Voluntary Exercise Suppresses Choroidal Neovascularization in Mice

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Purpose. To determine the effect of voluntary exercise on choroidal neovascularization (CNV) in mice.

Methods. Age-matched wild-type C57BL/6J mice were housed in cages equipped with or without running wheels. After four weeks of voluntary running or sedentariness, mice were subjected to laser injury to induce CNV. After surgical recovery, mice were placed back in cages with or without exercise wheels for seven days. CNV lesion volumes were measured by confocal microscopy. The effect of wheel running only in the seven days after injury was also evaluated. Macrophage abundance and cytokine expression were quantified.

Results. In the first study, exercise-trained mice exhibited a 45% reduction in CNV volume compared to sedentary mice. In the replication study, a 32% reduction in CNV volume in exercise-trained mice was observed ($P = 0.029$). Combining these two studies, voluntary exercise was found to reduce CNV by 41% ($P = 0.0005$). Exercise-trained male and female mice had similar CNV volumes ($P = 0.99$). The daily running distance did not correlate with CNV lesion size. Exercise only after the laser injury without a preconditioning period did not reduce CNV size ($P = 0.41$). CNV lesions of exercise-trained mice also exhibited significantly lower F4/80+ macrophage staining and *Vegfa* and *Ccl2* mRNA expression.

Conclusions. These findings provide the first experimental evidence that voluntary exercise improves CNV outcomes. These studies indicate that exercise before laser treatment is required to improve CNV outcomes.

Keywords: exercise, choroidal neovascularization, angiogenesis

Pathological neovascularization underlies dozens of vision-threatening diseases including age-related macular degeneration (AMD), corneal neovascularization, glaucoma, diabetic retinopathy, and retinopathy of prematurity. Although intraocular anti-Vascular Endothelial Growth Factor A (VEGFA) therapies are a clinical success, they are not a panacea. For example, 12% to 25% of neovascular AMD patients, representing hundreds of thousands of individuals in the United States,[1] have 20/200 vision or worse despite treatment.[2–4] Prolonged exposure to anti-VEGFA is accompanied by loss of initial visual acuity gains,[5–8] and a significant portion of anti-VEGFA-exposed eyes develop untreatable central retinal atrophy.[4,9] Moreover, between 2013 and 2015, 3.75 million doses of anti-VEGFA drugs approved by the Food and Drug Administration were administered in the United States, costing patients, taxpayers, insurers, and providers approximately $7.5B.[10] Thus there is a compelling need for new, inexpensive antiangiogenic strategies that can target the molecular drivers of neovascularization.

Physical activity is a noninvasive, patient-controlled, and inexpensive intervention that improves numerous health outcomes both in healthy people and in those suffering from diverse clinical conditions (systematically reviewed in).[11,12] In contrast to prevalent conditions, such as diabetes,[13]

cardiovascular disease,[14,15] and neurocognitive disease,[16] the relationship between exercise and AMD is far less established. Numerous epidemiologic studies have attempted to characterize this impact, with the majority reporting a positive influence of physical activity on AMD and related outcomes (e.g., large macular drusen).[17–33] A recent systematic meta-analysis of nine studies on exercise and AMD in white subjects found that physical activity was associated with modest reduction in early AMD (odds ratio 0.92; 95% confidence interval [CI], 0.86–0.98) and a dramatic reduction in late AMD (odds ratio 0.59; CI, 0.49–0.72).[34] Together, these findings suggest that physical activity may represent a significant modifiable risk factor for AMD.

Voluntary wheel running, a model of endurance exercise in mice, has been widely used to study physiological adaptations, including muscle fiber transformation, angiogenesis, mitochondrial biogenesis, and mitophagy with significantly improved physiological and metabolic functions and protection against chronic diseases.[35–41] A voluntary regimen allows mice to exercise during their normal active dark cycle,[42] which would be disrupted by forced exercise, such as treadmill running and swimming.[43–45] Forced exercise is reported to cause acute and chronic stress responses that can manifest systemically[46–49] and may confound results. Finally, perhaps because of these issues, direct comparison of voluntary and forced exercises has found voluntary exercise superior in improving other pathological phenotypes.[50–52] Therefore we sought to examine the effects of voluntary wheel running on laser photocoagulation-induced CNV in a rigorous and controlled experimental setting.

Materials and Methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Virginia. Animal studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male and female C57BL/6J mice were housed in temperature-controlled (21°C) cages in a pathogen-free room with a 12:12-hour light/dark cycle and free access to water and normal chow.

Voluntary Running

Voluntary running was conducted as established previously.[41] Briefly, mice in the exercise group were housed individually in cages equipped with running wheels and sedentary mice were housed in cages not equipped with running wheels. Daily running was recorded via a computerized monitoring system, as described in previous studies.[35,37]

Laser Photocoagulation Induced Choroidal Neovascularization

Laser photocoagulation (532 nm, 180 mW, 100 ms, 75 µm) (OcuLight GL; IRIDEX Corp., Mountain View, CA, USA) was performed bilaterally (four spots per eye) on day 0 to induce CNV as previously described.[53] Irrespective of the exercise protocol, mice were three months old at the time of laser injury.

CNV Volume and F4/80 Labeling

After laser injury, mice were euthanized, and eyes were enucleated and fixed with 4% paraformaldehyde for 30 minutes at 4°C. Eyecups were incubated with 0.7% fluorescein isothiocyanate (FITC)-isolectin B4 (Vector Laboratories, Burlingame, CA, USA), and R-phycoerythrin-conjugated anti-F4/80 (Bio-Rad, Hercules, CA, USA) and the flat mounts of RPE-choroid-sclera were mounted in antifade medium (Immu-Mount Vectashield Mounting Medium; Vector Laboratories). CNV volume was visualized using a scanning laser confocal microscope (Nikon A1R, Nikon Instruments). Volumes were quantified using Image J software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) as previously reported.[53] F4/80 labeling was quantified by densitometry of the F4/80 signal in the maximum z-projection of the CNV lesion.

Fluorescent in Situ Hybridization

Enucleated mouse eyes were embedded in optimal cutting temperature medium (Sakura Finetek USA, Torrance, CA, USA) and snap-frozen in liquid nitrogen-supercooled isopentane. Seven-micrometer–thick sections were hybridized with RNAscope probes for Ccl2 (ID: 311791), Il6 (ID: 315891), and Vegfa (ID: 412261) according to manufacturer's instructions (ACDBio, Newark, CA, USA). Sections were mounted in Invitrogen ProLong Gold Antifade Mountant with DAPI (Thermo Scientific, Waltham, MA, USA) and imaged on a Nikon A1R inverted confocal microscope (Nikon Instruments Inc., Melville, NY, USA). Quantification of absolute transcripts was performed in ImageJ. The integrated density of an individual punctum was measured as the first peak in the intensity histogram of each 8-bit grayscale image, thresholded to reduce background. Then, the following equation was used to calculate the total number of transcripts: $\frac{\sum integrated\ density}{average\ intensity\ of\ single\ dot} \times area\ of\ image \times section\ thickness$.

Results

Effect of Voluntary Exercise on CNV in Mice

The first study design is depicted in Figure 1A. Age- and sex-matched wild-type C57BL/6J mice were singly housed in cages equipped with or without running wheels (N = 3 male sedentary, N = 3 female sedentary, N = 3 male exercise, N = 3 female exercise). After four weeks of voluntary running, mice were anesthetized and subjected to laser injury to induce CNV. After surgical recovery, mice were placed back in cages with or without exercise wheels for seven days, at which time animals were euthanized, and CNV lesions were analyzed. Seven days after injury was selected as an endpoint because the lesion is sufficiently large to measure accurately, and the lesion is actively expanding, with a peak volume occurring at 14 days,[54] allowing us to quantify pathology in a state that is both established and expanding.

In total, N = 40 CNV lesions from sedentary and N = 48 lesions from exercise-trained mice were included for analysis in Study 1. One mouse in the sedentary group was excluded because the procedure failed, possibly due to its poor health. We observed a 45% reduction in CNV volume in exercise-trained mice compared with sedentary mice ($P = 0.017$ by two-tailed Mann-Whitney U test, Fig. 1B). We did not find a

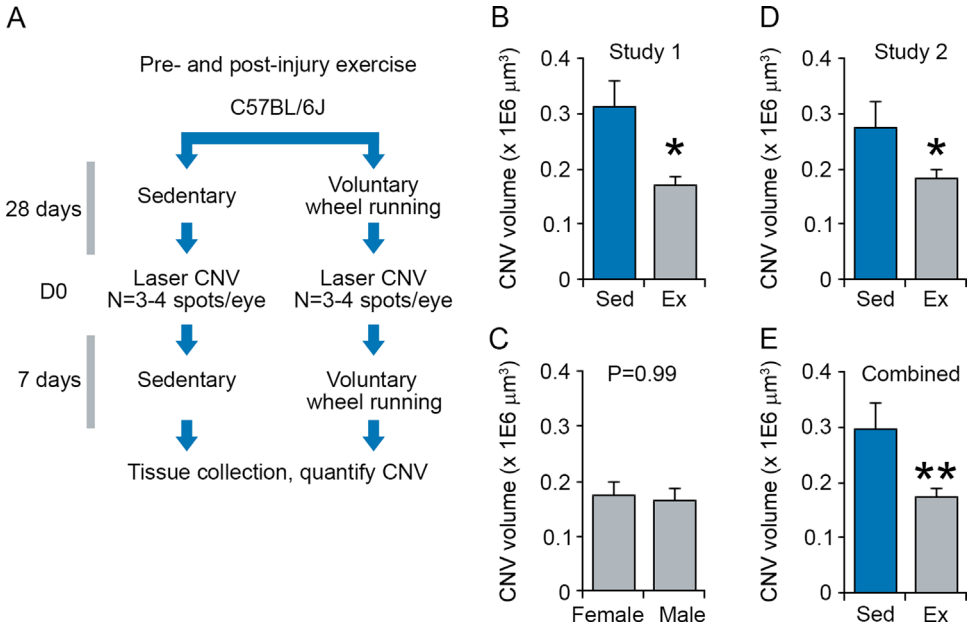

Figure 1. Exercise-trained mice develop less CNV than sedentary mice, independent of sex. (**A**) Study design for Studies 1 and 2: C57BL/6J mice were housed with (voluntary wheel running) or without (sedentary) an exercise wheel for 28 days. After laser photocoagulation on day 29, mice were returned to their respective cages for seven days. (**B**) CNV volume in sedentary and exercise-trained mice in Study 1. (**C**) CNV volume in exercise-trained male and female mice ($P = 0.99$, Mann-Whitney U test). (**D**) CNV volume in sedentary and exercise-trained mice in Study 2. (E) CNV volumes from Studies 1 and 2 combined. N = 65 sedentary, N = 67 exercise. *$P < 0.05$, ** $P < 0.01$.

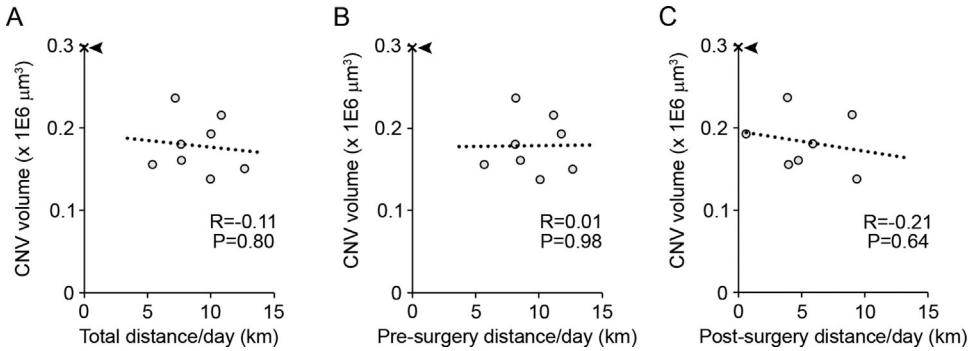

Figure 2. CNV volume and average distance traveled. (**A**) The average CNV volume of each mouse plotted against its average distance traveled throughout the duration of the experiment. Arrow on y-axis denotes the average CNV volume in sedentary mice. (**B**) The average CNV volume of each mouse plotted against its average distance traveled prior to laser photocoagulation surgery. (**C**) The average CNV volume of each mouse plotted against its average distance traveled after laser photocoagulation surgery.

significant difference in body weights of exercise or sedentary mice (25.1 ± 0.8 g vs. 25.5 ± 1.1 g, $P = 0.76$).

Exercise-trained male and female mice had similar CNV volumes ($P = 0.99$, Fig. 1C). No significant difference was observed between CNV in sedentary male and female mice in Study 1 ($P = 0.42$, Supplementary Figure S1). We validated this interpretation by comparing male and female mice in the remaining studies finding no significant difference ($P = 0.70$, Supplementary Figure S1). This finding is consistent with a previous study reporting no significant effect of sex on CNV volumes in mice younger than nine months of age.[55]

We conducted a replication study of similar design, with the exceptions that only male mice were used and that sedentary mice were not individual-housed, because CNV volumes from individual-housed mice were not significantly different from group-housed mice as established in prior baseline studies ($P = 0.81$, Supplementary Figure S2). We validated this interpretation by comparing individual- and group-housed mice in the remaining studies finding no significant difference ($P = 0.96$, Supplementary Figure S2). In the replication study, an additional N = 25 CNV lesions from sedentary mice and N = 19 CNV lesions from exercise-trained mice were analyzed. In this second study, we again observed a reduction in CNV volumes in exercise-trained compared to sedentary mice ($P = 0.029$, Fig. 1D). Combining these two studies, voluntary exercise was found to reduce CNV by 41% ($P = 0.0005$, Fig. 1E).

Dose Effect of Wheel Running on CNV

Throughout both studies, mice with exercise wheels traveled an average of 8.2 km/day, comparable to C57BL/6J in

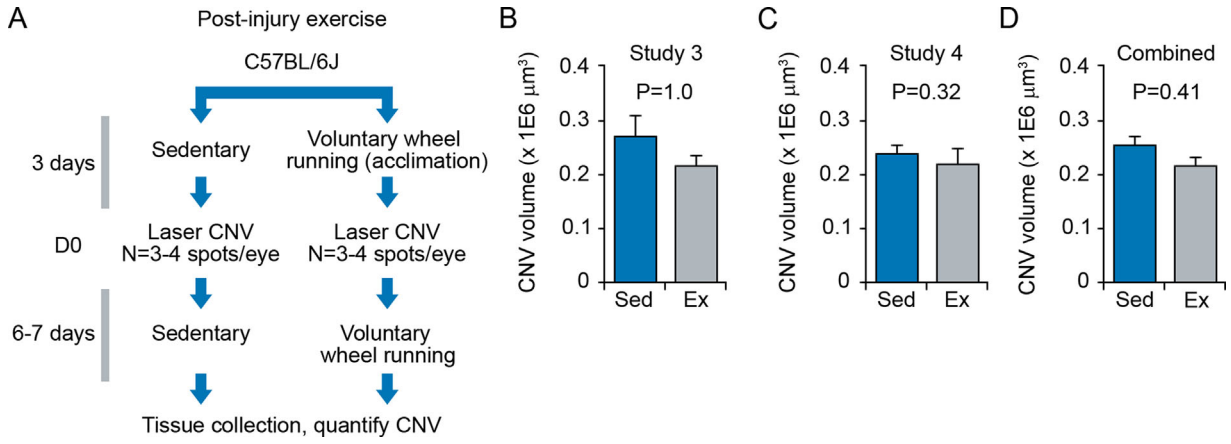

Figure 3. Post-injury exercise and CNV. (**A**) Study design for Studies 3 and 4: C57BL/6J mice were housed with or without an exercise wheel for 3 days to acclimate. Then, mice were subjected to laser photocoagulation surgery, and returned to their respective cages for seven (Study 3) or six days (Study 4). (**B**) CNV volume in sedentary and post-injury, exercise-trained mice in Study 3. N = 44 sedentary, N = 45 post-injury, exercise-trained, $P = 1.0$ by Mann-Whitney U test. (**C**) CNV volume in sedentary and post-injury, exercise-trained mice in Study 4. N = 54 sedentary, N = 23 post-injury, exercise-trained. $P = 0.32$ by Mann-Whitney U test. (**D**) CNV volumes from Studies 3 and 4 combined. $P = 0.41$ by Mann-Whitney U test.

previous studies.[42,56] Quantifying the relationship between running activity and CNV volume in individual mice, the average daily distance traveled did not correlate strongly with CNV volume ($R = -0.11$, $P = 0.80$, Fig. 2A). Daily distance traveled was significantly greater in mice before laser photocoagulation surgery ($P = 0.03$ by two-tailed paired Student's *t*-test). Neither the daily distance traveled before nor after surgery significantly correlated with CNV volume (Figs. 2B, 2C), although there was a slight negative relationship between run distance after surgery and CNV volume that did not reach statistical significance ($R = -0.21$, $P = 0.64$).

Effect of Postinjury Voluntary Exercise on CNV in Mice

We sought to determine whether exercise undertaken concurrent with pathology, without preinjury preconditioning, was sufficient to improve CNV outcomes. To isolate the effects of postinjury exercise, a second study design was conducted as depicted in Figure 3A. Here, mice were allowed a brief three-day acclimation period with the exercise wheel, followed by laser injury to induce CNV, and then permitted to exercise throughout the recovery period with or without exercise wheels. Once again, a replication study of similar design was performed. In the first of two independent postinjury exercise trials (Study 3), a total of N = 44 CNV lesions from sedentary and N = 45 lesions from postinjury exercise-trained mice were included for analysis. We observed a 21% reduction in CNV volume in exercise-trained mice compared with sedentary mice, although this effect did not achieve statistical significance ($P = 1.0$ by two-tailed Mann-Whitney U test, Fig. 3B). In a replication study of similar design (Study 4), an additional N = 54 CNV lesions from sedentary mice and N = 23 CNV lesions from exercise-trained mice were analyzed. In this second study, we again observed a nonsignificant reduction in CNV volumes in exercise-trained mice compared with sedentary mice (8% reduction, $P = 0.32$, Fig. 3C). Combining these two studies, postinjury exercise did not significantly reduce CNV ($P = 0.41$, Fig. 3D).

Reduced F4/80+ Cells and Cytokine Transcription in CNV in Exercise-Trained Mice

Immune cells, including macrophages, are prevalent in human CNV[57–60] and critically contribute to experimental CNV.[61–63] We quantified the effect of exercise training on immune cell infiltration in CNV seven days after injury by measuring F4/80 immunolabeling in RPE/CNV whole mounts. In mice undergoing preinjury and postinjury exercise, we observed a dramatic 72% reduction in F4/80 positive staining in the CNV lesions of exercise-trained mice compared with sedentary mice ($P = 0.037$, Fig. 4A). Additionally, we used in situ hybridization to quantify the absolute number of transcripts of angiogenic cytokines in CNV lesions of exercise-trained and sedentary mice. In exercise-trained eyes, we observed a 38% reduction in *Vegfa* mRNA ($P = 0.012$ by two-tailed *t*-test) and 71% reduction in *Ccl2* mRNA ($P = 0.021$) in CNV lesions of exercise-trained eyes compared with lesions from sedentary mice (Fig. 4B). We also observed a 32% reduction in *Il6* mRNA in lesions of exercise-trained eyes, although this was not statistically significant ($P = 0.18$).

Discussion

This study provides the first experimental evidence on the influence of physical activity on CNV, supporting the findings of epidemiologic studies reporting beneficial effects of exercise on AMD-related pathologies. The dose effect of exercise was modest and did not achieve statistical significance. We interpret these findings to mean that the amount of exercise undertaken in this experimental design exceeded the threshold to achieve the maximal effect. Limiting exercise training to the CNV lesion growth period did not significantly reduce lesion size. We interpret this finding to mean that exercise preconditioning before the initiation of CNV is necessary to achieve a salutary effect.

In contrast to CNV, prior studies in mice report that exercise promotes angiogenesis and vascularity in skeletal muscle,[31] brain,[57] and subcutaneous adipose tissue.[58] It appears that the mechanisms by which exercise affects blood vessel homeostasis in these tissues may differ from CNV.

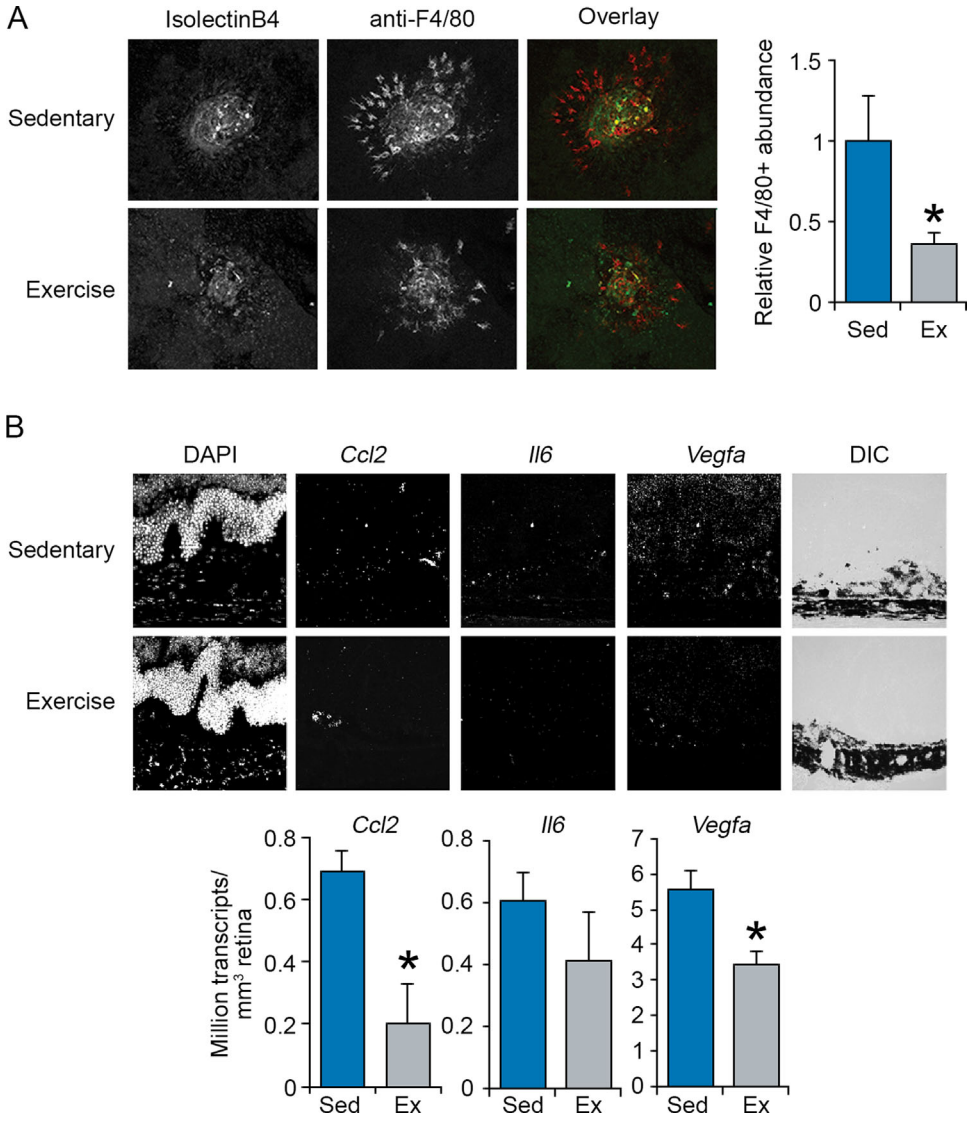

Figure 4. Voluntary exercised-trained mice and macrophage infiltration in CNV. (**A**) Fluorescent micrographs of choroid-RPE-sclera flat mounts from sedentary (*top*) and exercise-trained (*bottom*) mice seven days after laser injury. Isolectin-B4 depicted in green in overlay and F4/80 depicted in red in overlay. Quantification of F4/80+ staining depicted at right. N = 14 lesions from sedentary and N = 15 from exercise-trained mice (following the protocol in Fig. 1A). (**B**) Representative images (*top*) and quantification (*bottom*) of in situ hybridization of mRNA in retina of sedentary and exercise-trained mice (following the protocol in Fig. 1A) seven days after laser injury. N = 3 lesions per condition. *$P < 0.05$ by two-tailed Student's *t*-test.

We observed that lesions of exercise-trained mice exhibited reduced F4/80+ labeling and cytokine expression, suggesting that exercise may impart immunomodulatory effects. Indeed, exercise has been shown to ameliorate macrophage mobilization in a murine aging model[64] and in high-fat diet-induced inflammation.[65–67] Whether reduced immune cell recruitment is a driver of the beneficial effects of voluntary exercise on CNV is an important avenue of future study, as is identifying molecular intermediates of this effect.

Voluntary exercise induces a variety of systemic changes that may modulate CNV size, including food consumption and plasma cholesterol. Interestingly, short-term voluntary exercise is reported to induce an anorexic effect in mice, with reduced food consumption[68,69] whereas prolonged voluntary wheel running increases food consumption.[70,71] Voluntary exercise is reported to lower plasma triglycerides in humans[72] and triglycerides and cholesterol turnover in mice.[73]

Prior studies have found that voluntary exercise does not affect fasting blood glucose levels in normal, nondiabetic mice.[74,75] We found no significant difference in body weights of exercise and sedentary mice. Therefore we find it unlikely that blood glucose or body weight per se is responsible for the effect of exercise on CNV we observed. The extent to which these exercise-modifiable biomarkers correlate with CNV lesion size is an important avenue of future study.

Apart from our findings in CNV, exercise has also been reported to prevent retinal degeneration in normal aged mice,[76,77] in light-induced retinal degeneration,[78] and in a light injury model of retinitis pigmentosa.[79] Thus the beneficial effects of exercise on the retina may extend beyond suppressing pathologic angiogenesis.

A recent study found that Korean men, but not women, self-reporting five or more sessions of vigorous exercise per week were significantly more likely to develop neovascular AMD (hazard ratio, 1.54; CI, 1.15-2.06).[80] However, limitations in the methodology of this study include survival bias of the low physical activity cohort ("left truncation"), and potential disproportionate underreporting of neovascular AMD in the nonactive group.[81] Other studies have also reported marginal positive associations between physical activity and risk of developing AMD.[82,83] It should also be noted that the definitions of "adequate," "moderate," "strenuous," and "vigorous" physical activity are nonuniform between studies. In general, it is challenging to draw conclusions from epidemiologic studies of this nature because of the potential unreliability of questionnaire-based data[84] and the confounding effects that vision loss may have on the amount and type of exercise an individual undertakes.[85] Thus the continued study of exercise on AMD-relevant phenotypes in experimental models may provide clarity as to the nature of the effect and mechanistic drivers of physical activity in this condition.

Physical activity may be a low-cost, effective, and noninvasive treatment option in prevention of a number of eye diseases, including AMD. Identifying the molecular mediators that couple physical activity and CNV is an important avenue of research to understand the relationship between this complex modifiable risk factor and retinal disease. This study presents an experimental platform from which such investigations may be undertaken in future studies. The translational relevance of this study must be considered in the context of the limitations of mouse voluntary wheel running as a model for human exercise and laser photocoagulation as a model of CNV in human patients. Ultimately, the extent to which exercise proves beneficial for humans suffering with or at risk for development of CNV must be tested in the context of controlled, prospective clinical trials.

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