The Journal of PHARMACOLOGY And Experimental Therapeutics

Age- and Sex-Specific Plasticity in Dopamine Transporter Function Revealed by Food Restriction and Exercise in a Rat Activity-Based Anorexia Paradigm[S]

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Received June 13, 2019; accepted August 21, 2019

ABSTRACT

Eating disorders such as anorexia typically emerge during adolescence, are characterized by engagement in compulsive and detrimental behaviors, and are often comorbid with neuropsychiatric disorders and drug abuse. No effective treatments exist. Moreover, anorexia lacks adolescent animal models, contributing to a poor understanding of underlying age-specific neurophysiological disruptions. To evaluate the contribution of dopaminergic signaling to the emergence of anorexia-related behaviors during the vulnerable adolescent period, we applied an established adult activity-based anorexia (ABA) paradigm (food restriction plus unlimited exercise access for 4 to 5 days) to adult and adolescent rats of both sexes. At the end of the paradigm, measures of plasma volume, blood hormone levels, dopamine transporter (DAT) expression and function, acute cocaine-induced locomotion, and brain water weight were taken. Adolescents were dramatically more affected by the ABA paradigm than adults in all measures. In vivo chronoamperometry and cocaine locomotor responses revealed sex-specific changes in adolescent DAT function after ABA that were independent of DAT expression differences. Hematocrit, insulin, ghrelin, and corticosterone levels did not resemble shifts typically observed in patients with anorexia, though decreases in leptin levels aligned with human reports. These findings are the first to suggest that food restriction in conjunction with excessive exercise sex-dependently and age-specifically modulate DAT functional plasticity during adolescence. The adolescent vulnerability to this relatively short manipulation, combined with blood measures, evidence need for an optimized age-appropriate ABA paradigm with greater face and predictive validity for the study of the pathophysiology and treatment of anorexia.

SIGNIFICANCE STATEMENT

Adolescent rats exhibit a distinctive, sex-specific plasticity in dopamine transporter function and cocaine response after food restriction and exercise access; this plasticity is both absent in adults and not attributable to changes in dopamine transporter expression levels. These novel findings may help explain sex differences in vulnerability to eating disorders and drug abuse during adolescence.

Introduction

Eating disorders afflict ≥3% of individuals in developed countries, and disproportionately impact females over males by more than double (Smink et al., 2014; Keski-Rahkonen and Mustelin, 2016). These disorders, which include anorexia nervosa (AN), bulimia nervosa, and binge-eating disorder, most often emerge during adolescence (Herpertz-Dahlmann, 2015). Indeed, adolescence is characterized not only by an increased risk of eating disorders, but also other neuropsychiatric disorders, as well as drug abuse and similar risky or compulsive behaviors (Andersen, 2003; Chambers et al., 2003; Paus et al., 2008; Arain et al., 2013; Holder and Blaustein, 2014).

Adolescence coincides with a particularly sensitive maturation period for the dopaminergic system (Chambers et al., 2003; Casey et al., 2008; Ernst et al., 2009; Novick et al., 2011; Sinclair et al., 2014; Fuhrmann et al., 2015; Niwa et al., 2016). Dopamine (DA) is a catecholamine neurotransmitter

important for modulation of eating (Rolls et al., 1974; Yoshida et al., 1992; Volkow et al., 2003; Davis et al., 2009), motor activity (Andén et al., 1973; Baldo et al., 2002; Gallardo et al., 2014; Robertson et al., 2015; Felger and Treadway, 2017), emotion (Herman et al., 1982; Salgado-Pineda et al., 2005; Kienast et al., 2008; Belujon and Grace, 2015), impulsivity (van Gaalen et al., 2006; Buckholtz et al., 2010; Pine et al., 2010; Dalley and Robbins, 2017), and reward (Nestler and Carlezon, 2006; Johnson and Kenny, 2010; Eshel et al., 2016). Eating disorders, particularly AN and bulimia nervosa, are characterized by drastic changes in eating and/or activity behaviors that, when sufficiently rewarding to the individual's desired outcome (e.g., dramatic weight loss), can lead to compulsive engagement in such behavior despite severe deterioration in health or lifestyle. Consequently, DA pathophysiology is suspected to underlie, at least in part, adolescent-onset eating disorders (Barry and Klawans, 1976; Golden and Shenker, 1994; Frank et al., 2005; Frieling et al., 2010; Scherag et al., 2010; Kaye et al., 2013).

AN consistently has the highest mortality rate of all eating disorders (Arcelus et al., 2011; Preti et al., 2011; Keshaviah et al., 2014). Yet effective pharmacological treatments do not exist for *any* eating disorders. Antidepressant drugs, though beneficial for frequently comorbid mood disturbances, appear otherwise ineffective in treating AN, and antipsychotic drugs have marginal if any effectiveness [see reviews by Carrera et al. (2014), Zipfel et al. (2015)]. One potential explanation for such weak treatment effectiveness is a lack of AN animal models. As with most neuropsychiatric disorders, modeling pathophysiology in animals such as rodents is inherently constrained by species differences and questions of emotional and intellectual complexity [refer to Gutierrez (2013), Bale et al. (2019)]. Nonetheless, introduction of specific disorder components, such as the varied physical and environmental stressors in the chronic mild stress model of depression (Willner, 2016), can be advantageous both for understanding neurophysiological disruptions (face validity) and for screening potential therapeutics (predictive validity). Here, we explored use of an adult activity-based anorexia (ABA) paradigm in early adolescence (postnatal day 30, P30) to examine how dopaminergic disruptions from food restriction and/or exercise might differ as a function of sex in adolescents and adults. Until now, animal investigations into anorexia have predominantly used adults, with the few examining adolescent animals missing the vulnerable *early* adolescent period (Kinzig and Hargrave, 2010; Aoki et al., 2012; Barbarich-Marsteller et al., 2013a,b; Chowdhury et al., 2014; Frintrop et al., 2018a,b) during which these behaviors typically emerge in humans (approx. age 13 years) (Micali et al., 2014; Nagl et al., 2016).

The ABA paradigm is based upon work in the 1950s and 1960s that observed significantly enhanced running wheel activity in adult male rats under conditions of food restriction (Hall et al., 1953), to the extent that after approximately 2 weeks, animals ran themselves to death rather than eat when food was available (Routtenberg and Kuznesof, 1967). Current paradigm iterations closely resemble these original

findings, involving unlimited running wheel access (exercise) plus duration-based (rather than quantity-based) food restriction (1 hour/day) (Carrera et al., 2014). To assess dopaminergic disruptions in the ABA paradigm, we measured DA transporter (DAT) function after food restriction, exercise, or their combination (ABA) in adult and adolescent rats of both sexes. Because DA uptake by DAT is a primary regulatory mechanism of dopamine signaling duration, measuring DAT function provides an excellent indication of dopaminergic tone (Cass and Gerhardt, 1994; Zahniser et al., 1999; Daws et al., 2002; Sabeti et al., 2003). To our knowledge, this is the first assessment of DAT function in an ABA paradigm.

Materials and Methods

Animals. Sprague-Dawley rats were bred in-house from breeders purchased through Taconic (NTac:SD; Rensselaer, NY), and all animals were maintained at 24°C on a 12:12 light/dark cycle, lights on at 0400 hours. Rats were weaned at P21, with P0 as day of birth. Rats were group-housed, two to three per cage, with same-sex littermates on 7090 Teklad sani-chip bedding (Envigo, East Millstone, NJ) and provided ad libitum access to water and Teklad LM-485 mouse/rat sterilizable diet 7012 chow (Envigo) until commencement of experimental manipulations. All experiments were approved by the University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committee, and complied with the National Research Council's *Guide for the Care and Use of Laboratory Animals*, 8th Ed.

Activity-Based Anorexia Paradigm. Adolescent (P30 ± 1 day) and adult (P90 ± 5 days) male and female rats were randomly assigned to one of four treatment conditions: cage control (CC), exercise control (EC), food restricted control (FC), and ABA. Initial body weights were not different across treatment conditions within any age group (see Supplemental Table S1). Body weights, and food and water consumed, were measured once daily between 1500 and 1600 hours. Animals always began in the paradigm at 1600 hours; adults continued for 5 days [a standard adult paradigm lasts for between 8 and 14 days (Routtenberg and Kuznesof, 1967; Carrera et al., 2014)], but adolescents were restricted to 4 days due to survival issues during pilot chronoamperometry and cocaine-induced locomotion endpoints at 5 days. On the final day of their respective paradigm, rats were assigned to one of three endpoints (Fig. 1): blood and brain collection from drug-naive rats (i.e., not previously used in chronoamperometry or locomotor experiments) for blood hormone and quantitative autoradiography analyses; locomotor assay to measure acute, dose-dependent effects of cocaine, a DAT blocker; or in vivo high-speed chronoamperometry for measurement of DA uptake in the DAT-rich dorsal striatum, a brain region important for feeding behavior (Palmiter, 2008). Additional details are in the Supplemental Material.

Chemicals and Reagents. All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated.

Locomotor Assay. Testing occurred between 1000 and 1500 hours on the final paradigm day. Locomotor activity was measured using beam breaks quantified in 1-minute bins with Multi-Varimex software (v2.10; Columbus Instruments, Columbus, OH). Animals first underwent a 45-minute habituation session; thereafter, rats received injections of vehicle (0.9% NaCl, saline; 1 ml/kg, i.p.), then cocaine at increasing doses (3.2, 5.6, and 10 mg/kg) such that cumulative doses were 3.2, 8.8, and 18.8 mg/kg, with locomotor activity recorded for

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ABBREVIATIONS: ABA, activity-based anorexia; AN, anorexia nervosa; ANOVA, analysis of variance; AUC, area under the curve; CC, cage control; DA, dopamine; DAT, dopamine transporter; EC, exercise control; FC, food control; NAc, nucleus accumbens; RTI-55, methyl (1*R*,2*S*,3*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; SERT, serotonin transporter.
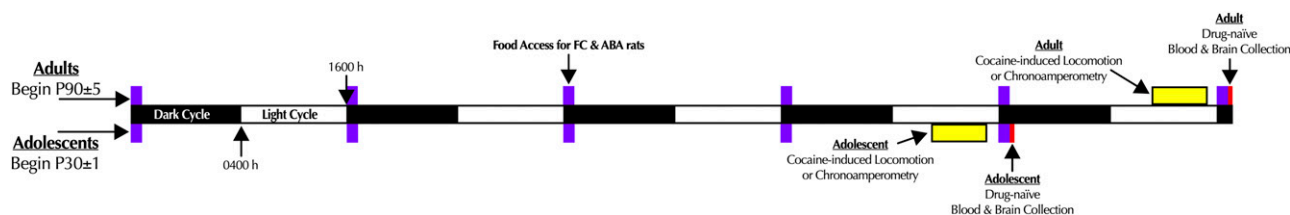
Fig. 1. Timeline of experiments. Adult male and female rats were singly housed in cages directly adjoining running wheels at postnatal day 90 (P90, ±5 days), whereas adolescent male and female rats were housed in the same setup at P30 (±1 day). Cage control (CC) and exercise control (EC) animals had 24-hour access to food, whereas food restricted control (FC) and activity-based anorexia (ABA) animals were given access to food for 1 hour per day (purple blocks) at the onset of the dark cycle (1600 hours, 12:12 dark/light cycle, lights on at 0400 hours). Adolescents went through the paradigm for 4 days, adults for 5 days. At the end of the paradigm, animals underwent one of three endpoints: chronoamperometry (between 0900 and 1500 hours; yellow block), cocaine-induced locomotion (between 1000 and 1500 hours; yellow block), or postprandial drug-naive blood and brain collection (1700 hours, red block).

15 minutes following each injection. For statistical comparisons, area under the curve (AUC) was calculated for the 15 minute period after saline and each cocaine injection using GraphPad Prism (v7.0e; GraphPad Software, La Jolla, CA). Additional details are in the Supplemental Material.

In Vivo High-Speed Chronoamperometry. Chronoamperometry involves measurement of the clearance of increasing amounts of exogenously applied DA from the extracellular space, at a time resolution of 100 milliseconds and spatial resolution of micrometers. This technique is an established tool for measuring DAT function in vivo and was performed as previously described (Baladi *et al.*, 2015; Daws *et al.*, 2016). Additional details are in the Supplemental Material.

[^{125}I] RTI-55 Quantitative Autoradiography. Autoradiography was performed as previously detailed (Coulter et al., 1996). Additional details are in the Supplemental Material.

Statistical Analyses. Data were analyzed in each age group with a two-way (sex × condition) analysis of variance (ANOVA) in NCSS (v12.0.9; NCSS, LLC, Kaysville, UT) with Tukey-Kramer's post-hoc tests where appropriate to a priori compare across sex within condition, or across condition within sex. Statistical outcomes of three-way (day × sex × condition) repeated-measures ANOVA analyses of weights, with Geisser-Greenhouse correction for within-subjects analyses, are provided in Supplemental Table S2 for the drug-naive cohort of animals. Three-way (pmol of DA × sex × condition) repeated-measures ANOVA analyses of DA clearance rate, with Geisser-Greenhouse correction for within-subjects analyses, are provided in Supplemental Table S8. Data were graphed using GraphPad Prism (v7.0e; GraphPad Software). Significance was set a priori at $P < 0.05$. Additional details are in the Supplemental Material.

Results

Body Weights

Initial weights were not different across treatment conditions in the drug-naive cohort, though expected sex differences in initial (day 0) weights were observed (Supplemental Table S1). By the end of the 4 or 5 day paradigm for adolescents and adults, respectively, significant weight differences across treatment conditions were noted. Day-by-day graphs of the time courses of body weights are provided in Supplemental Fig. S1, A–D, and three-way repeated-measure ANOVA outcomes relevant to these day-by-day measures are reported in Supplemental Table S2. Because all blood hormone, acute cocaine-induced locomotion, chronoamperometry, and autoradiography outcome measures took place on the final day of the paradigm, we focus here on final day weights, shown in Fig. 2.

Adolescents. As with day 0 weights, a significant main effect of sex was observed for day 4 body weights [F(1,68) = 55.7,

$P < 0.001$), but unlike day 0 weights, a significant main effect of treatment condition was also detected [F(3,68) = 69.3, $P < 0.001$]. No interactions between treatment and sex were detected. FC and ABA day 4 body weights were lower than both CC and EC groups within the same sex.

Adults. Though no significant sex × condition interaction was observed, significant main effects of sex (F(1,57) = 1039, $P < 0.001$) and condition (F(3,57) = 15.0, $P < 0.001$) were found for day 5 body weights. Within adult females, only ABA animals had day 5 body weights different from CC animals. Weights of adult male ABA animals at day 5 were less than both CC and EC males.

Food Consumption as Percent Body Weight

Given the significant differences in body weights at the end of the paradigms for adolescent and adult rats in the drug-naive cohort, food consumption for each 24 hour period of the paradigm was calculated for each animal as a percentage of their previous day's body weight. Time-course graphs of these measures are presented in Supplemental Fig. S1, E–H, and as with body weights, we focus here on the final day of the adolescent (day 4) and adult (day 5) paradigms. Three-way repeated-measure ANOVA outcomes are reported in Supplemental Table S2.

Adolescents. A significant main effect of condition [(F3,68) = 222, $P < 0.001$] was observed, with FC and ABA adolescents consuming less food than their EC and CC counterparts. No significant interaction between condition and sex, and no significant main effect of sex, were found. Adolescent EC males also consumed less food based on their body weight than CCs (Fig. 2C).

Adults. As with adolescents, condition displayed a significant main effect [F(3,57) = 92.5, $P < 0.001$]. No significant main effect of sex was indicated, nor was a significant condition × sex interaction. Food intake was reduced in female FC and ABA conditions compared with female EC and CC conditions. Adult male body weight–based food intake on day 5 was reduced in EC, FC, and ABA animals relative to CC males. Male FC and ABA rats further exhibited reduced food intake relative to male EC animals (Fig. 2D).

Running Wheel Activity

Only animals in the EC and ABA conditions had unlocked running wheels (Fig. 3, A–D), so these two conditions were analyzed within age and across sex and condition. Area under the curve (AUC) was calculated for 23 hours during each paradigm day, and these AUC data per day are shown in
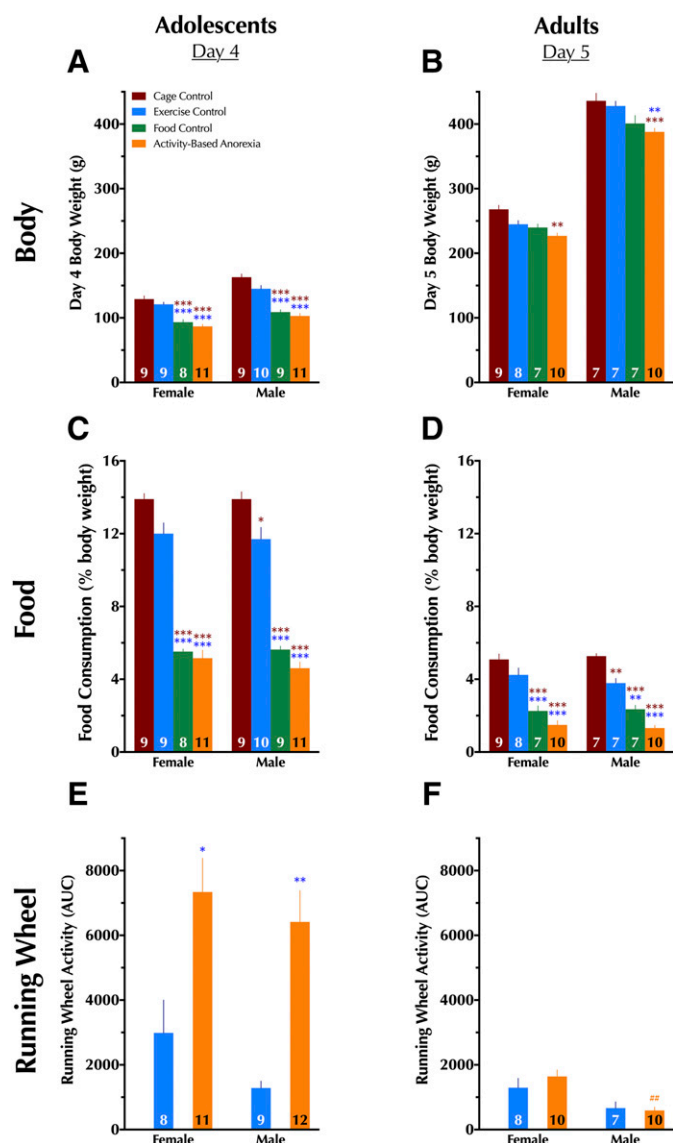
Fig. 2. Final day body weight, food consumption, and running wheel measures in drug-naive rats. The final day in the experimental paradigm for adolescents was day 4, whereas the final day for adults was day 5. Because animals used for chronoamperometry and cocaine-induced locomotion were removed prior to completion of their full fourth (adolescents) or fifth (adults) day, only data from drug-naive rats are shown here. (A) Adolescent day 4 body weight; (B) adult day 5 body weight; (C) adolescent and (D) adult food consumption over the past 23 hours as a percentage of the individual animal's body weight; (E) adolescent and (F) adult running wheel activity over the last 23 hours for EC and ABA animals. No running wheel activity is shown for CC and FC animals because their wheels were locked and therefore they could not run in the wheel. Data were analyzed within each age group with a two-way (sex × condition) ANOVA with Tukey-Kramer's post-hoc tests where appropriate. Data are graphed as mean + S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. same-sex condition, indicated by color of asterisk(s), within the same age group. $^{\#\#}P < 0.01$ vs. same condition in females of same age group. Number of animals is indicated at base of each bar.

Fig. 3, E–H, with corresponding three-way repeated-measure ANOVA outcomes reported in Supplemental Table S3. Final paradigm day 4 AUCs for adolescents are in Fig. 2E, and final day 5 for adults are in Fig. 2F.

Adolescents. A significant main effect of condition was detected [$F_{(1,36)} = 25.9$, $P < 0.001$], with both female and

male ABA animals exhibiting greater day 4 running wheel activity relative to their same-sex EC counterparts (Fig. 2E). Sex × condition interaction did not reach significance, nor did a main effect of sex.

Adults. There was no significant main effect of condition in adults, nor was there a significant sex × condition interaction. However, a main effect of sex was significant [$F_{(1,31)} = 17.1$, $P < 0.001$], with this driven by greater running wheel activity in female ABA animals compared with male ABA (Fig. 2F).

Blood Hormone and Other Physiologic Measures

See Supplemental Results and Supplemental Tables S4 and S5 for full details. Briefly, relative to same-sex CCs, circulating leptin and corticosterone were reduced in adolescent female ABA and FC groups, and adolescent female FCs also exhibited reduced ghrelin and increased insulin postprandial. Adolescent male blood hormone changes were similar for leptin and insulin, though no ghrelin or corticosterone differences relative to same-sex CCs were detected. In adults, only leptin levels were reduced compared to CCs in ABA animals (both sexes) and FCs (males). Brain water weights and plasma osmolality were unaffected in all groups. Hematocrit was elevated in adolescent ABAs (both sexes) compared to CCs, whereas only plasma protein was reduced selectively in adolescent female ABAs. No changes in either hematocrit or plasma protein were detected in any adults.

Locomotor Activity

AUC for locomotor activity, measured by minute-to-minute beam breaks, was calculated for the 15 minutes immediately following a saline injection, and for the combined three 15 minute periods immediately following three successive individual doses of cocaine (3.2, 5.6, 10 mg/kg). These AUCs were then analyzed to compare locomotor responses after injections.

Locomotor Activity after Saline. Saline AUCs are described in Supplemental Table S6, with corresponding time courses for saline and cocaine graphed in Fig. 4. For saline AUCs, no significant differences across treatment conditions were observed in either age group (Supplemental Table S6).

Locomotor Activity after Cocaine. Cocaine AUCs are inclusive of all three individual doses (3.2, 5.6, 10 mg/kg).

Adolescents. A significant sex × condition interaction was observed in the cocaine AUCs of adolescent rats [$F_{(3,64)} = 5.11$, $P < 0.01$]. Adolescent female ABA animals exhibited greater cocaine AUCs than EC counterparts (Fig. 4A). In contrast to adolescent females, adolescent males in the FC condition displayed greater cocaine AUCs than all other male conditions, and compared to female FC counterparts (Fig. 4B). Adolescent male ABA animals also exhibited higher cocaine-induced locomotion than male CC rats.

Adults. A main effect of sex was detected [$F_{(1,66)} = 25.4$, $P < 0.001$], with female CC rats displaying a higher cocaine AUC than male CCs (Fig. 4, C and D). Condition did not show a significant main effect, nor was there a significant sex × condition interaction.

In Vivo High-Speed Chronoamperometry

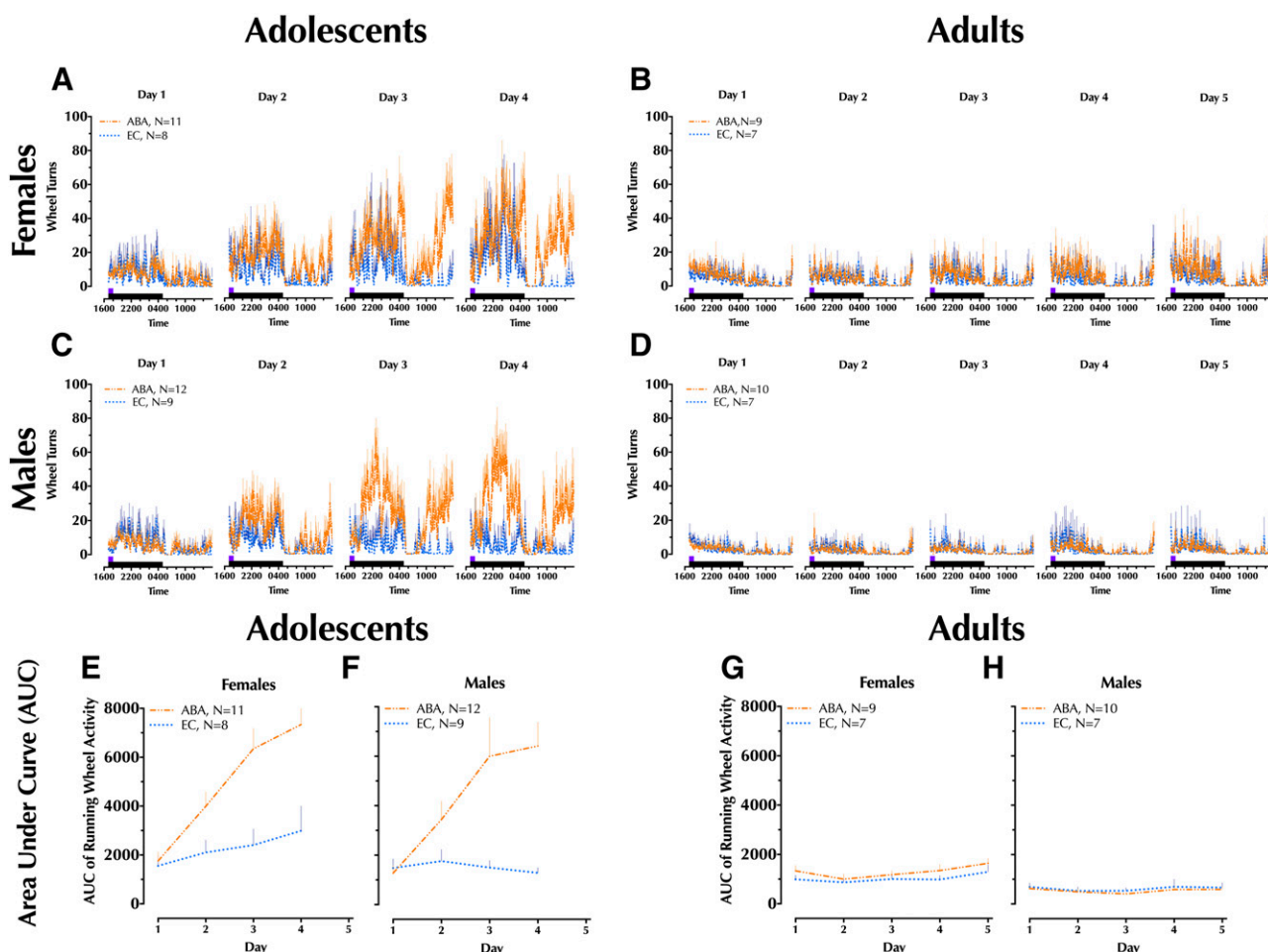For chronoamperometry, signal amplitudes at the highest amount of DA infused (100 pmol) were first evaluated to

Fig. 3. Daily running wheel measures in drug-naive rats. Adolescents were run in the experimental paradigm for 4 days, whereas adults were run for 5 days. Because animals used for chronoamperometry and cocaine-induced locomotion were removed prior to completion of their full fourth (adolescents) or fifth (adults) day, only data from drug-naive rats are shown here. Collected in 5 minute bins, 23 hours of running wheel activity were recorded each day for (A) adolescent female and (B) male running wheel activity over four paradigm days; (C) adult female and (D) male running wheel activity over five paradigm days. Area under the curve (AUC) was calculated for each day from the graphs in (A–D), and are shown for (E) adolescent female and (F) male animals; (G) adult female and (H) male animals. No running wheel activity is shown for CC and FC animals, because their wheels were locked and therefore they could not run in the wheel. Only the final day of wheel running activity was analyzed (refer to Fig. 2, E and F). Data are graphed as mean + S.E.M. Number of animals is indicated in line legend: exercise control (EC), activity-based anorexia (ABA).

confirm that these were not significantly affected by treatment condition (Supplemental Table S7). The clearance rate of DA from the extracellular space was then plotted against picomoles of DA infused.

Adolescents. Significant interactions between sex × condition and condition × pmol of DA were observed (see Supplemental Table S8 for statistics). For comparisons across each treatment condition, a repeated-measures ANOVA was performed within each sex. In adolescent females, a significant main effect of condition was detected [$F_{(3,185)} = 2.97, P < 0.05$]. Specifically, at 40 and 50 pmol of DA, female EC and FC rats had slower DA clearance relative to same-sex CCs (Fig. 5A). Clearance rates in female ABA rats were also greater at 40 and 50 pmol of DA relative to FC rats. Adolescent males likewise exhibited a significant main effect of condition [$F_{(3,155)} = 5.86, P < 0.01$], but in stark contrast to females, male ABA rats exhibited significantly impaired DA clearance relative to CC at DA levels of 20 pmol and higher (Fig. 5B). As with females, male EC and FC rats exhibited significantly impaired DA clearance at 20, 40, and 50 pmol, compared with male CCs.

Adults. Adult rats exhibited no significant interactions across sex, condition, and/or picomoles of DA (Supplemental Table S8; all $P > 0.57$), though there was an expected significant main effect of picomoles of DA [$F_{(5,235)} = 143, P < 0.001$] (Fig. 5, C and D).

Adolescent DAT and Serotonin Transporter Expression

Given that only adolescent rats exhibited functional changes in DAT as a result of condition, expression of DAT and serotonin transporter (SERT) was quantified using autoradiography in striatum and nucleus accumbens (NAc) of only adolescent animals. No significant differences in DAT or SERT expression were observed in either brain region in either sex, and no significant interactions between sex × condition were found (Fig. 6).

Discussion

Our key findings indicate that during adolescence, the dopaminergic system possesses a functional plasticity impacted not only by sex but also by dietary and behavioral patterns, such as
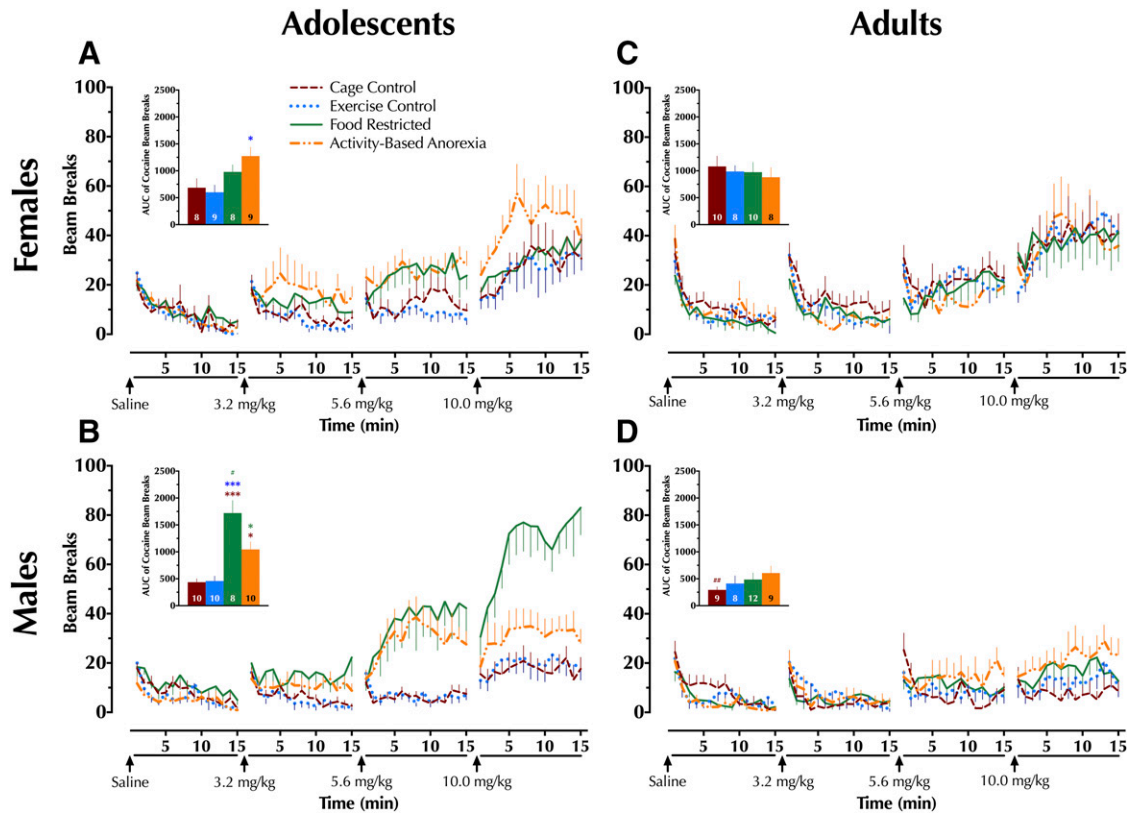
Fig. 4. Cocaine-induced locomotion. Rats were habituated to activity chambers for 45 minutes, then injected first with saline (1 ml/kg), then increasing individual doses of cocaine (3.2, 5.6, and 10 mg/kg) at 15 minute intervals in a cumulative-dosing paradigm. Activity was quantified as the number of beam breaks in 1 minute bins (lines in graphs), and the area under the curve (AUC) for all three cocaine doses is cumulatively shown in inset bar graphs for adolescent (A) female and (B) male and adult (C) female and (D) male rats. AUC data (inset bar graphs) were analyzed within each age group with a two-way (sex × condition) ANOVA with Tukey-Kramer's post-hoc tests where appropriate. Data are graphed as mean ± S.E.M. $*P < 0.05$; $***P < 0.001$ vs. same-sex condition, indicated by color of asterisk(s), within the same age group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ vs. same condition in females of same age group. Number of animals is indicated at base of each bar.

food restriction and exercise, in a manner not confounded by homeostatic body fluid disruptions or transporter expression changes. This responsive plasticity may feed forward to neuro-behaviorally accelerate engagement in additional or continued unhealthy activities or compulsions, reshaping dopaminergic circuitry such that vulnerability to pervasive eating disorders or substance use disorders is perpetually augmented.

Being the first, to our knowledge, to apply the established adult ABA paradigm to *early* adolescence (P30), we observed striking vulnerability in adolescents compared with adults. Adult rats require at least 7 days to exhibit ≥20% starting body weight loss under these conditions (Routtenberg and Kuznesof, 1967); indeed, we observed minimal to no differences among treatment conditions in most adult endpoints. In contrast, adolescents responded so robustly that our experimentally planned 5-day paradigm was shortened to 4 days to facilitate survival for endpoint measures. This susceptibility of *early* adolescent rats parallels emergence in humans of pathologic behaviors and symptoms relating to eating disorders in early adolescence (age 13 years) (Micali et al., 2014; Nagl et al., 2016). The few studies using adolescent rats (all > P35) in an ABA paradigm have not validated the model (i.e., confirmed that key AN metabolic and hormonal manifestations are present), and seldom included all relevant controls, limiting interpretability of data (Kinzig and Hargrave, 2010; Aoki et al., 2012; Barbarich-Marsteller et al., 2013a,b; Chowdhury et al., 2014; Frintrop et al., 2018a,b). Moreover,

the rapidity with which adolescent rats succumb to the adult-based ABA paradigm highlights the necessity for age-appropriate modifications that expand the experimental window beyond 4 days. Such a truncated window precludes the ability to investigate neurophysiological changes resulting from chronic ABA, and likewise hinders exploration of novel pharmacological interventions. Further, studies in adult rodents fail to reflect neural mechanisms active in the most vulnerable population, adolescents, during a time when brain maturation occurs. Considering AN's high mortality (Arcelus et al., 2011; Preti et al., 2011; Keshaviah et al., 2014), as well as its long-term stability in those who survive (Nagl et al., 2016), a chronic ABA paradigm commencing during the vulnerable early adolescent period could provide a much-needed model in AN pathophysiology and treatment studies.

Blood hormone measurements further evidence the need for an optimized early adolescent ABA model. AN is characterized by substantial baseline reductions in circulating insulin and leptin, along with increases in ghrelin and cortisol (Monteleone et al., 2000; Nakahara et al., 2008; Karczewska-Kupczewska et al., 2010; Korek et al., 2013). With the exception of reduced leptin levels, none of these hormonal disruptions were mirrored in adolescents, underscoring the poor face validity of the current adult-based paradigm in adolescents. Recent efforts have prolonged survival of adolescent female rats in a modified ABA protocol (Frintrop et al., 2018b), but it remains to be seen whether this procedure elicits any
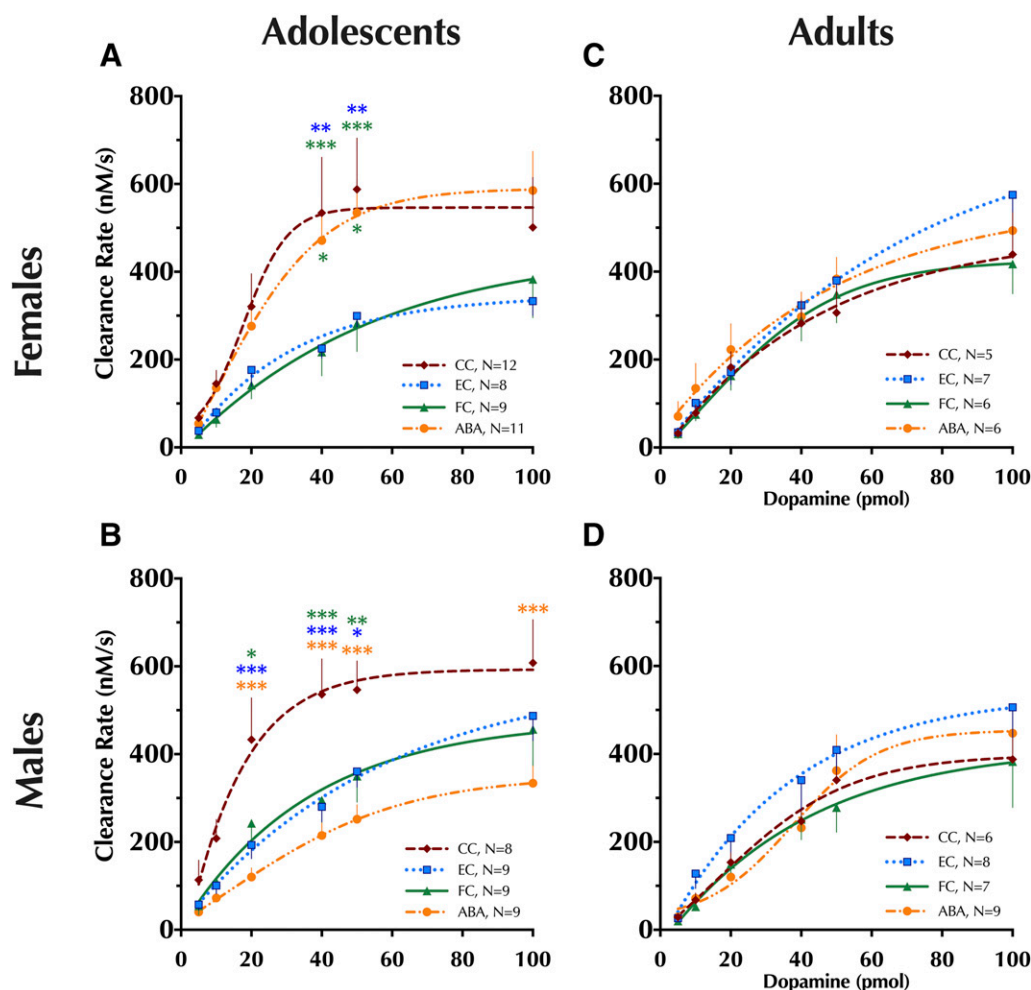
Adolescents

Adults

Fig. 5. Striatal dopamine clearance measured using in vivo high-speed chronoamperometry. Clearance rate of dopamine in the striatum of adolescent (A) female and (B) male rats and adult (C) female and (D) male rats is shown for increasing amounts of exogenously infused dopamine. Four-parameter logistic regressions with baseline constrained at 0 were used to generate curve fits. Data analyzed within each age and sex group using a repeated-measures (pmol DA × condition) ANOVA with Tukey-Kramer's post-hoc tests where appropriate. Data are graphed as mean ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. same-sex condition, indicated by color of asterisk(s), within the same picomole amount. Number of animals is indicated in respective legends: cage control (CC), exercise control (EC), food restricted control (FC), activity-based anorexia (ABA).

sex-dependent effects, or reflects any hallmark blood hormone disruptions observed in AN patients. Another alternative approach to modeling AN uses a behavioral economics framework (Rowland et al., 2018), though how well this can be adapted to adolescents is undetermined.

Homeostatic body fluid maintenance, as evidenced by plasma osmolality and brain water weight, was not disrupted in FC and ABA adolescents, despite dramatic reductions in food and water consumption. Importantly, this suggests that the observed plasticity in DAT function is not confounded by dehydration, differences in DA diffusion, or changes in tortuosity at the carbon fiber microelectrode site.

The elevated hematocrit observed in adolescent ABA rats of both sexes, and adolescent male FC rats, is at odds with reports of reduced hematocrit levels in food restricted rats (Wojciak, 2014) and patients with severe AN [but see Symreng et al. (1985), Sabel et al. (2013)]. Instead, increased hematocrit is usually observed after excessive exercise in humans (Skarpańska-Stejnborn et al., 2015) but not rodents (Tian et al., 2012; Tang et al., 2019). Plasma protein levels were diminished only in adolescent female ABA rats, which could be attributable to muscle wasting or liver inflammation, both

commonly observed in patients with AN (Rosen et al., 2017).

Despite the limitations of applying the adult ABA paradigm to adolescents, the current investigation revealed striking age- and sex-specific effects of exercise access plus food restriction on the locomotor-stimulating effects of the drug of abuse, cocaine. Cocaine-mediated blockade of DA uptake through DAT corresponds to cocaine-induced locomotor responses in Sprague-Dawley rats (Sabeti et al., 2002; Gulley et al., 2003). Thus, this behavior assay can serve as a proxy, at least in part, to indicate individual differences in DAT function as revealed by cocaine blockade.

To our knowledge, the influence of food restriction alone on adolescent or female locomotor activity in response to cocaine had not previously been explored, though the observed augmentation of cocaine response in adolescent males fits with previous, more extended periods of food restriction (1–2 weeks) in adult males (Bell et al., 1997). The absence of changes here in cocaine locomotor response after exercise alone across age and sex indicate that its effects take more than 4–5 days to emerge behaviorally, in agreement with multiple reports (Cosgrove et al., 2002; Smith et al., 2008a,b;
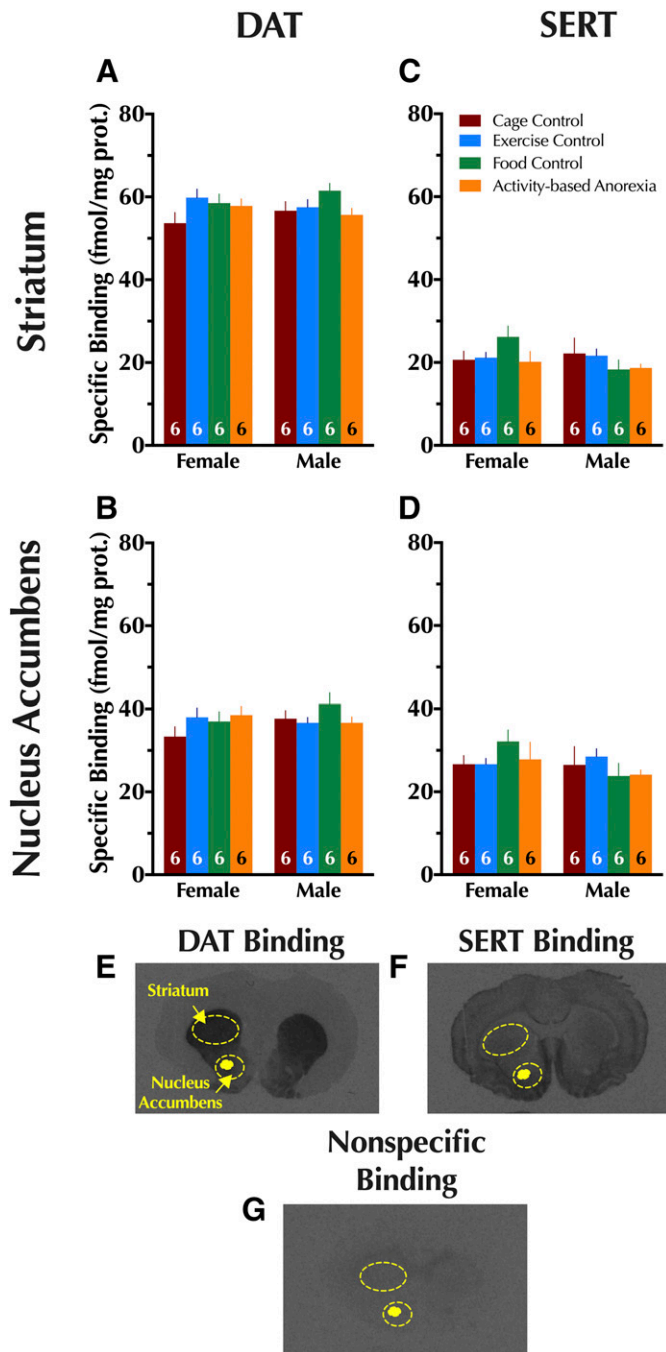
Fig. 6. Quantitative autoradiography in adolescents for dopamine and serotonin transporter expression. Specific binding for dopamine transporter is shown in adolescent (A) striatum and (B) nucleus accumbens. Specific binding for serotonin transporter is also shown in adolescent (C) striatum and (D) nucleus accumbens. Representative brain sections illustrating (E) DAT, (F) SERT, and (G) nonspecific binding are shown with the analyzed regions for striatum and nucleus accumbens illustrated with dashed circles. Data were analyzed within each age group with a two-way (sex × condition) ANOVA. Data are graphed as mean + S.E.M. Number of animals is indicated at base of each bar.

a disproportionately high prevalence of eating disorder diagnoses, particularly of AN, among females with comorbid substance use disorders [see Harrop and Marlatt (2010) for review]. The seemingly augmented response of ABA adolescent females to cocaine, versus the moderated response in ABA adolescent males, likely reflects underlying sex- and age-dependent plasticity in DAT function in response to the combination of food restriction and exercise.

Indeed, we directly investigated DAT function in dorsal striatum, where DAT expression is high, and DA signaling influences reward, activity, and eating behaviors (Sotak et al., 2005; Palmiter, 2007, 2008; Tomasi and Volkow, 2013). To our knowledge, this is the first characterization of in vivo DAT function in adolescent females, and the first evaluation of DAT functional changes after an ABA paradigm. We found a robust age difference in DA uptake across sexes, with faster clearance in adolescents compared with adults. Moreover, only in adolescents did ABA reveal a sex-specific effect, with dramatic functional DAT reductions observed in males but not females. Reductions in adolescent DAT function in ECs and FCs support the observed slowing of DA clearance in adolescent male ABAs, making the lack of attenuated DAT function in adolescent ABA females remarkable. Under prolonged (>4 days) ABA conditions, DAT function in adolescent females might eventually either increase, or persist at an adolescent CC-like level, reflecting clinical reports of significant DAT upregulation in female patients suffering from AN for an average of 10 years (Frieling et al., 2010). Optimization of the ABA paradigm for adolescents, to enable prolonged study with more of the characteristic hormonal disruptions in place, will permit study of this possibility. Importantly, the functional changes in DAT are not mirrored by any significant changes in total striatal DAT or SERT expression, the latter of which can transport DA under conditions of impaired DAT function (Larsen et al., 2011). These results suggest that DA clearance rate shifts are probably the result of changes in intrinsic activity and/or plasma membrane expression of DAT.

The sex-specific adolescent plasticity in dorsal striatum DAT function does not directly map on to cocaine-induced locomotor activity. This could be attributable to brain region–specific effects of cocaine on DA clearance, as cocaine-mediated blockade of DAT in NAc corresponds more closely to drug-induced changes in locomotor activity, compared to dorsal striatum (Sabeti et al., 2002). However, this is not attributable to any detectable changes in DAT expression or cocaine affinity in NAc (Gulley et al., 2003); indeed, we observed no significant differences in NAc DAT expression here. Thus, different cocaine-induced locomotor responses across conditions in female and male adolescents might reflect functional NAc DAT differences, whereas the functional changes measured using chronoamperometry in dorsal striatum could indicate DA clearance shifts that impact more habitual behaviors (e.g., home cage or running wheel activity).

Certainly, this plasticity could instead be secondary to other neurophysiological disruptions in the dopaminergic system (e.g., changes in DA release) or other neurotransmitter systems (Hillebrand et al., 2005; Atchley and Eckel, 2006), and could be influenced by apparent circadian changes in the adolescent ABA groups during the final 2 days of the paradigm (see Fig. 3). Nonetheless, the present findings suggest that striatal DAT function should be a focus of future studies into the mechanisms underlying eating disorder and drug abuse

Smith et al., 2011; Smith and Witte, 2012; Zlebnik et al., 2012). Most striking is that the combination of exercise plus food restriction in adolescent males substantially attenuated the locomotor response to cocaine, relative to FCs, but that this combination produced the largest behavioral response to cocaine in adolescent females. This is in accord with

vulnerability. Moreover, a chronic ABA paradigm commencing during the vulnerable early adolescent period would provide a much-needed model to study AN pathophysiology and treatment.

Authorship Contributions

Participated in research design: Gilman, George, Toney, Daws.

Conducted experiments: Gilman, Owens, George, Metzel, Vitela, Ferreira, Bowman, Gould.

Performed data analysis: Gilman, Daws.

Wrote or contributed to the writing of the manuscript: Gilman, Gould, Toney, Daws.

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Supplement for:

Age- and sex-specific plasticity in dopamine transporter function revealed by food restriction and exercise in a rat activity-based anorexia paradigm.

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Supplemental Methods and Materials

Activity-based anorexia paradigm. Adolescent (P30±1 day) and adult (P90±5 days) male and female rats were randomly assigned to one of four treatment conditions: cage control (CC), exercise control (EC), food restricted control (FC), and activity-based anorexia (ABA). Initial body weights were not significantly different across treatment conditions within any age group (see Supplemental Table S1 for weights and statistics). Commencement of these experimental manipulations consisted of singly housing each rat in a standard plastic rat cage (45.5 cm L × 24 cm W × 20 cm H) adjoining an activity wheel (Med Associates product number ENV-046; Fairfax, VT). Activity wheels were connected to a computer through a Med Associates 28V T/T interface cabinet, and running wheel activity was recorded in 5 min bins for 23 h each day with Med PC IV (v4.2, release 1, build 48) software. Running wheel data between 1500-1600 h was omitted given the presence of an experimenter during this hour to record food, water, and body weight measurements and to reset the running wheel software. Body weights and water consumed were measured once daily between 1500-1600 h, and food consumed was measured after the first hour of the dark cycle at 1700 h for all animals, as well as at 1600 h for 23 h consumption in CC and EC control animals. Animals always began in the paradigm at 1600 h; adults continued for 5 days, but adolescents were restricted to 4 days due to survival issues during pilot chronoamperometry and cocaine-induced locomotion endpoints at 5 days. On the final day of their respective paradigm, rats were assigned to one of three endpoints (Fig. 1): blood and brain collection from drug-naïve rats (i.e. not previously used in chronoamperometry or locomotor experiments) for blood hormone and quantitative autoradiography analyses; locomotor assay to measure acute, dose-dependent effects of cocaine, a DAT blocker; or *in vivo* high-speed chronoamperometry for measurement of DA uptake in the DAT rich dorsal striatum, a brain region important for feeding behavior (1). Brains and blood used for brain water weight and blood osmolality, hematocrit, and plasma protein were randomly sampled from rats assigned to all 3 endpoints.

Locomotor assay. Rats were moved to the testing room at least 1 h before testing commenced. Testing occurred between 1000 and 1500 h on the final day of the paradigm, and locomotor activity was measured using beam breaks quantified in 1 min bins with Multi-Varimex software (v2.10; Columbus Instruments, Columbus, OH). Animals first underwent a 45 min habituation session in the locomotor chambers (26 cm W × 60 cm L × 23 cm H), situated in sound-attenuating chambers. Thereafter, rats received injections of vehicle (0.9% NaCl, saline; 1 mL/kg, ip.) then cocaine at increasing individual doses (3.2, 5.6, 10, 17, 32 mg/kg) administered cumulatively (3.2, 8.8, 18.8, 35.8, and 67.8 mg/kg cumulative doses), with locomotor activity recorded for 15 min following each injection. Adverse outcomes, including bloody nose, seizure, and death, occurred at the highest two doses in both ages and sexes of ABA and FC animals, so for ethical reasons these individual doses (17 and 32 mg/kg) were discontinued for all conditions. For statistical comparisons, area under the curve (AUC) was calculated for the 15 min period after saline and each cocaine injection using GraphPad Prism (v7.0e; GraphPad Software, La Jolla, CA).

Blood collection and processing for hormone measures. Blood was collected from animals that did not undergo chronoamperometry or locomotor testing (referred to as "drug-naïve"). Collection occurred at 1700 h so that animals were postprandial, as pilot experiments indicated that insulin levels in P30 animals were undetectable after an adult-standard 20 h fast. Animals were briefly anesthetized with isoflurane, then decapitated with a guillotine for trunk blood collection. Blood aliquots from each animal were processed separately as plasma or serum. For plasma, samples were collected on wet ice, then immediately centrifuged at 3500 rpm for 10 min at 4°C. For serum, samples were allowed to clot at room temperature for 1 h, then were centrifuged at 3500 rpm for 1 h at 4°C. Plasma or serum supernate from these samples were stored in 100 μL aliquots at

−80°C until blood hormone analyses with ELISAs. Plasma insulin (Ultrasensitive Rat Insulin ELISA, Mercodia, Uppsala, Sweden), plasma ghrelin (Rat/mouse Ghrelin (total) ELISA, Millipore, Burlington, MA), serum leptin (Rat Leptin ELISA, Millipore), and serum corticosterone (Corticosterone ELISA, Enzo Life Sciences, Inc., Farmingdale, NY) were quantified according to manufacturer's instructions.

Blood and brain collection and processing for physiology measures. Across drug-naïve, locomotor, and chronoamperometry endpoint animals, trunk blood was collected at time of euthanasia for assessment of hematocrit, plasma protein, and osmolality. Brains were also collected to determine water content by mass. For hematocrit, heparinized capillary tubes (Fisher Scientific, Hampton, NH) were used to collect blood, sealed with Critoseal (Leica Biosystems, Wetzlar, Germany) and centrifuged at 17,000×g for 5 min. Red blood cell count/percentage was estimated using a capillary tube reader. For osmolality and plasma protein, trunk blood was collected into microcentrifuge tubes, centrifuged at 2,000×g for 10min, and plasma supernatant was removed to a clean microcentrifuge tube. Plasma osmolality was measured in duplicate using a digital, single-sample micro-osmometer (Advanced Instruments Model 3320, Norwood MA). Plasma protein was quantified by pipetting 50µL of plasma onto a calibrated hand-held manual refractometer (AtagoUSA, Inc., Bellevue, WA) to determine specific gravity (g/100ml). Brain water weight was assessed by weighing the brain upon initial extraction, then storing in a room temperature desiccator for 60 d, an interval we empirically determined sufficient for complete brain dehydration.

In vivo high-speed chronoamperometry. Chronoamperometry was performed as previously described (2; 3). Briefly, animals were removed from the housing room between 0900 and 1500 h on the final paradigm day and anesthetized with urethane (850 mg/kg, ip.) and α-chloralose (85 mg/kg, ip.). Once fully anesthetized, they were mounted in a stereotaxic headframe and a Nafion coated, precalibrated 30 µm diameter carbon fiber microelectrode attached to a multibarrel micropipette (FHC, Bowdoin, ME) was lowered into the right striatum (AP +1.5, ML +2.2, DV −4.0). Tips of the microelectrode and micropipette were separated by 325 µm. Local application of DA (200 μM), preloaded into the micropipette, was performed using pressure ejection of volumes of 25-500 nL (5-100 pmol). The order in which pmol amounts of DA were delivered was randomized among rats in each treatment group. High-speed chronoamperometric recordings of DA uptake were made using the FAST-12 system (Quanteon, Nicholasville, KY), with oxidation currents determined as the stable current following decay of the capacitive transient produced by 100 ms oxidation potential pulses to +550 mV. Oxidation pulses were separated by 100 ms at 0 mV.

[^{125}I] RTI-55 Quantitative Autoradiography. Brains from adolescent drug-naïve rats were collected concurrent with blood collection for hormone measures, and rapidly flash frozen on powdered dry ice. Brains were stored at −80°C until sectioning at AP 1.60 ± 0.4 mm from bregma to capture striatum (i.e., caudate putamen(4) in 20 μm coronal slices in a -20 ± 1°C cryostat (Leica CM1860, Bannockburn, IL). Slices were thaw-mounted sequentially onto a series of 10 gelatin coated microscope slides until there were 4 to 5 sections per slide, desiccated overnight (18h) at 4°C, then frozen at -80°C until use. Slides with sections were defrosted at 22°C (room temperature) for 30 min and pre-incubated in 30 mM sodium phosphate, 120 mM sodium chloride buffer pH 7.4 at 22°C for 30 min. For incubation, 100 mM sucrose and 60 pM [^{125}I] RTI-55 (NEX272, Perkin Elmer, Boston, MA) was added to the buffer. This concentration was 5x the K_D value of 12 ± 1 nM for RTI-55 in rat striatum (5). Four sets of serial sections on slides from each rat were used under different incubation conditions for each rat: 1) for DAT binding, 100 nM sertraline HCl was added to the buffer for blockade of serotonin transporter (SERT) binding; 2) for SERT binding, 100 nM GBR12909 was added to the buffer to block DAT; 3) for total binding, nothing was added

to buffer; 4) for non-specific binding, 10 µM mazindol (to block both SERT and DAT binding) was added. All unlabeled ligands were from Sigma. Incubation was carried out for 2 h at 22ºC. Sections were rinsed twice for 1 min in 4ºC buffer (without sucrose), then dipped in 4ºC deionized water, drained and placed on a slide warmer (Lab-Line, Fisher Scientific, USA), with a moderate setting (4 on 10 scale) for 2 h. Slides were opposed to Biomax MR film for 48 hours, with tritium standards (ART 123A American Radiolabeled Chemicals, St. Louis, MO, USA) calibrated by propagation to [^{125}I] incubated brain mash as previously described (6). Films were developed in an automatic film processor. Digital images were captured using a stabilized fluorescent light box (Northern Lights, Imaging Research, Ontario, Canada), CCD monochrome camera (CFW-1612M, Scion, Frederick, MD) with C-mounted 60 mM lens (Nikon, Melville, NY) on a copy stand (Kaiser RS-1,White Plains, NY). Film images were captured as digital images and calibrated to measure density in units of fmol/mg protein using a linear function with ImageJ (http://rsb.info.nih.gov/ij/download.html). Images were quantified using ImageJ by measuring density from the left and right hemisphere of at least 3 sections per slide, taking their average (n=1) for measures of binding site density in striatum and nucleus accumbens of each rat brain.

Statistical analyses. Previous work by our lab and others has demonstrated age-specific differences in DAT function as measured with chronoamperometry and acute cocaine-induced locomotion in male rats (2). Consequently, we specifically investigated herein the effects that exercise and/or food restriction had on DAT function, DAT and SERT expression, or other physiological measures, within each age group (adolescent or adult) in both males and females. Data were therefore analyzed in each age group with a two-way (sex × condition) ANOVA in NCSS (v12.0.9; NCSS, LLC, Kaysville, UT) with Tukey-Kramer's post-hoc tests where appropriate to *a priori* compare across sex within condition, or across condition within sex. Statistical outcomes of three-way (day × sex × condition) repeated measures ANOVA analyses of weights, with Geisser–Greenhouse correction for within-subjects analyses, are provided in Supplemental Table S2 for the drug-naïve cohort of animals as only this cohort completed the entirety of the final day of the paradigm. Animals used for locomotor assays or chronoamperometry were removed from the housing room for experiments before completion of the final 24 h, and are therefore not included in the repeated measures analyses of weights or running wheel activity. Three-way (pmol DA × sex × condition) repeated measures ANOVA analyses of DA clearance rate, with Geisser–Greenhouse correction for within-subjects analyses, are provided in Supplemental Table S6. Data were graphed using GraphPad Prism (v7.0e; GraphPad Software, La Jolla, CA). Significance was set *a priori* at p<0.05.

Supplemental Results

Water consumption as percent body weight
Water (mL) consumption for each 24 h period of the paradigm was calculated for each individual animal as a percentage of their previous day's body weight (g). Time course graphs of these measures are presented in Supplemental Figure S1I-L, and as with body weights, we focus here on the final day of the adolescent (day 4) and adult (day 5) paradigms. Three-way repeated measure ANOVA outcomes are reported in Supplemental Table S2.

Water consumption
Adolescents
On the final day of the 4 day adolescent paradigm, water consumption as a percent body weight in adolescent females did not indicate a sex × condition interaction ($F(3,68)=0.62$, $p>0.60$). However, both main effects of sex ($F(1,68)=10.5$, $p<0.01$) and condition ($F(3,68)=28.8$, $p<0.001$) were found. Across sexes, post-hocs did not indicate any significant differences within treatment conditions. Within adolescent females, FC ($p<0.01$) and ABA ($p<0.001$) animals consumed significantly less water as a percent of body weight compared to CC females (Supplemental Fig. S2A). Relative to EC females, FC ($p<0.05$) and ABA ($p<0.001$) females also consumed less water (Supplemental Fig. S2A). Similar patterns were observed for adolescent males, with FC ($p<0.01$, $p<0.001$) and ABA ($p<0.001$, $p<0.001$) males consuming significantly less than both CC and EC males, respectively (Supplemental Fig. S2A).

Adults
Adult water consumption on day 5 as a percentage of body weight approached significance for a sex × condition interaction ($F(3,57)=2.64$, $p=0.058$). A main effect of sex was significant ($F(1,57)=12.5$, $p<0.001$), whereas condition was not ($F(3,57)=0.35$, $p>0.79$). Post-hocs revealed that the only significant difference across sexes within condition was between female and male FC animals ($p<0.01$), with no significant differences detected across treatment groups within either sex (Supplemental Fig. S2B).

Blood hormone levels
Levels of insulin, leptin, ghrelin, and corticosterone were analyzed from rats in the drug-naïve cohort (Supplemental Table S4). Postprandial blood was collected at 1700 h at the end of the final day in the paradigm (day 4 for adolescents, day 5 for adults), and analyzed with appropriate ELISA kits for the different blood hormones.

Adolescent insulin levels
In adolescent rats, no significant interaction of sex × condition was detected ($F(3,59)=0.79$, $p>0.50$), nor was a main effect of sex observed ($F(1,59)=0.01$, $p>0.90$). A significant main effect of condition was detected ($F(3,59)=15.9$, $p<0.001$), with post-hocs indicating that insulin levels were significantly higher in female FC animals relative to female EC and CC counterparts ($p<0.01$; Supplemental Table S4A). Post-hoc tests similarly indicated that adolescent FC male rats had insulin levels significantly higher than those of CC ($p<0.05$) and EC ($p<0.01$) males, and a non-significant trend was noted between male FC and ABA insulin levels ($p=0.064$; Supplemental Table S4A).

Adolescent leptin levels
A significant sex × condition interaction was detected in adolescents for leptin levels ($F(3,60)=8.65$, $p<0.001$). For both sexes, leptin levels in FC and ABA animals were significantly less than levels in same-sex CC and EC groups ($p<0.001$, Supplemental Table S4A), as determined by post-hoc analyses. Male EC animals had significantly less serum leptin than male CC animals ($p<0.01$), and female CC rats had significantly less leptin than male CCs ($p<0.001$, Supplemental Table S4A).

Adolescent ghrelin levels

Ghrelin levels in adolescents were not affected by an interaction between sex × condition ($F_{(3,60)}=0.55$, $p>0.64$), or by sex alone ($F_{(1,60)}=0.13$, $p>0.71$), but were altered by condition ($F_{(3,60)}=10.1$, $p<0.001$). Compared to CC females, adolescent FC females had significantly ($p<0.05$) lower ghrelin levels at the time of blood collection according to post-hoc tests (Table 1A). In contrast, male FC rats exhibited significantly lower ghrelin levels only compared to EC males ($p<0.01$), though a non-significant trend was noted between adolescent ABA and EC males ($p=0.051$; Supplemental Table S4A).

Adolescent corticosterone levels

Circulating corticosterone in adolescents was significantly affected by a sex × condition interaction ($F_{(3,60)}=3.01$, $p<0.05$). Follow-up testing with post-hocs revealed that relative to both CC and EC treatment conditions, ABA ($p<0.05$) and FC ($p<0.05$) adolescent females had significantly lower levels of corticosterone (Supplemental Table S4A). In contrast to females, adolescent males displayed no differences in corticosterone levels across treatment conditions, though a non-significant trend ($p=0.056$) was noted between female and male CC rats.

Adult insulin levels

As with adolescents, adult insulin levels did not show an interaction of sex × condition ($F_{(3,48)}=2.06$, $p>0.11$) or a main effect of sex ($F_{(1,48)}=1.79$, $p>0.18$), but a significant main effect of condition was found ($F_{(3,48)}=8.99$, $p<0.001$; Supplemental Table S4B). However, no significant differences between treatment conditions in females were indicated by post-hoc tests. Post-hoc tests did indicate that male adult EC rats had significantly lower insulin levels than CCs ($p<0.05$), whereas male FC animals had insulin levels significantly higher than ABA ($p<0.05$) and EC ($p<0.001$) counterparts (Supplemental Table S4B).

Adult leptin levels

Levels of leptin in adults were not affected by a sex × condition interaction ($F_{(3,52)}=1.18$, $p>0.32$) or a main effect of sex ($F_{(1,52)}=1.81$, $p>0.18$), but were significantly influenced by condition ($F_{(3,52)}=12.1$, $p<0.001$). Adult female ABA animals were revealed by post-hoc testing to have significantly lower leptin levels than CC ($p<0.05$) animals (Supplemental Table S4B), and a non-significant trend was noted between female ABA and EC rats ($p=0.085$). In adult males, post-hoc tests showed that FC ($p<0.01$) and ABA ($p<0.001$) treatment conditions produced significantly lower leptin levels than in adult male CC animals (Supplemental Table S4B).

Adult ghrelin levels

No interaction between sex × condition was observed in adults ($F_{(3,56)}=1.11$, $p>0.35$), but main effects of sex ($F_{(1,56)}=12.9$, $p<0.001$) and condition ($F_{(3,56)}=11.3$, $p<0.001$) were significant. Follow-up post-hocs revealed that adult female EC animals had significantly higher ghrelin levels than FC ($p<0.01$) and ABA animals ($p<0.05$; Supplemental Table S4B), with a non-significant trend ($p=0.10$) noted between adult female EC and CC rats. In adult males, ghrelin levels were significantly higher in EC rats compared to FCs ($p<0.01$; Supplemental Table S4B). Another non-significant trend was noted in adult males between ABA and FC conditions ($p=0.054$).

Adult corticosterone levels

Similar to adolescents, adult rats had a significant sex × condition interaction ($F_{(3,54)}=4.27$, $p<0.01$), with post-hocs suggesting that this was driven by significantly higher corticosterone levels in female FC rats compared to male FCs ($p<0.05$; Supplemental Table S4B). In addition, female ABA animals had significantly lower corticosterone levels than their FC counterparts ($p<0.01$; Supplemental Table S4B).

Physiology measures

Whole blood hematocrit, plasma protein and osmolality, and brain water weight were assessed across animals of both ages from different endpoints (Supplemental Table S5).

Adolescent hematocrit

No significant sex × condition interaction was observed ($F_{(3,93)}=0.41$, $p>0.74$) for adolescent hematocrit, nor was an effect of sex ($F_{(1,93)}=1.66$, $p>0.20$). However, a significant main effect

of condition was detected for hematocrit in adolescent rats ($F_{(3,93)}=26.0$, $p<0.001$). Post-hoc tests indicated that both male and female ABA rats displayed significantly greater hematocrit than CC and EC rats of the same sex ($p<0.001$; Supplemental Table S5A). In males, FC rats also exhibited elevated hematocrit relative to male CC and EC animals ($p<0.05$). A non-significant trend for a difference between hematocrit in female ABA and FC rats was noted ($p=0.06$).

Adolescent plasma protein

Unlike hematocrit, a significant sex × condition interaction was observed for adolescent plasma protein ($F_{(3,94)}=6.38$, $p<0.001$; Supplemental Table S5A). Post-hoc testing revealed that this was primarily driven by a significant reduction in plasma protein in female ABA rats relative to same-sex CC ($p<0.001$), EC ($p<0.01$), and FC ($p<0.01$) animals. Moreover, plasma protein levels in female ABA rats were also significantly lower than those measured in male ABA rats ($p<0.01$).

Adolescent osmolality

A non-significant trend for an interaction between sex × condition was noted for adolescent osmolality ($F_{(3,92)}=2.15$, $p=0.10$; Supplemental Table S5A). A non-significant trend was also present for condition ($F_{(3,92)}=2.26$, $p=0.09$), but no main effect of sex was found ($F_{(1,92)}=0.00$, $p>0.97$).

Adolescent brain water weight

No significant interaction ($F_{(3,60)}=1.61$, $p>0.19$) of sex × condition was observed for adolescent brain water weight, nor were any main effects of sex ($F_{(1,60)}=0.74$, $p>0.39$) or condition ($F_{(3,60)}=0.14$, $p>0.93$; Supplemental Table S5A).

Adult hematocrit

The interaction of sex × condition was not significant for adult hematocrit ($F_{(3,93)}=0.10$, $p>0.95$), nor was a main effect of condition ($F_{(3,93)}=1.49$, $p>0.22$). Though a main effect of sex was significant ($F_{(1,93)}=22.4$, $p<0.001$), post-hoc tests did not indicate any significant differences within treatment groups across sex (Supplemental Table S5B).

Adult plasma protein

Neither a sex × condition interaction ($F_{(3,96)}=0.49$, $p>0.69$), nor a main effect of sex ($F_{(1,96)}=0.16$, $p>0.69$), were significant for adult plasma protein levels. Similar to adult hematocrit, though a main effect of condition was significant for adult plasma protein ($F_{(3,96)}=4.51$, $p<0.01$), post-hoc testing did not reveal any significant differences within sex across treatment condition (Supplemental Table S5B).

Adult osmolality

No significant differences for adult osmolality were observed for a sex × condition interaction ($F_{(3,95)}=1.06$, $p>0.37$), or for main effects of sex ($F_{(1,95)}=0.08$, $p>0.78$) or condition ($F_{(3,95)}=1.03$, $p>0.38$; Supplemental Table S5B).

Adult brain water weight

The interaction of sex × condition was not significant for adult brain water weights ($F_{(3,45)}=0.67$, $p>0.57$). A main effect of sex was also not significant ($F_{(1,45)}=0.15$, $p>0.70$). Despite a main effect of condition reaching significance ($F_{(3,45)}=3.10$, $p<0.05$; Supplemental Table S5B), post-hocs did not indicate any significant differences between treatment conditions in either sex.

Supplemental Tables

Supplemental Table S1
Starting body weights for different endpoints

Table S1A	Day 0 Body Weight (g)					
Adolescents	**Naïve**		**Locomotor**		**Chrono**	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females						
Cage Control	108 ± 14	9	98.5 ± 17	8	102 ± 13	12
Exercise Control	106 ± 11	9	101 ± 15	9	89 ± 14	8
Food Control	111 ± 12	8	96.6 ± 18	8	101 ± 18	10
ABA	110 ± 14	11	102 ± 16	9	103 ± 10	12
Males						
Cage Control	130 ± 14	9	99.6 ± 8.5	10	108 ± 11	8
Exercise Control	123 ± 14	10	113 ± 17	10	113 ± 23	10
Food Control	126 ± 12	9	106 ± 20	8	117 ± 18	9
ABA	127 ± 13	11	107 ± 21	10	107 ± 15	9

		p value		*p value*		*p value*
Interaction	$F_{(3,68)}=0.21$	0.89	$F_{(3,64)}=0.38$	0.77	$F_{(3,70)}=1.58$	0.20
Sex	$F_{(1,68)}=35.1$	0.001	$F_{(1,64)}=3.06$	0.08	$F_{(1,70)}=12.4$	0.001
Condition	$F_{(3,68)}=0.53$	0.66	$F_{(3,64)}=0.77$	0.51	$F_{(3,70)}=0.78$	0.51

Table S1B	Day 0 Body Weight (g)					
Adults	**Naïve**		**Locomotor**		**Chrono**	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females						
Cage Control	269 ± 20	9	258 ± 22	10	244 ± 10	6
Exercise Control	260 ± 16	8	244 ± 24	8	246 ± 13	7
Food Control	273 ± 15	7	244 ± 24	11	242 ± 13	6
ABA	262 ± 15	10	264 ± 30	8	243 ± 16	6
Males						
Cage Control	437 ± 33	7	389 ± 43	9	367 ± 16	6
Exercise Control	447 ± 24	7	387 ± 33	8	366 ± 24	8
Food Control	444 ± 30	7	381 ± 69	12	377 ± 25	7
ABA	446 ± 25	10	409 ± 67	9	371 ± 21	9

		p value		*p value*		*p value*
Interaction	$F_{(3,57)}=0.70$	0.55	$F_{(3,67)}=0.09$	0.97	$F_{(3,47)}=0.40$	0.75
Sex	$F_{(1,57)}=973$	0.001	$F_{(1,67)}=182$	0.001	$F_{(1,47)}=626$	0.001
Condition	$F_{(3,57)}=0.16$	0.92	$F_{(3,67)}=1.09$	0.36	$F_{(3,47)}=0.09$	0.96

Supplemental Table S2
Time course analyses of drug-naïve body, food, and water weights (see also Supplemental Fig. S1)

Table S2A: Adolescents							
Sex × Condition	p	Sex × Day	p	Condition × Day	p	Sex × Condition × Day	p
Body weights							
F(4,272)=0.86	0.46	F(4,272)=14.6	0.001	F(12,272)=367	0.001	F(12,272)=5.80	0.001
Food consumption (as percent of body weight)							
F(3,203)=0.51	0.68	F(3,203)=1.22	0.30	F(9,203)=0.81	0.001	F(9,203)=0.81	0.60
Water consumption (as percent of body weight)							
F(3,204)=0.45	0.72	F(3,204)=0.45	0.66	F(9,204)=4.54	0.001	F(9,204)=1.60	0.14

Table S2B: Adults							
Sex × Condition	p	Sex × Day	p	Condition × Day	p	Sex × Condition × Day	p
Body weights							
F(3,285)=0.98	0.41	F(5,285)=2.50	0.07	F(15,285)=18.7	0.001	F(15,385)=2.57	0.01
Food consumption (as percent of body weight)							
F(3,227)=1.21	0.32	F(4,227)=0.69	0.56	F(12,227)=6.21	0.001	F(12,227)=0.99	0.45
Water consumption (as percent of body weight)							
F(3,228)=1.68	0.18	F(4,228)=0.37	0.80	F(12,228)=1.61	0.10	F(12,228)=0.93	0.51

Supplemental Table S3
Drug-naïve running wheel AUCs

Table S3								
	Sex × Condition	**p**	**Sex × Day**	**p**	**Condition × Day**	**p**	**Sex × Condition × Day**	**p**
Adolescents								
	F(1,108)=0.02	0.88	F(3,108)=0.55	0.65	F(3,108)=13.1	0.001	F(3,108)=0.23	0.88
Adults								
	F(1,116)=1.24	0.27	F(4,116)=2.97	0.02	F(4,116)=0.33	0.85	F(4,116)=0.41	0.80

Supplemental Table S4
Blood hormone levels

Table S4A Adolescents

	Insulin (ng/mL)		Leptin (ng/mL)		Ghrelin (ng/mL)		Corticosterone (ng/mL)	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females								
Cage Control	0.443 ± 0.22	7	1.37 ± 0.41	8	2.23 ± 0.35	8	38.8 ± 16	8
Exercise Control	0.454 ± 0.24	8	1.31 ± 0.74	8	2.11 ± 0.53	8	39.9 ± 18	8
Food Control	1.02 ± 0.29^{EE,**}	7	0.176 ± 0.18^{EEE,***}	8	1.46 ± 0.35*	8	9.57 ± 5.3^{E,*}	7
ABA	0.742 ± 0.22	9	0.00 ± 0.00^{EEE,***}	8	1.75 ± 0.29	9	11.0 ± 9.9^{E,*}	8
Males								
Cage Control	0.599 ± 0.26	9	2.88 ± 0.72$^{\#\#\#}$	9	2.14 ± 0.57	9	12.3 ± 9.3	9
Exercise Control	0.322 ± 0.18	8	1.97 ± 0.70**	9	2.39 ± 0.85	9	28.7 ± 36	9
Food Control	1.09 ± 0.39 ^{EEE,*}	9	0.251 ± 0.29^{EEE,***}	9	1.53 ± 0.34^{EE}	9	14.3 ± 11	9
ABA	0.684 ± 0.39	10	0.0466 ± 0.081^{EEE,***}	9	1.66 ± 0.21	8	15.4 ± 12	10

Table S4B: Adults

	Insulin (ng/mL)		Leptin (ng/mL)		Ghrelin (ng/mL)		Corticosterone (ng/mL)	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females								
Cage Control	0.586 ± 0.26	8	2.79 ± 0.96	8	1.79 ± 0.38	9	107 ± 53	9
Exercise Control	0.551 ± 0.25	7	2.64 ± 1.1	8	2.37 ± 0.52	8	109 ± 89	8
Food Control	0.980 ± 0.44	7	1.81 ± 1.3	7	1.44 ± 0.50^{EE}	7	180 ± 125$^{\#}$	7
ABA	0.711 ± 0.33	7	0.978 ± 0.57*	8	1.72 ± 0.48^{E}	10	47.4 ± 40^{FF}	8
Males								
Cage Control	0.960 ± 0.33	7	4.05 ± 1.8	7	1.33 ± 0.36	7	41.1 ± 15	7
Exercise Control	0.410 ± 0.093*	6	2.75 ± 1.2	7	1.86 ± 0.35	7	90.1 ± 35	7
Food Control	1.21 ± 0.44^{EEE}	6	1.55 ± 0.57**	6	0.975 ± 0.39^{EE}	6	53.5 ± 28	6
ABA	0.687 ± 0.18^{F}	8	1.43 ± 0.94***	9	1.65 ± 0.29	10	85.5 ± 64	10

*p<0.05, **p<0.01, ***p<0.001 vs. same-sex cage control; ^{E}<0.05, ^{EE}<0.01, ^{EEE}<0.001 vs. same-sex exercise control; ^{F}<0.05, ^{FF}<0.01 vs. same-sex food control; $^{\#}$<0.05, $^{\#\#\#}$<0.001 vs. same condition in opposite sex.

Supplemental Table S5
Physiology measures

Table S5A: Adolescents

	Hematocrit (%)		Plasma Protein (g/100mL)		Osmolality (mOsm/kg)		Brain Water Weight (%)	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females								
Cage Control	42.1 ± 2.8	11	4.88 ± 0.55	11	284 ± 38	11	77.8 ± 0.93	8
Exercise Control	41.2 ± 2.5	8	4.80 ± 0.54	8	291 ± 11	8	78.0 ± 0.68	8
Food Control	46.4 ± 4.9	9	4.68 ± 0.70	10	310 ± 36	10	77.5 ± 0.63	7
ABA	52.6 ± 5.3***,EEE	13	3.70 ± 0.81***,EE,FF	13	306 ± 36	13	78.7 ± 1.4	7
Males								
Cage Control	43.2 ± 3.9	18	4.60 ± 0.48	18	300 ± 17	18	77.8 ± 1.9	9
Exercise Control	43.0 ± 5.3	15	4.42 ± 0.54	15	292 ± 19	15	77.8 ± 1.1	11
Food Control	48.9 ± 5.2*,E	13	4.26 ± 0.65	13	286 ± 39	12	78.1 ± 0.76	10
ABA	52.2 ± 6.1***,EEE	14	4.59 ± 0.71##	14	314 ± 21	13	77.4 ± 1.4	8

*p<0.05, ***p<0.001 vs. same-sex cage control; ^{E}<0.05, ^{EE}<0.01, ^{EEE}<0.001 vs. same-sex exercise control; ^{FF}<0.01 vs. same-sex food control; $^{\#\#}$<0.01 vs. same condition in opposite sex.

Table S5B: Adults

	Hematocrit (%)		Plasma Protein (g/100mL)		Osmolality (mOsm/kg)		Brain Water Weight (%)	
	Mean ± SD	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*	*Mean ± SD*	*N*
Females								
Cage Control	48.1 ± 5.6	16	5.38 ± 1.1	16	311 ± 20	16	76.5 ± 0.91	6
Exercise Control	48.7 ± 4.0	11	5.59 ± 0.82	11	311 ± 22	11	77.1 ± 0.49	7
Food Control	47.9 ± 5.8	14	5.05 ± 0.60	15	308 ± 17	15	77.3 ± 0.40	9
ABA	50.7 ± 6.7	11	4.75 ± 0.61	11	316 ± 18	11	77.6 ± 0.43	6
Males								
Cage Control	53.1 ± 6.5	12	5.53 ± 0.90	12	310 ± 17	12	76.6 ± 1.2	7
Exercise Control	55.4 ± 5.3	9	5.39 ± 1.0	9	304 ± 21	9	77.3 ± 0.63	5
Food Control	54.0 ± 9.7	15	4.74 ± 0.78	16	318 ± 15	15	77.0 ± 0.29	7
ABA	57.5 ± 5.5	13	4.85 ± 0.94	14	317 ± 15	14	77.1 ± 0.81	6

Supplemental Table S6
Post-saline injection AUCs prior to cocaine-induced locomotion

Table S6: Saline AUCs	Adolescent		Adult	
	Mean ± SD	N	Mean ± SD	N
Females				
Cage Control	113 ± 63	8	146 ± 93	10
Exercise Control	103 ± 42	9	125 ± 74	8
Food Control	132 ± 74	8	95 ± 70	11
ABA	104 ± 54	9	133 ± 77	8
Males				
Cage Control	112 ± 49	10	115 ± 72	9
Exercise Control	85 ± 46	10	84 ± 51	8
Food Control	152 ± 140	8	75 ± 55	12
ABA	71 ± 34	10	56 ± 25	9

		p		p
Interaction	$F_{(3,64)}=0.51$	0.68	$F_{(3,67)}=0.62$	0.61
Sex	$F_{(1,64)}=0.26$	0.61	$F_{(1,67)}=7.28$	0.01*
Condition	$F_{(3,64)}=2.24$	0.09	$F_{(3,67)}=1.70$	0.18

*no significant differences within treatment condition across sex indicated by Tukey-Kramer's post-hoc test

Supplemental Table S7
Chronoamperometry 100 pmol DA amplitudes

Table S7: 100 pmol DA Amplitudes				
	Adolescents		Adults	
	Mean ± SD	*N*	*Mean ± SD*	*N*
Females				
Cage Control	22.9 ± 11	12	22.9 ± 12	6
Exercise Control	18.1 ± 7.9	8	25.6 ± 8.7	7
Food Control	19.3 ± 7.3	10	23.3 ± 12	6
ABA	26.6 ± 9.4	11	21.6 ± 8.8	6
Males				
Cage Control	25.1 ± 9.2	8	15.1 ± 8.6	6
Exercise Control	21.7 ± 10	9	17.0 ± 6.6	8
Food Control	19.9 ± 6.5	9	16.9 ± 8.7	7
ABA	18.8 ± 6.2	9	20.5 ± 11	9

|		p		p
Interaction	$F_{(3,68)}=1.69$	0.18	$F_{(3,47)}=0.43$	0.73
Sex	$F_{(1,68)}=0.04$	0.85	$F_{(1,47)}=5.30$	0.03*
Condition	$F_{(3,68)}=1.19$	0.32	$F_{(3,47)}=0.16$	0.92

*no significant differences within treatment condition across sex indicated by Tukey-Kramer's post-hoc test

Supplemental Table S8
Chronoamperometry rate of clearance versus pmol DA

Table S8								
	Sex × Condition	p	Sex × pmol DA	p	Condition × pmol DA	p	Sex × Condition × pmol DA	p
Adolescents								
	F(3,340)=2.74	0.05	F(15,340)=1.07	0.35	F(15,340)=2.20	0.04	F(15,340)=1.52	0.17
Adults								
	F(3,235)=0.37	0.78	F(5,235)=0.52	0.58	F(15,235)=0.69	0.64	F(15,235)=0.40	0.86

Supplemental Figures
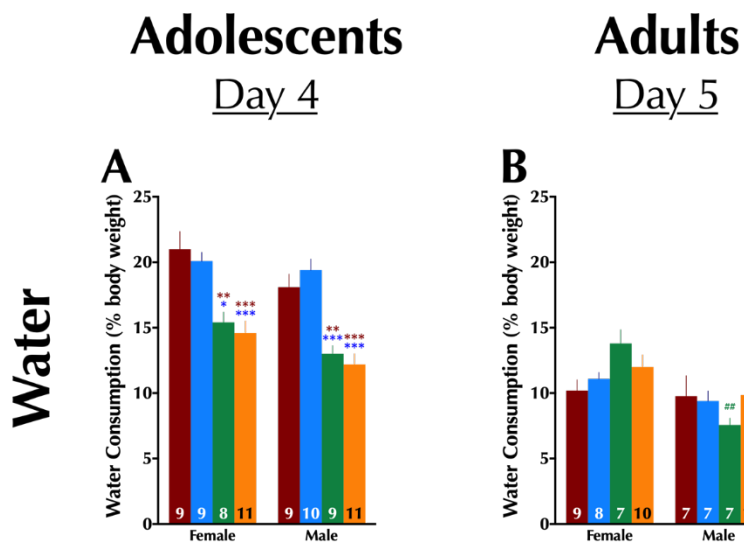
Supplemental Figure S1
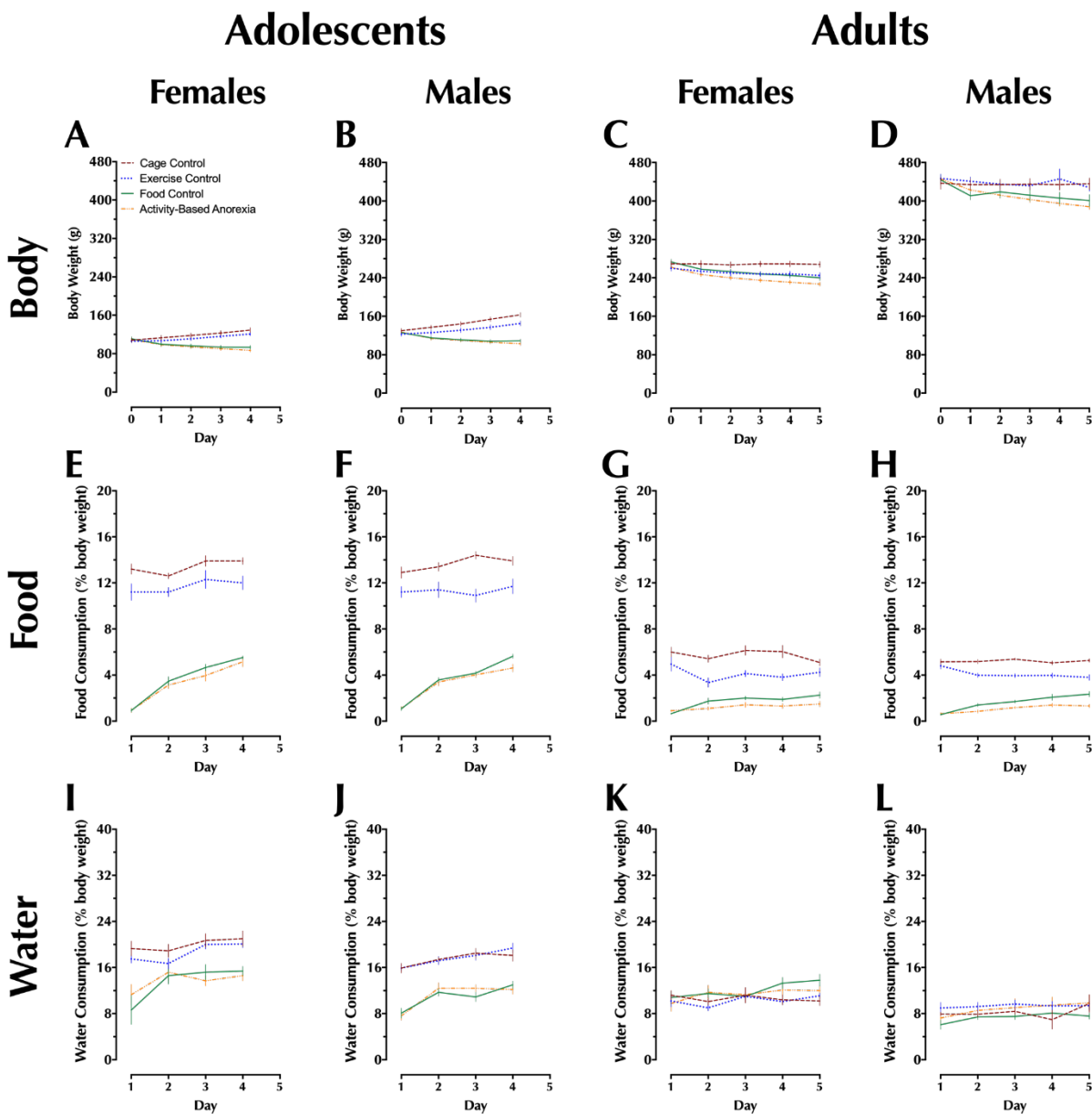
Supplemental Figure S1
Time course data for weight and consumption measures
Because animals used for chronoamperometry and cocaine-induced locomotion were removed prior to completion of their full 4ᵗʰ (adolescents) or 5ᵗʰ (adults) day, only data from drug-naïve rats are shown here. Adolescent **A**) female and **B**) male and adult **C**) female and **D**) male body weights; adolescent **E**) female and **F**) male and adult **G**) female and **H**) male food consumption over the past 24 h as a percentage of the individual animal's body weight; adolescent **I**) female and **J**) male and adult **K**) female and **L**) male water consumption over the past 24 h as a percentage of the individual animal's body weight. Data are graphed as mean ± SEM. Ns: female adolescent (8-11), male adolescent (9-11), female adult (7-10), male adult (7-10)

Supplemental Figure S2

Supplemental Figure S2
Final day water consumption in drug-naïve rats
The final day in the experimental paradigm for adolescents was day 4, whereas the final day for adults was day 5. Because animals used for chronoamperometry and cocaine-induced locomotion were removed prior to completion of their full 4th (adolescents) or 5th (adults) day, only data from drug-naïve rats are shown here. **A**) adolescent and **B**) adult water consumption over the past 23 h as a percentage of the individual animal's body weight. Data are graphed as mean + SEM. *p<0.05, **p<0.01, ***p<0.001 vs same-sex condition, indicated by color of asterisk(s), within the same age group. ##p<0.01 vs. same condition in females of same age group. Ns are indicated at base of each bar.

Supplement References

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