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Activating autophagy by aerobic exercise in mice

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Abstract:	Autophagy is a lysosomal degradation pathway essential for cell homeostasis, function and differentiation. Under stress conditions, autophagy is induced and targets various cargos, such as bulk cytosol, damaged organelles and misfolded proteins, for degradation in lysosomes. Resulting nutrient molecules are recycled back to the cytosol for new protein synthesis and ATP production. Upregulation of autophagy has beneficial effects against the pathogenesis of many diseases, and pharmacological and physiological strategies to activate autophagy have been reported. Aerobic exercise is recently identified as an efficient autophagy inducer in multiple organs in mice, including muscle, liver, heart and brain. Here we show procedures to induce autophagy in vivo by either forced treadmill exercise or voluntary wheel running. We also demonstrate microscopic and biochemical methods to quantitatively analyze autophagy levels in mouse tissues, using the marker proteins LC3 and p62 that are transported to and degraded in lysosomes along with autophagosomes.
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TITLE:
Activating autophagy by aerobic exercise in mice

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KEYWORDS:
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SHORT ABSTRACT:
Autophagy activation is beneficial in the prevention of a number of diseases. One of the physiological approaches to induce autophagy *in vivo* is physical exercise. Here we show how to activate autophagy by aerobic exercise and measure autophagy levels in mice.

LONG ABSTRACT:
Autophagy is a lysosomal degradation pathway essential for cell homeostasis, function and differentiation. Under stress conditions, autophagy is induced and targets various cargos, such as bulk cytosol, damaged organelles and misfolded proteins, for degradation in lysosomes. Resulting nutrient molecules are recycled back to the cytosol for new protein synthesis and ATP production. Upregulation of autophagy has beneficial effects against the pathogenesis of many diseases, and pharmacological and physiological strategies to activate autophagy have been reported. Aerobic exercise is recently identified as an efficient autophagy inducer in multiple organs in mice, including muscle, liver, heart and brain. Here we show procedures to induce autophagy *in vivo* by either forced treadmill exercise or voluntary wheel running. We also demonstrate microscopic and biochemical methods to quantitatively analyze autophagy levels in mouse tissues, using the marker proteins LC3 and p62 that are transported to and degraded in lysosomes along with autophagosomes.

INTRODUCTION:
Autophagy is an evolutionarily conserved degradation pathway, which is induced in response to various stress conditions such as starvation and hypoxia[1,2]. During

autophagy, double-membrane vesicles, called autophagosomes, incorporate unnecessary or damaged subcellular components and transport them into lysosomes for degradation[3]. Basal autophagy is essential for cellular function and organism development, and impaired basal autophagy is implicated in many disorders, including neurodegeneration, tumorigenesis and type 2 diabetes[4-6].

The best-known physiological autophagy inducer is starvation. However, it has two major limitations. First, starvation takes a long period to effectively induce autophagy in animals, *e.g.*, 48 hours of food restriction in mice in most organs. Second, starvation barely induces brain autophagy, due to a relatively stable nutrient supply in the brain. In fact, it is also difficult to detect autophagy induction by small-molecule inducers, as many drugs cannot pass the blood brain barrier. Thus, to better analyze the function of autophagy activation in disease pathogenesis, we recently discovered that exercise is a more potent physiological method to induce autophagy in a short period of time[7-9]. Compared with starvation, autophagy is effectively induced by treadmill running as fast as 30 min. Thus, exercise is a convenient and potent physiological approach to study the mechanism of autophagy in mediating health benefits and preventing diseases.

There are several protein markers for the detection of autophagy activity, including LC3 and p62. LC3 (microtubule-associated protein 1A/1B-light chain 3) is a cytosolic protein (LC3-I form) that is conjugated to PE (phosphatidylethanolamine) upon autophagy induction. PE-lipidated LC3 (LC3-II form) is recruited onto autophagosomal membranes and can be used to visualize autophagosomes when labeled with GFP. Its translocation from the cytosol to punctate structures of autophagosomes under microscopy is an indication of autophagy induction. p62 is a cargo receptor for autophagy substrates (such as ubiquitination proteins), and is incorporated into autophagosomes as well. Since the protein is degraded in lysosomes along with autophagosomes, its levels can be used to measure the autophagy flux. Here we show how to use these markers to quantify autophagy in different mouse tissues induced by aerobic exercise, including forced exercise (treadmill) and voluntary exercise (running wheels). The same procedures can also be applied to *in vivo* measurement of autophagy after treatment of other inducers.

PROTOCOL:

All procedures involving animals were performed according to guidelines approved by the Northwestern University Institutional Animal Care and Use Committee (IACUC).

1. Mouse models

1.1. Use 8-12 week-old mice in the exercise training. To detect exercised-induced autophagy *in vivo*, use the GFP-LC3 transgenic mice (C57BL/6 background) for imaging studies and C57BL/6 mice for biochemical analyses.

2. Exercise-induces autophagy

2.1. Treadmill setup – forced exercise

2.1.1. Acclimate and train the mice on a 10° uphill open treadmill for 2 days. On day 1, exercise the mice for 5 min at 8 m/min, and on day 2, exercise the mice for 5 min at 8 m/min followed by another 5 min at 10 m/min. Encourage the mice to run by gentle hand nudge.

2.1.2. On the 3rd day, let the mice undergo a single bout of running for 90 minutes on the 10° uphill treadmill, starting at the speed of 10 m/min. After 40 minutes, increase the treadmill speed at a rate of 1 m/min every 10 min for a total of 30 min, and then increase the speed at the rate of 1 m/min every 5 min until it reaches 17 m/min, for a total of 90 minutes of exercise time and 1070 meters of running distance.

2.1.3. Set the electric stimulus at a low intensity range (1.2 Hz), and encourage the mice to run to avoid repeated foot shock.

2.1.4. For tissue collection, euthanize the mice by isoflurane inhalation, followed by cervical dislocation immediately after exercise.

2.2. **Running wheel setup – voluntary exercise**

2.2.1. Place a mouse-running wheel (11.4 cm diameter) inside a cage with a single-housed mouse.

2.2.2. Verify the running capacity using a bike odometer. Set up the wheel parameter [distance (in mm) per full rotation of the wheel] at 358 (11.4*π). Measure the running distance after 24 hours.

2.2.3. Let the mouse run voluntarily for 2 weeks with the running wheel. For tissue collection, sacrifice the mouse in the morning after the running period.

3. Autophagy flux evaluation

3.1. To measure the autophagic flux under resting and exercise conditions, treat mice with the autophagy inhibitor chloroquine for three days and compare them to mice treated with PBS.

3.1.1. Dissolve chloroquine in PBS (5 mg/mL), and inject chloroquine into mice intraperitoneally at the dose of 50 mg/kg/day for three consecutive days. Sacrifice mice and collect tissues 3 hours after the last injection.

3.1.2. For exercised mice, pretreat them with chloroquine for two consecutive days at the dose of 50 mg/kg/day. On the third day, inject the mice with the same concentration of chloroquine, and after 90 minutes let them run on the treadmill for another 90 minutes.

3.1.3. Euthanize the mice by isoflurane inhalation, followed by cervical dislocation. Collect tissues immediately after running, so that the total incubation time of chloroquine is the same as control (resting) mice (3 hours).

Note: Alternatively, use a single injection of chloroquine to determine the autophagy flux in mouse tissues.

4. Tissue harvest and fixation

4.1. Prior to tissue harvest prepare phosphate buffered saline (PBS) and fresh 4% paraformaldehyde (PFA, caution: personal protection equipment required) in PBS at a final pH of 7.4. Store both solutions at 4 °C.

4.2. Fill 30 mL syringe with 15-20 mL of 4% PFA (for GFP-LC3 mice). Connect the syringe with a 20G catheter needle.

4.3. After the exercise, euthanize the mice by isoflurane inhalation, followed by cervical dislocation.

4.4. Place the animal on a clean work surface on its back. Cut and open the thoracic cavity by cutting the diaphragm to expose the heart.

4.5. Make a small incision on the liver to let the blood come out, and inject a total of 15 mL 4% PFA slowly into the right ventricle of the heart via the catheter, until the lung and liver turn completely pale. Perfuse the mouse immediately after the exercise session, using a syringe pump with a constant flow rate of 90 mL/h.

4.6. After perfusion, remove the needle and collect tissues of interest (*e.g.* brain[10] and skeletal muscle). For skeletal muscle, dissect the vastus lateralis. Briefly, pull the skin of the leg backwards to expose the muscles, localize the quadriceps femoris[11], and dissect out vastus lateralis, which is the external muscle attached to the upper portion of the femoral bone.

4.7. For further fixation and dehydration, place the tissues of GFP-LC3 mice in 4% PFA for 24 h at 4 °C, and then transfer them to 15% sucrose-PBS at 4 °C overnight or until the tissues have settled, and then to 30% sucrose-PBS at 4 °C overnight or for longer storage. Keep the samples in the dark as much as possible as they may be sensitive to strong light.

4.8. For western blot analysis snap freeze the tissues from C57BL/6 mice directly in liquid nitrogen, and store them at -80 °C.

5. Imaging analysis of GFP-LC3 puncta

5.1. Tissue process

5.1.1. Acclimate the tissues (previously fixed in PFA and stored in 30% sucrose) in embedding medium such as “Optimal Cutting Temperature compound” (OCT) for a few minutes in a labeled small petri dish.

5.1.2. Transfer the tissue in a labeled cryomold containing enough fresh OCT to cover the tissue. Orient the sectioning surface toward the bottom of the cryomold (cross

section for the vastus lateralis muscle and sagittal section for the hemi-brain). Avoid formation of bubbles.

5.1.3. Freeze the samples by placing the cryomold for a few minutes in a covered foam cooler filled with dry ice, until the OCT turns white. Wrap individual samples in labeled foils, seal them in a plastic bag, and store them at -80 °C overnight or longer.

5.1.4. Use a cryostat to cut sample sections at a thickness of 10 µm, and mount the sections on the slides. Store the slides at -20 °C, and always keep the slides away from light whenever possible.

5.2. **Slide preparation**

5.2.1. Remove slides from the freezer and thaw them at room temperature. Cover the tissue section with a drop of mounting media with DAPI. Place a coverslip of appropriate size on top and avoid bubble formation.

5.2.2. Use nail polish to seal the edges of the coverslip, allow nail polish to dry in the dark at room temperature, and then store the slides in a light-tight container at 4 °C, or proceed with imaging capture of GFP-LC3 puncta.

5.3. **Quantification of GFP–LC3 puncta by fluorescence microscopy**

5.3.1. Perform epifluorescence microscopy, capturing pictures in the same condition (exposure and settings) for all the samples. For GFP, use an emission wavelength of 525 nm; for DAPI, use 490 nm. Acquire at least ten images per sample using a 60X objective for vastus lateralis and the frontal cortex region of the brain.

5.3.2. Quantify the number of GFP-LC3 puncta in a tissue area of 2,500 μm^2 in each image for statistical analysis, blinded for experimental groups. Calculate the average number from 10 images of each sample, using the automatic measurement function of “Object Count”. Settings for the vastus lateralis muscle are: Threshold L=10 H= 200; 2X smooth; 1X clean, and settings for the brain frontal cortex are: Threshold L=11 H= 200; 2X smooth; 1X clean.

6. Western Blot analysis on autophagy markers

6.1. Tissue process

6.1.1. Prepare the lysis buffer: 50 mM Tris-HCl; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; protease inhibitor and phosphatase inhibitor (freshly added).

6.1.2. Remove the tissue of C57BL/6 mice from -80 °C storage. Cut the muscle tissue into small pieces with a razor blade prior to the following process. Add 800 µL (for hemi-brain) or 500 µL (for vastus lateralis) of cold lysis buffer to the sample. Homogenize the sample at 4 °C with a homogenizer at a medium speed.

6.1.3. Fully lyse the homogenized mixture for another 30 min to 1 h at 4 °C with rotation.

6.1.4. Spin down the homogenate at 12,000 x g for 10 min at 4 °C, discard the pellet, and collect the supernatant in a new tube.

6.2. **Autophagy analysis**

6.2.1. Determine the protein concentration of tissue lysates using the BCA Protein assay kit according to the manufacturer's specifications. Normalize each sample to the same concentration.

6.2.2. Combine the sample with the 2X Laemmli sample buffer at a 1:1 ratio, boil it at 95 °C for 5-10 min, and proceed with western blot analysis on autophagy markers[12,13], such as p62 (anti-p62 antibody, 1:500 dilution) and LC3 (anti-LC3 antibody, 1:500 dilution). Boil the samples immediately after addition of the sample buffer, as longer incubation on ice may generate multiple degraded bands of LC3.

6.2.3. Quantify p62 and LC3 bands by densitometry analysis using ImageJ, and normalize the value to the corresponding actin band.

REPRESENTATIVE RESULTS:

This protocol describes two different methods to induce autophagy in mouse tissues by aerobic exercise: a total of 90 minutes of forced exercise on a multi-lane treadmill proceeded by two days of acclimation; or two weeks of voluntary exercise on a running wheel used by single-housed mice. In each exercise protocol, we can measure the autophagy flux by fluorescence microscopy and western blot analysis in various organs. We used a transgenic mouse line expressing GFP-tagged LC3 as a reporter system to monitor autophagy by exercise[1]. Upon autophagy induction, LC3 translocates from the cytosol to the autophagosome in punctate structures. After sectioning, formation of GFP-LC3 puncta can be directly visualized by fluorescence microscopy (Figure 1A). Alternatively, autophagosome structures can also be immunostained by an LC3 antibody for imaging. Either 90 minutes of treadmill exercise or 2 weeks of voluntary running increased the number of GFP-LC3 puncta in both skeletal muscle (vastus lateralis) and cerebral cortex, compared to the resting condition (Figure 1B). It should be noted that the frontal cortex region has been the major region in the brain where autophagy is clearly induced by either method so far. Exercise also induced the conversion of LC3 from the cytosolic form (LC3-I) to the lipid-conjugated form (LC3-II), which can be detected by western blot analysis (Figure 1C).

Exercise-induced increment of autophagosomes (represented by LC3-II and GFP-LC3 puncta) is due to an elevated autophagic flux, rather than a block in autophagosome degradation, assessed by the use of inhibitors of lysosomal degradation, such as bafilomycin A1 or chloroquine. Here we measured the degradation of the autophagic cargo receptor p62 as an example. Exercise (90 minutes of treadmill) caused a higher degradation of p62 in skeletal muscle than the resting condition, which was rescued by injection with chloroquine prior to exercise (Figure 2). The similar results are also observed with voluntary exercise by running wheels. Thus, aerobic exercise by treadmill or running wheels induces autophagy *in vivo*, measured by the steady-state level of LC3 and the degradation of p62.

Figure 1. Aerobic exercise induces autophagy in mouse tissues.
Representative images (A) and quantification (B) of GFP-LC3 puncta in skeletal muscle (vastus lateralis) and brain (frontal cortex) of GFP-LC3 transgenic mice under the control condition (resting), after 90 minutes of forced exercise (treadmill) or after 2 weeks of voluntary exercise (wheel). Results represent mean ± s.e.m of 10 pictures per mouse. N=5 mice. The following emission wavelengths were used: GFP-525 nm; DAPI-490 nm. (C) Western blot detection of LC3 in skeletal muscle (vastus lateralis) from rested and exercised (by treadmill) mice. Quantification data represent the level of LC3-II normalized to actin (left) and the ratio of LC3-II to LC3-I (right). N=3 mice. Statistic is comparing each value to the control sample. Results represent mean ± s.e.m. *, P<0.05; **, P<0.01; ***, P<0.001, t-test. Scale bar, 25 μm.

Figure 2. Exercise increases autophagy flux in mouse skeletal muscle.
(A) Western blot detection of p62 in vastus lateralis from rested and exercised mice in the presence or absence of the lysosomal inhibitors chloroquine. (B) (Left) Quantification analysis of p62 normalized to the corresponding actin band. (Right) The p62 flux is determined by subtracting the normalized densitometric value of PBS-treated p62 from that of chloroquine-treated p62. Results represent mean ± s.e.m. N=3 mice. *, P<0.05, t-test.

DISCUSSION:
Autophagy is a catabolic process that provides energy and reduces cytotoxicity by lysosomal degradation of cytoplasm components or damaged organelles. Studying autophagy is important to understand the regulation of cellular homeostasis and the mechanisms of stress response. New models and methodologies are emerging in the research field[14], to study how impaired autophagy contributes to numerous pathological processes[15,16].

Nutrient deprivation (starvation) and pharmacological induction are commonly used approaches to induce autophagy *in vitro* and *in vivo*. These methods, especially for animal models, may show adverse effects that can influence the overall results. For example, since it requires at least 48 hours of starvation to induce a detectable level of autophagy, the animals may not have energy needed for regular motor activity, which affects the outcome of many subsequent behavioral studies. Yet pharmacological inducers may also lead to side effects due to their lack of specificity to the autophagy pathway. The major autophagy inducer rapamycin and its derivatives suppress mTOR activity and cause metabolic dysfunction and immunosuppression[17-19], which should be taken into consideration in the experimental design for long-term treatment. Thus, we have been working on more physiological and robust ways for autophagy activation in animal models.

Recently we and others have identified exercise as an effective, faster and safer autophagy inducer *in vivo*[7-9]. Both forced exercise by treadmill and voluntary exercise by running wheel have been used to analyze the effects of exercise on autophagy activation, with variations in exercise duration and intensity[20,21]. For example, an hour of

treadmill running (starting at speed of 10 m/min to a maximum of 40 m/min) induces abundant LC3 lipidation in skeletal muscle[20], and longer-term voluntary wheel running for 3 months or 4-5 weeks also increases basal autophagy and enhances the expression of autophagy proteins in skeletal muscle[20,21].

Here we described and compared the two methods (treadmill and running wheel) to exercise mice, and presented optimized shorter protocols with high efficiency in autophagy induction. Each approach has pros and cons. A single bout of 90 minutes of forced exercise on treadmill is sufficient to induce autophagy in skeletal muscle and brain. We have done a time course of treadmill exercise[7], and found that the exercise conditions described here induce the maximal level of autophagy in mouse skeletal muscle, which is also validated by others[22]. Under these conditions, mice undergo aerobic exercise; as previously reported, aerobic exercise is induced by prolonged running with gradual increases in speeds on a treadmill [23-25], whereas the anaerobic condition is obtained by a short duration of high intensity exercise, which increases muscle mass but does not necessarily enhance exercise endurance[26,27]. Furthermore, the running speed reported here in this protocol positively correlates with oxygen consumption capacity, by indirect methods to assess the aerobic capacity[28]. Therefore, aerobic exercise on a treadmill is a fast and effective way to induce autophagy.

However, forced running in an enclosed space may exert stress to mice. Thus, to avoid giving additional stress to animals, we use finger nudges or wire tassels as a method to keep mice running, instead of the built-in electric shock. Compared to the treadmill, the use of running wheel has many advantages. It is less stressful, does not require researchers' observation time, and is convenient to study long-term effects of autophagy activation. Yet exercise on a running wheel is voluntary and thus generates variability in terms of running distance and speed among different mice or the same mouse on different days. Therefore, it is necessary to run mice on a wheel for an extended period of time (e.g., several weeks) to minimize the individual variability. Importantly, the approach also requires using an odometer at night to verify that the mouse is actually running. We measured the average running distance of wild-type C57BL/6 mice over a period of 2 weeks, and found that they run approximately 1 km/night at the beginning of training (day 1) and can run up to 8-10 km/night at day 14. Overall, the running wheel is an effective method to stimulate autophagy in most organs, although, it is harder to capture autophagosome accumulation in the skeletal muscle compared to using the treadmill. The reason is that skeletal muscle has a high autophagic flux and a fast autophagosome degradation rate, which requires immediate tissue harvesting after running to detect the autophagy induction by GFP-LC3 puncta measurement. In either protocol, rapid PFA perfusion and fixation of the tissues after animal euthanasia is a critical step of the procedure to preserve the autophagosome structures that are otherwise easily degraded during dissection.

This protocol demonstrates how to use aerobic exercise to stimulate autophagy in mouse tissues, including brain and skeletal muscle. These methods can be used to study the mechanisms of the autophagy pathway in the maintenance of tissue function, such as mitochondrial maintenance[22], and to study the long-term effects of autophagy

induction on the regulation of behavior and health of animal disease models, such as enhancing the analgesic effects of cannabinoids[9]. Both the treadmill and running wheel methods induce a good level of autophagy; yet it is important to consider their differences when choosing the best approach to meet different research goals.

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DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure 1

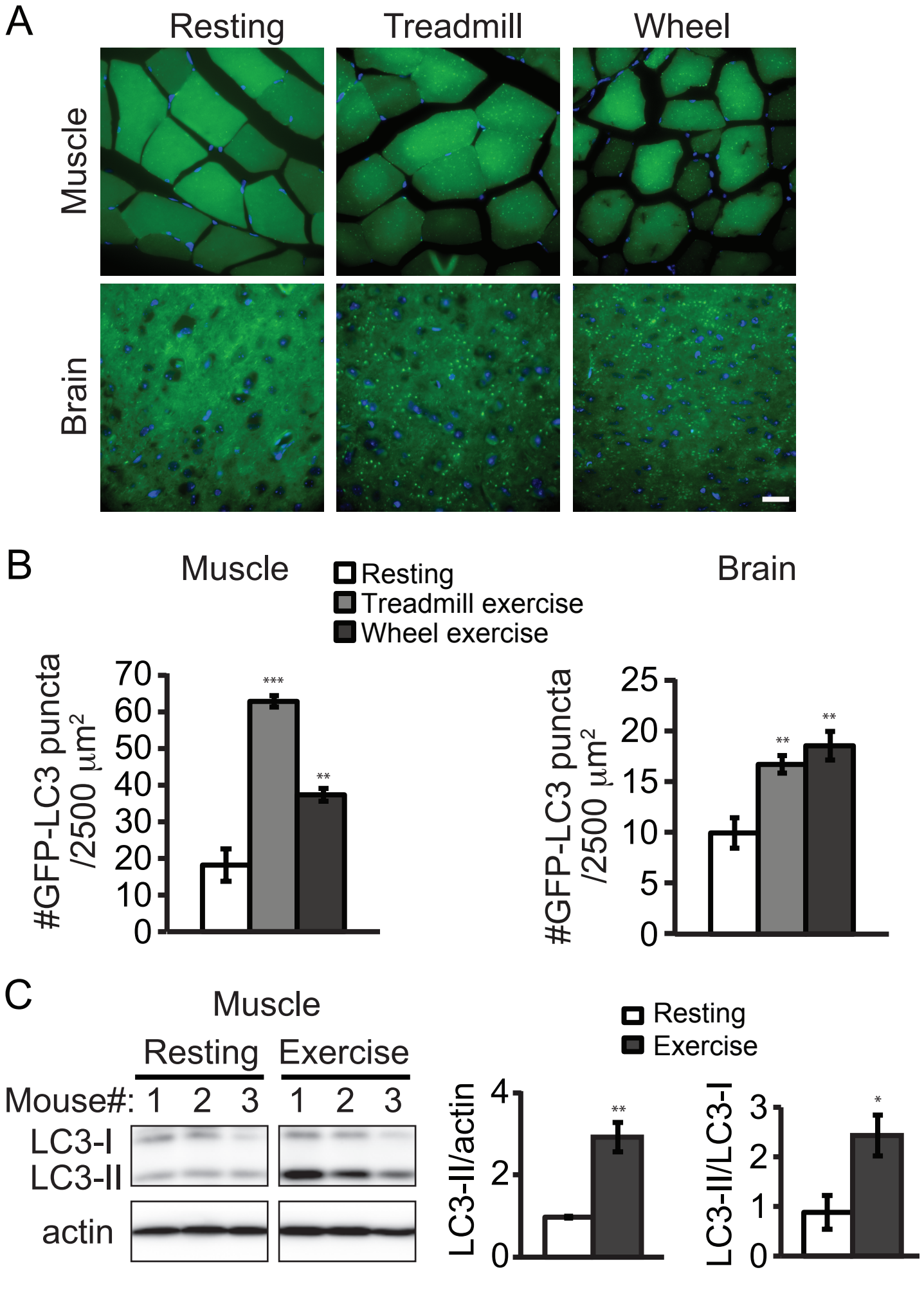
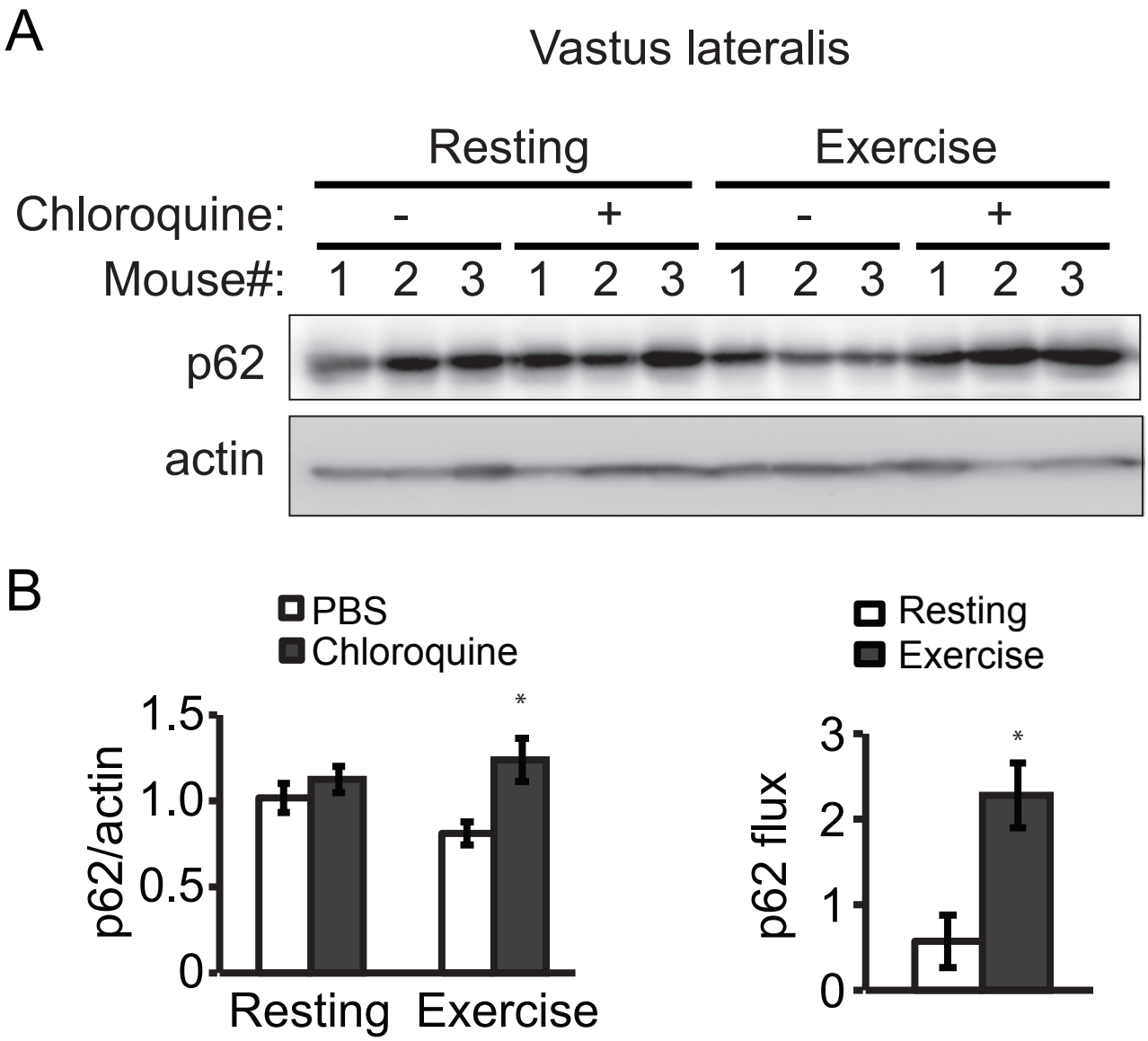


Figure 2



Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Treadmill	Columbus Instruments	150-RM Exer 3/6	
Mouse running wheel	Super Pet	100079365	diameter 11.4 cm
Odometer	Bell	DASHBOARD 100	
Syringe pump	KD Scientific	KDS100	
Fluorescence microscope	Nikon	Model: inverted microscope ECLIPSE	
Cryostat	Leica	CM 1850UV	
Homogenizer	IKA	003737001 / Model: T10 Basic S1	
Chloroquine	CAYMAN CHEMICAL COMPANY	14194	
Parafolmaldehye	SIGMA-ALDRICH	P6148	Personal protection equipment required.
Mounting media	Vector Laboratories	H-1200	
p62 antibody	BD Biosciences	610833	
LC3 antibody	Novus Biologicals	NB100-2220	
2X Laemmli Sample Buffer	Bio-Rad Laboratories	161-0737	
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Authors' Response Letter

We thank the editor and the reviewers for the very helpful comments. In response, we have added new discussions, references and revisions to address the concerns. **All the changes have been tracked in the manuscript**. We believe that the revisions considerably strengthen the manuscript. Below we provide a detailed point-by-point response to each comment.

Detailed Point-by-Point Response:

Editorial comments:
The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55099_R0_062316.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
Authors' Response:
We have proofread the manuscript throughout and made necessary corrections.

2. Please provide an email address for each author.
Authors' Response:
We have included the email addresses for both authors.

3. How are the mice sacrificed?
Authors' Response:
The mice are euthanized by isoflurane inhalation, followed by cervical dislocation. It is described in 4.3. We have added "as described in 4.3" in Sections 2.1.3 and 3.1.3.

4. Formatting:
-Please use italics for Latin phrases (ex vivo, in vivo, etc.).
Authors' Response:
We have used italics for all the Latin phrases.

-PFA is toxic and requires a caution statement.
Authors' Response:
We have added "caution: personal protection equipment required" in the texts 4.1, and added notes "Personal protection equipment required. This product may release formaldehyde gas, a chemical known to cause cancer" in the Table of Materials and Equipment.

5. Grammar: 6.1.1 – "Triton TX-100"
Authors' Response:
We have corrected it to "Triton X-100".

6. Additional detail is required:
-2.1.1 – How is initial acclimation and training performed (ie length of time, rewards, speed etc.)? For the third day, what speed and incline is used?

Authors' Response:
We have revised the texts in 2.1.1 and 2.1.2 to be clearer on the initial acclimation and training on the first two days and the speed and incline on the third day.

-4.6 – How are tissues extracted? Please describe or provide a citation.
Authors' Response:
We have cited the following references on brain and muscle dissection, and added new descriptions in Section 4.6:

1. Nat Protoc. 2013 Sep;8(9):1680-93. doi: 10.1038/nprot.2013.107. Epub 2013 Aug 8. isolation and expansion of human and mouse brain microvascular endothelial cells. Navone SE, Marfia G, Invernici G, Cristini S, Nava S, Balbi S, Sangiorgi S, Ciusani E, Bosutti A, Alessandri G, Slevin M, Parati EA
2. Nat Protoc. 2015 Oct;10(10):1612-24. doi: 10.1038/nprot.2015.110. Epub 2015 Sep 24. Isolation of skeletal muscle stem cells by fluorescence-activated cell sorting. Liu L, Cheung TH, Charville GW, Rando TA.

-5.1.4 – Is a cryostat used for cutting? Are tissues oriented a certain way?
Authors' Response:
We used the cryostat Leica CM 1850UV machine for cutting, and have added the information in the texts (5.1.4) and in the Table of Materials and Equipment. The orientation of the tissue is described in 5.1.2: cross section for the vastus lateralis muscle and sagittal section for the hemi-brain.

-5.3.1 – What wavelengths are used?
Authors' Response:
For GFP, the emission wavelength is 525 nm; for DAPI, the emission wavelength is 490 nm. We have added the information in 5.3.1 and in the legends of Fig 1.

-5.3.2 – Is ImageJ used here? Please indicate the software in the materials table.
Authors' Response:
No. ImageJ is not used here in 5.3.2, but is used in 6.2.3 for quantification. We have added the software in the Table of Materials and Equipment.

-6.2.2 – What is the composition of the sample buffer? Please include in the materials table is used as purchased. Please also include a citation for how to perform the western blotting, as this is a multistep process.
Authors' Response:
We used the following sample buffer: 2X Laemmli Sample Buffer (Bio-Rad Laboratories; Cat #: 161-0737), and have included the information in 6.2.2 and the Table of Materials and Equipment.

We have added the following references for western blotting analyses in Section 6.2.2:

1. J Vis Exp. 2010 Oct 14;(44). pii: 2359. doi: 10.3791/2359. Western blotting: sample preparation to detection. Eslami A and Lujan J.
2. Methods Enzymol. 2009;452:181-97. doi: 10.1016/S0076-6879(08)03612-4. Monitoring autophagic degradation of p62/SQSTM1. Bjørkøy G1, Lamark T, Pankiv S, Øvervatn A, Brech A and Johansen T.

7. Branding: 5.1.2 – Please reduce the number of mentions of OCT to 1 in this step.
Authors' Response:

We have revised the texts and mentioned “OCT” only once.

8. Results: Please define the error bars in Figure 1C (SD, SEM, etc.).
Authors’ Response:
We have defined the error bars as the standard error of the mean (s.e.m.), by adding “Results represent mean±s.e.m.” in Fig 1C legends.

9. Discussion: Please discuss any modifications/troubleshooting that can be performed as well as the future applications of the protocol.
Authors’ Response:
Modifications/troubleshooting is included in the discussion section. We have discussed the future applications in the last paragraph and cited two recent references as examples (Lo Verso et al. 2014; Kuramoto et al. 2016). The usage of treadmill or running wheel in autophagy regulation is relatively new, and we believe will be utilized in more studies in the near future.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
In the manuscript the authors described two different way to induce autophagy through fiscal exercise: forced run on the treadmill and spontaneous run on the wheel. Beside the technical aspects related to the physiology of the experiments, the authors clarify the methods to monitor autophagy flux through histology and biochemistry.
The major issue in the manuscript is that these methods are not new. Several papers reported how autophagy is induced through physical exercise, in mice and humans as well. Surprisedly, the authors didn't comment or compare their methods with the set up reported in the other papers. A more deep critical analysis of the literature is needed.
Authors’ Response:
To address the reviewer’s comments, we have added the following texts in the discussion:

“Recently we and others have identified exercise as an effective, faster and safer autophagy inducer *in vivo*. Both forced exercise by treadmill and voluntary exercise by running wheel have been used to analyze the effects of exercise on autophagy activation, with variations in exercise duration and intensity. For example, an hour of treadmill running (starting at speed of 10 m/min to a maximum of 40 m/min) induces abundant LC3 lipidation in skeletal muscle, and longer-term voluntary wheel running for 3 months or 4-5 weeks also increases basal autophagy and enhances the expression of autophagy proteins in skeletal muscle.”

We also cited the following references to discuss and compare the published methods on autophagy induction by exercise.

1. Autophagy. 2011 Dec;7(12):1415-23. Physical exercise stimulates autophagy in normal skeletal muscles but is detrimental for collagen VI-deficient muscles. Grumati P, Coletto L, Schiavinato A, Castagnaro S, Bertaggia E, Sandri M, Bonaldo P.
2. FASEB J. 2013 Oct;27(10):4184-93. doi: 10.1096/fj.13-228486. Epub 2013 Jun 27. Autophagy is required for exercise training-induced skeletal muscle adaptation and improvement of physical performance. Lira VA1, Okutsu M, Zhang M, Greene NP, Laker RC, Breen DS, Hoehn KL, Yan Z.
3. Autophagy. 2014;10(11):1883-94. doi: 10.4161/auto.32154. Epub 2014 Oct 30. Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is

important to prevent mitochondrial damage during physical activity. Lo Verso F, Carnio S, Vainshtein A, Sandri M.

Reviewer #2:

Manuscript Summary:

This manuscript is nice. The authors described the exercise pathway for mice and the autophagy detective methods in detail.

Major Concerns:

1. About the definition of "aerobic exercise" and the amount of exercise for mice, the authors should explain why the exercise conditions described in this manuscript were used in the research, and why the exercise condition is belonging to aerobic exercise. The different exercise amount maybe causes different autophagy level.

Authors' Response:

Based on a time course of treadmill exercise in one of our literatures (He C, et al. 2012), we conclude that the exercise conditions described in this manuscript induce the maximal level of autophagy in mouse skeletal muscle, which is also validated by others (Lo Verso et al. 2014). Thus, we used these conditions for autophagy induction in this protocol. Treadmill can be used for either aerobic or anaerobic exercise in mice, which is determined by different exercise duration and intensity. Specifically, aerobic exercise is induced by prolonged running with gradual increases in speed (references listed below), whereas the anaerobic condition is obtained by a short duration of high intensity exercise, which increases muscle mass but does not necessarily enhance exercise endurance (references listed below).

References on aerobic exercise:

- Kregel KC, Allen DL, Booth FW, Fleshner MR, Henrikson EJ, Musch TI, O'Leary DS, Parks CM, Poole DC, Ra'anan AW, Sheriff DD, Sturek MS, Toth LA. Resource book for the design of animal exercise protocols. American Physiological Society; 2006. Exercise Protocols Using Rats and Mice.
- Lightfoot J.T., Turner M.J., Debated K.S. & Kleeberg S.R. Interstrain variation in murine aerobic capacity. Med Sci Sports Exerc. 2001: 33, 5.
- Rezende EL, Chappell MA, Gomes FR, Malisch JL, Garland T., Jr Maximal metabolic rates during voluntary exercise, forced exercise, and cold exposure in house mice selectively bred for high wheel-running. J. Exp. Biol. 2005;208:2447–2458.

References on anaerobic exercise:

- Eur J Appl Physiol. 2002 Jun;87(2):141-4. Epub 2002 Apr 12. Effects of sprint exercise on oxidative stress in skeletal muscle and liver. Kayatekin BM, Gönenç S, Açikgöz O, Uysal N, Dayi A.
- J Appl Physiol. 1998 Jun;84(6):1852-7. Effects of high-intensity intermittent swimming on glucose transport in rat epitrochlearis muscle. Kawanaka K, Tabata I, Tanaka A, Higuchi M.

Moreover, the running speed reported in this protocol has been positively correlated with oxygen consumption capacity, by indirect methods to assess the aerobic capacity (Fernando, P., A. Bonen, and L. Hoffman-Goetz. Predicting submaximal oxygen consumption during treadmill running mice. Can. J. Physiol. Pharmacol. 71: 854–857, 1993). Therefore, the exercise protocol described here belongs to aerobic exercise. We have added the above texts and references in Discussion.

Additionally, white muscle and red muscle maybe have different responses to the same exercise condition. There are lot of questions are not clear as so far. So, I suggest the authors to remind the readers that the name or the part of the muscles should be defined in their research focused on skeletal muscle. With the same reason, I suggest the author to define the name or the part of skeletal muscle and brain in the two graphs in this manuscript. "Skeletal muscle" and "brain" is ambiguous.

Authors' Response:

We agree with the reviewer that glycolytic and oxidative muscles may have different levels of autophagy in response to exercise. Thus, we have specified in the texts and figure legends that the muscle group used for autophagy analyses is "vastus lateralis", and that the brain images taken are in the region of the frontal cerebral cortex, as this is the main region of the brain where we detected clear exercise-induced autophagy.

Minor Concerns:

1. Line 295 in Page 8: "adverse side effects" should be revised as "adverse effects" or "side effects".

Authors' Response:

We have corrected it to "adverse effects".

2. Line 166 in Page 5. In preparing the tissue for western blot analysis, why the tissues were collected after cold PBS perfusion? This step maybe causes the increment of autophagy. Autophagy is a physiological process, so, any changes of physiological condition may cause the activation of autophagy. I think the tissues for Western blot should be collected on ice directly after the mice were euthanized.

Authors' Response:

We would like to thank the reviewer to point it out. We have mistakenly included the step in this protocol. This step (perfusion with ice-cold PBS) is used only if we need to perform immunostaining and western blotting analyses on tissues of the same mouse. We have now corrected it in Sections 4.2 and 4.8.

3. Fig 1A, Could the authors supply the GFP-LC3 images with DAPI staining?

Authors' Response:

We have replaced the images with the new ones with DAPI staining in Fig 1A.

4. Fig 2A, the picture of Western blot is not very clear. Could the authors supply a lighter exposure picture of actin?

Authors' Response:

We have replaced the original actin image with a lighter exposure one in Fig 2A.