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Original Article

Dopamine D2 receptors in the dorsomedial prefrontal cortex modulate social hierarchy in male mice

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Abstract

Social hierarchy greatly influences behavior and health. Both human and animal studies have signaled the medial prefrontal cortex (mPFC) as specifically related to social hierarchy. Dopamine D1 receptors (D1Rs) and D2 receptors (D2Rs) are abundantly expressed in the mPFC, modulating its functions. However, it is unclear how DR-expressing neurons in the mPFC regulate social hierarchy. Here, using a confrontation tube test, we found that most adult C57BL/6J male mice could establish a linear social rank after 1 week of cohabitation. Lower rank individuals showed social anxiety together with decreased serum testosterone levels. D2R expression was significantly downregulated in the dorsal part of mPFC (dmPFC) in lower rank individuals, whereas D1R expression showed no significant difference among the rank groups in the whole mPFC. Virus knockdown of D2Rs in the dmPFC led to mice being particularly prone to lose the contests in the confrontation tube test. Finally, simultaneous D2R activation in the subordinates and D2R inhibition in the dominants in a pair switched their dominant–subordinate relationship. The above results indicate that D2Rs in the dmPFC play an important role in social dominance. Our findings provide novel insights into the divergent functions of prefrontal D1Rs and D2Rs in social dominance, which may contribute to ameliorating social dysfunctions along with abnormal social hierarchy.

Key words: dopamine receptors, medial prefrontal cortex, social dominance, social hierarchy, tube test

Social rank/hierarchy are ubiquitous features of virtually all group-living animals (Qu et al. 2017; Hou et al. 2022; Murlanova et al. 2022). The organizing principle of social hierarchy is to provide dominant individuals with priority access to limited resources, such as food, territory, and mates; on the contrary, subordinates are forced to live with social stress, which induces various mental and physical health problems through disruption of the immune and endocrine systems, and brain functions (van der Kooij and Sandi 2015; Uchida et al. 2021). Therefore, understanding the neural mechanisms underlying social hierarchy may be not only benefiting the animal's social life but also their health (Komori et al. 2019).

In the last decade, researchers have attempted to decipher the neural mechanisms underlying social dominance. Dominance perception is represented in broad brain regions, including but not limited to the amygdala, hippocampus, nucleus accumbens (NAC), and the prefrontal cortex (Watanabe and Yamamoto 2015). The medial prefrontal cortex (mPFC) is best known for its role in higher cognitive functions and emotion disorders (Yizhar and Levy 2021). Both animal and human studies have indicated that this brain structure is specifically related to social dominance. For example, Kumaran et al. (2016) found that the mPFC selectively mediates updating the knowledge on the own hierarchy in humans; Wang et al. (2011) found that the strength of excitatory transmission in the mPFC is a critical determinant of intermale hierarchy in C57 male mice; and Padilla-Coreano et al. (2022) found that the mPFC's neural activity reflects social rank and winning in mice. Rodents' mPFC can be roughly divided into dorsal and ventral regions (Le Merre et al. 2021). The neuron types, neurotransmitters, neural pathways, and receptor distributions of these two regions show obvious differences and differentially contribute to their distinct biological functions (Laubach et al. 2018; Jing et al. 2021). It is, therefore, interesting to clarify the functional role of these two subregions in the formation and maintenance of social hierarchy.

Additionally, explored neurotransmitter or modulator systems underlying the formation and maintenance of social hierarchy in a group include, but are not limited to, the dopamine (DA) and serotonin (5-HT) systems, the oxytocin and neuropeptide B/W systems, and steroid hormones (Wang et al. 2014; Watanabe and Yamamoto, 2015; Papilloud et al. 2020). The DA system has long been involved in social motivation and rewarding, both closely linked to social dominance (Ghosal et al. 2019). Moreover, there is ample evidence

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across taxa for the involvement of the mesolimbic DA system in social dominance. For example, in lizards, increased DA concentrations in the ventral tegmental area and NAC have been associated with higher dominance (Korzan et al. 2006); in mice, Balog et al. (2019) found that prefrontal DA concentrations were positively correlated with their dominant status and systemic manipulation of DA transmission changed their dominance plasticity bidirectionally; in cynomolgus macaques, Kaplan et al. (2002) found that dominant subjects tend to have higher levels of DA metabolites in cerebrospinal fluid than subordinates. DA functions through two groups of G-protein-coupled receptors, DA receptors 1 and 2 (D1Rs and D2Rs), which exert divergent functions due to different intracellular signaling pathways and properties (Li et al. 2020; Kourosh-Arami et al. 2022). Numerous studies investigated the contributions of DA receptors in social dominance but with inconclusive results depending on the intervening measures, related brain structures, species, or many other unknown reasons (Ghosal et al. 2019; Amaral et al. 2021). D1Rs and D2Rs are abundantly expressed in the mPFC, which play critical but distinct roles in social information processing in rodents (Shinohara et al. 2018; Xing et al. 2021). It is, therefore, important to explore their contributions to social dominance within different subregions of the mPFC.

Various paradigms have been developed to identify social dominance in laboratory rodents, such as the home-cage behavior observation test, the tube test, the warm spot test, and the territory urine marking test, etc (Wang et al. 2014; Fulenwider et al. 2022; Murlanova et al. 2022). Despite limitations such as the need to match age and weight or habituation, among others, the tube test has been validated as a reliable measure of social hierarchy in mice due to its ease of use, stability, consistency, and transitivity (if A > B, B > C, then A > C) (Fan et al. 2019).

Therefore, in this study, we investigated the potential role of DA receptors within the mPFC in social dominance in mice. We first investigated the behavioral variability among individuals in a social group. Then, D1R and D2R expression levels were analyzed in different parts of the mPFC among rank groups. Next, the causal involvement of DA receptors in rank attainment was explored either by genetic modulation of its expression or pharmacological approaches. Considering the important effects of steroid hormones in social rank, serum corticosterone (CORT) and testosterone (T) levels were also compared among the rank groups.

Materials and Methods

Animals

We used 8–10-week-old C57BL/6J (strain number: 000664) male mice purchased from Huaxing Experimental Animal Husbandry (Zhengzhou, China). Unless otherwise stated, animals were housed (4 per cage) and allowed to acclimate to the environment for at least 1 week after arrival. They were maintained under standard housing conditions on a 12 h light:dark cycle (lights on at 20:00 h) and fed ad libitum. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of China and approved by the Animal Care and Use Committee of Nanyang Normal University (approved number: 20201202005).

Behavioral tests

For all behavioral tests, mice were habituated to the testing room at least 30 min prior to the beginning of each test. All subjects were randomly selected during the tests. Unless other indicated, all tests were performed between 9:00 a.m. and 13:00 p.m. Between sessions, the apparatus was cleaned with 30% ethanol and dried with napkins to avoid olfactory cues. Three animal cohorts (for 4 animals per cage's study) were used for behavioral tests (n = 96 in total; 24 cages, 3 cages were excluded from data analysis due to unstable performance in the tube test).

Tube test

Tube test was conducted according to standard protocols with slight modifications (Fan et al. 2019). In brief, mice were first trained to move forward in a transparent Plexiglas tube (diameter: 2.5 cm and length: 30 cm) for 2 consecutive days, with 5 trials per day. After the training phase, testing was performed over 6 days. On each day of the testing phase, each mouse explored the tube once from each side before the confrontation trials. Then, a pair of cage mates was allowed to enter the tube from opposite ends and meet in the middle. The trial ended when 1 of the mice retreated with all 4 paws out of the tube, becoming the "loser" or subordinate. The mouse that forced its cage mate to retreat was termed "winner" or dominant. The order of confrontations was randomized day after day using a round-robin design. In the following pharmacological and virus knockdown (KD) D2Rs experiments, as the animals were raised in pairs, the tube test was conducted twice daily and the time interval between the test is about 1 h. Social rank was determined by the total score of the "winning–lose relationship." During the test, if 1 of the parties could not push their partner out of the tube within 5 min (frequency of about 6% for all trials in our study), the tube tests were terminated and the pairs were put back into their original cages to rest and retest again 1 h later. Only mice with stable ranks (same rank position over 3 days) were used for further tests.

Open-field test

The open-field test (OFT) was used to assess the mice's emotional and exploratory/locomotive behavior (Wang et al. 2022). Briefly, a rectangular open arena was utilized (50 × 50 × 30 cm). In the beginning, mice were individually introduced in the center zone of the open-field chamber (40 × 40 cm) under dim light for 5 min. Their path was automatically recorded by a video-tracking system (SuperMaze Systems, Shanghai Xinruan, China). Both the total distance and time spent in the center area were analyzed.

Elevated plus maze test

The elevated plus maze (EPM) test is frequently used to evaluate anxiety-like behaviors in rodents (Li et al. 2021). The maze is elevated 70 cm from the floor and consists of 2 open arms (35 × 5 × 1 cm), 2 closed arms (35 × 5 × 15 cm), and an open square 5 × 5 cm in the center. At first, mice were individually introduced in the open center with the head facing the open arm and allowed to freely explore for 5 min. Entries, explorations, and the time spent in each arm were automatically recorded via the SuperMaze System.

Three-chamber test

The 3-chamber test was used to assess sociability. The apparatus consisted of a rectangular box with 3 separate chambers (20 × 40 × 20 cm each). Each lateral chamber contained a small cylindrical cage (10 cm high and 8 cm in diameter). One day before the test, all subjects were habituated to the arena for 5 min. During the test, an unfamiliar age-matched C57 male mouse (stimulus animal) was confined into a cage in 1 chamber of the apparatus, while the cage in the other chamber was empty. The subjects were then placed in the center chamber and allowed to explore the apparatus for 5 min. The time spent in each chamber was recorded using the SuperMaze System. The social interaction (SI) ratio, calculated as the time spent in the stimulus chamber divided by the time spent in the empty chamber, was used to evaluate sociability.

c-Fos immunohistochemistry staining

One day after the behavioral tests, 1 animal cohort was tested again in the tube test. After 90 min, the animals were anesthetized with 2% sodium pentobarbital and transcardially perfused with 0.1 M PBS, followed by 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 24 h, followed by 15%, and 30% freshly prepared sucrose solution for dehydration. Coronal brain slices (40 μm) were serially cut with a cryostat (CM-1850, Leica, Germany).

For c-Fos IHC staining, sections were dried at room temperature for 10 min, washed in PBS (0.1 M, pH 7.4, 3 × 5 min), and incubated in 0.3% H_2O_2 for 20 min. After thoroughly washing in PBS, the sections were blocked in 5% normal goat serum blocking solution (containing 0.2% Triton X-100, Boster, Wuhan, China) and incubated in rabbit anti-c-Fos (1:1,000, ab190289, Abcam, UK) at 4 °C overnight. The following day, the sections were washed with PBS (3 × 5 min) and incubated with the secondary antibody (sheep anti-rabbit, Boster Company, SA1022, China) at room temperature for 1 h. Sections were then incubated in avidin-biotin-complex (ABC Kit, Boster Company, SA1052) for 60 min. The peroxidase reaction was finally visualized using a DAB Immunohistochemistry Kit (AR1022, Boster, China) following manufacturer's instructions. After air-drying for 48 h, sections were dehydrated using an alcohol series, cleared with dimethylbenzene, and cover-slipped using Permount (AR0038, Boster, China). Photomicrographs were captured at 20× magnification using a light microscope attached to a Leica camera. The number of c-Fos-positive cells was quantified in the dorsal and ventral parts of the mPFC (dmPFC and vmPFC) using Image J software. Cell counts were performed by 2 independent viewers; the border of the brain structures was tailored to each brain region's size according to Franklin and Paxinos' mouse brain atlas (2nd edition). Individual means were obtained by counting positive c-Fos numbers in 3 representative sections from anterior to posterior of the mPFC.

Quantitative RT-PCR and western blot

Brain tissue sampling

Two animal cohorts were used, 1 for qRT-PCR (n = 24) and the other for western blot (n = 24). One day after the behavioral tests, the animals were anesthetized with 2% sodium pentobarbital and decapitated. Trunk blood samples were collected for the following ELISA experiment. The brains were immediately extracted and frozen in liquid nitrogen. Subsequently, the brains were sectioned (100 μm) on a cryostat and mounted onto slides. The bilateral dmPFC and vmPFC (bregma: 2.34–0.86) were dissected using a tissue punch (1 mm in diameter) according to Franklin and Paxinos' mouse brain atlas. The tissue samples were frozen in liquid nitrogen immediately and stored at −80 °C until further use.

qRT-PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Cat# 15596018), quantified by a NanoDrop spectrophotometer, and finally reverse transcribed to cDNA using a PrimeScript RT reagent kit (Accurate Biology, Cat# AG11706) according to manufacturer's instructions. PCR reactions were carried out in 10 μL total volume using Pro Taq HS SYBR Green qPCR Master mix reagent kit (Accurate Biology, Cat# AG11718). GAPDH was used as an internal control for normalization. The primers for D1Rs (D1Rs, NM_010076.3), D2Rs (NM_010077.3) and the housekeeping gene GAPDH (NM_001289726.1) were designed and synthesized by Tsingke Biotech (Zhengzhou, China). The primer sequences are listed in Table 1. The amplification conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melting curve analysis was performed to eliminate nonspecific products by collecting data during an incremental temperature change from 65 to 95 °C at a rate of 0.2 °C/s. The C_t value of each reaction tube was recorded, and the data were analyzed by the $2^{-\Delta\Delta}C_t$ method.

Western blot

Total protein extraction was performed as described in our previous study (Zou et al. 2021). In brief, the protein was extracted with RIPA lysis buffer (Solarbio, R0020, China). The samples were then sonicated for 60 s on ice, and centrifuged at 4 °C (12,000 rpm, 10 min). The protein extracts were quantified by a BCA assay (Tiangen, PA115, China) and diluted to a final concentration of 1 mg/μL.

For western blot, protein samples (15 μg/well) were separated by 10% SDS-polyacrylamide gels. The proteins were then transferred to 0.45 μm PVDF membranes (Millipore, Billerica, USA), blocked with 5% milk for 2 h, and incubated with the

Table 1 Nucleotide sequences of primers used for qRT-PCR amplification

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
GAPDH	CAGTGGCAAAGTGGAGATTGTTG	TCGCTCCTGGAAGATGGTGAT
D1Rs	CTGTGCCTCATCGTGGAAGT	ACTGTTGCAATACCCCCACC
D2Rs	GACTCGGCCTTCTCTTGACC	CTAGCTTCCGTGCCTGAGAG

following diluted primary antibodies at 4 °C overnight: rabbit anti-D1Rs (1:500, DF7097, Affinity, USA), rabbit anti-D2Rs (1:1,000, ab85367, Abcam, UK), and mouse anti-GAPDH (1:2,000, T0004, Affinity, USA). On the next day, membranes were washed with TBST (5 min × 3), and incubated with the following secondary antibodies for 2 h at room temperature: goat anti-rabbit lgG (H + L) HRP (1:5,000, Affinity, S0001, USA) for D1Rs and D2Rs, goat anti-mouse lgG (H + L) HRP (1:5,000, Affinity, S0002, USA) for GAPDH. After thorough washing with TBST, the membranes reacted with ECL detection reagents (WBKLS0500, Millipore, USA). Protein band density was quantified by Image-Pro Plus 6 software; all signals within the same membrane were normalized to GAPDH.

ELISA measurement of serum CORT and T

The blood samples were collected between 9:00 a.m. and 12:00 p.m. The samples were then centrifuged at 4,000 r/min for 10 min at 4 °C, and the supernatant was collected and stored at −80 °C before ELISA assay. CORT and T serum levels were quantified with commercially available ELISA kits (CORT: Nanjing Jiancheng Bio, H094; testosterone: Nanjing Jiancheng Bio, H090-1-1) conducted using the manufacturer's specifications. All samples were analyzed in duplicate. The intra- and interassay coefficients of variation were 9% and 11% for CORT and 8.5% and 12% for T.

Viral D2R downregulation

The mice were raised in pairs for this experiment. In detail, mice were subjected to surgery for viral D2R downregulation in the dmPFC and injected with an adeno-associated AAV2/9 vector containing an U6-shRNA(D2R)-CMV-mCherry-SV40-pA cassette. Control animals were injected with a scrambled shRNA construct (AAV2/9-U6-shRNA(scramble)-CMV-mCherry-SV40-pA). The D2R shRNA sequence is available at the Genetic Perturbation Platform (TRCN0000025738). All viral constructs were designed and produced by BrainVTA™, China (shRNA: PT4618; scramble: PT 0925). The virus titer was about 2.0×10^{12} vg/mL.

For virus injection, the animals were anesthetized by isoflurane inhalation (1.5–3.0%) and placed in a stereotaxic instrument. A volume of 500 nL of viruses was bilaterally injected into the dmPFC (AP: +1.7 mm; ML: ±0.5 mm; DV: −1.6 mm) using a 10-μL syringe with a 33-gauge needle (Hamilton). The injection rate was set at 50 nL/min. After each injection, the needle remained in the brain for another 5 min before being slowly withdrawn to prevent virus leakage. A minimum of 4 weeks was allowed for virus expression before starting with the experiments. Successful KD was verified by gene expression analysis.

Pharmacological studies

Animals were raised in pairs for this experiment and housed together for at least 1 week before undergoing a tube test to determine their social rank. Thereafter, all subjects except the control pairs (used to determine the natural occurrence of the social rank switching rate without manipulation) were implanted with bilateral guide cannulas (0.41 mm o.d. × 0.25 mm i.d.) into the dmPFC (AP: +1.7 mm; ML: ± 0.5 mm; DV: −1.3 mm). Dental cement was used to secure the implants. The animals were allowed to recover for 1 week together with their partners before the following pharmacological studies.

The animals were first administered with saline, and 30 min later, a tube test was conducted to determine their relative ranks. In consideration of the subjects having undergone a pre-tube test and also for the security of the cannulas in the head, the tube test was shortened to 3 days. To check the emotional and movement effects of the drugs, the OFT, EPM, and 3-chamber test were conducted separately 30 min after injection over the next 3 days. To test the drug effects, in the following 6 days, high-rank individuals received a bilateral injection of raclopride (D2R antagonist; 0.5 μg/0.2 μL per side; Sigma), whereas their low-rank partners were injected with quinpirole (D2R agonist; 100 ng/0.2 μL per side; Sigma). The relative social ranks and other behavioral performances were examined 30 min after drug administration. The injection speed was 0.1 μL/min. Internal cannulas were left in place for 2 min after the end of the injection to allow diffusion. The dose and timing of drug administration were based on previous studies and adjusted according to a preliminary study (Watt et al. 2014; Li et al. 2020). Cannula position was verified postmortem. Only subjects with correct cannula placements were included in the final data analysis.

Statistical analysis

Before analysis, all data were assessed for normality using a 1-sample Shapiro–Wilk test; Levene's test was used to confirm the homogeneity of variance. Comparisons between 2 groups were performed by independent-samples *t*-test. Comparisons among 3 or more groups were performed using a 1-way ANOVA. Two-way repeated-measures ANOVA was used to compare multiple groups under multiple testing conditions. Post-hoc comparisons were conducted using Tukey. If the data did not meet the criteria for normality and homogeneity of variance, Kruskal–Wallis test (≥3 groups) or Mann–Whitney *U* test (2 groups) was conducted. To confirm the significance and calculate the effect size, Bayesian *t*-test (paired or unpaired) or Bayesian ANOVA was conducted using default priors. For more detailed information about the Bayesian test, please refer to Dr. Keysers' work (Keysers et al. 2020). Data are presented as mean ± *SE*. Significance was set at $P < 0.05$.

Results

Bottom-rank individuals showed reduced sociability

To assess social hierarchy and dynamics over time, groups of 4 C57BL6/J male mice were housed together for 1 week before the tube test (Figure 1B). After habituation and training, the tube tests were conducted using a round-robin design, in which each mouse was paired daily with the 3 other cage mates. The results show that most of the caged mice could form a stable hierarchy from day 4 of testing (Figure 1C). To gather further information on the behavioral profile related to the social rank, we tested mice for anxiety-like behaviors. In the 3-chamber test, there was a significant difference in the SI ratio ($F_{3,83} = 4.067$, $P = 0.010$, $BF_{incl} = 4.679$). The following post-hoc comparison indicated that rank 4 (R4) individuals showed reduced sociability compared with R1 individuals (R1 *vs.* R4, $P = 0.005$, Figure 1G). This difference may not be due to exploration motivation as there was no significant difference in the total exploration time ($F_{3,83} = 0.657$, $P = 0.241$, $BF_{incl} = 0.130$; data not shown). There were no significant differences in behavioral performance in the OFT and EPM (OFT_total distance: $F_{3,83} = 0.121$, $P = 0.948$, $BF_{incl} = 0.074$, Figure 1D; OFT_central area time: $F_{3,83} = 1.427$, $P = 0.241$, $BF_{incl} = 0.293$, Figure 1E; EPM_open arm time: $F_{3,83} = 2.261$, $P = 0.088$, $BF_{incl} = 0.705$, Figure 1F).

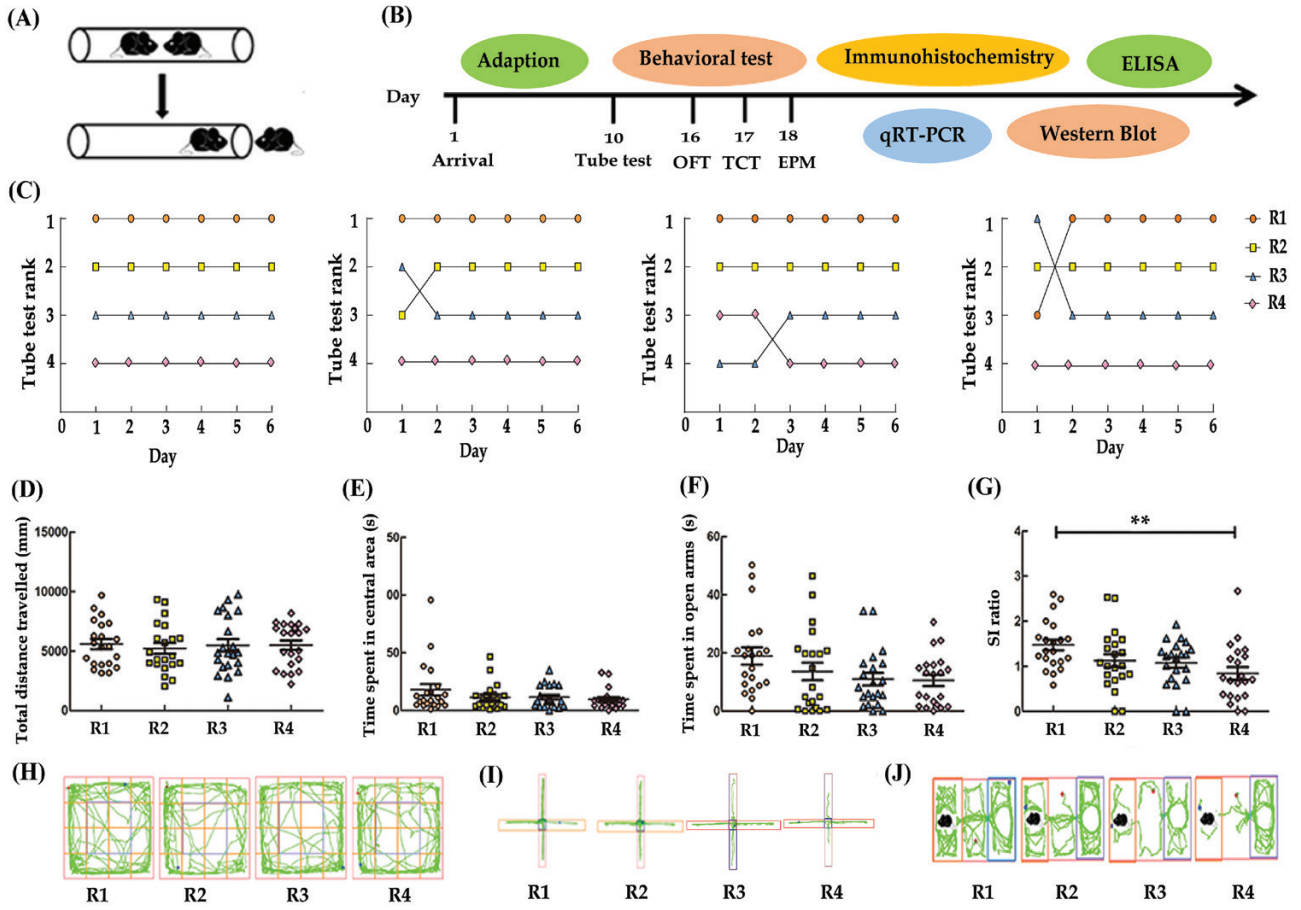

Figure 1 Social rank characterization and behavioral performance. (A) Schematic of the tube test. (B) Study timeline. (C) Representative hierarchies over 6 days of tube test. Each line represents a single animal and its rank position based on summed tube test wins. (D, E) Total distance traveled and time spent in central areas in the open-field test. (F) Time spent in the open arms in the elevated plus maze test. (G) SI ratio in the 3-chamber test. (H–J) Representative tracks in an open-field test, elevated plus maze test, and 3-chamber test. Data are analyzed by 1-way ANOVA and presented as mean ± *SE*. $N = 21$ in each group; $^{**}P < 0.01$. SI ratio: social interaction ratio.

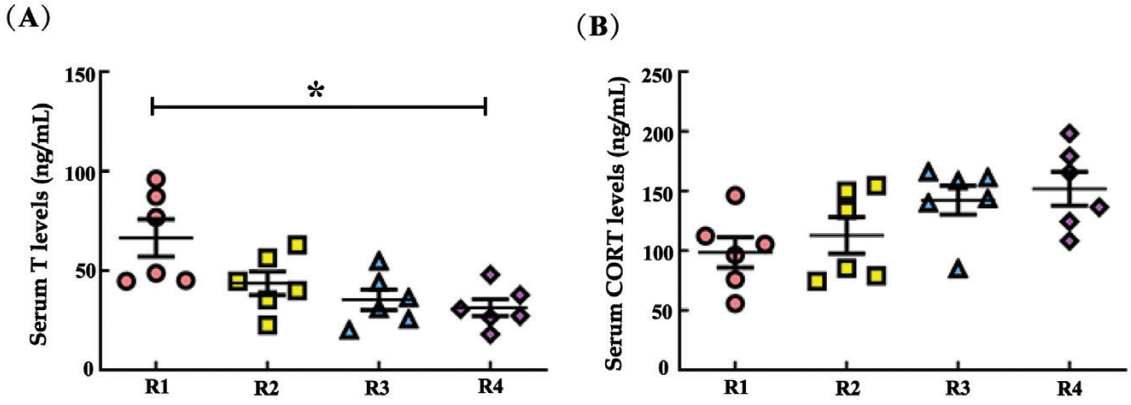

Figure 2 Serum T (A) and CORT (B) levels in different social rank individuals. Kruskal–Wallis test (panel A) or 1-way ANOVA (panel B) was used for analysis. Data are presented as the mean ± *SE*; $n = 6$ in each group. $^{*}P < 0.05$. CORT: corticosterone; T: testosterone.

Bottom-rank individuals showed reduced serum testosterone levels

T and CORT are two steroid hormones, which play an important organizational role during social rank formation and behavioral performance. We then analyzed variations in these 2 hormones among ranks. For T, the results showed a significant difference among rank groups (Kruskal–Wallis test, $H = 9.909$, $P = 0.02$). The follow-up pairwise comparison results showed that R4 individuals had lower T levels than R1 (R4 vs. R1, adjusted $P = 0.024$, Figure 2A). For CORT, ANOVA revealed inconclusive evidence regarding group difference ($F_{3,23} = 3.34$, $P = 0.040$, $BF_{incl} = 2.127$), despite a trend

for R4 individuals showed higher CORT levels compared R1 group (R1 vs. R4, $P = 0.053$, Figure 2B).

Low-rank individuals showed increased neural activity in the mPFC after a tube test

To investigate which mPFC subregion is more involved in the social rank competition, c-Fos IHC staining was conducted 90 min after the tube test. There was a significant difference among rank groups in the dmPFC ($F_{3,23} = 6.380$, $P = 0.003$, $BF_{incl} = 13.639$). R4 individuals showed increased c-Fos expression compared with both R1 and R2 groups (R4 vs. R1, $P = 0.002$; R4 vs. R2, $P = 0.032$; Figure 3B). This was similar to the vmPFC, where there was a significant difference among rank groups (Kruskal–Wallis test, $H = 16.365$, $P = 0.001$), with subordinate individuals showing increased c-Fos expression (R4 vs. R1, $P = 0.001$; R4 vs. R2, $P = 0.004$; R3 vs. R1, $P = 0.033$; Figure 3C).

Lower D2R expression in the dmPFC in low-rank individuals

To investigate the involvement of DA receptors in social rank formation and maintenance, we investigated D1R and D2R expression profiles in the mPFC with western blot and qRT-PCR. Western-blot results showed a significant difference in D2R expression in the dmPFC ($F_{3,23} = 3.513$, $P = 0.021$; $BF_{incl} = 3.397$), but not in the vmPFC (Kruskal–Wallis test, $H = 4.547$, $P = 0.208$). Follow-up analysis indicated that bottom-rank individuals had significantly lower D2R expression in the dmPFC compared with their top-rank partners (R4 vs. R1, $P = 0.014$; Figure 4D). There was no significant difference in D1R expression in either the dmPFC or the vmPFC (dmPFC: $F_{3,23} = 0.378$, $P = 0.770$, $BF_{incl} = 0.257$; vmPFC: $F_{3,23} = 2.321$, $P = 0.106$, $BF_{incl} = 0.267$; Figure 4C). The qRT-PCR results are shown in Figure 4E–H. The Kruskal–Wallis test showed a significant difference in D2R expression in the whole mPFC (dmPFC: $H = 8.287$, $P = 0.04$; vmPFC: $H = 8.370$, $P = 0.039$). However, the follow-up analysis indicated only a significant difference in the R4 versus R1 comparison in the dmPFC (adjust P value = 0.029, Figure 4F). Moreover, we found no significant difference in D1R mRNA expression among groups in the whole mPFC (dmPFC: $F_{3,23} = 1.388$, $P = 0.257$, $BF_{incl} = 0.551$; vmPFC: $F_{3,23} = 0.708$, $P = 0.558$, $BF_{incl} = 0.332$; Figure 4E).

Knocking down D2R expression in the dmPFC lowers social rank

Next, we assessed whether a D2R-KD in the dmPFC affected social dominance in mouse dyads. After determining anxiety-like and exploratory behaviors, a pair was randomly selected and injected with an AAV to downregulate D2R (D2R-KD) expression in the dmPFC. Its partner was correspondingly injected with a scrambled virus control. Four weeks later, the pairs were tested for their relative ranks and anxiety-like and exploratory behaviors. The study protocol is

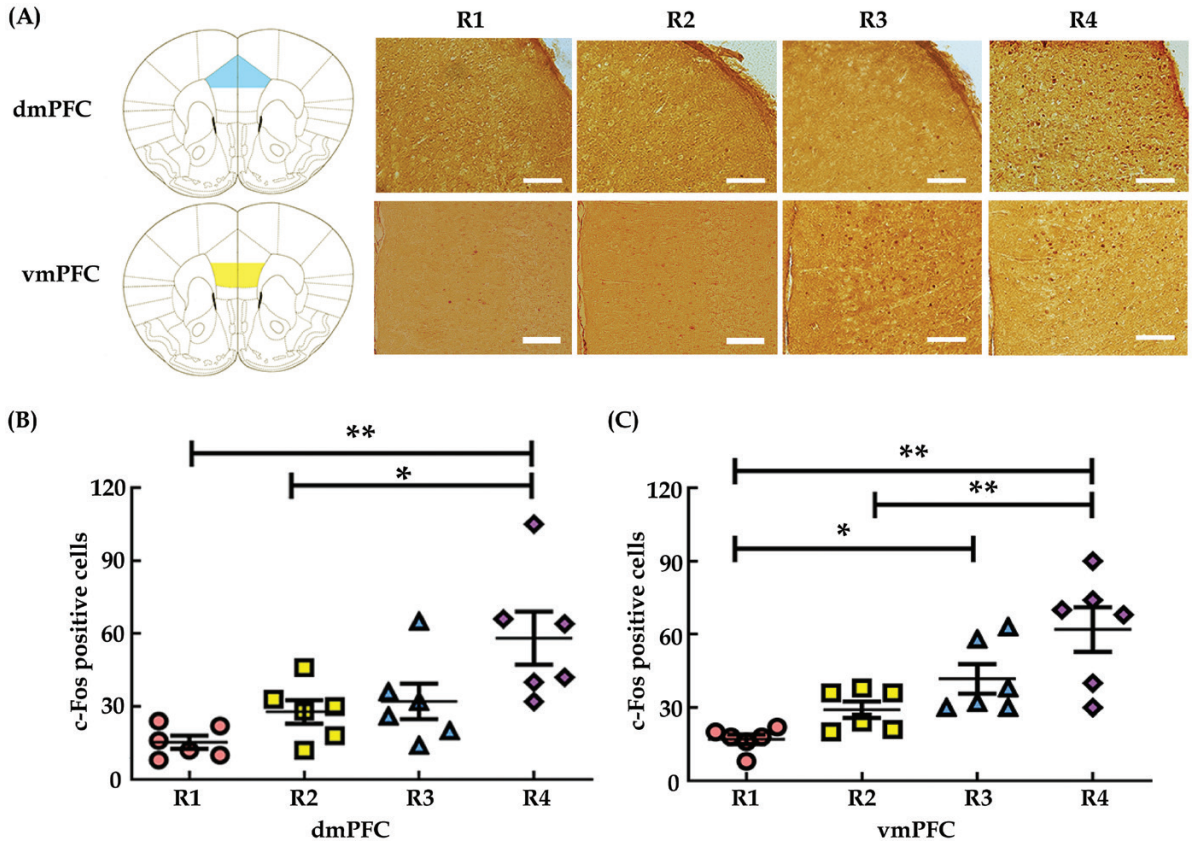

Figure 3 c-Fos expression profiles in the mPFC after a tube confrontation test. (A) Schematic location of the dmPFC and vmPFC and representative images of c-Fos expression in each rank group (scale bars = 100 μm). (B) Quantification of c-Fos expression in the dmPFC (one-way ANOVA). (C) Quantification of c-Fos expression in the vmPFC (Kruskal–Wallis test). Data are presented as the mean ± *SE*; $n = 6$ in each group; $^{*}P < 0.05$, $^{**}P < 0.01$. dmPFC: dorsal part of the medial prefrontal cortex; vmPFC: ventral part of the medial prefrontal cortex.

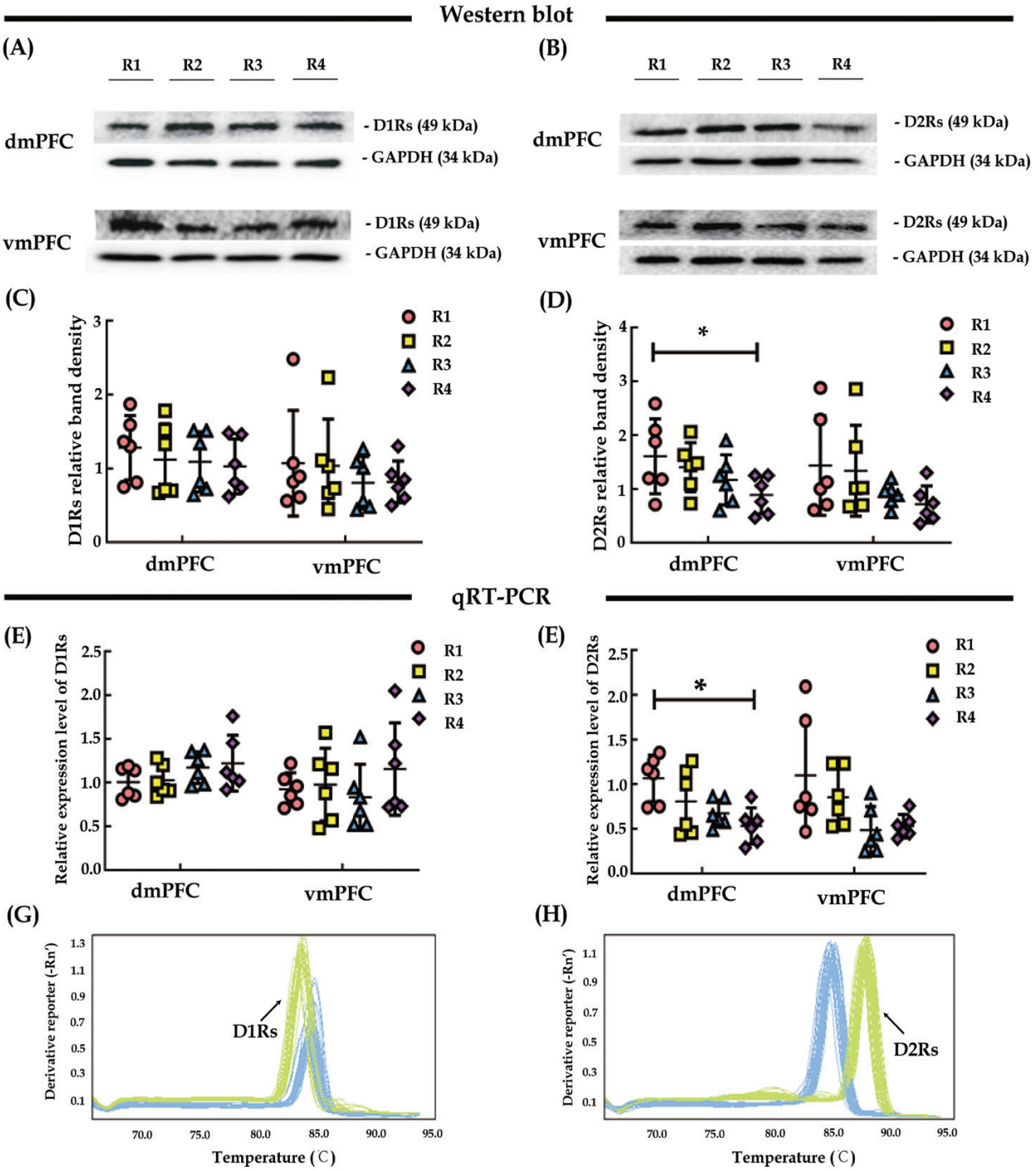

Figure 4 D1R and D2R expression profiles in the mPFC in different rank groups. (A, B) Representative western-blot images of D1Rs and D2Rs. (C, D) Quantitative analysis of D1R and D2R expression in the mPFC in different rank groups. (E, F) qRT-PCR results showing the relative expression of D1R and D2R mRNA in different rank groups. (G, H) Representative melting curves of D1R and D2R expression during qRT-PCR, showing primer availability and PCR product homogeneity (lines without arrows are β-actin). Panels C–E are analyzed with 1-way ANOVA. Panel F was analyzed with the Kruskal–Wallis test. Data are shown as the mean ± *SE*; $n = 6$ in each group; $^{*}P < 0.05$. dmPFC: dorsal part of the medial prefrontal cortex; vmPFC: ventral part of the medial prefrontal cortex.

shown in Figure 5A–B. The KD efficiency in the dmPFC was confirmed through qRT-PCR quantification of D2R mRNA levels (dmPFC: D2R-KD vs. virus control, $t_{10} = -2.291$, $P = 0.022$, $BF_{+0} = 3.683$, Figure 5D; vmPFC: $Z = -1.601$, $P = 0.109$, Figure 5E).

In the tube test, we found that D2R-KD mice had a lower number of winning trials, exhibiting less dominance than their control partners (D2R-KD vs. virus control, $t_{10} = 4.767$, $P < 0.01$, $BF_{+0} = 34.846$; Figure 5F). In the OFT, 2-way repeated ANOVA results showed significant main effects of "time" ($F_{1,10} = 20.100$, $P = 0.001$, $BF_{incl} = 95.847$), but not "virus" ($F_{1,10} = 3.986$, $P = 0.074$, $BF_{incl} = 1.367$) or their interaction ($F_{1,10} = 1.049$, $P = 0.330$, $BF_{incl} = 0.644$) on time spent in the central area. The following post-hoc comparison showed that D2R-KD

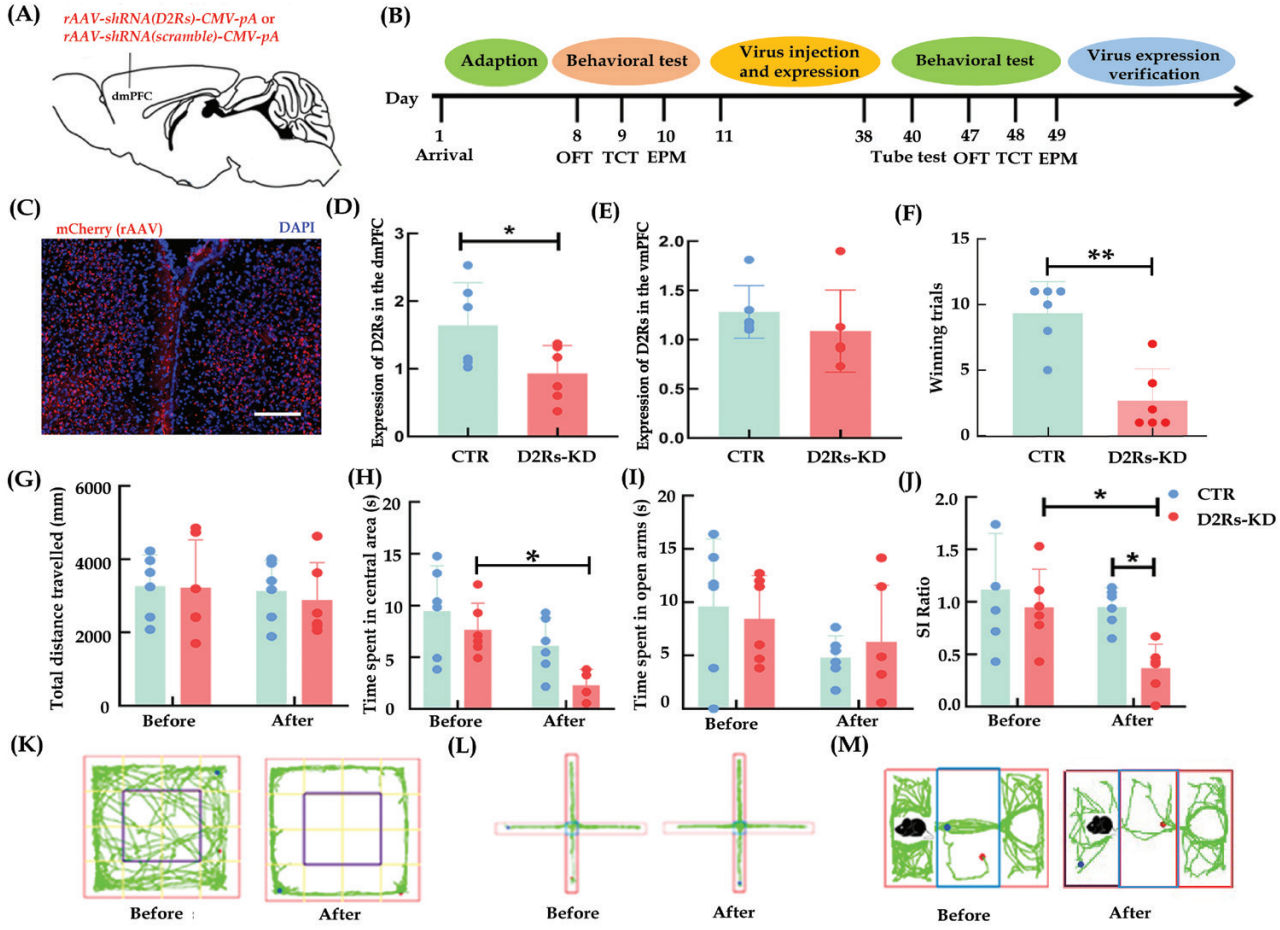

Figure 5 Effects of D2Rs knockdown in the dmPFC on social rank attainment and anxiety-like behavioral performance in a dyadic hierarchy. (A) Schematic representation of dmPFC infusion sites and virus strategy. (B) General study timeline. (C) Representative image showing virus expression (scale bar = 100 μm). (D, E) D2Rs mRNA expression in the dmPFC but not in the vmPFC decreased in mice injected with the AAV-U6-shRNA(D2Rs)-mCherry virus (D2Rs-KD) compared to scrambled-infused mice (control). (F) D2Rs-KD mice had less winning trials than scramble controls. (G, H) Total distance traveled and time spent in central areas in the open-field test before and after virus injection. (I) Time spent in open arms in the elevated plus maze test before and after virus injection. (J) SI ratio in the 3-chamber test before and after virus injection. (K–M) Representative tracks for the D2R-KD group in an OFT, EPM test, and 3-chamber test before and after virus injection. Panels D and F were analyzed with an independent *t*-test, panel E with the Mann–Whitney *U* test, and panels G–J with a 2-way repeated-measures ANOVA. Data are presented as the mean ± *SE*; $n = 6$ in each group; $^{*}P < 0.05$, $^{**}P < 0.01$. CTR: control; dmPFC: dorsal part of the medial prefrontal cortex; SI ratio: social interaction ratio.

mice spent less time in the central area in the OFT (D2R-KD: pre vs. post, $P = 0.013$, Figure 5H). However, there was no significant main effect of "virus" ($F_{1,10} = 0.098$, $P = 0.761$, $BF_{incl} = 0.503$), "time" ($F_{1,10} = 0.447$, $P = 0.519$, $BF_{incl} = 0.450$), or their interaction ($F_{1,10} = 0.091$, $P = 0.769$, $BF_{incl} = 0.489$) on the total distance traveled in the 5-min OFT (Figure 5G).

In the EPM test, we found no significant main effect of "virus" ($F_{1,10} = 0.029$, $P = 0.869$, $BF_{incl} = 0.531$), "time" ($F_{1,10} = 4.771$, $P = 0.054$, $BF_{incl} = 1.964$) or their interaction ($F_{1,10} = 0.224$, $P = 0.646$, $BF_{incl} = 0.491$) on time spent in the open arms (Figure 5I). In the 3-chamber test, there were significant main effects of "time" ($F_{1,10} = 9.601$, $P = 0.011$, $BF_{incl} = 5.424$), but not "virus" ($F_{1,10} = 3.874$, $P = 0.077$, $BF_{incl} = 1.282$) or their interaction ($F_{1,10} = 2.867$, $P = 0.121$, $BF_{incl} = 1.077$) on the SI ratio. The post-hoc comparison showed that D2R-KD mice had reduced sociability compared with both their control partners and the baseline (D2R-KD: before vs. after, $P = 0.030$; after virus injection: D2R-KD vs. virus control, $P = 0.034$; Figure 5J). As there was no significant difference in total exploration time, the difference may not be due to motivation (virus: $F_{1,10} = 0.02$, $P = 0.891$, $BF_{incl} = 0.467$; time: $F_{1,10} = 0.123$, $P = 0.733$, $BF_{incl} = 0.395$; virus × time: $F_{1,10} = 0.225$, $P = 0.645$, $BF_{incl} = 0.515$; data not shown).

Simultaneous D2R activation in the subordinate and DR2 inactivation in their dominant partners reversed their social rank relationship

To further verify D2R functions in social dominance, we next determined whether manipulating D2Rs' activity in a pair of mice could alter a dominant–subordinate relationship. To this end, after determining social rank, a D2R agonist, quinpirole, was injected into the dmPFC of subordinate individuals, and a D2R antagonist, raclopride, was simultaneously injected into the dmPFC of their dominant partners. The whole study protocol is shown in Figure 6B. Fisher's Exact test showed a significant difference in rank switching rate (dominant reversed to subordinate) among groups (Control_Day 22: 1/12 (8.33%), Control_Day 28: 2/12 (16.7%), Saline_Day 22: 2/12, 16.7%, Drugs_Day 28: 9/12 (75%); $X^2 = 14.437$, $P = 0.001$). The follow-up analysis indicated that these drug treatments significantly

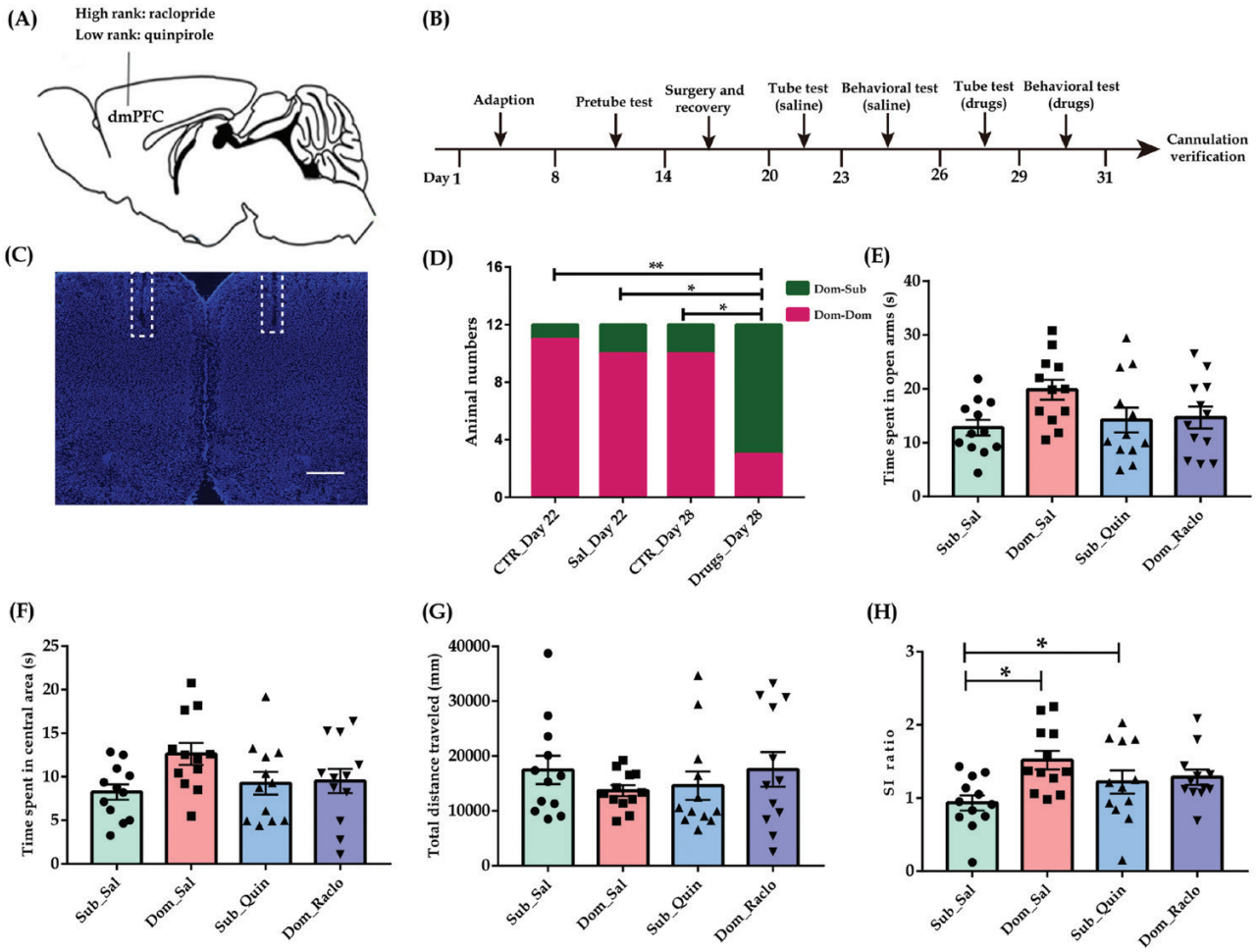

Figure 6 Effects of microinjection of a D2Rs agonist or antagonist in the dmPFC on social rank attainment and anxiety-like behavioral performance in a dyadic hierarchy. (A) Schematic representation of dmPFC infusion sites. (B) Experimental timeline. (C) Representative photomicrograph of the injection site (dashed boxes; scale bar = 200 μm). (D) Social dominance switching rate across days and treatments. The switching rates were consistently determined in a pre-tube test before the stereotaxic surgery. (E) Time spent in the open arms in the elevated plus maze test after saline or drug injection. (F, G) Time spent in the central areas and total distance traveled in the open-field test after saline or drug injection. (H) SI ratio in the social interaction test after saline or drug injection. Fisher's Exact test (panel D) or 2-way repeated-measures ANOVA (panels E–H) were conducted for analysis; $n = 12$ in each group; $^{*}P < 0.05$; $^{**}P < 0.01$. dmPFC: dorsal part of the medial prefrontal cortex; SI ratio: social interaction ratio; Sub: subordinate; Dom: dominant; CTR: control; Sal: saline; Raclo: raclopride (a D2R antagonist); Quin: quinpirole (a D2R agonist).

increased the switching rate compared with both saline treatment and the naturally occurring rate (Drugs_Day 28 vs. Saline_Day 22, Drugs_Day 28 vs. Control_Day 28, all adjusted $P = 0.012$; Figure 6D). In the behavioral tests, a repeated-measures ANOVA revealed strong evidence of an interaction of "Time × Drug" in the SI ratio ($F_{1, 22} = 24.918$, $P < 0.001$, $BF_{incl} = 60.869$). Consistent with the above results, subordinate individuals showed lower sociability than their high-rank partners (saline_subordinate vs. saline_dominant, $P = 0.013$), but quinpirole administration reversed this trend (saline_subordinate vs. quinpirole_subordinate, $P = 0.016$; quinpirole_subordinate *vs*. saline_dominant, $P = 0.418$; Figure 6H). In addition, we found a minimal "Time × Drug" interaction effect on the time spent in the open arms ($F_{1, 22} = 4.588$, $P = 0.044$, $BF_{incl} = 2.424$; Figure 6E) and time spent in the central area ($F_{1, 22} = 5.678$, $P = 0.026$, $BF_{incl} = 2.910$; Figure 6F), but the result was inclusive. No "Time × Drug" interaction effect was found on the total distance traveled in the OFT ($F_{1, 22} = 2.266$, $P = 0.146$, $BF_{incl} = 0.964$; Figure 6G).

Discussion

In this study, we investigated the functional role of DA receptors within different subregions of the mPFC in social rank attainment. Using various tests, we found that D2Rs within the dmPFC may play an important role in social dominance in C57 male mice, as manifested by (1) D2R expression was significantly downregulated in the dmPFC in lower rank individuals; (2) viral D2R-KD in the dmPFC led to mice being particularly prone to lose the contests in the tube test; and (3) simultaneous activation of D2Rs in subordinates and D2R inhibition in the dominants in a pair reversed their previous social rank relationship.

Consistent with Kunkel's study (Kunkel and Wang 2018), we found that subordinate mice showed increased social anxiety in the 3-chamber test (Figure 1G). Further, Horii et al. (2017) found that subordinate mice exhibited higher anxiety- and depression-like behaviors in the EPM test and forced swim test compared with dominant mice. Nevertheless, other studies reported that dominant animals could show higher

(Kudryavtseva et al. 2002; Larrieu et al. 2017) or comparable anxiety levels (Pallé et al. 2019). The reasons for this discrepancy are not clear. Maybe, just as a few researchers had suggested, social rank stability in a social group rather than subordination per se, is particularly relevant for emotional disorders (Williamson et al. 2017; Larrieu and Sandi 2018; Varholick et al. 2020). We found that social rank was not linked to differences in total locomotion in the OFT (Figure 1D); this result adds to the mixed results from previous studies (Larrieu et al. 2017; Zhou et al. 2017; Pallé et al. 2019; Varholick et al. 2019).

Consistent with previous studies (Zielinski and Vandenbergh 1993; Clinard et al. 2016), we found higher basal serum T levels in dominant individuals (Figure 2A), which could partly explain their more competitive in the tube test. For CORT, our results were inconclusive, despite a trend for subordinate individuals to have higher CORT levels than dominant ones (Figure 2B). Currently, the literature reporting the relationship between steroidal hormones and social dominance is also mixed (Barnard et al. 1996; Blanchard et al. 2001; Hardy et al. 2002; Merlot et al. 2004; Larrieu et al. 2017; Williamson et al. 2017; Varholick et al. 2019; Tibbetts et al. 2022). Methodological discrepancies, differences between species or social factors may account for these disparities. As Williamson et al. (2017) indicated, the unique contextual characteristics of a specific social network are important when examining the physiological correlates of dominant or subordinate social status.

Recently, using in vivo single-unit electrophysiological recording, Zhou et al. (2017) found that dmPFC neurons in mice are activated during effortful behaviors in the tube test and that optogenetic activation of the dmPFC induces instantaneous winning. However, we found significantly lower c-Fos in the dmPFC in top-rank individuals compared with their subordinate partners after a tube test (Figure 3B). Methodological differences may account for these discrepancies, that is, the c-Fos-positive neurons may not be the same as those electrophysiologically recorded or those optogenetically manipulated neurons in Zhou's study (Zhou et al. 2017). Considering the low time resolution of c-Fos immunohistochemistry, it would be recommended to further study the relationship between dmPFC's activity and social dominance by using high-time-resolution technique such as fiber photometry. The number of c-Fos-positive neurons was also higher in the vmPFC in low-rank individuals (Figure 3C). Growing evidence suggests that the vmPFC has a key role in mediating affective disorders and adaptive responses to stress (Seese et al. 2013; Suzuki et al. 2016). The increased c-Fos expression may, therefore, reflect stress suffered by the subordinate during the tube test. Consistently, we found stress-vulnerable mice showed elevated c-Fos expression in the infralimbic cortex (IL, part of the vmPFC) when exposed to a social stressor (Zou et al. 2021). However, Cooper et al. (2017) found that subordinate hamsters had lower c-Fos immunoreactivity compared to dominants in the IL. The use of different stressors (restraint stress vs. tube test) or species (C57 male mice vs. male Syrian hamsters) may account for these discrepancies.

DA is well known for modulating appetitive and aversive motivational processes through binding to D1Rs or D2Rs (Salamone and Correa 2012; Kesner et al. 2022). For social dominance, van der Kooij et al. (2018) found that intra-NAC infusion of a D1R antagonist abolishes the animals' chances to become dominant; Shan et al. (2022) found that chemogenetic activation of D1R-expressing neurons in the NAC shell reduces social dominance, while chemogenetic activation of D2R-expressing neurons in the NAC core promotes social dominance in mice; and Yamaguchi et al. (2017) found that systemic administration of a D2R but not a D1R antagonist attenuated social dominance in both mice and macaques. As in the NAC, DA similarly modulates prefrontal functions through D1Rs and D2Rs, which have critical but distinct roles in social information processing in rodents (Xing et al. 2016; Shinohara et al. 2018; Li et al. 2020). We found significant D2Rs downregulated in the dmPFC of bottom-rank individuals (Figure 4D and F); moreover, viral downregulation of D2Rs in the dmPFC lowered their social rank (Figure 5F). In line with these results, simultaneously activating D2Rs in subordinates and inhibiting D2Rs in dominants switched their dominant–subordinate relationship (Figure 6D). Therefore, DR function in social dominance might depend on manipulation route or pattern, as well as on the targeted brain regions. However, Xing et al. (2022) found recently that the synaptic strength of D1R-expressing neurons in the mPFC determines the dominant status, while hyperactive D2R-expressing neurons are associated with subordinate status in C57 male mice. It should be noted that in Xing's study, they did not distinguish between different parts of the mPFC, which may account for the discrepancy. Furthermore, a previous study indicated that D1Rs are most abundantly expressed in deep layers whereas D2Rs are more densely expressed in superficial layers of the mPFC (Wei et al. 2018). These variations in the distribution of these 2 receptors may also account for the discrepancy.

We found that viral downregulation of D2Rs in the dmPMC increased social anxiety in SI tests (Figure 5J). These results are consistent with the social dominance performance in the tube test and with the pharmacological study, which showed that dmPMC administration of a D2R agonist increased subordinates' sociability (Figure 6H). We also found D2R-KD mice showed anxiety in the OFT (Figure 5H), which was not observed either in the pharmacological or behavioral study in Figure 1. These inconsistent results may be explained by intervening measures, group or sample sizes, or unknown reasons. The results are generally in line with Chen's study, which showed an antidepressant-like effect of exercise mediated by D2Rs in the mPFC (Chen et al. 2016).

Overall, the above results suggest that D2Rs within the dmPFC likely play an important role in social dominance. This study has some limitations worth noting. First, we only studied groups of mice with stable dominance hierarchies, which excluded other dominance organizations such as despotic or unclear (Varholick et al. 2019). Although a common practice in current social rank studies (Varholick et al. 2020), it might bias our understanding of the general relationship between social dominance and other behavioral tests. Second, we did not verify social dominance in other paradigms such as the cage observation test, warm spot test, or scent-marking test (Fulenwider et al. 2022), even if the availability and transitivity of tube test in social dominance has been verified in numerous studies (Wang et al. 2011; Larrieu et al. 2017). Third, according to Dr. Colegrave and Dr. Ruxton's view, cage assignment might be an independent factor in some animal studies, which means testing animals from the same cage is pseudo-replication and cage factors should be taken into account during study design or data analysis (Colegrave and Ruxton 2018). In support of this, Varholick et al. found that

although social rank more consistently accounted for data variability, cage assignment sometimes accounted for a significant portion of the variability (Varholick et al. 2018, 2019). The results in this study, therefore, should be interpreted cautiously to avoid overstating the effect of social rank. In spite of this, our study provides a new understanding on the neurochemical mechanisms underlying social dominance in male mice, and might shed new light on the treatment of social stress-related psychiatric diseases such as depression, autism, and schizophrenia.

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Conflict of Interest

None.

Ethics Statement

This study protocol was reviewed and approved by Animal Care and Use Committee of Nanyang Normal University (approved number: 20201202005).

Author Contributions

Liu YJ, Li LF, and Yao LG participated in designing the study; Li ZL conducted the majorities of experiments; LI LF wrote the original draft; Song BL, Wang Y, Jiang Y, and Zou HW participated in the behaviors' experiment and helped to collect and analyze the data; Yao LG participated in the manuscript revision. All authors contributed to and have approved the final manuscript.

Data Availability

All data generated or analyzed during this study are available upon reasonable request from the corresponding authors.

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