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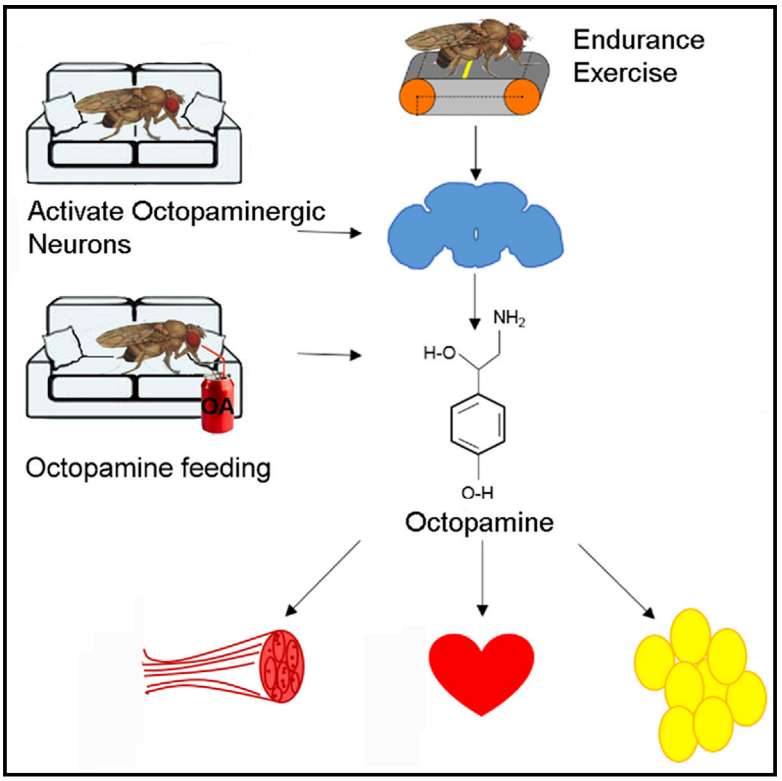
Octopamine Drives Endurance Exercise Adaptations in *Drosophila*

Graphical Abstract



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In Brief

Chronic exercise causes stereotypical adaptations in muscle and adipose tissue of *Drosophila*. Sujkowski et al. show that these adaptations require the activity of octopaminergic neurons. Differences in octopaminergic activity control sexual dimorphism in exercise response. Both octopamine feeding and stimulation of octopaminergic neurons can substitute for endurance exercise.

Highlights

- Exercise adaptations in *Drosophila* are sexually dimorphic
- Octopamine (OA) is necessary and sufficient for exercise adaptation in *Drosophila*
- Stimulation of OA-ergic neurons fully mimics exercise in sedentary flies

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Octopamine Drives Endurance Exercise Adaptations in *Drosophila*

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SUMMARY

Endurance exercise is an effective therapeutic intervention with substantial pro-healthspan effects. Male *Drosophila* respond to a ramped daily program of exercise by inducing conserved physiological responses similar to those seen in mice and humans. Female flies respond to an exercise stimulus but do not experience the adaptive training response seen in males. Here, we use female flies as a model to demonstrate that differences in exercise response are mediated by differences in neuronal activity. The activity of octopaminergic neurons is specifically required to induce the conserved cellular and physiological changes seen following endurance training. Furthermore, either intermittent, scheduled activation of octopaminergic neurons or octopamine feeding is able to fully substitute for exercise, conferring a suite of pro-healthspan benefits to sedentary *Drosophila*. These experiments indicate that octopamine is a critical mediator of adaptation to endurance exercise in *Drosophila*.

INTRODUCTION

Endurance exercise is a powerful, low-cost intervention effective in both disease prevention and healthspan promotion (Topp et al., 2004). Endurance training improves the healthy function of multiple organ systems including skeletal muscle, heart, and brain (Boström et al., 2013). These functional improvements are associated with conserved shifts in metabolism (Booth et al., 2015) and changes in gene expression in both vertebrate models (Coffey and Hawley, 2007) and *Drosophila* (Sujkowski et al., 2015).

Neither exercise capacity nor adaptations to chronic exercise are universal, however, and the mechanisms that regulate differences in individual exercise adaptations are not well understood. Changes in gene expression are variable in trained individuals, and metabolic remodeling is not uniform in training humans (Johnson et al., 2014). Moreover, exercise is inherently hormetic. Continuous, repeated exposure to a stressor (endurance training) promotes adaptive cellular changes that enable better tolerance to the stress (beneficial adaptations) (Merry and Ristow, 2015). Therefore, variation in stress response may influence how well an individual responds and adapts during endurance training.

Although several important hormesis-inducing factors have been identified in flies and vertebrate models (Owusu-Ansah and Perrimon, 2014), the lack of an endurance exercise paradigm for an invertebrate species has impaired the use of large-scale genetic discovery of exercise-induced pathways. We have previously described an endurance training paradigm for *Drosophila* that uses reiterated induction of negative geotaxis to allow controlled, daily training of fruit fly cohorts (Tinkerhess et al., 2012a). Following a 3-week period of ramped endurance exercise activity, wild-type male flies display increased climbing speed (Piazza et al., 2009), endurance (Tinkerhess et al., 2012b), cardiac performance (Piazza et al., 2009; Sujkowski et al., 2012), flight (Sujkowski et al., 2015), and mitochondrial enzyme activity (Piazza et al., 2009). Furthermore, endurance training increases mitochondrial number and reduces accumulated oxidative stress in fly cardiac muscle (Laker et al., 2014). Exercise also induces consumption of accumulated fat stores in flies with excess lipid (Sujkowski et al., 2012). Finally, exercised male *Drosophila* exhibit healthspan-associated changes in gene expression, including upregulation of stress defense genes (Sujkowski et al., 2015). Taken together, these observations support the idea that endurance training in *Drosophila* induces similar effects as training in humans (Gaman et al., 2011) and vertebrate models (Flück, 2006) and reinforce the concept of exercise training as a conserved hormetic intervention.

Serendipitously, *D. melanogaster* have strong sex-dependent differences in response to exercise. Female *Drosophila* respond poorly to chronic exercise and do not exhibit training-dependent adaptations. This provides an excellent opportunity for the study of individual variation, with females used as a model for poor trainers. Here, we separately feminize/masculinize target tissues in order to identify the cellular source of the variation between males and females.

We find that octopaminergic neurons are critical for the response to chronic exercise in *Drosophila*. Furthermore, we report that exercise adaptations require octopamine, the *Drosophila* counterpart to norepinephrine. Conversely, octopamine feeding is sufficient to fully replace exercise training in males or females. Excitingly, we discover that intermittent activation of octopaminergic neurons fully conveys the beneficial cellular and functional adaptations of endurance exercise, even in sedentary *Drosophila.*

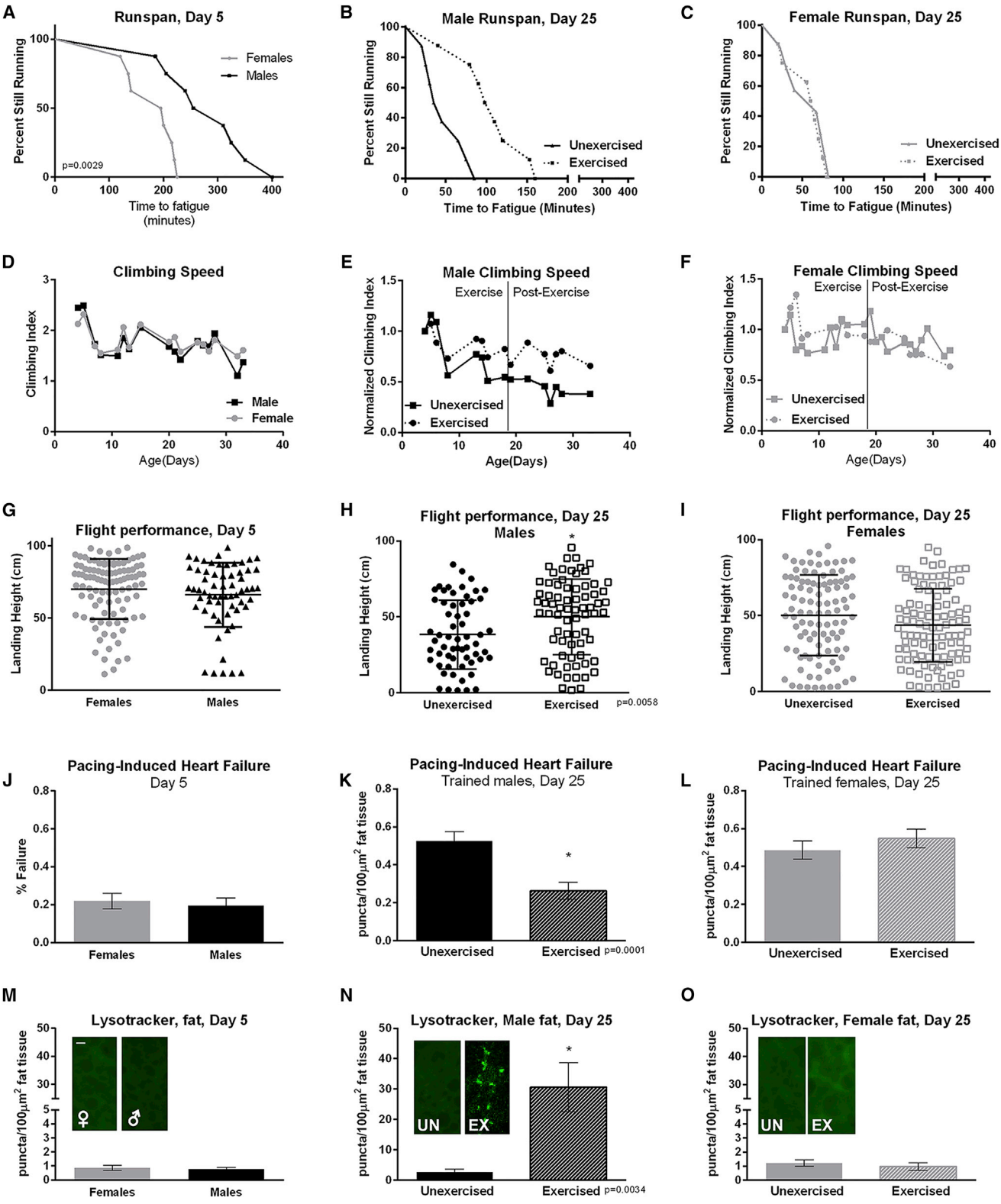

Figure 1. Adaptation to Endurance Exercise Is Sexually Dimorphic in *Drosophila*

(A) Endurance is greater in males than in age-matched, 5-day-old female siblings ($p = 0.029$, $n = 160$).

(B) Runspan declines with age (compare to A). Exercise trained males have greater endurance than unexercised males ($p < 0.0001$, $n = 160$).

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RESULTS

Females Are Not Deficient in Acute Performance

Runspan, a measure of *Drosophila* climbing endurance in which time-to-fatigue is charted by vial and plotted as a survival curve, is greater in male flies than female siblings at day 5 of adult life (Figure 1A). However, female flies have similar climbing speed to males in an acute test of distance climbed in 2 s (Figure 1D). Young, untrained male and female flies also have similar flight performance, measured by recording landing height after ejection from a fixed platform (Figure 1G) and similar response to cardiac pacing, as measured by percentage of flies experiencing cardiac fibrillation or arrest after 30 s of external electrical stimulation (Wessells and Bodmer, 2004). In young, untrained adults, lysosomal activity is similarly low in both male and female fat bodies (Figure 1M).

Adaptation to Chronic Exercise Is Greater in Wild-Type Males Than in Females

Wild-type males show a distinct age-related decline in endurance, but exercise-trained cohorts have much higher endurance than unexercised siblings at day 25 (Bazzell et al., 2013; Sujkowski et al., 2015) (Figure 1B). In addition, exercised wild-type male *Drosophila* retain higher climbing speed across ages than unexercised siblings (Figure 1E), as previously observed (Sujkowski et al., 2012, 2015; Tinkerhess et al., 2012a, 2012b). Flight performance (Figure 1H), resistance to cardiac pacing stress (Figure 1K), and adipose-specific LysoTracker staining (Figure 1N) are also higher in trained male flies, as previously observed (Sujkowski et al., 2012, 2015). Thus, climbing induces systemic adaptations that are not limited to climbing behavior, to startle responses, or to skeletal muscle. Increased non-muscle lipolysis and increased cardiac stress resistance are also commonly observed vertebrate endurance exercise adaptations (Ascensão et al., 2007).

After 3 weeks of training stimulus identical to males, females do not display enhanced runspan (Figure 1C), climbing speed (Figure 1F), flight performance (Figure 1I), resistance to cardiac pacing (Figure 1L), or adipose lysosomal activity (Figure 1O), suggesting a general failure to induce systemic adaptations. This difference in adaptation is not due to reduced female endurance, as our maximum daily exercise regime is well within the endurance of untrained female flies (Figure 1A).

Octopaminergic Neurons Mediate Sex-Specific Exercise Adaptations

Sex determination in *D. melanogaster* is regulated cell-autonomously. Expression of a single gene, *transformer* (tra^F), is sufficient to feminize gene expression in male cells (Verhulst et al., 2010), while expression of RNAi against tra^F is sufficient to masculinize gene expression in female cells (Chan and Kravitz, 2007) (Figures S1A and S1B). To identify which tissues mediate sex differences in performance, we feminized or masculinized muscle, adipose, or nervous tissue.

We found that pan-neuronal expression of tra^F was sufficient to feminize male baseline performance and training response (Table S1). Conversely, pan-neuronal expression of tra^F RNAi was sufficient to masculinize female baseline performance and training response (Table S1). As induction of masculinizing RNAi did not occur until after development was complete, sex differences in endurance are not dependent on developmental differences in neurons but instead on differences in gene expression and/or neuronal activity in response to exercise stimuli.

We used tra^F expression as the basis of a targeted screen, using tissue-specific masculinization and feminization of subsets of neurons, to identify neurons that govern runspan. Gal4 lines were selected based on their expression in neurons previously observed to affect *Drosophila* behavior (Bhandari et al., 2006; Ceriani et al., 2002; Dauwalder et al., 2002; Edwards et al., 2006; Jordan et al., 2006; Mertens et al., 2005; Rezával et al., 2014; Riemensperger et al., 2011; Wittmann et al., 2001; Wu et al., 2003; Yu et al., 2010) and are listed in Table S1.

Day-5 endurance was assessed in flies expressing either tra^F or tra^F RNAi in each of the neuronal subsets listed in Table S1. Manipulating tra^F expression with *Tdc2-Gal4*, which drives expression in 136 octopaminergic neurons (Busch et al., 2009), was sufficient to feminize male runspan and fully masculinize female runspan in young flies (Figures 2A and 2G; Table S1). *Gal4* drivers that express in smaller subsets of *Tdc2*-positive cells were unable to fully feminize male runspan (Table S2).

Males with feminized octopaminergic neurons were exercise trained for 3 weeks and post-training performance was assessed. *Tdc2>UAS* tra^F male flies displayed feminized day-25 runspan, climbing speed, LysoTracker staining, and cardiac stress tolerance across ages (Figures 2B–2F). Thus, feminizing octopaminergic neurons renders male flies resistant to exercise training.

In the converse experiment, female flies with masculinized octopaminergic neurons (*Tdc2>UAS* tra^F RNAi) displayed fully

(C) Females do not increase endurance following exercise training.
(D) Climbing speed across ages is equivalent in males and females.
(E) Exercise-trained males maintain greater climbing speed across ages than untrained males ($p < 0.0001$, $n > 100$).
(F) Climbing speed in exercised females is similar to unexercised female siblings across ages.
(G) Day-5 flight performance is equivalent in males and females.
(H) Flight performance declines with age (compare to G). Trained males retain higher flight performance than unexercised male siblings ($p = 0.006$, $n > 60$).
(I) Flight performance is not different between exercised and unexercised females at day 25. Error bars, mean ± SD for all flight graphs.
(J) 5-day-old males and females have equal cardiac response to electrical pacing stress.
(K) Exercise-trained males display less stress-induced cardiac failure than unexercised siblings.
(L) Trained females receive no benefit to cardiac failure in response to pacing. Error bars, mean ± SEM for all pacing graphs.
(M) Baseline lysosomal activity in adipose tissue is low in 5-day-old males and females.
(N) Exercise-trained males have an increase in Lysotracker-positive puncta (inset, scale bar, 5 μm) in the fat body relative to unexercised flies ($p = 0.003$, $n = 10$).
(O) Exercised females do not induce lysosomal activity post-training. Error bars, mean ± SEM for all LysoTracker graphs.
Error bars for (G)–(I) represent mean ± SD. Error bars for (J)–(O) represent mean ± SEM.

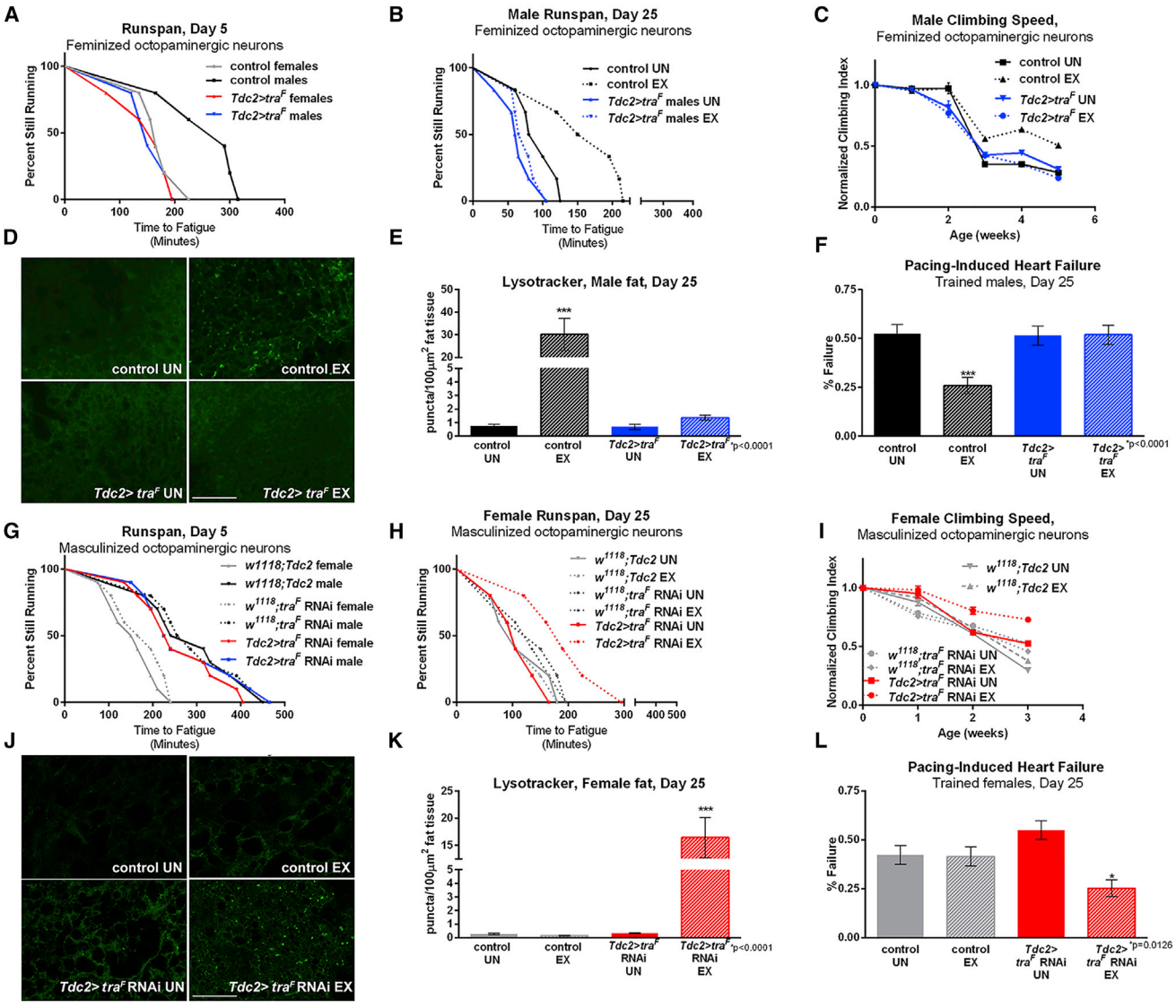


Figure 2. Octopaminergic Neurons Mediate Sex-Specific Exercise Adaptations

(A) *transformer* expression in octopaminergic neurons (*Tdc2>UAS tra*^{F}) feminizes endurance of 5-day-old males ($p = 0.186$, $n = 160$). Age-matched controls have typical male endurance.

(B and C) Feminizing male octopaminergic neurons abolishes training-induced increases in endurance (B) at day 25 ($p = 0.058$, $n = 160$), as well as (C) enhancements in climbing speed across ages ($p = 0.229$, $n > 100$). Control males respond normally to training ($p < 0.0001$, $n > 100$).

(D and E) Control males have increased LysoTracker staining in abdominal fat (D) after exercise training (scale bar, 50 μm) quantified in (E) ($p = 0.0006$, $n = 10$). *Tdc2>tra*^{F} males do not increase lysosomal activity with exercise.

(F) *Tdc2>tra*^{F} males do not increase resistance to cardiac pacing stress after training. Exercised genetic background controls respond to training normally with decreased cardiac failure rate ($p < 0.0001$, $n = 103$).

(G) *Tdc2>tra*^{F} RNAi females have increased endurance relative to control females ($p > 0.0001$, $n = 160$), but are similar to experimental or control males.

(H) Post-training runspan is increased in exercised *Tdc2>tra*^{F} RNAi females ($p = 0.004$, $n = 160$).

(I) *Tdc2>tra*^{F} RNAi females increase climbing speed after exercise training ($p = 0.005$, $n > 100$).

(J and K) *Tdc2>tra*^{F} RNAi increase fat-body lysosomal activity (J) after exercise (quantified in K). Control females exhibit very low LysoTracker staining in the fat body regardless of training status, scale bar, 50 μm (quantitation in K).

(L) Exercise-trained *Tdc2>tra*^{F} RNAi females reduce cardiac failure rate after electrical pacing ($p = 0.013$, $n > 100$), while control females do not. See the Supplemental Experimental Procedures for genetics of background controls.

All error bars represent mean ± SEM.

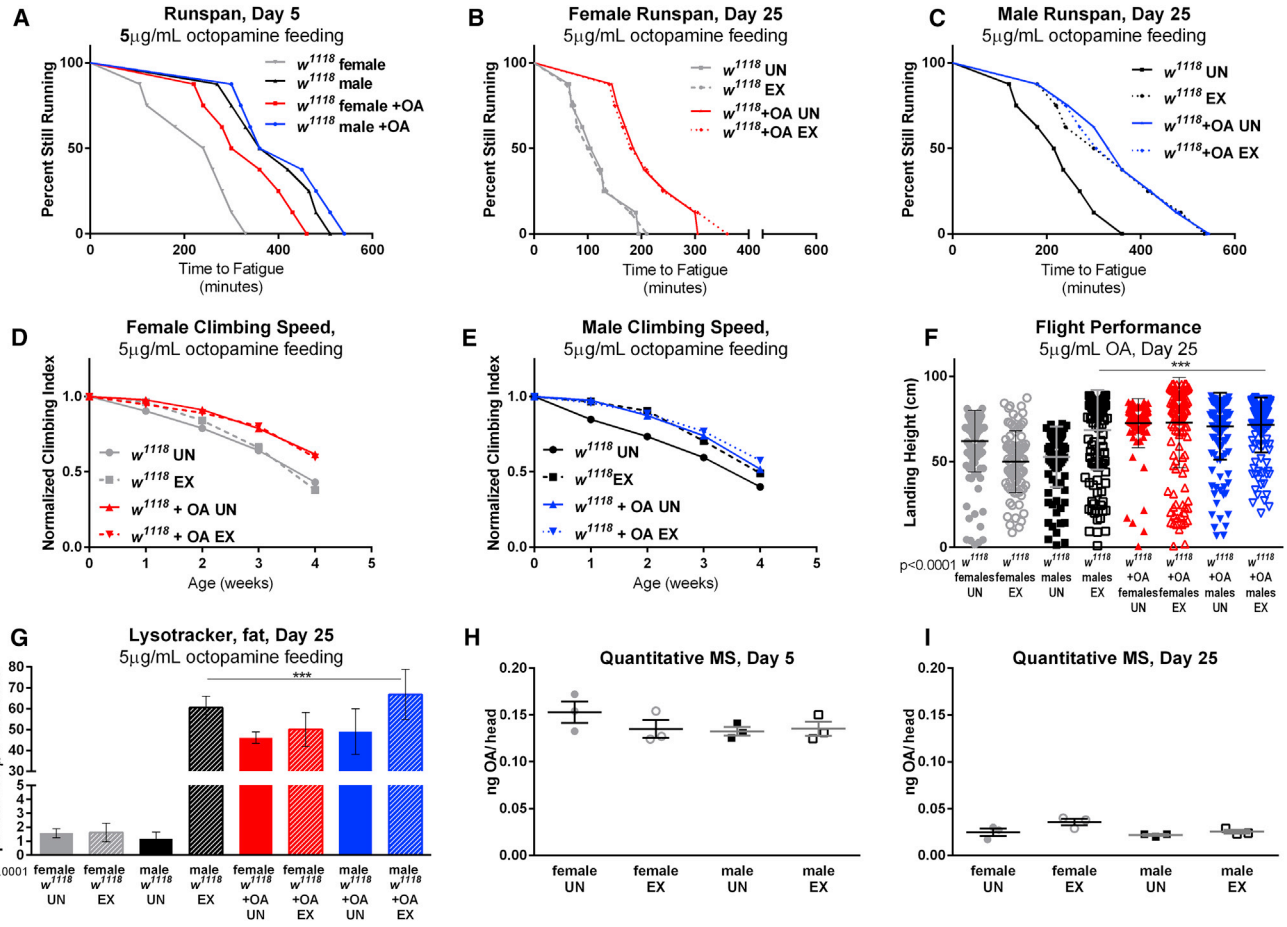


Figure 3. Octopamine Feeding Mimics Exercise Training in Males or Females

(A) Wild-type w^{1118} females fed 5 μg/mL octopamine (OA) for the first 5 days of adulthood increase runspan ($p = 0.026$, $n = 160$), but male endurance at day 5 is not altered by octopamine feeding.

(B) Day-25 runspan is increased in w^{1118} females housed on a diet containing 5 μg/mL octopamine ($p = 0.003$, $n = 160$). Exercise provided no further benefit to OA-fed females.

(C) Octopamine-fed w^{1118} males have increased endurance compared to controls ($p = 0.030$, $n = 160$), similar to exercise-trained males.

(D) w^{1118} females fed 5 μg/mL OA have enhanced climbing speed across ages independent of training status ($p < 0.001$, $n = 100$).

(E) Trained and untrained OA-fed males have climbing speed similar to exercise-trained controls.

(F) Flight performance is enhanced independent of training in OA-fed flies ($p < 0.001$, $n > 89$).

(G) OA-fed males and females have a similar increase in lysosomal activity in the fat body ($p < 0.0001$, $n = 10$).

(H) Mass spectrometry of fly heads reveals no change in OA titers after a single bout of exercise in either females or males.

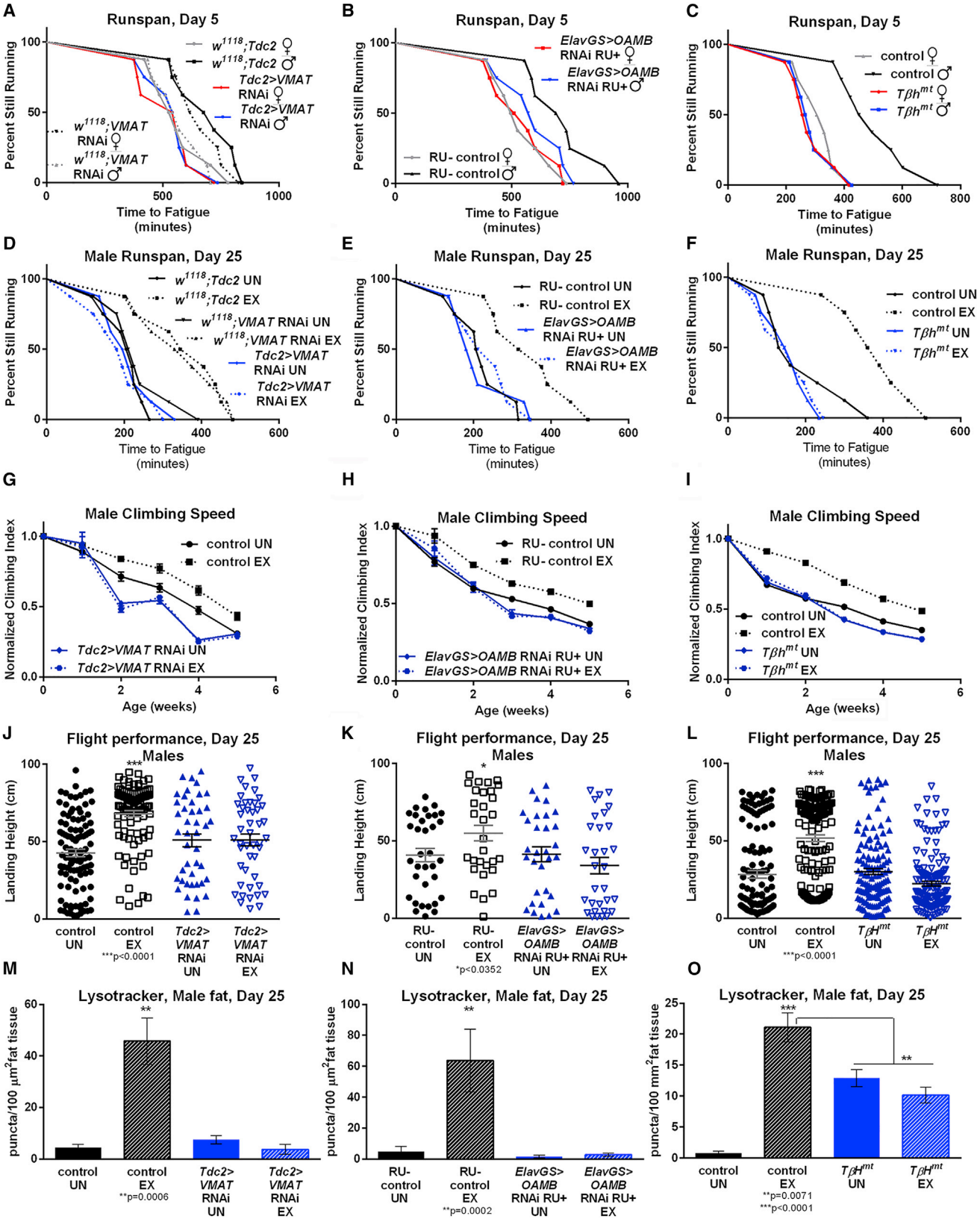
(I) After 3 weeks of training, absolute levels of OA decrease in both male and female heads in comparison to young flies, but no exercise-dependent change in OA is observed.

Error bars in (F) represent mean ± SD. Error bars in (G)–(I) represent mean ± SEM.

masculinized exercise response, with enhancements to day-25 runspan, climbing speed across ages, adipose lysosomal activity, and cardiac stress tolerance post-training (Figures 2H and 2I). Masculinization of octopaminergic neurons in males and feminization of octopaminergic neurons in females had no effect on exercise response (Figure S2).

Octopamine Feeding Mimics Exercise Training in *Drosophila*

We fed flies octopamine (OA) to test whether OA levels drive *Drosophila* exercise adaptations. w^{1118} females increase endurance after 3 days of OA-feeding, while OA-fed males receive no apparent benefit (Figure 3A). After 3 weeks of endurance exercise, OA-fed w^{1118} females have enhanced endurance in comparison to control siblings independent of training (Figure 3B). When males were fed 5 μg/mL OA, runspan increased to resemble exercise-trained males (Figure 3C). Climbing speed increased in OA-fed female flies across ages independent of training status (Figure 3D). OA-fed male w^{1118} flies displayed climbing speed similar to exercise-trained male cohorts (Figure 3E). Similar results were seen in OA-fed female and male flies assessed for flight performance (Figure 3F) and fat body lysosomal activity (Figure 3G). In the absence of OA supplementation, we were unable to detect a change in total OA



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levels in exercised male or female heads using mass spectrometry (Figures 3H and 3I), although an age-related decrease in total OA was observed. (Figures 3H and 3I). This result is consistent with prior reports in which differences in raw levels of OA were not detected even when octopaminergic neurons were directly stimulated by optogenetics, attributed to rapid cycling of the compound (Pyakurel et al., 2016). Taken together, these results identify OA as a pharmacological exercise mimetic in *Drosophila*.

Octopamine Is Required for Adaptations to Exercise Training

Vesicular monoamine transport proteins (VMATs) are required for monoamine transport and release at the synaptic membrane (Greer et al., 2005). Male and female flies expressing RNAi against VMAT in octopaminergic neurons (*Tdc2>UAS VMAT* RNAi) have a runspan typical of female control siblings and are more sensitive to fatigue than male controls at day 5 of adulthood (Figure 4A). *Tdc2>VMAT* RNAi males do not increase climbing speed after training and instead have similar runspan to untrained wild-type males (Figures 4D and 4G). Flight performance (Figure 4J), cardiac stress resistance (Figure S4B), and lysosomal activity in the fat body (Figure 4M) also fail to improve following training in *Tdc2>VMAT* RNAi flies, while control male siblings show wild-type training improvements in each assay.

Octopamine receptor in mushroom bodies (*OAMB*) is highly expressed in the *Drosophila* mushroom body and the ellipsoid body of the central complex, a brain region critical to motor control (El-Kholy et al., 2015). Using *ElavGS-Gal4*, we expressed *OAMB* RNAi in the adult neurons of male and female flies. *ElavGS>OAMB* RNAi RU^+ males and females had reduced endurance at day 5 in comparison to male RU^- controls and both were indistinguishable from RU^- female siblings (Figure 4B). Trained or untrained *ElavGS>OAMB* RNAi RU^+ males had identical runspan (Figure 4E) to untrained controls. Similar results were seen for climbing speed (Figure 4H), flight (Figure 4K), and lysosomal activity (Figure 4N).

Flies with a null mutation in the gene encoding tyramine β-hydroxylase ($T\beta h^{nM18}$, abbreviated $T\beta h^{mt}$ here) are devoid of octopamine and defective in several behaviors (Saraswati et al., 2004). Both sexes of $T\beta h^{mt}$ flies display female-like runspan at day 5, while background control males have wild-type endurance (Figure 4C). Trained $T\beta h^{mt}$ flies do not improve runspan (Figure 4F), climbing speed (Figure 4I), or flight performance (Figure 4L), instead behaving identically to untrained controls. Female cohorts behave normally in speed, endurance, and flight (Figures S3B, S3D, S3F, and S3H). Both male and female $T\beta h^{mt}$ flies, however, showed high adipose LysoTracker staining whether exercised or not (Figures 5O, S3J, and S3K), indicating that the lysosomal activity in these mutants is exercise-independent.

Tdc2 is expressed in both octopaminergic and tyraminergic neurons (El-Kholy et al., 2015), and it is therefore possible that tyramine plays a contributing role in exercise behavior and adaptation in *Drosophila*. To confirm that loss of octopamine is the sole cause of the $T\beta h^{mt}$ exercise phenotype, we fed $T\beta h^{mt}$ flies 5 μg/mL OA beginning at day 3 of adulthood and throughout the training period. Both male and female $T\beta h^{mt}$ flies responded to OA feeding with enhanced endurance as early as day 5 (Figure S3A) and returned to wild-type physiology by day 25 (Figures S3B–S3K) in all assays. OA-fed mutants did not receive further increase to runspan following exercise (Figures S3B and S3C). However, exercise-dependent improvements in speed, flight, and cardiac function were fully restored by OA feeding (Figures S3F–S3I). Taken together, these data support the idea that octopamine is required for exercise adaptations.

Activating Adult Octopaminergic Neurons Is Sufficient to Cause *Drosophila* Exercise Adaptations

To increase the function of octopaminergic neurons, we expressed the temperature-sensitive TrpA1 channel in octopaminergic neurons. TrpA1 proteins are highly selective cation channels that open at increased temperatures (≥25+°C) allowing for temporal control of conductance and excitability (Hamada et al., 2008). Female and male flies expressing TrpA1 in octopaminergic neurons were kept at 25°C throughout a 3-week period, whether exercising or not. Both female and male *Tdc2>UAS TrpA1* flies had markedly enhanced endurance at day 5 in comparison to age-matched outcrossed siblings of the same sex

Figure 4. Octopamine Is Required for Exercise Adaptations

(A) Day-5 runspan of males with blocked vesicle release at octopaminergic synapses (*Tdc2>UAS VMAT* RNAi) is typical of wild-type females and reduced relative to male controls ($p = 0.048$, $n = 160$).

(B and C) Endurance is similarly reduced in (B) males expressing adult-specific pan-neural RNAi against an octopamine receptor (*ElavGS>UAS OAMB* RNAi RU^+), or in (C) *Tβh* mutant males.

(D–F) Post-training day-25 runspan is reduced in trained (D) *Tdc2>VMAT* RNAi males, (E) *ElavGS>UAS OAMB* RNAi males, and (F) *Tβh* mutant males. Trained and untrained experimental males resemble untrained controls and have reduced endurance compared to exercised controls ($p = 0.003$, 0.023, 0.0003, respectively, $n = 160$).

(G) Exercised *Tdc2>UAS VMAT* RNAi males have reduced climbing speed across ages compared to unexercised controls ($p = 0.002$, $n > 100$). *Tdc2>UAS VMAT* RNAi males do not increase climbing speed with training but controls respond normally.

(H) *ElavGS>UAS OAMB* RNAi males do not increase climbing speed with training and are similar to RU^- unexercised controls. RU^- exercised controls increase climbing speed normally.

(I) *Tβh* mutants have climbing speed similar to unexercised controls and do not improve with training. Exercised controls improve climbing speed normally.

(J–L) Trained *Tdc2>UAS VMAT* RNAi (J), *ElavGS>UAS OAMB* RNAi (K), and *Tβh* mutant males (L) do not increase flight performance with exercise, and are similar to untrained controls.

(M) Trained *Tdc2>UAS VMAT* RNAi males have low levels of LysoTracker staining in abdominal fat, while controls display normal increases post-exercise.

(N) Lysosomal activity remains low in adipose tissue of trained *ElavGS>UAS OAMB* RNAi males.

(O) LysoTracker in abdominal fat of $T\beta h^{mt}$ males is higher than unexercised controls ($p = 0.007$, $n = 10$) but does not increase with exercise.

All error bars represent mean ± SEM.

(Figure 5A). *Tdc2>UAS TrpA1* females far surpassed female controls in endurance assessments and were indistinguishable from male control groups. *Tdc2>UAS TrpA1* males had even greater endurance, with the last vial reaching exhaustion after nearly 18 hr from induction of the first climbing stimulus (Figure 5A).

When exercise trained, *Tdc2>UAS TrpA1* female flies increased runspan over female controls (Figure 5B). Meanwhile, *Tdc2>UAS TrpA1* male flies displayed endurance identical to trained male control groups whether exercised or not (Figure 5C). Although activated octopaminergic neurons increased the baseline endurance of both males and females, trained males did not receive further benefit from activation of octopaminergic neurons.

Tdc2>TrpA1 flies of both sexes display greater climbing speed across ages when compared to background controls (Figure 5D). *Tdc2>TrpA1* females consistently perform better than control females in longitudinal speed assessments, whether exercised or not (Figure 5E). *Tdc2>TrpA1* male flies have higher climbing speed than unexercised control males and are indistinguishable from trained male controls (Figure 5F).

Exercised wild-type females do not have higher flight performance or cardiac stress resistance than unexercised females. However, *Tdc2>TrpA1* female flies perform significantly better than outcrossed control female cohorts in both flight and cardiac pacing assays whether exercised or not (Figure 5G, I). Male *Tdc2>TrpA1* flies have improved flight performance, (Figure 5H), cardiac performance (Figure 5I), and increased LysoTracker staining, resembling trained control males in all assays (Figure 5J).

Intermittent Stimulation of Octopaminergic Neurons Fully Mimics Endurance Exercise

The previous experiments established that continuous octopaminergic neuron activity mimics the benefits of exercise training. However, continuous octopaminergic activity is an artificial situation that is not likely to occur in wild-type exercisers. Rather, we expect that octopaminergic neurons are activated during actual exercise bouts. In order to better simulate training conditions, we transferred *Tdc2>TrpA1* males and females from 18°C to 25°C five times a week (designated as TT for temperature-trained). The duration of temperature training was exactly the same number of minutes that control flies were exercised. The training machine was placed in a 25°C room. We also included controls that were placed on the machine but prevented from climbing by a foam plug (UN), control *Tdc2>TrpA1* flies that were temperature shifted but prevented from climbing by a foam plug (TT + restrained), and wild-type controls that were temperature shifted side-by-side with *Tdc2>TrpA1* flies as a control for non-specific response to frequent temperature shift. Because flies in this experiment were kept at 18°C when not actively shifting or training, they experienced less age-related decline in runspan and climbing speed than flies in the previous experiments where flies were kept at 25°C throughout.

We first asked whether acute induction of octopaminergic neurons during a single bout of exercise would significantly alter endurance by providing a motivational stimulus. To do this, we tested the runspan of young, untrained *Tdc2>TrpA1* males and females. All cohorts were placed on the training machine as their initial exposure to 25°C. Neither males nor females showed significant extension of untrained runspan (Figure 6A) compared to controls. We next measured runspan of temperature-trained *Tdc2>TrpA1* males and females. Male or female flies with periodic activation of octopaminergic neurons had identical endurance and both were much higher than controls (Figures 6B and 6C) and similar to exercised males (Figures S5A and S5E). Restraint to prevent climbing abrogated the effect of the exercise (Figures S5A and S5E), but had no effect on temperature trained *Tdc2>TrpA1* males or females (Figures 6B and 6C). Similar results were obtained for climbing speed (Figures 6D, 6G, S5B, and S5F), flight performance (Figures 6E, 6H, S5C, and S5G), cardiac pacing (Figures 6F, 6I, S5D, and S5H), and adipose LysoTracker staining (Figure 6J).

Taken together, these results strongly indicate that intermittent activation of octopaminergic neurons on a specific training schedule can produce the same effects as actual exercise during the same time period.

Adaptations to Endurance Exercise Are Separable

In order to examine the role of separate adrenergic receptors on *Drosophila* exercise adaptation, we used *Tub5 Gene Switch Gal4* to adult-specifically express RNAi against *Octβ1*, *Octβ2*, *Octβ3*, and *Octβ3* and *Octβ1* simultaneously (*Octβ1*R, *Octβ2*R, *Octβ3*R, and *Octβ3/1*R). RNAi knockdown was confirmed with qRT-PCR (Figures S1C–S1F). At day 5, only *Octβ2*R was required for normal endurance (Figure 7B), with male *Tub5GS>Octβ2*R RNAi RU^+ flies displaying endurance similar to female siblings. Flies expressing RNAi against *Octβ1*R, *Octβ3*R, and *Octβ3/1*R all had wild-type day-5 endurance (Figures 7A, 7C, and 7D). After exercise training, however, only *Tub5GS>Octβ1*R RNAi flies had normal endurance (Figure 7E). Male flies expressing RNAi against *Octβ2*R, *Octβ3*R, and *Octβ3/1*R do not improve endurance following exercise and have runspan similar to untrained RU^- control flies (Figures 7F–7H). All 4 octopamine receptors tested were required for exercise-dependent enhancements in climbing speed (Figures 7I–7L), while RU^- controls all responded normally.

Male flies expressing RNAi against *Octβ1*R have normal post-training improvements in flight performance (Figure 7M). *Tub5GS>Octβ2*R RNAi RU^+ male flies do not improve flight with training, however (Figure 7N). Male flies expressing RNAi against *Octβ3*R and *Octβ3/1*R have higher flight capacity than untrained RU^- control siblings, but decrease performance post training (Figures 7O and 7P). Male *Octβ3*R RNAi flies displayed wild-type exercise adaptations in both cardiac performance (Figure 7S) and LysoTracker (Figure 7W), while *Octβ1*R, *Octβ2*R, and *Octβ3/1*R RNAi flies displayed aberrant phenotypes in both assays (Figures 7Q, 7R, 7T–7V, and 7X). These results suggest that, while *Octβ3*R and *Octβ1*R play a significant role, *Octβ2*R is the most broadly required receptor for the *Drosophila* exercise response.

Exercise Adaptations Do Not Correlate with Spontaneous Activity

We addressed the possibility that flies with increased baseline activity levels might increase normal daily movement enough to account for improvements in induced mobility and endurance.

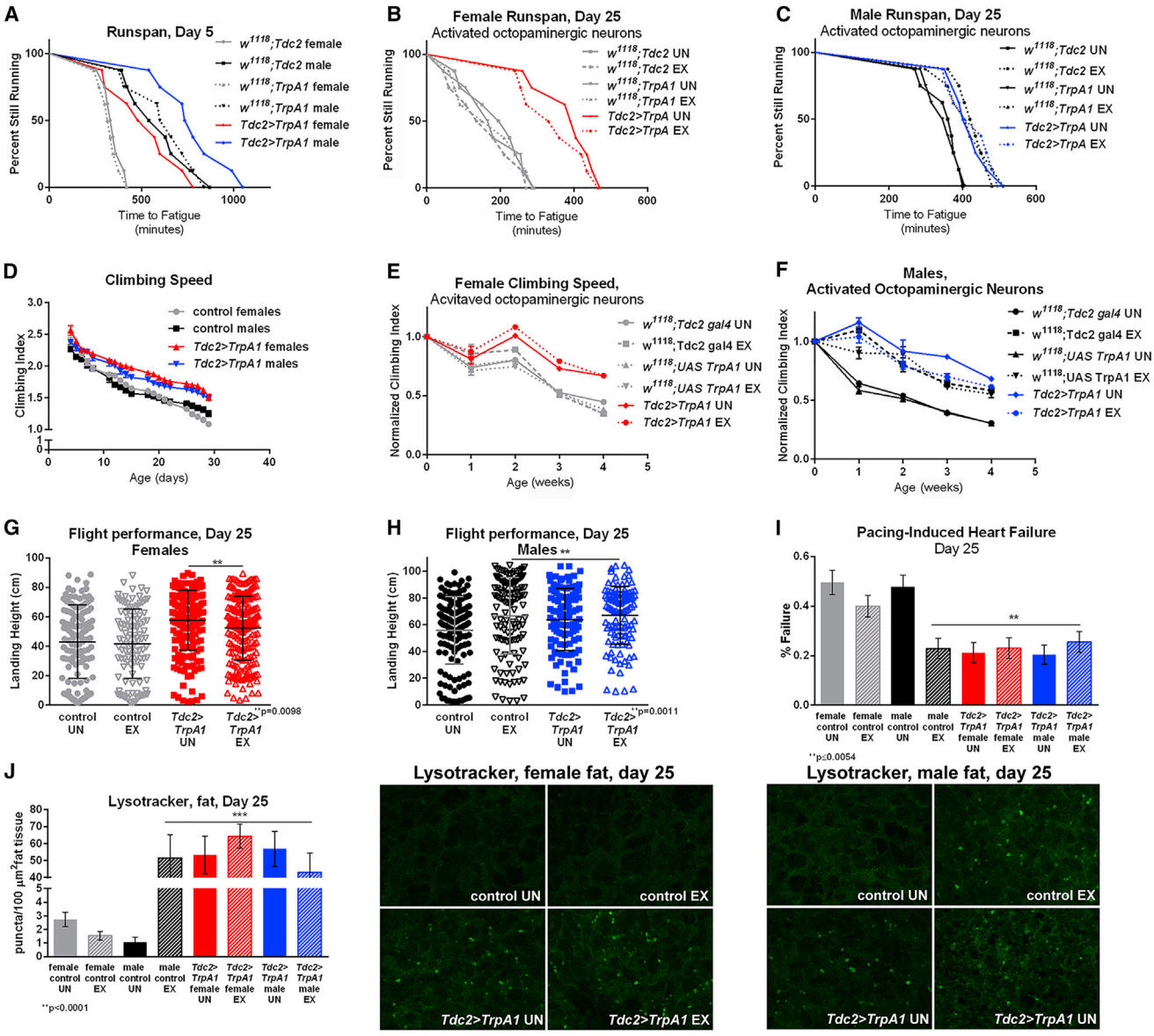

Figure 5. Increased Octopaminergic Neuron Activity throughout Adulthood Mimics Exercise Training

(A) Both male and female *Tdc2>UAS TrpA1* flies display increased endurance relative to outcrossed controls at day 5 ($p = 0.0004$, $p < 0.0001$, respectively).

(B) *Tdc2>UAS TrpA1* females display enhanced endurance at day 25 whether exercised or not ($p < 0.0001$, $n = 160$).

(C) Both trained and untrained *Tdc2>UAS TrpA1* males display higher endurance than unexercised controls ($p < 0.0001$, $n = 160$) and are not different from exercised control males.

(D) Climbing speed of *Tdc2>UAS TrpA1* flies is greater than outcrossed controls in both sexes ($p < 0.0001$, $n = 100$).

(E) *Tdc2>UAS TrpA1* females have increased climbing speed relative to control females ($p < 0.0001$, $n > 100$) regardless of training status.

(F) *Tdc2>UAS TrpA1* males display increased climbing speed independent of training ($p < 0.0001$, $n > 100$) and perform similarly to exercise-trained controls.

(G) *Tdc2>UAS TrpA1* females display increased flight performance at day 25 in comparison to female controls, ($p < 0.010$, $n > 110$) regardless of training.

(H) *Tdc2>UAS TrpA1* males have enhanced flight performance relative to unexercised controls ($p < 0.001$, $n > 104$) and are not different from exercised controls.

(I) Pacing-induced heart failure in trained and untrained *Tdc2>TrpA1* males and females is similar to exercised control males.

(J) Representative 100× images of LysoTracker staining in day-25 adult fat body, quantified at left. Exercise-trained control males have higher adipose-specific lysosomal activity than control females or unexercised control males ($p = 0.002$, $n = 10$). Equivalent increases in LysoTracker staining are observed in both trained and untrained male and female *Tdc2>TrpA1* flies. LysoTracker-positive fat bodies are shown in representative 100× micrographs of trained and untrained *Tdc2>TrpA1* females, exercised control males, and trained and untrained *Tdc2>TrpA1* males (scale bar, 50 μm).

Error bars for (G) and (H) represent mean ± SD. Error bars for (I) and (J) represent mean ± SEM.

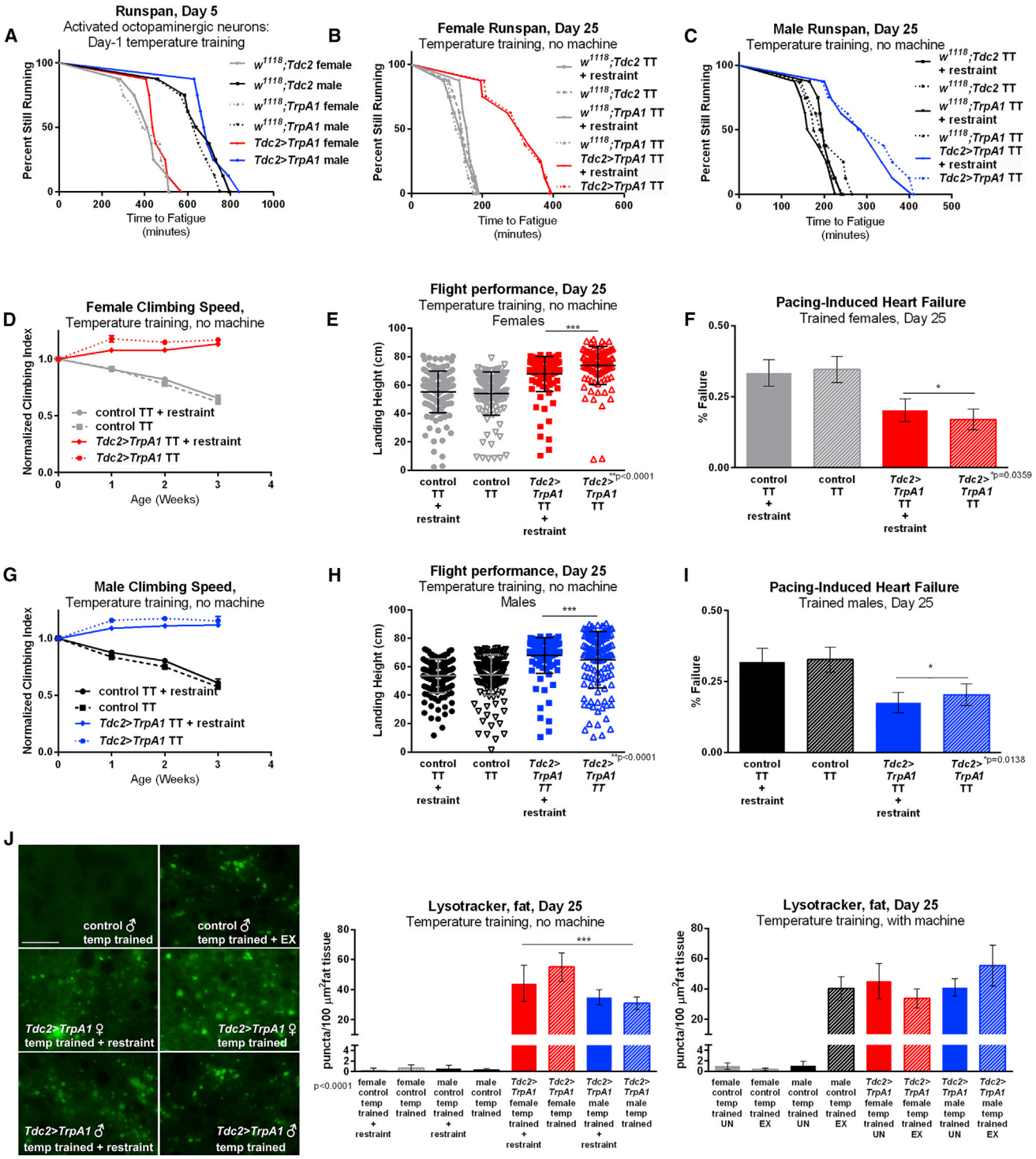

Figure 6. Intermittent Octopaminergic Activation Mimics Exercise Training

(A) *Tdc2>TrpA1* females reared at restrictive temperature (18°C) and tested for endurance upon their first exposure to activating temperature (25°C) display normal endurance. *Tdc2>TrpA1* males also have normal endurance upon first exposure to activating temperature.

(B) After 25 days of temperature training (placed at activating temperature at the same time as controls were placed on exercise machine), *Tdc2>TrpA1* females had much greater endurance than background controls under the same conditions, with or without restraint ($p < 0.0001$, $n = 160$). "TT + restraint" indicates flies under identical intermittent temperature training conditions, but with a stopper placed low in the vial to restrict movement.

(C) *Tdc2>TrpA1* males also have enhanced endurance, with or without restraint, relative to controls ($p < 0.0001$, $n = 160$).

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We measured spontaneous activity of wild-type, *Tdc2>UAS TrpA1*, and $T\beta h^{mt}$ flies and all relevant controls both at day 5 and again post-training at day 25 of adult life (Figures S7A–S7H). Although sex- and genotype-specific differences were observed in some groups, none were directly correlated with or could account for post-training enhancements in muscle or adipose tissue physiology.

DISCUSSION

Octopamine, a biogenic amine similar to the excitatory neurotransmitter norepinephrine (Roeder, 2005), has been previously implicated in diverse behaviors in *Drosophila* and other invertebrate species (Roeder, 2005). Octopamine is required for initiation and generation of coordinated bursts of neural activity resulting in body-wall muscle contraction in *Drosophila* larvae (Fox et al., 2006). Octopaminergic neurons are also thought to modify muscle metabolism in insects prior to flight (Pflüger and Duch, 2011).

Octopamine is required for the *Drosophila* fight-or-flight response, a function similar to norepinephrine in vertebrate species. *Drosophila* mutants with decreased levels of octopamine have increased sensitivity to environmental stress (Chentsova et al., 2002) and normal flies increase octopamine in response to a stressor (Hirashima et al., 2000). In crickets, octopamine release correlates with periods of extended activity and times of recovery from increased energy demand (Adamo et al., 1995). Thus, it makes sense that octopamine would be involved in the adaptive response of flies to repetitive bouts of hormetic endurance exercise.

An important question, however, is whether the effects we see are a legitimate adaptation to exercise or are they just the effects of non-specific stress? In other words, are the effects driven by the stress of endurance exercise or by machine stress? Wild-type flies placed on the training machine but prevented from running show no improvements in any assessment, indicating that machine stress by itself induces no hormesis effect. Furthermore, intermittent stimulation of octopaminergic neurons produces identical effects to exercise training even in restrained animals, indicating that movement itself is not required for improvements, provided octopaminergic neurons are stimulated by other means. Rather, these results are consistent with a model in which exercise training acts primarily through octopaminergic neurons to provoke an adaptive response.

Taken together, these data suggest a model in which exercise stimulates octopaminergic neuron activity more effectively in males than females. This male-specific response can be fully mimicked, in either males or females, either by genetically stimulating octopaminergic neurons or by octopamine feeding. Chronic endurance exercise induces the activity of octopaminergic neurons for short periods each day, resulting in systemic adaptations that give rise to increased performance. Females' reduced octopaminergic neuron activity may prevent this effect in two ways. First, reduced octopamine may result in reduced motivation to complete daily training runs, thus reducing the total exercise. Second, octopamine may itself mediate the benefits of endurance exercise, in which case females would not receive these benefits no matter how much they run. Our experiments with temperature shifted sedentary flies strongly support the second of these possibilities.

During extended periods of locust flight, muscle metabolism shifts from glycolytic to lipolytic to account for increased energy demand. Octopamine acts as both a neurotransmitter and neuropeptide, controlling adipokinetic activity in the fat body and oxidative lipid metabolism in flight muscles. Octopamine also exerts neuro-hormonal action in locusts, mobilizing stored carbohydrate and lipid to fuel increases in activity (Roeder, 2005). In fact, increased octopamine has been observed to directly stimulate lipolysis both *in vitro* and *in vivo* (Wang et al., 1990).

Here, activating octopaminergic signaling causes an increase in LysoTracker-positive puncta in the *Drosophila* fat body independent of sex or exercise training, suggesting a tissue non-autonomous effect on lysosomal activity in adipose tissue. Based on the aforementioned insect studies, we believe that increases in lysosomal activity indicate enhanced lipolysis in these animals. Previous studies show exercise-dependent activation of transcripts required for lipid metabolism (Sujkowski et al., 2015) as well as consumption of accumulated fat stores in animals with excess lipid post training (Sujkowski et al., 2012), lending further support to the idea that endurance exercise acts through octopamine in *Drosophila* to activate lipolysis and alter metabolism.

In contrast to recent findings that elegantly narrow down a specific subset of octopaminergic neurons that are required to modulate male aggression (Watanabe et al., 2017), we were unable to detect a smaller subset of octopaminergic neurons that govern the exercise response. These results are consistent with a model in which a requisite level of circulating octopamine must be induced for adaptation to chronic exercise. This could reflect the variegated response to exercise in multiple tissues, which may require multiple inputs, in contrast to the behavioral change in aggression.

Individual differences in endurance exercise adaptations are frequently observed in humans, but the mechanisms at play are varied and poorly understood (Puthucheary et al., 2011). Exercise-induced adaptations vary between sex, age, and individual (Deschenes and Kraemer, 2002). Sympathetic signaling pathways may contribute to variations in exercise performance and adaptation, as β-adrenergic response is sexually dimorphic in both animal models and humans (Hedrington and Davis, 2015). It is interesting to consider the possibility that differences in excitatory α- and/or

(D–F) Temperature trained *Tdc2>TrpA1* females displayed increased climbing speed ($p < 0.0001$, $n = 100$) (D), flight performance ($p < 0.0001$, $n = 100$) (E), and enhanced cardiac performance ($p = 0.036$, $n = 100$) (F), with or without restraint.

(G–I) Temperature trained experimental male cohorts displayed similar enhancements in speed ($p < 0.0001$) (G), flight ($p < 0.0001$) (H), and cardiac performance ($p = 0.014$) (I), with or without restraint.

(J) Lysosomal activity was increased in adult fat bodies of male and female temperature trained *Tdc2>TrpA1* flies, with or without restraint. Representative 100× image, scale bar, 50 μm, quantified at left ($p < 0.0001$).

Error bars for (E)–(H) represent mean ± SD. Error bars for (F), (I), and (J) represent mean ± SEM.

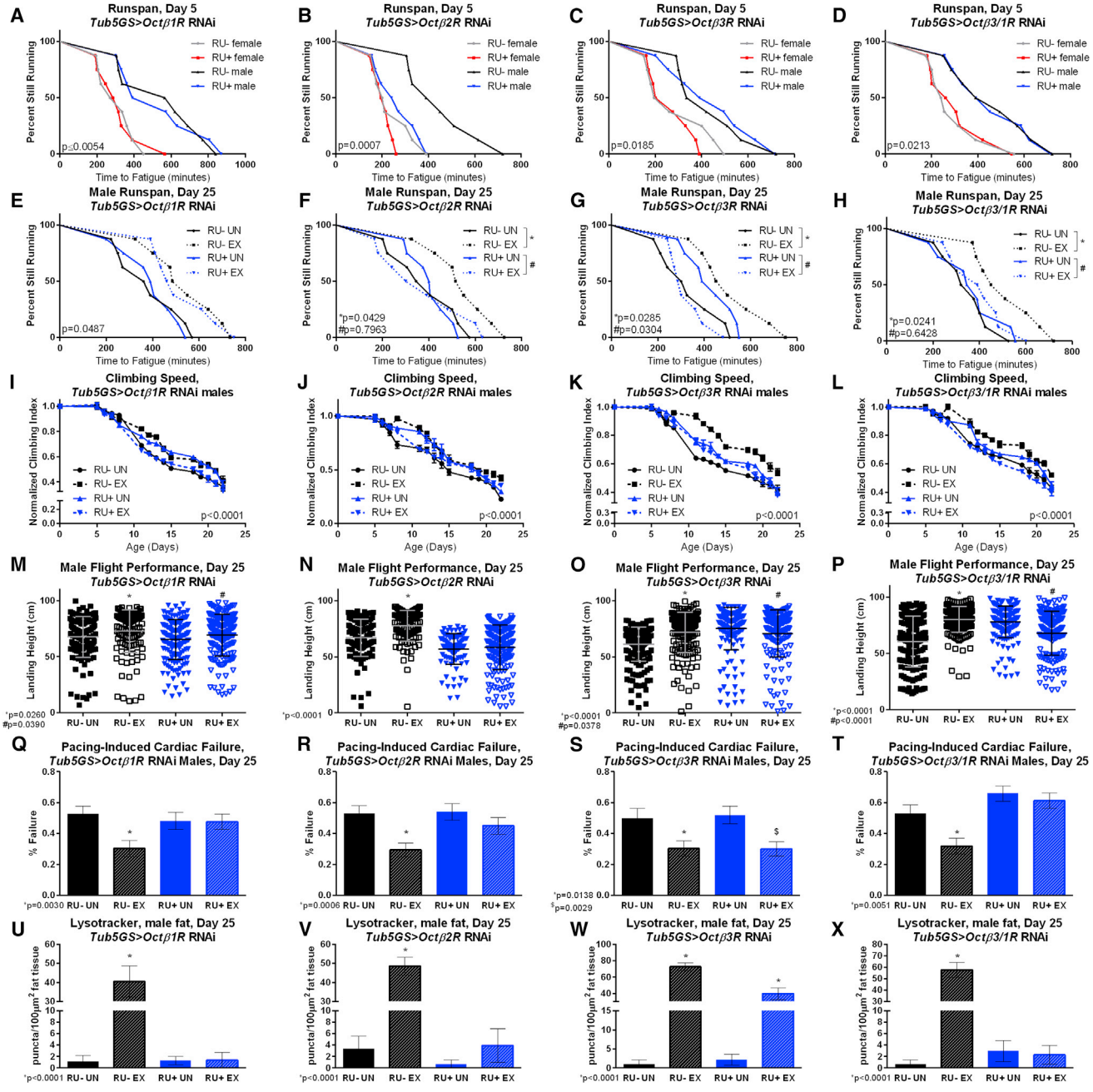

Figure 7. Octopamine Receptors Are Required for Exercise Adaptations

(A) Ubiquitous, adult-specific RNAi against *Octβ1R* does not affect male or female endurance at day 5.

(B) *Tub5GS>Octβ2R* RNAi RU$^+$ male flies have decreased endurance compared to male RU$^-$ controls ($p = 0.0007$), and similar endurance to *Tub5GS>Octβ2R* RNAi females.

(C and D) RNAi against *Octβ3R* (C) and *Octβ3/1R* (D) does not affect day-5 endurance in male or female flies.

(E–H) Following exercise training, RNAi against *Octβ2R* (F), *Octβ3R* (G) or *Octβ3/1R* (H) reduces endurance performance in trained males, while *Tub5GS>Octβ1R* (E) RNAi flies have wild-type endurance after exercise-training. $n \geq 160$ for all endurance experiments.

(I–L) All octopamine receptors tested, (I) *Octβ1R*, (J) *Octβ2R*, (K) *Octβ3R*, (L) *Octβ3/1R*, were required for normal training-dependent improvements in climbing speed. RU$^-$ controls responded normally to training with improved climbing speed across ages. $n \geq 100$ for all climbing experiments.

(M) *Tub5GS>Octβ1R* RNAi RU$^+$ had increased landing height compared to unexercised siblings following endurance training ($p = 0.026$), as did exercise-trained RU$^-$ controls.

(N–P) In contrast, *Tub5GS>Octβ2R* (N) RNAi RU$^+$ flies failed to respond to training with increased landing height, while RU$^-$ controls behaved normally. Both *Tub5GS>Octβ3R* (O) RNAi RU$^+$ and *Tub5GS>Octβ3/1R* (P) RNAi RU$^+$ responded abnormally to exercise a decreased landing height in trained RU$^+$ male flies ($p < 0.0001$). $n \geq 103$ for all flight experiments.

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β-adrenergic response pathways may play a role in individual differences in endurance exercise and resulting adaptations.

These results indicate that conserved, "fight-or-flight"-related responses are required and sufficient for beneficial hormetic adaptations to exercise training in *Drosophila* and open the door for identification of similar responses in other model systems. It will be interesting in the future to examine whether it is also possible in vertebrates to stimulate exercise-like effects by periodic activation of noradrenergic neurons, possibly opening a door to providing benefits of exercise to patients with restricted mobility.

EXPERIMENTAL PROCEDURES

Fly Stocks and Maintenance

All fly lines were reared and aged at 25°C; 50% humidity with a 12-hr light-dark cycle and provided with a standard 10% yeast/10% sucrose diet unless otherwise indicated. All females were virgins. For stock sources and background controls, see the Supplemental Experimental Procedures.

Drug Treatment

For gene-switch experiments, adult progeny were age-matched by collecting within 2 hr of eclosion over a 72-hr time period and immediately transferred into vials containing 5 mL standard medium. Populations were split into control RU^- and experimental RU^+ groups on the 3rd day and transferred into vials containing 5 mL medium containing either 70% ethanol vehicle or 100 μM mifepristone (RU486) (Cayman Chemical, Ann Arbor, MI), respectively. Experimental and control flies were then housed at 25°C on either RU486 or vehicle until experimentation.

Flies fed octopamine were treated similarly to gene-switch experiments, but were collected within a single 24-hr window immediately after eclosion and housed on SY10 food containing 5 μg/mL octopamine (Sigma-Aldrich, St. Louis, MO) or an equal volume of ddH_2O vehicle.

Exercise Training

Cohorts of at least 800 flies were collected under light CO_2 anesthesia within 2 hr of eclosion and separated into vials of 20. Flies were then further separated into 2 large cohorts of at least 400 flies divided into exercised and unexercised groups. The unexercised groups were placed on the exercise training device, but were prevented from running by the placement of a foam stopper. The exercise device drops the vials of flies every 15 s, inducing a repetitive negative geotaxis response. Exercised flies are free to run to the top of the vial. Daily time of exercise followed the previously described ramped program (Piazza et al., 2009).

All exercised and unexercised cohorts were assessed for speed, endurance, cardiac performance, flight, and LysoTracker as described in detail in the Supplemental Experimental Procedures.

Temperature-Shift

For temperature-sensitive *UAS-TrpA1* experiments, parental lines were mated and experimental F1 progeny allowed to eclose at the restrictive temperature (18°C). Adult progeny were age-matched by collecting within 2 hr of eclosion and shifted to the inducing temperature (25°C) where they were maintained until time of assessment.

In temperature-training experiments, all experimental and control groups were housed at constant density at the restrictive temperature (18°C) until time of F1 eclosion. A minimum of 5,000 adult progeny from each sex and genotype were age-matched by collecting within 2 hr of eclosion over a 48-hr time period. Each cohort was then split into the following 4 groups: temperature-trained (TT), temperature-trained with a foam stopper placed low in the vial (TT + restraint), temperature-trained and exercised on the Power Tower (TT EX), and temperature-trained and unexercised (placed on the machine while mobility-restricted, TT UN). For treatment details, see the Supplemental Experimental Procedures.

qRT-PCR

Relative message abundance was determined by amplification and staining with SYBR Green I using an ABI 7300 Real Time PCR System (Applied Biosystems). Expression of *Rp49*, *Actin5c*, and corresponding control (either RU^- or gal4/+, UAS-RNAi/+) flies were used for normalization. For detailed methods and primer sequences, see the Supplemental Experimental Procedures.

OA Quantitation

OA was quantified using mass spectrometry (MS) from 5 homogenized heads for each repetition. Each sample was analyzed in three biological replicates. For detailed protocol, see the Supplemental Experimental Procedures.

Statistical Analysis

Climbing speed was analyzed longitudinally by 2-way ANOVA with post hoc Tukey multiple comparison test. Endurance was analyzed by log-rank. Flight was analyzed by t test. Cardiac pacing was measured by percentage failure in each group and analyzed by F-test for binary measures. LysoTracker, RT-PCR, and mass spectrometry were analyzed by t test. P values were considered significant at $p < 0.005$. For more detail, see the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.065.

AUTHOR CONTRIBUTIONS

A.S. and R.W. designed the experiments, analyzed data, and wrote the manuscript. A.S. performed the experiments, except for mass spectrometry, which was performed, analyzed, and described by D.R. and A.B.

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(Q–X) *Octβ1R* (Q), *Octβ2R* (R), and *Octβ3/1R* (T) RNAi all resulted in aberrant cardiac responses to endurance training, as no group improved after exercise. Trained RU^- controls respond normally with reduced failure rate. *Tub5GS > Octβ3R* RNAi RU^+ flies (S) respond normally with reduced cardiac failure after pacing stress ($p = 0.0029$). $n \geq 87$ for pacing experiments. Fat body lysosomal activity was also normal in *Tub5GS>Octβ3R* RNAi RU^+ flies ($p < 0.0001$) (W) and background controls flies, but RNAi against *Octβ1R* (U), *Octβ2R* (V), and *Octβ3/1R* (X) resulted in low levels of lysosomal activity in the fat body of exercise-trained male flies. n = 5 for LysoTracker experiments.
Error bars for (M)–(P) represent mean ± SD. Error bars for (Q)–(X) represent mean ± SEM.

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