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Dear Editors of JoVE,

We are pleased to submit our manuscript titled "*Nest building behavior: An early indicator of behavioral deficits in mice*" for publication in The Journal of Visualized Experiments. Nesting serves as an informative, ancillary assessment for activities of daily living in rodents, especially those modeling pathological disease states including, but not limited to, Alzheimer's disease, traumatic brain injury, and depression. But, as the utility and popularity of nesting increase, so do the various ways to conduct it, leading to variable and sometimes, null, results. We believe that this manuscript can provide insight into some methodological and statistical considerations that improve the validity and reliability of this assay.

In this protocol, we offer the opportunity for researchers to use our adapted scoring system that semi-quantifies nest quality. We use this scoring system with four different nesting materials – the commonly used cotton square, as well as diamond twists, a softcob-nesting blend, and shredded paper. We provided the four materials to wildtype C57BL/6J mice and homozygous APOE4 mice, a model for late onset-Alzheimer's disease. More importantly, we implemented the use of an intraclass correlation to assess the agreement among nest ratings of three blind scorers. Our representative results demonstrate the superiority of one nesting material in this sample – shredded paper – in terms of nest quality and scorer agreement. Overall, wildtype mice built significantly better nests compared to APOE4 mice regardless of the nesting type; however, both wildtype and APOE4 mice build significantly higher quality nests out of shredded paper compared to the three other nesting materials. Scorers had stronger agreement when scoring nests constructed out of shredded paper, followed by cotton squares, as determined by intraclass correlation. Taken together, we believe that our method offers alternative, cost-effective, and practical solutions for experiments that do not yield the typical floor and ceiling effects in nesting.

All conducted procedures to materialize this protocol were approved by the George Mason University Institutional Animal Care and Use Committee. The images provided in Figure 4 have been published in Craven et al., 2018. This manuscript is not currently under review, nor will it be submitted to any other journal, while under consideration for JoVE. Caroline L.C. Neely, Ph.D. will serve as the corresponding author and accepts full responsibility for informing the co-authors about the submission, review process, and content.

Kind regards,

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TITLE:

Nest Building Behavior as an Early Indicator of Behavioral Deficits in Mice

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SUMMARY:
Here, we present a protocol to quantify the nest building behavior, which is known to be impaired in several neurological disorders and diseases. This protocol examines the utility of four materials and offers the opportunity to quantify the rater agreement in scoring, improving the validity and reliability of the assay.

ABSTRACT:
Nest building is an innate behavior in male and female rodents, even when raised in laboratory settings. As such, many researchers provide rodents synthetic and/or natural materials (such as twine, tissue, cotton, paper, and hay) as a gauge of their overall well-being and as an ancillary assessment to predict the possible decline in cognition. Typically, changes in nesting behaviors, such as failure to create a nest, indicate a change in health or welfare. In addition, nesting behavior is sensitive to many environmental and physiological challenges, as well as many genetic mutations underlying pathological disease states. The following protocol describes a nesting behavior paradigm that explores the usage of four types of nesting material. In addition, the protocol utilizes intraclass correlations to demonstrate that inter-rater reliability is higher when nests are constructed out of shredded paper compared to other common nesting materials such as cotton squares, paper twists, and soft cob bedding. The chosen methodology and statistical considerations (i.e., intraclass correlation) for this assay may be of interest for those conducting experiments assessing the quality of living of mice.

INTRODUCTION:
Nest building is an innate behavior in many animals such as birds, fish, rodents, and great apes, and it is attracting more attention for its potential utility in the study of neurological diseases and disorders characterized by diminished well-being and impaired activities of daily living[1]. Mice,

both male and female, build similar-sized nests for reproductive purposes, heat conservation, and shelter; more importantly, they continue to do so even when raised in laboratory settings[2]. Hess, Petrovich, and Goodwin[3] argue that biologically-appropriate stimuli are paramount to induce biologically-appropriate behaviors such as nesting; however, a variety of natural and artificial materials, such as hay, hemp, cotton twine, paper strips, cotton dental balls, and pressed cotton squares, have been used to assess nest building behavior[2]. Changes in these nesting behaviors (i.e., failure to create a nest out of the provided material) generally indicate a change in health or welfare. In most cases, a lack of nest building is attributed to several factors that negatively impact well-being. Such examples that have all effectively decreased the quality of nest building in mice include temperature extremes; painful stimulation; induced illness and infection; genetic mutations; and brain lesions in the medial preoptic areas, septum, and hippocampus[4,5,6].

Alzheimer's disease is a progressive neurodegenerative disease characterized by loss of brain tissue, accumulation of extracellular amyloid-β plaques, and intracellular neurofibrillary tangles composed hyperphosphorylated microtubule-associated protein, tau[7]. Additionally, Alzheimer's disease is characterized by, most notably, deficits in learning and memory and impairments of activities of daily living. In mice, activities of daily living are commonly examined via circadian wheel-running[8,9,10,11], although new alternatives, such as nesting, are growing in popularity. Nesting has been shown to be sensitive to manipulations (e.g., genetic mutations, environmental stressors) that have been identified as risk factors and/or causes of Alzheimer's disease. As such, nesting can be used as an additional or complementary assay in many mouse strains that model those characteristics of Alzheimer's disease. For example, Deacon and colleagues[12] measured nest building of Tg2576 mice with the Swedish amyloid precursor protein (APPswe) and presenilin 1 (PS1) genetic mutations. The quality of nests constructed by group-housed Tg2576 mice was significantly worse than wildtype controls at both 3- and 12-months of age. In agreement with these findings, Filali et al.[13] reported that singly housed APPswe/PS1 male mice given two pieces of 5 x 5 cm cotton built significantly poorer nests as rated on a scale of 1 - 5 (1 = nestlet not touched, 5 = a near perfect nest). Transgenic mice consistently built worse nests at 6-, 9-, and 12-months of age compared to their wildtype counterparts, and in some cases, the Appswe/PS1 mice completely failed to build a nest out of the provided cotton.

Previous research from our lab has demonstrated that wildtype C57BL/6J mice build significantly better nests out of cotton squares compared to CRND8 and CRND8/E4 mice[9]. However, the majority of experiments using pressed cotton squares appear to be variable, with wildtype mice failing to show expected high scores compared to transgenic mice that are expected to show very low scores in nesting[2], which may in part lead to a lack of differences in estimated parameters (i.e., mean differences) and statistical significance. The lack of differences may be due in part to inadequate aging of mice or time allotted for nesting. Alternatively, nesting material may be an additional challenge, resulting in more variability due to researchers' methodological preferences in the quantity and type of material, which may even interact with mouse strain. For example, Robinson-Junker and colleagues[14] provided processed or unprocessed bedding material of different sizes (i.e., small or large flakes) to C3H/HeNCrl mice and BALB/cAnNCrl mice, which are commonly observed, respectively, as poor and strong nest builders. When provided unprocessed bedding, C3H mice built less complex, yet similar in quality nests compared to those of BALB/c mice. Likewise, Martin and colleagues[15] compared nest complexity of different nesting materials given to deer mice, a distant relative of the *Mus musculus* species that have distinct evolutionary differences (i.e., more likely to burrow in trees and underbrush and are more active in captivity), but receive similar husbandry care as common laboratory mice and build nests out of any available soft, fibrous material[16,17,18]. Females and breeders with pups in the home cage built more complex nests than males, and the authors suggest that these behavioral differences may be due to associated changes in progesterone concentrations in deer mice[15]. More importantly, mice built more complex nests composed of brown paper followed by cotton squares and cotton cylinders, and the least complex nests were constructed out of white paper and dispersed mini-cotton squares.

Despite the growing popularity of nesting, considerations regarding scientifically valid, cost-effective, and time-sensitive practices are minimally discussed. Given the aforementioned methodological and economical challenges, this protocol investigates the utility of different nesting materials – cotton squares, paper twists, shredded paper, and processed bedding – in nesting behavior. Specifically, we provided all nesting materials to both aged C57BL/6J wildtype controls and Alzheimer's disease APOE e4 mice in order to investigate any potential genotype by material interactions in nesting quality. Additionally, the experiment sought to assess inter-rater reliability of nests constructed out of different materials. Taken together, this protocol demonstrates the superiority of one nesting material in this sample – shredded paper – in terms of nest quality and scorer agreement, with the intention of improving the validity and reliability of the nesting assay.

PROTOCOL:

All procedures were approved by the George Mason University Institutional Animal Care and Use Committee and are in accordance with guidelines set forth by the Assessment and Accreditation of Laboratory Animal Care.

1. Animals and considerations prior to the assessment

1.1. For this protocol, use adult, 9 - 12-month-old C57BL/6J (n = 10) (stock # 000664) wildtype mice and APOE e4 mice (n = 11) from a hemizygous J20 (stock #006293) x homozygous APOE e4 (stock #012307) cross.

1.2. In the housing room, group-house mice with littermates of the same sex with appropriate enrichment (e.g., for this protocol, mice were provided a running wheel, igloo, and a small nylon chew toy). Group-house 4 - 6 female mice, and 4 males in a 356 mm L x 485 mm W x 218 mm H home cage.

NOTE: Researchers may consider implementing strategies prior to and/or after nesting in order to avoid cage-mate aggression when mice are reintroduced after nesting trials. Such strategies may include, but are not limited to, daily handling prior to behavioral testing to better acclimate mice to handling during behavioral testing, researchers, and husbandry staff[19], separating and individually housing aggressive mice, or reducing the number of mice in the homecage[20], depending on the severity of in-cage fighting, observed wounds[21], etc.

2. Room and nesting set-up

2.1. Ensure that each mouse completes four trials (1 material per trial). Randomize the order of nesting material for each mouse to avoid an order effect.

2.2. Prepare the cages in a separate testing room. Record environmental conditions (22.2 – 22.3°C, 45% humidity, lights on 9:00 AM – 9:00 PM) such that they are consistent across trials and are identical to the housing room. Provide food and water *ad libitum*.

2.2.1. Assign each mouse a random identification (ID) number or letter. Attach the random identifier card to a 29.2 x 18.4 cm cage.

2.2.2. Record the original animal ID/tag and other necessary identification in a colony record to ensure that assistants and animal husbandry staff remain blind to conditions.

2.3. Randomly order cage placement in the testing room such that wildtype and transgenic mice are not inappropriately separated (i.e., on opposite sides of the room, on separate shelves, etc.).

2.4. Prepare the nesting materials by sufficiently covering the bottom of the cage. Use approximately 100 g dry weight of corncob bedding for the square, twist, and shredded paper trials, and approximately 100 g of the soft cob bedding for the soft cob trial).

2.5. If using a beaker to disperse the nesting material, then fill the beaker no more than 100 mL with corncob bedding or soft cob bedding.

2.6. Place the first nesting material in the sequence prior to introducing the mouse into the cage. This protocol utilized (1) a single pressed cotton square, (2) a single paper twist, (3) 50 g of soft cob bedding, and (4) 2.5 g of clean (no ink), shredded white printer paper cut into 5 – 7cm strips. Disperse nesting materials as shown in **Figure 1** (baseline).

[Place **Figure 1** Here]

3. Nesting trial

3.1. Begin the first nesting trial at the same time of day at the start of the light cycle (e.g., 9:00 AM).

3.2. Bring the homecage containing the mice into the testing room. Remove each mouse from the homecage and place it into its assigned nesting cage with the material already placed in the cage. Return the homecages to the housing room.

3.3. Allow mice to complete 1 trial for 24 h undisturbed.

3.4. 24 h after the start of the first trial, return to the testing room.

3.5. Carefully remove the lid of the cage and photograph each mouse's nest, capturing the assigned ID in the photograph and minimizing the appearance of any materials outside of the cage.

NOTE: Do not remove the mouse from the cage and wait to photograph once the mouse is off the nest. Attempting to remove the mouse from the cage before photographing can potentially cause the mouse to become startled, thus move on top of, and disperse, nesting materials.

3.6. Gently remove the mouse from the nesting cage and place it in a temporary holding cage. Dispose of the corncob bedding and any nesting materials, replace the corncob bedding and provide the next nesting material in the sequence, and return the mouse to the nesting cage.

3.7. Repeat as necessary to obtain scores for all 4 trials. It is recommended to use shredded paper and the accompanying scoring criteria, although researchers may be interested in using the alternative materials discussed in this protocol.

3.8. When all trials are complete, return mice to their home cages. Observe the mice for any potential aggressive behavior. Aggression may occur in older wildtype males.

NOTE: For the purposes of this protocol, mice were tested once at approximately 9 – 12 months of age. Nesting should be conducted at several ages (i.e., at an earlier age, dependent on the chosen strain, prior to the onset of phenotypic traits) to document the decrease in nesting capacity over time and demonstrate the likely causative role of neurodegeneration.

4. Scoring and assessing inter-rater reliability

4.1. Provide baseline images for each nesting material to at least 2 individuals blind to the study. Although not required, ensure that the scorers are familiar with the concept of nesting. When training scorers, provide a series of example nests (e.g., **Figure 2** for shredded paper) to familiarize the scorers with each type of material (if applicable) and the scoring criteria.

4.1.1. Evaluate each nest on a scale of 1 – 5 using the following scale information (adapted from Deacon, 2006)[2]. See **Figure 2** as example scoring using shredded paper.

4.1.2. Assign a score of 1 when the shredded paper or small squares remained scattered throughout the cage, or the cotton square or twist remained untouched;

4.1.3. Assign a score of 2 when some of the material was constructed into a nest, but over 50% of the material was not used for nest construction (i.e., remained scattered or the majority of the original material remained untouched);

4.1.4. Assign a score of 3 when a noticeable nest was constructed, but several pieces were still scattered;

4.1.5. Assign a score of 4 when almost all the material was used for the nest, but few pieces of material remained scattered or were near the nest;

4.1.6. Assign a score of 5 when all material was used to make an identifiable nest.

[Place **Figure 2** here]

NOTE: Scorers should take breaks and revisit baseline photos in order to avoid fatigue and bias during the scoring procedure. Scorers should not discuss scores with one another to avoid bias. Scorers should discuss scores after scoring is complete, or if further discussion is needed to change scores, which can potentially resolve issues with intraclass correlations (ICC). Intraclass correlations will be conducted using the steps listed in section 4.2.

4.2. Collect and input scores and format the data file such that each scorer's scores for each material are in separate columns.

NOTE: If using **Supplementary File 1**, please note that the coding script for this analysis requires free, open source softwares (e.g., *irr* package in RStudio)[22,23]**.** The script removes headers and subjects variables (i.e., the top row and animal IDs) to conduct the intraclass correlation (ICC). Run the script to conduct a two-way, agreement, averages measures ICC by highlighting the demarcated section of the code and hitting Ctrl+Enter or Command+Enter.

4.3. Compare the ICC to existing criteria[24,25,26]. Typically, ICC values above 0.80 correspond to strong inter-rater reliability, justifying scores to be averaged at a given time point (**Supplementary File 2**).

4.4. Use the same ICC criteria for intra-rater reliability, which may be of interest for those conducting scoring for the first time.

CAUTION: Scorers should score the same photographed nests within a week of the initial assessment. Proceed with caution with averaging data when ICC is low, as this may skew data or produce null findings. If ICC values are low, then (1) additional training for scorers may be required, (2) scorers may need to discuss their reasoning for nest scores, (3) another independent reviewer may be needed to make a judgment call regarding the scores, or (4) intra-rater reliability may need to be assessed. If intra-rater reliability is poor, then additional training or a different scorer may be required.

5. Statistical considerations

5.1. Conduct statistical analyses, as appropriate. For data that do not violate normality assumptions but use multiple materials for each mouse (i.e., a within-subjects effect), use a mixed analysis of variance (ANOVA).

5.1.1. If using codes provided as supplemental files, please download additional add-on packages are specified in the script[27,28,29,30].

5.1.2. If conducting a mixed ANOVA using the provided script, run the script to convert any wide-format data files into long-format (i.e., instead of time variables repeated across Columns, convert the cells to repeat across Rows). An example data file is included in this manuscript to demonstrate how to convert a data file from wide-format to long-format.

5.1.3. Conduct the mixed ANOVA, as specified in the script. Note that if Mauchly's test of Sphericity is violated, then implement correction factors such as Greenhouse-Geisser correction.

5.1.4. Conduct any necessary posthoc tests to examine differences among factors with more than 2 levels, as specified in the coding script. In this example, conduct Bonferroni posthoc tests to compare the different types of nesting material.

NOTE: If only using one nesting material, then do not incorporate a within-subjects factor. If collecting data at multiple time points, however, incorporate these repeated-measures as if levels of a within-subjects factor.

NOTE: In some cases, wildtype mice may have perfect scores and thus will exhibit a ceiling effect, leading to a non-normal distribution[2]. Consider appropriate statistical tests (e.g., nonparametric tests with alternative measures of central tendency and dispersion), normalization methods, or robust approaches (mixed effects modeling for repeated measures) when analyzing data.

REPRESENTATIVE RESULTS:

Results from the four different nesting materials provided to wildtype and APOE e4 mice are explained as follows. Based on existing criteria, the ICC showed a strong agreement among all three coders for all four nesting materials (shredded paper $ICC = 0.94$; square $ICC = 0.91$; bedding $ICC = 0.87$; twist $ICC = 0.87$); therefore, the three scores were averaged together to create a single score for each material provided. A 2 x 4 mixed ANOVA yielded a significant main effect of genotype, $F(1, 19) = 31.30$, $p < 0.01$, $\eta p^2 = 0.62$. Across all four provided materials, wildtype mice scored higher on nest quality (3.18 ± 0.20) compared to APOE e4 mice (1.98 ± 0.16). In addition, the mixed ANOVA yielded a significant main effect of material, $F(3, 57) = 57.48$, $p < 0.01$, $\eta p^2 = 0.75$. Pairwise comparisons with Bonferroni correction showed that the shredded paper (4.11 ± 0.20) was rated significantly higher ($p < 0.05$) in quality than the square (1.95 ± 0.21), bedding (2.21 ± 0.21), or twist (1.94 ± 0.20) materials, with no differences seen between the square, bedding, and twist materials (all $p > 0.99$). There was no significant interaction between genotype and material. Data are shown in **Figure 3**.

Other experiments from our laboratory have yielded similar results in early-onset models of

Alzheimer's disease. For example, as demonstrated in **Figure 4**, 5.5-month-old P301L rTg4510 (hTau) mice built significantly worse nests out of shredded paper compared to their age-matched wildtype counterparts[31]. Likewise, dual J20 (hAPP)/hTau and single hTau mice built poorer nests out of shredded paper compared to their wildtype and CAMKIIa-promoter only counterparts, both at 3.5- and 7-months of age[32] (data not shown).

FIGURE LEGENDS:

Figure 1: **Cage set-up for each material.** All mice completed one trial with each type of material for a total of four trials. Corncob bedding lined the entire bottom of the cages containing a paper twist, a pressed cotton square, and shredded paper. Soft cob bedding was evenly dispersed across the cage to encourage mice to separate out the small cotton squares from the corncob.

Figure 2: **Example of scoring using the criteria for shredded paper, the preferred material.** From left to right: **1** – the shredded paper remained untouched; **2** – some of the paper was constructed into a nest but most of the pieces remained scattered; **3** – a noticeable nest was constructed, but several pieces were still scattered; **4** – most pieces were used for the nest, but some pieces were scattered near the nest; **5** – all pieces were used to make the nest. Note that in the photographs, the animal's assigned number is shown to avoid unblinding. The color of the card is random. Researchers should allow the mouse to remain in the cage in order to avoid startling the mouse, which could potentially disperse the nest.

Figure 3: Rated quality of nests made from different materials provided to wildtype and APOE e4 mice. A main effect of genotype (*$p < 0.01$) demonstrated that wildtype mice consistently built better nests made from the shredded paper, pressed cotton squares, paper twist, and soft cob bedding compared to APOE e4 mice. The main effect of material (**$p < 0.01$) also demonstrated that nests constructed from the shredded paper were rated higher in quality compared to the three other materials. The shredded paper had the highest inter-rater reliability measure as assessed by ICC.

Figure 4: Shredded paper nests built by wildtype and htau mice, on or off zinc water. (**A**) Representative nests constructed by 5.5-month-old wildtype and htau mice, on control (tap) water or water supplemented with 10 parts per million zinc (a biometal implicated in Alzheimer's disease). Nests were scored on the 1 - 5 scale using the specified criteria. From left to right: wildtype + zinc water (4.54±0.52); wildtype + control water (4.15±0.80); htau + control water (3.08 ± 0.64); htau + zinc water (2.46±0.97). (**B**) A genotype*water interaction demonstrated that htau mice on zinc water constructed significantly worse nests compared to the other groups (*); in addition, simple effects showed that htau mice on zinc water built worse nests than htau mice on control water. Wildtype mice on control water and zinc water-built nests of higher, and similar quality, compared to their htau counterparts. Reprinted from Journal of Alzheimer's Disease, Vol 64, Craven, KM, Kochen WR, Hernandez CM, Flinn JM, Zinc Exacerbates Tau Pathology in a Tau Mouse Model, 671-630, Copyright (2018), with permission from IOS Press[31]. The publication is available at IOS Press through http://dx.doi.org/10.3233/JAD-180151.

Supplementary File 1: Supplemental Coding File – nesting.R

Supplementary File 2: AD Study nesting scores.csv

DISCUSSION:

Nesting is an evolutionarily important rodent behavior and has been used to assess the activity of daily living and general well-being in mice[2]. The ease to conduct the test, its reliability, and its face validity makes nesting a practical complement to many behavioral tests such as burrowing, circadian rhythm, and grooming. But, as nesting becomes more commonly utilized in the laboratory, the various combinations to conduct, quantify, and interpret nesting increase. As such, further research is needed to explore the best methodologically and practically sound procedures for nesting, such that the validity and reliability of the assay are not sacrificed for costs, time of testing, and other procedures that reduce testing burden.

Nest building quality is indeed sensitive to the type of bedding provided as well as genotype. Overall, wildtype mice built significantly better nests compared to APOE e4 mice regardless of the nesting material; however, both wildtype and APOE e4 mice build significantly higher quality nests out of shredded paper compared to the three other nesting materials. Other studies provide corroborating evidence regarding the shredded paper: mice constructed more complex nests out of shredded paper strips than with pressed cotton squares, tissue, and aspen bedding[33]. Furthermore, nests constructed out of shredded paper strips have been qualitatively evaluated as more "naturalistic" than those made of other materials, which are characterized by the shape of the nest itself and the height of the walls around the nest cavity in order to form a dome[6,33]. As such, selection of proper material for this assay is critical in order to better observe natural behavior in a relatively controlled environment, i.e., the laboratory setting. More importantly, although this protocol assessed nesting once at 9 – 12 months of age, we emphasize that nesting should be conducted at several ages. The simplicity of this protocol permits it to be conducted several times, ideally before the onset of deficits that accompany neurodegeneration. Repeated measurements afford the opportunity to document the likely causative role of neurodegeneration in decreased nesting ability.

Natural nest building has been shown to differ among mice of different background strains[34], and as such, the overall quality and shape of the nest may differ not due to the transgene of interest, but due to the background strain. For example, the ancestors of C57BL/6 mice were considered "hole" nesters, whereas the ancestors of BALB/C mice were considered "surface" nesters[35]. Mice on the C3H background or crosses with this strain, such as the hybrid C3H/He-C57BL/6 with E4 used in Graybeal et al.[9] are also considered to be poor nesters; thus, researchers should strongly consider using control mice on the same background as transgenic mice, which would overall improve the direct, causative role of the transgene(s), rather than the background, in neurodegeneration and subsequent deficits in nest building behavior.

Some experiments often utilize a single scorer to qualitatively judge the complexity of the nest; however, we make the argument to include more scorers, and more importantly, scorers blind to experimental conditions. Through this approach, we utilized three independent, blind scorers and an intraclass correlation to assess the agreement among the scorers, who, with basic training, yielded high intraclass correlations which were indicative of high inter-rater reliability and strong agreement regarding nest quality. Furthermore, scores of nests composed of shredded paper and cotton squares had the highest intraclass correlations, an indication of stronger agreement and greater consistency among the scorers. The strong agreement also provides justification to average the multiple scores together, a strategy haphazardly implemented in behavioral research. Although this strategy requires more individuals and, presumably, more time to score nests, it effectively reduces bias in qualitative assessments such as nesting.

The nesting materials used in this assay were approximately equal in price except for the soft cob bedding. Commercial bedding for nesting may be economically resourceful for some experimenters; however, Martin et al.[15] note that cotton squares, when compared to other materials such as crinkled paper, is one of the most expensive materials available for purchase. This may be due to easy availability, storage, and administration, but other researchers may opt for similarly valid and reliable materials, especially when challenged by large numbers of animals in a facility, the number of nesting trials, time restrictions for scoring nests, and high cage costs. Thus, shredded paper may be a more feasible and appropriate option. In addition, data capture for our method can be conducted immediately (i.e., scoring in person), although photographs should be highly considered in order to record, save, and re-quantify nests, if desired for the purposes of assessing inter- and intra-rater reliability at later times. As noted, we strongly emphasize the inclusion of multiple scorers for “test, re-test” practices to assess for agreement, as these methodologically sound procedures are often overlooked.

In conclusion, we believe that the methodology and statistical considerations (i.e., intraclass correlation) for this assay may be of interest for experiments assessing the quality of living, general well-being, and activities of daily living in mice.

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DISCLOSURES:
The authors have nothing to disclose.

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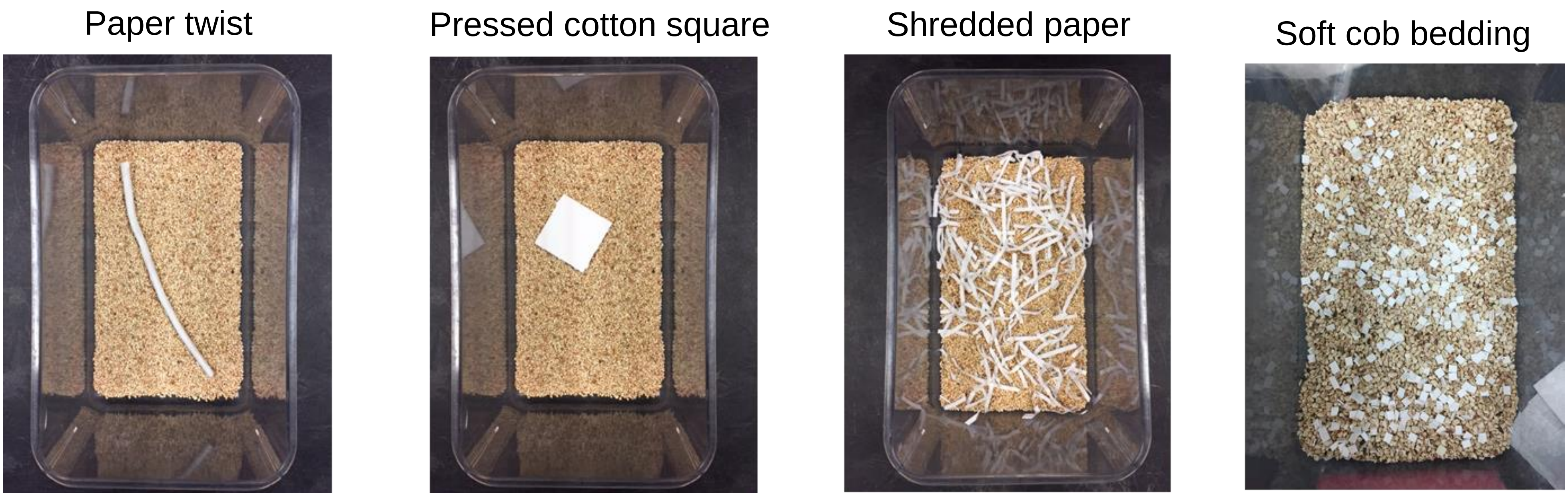

Paper twist
Pressed cotton square
Shredded paper
Soft cob bedding

Figure 2 - scoring for shredded paper

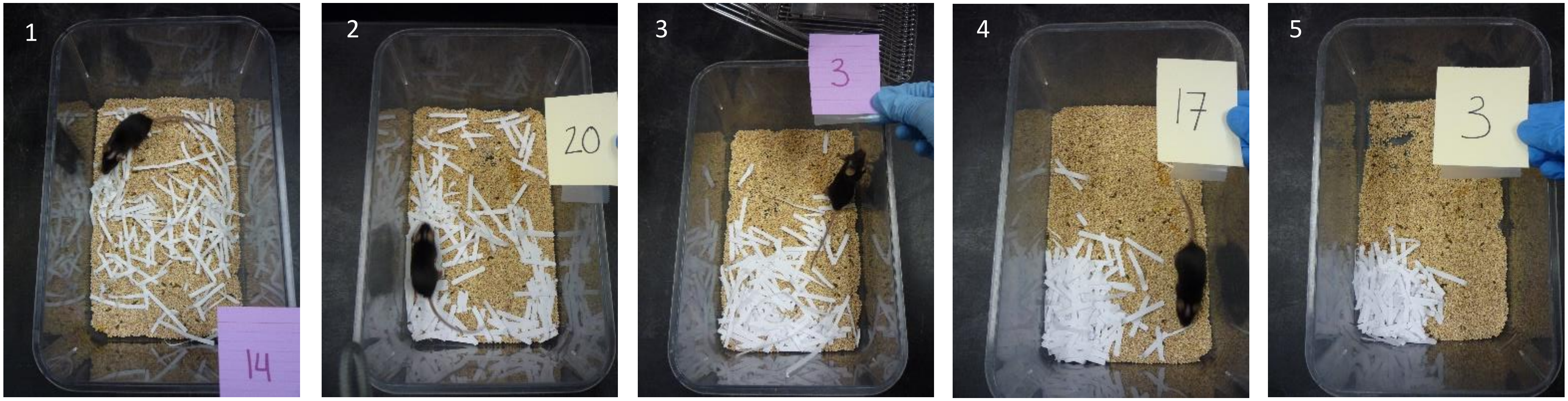
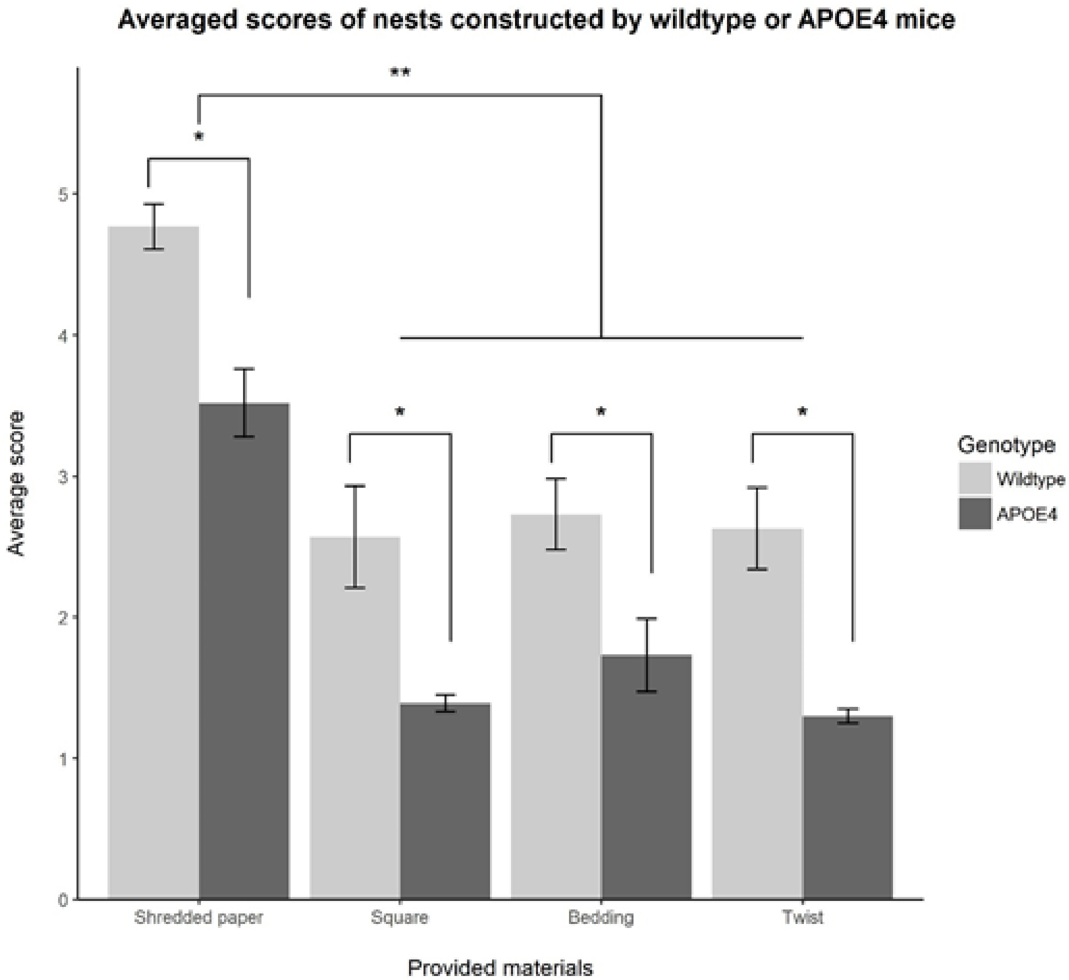
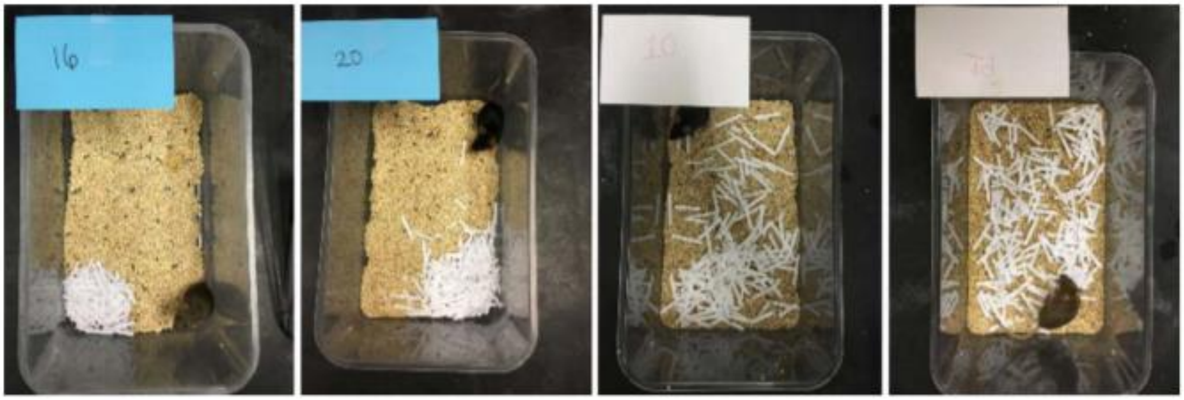


Figure 3 - representative results



A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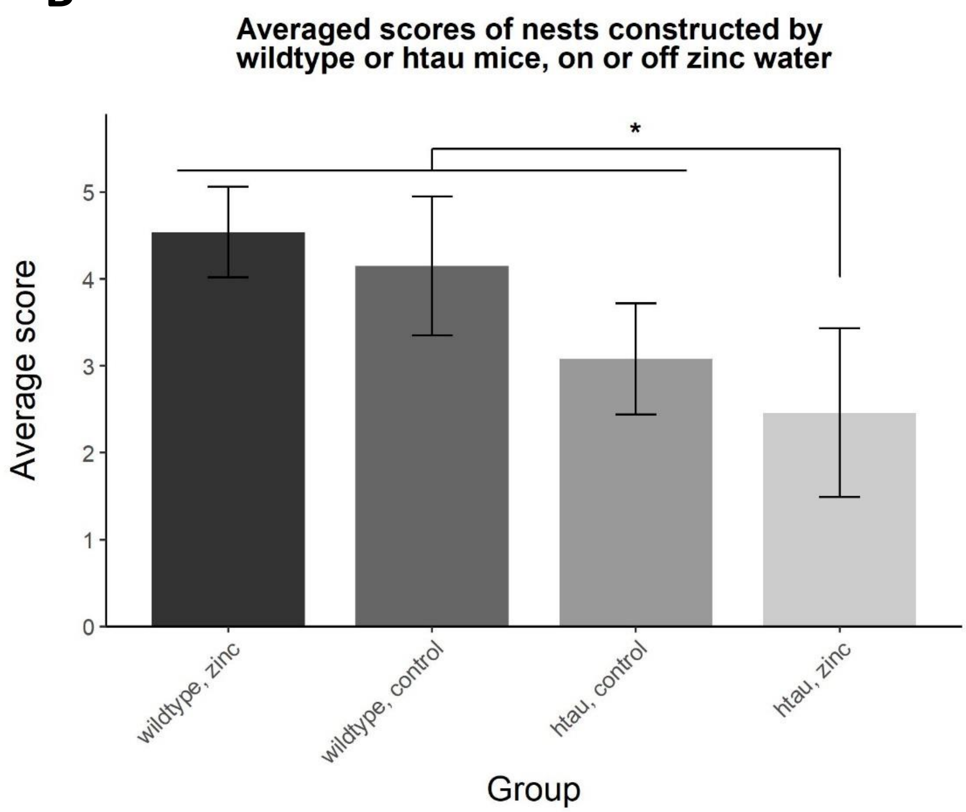
B


Averaged scores of nests constructed by wildtype or htau mice, on or off zinc water
*
Average score
0
1
2
3
4
5
wildtype, zinc
wildtype, control
htau, control
htau, zinc
Group

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Corncob bedding	Envigo	7092	1/8 in bedding for cotton squares, shredded paper, and paper twist trials
Cotton Squares	Envigo	Iso-Blox	
Diamond Twists	Envigo	7979C.CS	Paper twists used in protocol
Mouse - APOE4 e4	Jackson Laboratories	#012307	Homozygous APEO4 e4 mouse bred with hemizyous J20 mouse
Mouse - C57BL/6J	Jackson Laboratories	#000664	Wildtype mouse for controls
Mouse - J20	Jackson Laboratories	#006293	Hemizygous mouse bred with the homozygous APOE e4 mouse to generate cross
Rstudio	R Core Team	V1.1.463	Run with R version 3.5.3 (2019-03-11) -- "Great Truth". Note: additional R Packages are included in provided code and can be installed from CRAN
Soft Cob	Envigo	7087C	

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Reviewers' comments:

We would like to thank our reviewers for their thorough review and thoughtful feedback for our manuscript. We have revised the manuscript based on their suggestions and have addressed their comments below.

Reviewer #1:

1. In the introduction is rat and mouse nest building behavior is commented and it is referred to a citation by Deacon. From my experience one cannot say rats and mice, both, build nests in the same way - suitable to be taken as an indicator. In my institution we, contrary, observe no real nest building behavior in rats and search for new ways how to enrich their cage environment.
    - We have removed the reference to rats, as we agree that the provided citation does not accurately apply to rats, and overall, to this protocol.

2. Paragraph 1.1.: was there any consideration of same-sex husbandry?
    - We note that literature has suggested that the sex of personnel involved in animal husbandry can influence animal behavior (Sorge et al., 2014), and should be taken into consideration for behaviors that require active involvement of a human experimenter (for example, Morris water maze, open field test, or elevated zero maze usually requires an experimenter to place the mouse in the pool, arena, or maze, respectively). In our protocol, however, mice construct their nests over a period of 24 hours undisturbed; thus, we believe that husbandry staff may not have an immediate effect on their behavior. In addition, we did not include literature regarding the effect of sex of human experimenters/husbandry staff in the manuscript for brevity.

3. Paragraph 2: I was wondering if agonistic behaviors of males were considered. Indeed this is discussed later in the manuscript. Is there any way to avoid aggressive behavior when using the protocol?
    - We have added an additional section in the protocol that provides suggestions to acclimate mice and to potentially avoid aggression among cagemates, particular older males.

General:

4. The nest quality is measured by observers - would it be possible to measure "densitiy", i.e. quality of use by photographic measures?
    - Several methods have been used to score nesting such as using different scaling criteria (Deacon, 2006), a combination of scoring and weighing the nesting material (Deacon, 2012), and scoring the height of domes (Gaskill et al., 2013). We have attempted to use semi-quantitative methods in ImageJ to better quantify the density of nests; however, thresholding images and converting images into 8-bit, gray-scale images not only proves futile, but also increases workload and data processing time. Here, we emphasize that our approach not only facilitates data collection and analysis, but also will potentially reduce the cost associated with the test (if using shredded paper), reduce time burden, and improve the validity and reliability of the assay (again, if using shredded paper).

5. I didn't find any information about the abiotic conditions of the room, which may be very informative - especially with regard to nest building

- We have added a statement at the beginning of the protocol stating that all procedures followed the guidelines set forth by the Assessment and Accreditation of Laboratory Animal Care (AAALAC). We hope that this provides some idea of the criteria of our laboratory standards.
- We have also included information about the typical environmental conditions in the testing room (which are identical to the housing room).

6. Also I miss information about group sizes.
    - This information was added to the protocol section.

Reviewer #2:

Major Concerns:
None

Minor Concerns:

Some breeds might go off the top of the current scale as they tend to create more fully formed nests that would be best captured by having a level 6 in the scoring.

- We believe that shredded paper (out of the tested materials in this protocol) yields the most consistent results and is a more appropriate nesting material. As such, we have used only shredded paper in our behavioral experiments, but occasionally, we provide pressed cotton squares and paper twists as forms of enrichment. From numerous observations in our lab, the nests constructed out of shredded paper remain flat; likewise, any nest constructed from the square or twist remains flat (although nest building out of these materials are not consistent). Overall, these nests are usually not constructed into domes (see Gaskill et al., 2013 for dome scoring). We restricted the potential scores to 1 – 5 to be as consistent as possible with previous literature (see Deacon (2006) on which our criteria are based) and to avoid increasing the complexity of nest scoring. Overall, we believe that this method will streamline the testing set-up, data collection, and subsequent analyses.
- We note that we have included additional references regarding alternative nesting assessments and scoring procedures (Hess et al., 2008; Gaskill et al., 2013).

Reviewer #3:

Major Concerns:

1. Lines 65-93: The authors provide evidence why nest building might be a good indicator for neurodegenerative behavior. However, some very important background information is missing here:
   - Natural nest building behavior differs between mice that derive from different background strain. For example, the wild ancestors of C57BL/6 mice were hole nestlers whereas the ancestors from BALB/c mice were surface nestlers. Hence, the shape of the nests from these mice differ (plate shape versus dome shape; Van de Weerd et al, 1998), and a cross from C57B/6 with BALB/c is a generally very poor nestbuilder (Van Oortmerssen, 1970).
     - We thank the reviewer for providing references and bringing background strain to our attention. We have added the references to the discussion section and emphasize that researchers should heavily consider using mice of the same background strain for their studies.
   - The C3H strain, mentioned in line 83 to be a poor nester, originates from a cross between Bagg and DBA mice. It may be interesting to find out the origin of Bagg and DBA to see whether the poor nesting behavior of the C3H is similarly due to mixing up genes from surface and hole nestlers.
   - The research mentioned in lines 73-75 compares C57BL/6 with transgenic mice that have a mixed background (C57BL/6 with C3H). Again, the mixed background, rather than the transgene might be responsible or will at least be a confounder for poor nesting behavior.
     - We include commentary regarding the C3H mouse as a poor nester (in reference to prior research from our lab); however, we have opted to not include additional background information on the C3H mouse for brevity. In addition, the sources of the breeders used to generate the APOE4 transgenic mice recommend that the C57BL/6J mouse be used as a control mouse.
   - Deer mice (mentioned in lines 86-93) are distant relatives from Mus Musculus all together, wild deer mice may build nests in trees or burrows so comparisons with Mus Musculus based on innate behavior should be made with caution.
     - The Introduction has been revised to further clarify that deer mice are distant relatives to the C57 mouse. We also included, as suggested in the original publication (Martin et al., 2016), that deer mice often receive the same husbandry care as typical laboratory mice, and that these mice are capable of building nests despite differences in their natural habitats (that the reviewer has kindly provided). We have also provided additional citations regarding deer mice.
   - Main point is that it is very important to know the background strain of the transgenic mice that are tested, know how they will make their nests and make sure that the wildtype counterparts you use are the right controls in terms of genetic background.
     - We hope that our revisions to the Introduction and the Discussion sections adequately address the need to use mice of the same background strain.

2. Based on the issues raised above, it is very important that if nest building is used to measure neurodegenerative disease, then the behavior should be tested at several ages within the strain with neurodegeneration and show a decrease in nest building capacity. Merely comparing wild type with transgenic strains may yield differences, but whether this is the result of neurodegeneration, cannot be concluded.

- It was our intention for our protocol to primarily emphasize the selection of the best nesting material and appropriate scoring methods and statistical analyses. We also agree with the reviewer's comment and have added it to the Protocol and Discussion sections.

Protocol

1. The following information is missing:
   - How many animals were used in this example
   - Details of the animals (gender, age)
   - How were animals housed in the stock room: social? How many animals per cage?
   - Were animals provided with enrichment? If so what type? Etc.
     - Additional information has been added to the Protocol section.

2. Animals are transferred to the room and cage with nesting material. The authors should be aware that mice, transferred to a novel environment, need time to adjust. In this experiment, they are transferred into a novel cage without their cage mates, provided with nesting material they may or may not be familiar with. The mice will need time to adjust. Adjustment to new surroundings, or test setup may take up to 2 weeks for all physiological levels to return to normal. This may seriously interfere with te mice behavior and hence with the outcome of the test. A far more elegant way would be to leave the mice in their home cage and provide them with the different nesting materials in randomized order. What is the reason that the researchers chose to move the animals out of their familiar surroundings? Did the authors consider training sessions before performing the actual test?
   - We have added to the Protocol section potential methods to reduce anxiety and aggression in mice.
   - There are several issues with some of the comments above. First, it would be ineffective to allow 2 weeks for all physiological levels to return to normal after transport, as nesting can be done in 24 hours. This would prove to be exceptionally difficult for studies that require multiple behavioral assessments that require a strict time schedule, or for studies that use manipulations that have acute effects that may dissipate with time (i.e., the effects of a single mild traumatic brain injury).
   - Second, group-housing would not allow for each individual mouse to be assessed unless 24 hour surveillance and animal tagging (such as implanted chips and tracking software) were utilized. Although possible, it is not feasible for all labs, especially for those that lack such equipment and personnel.
   - We did not consider training sessions, as nesting is considered an innate behavior in mice (Deacon, 2006).

3. (lines 164-167): The authors mention: …although not required… and ..if desired… Why? It is very important that scorers are trained together, that they have the opportunity to discuss several test nests together prior to their independent scoring of the real nests. This will increase interobserver reliability. Furthermore, to test intra-observer reliability, several nests that were scored at the beginning of the scoring session should (blindly) be rescored again at/near the end of all scoring sessions. This reliability should also be at least 0.8
   - We have conducted and published several studies using scorers that have yielded valid, reliable scoring using our criteria, even with minimal training (i.e., shown examples of scores, as provided in Figure 2); however, we agree with the reviewer

that scorers should ideally be trained together, or receive training in the same manner. We have revised the Protocol section to reflect this suggestion.
- Test-retest considerations have been added to the Protocol section.
- The *ICC* value corresponding to strong agreement has been specified in the Protocol section.

Scoring

1. To what extend can be assumed that normality of data is present since the range in possible scores is very small (1-5), which may lead to skewed data? See also lines 76-77: If the floor-ceiling effect means that there are no or hardly any 1 and 5 scores, this would mean an even smaller range in scores.
   - We have included additional information on potential ceiling effects that some wildtype mice exhibit. As suggested by Deacon (2006), non-parametric methods may be used to address some of the issues with normality and small sample sizes. In our data, we did not violate normality; therefore, we were able to conduct a mixed ANOVA.

2. Merely comparing wild type with transgenic strains may yield differences in nest scores, but whether this is the result of neurodegeneration, cannot be concluded. Based on the issues raised in the introduction part, it is very important that if nest building is used to measure neurodegenerative disease, then the behavior should be tested at several ages within the strain with neurodegeneration and show a decrease in nest building capacity. The authors do not provide evidence for this, although they may have performed these experiments?
   - We have included this in the Protocol section and have addressed the fact that we conducted this assay once (at 9-12 months of age) as a limitation in the Discussion section.

Minor Concerns:

1. Line 60: In mice, activities of daily living are commonly examined via circadian wheel-running,…: Please provide references, preferably from a review since the authors use the word 'common'. If common in neurodegenerative research, please add.
   - We have added four citations (2 experiments from our lab, 1 outside of our lab, and 1 review paper).

2. Lines 76-77: What is meant with: the typical ceiling and floor effect? Do the authors mean that these mice do not score 1 or 5? This needs a bit explanation for the reader that is new to these tests.
   - This has been revised into lay terms. In addition, we have rephrased this section to be more specific regarding how variability (i.e., lack of typical high scores for wildtype mice and low scores for transgenic mice) may lead to lack of statistical significance.

3. (lines 169-174): The scale leaves room for interpretation. It is written for the shredded paper. For the diamond twist for example level 2: '…most of the pieces remained scattered', is not a correct description and may lead to confusion for the scorer. Why not say:…most of the nesting material was not used for nest construction.

- We have revised the language such that it is applicable to all materials used in the study.

4. Examples of the other materials scoring levels should be added as well. Alternatively, the authors should consider to advice using the shredded paper as the only nesting material suitable for a proper test set-up.
    - We have explicitly clarified in the protocol that shredded paper is the preferred material for this experimental set up.

5. Will scorers be allowed to score e.g. 1.5 as a score? And if so, how consistent is that?
    - As discussed in Reviewer 2's comments, we restricted the potential scores to 1 – 5 and used whole numbers in order to be as consistent as possible with previous literature (see Deacon, 2006, on which our criteria are based). Overall, we believe that this approach will avoid unnecessary complexity to nesting, and will streamline the procedure, data collection, and subsequent analyses.

6. Figure 2: The pictures show purple and yellow pieces of paper. Although these may have been used randomly, this may lead to the assumption that a color is assigned to the strains. To avoid unwanted bias in this respect, use same colored paper in the photographs.
    - The reviewer is correct – the colors of the cards are all random (those notecards happened to be available in the lab that day). These are some of the best pictures that exemplify the criteria for our scoring system, and other photos that are similar also have random colors. Unfortunately, we cannot change these.

7. Figure 2: Do not give researchers the choice to remove the mouse prior to photographing. The authors give strong reasons why this may interfere with the nest.
    - We thank the reviewer for agreeing with us – the option to remove the mouse has been removed from the protocol.

Discussion:

1. Line 256: ….which the authors note that cotton squares remain one of the most commonly used nesting materials. … This is new to me, and may be very institute dependent, or possibly country dependent? What source did the authors use for this information?
    - This has been revised to appropriately describe a key point discussed in Martin et al. (2016) – that the cotton square, compared to the other tested materials in their experiment, is a common material used for nesting, and it was considered one of the more expensive materials tested (compared to crinkled paper).

Vineeta Bajaj <vineeta.bajaj@jove.com>

Permission for reprinting published figures; updated manuscript

1 message

Caroline Neely <cneely3@masonlive.gmu.edu> Mon, Jun 24, 2019 at 9:10 PM
To: Vineeta Bajaj <vineeta.bajaj@jove.com>

Good evening, Vineeta,

My apologies for the delay, and thank you for your patience. Attached is our updated manuscript which we believe contains all additions and edits that addressed the comments.

In addition, please see the email exchange between Mrs. Koolbergen of IOS Press and myself below concerning the permission to reuse figures, which we were able to obtain.

Please let me know if you require any additional information. Thank you!

Best,

Caroline L.C. Neely, PhD
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From: Carry Koolbergen <C.Koolbergen@iospress.nl>
Sent: Friday, June 21, 2019 8:23 AM
To: Caroline Neely
Subject: RE: Permission for reprinting published figures

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Yours sincerely

Carry Koolbergen (Mrs.)
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Van: Caroline Neely [mailto:cneely3@masonlive.gmu.edu]
Verzonden: woensdag 12 juni 2019 14:52
Aan: Carry Koolbergen <C.Koolbergen@iospress.nl>
CC: Kristen Craven <kcraven2@masonlive.gmu.edu>; Jane Flinn <jflinn@gmu.edu>; Carlos Hernandez <chernan8@masonlive.gmu.edu>; Bill Kochen <wkochen@masonlive.gmu.edu>
Onderwerp: Permission for reprinting published figures

Good morning, Mrs. Koolbergen,

I'm emailing in regard to the reprint permission of one of my colleague's publication in the *Journal of Alzheimer's Disease* (doi: 10.3233/JAD-180151). We would like to re-use Figure 2 (Title: Nests built from crosscut paper.) for a methods publication in a different journal, which requires email correspondence granting permission. The authors on the paper are cc'ed, and the first author has given permission to use Figure 2 in this paper.

Please let me know what the proper steps are in order to obtain permission.

Thank you.

Best regards,

Caroline Neely, PhD

--
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