Exposure to varied cage-size habitats alters pain sensitivity and inflammation-related biomarkers

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Keywords: Exposure, varied cage-size habitats, alters pain sensitivity, inflammation-related biomarkers

Posted Date: January 28th, 2020

DOI: https://doi.org/10.21203/rs.2.22089/v1

Abstract

Background Nature and size of rodent cages vary from one laboratory or country to another. Little is however known about the physiological implications of exposure to diverse cage sizes in animal-based experiment. Here, we exposed male Swiss mice to various cage sizes used across laboratories in Nigeria, top-rated paradigms were used to profile changes in physiological behaviours, and this was followed by evaluation of modified biochemical metrics.

Results The study showed a better systemic regulation of glucose metabolism in cage migrated mice compared to cage stationed. Strikingly, peripheral oxidative stress and pain sensitivity decreased significantly in cage-to-cage migrated mice despite increased pro-inflammation mediators (IL-6 and NF-κB) which contrast the norm reported in inflammatory conditions. Interestingly, emotion-linked behaviours, neurotransmitters (serotonin, noradrenaline and GABA) and body electrolytes were not altered by cage-to-cage migration.

Conclusion Taken together, these results suggest that varied size cage-to-cage migration of experimental mice could affect targeted behavioural and biomolecular parameters of pain and inflammation, thus diminishing research reproducibility, precipitating false negative/positive results and leading to poor translational outcomes.

Background

Animal experimentation remains an indispensable component in science-powered medicine despite ethical controversy and disputed public philosophy. Most recorded advances in medicine commenced as controlled translational scientific endeavors in animals. Even where *Replacement* (from “Three Rs”) principle is adopted, efficacy of new medical device or novel therapeutic is assessed in animals prior to clinical use in humans [1]. However, success rate of most therapeutics during clinical trial in human remain low [2]. Persuasive claims for these failure-littered trials bordered on inadequate study design, improper dose selection, inappropriate efficacy markers, wrong approach to data analysis and erroneous interpretation. The heterogeneity in habitat during laboratory study is mostly ignored when considering factors that could elicit flawed extrapolation or translational success in human.

Diverse habitats exposure during preclinical/laboratory studies is usually in bid to fulfill ethical guidelines on provision of adequately cleaned and conducive animal housing [3], as well as accomplishing other laboratory procedures. Procedures such as administration of substances, change of bedding, animal training and so on, require temporary or permanent transfer of research animals to new cages [4] or environment. While acute migration exposes the animals to various physiological stressors [5], effects of consistent long term cage-migration receives little or no attention from scientists. The possibility of frequent long-term cage-to-cage migration of experimental animals initiating physiological adaptation that could compromise targeted biological variables in a study has paucity of data.

In human, migration is a social phenomenon involving shifts of residence from one administrative boundary or geopolitical location to another [6]. Diverse array of studies implicated migration in human as a precipitating factor for mental illnesses [7], metabolic related disorders in children [8] and other forms of health deteriorations depending on the stressors experienced before, during and after changing the environment [9]. Because human and animals share similar organ systems, and many therapeutics or diseases showed similar expressions in them [1], there is a possibility that long-term cage-to-cage migration of experimental animals could trigger similar stressors-induced changes for certain health indices. This likelihood is further fortified by the fact that environment is a critical stressor for all species and any form of environmental change may have a consequential effect on health [9].

In this study, we examined how exposure to diverse cage sizes hereafter called cage-to-cage migration could influence certain metric in emotion- and pain-related behavioural studies. Using Swiss mice, behavioural paradigms and relevant biochemical parameters, we tested our hypothesis that cage-to-cage migration modifies certain outcomes in animal based experiments. Our findings revealed that this least considered procedural act could mar certain animal experimentations, diminish reproducibility and precipitate poor translational outcome in animal-based research.

Results

Movement of animals from one environment to another is arguably unavoidable practice in animal-based experiments. Insight into procedural act of consistent cage-to-cage migration of Swiss mice during experiment showed consequential change in functional and biochemical annotations. First, varied cage size-migrated animals presented with elevated fasting blood glucose and closely-reversed oral glucose load (in tolerance test). Second, consistent exposure to different cage size in experimental animals precipitated decrease in peripheral oxidative stress but increase in specific central pro-inflammatory mediators. Third, cage-to-cage migration of Swiss mice resulted in a remarkable decrease in pain sensitivity to noxious chemical, thermal and mechanical stimuli. Fourth, neural cells distribution and morphology, mood related behaviours, central and peripheral nonspecific protein or electrolytes level, and certain transmitters (serotonin, noradrenaline and GABA) were not perturbed by diverse cage size exposure in male Swiss mice.

The ideal cage size and density for experimental animals has always been a welfare concern to scientists, though consensus seems to have been reached in this schema [16]. On the other hand, exposing experimental animals to different cage sizes has paucity of data, thus profiling the physiology of such exposed animals will benefit biological research. The present experiment showed no mortality among the mice exposed to diverse cage size, body weight showed improving trend and body/organs weights did not differ significantly. Claims of lower body and organ weights upon exposure to increasing cage size [17] was not observed, though this was reported in rats where targeted interest was the links between cage size and density of experimental animals. The relatively constant weight recorded might be

pertinent to appreciable maintenance of good ventilation and internal cage environment. Poor housing conditions were documented to depress growth and development [18].

Examining the metabolic profile of mice exposed to various cage sizes showed elevated fasting blood glucose compared to its stationed counterpart, despite this, migrated mice responded appropriately by reversing oral glucose load at the end of 120 minutes showing intact gut hormone releasing cells and beta cell. Supporting evidences in the literature revealed that fasted mice usually presented with significant lower blood glucose compared to least fasted or unfasted mice [19, 20]. Thus, the striking increase in the recorded blood glucose level in spite of equal period of fasting for both groups was assumed to be cage migration-induced or a response to stress. Since varying degrees of stress is known to increase fasting blood glucose [21], experimental animals were screened for one of the cellular responses to stress, oxidative stress. In evaluating stress, we reckoned that since mice have 18- to 22-day gestation period [22], then if the 30 days exposure to varied cage sizes could induce stress, such stress should have exceeded from the first two stages of responses to stress [that is, the alarm reaction and the resistance stage] to the exhaustion stage which is associated with oxidative stress [23]. Quantifiable marker of oxidative stress, malondialdehyde was evaluated and data showed noticeable reduction in peripheral oxidation with no clear difference centrally. Also, exposure to stress conditioning was shown to modify neuronal changes in anterior cingulate cortex, induced anxiety or depression and enhance anterior cingulate cortex excitatory nociceptive responses [24, 25]. Our histological studies showed no striking neuronal modification, behavioural results did not support presence of anxiety or depression condition and pain perception was decreased in the cage migrated mice. Together, these suggested that elevated blood glucose level was not in response to stress. Then, protein content of the plasma and other selected organs was determined and results did not appreciably differ compared to cage stationed mice. Measured brain and plasma electrolytes in cage-to-cage migrated mice were also not different compared to stationed animals. Summarily, protein and electrolytes profiles were not susceptible to diverse cage size exposure in Swiss mice.

Results from past studies showed cage crowding could result in high plasma IgG [26] and brain dopamine level [27]. The possibility of cage-to-cage migration causing alteration in concentrations of neurotransmitters and inflammatory mediators was then investigated. Concentrations of noradrenaline, serotonin and GABA were not significantly different in migrated mice compared to its stationed counterpart. Also, with the exception of IL-6 and NF-κB, all other quantitative data on anti-and pro-inflammatory mediators (NGF, IL-10, IL-1β and TNF-α) were not significantly different in migrated mice compared to cage stationed mice. High IL-6 concentration showed that neural defense was not suppressed, though the reason for preferential increase of IL-6 was not known. This is more surprising as measured TNF- α was not significantly different. TNF- α prompts production of proinflammatory mediators like 1L-6 and IL-1 [28, 29] during inflammation. IL-6 with other activators in turn induce NF-kB, a protein that plays vital roles in inflammatory responses [30]. However, there are compelling evidences of exercise-induced increase in IL-6 concentration [31]. Thus, there is a possibility that daily exposure of cage-migrated mice to new cage sizes encouraged increase muscular exertion in an attempt to explore the new environments.

Given the strong link between biochemical and functional metrics, phenotypic behaviours relating to mood and pain were profiled. Despite decreased ambulation and increased grooming on OFM, there was no enough data to support claim for mood (anxiety- or depression-related) change in migrated mice compared to its stationed counterpart. Data from other tests (light and dark test, and tail suspension test) also reinforced this assertion. Emotional deterioration had been convincingly linked with adverse pain sensitivity in the literature [32, 33]. In view of this, pain sensitivity in migrated mice was postulated to remain unaltered since evaluated emotional behaviours did not vary in cage-to-cage migrated mice. Contrast to the assumption, there was a remarkable decrease in pain sensitivity of cage migrated mice compared to cage stationed animals. The increased pain threshold was similar when exposed to either noxious chemical, thermal or mechanical stimuli. Structural evidences could not explain why pain sensitivity decreased. All routine-, special stain- and immuno-histological studies showed no prominent morphological, cytoarchitectural or expressional difference in the cells at the anterior cingulate cortex of the cage migrated mice compared to cage stationed animals. We then investigated the motor area, as this is where motor commands needed for coordinated movements are being generated. Some of these movements are main activities of evaluation in most behavioural studies. Using routine H&E stain, CFV stain, Gfap- and Ki-67-processed immuno-histochemistry on sections of primary motor cortices, we could not establish noticeable difference in morphology, cytoarchitecture or protein (Gfap and Ki-67 antigens) expression in the motor neurons of cage migrated mice compared to cage station animals. Summarily, there was no structural modification that could be associated with the recorded decreased pain sensitivity. This observation is contrary to the obtainable in the literature where animals with elevated pro-inflammatory mediators were prone to increase pain sensitivity [34, 35]. With elevated IL-6 and NF-κB recorded in this group, increase pain sensitivity is theoretically favoured.

In conclusion, emphasis on translational results, reproducibility of research and robustness of data makes findings from this study of utmost importance for animal-based experiments. Consistent cage-to-cage migration of mice into varied cage sizes alters metrics associated with blood glucose, pain, inflammation and oxidative capacity. Owing to the observed closely regulated oral glucose load, decreased marker for oxidative stress, increased mediators of innate immunity and decrease pain sensitivity, exposure to various cage size habitats had beneficial effects on experimental animals but could precipitate false positive in studies where these parameters are targeted.

Methods

Mice. Male Swiss mice (11-week old, 29-34 g and at least five times backcrossed) obtained from Central Animal Facility, University of Ilorin, Nigeria were used for this study. The mice were housed in the animals' facility of Bioresearch Hub Laboratory where the experiment was carried out. Prior to any procedure, the mice were allowed to acclimatise for 1 week in a standard housing condition at a relatively constant temperature with regular light-dark cycle. Mouse pellet and water were accessible *ad libitum* before and during the experiment. All procedures here stated were as approved by the University Ethical Review Committee, University of Ilorin, Nigeria; and the proposal was issued with approval number – UERC/ASN/2019/1923.

Exposure to varied home-cage sizes. Mice were randomly divided into two, cage stationed (Control Group) and cage migrated (Test Group). For all the experiments performed, animals were housed in six (n=6) per group. Mice assigned to cage stationed group were kept in the same size cage of 45 x 25 x 15 cm (length x width x height) for 30-day duration of the experiment. These mice were transferred to clean same size cage within a period of 60-second at 8 am daily. The same toys and water bottle were returned into the cage after each transfer. Mice allotted to cage migrated were exposed to cage of diverse sizes. Exposure to new cage size was done daily (every 24 hours) at 8 am and all mice were moved into the new size cage within an average time of 60 seconds. The same toys and water bottle were returned to the new cage. From Days 1 to 15, mice were exposed to the following cage respectively - 45 x 25 x 15 cm, 35 x 20 x 15 cm, 60 x 30 x 20 cm, 45 x 15 x 15 cm, 50 x 25 x 20 cm, 30 x 20 x 14 cm, 45 x 20 x 20 cm, 40 x 25 x 14 cm, 60 x 15 x 30 cm, 35 x 30 x 15 cm, 30 x 30 x 14 cm, 40 x 24 x 20 cm, 50 x 30 x 25 cm, 35 x 15 x 8 cm, and 30 x 15 x 15 cm. On Days 16 to 30, mice were re-exposed to the above cages respectively starting from the earliest. All cages were transparent plastics with thick gauze-like galvanized metal lids.

Weight. The body weights were measured weekly with Kerro weighing scale (Kerro Scale BL3002, Taiwan) starting from the first day of the experiment. Body weights were monitored for four (4) weeks. At expiration of different cage size exposure (Day 30), mice were sacrificed under anesthesia and weights of vital organs were taken in relative to the body weight of each mouse (organosomatic ratio).

Fasting and Oral glucose tolerance test. *Fasting blood glucose* levels were determined in 12-hour fasted mice using blood from their tail veins. The glucose levels were quantified using ACCU-CHEK glucometer. *Oral glucose tolerance test* was carried out following a preceding oral glucose load of 50mg in 12-hour fasted mice and subsequence measurement of blood glucose levels at 0, 60 and 120 min using ACCU-CHEK glucometer machine. Values were presented in mg/dl.

Emotion-associated Behavioural Studies. Emotion-related behaviours were evaluated using paradigms such as open field maze, light and dark box, and tail suspension.

Open field maze was first described by Hall in 1934 and it provides means for simple assessment of activities, and general behaviours in rodents [10]. The open field maze used was 40 x 40 x 30 cm in dimensions with a dirty white tile floor. Using black paint, the floor was divided into a 16-cell of 10 cm^2 each. Each mouse was placed at the centre of the maze with the animal facing forward away from its handler. Five (5) minutes of exploration was allowed and the entire maze was cleaned with 70% ethanol before another mouse was introduced. Total line crossing [ambulation], frequency of rearing, grooming time and time spent probing the central part of the maze were used in assessing emotional state of the mice.

Light/dark box test explores the inherent aversion to illuminated arena in rodents. The paradigm used was a two-compartment (a dark and a light) box with a total size of 83 x 42 x 30 cm. Each compartment was 40 x 40 x 30 cm in size and the two compartments were linked with a tunnel of 2 x 2 cm. Mice were individually placed in the brightly illuminated light compartment and allowed to explore the maze for 5

minutes. The total number of visits and time expended in the light or dark compartment were used to determine the emotional state of the animals as earlier described in the literature [11,12].

Tail suspension test was used to evaluate the behavioural and physiological aspect of emotional state in experimental mice [13]. Briefly, each mouse was suspended by its tail with paper tape on a fixed retort stand. Every mouse spent 5 minutes on tail suspension, total number of attempt to turn against the gravity (tail climbing) and time spent inactive were used to quantify mood of the animals.

Pain-related Behavioural Studies. Pain-linked behavioural responses were used to assess pain sensitivity in experimental mice subjected to noxious chemical, thermal and mechanical stimuli.

Formalin pain test was used to assess animals' responses to noxious chemical. After 15 minutes habituation in a transparent glass arena, 20µl of 2.5% PBS-formalin solution was injected subcutaneously in the plantar surface of right hindpaw of each mouse. The total time spent on paw licking was monitored for 50 minutes and was used to evaluate pain perception as described in the literature [14]. The time spent were eventually grouped into 0-5 minutes for nociceptive [early phase] pain and 20-30 for inflammatory (late phase) pain.

Hot- and cold- plate test were used to quantify pain sensitivity of mice to noxious thermal stimuli of 55 ^{o}C and 0 ^{o}C respectively. Each mouse was placed within the plexiglass walls positioned on a preheated iron plate surface maintained at 55 ^{o}C and the time taken for the mouse to jump was detailed as nociception threshold point. The cold plate used was a thin glass surface stand with attached perforated restriction glass chambers on it. The restriction boxes were blinded to prevent mice from seeing one another. Following 15 minutes acclimatisation, ice was applied to the plantar surface of the right hindpaw till animal demonstrated nocifensive behaviour such as lifting the affected limb or moving away from the stimulus. Time taken to exhibit these behaviours was accepted as nociceptive end-point and the average of three trials within 3 minutes apart was recorded for each mouse.

Von Frey Test. Pain sensitivity to mechanical noxious stimulus was assessed via quantifying withdrawal thresholds of the right hind paw to calibrated filament [15]. The filament was applied with enough pressure to cause bucking against the ventral surface of hind paw and was allowed to remain for 5 seconds before its removal for an unresponsive mouse. From the least filaments of 0.008 g to 6 g, the twelve von Frey filaments were used to probe for pain sensitivity in a continuous ascending order starting from the smallest calibration. The withdrawal threshold was accepted for a calibrated von Frey filaments if animal withdrew its hind paw at least five times from seven applied stimuli.

Note – Every maze was cleaned with 70% ethanol solution before introducing another mouse. All behavioural studies were video-recorded and extraction of data was done by three trained persons blinded to the experiment.

Biochemical assays. Mice were sacrificed at experimental endpoint (Day 30) under continuous inhalation of anesthetic drug, isoflurane. Blood was collected with 1 ml syringe via cardiac puncture and was

emptied into lithium-heparinized Eppendorf bottle. This was then centrifuged at 3000 rpm for 10 minutes under 4 ^{o}C. The plasma extracted from all samples were used for chemical analysis. Immediately after blood collection, mouse was transcardially perfused with 20 ml of cold PBS, the brain was carefully removed, homogenised, centrifuged (3000 rpm, 10 minutes and at 4 ^{o}C) and the supernatant was collected into plane bottle.

Protein assay. Bradford protein assay was used to evaluate the total protein content of the plasma, harvested organs and brain homogenates. The principle of this assay borders on capacity of protein molecule to bind on Coomassie dye. The reaction gives best result under acidic conditions and it is characterised with a colour change from brown to blue. In this study, the homogenate was diluted to obtain 50µg protein/30µl. 30µl of the sample or 30µl of the standard solution was added to separate test tubes. 30µl of water was added to two other test tubes for the standard curve. 30µl of buffer was added to the test tube containing the sample, followed by the addition 1.5ml of Bradford reagent to each test tube. The test tubes were incubated at room temperature for 5minutes after which the absorbance was read at 595nm.

Inflammatory mediators and neurotransmitters assays. NGF, 1L-10, 1L-6, 1L-1β, TNF-α, NF-Kb, serotonin, noradrenaline and GABA were assessed in the brain in duplicate using ELISA kits manufactured by Elabscience, China. The samples were appropriately added to the varied kit plates and the assays were performed in accordance to the manufacturer instructions.

Malondialdehye. This assay is based on the reaction of a chromogenic reagent, 2-thiobarbituric acid with MDA at 25^{o} C. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid via knoevenagel-type condensation to yield a chromophore with absorbance maximum at 532 nm. Standard and samples solutions were prepared and 300µl of each was added into microwell, followed by the addition of 300µl of indicator to the sample and standard wells which were mixed. The mixtures were incubated for 45 minutes at room temperature. The absorbance of the resulting solution was taken at 532 nm using microplate reader.

Histological Studies. On the last day of the experiment, blood was collected from anaesthetised mice, perfused with cold phosphate-buffered saline and then followed by perfusion with 4% phosphate-buffered paraformaldehyde via cardiac puncture. Sections of the brain showing motor and anterior cingulate cortices were exercised, fixed in 4% phosphate-buffered paraformaldehyde, stored under 4^{o}C for 24 hours, processed in ascending grades of ethanol, cleared in xylene and embedded in paraffin. Sections (5µm; MK 1110 rotary microtome) of the cortices were stained with hematoxylin and eosin (H&E), and Cresyl fast violet (CFV). Immunohistochemistry was also performed on similar coronal sections of paraffin-embedded cortices tissues using anti-mouse-Ki-67 (1:100, BIO-RAD, UK) and GfaP (1:100, BIO-RAD, UK). Sections were deparaffinised, subjected to antigen retrieval and immunostained with the Ki-67 and Gfab antibodies. Photomicrographs of the processed tissues were captured under 100X objective len using the Zeiss Axiostar plus light microscope.

Statistical Analysis. The present data were analyzed with 7.0 version of Graph Pad Prism. The specific tool used include unpaired Student's t-test and EC50 shift. Data were reported in mean ± SEM and statistical significance was accepted at $p < 0.05$.

Discussion

Movement of animals from one environment to another is arguably unavoidable practice in animal-based experiments. Insight into procedural act of consistent cage-to-cage migration of Swiss mice during experiment showed consequential change in functional and biochemical annotations. First, varied cage size-migrated animals presented with elevated fasting blood glucose and closely-reversed oral glucose load (in tolerance test). Second, consistent exposure to different cage size in experimental animals precipitated decrease in peripheral oxidative stress but increase in specific central pro-inflammatory mediators. Third, cage-to-cage migration of Swiss mice resulted in a remarkable decrease in pain sensitivity to noxious chemical, thermal and mechanical stimuli. Fourth, neural cells distribution and morphology, mood related behaviours, central and peripheral nonspecific protein or electrolytes level, and certain transmitters (serotonin, noradrenaline and GABA) were not perturbed by diverse cage size exposure in male Swiss mice.

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Declarations

Ethical Approval

The protocol for the present experiment was approved and issued with approval number – UERC/ASN/2019/1923 by University Ethical Review Committee, University of Ilorin, Nigeria.

Consent for Publication

Not Applicable

Availability of data and materials

The datasets used in the present study are available from the corresponding author on request.

Competing interests

All the authors declare that they have no competing interests.

Authors' contribution

ALO designed the study, performed and supervised the experiments, and wrote the manuscript. KOO, KSB, JOO, MFA, OA-R, AA, DLL, AOI, AOY and SSI carried out the experiments. GLO, FAS and OJO supervised the

histological studies, and reviewed the manuscript. AOA, ABN, OA, AA, WIA, OSM and OAA acquired and analyzed data, and reviewed the manuscript.

Acknowledgements

The authors wish to thank Precious O. Thomas, Oluwafeyisayo O. Ayoola, Abimbola A. Olabisi, Moruf J. Owoeye, Adebola D. Koya and Oluwatosin D. Ogunleye for accepted to be the double blind individuals during the extraction of data from the videos.

References

1. Reynolds RP, Li Y, Garner A, Norton JN. Vibration in mice: A review of comparative effects and use in translational research. Anim Model Exp Med. 2018;1(2):116–24.
2. Arrowsmith J. Trial watch: Phase II failures: 2008-2010. Nat Rev Drug Discov. 2011;10(5):328–9.
3. Russell WMS., Burch RL. The principles of humane experimental technique. [Internet]. 18th ed. Methuen, editor. London: http://books.google.com/books?id=j75qAAAAMAAJ.; 1956. Available from: http://books.google.com/books?id=j75qAAAAMAAJ.
4. Reeb-Whitaker CK, Paigen B, Beamer WG, Bronson RT, Churchill GA, Schweitzer IB, et al. The impact of reduced frequency of cage changes on the health of mice housed in ventilated cages. Lab Anim. 2001;35(1):58–73.
5. Balcombe JP., Barnard ND., Sandusky C. Laboratory routines cause animal stress. Vol. 43, Contemporary Topics in Laboratory Animal Science. 2004. p. 42–51.
6. Virupaksha HG, Kumar A, Nirmala BP. Migration and mental health: An interface. J Nat Sci Biol Med. 2014;5(2):233–9.
7. Bhugra D, Gupta S, Bhui K, Craig T, Dogra N, Ingleby JD, et al. WPA guidance on mental health and mental health care in migrants. World Psychiatry. 2011;10(1):2–10.
8. Smith PK, Bogin B, Varela-Silva MI, Loucky J. Economic and anthropological assessments of the health of children in Maya immigrant families in the US. Econ Hum Biol. 2003;1(2):145–60.
9. Tuggle AC, Cohen JH, Crews DE. Stress, migration, and allostatic load: A model based on Mexican migrants in Columbus, Ohio. J Physiol Anthropol. 2018;37(1):1–11.
10. Hall CS. Emotional Behavior in the Rat. J Comp Psychol. 1941;18(5):385–403.
11. Crawley J, Goodwin FK. Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. Pharmacol Biochem Behav. 1980;13(2):167–70.
12. Bourin M, Hascoët M. The mouse light/dark box test. Eur J Pharmacol. 2003;463(1–3):55–65.

13. Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: Review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev. 2005;29(4–5):571–625.
14. Hunskaar S, Fasmer OB, Hole K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci Methods. 1985;14(1):69–76.
15. Lambert GA, Mallos G, Zagami AS. Von Frey's hairs - a review of their technology and use - a novel automated von Frey device for improved testing for hyperalgesia. J Neurosci Methods. 2009;177(2):420–6.
16. Care Canadian Council on Animal C. Guide To the Care and Use of Experimental Animals, Volume 2. Vol. 49, Canadian Journal of Comparative Medicine. 1985. 49 p.
17. Yildiz A, Hayirli A, Okumus Z, Kaynar Ö, Kisa F. Physiological profile of juvenile rats: Effects of cage size and cage density. Lab Anim (NY). 2007;36(2):28–38.
18. Dawkins MS, Donnelly CA, Jones TA. Chicken welfare is influenced more by housing conditions than by stocking density. Nature. 2004;427(6972):342–4.
19. Heijboer AC, Donga E, Voshol PJ, Dang ZC, Havekes LM, Romijn JA, et al. Sixteen hours of fasting differentially affects hepatic and muscle insulin sensitivity in mice. J Lipid Res. 2005;46(3):582–8.
20. Oosterveer MH, Van Dijk TH, Grefhorst A, Bloks VW, Havinga R, Kuipers F, et al. Lxrα deficiency hampers the hepatic adaptive response to fasting in mice. J Biol Chem. 2008;283(37):25437–45.
21. Ghosal S, Nunley A, Mahbod P, Lewis AG, Smith EP, Tong J, et al. Mouse Handling Limits the Impact of Stress on Metabolic Endpoints HHS Public Access Author manuscript. Physiol Behav [Internet]. 2015;150:31–7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4546855/pdf/nihms700386.pdf
22. Murray SA, Morgan JL, Kane C, Sharma Y, Heffner CS, Lake J, et al. Mouse gestation length is genetically determined. PLoS One. 2010;5(8).
23. Schiavone S, Jaquet V, Trabace L, Krause KH. Severe life stress and oxidative stress in the brain: From animal models to human pathology. Antioxidants Redox Signal. 2013;18(12):1475–90.
24. Yamashita H, Zeredo JL, Kaida K, Kimoto M, Asahina I, Toda K. Stress-induced modulation of nociceptive responses in the rat anterior cingulate cortex. J Integr Neurosci. 2013;12(2):235–46.
25. Davidson RJ, Lewis DA, Alloy LB, Amaral DG, Bush G, Cohen JD, et al. Neural and behavioral substrates of mood and mood regulation. Biol Psychiatry. 2002;52(6):478–502.
26. Tsukamoto K, Machida K. Effects of life events and stress on neutrophil functions in elderly men. Immun Ageing. 2012; 9:1–5.
27. Holladay SD, Edens FW. Effect of cage density and rank in peck order on brain regional monoamines in adult male Coturnix coturnix japonica. Comp Biochem Physiol – Part A Physiol. 1987;87(2):261–5.
28. Le Bitoux MA, Stamenkovic I. Tumor-host interactions: The role of inflammation. Histochem Cell Biol. 2008;130(6):1079–90.

29. Soria G, Ofri-Shahak M, Haas I, Yaal-Hahoshen N, Leider-Trejo L, Leibovich-Rivkin T, et al. Inflammatory mediators in breast cancer: Coordinated expression of TNFα & IL-1β with CCL2 & CCL5 and effects on epithelial-to-mesenchymal transition. BMC Cancer. 2011;11:1–20.
30. Karin M. Nuclear factor-κB in cancer development and progression. Nature. 2006;441(7092):431–6.
31. Ellingsgaard H, Hojman P, Pedersen BK. Exercise and health — emerging roles of IL-6. Curr Opin Physiol [Internet]. 2019;10:49–54. Available from: https://doi.org/10.1016/j.cophys.2019.03.009
32. Lumley MA., Cohen JL., Borszcz GS., Cano A, Radcliffe AM., Porter LS., et al. Pain and Emotion: A Biopsychosocial Review of Recent Research. J Clin Psychol [Internet]. 2011;67(9):942–68. Available from: http://www.accessmedicine.com.proxy.kumc.edu:2048/content.aspx?aID=576916
33. Cai YQ, Wang W, Paulucci-Holthauzen A, Pan ZZ. Brain circuits mediating opposing effects on emotion and pain. J Neurosci. 2018;38(28):6340–9.
34. Zhang J-M, An J. NOT RIGHT REFERENCECytokines, Inflammation and Pain. Int Anesth Clin. 2009;69(2):482–9.
35. Ronchetti S, Migliorati G, Delfino D V. Association of inflammatory mediators with pain perception. Biomed Pharmacother. 2017;96(August):1445–52.

Figures

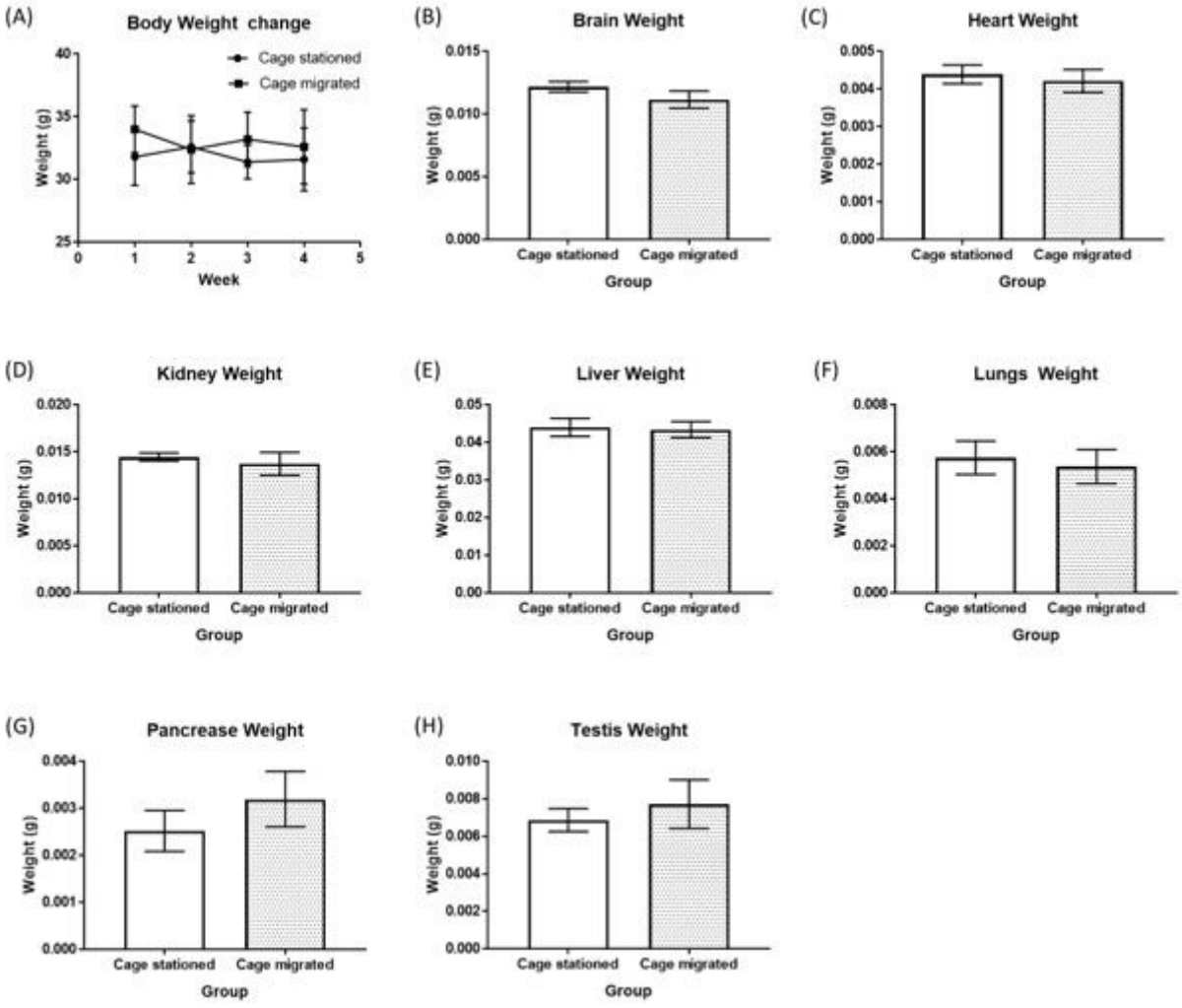

Figure 1

Overview of changes in body weights and organ/body weight ratios. (A) Changes in Body weight over four weeks. (B) to (H) Changes in organosomatic weight ratio in the brain, heart, kidney, liver, lungs, pancreas and testis respectively; (n = 6).

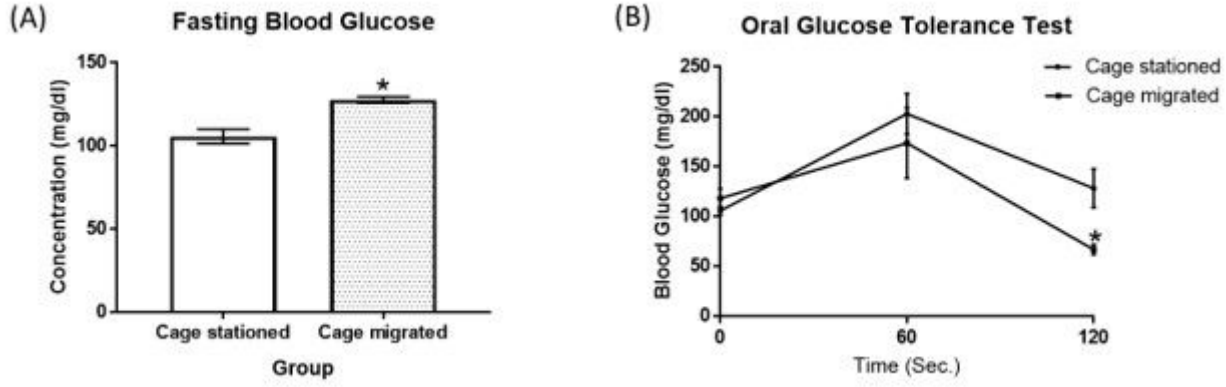

Figure 2

Blood glucose homeostasis. (A) Fasting blood glucose (B) Glucose regulation in oral glucose tolerance test. *Significant at p<0.05 compared to Cage stationed group, (n = 6).

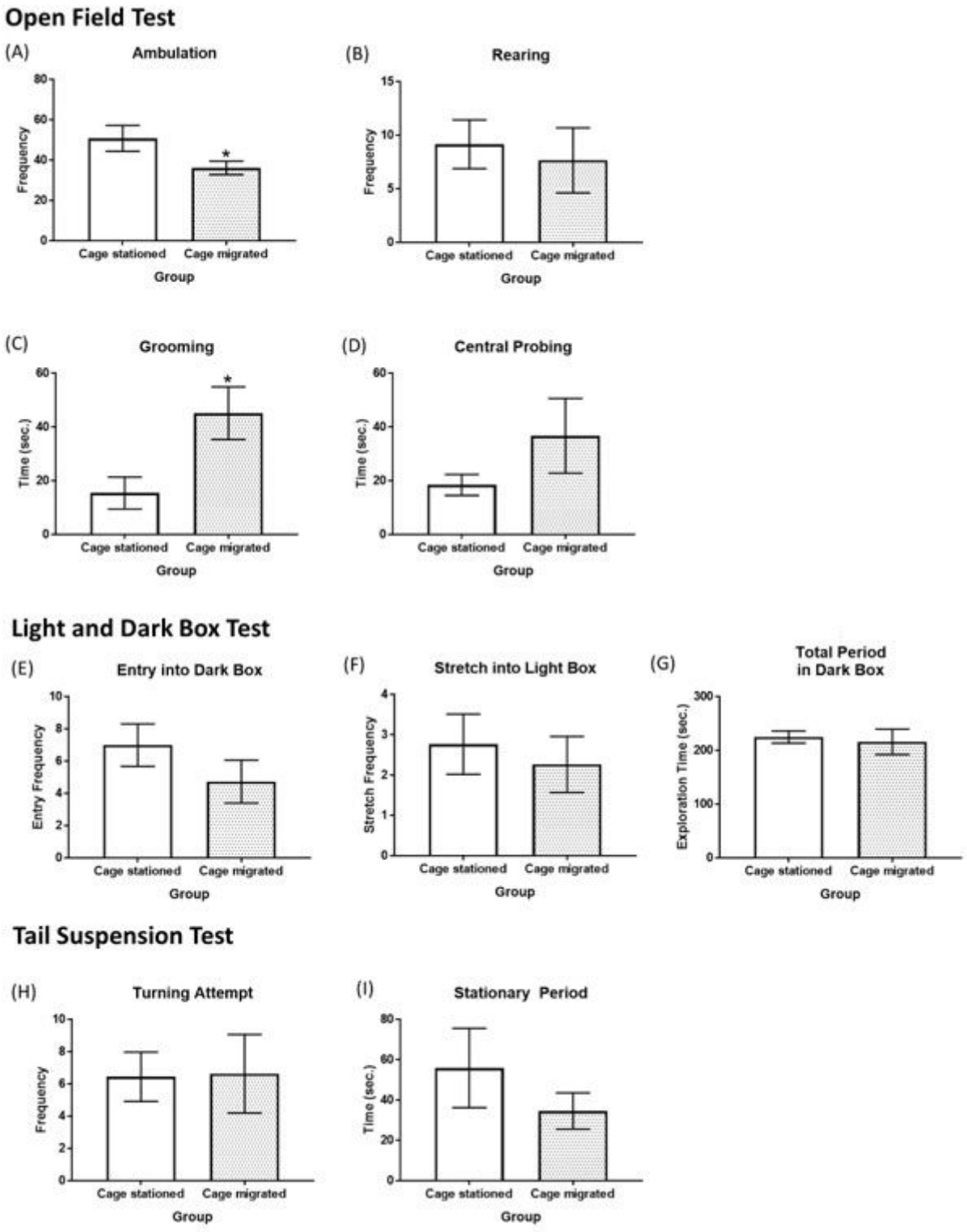

Figure 3

Evaluation of mood-related behaviours. (A) Line crossing (horizontal locomotion) on OFM, (B) Vertical locomotion (Rearing) on OFM rearing frequency, (C) and (D) Grooming and time spent probing the central area of OFM respectively, (E) Total number of entry into dark compartment of light/dark box, (F) Number

of neck stretch into the light box compartment in attempt of assessing risk, (G) Total time spent seeking safety in dark compartment of light/dark box, (H) Attempt of tail climbing on tail suspension, (I) Time spent inactive during tail suspension. *Significant at p<0.05 compared to Cage stationed group, (n = 6).

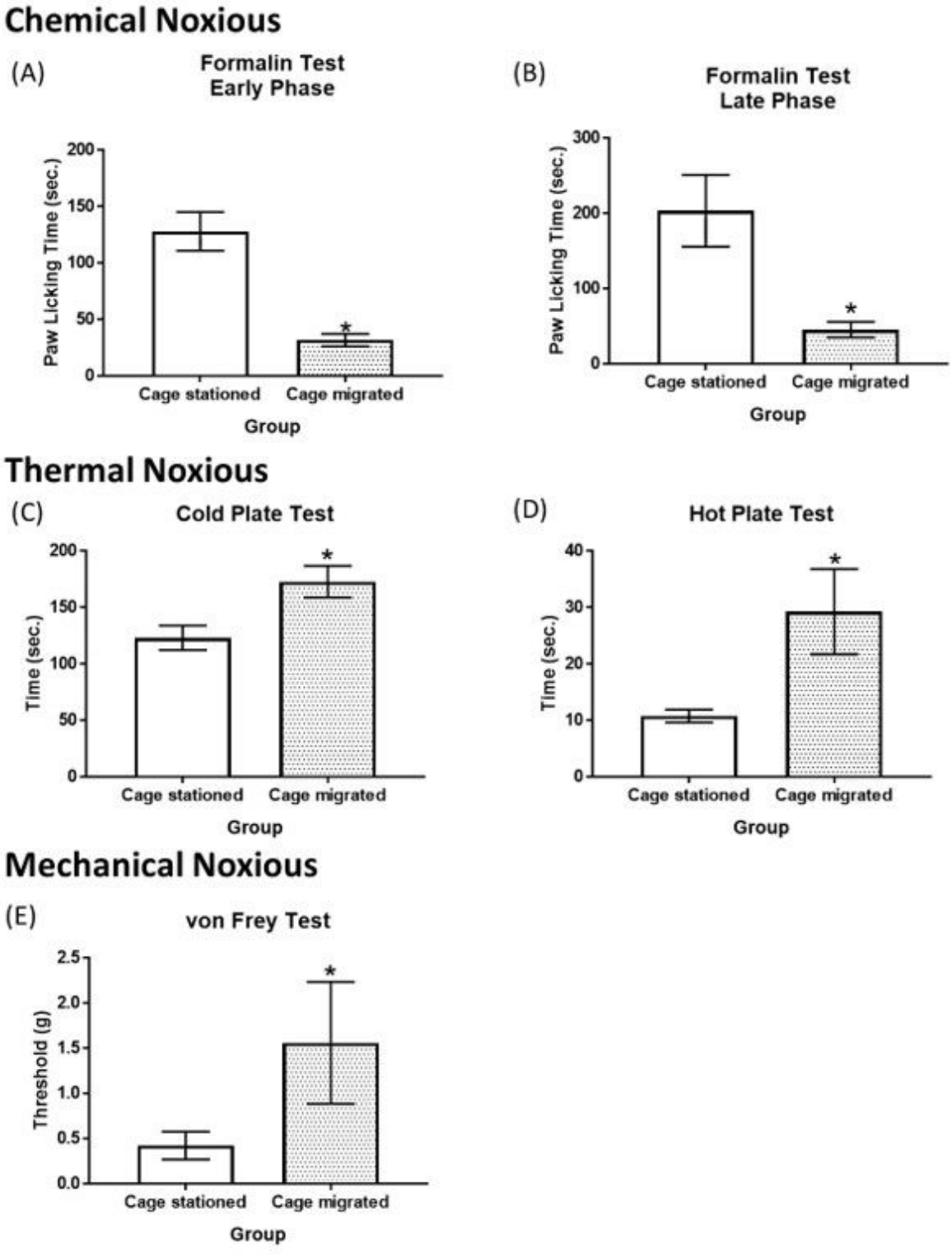

Figure 4

Pain sensitivity in cage-to-cage migrated mice. (A) and (B) Decreased paw licking during early and late phase in formalin pain test, (C) and (D) Total time spent on hot and cold plate, (E) Response to

mechanical pain stimulus. *Significant at p<0.05 compared to Cage stationed group, (n = 6).

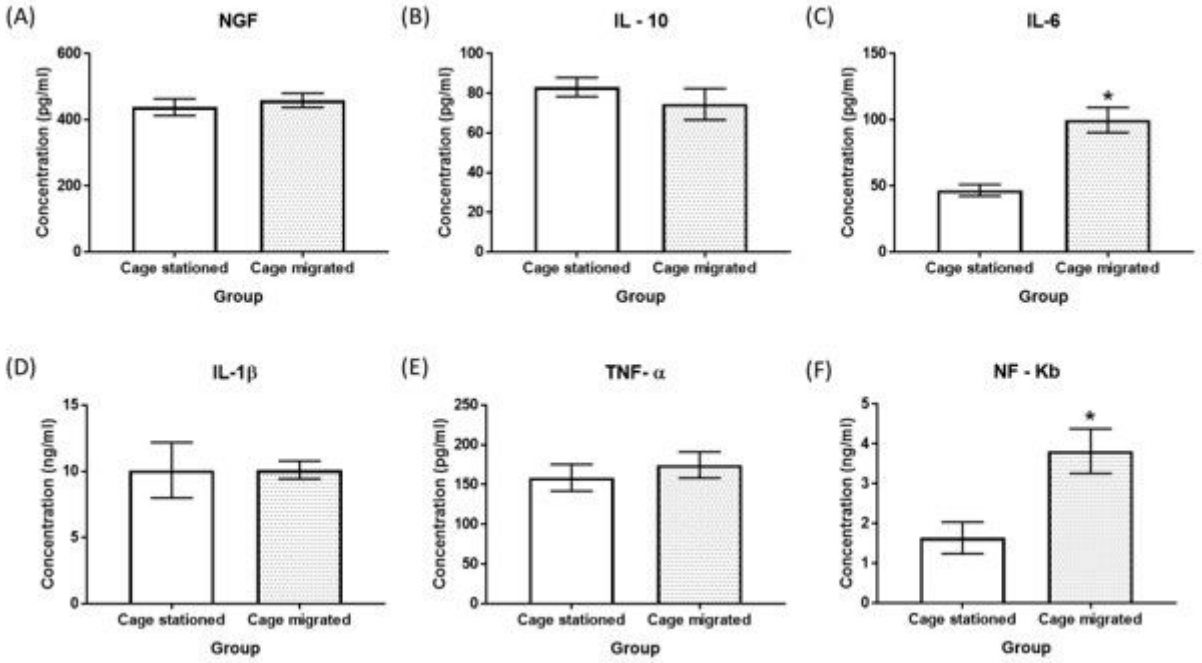

Figure 5

Changes in anti- and pro-inflammatory markers. (A) and (B) Neuropretective mediators, (C) to (F) Pro-inflammatory markers. *Significant at p<0.05 compared to Cage stationed group, (n = 6).

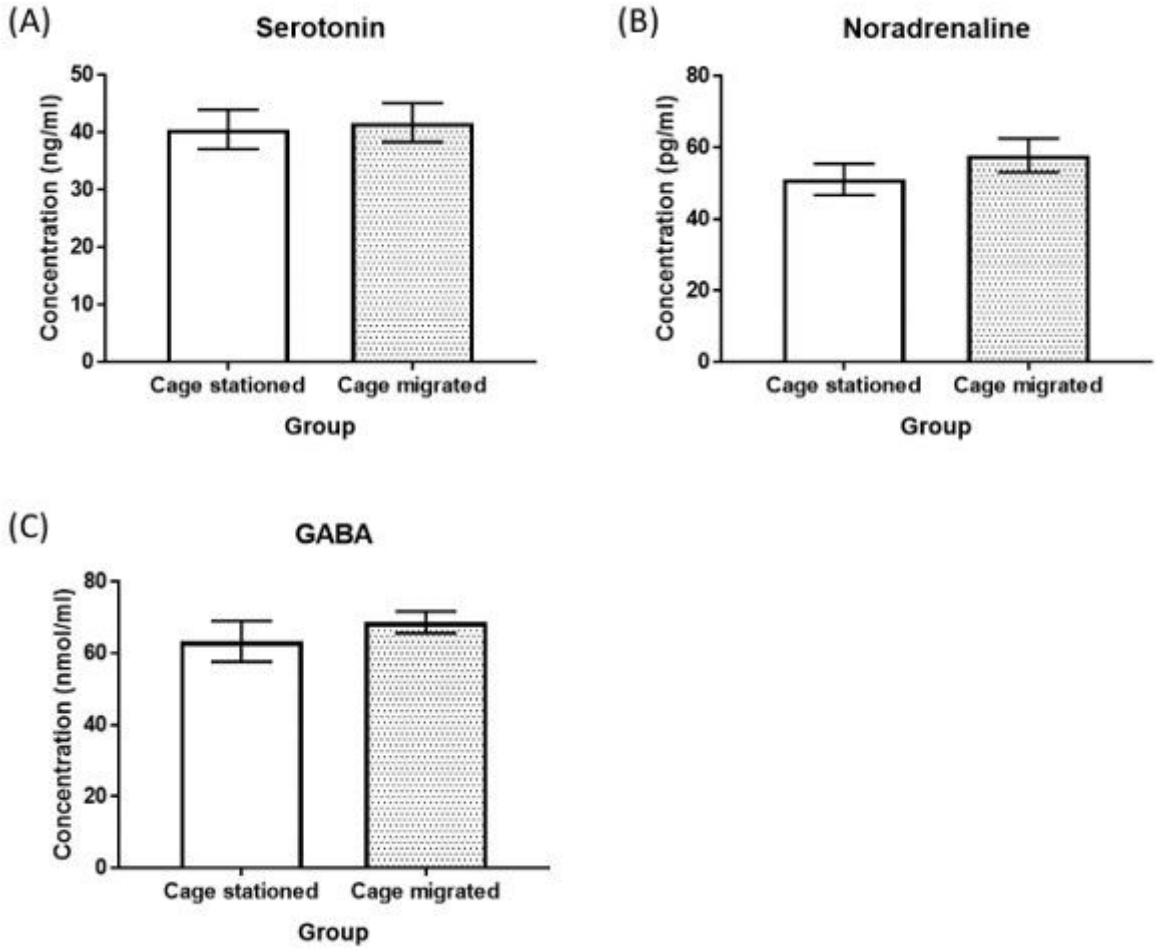

Figure 6

Levels of relevant neurotransmitters. (A) and (B) Excitatory neurotransmitter, serotonin and noradrenaline respectively, (C) Inhibitory neurotransmitter, GABA

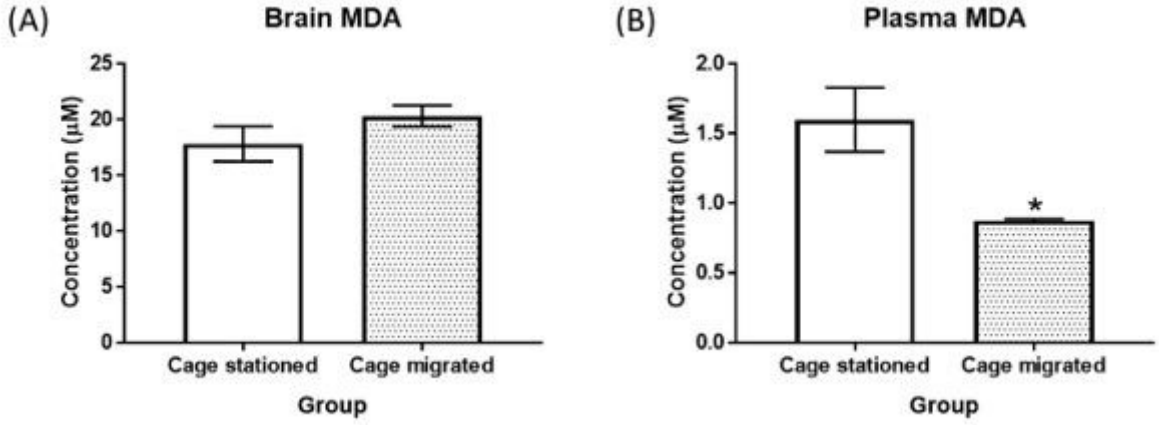

Figure 7

Oxidative stress markers. (A) and (B) Concentration of malondialdehyde in the brain and plasma respectively. *Significant at $p<0.05$ compared to Cage stationed group, (n = 6).

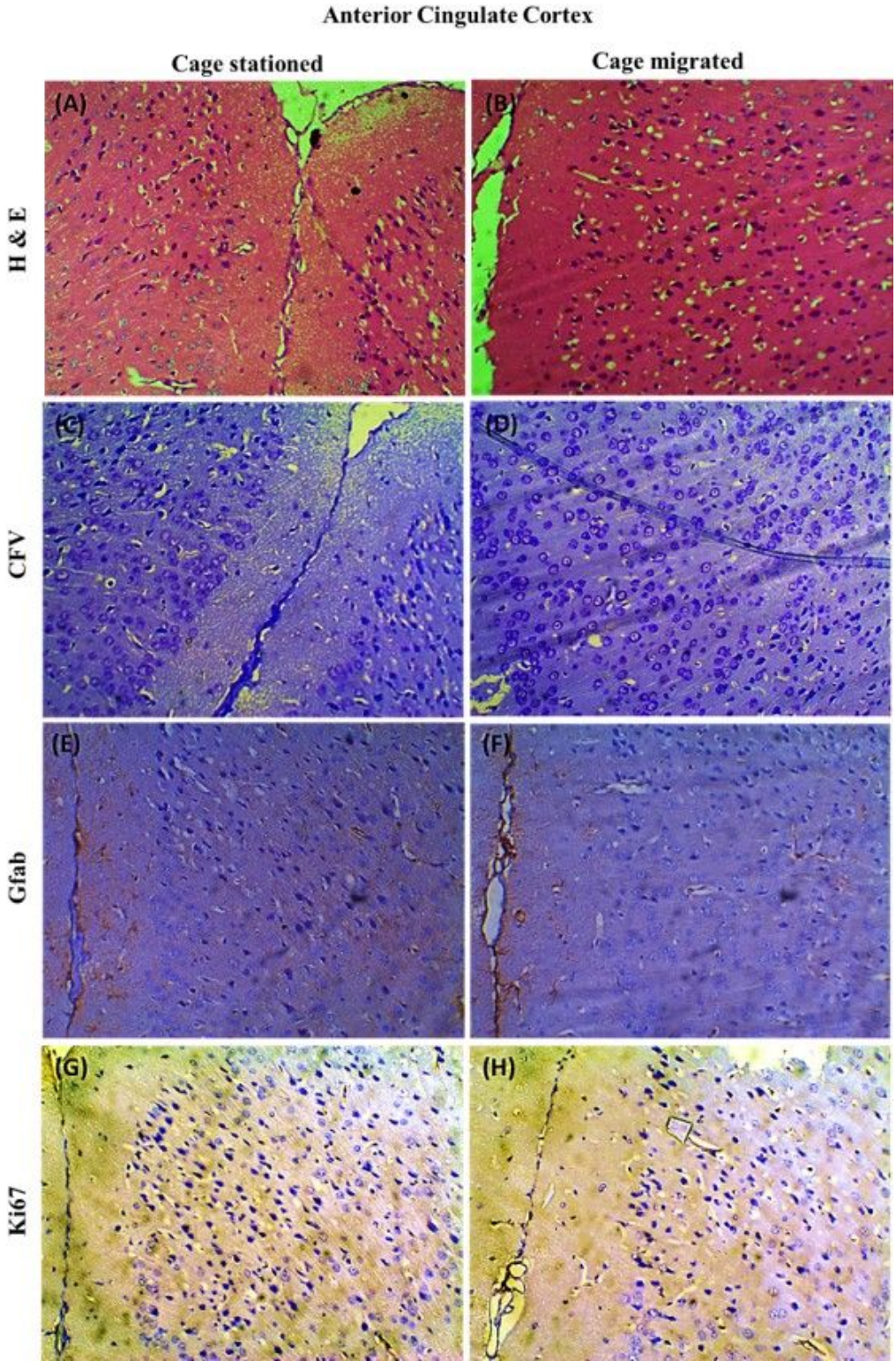

Figure 8

Histological investigation within the anterior cingulate cortices. (A) and (B) H&E-stained sections of cingulate cortex in cage migrated and cage stationed mice respectively. (C) and (D) CFV-Stained sections of cingulate cortex area in cage migrated and cage stationed mice respectively. (E) and (F) Immunostaining of the cingulate cortex with Gfap antibody in cage migrated and cage stationed mice

respectively. (G) and (H) Immunostaining of the cingulate cortex with Ki-67 antibody in cage migrated and cage stationed mice respectively. (Final magnifications (A), (B), (C), (D), (E), (F), (G) and (H) = 100x).

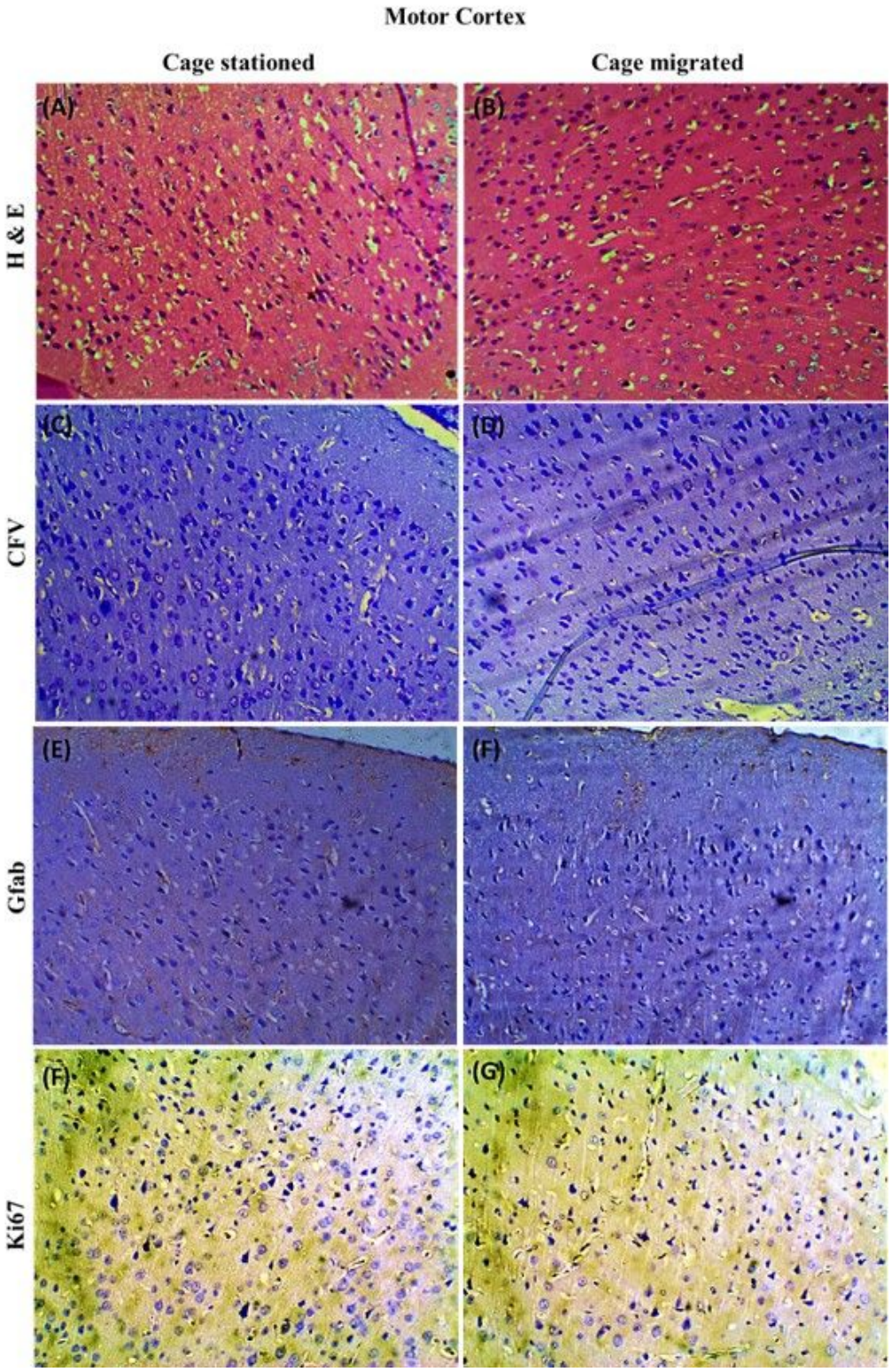

Figure 9

Structural outcomes of motor areas. (A) and (B) H&E-stained sections of motor cortex in cage migrated and cage stationed mice respectively. (C) and (D) CFV-Stained sections of motor cortex area in cage migrated and cage stationed mice respectively. (E) and (F) Immunostaining of the motor cortex with Gfap

antibody in cage migrated and cage stationed mice respectively. (G) and (H) Immunostaining of the motor cortex with Ki-67 antibody in cage migrated and cage stationed mice respectively. (Final magnifications (A), (B), (C), (D), (E), (F), (G) and (H) = 100x).

Supplementary Files

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- OyewoleetalSupplementaryFigure13.pdf