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Wheel running and environmental complexity as a therapeutic intervention in an animal model of FASD

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Abstract:	Aerobic exercise (e.g., wheel running (WR) extensively used in animal research) positively impacts many measures of neuroplastic potential in the brain, such as rates of adult neurogenesis, angiogenesis, and expression of neurotrophic factors in rodents. This intervention has also been shown to mitigate behavioral and neuroanatomical aspects of the negative impacts of teratogens (i.e., developmental exposure to alcohol) and age-related neurodegeneration in rodents. Environmental complexity (EC) has been shown to produce numerous neuroplastic benefits in cortical and subcortical structures and can be coupled with wheel running to increase the proliferation and survival of new cells in the adult hippocampus. The combination of these two interventions provides a robust "superintervention" (WR-EC) that can be implemented in a range of rodent models of neurological disorders. We will discuss the implementation of WR/EC and its constituent interventions for use as a more powerful therapeutic intervention in rats using the animal model of prenatal exposure to alcohol in humans. We will also discuss which elements of the procedures are absolutely necessary for the interventions and which ones may be altered depending on the experimenter's question or facilities.
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Wheel running and environmental complexity as a therapeutic intervention in an animal model of FASD

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SHORT ABSTRACT:
Cardiovascular exercise and stimulating experiences in a complex environment have positive benefits on multiple measures of neuroplasticity within the rodent brain. This article will discuss the implementation of these interventions as a "superintervention" which combines wheel running and environmental complexity and will address the limitations of these interventions.

LONG ABSTRACT:
Aerobic exercise (e.g., wheel running (WR) extensively used in animal research) positively impacts many measures of neuroplastic potential in the brain, such as rates of adult neurogenesis, angiogenesis, and expression of neurotrophic factors in rodents. This intervention has also been shown to mitigate behavioral and neuroanatomical aspects of the negative impacts of teratogens (i.e., developmental exposure to alcohol) and age-related neurodegeneration in rodents. Environmental complexity (EC) has been shown to produce numerous neuroplastic benefits in cortical and subcortical structures and can be coupled with wheel running to increase the proliferation and survival of new cells in the adult hippocampus. The combination of these two interventions provides a robust "superintervention" (WR-EC) that can be implemented in a range of rodent models of neurological disorders. We will discuss the implementation of WR/EC and its constituent interventions for use as a more powerful therapeutic intervention in rats using the animal model of prenatal exposure to alcohol in humans. We will also discuss which elements of the procedures are absolutely necessary for the interventions and which ones may be altered depending on the experimenter's question or facilities.

INTRODUCTION:

Rearing in different environments has long been known to cause changes in various measures of neurological wellness. Many studies look at the beneficial effects of rearing in a complex environment (EC) starting with groundbreaking research by Diamond and Rosenzweig e.g., [1,2] and Greenough e.g., [3,4]. EC has been demonstrated to have an undeniable positive effects on synaptic and cellular changes in the brain [5-7]. EC can affect a multiplicity of brain regions including the hippocampus [8,9] and visual cortex [10,11], ventral striatum [12,13], as well as brain-wide neuroimmune function [reviewed in 14]. Particular interest has developed from the studies on hippocampus when it was demonstrated that EC can increase the survival rate of adult-born granule cells of the dentate gyrus through dendritic plasticity [9,13]. This last point has gathered much interest due to the growing body of literature indicating that cardiovascular exercise promotes adult neurogenesis in both the healthy and damaged brain [15-18]. Wheel running (WR) is an easy to implement form of voluntary cardiovascular activity that has been shown to be beneficial in rodent models of neurological disorders or aging [17,19,20]. WR affects the expression of growth factors in both the central and peripheral nervous system [21-23].

Combining (subsequently) WR and EC into a "superintervention" (WR-EC) (i.e., 12 days of WR followed by 30 days in EC) provides a robust increase in hippocampal adult neurogenesis and increased survival of the newly proliferated cells [8], the effect that in the animal model of FASD is not achieved by individual components (see below). Since both components of WR-EC affect a diverse array of structures within the brain [13] (WR reviewed in [22], EC reviewed in [24]), implementation of this intervention can easily be applied to rodent models of both developmental and later life onset models of neurological impairment (e.g., neonatal alcohol exposure, aging, early life stress).

Integration of WR-EC in the adolescent and early adult periods (i.e., postnatal days 30-72) can ameliorate some of the negative effects of a rat model of fetal alcohol spectrum disorders (FASDs) [8]. A collection of studies have demonstrated that rodents exposed to alcohol from postnatal day (PD) 4 through 9 display significant deficits in neuroanatomical measures such as dendritic complexity [25], cerebellar development [26,27] and neuroimmune responsiveness [28] as well as manifestations of impaired learning and memory [29-31]. Even a reduced amount of alcohol exposure within this time window (i.e., PD 7 through 9) can lead to deficits in learning and memory in adolescent and adult rats [32] while some structures no longer see significant neuroanatomical impairment [27]. Many of these deficits - in addition to behavioral impairments in hippocampus-dependent tasks - have been mitigated following exposure to this WR-EC paradigm [8,33] or WR alone [25,31]. Although WR alone has been a widely used intervention, the combination of WR-EC has not yet been utilized in the literature despite its ability to sustain the relatively shorter-term benefits of WR [8]. This article will discuss the implementation of the WR-EC intervention during adolescence. Although this paradigm is used in the context of early postnatal alcohol exposure, it can be introduced to various rodent models to assess brain potential for neuroplasticity in the models of brain disorders.

PROTOCOL:

Ethics Statement: The following protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Delaware.

1. Developmental Exposure (or Model of Binge-Like Ethanol Exposure)

1.1. On PD3, determine the sex of each animal and cross-foster any animals if necessary to keep litter size (8 animals) and sex distribution (4 males : 4 females) consistent within each litter.

Note: It is important to keep litter size and sex distribution as consistent as possible to avoid experimental confounds. Although this protocol uses 8 pups (4 males and 4 females) per litter, alternative litter sizes or sex distributions may be tailored to the needs of the experimental design.

1.2. Subcutaneously inject a small amount of black India ink into the paws to identify animals within each litter.

1.3. Pseudo-randomly assign litters as experimental (containing 50 % alcohol-exposed (AE) and 50 % sham-intubated control (SI) pups) or suckle control (SC) (animals that do not undergo any intubation, tail clipping, or separation protocols from PD 4-9 except for daily weighing and ear-punching).

1.3.1. To retain consistent group size, assign twice as many experimental litters as SC litters.

1.4. Weigh each animal then return it to its home cage. Animal weighing should occur daily during the intubation period (PD 4-9).

1.4.1. Remove the whole litter from the dam.

1.4.2. Place pups on heated pad.

1.4.3. Record the weight of each individual pup.

1.5. On PD4, after weighing each animal calculate the necessary alcohol amount for a total of 5.25 g/kg/day per each animal (based on pup weight from step 1.4)[8].

1.5.1. Administer alcohol as 11.9 % ethanol-in-milk substitute (vol/vol).

1.6. Starting at 9 AM, remove one litter's pups from the mother at a time.

1.7. Administer the ethanol-in-milk to each AE pup[34].

1.8. Sham-intubate each SI pup[8].

1.9. Repeat steps 1.5. through 1.8. for each experimental litter.

1.10. Two hours following the first dose, repeat the dosing procedure (steps 1.5 through 1.8) for a second alcohol dose.

1.11. One and a half hours after the second alcohol dose (the point at which peak daily blood alcohol content is achieved), collect and centrifuge blood from the AE and SI pups via tail clipping for future blood alcohol content analysis [35].

1.11.1. Collect 60 µL of blood.

1.11.2. Place blood in a 1 mL microcentrifuge tube. Centrifuge blood at 1.5 x g for 25 min.

1.11.3. Carefully collect the supernatant serum from the centrifuge tube and save for future blood alcohol content analysis.

1.12. Repeat the dosing procedure (steps 1.5 through 1.8) using milk instead of ethanol-in-milk to prevent nutritional deficits from nursing inability in AE pups.

1.12.1. Perform a total of 2 supplemental milk doses two hours apart on PD4.

1.13. Repeat steps 1.4 through 1.12 on PD5-9.

1.14. Following the final supplemental milk dose on PD9, ear punch all pups for identification in the EC cage.

1.14.1. Coordinate punched ear with some measure of litter number or identifier (e.g., odd numbered litters within a cohort would get their left ear punched while animals from even numbered litters would get their right ears punched). This will make it easier to identify animals in the EC cage should multiple animals from different litters have the same pawmark pattern.

2. Weaning

2.1. On PD23, house all animals in cages of 2 - 3.

2.1.1. Ensure that all animals housed in the same cage are the same sex.

2.1.2. Include one SC, one SI, and one AE animal per cage when possible.

2.1.3. Minimize the number of cage mates that are from the same litter.

2.1.4. Make sure all animals are capable of accessing food and water.

3. Wheel Running

3.1. On PD30, allocate half of the cages with animals to WR. House these animals in cages with a free access to attached stainless steel running wheel.

3.1.1. Ensure that wheels have a counter to assess the total number of revolutions.

3.2. Weigh all animals on PD30 and PD36.

3.3. Check the number of revolutions of each wheel at 9 AM every day.

3.4. Leave animals in their respective housing condition for 12 days.

4. Environmental Complexity

4.1. Prepare the EC cage before 9 AM on the day that corresponds to PD 42 for experimental animals.

4.1.1. Get a 30 ” x 18 ” x 36 ” galvanized steel cage.

Note: The cage should have multiple levels, be capable of supporting the weight of multiple rats, be filled with standard bedding, and have multiple locations to attach water bottles and food dispensers.

4.1.2. Place novel, colorful objects of variable sizes and shapes in the cage.

4.1.2.1. Place 6 large toys in the EC cage. Ensure that each toy is big enough for 3 or more rats to interact with concurrently.

4.1.2.2. Place 6 medium toys in the EC cage. Ensure that each toy is big enough for 3 - 4 rats to interact with concurrently.

4.1.2.3. Place a lot (at least 20) of smaller toys in the EC cage.

4.1.2.4. Use toys of varying colors, shapes, size, etc. Novelty is critical to this intervention (see discussion).

4.1.3. Place two dishes of food at opposite ends of the cage.

4.1.4. Place two bottles of water at opposite ends of the cage.

4.2. At 9 AM on PD 42, weigh all animals and relocate the WR animals to the EC cage. Each EC cage should contain 9 - 12 animals.

4.2.1. Make sure that no animals have both the same pawmark and ear-punch patterns.

4.3. Check all food and water daily.

4.4. Every two days, remove the toys from the EC cage and replace them (according to step 4.1.2.).

4.5. Every three days, clean the EC cage.

4.5.1. Remove the animals from the EC cage and put them in temporary holding cages of 2 - 3 animals.

4.5.2. Remove all of the bedding from the bottom of the cage.

4.5.3. Replace all of the toys according to step 4.1.2.

4.5.4. Replace all of the food and water.

4.5.5. Replace the rats into the EC cage.

5. Collect Tissue

Note: Tissue collection (e.g., perfusion with paraformaldehyde, rapid decapitation) and storage (e.g., freezing, paraffin embedding) can be performed with a variety of methods. The following

will explain the process of perfusion with 4 % paraformaldehyde in 0.1 M phosphate buffered saline (4 % paraformaldehyde in PBS) solution [8].

Caution: Paraformaldehyde is carcinogenic and may also cause skin irritation, allergic skin reaction, or eye damage. Use appropriate eye/skin protection.

5.1 Expose one rat at a time to isoflurane to lightly anesthetize the animal.

5.2 Intraperitoneally inject the rat with 2 mL/kg of Ketamine/xylazine mixture (1.5 mL xylazine mixed with 10 mL of ketamine).

Note: Ketamine and xylazine are both at stock concentrations of 100 mg/mL before combining for injection mixture.

5.3 Once rat is no longer responsive, perfuse the animal with 0.1 M phosphate buffered saline (PBS; pH = 7.2) followed by 4 % paraformaldehyde in PBS (pH = 7.2).

5.4 Remove brain and store in 4 % paraformaldehyde in PBS at 4 °C for 48 h.

5.5 After 2 days, transfer to solution of 30 % sucrose added to 4 % paraformaldehyde in PBS at 4 °C.

REPRESENTATIVE RESULTS:
In order to assess the effect of the super intervention, we must look at the effects of each of its constituent elements - WR and EC – on our measures of interest. Figures 1 through 3 (below) appeared in a previous publication utilizing this paradigm [8]. Figure 4 appeared in a doctoral dissertation [36]. These data illustrate the impact of WR-EC on hippocampal adult neurogenesis in the dentate gyrus. All graphs illustrate group means, with error bars indicating a single standard error from mean. Figure 1 demonstrates increases in cell proliferation following the WR portion of our intervention, indicating that the WR component is robustly capable of increasing cellular proliferation in the DG of the hippocampus in normally developing, early-life stressed, and alcohol-exposed animals. Figure 2 demonstrates the ability of EC to increase survival of adult generated cells in the DG in animals that were exposed to either stress or alcohol neonatally. Figure 3 demonstrates the increase in cells that differentiate into a neuronal phenotype, indicating that WR-EC can increase proliferation and survival of adult-born dentate gyrus granule cells in animals that undergo neonatal exposure to alcohol or intubation stress, implicating it as a therapeutic to rescue deficits in hippocampal adult neurogenesis. Finally, Figure 4 confirms the WR-EC effect on dendritic plasticity: the length of doublecortin-positive dendrites of dentate gyrus' granule cells in AE rats is no longer different from control. Blood alcohol content (BAC) on PD 4 was 321.19 ± 14.03 mg/dl (mean ± SEM), comparable to other studies using this exposure paradigm [28,37]. Previous studies have demonstrated that animals across these treatment groups do not differ in distances run during WR [15].

Figure 1. WR robustly increases cell proliferation in the hippocampal DG. Photomicrographs illustrate differences in cell proliferation in the DG on PD42 (the cessation of WR) as labeled with Bromo-deoxyuridine (BrdU) in AE animals following WR (A) and social housing (B). WR robustly increases cell proliferations irrespective of neonatal treatment (C). A two-way ANOVA

revealed a main effect of housing condition (WR vs. SH) ($F_{1,40}$ = 19.703, $p < 0.001$), while no significant main effect of postnatal treatment (SC vs. SI vs. AE) or interaction between the two factors were observed. Post hoc comparisons were performed as Tukey's tests. All values represent mean ± standard error of the mean (SEM). *$p < 0.05$, #$p < 0.01$. This figure has been reproduced from Hamilton et al., 2012[8].

Figure 2. WR followed by EC rescues deficits in cell survival following neonatal alcohol exposure or sham stress. Photomicrographs illustrate differences in cells labeled with BrdU in AE animals from WR-EC (A) and social housed conditions (B) injected with BrdU on PD41. Socially housed animals displayed a decrease following alcohol exposure relative to suckle controls. Animals undergoing the WR-EC superintervention display increased survival rates of cells proliferating after PD41 in both SI and AE groups (C). A two-way ANOVA revealed a main effect of housing condition (WR vs. SH) ($F_{1,29}$ = 11.402, $p < 0.01$) and a significant interaction between postnatal treatment and housing condition ($F_{1,29}$ = 3.870, $p < 0.05$), while no significant main effect of postnatal treatment (SC vs. SI vs. AE) was observed. A one-way ANOVA within SH animals revealed a main effect of postnatal treatment ($F_{1,19}$ = 3.727, $p < 0.05$) whereas a one-way ANOVA within WREC animals revealed no significant differences between postnatal treatments. Post hoc comparisons were performed as Tukey's tests. All values represent mean ± SEM. *$p < 0.05$, #$p < 0.01$. This figure has been reproduced from Hamilton et al., 2012[8].

Figure 3. WR-EC rescues deficits in neurogenesis following neonatal alcohol exposure or sham stress. Co-localization of BrdU (green) expression and NeuN (red) in hippocampal granule cells. Fluorescent confocal images were acquired following immunohistochemical procedures. BrdU was injected on PD41 tissue was collected on PD72. Both BrdU and NeuN were observed in the DG (A, B). Although SC animals did not show a significant increase in number of proliferating neurons, both AE and SI animals showed an increase in neurogenesis (as indicated by double labeling with BrdU and NeuN) following the WR-EC paradigm compared to socially housed animals (C). A two-way ANOVA revealed a main effect of housing condition (WR vs. SH) ($F_{1,28}$ = 20.48, $p < 0.001$), while no significant main effect of postnatal treatment (SC vs. SI vs. AE) or interaction between the two factors were observed. Post hoc comparisons were performed as Tukey's tests. All values represent mean ± SEM. *$p < 0.05$, #$p < 0.01$. This figure has been reproduced from Hamilton et al., 2012[8].

Figure 4. WR-EC rescues deficits in dendritic complexity of hippocampal DG granule cells. Sholl analyses of dendritic intersections illustrate WR-EC's ameliorative effects on dendritic complexity in the dentate gyrus of adult rats following neonatal alcohol exposure. In social housing conditions, AE animals have a decreased number of DG granule cell dendrite intersections relative to control animals (A). Housing in WREC increases the number of intersections in AE animals relative to socially housed controls (B). AE animals reared in our WREC paradigm display similar numbers of intersections relative to control animals housed in WREC (C). Repeated measure ANOVAs were performed on the data in each graph. Panel A demonstrates a main effect of postnatal treatment ($F_{1,11}$ = 6.265, $p = 0.029$). Panel B demonstrates a trend toward a main effect between housing conditions ($F_{1,6}$ = 4.181,$p = 0.087$). Panel C demonstrates no significant difference between SC and AE animals within the WREC housing condition. All post hoc comparisons were performed as Tukey's tests. All values represent mean ± SEM. ^$p < 0.01$, *$p < 0.05$. This figure has been reproduced from Hamilton, 2012[36].

DISCUSSION:

In the above protocol, we demonstrated an expedient intervention to rescue neuroanatomical deficits following neonatal alcohol exposure. This intervention can be used as a therapeutic in other animal models due to the robustness of each of the components of the intervention. Voluntary cardiovascular activity in the form of WR has been shown to benefit several behavioral outcomes [38,39] and induce functional plastic alterations in brain regions such as the hippocampus (reviewed in [40]). This is in part due to expression of growth factors and other neuroprotective mechanisms in the brain parenchyma in both rodents and humans [21,41]. Supplementing these effects, EC can induce beneficial cellular [6,11,42,43], structural [2] and pharmacological [12,44] change in rodents.

In order for WR to be maximally effective in this particular model of human syndrome, it is critical for animals to have voluntary access to a functional running wheel; daily wheel access should last for an extended amount of time[45] at least 10-12 h per day and preferably 24 h (some adverse effects of withdrawal from the running wheel were reported). This WR paradigm lasts for 12 days to allow for the combination of WR and EC to fit into adolescence and early adulthood. The duration, age at exposure, and modality of exercise (among other factors) can affect the efficacy of exercise as a therapeutic intervention[46], and such critical factors should be considered when planning to implement this protocol or any other WREC paradigm. A key component of this EC paradigm is the novelty of the multiple objects in the environment and social interaction (reviewed in [14,47]). Therefore, it is critical for the items in this paradigm to be replaced every 48 h. Based on the need for multiple items, the interaction with the items and their exploration, and social interaction, we find that our number of unique items, frequency of item replacement, and number of cage mates is sufficient to induce therapeutic outcomes on the neuroanatomical measures that we assess. We found that continuous exposure for 30 days is more appropriate to overcome deficits induced by neonatal alcohol exposure than limited exposure interaction to a novel environment.

The goal of this protocol is to introduce a WREC paradigm that addresses both the cardiovascular exercise and environmental novelty components of plastic intervention. For this reason, we will address the modification that can be made to the paradigm but would caution use of modifications that may alter the ways that animals interact within the paradigm as well as the experimental conclusions that can be drawn. One possible alteration would be the introduction of running wheels to the EC environment. In doing so, it would be difficult to determine the relative contributions of each component. It would additionally be difficult to assure that all animals participate in both the WR components and EC components of the paradigm as housing of 8-10 animals together is required for EC. However, since long-term access to exercise is critical in the efficacy of this intervention [45], further research may address the optimal ratio of WR access to EC access (although the methods in this protocol have shown robust neuroanatomical and behavioral implications [8,33]). Modifications to individual items used within the EC environment are acceptable, but it is critical for the items to be interesting, complex, novel, stimulating and frequently refreshed [14].

This paradigm does contain several innate limitations in our hands, which should be considered when planning to implement this "super intervention". One limitation to the WR component of the paradigm is the inability to assess the distance run by individual animals. One of the obvious and straightforward solutions would be an individual housing of the animals during WR component.

However, it needs to be stressed that individual housing is widely accepted as detrimental to animals and can even directly counteract the beneficial effects of wheel running [48]. An additional alternative (although time-consuming and imperfect) would be to video record the running wheel at all times that the animals have access. This would require a unique identifier for each animal in a cage (e.g., painting unique colors or patterns on the fur of each animal)[49]. This technique would still be subject to confounds of multiple animals utilizing the wheel concurrently. A similar difficulty carries to EC where it becomes difficult to food restrict individual animals (without limiting the time period of food consumption). To reduce the impact of this, we would recommend housing in EC for a full 30 days followed by an immediate food restriction paradigm. Extended amounts of time out of EC could inhibit induced plasticity that occurs during this paradigm.

As mentioned previously, the importance of this article is to allow for consistent characterization of the EC paradigm and its implementation following cardiovascular exercise in the form of WR. Previous EC paradigms have exposed animals to EC housing without exposure to WR [12,50], WR inside of the EC cage for a shorter amount of time [51] or with less animals [52], or the animals were exposed to an EC environment for a longer amount of time with less frequent changing of cage items [13]. It is likely that the beneficial effects of EC require the induced plasticity from WR in a temporally relevant time window to show long-term benefit. In this way, we believe that coupling WR and EC for 12 and 30 days respectively allows for a maximally beneficial and concise intervention.

At this point, the use of this model has been limited to the adolescent and early adult time periods. Further examination of the robustness of this intervention at different stages, and the ontogeny of neuroplastic benefit should be examined further in the future. Additionally, the use of different developmental deficits is greatly encouraged, as this will assist in developing effective therapeutic interventions for individuals afflicted by such disorders. Previous literature has demonstrated independent effects of WR or EC on adult neurogenesis, learning and memory, or anxiety-like behaviors in a genetic mouse model of anxiety [53]. The robustness of these two interventions and the synergistic effect of EC to sustain the short-term effects of increased WR-induced benefits (i.e., hippocampal cell proliferation and neurogenesis) makes it well poised for integration into a diverse range of research questions.

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DISCLOSURES:
The authors have nothing to disclose.

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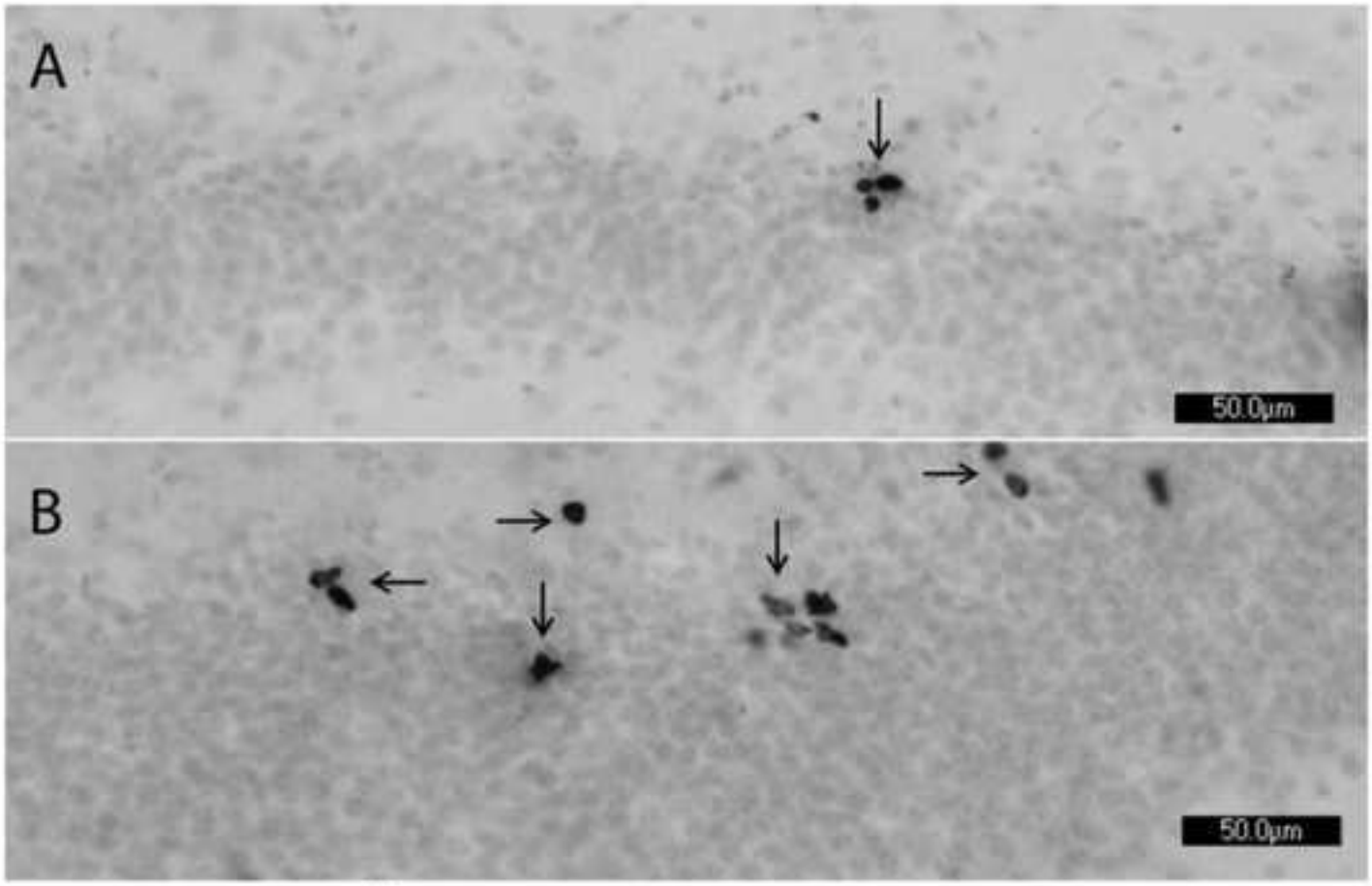

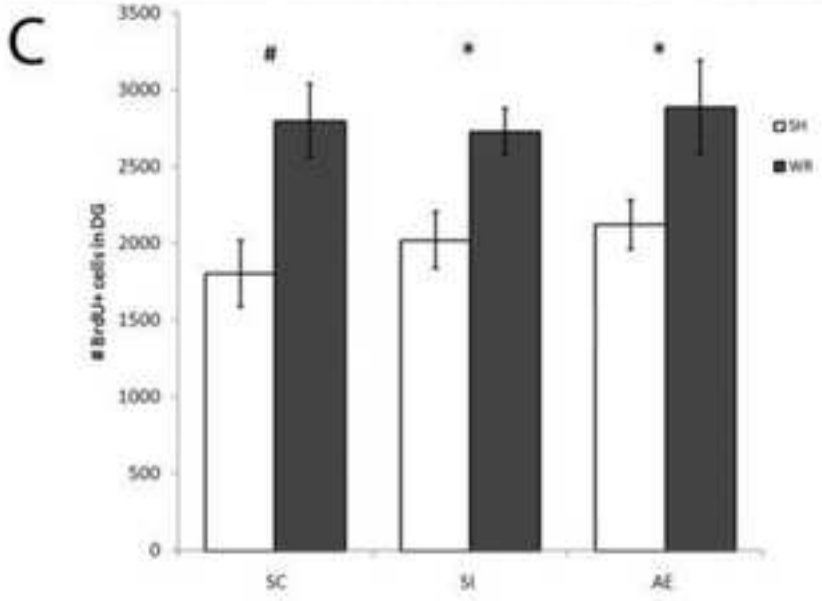

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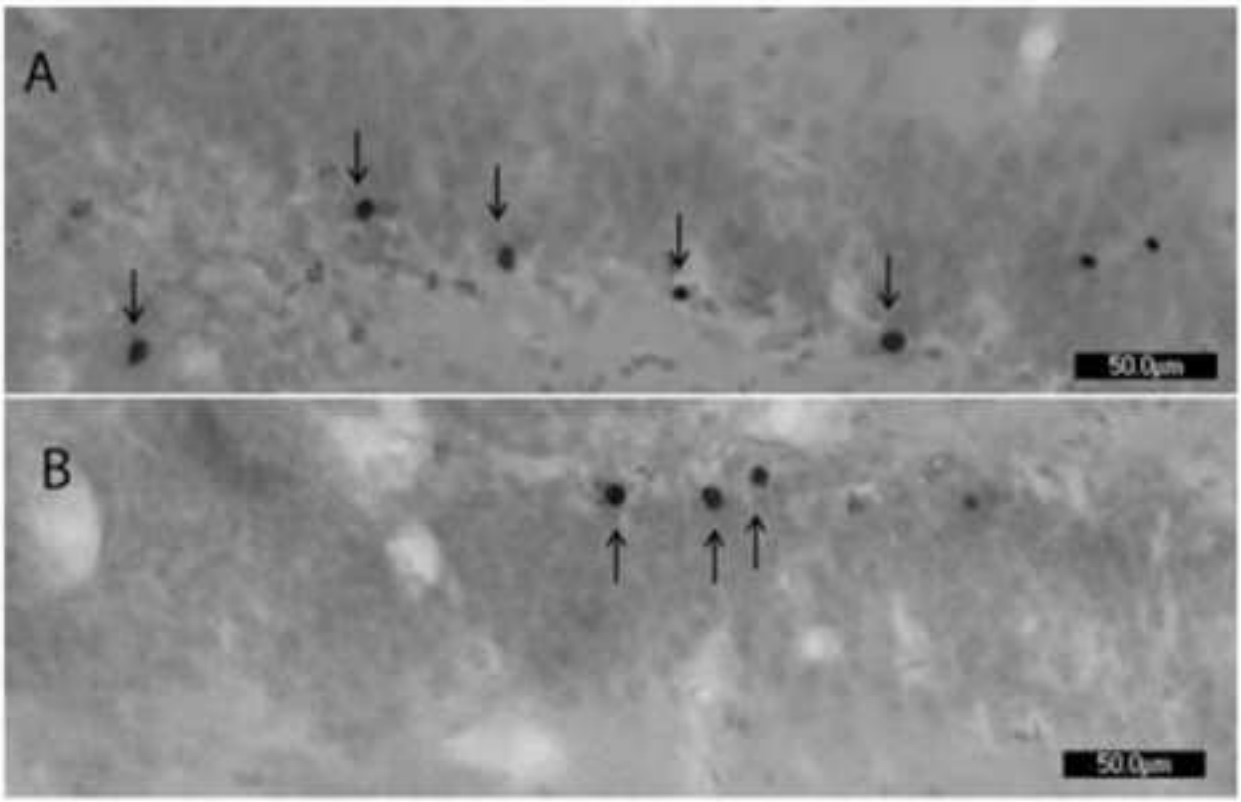

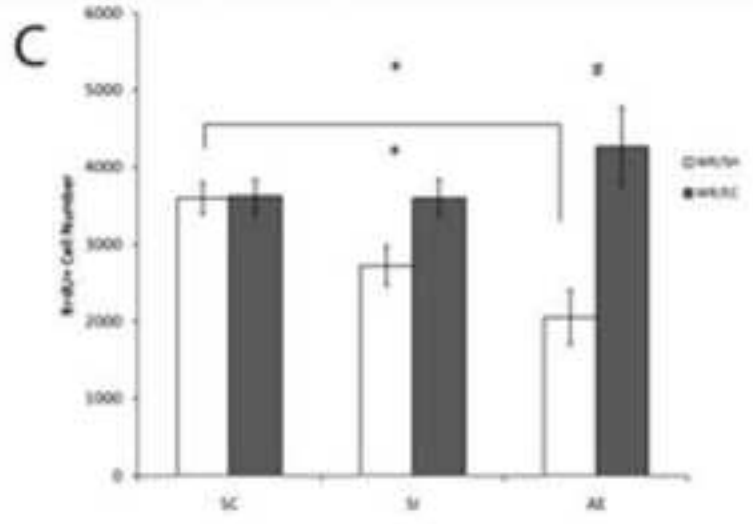

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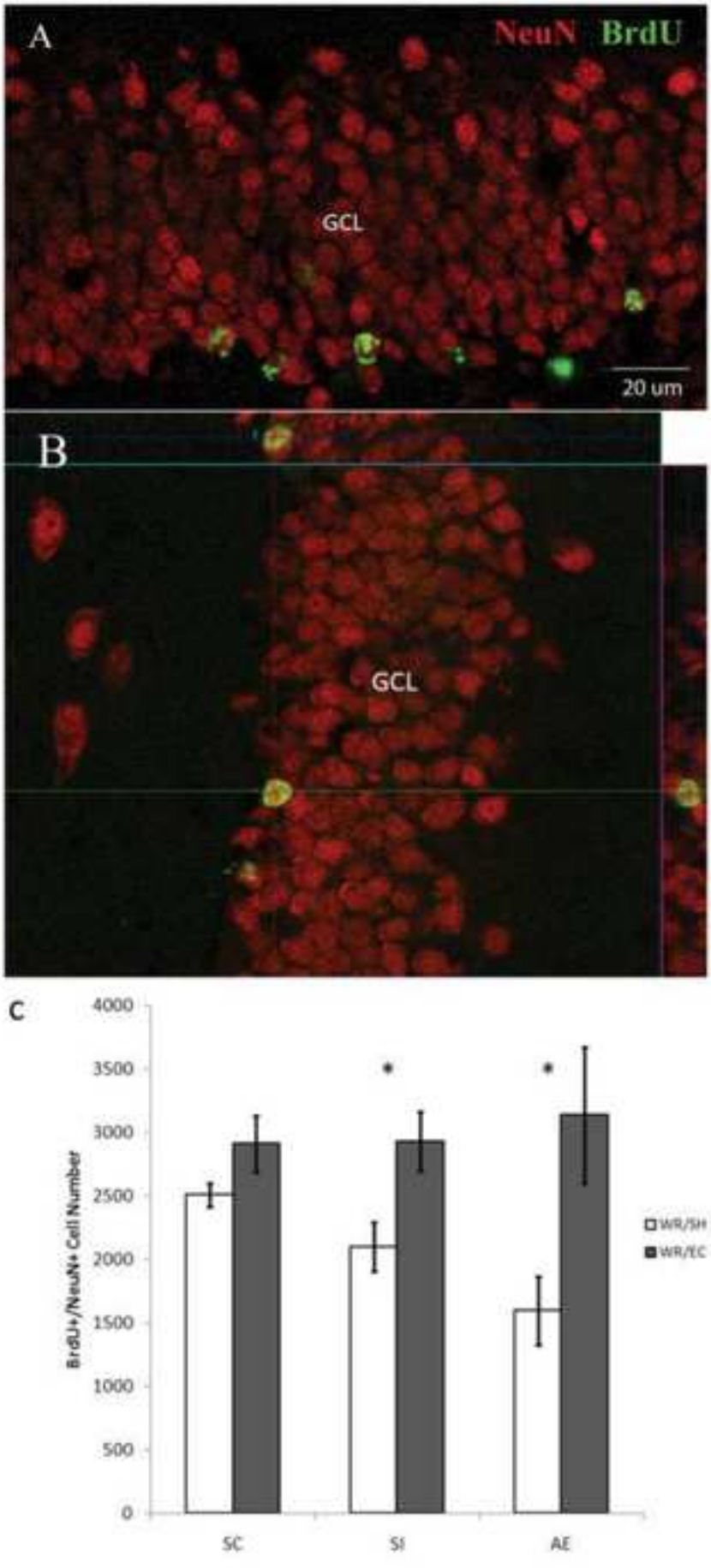

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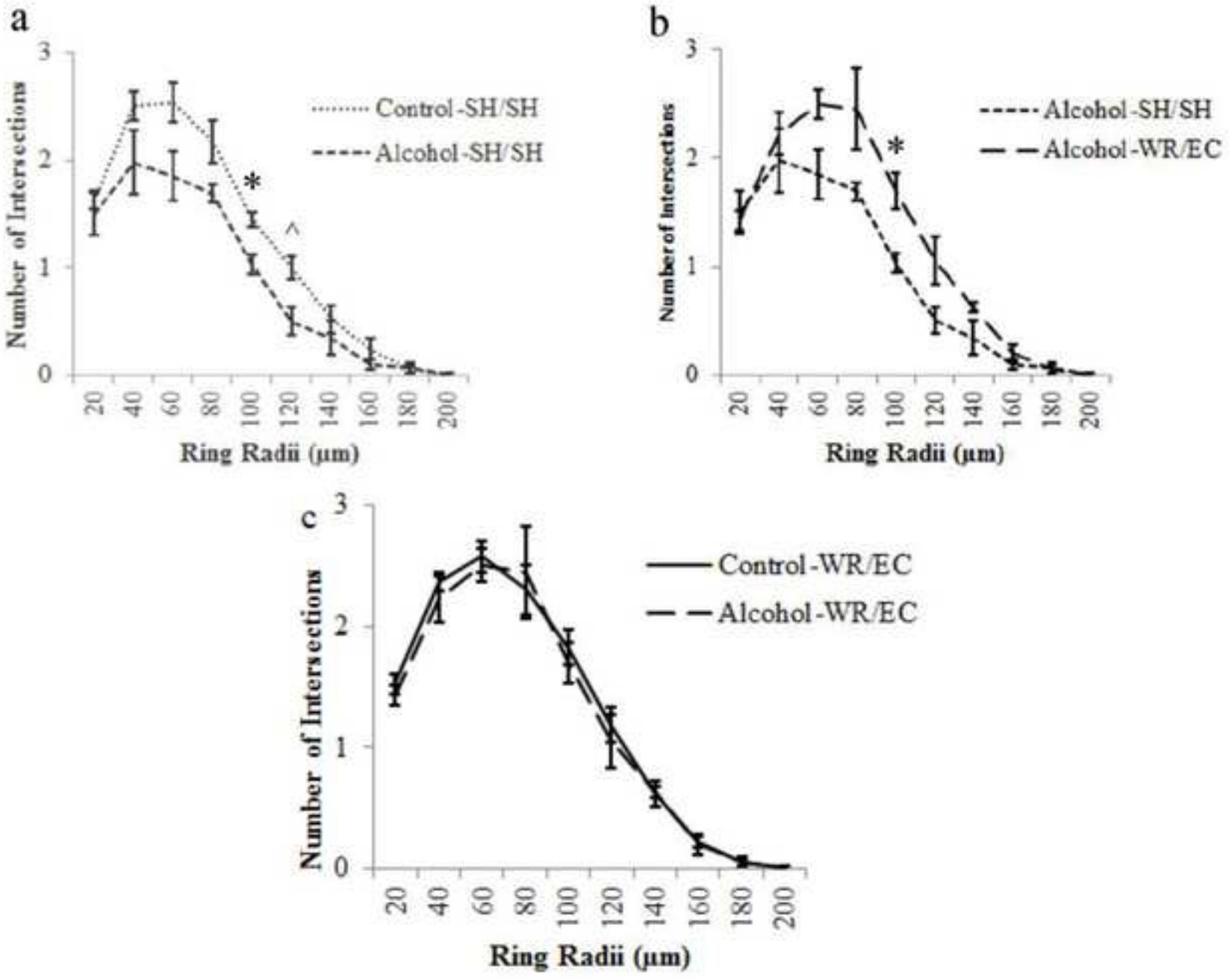

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Female Time-pregnant Long Evans Rats	Envigo (Formerly: Harlan, Inc.)		Average litter size is 8-10 pups
Black India Ink	Higgins (Chartpak, Inc.)	44201	
Syringes and Injection Needles	Becton, Dickinson and Company (BD)	Assorted	For injection of pawmarking ink, administration of milk-alcohol solution
Ear Punch	Kent Scientific Corporation	INS750076	
Running Wheels	Wahmann Labs		Wahmann Running Wheel is discontinued. Substitute with One per cage
EC Cage	Martin's Cages, Inc.	R-695	
Small EC Toys	Assorted		
Medium EC Toys	Assorted		Should be able to fit 1-2 rats inside of/ on top of object
Large EC Toys	Assorted		Should be able to fit 3 or more rats inside of/on top of object

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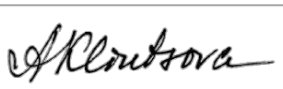
Signature:	Anna Y. Klintsova  Date: April 30, 2016

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The authors would like to thank the Editors and Reviewers for their comments on our manuscript. Below, we have included a list of changes made to the manuscript in response to comments that we have received. All line references mentioned in this summary correspond to the corrected and newly submitted manuscript (when tracked changes are not visible).

Editorial comments:

1. All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word.
   - ✓ We used the manuscript mentioned above for our edits. Due to copy edits and formatting on the "Protocol" section, the numbering for lines 98 and lines 180 to 189 could not be corrected by the authors when steps were removed or added to the protocol.

2. Editor made formatting changes (line spacing), please maintain these changes moving forward.
   - ✓ Changes to line spacing were maintained.

3. Formatting:
   a. The protocol should be formatted into Sections separated by section headings. Ethics approval should not be an experimental step. Only an ethics statement at the beginning of the Protocol section (below the "Protocol" sub-heading) is required.
      - ✓ We added a separate statement regarding ethics approval to the manuscript as the opening sentence in the Protocol (lines 83 and 84). When this change was made, subsequent numbering of the protocol section was changed (see earlier statement regarding the several protocol steps in which this wasn't the case).
   b. Please express centrifuge speeds as "x g" rather than rpm (see 2.11.2).
      - ✓ Line 118 now states "Centrifuge blood at 1.5 x g for 25 minutes."

4. Paraformaldehyde is toxic and requires a caution statement.
   - ✓ We included the following statement on lines 177 to 179: "CAUTION: 4 % paraformaldehyde in PBS is carcinogenic and may also cause skin irritation, allergic skin reaction, or eye damage. Use appropriate eye/skin protection."

5. Please include spaces between all numbers and units.
   - ✓ Thank you, this has been corrected.

6. Please remove references to the video.
   - ✓ The reference to the video has been removed.

7. Grammar:

a. Title should be “Wheel running and environmental complexity as a therapeutic intervention in an animal model of FASD”
   - ✓ The title has been corrected.
b. Line 121 – “make them easier to identify animals”
   - ✓ Line 129 (previously line 121) has been changed to “…make it easier to identify animals…”
c. 5.1.2.3 – “Place lots” should be “Place a lot”
   - ✓ Line 155 has been changed as recommended.
d. Line 253 – “is introduce a”
   - ✓ Line 290 (previously line 253) has been changed to “…is to introduce a…”
e. Line 271 – “individual housing is widely accepted as detrimental to animals can even be directly detrimental”
   - ✓ Lines 309 and 310 have been changed to “…individual housing is widely accepted as detrimental to animals and can even directly counteract the beneficial effects of…”

8. Additional detail is required:
   a. 2.7, 2.8 – How is intubation performed? This should be described or a citation should be provided.
      - ✓ A citation was added on line 108 (Kelly & Lawrence, 2008) which provides a detailed protocol for the dosing procedure.
   b. 2.11.1 – Please describe how blood is collected or provide a citation.
      - ✓ A citation was added on line 115 (Helfer et al., 2009) which describes the process of blood collection in its methods.
   c. Results:
      i. Please define the error bars (SD, SEM, etc.) in the figure legends. Please also define any statistical differences and indicate the statistical test used.
         - ✓ The figure captions were revised to include descriptions of error bars, statistical tests performed, and statistical results.
      ii. Figure 3 – What treatments were used in A and B?
         - ✓ The postnatal treatment on animals used in the fluorescent photomicrographs in Figure 3 was AE (alcohol exposure), based on the laboratory records. Figure 3 was reproduced from a publication, and the initial publication does not include any details regarding the animal(s) displayed in the figure.

9. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.
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Reviewers' comments:

1. Reviewer #1:
    a. Minor Concerns:
        i. One suggestion is related to the role of the type of exercise. Currently she studies on exercise and its effects on brain plasticity show that the type of exercise and the duration of the program must be taken into account. I think it should include some mention of these topics of interest (see Ryan, S and Kelly(2016) Ageing Research Reviews, for example).
            ✓ This was briefly addressed on lines 273 to 278. Further discussion and a citation discussing the implementation of these factors into rodent models of disease has been added on lines 278 to 280.
        ii. On the other hand I consider relevant to mention the statistical analysis used in the different phases of study.
            ✓ This has been added to the figure captions in the "Representative Results" section.
    b. Additional Comments to Authors:
    it should also include some studies that show the different therapeutic effects of both interventions. So appears to be EC could have more influence on the anxiolytic responses while the voluntary exercise appears to modulate responses more related to cognitive functions (Rogers, J., et al 2016, Transl Psychiatry; doi:. 10.1038 / tp2016.52)
        ✓ Discussion was added on lines 334 to 336 adding promise to the use of this paradigm in other models of rodent behavioral and neuroanatomical deficits.

2. Reviewer #2:
    a. Major Concerns:

i. The major flaw of this protocol is that there is a lack of cell survival and neurogenesis effects by WR/EC in the control group. The pro-survival and neurogenic effects of enrichment are well documented and reproducible by independent labs. Therefore, the absence of that observation here indicates a major flaw to the proposed protocol. This has to be discussed as a major limitation to their approach, regardless of the impressive rescuing effects observed in the alcohol-exposed rats.
    ✓ The neurogenic effects of "enrichment" in the field are dependent primarily on the inclusion of a running wheel or some alternative form of cardiovascular exercise in the environment (e.g., references [9,51]). The purpose of this manuscript is to propose an intervention that has been demonstrated in the literature and allows for the contribution from each of the two elements to be independently identified. Additionally, this protocol proposes the use of this "superintervention" as a therapeutic to rescue alcohol-induced deficit. For this reason, the authors

respectfully disagree with reviewer #2's comments above as major limitations to the proposed protocol.

ii. I suspect it may be due to the lack of detail in describing Section 5. Environmental Complexity. There is only mention of different sized toys, but these could be solid toys rather than objects which provide the rats housing opportunities. There is also no mention of nesting material (e.g. shredded paper, straw) to promote nest building and digging behaviour, tunnels to promote exploratory behaviour.
   - ✓ The items in the environmental complexity paradigm should be highly varied (as mentioned on line 150). Since there should be a variety of different objects that should not be consistent, the authors decided that it would be too cumbersome to list the wide variety of objects used, and believed that individuals interested in the specific items would be able to see the items in the accompanying video protocol. Novel nesting material is not used in this paradigm, and a statement has been added on line 148 that states that the cage should include standard bedding. Among the various items used in this protocol, some are tunnel-like, but the authors' protocol does not mandate a certain number of tunnel-like objects. Animals in all treatment groups explore the objects used in this paradigm (see reference [49]) and thus it is unnecessary to state that tunnels would be required to promote exploratory behavior.

iii. The constant configuration changes (every 2 days) and cage cleaning (3 days), but especially the latter, is stressful to rodents. I cannot help wonder if the absence of a significant up-regulation of neurogenesis reflects this since stress is well-established to reduce neurogenesis.
   - ✓ The citation proposed by Reviewer #1 (Rogers et al., 2016; reference number [53]) added in response to a comment by Reviewer #1 (comment 1.b., above) discusses anxiety in response to an EC paradigm. Specifically, this reference addresses that EC reduces anxiety-like behavior. Further, we could not determine an empirical basis that would implicate any stressful effects of cage cleaning as responsible for the lack of survival in adult-born hippocampal granule cells in suckle control animals (but not sham-intubated or alcohol exposed animals).

b. Minor Concerns:

i. The authors should substantiate the dosage indicated in point 2.5, 2.5.1. Are there references for these doses?
   - ✓ A citation was added to line 105 to indicate the model from which this dose is derived.

ii. 2.11.1 Is blood collected in an EDTA tube for plasma or serum? Authors should mention that blood sampling 1.5hr after intubation gives the maximal blood alcohol readout.
   - ✓ An additional protocol step was added (1.11.4.) to address that the blood collected provides serum for further analyses. Additionally, a parenthetical statement was added on lines 113 and 114 addressing the reason for collecting blood at 1.5 hours after exposure.

iii. The stringent need for litter sizes to be 8, makes the flexibility for weaning numbers (2-3) odd. Especially since 3.1.2. suggests at least 3 rats per cage. Surely a minimum of 3 would be a preferable approach.

- ✓ This is explicitly addressed in section "2. Weaning" (previously, "3. Weaning"). Line 134 states that each cage only contains one animal from each condition. Line 135 further describes that the 3 animals in a single cage should not be from the same litter if possible, thus the use of 2-3 animals per cage is not unusual. To address the possible need for an experiment to use different litter sizes, step 1.1.1. was added to discuss the considerations that led to the decision of 8 animals per litter.

iv. More detail might be required for 4.1. WR description. I believe there are commercially available rat cages with wheels that automatically log the usage.
- ✓ The "Instructions for Authors" document says to avoid the use of commercial language or company brand names in the document. Additionally, the authors included the wheel that their lab uses which is not similar to the item suggested by Reviewer #2 and thus it was not included in the manuscript or materials list. It is also unclear in what way a wheel would "log usage" that the model listed by the authors (in their materials list) does not, as all animals in the cage will have an access to the wheel and no individual recording is possible.

v. There should be an additional point 6.6 to conclude the section of tissue collection. 'Fixed brains are cryopreserved or paraffin embedded in accordance to teh requirements of the subsequent sectioning and immunohistochemical analyses.'
- ✓ The reference given in the note below step 5 (line 177) includes a detailed description of the procedures following the tissue collection protocol explained in this manuscript. Although this manuscript describes perfusion with paraformaldehyde, the authors had previously included the note that, "Tissue collection can be performed with a variety of methods (e.g., perfusion with paraformaldehyde, rapid decapitation, etc.)." This note was revised to include flexibility in tissue storage techniques as well.

vi. Asterisks in Fig 1C, 2C, and 3C are misaligned.
- ✓ Figures 1, 2, and 3 are reproduced from a previously published article. The authors reproduced these figures and did not alter them in any way.

vii. The table needs to be aligned properly. Also the description of small, medium and large objects is poorly included, as it provides no information to the reader.
- ✓ The cells in the excel spreadsheet materials table have been aligned to the top left corner. Details regarding the specific objects are intentionally left vague as a wide variety of objects are used in the environmental complexity cage, and quantification of each of these objects (and the specificity of the brand and item) would make the list incredibly cumbersome. The authors decided (as addressed above) that the accompanying video protocol would provide an appropriate supplement to the list and manuscript.

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