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The Rewarding Effects of Exercise are Independent of Wheel Running Controllability and Involve Activation of Nigrostriatal Dopamine Circuits

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By

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has been approved for the Department of Integrative Physiology

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

The Rewarding Effects of Exercise are Independent of Wheel Running Controllability and Involve Activation of Nigrostriatal Dopamine Circuits

Jonathan Joseph Herrera

Thesis directed by Professor Monika Fleshner, Ph.D

Department of Integrative Physiology, 2014

The mesocorticolimbic reward pathway is implicated in the development and treatment of stress-related psychiatric disorders such as anxiety and depression. Exercise can reduce the incidence of stress-related disorders, but the contribution of exercise reward to exercise-induced stress resistance is unknown. We have previously reported that the anxiolytic and antidepressant-like effects of exercise are independent of exercise controllability; whereby both voluntary and forced wheel running protect rats against behavioral consequences of stress. Voluntary wheel running has previously been shown to be rewarding, however, whether rats find forced wheel running rewarding is unknown. The goal of the current studies was to test the novel hypothesis that both voluntary and forced wheel running are similarly rewarding. Young adult, male Fischer 344 rats allowed voluntary or forced wheel access found running rewarding as measured by conditioned placed preference (CPP). In addition, the rewarding effects of wheel running were examined neurobiologically. Utilizing double label fluorescence *in situ* hybridization (FISH), voluntary and forced running rats re-exposed to the side of the CPP chamber previously paired with wheel running displayed greater conditioned activation (c-*fos*) of dynorphin-expressing direct pathway striatal neurons and tyrosine hydroxylase

(TH)-expressing lateral ventral tegmental area (VTA) neurons compared to rats re-exposed to the side of the CPP chamber previously paired with the lack of a running wheel. These results demonstrate that both voluntary and forced wheel running elicit c-*fos* activity in both classic mesocorticolimbic reward circuitry, as well as dorsal striatal direct pathway circuitry more recently implicated in reward processing. The activation of these particular neural circuits could be instrumental to the rewarding effects of exercise. Moreover, the implications of these data suggest that rewarding pathways may contribute to the mechanisms by which exercise increases stress resistance. Our findings warrant the need for greater investigation into central reward circuitry not only to seek novel pharmacological targets, but also to improve and tailor exercise interventions in the prevention and treatment of stress related psychiatric disorders.

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CHAPTER I: INTRODUCTION

Investigating the neurobiology of reward is a critical area of active research as findings have implicated the involvement of brain reward circuitry in the pathophysiology of stress-related psychiatric disorders such as anxiety and depression [1, 2, 3]. The number of American adults suffering from these conditions, along with their comorbidities, is major cause for concern. Depression is considered one of the most common types of mental illness, affecting around 25% of the US population [4]. Anxiety disorders are similarly detrimental, impacting around 18% of Americans [5]. Although the prevalence of these stress-related psychiatric disorders in the population is staggering and a major public health issue, the best-established treatments have limited and controversial efficacy [6, 7]. Furthermore, medication use carries a significant economic burden. For example, anti-depressant medication represents one of the three most prescribed categories of drugs nationally [8].

The majority of interventions to prevent stress-related psychiatric disorders have not focused on central reward circuitry. Interestingly, physical activity, in the form of exercise, has not only been shown to activate and elicit changes in gene expression in central reward neural circuitry [9, 10, 11], but is also protective against stress-related psychiatric disorders like anxiety and depression, including diminishing stress-related symptoms such as anhedonia [12, 13, 14, 15, 16, 17, 18]. Therefore, further investigations into the mechanisms underlying the neurobiology of reward, including those brought about by exercise, are warranted and can provide additional insight into helping prevent and treat stress-related psychiatric disorders.

Particular neural circuits underlie processing of reward. These brain circuits likely developed in response to natural rewards, such as food or sex, as an adaptive evolutionary function important for survival, reproduction, and fitness [19]. The classically established brain circuitry involved in reward is the mesocorticolimbic dopamine system, which includes the midbrain ventral tegmental area (VTA), as well as its efferent projections: orbital and medial prefrontal cortices, amygdala, thalamus, ventral striatum [nucleus accumbens (NAc)], and the hippocampus [20]. The mesocorticolimbic system is the circuitry proposed to be activated by and involved in the processing of natural rewards such as exercise [21].

Recent studies have more clearly elucidated specific regions within the mesocorticolimbic circuitry that are involved in reward processing, as well as other non-classical reward neurocircuitry. More specifically, distinct VTA circuits can encode reward and aversion [22, 23]. Whereas, laterodorsal tegmentum (LDT) optogentic stimulation in rodents caused greater Fos expression in the lateral VTA resulting in conditioned placed preference (CPP; a behavioral measure of reward), lateral habenula (LHb) optogentic stimulation caused greater Fos expression in the medial VTA, resulting in conditioned place aversion (CPA). In addition to the classical mesocorticolimbic reward system, direct and indirect pathways of the dorsal striatum can also influence reinforcing behavior in rodent models. Indeed, optogenetic stimulation of dorsal striatal direct pathways neurons induced persistent reinforcement, while stimulation of indirect pathway neurons induced transient punishment as indicated by an operant task and place preference [24]. Whether exercise elicits changes in specific sub-regions of the VTA or the dorsal striatum has not been previously examined.

Both animals and humans find exercise to be a rewarding behavior. Rats and mice, for example, will choose to run spontaneously on running wheels, and will learn to press levers in order to access a running wheel [25, 26, 27]. Furthermore, rats develop CPP to environments associated or paired with the after effects of brief voluntary running wheel bouts, as well as to nightly, long-term voluntary wheel running [10, 28, 29, 30]. Humans have reported an improvement in a positive affective state (positive feelings) and reduction in a negative affective state (negative feelings) immediately following both anaerobic and aerobic exercise bouts [12, 31, 32, 33].

Accompanying such behavioral outcomes is neurobiological evidence that exercise is rewarding. Voluntary wheel running increases the reward-related plasticity marker ΔFosB [34], as well as c-Fos expression in the NAc core [35, 36]. Voluntary wheel running also elicits changes in gene expression in dopaminergic and opioidergic reward systems including increasing tyrosine hydroxylase (TH) mRNA levels in the VTA, increasing delta opioid receptor (DOR) mRNA levels in the NAc shell, and reducing levels of dopamine receptor (DR)-D2 mRNA in the NAc core of rodents [10]. If opioid receptor systems are blocked in humans prior to acute physical activity, overall positive mood change following exercise was blocked compared to a placebo group [37].

The majority of work to date has focused on voluntary exercise, i.e., exercise that is initiated and terminated by one's own volition. We have recently demonstrated that wheel running can prevent the depression- and anxiety-like behavioral consequences of uncontrollable stress regardless of exercise controllability [38]. In that study, rats were forced to run in wheels following a running pattern that closely matched the distance, speed, and bout length of voluntary runners. If activation and plasticity in reward systems

is a central component to the mechanisms underlying the anxiolytic and antidepressant effect of exercise, then one might expect that forced wheel running would also be rewarding.

Intuitively, forced exercise would not be expected to be a reinforcing or rewarding behavior. Indeed, rats forced to run on wheels display classic signs of chronic stress, including adrenal hypertrophy and thymic involution [38]. Despite a potential stress response, however, forced wheel running produced stress resistance, and one report indicates that rats will learn to press levers for access to a rotating drum [39]. These data suggest that forced exercise and voluntary exercise might be similarly rewarding, both behaviorally and neurobiologically.

To test whether the rewarding properties of wheel running is independent of controllability, as well as to determine whether wheel running elicits differential activation in VTA sub-regions or the dorsal striatum, both Voluntary Wheel Running (VW) and Forced Wheel Running (FW) groups were subjected to a 38 day regimen of wheel running, and assessed for reward behaviorally and neurobiologically. Both groups were subjected to CPP training to assess preference towards a context either associated with a exercise or non-exercise condition. Following CPP, we assessed whether voluntary and forced wheel running recruit similar reward circuitry by assessing conditioned activation of reward circuitry elicited by re-exposure to either the side of the CPP chamber paired with prior exercise or non-exercise conditions. Our results suggest that both voluntary and forced exercise are rewarding, and the rewarding effects of exercise include activation of dopamine neurons in the lateral VTA and direct pathway neurons in the dorsal striatum.

CHAPTER II: MATERIAL AND METHODS

i. Animals

Young adult (6 to 7 weeks upon arrival), male Fischer 344 rats (Harlan SPF, Indianapolis, IN., USA) were used in all experimental procedures (n=24). Rats were housed in a temperature (22°C) and humidity-controlled environment; were maintained on a 12:12 hour light:dark cycle (lights on 07:00 to 19:00 hour); had *ad libitum* access to food (lab chow) and water; and were weighed at junctures in experimental timeline (see Figure 1). All animals were individually housed in Nalgene Plexiglas cages (45 cm × 25.2 cm × 14.7 cm). Animals acclimated to these housing conditions for 7 days prior to any experimental manipulation. Care was taken to minimize animal discomfort during all procedures. The University of Colorado Animal Care and Use Committee approved all experimental protocols.

ii. Exercise Protocols

Following one week of environment acclimation, rats were transported prior to lights out from home cages to running wheels each night. For the first 7 days of the experiment, all rats were allowed access to voluntary wheel (1.1 m circumference; Lafayette Instruments, Lafayette, IN., USA) running overnight (during light off 19:00 to 7:00 hour). Prior experiments revealed that rats forced to wheel-run, with no prior running experience, were tumbled about in the wheel and hung onto the wheel rungs rather than running [34]. Experience with voluntary running minimized these non-running behaviors.

After voluntary wheel acclimation, rats were assigned to either a Voluntary Wheel Running (VW, n=12) or Forced Wheel Running (FW, n=12) group. Groups were

assigned such that there was an equal distribution of high distance and low distance runners (based upon the prior 7 day voluntary wheel running data) in each group. Following conditioned placed preference (CPP) baseline testing (see below), rats ran every other night for two hours per night. This specific running duration was chosen as we have previously reported that rats allowed voluntary access to wheels run the most during the first two hours of their active cycle [10]. At the beginning of the active cycle, rats in either the VW or FW group were removed from their home cages and transported to their assigned voluntary or motorized running wheel (Lafayette Instruments, Lafayette, IN., USA). The forced running wheels (1.1 m circumference) could not be turned voluntarily by rats. Instead, forced wheels were driven by a motor controlled by the Activity Wheel Monitor software (Lafayette Instruments, Lafayette, IN., USA) according to a protocol pre-programed by the experimenters and designed to closely approximate a rat's natural voluntary running behavior based on analyses of prior experiments [34]. This pattern is characterized by brief bouts of running (average of 2.04 ± 1.95 minutes) at various speeds (range 4–17 meters/minute) interspersed with frequent periods of no running (range 0.33–30 minutes). Both VW and FW rats were confined to their wheels for their running bouts, were immediately conditioned thereafter (see below), and were returned to their home cages after conditioning (see below). Total revolutions of the running wheels were automatically recorded by the Activity Wheel Monitor software.

iii. Conditioned Placed Preference Apparatus

The CPP apparatus (38 cm tall x 57 cm long x 38 cm high) was composed of opaque black Plexiglas (5 mm thick) and contained two joined compartments. The two compartments of the conditioning apparatus were separated by a removable black

Plexiglas divider (10 cm tall x 12 cm wide x 5 mm thick), and had distinctive flooring. The flooring on the "grill" side consisted of black, anodized stainless steel bars that were 29 cm long and 5 mm thick, and separated by 4 mm of space between each bar. The flooring on the "hole" side consisted of a perforated, black anodized steel sheet covered in small holes 6.5 mm in diameter, and spaced 3.5 mm apart edge-to-edge.

Figure 1. Experimental Timeline

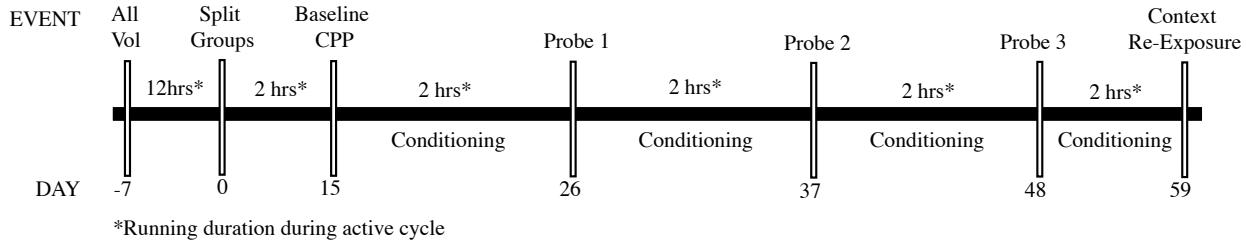

Figure 1. Experimental timeline. All rats were given voluntarily wheel running access overnight for 7 days to get accustomed to running prior to being split into two groups for CPP: forced wheel running (FW) or voluntary wheel running (VW). Each rat ran a total of 2 hours per night for 5 nights after Baseline CPP and between each Probe trial. Following Probe 3, rats participated in one more night of a wheel running/paired training bout before Context Re-Exposure.

iv. Conditioned Placed Preference Protocol

Baseline

Immediately following the 7 days of voluntary wheel running acclimation, rats were placed into the CPP apparatus for 20 minutes. A black Plexiglass divider (10 cm tall x 12 cm wide x 5 mm thick) with a doorway cutout (10 cm tall X 12 cm wide X 5 mm thick) was employed to allow animals free access to both sides of apparatus to assess baseline preference (Baseline). Behavior was videotaped for time spent on each side of the CPP apparatus and scored automatically using TopScan (CleverSys Inc., Reston, VA., USA) software. Following baseline preference testing, the rats underwent CPP training.

Training

During CPP training, rats ran every other night. On running nights, rats were exposed to their assigned running wheel (voluntary or forced) for 2 hours. Following 2 hours of running, rats were removed from their wheels and immediately placed into one side of the CPP apparatus for 20 minutes. Note that rats were assigned to a paired side such that they had to overcome their baseline preference (e.g. if a rat preferred grill, the assigned side paired with exercise would be hole). Interestingly, the groups were nearly counterbalanced for grill or hole as determined by their baseline preference. During CPP training the doorway between the two CPP chambers remained closed, so rats were confined to one side. The side of the CPP apparatus paired with wheel running was deemed the "paired side". On alternating nights, rats were placed individually into a sedentary cage (Nalgene Plexiglas cage; 45 cm × 25.2 cm × 14.7 cm) for 2 hours, rather than into their assigned running wheel. After 2 hours in this sedentary cage, rats were placed for 20 minutes onto the opposite side of the CPP chamber, deemed the "unpaired side." This protocol insured that each rat was exposed to both sides of the CPP apparatus equally, but only one side was associated with wheel running. Rats were returned to their home cages following the CPP training. The CPP chambers were thoroughly cleaned with water between each rat exposure. CPP training was conducted for a total of 30 days (3, 10 day periods in between probe trials; see below).

Assessment of Conditioned Place Preference (Probe Trials)

To determine which side of the CPP apparatus (unpaired or paired side) the rat preferred, the Plexiglas insert with the doorway cutout separating the two halves of the

CPP apparatus was utilized and rats were placed (one at a time) onto one side of the apparatus in a counterbalanced manner. Rats were allowed to explore both sides of the apparatus for 10 minutes. Behavior was recorded and scored using TopScan (CleverSys Inc., Reston, VA., USA). There were three probe trials, each separated by 10 days of CPP training. Probe trials occurred 24 hours following a wheel running/paired training bout, and occurred immediately following the start of the active cycle at 17:00 hour.

v. Context Re-Exposure

Rats were exposed to one more night of a wheel running/paired CPP training bout following the third and final probe trial. In order to determine whether exposure to the paired side of the CPP chamber elicited conditioned activation of reward circuitry in FW and VW groups, rats were re-exposed to either the paired or unpaired side of the CPP chamber 24 hours after the last running bout. Rats were randomly assigned to the following groups: Voluntary Unpaired (VUP), Voluntary Paired (VP), Forced Unpaired (FUP), and Forced Paired (FP). Rats were exposed to the assigned unpaired or unpaired side for 30 minutes, during which time locomotor activity was recorded and scored for a subset of rats (n=4 /group) using TopScan (CleverSys Inc., Reston, VA., USA). Following context re-exposure, rats were sacrificed by rapid decapitation, brains were extracted, frozen in chilled isopentane (-20° C) for 4 minutes, and stored at -80° C until sectioning. Brains were sliced through the rostral to caudal extent of the striatum and ventral tegmental area at 10 μm coronal sections using a cryostat. Slices were thaw-mounted directly on to Superfrost Plus slides (Fisherbrand, Pittsburg, PA., USA) and stored at -80° C until processing for double label fluorescent *in situ* hybridization.

vi. Fluorescent *In Situ* Hybridization

A. Fluorescent In Situ Hybridization Protocol

Double radioactive *in situ* hybridization was used to detect the proportion of dynorphin (direct pathway) and enkephalin (indirect pathway) containing neurons co-expressing c-*fos* (neural activation) in the dorsal striatum, as well as tyrosine hydroxylase (TH) neurons co-expressing c-*fos* in the ventral tegmental area (VTA) following exposure to a context paired with or unpaired with wheel running. Briefly, on Day 1, a 1-in-20 series (separated by 200 μm) of sections containing the striatum or VTA were washed in 4% paraformaldehyde for 1 hour, 0.1 M triethanolamine with 0.25% acetic anhydride for 10 minutes, and then dehydrated in graded ethanol. Complementary (c)RNA riboprobes for dynorphin (744 bp), enkephalin (693bp), and tyrosine hydroxylase (300bp) were labeled with digoxigenin UTP (Roche, Indianapolis, IN, USA) and c-*fos* (680bp) was labeled with fluorescein UTP (Roche, Indianapolis, IN., USA). Riboprobes were prepared from cDNA subclones in transcription vectors and labeled with [S-35] UTP (Perkin-Elmer, Waltham, MA, USA), using standard transcription methods. Riboprobes were diluted in 50% hybridization buffer containing 50% formamide, 10% dextran sulfate, 2X Sodium Citrate (SSC), 50 mM Phosphate Buffer (PBS) at pH 7.4, 1X Denhardt's solution, and 0.1 mg/ml yeast tRNA. Slides containing sections of the striatum or VTA were hybridized with the respective probe for approximately 18 hours at 55°C. On Day 2, sections were washed in 2X SSC, treated in RNaseA (200 μg/ml) for 1 hour at 37°C. Sections were treated with graded SCC washes (2X, 1X, 0.5X, 0.1X) and placed in 0.1X SSC at 65°C for 1 hour. Following 0.1X SSC incubation, sections were brought back to room temperature in distilled water and washed

with 0.05 M PBS. Sections were then quenched in hydrogen peroxide diluted to 2% in 0.05 M PBS for 30 minutes, washed in 1X Tris-Buffered Saline containing Tween pH 7.5 (TBS-T), and incubated in 0.5% blocking buffer (Perkin-Elmer, Waltman, MA., USA) in 1X TBS for 1 hour. Sections were immediately incubated in anti-fluorescein-horseradish peroxidase (Perkin-Elmer, Waltham, MA., USA) at 1:750 in TBS-T for 30 minutes. Sections were next washed in TBS-T and then incubated in cyanine 3 (CY3) amplification reagent solution at a 1:100 dilution in 1X amplification diluent (Perkin-Elmer, Waltham, MA., USA). Sections were washed and stored overnight in 0.05 M PBS at 4°C. On Day 3, sections were again quenched in 2% hydrogen peroxide in 0.05 M PBS for 30 minutes. Sections were next washed in TBS-T and incubated for 90 minutes in anti-digoxigenin-horseradish peroxidase (Perkin-Elmer, Waltham, MA, USA) at 1:100 dilution in TBS-T. Next, sections were again washed in TBS-T and incubated in fluorescein amplification reagent at a 1:100 diultion in 1X amplification diluent (Perkin-Elmer, Waltham, MA, USA) for 1 hour. Slides were then washed in PBS and air dried for approximately 30 minutes. Coverslips were set on slides using ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY., USA).

B. Image Analysis for Fluorescent In Situ Hybridization

Images were obtained from a Zeiss AX10 with axioscan Z1 fluorescent microscope, interfaced to a computer operating AxioVision software (Zeiss, Oberkochen, Germany), at 200X total magnification. Digoxigenin (dynorphin, enkephalin, or TH), Cy3 (c-*fos*), and DAPI (nuclei) emission channels were merged to construct a single image. For each rat, two images were acquired in the dorsal medial striatum (DMS) and dorsal lateral striatum (DLS) from two sections across the rostral to caudal axis of the

striatum (total 4 images per slice; 16 images per animal)(coordinates between 1.2 mm to 0.2 mm from bregma). Additionally for each rat, two images were acquired in the lateral and medial VTA over two sections across the rostral to caudal axis of the VTA (total 4 images per slice; 16 images per animal)(coordinates between -5.80 mm to -6.04 mm from bregma). Medial and lateral VTA boundaries were established based on a prior experiment [20]. Images were taken bilaterally and encompassed the right and left striatum, as well as the right lateral/medial and left lateral/medial VTA for each rat. The number of c-*fos*, enkephalin, dynorphin, tyrosine hydroxylase, and co-labeled (dynorphin/c-*fos*, enkephalin/c-*fos*, or TH/c-*fos*) neurons were counted in each image using ImageJ. A co-label was confirmed when a dynorphin-, enkephalin-, or TH-expressing neuron near completely covered and had a similar morphology to a c-*fos* reactive cell.

vii. Statistics

Group differences in body weight and nightly running distance were analyzed utilizing repeated measures analysis of variance (ANOVA). CPP for Baseline and each Probe test were expressed as a percentage preference for the side of the CPP chamber paired with wheel running, using the following formula: (time spent on the paired side/total time spent on both sides) x 100. Preference scores were compared with repeated measured ANOVA. VUP, FUP, VP, and FP group differences in total distance traveled were analyzed using two-by-two ANOVA. Percentage of (dynorphin, enkephalin, or TH) neurons expressing c-*fos* was calculated as per the following formula: [# of double positive cells (dynorphin/c-*fos*, enkephalin/c-*fos*, or TH/c-*fos*)] / total # of cells (dynorphin, enkephalin, or TH)]. Group differences in percentage of neurons expressing

c-*fos* were analyzed using two-by-two ANOVA. Analyses were followed by Fisher's protected least significant difference (PLSD) *post hoc* tests when appropriate. Group differences were considered different when $p < 0.05$.

CHAPTER III: RESULTS

i. Average Body Weights

Figure 2 shows average body weights measured at specific time points throughout the duration of the experiment. Body weight increased steadily over the duration of the experiment in both VW and FW groups. Repeated measures ANOVA revealed a significant main effect of time [$F(5, 105) = 279.56$, $p < 0.0001$] and a significant time-by-exercise interaction [$F(5, 105) = 3.35$, $p < 0.0076$] on body weight. VW and FW groups significantly differed only at the Baseline CPP body weight measurement ($p < 0.05$). There was not a main effect of exercise [$F(1, 21) = 3.32$, $p = 0.087$]

FIGURE 2. Average Body Weights

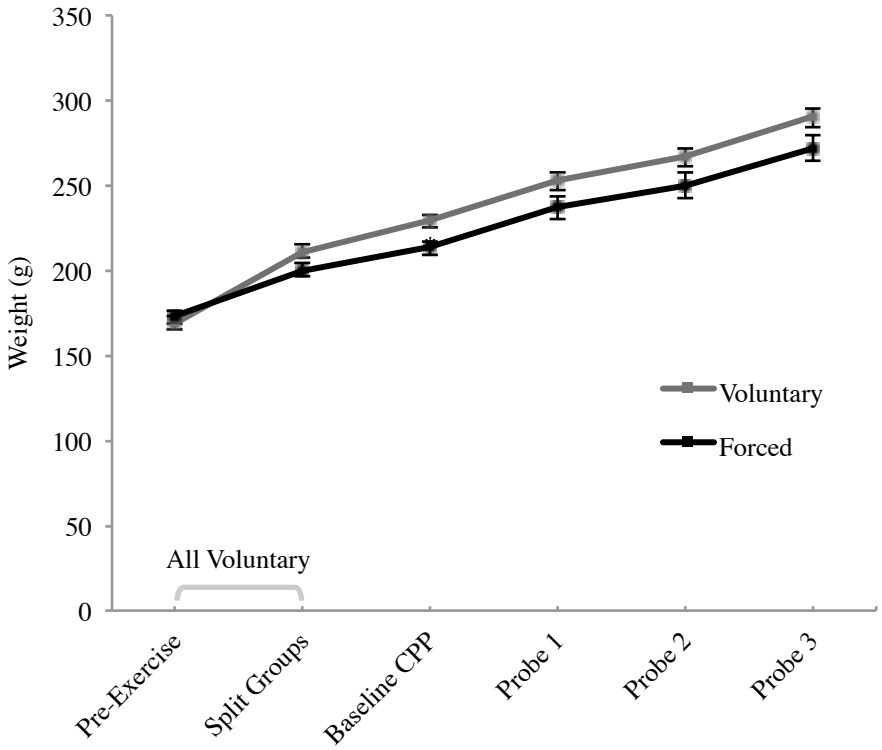

Figure 2. Average body weights measured at specific junctions during the experiment. Body weight increased steadily over the duration of the experiment in both VW and FW groups. VW and FW groups significantly differed only at the Baseline CPP body weight measurement ($p < 0.05$).

ii. Average Daily Running Distance

All rats participated in 38 total days of wheel running. During the first 7 days when all rats were placed in voluntary running wheels for the entire active cycle, VW rats averaged 2071 ± 350.75 m/night and FW rats averaged 1575 ± 224.55 m/night. Repeated measures ANOVA revealed no significant difference in wheel running distance based upon the exercise condition [$F(1, 21) = 1.722$, $p = 0.204$].

Figure 3 shows the average wheel running distance once animals were split into VW and FW groups and includes the remaining 30 running days (2 hr/night) up until the last CPP probe (the single bout of wheel running prior to Context Re-Exposure is not shown). In order to familiarize FW with forced wheel running, the distance and speed were increased progressively as we have previously reported [34]. Repeated measures ANOVA revealed a significant main effect of time [$F(29, 609) = 6.47$, $p < 0.0001$], and a significant time-by-exercise interaction [$F(29, 609) = 18.73$, $p < 0.0001$] on distance run. The main effect of exercise [$F(1, 22) = 10.42$, $p < 0.05$] on distance run was not significant. Interestingly, on the 22nd day of wheel running, distance run by the VW group began to decline below the distance run by the FW group. This pattern continued through the remainder of the study. Results of the post-hoc comparisons are reported in Figure 3.

FIGURE 3. Average Wheel Running Distance per Day

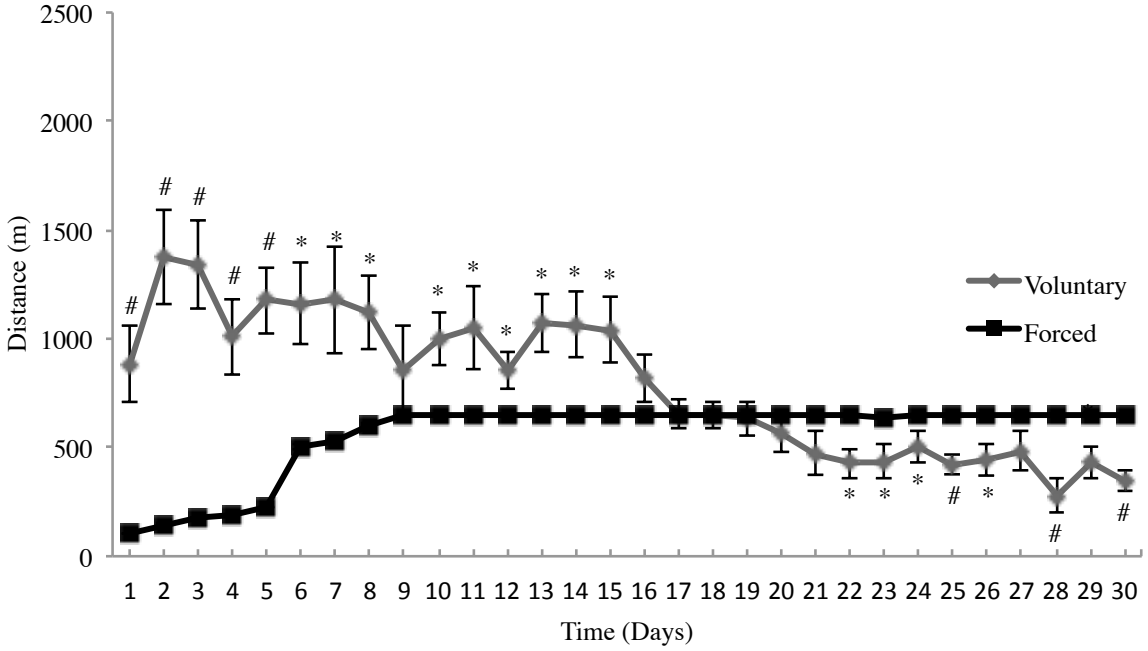

Figure 3. Average wheel running distance during each 2 hours of wheel exposure starting after the animals were split into VW and FW groups. In order to familiarize FW with forced wheel running, the distance and speed were increased progressively to eventually approximate a rat's natural voluntary running behavior based on analyses of prior experiments. # $p < 0.0001$ relative to FW group; * $p < 0.05$ relative to the FW group.

iii. Conditioned Placed Preference

Rats were trained and tested for CPP according the schedule presented in Figure 1. As presented in Figure 4 rats developed a preference for the side paired with wheel running over time regardless of the controllability of exercise. Repeated measures ANOVA revealed a significant effect of time on preference for side paired with wheel running [$F(3, 63) = 6.08$, $p < 0.05$].

FIGURE 4. Conditioned Place Preference

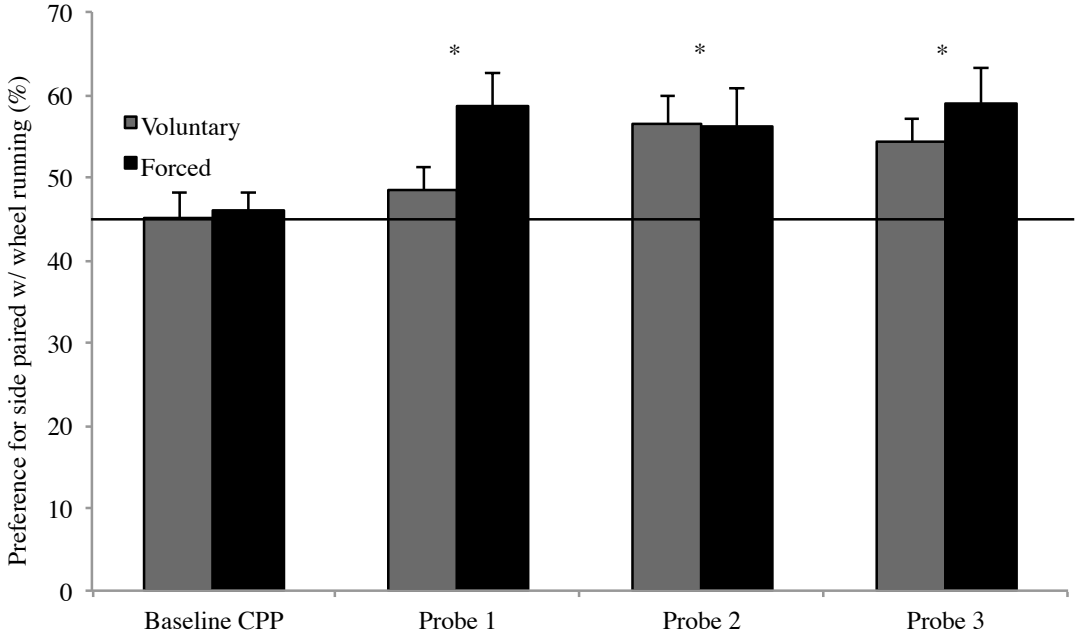

Figure 4. Preference for side paired with wheel running. Rats were assigned to paired side against their Baseline CPP preference and developed a preference for the side paired with wheel running over time ($p < 0.05$).

iv. Context Re-Exposure

Following Probe 3, and one more night of a wheel running/paired training bout, rats were re-exposed to either the paired or unpaired side of the CPP chamber for 20 minutes. Figure 5 shows the total distance traveled in meters during the context re-exposure. ANOVA revealed an exercise-by-side interaction [$F(1,10) = 6.70$, $p < 0.05$] on total distance traveled. However, post hoc analysis determined that there were no significant differences in total distance traveled between any of the groups.

FIGURE 5. Context Re-Exposure Locomotion

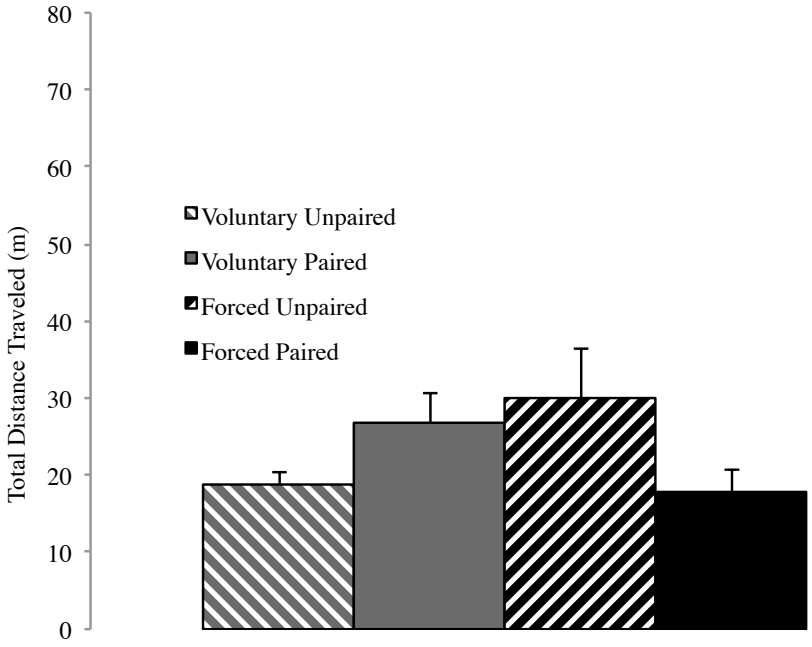

Figure 5. Average total distance traveled during re-exposure to the context previously paired (VP or FP) or unpaired (VUP or FUP) with voluntary or forced wheel running. Rats were exposed to the paired or unpaired side 24 hours following the last wheel running bout. There were no significant differences in total distance traveled between any of the groups.

v. c-*fos* mRNA expression in Dynorphin and Enkephalin Neurons of the Dorsal Striatum

Due to damage incurred during removal, one brain had to be dropped from analyses. This resulted in the following group sizes: VUP, n=5; FUP, n=5; VP, n=6; FP, n=6. Figure 6 displays the percentage of dynorphin neurons expressing c-*fos* mRNA in the dorsal medial (DMS) and dorsal lateral (DLS) striatum after Context Re-exposure. Regardless of exercise controllability, rats re-exposed to the side of the CPP chamber previously paired with wheel running displayed a significantly greater percentage of dynorphin neurons expressing c-*fos* mRNA in both the DMS and DLS compared to rats re-exposed to the unpaired side. ANOVA revealed a main effect of re-exposure side in

the DMS [F (1, 18) = 62.22, p < 0.0001] and the DLS [F (1, 18) = 59.23, p < 0.0001] on percentage of dynorphin neurons expressing c-*fos* mRNA. The main effects of exercise did not reach significance.

FIGURE 6. Dynorphin/c-*fos* Double Label in Dorsal Striatum

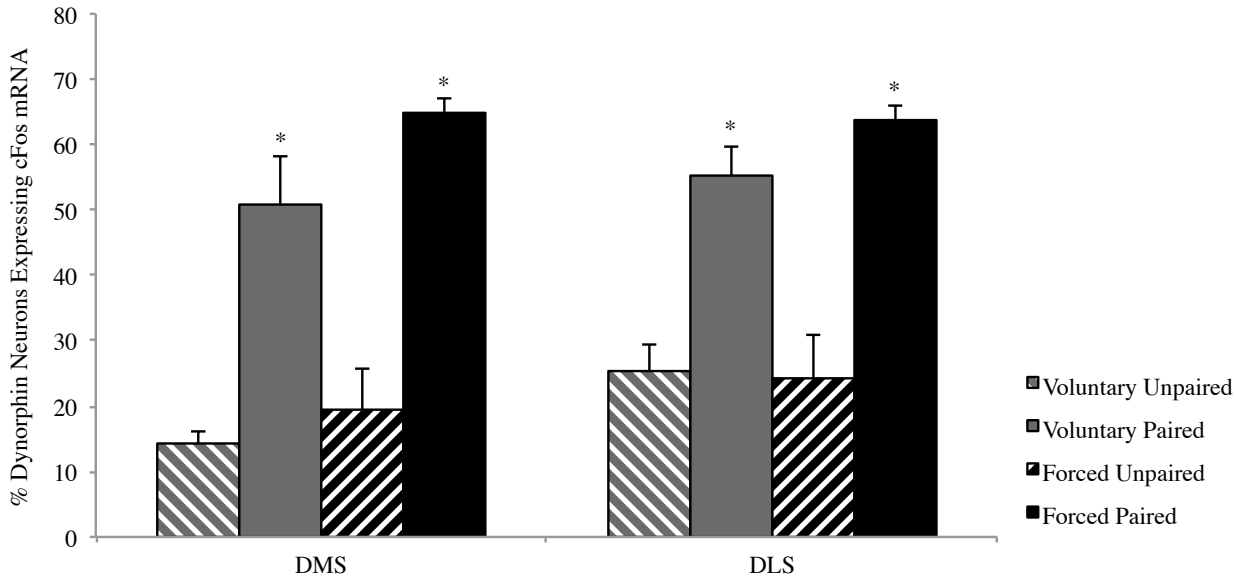

Figure 6. Percentage of dynorphin neurons expressing c-*fos* mRNA after rats were re-exposed to a side of the CPP apparatus either paired (VP or FP) with wheel running or unpaired to wheel running (VUP or FUP) for 30 minutes. Values represent mean number of % dynorphin neurons expressing c-*fos* mRNA ± SEM. Groups paired with wheel running (VP or FP), regardless of controllability, had a significant increase in % c-*fos* positive dynorphin neurons compared to unpaired counterparts (VUP or FUP) (*p < 0.0001).

Figure 7 shows the percentage of enkephalin neurons expressing c-*fos* mRNA in dorsal striatum after re-exposure to either the paired or unpaired side of the CPP chamber. In contrast to the dynorphin results, rats exposed to the side of the CPP chamber previously paired with either voluntary or forced wheel running displayed a significantly smaller percentage of enkephalin neurons expressing c-*fos* mRNA in both the DMS and DLS compared to the rats re-exposed to the unpaired side. ANOVA revealed a main effect of re-exposure side in the DMS [F (1, 18) = 118.59, p < 0.0001]

and DLS [F (1, 18) = 102.04, p < 0.0001] on percentage of enkephalin neurons expressing c-*fos* mRNA. The main effects of exercise on percentage of enkephalin neurons expressing c-*fos* mRNA were not significant.

FIGURE 7. Enkephalin/c-*fos* Double Label in Dorsal Striatum

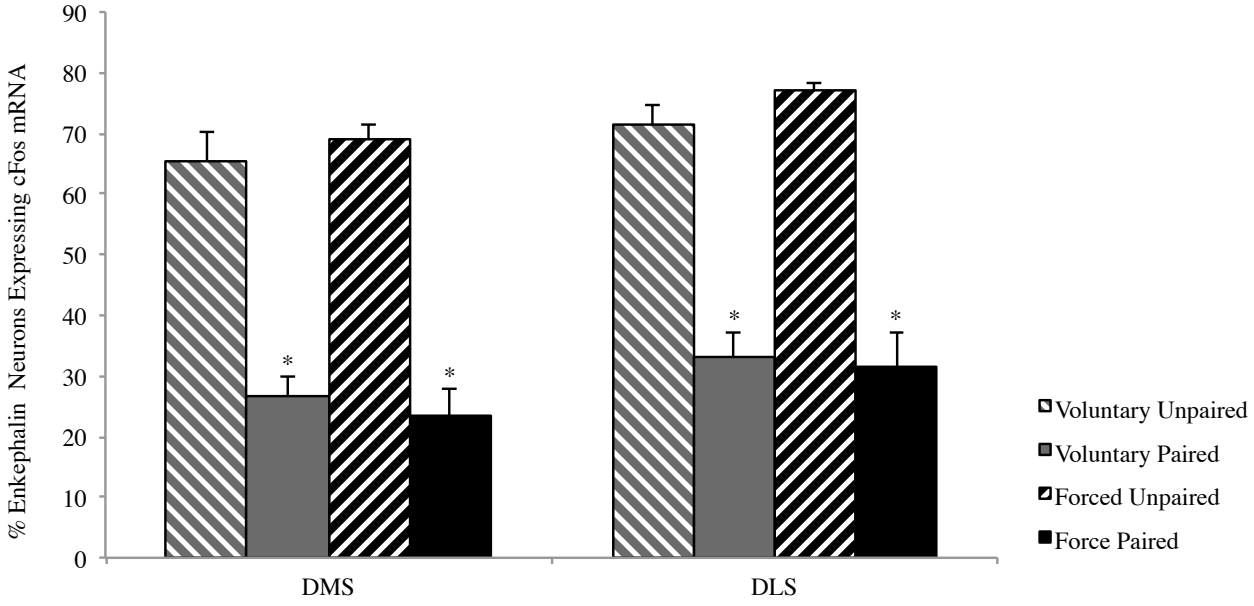

Figure 7. Percentage of enkephalin neurons expressing c-*fos* mRNA after rats were re-exposed to a side of the CPP apparatus either paired (VP or FP) with wheel running or unpaired to wheel running (VUP or FUP) for 30 minutes. Values represent mean number of % enkephalin neurons expressing c-*fos* mRNA ± SEM. Groups paired with wheel running (VUP or FUP), regardless of controllability, displayed a significantly smaller % of c-*fos* positive enkephalin neurons compared to unpaired counterparts (VP or FP) (*p < 0.0001).

vi. c-*fos* mRNA expression in TH Neurons of the VTA

Figure 8 represents the percentage of TH neurons expressing c-*fos* mRNA in the lateral VTA and medial VTA. Rats exposed to the side of the CPP chamber previously paired with either voluntary or forced wheel running displayed a significantly greater percentage of TH neurons expressing c-*fos* in the lateral VTA compared to the rats re-exposed to the unpaired side. ANOVA revealed a main effect of side re-exposure on

percentage of TH neurons expressing c-*fos* in the lateral VTA [F (1, 18) = 101.49, p < 0.0001]. The main effect of exercise on percentage of TH neurons expression c-*fos* mRNA in the lateral VTA was not significant [F (1, 18) = .790, p = 0.386]. In contrast to the lateral VTA, rats re-exposed to the side of the CPP chamber paired with either voluntary or forced wheel running displayed a significantly smaller percentage of TH neurons expressing c-*fos* in the medial VTA compared to the rats re-exposed to the unpaired side. ANOVA revealed a significant main effect of side re-exposure on percentage of TH neurons expressing c-*fos* in the medial VTA [F (1, 17) = 40.76, p < 0.0001]. Similar to the lateral VTA, the main effect of exercise on percentage of TH neurons expressing c-*fos* in the medial VTA was not significant [F (1,17) = .724, p = 0.407] (Note: One medial VTA belonging to a rat in the FUP group was damaged during sectioning rendering it unusable; n=4).

FIGURE 8. Tyrosine Hydroxylase/c-*fos* Double Label in VTA

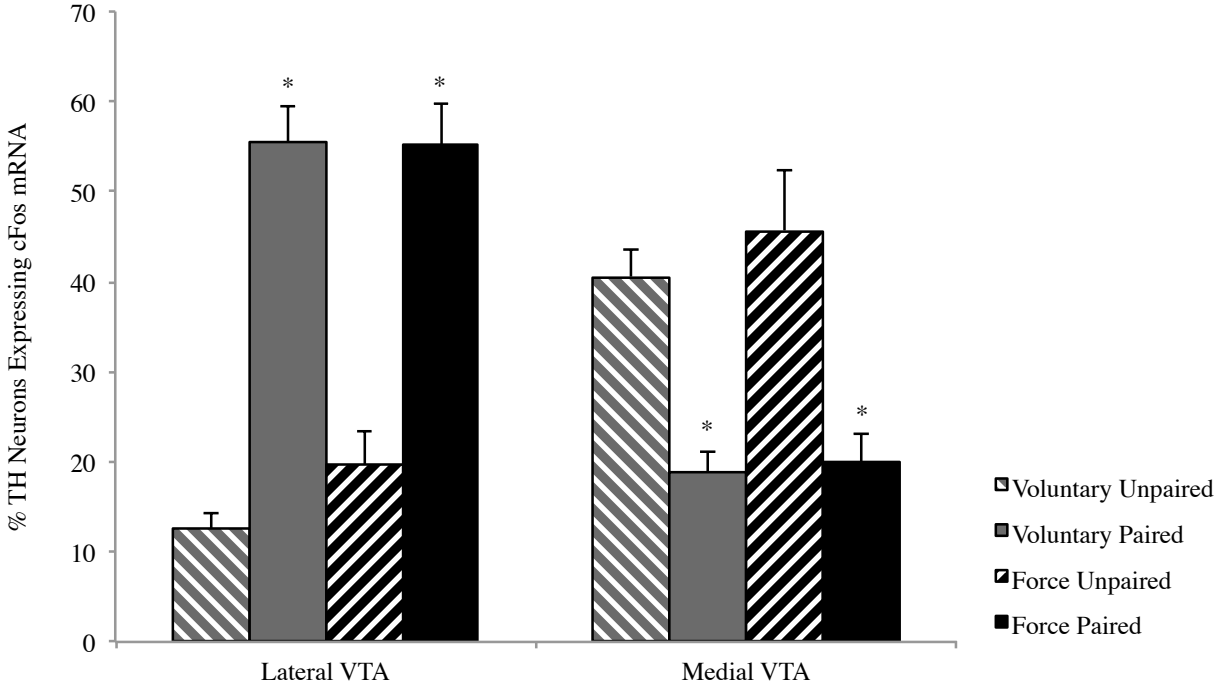

Figure 8. Percentage of tyrosine hydroxylase (TH) neurons expressing c-*fos* mRNA after rats were re-exposed to the side of the CPP apparatus either previously paired with wheel running (VP or FP) or unpaired with wheel running (VUP or FUP) for 30 minutes. Values represent mean number of % TH neurons expressing c-*fos* mRNA ± SEM. Rats re-exposed to the side of the CPP chamber previously paired with wheel running displayed a significantly greater percentage of TH neurons expressing c-*fos* in the lateral VTA compared to their unpaired counterparts; (* $p < 0.0001$). Contrarily, rats re-exposed to the side of the CPP chamber previously paired with wheel running displayed a significantly smaller percentage of TH neurons expressing c-*fos* in the medial VTA compared to their unpaired counterparts; (* $p < 0.0001$).

CHAPTER IV: DISCUSSION

i. Effects of Voluntary and Forced Wheel Running on Average Body Weight

We had previously reported that rats having undergone six weeks of wheel running showed significant changes in body weight dependent upon exercise controllabilty [38]. In fact, FW rats gained less weight over time compared to VW. The reduction of body weight gain, as well as thymic involution and splenic hypertrophy, suggested the FW condition imparted a state of chronic stress. Interestingly, although the FW condition induced peripheral physiological changes indicative of repeated activation of the stress response, the wheel running behavior still produced resistance against the consequences of uncontrollable stressor exposure including exaggerated fear conditioning and interference with escape learning. Due to these previous findings, we would expect the FW condition in the current experiment to demonstrate similar effects. However, no significant effect of exercise condition on body weight gain was detected (thymus and spleen weights were not measured). Importantly, there were methodological differences between the two studies. Whereas in our previous study VW and FW rats were allowed access during their entire active cycle, rats were only given two hours of wheel access in the current experiment. Therefore, the absence of body weight changes due to the FW condition could have been a result of several factors including the shorter duration of exercise bout compared to previous findings.

ii. Running Behavior of VW and FW Groups

In the current study, we utilized a protocol that forced rats to run in wheels following a duration and a pattern closely resembling voluntary wheel running, which was just as effective at preventing consequences of stress as voluntary wheel running

[38]. Furthermore, wheel access was restricted to two hours of wheel running due to the fact that rats run a greater hourly distance during the first two hours of the active cycle [10]. Although there was not a main effect of the exercise condition on running distance, VW rats ran more 14 out of first 15 days and FW rats ran more 7 out of the final 9 days. This was a surprising finding, as we had previously observed that VW rats allowed access for the entire active cycle ran a greater distance than FW over the last few weeks of running [38]. Moreover, we have also observed that VW rats maintain their average distance run in the first two hours of the active cycle between two and six weeks of running [10]. The unexpected decline in running distance displayed by VW rats may have been a consequence of the wheel access restriction, however this possibility has yet to be tested.

iii. Voluntary and Forced Running is Rewarding

The current data indicate, for the first time to our knowledge, that rats display place preference for forced wheel running. Additionally, the present data provide evidence that preference for both voluntary and forced wheel running occurs with as little as 10 days of CPP training and is sustained for over six weeks from the onset of CPP training. A prior report demonstrated that preference towards a context paired with voluntary wheel running occurred as an aftereffect of the wheel running, or immediately following two hours of running access [28]. Our VW results are similar to this finding in that we also restricted rats running access to two hours but differ in several aspects including chamber design, food access, training duration, as well as the time of day when running, training, and preference testing occurred. We had previously reported that long-term voluntary wheel running rats, allowed wheel access for the entire active cycle,

developed preference for wheel running at 6 weeks, but not at 2 weeks, following CPP training. Thus, restricting access to the wheels and exposing rats to the CPP chambers immediately following peak running may have facilitated the learning of CPP.

iv. Direct Dorsal Striatal Pathways may Contribute to Exercise Reward

It is well known that the dorsal striatum, a subcortical forebrain structure, is important for the organization and generation of voluntary movement [40, 41, 42]. It is only recently, however, that the dorsal striatum has been implicated in mechanisms underlying reward and aversion [24, 43, 44, 45, 46]. Rodents, for example, will learn to press touch triggers resulting in optogenetic activation of the direct pathway of the dorsal striatum, and will learn to avoid touch triggers resulting in optogenetic activation of the indirect pathway [24]. Moreover, rodents will develop a place preference for a compartment paired previously with laser illumination that stimulated direct pathway neurons [24]. Importantly, these results occur in the absence of effects of optogenetic stimulation on locomotor activity, indicating that reward elicited by direct pathway activation can be independent of movement. The present data support these recent studies. Rats re-exposed to the side of the CPP chamber paired with wheel running demonstrated greater activation of direct pathway dorsal striatum neurons, as indicated by a greater percentage of dynorphin neurons expressing the neuronal activation marker c-*fos* mRNA, compared to rats re-exposed to the unpaired side. Moreover, rats exposed to the paired side demonstrated reduced activation of the indirect pathway, as indicated by a lesser percentage of enkephalin neurons expressing c-*fos* mRNA, compared to rats re-exposed to the unpaired side. Analyses of locomotor activity with Top Scan indicated that rats re-exposed to the paired and unpaired sides moved around equally during re-

exposure; suggesting that difference in movement did not contribute to the observed differential activation of the direct and indirect pathways. These data, therefore, suggest that rats exposed to an environment previously paired with a rewarding stimulus such as wheel running, express conditioned activation of the dorsal striatum direct pathway and suppression of the indirect pathway. One implication of these data is that activation of the direct pathway and suppression of the indirect pathway may contribute to the rewarding effects of exercise and the development of exercise CPP. This novel finding implies that non-classical reward pathways, in addition to classical reward pathways associated with the mesocorticolimbic dopamine circuitry, may mediate exercise reward. Consistent with these observations, reward-related dopaminergic agents (e.g.-cocaine & apomorphine) induce dorsal striatal c-*fos* induction in rats [42]. Furthermore, current studies have found that optogenetic excitation and inhibition of substania nigra pars compacta (SNc) neurons, which provide dopamine innervation of the dorsal but not ventral striatum, elicit positive (reward) and negative (aversion) affects as determined by operant place preference [48]. Therefore, it is possible that activation of SNc neurons and subsequent dopamine release on direct pathway striatal neurons not only contributes to movement organization and generation, but may also play a significant role encoding reward including reward associated with exercise. Analyses of conditioned activation of SNc dopaminergic neurons during re-exposure to the paired and unpaired sides of the CPP chamber are currently underway.

v. Voluntary and Forced Wheel Running Causes Conditioned Activation of the Mesocorticolimbic Dopamine Reward System

In addition to the novel role of nigrostriatal dopamine circuitry in exercise reward, the current data also implicate the more traditional, mesocorticolimbic dopamine system

in exercise reward. Other studies have previously implicated the mesolimbic system in the rewarding effects of wheel running, but all prior studies of which we are aware only assessed the downstream effects of midbrain dopamine neurons in the NAc on gene expression changes and neuronal activation [10, 35]. Our current experiment demonstrates the novel finding that subregions of midbrain VTA dopamine neurons may contribute unique roles to exercise reward. Rats re-exposed to the side of the CPP chamber previously paired with wheel running displayed greater conditioned activation of lateral midbrain VTA dopamine neurons as indicated by a higher percentage of TH neurons expressing the neuronal activation marker c-*fos*. The present data are consistent with prior reports showing that optogenetic activation of lateral VTA can elicit CPP [20]. Conversely, rats re-exposed to the side of the CPP chamber paired with the absence of wheel running exhibited a greater percentage of double c-*fos*/TH neurons in the medial VTA. Lamell et al. (2011) reported that c-*fos* expression in the medial VTA, as a result of upstream optogenetic stimulation of the lateral habenula, was associated with CPA. The current data, along with this prior report, suggest that rats may find exposure to the unpaired side of the CPP chamber following 2 hours of exposure to a cage lacking a running wheel (during "unpaired" CPP training trials) aversive. This aversive memory could have been recalled during re-exposure to the unpaired side of the CPP chamber during context re-exposure, resulting in activation of the medial VTA. Thus, we have now provided evidence that specific midbrain VTA dopamine neurons encoding reward are conditionally activated upon re-exposure to a wheel running context. Even though this finding is novel in the context of exercise reward, it is congruous with earlier, well-established studies that revealed the importance of VTA dopamine activity in the

processing of a conditioned stimulus associated with a rewarding outcome. More specifically, it is known that VTA neurons in monkeys elicit phasic responses to the onset of a stimulus predicting other natural rewards, such as food [49, 50, 51].

vi. Conclusion

Here we report for the first time that the rewarding effects of exercise, in the form of wheel running, are independent of controllability. Our data support the novel hypothesis that both voluntary and forced exercise are similarly rewarding, as demonstrated through both behavioral and neurobiological measures. Importantly, we were also able to reveal activation of the both the classical and non-traditional reward pathways after exposure to conditioned cue, i.e., in the absence of any physical running *per se*. Specifically, we have provided evidence that the rewarding effects of exercise could be mediated through both classical mesocorticolimbic neural circuitries, via activation of reward-related lateral VTA dopamine neurons, and through activation of non-traditional direct pathway dorsal striatal neural circuits. Because both voluntary and forced exercise are not only similarly rewarding but also stress protective [34], the present data provide additional reason to suspect that the activation and plasticity in neural reward circuitry may impart critical mechanistic contributions to both the stress protective effects and the anxiolytic and antidepressant effects of exercise. This idea has profound translational significance such that greater investigation into the relationships between reward neural circuitry, exercise, and stress-related psychiatric disorders could yield not only unique targets for pharmacological intervention, but might also spur basic scientists and clinicians to recommend and facilitate physical activity interventions and

treatments for greater numbers of people at risk or afflicted by such mental health disorders.

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