# Vulnerable and Resilient Phenotypes in a Mouse Model of Anorexia Nervosa

## *Supplemental Information*

CONTENTS

SUPPLEMENTAL DATA

## ***DAT KD in wheel control condition (WH)***

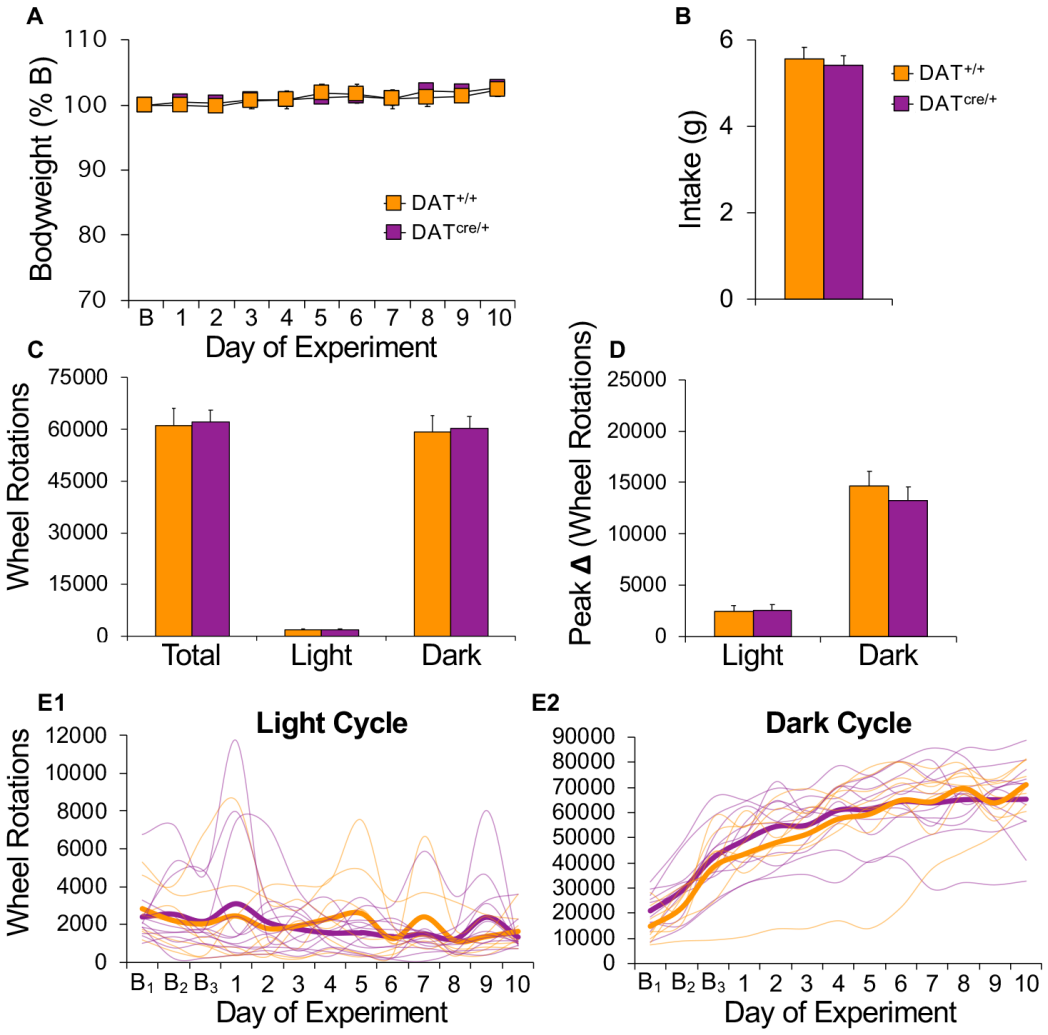


**Figure S1.** *No differences between hyperdopaminergic DAT$^{cre/+}$ KD mice and control DAT$^{+/+}$ WT littermates in the wheel control (WH) condition.* **(A)** Bodyweight, **(B)** average food intake and **(C)** average wheel running across 10 days of unlimited access to food and running wheel. **(D)** Maximum increase in wheel running across two consecutive days (averaged by group) during the light cycle and dark cycle. Wheel running of individual mice (light traces) and group mean (bold traces) during the **(E1)** light and **(E2)** dark cycle of each experimental day. n=8 (DAT$^{+/+}$), n=12 (DAT$^{cre/+}$). Error bars, ±SEM.

## ***Basal dopamine in adolescent and young adult mice***

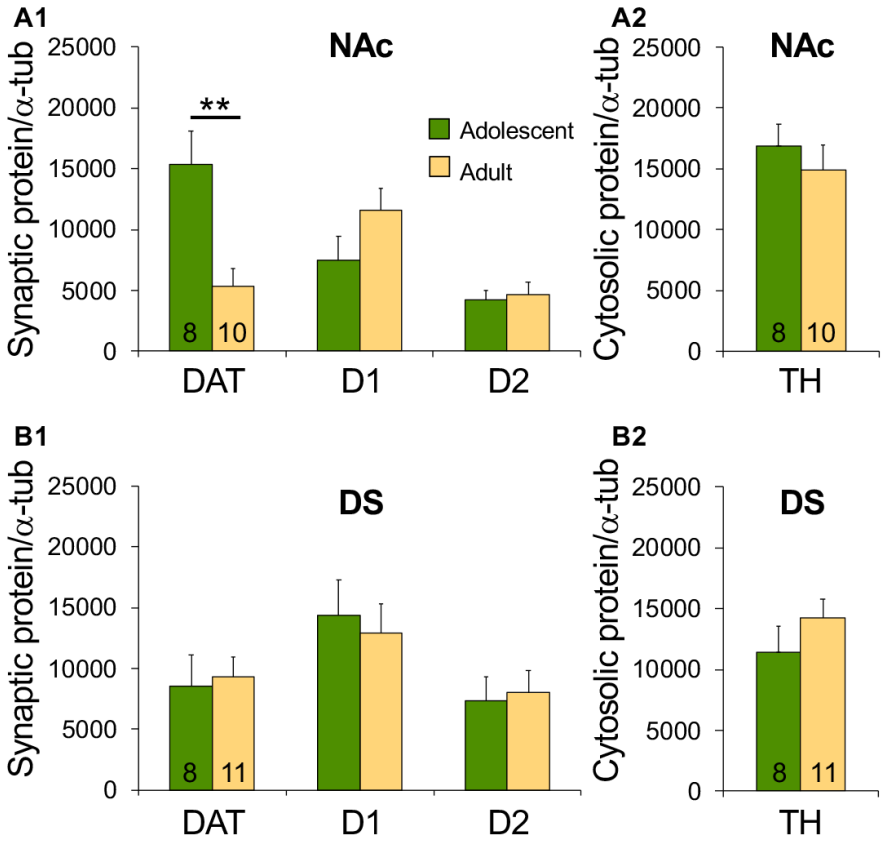


**Figure S2.** *Basal expression of dopamine markers in adolescent and young adult female C57Bl/6N mice.* Dopamine transporter (DAT), dopamine D1 receptor (D1), dopamine D2 receptor (D2) and tyrosine hydroxylase (TH) in the **(A)** nucleus accumbens (NAc) and **(B)** dorsal striatum (DS). Number of animals indicated inside bars. NAc: **(A1)** DAT, $t_{(16)} = 3.42$, $^{**}p < 0.01$; D1, $t_{(16)} = 1.52$, $p = 0.15$; D2, $t_{(16)} = 0.29$, $p = 0.77$, **(A2)** TH, $t_{(16)} = 0.67$, $p = 0.51$, n=8 (adolescent), n=10 (adult). DS: **(B1)** DAT, $t_{(17)} = 0.27$, $p = 0.79$; D1, $t_{(17)} = 0.38$, $p = 0.71$; D2, $t_{(17)} = 0.23$, $p = 0.82$, **(B2)** TH, $t_{(17)} = 1.10$, $p = 0.29$, n=8 (adolescent), n=11 (adult). Error bars, ±SEM.

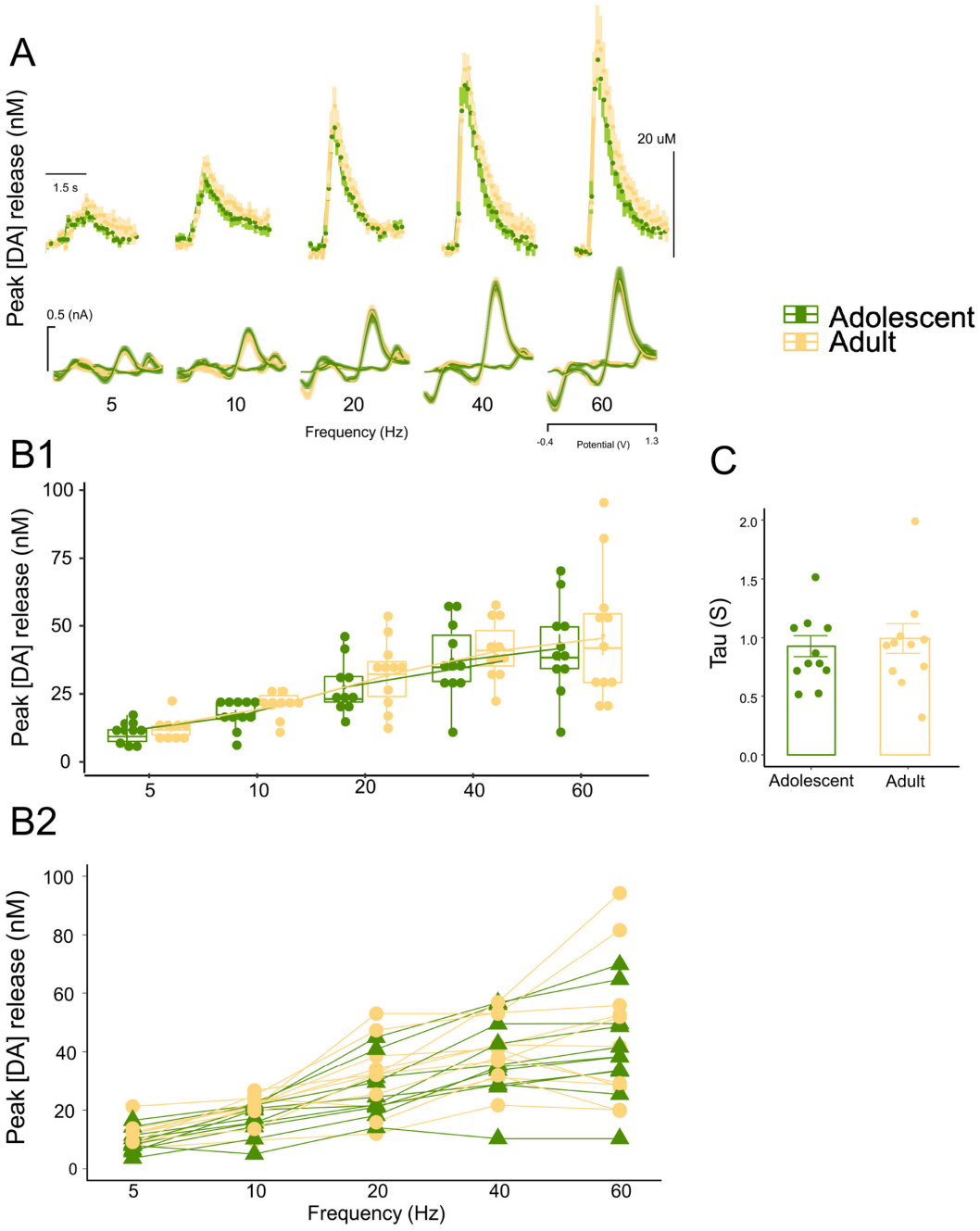


**Figure S3.** *Fast-scan cyclic voltammetry comparing adolescent and young adult female mice.* **(A)** Averaged evoked dopamine release (top) and cyclic voltammagrams recorded in the nucleus accumbens at the frequencies indicated (5 pulses). Timecourse of traces, LME age x time, $F_{(1,32)}$ = 0.78, p = 0.38. **(B1-B2)** Peak dopamine as a function of frequency in box/line plot (B1, LME, age: $F_{(1,19)}$ = 1.88, p = 0.18, age x frequency $F_{(1,20)}$ = 0.03, p = 0.86) and showing individual traces for each mouse (B2). **(C)** DA clearance following stimulated release (tau, $F_{(1,20)}$ = 0.18, p = 0.68). n=11 (adolescent), n=11(adult). Error bars, ±SEM.

# SUPPLEMENTAL DISCUSSION

## ***Vulnerability in food-restricted control mice***

Early studies conducted by Routtenberg and Kuznesof in 1967 (1) found that food restricted adult male albino rats without access to a running wheel (FR control group) maintain a stable body weight in an average of 15.9 days. The idea that catastrophic weight loss only results from the combination of food restriction and wheel running is so fundamental that it is reflected in the name of the paradigm, *activity-based* anorexia. This idea has been reinforced in studies performed across several decades, including recent work by Foldi et al. (2) and Scharner et al (3) who showed that food restriction (90 minutes/day unlimited food access) leads to less weight loss in female Sprague-Dawley rats (130-180g) when the wheel is locked. However, this was not found in two other recent studies in which adolescent female Sprague-Dawley rats were given 60 minutes/day of unlimited food access. Similar to what we show in Figure 4A (days 1-5), they found no differences in weight loss between those with and without a wheel (4,5). The most obvious methodological difference between those studies is the amount of time food was available (90 vs. 60 minutes), with less time potentially intensifying the adaptive challenge to food restricted rats (no wheel). Indeed, Klenotich et al. (6) and others have reported that animals survive longer in the ABA model the longer food is available. The strain of the animal is another important factor, as indicated by the finding that Balb/cJ mice given 2 hours of unlimited food access (same amount of time used in our study) all lose enough weight to require removal from the model within 4 days, regardless of wheel access (6). This is in contrast to

the resilient C57/Bl6 mice used in our study. Although these findings are at odds with the original description of the ABA model, the papers above indicate that a number of variables determine how animals respond to food restriction. These include (but are not limited to) age, species, strain, and length of time food is available. Therefore, the inability to adapt to restrictive feeding is not always dependent upon wheel running. However, as we show, when animals have enough time to eat, wheel running serves to tip the balance, accelerating the deleterious effects of food restriction. Setting experimental parameters so that vulnerability appears in both the ABA and FR groups may be more comparable to the human phenotype than when vulnerability is only observed in the ABA group.

***Adolescent vs young adult mice: A comparison of dopamine function***

The increased DAT we observe in the NAc of adolescent mice does not lead to more rapid clearance in evoked dopamine (Fig S3C). While visually the traces suggest a slightly more rapid rate of clearance (Fig S2A1), this difference is not statistically significant ($p = 0.38$), is of small magnitude and notably, is not apparent at 20 Hz, the frequency of phasic dopamine where DAT is most influential on and relevant to dopamine signaling.

To our knowledge, no other studies have examined changes in striatal dopamine from adolescence to young adulthood. The few studies done in rats have not, in aggregate, generated a clear picture of how striatal dopamine changes during this developmental time period. Consistent with our FSCV results, a study by Matthews et al (7) in male Sprague-Dawley rats found no difference between

adolescents (PND 34-38) and adults (PND 70-80) in basal extracellular dopamine in either the nucleus accumbens (NAc) or dorsal striatum, as measured with microdialysis. They also observed differential DAT expression in the NAc, but their findings were the opposite of ours, with the adults expressing higher levels of protein. However, similar to our results, this difference in DAT did not result in an increase in basal extracellular dopamine or an increased response to amphetamine challenge. In a receptor binding study, Teichner et al (8) observed that adolescent (PND 40) male rats had higher levels of D1 and D2 binding in the NAc and dorsal striatum than young adults (PND 60), though this was not found in female rats in a subsequent study (9).

## SUPPLEMENTAL METHODS

### ***Detailed ABA protocol***

ABA experiments were conducted in a room dedicated to ABA testing, where mice were never exposed to male mice. Wheel running was tracked with wireless running wheels (ENV-044, Med Associates, Inc., St. Albans, VT) that continuously transmitted running data to a computer. On each day of food restriction, pre-weighed pellets were place in the overhead food bin of the wire lid and ABA and FR mice were given unlimited access to food during the first 2 hours of the dark cycle. Fresh pellets were weighed and provided to ABA and FR mice each night of food restriction.

*Groups:* For experiments with adolescent and young adults, female mice were evenly distributed by body weight into four groups: activity-based anorexia (ABA),

wheel control (WH), food restricted control (FR) and homecage control (HC). Running in the WH and ABA groups was compared to evaluate how food restriction changes running behavior. Food intake in the FR and ABA groups was compared to evaluate how running affects food intake when the availability of food is restricted. For experiments with the heterozygote DAT-cre mice, separate cohorts of female KD and WT littermates were tested in each condition (ABA, FR, WH).

*Timeline:* Mice were individually housed with unlimited food and wheel access (locked or freely turning based on group assignment). They were given the next day to adapt to their housing conditions and were not touched. Baseline measurements were taken on the subsequent three days (body weight, food intake, wheel running). On baseline day 3, food was removed from ABA and FR mice 2 hours after the onset of the dark cycle. Starting the next day (ABA day 1), ABA and FR mice were given unrestricted access to food during the first 2 hours of the dark cycle, during which time the wheels remained in the cage (freely moving for ABA; locked for FR). Cages were checked daily to ensure mice were not hiding food. Mice were removed from the experiment when they lost 25% of their baseline weight, as measured on baseline day 3, and were characterized as “vulnerable.” Mice that survived in the model throughout the duration of the experiment were characterized as “resilient.”

## ***Characterization of dopamine transporter knockdown mice***

### Mice

Both males and females (postnatal day, PND 63-96) were used for characterizing the $DAT^{cre/+}$ mice with real-time quantitative PCR, Western blots, immunohistochemistry, and fast-scan cyclic voltammetry.

### RNA extraction and reverse transcription quantitative PCR (RT-qPCR)

We measured gene mRNA in brain dissections of the ventral midbrain by quantitative real time polymerase chain reaction (qRT-PCR) using mRNA purification kits, RT kits and microarray plates with primers designed by Qiagen (Germantown, MD). The primers were custom designed to recognize cDNA for DAT (gene *Slc6a3*), D1 (*gene Drd1a*) and D2 (*gene Drd2*) DA receptors, tyrosine hydroxylase (TH; *gene Th*), vesicular monoamine transporter 2 (VMAT2, *gene Slc18a2*). Adult male and female $DAT^{cre/+}$ and $DAT^{+/+}$ littermate controls were anesthetized with ketamine (90 mg/kg) + xylazine (7 mg/kg) and sacrificed by cerebral dislocation. A 1mm section of the ventral midbrain was dissected -2.79 to -3.79 mm posterior to Bregma, as shown in figure 8A, and homogenized in 1ml of Phenol/guanidine-based QIAzol lysis reagent. The mRNA was extracted and purified using the RNeasy Lipid Tissue Mini kit with an extra DNAse digestion step to eliminate contamination from genomic DNA (RNase-Free DNase set), following the manufacturer's protocol. Before the reverse transcription step all samples were normalized to the same mRNA concentration using NanoDrop 1000 Spectrophometer (Thermo Scientific, Waltham, MA). The 260:280 nm absorbance

ratio was measured to assess RNA quality; samples were excluded if the ratio was outside the range of 2.0 ± 0.2, or if the RNA concentration was too low. The reverse transcription was carried out using the RT First Strand Kit and following the manufacturer's protocol. The cDNA product was then mixed with SYBR green using the RT SYBR Green qPCR Mastermix and put in PCR plates with the custom designed primers. Quantitative PCR (qPCR) was performed as using a Opticon 2 DNA Engine (BioRad, Hercules, CA) and using universal PCR conditions (65-59°C touch down, followed by 40 cycles of 95°C for 15 s, 55°C for 10 s, and 72°C for 10 s). cDNA was amplified in a 25 µL reaction and primer suitability was determined using standard curve analysis and melting curve analysis. The cycle threshold (Ct) values were normalized to GAPDH (ΔCt). Relative copy number was obtained by exponentiation of ΔCt values (function $2^{-\Delta CT}$).

DAT protein determination

Protein analysis was performed using the automated Simple-Western assay (ProteinSimple, San Jose, CA). Adult male and female $DAT^{cre/+}$ mice and $DAT^{+/+}$ littermate controls were sacrificed by cervical dislocation. Dorsal striatum tissue samples were dissected as shown in figure 8B. Protein was extracted by incubation on ice for 60 min in 100 µl protein extraction buffer prepared from a Bicine/CHAPS extraction kit with proteases and phosphatases inhibitors (# CBC 403, Protein Simple, San Jose CA). After incubation, the lysates were centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was transferred to new tubes and frozen at −20°C pending subsequent analysis. Tissue samples were diluted to

a concentration of 1.0 mg/mL in lysis buffer and 4 volumes of it were mixed with 1 volume of a 5x master mix prepared from designated kits (Anti Rabbit Detection Module #DM-001) according to supplier instructions (ProteinSimple). The kits contained sample buffer, DTT, and internal molecular weights standards in all samples. The samples were boiled for 5min and were then loaded (5μl/well) in the first row of ProteinSimple provided plates (WES Separation Module #SM-W004). The rest of the reagents were loaded onto well rows in the following order: antibody diluent, primary antibody (1:100 rabbit anti-DAT, Abcam Cat# ab111468), HRP conjugated anti-rabbit antibody, and a luminol-S/peroxide chemiluminescent detection mixture. The plate and a capillary cartridge was loaded onto the WES instrument and, after the run started, all steps of the immuno-electrophoresis proceeded automatically. Chemiluminescence was measured along the length of the capillary over time, and analyzed using ProteinSimple Compass software.

DAT protein levels were calculated relative to the total amount of protein in the sample, which was measured separately using a specific kit for in-capillary electrophoretic quantitation of fractionated protein bands (Total Protein Detection Module #DM-TPO1, ProteinSimple). This WES procedure includes a catalytic protein biotinylation process of all the proteins in the loaded samples. Proteins were then size-based separated by electrophoresis, bond to a streptavidin-HRP conjugate and finally detected by luminol-S/peroxide chemiluminescent to obtain a total protein density measurement. To determine the linearity-range of protein content among various samples we ran a premeasured standard in parallel to brain extracts. The premeasured BSA (bovine serum albumin) standards underwent the

same in-capillary quantitation. The regression line of the densities of various brain nuclei samples, plotted against BSA standard dilutions, shows that linearity exists between 1000ng to 10ng of brain protein/sample, restricting its fidelity to this range. The DAT quantities detected by immuno-electrophoresis in corresponding sample aliquots, expressed in arbitrary chemiluminescent units, were corrected by the arbitrary units of total protein of bands in respective capillaries. Finally, the DAT protein levels were present as % of DAT-WT control values.

Immunohistochemistry

Adult $DAT^{cre/+}$ mice and $DAT^{+/+}$ littermate controls were anesthetized with ketamine (90 mg/kg) + xylazine (7 mg/kg) and perfused with cold PBS followed by 4% paraformaldehyde (PFA), the brains removed, post-fixed overnight in 4% PFA, and cut at 50 µm with a vibrating microtome (Leica VT1200S). Coronal slices were collected into a cryoprotectant solution (30% glycerol, 30% ethylene glycol in 0.1 M Tris HCl [pH 7.4]) and kept at −20°C until processing. Sections were washed in PBS (100 mM; pH 7.4) and incubated in glycine (100 mM) for 30 min to quench aldehydes. Non-specific binding was blocked with 10% normal goat serum (NGS; Millipore) in 0.1% PBS Triton X-100 for 2 hr (PBS-T). The brains were then incubated in anti-DAT primary antibody (1:5,000 dilution; rabbit polyclonal, Abcam Cat# ab111468) for 2 days at 4°C. Primary antibody was diluted in 0.02% PBS-T and 2% NGS solution. Sections were then washed with PBS and incubated in a secondary antibody (1:200 dilution; anti-rabbit Alexa Fluor 594 made in goat; ThermoFisher Scientific Cat# A32740) for 45 min at room temperature. Secondary

antibody was diluted in 0.02% PBS-T solution. Sections were then further washed in PBS and mounted on slides, cover slipped with Prolong Gold aqueous medium (ThermoFisher Scientific) and stored at 4°C. Fluorescence images were acquired with Axiovert 35M (Zeiss) epifluorescence microscope.

Fast-scan cyclic voltammetry (slice, characterizing KD mice)

Recordings were done in adult $DAT^{cre/+}$ and $DAT^{+/+}$ littermate controls in 300 µm coronal slices taken from the striatum. Animals were anesthetized with ketamine (90 mg/kg) + xylazine (7 mg/kg) and brains removed into ice-cold high-glucose aCSF saturated with carbogen (95% $O_2$5% $CO_2$) and sectioned using a vibratome. The composition of the high-glucose aCSF (cutting solution) was, in mM: 75 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4$, 0.7 $CaCl_2$, 2 $MgCl_2$ and 100 glucose, adjusted to pH 7.4. After 1 hr incubation in high-sucrose aCSF at room temperature, slices were placed in a recording chamber with continuous perfusion of standard aCSF (in mM: 125 NaCl, 2.5 KCl, 25 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, and 25 glucose, pH 7.4) saturated with carbogen and maintained at 30–32°C (TC 344B Temperature Controller, Warner Instruments). DA release was evoked by electric stimulation either at 0.1 Hz (single stimulation) or at 20 Hz (5 pulses, burst stimulation). DA release was measured in the nucleus accumbens (NAc), dorsomedial (DMS) and dorsolateral (DLS) striatum using carbon fiber working electrodes. A triangle wave (−450 to +800 mV at 312.5 V/sec vs. Ag/AgCl) was applied to the recording electrode at 10 Hz. Fibers were conditioned in the brain slice prior to recording by cycling the fiber for 20–30 min or until the current

stabilized. Working electrodes were calibrated against 1 μM DA at the end of the experiment and recorded current reported as DA concentration. Recordings were filtered at 10 kHz with a 4-pole Bessel filter, digitized at 25 kHz (ITC-18) using Igor Pro 6 (WaveMetrics) and analyzed with MATLAB R2014b (MathWorks).

## ***Dopamine Function in Adolescent and Young Adult Female Mice***

### Mice

Female C57BL/6N mice were purchased at PND 28 and 56 (Taconic Biosciences, Germantown, NY).

### Sample Preparation and Tissue Fractions

Brains were removed from group housed adolescent (PND 43) and young adult (PND 71) mice and immediately frozen on dry ice for Western blot analysis. Dorsal striatum and nucleus accumbens were dissected under a dissecting microscope and prepared into cytosolic and synaptic fractions. Tissue was thawed and immersed in a TEE (Tris 50 mM; EDTA 1 mM; EGTA 1 mM) buffer containing a SigmaFast, protease inhibitor cocktail (Sigma Aldrich) diluted to contain AEBSF (2 mM), Phosphoramidon (1 μM), Bestatin (130 μM), E-64 (14 μM), Leupeptin (1 μM), Aprotinin (0.2 μM), and Pepstatin A (10 μM). Tissue was homogenized in 150 μl of the TEE-homogenization buffer using 20 pumps with a motorized pestle. Homogenates were transferred to Eppendorf tubes and centrifuged at 3,000 g (5 min at 4$^{o}$ C), to remove pellet. The resulting supernatant was centrifuged at 100,000 g for 30 min. After ultracentrifugation, the supernatant was collected and

stored as the cytosolic fraction. The remaining pellet was resuspended in 75 μl of homogenizing TEE buffer containing 0.001% Triton X-100, incubated on ice for 1h and then centrifuged at 100,000 g for 1h at 4° C. The resulting pellet was resuspended in 25 μl of TEE buffer and stored as the synaptic fraction. The Pierce bicinchoninic acid assay (BCA) (Thermo Scientific, Rockford, IL) was used to determine protein concentration for each sample. Samples were reduced with 4x Laemmli sample buffer equivalent to 25% of the total volume of the sample and then boiled and stored frozen at -80$^{o}$C.

Immunoblots

Samples (13.3 μg) were loaded onto a Tris/Gly 4-20% midi™ gel to resolve alpha-tubulin (55 kDa), D1R (49 kDa), D2R (49 kDa), DAT (69 kDa), and TH (62 kDa). Every gel contained 3-4 lanes loaded with the same control sample, (all brain samples, ABS). ABS were used to standardize protein signals between gels. Gels were transferred to nitrocellulose membranes in IBlot® Dry Blotting System (Life Technologies; Carlsbad, CA) for 7 minutes. Nitrocellulose membranes were then incubated in blocking solution containing 5% sucrose in Tris Buffered Saline with Tween-20 (TBST; 0.1% Tween-20 in TBS) for 30 minutes at room temperature. Samples were incubated with the following primary antibodies overnight: alpha-tubulin (1:2000; Millipore, Burlington, MA), D1R (1:1000; AbCam, Cambridge, MA), D2R (1:1000; AbCam, Cambridge, MA), DAT (1:1000, Millipore, Burlington, MA), TH (1:2000; Millipore, Burlington, MA). Membranes were washed in TBST for 20 min and probed with Horseradish Peroxidase (HRP) conjugated

secondary antibody. Membranes were incubated with Enhanced Chemiluminescence (ECL) substrate and exposed on CL-X Posure Film (Thermo Scientific; Rockford, IL). Alpha-tubulin was used as a control to standardize for protein concentration loaded on gels. Films were scanned for quantification with NIH Image J.

Fast-scan cyclic voltammetry (whole animal, anesthetized)

Following approximately 1 week of acclimation, adolescent (PND 43) and young adult (PND 71) female mice were individually housed with ad libitum food, left untouched for one day, and then weighed for baseline measurements the following three days. On what would be ABA day 1, mice were anesthetized (2 g/kg i.p.) and placed into a stereotaxic frame and fast-scan cyclic voltammetry (FSCV) was performed. A carbon fiber microelectrode (CFME) was lowered into the nucleus accumbens (NAc: AP, +1.3 mm; LAT, .5 mm; DV, 4.1 mm, from the cortical surface) and stimulated dopamine release was measured as described previously (10,11). Briefly, a bipolar stimulating electrode was lowered into the ventral tegmental area (VTA, AP, -3.16 mm; LAT, 0.5 mm; DV, 4.3 mm). A chloride-coated silver wire (Ag/AgCl) reference electrode was implanted contralateral to the CFME and secured with a stainless-steel screw and dental cement. A potential applied to the CFME at -.4 V was ramped up to 1.3 V and back, compared to the reference (Ag/AgCl) electrode, with a scan rate of 400 V/s held at -0.4V between scans. CFMEs were cycled at 60 Hz for 20 minutes and then returned to 10 Hz for ten minutes to stabilize background current. Experimental measurements were made

at a scan rate of 10 Hz. During stimulation protocols, for each 15s recording, background was digitally subtracted by averaging the background current obtained from ten scans selected from the period prior to stimulation onset. To optimize dopamine signals, both the CFMEs and stimulating electrodes were systematically lowered in .1 mm increments. At each increment, a train of current (24 pulses, 4 ms per pulse, 60 Hz, 150 μA) were used to evoke a reproducible dopamine release. After optimization, dopamine was evoked using 5 pulses of stimulation administered at 5, 10, 20, 40 and 60 Hz, with 2 minutes between stimulations. A cyclic voltammogram electrochemically identified dopamine with peak oxidation at .6 V and a reduction peak at .2 V. The CFMEs were calibrated following the experiment using a micro flow cell and 1 μM dopamine solution.

Demon Voltammetry Analysis Software was used to model dopamine reuptake kinetics (Wake Forest University, Winston-Salem NC). The decay constant tau was used as a measure of dopamine reuptake. Tau was calculated from an exponential curve fit and is highly correlated with changes in Km ($r = .9899$), suggesting tau is an accurate measure of DA clearance (12).

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