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Wheel running alters patterns of uncontrollable stress-induced *cfos* mRNA expression in rat dorsal striatum direct and indirect pathways: a possible role for plasticity in adenosine receptors

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Abstract

Emerging evidence indicates that adenosine is a major regulator of striatum activity, in part, through the antagonistic modulation of dopaminergic function. Exercise can influence adenosine and dopamine activity, which may subsequently promote plasticity in striatum adenosine and dopamine systems. Such changes could alter activity of medium spiny neurons and impact striatum function. The purpose of this study was two-fold. The first was to characterize the effect of long-term wheel running on adenosine 1 (A_1R), adenosine 2A ($A_{2A}R$), dopamine 1 (D_1R), and dopamine 2 (D_2R) receptor mRNA expression in adult rat dorsal and ventral striatum structures using *in situ* hybridization. The second was to determine if changes to adenosine and dopamine receptor mRNA from running are associated with altered *cfos* mRNA induction in dynorphin- (direct pathway) and enkephalin- (indirect pathway) expressing neurons of the dorsal striatum following stress exposure. We report that chronic running, as well as acute uncontrollable stress, reduced A_1R and $A_{2A}R$ mRNA levels in the dorsal and ventral striatum. Running also modestly elevated D_2R mRNA levels in striatum regions. Finally, stress-induced *cfos* was potentiated in dynorphin and attenuated in enkephalin expressing neurons of running rats. These data suggest striatum adenosine and dopamine systems are targets for neuroplasticity from exercise, which may contribute to changes in direct and indirect pathway activity. These findings may have implications for striatum mediated motor and cognitive processes, as well as exercise facilitated stress-resistance.

Keywords

exercise; striatum; adenosine; dopamine; stress; running

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1. Introduction

The striatum is a basal ganglia structure critically involved in a wide range of motor, learning, and motivational processes. Abnormalities in the striatum have been suggested to contribute to a diverse array of motor and mental health pathologies including Huntington's disease, Parkinson's disease, schizophrenia, drug abuse, and depression (for review see [1-5]). A growing literature suggests that exercise promotes neuroplasticity in the striatum, which may have therapeutic benefits for mental and physical health [6-11]. Moreover, recent findings indicate rodents that engage in running are protected against uncontrollable stress-induced enhancement of drug preference and cognitive deficits that involve the striatum [12, 13]. Therefore, exercise may promote neuroplasticity in the striatum that protects against the deleterious effects of stress. However, few studies have characterized exercise-induced plasticity in the striatum that may contribute to stress-resistance [7, 11, 14].

Emerging evidence indicates that the endogenous purine nucleoside adenosine is a major regulator of striatum activity under periods of increased metabolic demand, in part through its potent modulation of dopamine signaling (for review see [15, 16]). Presynaptic adenosine A₁ and A_{2A} receptors located on terminals of glutamate, dopamine, and cholinergic neurons in the striatum can fine tune neurotransmission at synapses of medium spiny neurons (for further review see [16]). Additionally, post-synaptic adenosine A₁R and A_{2A}R in the striatum are located in abundance on distinct networks of medium spiny neurons [17, 18], where they antagonistically modulate the binding and function of dopamine D₁ and D₂ receptors respectively [17-21]. Dopamine transmission in the striatum is critically involved in many cognitive and motor processes including instrumental learning, working memory, reward, motivation, and movement (as reviewed in [22, 23]). Therefore, neuroplasticity in adenosine receptor systems in the striatum may have profound effects on cognitive and motor processes that require normal dopamine signaling, and may be a target for the stress protective properties of exercise.

Prolonged or acute exposure to A₁R and A_{2A}R agonists are associated with increases or decreases of receptor mRNA in both cell culture and *in vivo* models [24-29]. Therefore, treatments that increase adenosine, such as exercise, may result in changes to A₁R and A_{2A}R receptor mRNA in the striatum. Acute bouts of running increase brain-wide adenosine concentrations [30] and metabolic activity in the striatum [31, 32]. Repeated increases of adenosine that may follow several bouts of wheel running could thus be sufficient to change adenosine receptor mRNA levels in the striatum. Additionally, evidence suggests that a particularly augmented adenosine activity in the striatum may contribute to the development of depression-like behaviors in rats following uncontrollable stress [33-37]. Indeed, brain adenosine receptor activation or blockade can, respectively, mimic or prevent depression-like deficits in shuttle box escape following exposure to uncontrollable tail shock [36, 37] that are thought to involve the striatum [13] and are prevented by exercise [38, 39]. Therefore, wheel running may alter adenosine receptor mRNA in a manner consistent with protection against the development of depression-like behavior. Changes to A₁R and A_{2A}R mRNA levels are commonly associated with similar changes in radioligand binding affinity or protein concentration in the brain [40-56]. Thus, altered A₁R and A_{2A}R mRNA levels may have importance for adenosine receptor expression, which may ultimately influence

dopamine-mediated medium spiny neuron activity and striatum function. However, to the best of our knowledge, the impact of long-term wheel running on A₁R and A_{2A}R mRNA levels in the striatum remains unknown.

The first aim of this study was to characterize the effects of long-term wheel running on A₁R, A_{2A}R, D₁R, and D₂R mRNA expression in the rat dorsal medial (DMS) and lateral striatum (DLS), as well as structures in the ventral striatum including the core (AcbC), lateral shell (AcbShL), and medial shell (AcbShM) of the nucleus accumbens. We examined dorsal and ventral structures, because the striatum has been traditionally separated into functionally distinct components. The dorsal striatum has been generally implicated in control of movement and motor learning processes, whereas the ventral striatum has been thought to be involved in motivation and reinforcement, although considerable functional overlap has been reported between striatal divisions (for review see [57]). Moreover, structures within the dorsal and ventral striatum have been further subdivided by function. The DMS has been associated with goal directed motor behavior [58, 59], whereas the DLS has been implicated in habit learning [60]. Furthermore, evidence suggests ventral striatum subdivisions including AcbC, AcbShL, and AcbShM may serve distinct roles in processing reward [61-65]. Therefore, running-induced regional changes to A₁R, A_{2A}R, D₁R, and D₂R gene expression in the striatum may have significance for different striatum-mediated processes.

The second aim of this study was to determine if potential changes to A₁R, A_{2A}R, D₁R, or D₂R gene expression following wheel running are associated with differences in *cfos* (neural activity marker) induction from uncontrollable tail shock in two distinct classes of dorsal striatum medium spiny neurons. The first class, striatonigral “direct pathway” neurons, almost exclusively express the opioid dynorphin and post-synaptic D₁R [66]. The second class, striatopallidal “indirect pathway” neurons, nearly exclusively express the opioid enkephalin, as well as post-synaptic A_{2A}R and D₂R [67]. Through antagonistic A₁R-D₁R and A_{2A}R-D₂R interactions, adenosine can reduce post-synaptic D₁R-mediated activation of direct pathway and D₂R-mediated inactivation of indirect pathway neurons in the dorsal striatum [17-21]. Resulting changes to dorsal striatum A₁R, A_{2A}R, D₁R, or D₂R mRNA following running may thereby be related to differential stimulation of dynorphin-expressing direct and enkephalin-expressing indirect pathway neurons during exposure to a stressor that has been suggested to increase adenosine and dopamine activity [35, 68]. Direct and indirect pathways have been suggested to serve opposing roles for processing reward [69], with increased direct pathway activity promoting rewarding and indirect pathway activity signaling aversive experiences [70]. Therefore, potential changes to net activity in direct or indirect pathway neurons during tail shock exposure in running rats could play a role in signaling the degree of reward or aversion experienced during a stressful event, and thus have important implications for stress buffering properties of exercise [69, 71].

We report that 6 weeks of wheel running reduced A₁R and A_{2A}R mRNAs in the rat dorsal and ventral striatum. Wheel running had no impact on D₁R mRNA levels, but modestly increased D₂R mRNA striatum sub-regions. Finally, *cfos* mRNA induction was potentiated in direct pathway and attenuated in indirect pathway neurons of running rats following tail shock. These data are consistent with a reduced efficacy of adenosine-mediated inhibition of

dopamine signaling which could be related to observed reductions of A₁R and A_{2A}R mRNA in medium spiny neurons of exercising animals. Taken together, these data suggest the striatum adenosine system may be a target for neuroplasticity from exercise. Therefore, these findings may have implications for striatum-mediated motor and cognitive processes, as well as exercise facilitated stress-resistance.

2. Materials and methods

2.1 Subjects and husbandry

Upon arrival, 32 male Fischer344 rats (obtained from Harlan SPF, Indianapolis, IN, USA) weighing approximately 148g were individually housed in standard laboratory cages (45×25.2×14.7 cm) (n=16) or cages with locked running wheels (45×25.2×14.7 cm) (n=16). Although stress induced by single housing could potentially impact our dependent measures, rats were single housed in order to accurately monitor individual running distance. Moreover, evidence suggests corticosterone is equivalently low in single-housed sedentary and running rats (see results section 3.2 & [72]), and single housing during adulthood does not exacerbate the development of the depression- and anxiety-like behavioral consequences of uncontrollable tail shock [73]. After 1 week, running wheels were unlocked and rats received free access to wheels for the 6 week remainder of the study. Daily wheel revolutions were recorded digitally using Vital View software (Mini Mitter, Bend, OR, USA). Rooms were controlled for temperature (21 ± 1°C) and photo-period (12:12 L:D) for the entire study. Food (Harlan Teklad 7012) and water were provided *ad libitum*. All procedures were approved by the University of Colorado Institutional Animal Care and Use Committee and adhered to NIH guidelines. Special care was taken to minimize animal discomfort during all procedures.

2.2 Uncontrollable tail shock

After 6 weeks of running or sedentary conditions, rats were randomly assigned to either receive uncontrollable tail shocks (n=8 Sedentary Stress, n=8 Running Stress) or no stress (n=8 Sedentary Control; n=8 Running Control). Approximately 2-5 hours into the light cycle, rats that received tail shocks were restrained in a Plexiglas tube with the tail protruding from the back where electrodes were placed to deliver 100, 5-s tail shocks on a variable 1 min inter-shock interval. Rats received 1.0mA tail shocks for 50 mins. Shock intensity was then increased to 1.5mA for the remainder of the session. Uncontrollable tail shock is a well characterized stressor that produces depression- and anxiety-like behaviors after a single episode [74]. Moreover, evidence indicates depression-like behaviors following uncontrollable tail shock involves a *particularly* augmented adenosine signaling in the brain [36, 37, 75], and can be prevented by 6 weeks of running [38, 39]. Control rats that did not receive stress were left undisturbed in home cages.

2.3 Tissue preparation

All rats were euthanized immediately after the termination of the final tail shock (between 4-7 hours into the light cycle) in pairs (one rat from the Stress and Control groups, alternating exercise condition) by rapid decapitation. Trunk blood was collected in EDTA tubes (Greiner Vacuette, Monroe, NC) and spun for 15 min. at 3000 g in a refrigerated

centrifuge (4 °C). Plasma supernatant was removed and stored at –80°C until processing for corticosterone ELISA. Brains were rapidly extracted, frozen in chilled isopentene (–20°C) for 4 mins, and stored at –80°C until sectioning. Brains were sliced through the rostral to caudal extent of the striatum at 10µm coronal sections using a cryostat. Slices were thaw-mounted directly on to Superfrost Plus slides (Fisherbrand, Pittsburgh, PA) and stored at –80°C until processing for radioactive or dual label fluorescent *in situ* hybridization.

2.4 Corticosterone ELISA

Plasma corticosterone was measured using a commercially available ELISA (Enzo Life Sciences, Farmingdale, NY) in accordance with the provided instructions.

2.5 Radioactive *in situ* hybridization

Radioactive *in situ* hybridization was used to detect relative group differences in striatal A₁R, A_{2A}R, D₁R, or D₂R mRNA. Radioactive *in situ* hybridization followed our previously published protocols [11]. Briefly, on day 1, a 1-in-20 series (separated by 200 µm) of sections containing the striatum were washed in 4% paraformaldehyde for 1h, 0.1 M triethanolamine with 0.25% acetic anhydride for 10 min, and then dehydrated in graded ethanol. Complementary (c)RNA riboprobes to A₁R (743 bp, 127-869 coding region), A_{2A}R (949 bp, 1316-2264 coding region), D₁R (460 bp, 383-843 coding region), and D₂R (495 bp, 1174-1665 coding region) 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), 50mM Phosphate Buffer (PBS) at pH 7.4, 1x Denhardt's solution, and 0.1 mg/ml yeast tRNA. Slides containing sections of the striatum were hybridized with the respective probe for approximately 18h at 55°C. On day 2, sections were washed in 2x SSC, treated in RNaseA (200 µg/ml) for 1 h 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 h. Sections were then dehydrated in graded ethanol, air dried for approximately 40 min. Finally, slides were placed into X-ray film cassettes and covered with X-ray film (Biomax-MR; Eastman Kodak, Rochester, NY, USA) for 3 days (for A_{2A}R, D₁, D₂) or 7 days (for A₁R). For individual probes (A₁R, A_{2A}R, D₁, or D₂), slides from all rats were processed together to allow for direct comparisons across experimental groups. Labeling with “sense” probes indicated that the signal observed with the “antisense” probes was specific.

2.6 Double fluorescent *in situ* hybridization (non-radioactive)

Double radioactive *in situ* hybridization was used to detect the proportion of dynorphin (direct pathway) and enkephalin (indirect pathway) containing neurons that co-express cfos (neural activation) in the dorsal striatum following exposure to uncontrollable tail shock. The protocol was similar to *radioactive in situ hybridization* except (c)RNA riboprobes for dynorphin (744 bp, 164-907 coding region) or enkephalin (558 bp, 493-1052 coding region) were labeled with fluorescein UTP (purchased from Roche, Indianapolis, IN) and cfos (574 bp, 596-1171 coding region) was labeled with digoxigenin UTP (purchased from Roche, Indianapolis, IN). Moreover, sections were not exposed to the final graded ethanol dehydration on day 2. Instead, immediately following the 0.1x SSC wash at 65°C, 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 0.05M PBS for 30 min, washed in 1x Tris-Buffered Saline containing Tween pH 7.5 (TBS-T), and incubated in 0.5% blocking buffer (Perkin-Elmer, Waltham, MA, USA) in 1x TBS for 1 h. Sections were immediately incubated in anti-digoxigenin-horseradish peroxidase (Perkin-Elmer, Waltham, MA, USA) at 1:750 in TBS-T for 30 mins. 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 0.05M PBS at 4°C. On day 3, sections were again quenched in 2% hydrogen peroxide in 0.05M PBS for 30 min. Sections were next washed in TBS-T and incubated for 90 min in anti-fluorescein-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 dilution in 1x amplification diluent (Perkin-Elmer, Waltham, MA, USA) for 1 h. Slides were then washed in PBS and air dried for approximately 30 min. Coverslips were set on slides using ProLong Gold antifade reagent with DAPI (Life Technologies, Grand Island, NY).

2.7 Image analysis for radioactive *in situ* hybridization

Levels of A₁R, A_{2A}R, D₁R, and D₂R were semi-quantitatively analyzed following our previously published protocols [11]. Briefly, images of brain sections on X-Ray film were digitally captured using camera (CCD camera, model XC-77; Sony, Tokyo, Japan) interfaced to a computer (see Figure 1). The relative optical density of the probe on each captured image was determined using Scion Image version 4.0 (Scion, Fredrick, MD, USA). For each section, a background sample was taken over an area of white matter (for A_{2A}R, D₁R, and D₂R) or off the section (for A₁R, as it is expressed in oligodendrocytes). Next, a sample was taken over the respective brain areas that include DMS, DLS, AcbC, AcbShM, or AcbShL (boundaries defined in Paxinos and Watson rat brain atlas). The signal threshold was calculated as mean gray value of background +3.5 SD. The section was automatically density-sliced at this value, so that only pixels with gray values above these criteria were included in the analyses. The results reflect the mean signal produced by the cRNA probe above the background multiplied by the number of pixels above the assigned background. Each rat's relative optical density for a particular cRNA probe was measured across rostral to caudal axis of the striatum containing 5 sections in the DLS and DMS (coordinates 2.2 to 1.7mm, 1.6 to 1mm, 0.2 to -0.3mm, -0.4 to 0.8mm, and -0.9 to -1.5mm from bregma), as well as 4 sections in the AcbC, AcbShM, and AcbShL (coordinates 2.2 to 2.0mm, 1.9 to 1.6mm, 1.5 to 1.2mm, and 1.1 to 0.8mm from bregma).

2.8 Image analysis for double fluorescent *in situ* hybridization (non-radioactive)

Images were acquired from a Zeiss AX10 with axioscan Z1 fluorescent microscope interfaced to a computer running axiovision software (Zeiss, Oberkochen, Germany) at 200x total magnification. Fluorescein (dynorphin or enkephalin), Cy3 (cfos), and DAPI (nuclei) emission channels were merged to create a single image. For each rat, two non-overlapping adjacent images were acquired in the DMS and DLS over 3 sections across the rostral to caudal axis of the striatum (coordinates between 2.0 to 1.8mm, 1.2 to 1.0mm, and 0 to -0.2mm from bregma). Images were taken unilaterally alternating right and left striatum for

each rat. Each image captured the entire z-plane of a striatum. The number of cfos, enkephalin or dynorphin, and co-labeled (enkephalin/cfos or dynorphin/cfos) neurons were counted in each image by an individual blind to rat group assignments. A co-label was confirmed when a dynorphin- or enkephalin-expressing neuron near completely covered and had a similar morphology to a cfos-reactive cell.

2.9 Data analysis

Overall distance run was compared between Running Control and Running Stress groups using Student's *t*-test. Serum corticosterone concentrations were compared by a 2-way ANOVA with exercise (Sedentary vs. Running) and stress conditions (Control vs. Stress) as factors. Trends in A₁R, A_{2A}R, D₁R, and D₂R mRNA expression were generally consistent across the rostral to caudal extent of each striatal area. Therefore, relative optical density for A₁R, A_{2A}R, D₁R, and D₂R mRNA levels were reported as an average across analyzed rostral to caudal sections for each brain area (DMS, DLS, AcbC, AcbShM, AcbShL) and compared by a 2-way ANOVA with exercise and stress condition as factors. Relative optical density for each rostral to caudal sub region analyzed (as previously described in the methods) in dorsal and ventral striatum structures were also compared using 2-way ANOVAs with exercise and stress conditions as factors. These data are included in supplementary tables 1-5. A small number of sections contained damage over the region of interest and were subsequently removed from analysis. Moreover, 1-2 of the rats in each group were missing sections from the most rostral level of the nucleus accumbens (2.2 to 2.0 mm from bregma). However, each group maintained at least a sample size of 6 for comparison at every level of the striatum. Discrepancies in sample sizes are noted in the degrees of freedom in supplementary tables.

The densities of cfos positive cells, as well as the proportions of enkephalin or dynorphin neurons that co-expressed cfos in the dorsal striatum were compared by a 2-Way ANOVA with exercise and stress condition as factors. For ANOVAs, *post hoc* analyses using Fisher projected least significant difference (PLSD) corrections were completed when appropriate. For all analyses $P < 0.05$ was considered statistically significant.

3. Results

3.1 Wheel running

Daily wheel running distance increased steadily for the first 3 weeks and thereafter maintained a plateau averaging 4.49 km/day (± 0.09 SE) the final 3 weeks. Average distance run during the entire experiment was 3.66 km/day (± 0.20 SE). Average distance traveled on wheels did not differ between Running Control and Running Stress groups.

3.2 Corticosterone concentrations

Exposure to uncontrollable tail shock potently increased corticosterone concentrations in both sedentary and running conditions [$F(1,28)=351.8, P<0.0001$]. No effect of exercise condition or significant interaction between exercise and stress condition was observed. Average corticosterone concentrations were 1.9 ug/dl (± 0.4 SE) for Sedentary Control, 1.4

ug/dl (± 0.4 SE) for Running Control, 61.4 ug/dl (± 1.8 SE) for Sedentary Stress, and 72.3 (± 6.5 SE) ug/dl for Running Stress groups.

3.3 Dorsal Striatum

3.3.1 A₁R Gene Expression—In the DMS, exercise decreased A₁R mRNA levels [$F(1,28)=25.5$, $P<0.0001$](Figure 2A). A significant interaction was observed between exercise and stress conditions [$F(1,28)=5.9$, $P=0.02$]. *Post hoc* analysis revealed that exposure to stress decreased A₁R mRNA in sedentary rats ($P=0.007$). Moreover, Sedentary Control maintained greater A₁R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups. Additionally, Sedentary Stress had a greater A₁R mRNA expression than the Running Control group ($P=0.03$).

In the DLS, exercise decreased A₁R mRNA levels [$F(1,28)=23.0$, $P<0.0001$](Figure 2B). A significant interaction was found between exercise and stress conditions [$F(1,28)=5.8$, $P=0.02$]. *Post hoc* analysis revealed that exposure to stress decreased A₁R mRNA in sedentary rats ($P=0.003$). Sedentary Control maintained greater A₁R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups.

3.3.2 A_{2A}R Gene Expression—In the DMS, exercise decreased A_{2A}R mRNA levels [$F(1,28)=94.5$, $P<0.0001$](Figure 2A). A significant interaction was observed between exercise and stress conditions [$F(1,28)=28.4$, $P<0.0001$]. *Post hoc* analysis revealed that exposure to stress decreased A_{2A}R mRNA in sedentary rats ($P<0.0001$). Moreover, Sedentary Control displayed greater A_{2A}R mRNA expression than Running Control ($P<0.0001$) and Running stress ($P<0.0001$) groups. In addition, Sedentary Stress had a greater A_{2A}R mRNA expression than Running Control ($P=0.04$) and Running Stress ($P=0.004$) groups.

In the DLS, exercise decreased A_{2A}R mRNA expression [$F(1,28)=111.3$, $P<0.0001$](Figure 2B). A significant interaction was observed between exercise and stress conditions [$F(1,28)=29.5$, $P<0.0001$]. *Post hoc* analysis revealed that exposure to stress decreased A_{2A}R mRNA in sedentary rats ($P<0.0001$). Sedentary Control had greater A_{2A}R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups. Moreover, Sedentary Stress rats had more A_{2A}R mRNA than Running Control ($P=0.009$) and Running Stress ($P=0.001$) groups.

3.3.3 D₁R Gene Expression—No significant effects of exercise, stress, or interaction between exercise and stress were observed for D₁R mRNA in the DMS or DLS (Figure 2A & B).

3.3.4 D₂R Gene Expression—Exercise increased D₂R mRNA in the DMS [$F(1,28)=7.8$, $P=0.009$] (Figure 2A), as well as in the DLS [$F(1,28)=4.7$, $P=0.03$] (Figure 2B). No significant effect of stress or interaction between exercise and stress was observed.

Values and statistical analysis for A₁R, A_{2A}R, D₁R, and D₂R mRNA expression at each level of the DMS and DLS are located in supplementary tables 1 & 2.

3.4 Ventral Striatum

3.4.1 A₁R Gene Expression—In the AcbC, exercise decreased A₁R mRNA levels [$F(1,28)=24.4$, $P<0.0001$](Figure 3A). A significant interaction was observed between exercise and stress conditions [$F(1,28)=5.5$, $P=0.03$]. *Post hoc* analysis revealed that exposure to stress decreased A₁R mRNA in sedentary rats ($P=0.007$). Sedentary Control had greater A₁R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups. Moreover, Sedentary Stress rats had greater A₁R mRNA expression than Running Control rats ($P=0.03$).

In the AcbShM, exercise decreased A₁R mRNA levels [$F(1,28)=36.1$, $P<0.0001$](Figure 3B). A significant interaction was found between exercise and stress conditions [$F(1,28)=6.6$, $P=0.02$]. *Post hoc* analysis revealed that exposure to stress decreased A₁R mRNA in sedentary rats ($P=0.002$). Sedentary Control had greater A₁R mRNA expression than Running Control ($P<0.0001$), and Running Stress ($P<0.0001$) groups. Moreover, Sedentary Stress rats had greater A₁R mRNA expression than Running Control ($P=0.01$) and Running Stress ($P=0.02$) groups.

In the AcbShL, exercise decreased A₁R mRNA levels [$F(1,28)=36.8$, $P<0.0001$](Figure 3C). A significant interaction was found between exercise and stress conditions [$F(1,28)=7.3$, $P=0.01$]. *Post hoc* analysis revealed that exposure to stress decreased A₁R mRNA in sedentary rats ($P<0.0001$). Sedentary Control had greater A₁R mRNA expression than Running Control ($P<0.0001$), and Running IS ($P<0.0001$) groups. Moreover, Sedentary Stress had greater A₁R mRNA expression than Running Control ($P=0.02$) and Running Stress ($P=0.02$) groups.

3.4.2 A_{2A}R Gene Expression—In the AcbC, exercise decreased A_{2A}R mRNA levels [$F(1,28)=111.8$, $P<0.0001$](Figure 3A). A significant interaction was observed between exercise and stress conditions [$F(1,28)=19.2$, $P=0.0001$]. *Post hoc* analysis revealed that exposure to stress decreased A_{2A}R mRNA in sedentary rats ($P<0.0001$). Sedentary Control maintained greater A_{2A}R mRNA expression than Running Control ($P<0.0001$) and Running IS ($P<0.0001$) groups. Sedentary Stress had more A_{2A}R mRNA than Running Stress rats ($P=0.002$). Additionally, Running Control had significantly greater expression A_{2A}R mRNA than Running Stress rats ($P=0.02$).

In the AcbShM, exercise decreased A_{2A}R mRNA levels [$F(1,28)=68.5$, $P<0.0001$](Figure 3B). A significant interaction was observed between exercise and stress conditions [$F(1,28)=27.0$, $P<0.0001$]. *Post hoc* analysis revealed that exposure to stress decreased A_{2A}R mRNA in sedentary rats ($P<0.0001$). Sedentary Control maintained greater A_{2A}R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups. Sedentary Stress rats had more A_{2A}R mRNA than Running Stress rats ($P=0.04$). Moreover, Running Control had greater mRNA expression than Running Stress rats ($P=0.04$).

In the AcbShL, exercise decreased A_{2A}R mRNA levels [$F(1,28)=78.6$, $P<0.0001$](Figure 3C). A significant interaction was observed between exercise and stress conditions [$F(1,28)=24.7$, $P<0.0001$]. *Post hoc* analysis revealed that exposure to stress decreased

A_{2A}R mRNA in sedentary rats ($P<0.0001$). Sedentary Control maintained greater A_{2A}R mRNA expression than Running Control ($P<0.0001$) and Running Stress ($P<0.0001$) groups. Moreover, Sedentary Stress rats had greater A_{2A}R mRNA expression than Running Control ($P=0.04$) and Running Stress ($P=0.01$) groups.

3.4.3 D₁R Gene Expression—No significant effect of exercise, stress, or interaction between exercise and stress was observed for D₁R mRNA in the AcbC, AcbShM, and AcbShL (Figure 3A-C).

3.4.4 D₂R Gene Expression—Exercise modestly increased D₂R mRNA [$F(1,28)=9.4$, $P=0.005$] in the AcbShM (Figure 3B), as well as in the AcbShL [$F(1,28)=11.8$, $P=0.002$] (Figure 3C). No significant effect of stress or interaction between exercise and stress was found in AcbC, AcbShM, or AcbShL.

Values and statistical analysis for A₁R, A_{2A}R, D₁R, and D₂R mRNA expression at each level of the AcbC, AcbShM, and AcbShL are located in supplementary tables 3-5.

3.5 Fluorescent *in situ* Hybridization

3.5.1 cfos mRNA Induction in the Dorsal Striatum Following Acute Stress—No statistically significant differences were observed for density of cfos mRNA between DLS and DMS in any group. Moreover, cfos induction did not differ through the rostral to caudal striatum. Therefore, cfos density was averaged across the DLS and DMS at all levels (see Figure 4A). Exposure to stress caused a marked increase in cfos induction in the dorsal striatum [$F(1,28)=268.0$, $P<0.0001$] (Figure 4B). No effect of exercise or interaction between exercise and stress condition was observed.

3.5.2 Effect of Acute Stress on cfos Induction in Dynorphin Neurons in the Dorsal Striatum (see Figure 4C)—The proportion of dynorphin neurons that co-expressed cfos did not differ between the DMS and DLS. Moreover, proportion of dynorphin neurons expressing cfos was similar throughout sections containing rostral to caudal striatum within each group. Therefore, proportion of dynorphin neurons that co-expressed cfos were averaged across the DLS and DMS at all levels. Stress increased the proportion of dynorphin neurons that expressed cfos [$F(1,28)=193.2$, $P<0.0001$] (Figure 4D). Moreover, a statistically significant interaction as observed between stress and exercise condition [$F(1,28)=7.4$, $P=0.01$] (Figure 4D). *Post hoc* analysis revealed stress-induced cfos expression in dynorphin neurons was augmented in running rats ($P=0.0003$).

3.5.3 Activation of Enkephalin Neurons in the Dorsal Striatum from Acute Stress (see Figure 4E)—The proportion of enkephalin neurons that expressed cfos did not differ between the DMS and DLS. Moreover, proportion of enkephalin and cfos double labeled neurons were similar throughout sections containing rostral to caudal striatum within each group. Therefore, the proportion of enkephalin cells that co-expressed cfos were averaged across the DLS and DMS at all levels for each group. Stress increased the proportion of enkephalin neurons that expressed cfos [$F(1,28)=162.9$, $P<0.0001$] (Figure 4F). A statistically significant interaction was observed between stress and exercise

conditions [$F(1,28)=5.7$, $P=0.02$] (Figure 4F). *Post hoc* analysis revealed stress-induced *cfos* expression in enkephalin neurons was reduced in running rats ($P=0.01$).

4. Discussion

The results of the current study show long-term wheel running and acute uncontrollable tail shock decreased striatal A₁R and A_{2A}R mRNA levels across the DMS, DLS, AcbC, AcbShM, and AcbShL (see Figure 2 & 3). Acute uncontrollable tail shock exposure further reduced A_{2A}R mRNA levels in the AcbC and AcbShM in running rats (see Figure 3A & B). D₂R mRNA was modestly increased in striatum sub-regions of running rats (see Figure 2 & 3). Neither running nor stress affected striatal D₁R mRNA expression (see Figure 2 & 3). Changes in A₁R, A_{2A}R, and D₂R gene expression in the dorsal striatum from running were associated with greater stress-induced *cfos* induction in dynorphin expressing “direct pathway” neurons and attenuated stress-induced *cfos* induction in enkephalin expressing “indirect pathway” neurons (see Figure 4D & F). Taken together, these data provide novel evidence indicating running alters striatum A₁R and A_{2A}R mRNA levels, which may affect adenosine-mediated antagonism of dopamine signaling in the striatum. These data suggest the striatum adenosine system may be a target for neuroplasticity following exercise, which may have significance for several behavioral and cognitive processes that involve the striatum.

Changes to A₁R and A_{2A}R mRNA levels are commonly associated with similar changes to protein or radioligand binding [40-56]. If reported decreases of A₁R and A_{2A}R gene expression from running translate to functional reductions in protein, these data may have implications for both normal striatum function and potentially the prevention or treatment of disorders that involve abnormal dopamine signaling. Indeed, A₁R and A_{2A}R are now recognized as major modulators of striatum function. Their relatively high degree of expression in the striatum, predominate post-synaptic location on medium spiny neurons, and antagonistic interaction with dopamine receptors are consistent with a role in motor control, instrumental learning, habit learning, working memory, reward, and motivation [76-85]. Thus, potential changes to A₁R and A_{2A}R expression, related to reduced mRNA from running, may influence a number of striatum-involved cognitive modalities. In addition to normal physiological processes, the A₁R and A_{2A}R may serve as novel targets to treat pathologies associated with striatum dysfunction (as reviewed in [86]). Striatal dopamine receptors have been widely studied as targets for therapeutic treatment of drug abuse, depression, schizophrenia, cognitive dysfunction, and motor control disorders (e.g. Parkinson's and Huntington's Disease) with mixed efficacy. Due to their ability to reduce dopamine signaling, adenosine receptors have provided a new direction in the search for novel drugs to treat these disorders [87-95]. In particular, A_{2A}R antagonists have entered clinical trials for treatment of motor degenerative Parkinson's disease, due to its ability to stimulate motor enhancement with a reduced propensity to cause dyskinesia compared to dopamine agents [96]. Given the potential for A_{2A}R antagonist treatment of Parkinson's disease, the reported decrease of A_{2A}R mRNA in the striatum (see Figure 2 & 3) could contribute to delayed onset of Parkinson's disease symptoms in individuals that exercise (as reviewed in [97]).

In addition to motor control, data from pre-clinical work suggest striatal A₁R and A_{2A}R may represent novel targets to treat mental health disorders like drug abuse or depression. Therefore, decreased striatum adenosine receptor mRNA levels from exercise could be related to reductions in the abusive properties of certain drugs and protect against incidences of depression. Indeed, A_{2A}R antagonism or knockdown has been reported to suppress motivation and drug seeking for morphine in rodents [98-100]. Therefore, reduced A_{2A}R mRNA levels in wheel running rats (see Figure 2 & 3) may be related to protection from uncontrollable tail shock-induced enhancement of morphine preference in rats [12]. Moreover, the exercise-induced decrease in A_{2A}R mRNA could be relevant for reduced abusive properties of other drugs, as A_{2A}R knockdown or blockade has also been reported to attenuate the reinforcing properties of nicotine and cannabinoid receptor stimulation [101, 102]. Finally, striatum adenosine receptors may be involved in the expression of behavioral despair [34, 92, 93, 103], as well as the development of depression-like behavior that follows uncontrollable stress [33, 35-37]. Indeed, A_{2A}R knockdown or antagonist treatment has been reported to increase rodent mobility on forced swim and tail suspension tests [92, 93], as well as prevent depression-like behavior that develops following reserpine treatment [34]. Furthermore, blockade of brain adenosine receptors has been reported to prevent the development of depression-like shuttle box escape deficits following uncontrollable tail shock [33, 35-37] that are known to involve the striatum [13]. Therefore, reduced adenosine receptor mRNA levels in the striatum following wheel running (see Figure 2 & 3) may contribute to the stress- buffering properties of exercise [38]. Future analyses are required to determine if reported decreases of A₁R and A_{2A}R mRNA from wheel running are translated to functional reduction in receptors, as these changes could have significance for striatum function and dysfunction.

The mechanism by which wheel running reduces A₁R and A_{2A}R mRNA is unknown; however, repeated or excessive ligand binding during exercise could contribute to the observed reduction in A₁R and A_{2A}R mRNA levels. Indeed, prior work indicates that A₁R or A_{2A}R activation can lead to changes in receptor mRNA both *in vitro* and *in vivo*, although inconsistencies in the literature exist in the direction and magnitude of such changes [24-29, 104, 105]. Moreover, neither of these receptor mRNAs have been well characterized in the brain following *in vivo* exposure to agonists, as only one report exists, to the best of our knowledge, suggesting chronic A₁R activation with the agonist R-PIA reduces mRNA and surface expression of A₁R in the brain *in vivo* [26]. However, prolonged exposure to agonists of other brain G-protein coupled receptors, like A₁R and A_{2A}R, commonly result in decreased receptor mRNA and protein (as reviewed in [106]). Multiple lines of evidence suggest wheel running can increase adenosine activity in the striatum, which may have contributed to the observed reduction of A₁R and A_{2A}R mRNA levels. Adenosine is released into the extracellular space when the rate of adenosine triphosphate (ATP) hydrolysis exceeds synthesis. Thus, adenosine concentrations increase as a function of cellular workload (as reviewed in [16]). Exercise promotes activity-dependent increases of ATP utilization and adenosine concentrations in both peripheral and central tissues [30, 107-109]. However, the source of brain adenosine from exercise that may contribute to altered A₁R and A_{2A}R mRNA expression is not well understood. One possibility is that adenosine becomes released into extracellular space during brain area-specific increases in

energy demand. Previous work has shown that bouts of wheel and treadmill running potentially increase neural activity and markers of metabolic demand in the striatum [8, 31, 32, 110-112], which may be sufficient to increase local adenosine levels. Consistent with this idea, extracellular adenosine concentrations measured by microdialysis are slightly elevated in the rat striatum across the dark-cycle, a period of increased physical activity, suggesting even moderate increases of locomotor activity can augment striatum adenosine signaling [113]. In addition, adenosine can cross the blood brain barrier [114, 115]. Therefore, peripheral increases of adenosine during running could also contribute to the altered receptor mRNA levels by elevating brain-wide adenosine concentrations [30]. Taken together, these data suggest that repeated increases of local or brain-wide adenosine levels during running may be a contributing factor to reduced A₁R and A_{2A}R mRNA levels, and could be a topic for future investigations.

The potential factors contributing to and significance of the rapidly reduced striatum adenosine receptor mRNA immediately following uncontrollable tail shock is more difficult to hypothesize (see Figures 2 & 3). It is possible that rapid and excessive increases of adenosine in the striatum related to increased neural activity during stress could be a factor contributing to decreased receptor mRNAs (see Figure 4B) [35], as levels of mRNA coding for the adenosine receptors can become altered within hours following agonist binding in cell culture models [25, 27]. However, this effect has not been well characterized *in vivo*. Other factors related to uncontrollable tail shock such as increases of corticosterone (see results section 3.2) [40] and proinflammatory cytokines [116] likely do not contribute to observed reductions because evidence suggests these factors increase, not decrease adenosine receptor mRNA [54, 117, 118]. Therefore, factors contributing to rapid reductions of both adenosine receptor mRNAs following uncontrollable stress requires further investigation. Additionally, stress has been reported to up-regulate A_{2A}R in the striatum, which is related to the development of depression-like behaviors in rats [103]. Therefore, rapid changes to striatum adenosine receptor mRNA observed immediately following tail shock may not be representative of later time points. Other G-protein coupled receptors that display altered mRNA levels immediately following uncontrollable tail shock return to baseline levels within hours [119], or can continue to change expression days later [73]. It is possible that A₁R or A_{2A}R mRNAs may also display differing patterns of expression overtime. The stability of reported adenosine receptor mRNA reductions immediately following uncontrollable stress also requires further exploration in order to better understand how these changes may influence striatum function.

Factors contributing to the activation of medium spiny neurons comprising the direct and indirect pathways are complex, and involve coordinated interactions between several neurotransmitter systems that act both pre- and post-synaptically. However, several lines of evidence suggest potentiated stress-induced *cfos* in dynorphin-expressing direct pathway and attenuated stress-induced *cfos* in enkephalin-expressing indirect pathway neurons of physically active rats (see Figure 4D & F) are consistent with observed changes to A₁R, A_{2A}R, and D₂R gene expression (see Figure 2). Evidence suggests uncontrollable tail shock can increase adenosine and dopamine signaling in the striatum [33, 35-37, 68]; therefore, potential exercise-induced changes to adenosine or dopamine receptor expression, related

altered mRNA, could lead to the observed differences in stress-induced activation of direct and indirect pathway neurons between running and sedentary rats. Indeed, A_{2A}R and D₂R mRNA is nearly exclusively found in enkephalin-expressing striatum indirect pathway neurons, where these receptors are predominately located on dendrites and cell bodies [18, 67]. A_{2A}R activation decreases the binding affinity and function of D₂R in indirect pathway neurons [120, 121]. Therefore, potential reductions of A_{2A}R and greater D₂R expression [122-124], associated with observed changes to receptor mRNA, in the striatum of physically active rats could lead to a greater inhibitory influence on indirect pathway neurons during tail shock. Furthermore, adenosine can reduce dopamine-mediated activation of direct pathway neurons through post-synaptic A₁R-D₁R heterodimer interactions [19, 125, 126]. Therefore, decreased A₁R mediated inhibition of D₁R could lead to a greater excitatory influence on direct pathway neurons. Together, observations from the literature along with reported changes in adenosine and dopamine receptor mRNA levels are consistent with a reduced efficacy of adenosine-mediated inhibition of dopamine signaling in medium spiny neurons of exercising animals, thus resulting in potentiated direct pathway and attenuated indirect pathway activation during stress in physically active rats.

This interpretation, albeit consistent with our data, requires caution. A₁R mRNA expression has been reported in direct and indirect pathway neurons, and presumably both A₁R and A_{2A}R mRNA are located in striatum cholinergic interneurons [127]. Therefore, the sources contributing to observed decreases in adenosine receptor mRNAs are not clear. However, given the small proportion of striatum neurons that are estimated to be cholinergic (~0.4%) [128], this source would likely only contribute modestly to the large decreases in adenosine receptor mRNA observed in this study (see Figures 2 & 3). Furthermore, exposure to uncontrollable tail shock also rapidly reduced A₁R and A_{2A}R mRNA (see Figure 2), suggesting that functional differences in protein level could be minimal between running and sedentary rats following tail shock. However, loss of G-protein coupled receptors, like A₁R and A_{2A}R, that are associated with reductions of mRNA normally occurs in response to a stimulus treatment for several hours or longer (as reviewed in [106, 129-131]). Therefore, it is unlikely that the reductions in mRNA levels observed immediately after stress would also be reflected in a change in protein at this time point, whereas potential protein changes in exercising rats would have been in place prior to stress. Finally, activation of the direct pathway and indirect pathways has been shown to promote and inhibit movement respectively [69]. If running rats struggled to a greater degree in restraint tubes during tail shock than sedentary rats, this could be an alternative source for the observed changes in patterns of *cfos* induction in direct and indirect pathways. However, we have observed no difference in movement between sedentary and running rats during uncontrollable tail shock using accurate biotelemetric recording (*unpublished data*). Therefore, this alternative explanation seems unlikely. While it is difficult to make strong conclusions from the current data that altered striatum A₁R, A_{2A}R, or D₂R gene expression from running contributes to increased direct and decreased indirect pathway activation during tail shock, the data are consistent with this hypothesis and should be explored in greater detail by future studies.

Recent evidence has emerged suggesting excitation of direct and indirect pathway neurons in the striatum serve opposing roles for reinforcement learning, which may have significance

for stress-buffering properties of exercise. Data from studies using gene modification combined with rodent behavior suggest activation of striatum direct and indirect pathway neurons can contribute to reward and aversion, respectively [70, 132]. Differences in the amount of direct and indirect pathway activation; therefore, may be important for varying expression of hedonic states during exposure to rewarding or noxious stimuli [69]. In light of recent findings, the current observation of greater activation of the direct pathway and attenuated activation of the indirect pathway during stress in physically active, compared to sedentary, rats suggests that uncontrollable stress could be a more aversive experience for sedentary compared to running rats. Consistent with this idea, selective induction of the chronic activity marker FosB in direct and indirect pathway neurons are, respectively, related to stress-resistance and -susceptibility in mice that were previously exposed to repeated social defeat [71]. Together, these data raise the interesting possibility that exercise-induced neuroplasticity in the striatum may facilitate a shift from aversion-related to reward-related neural activation during exposure to stressful events. Such changes in neural activity could play a role in exercise-induced stress resistance and resilience, and should be topics for further investigation.

Conclusion

In conclusion, the current data provide novel evidence that the adenosine system in the striatum may be sensitive to physical activity status and acute uncontrollable stress. The observed reduction in A₁R and A_{2A}R mRNA levels following exercise could potentially contribute to reduced efficacy of adenosine-mediated inhibition of dopamine signaling in the striatum. Indeed, the observed changes to direct and indirect pathway neuron activation during uncontrollable stress in running rats are compatible with this hypothesis. These findings could have important implications for striatum function, as well as the prevention and treatment of mental health and motor pathologies involving the striatum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Long-term wheel running and acute uncontrollable stress decrease adenosine 1 and 2A receptor mRNA levels in the dorsal and ventral striatum

Wheel running modestly increases dopamine 2 receptor mRNA levels in the dorsal and ventral striatum

Long-term running potentiates stress-induced cfos in direct pathway and attenuates stress-induced cfos in indirect pathway neurons of the dorsal striatum

Figure 1.

Radioactive *in situ* hybridization in the striatum. Representative autoradiographs showing *in situ* hybridization for A) A₁R, B) A_{2A}R, C) D₁R, and D) D₂R of Sedentary Control (top left), Sedentary Stress (top right), Running Control (bottom left), and Running Stress (bottom right) in the (a.) dorsal medial striatum, (b.) dorsal lateral striatum, (c.) nucleus accumbens core, (d.) medial shell of the nucleus accumbens, and (e.) lateral shell of the nucleus accumbens.

Figure 2.

A₁R, A_{2A}R, D₁R, and D₂R gene expression in the dorsal striatum following stress and exercise. Relative levels of A₁R, A_{2A}R, D₁R, and D₂R messenger ribonucleic acid (mRNA) averaged across five sections (coordinates 2.2 to 1.7mm, 1.6 to 1mm, 0.2 to -0.3mm, -0.4 to 0.8mm, and -0.9 to -1.5mm from bregma) of the A) Dorsal Medial Striatum and B) Dorsal Lateral Striatum. Please note, the presented data are not normalized across receptors, as *in situ* hybridization for each receptor-type was completed on different days (see Methods for details). ** $P < 0.001$ from Running Control, Sedentary Stress, and Running Control within respective receptor, ^ $P < 0.05$ from Sedentary Stress within respective receptor, # $P < 0.05$ main effect of exercise within respective receptor

Figure 3.

A₁R, A_{2A}R, D₁R, and D₂R gene expression in the ventral striatum following stress and exercise. Relative levels of A₁R, A_{2A}R, D₁R, and D₂R messenger ribonucleic acid (mRNA) averaged across four sections (coordinates 2.2 to 2.0mm, 1.9 to 1.6mm, 1.5 to 1.2mm, and 1.1 to 0.8mm from bregma) of the A) Nucleus Accumbens Core, B) Medial Shell of the Nucleus Accumbens, and C) Lateral Shell of the Nucleus Accumbens. Please note, the presented data are not normalized across receptors, as *in situ* hybridization for each receptor-type was completed on different days (see Methods for details). * $P < 0.01$ from Running

Control, Sedentary Stress, and Running Control within respective receptor $**P<0.001$ from Running Control, Sedentary Stress, and Running Control within respective receptor; $^{\wedge}P<0.05$ from Sedentary Stress within respective receptor, $\sim P<0.05$ from Running control within respective receptor; $P=0.05$ main effect of exercise within respective receptor

Figure 4.

cfos induction in direct and indirect pathway neurons of the dorsal striatum. A) Approximate location of images capturing the dorsal lateral and dorsal medial striatum used for analysis. Pictures modified from the Paxinos and Watson rat brain atlas. B) Number of *cfos* expressing neurons in the dorsal striatum per square mm from uncontrollable tail shock stress in sedentary and running rats. C) Representative coronal section of the dorsal striatum stained for dynorphin mRNA (green), *cfos* mRNA (red), and DAPI (blue). The white box contains a zoomed in image of a dynorphin-positive cell within the section co-expressing

cfos. D.) The proportion of dynorphin-positive cells co-expressing cfos in the dorsal striatum following uncontrollable tail shock stress in sedentary and running rats. E. Representative coronal section of the dorsal striatum stained for enkephalin mRNA (green), cfos mRNA (red), and DAPI (blue). The white box contains a zoomed in image of an enkephalin-positive cell within the section co-expressing cfos. F) The proportion of enkephalin-positive cells co-expressing cfos in the dorsal striatum following uncontrollable tail shock stress in sedentary and running rats. ^{##} $P < 0.0001$ main effect of stress; * $P < 0.05$ or ** $P < 0.001$ from Running Stress