Using Drosophila to Understand Biochemical and Behavioral Responses to Exercise

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SUJKOWSKI, A. and R. WESSELLS. Using drosophila to understand biochemical and behavioral responses to exercise. *Exerc. Sport Sci. Rev.*, Vol. 46, No. 2, pp. 112–120, 2018. *The development of endurance exercise paradigms in* Drosophila *has facilitated study of genetic factors that control individual response to exercise. Recent work in* Drosophila *has demonstrated that activation of octopaminergic neurons is alone sufficient to confer exercise adaptations to sedentary flies. These results suggest that adrenergic activity is both necessary and sufficient to promote endurance exercise adaptations.* **Key Words:** *Drosophila*, endurance, adaptation, octopamine, norepinephrine, neuron

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Key Points

- Working models for endurance training in *Drosophila* are described.
- Training induces improvements in climbing speed, endurance, flight, and cardiac performance.
- Octopaminergic neurons are absolutely required for endurance adaptations.
- Stimulation of octopaminergic neurons is sufficient to drive adaptation in sedentary animals.

INTRODUCTION

Genetic variation has long been supposed to play an important role in individual response to exercise training programs, yet the specific factors that govern these differences are not well understood. One obstacle to a detailed study of the role of genetic variation in exercise response has been the lack of an exercise training paradigm in an invertebrate genetic model organism. Here, we describe recently developed methods for endurance training *Drosophila*. Further, we describe a novel hypothesis that adrenergic activity is both necessary and sufficient to promote endurance exercise adaptations.

Power Tower

Recently, we have developed an automated endurance exercise device for *Drosophila* capable of training several thousand flies simultaneously. Known as the Power Tower (Fig. 1), it takes advantage of the flies' natural instinct for negative geotaxis to stimulate repetitive climbing behavior. Fly vials on a platform are raised and dropped repetitively by a rotating lever arm. Each time the platform drops, flies climb up the side of the tube. This allows stimulation of mobility for defined times. Because the noise and physical jolt of the machine may cause some degree of nonspecific stress, control flies also are placed in vials on the machine, but have the foam plug in the top pushed down to limit the available area of movement. Thus, control files experience all the sensory stress of the exercise device without actually exercising.

After a ramped training program that increases the training time once per week for 3 wk, trained flies experience a suite of adaptations (Fig. 2). These include increased climbing speed, increased endurance, increased flight performance, increased mitochondrial activity, increased mitochondrial quality, increased lipolysis, increased cardiac fractional shortening, and increased resistance to cardiac pacing stress (4–8). Age-matched controls experience none of these adaptations, indicating that movement is required to induce these physiological changes; merely experiencing the stress of the machine exposure produces no positive effects.

Training effects are robust across genotypes, although the degree of change varies between genotypes (4,9). Diet also has been shown to have a strong effect on performance of flies on the Power Tower, with flies generally responding well to balanced amounts of protein and carbohydrate (4,9). Microarray experiments have demonstrated a high degree of overlap between changes in gene expression in trained flies and changes in flies selected for increased longevity (5). This strongly suggests that training on the Power Tower induces a pro-healthspan gene expression program.

Figure 1. The Power Tower, a motor-operated enforced climbing apparatus that exercise trains *Drosophila*. As the rotating arm circles around in a clockwise direction, the lever pushes down and the two platforms of vial holders rise up. As the rotating arm rolls off, the lever lifts and the platform of vial holders drops down. The flies drop to the bottom of the vial, inducing their negative geotaxis instinct. The flies continue to repeat this process until the motor is shut off. Exercise training thus occurs through continuous climbing.

TreadWheel

A second paradigm for exercising flies has now emerged, using a machine called the TreadWheel, which systematically inverts fly vials to stimulate negative geotaxis without lifting and dropping them (Fig. 3). Flies have been shown to respond to a ramped training program using the TreadWheel by increasing climbing response, reducing triglycerides, and upregulating genes related to mitochondrial biogenesis (10). Flies from different genetic backgrounds have been tested on this machine and have been shown to respond with different metabolic alterations, suggesting that the TreadWheel will be an effective means of studying the complete metabolic and physiological effects of exercise.

ASSAYS FOR TRAINING OUTCOMES IN *DROSOPHILA*

To maximize the utility of an invertebrate genetic model for exercise training, it is not sufficient to rely on genetics alone. One must have clear methods for quantitating physiological outputs of training to dissect pathways that are necessary or sufficient for various adaptations. Optimally, to take advantage of large numbers and short lifespan in invertebrate systems, noninvasive assays that can be measured longitudinally should be included. These methods can be blended with methods that require sacrifice and dissection of members of the cohort to generate a broad picture of physiological changes occurring during training. Here, we present a brief overview of the main methods of assessment currently in use.

Rapidly Inverted Negative Geotaxis (RING)

Because training on either the Power Tower or the TreadWheel relies on induction of negative geotaxis, a minimum expectation is that negative geotaxis ability should improve during the training. Negative geotaxis is easily assessable in noninvasive methods, making climbing ability an excellent indicator assay for daily tracking of training progress.

Flies are kept in groups of 20 throughout a training program because this degree of density provides best results. Each group of 20 can be conveniently transferred into a fresh vial for assessment each day just before their training bout. A rack of several vials is tapped down to induce negative geotaxis, with a photo taken at 2 s after stimulation. Photos are taken with a bright backdrop to facilitate automated quantitation of average height climbed by software that recognizes dark spots on bright background (11).

The brief time given for climbing allows comparisons of speed, as opposed to other versions of this assay that measure how many flies climb over a certain line when given plenty of time to do so. Assessment of percent of flies over the line is excellent for measuring general vigor and neuromotor coordination, as, for example, in neurodegenerative mutants or during normal aging (11,12), but more stringent measurements are needed to assess gradual improvements in speed. Daily assessments allow for measurements of the slope of change in individual vials and in the entire cohort.

Assessments of slope of change in the short-lived fly system are somewhat complicated because of the strong effect of aging during a 3-wk training program. Although 3 wk is unlikely to produce significant aging effects in a vertebrate model, this period represents a large fraction of the median lifespan for most *Drosophila* laboratory strains. Because positive effects of training are overlaid on negative effects of aging, it is important to compare slope of change to age-matched, unexercised controls to isolate the effects of exercise. Depending on genotype and treatment, exercised cohorts may finish with less raw speed than they started because of the large aging effect. However, wild-type cohorts always finish a training program with significantly higher speed than age-matched controls and show a substantially reduced slope of age-related decline. To extend a comparison to humans, this is equivalent to a 60-year-old marathoner who is slower than he or she was at the age of 25, but has much higher speed and endurance than an untrained 60-year-old.

Runspan

Increased endurance is a fundamental trademark of endurance training, making a method to measure endurance longitudinally essential for any training model. To do this, the Power Tower system uses a visual measurement of climbing response over time. A cohort of flies is observed during continuous stimulation by the Power Tower. Whenever less than 25% of flies in a vial are responding to the stimulus, the vial is considered fatigued and removed. The assay can then be quantitated as a survival curve, with each vial's time of removal treated as a *time of death*. This assessment, referred to as a *runspan*, has proven to be an extraordinarily sensitive and robust assay for tracking improved endurance in a variety of contexts (5,9,12,13). The TreadWheel system also measures the length of time that flies respond to its stimulus, in this case by an innovative automated measurement of distance travelled during stimulation (10).

Flight

It often is desirable to establish that a training program has systemic effects by testing a performance capacity using separate muscle groups from those used in the training behavior. For flies, flight offers a convenient, climbing-independent assay of mobility. There are several ways to measure flight performance (14,15). We typically use a drop tube that ejects flies into a cylindrical chamber that contains a rolled-up sticky trap (5). When flies are ejected into the sticky tube, they fly to the side and become stuck. Thus, the best flyers are stuck near the top and those with poor flight performance drift farther toward the bottom. The sticky trap can then be unrolled
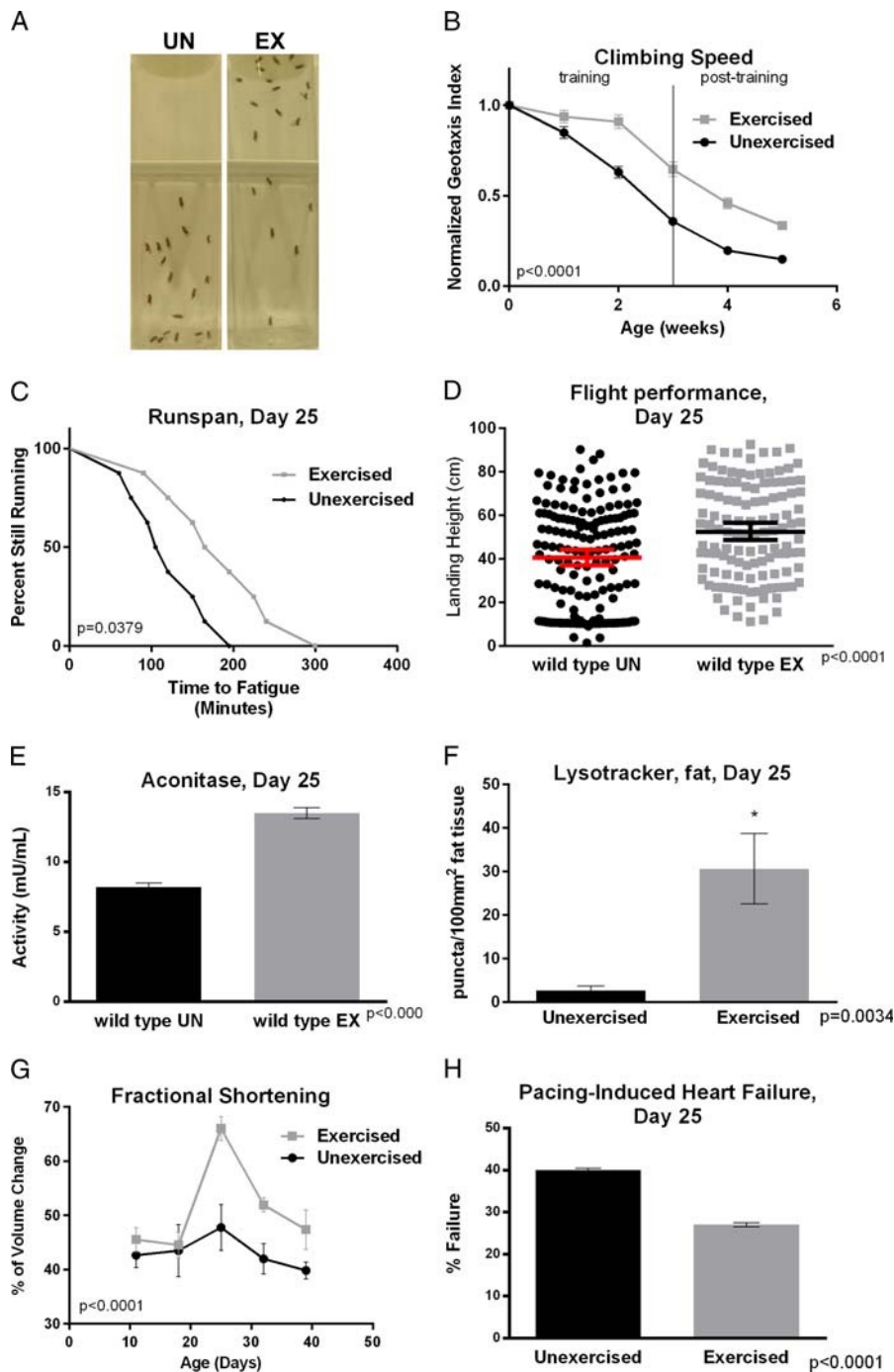
Figure 2. Exercise-trained (EX) *Drosophila* display conserved improvements in multiple physiological measures. A. Exercise-trained flies have enhanced climbing speed in comparison with unexercised cohorts also placed on the machine but prevented from climbing by a foam stopper placed low in the vial, quantified across ages in (B). C. Exercise-trained flies display increased endurance. Each point represents the time at which a vial of 20 flies reaches fatigue. D. Flight performance is increased in exercise-trained flies. E. Aconitase levels are increased in exercise-trained flies. F. Lysosomal activity in the adult fat body is increased in exercise-trained *Drosophila*. G. Exercise-trained flies have enhanced cardiac fractional shortening, an indirect measure of cardiac output. H. Exercise-trained flies exhibit reduced cardiac failure in response to electrical pacing-stress in comparison with age-matched, unexercised cohorts (UN). Data shown are independent repetitions of original data from (1–3).

and photographed, with the resulting spread of stuck flies quantitated by imaging software.

Exercise training has a clear effect in this assay, with trained flies landing higher in the tube on average than untrained (5), demonstrating that training has produced general improvements to motor function, not limited to negative geotaxis.

Cardiac Video Analysis

Along with longitudinal assays, it also is possible to take advantage of the large numbers available in *Drosophila* experiments by removing cohorts of 100 or more from the training regimen at particular time points for analyses that require dissection and sacrifice of the animals. Cardiac assays have been established

Figure 3. The TreadWheel provides a low-impact exercise stimulus for *Drosophila*. Rotating arms induce continuous walking in several *Drosophila* vials simultaneously. Unexercised cohorts have foam stoppers inserted close together to restrict movement and control for machine exposure, visible in close-up photo (below).

as worthwhile markers for physiological aging and robust assays for general health (16–18).

A great deal of information about cardiac performance can be extracted from live high-speed videos of cardiac performance. Such videos have been successfully collected from intact animals imaged through the transparent abdominal cuticle (6), or from animals dissected in physiological media to expose the heart (19). Because the fly heart is tube-shaped, it is relatively easy to track movements in two dimensions from a dorsal view. Live videos tracking these movements with a timestamp can be used to quantify rate, rhythmicity, contractile volume change, contractile velocity, and relaxation velocity. Such methods have been used to study numerous genetic models of development and disease in the fly system (17,20). Trained flies typically show increased contractile volume change and contractile velocity compared with age-matched controls.

Cardiac Pacing

Because exercise tolerance in vertebrates is closely related to the heart's ability to tolerate increased heart rate, testing flies' response to cardiac pacing is a useful assay to track changes during training. Flies can be connected to a square-wave stimulator using modified microscope slides with soldered wire leads. Using a 40 V pulse, the heart is stimulated to about twice its normal heart rate for 30 s, then hearts are visually scored through the transparent cuticle for their ability to successfully resume normal heart rate and rhythm (16,21). The percentage of hearts that fail to regain normal rate, going instead into arrest or fibrillation, is reported as the failure rate.

Failure rate in response to pacing is a powerful measure of physiological aging because it increases steadily with age (20), is reduced by treatments that slow aging (1,5,6), and is a sensitive indicator of decline before any obvious increase in mortality. Exercise training slows the increase of failure rate with age

in multiple genotypes (4) and can even rescue the failure rate of mutants with poor heart performance (6). Like flight, cardiac performance is useful for distinguishing interventions that genuinely mimic exercise from those that narrowly facilitate negative geotaxis.

MitoTimer

Transgenic flies have been made that express the MitoTimer construct, a mitochondrially targeted fluorophore that irreversibly changes color from green to red when it is oxidized. Because the color change is irreversible, the red-to-green ratio of MitoTimer expression in a given tissue typically always increases with time, as suggested by the name. The rate of change in this ratio can be used to calculate the rate at which a particular tissue or cell has been exposed to oxidizing agents.

Interestingly, in *Drosophila*, exercise training seems to preserve green-colored mitochondria in the heart compared with untrained controls (7). This seems to correlate with autophagosome activity in cardiomyocytes, consistent with the idea that exercise induces selective mitophagy with oxidized mitochondria preferentially removed. This assay can be used as a sensitive way to track mitochondrial quality in particular tissue types during training.

Other

The typical array of biochemical and metabolomic assays also can be used in the *Drosophila* system, either in whole flies or in isolated flight muscle or hearts. So far, Power Tower–trained flies have been assayed for total triglycerides, lysosomal activity, and mitochondrial enzyme activity (5,6,8) and TreadWheel–trained flies have been assessed for glucose levels, triglycerides, and mitochondrial gene expression (10).

Global changes in gene expression have been assessed by microarray, with many conserved genes and pathways identified, including changes to carbohydrate metabolism, fatty acid metabolism, and folate biosynthesis (5). In addition, a few novel genes have been identified in flies as regulators of the exercise response, including the G-protein–coupled receptor *methuselah-like 3* and the conserved Target of Rapamycin Complex-1 regulator *sestrin* (5).

The *Drosophila* model is an attractive option for rapid testing of candidate genes because of the large numbers that can be simultaneously tested, giving high statistical power, as well as the feasibility of genetic manipulation. Epistasis experiments and use of multiple genetic backgrounds are routine in fly experiments, lending rigor to assessments of relevance to particular genes or genetic interactions. Importantly, the *Drosophila* model is highly advantageous for experiments that include long-term assessments across the lifespan, an experimental design that is time consuming and expensive in vertebrate systems.

ARE EXERCISED FLIES "JUST STRESSED OUT"?

One concern about fly exercise is whether the changes that are seen are driven by the stressful environment of the exercise machine. This concern also has been applied to other exercise models, such as mouse exercise with a treadmill, enforced by a small electric shock, or swim training in mice (22). However, the effects of nonspecific treatment stress tend to be negative effects that reduce, if anything, the prohealthspan effects of exercise. Experiments with both the Power Tower and TreadWheel *Drosophila* exercise routinely use control cohorts that are

exposed to machine stress while prevented from increased running by a restraining foam plug. Comparing trained flies with controls that experience machine stress allows experimenters to isolate the effects of actual movement from any nonspecific machine effects. In no cases have the machine-control cohorts experienced any metabolic, mobility, or cardiac improvements.

Endurance exercise itself is a stressful experience, however, and is widely believed to act through hormesis, in which chronic exposure to low-level stresses builds up stress resistance to protect healthspan (23). Consistent with this point of view, we have found that maximum improvements are produced in flies by a ramped program of gradually increasing times of exercise, with interspersed rest days (4).

What factors mediate the effects of this hormetic stimulus, and are they conserved throughout the animal kingdom? Several factors that are secreted from skeletal or cardiac muscle have been identified that cause systemic prohealthspan effects in mammals and flies (24). These factors are currently the focus of intense study as potential mediators or mimetics of the exercise response. Differential secretion or activity of such factors may well mediate some of the differences between individual exercise responses. However, we reasoned that another important source of variation may be neuronal activity induced by exercise that triggers systemic adaptive responses. If so, this activity could be another important target for prohealthspan interventions. Next, we discuss recent work in the fly model that strongly supports this view.

NEURONAL CONTROL OF DROSOPHILA EXERCISE TRAINING

We and others (4,10) have observed substantial differences in exercise response between *Drosophila* genotypes, indicating that genetic differences are likely to play a substantial role in modulating training adaptations. However, we also routinely observe substantial individual variation among flies that are genetically identical, age matched, reared in the same environment, and fed the same diet. What is the source for this variation?
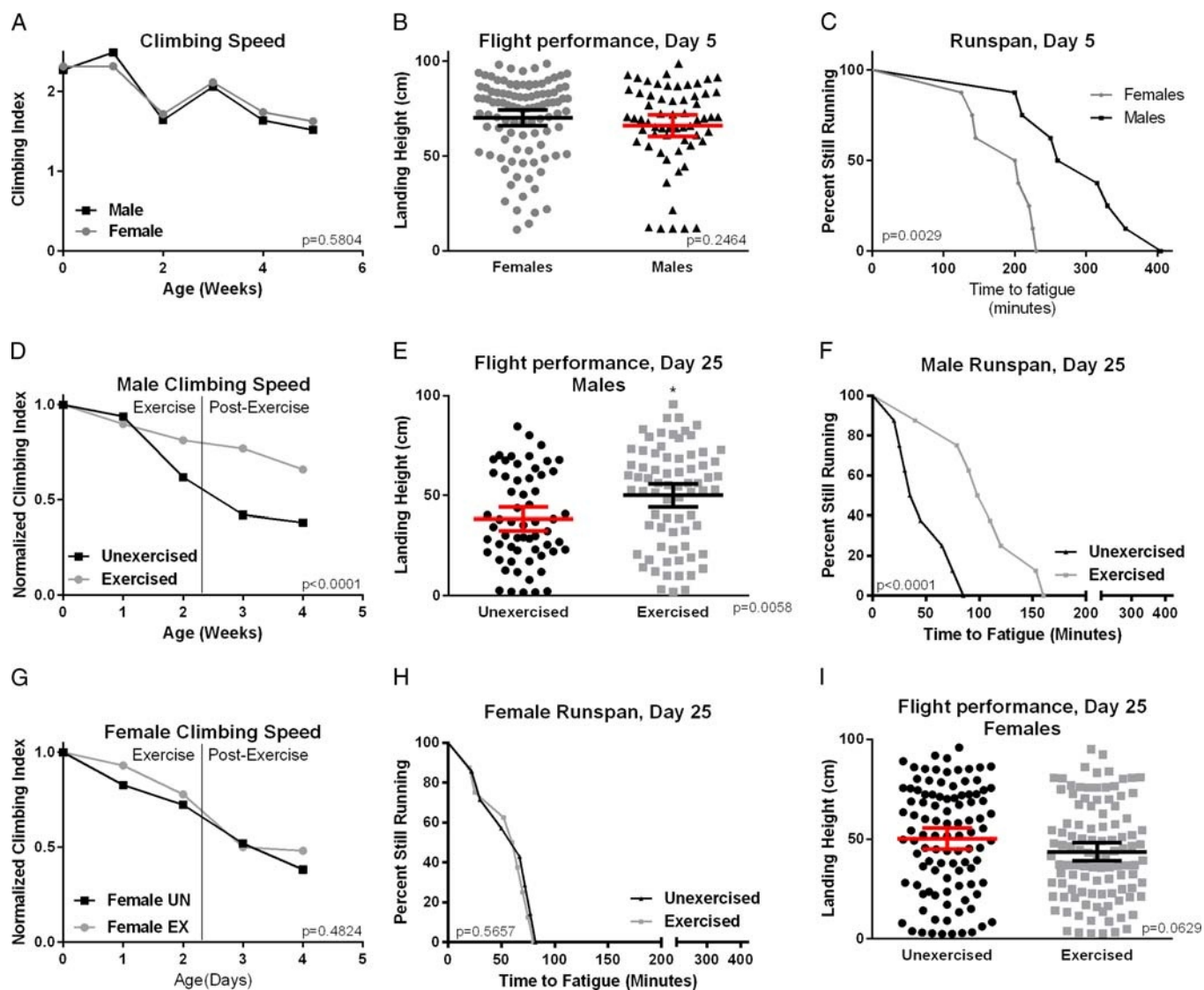
Figure 4. Exercise adaptations are sexually dimorphic in *Drosophila*. Male and female *Drosophila* perform equally in acute tests of (A) climbing speed and (B) flight capacity, but females stop responding to climbing stimulus sooner, as reflected in (C) decreased endurance. As a result, male flies adapt to endurance exercise with increased (D) climbing speed, (E) flight performance, and (F) endurance, whereas trained female siblings do not (G–I). Data shown are independent repetitions of original data from (24,26).

One possibility is that a neuronal response to sensory input during the training period is required for adaptations, and this neuronal response is quantitatively different among individuals as they experience training. To test this possibility and identify a mechanistic explanation, we took advantage of a species-specific peculiarity about *Drosophila melanogaster*, a marked sexual dimorphism in the response to the training stimulus provided by the Power Tower.

Sexual Dimorphism in The Power Tower Training Response

Although female flies have equal climbing speed and flight ability with males in an acute test (25), they do not continue to respond to the Power Tower stimulus as long as males (Fig. 4). This could be because of a difference in muscle metabolism that provides greater endurance to males, or could be due

to behavioral differences between males and females. To distinguish between these possibilities, we used cell-autonomous, inducible expression of feminizing or masculinizing constructs (2,27) to identify the tissues that mediate this sexual dimorphism.

We first determined that differences between male and female muscle did not account for the difference in exercise response, then ascertained that pan-neuronal masculinization was sufficient to confer a male exercise response on female flies (Fig. 5). Using a series of expression drivers, we then narrowed down the set of neurons responsible for this effect to those that produce and secrete the norepinephrine-like biogenic amine octopamine (Fig. 5). Although both males and females contain octopaminergic neurons, octopaminergic neurons seem to respond to an identical exercise protocol more readily in males. The specific genes that control this sex difference are not yet
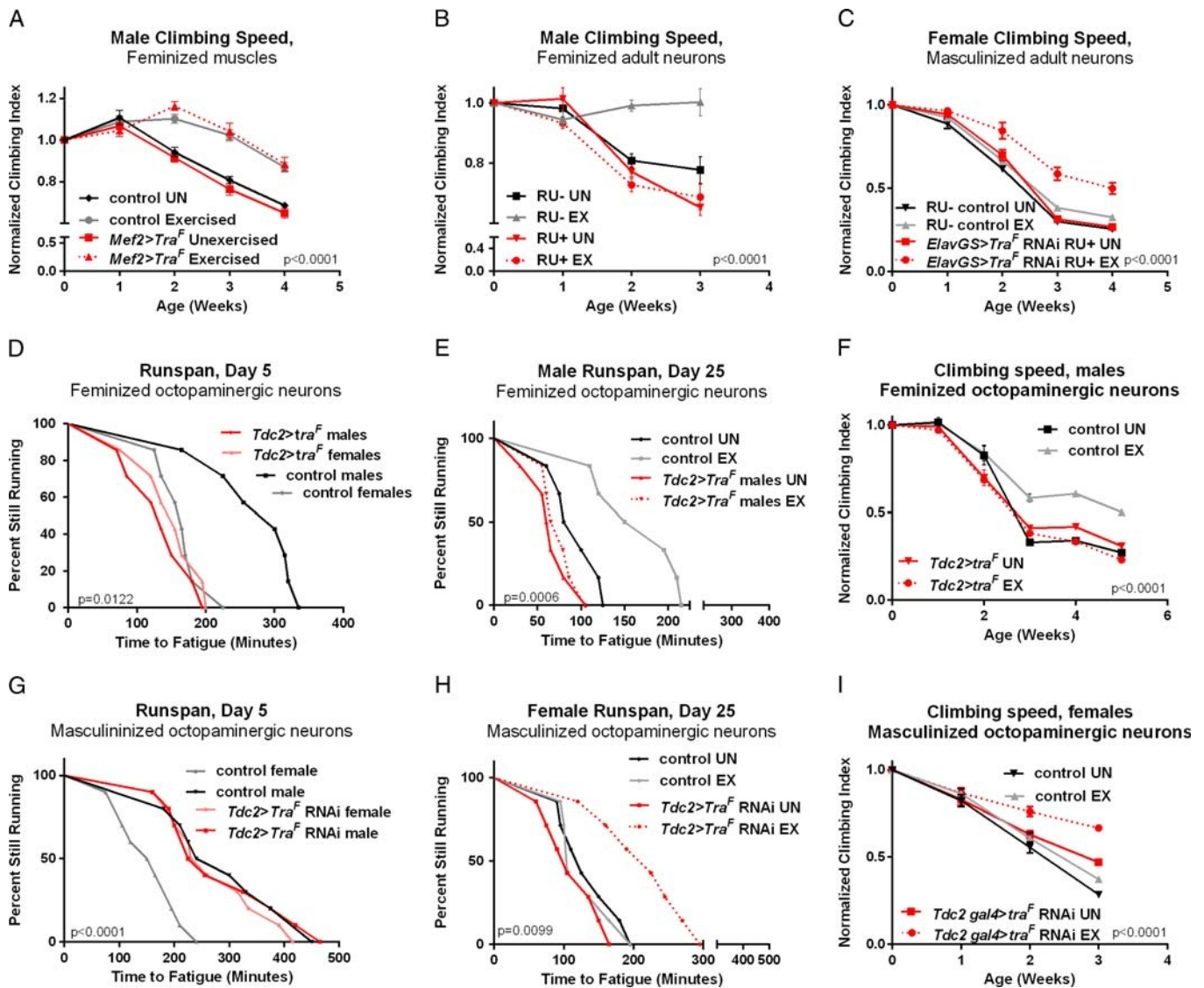
Figure 5. Sexually dimorphic exercise response can be reversed by feminization and masculinization of neurons. Sex determination in *Drosophila* is controlled by a single, alternatively spliced factor, *transformer* (tra). A. Males with feminized muscle tissue (*Mef2 > tra^F*) have normal male exercise response. B. Male flies with neurons feminized after development is complete (*ElavGS > tra^F* RU+) have female exercise response. Genetically identical controls in the absence of RU-induced adult-specific pan-neural feminization (*ElavGS > tra^F* RU-, abbreviated RU- here) respond to training normally (See RU- EX). C. Females with masculinized adult neurons (*ElavGS > tra^F* RNAi RU+) increase climbing speed with exercise, whereas genetic controls in the absence of drug do not. A targeted screen of neural populations revealed that feminization of octopaminergic neurons in males (*Tdc2 > tra^F*) is sufficient to reduce both young (D) and trained (E) endurance, as well as (F) improvements in climbing speed with exercise. Converse experiments were able to improve endurance and climbing speed in trained females (G–I). Data shown are independent repetitions of original data from (26). EX, exercised; UN, untrained.

known, but they must be under the regulation of the cell-autonomous master sex control gene *transformer* because altering transformer expression in octopaminergic neurons is sufficient to drive the effect (Fig. 5).

Octopamine is Necessary and Sufficient For Training Adaptations

When octopaminergic neurons were inhibited by expressing RNAi against *VMAT*, a gene necessary for vesicular release (26), neither male nor female flies were able to generate adaptations in response to exercise (Fig. 6). Similar results were seen when flies were fed mianserin, an inhibitor of octopamine activity (28). These results strongly suggest that secretion of octopamine from octopaminergic neurons is absolutely required for chronic

exercise to induce changes to speed, endurance, flight, and cardiac performance.

To do the converse experiment, a temperature-sensitive, depolarizing *trpA1* channel (3) was expressed in octopaminergic neurons. This allowed constitutive activation of octopaminergic neurons when flies were placed at 25°C. When octopaminergic neurons were constitutively activated in sedentary flies throughout the training period, those sedentary flies received the same increases in each assay as flies that had exercised daily at the same temperature. Similar results were achieved by feeding flies octopamine (25). Taken together, these results indicate that increased octopamine levels are indeed sufficient to drive adaptations to speed, endurance, flight, and cardiac performance, even in sedentary flies.
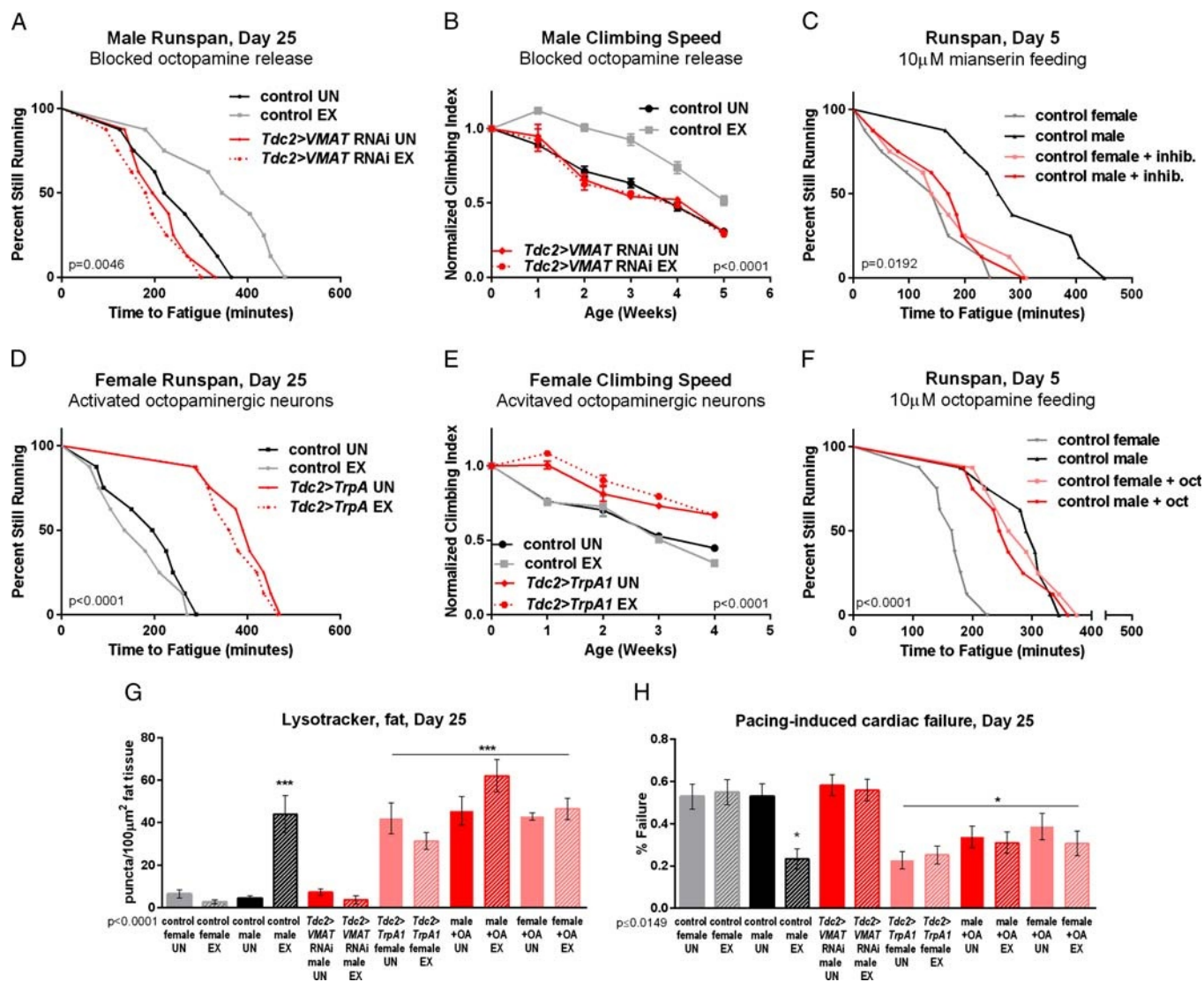
Figure 6. Octopamine (oct) phenocopies adaptations to endurance exercise even in untrained flies (UN). A. Males with blocked synaptic release at octopaminergic synapses do not increase endurance or (B) climbing speed with training. C. Similar results are observed when flies are fed the octopamine inhibitor mianserin. D. Adult females with increased excitability in octopaminergic neurons display increased endurance independent of training status. E. Climbing speed also is increased in adult females with enhanced octopaminergic neuron activity. Similar results were seen in males (see (27)). F. Octopamine feeding increases baseline endurance in young flies. G. Lysosomal activity is upregulated in the fat body of exercised male flies. Flies with RNAi against *VMAT* at octopaminergic synapses fail to increase fat body lysosomal activity. Females with increased octopaminergic neuron activity have high lysosomal activity in the fat body whether exercised or not. Males and females fed octopamine also have high lysosomal activity in the fat body independent of endurance exercise. H. Exercise training reduces cardiac failure in response to electrical pacing in male flies. RNAi against *VMAT* in octopaminergic neurons prevents cardiac adaptation to endurance training in exercised males. Female flies with increased octopaminergic neuron activity have increased resistance to cardiac pacing stress whether exercised or not. Males and females fed octopamine receive similar cardiac benefits. Data shown are independent repetitions of original data from (27).

Intermittent Stimulation of Octopaminergic Neurons Mimics Training

Because endurance exercise is an intermittent stimulation rather than a continuous activity throughout life, we wondered if we could mimic the effects of exercise training in flies by intermittent induction of octopaminergic neuron activity. To do this, we used flies expressing a temperature-sensitive depolarizing channel specifically in octopaminergic neurons. We performed temperature shifts at the same time as our standard times for exercise training throughout a 3 wk training protocol. Control flies were temperature shifted at the same time as the *trpA1-octopamine* flies, whereas another control group was actually exercised during the temperature shift time. Flies with daily induction of octopaminergic neuron activity received the same benefits to speed, endurance, flight, and cardiac performance as the group that performed daily exercise (25). These results indicate that induction of octopaminergic activity for defined times each day can fully substitute for a chronic endurance exercise program.

CONCLUSIONS

Here, we have described data in support of the hypothesis that adrenergic activity is necessary and sufficient to drive adaptations to exercise (Fig. 7). The adrenergic agonist octopamine has been observed to play sexually dimorphic roles in multiple behaviors (29) and is known to stimulate lipolysis (30), increase heart rate (31), and modify neuromuscular junctions (32). Each of these outputs are expected responses to exercise in many organisms, including humans (33). The vertebrate equivalent of octopamine is norepinephrine, a monoamine that is upregulated by exercise and also is known to stimulate heart rate, stimulate lipolysis, and modulate blood pressure (34). Because insects have an open circulatory system, continuous secretion of a pro-vasoconstriction compound has no effect on circulation *per se*. However, continuous administration of norepinephrine in a mammalian model or a human would be likely to have substantial hypertension-related adverse effects.

Thus, it is exciting that intermittent stimulation of octopaminergic signaling is sufficient to generate endurance adaptations in flies. Because endurance exercise is known to stimulate norepinephrine release in mammals (35), it is likely that intermittent secretion of norepinephrine does in fact occur in mammals undergoing chronic exercise. This raises the question whether endurance adaptations could be generated in patients with enforced sedentary conditions. Because chronic exercise is known to have numerous salutary effects on metabolism and provides protection against multiple age-related diseases, an intervention to provide such benefits would not be trivial.

For this to occur, further work will be necessary in both mammalian and invertebrate models to identify the minimum
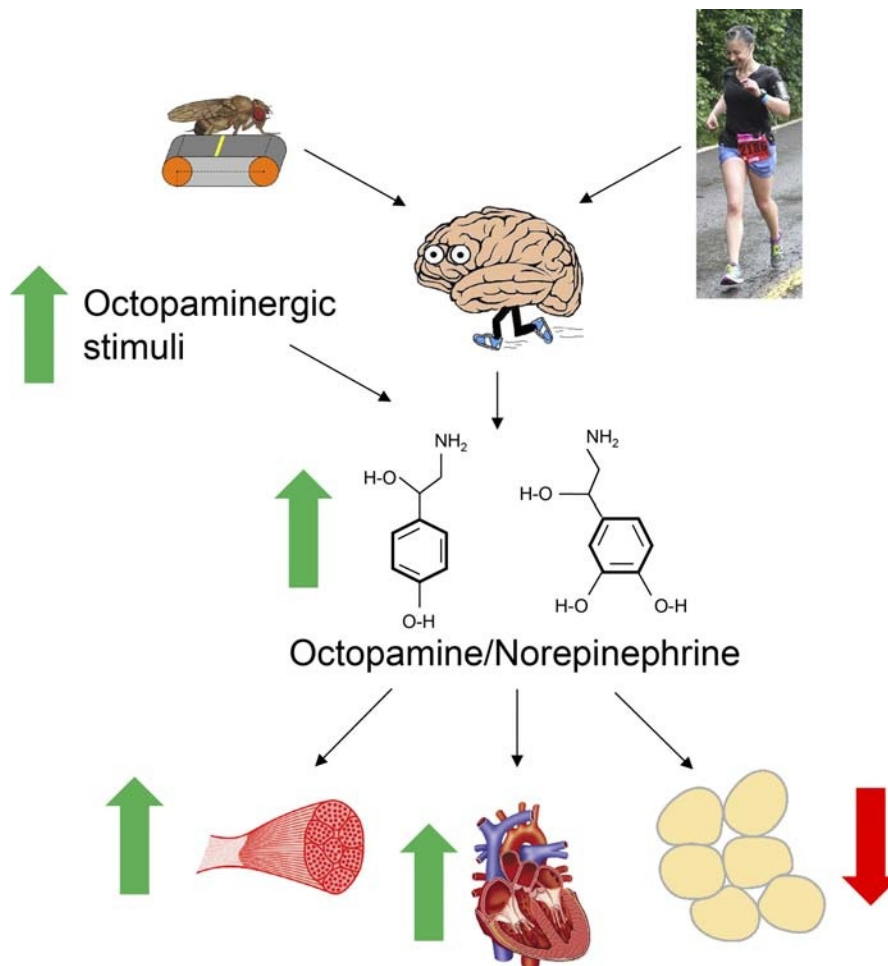
Figure 7. Octopamine drives exercise adaptations in *Drosophila*. Schematic diagram of the role of octopamine during endurance training. Running provides a signal to the brain to increase octopamine secretion (norepinephrine in vertebrates), which signals to target tissues and induces beneficial hormetic adaptations.

necessary downstream targets and their tissue requirements, or to identify downstream targets that have known pharmaceutical activators. One promising possibility, for example, is the conserved gene *sestrin*, which is activated by acute bursts of reactive oxygen species, and responds by upregulating AMP-activated kinase (AMPK) and downregulating target of rapamycin complex 1 (TORC1) activity (36). This combination of activities is similar to observed effects of endurance exercise in vertebrates (37). Thus, activity of the Sestrin protein is a strong candidate for a downstream regulator stimulated by exercise that could act as a mimetic. There are three Sestrin proteins in mice and humans, but only one in *Drosophila*, previously shown to be cardioprotective in *Drosophila* (38), making the fly an attractive model for study of the mimetic potential of this protein family.

Another possibility for future efforts will be to identify the minimum required sensory inputs in model organisms and in humans to generate these effects. Because control flies that experience the Power Tower machine without running do not receive adaptations, it is clear that simple exposure to stress cannot be sufficient to drive these adaptations. What then is the minimum experience of movement that induces the brain to upregulate octopamine secretion in the fly? Future experiments blocking certain subsets of sensory neurons will shed light on this problem. Given the tremendous progress in recent years in the development of virtual reality technology, the exciting possibility may exist in the near future to provide minimum sensory inputs to bedridden or disabled patients that would induce downstream effects that mimic exercise, allowing such patients, and potentially astronauts as well, to maintain a healthier life.

Acknowledgments

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