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Both Voluntary And Forced Wheel Running Activate Reward-Related Dopamine Neurons In The Lateral VTA

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Introduction

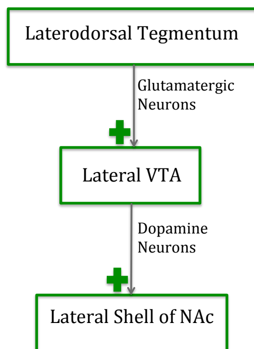
Stress related psychiatric disorders, such as anxiety and depression, are a growing problem among American adults. According to the Centers for Disease Control and Prevention, an estimated 1 in 10 adults have reported feeling depressed (http://www.cdc.gov/features/dsdepression). Current pharmacological treatments of anxiety and depression, two highly comorbid psychiatric disorders, have had limited efficacy in treating the signs and symptoms associated with anxiety and depression [1,4]. Thus, there is a definite need for more effective treatments and therapies for these disorders. Current data demonstrate that exercise, a natural reward, reduces the incidence of stress related psychiatric disorders.

Exercise is known to produce plastic changes in the mesolimbic reward pathway, which includes dopamine (DA) neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAc). This neuroplasticity in the mesolimbic reward pathway, as a result of exercise, or wheel running for rats, is a potential mechanism thought to contribute to stress resistance [2]. Indeed, stress-related psychiatric disorders are thought to be disorders of the reward system. For example, anhedonia, or the lack of pleasure, is a primary symptom of depression [7]. Moreover, rats exposed to repeated stress and displaying signs of anhedonia have altered gene expression in the mesolimbic reward pathway [7,8]. It is possible that the rewarding effects of exercise contribute to the anxiolytic and antidepressant effects of exercise [2].

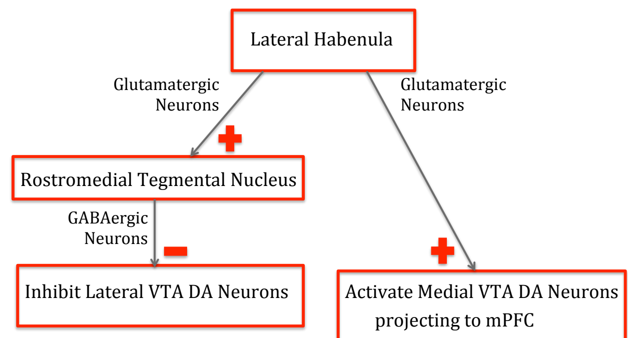
When rodents are given access to free wheel running, it has been shown that this type of voluntary exercise is rewarding both behaviorally and neurobiologically [2,6,10,11]. Behaviorally, reward has been demonstrated through conditioned place preference (CPP)

[2]. At a week 6 CPP probe test, experimenters observed that rodents displayed a preference for a context paired with exercise compared to both baseline and 2 week preference measurements [2]. Neurobiologically, Werme et al. and Greenwood et al. demonstrated that rodents allowed free wheel running access for 6 weeks showed significant increases in a reward-related plasticity marker, ΔFosB, in the NAc [2,12]. The NAc is a target of VTA DA neurons, which respond to reward. It remains unknown, however, whether the reward-related plasticity in the NAc that occurs as a result of exercise is a result of DA neuron activation.

Lammel et al. have recently reported new findings of regional specificity in the mesolimbic reward pathway. This study showed that distinct VTA circuits generate reward



and aversion [6]. Glutamatergic neurons from the laterodorsal tegmentum project onto the lateral VTA, activating VTA DA neurons that project onto the lateral shell of the NAc, resulting in reward elicitation [6]. Contrarily, glutamatergic neurons from the lateral habenula project onto 1) the rostromedial tegmental nucleus, activating GABAergic neurons which inhibit lateral VTA DA neurons; and 2) the medial VTA, activating VTA DA neurons that project onto the medial prefrontal cortex, resulting in aversion [6]. These findings have provided a clearer understanding of VTA regional specificity that encodes for reward and aversion.



With the finding of these two distinct circuits, the neurobiological basis of reward in response to specific stimuli can be more confidently studied.

Recently, Greenwood et al. reported that forced exercise was just as effective as voluntary exercise at preventing anxiety and depression-like behaviors produced by uncontrollable stress [3]. This study utilized a design in which the distance and pattern of forced wheel running closely matched that of voluntary wheel running [3]. Although forced wheel running in this study was able to prevent the behavioral consequences of stress, whether rats found forced wheel running to be rewarding is unknown. Intuitively, one would not expect forced wheel running to activate the reward pathway. In fact, in Greenwood's study, the rats forced to run on wheels showed classic signs of chronic stress, such as thymic involution, splenic contraction, and adrenal hypertrophy [3]. However, a previous report has shown that rats will learn to press a lever for forced wheel running [5]. These data suggest that like voluntary wheel running, forced wheel running might be rewarding. These results call for a further investigation into the relationship between exercise reward and the protective effects of exercise against stress related psychiatric disorders. First, it is necessary to determine whether forced wheel running is rewarding neurobiologically, as has been determined in voluntary wheel run rodents.

To test whether rats forced to run on wheels find forced exercise rewarding, rats were either allowed to run voluntarily on their running wheels for 6 weeks, or were forced to run on wheels following a pre-programmed running pattern that is similar to voluntary running and shown previously to prevent anxiety and depressive-like behaviors following stress [3]. Following 6 weeks of wheel running, the rats were sacrificed and TH / pCREB were used to examine activity of DA neurons in both the lateral and medial VTA to assess

the effects of voluntary and forced wheel running on activity of reward and aversion. Results suggest that both voluntary and forced wheel running are rewarding neurobiologically.

Materials and Methods

Animals

Young adult (6 weeks old upon arrival) male Fischer 344 rats (Harlan, Indianapolis, IN, USA) were housed in a temperature (22°C) and humidity controlled environment on a 12:12 hour light:dark cycle. Rats were acclimated to the housing conditions one full week prior to the experiment. All animals were individually housed in Nalgene Plexiglas cages. Each runner had its own wheel separate from its home cage. Sedentary animals had a separate cage apart from their home cage during the active cycle to control for the stress created from introducing the animals into a novel environment. Rats were weighed weekly and had ad libitum access to food (Lab Chow) and water during their active and inactive cycles. During the third week of the experiment, rat #14, which was in the forced running group, developed ulcerations on all four of its paws. The animal stopped running and was eventually euthanized. The University of Colorado Animal Care and Use Committee approved all experimental protocols.

Exercise Protocols

After the first acclimation week, animals 1-16 were all allowed voluntary access to Lafayette running wheels (Lafayette Instruments, Lafayette, IN, USA) for one week. At the end of this week, the animals were assigned to either the voluntary or forced running groups. The forced Lafayette wheels are controlled by a motor coordinated by the Activity

Wheel Monitor software (Lafayette Instruments) according to a protocol pre-programmed by the experimenters and designed to closely approximate rats' natural voluntary running behavior as possible [3]. The pattern of forced running is characterized by short stretches of running at various speeds combined with frequent periods of rest. The motors were turned on at the beginning of the dark (active) cycle and turned off at the end of the active cycle. These forced wheels could not be turned voluntarily by the rats. Both the voluntary wheel running (VW) and forced wheel running (FW) groups ran during the active cycle (7:00 PM – 7:00 AM) and did not run on Saturdays and Sundays. Sedentary animals were put into their separate cages during the active cycle and were returned to their home cages along with both run groups at the end of the active cycle. Daily wheel revolutions were recorded digitally using Vital View software, and distance was calculated automatically.

Immunohistochemistry

All brains were stained using immunohistochemistry. After six weeks of running, the animals were deeply anesthetized with sodium pentobarbital and sacrificed two hours after lights out (during peak running). Rat brains were perfused transcardially with cold saline, followed by 400-500 ml of 4% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB). Brains were extracted, rapidly frozen in isopentane, and stored at -70°C. Then, using a cryostat, brains were sliced into 35 μm coronal sections and stored at 4°C until staining.

Immunohistochemistry staining following Fleshner lab protocol was used to examine the presence of pCREB and tyrosine hydroxylase (TH) neurons in the VTA. Floating, 35 μm sections of the VTA were rinsed 3 times for 5 minutes each in 0.01 M PBS and were then treated with 0.6% H2O2 for 15 minutes. The tissue was again rinsed 3 times for 5 minutes each in 0.01 M PBS, incubated in PBS-X with 5% Normal Goat Serum (NGS)

for 1 hour, and then incubated in PBS-X, 5% NGS, and rabbit anti-pCREB antibody at a dilution of 1:5000 for 48 hours. Then, the tissue was rinsed 4 times for 5 minutes each in PBS-X with 2% NGS, incubated in PBS-X, 2% NGS, and anti-rabbit secondary IgG at a dilution of 1:300 for 90 minutes at room temperature, and rinsed again 4 times for 5 minutes each in PBS-X. Next, the tissue was incubated in AB solution (123.3 μl of solution A and 123.3 μl of solution B in 50 ml PBS-X) for 1 hour and rinsed 4 times for 5 minutes each in PBS-X. Then, the tissue was incubated in diaminobenzidine (DAB) solution for 13 minutes, when the desired staining was achieved. The tissue was rinsed 4 times for 5 minutes each in PBS-X. Next, the tissue was incubated in PBS-X with 5% NGS for 20 minutes and then incubated in PBS-X, 5% NGS, and rabbit anti-TH antibody at a dilution of 1:100,000 for 48 hours. The tissue was rinsed 4 times for 5 minutes each in PBS-X with 2% NGS and then incubated in PBS-X, 2% NGS, and anti-rabbit secondary IgG at a dilution of 1:300 for 90 minutes at room temperature. Again, the tissue was rinsed 4 times for 5 minutes each in PBS-X, incubated in AB solution for 1 hour, and rinsed 4 times for 5 minutes each in PBS-X. Lastly, the tissue was incubated in DAB for 11 minutes until the desired staining was achieved and rinsed 4 times for 5 minutes each in PBS. The tissue was left in the last wash of PBS overnight and was mounted on slides the next day.

Quantification

Immunohistochemistry quantification was done using the computer program Image J. Images of brain slices were captured digitally and those 10X images of the left and right sides of the slice were split up into 40X images of the lateral and medial VTA, resulting in 4 images per brain slice: lateral left, medial left, lateral right, medial right. In each image, a 450 μm by 450 μm square was drawn and quantification took place in that area. The light

staining represented TH and the dark staining represented pCREB. Double-labeled neurons expressed both TH and pCREB. Results are expressed as the percentage of double-labeled TH / pCREB neurons of all neurons that expressed TH in that specific area.

Statistical Analysis

Group differences in body weight were analyzed using a repeated measures analysis of variance (ANOVA). Nightly running distance was analyzed using a repeated measures ANOVA with exercise group (forced vs. voluntary) as the factor, followed by Fisher's protected least significant difference (PLSD) *post hoc* test. The percentage of double-labeled pCREB and TH neurons in the VTA were analyzed using a 1x3 ANOVA for the lateral and the medial VTA.

Results

Body Weight

Final group sizes were as follows: FW group, n=7; VW group, n=8; sedentary group, n=9. Upon arrival, rats in the forced wheel running (FW) group weighed 133.43 ± 4.27g, rats in the voluntary wheel running (VW) group weighed 136.875 ± 3.01g, and rats in the sedentary group weighed 133.67 ± 2.00g. The body weights of rats increased steadily over the duration of the study (Figure 1). At the end of the 6 week running period, rats in the FW group weighed 233.571 ± 5.42g, rats in the VW group weighed 259.00 ± 9.02g, and rats in the sedentary group weighed 269.44 ± 4.61g. Body weight gain was also influenced by exercise, as rats in the FW group gained the least amount of weight, while rats in the sedentary group gained the most amount of weight in the 6 weeks. Repeated measures ANOVA revealed a significant main effect of time [$F(6,126) = 524.486$, $p<0.0001$],

significant main effect of exercise [F (2,21) = 4.502, p=0.0236], and a time-by-exercise interaction [F (12,126) = 9.908, p<0.0001] on body weight. See graph for *post hoc* analyses (Figure 1).

Running Behavior

Overall, there was not a significant main effect of exercise between VW and FW groups [F (1,13) = 4.530, p=0.0530] on total distance run. Repeated measures ANOVA revealed a significant main effect of time [F (25,25) = 5.761, p<0.0001] and a significant time-by-exercise interaction [F (25,325) = 2.551, p<0.0001] on total distance run. The VW and FW groups differed in their total running distance on certain days. See graph for *post hoc* analyses (Figure 2).

Percentage of Double-Labeled Neurons in the VTA

ANOVA revealed a significant main effect of exercise [F (2,21) = 5.436, p=0.0125] on the percentage of double TH / pCREB-labeled neurons in the lateral VTA. Both the VW (p=0.0490) and FW (p=0.0041) groups had a higher percentage of double-labeled neurons in the lateral VTA compared to the sedentary group (Figure 3). The percentage of double-labeled neurons in the lateral VTA did not differ significantly between the VW and FW groups (p=0.251). The percentage of double-labeled neurons in the medial VTA did not differ between groups (Figure 4). These data suggest that exercise, both forced and voluntary, has an effect on the activation of DA neurons in the lateral VTA but not the medial VTA (Figure 5).

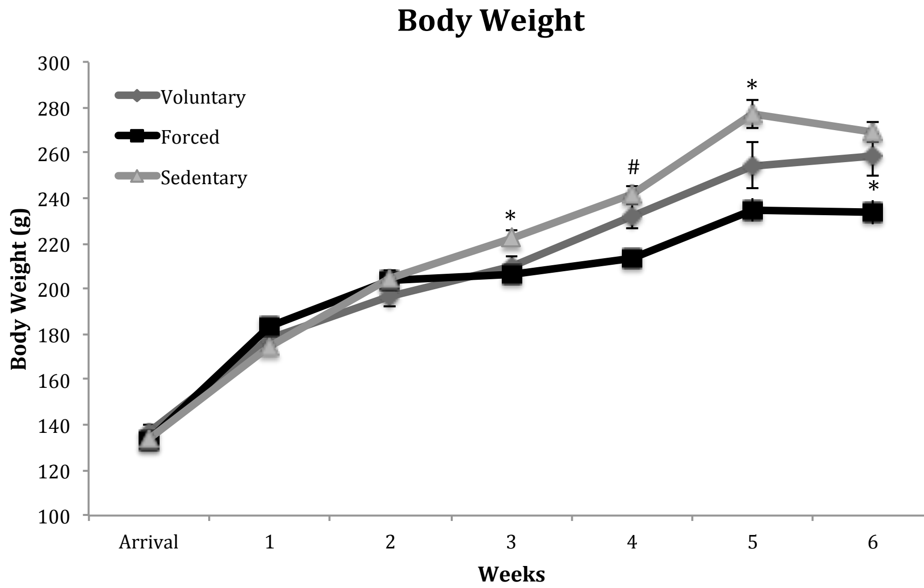


Figure 1. Body weight data for adult male Fisher 344 rats through the duration of the study. Body weights increased steadily over the duration of the study. Repeated measures ANOVA revealed a significant main effect of time [F (6,126) = 524.486, p<0.0001], significant main effect of exercise [F (2,21) = 4.502, p=0.0236], and a time-by-exercise interaction [F (12,126) = 9.908, p<0.0001] on body weight. *Post hoc* analyses show that body weights differed between groups on weeks 3, 4, 5, and 6. On weeks 3 and 5, the sedentary group's body weight was significantly different from both the VW and FW groups. On week 4, the sedentary group's body weight was significantly different from the FW group but not the VW group. On week 6, the FW group's body weight was significantly difference from the VW and sedentary groups.

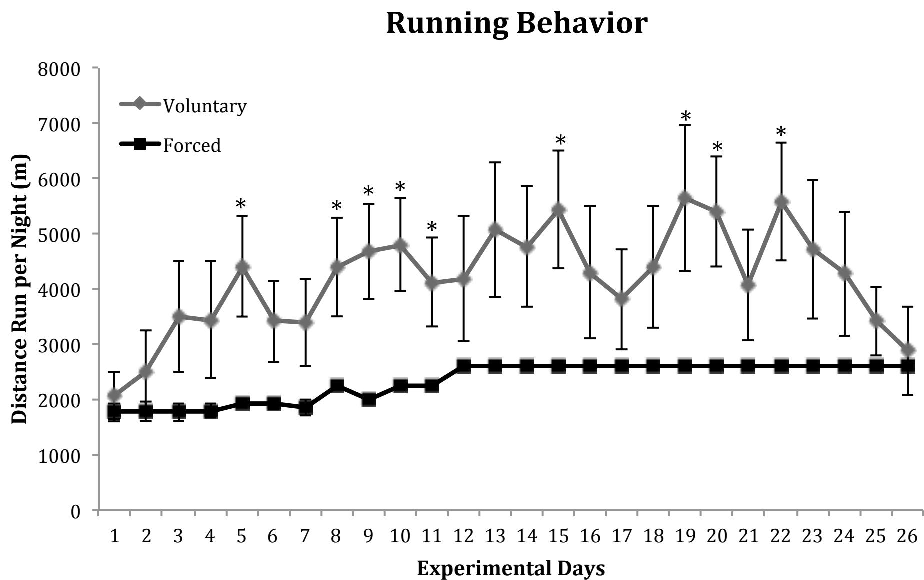


Figure 2. Mean distance in meters run each night by adult male Fisher 344 rats in the VW and FW groups. Rats ran for 5 nights each week for 12 hours each night during the dark part of the light cycle. There was not a significant main effect of exercise between VW and FW groups [F (1,13) = 4.530, p=0.0530] on total distance run. *Post hoc* analyses show that the distance run significantly differed between groups on days 5, 8, 9, 10, 11, 15, 19, 20, and 22.

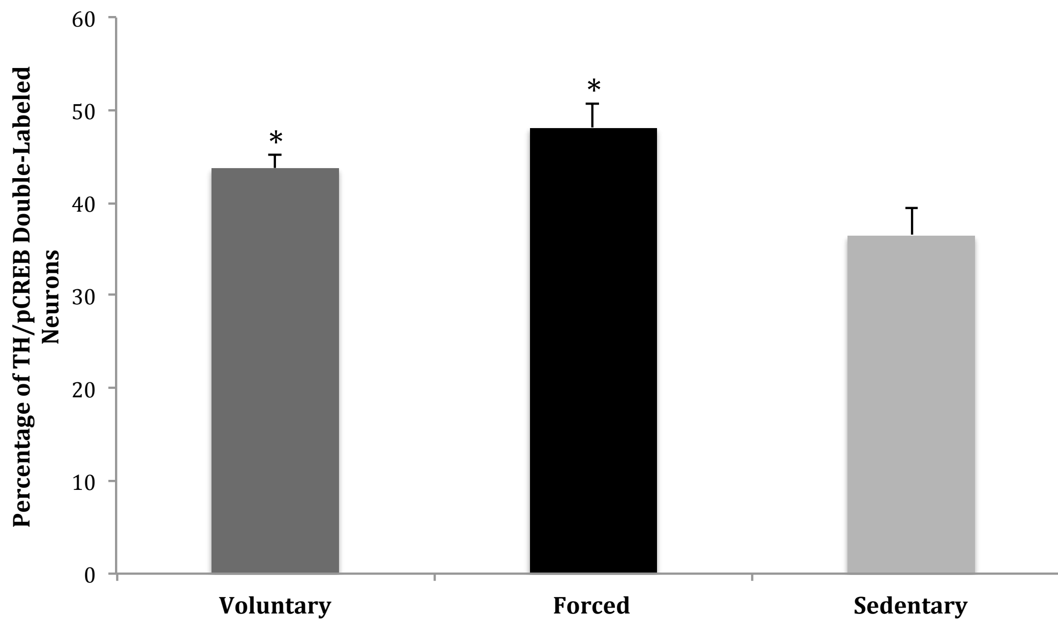


Figure 3. Both VW (p=0.0490) and FW (p=0.0041) groups showed significantly more dopamine neuron activation than the sedentary group in the lateral VTA: ANOVA revealed a significant main effect of exercise [$F(2,21) = 5.436$, $p=0.0125$] on the percentage of double TH / pCREB-labeled neurons in the lateral VTA.

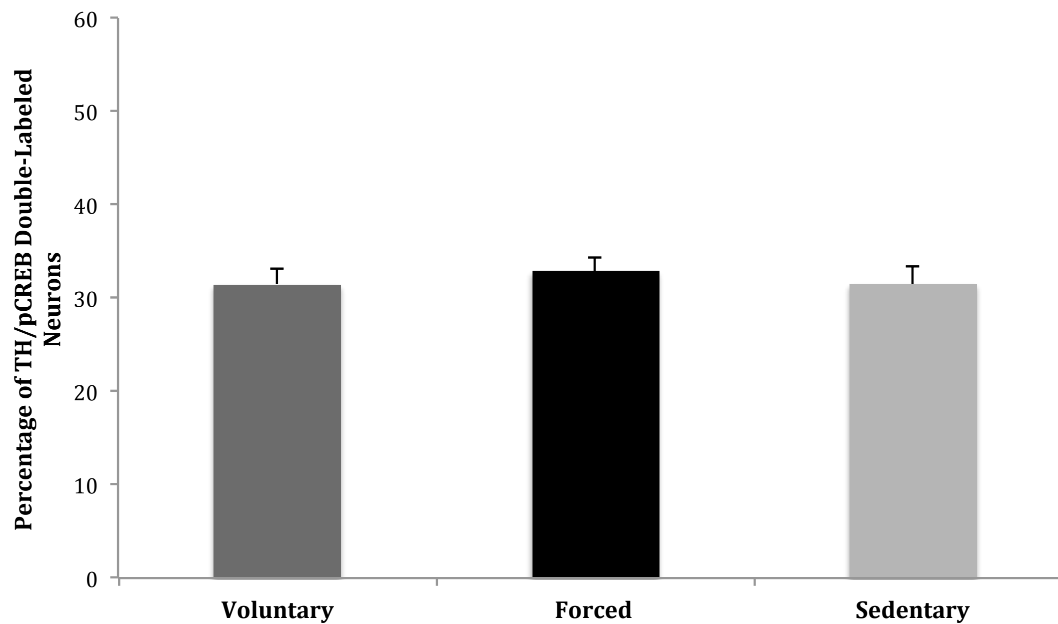


Figure 4. Dopamine neuron activation in the medial VTA did not differ between groups.

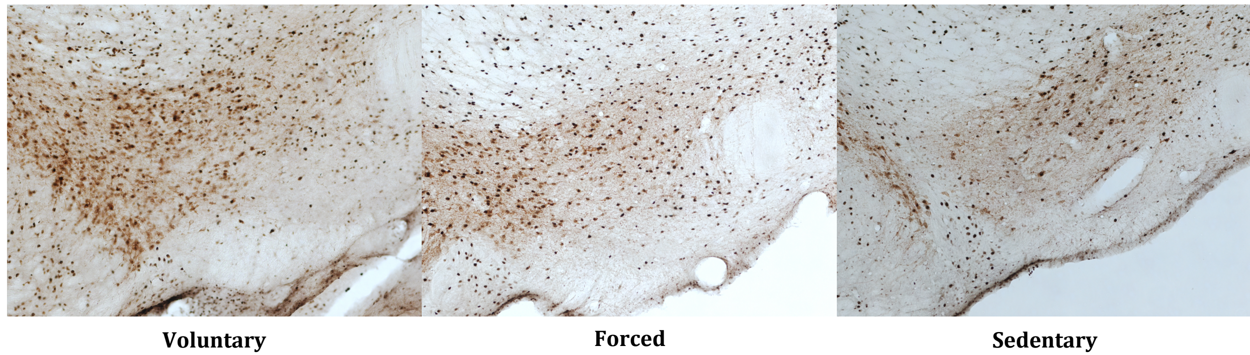


Figure 5. 10X images of the left side of the VTA for all groups. VW and FW groups show more dopamine neuron activation in the lateral VTA than the sedentary group.

Discussion

This study examined the role of exercise controllability on eliciting neurobiological reward. Consistent with previous studies, VW was found to be rewarding neurobiologically [2]. The novel finding of this study was that FW was also found to be rewarding neurobiologically.

Although there was not a main effect of exercise condition (VW or FW) on the total distance run, there was a time-by-exercise condition interaction on the total distance run. In order to evaluate this interaction, a correlation will be performed between the last two hours of running and the percentage of double-labeled neurons to see if run distance has an effect on the amount of activation of DA neurons in the VTA. VW rats ran longer distances than FW rats on some days, but FW rats showed just as many double-labeled neurons in the lateral VTA as VW rats. These results suggest that running distance is not a primary factor that influences exercise-induced reward.

FW rats gained the least amount of body weight even though they ran less than VW rats on some days (Figure 1). Low body weight could be a sign that the FW rats were stressed throughout the study. Greenwood et al. had an identical forced exercise paradigm,

and they also found that the FW group gained the least amount of body weight compared to any other group [3]. They measured thymus weight, spleen weight, and adrenal weight for all rats and found thymic involution, splenic contraction, and adrenal hypertrophy in both VW and FW groups, but significantly more so in FW rats [3]. These are all signs of chronic stress, suggesting that the animals found forced exercise to be stressful, but still rewarding.

It has been shown that elevated expression of ΔFosB accompanies repeated exposure to drugs of abuse, particularly in brain areas associated with reward and motivation, such as the VTA and NAc [7,9]. Because of this study, we now know that exercise, a natural reward, activates the lateral VTA specifically. These results build on Lammel et al.'s work on regional specificity within the VTA, and suggest that regardless of controllability, exercise induces greater activation of glutamatergic neurons projecting onto the lateral VTA from the laterodorsal tegmentum than glutamatergic neurons from the lateral habenula projecting onto the medial VTA. Further work should be done to look at neural activation in both the laterodorsal tegmentum and the NAc following forced exercise since these structures are upstream and downstream from the lateral VTA, respectively. It is interesting that there was a significant main effect of exercise on the percentage of double-labeled neurons in the lateral VTA but no difference between groups in the medial VTA because one would not expect forced exercise to activate the reward pathway, but to instead be aversive.

Previous studies have determined that voluntary exercise is rewarding both behaviorally and neurobiologically [2,6,10,11]. Herrera et al. showed that forced exercise is rewarding behaviorally [Herrera et al. Unpublished data], and now this work contributes that forced exercise is also rewarding neurobiologically. Because of this novel finding,

more work can be done to determine the neurobiological basis of reward in response to specific stimuli and whether the rewarding effects of exercise contribute to the anxiolytic and antidepressant effects of exercise, which this study suggests is the case. Even though FW rats showed signs of chronic stress (low body weight), the high amount of DA neuron activation they showed in the lateral VTA demonstrates that they still found forced exercise rewarding. This has important, real life implications. For example, people prescribed exercise by a doctor may perceive the exercise as forced, but their reward circuitry may still be activated. If the rewarding effects of exercise contribute to the stress protective effects of exercise, then those exercising regardless of perceived controllability may still experience exercise-induced reward to counter the anxiolytic and antidepressant effects of stress. In fact, it has been shown that exercise-induced stress resistance is independent of exercise controllability [3].

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