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Original Article

Cage Migration and Memory: Investigating the Impact of Varied Cage Sizes on Memory Function in Swiss Mice

[1,2]Oyewole, A.L., [1]Ishola S.S., [1]Oyafemi K.O., [1]Badmus K.S., [1]Omoleye J.O., [1]Abubakar M.F., [1]Adeniyi-Raheem O., [1]Amedu A., [1]Lawal D.L., [1]Ijiyode A.O., [1]Yussuf A.O., [1]Yakub Y.O., [2]Ogunjimi G.L., [3]Sulaimon F.A.

Abstract

BACKGROUND AND AIM: Diverse cage sizes have been implicated in the alteration of pain sensitivity and inflammation parameters in animal-based experiments. Sparse information is available on the effect of exposure to different cage sizes on memory in animal studies. This study thus aimed to investigate the effect of varied cage size exposure on memory.
METHODOLOGY: Twelve adult male Swiss mice (29-34 g) divided into two groups (n=6) were used for this study, comprising a Control group (stationary cage) and a Test group (migrated cage). The cage-migrated mice were exposed daily to various cage sizes typically used in Nigerian laboratories for 30 days. In contrast, the cage-stationed mice were daily exposed to new but the same size and shape cage. After 30 days of exposure, memory functions were assessed in the animals using memory-related behavioural paradigms (such as novel object recognition test), acetylcholinesterase activity and histological evaluation.
RESULTS: The results showed no significant difference in the recognition and spatial memory of cage-migrated animals compared to its cage stationed counterparts. The brain acetylcholinesterase activity significantly ($p < 0.05$) decreased in cage-migrated mice compared to cage-stationed animals but there was no difference in plasma acetylcholinesterase activity in both groups. Also, histological evaluation of all regions of the hippocampus in both groups of animals did not show any significant difference. Though cornu ammonis regions appeared to be enlarged in cage migrated animals compared to cage stationed.
CONCLUSION: Repeated exposure of experimental mice to varied cage sizes could selectively decrease brain acetylcholinesterase activity without affecting the animals' memory.

Keywords:
cage, memory, hippocampus, acetylcholinesterase

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[1]Department of Physiology, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria; [2]Bioresearch Hub Laboratory, Ilorin, Nigeria; [3]Department of Anatomy, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria.

Address for Correspondence:
Sulaimon F.A.
Department of Anatomy, Faculty of Basic Medical Sciences, University of Ilorin, Ilorin, Nigeria.
sulaiman.fa@unilorin.edu.ng
+2348189673881

INTRODUCTION

Animal experimentation remains maligned by ethical and philosophical submissions despite the rich history of ground-breaking feats garnered from animal studies. The general aim of biomedical research is to improve the quality of life, increase life expectancy and develop possible treatments for diseases. The use of animals as partial models for humans' physiological and pathological states has enabled scientists to achieve an aspect of this general aim, through the deployment of various revolutionary cutting-edge techniques during research. Despite this, many animal-based experiments remain non-translational in humans. The rate of successful translation of animal-based findings during human clinical trials is low (van Luijk *et al.*, 2014). This failure is sometimes due to differences in epigenetics, physiology, and genetics (Pound *et al.*, 2018). The micro-environment of the experimental animals and their handling during research are usually overlooked as a cause of skewed translational outcomes in humans. But advances in epigenetics now show that animals' micro-environment could

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impact their biology, thus, ground-breaking findings that are epigenetically marred by animals' micro-environment might fail in humans' clinical trial.

Various laboratory practices could facilitate animals' micro-environment interference with the experiment. In countries where research funding is grossly poor for instance, it is common to see animals meant for the same experiment (though belong to different groups) being housed in cages of different sizes and shapes. Also, the housing condition is sometimes adjusted from time to time during the experiment without considering its possible impact on the findings. We believe that repeated exposure to varied cage sizes during experiments contributes to poor reproducibility in most poorly funded laboratories. This is because of the epigenetic effect of the cage-housing environment on animal physiology and behaviours. Frequent cleaning of the home cage and variation in noise or rack position are known to affect reproduction, anxiety (Izídio *et al.*, 2005; Burn *et al*., 2008) and cannibalism, thus the overall experimental outcome.

Recently, we established that repeated alternation of cage micro-environment sizes in animal-based experiments could decrease pain sensitivity and increase selected inflammatory factors in mice (Oyewole *et al*., 2020). In the present study, we probed further to know if varied sizes of cage-to-cage exposure could impact memory function in Swiss mice. This new direction is opined from the fact that exposing rodents to different cages motivates the animal to explore their new micro-environment, an action that leads to increased cage activities and sometimes increased aggression in mice (Van Loo *et al*., 2000). Increased cage activities in rodents imply an increase in muscular exertion similar to mild exercise. Mild physical exercise is known to improve learning and memory in rodents (Aderbal *et al*., 2011). Apart from this, environmental factors are known to play a crucial role in cognition and memory (Valero *et al*., 2011). Thus, we hypothesised that "animals repeatedly exposed to varied cage sizes would have improved memory function".

Investigating this hypothesis, memory function was assessed via evaluation of various memory-related behaviours during cognitive tests (Y-maze test, Novel object recognition test, social recognition test, and modified light and dark box test). Also, the activity of acetylcholinesterase, an enzyme that breaks down acetylcholine (a neurotransmitter for memory formation) was determined. Lastly, we carried out a histological technique on the hippocampus (the part of the brain where memory formation occurs) to observe possible structural changes.

MATERIALS AND METHODS

Mice. Twelve adult male Swiss mice with a weight range of 29-34 g were obtained from Central Animal Facility, University of Ilorin, Nigeria and were used for this study. The mice were transferred to the animals' facility of Bioresearch Hub Laboratory, Department of Physiology, University of Ilorin where the study was carried out. The mice were allowed to acclimatize for 7 days in a standard housing condition at a relatively constant temperature with a regular light-dark cycle. During the period of acclimatization, each mouse was handled for 1 minute to allow the animals to get familiarized with handling before the experiment. Mouse pellets and water were available *ad libitum* before and during the study. The procedures here used were as approved by the University Ethical Review Committee, University of Ilorin, Nigeria; (Approval number – UERC/ASN/2019/1/923).

Exposure to varied home-cage sizes: Animals were divided randomly into two groups (containing 6 mice each) of cage stationed (Control Group) and cage migrated (Test Group). For all the studies carried out, mice were housed in six per group, (n=6). Mice allocated to cage stationed group were kept in the same size cage of 45 x 25 x 15 cm (length x width x height) for the 30-day duration of the experiment. These control mice were transferred to a clean but the same size cage within a period of 60-second at 8 am daily. The same water bottle and toys were returned to the new same size-cage after each transfer. Mice in the test group (cage migrated) were exposed to cages of different sizes. Just like in the cage station group, exposure to new cage size was done daily (every 24 hours) at 8 am with all mice moved into the new size cage within an average time of 60 seconds. Also, the same water bottle and toys were returned to the new cage. From Days 1 to 15, animals 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-introduced to the above respective cages starting from the earliest. All cages used were transparent plastics with metallic lids.

Memory-linked Behavioural Studies. The ability of mice to use their immediate environmental cues to form representations of the outer world was tested with memory-related behavioural paradigms that include novel object recognition, social cognition, Y-maze and modified light/dark box.

Novel object recognition test was carried out in a 4-wall square arena (40 x 40 x 40 cm) apparatus. Two identical objects were placed in a symmetrical position about 20 cm away from each other and away from the wall. Mice were individually placed in between the familiar and novel objects and allowed to explore them for 5 minutes. At the end of this trial, one of the identical objects was replaced with different object and animals were allowed to explore it for another 5 minutes. The time spent on both novel and familiar objects were documented. Time spent was then used to calculate discrimination index as follows.

$$\text{Discrimination index} = \frac{TNO}{TNFO} \times 100$$

TNO - Time spent exploring Novel Object; TNFO – Total time spent exploring both Novel and Familiar Object (i.e. time spent exploring the familiar object + time spent exploring the novel object) (Lueptow, 2017)

Social recognition test aimed to investigate ability of the mice to discriminate their cage-mate (familiar mouse) from a strange mouse. The same arena used for novel object cognition was adopted and used for this test. Two mice (a familiar and a strange)

were placed individually in top-perforated plexiglass restriction boxes about 20 cm apart. The familiar mouse is a cage mate of the mouse that is being tested. The number of visits and time spent with each mouse were used to evaluate the animals' memory. The discrimination index was calculated similarly to the formula mentioned above in the Novel Object Test as stated below:

Discrimination index = $\frac{TSM}{TSFM} \times 100$

TSM - Time spent visiting the strange mouse; TSFM – Total time spent visiting both strange and Familiar mouse (i.e. time spent visiting the strange mouse + time spent visiting the familiar mouse) Adapted from Lueptow, 2017

Y-maze test was used to assess short-term spatial working memory in the experimental animals. The apparatus used had a typical Y-shape design with about 30 cm wall. Spatial working memory was assessed by quantifying spontaneous alternation of each mouse in the maze. A mouse with good working memory is expected to show a high tendency to explore less recently visited arms. In this study, the mouse was placed into the long tail of the Y-maze and was allowed to freely explore all three arms within 5 minutes. A correct alternation is achieved when entries are made into all three arms in order of previously unvisited arms. The relative ratio of correct alternation to the total entries was used as a memory index. A high ratio showed good working memory and an indication that the mouse could recall the arms it had already visited (Kraeuter *et al.*, 2019).

Light/dark box, a paradigm primarily used in the evaluation of anxiety in rodents was modified to assess short-term spatial working memory. Briefly, each mouse was placed into the light compartment of the maze and was allowed to explore the maze for 5 minutes. During this exploration period, the animals learnt about the tunnel via which they shuttled to and from the light and the dark boxes. At the expiration of 5 minutes, the tunnel was blocked and the mouse was permitted to explore the light box for another 5 minutes. The number of visits to the location of the blocked tunnel and the total time spent probing the tunnel area were used to evaluate memory in mice (Bloch and Belzung, 2023).

Note – Each maze used for all the tests was cleaned with 70% ethanol solution and allowed to dry before introducing another mouse during the behavioural tests. All behavioural studies were video-recorded using a webcam attached to the paradigm and extraction of data were done by three trained persons blinded to the experiment and the average was accepted as the value of each parameter.

Biochemical assays. Acetylcholinesterase (AChE).

Mice were sacrificed at experimental end point (Day 30) under continuous inhalation of an anesthetic drug, isoflurane. Blood was collected with a 1 mL syringe via cardiac puncture and was emptied into lithium-heparinized Eppendorf bottle. This was then centrifuged at 3000 rpm for 10 min under 4 °C to get the plasma. Immediately after blood collection, mouse was transcardially perfused with 20 mL of cold PBS, the cerebrum was carefully removed, homogenised, centrifuged (3000 rpm, 10 min and at 4 °C) and the supernatant was collected into plane bottle. Both the plasma and cerebral homogenates were used for AChE assay. The following reagents were used to assay for brain and plasma AChE; 35 µL of 5mMdithio-bisnitrobenzoic acid, also known as Ellman's reagent (DTNB), 10 µL of 75 mM acetylthiocholine (ATCh) and 50 mM phosphate buffer (pH 8.0). Protein concentration in brain homogenates was quantified using a Bradford assay and AChE activity was calculated in micromoles of ATCh hydrolysed per hour per milligram of protein and was expressed as a percentage of control activity and measured values in micromole per hour per milligram of protein.

Histological studies. At the end of the experiment, following blood collection from the right ventricle of the mice under isofluorane anaesthesia, animals assigned for the histological study were successively perfused with cold phosphate-buffered saline and 4% phosphate-buffered paraformaldehyde through cardiac puncture. Sections of the brain containing hippocampus cells were exercised, fixed in 4% phosphate-buffered paraformaldehyde and stored for 24 hours under 4 °C. The tissues were later processed in ascending grades of ethanol, cleared in xylene and finally embedded in paraffin. Sections (5 µm; MK 1110 rotary microtome) of the cortex were stained with Cresyl fast violet (CFV) to demonstrate the Nissl substance. The photomicrographs of the CFV-stained tissues were captured under 40X objective lens using the Zeiss Axiostar plus light microscope manufactured by Amscope.

Statistical Analysis. The present data were analyzed with 7.0 version of Graph Pad Prism software using its unpaired Student's t-test tool. Data were stated in mean ± SEM and the statistical significance used was $p < 0.05$.

RESULTS

The impact of cage migration on the memory of experimental mice

Memory assessment using Y maze showed that there was no significant difference ($p < 0.05$) in Cage-migrated animals when compared with Cage-stationed mice (Figure 1A). The frequency for novel object probing was however significantly ($p < 0.05$) decreased in Cage migrated mice when compared to Cage stationed mice (Figure 1B). This gave the impression that the Cage-migrated mice paid less attention to the details of the novel object. However, when time spent on each object was used, the discrimination index was not significantly ($p < 0.05$) different in cage-migrated mice compared to their cage-stationed counterparts (Figure 1C).

The above was followed by subjecting the animals to a memory test that entails discriminating between wild mice from familiar mice. Again, the probing rate and discrimination index in cage-to-cage migrated mice were not significantly different compared to cage-stationed animals (Figures 1D and 1E). Lastly, the mice were subjected to another memory test to validate accumulated data on the impact of consistent cage-to-cage migration of mice on memory. Once more, the results on frequency and time spent probing for the tunnel in modified light and dark box (Figures 1F

and 1G) were not significantly different in cage migrated mice compared to cage stationed mice.

Brain acetylcholinesterase activity was higher the cage migrated group

Acetylcholinesterase activity was then evaluated as the enzyme has been profiled as a marker for memory index. In the plasma, acetylcholinesterase activity in cage migrated mice compared to cage stationed animals was not significantly different compared to cage stationed mice (Figure 3A). However, brain acetylcholinesterase activity decreased significantly in cage-to-cage migrated mice compared to cage stationed animals (Figure 2B).

The effect of cage migration hippocampal histoarchitecture

The histology of the hippocampus was revealed through staining with cresyl fast violet. Analysis of the histological data indicated no significant variance in histoarchitecture of hippocampus between the cage-migrated mice and those stationed within cages. Furthermore, the distribution of Nissl substance granules appeared comparable in both groups. Nevertheless, an observable enlargement of the regions of cornu ammonis was noted in cage-migrated mice in comparison to their cage-stationed counterparts (Figure 2A and B).

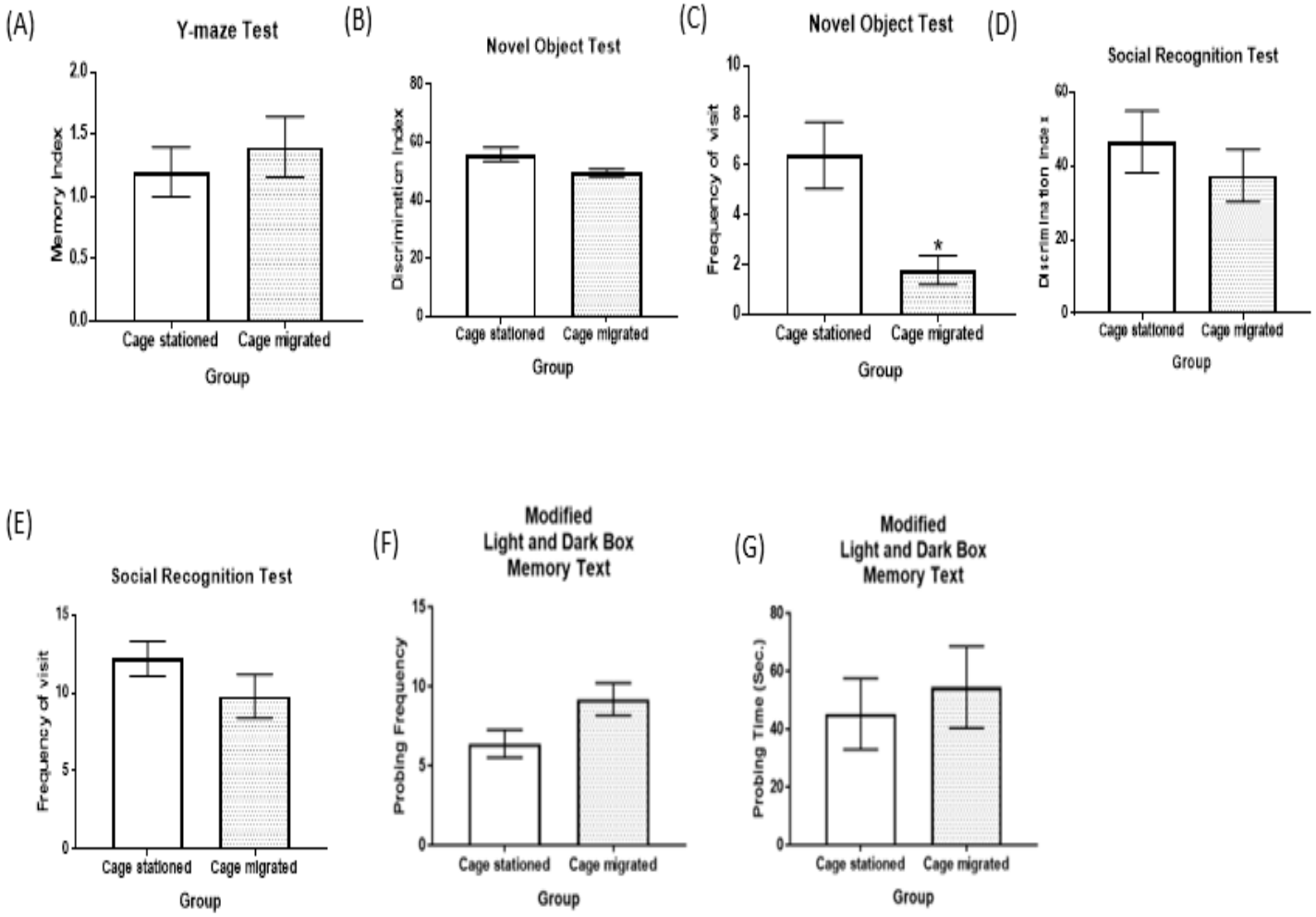

Figure 1. Spatial Working and Recognition Memory Tests. (A)Memory index in Y maze (B) and (C) Probing frequency and discrimination index of novel object respectively (D) and (E) Probing frequency and discrimination index of unfamiliar mouse respectively, (F) and (G) Tunnel probing frequency and time in modified light and dark boxes respectively.

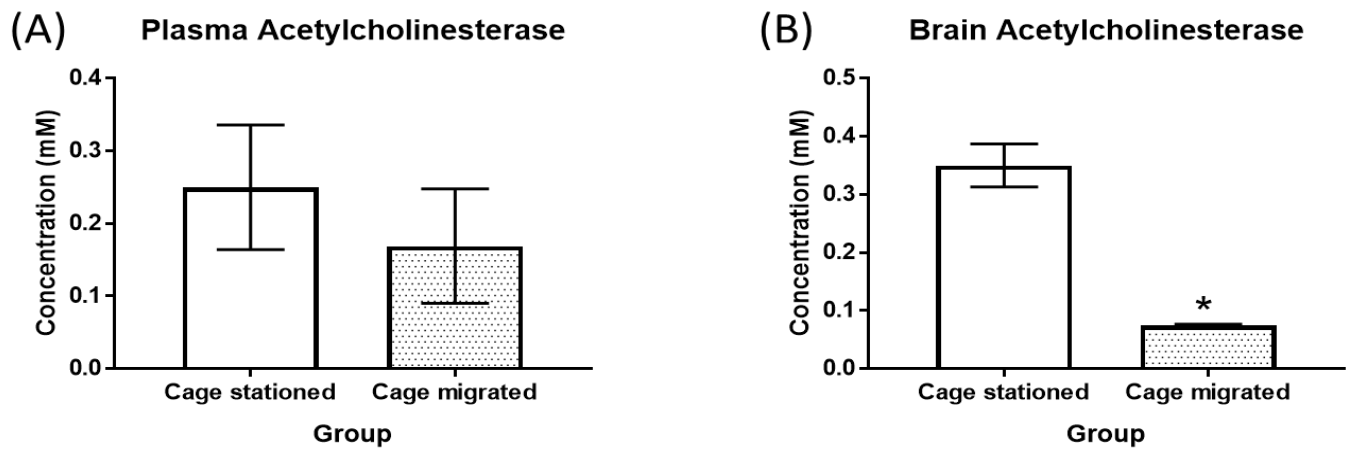

Figure 2. Acetylcholinesterase Activity. (A) and (B) Activity of acetylcholinesterase in the plasma and brain respectively.

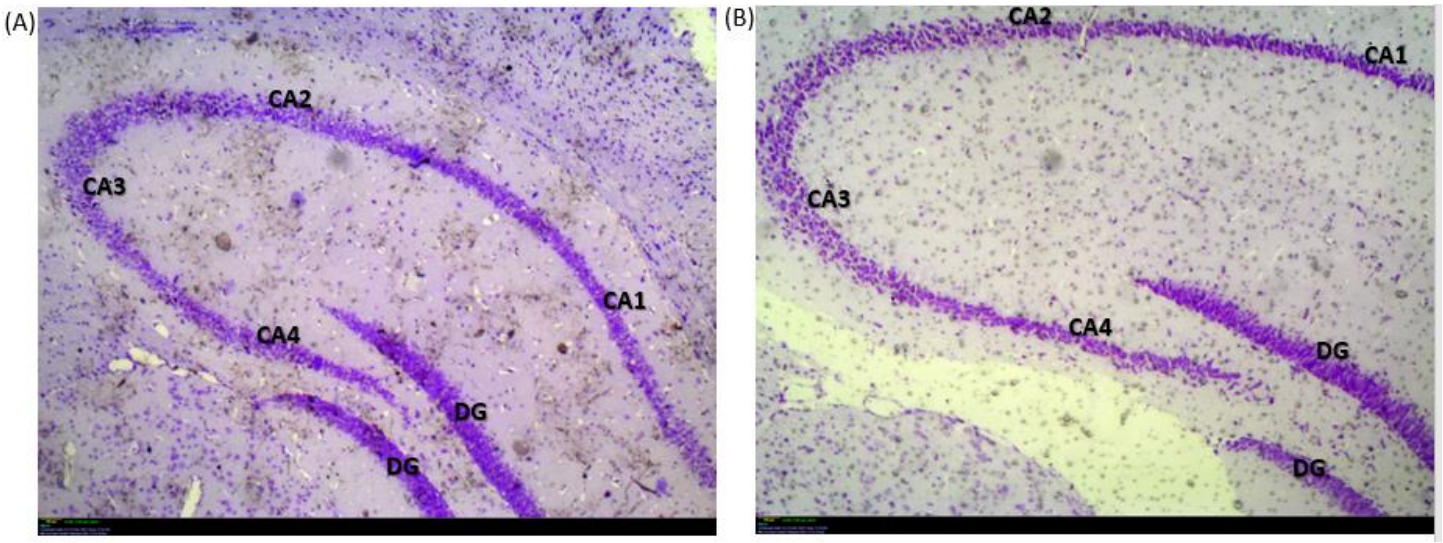

Figure 3. CFV-Stained sections of Hippocampus. (A) and (B) Cornu Ammonis regions of hippocampus in cage stationed and cage migrated mice respectively. CFV implies Cresyl Fast Violet; CA1, CA2, CA3 and CA4 denote Cornu Ammonis (CA) 1, 2, 3 and 4; DG signifies dentate gyrus with its upper and lower limbs surrounding the CA4; M denotes regions within the enclosed Cornu Ammonis, mostly filled with axons and supporting cells. (Objective Lens: 4×; scale bar = 100 µm).

DISCUSSION

In the assessment of the recognition memory test, the discrimination index for novel object was not significantly different in cage migrated mice compared to cage stationed animals. To corroborate this result, the animals were further subjected to another memory test that entails discriminating between wild (unfamiliar) mouse from familiar (cage-mate) mouse. Again, both the probing/visiting frequency and discrimination index in cage migrated mice were not significantly different compared to cage stationed animals. Though statistically, there was no difference in recognition memory-related behaviours in cage migrated mice compared to cage stationed mice. The graph bars representing the cage migrated mice however showed a decreasing direction for recognition memory. This is surprising since exposing rodents to new cages generally increases their cage activities (Van Loo *et al.,* 2000) with accompanied muscular exertion which we assumed is equivalent to a mild exercise. Exercise has been shown in the literature to improve memory, including recognition memory (Grace *et al.,* 2009; Aderbal *et al*., 2011). To know whether our observation is only limited to recognition memory or not, we investigated the impact of varied cage size exposure on another form of memory, spatial working memory. Again, data collated from the Y maze test showed that there was no significant difference in spatial memory of cage-migrated animals when compared with cage-strapped mice. This result was then validated by subjecting the mice to another form of spatial working memory test. Once more, the results on frequency and time spent probing for tunnel in modified light and dark box were not significantly different in cage-migrated mice compared to cage-stationed mice. However, unlike in the graphs for the recognition memory test where bars representing the cage-migrated mice showed a decreasing direction, bar representatives of cage-migrated mice in spatial working memory test was on an increasing direction. As stated earlier, the results from the two forms of memory tested were not statistically significant. However, this opposing direction in cage-migrated mice suggested that if the period of exposure is extended beyond what was used in this study, there is a possibility

that the spatial memory may be significantly enhanced while the recognition memory may be significantly impaired. This further suggests different neural circuits for both recognition and spatial memory, and in mice repeatedly exposed to varied cage sizes, spatial memory may be favoured over recognition memory. The reason for this difference in direction for both forms of memory is not known and we do not investigate this further since all data are not statistically significant. Nonetheless, we are surprised that none of the data from the memory-related behaviours was significant owing to the contrasting result from the measured acetylcholinesterase activity.

Acetylcholinesterase activity was profiled owing to it vital role in acetylcholine function which is one of the important neurotransmitters important for memory index (Farzi *et al.,* 2018). In the plasma, acetylcholinesterase activity in the cage-migrated mice was not significantly different compared to cage-stationed mice. However, brain acetylcholinesterase activity decreased significantly in cage-to-cage migrated mice compared to cage-stationed animals. Previous findings showed hippocampal acetylcholine as the central neurotransmitter that orchestrates memory formation (Drachman, 1977; Bartus, 1978). This transmitter is released into the synaptic cleft of the hippocampal neurons to enable communication between pre- and post-synaptic neurons. The action of synaptic acetylcholine is inactivated by the acetylcholinesterase activity (Soreq, and Seidman, 2001), an enzyme that breaks the transmitter into choline and acetic acid. Increased acetylcholinesterase activity signifies inadequate duration for acetylcholine action (as the transmitter is broken down rapidly) while decrease acetylcholinesterase activity implies sufficient time for acetylcholine action before it is terminated. Relating to memory, increased acetylcholinesterase activity is associated with impaired memory formation (Soreq and Seidman, 2001) while decreased acetylcholinesterase activity is linked to enhanced memory (Farzi *et al.,* 2018). In this study, there was a significant decreased in brain acetylcholinesterase activity of the cage-migrated mice compared to its cage stationed counterpart. Given this, one expects all forms of memory in this group of mice to be significantly enhanced as reported in the literature (Farzi *et al.,* 2018). Our observations contradict this norm. We do not know why the memory-related behaviours evaluated were not significant despite viable acetylcholinesterase activity index. However, in our recent study published in Journal of Neuroscience Methods, we established an increase in IL-6 and NF-κB concentration in cage migrated mice compared to cage stationed mice (Oyewole *et al*., 2020). Elevated levels of IL-6 and other pro-inflammatory markers have substantial correlation with declined memory and cognitive performance (Virginia et al., 2012; Newcombe *et al.,* 2018). This might be a plausible reason why decreased brain acetylcholinesterase activity was not commensurate with the expected enhanced memory. The observed contrast in the results of memory-related behaviours and acetylcholinesterase activity further buttresses how frequent exposure to varied cage sizes could affect targeted parameters and prevent results reproducibility in memory-related studies.

Lastly, histological technique was carried out on the hippocampus of the animals in the two groups using cresyl fast violet stain. The cresyl fast violet stain was used because of its additional advantage of staining the Nissl substance as well as the cell morphology. The histological results showed no significant difference in the cytoarchitecture and morphology of hippocampus in the cage-migrated mice compared to cage-stationed animals. The distribution of the Nissl substance was also similar in both groups evidenced by their similar CFV staining intensities. However, the regions of cornu ammmonis appeared to be enlarged in cage-migrated mice compared to cage-stationed animals. This enlargement increased the distance between CA1 and CA4. What led to this difference is not known but it appears to not influence memory-related behaviours since these behaviours are not significantly different in cage-migrated mice compared to cage-stationed animals.

In conclusion, consistent exposure of mice to varied cage sizes decreased acetylcholinesterase activity without affecting the animals' memory.

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