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Supplementary information

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Social rank modulates methamphetamine-seeking in dominant and subordinate male rodents via distinct dopaminergic pathways

In the format provided by the authors and unedited

Supplementary Figure 1.

Fig3i uncropped images

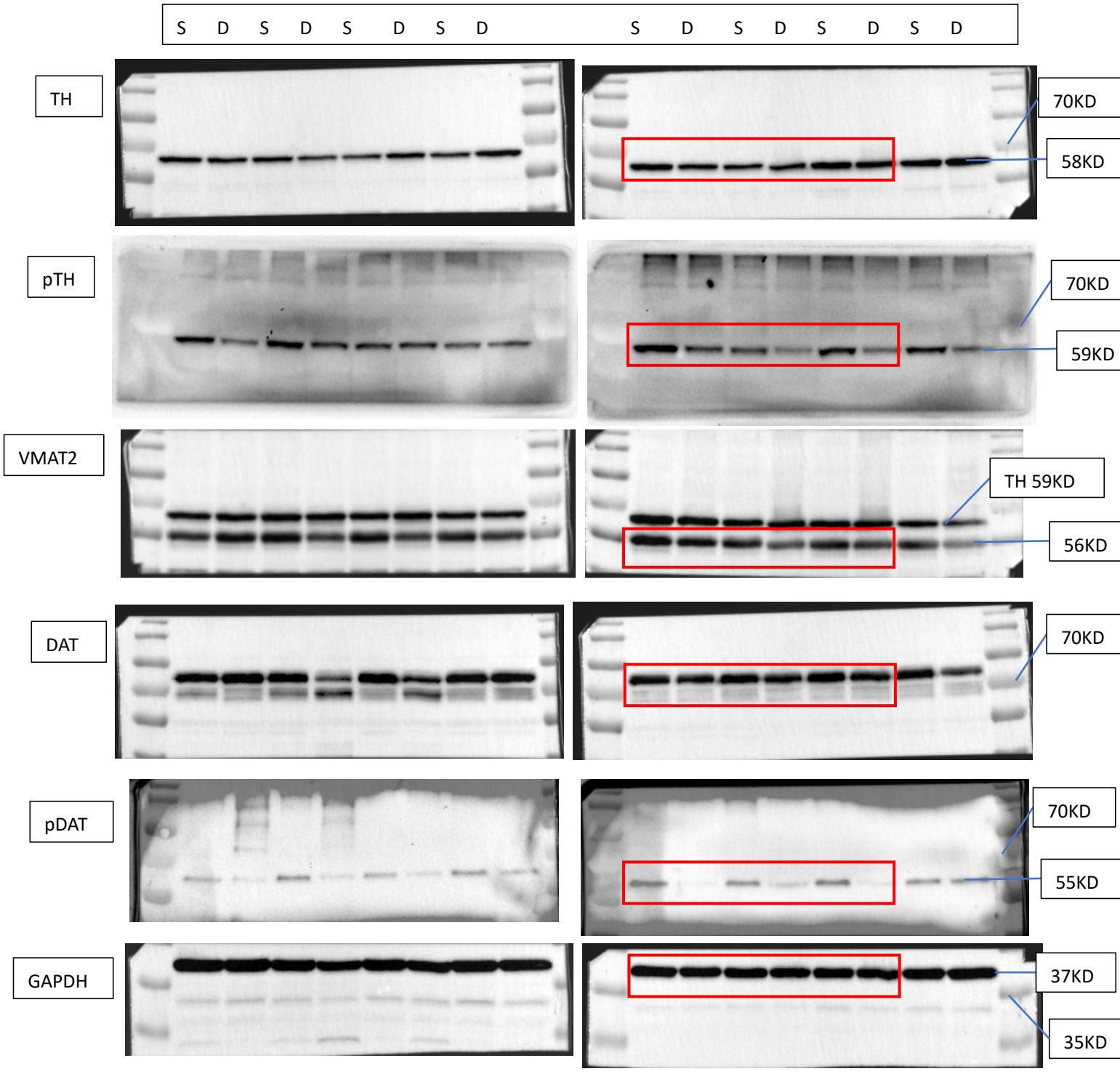


Fig4f uncropped images

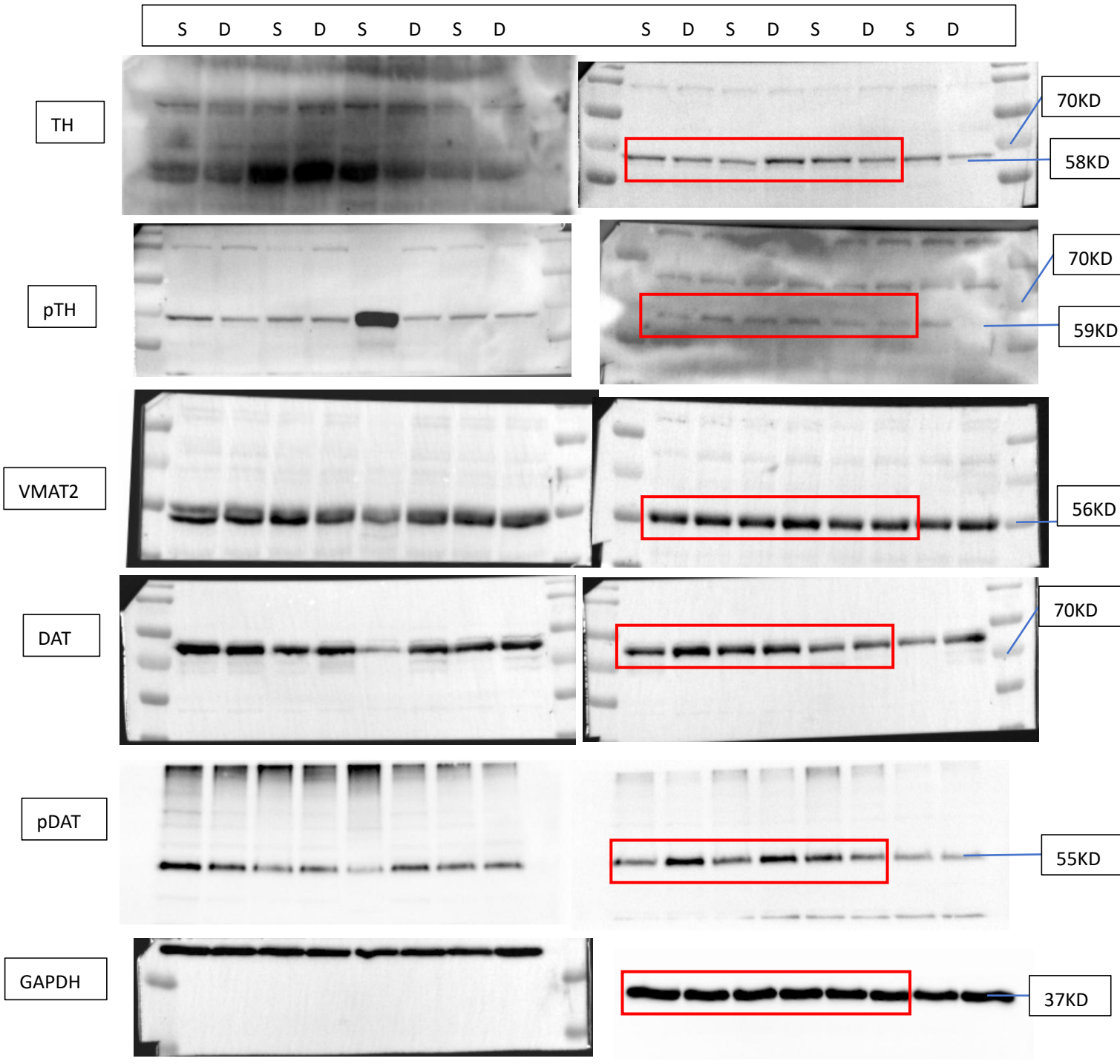


Fig5b uncropped images

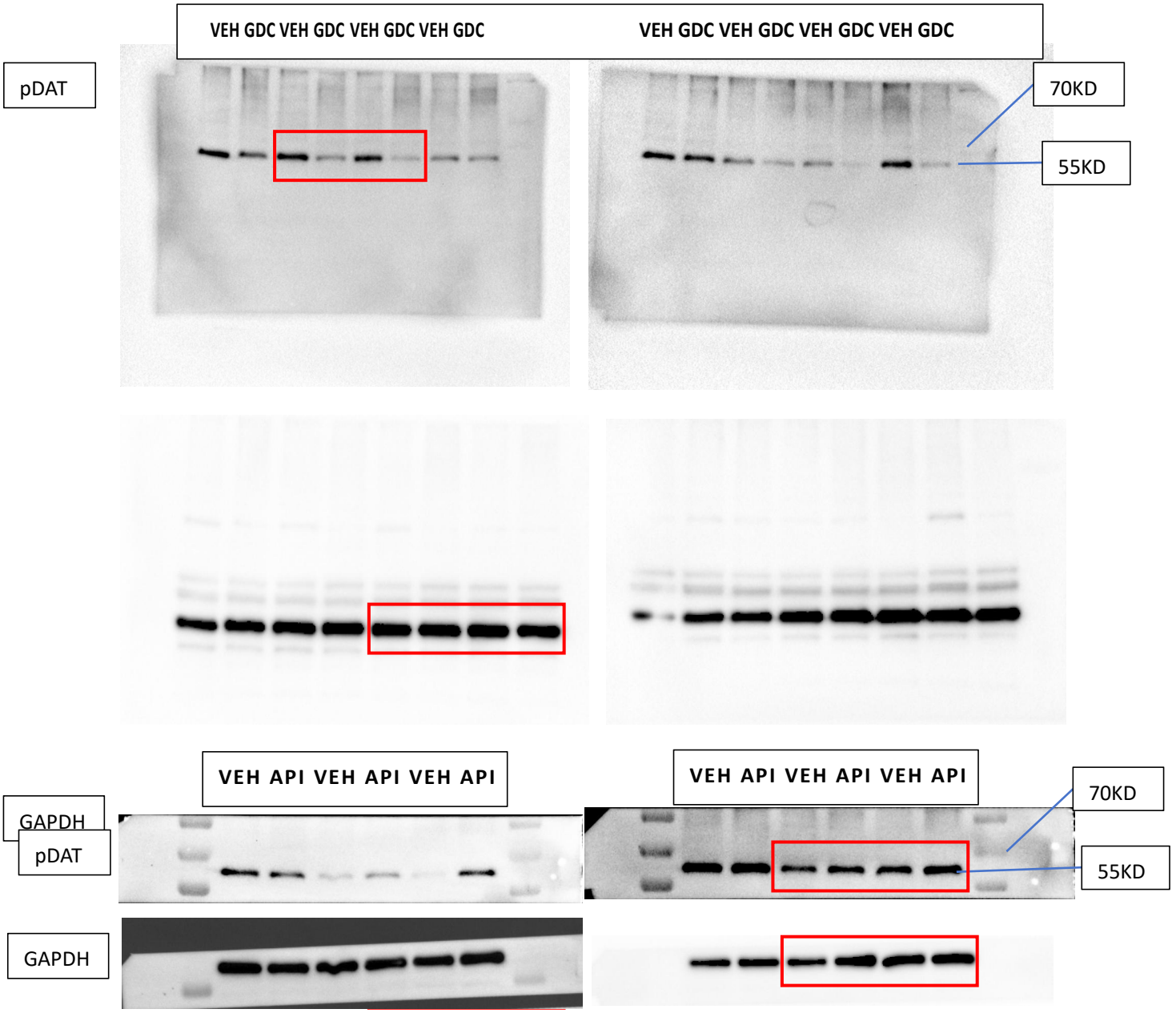
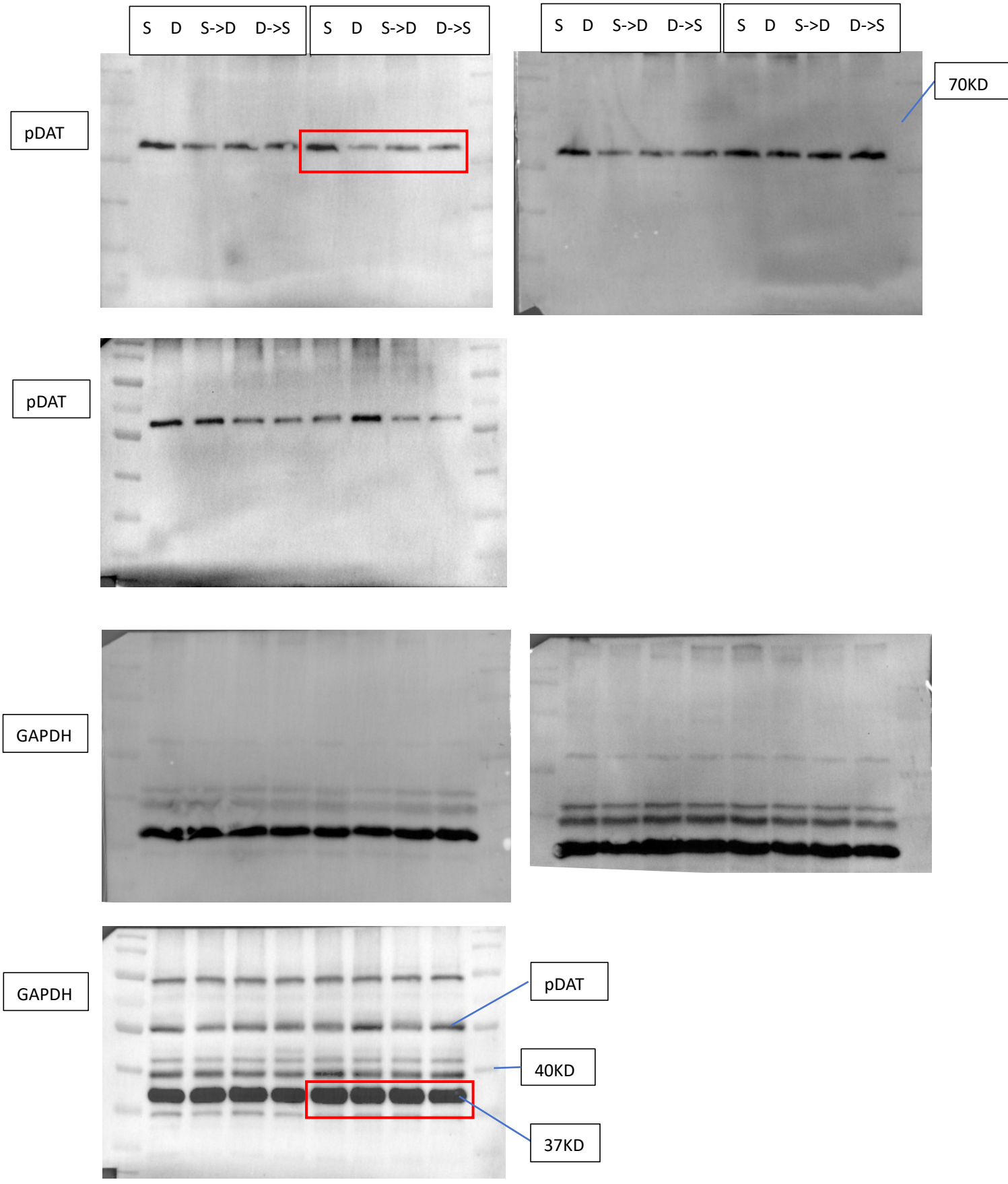
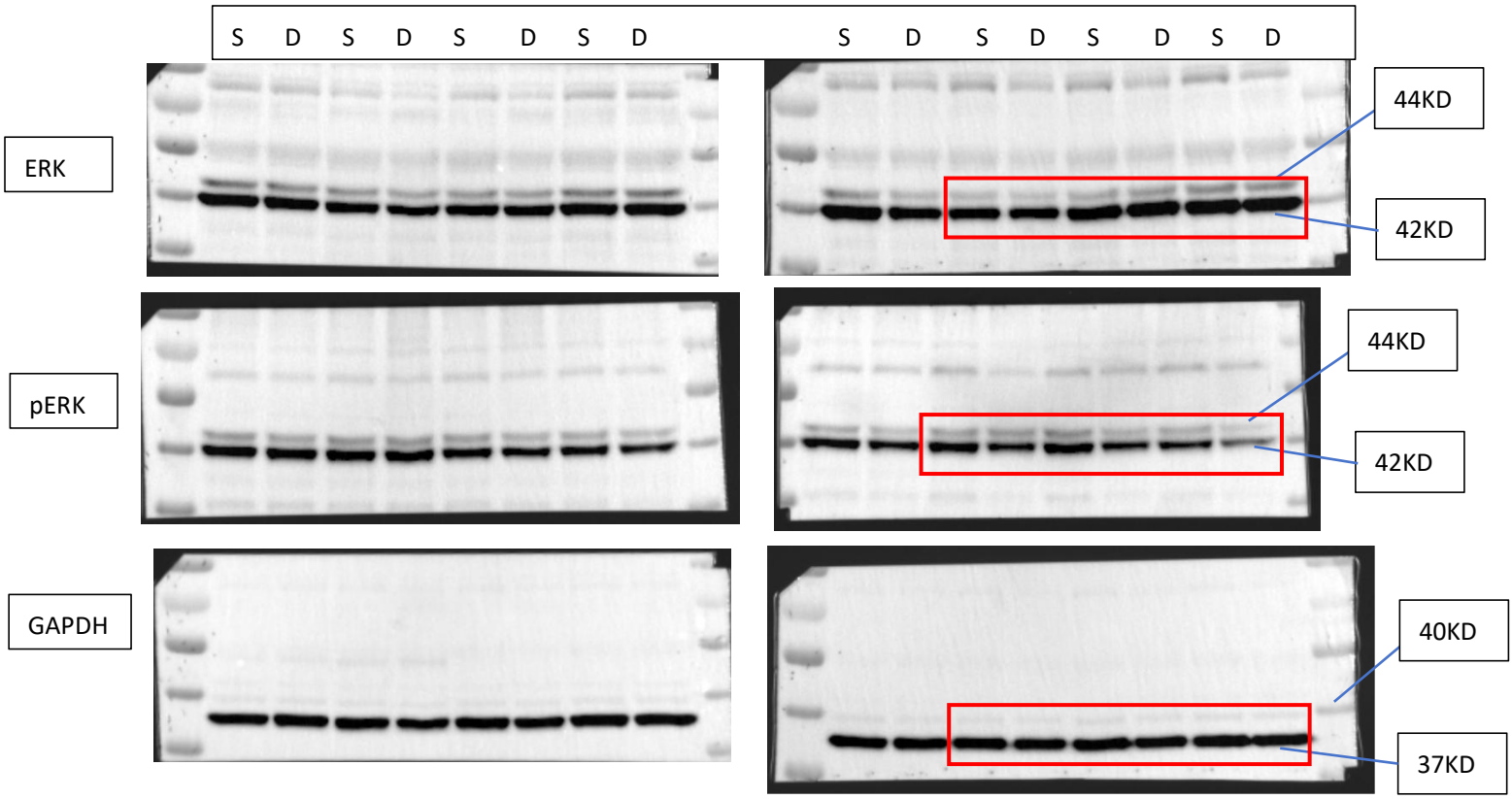


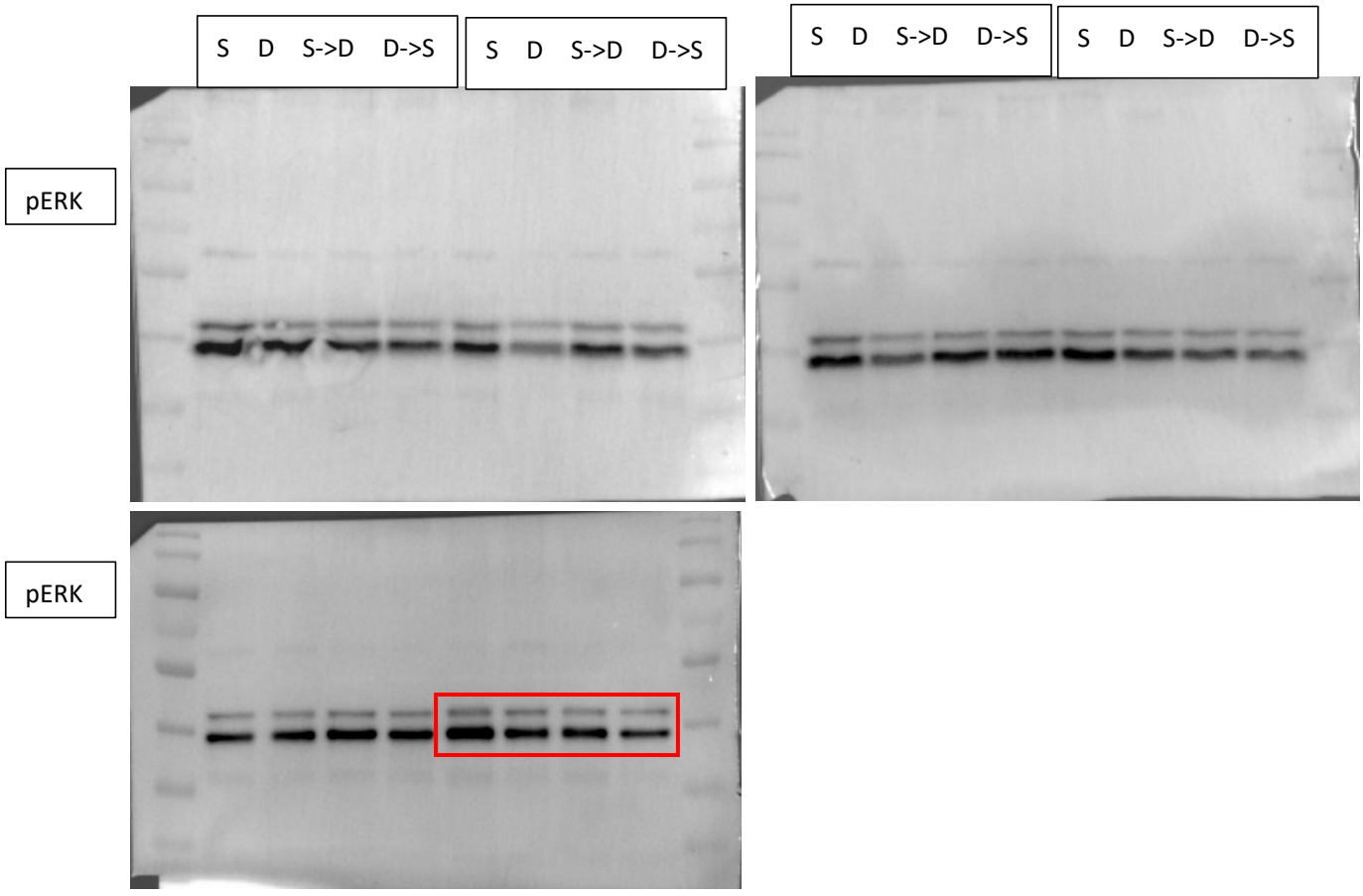
Fig7k uncropped images

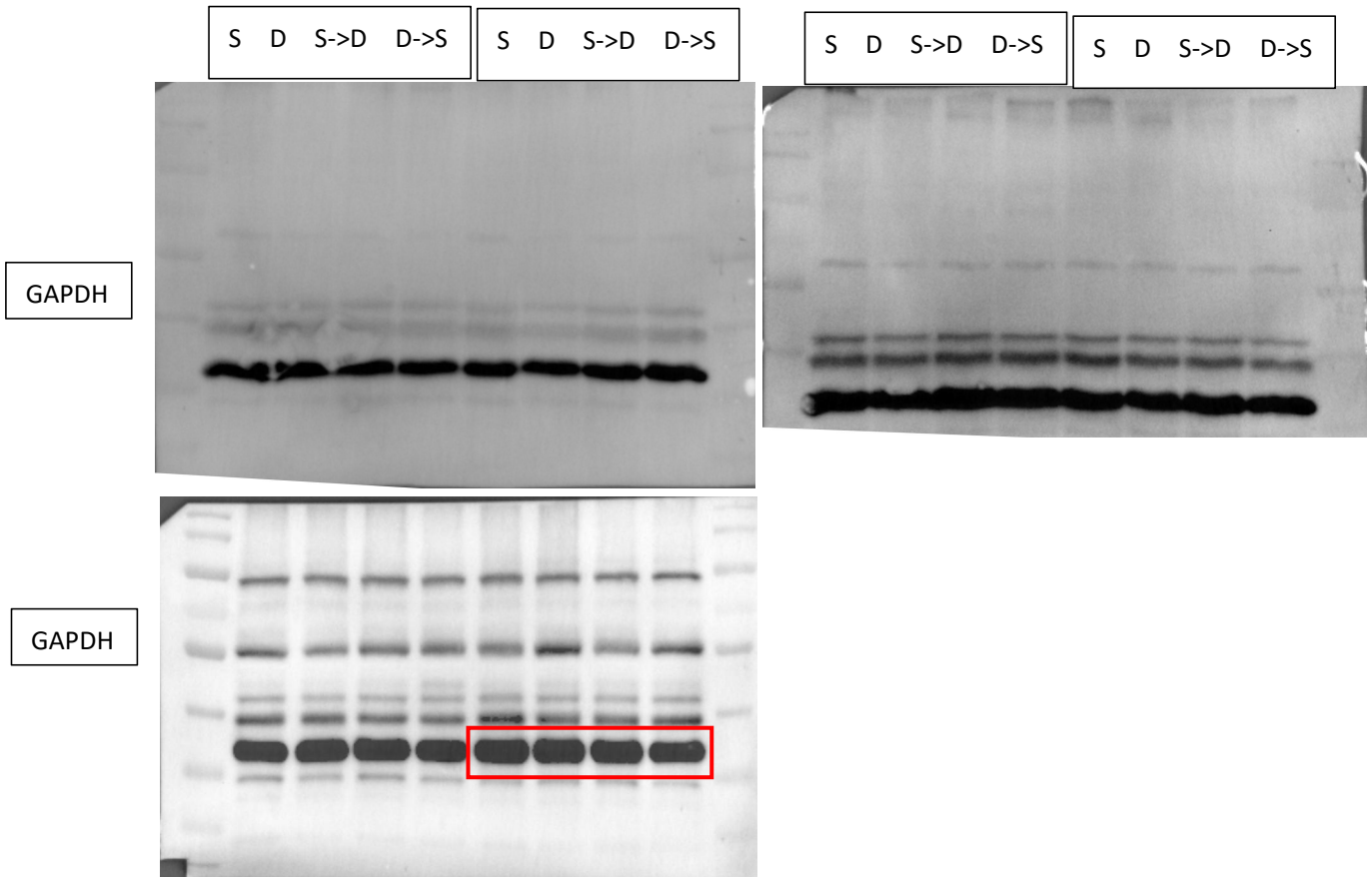


Extended data Figure 5e uncropped images

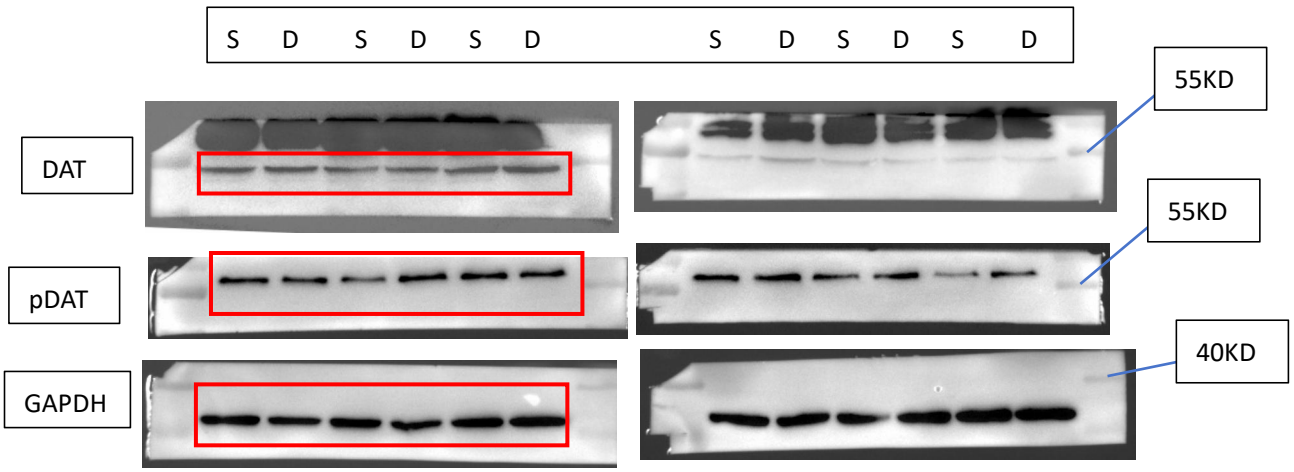


Extended data Figure 9o uncropped images





Extended data Figure 11h uncropped images



Supplementary Protocols

Agonistic Behavior Test

The agonistic behavior test [1] was performed following the completion of 20 trials of the tube test. Briefly, each pair of group-housed mice was placed together in a neutral cage, and observed for any fighting behavior over a period of 20 minutes. The dominance score for each mouse was calculated by subtracting the number of defensive behaviors from the number of offensive behaviors. Offensive behaviors included lateral attacks, boxing, mounting, and chasing, while defensive behaviors were identified as fleeing, freezing, and underside exposure during a fight.

Warm Spot-test

We validated the social dominance identified by the tube test using the warm spot-test [2]. Mice housed in each cage were placed into a new cage where the floor was cooled to 0℃. Prior to being transferred to the test cage, which had a small heating coil under one corner to maintain a temperature of 34℃, the mice were first kept in the cooled cage for 30 minutes. As animals would naturally compete for the warm spot, the dominant mice spent more time in this area, while the subordinate mice spent less time there. We recorded and compared the time spent in the warm spot by the dominant and subordinate mice during the 20-minute test.

Methamphetamine-induced hyperlocomotion

Mice were acclimated in an open field for 30 minutes over two days to minimize potential influences from novelty. On the third day, mice received an intraperitoneal injection of 0.2 ml saline solution before being introduced into the open field for a 45-minute recording using a suspended digital camera. After 24 hours, mice were intraperitoneally injected with METH (2.5 mg/kg), and their locomotor activity was recorded for 45 minutes. Subsequently, the recorded videos were imported into a custom MATLAB-based program for further analysis.

Dopamine recording during METH self-administration in freely moving mice

To monitor dopamine dynamics during a freely moving METH self-administration behavioral regimen, we custom-designed a rotator that allows coaxial rotation of both the recording cable and the drug delivery catheter, preventing them from becoming entangled. We connected sensors for the active and inactive holes to the fiber photometry system so that when the animal poked either hole, event signals were recorded and used as timestamps for signal analysis. For the experiment, AAV-hSyn-$GRAB_{D2m}$ was injected into both the mPFC and NAc of the same mouse, and optical fibers were implanted at these sites. During the experiment, the animals were connected to the fiber photometry recording device and the drug delivery system via their pre-implanted catheters, and then placed in the self-administration operant chamber. The experimental procedure consisted of two sessions, each lasting 2 hours: one session recorded from the mPFC, and the other from the NAc, with the order balanced across animals. All other procedures were consistent with standard self-administration experiments.

Ex vivo electrophysiological recordings

Coronal slices of 250–300 μm thickness containing the VTA were crafted utilizing a VT-1000S vibratome (Leica) in an ice-cold choline-based solution, saturated with 95% O_2 and 5% CO_2, which consisted of the following components (in mM): 110 choline chloride, 2.5 KCl, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.3 NaH_2PO_4, 1.3 Na-ascorbate, 0.6 Na-pyruvate, 25 glucose, and 25 $NaHCO_3$. Then slices underwent a 1-hour incubation at 32 °C in oxygenated aCSF (in mM: 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 1.3 NaH_2PO_4, 1.3 Na-ascorbate, 0.6 Na-pyruvate, 25 glucose, and 25 $NaHCO_3$) before recording.

The slices were then transferred to a recording chamber and superfused with artificial cerebrospinal fluid at a rate of 2 ml/min. Patch pipettes (2–5 MΩ) created from borosilicate glass (PG10150-4, World Precision Instruments) were filled with a Cs-based low Cl– internal solution containing (in mM): 135 $CsMeSO_3$, 10 HEPES,

1 EGTA, 3.3 QX-314, 4 Mg-ATP, 0.3 Na-GTP, 8 Na2-phosphocreatine, with an osmolality of 290 mOsm kg−1 and pH adjusted to 7.3 with CsOH.

For intrinsic excitability, neurons were studied under current-clamp conditions. Specifically, Ai14-expressing dopamine neurons in the VTA were selected for recording. Action potential firing was induced by injecting step currents ranging from 0 pA to 160 pA (with intervals of -40 pA and a duration of 500 ms). The input resistance was derived from the linear relationship observed in the neuronal voltage response against the applied step current. Calculations of membrane capacitance were based on the ratio of the membrane time constant to the input resistance. Spontaneous firings were documented in a current-clamp configuration with a zero-current mode. Data were sampled at 10 kHz and subjected to analysis using Clampfit (Molecular Devices) or MATLAB (MathWorks).

Real-time place preference test

In the real-time place preference test (RTPP), we utilized a custom-made two-chamber apparatus. On the initial day, mice freely explored the apparatus for 15 minutes without any light stimulation. The following day, one of the chambers—chosen in a counterbalanced manner—was designated as the stimulation side. Upon entering this side, mice received laser stimulation. The stimulation ceased once the mouse moved to the opposite chamber. The place preference score was derived by subtracting the baseline time from the time spent on the stimulation side during the test.

Retrograde Cholera Toxin-B Tracing

To selectively retrogradely label dopamine neurons projecting to the mPFC and NAc, we administered 0.3 μl of cholera toxin subunit B (CTB) conjugated to Alexa Fluor 488 (Invitrogen™,C34775) or 647 (Invitrogen™, C34778) into the mPFC (AP, 2.0, ML:-0.45, DV:-2.4) and NAc (AP:1.40, ML:-0.75, DV:-4.6) of DAT:Ai14 transgenic mice. One-week post-injection, the mice were transcardially perfused and brain tissues were processed for subsequent histological analysis. Imaging was carried out using an Olympus Virtual Slide Microscope (VS120-S6-W). Both CTB-positive

neurons and those co-labeled with dopamine markers in the VTA were manually quantified through visual inspection.

Immunoassays

Blood was collected from the heart and immediately stored in ice-cold tubes pre-coated with heparin, a protease inhibitor cocktail, and ethylenediaminetetraacetic acid (EDTA). Following a swift centrifugation at 2000 g at 4 °C for 10 minutes, the supernatants (serum) were harvested, portioned out, and preserved at –80 °C until further analysis. Corticosterone concentrations were determined using an ELISA kit (abcam, ab108821), while testosterone and estrogen levels were quantified using RIA (radioimmunoassay) kits from Beijing North Institute Of Biotechnology (testosterone kit: 191220, estrogen kit: 191020).

Pharmacokinetic Analysis of Methamphetamine

Sprague–Dawley rats were housed in pairs for at least three weeks before the experiment. After this period, the rats underwent a tube test to determine their social hierarchy, classifying them into dominant or subordinate groups. Surgical implantation of catheters was performed as follows: a femoral vein catheter for METH administration and an external jugular vein catheter for blood sampling (Platinum-cured silicone pharmaceutical tubing, 0.020 in. inner diameter and 0.037 in. outer diameter; Scientific Commodities, Lake Havasu City, AZ).

METH was administered at a dose of 3.0 mg/kg via the femoral vein catheter as a 15-second bolus. Blood samples were collected from the jugular vein catheter at baseline (5 minutes pre-injection) and at 2, 5, 10, 20, 40, 60, 90, 120 minutes post-injection. To compensate for the volume of blood withdrawn, equivalent volumes of sterile saline were infused. The total blood volume collected per experiment did not exceed 10% of the rat's total blood volume (Leonard and Ruben, 1986), with each sample measuring 300 μL. Serum was isolated by centrifugation after allowing the blood to clot at room temperature, and all samples were stored at −80 °C until further analysis.

Liquid Chromatography-Tandem Mass Spectrometry (LC/MS/MS): METH concentrations were quantified using a liquid chromatography-tandem mass spectrometry (LC/MS/MS) method adapted from the Chinese national standard for METH detection (GB/T 29636-2023). Blood samples collected within 90 minutes post-METH injection were diluted 1:80 (v/v), while later samples were diluted 1:4. The dilution buffer consisted of a mixture of acetonitrile, water, and methanol in a 5:4:1 ratio. For each analysis, 10 μL of the diluted sample was injected into the LC column.

Chromatographic separation was achieved using a Shimadzu LCMS-8060 system (Kyoto, Japan) equipped with a reversed-phase ACQUITY UPLC PFP column (2.1 mm × 100 mm, 1.8 μm), maintained at 40°C. The mobile phases consisted of water with 0.1% formic acid (A) and methanol (B). A 10-minute gradient elution was employed with the following profile: 0–3.0 min, 20–60% B; 3.0–7.0 min, 60–95% B; 7.0–7.1 min, 95–20% B; 7.1–10.0 min, 20% B. The flow rate was set at 0.3 mL/min, with an injection volume of 10 μL.

The analysis was conducted on a triple quadrupole mass spectrometer within the LCMS-8060 system, operating in positive ionization mode under multiple reaction monitoring (MRM). Two transitions were monitored for METH (150.1 > 91.0 and 119.0). Quantification was performed using LabSolution software (Shimadzu), with calibration curves constructed by plotting calibrator concentrations against external standard peak areas. The calibration curves demonstrated linearity, and the quality control sample results were within acceptable limits (≤±20% deviation from nominal values).

Pharmacokinetic Analysis: Pharmacokinetic analysis of the METH concentration-time data for each rat was conducted using the non-compartmental model module in Phoenix WinNonlin 8.2 (Pharsight Corporation, Mountain View, CA). The primary pharmacokinetic parameters C_{max}, and $T_{1/2}$ were calculated by the software. C_{max} values were obtained from observed data. The terminal elimination half-life ($T_{1/2}$) was calculated as $T_{1/2} = 0.693/\lambda z$, with an adjusted $R^2 > 0.7$.

Depressive-like Behaviors

We utilized two cohorts of mice to examine depressive-like behaviors in paired-house mice (without any extra treatments) and forced-loss (or naturally losing) mice, respectively. In the first experiment with paired-house mice, we compared the immobility time in the tail suspension test (TST), forced swimming test (FST), and sucrose preference test (SPT) between dominant and subordinate mice. In the second experiment, we compared TST, FST, and SPT results among naïve, naturally losing, and forced-loss mice. The naïve group consisted of mice that underwent no additional experiments beyond the tube tests. The naturally losing group was identified as the dominant mice that had experienced a loss to other dominant mice from different cages in previous inter-cage tests (at least 20 trials). The experimental procedure followed the protocol described in Fan et al [3].

Tail Suspension Test (TST): Mice were suspended by their tails using tape, with one end of the tape secured to a horizontal bar 55 cm from the ground. Clear hollow cylinders were placed around the tails to prevent tail-climbing behavior during the test. To prevent the mice from observing or interacting with each other, physical barriers were placed between each suspended mouse. The test lasted for 6 minutes, during which the immobility time was recorded for the entire duration.

Forced Swimming Test (FST): Mice were placed into a glass cylinder (30 cm height × 20 cm diameter) filled with water at 22 ℃ to a depth of 15 cm. The behavioral test lasted for 6 minutes. Typically, mice struggle vigorously at the beginning of the test, so only the last 4 minutes were analyzed, as most mice are very active in the first 2 minutes.

Sucrose Preference Test (SPT): The SPT was used to test anhedonia and was performed once all the above tests were completed. Mice were initially habituated to a two-bottle choice with drinking water one day prior to the sucrose preference measurement. For the following 72 hours, mice were given access to a two-bottle choice of water or 1% sucrose solution. The bottles containing water and sucrose were weighed at 24-hour, 48-hour, and 72-hour time points. The positions of the bottles were interchanged after each measurement to prevent side preference. Sucrose preference was determined as (Sucrose intake/total fluid intake) × 100.

Open Field

Prior to testing, animals were transported to the experimental room and given at least 1 hour to habituate to the new environment. For mice, the open-field test was conducted in a square plastic box (40 cm length × 40 cm width × 30 cm height), with a designated central area (20 cm length × 20 cm width). Meanwhile, for rats, the open-field test took place in a larger square plastic box (60 cm × 60 cm × 50 cm), with the central area defined as a 30 cm × 30 cm square. Animals were individually placed in the central area of the chamber and allowed 10 minutes to freely explore the entire box. An overhead industrial camera was used to record behavior. After each test, the box was cleaned with a 20% ethanol solution and then wiped down with paper towels. Video data were analyzed using software (Anymaze, Stoelting), with a focus on total locomotion and the proportion of time spent in the central area.

Reference:

1 Wang, F. *et al.* Bidirectional control of social hierarchy by synaptic efficacy in medial prefrontal cortex. *Science* **334**, 693-697 (2011).

2 Zhou, T. *et al.* History of winning remodels thalamo-PFC circuit to reinforce social dominance. *Science* **357**, 162-168 (2017).

3 Fan, Z. *et al.* Neural mechanism underlying depressive-like state associated with social status loss. *Cell* **186**, 560-576. e517 (2023).