has been noted across multiple studies (Schipper et al. 2018; Späni et al. 2003), suggested to stem from not only lack of opportunities

to socially thermoregulate, but also from emotional stress. The latter is further reinforced by multiple research groups noting behavior consistent with increased anxiety in single housed mice (e.g. Ferrari et al. 1998; Heredia et al. 2012; Voikar et al. 2005). This effect, seen across multiple strains, seems to be inconsistent and somewhat specific to the test that is employed. Ferrari et al. (1998) for example noted increased anxiety when testing single housed mice in the elevated plus maze, whereas Voikar et al. (2005) noted the opposite effect. The latter study did however note increased anxiety-like behavior in e.g. the light-dark box paradigm, conceptually corroborating the findings of Ferrari et al., even though they did not reproduce the exact findings. Moreover, Voikar et al. (2005) found evidence of impaired learning in spatial memory tests in single housed mice. Exacerbated memory impairments have also been noted in mice genetically engineered to mimic components of human Alzheimer's disease when they are housed alone (Huang et al. 2011). Published studies thus paint vague patterns suggestive of neurobehavioral changes brought about in socially isolated mice. Finally, in choice-based testing, mice will choose the company of a conspecific over e.g. nesting material (van Loo et al. 2004). Even male mice will voluntarily choose the company of a potentially abusive dominant male mouse over isolation when offered the choice (van Loo et al. 2001). The inability to consistently demonstrate a difference between single and group housed mice in tests of purported anxiety (e.g. Bartolomucci et al. 2009) complicates the picture, however. The fact that no differences can be found in acute stress parameters, such as glucocorticoid levels, has furthermore been used as evidence that mice do not suffer from being socially isolated (e.g. Hunt and Hambly 2006; Arndt et al. 2009).

An argument can be made that the problem with studying single housing is that no two single housing conditions are quite alike. Analogous to the standardization fallacy (Würbel 2000), the more one reduces the components of an experiment, the more ambient parameters – that which remains – influence the results of one's study, creating idiosyncratic results that do not translate well between laboratories (Richter et al. 2009). When studying single housing, we reduce our experimental setup to as simple as is possible within an *in vivo* science context: cages and different groupings of mice. Add to this subtle effects, requiring large numbers of animals in a sparsely funded field-of-study where costly studies seldom are an option. Varied results should thus not come as a surprise; they are to be expected. With meta-analyti-

cal pooling of data gaining momentum in the laboratory animal science community (Hooijmans and Ritkes-Hoitinga 2013; Avey et al. 2015) we may however overcome the problems of idiosyncratic results and under-powered studies. The key to addressing questions such as “do mice suffer from being housed alone” using a meta-analytical approach is however for all laboratories to report all of their findings, also when negative (Rosenthal 1979; Thornton and Lee 2000). It is in this vein we share the present report.

We have previously demonstrated an alarming effect on the serotonergic system of single housed male mice (Kalliokoski et al. 2014); a possible confounder of neurobiological investigations. Single housing for three weeks appeared to alter the reactivity of the mice's serotonergic system to an exogenous agonist when compared to group housed controls. A similar neurochemical change – an increased excitability of 5-HT_{1A} receptors – in humans is strongly associated with depressions (Savitz et al. 2009; Parsey et al. 2006). We have thus posited that our findings stem from a subtle depressive state, brought on by even a shorter duration of solitary housing and possibly the lack of stimulation in individually ventilated cages (the latter potentially act as sensory deprivation chambers, preventing mice from seeing, smelling and hearing their cage's surrounding environment). In order to better characterize the phenotype, we set out to combine our previous experiment with a battery of other common tests for characterizing depressive phenotypes; utilizing the hypothermic response to a challenge with the serotonin receptor agonist 8-OH-DPAT alone as a proxy of depressive affectedness can be construed as too abstract a measure. Our goal was consequently to better characterize the underlying phenotype, to find measures that were correlated with the exacerbated hypothermic response to 8-OH-DPAT following single housing, and measures that were not.

For our test battery we chose to investigate changes in sucrose preference, suggestive of anhedonia (Monleon et al. 1995), the immobility in a tail-suspension test (TST), suggestive of behavioral despair (Steru et al. 1985), and our previously validated method of measuring the hypothermic response to the serotonin receptor agonist 8-OH-DPAT, suggestive of increased 5-HT_{1A} activity (Bert et al. 2006). The sucrose preference test (SPT) and the TST have previously shown some promise in detecting depressive changes in isolated mice (e.g. D'Andrea et al. 2010; Martin and Brown 2010). In addition, we included an open field test to measure general activity level, which can be a confounder in

the tail-suspension test (Steru et al. 1985; Peng et al. 2007), and to measure serum levels of oxytocin. The latter was included to investigate whether the depressive phenotype was correlated with, or possibly modulated by, decreased oxytocin levels brought on by decreased social stimulation and contact.

Material and methods

Animals and housing

A total of 48 six-weeks-old C57BL/6J mice of both sexes ($n = 24/\text{sex}$) were acclimatized for a week after arriving from a commercial breeder (Janvier Labs, Le Genest-Saint-Isle, France). The animals were housed in groups of three in individually ventilated polycarbonate cages ("1290D001"; Tecniplast, Buggugiate, Italy). Diurnal rhythm was maintained with a 12:12 hour light-dark cycle with 30 minutes of twilight at transitions and lights on at 6 a.m. The ambient temperature was kept at 19-23 °C, humidity at 42-46 % and cages were set to be ventilated at a rate of 75 h⁻¹ air changes. Post-experiment, it was uncovered that the air-handling unit serving the cages in question had suffered a failure and ventilation rates had fallen to 63 h⁻¹ on cage level, suggesting that the ventilation rate may have been somewhat lower than expected. Cages were changed every 14 days and the positions on the racks were changed frequently to eliminate any

bias from cage position. Cages were lined with aspen chips (Tapvei, Kortteinen, Finland) with wooden gnawing sticks (Tapvei) intermixed, and shelters – red tinted translucent plastic shelters ("JAKO"; Molytex, Glostrup, Denmark) and cardboard tubes (Lillico, Horley, UK) – and nesting material ("Happi-mat" nestlets; Scanbur, Karlslunde, Denmark) were supplied and replaced as needed. Extruded feed ("Altromin 1314"; Brogaarden, Lynge, Denmark) and tap water was provided *ad libitum* throughout the study.

Single housing experiment

A week after arrival, baseline sucrose preference was established and twelve days post-arrival (Day 0 of the experiment) a baseline blood sample was obtained. Following the sampling, randomly selected (randomized on cage-level) mice of both sexes were single-housed. Body weights of the animals were recorded weekly throughout the study. After three weeks of single housing, a battery of tests were carried out, and on Day 28, following the hydroxytryptamine-induced hypothermia (HIH) challenge, the animals were euthanized by cervical dislocation (for an illustrated overview of the experimental timeline, refer to Figure 1). The group sizes in the study were determined using simplistic power calculations (Cohen, 1988) based on historical data (Kalliokoski et al. 2013; Kalliokoski

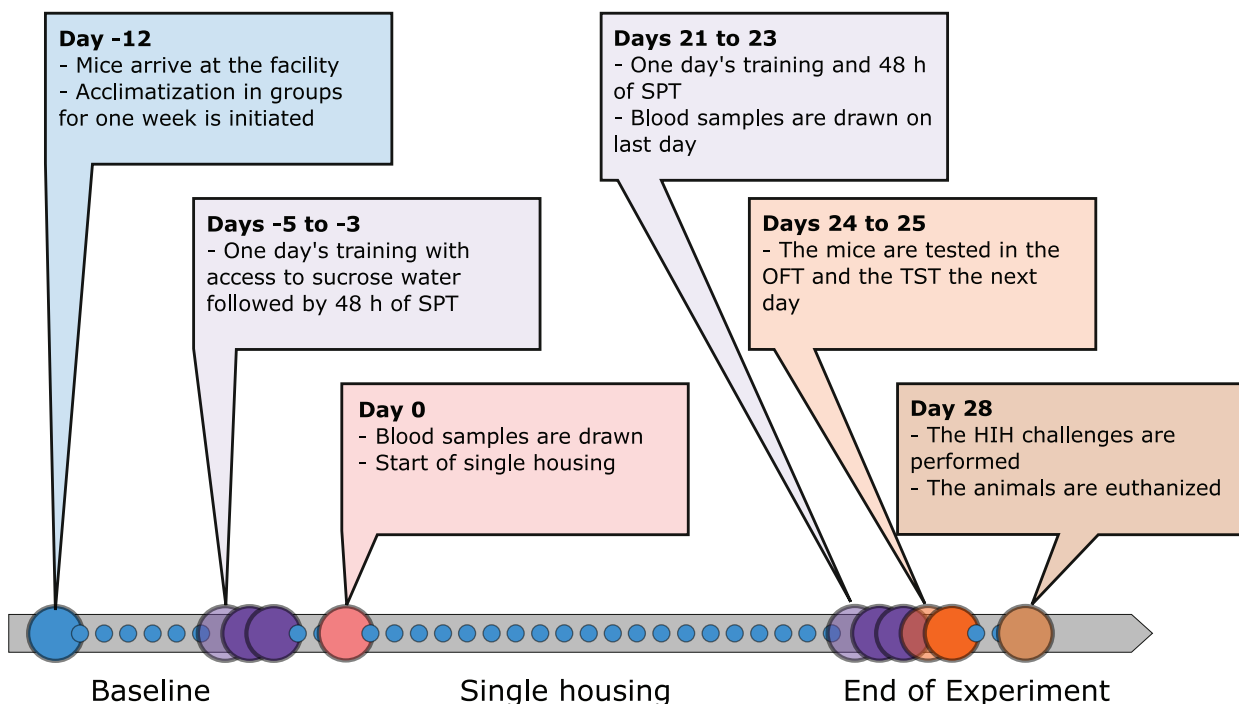


Figure 1. Timeline of the experiment. All dates are reported relative to the initiation of single housing. Group housed animals acting as negative controls were maintained group housed (in the same groups) throughout the study. Weekly recordings of body weights have been omitted from the timeline. SPT: Sucrose preference test; OFT: Open field test; TST: Tail-suspension test; HIH: Hydroxytryptamine-induced hypothermia.

et al. 2014). The study was scaled to detect a difference of 5 % in the sucrose preference tests and an absolute difference of 1 °C in the HIH challenge tests, with α and β levels set at 0.05 and 0.2, respectively. The design originally included a re-housing phase, following the period of single housing. The original plan for the study was to induce a depressive state that we have seen in the past (Kalliokoski et al. 2014), and then to attempt to reverse this by socially housing a cohort of isolated mice. As a consequence, uneven experimental cohorts were employed from the start (as the single housed mice were to be further subdivided). The female mice – tested first – were housed in two groups of six animals, and eighteen animals were housed alone. Based on the (initial) null findings in the females, the study design was modified. The re-housing period was removed from the study and a follow-up experiment in male mice was instead undertaken, where 12 animals were housed in groups of 3, and 12 animals were single-housed.

Tests of depressive phenotype

Prior to all the outlined tests, with exception of the sucrose preference tests, all animals were temporarily single-housed and transported to an adjacent laboratory to ensure blinding of the investigators. Testing orders were randomized for all of the tests.

Sucrose preference tests

The sucrose preference test (SPT) spanned 72 h and was initiated at 8 a.m. on Days -5 and 21 for baseline and endpoint measurements. In an introduction phase, the mice were introduced to the SPT setup with two water bottles mounted in the food hopper (feed pellets were moved to the cage floor). Both bottles were filled with a 1.5 % (w/v) sucrose solution. After 24 h, the bottles were substituted with one bottle containing tap water and one bottle containing an 8 % (w/v) sucrose solution (previously shown to be the preference maximum for C57BL/6 mice (Pothion et al. 2004)). The position of the sucrose-containing bottle was randomized and after 24 h, the position of the bottles was reversed in order to minimize the influence of place preference. The intake of sucrose solution, expressed as a fraction of total fluid intake, was recorded for a total of 48 h.

Open field tests

On Day 24, with testing initiated at 9 a.m., an open field test (OFT) was carried out similarly to other investigations (Sáenz et al. 2006; Valentinuzzi et al. 2000; Yoon et al. 2014). Briefly, a mouse was placed in the corner of a square arena (45 × 45 cm, with 41 cm

high opaque walls) and allowed to freely explore its surroundings for ten minutes (while the experimenters left the room). Activity was recorded by a camera suspended 40 cm above the OFT apparatus and automatically tracked in Ethovision XP (Noldus Information Technology, Wageningen, the Netherlands) with distance travelled as the endpoint measurement. Animals were acclimated to the testing laboratory for one hour prior to testing. Lighting was kept low and the testing apparatus was uniformly illuminated through only indirect lighting. The white OFT arena ensured a good contrast with the animals' fur color for optimal automated tracking, and a painted grid (9 × 9 cm squares) allowed for manual calculation of distance travelled through line-crossings (used to verify accurate tracking by the software in randomly chosen video recordings). The OFT arena was cleaned and wiped down with 70 % ethanol between tests.

Tail-suspension tests

The tail-suspension test (TST) was carried out approximately 24 h after the OFT, initiated at 9 a.m., and carried out according to Can et al. (2012). Briefly, mice were suspended from a transverse bar in an open testing box, 20-25 cm above the ground, using a piece of masking tape attached 2-3 mm from the tail tip. A plastic cylinder was threaded over the mouse's tail prior to suspension, preventing it from grabbing and holding on to its own tail once hanging; a common problem with C57BL/6 mice (Mayorga and Lucki 2001). Two mice were tested at a time in the TST apparatus; a solid divider prevented the animals from seeing one another. A six-minute video recording was obtained before the animals were returned to their home cages and the apparatus was cleaned and wiped down with 70 % ethanol between tests. Bouts of active struggling behavior were timed manually by two blinded observers and the immobility time calculated.

HIH challenge

On the last day of the study (Day 28) the HIH challenge was conducted similar to what we have described before (Kalliokoski et al. 2013; Kalliokoski et al. 2014). Briefly, the mouse's (baseline) rectal temperature was recorded ("BAT-12" temperature probe; Physitemp Instruments Inc., Clifton, NJ, USA) and a subcutaneous injection of 40 µg 8-OH-DPAT (R-(+)-8-hydroxy-2-(di-n-propylamino)-tetralin; Prod. No. H140; Sigma-Aldrich, St. Louis, MO, USA), delivered in 100 µl sterile isotonic saline, was deposited in the inguinal region. The animal was subsequently

placed in a barren cage, and 30 minutes post-injection its rectal temperature was again recorded (corresponding roughly to maximum hypothermia).

Oxytocin measurements

Tail vein blood samples were collected at 9 a.m. on Days 0 and 23. Briefly, animals were placed in a heated chamber (28–32 °C) for four minutes to ensure vasodilation, followed by placement in a restrainer. The tails were wiped down with 70 % ethanol, the lateral tail vein was punctured with a 25G hypodermic needle, and a 150 μ l (approximately) blood sample was drawn. Serum was separated out through centrifugation (15 minutes at 1,600 g, 4 °C) and stored frozen (-70 °C) until analysis. Oxytocin content of the serum was determined using a commercial ELISA kit (“ADI-900-153”; Enzo Lifesciences, Farmingdale, NY, USA); analyses were carried out according to the manufacturer’s instructions.

Hypothesis testing

End of experiment measures were compared using independent samples t-tests. Data were considered homoscedastic with the exception of the sucrose preference where obvious differences in group variances were noted. The SPT data were consequently tested using Welch’s t-test for unequal variances. OFT data were not tested for between-group differences, as the distance travelled is not considered a variable related to depressive states in its own right. Instead, the distance travelled was used as a proxy variable for an individual’s general activity level. To ensure that a higher activity level did not confound data from the TST, an ANCOVA model was constructed for the immobility in the TST; housing was considered a fixed effect, OFT distance travelled an explanatory covariate. For the SPT data and serum oxytocin levels, where baseline data were obtained, comparisons were made between baseline and end of experiment measures using paired-samples t-tests. To verify that a housing effect was not masked by individual differences at baseline, the oxytocin data were also analyzed using a repeated-measures ANOVA testing for a time \times housing interaction. Serum oxytocin levels appeared to conform to a log-normal distribution (confirmed using Q-Q plots) and were consequently log-transformed prior to all analyses. All tests were carried out in IBM SPSS 24 (Armonk, NY, USA).

Ethics statement

The study was conducted in an AAALACi (Association for Assessment and Accreditation of Laboratory Animal Care International) accredited facility under the supervision of a local ethics committee. All procedures were carried out in accordance with EU directive 2010/63/EU and approved by the Animal Experiments Inspectorate under the Danish Ministry of Food, Agriculture and Fisheries (license number 2016–15–0201–00991).

Results

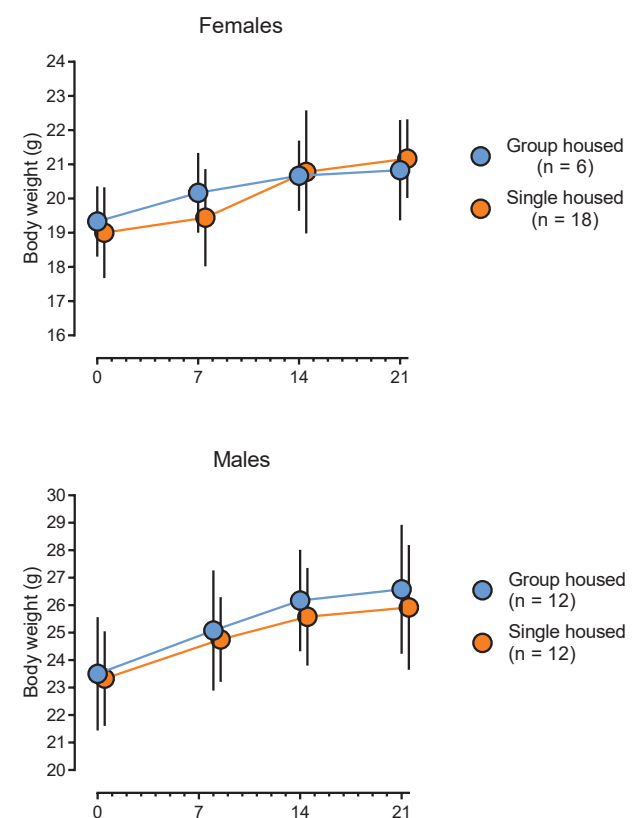


Figure 2. Body weight development in female and male mice over three weeks of differential housing. Data is displayed as means \pm SD. Animals were weighed on the same day but data points have been slightly shifted along the x-axis to prevent overlapping error bars. Single housed animals did not differ in body weight from their group housed counterparts throughout the study.

Following three weeks of single housing (on Day 21), neither female nor male mice differed in body weight (Figure 2) from their group housed counterparts (females: $t_{22} = -0.57$, $p = 0.57$; males: $t_{22} = 0.71$, $p = 0.49$). An ostensibly attenuated sucrose preference after three weeks’ single housing (Figure 3) was found for female but not male mice (females: $t_{21.3} = 6.84$, $p < 0.01$; males: $t_{17.5} = 0.42$, $p = 0.68$). No difference could be found in immobility time in the

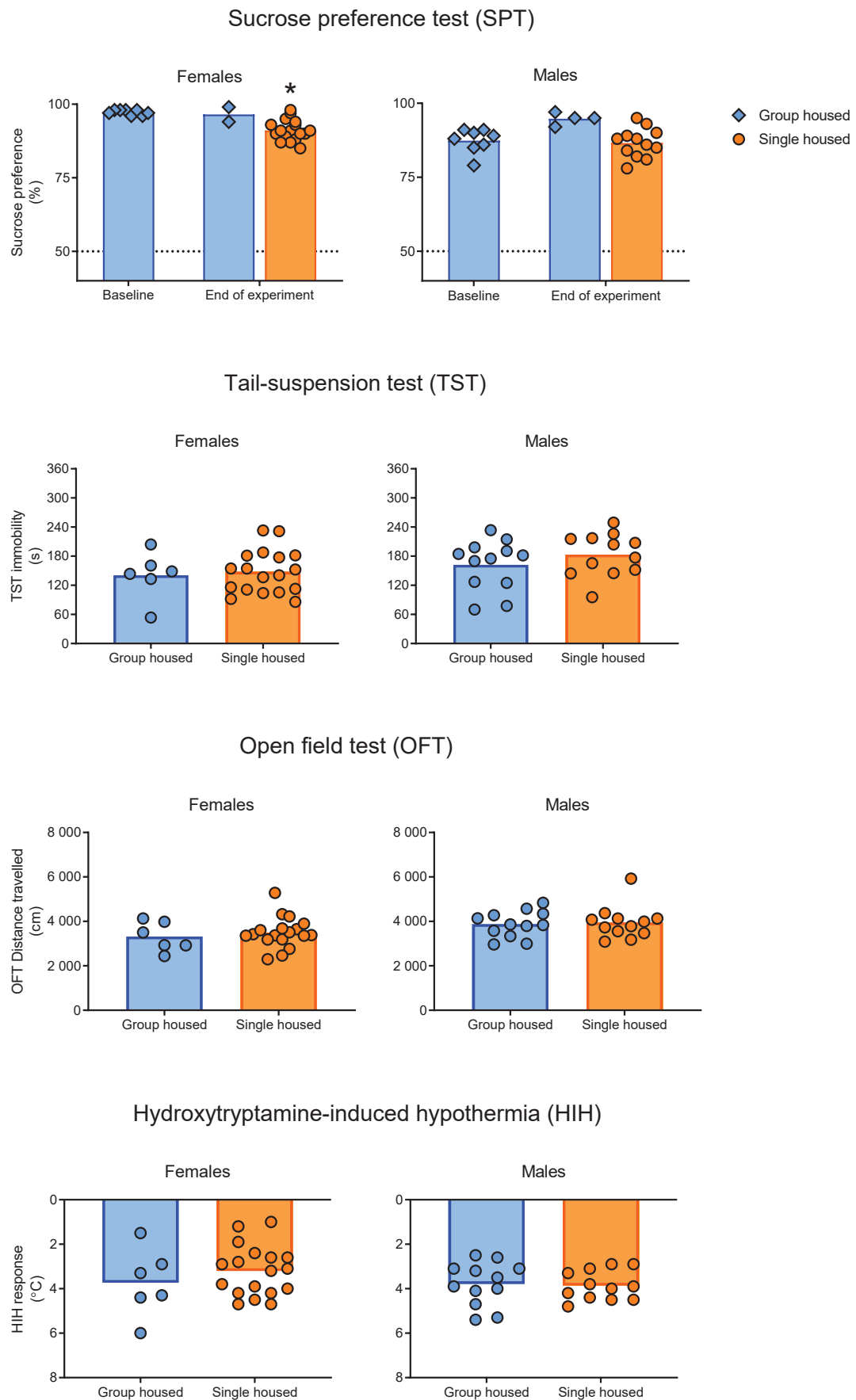


Figure 3. Results from a battery of tests carried out to assess depressive states in single housed mice. Figures listed in order from top to bottom: Sucrose preference in percent of total intake; Time spent immobile in the tail-suspension test (TST); Distance travelled in the open field test (OFT); Hydroxytryptamine-induced hypothermia (HIH) expressed as the drop in core body temperature from baseline. All of the data are shown as subject-level measurements with group means, with the exception of the sucrose preference where the measurements are displayed on cage-level (measurements collecting multiple individuals are shown as diamonds). The asterisk denotes a significant difference at a level of $p < 0.05$ from baseline values.

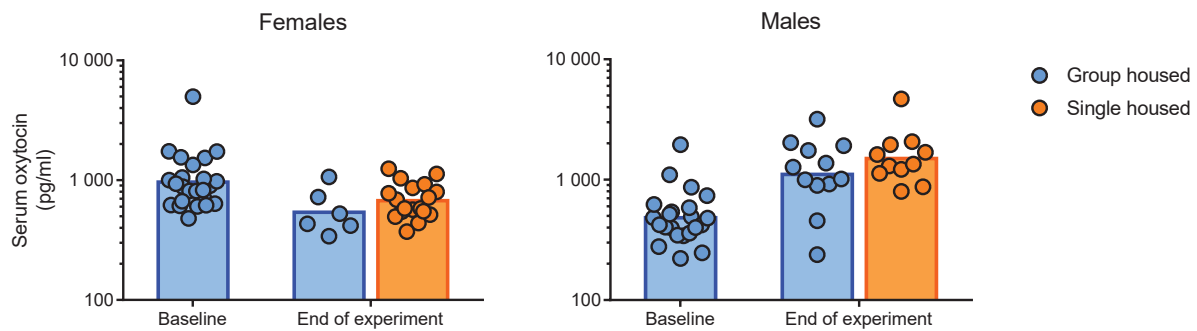


Figure 4. Serum oxytocin levels measured before (Day 0) and after (Day 23) differential housing. Data are displayed as individual replicates with geometric means for the groups on a logarithmic axis. Both for female and male mice, the oxytocin levels at the end of the experiment were found to be significantly altered from the baseline levels; but whereas the females had reduced their serum oxytocin for the second sampling, the males demonstrated an opposite trend.

TST between single housed and group housed mice of either sex (females: $t_{22} = -0.33$, $p = 0.74$; males: $t_{22} = -1.07$, $p = 0.30$). Utilizing distance travelled in the OFT as an explanatory co-variate when comparing TST immobility time between groups did not reveal any differences masked by differing activity levels (females: $F_{2,21} = 0.13$, $p = 0.73$; males: $F_{2,21} = 1.29$, $p = 0.27$). The hypothermic response to a challenge with 8-OH-DPAT did not differ following differential housing for three weeks for either sex (females: $t_{22} = 0.91$, $p = 0.46$; males: $t_{22} = -0.22$, $p = 0.83$).

No differences were found between single housed and group housed mice for either sex (over time) with respect to serum oxytocin levels (females: $F_{1,22} = 0.11$, $p = 0.74$; males: $F_{1,19} = 0.11$, $p = 0.74$). Differences were however found between baseline levels and the samples obtained on Day 23 (Figure 4), with female mice exhibiting lower levels of oxytocin at the end of the study (housing conditions combined,

paired data: $t_{23} = 3.29$, $p < 0.01$) and males demonstrating an inverse trend with higher levels toward the end of the study (housing conditions combined, paired data: $t_{20} = -6.63$, $p < 0.001$). Three serum samples (two baseline, one end of experiment) obtained from males were too small for analysis and were thus excluded (data considered missing at random).

Post hoc analysis

The reduced sucrose preference for single housed female mice in the SPT was considered questionable as there was no way of recording spillage from the bottles. Utilizing an empty cage to estimate how much fluid is lost to dripping bottles has been suggested (Eagle et al., 2015), but we considered this unreliable as it does not account for animal activity within the cage (differing with group size). With a very high affinity for the 8 % sucrose solution, much

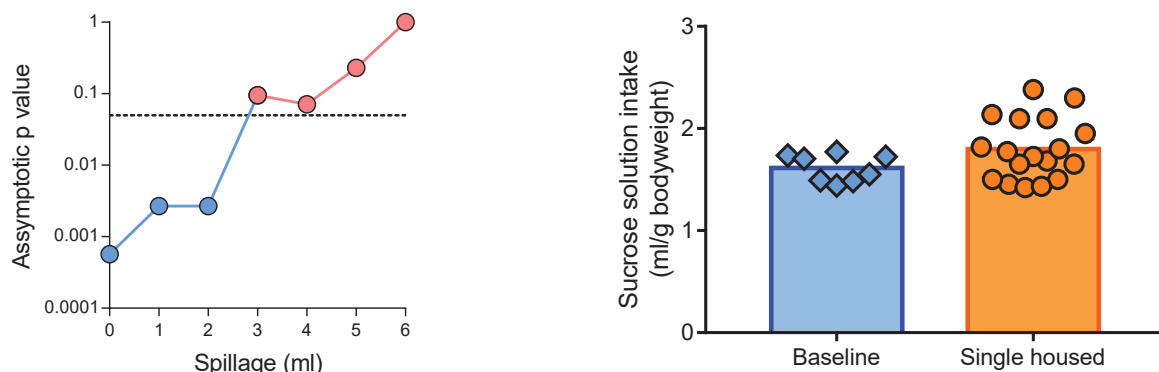


Figure 5. Sensitivity analysis (left) and total amount of sucrose solution consumed (per gram of animal) for the female mice (right). The p values were estimated by applying the Mann-Whitney U test (by ranks) in comparing baseline sucrose preference to the single housed end of experiment values. In increments of 1 ml (the measured accuracy), liquid was subtracted equally from each bottle. The seemingly low p value exceeds the critical level of 0.05 (dashed line) already after a loss of 3 ml (i.e. 1.5 ml per 24 h). When comparing the absolute intake of sucrose solution it becomes, furthermore, clear that the single housed animals did not consume less liquid than during baseline conditions.

of the tap water lost was potentially due to spillage. For the group housed animals any droplet lost would be shared between three animals, affecting the test less, whereas routine operations in the animal room rustling the racks and cages causing the water bottles to drip could potentially make it seem as if the single housed mice drank more tap water. As little as 1.5 ml lost, per 24 hours, due to dripping water bottles, we found employing some simplistic calculations (Figure 5), could produce the found significant difference. In a representative setup, a droplet was estimated at 54 μ l (10 droplets – average: 536 μ l; SD: 76 μ l) suggesting a loss of about a droplet an hour (1.17 h^{-1}) amounts to 3 ml over 48 hours. This is not an unrealistic loss given that just opening a cage lid was found to spill at least 4-5 droplets. Thus, to test whether the single housed female mice could truly be considered anhedonic, we chose to test the absolute amount of sucrose solution consumed, expressed per gram of mouse in the cage; after all, anhedonia is defined as a lowered affinity for sucrose, not as an increased interest in tap water.

Sucrose intake was not normally distributed when expressed per gram bodyweight, consequently rank testing was used. When expressed in absolute amounts, no reduction in sucrose intake was found for the female mice following single housing (Mann-Whitney, $U = 47$, $p = 0.17$, $r = 0.27$). It has, therefore, to be concluded that the female mice did not become anhedonic when housed alone, but rather that the difference is better explained as tap water lost due to dripping bottles.

Discussion

In the present study, we were unable to replicate our previous findings showing an exacerbated 8-OH-DPAT-inducible hypothermic response in single housed male C57BL/6 mice over their group housed counterparts (Kallioikoski et al. 2014). When combining the HIH challenge with sucrose preference testing, blood samples, tail-suspensions and time spent in open field arenas, the effect previously found for a number of substrains of C57BL/6 was wholly missing. Recently, focus has been brought to the fickle nature of the p-value as the deciding factor in hypothesis testing. Even in the face of highly significant statistics and miniscule p-values the chance of a non-significant finding in an identical replication study is close to the chances of a coin toss coming up heads (Halsey et al. 2015). Although we cannot fully rule it out, the outcome of the HIH challenge in the present study seems to be more than just the

case of chance deviation from a true difference. The data show not even the slightest trend toward a greater hypothermia in the single housed group. Whereas these data hardly refute our previous conclusions – the study collected data from 231 subjects across two experimental designs, after all – they do put them in a larger context. In another previous study (Kallioikoski et al. 2013), investigating the stressful nature of metabolism cage housing, we found the HIH challenge to produce erratic results when carried out shortly after a stressful behavioral test (the forced swimming test). In the present study, we suspect, similarly, that the stress response in relation to being suspended from one's tail, forced into an inescapable open arena, and blood samplings may have served to alter the hypothermic response to 8-OH-DPAT. The key follow-up question is whether the stressful nature of the battery of depression tests only confounds the HIH challenge, or whether it effectively destroys the purported depressive phenotype.

A reduced preference for a sweetened sucrose solution is in the SPT paradigm interpreted as an expression of anhedonia. With depressions comes a blunted feeling of joy, with previously enjoyable experiences losing their appeal. A depressed mouse presented with the SPT setup becomes less particular in choosing the sweetened solution over tap water when drinking. Single housing has previously been suggested to induce anhedonia in mice, demonstrable through the SPT (D'Andrea et al. 2010). Nevertheless, within the framework of the present study, we could not find any reliable evidence of anhedonia.

Similarly, our TST results did not differ between groups. Although the TST has been used in comparable contexts, sometimes to seemingly good effect (Martin and Brown 2010), the test has been suggested to be principally appropriate for screening pharmacological candidates for antidepressant potential, rather than for assessing depression/depressive states (Nestler and Hyman 2010). Findings such as the present ones, underscore the questionable appropriateness of utilizing the TST for evaluating naturally occurring depressive states. Not only is it unclear whether these are associated with the poorly defined concept of emotional despair, but the stressful nature of the test can potentially confound other tests and interfere with depressive phenotypes. Alternative behavioral tests, affecting the subjects' emotional state less, are under similar circumstances probably preferable. We combined the TST with an OFT, as it is considered best practice to combine the TST with a test of general activity. Without combining the tests, compounds such as caffeine and ampheta-

mines appear to have an antidepressant effect in the TST as they decrease immobility (Steru et al. 1987). The OFT can however be used to demonstrate that these compounds simply induce hyperactivity (Marriott 1968). In the case of evaluating emotional states, removed from the context of testing drug candidates, the distance travelled in the OFT is hard to interpret. Should more active mice be considered more or less likely to be depressed? Is this even a relevant factor? Luckily, we can refrain from excessive speculation, as all of our mice performed similarly across both the TST and OFT. Neither the TST nor the TST in combination with the OFT was able to tell us anything intelligible about the emotional state of our animals.

Oxytocin has a modulating effect in stress and anxiety. Studies in prairie voles (Grippe et al. 2007a; Grippe et al. 2007b) have demonstrated that circulating oxytocin levels change in response to social isolation in concert with the onset of anhedonia (measured using the SPT). We speculated that the negative outcome we previously had noticed from single housing might thus be accompanied by concomitant lowered levels of oxytocin. Our results, although not supportive of our initial hypothesis, do suggest that measuring circulating levels of oxytocin in laboratory mice may be of interest. The consequences of oxytocin supplementation has been studied at great lengths across a number of species (Smith et al. 2017), including mice (Teng et al. 2013; Harrison et al. 2016). However, data on endogenous circulating levels are all but completely missing. The fact that we find oxytocin to be a highly dynamic marker is interesting, even though we currently cannot interpret our results. There is cross-talk at the level of the hypothalamus between the HPA axis and oxytocin release (Neumann and Landgraf 2012). Elevated levels of oxytocin could thus have been caused by a stress response in relation to the blood samplings. With oxytocin levels having been shown to increase within five minutes from an acute stress response (Gibbs 1984), this is not unlikely. It may also be that collecting the blood samples immediately following the SPT may have influenced our results. Oxytocin modulates the feeling of satiation (Olszewski et al. 2010) and is thus likely to change in relation to *ad libitum* feeding of a sucrose solution (Song et al. 2014) with approximately the same caloric density as popular soft drinks. More data needs to be collected on the endogenous oxytocin levels in mice before we can explain our results in full; however, differential housing appeared to have nothing to do with it.

With no results suggestive of depressive states, we allow ourselves to speculate that it is most likely that the testing battery reversed the previously found phenotype, or prevented it from being induced altogether. Whereas the failure to reproduce previous findings could be construed as evidence that the previous findings were erroneous or a fluke (indications that we need to be watchful of, forging ahead), the size of the present study is not sufficient to refute previous evidence nor are the conditions of the experiments identical. Instead, we will suggest that changes in testing protocol are a more likely explanation to the difference in results. Whereas chronic prolonged stress is a well-recognized model of depression (Willner 1997) resembling human “burnout” syndrome (Wurm et al. 2016), it is not unlikely that stress induced by e.g. behavioral testing may counteract or over-shadow a social-isolation-induced depressive state. Although stressful interventions interfering with inducible phenotypes is certainly not a novel issue, it does present the question of how to approach studying models such as this. Utilizing fewer tests per subject reduces the amount of information we can gather from an animal, which is in poor agreement with the reduction ideal of the 3Rs, as this increases rather than reduces the number of animals used in a study. Perhaps it is time to update the tests we employ in studying depressive states in laboratory animals. Behavioral tests such as the TST, highly stressful tests likely to influence the subjects greatly, and with unclear underlying rationales (what even is “behavioral despair” in relation to human subjects?) to boot, should probably be replaced by less invasive tests with clearer frames of interpretation, e.g. judgement bias testing (Hoffman 2016; Boleij et al. 2012). The information we collect will never be more accurate than the means by which it is obtained.

Supplemental data

Complete raw data have been made freely available online for data synthesis, scrutiny, and reanalysis through the following link: <https://figshare.com/s/d9ff904e5b039ded9197>

References

- Arndt, S.S., Laarakker, M.C., van Lith, H.A., van der Staay, F.J., Gieling, E., Salomons, A.R., van 't Klooster, J., Ohl, F., (2009). Individual housing of mice—impact on behaviour and stress responses. *Physiology & Behavior*. **97**, 385-393.
- Avey, M.T., Fenwick, N., Griffin, G., (2015). The use of systematic reviews and reporting guidelines to advance the implementation of the 3Rs. *Journal of the American Association for Laboratory Animal Science*. **54**, 153-162.
- Bartolomucci, A., Parmigiani, S., Gioiosa, L., Ceresini, G., Palanza, P., (2009). Effects of housing social context on emotional behaviour and physiological responses in female mice. *Scandinavian Journal of Laboratory Animal Science*, **36**, 87-95.
- Bert, B., Fink, H., Hörtnagl, H., Veh, R.W., Davies, B., Theuring, F., Kusserow, H., (2006). Mice over-expressing the 5-HT_{1A} receptor in cortex and dentate gyrus display exaggerated locomotor and hypothermic response to 8-OH-DPAT. *Behavioural Brain Research*. **167**, 328-341.
- Boleij, H., van 't Klooster, J., Lavrijsen, M., Kirchhoff, S., Arndt, S.S., Ohl, F., (2012). A test to identify judgement bias in mice. *Behavioural Brain Research*. **233**, 45-54.
- Can, A., Dao, D.T., Terrillion, C.E., Piantadosi, S.C., Bhat, S., Gould, T.D., (2012). The tail suspension test. *Journal of Visualized Experiments*. **59**, e3769. doi: 10.3791/3769.
- Cohen, J., (1988). *Statistical power analysis for the behavioral sciences*. 2nd. Hillsdale, NJ: erlbaum.
- D'Andrea, I., Gracci, F., alleva, E., Branchi, I., (2010). Early social enrichment provided by communal nest increases resilience to depression-like behavior more in female than in male mice. *Behavioural Brain Research*. **215**, 71-76.
- Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, (2010). *Official Journal of the European Union*.
- Eagle, A.L., Mazei-Robison, M., Robison, A.J., (2015). Sucrose preference test to measure stress-induced anhedonia. *The Journal of Neuroscience*. **16**, e1822. doi: 10.21769/BioProtoc.1822.
- Ferrari, P., Palanza, P., Parmigiani, S., Rodgers, R., (1998). Interindividual variability in Swiss male mice: relationship between social factors, aggression, and anxiety. *Physiology & Behavior*. **63**, 821-827.
- Gibbs, D.M., (1984). Dissociation of oxytocin, vasopressin and corticotropin secretion during different types of stress. *Life Sciences*. **35**, 487-491.
- Grippe, A.J., Cushing, B.S., Carter, C.S., (2007a). Depression-like behavior and stressor-induced neuroendocrine activation in female prairie voles exposed to chronic social isolation. *Psychosomatic Medicine*. **69**, 149.
- Grippe, A.J., Gerena, D., Huang, J., Kumar, N., Shah, M., Ughreja, R., Carter, C.S., (2007b). Social isolation induces behavioral and neuroendocrine disturbances relevant to depression in female and male prairie voles. *Psychoneuroendocrinology*. **32**, 966-980.
- Halsey, L.G., Curran-Everett, D., Vowler, S.L., Drummond, G.B., (2015). The fickle P value generates irreproducible results. *Nature Methods*. **12**, 179.
- Harrison, N., Lopes, P.C., König, B., (2016). Oxytocin and social preference in female house mice (*Mus musculus domesticus*). *Ethology*. **122**, 571-581.
- Heredia, L., Torrente, M., Domingo, J.L., Colomina, M.T., (2012). Individual housing and handling procedures modify anxiety levels of Tg2576 mice assessed in the zero maze test. *Physiology & Behavior*. **107**, 187-191.
- Hoffman, K.L., (2016). New dimensions in the use of rodent behavioral tests for novel drug discovery and development. *Expert Opinion on Drug Discovery*. **11**, 343-353.
- Hooijmans, C., Ritskes-Hoitinga, M., (2013). Progress in using systematic reviews of animal studies to improve translational research. *PLoS Medicine*. **10**, e1001482.
- Huang, H.J., Liang, K.C., Ke, H.C., Chang, Y.Y., Hsieh-Li, H.M., (2011). Long-term social isolation exacerbates the impairment of spatial working memory in APP/PS1 transgenic mice. *Brain Research*. **1371**, 150-160.
- Hunt, C., Hambly, C., (2006). Faecal corticosterone concentrations indicate that separately housed male mice are not more stressed than group housed males. *Physiology & Behavior*. **87**, 519-526.
- Kalliokoski, O., Jacobsen, K.R., Darusman, H.S., Henriksen, T., Weimann, A., Poulsen, H E., Hau, J., Abelson, K.S., (2013). Mice do not habituate to metabolism cage housing—a three week study of male BALB/c mice. *PLoS One*. **8**, e58460.
- Kalliokoski, O., Teilmann, A.C., Jacobsen, K.R., Abelson, K.S., Hau, J., (2014). The lonely mouse – Single housing affects serotonergic signaling integrity measured by 8-OH-DPAT-induced hypothermia in male mice. *PLoS One*. **9**, e111065.
- Marriott, A., (1968). The effects of amphetamine, caffeine and methylphenidate on the locomotor activity of rats in an unfamiliar environment. *International Journal of Neuropsychopharmacology*. **7**, 487-491.
- Martin, A.L., Brown, R.E., (2010). The lonely mouse: verification of a separation-induced model of depression in female mice. *Behavioural Brain Research*. **207**, 196-207.
- Mayorga, A.J., Lucki, I., (2001). Limitations on the use of the C57BL/6 mouse in the tail suspension test. *Psychopharmacology*. **155**, 110-112.
- Monleon, S., Parra, A., Simon, V., Brain, P., D'Aquila, P., Willner, P., (1995). Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. *Psychopharmacology*. **117**, 453-457.
- Nestler, E.J., Hyman, S.E., (2010). Animal models of neuropsychiatric disorders. *Nature Neuroscience*. **13**, 1161.
- Neumann, I.D., Landgraf, R., (2012). Balance of brain oxytocin and vasopressin: implications for anxiety, depression, and social behaviors. *Trends in Neurosciences*. **35**, 649-659.

- Olszewski, P.K., Klockars, A., Schiöth, H.B., Levine, A.S., (2010). Oxytocin as feeding inhibitor: maintaining homeostasis in consummatory behavior. *Pharmacology Biochemistry and Behavior*. **97**, 47-54.
- Parsey, R.V., Oquendo, M.A., Ogden, R.T., Olvet, D.M., Simpson, N., Huang, Y.Y., van Heertum, R.L., Arango, V., Mann, J.J., (2006). Altered serotonin 1A binding in major depression: a [carbonyl-C-11] WAY100635 positron emission tomography study. *Biological Psychiatry*. **59**, 106-113.
- Peng, W.H., Lo, K.L., Lee, Y.H., Hung, T.H., Lin, Y.C., (2007). Berberine produces antidepressant-like effects in the forced swim test and in the tail suspension test in mice. *Life Sciences*. **81**, 933-938.
- Pothion, S., Bizot, J.C., Trovero, F., Belzung, C., (2004). Strain differences in sucrose preference and in the consequences of unpredictable chronic mild stress. *Behavioural Brain Research*. **155**, 135-146.
- Richter, S.H., Garner, J.P., Würbel, H., (2009). Environmental standardization: cure or cause of poor reproducibility in animal experiments? *Nature Methods*. **6**, 257.
- Rosenthal, R., (1979). The file drawer problem and tolerance for null results. *Psychological Bulletin*, **86**, 638.
- Sáenz, J.C.B., Villagra, O.R., Trías, J.F., (2006). Factor analysis of forced swimming test, sucrose preference test and open field test on enriched, social and isolated reared rats. *Behavioural Brain Research*. **169**, 57-65.
- Savitz, J., Lucki, I., Drevets, W.C., (2009). 5-HT(1A) receptor function in major depressive disorder. *Progress in Neurobiology*. **88**, 17-31.
- Schipper, L., Harvey, L., Beek, E., Dijk, G., (2018). Home alone: a systematic review and meta-analysis on the effects of individual housing on body weight, food intake and visceral fat mass in rodents. *Obesity Reviews*. **19**, 614-637. doi: 10.1111/obr.12663.
- Smith, J.E., Petelle, M.B., Jerome, E.L., Cristofari, H., Blumstein, D.T., (2017). Oxytocin experiments shed light on mechanisms shaping prosocial and antisocial behaviors in non-human mammals. *Integrative and Comparative Biology*. **57**, 619-630.
- Song, Z., Levin, B.E., Stevens, W., Sladek, C.D., (2014). Supraoptic oxytocin and vasopressin neurons function as glucose and metabolic sensors. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. **306**, R447-R456.
- Späni, D., Arras, M., König, B., Rüllicke, T., (2003). Higher heart rate of laboratory mice housed individually vs in pairs. *Laboratory Animals*. **37**, 54-62.
- Steru, L., Chermat, R., Thierry, B., Mico, J.A., Lenegre, A., Steru, M., Simon, P., Porsolt, R.D., (1987). The automated tail suspension test: a computerized device which differentiates psychotropic drugs. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. **11**, IN1-671.
- Steru, L., Chermat, R., Thierry, B., Simon, P., (1985). The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology*. **85**, 367-370.
- Teng, B.L., Nonneman, R.J., Agster, K.L., Nikolova, V.D., Davis, T.T., Riddick, N.V., Baker, L.K., Pedersen, C.A., Jarstfer, M.B., Moy, S.S., (2013). Prosocial effects of oxytocin in two mouse models of autism spectrum disorders. *Neuropharmacology*. **72**, 187-196.
- Thornton, A., Lee, P., (2000). Publication bias in meta-analysis: its causes and consequences. *Journal of Clinical Epidemiology*. **53**, 207-216.
- Valentinuzzi, V.S., Buxton, O.M., Chang, A.M., Scarbrough, K., Ferrari, E.A., Takahashi, J.S., Turek, F.W., (2000). Locomotor response to an open field during C57BL/6J active and inactive phases: differences dependent on conditions of illumination. *Physiology & Behavior*. **69**, 269-275.
- van Loo, P.L., de Groot, A.C., van Zutphen, B.F., Baumans, V., (2001). Do male mice prefer or avoid each other's company? Influence of hierarchy, kinship, and familiarity. *Journal of Applied Animal Welfare Science*. **4**, 91-103.
- van Loo, P.L., van de Weerd, H.A., van Zutphen, L.F., Baumans, V., (2004). Preference for social contact versus environmental enrichment in male laboratory mice. *Laboratory Animals*. **38**, 178-188.
- Voikar, V., Polus, A., Vasar, E., Rauvala, H., (2005). Long-term individual housing in C57BL/6J and DBA/2 mice: assessment of behavioral consequences. *Genes, Brain and Behavior*. **4**, 240-252.
- Willner, P., (1997). Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology*. **134**, 319-329.
- Würbel, H., (2000). Behaviour and the standardization fallacy. *Nature Genetics*. **26**, 263.
- Wurm, W., Vogel, K., Holl, A., Ebner, C., Bayer, D., Mörk, S., Szilagy, I.S., Hotter, E., Kapfhammer, H.P., Hofmann, P., (2016). Depression-burnout overlap in physicians. *PLoS One*. **11**, e0149913.
- Yoon, S.H., Kim, B.H., Ye, S.K., Kim, M.H., (2014). Chronic non-social stress affects depressive behaviors but not anxiety in mice. *The Korean Journal of Physiology & Pharmacology*. **18**, 263-268.