Published in final edited form as:

Physiol Behav. 2015 October 15; 150: 31–37. doi:10.1016/j.physbeh.2015.06.021.

Mouse Handling Limits the Impact of Stress on Metabolic Endpoints

Sriparna Ghosal^{1,*}, Amanda Nunley^{2,*}, Parinaz Mahbod², Alfor G. Lewis², Eric P. Smith², Jenny Tong², David A. D'Alessio², and James P. Herman¹

¹Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati, Cincinnati, Ohio-45237

²Division of Endocrinology, Diabetes and Metabolism, Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio-45237

Abstract

Studies focused on end-points that are confounded by stress are best performed under minimally stressful conditions. The objective of this study was to demonstrate the impact of handling designed to reduce animal stress on measurements of glucose tolerance. A cohort of mice (CD1.C57BL/6) naïve to any specific handling were subjected to either a previously described “cup” handling method, or a “tail-picked” method in which the animals were picked up by the tail (as is common for metabolic studies). Following training, an elevated plus maze (EPM) test was performed followed by measurement of blood glucose and plasma corticosterone. A second cohort (CD1.C57BL/6) was rendered obese by exposure to a high fat diet, handled with either the tail-picked or cup method and subjected to an intraperitoneal glucose tolerance test. A third cohort of C57BL/6 mice was exposed to a cup regimen that included a component of massage and was subjected to tests of anxiety-like behavior, glucose homeostasis, and corticosterone secretion. We found that the cup mice showed reduced anxiety-like behaviors in the EPM coupled with a reduction in blood glucose levels compared to mice handled by the tail-picked method. Additionally, cup mice on the high fat diet exhibited improved glucose tolerance compared to tail-picked controls. Finally, we found that the cup/massage group showed lower glucose levels following an overnight fast, and decreased anxiety-like behaviors associated with lower stress-induced plasma corticosterone concentration compared to tail-picked controls. These data demonstrate that application of handling methods that reduce anxiety-like behaviors in mice mitigates the confounding contribution of stress to interpretation of metabolic endpoints (such as glucose tolerance).

Address correspondence to: Sriparna Ghosal, Department of Psychiatry & Behavioral Neuroscience, University of Cincinnati, Cincinnati, Ohio 45237, ghosalsa@mail.uc.edu.

*Indicates equal contribution.

6. Author's Contribution:

S.G., A.G.L. and E.P.S. conceived and designed the study, collected and compiled data, and wrote the manuscript. P.M. originated the cup/massage method. A.N., E.P.S. and P.M. performed the experiments. J.T., D.A.D., and J.P.H. provided guidance and overall support for the studies. All authors contributed to discussion and reviewed the manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Corticosterone; handling; elevated plus maze; glucose tolerance test

1. Introduction

Metabolic studies in rodents can be significantly influenced by stress induced by experimental procedure [2, 3]. In particular, blood glucose, a common measure of metabolic studies, is affected by both activation of the hypothalamic-pituitary-adrenal (HPA) axis [14] and the autonomic nervous system (ANS) [40] to varying degrees. As such, recommendations are frequently instituted to minimize stress to the animals. Stress-reducing procedures including routine daily handling of mice before the experiment [5]; avoidance of olfactory stimuli associated with predatory animals (e.g., rats); reduction of ambient noise; atraumatic blood drawing; and use of dry bedding [2, 38] have been recommended as important adjuncts for optimizing experimental results. Attention to timing of the light/dark cycle is also important as glucose metabolism can vary across the diurnal period [28, 33, 41]. In the past it was common to perform metabolic testing on anesthetized mice to reduce stress. However, there is now ample evidence that the use of anesthesia in metabolic testing is itself a source of stress as well as potential direct metabolic consequences, and is probably more confounding than helpful [4, 6, 30, 39].

A commonly used approach for reducing stress during metabolic testing is regular handling of the mice post weaning to acclimatize the mice to the contact present during experiments [2]. However, depending on the type of handling and the animal strain, both positive and negative effects of handling on experimental outcomes have been reported. “Handling” in a general sense is thought to positively habituate the animals to human contact [18, 20, 35], but may also have some adverse effects (e.g., sleep disruption) [23]. Some handling procedures such as picking up animals by the tail may actually simulate the act of being captured and provoke stress responses [21]. Indeed, tail handling can cause seizures in susceptible strains [19]. In a recent study, Hurst and West [21] explored the degree to which a specific handling approach (referred to as a “cup” method) influenced the subsequent anxiety behaviors of mice. They showed that mice when handled by a potentially less threatening approach involving being “scooped up” (“cup” method) and permitted to roam unrestrained in the handler’s open gloved hands without direct physical restraint displayed 1) more voluntary interaction with the handlers, 2) lower anxiety-like behavior (as determined by elevated plus maze (EPM)) and 3) toleration of physical restraint compared to mice which were handled by traditional methods (e.g., picking up by the tail) [21]. Given the impact of “cup” method on reducing anxiety-like behaviors, we were interested in assessing whether there would be corresponding effects on metabolic parameters that are confounded by stress.

In the present study, we compared cohorts of mice handled by the standard tail-picked vs. the cup method and measured their anxiety-like behaviors, glucose homeostasis, and neuroendocrine indicators of stress. We also characterized a cup technique that included an additional massage component to determine whether this combination could mitigate effects of testing procedures on metabolic or stress endpoints. Our results indicate that both the cup

and cup/massage handling methods, in addition to reducing anxiety-like behaviors, can improve the results of metabolic studies that are confounded by stress experienced by the animal during the study.

2. Materials and methods

2.1. Animals

All procedures were approved by the University of Cincinnati Institutional Animal Care and Use Committee. In the present study, adult (10–16 weeks) male CD1.C57BL/6 (bred in house) and C57BL/6 mice (bred in house) were used. Mice were double housed at the Metabolic Diseases Institute of the University of Cincinnati under standard conditions in a temperature and humidity controlled room on a 12:12 hour light: dark cycle (lights on at 6:00 AM). The mice were given access to food and water *ad libitum* unless otherwise noted. Mice were fed either standard rodent chow (Teklad, Harlan, Indianapolis, IN; 3.1 kCal/g; ~5% fat) or a high fat diet (Research Diets, New Brunswick, NJ; 4.54 kcal/g; ~40% fat) as appropriate. Experiments were carried out on age-matched mice (5–10/group).

2.2. Handling procedure

Mice were handled by either (i) standard tail-picked, (ii) cup handling, or (iii) cup/massage. The tail-picked and the cup handling groups were trained as described previously by Hurst and West [21]. Briefly, mice were removed from their cage by the specified technique, and held for 30 seconds, returned to the cage for 60 seconds and then held again for 30 seconds. The cup mice were subjected to 10 training sessions over the course of two weeks similar to the Hurst and West method. The cup/massage group was trained similar to the cup group except for the addition of a massage, a variation of which has been previously described in rats [9, 11, 13, 34, 35]. During the first day of the training, the mice were confined or “cupped” with two hands for 30 seconds to prevent escape as described by Hurst and West [21]. This was followed by a massage involving 5–10 strokes with the second and third fingers of one hand starting at the level of the ears extending down the head/neck. If the mouse attempted to escape from the open hand of the handler, the cupping was briefly reapplied until there was no evidence of any attempt to escape. The massage was then repeated. This training was repeated at least daily for 5 days. For each session, the animal was not picked up by the tail but rather scooped up with the open palm as described by Hurst and West [21]. By the second or third day, most mice learned to interact voluntarily with the handler and did not require a second cupping. Following the 5 day of training period, the cup/massage sequence was repeated for approximately 2 sessions/week. Effective training was defined as (i) if the mouse “voluntarily” went from the cage into the open palm of the handler without need for chasing, and (ii) if the mouse displayed no effort to escape from the open palm of the handler. For this training, the mice were housed in individual cages. By the end of the two-week period, all mice were successfully trained. The control group for the cup/massage cohort were not handled except for being picked by the tail for cage changes occurring approximately once per week.

2.3. Experimental design and timeline

Experiment 1—A cohort of 20 mice (CD1.C57BL/6) was matched for body weight and divided into two groups: [tail-picked (n=10) or cup (n=10)]. The mice were trained for 10 sessions over a two-week period between 9:00 AM and 12:00 PM. During the course of handling and similar to Hurst and West [21], aggressive and stress-associated behaviors (including number of bites to the handlers, frequency of urination and defecation (Figure 2 A-C) were recorded. Following training, the mice were tested in the EPM for assessment of anxiety-like behaviors to confirm the observation of Hurst and West [21] but with the inclusion of the additional measures of blood glucose and plasma corticosterone. Figure 1 A shows the experimental time line.

Experiment 2—A cohort of 20 mice (CD1.C57BL/6) was matched for body weight and divided into two groups [tail-picked (n=10) or cup (n=10)]. These mice were maintained on a high fat diet (Research Diets, New Brunswick, NJ; 4.54 kcal/g; ~40% fat) for approximately 3 months prior to the handling to establish a state of obesity-induced mild glucose intolerance [15]. After training, the mice were subjected to an intraperitoneal (ip) glucose tolerance test (GTT) to assess the impact of cup handling on metabolic endpoints. Testing schedule is shown in Figure 1 B.

Experiment 3—A cohort of C57BL/6 mice was matched for body weight and divided into two groups handled either by the tail-picked (n=5) or cup/massage (n=10) methods. This cohort was trained using the cup/massage method as described above for two weeks. Following training, the mice were exposed to a 5-minute open-field test (OFT) at week 3 followed by exposure to a 5 minute elevated plus maze (EPM) with measures of blood glucose and plasma corticosterone at week 4. At week 5, the mice were fasted overnight for 16 hours followed by obtaining tail blood for glucose. The animals were allowed a week of recovery before each test. Testing schedule is shown in Figure 1 C.

2.4. Behavioral testing

2.4.1. Elevated plus maze—Mice were exposed to the EPM for 5 minutes to assess anxiety-related behaviors, as described previously [10]. The EPM experiment was carried out between 09:00 AM and 12:00 PM. In this test, mice were placed in the center of the plus maze facing an open arm and behavior was recorded for the entire time period using an overhead mounted camera. Recorded parameters included open- and closed- arm time, total distance traveled in the maze (locomotor activity), and number of entries into the open arm, and were scored using Topscan software (Clever System Inc.). Anxiety-related behavior is associated with less exploration of the open arm relative to overall exploration of all arms [42]. To investigate the effects of EPM exposure on blood glucose levels and HPA axis activation, blood samples were taken immediately after termination of the EPM. (See below for details regarding blood collection). Then the animals were returned to their home cages and a second blood sample was collected at 30 minutes after onset of the EPM.

2.4.2. Open field test (OFT)—To assess the handling effects on anxiety-like behaviors, mice were exposed to an open field apparatus for 5 minutes under red-light illumination. The OFT experiment was carried out between 09:00 AM and 12:00 PM. The open field

apparatus consisted of a white 50 x 50 x 22cm Plexiglas box as reported previously [10]. For testing, each mouse was placed in the border region of the open field apparatus and allowed to freely explore for 5 minutes, with behavior recorded from a camera mounted above the apparatus. Videos were scored for time spent in the border (82% of the total area) and center areas (18.34% of the inner area), latency to enter center regions, and total distance traveled.

2.5. GTT

The GTT was performed as previously described [36]. Briefly, mice were fasted for 5 hours in clean cages with water *ad libitum* after onset of the light phase. At $t = 0$ minutes, mice were given an ip injection of 1.5 g/kg, 25% dextrose. Tail blood glucose concentration from freely moving mice was measured at -30, 0, 30, 60, 90 and 120 minutes using a Roche Accu-Check glucometer.

2.6. Blood collection and plasma corticosterone analysis

Blood (25–30 μ l) samples were collected by the tail clip procedure as described previously [12] from freely moving mice. Briefly for tail clip, the distal millimeter of the tail is removed using a sterile scalpel blade. Each blood sample is collected into EDTA tubes within 2 minutes to minimize any increase in corticosterone concentration due to sampling, and was immediately placed on ice. Serial samples were obtained by gentle removal of the scab from the tip of the tail using sterile gauze. Blood samples were then centrifuged at $3,000 \times g$ for 15 minutes at 4°C and plasma was stored at -20°C for subsequent hormone analysis. Plasma corticosterone concentration was measured using a ^{125}I RIA kit (MP Biomedical, Solon, OH) as described previously [12]. All samples were run in duplicate in the same assay.

2.7. Statistical analysis

Data are shown as mean $\pm$ standard error of the mean (SEM). Values for integrated area under the curve (AUC) were calculated using the trapezoid rule. Two-way repeated measure analysis of variance (ANOVA) was used for comparisons of groups over time. Behavioral data from EPM and OFT were analyzed by *t*-test. All statistical analyses were performed using Sigma Stat (SYSTAT, San Jose, CA) software. Specific differences were determined by Bonferroni *post hoc* tests. When appropriate, outliers (determined using Grubb's test [7]) were removed. The homogeneity of variance was determined for each assay using Sigma Stat. The corticosterone assay required square root transformation to meet the criterion homogeneity. Effects were considered significant with a critical value α set at $p < 0.05$.

3. Results

3.1. Effects of cup handling on anxiety-like behavior

To confirm the impact of the cup method on measures of mouse anxiety-like behavior, animals were tested in the elevated plus maze (EPM). Cup mice spent significantly more time in the open arm ($t(18) = 3.93$; $p = 0.008$) (Figure 2 A) than standard tail-picked mice and had significantly higher total number of entries into the open arm ($t(18) = 2.47$; $p = 0.02$) (Figure 2 B). Total distance traveled in the maze revealed no difference between the two handling methods (Figure 2C).

3.2. Cup handling reduces stress-induced glucose levels, but does not affect plasma corticosterone levels in mice exposed to the EPM

Following EPM exposure (experiment 1), the time-course analysis of plasma glucose response to the EPM revealed a main effect of handling ($F_{1, 18} = 7.80$; $p < 0.01$) and time ($F_{1, 18} = 39.97$; $p < 0.001$). Although there was an increase in glucose levels in both cohorts at 30 minutes relative to 5 minutes following placement on the EPM, cup mice showed a significant reduction in the EPM-induced blood glucose response compared with the tail handled group (Figure 3 A).

The response of plasma corticosterone response to the EPM differed from that of blood glucose with the time course after EPM exposure showing only a main effect of time ($F_{1, 18} = 74.63$; $p < 0.001$) (Figure 3 B). Thus, there was no significant difference between the handling of the two groups on plasma HPA responses to EPM.

3.3. Impact of cup handling on IPGTT in high fat diet fed mice

The effect of handling was studied in diet-induced obese mice because the mild glucose intolerance [15] in these animals presents the advantage of greater glucose dynamics. Body weight was similar in both groups (tail-picked = 47.2 g, ± 1.18 ; cup = 45.1 g, ± 0.99 ; $p > 0.05$), as were baseline blood glucose levels (tail-picked = 195.2 gm/dl, ± 7.70 ; cup = 202 gm/dl, ± 10.3 0; $p > 0.05$). However, after the administration of ip glucose, glycemic response in the tail-held group was significantly elevated than the cup group (Figure 4 A), reflecting lower glucose tolerance ($t(18) = 1.76$; $p < 0.05$) (Figure 4 B).

3.4 Cup/massage handling reduces anxiety-like behavior

We next determined whether application of cupping in combination with stroking or massage (cup/massage) may influence anxiety-like behavior and improve metabolic outcome. A cohort of C57Bl/6 mice were either handled by the tail-picked method or a cup/massage approach as described in 2.2 in the Methods, (Experiment 3). The time spent in the center area of the open field apparatus was markedly increased in the cup/massage group relative to the tail-picked group ($t(13) = 3.38$; $p = 0.005$) (Figure 5 A). Moreover, the latency to enter the center zone from the peripheral region of the open field was increased in the cup/massage group ($t(13) = 1.93$; $p = 0.01$) (Figure 5 B). Notably, the total distance traveled, an index of locomotor activity was not affected by the handling (Figure 5 C). Importantly, cup/massage group also spent more time in open arm of the EPM ($t(13) = 3.72$; $p = 0.001$) (Figure 5D), and increased number of entries into the open arm ($t(13) = 2.31$; $p = 0.03$) (Figure 5E).

3.5 Cup/massage impact on glucose and corticosterone responses to EPM

Given the marked impact of cup/massage in decreasing the anxiety-like behaviors, we tested the effect of this handling intervention on endocrine parameters. The cup/massage method reduced fasted (16 hours of fasting) blood glucose levels ($t(13) = 7.72$; $p = 0.0001$) compared with tail-picked handling (Figure. 6 A). Moreover, the time-course analysis of plasma corticosterone following EPM exposure revealed a main effect of handling ($F_{1, 13} = 15.89$; $p < 0.002$), where by the cup/massage mice showed significantly reduced level of plasma corticosterone than respective tail-picked controls (Figure 6 B). Additionally,

animals in the cup/massage group did not show any glycemic response 30 minutes after an IP injection of saline (baseline glucose = 117 ± 8.0 ; at t (30) glucose = 115 ± 10.1).

4. Discussion

It is important to develop methods to minimize background stress in studies of metabolism [1–3, 5]. Prior studies note that stress stimulates glucose release [32] and thus can confound determination of metabolic phenotypes in animal models. The studies by Hurst and West suggested that a specific “cup” handling method that reduces anxiety-like behaviors in mice might serve to reduce background stress. The results of this study are in agreement with the previous study by Hurst and West [21] that cup handling decreases measures of anxiety-like behaviors. Further extending the behavioral findings, we show modest reduction in glucose following EPM exposure and improved glucose tolerance in diet-induced obese mice compared to tail-picked group. In a separate mouse cohort, we show that combining the cupping procedure with a massage of the animal’s dorsum also reduces basal fasting glucose and prevents glucose increase in response to a saline ip injection. Finally, we show that the cup/massage method reduces corticosterone responses to a novel EPM exposure, suggesting that this protocol is sufficient to reduce the HPA axis response to a stressor. The cup handling advocated by Hurst and West [21] and expanded on by Gouvias and West [16] demonstrated anxiety-reducing effects of the cup method. Our observations confirm the previous reports [21], and demonstrate that the cup handling method is generalizable among different laboratory settings. Further, the cup/massage method showed reduced corticosterone responses to elevated plus maze exposure, suggesting that this handling protocol was sufficient to limit both behavioral and neuroendocrine stress responses.

Reductions in EPM-induced corticosterone release were not observed in animals cupped without massage, whereas cup/massage method was effective in reducing the corticosterone response to a novel EPM. However, it is important to stress that our results do not directly address whether the cup/massage was a more effective handling strategy than cup alone for reducing the endocrine stress response. This would require direct side-by-side comparison in a same strain of mice [29].

Stress causes increases in circulating glucose. The influence of stress likely stems from stimulation of glucose release by stress-activated systems, in particular adrenal release epinephrine and glucocorticoids (reviewed in 26). Glucose and corticosterone concentrations are affected by even the time of fasting with respect to the day-night cycle [37]. Our results confirm that cup or cup/massage produce meaningful reductions in glucose responses in the context of both normal metabolic status and obesity. Indeed, in obese mice cupping improves glucose tolerance. Mice handled with cup/massage have reduced blood glucose after an overnight fast compared with tail-picked. Unlike humans, overnight fasting in mice leads to a more pronounced catabolic state with significant loss of total body, lean and fat mass and increased insulin sensitivity [1]. It is thought to be similar to starvation in humans [1] and is associated with higher corticosterone concentrations [29]. Handling interventions such as cup or cup/massage may mitigate endocrine stress and thereby improve glucose homeostasis. Altogether, our results support the general conclusion that if attention is not paid to limiting extraneous stress, data gathered on baseline glucose, glucose tolerance and

responses to metabolic challenge will include an influence of the glucose stress response [reviewed in 3, 26, 38]. The contribution of stress becomes particularly important if the mouse strain or line in question has a stress ‘phenotype’, wherein their responses to standard handling may be exaggerated or diminished relative to controls, resulting in illusory metabolic ‘phenotypes’.

Defining a regimen to minimize variable extraneous stress is not entirely straightforward (reviewed in 18). Seemingly mundane procedures, such as cage change, have been shown to increase serum corticosterone levels and anxiety behaviors [31]. Moreover, genetic background may also influence the extent to which a ‘handling’ procedure can enhance (or inhibit) stress responses [17, 20, 28, 34, 35]. These complex interactions between genetic background and environmental conditions that determine animal behavior [27] are difficult to differentiate. Regardless of these complexities, our studies highlight the need to systematically assess the impact of routine handling procedures in rodent studies, so as to insure the data collected to be free of ‘contamination’ by stress responses.

In summary, this work demonstrates the beneficial effects of mouse cup handling for metabolic studies and describes a combined cup/massage method that may be a further stabilizing maneuver for experimental protocols that would be enhanced by reducing background stress. We would now advocate the careful application of a very specific handling for all mouse cohorts to avoid the confounding effects of variable and unplanned handling on metabolic experimental outcomes.

Acknowledgments

We thank members of the D’Alessio and Herman laboratories for help and useful conversations. NIH R03 DK89090 (JT), NIH R01 DK057900 (DAD) and NIH R01 MH069860 (JPH) supported this work. SG is supported by an AHA fellowship and an Albert J Ryan Foundation award.

References

1. Andrikopoulos S, Blair AR, Deluca N, Fam BC, Proietto J. Evaluating the glucose tolerance test in mice. *Am J Physiol Endocrinol Metab.* 2008; 295:E1323–E1332. [PubMed: 18812462]
2. Ayala JE, Bracy DP, McGuinness OP, Wasserman DH. Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes.* 2006; 55:390–397. [PubMed: 16443772]
3. Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, Wasserman DH, McGuinness OP. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Dis Model Mech.* 2010; 3:525–534. [PubMed: 20713647]
4. Bailey CJ, Flatt PR. Insulin and glucagon during pentobarbitone anaesthesia. *Diabete Metab.* 1980; 6:91–95. [PubMed: 6997105]
5. Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. *Contemp Top Lab Anim Sci.* 2004; 43:42–51. [PubMed: 15669134]
6. Brown ET, Umino Y, Loi T, Solessio E, Barlow R. Anesthesia can cause sustained hyperglycemia in C57/BL6J mice. *Vis Neurosci.* 2005; 5:615–618. [PubMed: 16332272]
7. Barnett, V.; Lewis, T. *Outliers in statistical Data.* John Wiley & Sons; Newyork: 1994.
8. Cawley N. A critique of the methodology of research studies evaluating massage. *Eur J Cancer Care (Engl).* 1997; 6:23–31. [PubMed: 9238926]

9. Costa R, Tamascia ML, Nogueira MD, Casarini DE, Marcondes FK. Handling of adolescent rats improves learning and memory and decreases anxiety. *J Am Assoc Lab Anim Sci.* 2012; 51:548–553. [PubMed: 23312082]
10. Vollmer EL, Ghosal S, Rush AJ, Sallee RF, Herman PJ, Weinert M, Sah R. Attenuated stress-evoked anxiety, increased sucrose preference and delayed spatial learning in glucocorticoid-induced receptor-deficient mice. *Genes, Brain Behav.* 2013; 12:241–249. [PubMed: 23088626]
11. Field TM. Massage therapy effects. *Am Psychol.* 1998; 53:1270–1281. [PubMed: 9872050]
12. Furay AR, Bruestle AE, Herman JP. The role of the forebrain glucocorticoid receptor in acute and chronic stress. *Endocrinology.* 2008; 149:5482–5490. [PubMed: 18617609]
13. Genaro G, Schmidek WR. The influence of handling and isolation postweaning on open field, exploratory and maternal behavior of female rats. *Physiol Behav.* 2002; 5:681–688. [PubMed: 12020733]
14. Ghalami J, Zardooz H, Rostamkhani F, Farrokhi B, Hedayati M. Glucose-stimulated insulin secretion: Effects of high-fat diet and acute stress. *Endocrinol Invest.* 2013; 10:835–842.
15. Golson ML, Misfeldt AA, Kopsombut UC, Petersen CP, Gannon M. High fat diet regulation of β -cell proliferation and β -cell mass. *Open Endocrinol J.* 2010;4.
16. Gouveia K, Hurst JL. Reducing Mouse Anxiety during Handling: Effect of Experience with Handling Tunnels. *PLoS One.* 2013;8.
17. Fawcett A, Rose M. Guidelines for the housing of mice in scientific institutions. Animal Welfare Unit, NSW Department of Primary Industries, West Pennant Hills, Animal Research Review Panel 1, Guideline. 2012; 22:1–143.
18. Heidbreder CA, Weiss IC, Domeney AM, Pryce C, Homberg J, Hedou G, Feldon J, Moran MC, Nelson P. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. *Neuroscience.* 2000; 100:749–768. [PubMed: 11036209]
19. Heinrichs SC. Neurobehavioral consequences of stressor exposure in rodent models of epilepsy. *Prog Neuro-Psychopharmacology Biol Psychiatry.* 2010; 34:808–815.
20. Holson RR, Scallet AC, Ali SF, Turner BB. “Isolation stress” revisited: isolation-rearing effects depend on animal care methods. *Physiol Behav.* 1991; 49:1107–1118. [PubMed: 1896492]
21. Hurst JL, West RS. Taming anxiety in laboratory mice. *Nat Methods.* 2010; 7:825–826. [PubMed: 20835246]
22. Kohsaka A, Bass J. A sense of time: how molecular clocks organize metabolism. *Trends Endocrinol Metab.* 2007; 18:4–11. [PubMed: 17140805]
23. Longordo F, Fan J, Steimer T, Kopp C, Lüthi A. Do mice habituate to “gentle handling?” A comparison of resting behavior, corticosterone levels and synaptic function in handled and undisturbed C57BL/6J mice. *Sleep.* 2011; 34:679–681. [PubMed: 21532962]
24. Lund I, Yu LC, Uvnäs-Moberg K, Wang J, Yu C, Kurosawa M, Agren G, Rosén A, Lekman M, Lundeberg T. Repeated massage-like stimulation induces long-term effects on nociception: Contribution of oxytocinergic mechanisms. *Eur J Neurosci.* 2002; 16:330–338. [PubMed: 12169113]
25. Mantella RC, Vollmer RR, Amico JA. Corticosterone release is heightened in food or water deprived oxytocin deficient male mice. *Brain Res.* 2005; 1058:56–61. [PubMed: 16168966]
26. McGuinness OP, Ayala JE, Laughlin MR, Wasserman DH. NIH experiment in centralized mouse phenotyping: the Vanderbilt experience and recommendations for evaluating glucose homeostasis in the mouse. *Am J Physiol Endocrinol Metab.* 2009; 297:E 849–855.
27. Montgomery KC, Monkman JA. The relation between fear and exploratory behavior. *J Comp Physiol Psychol.* 1955; 48:132–136. [PubMed: 14367588]
28. Olfe J, Domanska G, Schuett C, Kiank C. Different stress-related phenotypes of BALB/c mice from in-house or vendor: alterations of the sympathetic and HPA axis responsiveness. *BMC Physiol.* 2010; 10:2. [PubMed: 20214799]
29. Parmigiani S, Palanza P, Rodgers J, Ferrari PF. Selection, evolution of behavior and animal models in behavioral neuroscience. *Neurosci Biobehav Rev.* 1999; 23:957–970. [PubMed: 10580310]
30. Pomplun D, Möhlig M, Spranger J, Pfeiffer AF, Ristow M. Elevation of blood glucose following anaesthetic treatment in C57BL/6 mice. *Horm Metab Res.* 2004; 36:67–69. [PubMed: 14983410]

31. Rasmussen S, Miller MM, Filipski SB, Tolwani RJ. Cage change influences serum corticosterone and anxiety-like behaviors in the mouse. *J Am Assoc Lab Anim Sci.* 2011; 50:479–483. [PubMed: 21838975]
32. Ravenelle R, Santolucito HB, Byrnes EM, Byrnes JJ, Donaldson ST. Housing environment modulates physiological and behavioral responses to anxiogenic stimuli in trait anxiety male rats. *Neuroscience.* 2014; 270:76–87. [PubMed: 24713371]
33. Rudic RD, McNamara P, Curtis AM, Boston RC, Panda S, Hogenesch JB, FitzGerald GA. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biol.* 2004;2.
34. Schmidt M, Oitzl MS, Levine S, De Kloet ER. The HPA system during the postnatal development of CD1 mice and the effects of maternal deprivation. *Dev Brain Res.* 2002; 139:39–49. [PubMed: 12414092]
35. Schmitt U, Hiemke C. Strain differences in open-field and elevated plus-maze behavior of rats without and with pretest handling. *Pharmacol Biochem Behav.* 1998; 59:807–811. [PubMed: 9586835]
36. Smith EP, An Z, Wagner C, Lewis AG, Cohen EB, Li B, Mahbod P, Sandoval D, Perez-Tilve D, Tamarina N, Philipson LH, Stoffers DA, Seeley RJ, D'Alessio DA. The role of β cell glucagon-like peptide-1 signaling in glucose regulation and response to diabetes drugs. *Cell Metab.* 2014; 19:1050–1057. [PubMed: 24836562]
37. Sommerville S, Perez VJ, Elias JW, Smith CJ. Weight, corticosterone and glucose: changes with time of day after food deprivation. *Physiol Behav.* 1988; 44:137–140. [PubMed: 3237808]
38. Tabata H, Kitamura T, Nagamatsu N. Comparison of effects of restraint, cage transportation, anaesthesia and repeated bleeding on plasma glucose levels between mice and rats. *Lab Anim.* 1998; 32:143–148. [PubMed: 9587896]
39. Tanaka K, Kawano T, Tomino T, Kawano H, Okada T, Oshita S, Takahashi A, Nakaya Y. Mechanisms of impaired glucose tolerance and insulin secretion during isoflurane anesthesia. *Anesthesiology.* 2009; 111:1044–1051. [PubMed: 19809283]
40. Thorens B. Brain glucose sensing and neural regulation of insulin and glucagon secretion. *Diabetes Obes Metab.* 2011; 13(Suppl 1):82–88. [PubMed: 21824260]
41. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science.* 2005; 308:1043–1045. [PubMed: 15845877]
42. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat Protoc.* 2007; 2:322–328. [PubMed: 17406592]

Highlights

- Cup handling reduces anxiety-like behaviors compared with tail-picked mice
- Diet-induced obese mice with prior cup handling showed improved glucose tolerance compared with those picked up by tail.
- Cup/massage handling in mice showed attenuated anxiety-like behaviors and less endocrine stress responses in comparison to tail-picked controls.

Figure 1. Time line of the experimental design

(A) For experiment 1, handling (tail-picked (n=10); cup (n=10)) began on week 1. Following two weeks, mice were tested for anxiety-like behavior in the elevated plus maze (EPM) with measures of blood glucose and later analysis of plasma corticosterone. (B) For experiment 2, handling (tail-picked (n=10); cup (n=10)) began on week 1. Mice were tested for ip glucose tolerance (ipGTT) on week 3. (DIO: diet induced obese) (C) For experiment 3, handling (tail-picked (n=5); cup/massage (n=10)) began on week 1. Following two weeks of training, mice were subjected to an open field test (OFT), followed by elevated plus maze on week 4 with collection of tail blood at 5 and 30 minutes for corticosterone. Fasted (16 hour fasting) blood glucose measurements were taken on week 5.

Figure 2. Cup handling increases open arm exploration in the elevated plus maze test
(A) Cup handling for two weeks increased time spent in the open arm of the EPM. **(B)** Cup handled mice exhibited higher number of entries into the open arm of the EPM. **(C)** Overall locomotor activity was not affected by any specific handling method. Data are presented as mean $\pm$ SEM. n= 10 (Tail-picked); n=10 (Cup). *P < 0.05 vs. tail-picked.

Figure 3. Cup handling attenuates blood glucose response to the elevated plus maze exposure (A) Cup handling for two weeks decreased glycemic response following exposure to the elevated plus maze. **(B)** Plasma corticosterone levels were elevated in both tail-picked and cupped groups following exposure to a 5 minute elevated plus maze test. Data are presented as mean $\pm$ SEM. $n=10$ (Tail), $n=10$ (Cup). * $P < 0.05$ vs. 5 minute time point, and # $P < 0.05$ vs. tail-picked in A.

Figure 4. Glucose tolerance to an ip injection of glucose (1.5g/kg) was significantly different in the tail-picked group vs. cup group

(A) Blood glucose levels were elevated in both tail-picked and cup groups following an ip injection of glucose. (B) The integrated glucose tolerance response to a glucose load was less in the cup group. Data are presented as mean $\pm$ SEM. $n=10$ (Tail), $n=10$ (Cup). * $P < 0.05$ vs. Tail-picked.

Figure 5. Effect of cup/massage on anxiety-like behavior

(A) Cup/Massage increased time spent in the center in an open-field test. (B) Latency to enter the center from the periphery was increased in the cup/massage group. (C) Total locomotion showed no significant differences between tail and cup/massage mice. (D) Cup/massage handling for two weeks increased time spent in the open arm of the EPM. (E) Cup/massage group had significantly higher entries into the open arm of the EPM. Data are mean $\pm$ SEM (n = 5–10/group). *P < 0.05 vs. tail-picked.

Figure 6. Impact of cup/massage on stress-induced glucose and corticosterone levels
(A) Cup/massage mice displayed reduced fasting (16 hours) blood glucose levels relative to tail-picked mice. **(B)** Plasma corticosterone response to a 5 and 30-minute exposure to a novel EPM was reduced in cup/massage. Data are mean $\pm$ SEM ($n = 5-10$ /group). * $P < 0.05$ vs. tail-picked.