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TITLE:

Assessing the Autonomic and Behavioral Effects of Passive Motion in Rats using Elevator Vertical Motion and Ferris-Wheel Rotation

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KEYWORDS:

motion sickness, balance beam, ferris-wheel rotation, elevator vertical motion, rodents, open-field examination

SUMMARY:

Protocols are presented to assess the autonomic and behavioral effects of passive motion in rodents using elevator vertical motion and Ferris-wheel rotation.

ABSTRACT:

The overall goal of this study is to assess the autonomic and behavioral effects of passive motion in rodents using the elevator vertical motion and Ferris-wheel rotation devices. These assays can help confirm the integrity and normal functioning of the autonomic system. They are coupled to

quantitative measures based on defecation counting, open-field examination, and balance beam crossing. The advantages of these assays are their simplicity, reproducibility, and quantitative behavioral measures. The limitations of these assays are that the autonomic reactions could be epiphenomena of non-vestibular disorders and that a functioning vestibular system is required. Examination of disorders such as motion sickness will be greatly aided by the detailed procedures of these assays.

INTRODUCTION:

Motion sickness (MS) due to abnormal visuo-vestibular stimulation leads to autonomic reaction, eliciting symptoms such epigastric discomfort, nausea and/or vomiting[1]. According to current theories, motion sickness may be caused by a sensory conflict or neuronal mismatch from receiving integrated motion information that differs from the anticipated internal model of the environment[2,3] or postural instability as would occur on a yawing ship[4,5]. Despite significant advances in the field of motion sickness and vestibular autonomic functioning[6-12], future research can be aided by standardized evaluation protocols. Assessing the autonomic effects of standard passive motions will greatly benefit investigations into the causes and prevention of motion sickness. The overall goal of this study is to assess the autonomic and behavioral effects of passive motion in rodents. Animal models, such as rodents, allow easy experimental manipulation (e.g., passive motion and pharmaceutical) and behavioral evaluation, which can be used to study the etiology of motion sickness. Here, we present a detailed assay battery for testing the effects of passive motion and the integrity of vestibular functioning.

The present study details two assays, elevator vertical motion (EVM) and Ferris-wheel rotation (FWR), that induce autonomic reactions to the passive motion. The assays are coupled to three quantitative behavioral measures, the balance beam (on mice[13] and rats[14-17]), open-field examination, and defecation counting. The EVM (similar to the pitch and roll of a ship encountering a wave) assesses vestibular functioning by stimulating the otolith sensory organs that encode linear accelerations (i.e., the saccule that responds to movements in the vertical plane)[18]. The FWR (centrifugal rotation or sinusoidal motion) device stimulates the otolith organs by linear acceleration and the semicircular canals by angular acceleration[19,20]. The Ferris-wheel/centrifugal rotation device is unique in its autonomic assessment. To date, the only similar device in the literature is the off-vertical axis rotation (OVAR) turntable, which is used to examine the vestibulo-ocular reflex (VOR)[18,21,22], conditioned avoidance[23,24], and the effects of hypergravity[25-27]. The EVM assay and the FWR device assay induce vestibular stimulation leading to autonomic reactions. We couple the EVM and FWR to quantitative measurements such as balance beam, defecation counting, and open-field analysis[28-30], to ensure robust and reproducible results. Similar to those previously described in mice[13] and rats[14-17], the balance beam assay is a 1.0 m long beam suspended 0.75 m from the ground between two wooden stools using a simple black-box modification at the goal end (finish). The balance beam has been used to assess anxiety (obscure black box)[14,17], traumatic injury[15-17], and here, autonomic reactions affecting balance. We have performed defecation counting for assessing autonomic response in the motion sickness model previously, and it is a reliable quantitative measurement that is easily performed and unequivocally assessed[6,8,9,11]. The open-field analysis employs a simple black box open-field behavior assessment using Ethovision[28], Bonsai[30], or a simple video analysis in

Matlab[29] to quantify behavior such as motion. In the current protocol, we use the total distance traveled, but we note several different paradigms exist (e.g., elongation, zone of movement, velocity, etc.)[28-Error! Reference source not found.0]. Collectively, these procedures form a short battery of assessments for the examination and evaluation of autonomic reactions to passive motion, for example in motion sickness[6-11]. The present assays can be adapted to a variety of animal models.

PROTOCOL:

The present study and procedures were approved by Ethics Committee for Animal Experimentation of the Second Military Medical University (Shanghai, China) in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996).

1. Animals

1.1. Use Sprague-Dawley (SD) rats of two months (200–250 g). For each behavioral assay, use a separate group of rats. Always use separate control and experimental groups.

NOTE: There were two autonomic tests: EVM and FWR. The EVM had three conditions in addition to a control group (= 4) with three behavioral assays (balance beam, defecation counting and open field = 3) with 8 rats in each for a total of 96 rats (4 x 3 x 8). The FWR had one condition in addition to a control group (= 2) with three behavioral assays (balance beam, defecation counting and open field = 3) with 8 rats in each for a total of 48 rats (2 x 3 x 8). In total, we report 144 rats.

1.2. Cage rodents under a constant 25 °C temperature and 60%–70% humidity.

1.3. House rodents in 12 h/12 h light/dark cycles with access to food and drinking water ad libitum.

NOTE: Since the following protocols are behavioral experiments, rats should be handled gently. Handling animals should be with both hands with body and rear support, so as not to induce anxiety.

1.4. Perform experiments (EVM and FWR) and evaluation assays (balance beam and open field evaluation) in the darkness to minimize visual cues.

2. Elevator vertical motion device

2.1. Perform the elevator vertical motion procedures in complete darkness to minimize visual cues.

2.2. Place the rodents in the Plexiglas box (22.5 cm x 26 cm x 20 cm). Here the Plexiglas box can accommodate four rodents (custom-made device).

2.3. Ensure the box is fastened shut and securely closed to avoid rodents falling out. Place the

Plexiglass box on the elevator pad of the elevator vertical motion device (custom-made device).

2.4. Turn on the elevator vertical motion device to the lowest setting for acclimatization.

2.5. Set the amplitude as 22 cm up and 22 cm down from neutral. Incrementally change elevator vertical motion as follows:

2.5.1. Set the initial periods as 2,500 ms for 5 min, 2,000 ms for 5 min, and 1,500 ms for 5 min.

2.5.2. Use a test period of 800 ms for 2 h.

2.5.3. Slow the device in reverse using periods of 1500 ms for 5 min, 2000 ms for 5 min, and 2500 ms for 5 min.

3. Ferris-wheel rotation device

3.1. Ferris-wheel rotation device setup

3.1.1. Place the plexiglass container (22.5 cm x 26 cm x 20 cm) on a wooden bench (custom-made device).

3.1.2. Place rodents in plexiglass container with the long axis of the body perpendicular to the horizontal rotation rod of the Ferris-wheel (custom-made device).

NOTE: The placement with body perpendicular to horizontal rod ensures stimulation of otolith organs (anterior-posterior and vertical direction) during rotation.

3.1.3. Close the plexiglass box securely.

3.1.4. Place the second set of rodents in plexiglass container with the long axis of the body perpendicular to the horizontal rotation rod on the second arm of the Ferris-wheel rotation device. Use a second set of rodents with similar mass to balance the Ferris-wheel.

3.1.5. Securely close the plexiglass box and place on the Ferris-wheel rotation device.

3.2. Ferris-wheel rotation procedure

3.2.1. Perform the Ferris-wheel rotation procedures in complete darkness to minimize visual cues.

3.2.2. Start the Ferris-wheel rotating in a clockwise direction at 16°/s^2 to reach an angular velocity of 120°/s, and then begin to decelerate at 48°/s^2 to reach 0°/s. After a 1 s pause, have the container continue to rotate in a counterclockwise direction in the same manner as above (acceleration at 16°/s^2 to reach an angular velocity of 120°/s and then deceleration at 48°/s^2 to

reach 0°/s). The clockwise-pause-counterclockwise cycle requires approximately 10 s to reach its initial position.

3.2.3. Continue the clockwise-counterclockwise rotation for 2 h per session for approximately 720 rotations.

4. **Evaluation of EVM and FWR**

NOTE: The evaluation of Ferris-wheel rotation device and elevator vertical motion is done by three procedures: balance beam testing, defecation counting, and open-field examination. Identical procedures are used to evaluate elevator vertical motion. These evaluation procedures should be done as soon as possible after Ferris-wheel rotation or elevator vertical motion.

4.1. Balance beam

4.1.1. Balance beam setup

4.1.1.1. Set up the balance beam[10-12] by placing two wooden stools (approximately 0.75 cm in height) in the experimental field, approximately 110 cm apart.

4.1.1.2. Place a black plastic box (15 cm x 15 cm x 8 cm) on the finish stool.

4.1.1.3. Place a narrow wooden beam (2.5 cm x 130 cm) between the two stools, leaving a 100 cm distance between the stool edges, from the start stool to the finish stool.

NOTE: The entrance to the black plastic box should be at the finish line of the 100 cm.

4.1.1.4. Place a lamp at the start stool. Turn on the lamp.

4.1.1.5. Turn off the room lights and ensure that the room is as dark as possible. This ensures the rodent follows the direction of the balance beam from the lighted region to the obscured region.

4.1.2. Balance beam procedures

NOTE: The motor coordination assay of the balance beam is assessed by measuring the time taken to traverse the elevated wooden beam.

4.1.2.1. Train each rodent daily for 3 consecutive days, before the examination period, in order to achieve stable performance on the balance beam[10]. Train by introducing the rat to the beam in the lighted corner and prompting it to cross the beam. Eventually the rat will cross of its own volition. Rats in the present protocol took 3.6 ± 0.9 seconds.

NOTE: Some rodents fail to achieve stable performance during training and should be excluded. Some rodents do not perform the task while others lack motivation to cross the beam. Stable

performance was two consecutive trial periods of crossing times less than 4 seconds. If a rat falls off during training or assessment it should be categorized as a rat 'fall' and not assessed further.

4.1.2.2. For the actual procedure, place the trained rodent on the start stool near the light and simultaneously press start on a stopwatch. The rodent should cross the balance beam rapidly and enter the black box on the finish stool.

4.1.2.3. Press start on the stopwatch once the rodent is in place and press stop when the nose enters the dark box on the finish stool. The time to traverse the beam is from start stool to finish stool.

NOTE: Once the rodent is trained, you may perform an intervention or manipulation, such as inducing motion sickness, prior to evaluation. You may also obtain a baseline measurement, prior to intervention, using the time to traverse of the last training session.

4.2. Defecation counting

4.2.1. Place the plexiglass container containing the four rodents on a bench after the Ferris-wheel test period.

4.2.2. Remove the rodents and place in individual open-field boxes (below).

4.2.3. Count the number of feces pellets in the plexiglass box attributed to each rodent.

NOTE: A baseline measurement can be obtained, for comparison with the evaluation after elevator motion, by counting feces pellets prior to undergoing elevator vertical motion.

4.3. Open-field examination

4.3.1. Place the rodents in the open-field box (40 cm x 40 cm x 45 cm).

4.3.2. Record open field behavior using an IR-video camera for 3 min[28-30].

4.3.3. Determine the total distance traveled.

NOTE: It is very important NOT to place the rodent in the open-field box before elevator vertical motion. The environment must be novel to the rodent. Therefore, baseline measurements should NOT be taken for open-field examination.

REPRESENTATIVE RESULTS:

Figure 2 demonstrates representative balance beam results of time taken to transverse. Rats were trained for 3 consecutive days in order to achieve stable performance on the balance beam[10]. The subsequent day, rats were evaluated for balance beam performance. In the y-axis of the figure, we have the number of seconds taken for rodents to cross the balance beam for

Ferris-wheel, elevator, and control groups for demonstrative purposes.

Figure 3 demonstrates representative defecation count results. For elevator vertical motion, rats were in one of three different rotation groups of 0.8 Hz, 0.4 Hz, and 0.2 Hz vertical motion, in addition to a control group, called the static group. The EVM significantly increased defecation (one-way ANOVA, $F(3,31) = 20.2306$, $p < 0.00001$). The change in Hz vertical motion increased defecation for 0.4 Hz ($t = 3.4064$, $df = 14$, $p = 0.0043$) and 0.8 Hz ($t = 10.6895$, $df = 14$, $p < 0.0001$). For Ferris-wheel rotation, rats were rotated in a clockwise-pause-counterclockwise cycle lasting approximately 10 s to reach its initial position. The entire session of rotation lasted for 2 h. The Ferris-wheel rotation group was compared to a control group, called the static group. The Ferris-wheel rotation group increased defecation as determined by a t-test ($t = 10.6895$, $df = 14$, $p < 0.0001$).

Figure 4 demonstrates the open field examination of total distance travelled results. These data were collected using commercial video tracking software for the analysis of open field behavior (**Table of Materials**)[28], but several open source software pipelines exist for behavioral video analysis such as Bonsai[30] and one our group has developed based on Matlab[29]. Also, here, the total distance traveled was assessed as a metric, but frame-by-frame differences can be used for determining other behaviors such as vertical motion. For elevator vertical motion, rats were in one of three different rotation groups of 0.8 Hz, 0.4 Hz, and 0.2 Hz vertical motion, in addition to a control group, called the static group. The EVM significantly decreased open field distance travelled (one-way ANOVA, $F(3,31) = 16.5994$, $p < 0.00001$). The change in Hz vertical motion decreased open-field locomotion for 0.4 Hz ($t = 3.1354$, $df = 14$, $p = 0.0073$) and 0.8 Hz ($t = 5.8929$, $df = 14$, $p < 0.001$). For Ferris-wheel rotation, rats were rotated in a clockwise-pause-counterclockwise cycle lasting approximately 10 s to reach its initial position. The entire session of rotation lasted for 2 h. The Ferris-wheel rotation group was compared to a control group, called the static group. The Ferris-wheel rotation group decreased open-field locomotion as determined by a t-test ($t = 4.3341$, $df = 14$, $p = 0.0007$).

A number of published studies have employed the protocols described here[6-12]. One recent example from our group studied the mechanisms behind anticholingenics mecamylamine and scopolamine alleviating motion sickness-induced gastrointestinal symptoms[12].

FIGURE AND TABLE LEGENDS:

Figure 1: Instrumentation used. (**a**) Balance Beam. The balance beam is a narrow wooden beam (2.5 cm x 130 cm) between the two stools placed 100 cm (approximately 0.75 cm in height) apart. A lamp is placed at the start stool and a black plastic box (15 cm x 15 cm x 8 cm) on the finish stool. (**b**) Elevator vertical motion device. The elevator vertical motion device amplitude is set at 22 cm up and 22 cm down from neutral. The warm-up vertical motion consists of 2500 ms period for 5 min, 2000 ms for 5 min, and 1500 ms for 5 min. The test motion consist of a 800 ms period for 2 h. The elevator vertical motion device is slowed in reverse using a 1500 ms period for 5 min, 2000 ms for 5 min, and 2500 ms for 5 min. Rats are placed head towards the front of the elevator vertical motion device. (**c**) Ferris-wheel rotation device. The Ferris-wheel rotates in a clockwise direction at $16°/s^2$ accelerating to 120°/s, subsequently decelerating at $48°/s^2$ to reach 0°/s,

pausing for 1 s, and then rotating in a counterclockwise (16°/s^2 accelerating to 120°/s, subsequently decelerating at 48°/s^2 to reach 0°/s). The clockwise-pause-counterclockwise cycle requires ~10 s to reach its initial position. Rats are placed head towards center of the Ferris-wheel rotation device.

Figure 2: Balance beam results. Time taken to transverse the beam (mean ± standard deviation). The y-axis indicates seconds taken to transverse the beam. Rats were trained for three days prior to evaluation in order to achieve stable performance on the balance beam[10]. Prior evaluation with the elevator motion or Ferris-wheel devices significantly increases crossing time. Statistical testing was performed by two-tailed t-test with Bonferroni correction between control and every other group. *** indicates $p < 0.001$.

Figure 3: Defecation count results. Elevator vertical motion results (**a**) Left panel – Defecation count (mean ± standard deviation) by group for 0.8 Hz, 0.4 Hz, and 0.2 Hz vertical motion, in addition to a control group, called the static group at 0 Hz. Note the significant increase in defecation for 0.8 Hz and 0.4 Hz as indicated by the asterisks. Ferris-wheel rotation results (**b**) Right panel – Defecation count (mean ± standard deviation) for Ferris-wheel rotation rat group (see description for angular velocity paradigm) and a control group (0 Hz), called the static group. Note the significant increase in defecation for the rotation group as indicated by the asterisk.

Figure 4: Total distance traveled. (**a**) Elevator vertical motion results. This panel consists of total distance traveled (mean ± standard deviation) by cm in the open field locomotion test by group for 0.8 Hz, 0.4 Hz, and 0.2 Hz vertical motion, in addition to a control (static) group. Note the significant decrease in total distance traveled for 0.8 Hz and 0.4 Hz as indicated by the asterisks. Statistical testing was performed by two-tailed t-test with Bonferroni correction between control and every other group. ** indicates $p < 0.01$ and *** indicates $p < 0.001$. (**b**) Ferris-wheel rotation results. This panel consists of total distance traveled (mean ± standard deviation) by cm in the open-field locomotion test for Ferris-wheel rotation rat group and a control (static) group. Note the significant decrease in total distance as indicated by the asterisk. Statistical testing was performed by two-tailed t-test between control and Ferris-wheel group. *** indicates $p < 0.001$.

DISCUSSION:

The present study describes assessing autonomic responses to passive motion in rodents using elevator vertical motion and Ferris-wheel rotation. These equipment and procedures can be easily adopted to other rodents and several modifications of the assays exist to confirm vestibular functioning in different circumstances, such as during in pharmacological challenge or surgical interventions. Research in MS elicited by vestibular stimulation has led to the theory that sensory conflict or neuronal mismatch caused by receiving visual information that differs from the anticipated internal model of the environment[2,3] leads to autonomic reaction eliciting symptoms such epigastric discomfort, nausea and/or vomiting[1]. Further theories have outlined that postural instability, as would occur on a yawing ship[4,5], elicits autonomic reaction. Despite these significant advances, questions remain that can be aided by evaluation protocols such as elevator vertical motion and Ferris-wheel rotation.

A critical step for balance beam is training. Rats must be motivated and have confidence to cross the beam; otherwise, balance (i.e., vestibular integrity) is not measured in an evaluation period. For researchers interested in examining anxiety[14,17] or traumatic injury[15-17], other behaviors during training or balance beam crossing may be relevant. For example, in anxiety research using the balance beam, defecation, urination, falls, and missteps can be enumerated[14]. Also in some research areas, rodents that lack motivation to cross the beam may be evaluated differently[13-17]. It is critical during elevator vertical motion and Ferris-wheel rotation to ensure that the box is fastened shut and securely closed, as rodents in an unsecured box may be propelled and injured. Also, ensure that rodents are evaluated in the open-field box[28-30] only once and immediately after the elevator and Ferris-wheel to ensure rapid evaluation of vestibular stimulation.

The abovementioned protocols use quantitative measures. Therefore, the limitations for balance beam include rodents that lack motivation to cross the beam, as balance is the behavior being evaluated. Limitations for the elevator vertical motion and Ferris-wheel rotation defecation assays include requiring a well-fed rodent. This is necessary; otherwise, the rodent may not experience a robust autonomic reaction to vestibular stimulation. It is good practice to observe baseline defecation count for a normal/control period of 2.5 h duration for comparative purposes.

Another important consideration when using the protocols, and interpreting results, is differences in motion sickness responses across species. In humans, and also other species like cats and dogs, retching and vomiting are two common symptoms[31-34]. Rats, on the other hand, cannot vomit. However, rats display motion sickness symptoms such as pica[35,36], defecation response[37], and spontaneous locomotion reduction[35,388]. Also, humans rely primarily on vision for sensory input and motion sickness is likely related to sensory conflict with the vestibular system[2,39]. In rats, especially albino rats (e.g., Sprague-Dawley), vision is not typically the primary sense, but rather somatosensory (whiskers). This may lead to inter-species differences in the relative contributions of different sensory inputs to the conflict. Lastly, there are inter-rodent species differences in motion sickness response. For example, the shrew mouse (Suncus murinus) is able to have an emetic response[400,41].

Collectively the procedures described form a short battery of assessments for the examination and evaluation of autonomic reactions in rodents during motion sickness[6-11]. The present techniques coupled to more physiological measures such as electrophysiology to determine the cortical consequences during vestibular stimulation would be of great interest.

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The authors declare no financial or non-financial conflicts of interests. The FWR device has a patent in China: ZL201120231912.1.

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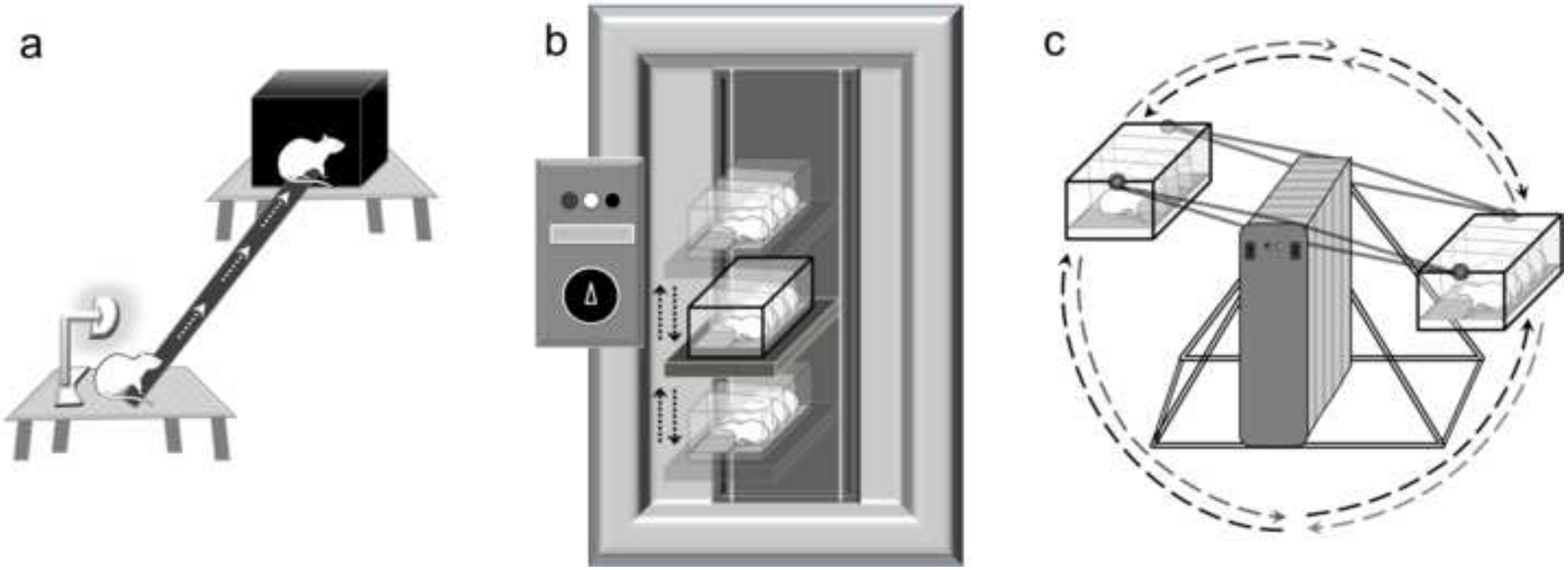
Figure



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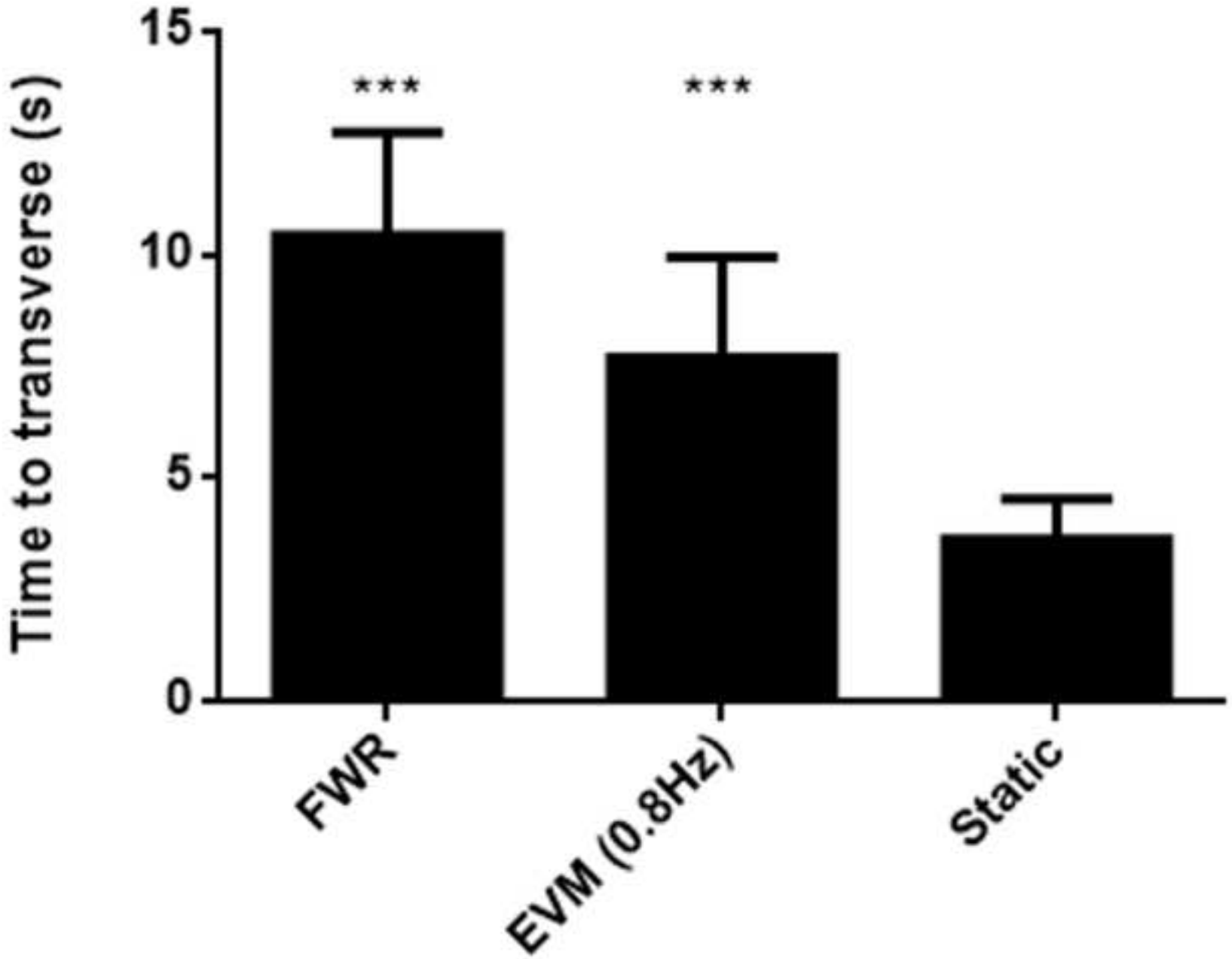

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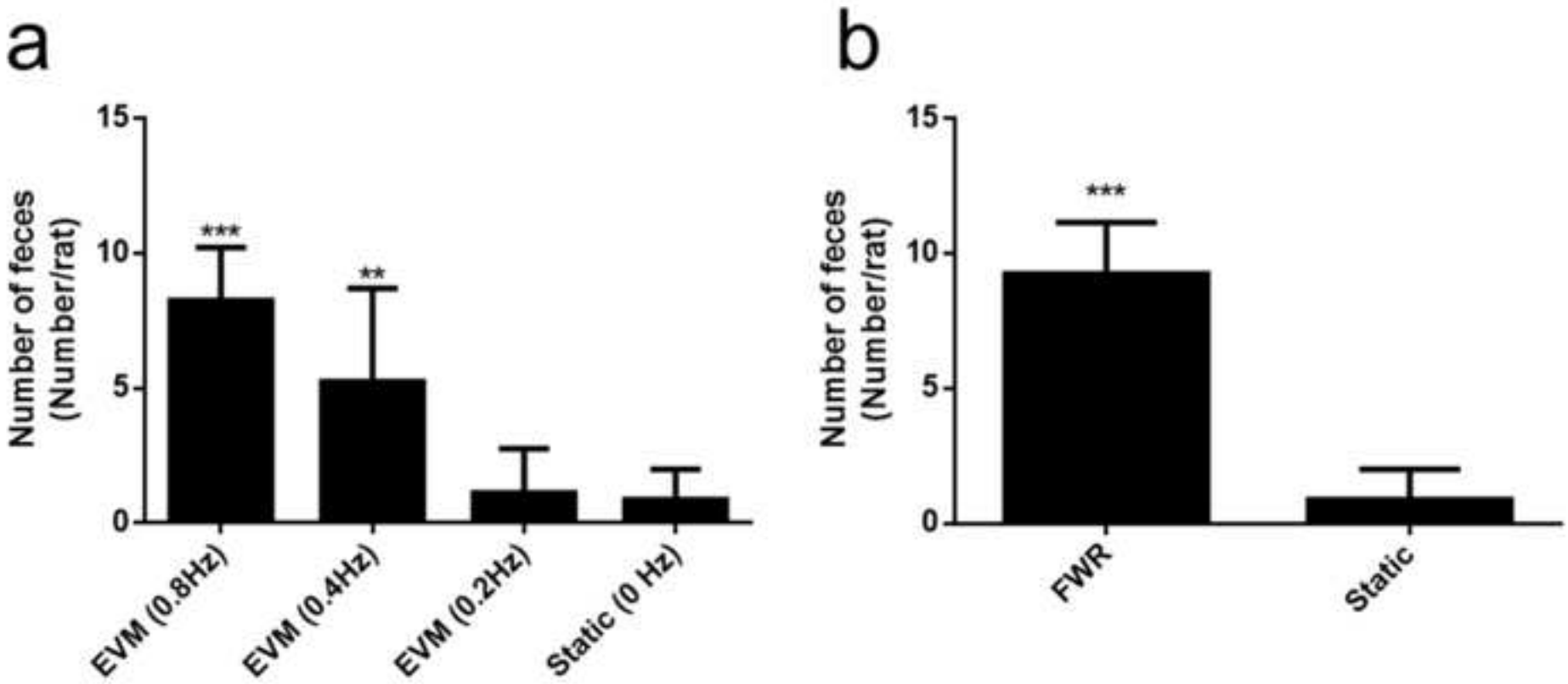

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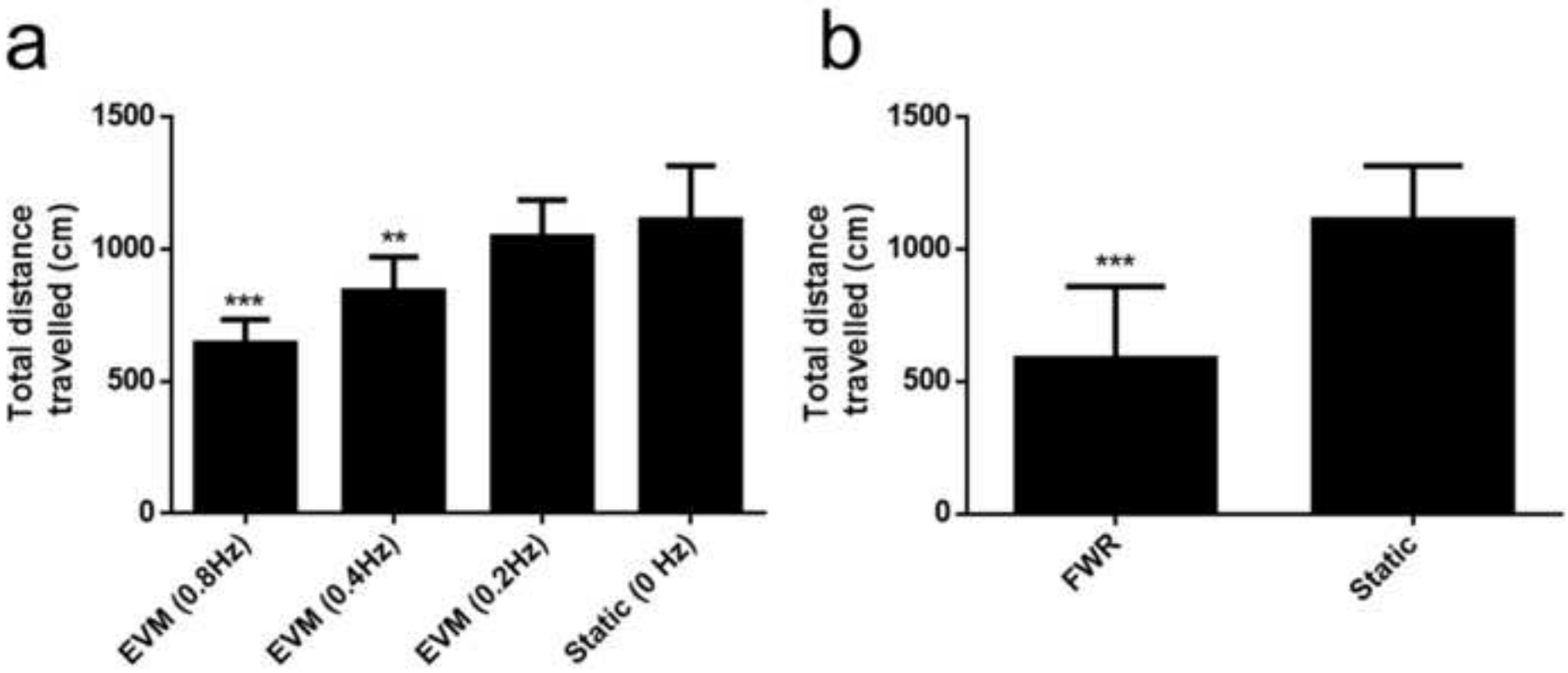

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials-Vestibular-03.02.2019.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Elevator vertical motion device	Custom		Custom-made Elevator vertical motion device to desired specification
Ethovision	Noldus Information Technology		Video tracking software
Ferris-wheel rotation device	Custom		Custom-made Ferris-wheel rotation device to desired specifications
Latex, polyvinyl or nitrile gloves	AMMEX		Use unpowdered gloves 8-mil
Open field box	Custom		Darkened plexiglass box with IR camera
Rat or mouse	JAX labs		Any small rodent
Small rodent cage	Tecniplast	1284L	
Wooden beam and stools	Custom		Custom-made wooden beam and stools to specifications indicated

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Title of Article: Assessing Vestibular Function in Rodents: Balance Beam, Elevator Vertical Motion and Ferris-Wheel Rotation

Author(s): Francis A. M. Manno, Leilei Pan, Yuqi Mao, Yang Su, Sinai H. C. Manno, Shuk Han Cheng, Condon Lau, Yiling Cai

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July 15th, 2019

Dear Dr Phillip Steindel,

Thank you very much for allowing the revision of our manuscript. Following the reviewers' suggestions, we have revised the title to "**Assessing the Autonomic and Behavioral Effects of Passive Motion in Rats: Elevator Vertical Motion and Ferris-wheel Rotation**". We have addressed the editor and reviewers' comments below, and revised the manuscript accordingly. The authors would also like to thank the editor and reviewers for their time and effort.

Sincerely yours, on behalf of all authors,

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Editorial comments:

The authors thank the editor for his time and effort.

1. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
   We have highlighted, in yellow, approximately 2.75 pages of essential protocol steps for the video.

2. Would it be possible to include more detailed schematics? I.e., something a machine shop could use to build one of the more complicated devices.
   These Devices are in the process of being patented. Nevertheless we note, the physical specifications of rotations and parameters etc would allow any machinist to design them.
3. 2.2.1: The statement to have the rats cross in less than 2.5 s and that the mean time was 3.6 s seem contradictory; please clarify.
   We have updated the statements to reflect our crossing time and stable performance.

4. 2.2.3: This still refers to the video; please remove or revise.
   Not sure the reference here. The video we are requesting from JOVE, therefore our video is no longer relevant.

5. Results, Figure 2: Please clarify more about the different groups here; e.g., how long before the balance-beam test were the Ferris wheel and elevator procedures done?
   We have clarified the groups. We always use separate groups.

6. Results: Please expand on results from previous studies; e.g., show a few numbers. You do not have to show everything but you should do more to address reviewer's concerns with showing this in more experimental contexts.
   We have explained our results with t-tests and ANOVA.

7. Figure 2: Please add " (s)" to the end of the y-axis label.
   "(s)" have been added to the axes.

8. Figure 4: Please remove the title from the figure itself.
   The titles have been removed.

Reviewer comments:

The authors thank the reviewers for their time and effort.

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Reviewer #1:

This model does not evaluate true motion sickness. Rats cannot vomit. Defaecation measures are not demonstrated to correlate with motion sickness. The authors do not appear to consider these factors, and I cannot therefore recommend acceptance, even though the Methods are adequately described.

We have revised the title and rest of the manuscript to focus on assessing autonomic and behavioral effects of motion rather than vestibular function specifically.

Reviewer #3:

Manuscript Summary:

Although a point to point reply to the referee comments has not been provided by the authors, the manuscript is now more clear and better focused. There are minor points that must be considered.

Major Concerns:

none

Minor Concerns:

1. line 69. acceleration.
   Revised.

2. Lines 167-172. I think that the motion of the device is sinusoidal. This information, which can be be inferred by the figures, must be clearly indicated in the text. This request was present also in my first revision and it has not be taken into account by the Authors.
   Sinusoidal motion is indicated, please clarify how you would like a better description.

3. lines 198-200. Since baseline measurements cannot be taken, whatever study must contemplate a control (no treatment) and an experimental (treated) group. Is better to specify this point here, rather than in the results section and in the related figures.
   We now specify the need for separate control and experimental groups, if baseline cannot be taken, in the protocol.

4. lines 377-379. Do the Authors have some idea how to deal with the lack of motivation?
   We have revised the manuscript. We feel our static group/stationary group is an adequate control.

Reviewer #4:

Manuscript Summary:

This manuscript is aimed at describing three procedures for rats. Two of them are designed to expose the rats to movements that may induce motion sickness and to record the autonomic response during the stimulation by counting defecation units. The other procedure is a beam crossing balance test that, together with an open field test, is used to evaluate the behavioral impact of the two stimulation procedures. The procedures and data are of interest, because they show an impact of the stimulation procedures on behavior and this indicated their interest for motion sickness research. However, this manuscript has in its present form several major and minor shortcomings.

Major Concerns:

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1. It is stated in the title ("Assessing vestibular function in rodents") and argued extensively in the text that the three procedures are "vestibular assays", but they are not. It is well possible that the recorded outcome of these procedures requires a proper vestibular function to be generated. However, a method to "assess vestibular function" should be a method to evaluate alterations in vestibular function, from optimal to complete loss. For instance, vestibular function is evaluated in humans by procedures like the video head impulse test (vHIT) or the vestibular-evoked myogenic potentials (VEMPs). In most species and strains of rodents, vestibular deficiency results in dramatic alterations in spontaneous motor behavior, including ataxia and hyperactivity (see for instance, Boadas-Vaello et al., Toxicol. Sci 2005 & 2017). In the balance beam, vestibular deficient rodents would simply fall from it and be unable to perform the task. In the open field, most vestibular deficient rodents would show hyperactivity irrespective of whether they had been exposed to the static or moving conditions in the elevator vertical motion device and the Ferris-wheel. That is, these procedures will only be useful in vestibular-competent rodents, and will not be useful to evaluate alterations in vestibular function. I would suggest to rename the article to something in the line of "Assessing the (autonomic and behavioral) effects of passive motion in rats:...", and to rewrite the text with this in mind. Stressing the utility of the procedures in motion sickness research would be much more accurate than to state that they assess vestibular function.
   The title has been revised to “Assessing the autonomic and behavioral effects of passive motion in rats: Elevator Vertical Motion and Ferris-wheel Rotation”. The remainder of the manuscript has been revised to focus on the above rather than vestibular function.

2. The organization of the manuscript is inadequate, because the procedures are presented as three methods to "assess vestibular function" when they are two procedures to stimulate rats by passive motion (and assess defecation), and two methods to assess the behavioral consequences of the previous. These are the balance beam (highlighted in title and text) and the open field (not highlighted). This is odd and should be rearranged. For instance, the balance beam data of the EVM animals (and their controls) should be presented with data in present Figure 3 (Elevator vertical motion results) and the balance data of the FWR animals (and their controls) should be presented with data in Figure 4 (FW rotation results). Also, both the balance beam and open field are widespread methods, but have many variants (length and width of the bean, size of the open field, illumination). In this article, the balance beam is described with detail, but the open field is not. The balance beam and open field should be described at a similar level, and differentiated, not confused, with the two methods of stimulation.
   The manuscript has been revised to present the elevator and Ferris-wheel as passive motion devices to induce autonomic reactions. Subsequently, the balance beam, defecation, and open-field examination are presented as methods to assess behavioral effects. We also elaborate the description of the open-field examination.
   Organization is: Balance beam results, defecation count results, and open field testing results. We have reorganized our figures by behavioral results.

3. Many important experimental details are missing:

- How many animals in each group fell from the balance beam? Was a maximum (cut-off) time imposed on the balance beam latencies?

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For our training protocols, no rats fell. We have now added a citation to include falls. We did not impose a cut off time for training or assessment.

- How many animals were included in each of the groups?
We indicate: Use Sprague-Dawley (SD) rats of two months (N = 12, 200-250g). Individuals employing this protocol may use whatever rodent desirable, but need to adapt the balance beam for size requirements.

- Where all animals independent or some animals were used in more than one of the experiments? It is unclear whether some of the "static" data may represent baseline levels of the same animals, or different animals.
We have specified: Use Sprague-Dawley (SD) rats of two months (N = 12, 200-250g). For each behavioral assay a separate group of rats was used: 3 behavioral assays (Balance beam, defecation counting, open field) X 2 autonomic tests (EVM and FWR) X N=12 rats = 72 rats total..

- The light cycle was 12/12 h. When were the experiments performed respect to the circadian cycle? Also, the EVM and FWR procedures take more than 2 hours. How was the time of the day balanced across treatment groups (e.g., were all control animals tested in the open field 3 h before all FWR animals?).
We tested all animals during the light cycle. .

- The motion devices seem to accommodate 4 or 8 animals, but the balance beam seems a one-by-one task, and it is unclear whether you used one or several open fields simultaneously. The question is how the animals were processed after the motion stimulation, when the first animal entered the beam (e.g. within one minute after motion) or the open field and when the last animal completed these tasks.
The EVM device can hold 4 animals (we did twice) and the FWR devise can hold 4 per cage two cages per side so 16 in total. One can stop the device, take out one animal, return the device to operation, and then test the animal on the protocol.

Minor Concerns:

4. There seems to be a lack of acknowledgment of the species differences in motion sickness and its outcome. For instance, species differences are not indicated in the first paragraph, when the sensory conflict theory is explained and supported by literature on human motion sickness, and then this seems to translate directly to rodents. However, the weight of different sensory systems is well different across species, with humans much more relying on visual cues than rats, in particular considering visually deficient albino rats such the Sprague-Dawley rats used here. Also, it is said that the present procedures may be easily adapted to any rodent. However, some rodents may respond quite differently, as the well characterized emetic responses of the house musk shrew (Suncus murinus) to chemical- or motion-induced sickness (Ueno et al. Life Sci., 1988).
   A paragraph has been added to the discussion describing possible inter-species differences. The sentence about adaptability to rodents has been revised to the equipment and procedures can be adjusted to different rodent species.

5. The description of the motion parameters at the EVM describes a single frequency of 1000 ms (1 Hz) as main frequency (together with increasing and decreasing frequencies towards this maximum).

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However, the data presented in the figures present data at 0.2, 0.4, and 0.8 Hz. This discrepancy should be resolved.
We thank the reviewer for catching this error. It was an error in noting the methods. We have corrected the writing error.

6. Statistics. Although the Bonferroni correction was used to correct for multiple comparisons, some of the data should be first better analyzed by one-way ANOVA.
One-way ANOVA has been applied to compare the elevator vertical motion where multiple frequencies are employed. We include the t-tests now.

7. Despite the repeated emphasis across the manuscript in the vestibular origin of the responses recorded, the need for well-feed rodents is cited as a limitation in lines 379-381. This raises the concern that other factors besides vestibular stimulation are involved in the responses, but this is not discussed. Also, the manuscript does not discuss how feeding was controlled to ensure that all animals in the different treatment groups were similarly feed at the moment of stimulation. This also links to the question raised above on the time of the day and duration of the stimulation cycle.
Revised.

Reviewer #5:

Manuscript Summary:

The paper described three interesting ways to test balance function. The measurements of defecation and travel distance in open field after vertical motion and rotation the vertical plan are novel. The paper is well written and will be an important contribution to the field.

Major Concerns:

1. It is important for the authors to add additional statements in the introduction and the discussion about contributions from other sensory cues on the measurements, such as visual cues and proprioceptive cues.
Information other sensory cues included in the discussion.

2. Since vestibular function is mentioned in the title, the authors need to make effort to make sure the measurements are vestibular-specific and not affected by other cues.
Following response 1 of reviewer 4, the manuscript has been revised to focus on autonomic and behavioral effects induced by the elevator and Ferris-wheel.

Minor Concerns:

3. Please indicate how visual cues are controlled in the tests.
All experiments were performed in the dark. We specified this in our previous manuscripts and briefly in the protocol, but now emphasized in the revised manuscript.