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5-HT_{2A} receptor in medial prefrontal cortex mediates pain-related empathic responses in rats

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ABSTRACT

Using a dyadic social interaction (DSI) paradigm, we validated a scenario of pain-related empathic responses in rats and mice, during which socially transferred pain was identified in naïve observers spontaneously engaging in caring behavior (allolicking and allogrooming, ALAG) toward a familiar pain sufferer. Although 5-hydroxytryptamine (5-HT) and its receptor subtype 5-HT_{2A}R are major players in mammalian social behaviors, whether they have specific modulatory actions in these processes remains unknown. Here, by genetic and pharmacological depletion of central 5-HT, we demonstrated that observers lacking 5-HT showed deficiencies not only in empathic responses but also in sniffing, huddling, and self-grooming during DSI with a familiar pain sufferer. However, bilateral intra-mPFC microinjections of M100907, a selective 5-HT_{2A}R antagonist, or downregulation of 5-HT_{2A}R expression in the bilateral mPFC, selectively inhibited socially transferred pain and ALAG without affecting other behaviors in observers. Moreover, systemic or intra-mPFC administration of 25CN-NBOH, a high-affinity and selective 5-HT_{2A}R agonist, selectively promoted empathic responses in observers toward a stranger pain sufferer in an antagonist-reversible manner, a phenomenon rarely observed under the naïve-pain DSI paradigm. Finally, 5-HT_{2A}R downregulation in the bilateral mPFC abolished the empathic responsiveness induced by 25CN-NBOH (i.p.) in stranger observers. Collectively, our findings establish that 5-HT_{2A}R activation in the mPFC is both necessary and sufficient for selectively mediating empathic responses in bystanders toward individuals in pain, identifying 5-HT_{2A}R as a promising therapeutic target for alleviating prosocial

and empathic impairments seen in disorders, such as autism spectrum disorder.

Keywords: 5-hydroxytryptamine; 5-HT_{2A} receptor; Medial prefrontal cortex; Empathy; Pain; Allolicking and Allogrooming

INTRODUCTION

Engaging in prosocial behavior is critical for enhancing mental health, strengthening social bonds, and promoting well-being in human and animal societies. One important form of prosocial behavior that remains inadequately understood is empathy toward others' distress (pain, fear, and catastrophe). When we see someone else in pain, we naturally feel sadness and compassion and want to offer support and comfort to help them overcome their difficulties (Chen, 2018; de Waal, 2008; de Waal & Preston, 2017). When empathy is impaired, the ability to perceive, respond to, and care for others' suffering becomes abnormal. This impairment has been proposed to be associated with the several psychiatric conditions, including autism spectrum disorder (Bird & Viding, 2014; Hadjikhani et al., 2014; Minio-Paluello et al., 2009), depression (Kupferberg et al., 2016; Schreier et al., 2013; Thoma et al., 2011), psychopathy and alexithymia (Bird & Viding, 2014), and schizophrenia (Bonfils et al., 2017; Haker et al., 2012). Ultimately, these conditions share underlying deficits in social perception, communication, emotional recognition, cooperativeness, and theory of mind. Genome-wide analyses have revealed high correlations of self-reported empathy with autism, schizophrenia, and anorexia nervosa (Warrier et al., 2018). Thus, assessment of the ability of empathy for distress is critically important for predicting whether patients are able to successfully return to normal social activities. In the past decades, featured empathic behaviors have been experimentally identified and validated qualitatively and

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quantitatively in both rats (Chen, 2018; Lü et al., 2017; Luo et al., 2020; Yu et al., 2019) and mice (Du et al., 2020, 2024a; Geng et al., 2020) under the dyadic social interaction (DSI) paradigm, in which a naïve observer (bystander) freely interacts with a conspecific demonstrator in pain. The empathic behaviors have the following characteristics: (1) naïve observers often show more distinct caring behavior toward a pain sufferer during 30-min DSI in terms of engagement of injury-site-targeted allolicking and body-targeted allogrooming (ALAG) (Du et al., 2020; Walsh et al., 2018), helping others cope with pain (Zhang et al., 2024); (2) pain hypersensitive state can be matched through social transfer from a pain sufferer to a naïve observer after 30-min DSI (Du et al., 2020; Walsh et al., 2018); (3) the caring behavior and socially transferred pain are basically dependent upon the extent of dyadic familiarity, but may be modulated by stress level (Li et al., 2014; Martin et al., 2015), visual and olfactory input (Geng et al., 2020; Smith et al., 2016), prior pain experience (Luo et al., 2020), sex hormones (Du et al., 2024a), and experimental procedure or paradigm (Du et al., 2024b; Yu et al., 2019).

Serotonin (5-hydroxytryptamine, 5-HT) is involved in numerous biological processes, including learning, memory, mood regulation, sleep, reproduction, circadian rhythm, and pain perception (Crockett et al., 2010; Kim et al., 2014; Mondoloni et al., 2024; Yang et al., 2022b). Interestingly, enhancing 5-HT levels, through dietary tryptophan or pharmacological agents like citalopram (a selective serotonin reuptake inhibitor), has been associated with increased emotional perception, moral decision-making, and prosocial behaviors (Attenburrow et al., 2003; Crockett et al., 2010; Knutson et al., 1998; Moul et al., 2018). A recent report has discovered that 5-HT regulates maternal affiliation by infants, conserved from mice and rats to monkeys (Liu et al., 2023). Moreover, bidirectional modulation of the release of 5-HT from the dorsal raphe neurons to the nucleus accumbens influences sociability in a reciprocal manner (Walsh et al., 2018). However, another study has also shown that increased 5-HT levels can disrupt socially transferred fear in mice (Kim et al., 2014). These findings highlight 5-HT as a master regulator in mammalian social behaviors.

The ability of 5-HT to mediate a wide range of behaviors may stem from various types of postsynaptic receptors, which determine the final response to 5-HT release (Barnes & Sharp, 1999; Wallach et al., 2023). The 5-HT_{2A} receptor (5-HT_{2A}R), which is densely expressed in the forebrain, plays a complex role in emotional behaviors (Aznar & Klein, 2013; Farias et al., 2024). While polymorphisms in the 5-HT_{2A}R gene have been linked to aggression, impulsivity, and antisocial traits (Burt & Mikolajewski, 2008; Butovskaya et al., 2018), other studies suggest its involvement in prosocial and affiliative behaviors (Gong et al., 2015; Unschuld et al., 2007; Wan et al., 2021). Interestingly, acute activation of 5-HT_{2A}R can disrupt maternal behaviors in rats, though this effect does not appear to be related to emotional or motivational processing (Gao et al., 2018, 2019; Wu et al., 2018). These findings suggest 5-HT receptors, particularly 5-HT_{2A}R, are major players in mammalian social behaviors. However, it remains unclear whether 5-HT and 5-HT_{2A}R specifically influence socially transferred pain and/or the caring behavior, due to limited evidence.

The medial prefrontal cortex (mPFC), which includes the anterior cingulate cortex (Cg1), prelimbic cortex (PrLC,

homologous to primate dmPFC), and infralimbic cortex (ILC, homologous to primate vmPFC), has been associated with a range of functions, including attention, decision-making, goal-directed behavior, and working memory (Carrillo et al., 2019; Li et al., 2014; Zhang et al., 2024). The existing literature, primarily from rodent and post-mortem primate studies, indicates that 5-HT_{2A}R shows significant expression in the PrLC, the ILC, and Cg1 (Du et al., 2024b). Several studies have attempted to explain socially transferred pain or distress at the cellular or molecular level, suggesting that the mPFC is crucial in processing socially transferred pain or distress and consolation behavior (Burkett et al., 2016; Li et al., 2014; Smith et al., 2021).

We therefore employed the DSI paradigm to define how 5-HT and its receptors in the mPFC mediate empathic responses in observer rats for a conspecific in pain. We aim to address the following questions: (1) How does endogenous 5-HT in the CNS modulate the empathic behaviors of observers toward a familiar pain sufferer? (2) What are the roles of 5-HT_{2A}R in the mPFC in the development of empathic behaviors toward a familiar pain sufferer? (3) Could activation of 5-HT_{2A}R in the mPFC elicit empathic behaviors toward an unfamiliar (stranger) pain sufferer?

METHODS

Animals

All Sprague-Dawley albino rats were provided by the Laboratory Animal Center of the Fourth Military Medical University (FMMU). Tryptophan hydroxylase 2 (Tph2) knockout (KO) rats were provided by Dr. Yi Rao of Peking University School of Life Sciences (<https://doi.org/10.1016/j.neuron.2023.02.010>) and bred in the Laboratory Animal Center of FMMU. The use of animals was reviewed and approved by the Animal Care and Use Committee of the FMMU. All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 1996) and followed the ethical guidelines for pain research in conscious animals of the IASP. Every effort was made to minimize the number and suffering of animals used. Female rodents exhibit estrous cycles characterized by fluctuating levels of estrogen and progesterone, which are known to modulate both 5-HT reuptake and function (Gu et al., 2022), as well as complex social behaviors (Asokan & Falkner, 2025). As no sex difference exists in pain-related empathic responses in rats (Du et al., 2020), male rats were used to reduce intrinsic variance in this study. Moreover, a double-blind design was employed for all behavioral testing. Rats were housed in a climate-controlled room (temperature 22 °C–26 °C, air humidity 40%–70%) under a 12 h light/12 h dark cycle with access to food and water *ad libitum*. The light was on at 0800h, and all handling and behavioral assays were carried out between 0900h and 1800h.

Tph2 KO rats

The details regarding the design and breeding of Tph2^{-/-} rats have been described previously (Liu et al., 2023). The Tph2^{-/-} line was maintained by crossing Tph2^{+/-} heterozygotes. Littermates included wild-type (Tph2^{+/+}), heterozygous (Tph2^{+/-}), and homozygous mutant (Tph2^{-/-}) rats. Only male rats were used for behavioral tests in this study.

Genotype identification and PCR

To identify the genotypes of wild-type and Tph2 KO alleles, rats were routinely identified using standard PCR conditions. Primers for genotyping were GACCATATGTCCTTGTCACG TTTC and CTTCTCGGGTGTCCCTTTGGGGCAG. Genomic DNA was isolated from tail tissue samples using a Mouse Tail Direct PCR Kit (FOREGENE, China). Premix Taq DNA Polymerase (TaKaRa, Japan) was used to simplify the setup of PCR reactions and minimize pipetting steps. PCR products were analyzed via agarose gel electrophoresis.

Bee venom test

The bee venom (BV) model is a well-established animal model of acute inflammatory pain. The model was established by injecting a volume of 50 μ L of solution (0.2 mg BV dissolved in 0.9% physiological saline) into the center of the plantar surface of the left hindpaw in rats with slight restraint (Chen et al., 1999, 2016; Du et al., 2020, 2024a; Yu et al., 2019).

Dyadic social interaction paradigm

As previously described (Du et al., 2024a; Li et al., 2014; Lü et al., 2017; Luo et al., 2020; Yu et al., 2019), a naïve rat, serving as a cagemate observer (CO) or non-cagemate observer (NCO), was allowed to move freely in a testing cage with clean bedding. After 5–10 min of acclimation, another rat, serving as a familiar (cagemate) demonstrator (CD) or unfamiliar (non-cagemate) demonstrator (NCD), was introduced into the testing cage for dyadic social interaction (DSI) with the CO or NCO in physical contact immediately after receiving the BV injection. The whole 30-min DSI process between the naïve observer and the pain-sufferer was recorded by a video camera (FDR-AX40, SONY, Japan) and analyzed off-line. Four sets of major behaviors were qualitatively identified and quantitatively analyzed as follows: (1) sniffing (SN), a social snout and anogenital investigation behavior engaged by the observer toward the demonstrator after union (Du et al., 2020, 2024a); (2) allogrooming (ALAG), referred to as caring behavior engaged by the observers toward a pain-sufferer by injury-site licking and body grooming (Burkett et al., 2016; Du et al., 2020, 2024a); (3) huddling (Hu), referred to as a prosocial behavior actively engaged by the observer toward the pain-sufferer by tight body contact against each other when resting (Du et al., 2024a); (4) self-grooming (SG), an innate programmed behavior engaged by the observer to maintain body hygiene (Du et al., 2024a; Kalueff et al., 2016). To quantify these behaviors, latency, total time, and bouts of each behavior were calculated for further statistical analysis. Latency was defined as the time lag from the starting point of the DSI to the first action of each behavior; total time, the cumulative time of each behavior during the 30-min DSI; and bouts, the counts of waves of each behavior during the 30-min DSI (Du et al., 2020, 2024a).

Quantification of pain-related behaviors in awake animals

Persistent spontaneous nociceptive behavior: As described in our previous works (Chen et al., 1999; Du et al., 2020, 2024a; Li et al., 2014), a 30×30×30 cm non-transparent plastic box was placed on a 30 cm high supporting frame above the experimental table. The rat was placed in the test box for at least 30 min before any treatments. After the acclimation period, an intraplantar injection of BV was made as described above (Chen et al., 1999; Du et al., 2024a; Luo et al., 2017; Yu et al., 2019). The quantitative score of persistent spontaneous nociception was obtained by counting

the number of paw flinches occurring in the injected hindlimb at each 5-min interval for 30 min following BV injection.

Mechanical pain sensitivity: The paw withdrawal mechanical threshold (PWMT) was used to evaluate mechanical pain sensitivity and was measured as previously described. Briefly, rats were placed on an elevated wire grid floor in a non-transparent plastic chamber (length×width×height=20×20×25 cm). A series of increasing forces of von Frey monofilaments (bending forces: 7.84, 19.60, 39.20, 58.80, 78.40, 98.00, 117.60, 137.20, 156.80, 176.40, 196.00, 245.00, 294.00, 441.00, and 588.00 mN) were used to stimulate the plantar surface of the rat's bilateral hind paws, respectively. The PWMT was obtained from the lowest forces enabling 50%–60% induction of paw withdrawal responses by 10 repetitive stimuli. The interval between each stimulus was no less than 5 s. The interval between stimuli of the left and right hind paws was no less than 5 min (Chen et al., 1999; Du et al., 2024a; Luo et al., 2020; Yu et al., 2019).

Cannula implantation and intra-mPFC microinjection

Intracerebral cannula implantation was performed as previously described (Geng et al., 2016; Okine et al., 2014). Rats were sufficiently anesthetized with 1% sodium pentobarbital (50 mg/kg, i.p.) and then fixed in a stereotaxic apparatus by ear bars (RWD Life Science Co., Ltd., Shenzhen, China). The eyes were covered with a drop of ophthalmic ointment to prevent drying. After a midline incision, two 0.8 mm diameter holes were drilled on two sides of the frontal skull. Two custom-designed stainless-steel guide cannulae (diameter 0.48 mm; RWD Life Science Co., Ltd., Shenzhen, China) were implanted into the medial prefrontal cortices (mPFC) according to the Paxinos and Watson atlas of The Rat Brain in Stereotaxic Coordinates (Bregma: rostral 3.2 mm, mediolateral 0.7 mm, dorsoventral 3.5 mm) (Geng et al., 2016). The cannulae were permanently fixed to the skull using stainless steel screws and dental cement. To prevent infection, penicillin and streptomycin (300 mg/kg, i.p., for each) were administered immediately after surgery for 3 days. Rats were allowed to recover from the surgery for 14 days before any treatments. In the last 5 days of recovery, rats were trained to acclimatize to handling and experimental environments for 5–10 min daily. Rats received bilateral microinjections of agents (0.5 μ L over 60 s for each side) using a microsyringe (1.0 μ L) connected with polyethylene tubing and a stylet to the implanted cannulae.

Drugs

To pharmacologically deplete endogenous 5-HT, 4-chloro-DL-phenylalanine (PCPA, C6505, Sigma, USA), a selective and irreversible inhibitor of tryptophan hydroxylase, which is a rate-limiting enzyme in the biosynthesis of 5-HT, was intraperitoneally administered for 3 days (i.p., 1 mL/kg body weight, 300 mg/mL dissolved in 2% Tween 80 saline solution). To selectively activate the 5-HT_{2A} receptor (5-HT_{2A}R), 4-[2-[[[(2-hydroxyphenyl)methyl]amino]ethyl]-2,5-dimethoxybenzonitrile hydrochloride (25CN-NBOH, Cat. No. 5171, Tocris, USA), a high-affinity and selective 5-HT_{2A}R agonist, was used as described in previous papers (Ferri et al., 2023). A stock concentration of 10 mmol/L 25CN-NBOH was prepared in sterile saline. For intraperitoneal (i.p., 1 mL/kg body weight) administration, 25CN-NBOH was diluted to a working concentration of 300 μ g/mL, while for intra-mPFC microinjections, a working concentration of 100 μ g/mL was used as described in previous papers (Odland et al., 2021). To

selectively inhibit 5-HT_{2A}R, (R)-(+)- α -(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol (M100907, formerly MDL 100907, Cat. No. 4173, Tocris, USA), a potent and selective 5-HT_{2A}R antagonist, was used as described in previous papers (Sorensen et al., 1993). A stock concentration of 100 mM M100907 was prepared in dimethyl sulfoxide (DMSO). When used for i.p. administration, a working concentration of 100 μ g/mL was used, while for intra-mPFC microinjections, a working concentration of 33.3 μ g/mL was used with 3% DMSO saline solution as a control. In the case of co-administration of agonist and antagonist, a solution of M100907 (100 μ g/mL, i.p., 1 mL/kg body weight) was administered 10 min prior to 25CN-NBOH (300 μ g/mL, i.p., 1 mL/kg body weight) for systemic effects, while a mixed solution of M100907 (33.3 μ g/mL)+25CN-NBOH (100 μ g/mL) was bilaterally microinjected into the mPFC for local effects.

Recombinant adeno-associated virus (rAAV) vectors

To locally interfere with the expression of 5-HT_{2A}R within the mPFC, rAAV-U6-shRNA (Htr2a: Rattus)-CMV-EGFP-SV40 polyA [rAAV-shRNA (5-HT_{2A}R), BrainVTA, 2.11 $\times$ 10¹² μ g/mL] and AAV-U6-shRNA (scramble)-CMV-EGFP-pA [rAAV-Scramble, BrainVTA, 5.61 $\times$ 10¹² μ g/mL] were designed. Intra-mPFC microinjections were performed with a 1 μ L syringe (Hamilton, Nevada, USA) with a 32-gauge needle. Syringe pumps (Longer Precision Pump Co., Ltd., China) were used to inject the virus vector (300 nL in total) at a speed of 1 nL/s.

Immunofluorescent labeling

Rats were anesthetized with 1% pentobarbital sodium (50 mg/kg, i.p.), then perfused with 0.1 mol/L PB solution, followed by 4% paraformaldehyde (PFA) in 0.1 mol/L PB solution. After perfusion, brains were carefully extracted from the skull and postfixed in 4% PFA for 24 hours at 4°C, and cryoprotected by immersion in 30% sucrose in 0.1 mol/L PB at 4°C until they sank to the bottom of the container. Continuous coronal sections (30 μ m thick) were sectioned on a CM1900 freezing microtome (Leica, Germany). Tissue sections were collected and stored in 0.1 mol/L PB solution at 4°C until immunocytochemical processing.

Free-floating sections undergoing immunohistochemical staining were washed in PBS three times and then washed in 0.5% PBST (0.5% Triton X-100 in PBS) three times. Sections were then incubated with blocking buffer containing 10% goat serum or 8% bovine serum albumin dissolved in 0.5% PBST for 1 h. Sections were then incubated with primary antibodies diluted in primary antibody diluent (P0103, Beyotime, China) overnight at 4°C. For primary antibodies, anti-c-Fos (1:500, mouse, NBP2-50037, Novus Biologicals, USA), anti-5-HT_{2A}R (1:200, rabbit, ab66049, Abcam, USA), and anti-5-HT (1:5000, goat, 20079, Immunostar, USA) were used. After incubation, the sections were rinsed four times (10 min each) with PBST and incubated with a fluorescent dye-conjugated secondary antibody diluted in secondary antibody diluent (P0103, Beyotime, China) for 3 h at room temperature. For secondary antibodies, Alexa Fluor 488-conjugated affinity-pure goat anti-mouse IgG (1:200, 115-545-003, Jackson ImmunoResearch, USA), Alexa Fluor 594-conjugated affinity-pure goat anti-rabbit IgG (1:200, 115-545-003, Jackson ImmunoResearch, USA), and anti-goat IgG (whole molecule)-Cy3 (1:200, C2821, Sigma, USA) were used. Afterward, the sections were washed in PBS three times (10 min each time), then transferred into DAPI solution (1:2000, D9542, Sigma, USA) and incubated for 3 min. After being stained with DAPI

and washed three times with PBS, the sections were mounted on glass microscope slides, dried, and covered with mounting media (FY1301, Xi'an Fei Yang Bio, China). When carrying out double immunofluorescent labeling, two primary or secondary antibodies were used in a mixture. The images were examined under a laser scanning confocal fluorescent microscope (Olympus FV1000, Japan).

Nissl stain and histological localization of the injection sites

The frozen sections (10 μ m), collected as described above, were subjected to Nissl staining with 1% cresyl violet solution. The localizations of intra-mPFC microinjections were observed and photomicrographed by a computer-based microscope CCD camera (DP-70, Olympus, Japan). Photomicrographs were processed and analyzed by Image-Pro-Plus 6.1 software (Media Cybernetics, USA).

Quantitative real-time polymerase chain reactions (qRT-PCR)

After behavioral assays, rats receiving intra-mPFC virus microinjections were sacrificed under anesthesia. Brains were rapidly extracted, and mPFC tissues were dissected out on dry ice. Total RNA was extracted from the brain tissue containing the mPFC using TRIzol reagent according to the manufacturer's instructions (Invitrogen, USA). RNA content was measured using a Nanodrop-2000 (Thermo Fisher Scientific, USA). qRT-PCR was performed as previously described (Zhang et al., 2017). Briefly, 1 μ g of ribonucleic acid (RNA) extracted from the mPFC tissue sample was reverse-transcribed to synthesize cDNA using a PrimeScript RT reagent Kit Perfect Real Time following the manufacturer's instructions (TaKaRa, Japan). PCR reactions were performed using FastStart Essential DNA Green Master using the LightCycler 96 system (Roche, Germany). The amount of cDNA was normalized based on the expression of the housekeeping gene β -actin. Data were calculated using the 2^{- $\Delta\Delta$ CT} formula.

Statistical analysis

All data were presented as mean $\pm$ SEM. Parametric and non-parametric statistical analysis methods were used depending on both the normality test and the equal variance test, and the consistency of statistical methods used. An unpaired two-tailed Student's *t*-test was used for comparison between two groups, while a two-tailed Mann-Whitney *U* test was used when the data were not normally distributed. For comparison of three or more groups, one-way ANOVA with Bonferroni post hoc comparison was used, while the Kruskal-Wallis one-way ANOVA test with Bonferroni post hoc comparison was used if the dataset did not pass the normality test and equal variance test. A two-way repeated measures test or the Greenhouse-Geisser method, based further on Mauchly's test of sphericity, was used for the data of time effects. All statistical analyses were performed using SPSS v.23.0. Significant differences were set at *P*<0.05.

RESULTS

Non-selective roles of central 5-HT in empathic responses toward a familiar pain-sufferer

As described in our previous reports (Du et al., 2020; Li et al., 2014; Luo et al., 2020), bystander (cagemate observer, CO) rats often show more distinct caring behavior toward a familiar

(cagemate demonstrator, CD), but not an unfamiliar (non-cagemate demonstrator, NCD), pain-sufferer during 30-min DSI in terms of engagement of injury-site targeted ALAG (Figure 1A). Although sniffing (SN) and huddling (Hu) can also be identified during 30-min DSI under the naïve-pain DSI paradigm (Figure 1A), these general prosocial behaviors have been demonstrated not to be empathy-related responses to a pain-sufferer (Du et al., 2024a). Self-grooming (SG), an anti-empathic behavior, was also used as a control because under the 30-min naïve-pain DSI paradigm, CO may engage more ALAG at the cost of sacrificing SG (Du et al., 2020, 2024a). The tryptophan hydroxylase 2 (Tph2) gene is essential for the synthesis of central 5-HT in the brain. To gain understanding of the exact roles of central 5-HT in pain-related empathic

responses, we first obtained three offspring genotypes of rats through hybridization between male and female *Tph2*^{+/-} heterozygotes bred by knockout of the 6th exon of the *Tph2* gene using sgRNA-Cas9 (Liu et al., 2023) (Figure 1B, C). Compared with the wild-type (*Tph2*^{+/+}), rats with *Tph2*^{-/-} had less 5-HT-positive neuronal labeling in the raphe system of the brain, where 5-HT neuronal cell bodies are mainly located (Figure 1D). Because 5-HT is known to be an important pain modulator in the CNS (Millan, 2002), we examined basal pain sensitivity using von Frey filaments and nociceptive responsiveness to peripheral inflammatory pain caused by subcutaneous (s.c.) injection of bee venom (BV) in the three genotypes. The results showed that there were no significant changes in either the basal paw withdrawal mechanical

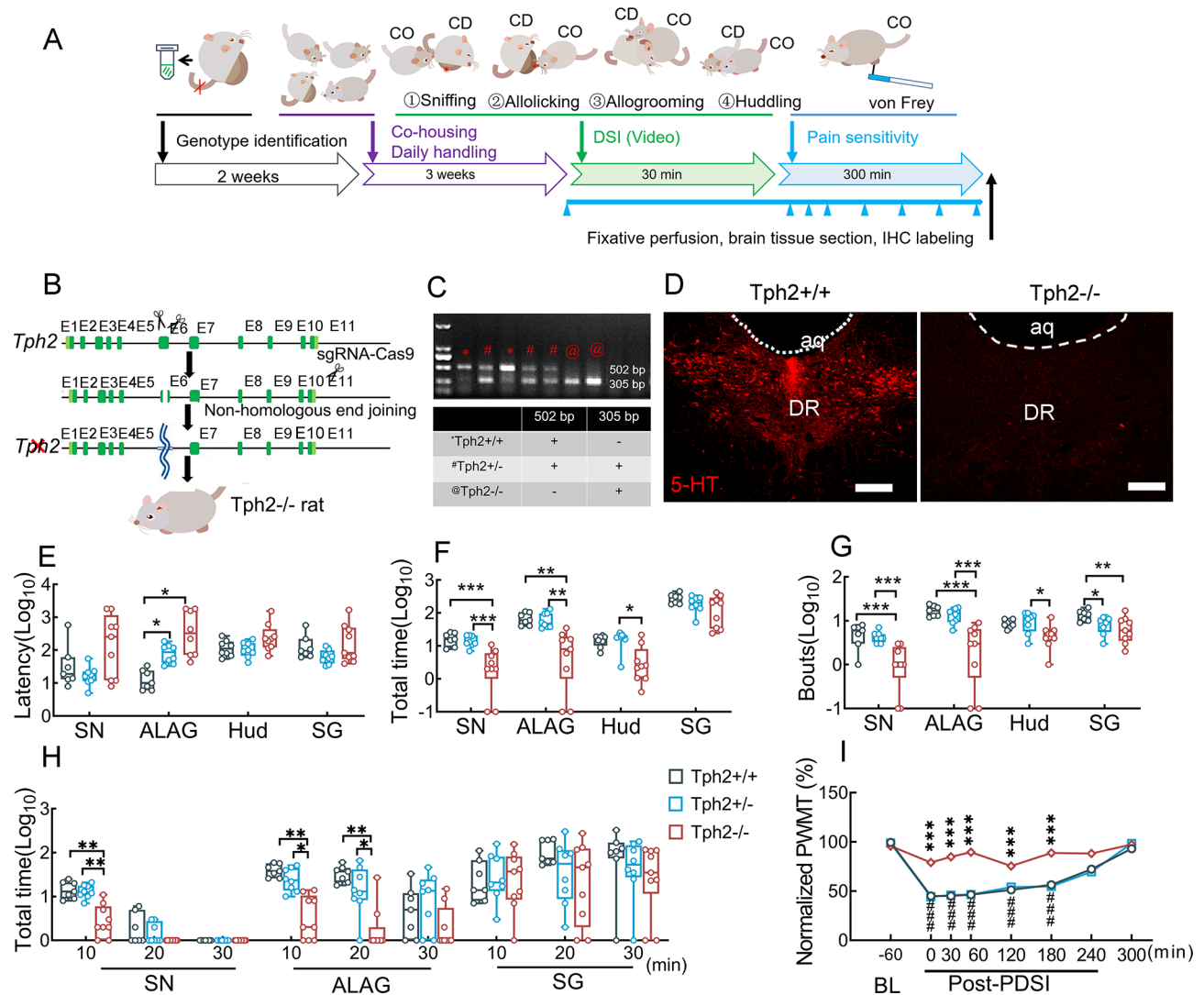


Figure 1 Identification of genotypes and behavioral phenotypes under dyadic social interaction paradigm in rats with *Tph2*^{+/+}, *Tph2*^{+/-} and *Tph2*^{-/-}

A: Timeline, experimental design and protocol of the study. B: Schematic design of *Tph2* KO rats whose 6th exon of *Tph2* gene was knocked out using sgRNA-Cas9. C: Genotype identification by DNA markers 502 bp (*Tph2* wild-type allele) and 305 bp (*Tph2* KO allele). *, *Tph2*^{+/+}; #, *Tph2*^{+/-}; @, *Tph2*^{-/-}. D: Images of 5-HT staining from a *Tph2*^{+/+} rat (top) and a *Tph2*^{-/-} rat (bottom). E–G: Latency, total time and bouts of SN, ALAG, SG and Hud in different genotypes of CO during DSI. Kruskal-Wallis one-way ANOVA test, *n*=7–9 per group. H: Time courses of the time spent on SN, ALAG and SG in CO. Kruskal-Wallis one-way ANOVA test, *n*=7–9 per group. I: Changes in PWMTs of different genotypes of CO pre- and post-DSI. Two-way ANOVA repeated measurement test, *n*=10–11 per group. Data shown as mean±SEM. *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001. Scale bar: 200 μm in D. Abbreviations: ALAG, allogrooming and allogrooming; aq, aqueduct; BL, baseline; CO, cagemate observers for naïve-pain DSI; DR, dorsal raphe nucleus; DSI, dyadic social interaction; E1–E11, exons of *Tph2* gene; Hud, huddling; PWMT, paw withdrawal mechanical threshold; SG, self-grooming; SN, sniffing.

threshold (PWMT) or the BV-induced nociceptive paw flinches and mechanical pain hypersensitivity occurring at the hind paw ipsilateral to the injection side when compared among the three genotypes of rats (Supplementary Figure S1). Under the naïve-pain DSI paradigm, CO rats with $Tph2^{-/-}$ had significantly longer latency for ALAG than $Tph2^{+/+}$ and $Tph2^{+/-}$ ($Tph2^{-/-}$ vs. $Tph2^{+/+}$, $P<0.001$; Supplementary Table S1), but without affecting SN, Hu, and SG (Figure 1E). However, in contrast, CO rats with $Tph2^{-/-}$ had significantly less total time spent on ALAG ($P<0.01$) as well as SN ($P=0.002$) and Hu ($P<0.05$), although without affecting SG, when compared with $Tph2^{+/+}$ and $Tph2^{+/-}$ (Figure 1F; Supplementary Table S1). The bouts of all four behaviors were significantly decreased in CO rats with $Tph2^{-/-}$ compared with $Tph2^{+/+}$ and $Tph2^{+/-}$ genotypes (Figure 1G). Time course observations revealed that the difference was statistically significant within the first 10 min for SN, and within the first 20 min for ALAG (Figure 1H). CO rats with $Tph2^{-/-}$ had no socially transferred mechanical pain hypersensitivity, while those with $Tph2^{+/+}$ and $Tph2^{+/-}$ did have similar time courses of this vicariously perceived pain as reported previously (Figure 1I; Supplementary Table S2) (Luo et al., 2020; Walsh et al., 2018).

To further confirm the central roles of 5-HT in SN, ALAG,

Hud and SG, we also depleted central 5-HT pharmacologically by intraperitoneal (i.p.) administration of 4-Chloro-DL-phenylalanine (PCPA), a 5-HT synthesis inhibitor in the CNS (Figure 2A). PCPA treatment (i.p.) in CO rats resulted in dramatically delayed latency in ALAG (PCPA vs. Vehicle, $P=0.005$) as well as in Hud ($n=9$ for each group, $P=0.038$) and SG ($P=0.033$) but without affecting SN, compared with vehicle treatment (Figure 2B; Supplementary Table S1). The total time was significantly reduced in CO rats with PCPA for ALAG ($P<0.001$), Hud ($P<0.001$), and SG ($P<0.001$) but not for SN, relative to those with vehicle (Figure 2C, E). However, bouts of ALAG ($P<0.001$) and SG ($P<0.001$) but not SN, Hud were decreased by PCPA compared with vehicle (Figure 2D). The depletion of central 5-HT by PCPA did not change the basal mechanical pain threshold relative to vehicle (Figure 2F; Supplementary Table S2). However, the mechanical pain threshold was significantly reduced in CO rats treated with vehicle but not changed with PCPA after 30-min naïve-pain DSI ($P<0.001$, Figure 2F).

Altogether, these data suggest that genetic and pharmacological depletion of central 5-HT in rats impairs both the caring behavior toward and socially transferred pain from a familiar conspecific pain-sufferer. However, the effects are not

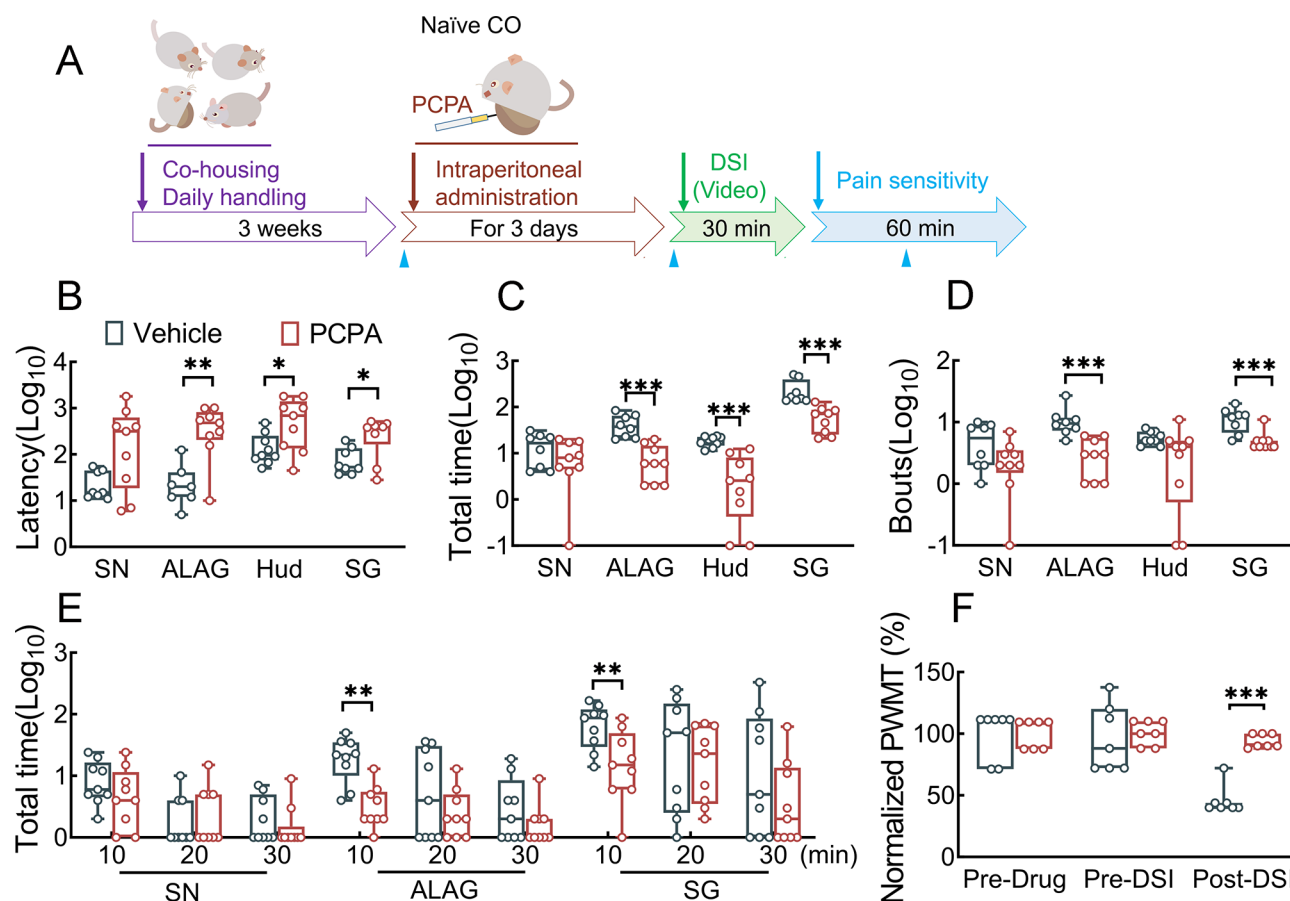


Figure 2 Effects of systemic administration of PCPA on social, non-social and pain-related behaviors in rat observers during and after DSI with a familiar pain-sufferer

A: Timeline, experimental design and protocol of the study. B–D: Latency, total time and bouts of SN, ALAG, SG and Hud in CO during 30-min DSI following i.p. vehicle and PCPA, a selective and irreversible inhibitor of TH (a rate-limiting enzyme in the biosynthesis of 5-HT). Mann-Whitney *U* test, $n=7-9$ per group. E: Time courses of the time spent on SN, ALAG and SG in CO. Mann-Whitney *U* test, $n=9$ per group. F: Changes in PWMT pre- and post-DSI, versus BL, in CO. Mann-Whitney *U* test, $n=7$ per group. Data shown as mean±SEM. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. Abbreviations: ALAG, allogrooming and allogrooming; BL, baseline; CO, cagemate observers for naïve-pain DSI; DSI, dyadic social interaction; Hud, huddling; i.p., intraperitoneal; PCPA, 4-Chloro-DL-phenylalanine; PWMT, paw withdrawal mechanical threshold; SG, self-grooming; SN, sniffing; TH, tryptophan hydroxylase.

specific because sniffing, huddling, and even self-grooming are affected as well, probably due to widespread projections of 5-HT neurons and multiple subtypes of 5-HT receptors in the brain.

Selective roles of 5-HT_{2A}R in the mPFC in empathic responses toward a familiar pain-sufferer

The mPFC, comprising Cg1, PrLC, and ILC, plays an indispensable role in modulating social contagion of pain as well as consolation behavior (Burkett et al., 2016; Li et al., 2014; Smith et al., 2021; Zhang et al., 2024). Here, we focused on the roles of 5-HT_{2A}R in the mPFC in empathic responses toward a familiar pain-sufferer (Figure 3A). Immunofluorescent staining showed high intensity of 5-HT_{2A}R labeling within the mPFC, with cortical layer V being the richest in density (Figure 3B, C), which was similar to previous reports (Price et al., 2019; Weisstaub et al., 2006). Compared with naïve-naïve (NN) control (cagemate observer control, CC), CO rats after naïve-pain (NP) DSI had more c-Fos labeling in 5-HT_{2A}R cellular profiles within the mPFC (CO vs. CC, $P=0.028$) (Figure 3D–H).

Bilateral intra-mPFC microinjections of M100907, a selective and potent 5-HT_{2A}R antagonist, selectively suppressed the empathic responses of CO rats toward a familiar pain-sufferer during 30-min DSI in terms of latency ($P<0.001$), total time ($P=0.013$) and bouts ($P<0.001$), without affecting other three behaviors (Figure 3J–L, Supplementary Table S1). Time course analysis showed the difference was statistically significant in the first 10-min section (Figure 3M). Moreover, bilateral intra-mPFC microinjections of M100907 blocked socially transferred pain in CO rats ($P=0.001$), whereas vehicle treatment failed to prevent its occurrence after 30-min DSI (Figure 3N). Finally, the basal mechanical pain threshold was not changed by bilateral intra-mPFC microinjections of M100907 (Figure 3N).

To further confirm the selective roles of 5-HT_{2A}R in the mPFC in mediating the empathic responses of rats toward a familiar pain-sufferer, bilateral microinjections of rAAV-U6-shRNA (Htr2a:Rattus)-CMV-EGFP-SV40 polyA (rAAV-shRNA-EGFP) were made to downregulate 5-HT_{2A}R expression in the mPFC (Figure 4A, B). Both immunofluorescent labeling and qRT-PCR showed that the rAAV-shRNA-EGFP strategy successfully downregulated the expression of 5-HT_{2A}R within the mPFC by 80% (rAAV-shRNA vs. rAAV-scramble, $P=0.004$), relative to rAAV-scramble (Figure 4D, E). Similar to the above local pharmacological antagonism against 5-HT_{2A}R, downregulation of 5-HT_{2A}R expression in the mPFC also selectively impaired the empathic responses of rats toward a familiar pain-sufferer in terms of caring behavior and socially transferred pain (Figure 4F–J; Supplementary Table S1).

Overall, these results indicate that 5-HT_{2A}R in the mPFC plays a critical role in mediating both social transfer of pain from and caring behavior toward a familiar conspecific pain sufferer, but has little impact on sniffing, huddling, and self-grooming behaviors.

Activation of 5-HT_{2A}R in the mPFC promotes empathic responses toward an unfamiliar pain sufferer

It is notable that pain-related empathic responses, especially the social transfer of pain, in both rats and mice are largely dependent upon the extent of familiarity between the observer and the pain sufferer (Chen, 2018; Gonzalez-Liencrez et al., 2014; Langford et al., 2006; Li et al., 2014; Luo et al., 2020; Martin et al., 2015). However, pain-related empathic

responses have been found to be improvable between unfamiliar conspecifics when bystander rats had experienced similar pain before 30-min DSI (Luo et al., 2020).

To test whether activation of 5-HT_{2A}R in the mPFC can improve pain-related empathic responses, non-cagemate rats were used as observers (NCO) and pain sufferers (non-cagemate demonstrators, NCD) for DSI (Figure 5A), as previously reported (Luo et al., 2020). First, we treated observers with 25CN-NBOH, a selective 5-HT_{2A}R agonist, via the i.p. route. The results showed that 25CN-NBOH shortened the latency ($P=0.003$; for details, see Supplementary Table S1), elongated the total time ($P=0.007$), and increased the bouts ($P=0.010$) of ALAG in naïve NCOs (Figure 5B–D). Moreover, the systemic administration of the 5-HT_{2A}R agonist 25CN-NBOH did not affect sniffing and huddling behaviors, indicating that ALAG was not influenced by non-empathic confounds (Figure 5B–D). Time course analysis showed that the difference was statistically significant in the first 10-min section (Figure 5E; Supplementary Table S1).

The same treatment of NCOs with 25CN-NBOH elicited socially transferred pain hypersensitivity after 30-min DSI with an NCD pain sufferer, which was rarely seen in the vehicle treatment group ($P=0.010$) (Figure 5F). In contrast, pre-antagonism of 5-HT_{2A}R by i.p. administration of M100907 selectively blocked the 25CN-NBOH-induced facilitatory effects on pain-related empathic responses in NCOs, without affecting the parameters of the other three behaviors (Figure 5B–F; Supplementary Table S1).

Then, we conducted bilateral intra-mPFC microinjections of 25CN-NBOH or a mixture of M100907 and 25CN-NBOH in naïve NCO rats (Figure 6A, B). The injection sites were confirmed to be mainly confined within the mPFC through Nissl staining (Figure 6C, D). Double immunofluorescence also revealed a significant increase in the number of c-Fos and 5-HT_{2A}R double-labeled profiles in the mPFC following bilateral intra-mPFC microinjections of 25CN-NBOH in NCOs, relative to the vehicle control (Figure 6E, $P=0.010$). Behavioral observations revealed that bilateral intra-mPFC microinjections of 25CN-NBOH selectively promoted pain-related empathic responses in naïve NCO rats in terms of both caring behavior and socially transferred pain, without affecting SN, Hu, and SG behaviors, relative to the vehicle control (Figure 6F–J; Supplementary Table S1). However, co-microinjections of a mixture of M100907 and 25CN-NBOH into the bilateral mPFC resulted in selective blockade of 5-HT_{2A}R neuronal activation (Figure 6E) and the empathic responses induced in naïve NCO rats (Figure 6F–J).

Finally, to confirm the selective action of systemic 25CN-NBOH through 5-HT_{2A}R within the mPFC, we conducted bilateral intra-mPFC microinjections of rAAV-shRNA-EGFP virus to downregulate 5-HT_{2A}R expression in the mPFC, followed by i.p. administration of 25CN-NBOH after 4 weeks of recovery (Figure 7A). The results showed that i.p. administration of 25CN-NBOH induced both caring behavior and socially transferred pain in NCO rats that had received bilateral intra-mPFC microinjections of rAAV-Scramble. Interestingly, intra-mPFC microinjections of rAAV-shRNA (5-HT_{2A}R) abolished the facilitation effects of 25CN-NBOH (i.p.) on the caring behavior and socially transferred pain towards stranger (Figure 7B–F; Supplementary Table S1).

Collectively, these results support that selective activation of 5-HT_{2A}R by an exogenous agonist in the mPFC promotes empathic responses toward an unfamiliar pain sufferer in rats.

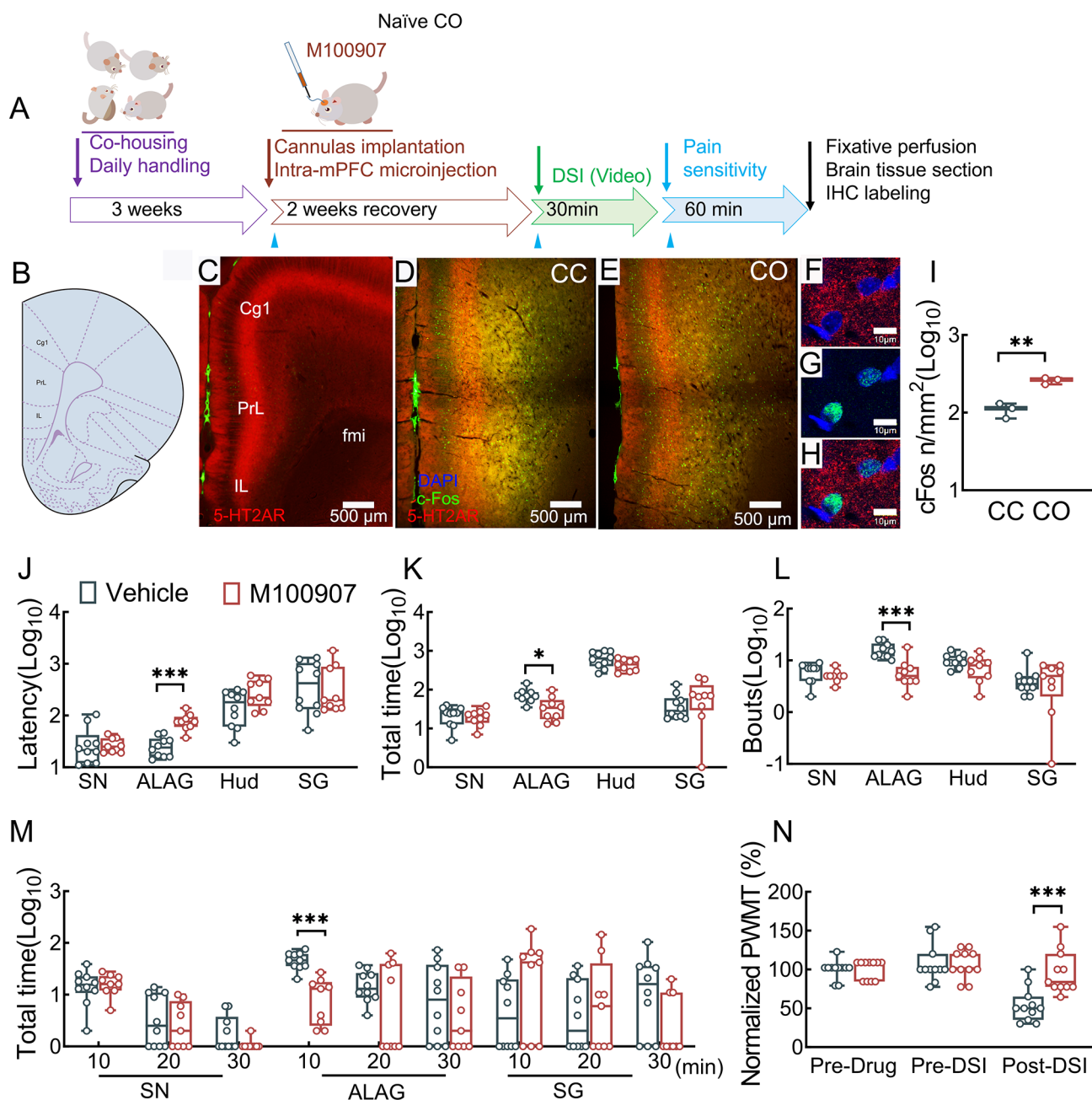


Figure 3 Selective effects of intra-mPFC microinjections of 5-HT_{2A}R antagonist on empathic responses in rat observers during and after DSI with a familiar pain-sufferer

A: Timeline, experimental design and protocol of the study. **B:** A scheme showing the location and subdivisions of the mPFC. **C:** Representative images of 5-HT_{2A}R expression in the mPFC with the highest density in layer V. **D–E:** Triple immunofluorescent labeling of c-Fos (green), DAPI (blue) and 5-HT_{2A}R (red) in the mPFC of CC and CO rats. **F–H:** Overlap of c-Fos, DAPI and 5-HT_{2A}R labelings in a neuronal profile. **I:** Number of c-Fos positive cells compared between CC and CO rats. t -test, $n=4$ per group. **J–L:** Latency, total time and bouts of SN, ALAG, SG and Hud during 30-min DSI following bilateral intra-mPFC administrations of vehicle and M100907, a potent and selective 5-HT_{2A}R antagonist. Mann-Whitney U test, $n=9-10$ per group. **M:** Time courses of the time spent on SN, ALAG and SG. Mann-Whitney U test, $n=9-10$ per group. **N:** The changes in PWMT pre- and post-DSI, versus BL. Mann-Whitney U test, $n=11$ per group. Data shown as mean $\pm$ SEM. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$. Scale bars: 500 μ m in (C–E) and 10 μ m in (F–H). Abbreviations: ALAG, allogrooming and allogrooming; BL, baseline; CC, cagemate control for naïve-naïve DSI; Cg1, cingulate cortex, area 1; CO, cagemate observer for naïve-pain DSI; DSI, dyadic social interaction; fmi, forceps minor of the corpus callosum; Hud, huddling; IL, infralimbic cortex; M100907, f(R)-(+)- α -(2,3-Dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4-piperinemethanol; PrL, prelimbic cortex; PWMT, paw withdrawal mechanical threshold; SG, self-grooming; SN, sniffing.

DISCUSSION

Empathy is an evolutionary behavior found in social animals and humans, associated with prosocial reciprocity, altruism, and morality. It involves the ability and capacity to feel, recognize, understand, and share the emotional states of

others (Chen, 2018; de Waal & Preston, 2017; Du et al., 2024b). Numerous observations in communities of non-human primates, such as chimpanzees and bonobos, and rodents have confirmed that empathy occurs primarily among familiar individuals (Chen, 2018; de Waal & Preston, 2017; Langford

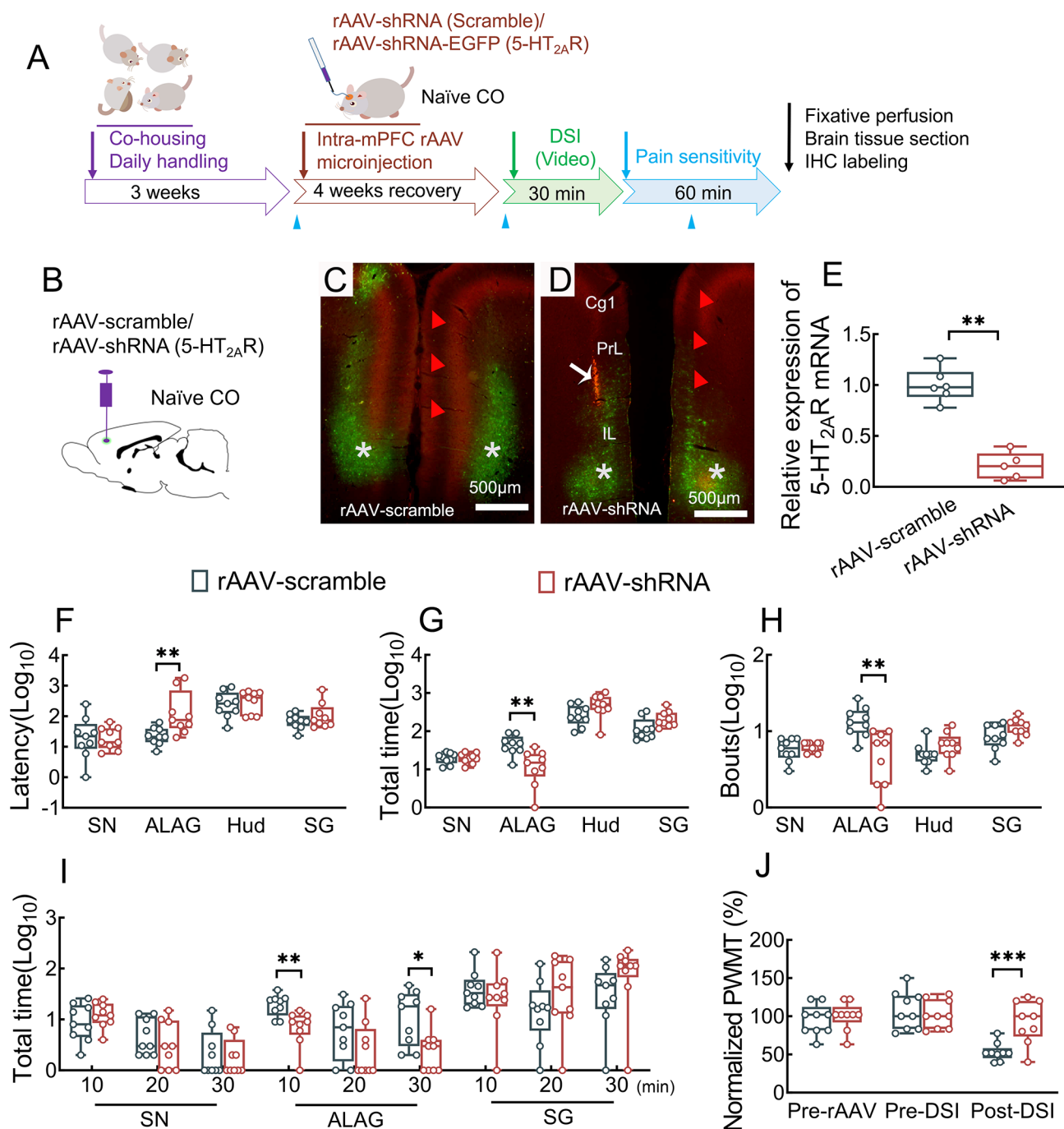


Figure 4 Selective effects of 5-HT_{2A}R down-regulation within bilateral mPFC on empathic responses in rat observers during and after DSI with a familiar pain-sufferer

A: Timeline, experimental design and protocol of the study. **B:** Schematic location of intra-mPFC injection of rAAV-shRNA-EGFP (scramble) or rAAV-U6-shRNA (Htr2a:Rattus)-CMV-EGFP-SV40 polyA (5-HT_{2A}R). **C, D:** Distribution of EGFP-expressing and immunofluorescent labeling of 5-HT_{2A}R (red) in the mPFC after rAAV-scramble or rAAV-shRNA (5-HT_{2A}R). **E:** Real time PCR results showing expression levels of 5-HT_{2A}R mRNA in the mPFC between rAAV-scramble and rAAV-shRNA (5-HT_{2A}R) groups. *t*-test, *n*=5–6 per group. **F–H:** Latency, total time and bouts of SN, ALAG, SG and Hud in CO between rAAV-scramble and rAAV-shRNA (5-HT_{2A}R) groups. Mann-Whitney *U* test, *n*=9 per group. **I:** Time courses of the time spent on SN, ALAG and SG in CO. Mann-Whitney *U* test, *n*=9 per group. **J:** Changes in PWMT pre- and post-DSI, versus BL, in CO. Mann-Whitney *U* test, *n*=9 per group. Data shown as mean±SEM. *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001. Scale bars: 500 μm in (C–D). Abbreviations: ALAG, allogrooming and allogrooming; BL, baseline; Cg1, cingulate cortex, area 1; CO, cagemate observer for naïve-pain DSI; DSI, dyadic social interaction; Hud, huddling; IL, infralimbic cortex; PrL, prelimbic cortex; PWMT, paw withdrawal mechanical threshold; SG, self-grooming; SN, sniffing.

et al., 2006; Li et al., 2014; Lü et al., 2017; Luo et al., 2020; Martin et al., 2015). More recent studies have demonstrated that the visibility of spontaneous pain-related behaviors

displayed by a familiar pain-sufferer can trigger robust ALAG behaviors, which serve as targeted caring behaviors aiming at helping others cope with pain (Du et al., 2020, 2024a; Li et al.,

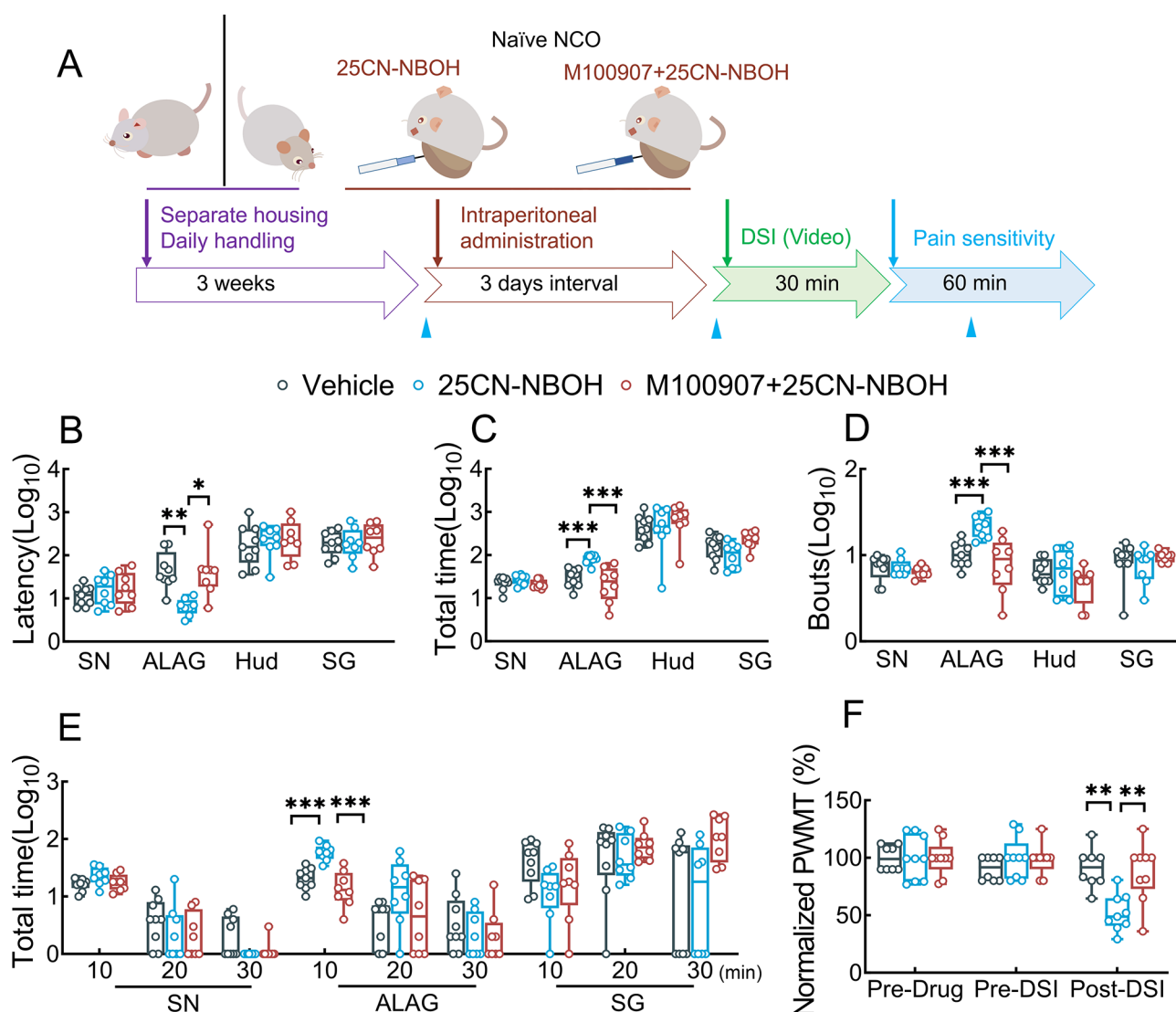


Figure 5 Antagonist reversible empathic responses induced by systemic 5-HT_{2A}R agonist in rat observers during and after DSI with a stranger pain-sufferer

A: Timeline, experimental design and protocol of the study. B–D: Latency, total time and bouts of SN, ALAG, SG and Hud in non-cagemate observers (NCO) during 30-min DSI following i.p. administration of vehicle, 5-HT_{2A}R agonist (25CN-NBOH), or agonist (25CN-NBOH)+antagonist (M100907) of 5-HT_{2A}R. Kruskal-Wallis one-way ANOVA test, $n=8-9$ per group. E: Time courses of the time spent on SN, ALAG and SG in NCO. Kruskal-Wallis one-way ANOVA test, $n=8-9$ per group. F: Changes in PWMT pre- and post-DSI, versus BL, in NCO. Kruskal-Wallis one-way ANOVA test, $n=8-9$ per group. Data shown as mean±SEM. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. For abbreviations see above.

2014; Walsh et al., 2018; Zhang et al., 2024). 5-HT reuptake inhibitors and 5-HT_{2A}R agonists are used as antidepressants in clinical settings (Kaplan et al., 2022). In the current study, we characterized the selective functional roles of 5-HT_{2A}R in the mPFC in mediating pain-related empathic responses in rat bystanders during social interaction with a familiar or an unfamiliar pain-sufferer. However, this receptor did not mediate general prosocial behaviors (sniffing and huddling) and self-grooming.

Studies in humans have demonstrated that promoting 5-HT release or inhibiting its reuptake enhances affiliation and prosocial behaviors (Crockett et al., 2010; Moul et al., 2018). Polymorphism studies indicate a link between 5-HT-related genes and autism (Patrick & Ames, 2014), and genetic or pharmacological depletion of brain 5-HT has been associated with behavioral symptoms of autism (Boylan et al., 2007). Additionally, research has shown that increased 5-HT in the anterior cingulate cortex (ACC) reduces observational fear,

potentially reflecting deficits in empathy observed in clinical populations (Kim et al., 2014). One recent report also highlights that 5-HT regulates maternal affiliation in a manner conserved across species, from rodents to primates (Liu et al., 2023). Furthermore, bidirectional modulation of 5-HT release from dorsal raphe neurons to the nucleus accumbens has been shown to influence sociability (Walsh et al., 2018). These findings collectively suggest that 5-HT plays a central role in regulating mammalian social behaviors. Supporting previous work, our results demonstrated that both genetic and pharmacological depletion of central 5-HT in rats suppressed ALAG, socially transferred pain, and general prosocial behaviors (sniffing and huddling). These observations indicate that central 5-HT mediates pain-related empathic responses as well as some general prosocial behaviors under the naïve-pain DSI paradigm, which may be attributable to the widespread axonal projections of 5-HT neuronal cell bodies from the raphe nuclei to other brain regions.

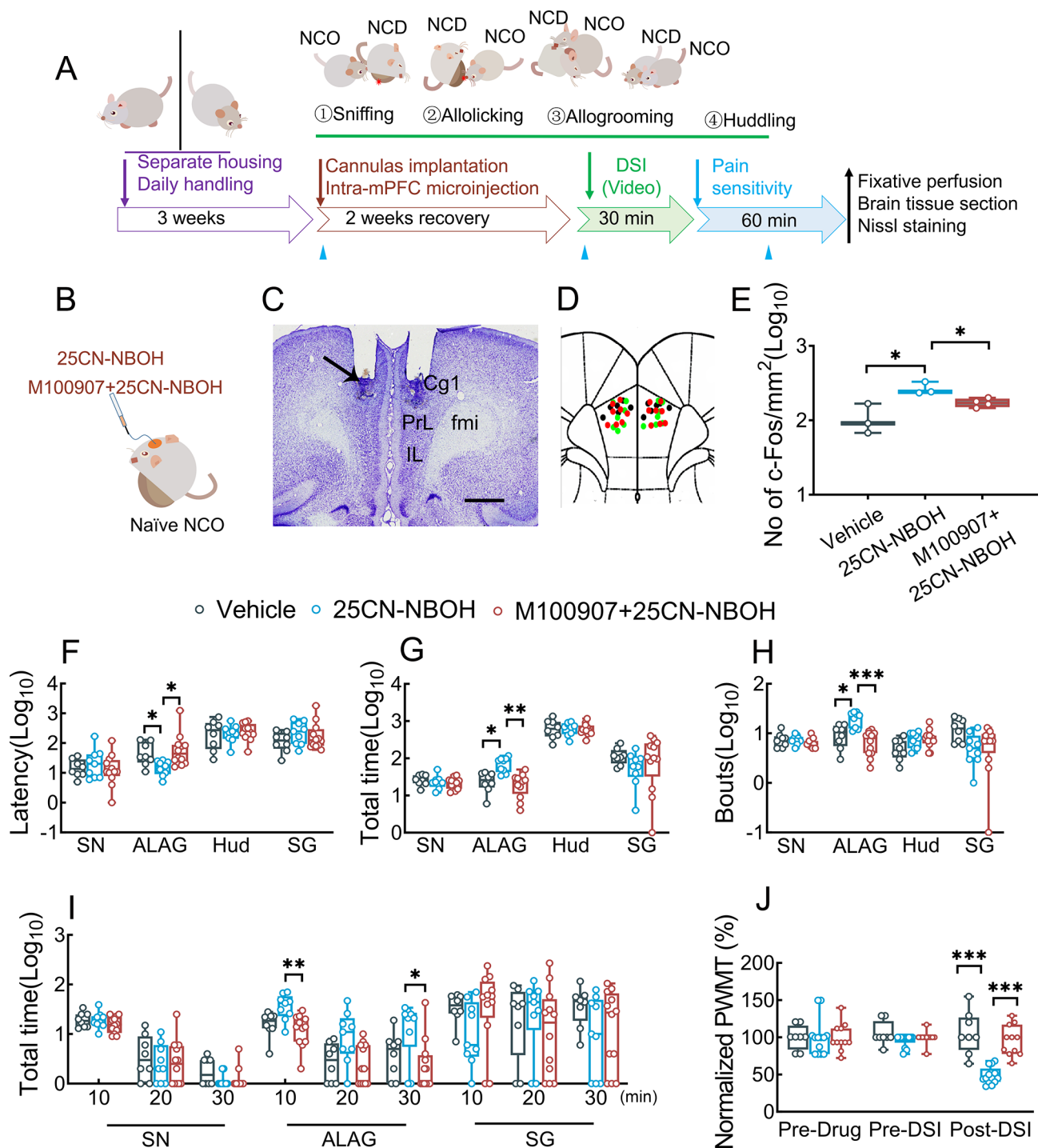


Figure 6 Antagonist reversible empathic responses induced by bilateral intra-mPFC microinjections of 5-HT_{2A} agonist in rat observers during and after DSI with a stranger pain-sufferer

A: Timeline, experimental design and protocol of the study. B: Schematic location of intra-mPFC injection. C: Nissl staining to confirm the injection loci within bilateral mPFC. D: Validated injection sites (dots) within bilateral mPFC, black for the vehicle, green for 25CN-NBOH and red for co-administration of 25CN-NBOH+M100907. E: The number of c-Fos positive cells within the mPFC following intra-mPFC administration of vehicle, 25CN-NBOH, 25CN-NBOH+M100907. One-way ANOVA test, $n=3-4$ per group. F–H: Latency, total time and bouts of SN, ALAG, SG and Hud in non-cagemate observers (NCO) following intra-mPFC administration of vehicle, 25CN-NBOH, and 25CN-NBOH+M100907. Kruskal-Wallis one-way ANOVA test, $n=8-12$ per group. I: Time courses of the time spent on SN, ALAG and SG in NCO. Kruskal-Wallis one-way ANOVA test, $n=8-12$ per group. J: Changes in PWMT pre- and post-DSI, versus BL, in NCO. Kruskal-Wallis one-way ANOVA test, $n=8-13$ per group. Data shown as mean±SEM. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$. For abbreviations see above.

5-HT_{2A}R, the major subtype of postsynaptic 5-HT receptors in the CNS, is known to be involved in memory, cognitive function, and mood (Aznar & Klein, 2013; Butovskaya et al., 2018; Farias et al., 2024). The drug 3,4-

methylenedioxymethamphetamine (MDMA, commonly known as ecstasy), which promotes 5-HT release, increases affiliative behaviors in squirrel monkeys in a 5-HT_{2A}R-dependent manner (Pitts et al., 2017). For the first time, we demonstrated

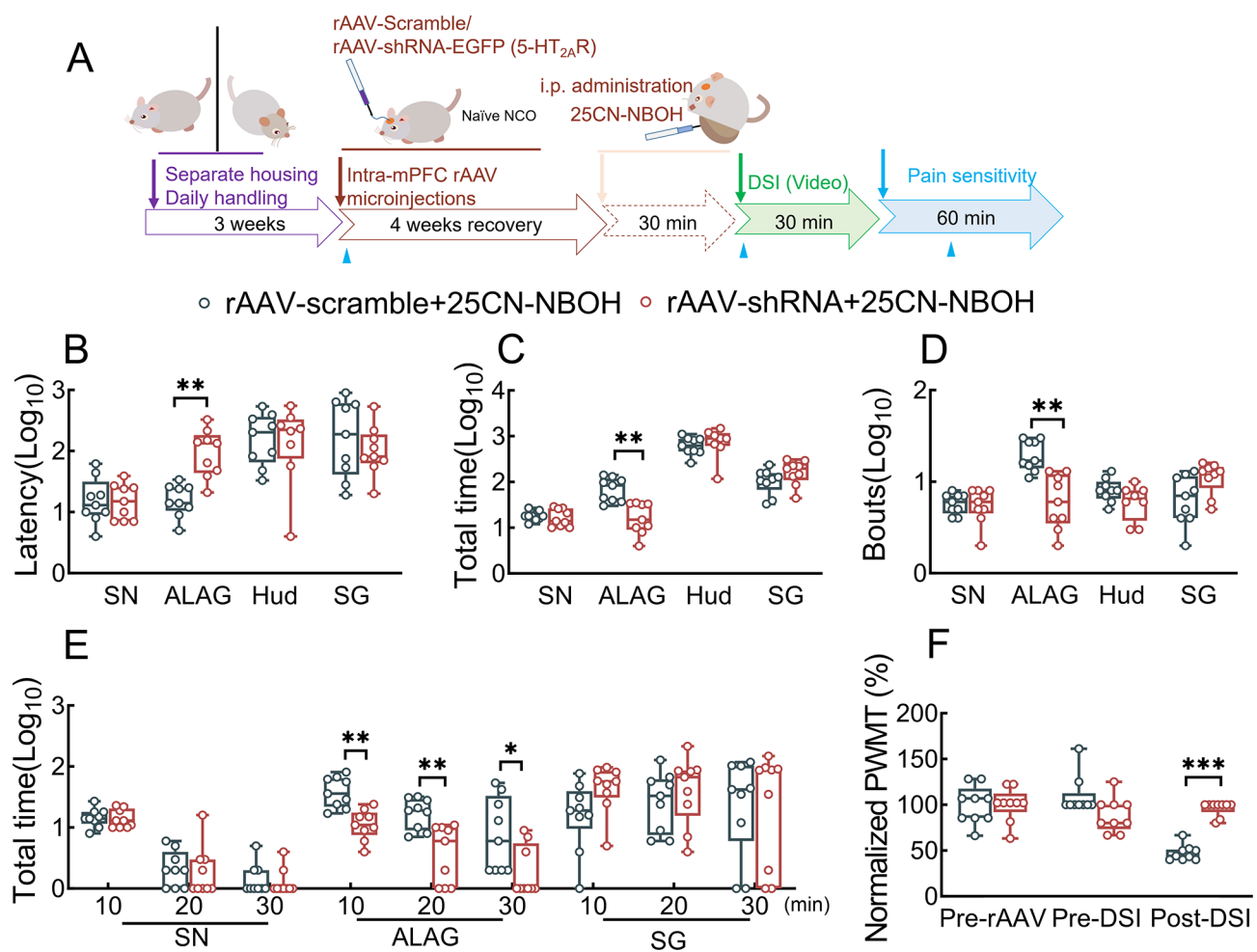


Figure 7 Effects of systemic 5-HT_{2A}R agonist on impaired empathic responsiveness caused by 5-HT_{2A}R down-regulation within bilateral mPFC in stranger rat observers

A: Timeline, experimental design and protocol of the study. B–D: Latency, total time and bouts of SN, ALAG, SG and Hud in non-cagemate observers (NCO) treated with i.p. 25CN-NBOH after bilateral intra-mPFC microinjections of rAAV-scramble or rAAV-shRNA (5-HT_{2A}R). Mann-Whitney *U* test, *n*=8–9 per group. E: Time courses of the time spent on SN, ALAG and SG in NCO. Mann-Whitney *U* test, *n*=9 per group. F: Changes in PWMT pre- and post-DSI, versus BL in NCO. Mann-Whitney *U* test, *n*=9 per group. Data shown as mean±SEM. *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001. For abbreviations see above.

that activation of 5-HT_{2A}R in the mPFC is selectively involved in mediating pain-related empathic responses in terms of caring behavior toward and socially transferred pain from a familiar pain-sufferer. Compared with the results from central 5-HT depletion, it is suggested that 5-HT_{2A}R in the mPFC is more specifically responsible for both observational pain contagion and caring behavior, which are related to empathic consolation. Meanwhile, receptors involved in sniffing and self-grooming might be other subtypes, such as 5-HT_{1A}R or 5-HT_{2C}R (Gráf, 2006; Kyzar et al., 2016; Li et al., 2021). Taken together, activation of 5-HT_{2A}R in the mPFC is necessary for social pain contagion and caring behavior toward a familiar conspecific in pain, with little impact on other common prosocial and non-social behaviors.

Familiarity among conspecifics appears to be essential for inducing emotional contagion and caring behavior in response to others' distress. Given the suppressing effects of 5-HT_{2A}R antagonism in the mPFC on emotional contagion from and caring behavior toward a familiar social partner, we investigated whether agonizing 5-HT_{2A}R in the mPFC could facilitate rats' empathic responses toward a stranger in pain. Unexpectedly, both systemic and intra-mPFC administration of

the 5-HT_{2A}R agonist 25CN-NBOH in observers significantly promoted social pain contagion and caring behavior toward unfamiliar conspecifics in pain, which could be completely blocked by selective pre-antagonism of 5-HT_{2A}R through either systemic or local administration of M100907. Additionally, 25CN-NBOH was ineffective when the expression of 5-HT_{2A}R in the mPFC had been cleared by rAAV-shRNA-EGFP virus, excluding the actions of 25CN-NBOH on 5-HT_{2A}R in other brain regions. Moreover, other common prosocial and non-social behaviors were not affected by either systemic or intra-mPFC agonism and/or antagonism of 5-HT_{2A}R under the naïve-pain DSI paradigm between strangers. These findings demonstrate that activation of 5-HT_{2A}R by exogenous agonists is sufficient to elicit pain-related empathic responses in unfamiliar rats, indicating that familiarity is not the only determining factor for the induction of empathy for pain.

The mPFC has been associated with a range of functions, including attention, decision-making, goal-directed behavior, and working memory (Euston et al., 2012; Shing et al., 2022; Yang et al., 2022a). Our previous study has shown that bilateral lesions of the mPFC eliminate social pain contagion

in rats (Li et al., 2014). Recent research has also demonstrated a causal role for the ACC in bidirectionally controlling targeted allogrooming towards others in pain (Zhang et al., 2024). It is interesting to note that the dorsal raphe-ACC neural circuit using 5-HT to activate 5-HT_{1A}R mediates allogrooming behavior between pair-bond male and female mandarin voles (Li et al., 2021). However, the activation of 5-HT_{1A}R in the ACC was not specific to allogrooming because it also mediates sniffing and social approach. Taking together this previous study and our current study, it suggests that pair-bond male-to-female allogrooming behavior may be quite different from the empathic responses we observed using familiar or unfamiliar male-to-male DSI paradigms.

In this study, we showed that activation of 5-HT_{2A}R in the mPFC is sufficient and necessary for the development of empathy for pain. It will be interesting to determine the effect of 5-HT_{2A}R on other forms of empathy (fear, reward sharing). Moreover, previous research has reported that 5-HT_{2A}R is densely expressed in both pyramidal neurons and interneurons of the mPFC. Studies have demonstrated that 5-HT_{2A}R on pyramidal neurons in the mPFC plays a key role in regulating cortical excitability (Celada et al., 2013; Wu et al., 2024), whereas 5-HT_{2A}R activation in interneurons produces bidirectional effects (Barzan et al., 2024). Whether the function of 5-HT_{2A}R on pyramidal neurons and interneurons differs during empathic responses remains unclear. To directly address this point, we will employ cell-type-specific approaches, such as using 5-HT_{2A}R-Cre mouse lines in combination with Cre-dependent viral vectors, to selectively manipulate 5-HT_{2A}R+ pyramidal neurons versus 5-HT_{2A}R+ interneurons and assess the functional outcome on empathic behaviors. In addition, in our previous data, we also found that OXT neurons in the paraventricular nucleus (PVN) respond to caring behavior (Du et al., 2024a). It would be intriguing to investigate how the neuronal circuits of OXT neurons in the PVN and 5-HT_{2A}R in the mPFC contribute to driving allolicking and allogrooming behaviors, as well as socially transferred pain.

Moreover, the intracellular mechanism by which 5-HT_{2A}R mediates pain-related empathic responses remains unknown and warrants further investigation. Firstly, 5-HT_{2A}R is a G protein-coupled receptor (GPCR). The activation of 5-HT_{2A}R receptors leads to the hydrolysis of phosphatidylinositol bisphosphate (PIP₂) and produces IP₃ and diacylglycerol, which in turn activates protein kinase C (PKC) (Bhattacharyya et al., 2002). This could modulate neuronal excitability in the mPFC. An alternative or parallel pathway could involve interaction with BDNF/TrkB signaling (Ceccarini et al., 2020; Mattson et al., 2004), which is known to be involved in plasticity related to social behaviors. We plan future studies to address the intracellular signaling pathways using targeted pharmacological disruption of these pathways or biosensors to visualize their activity *in vivo* during empathic tests.

In summary, we establish the naïve-pain DSI paradigm as a powerful tool for elucidating the neurobiological basis of empathy across varying social contexts. This model holds significant translational potential for uncovering the etiology of empathy deficits in neuropsychiatric disorders such as ASD, depression, and schizophrenia. Importantly, our work provides the first characterization of the functional roles of central 5-HT and 5-HT_{2A}R in the mPFC in socially transferred pain and caring behavior, offering a novel pharmacotherapeutic

strategy to directly target the psychological underpinnings of empathy deficits.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests

AUTHORS' CONTRIBUTIONS

Conceptualization, J.C., W.J.L., C.L.L.; methodology, W.-J.L., R.D., C.L.L., Y.W., Y.Y.; investigation, W.J.L., R.D., C.L.L., Y.W., Y.Y.; Data analysis and illustrations, J.C., W.J.L., S.W.; writing, W.J.L., S.W., J.C.; funding acquisition, J.C., C.L.L.; supervision, J.C. All authors read and approved the final version of the manuscript.

REFERENCES

- Asokan MM, Falkner AL. 2025. Hormonal regulation of behavioral and emotional persistence: novel insights from a systems-level approach to neuroendocrinology. *Neurobiology of Learning and Memory*, **220**: 108064.
- Attenburrow MJ, Williams C, Odontiadis J, et al. 2003. Acute administration of nutritionally sourced tryptophan increases fear recognition. *Psychopharmacology*, **169**(1): 104–107.
- Aznar S, Klein AB. 2013. Regulating prefrontal cortex activation: an emerging role for the 5-HT_{2A} serotonin receptor in the modulation of emotion-based actions?. *Molecular Neurobiology*, **48**(3): 841–853.
- Barnes NM, Sharp T. 1999. A review of central 5-HT receptors and their function. *Neuropharmacology*, **38**(8): 1083–1152.
- Barzan R, Bozkurt B, Nejad MM, et al. 2024. Gain control of sensory input across polysynaptic circuitries in mouse visual cortex by a single G protein-coupled receptor type (5-HT_{2A}). *Nature Communications*, **15**(1): 8078.
- Bhattacharyya S, Puri S, Miledi R, et al. 2002. Internalization and recycling of 5-HT_{2A} receptors activated by serotonin and protein kinase C-mediated mechanisms. *Proceedings of the National Academy of Sciences of the United States of America*, **99**(22): 14470–14475.
- Bird G, Viding E. 2014. The self to other model of empathy: providing a new framework for understanding empathy impairments in psychopathy, autism, and alexithymia. *Neuroscience & Biobehavioral Reviews*, **47**: 520–532.
- Bonfils KA, Lysaker PH, Minor KS, et al. 2017. Empathy in schizophrenia: a meta-analysis of the Interpersonal Reactivity Index. *Psychiatry Research*, **249**: 293–303.
- Boylan CB, Blue ME, Hohmann CF. 2007. Modeling early cortical serotonergic deficits in autism. *Behavioural Brain Research*, **176**(1): 94–108.
- Burkett JP, Andari E, Johnson ZV, et al. 2016. Oxytocin-dependent consolation behavior in rodents. *Science*, **351**(6271): 375–378.
- Burt SA, Mikolajewski AJ. 2008. Preliminary evidence that specific candidate genes are associated with adolescent-onset antisocial behavior. *Aggressive Behavior*, **34**(4): 437–445.
- Butovskaya ML, Butovskaya PR, Vasilyev VA, et al. 2018. Serotonergic gene polymorphisms (5-HTTLPR, 5HTT1A, 5HTT2A), and population differences in aggression: traditional (Hadza and Datoga) and industrial (Russians) populations compared. *Journal of Physiological Anthropology*, **37**(1): 10.
- Carrillo M, Han YG, Migliorati F, et al. 2019. Emotional mirror neurons in the rat's anterior cingulate cortex. *Current Biology*, **29**(8): 1301–1312. e6.
- Ceccarini MR, Tasegian A, Franzago M, et al. 2020. 5-HT_{2A}R and BDNF gene variants in eating disorders susceptibility. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, **183**(3): 155–163.
- Celada P, Puig MV, Artigas F. 2013. Serotonin modulation of cortical neurons and networks. *Frontiers in Integrative Neuroscience*, **7**: 25.
- Chen J. 2018. Empathy for distress in humans and rodents. *Neuroscience*

- Chen J, Guan SM, Sun W, et al. 2016. Melittin, the major pain-producing substance of bee venom. *Neuroscience Bulletin*, 32(3): 265–272.
- Chen J, Luo C, Li HL, et al. 1999. Primary hyperalgesia to mechanical and heat stimuli following subcutaneous bee venom injection into the plantar surface of hindpaw in the conscious rat: a comparative study with the formalin test. *Pain*, 83(1): 67–76.
- Crockett MJ, Clark L, Hauser MD, et al. 2010. Serotonin selectively influences moral judgment and behavior through effects on harm aversion. *Proceedings of the National Academy of Sciences of the United States of America*, 107(40): 17433–17438.
- de Waal FBM. 2008. Putting the altruism back into altruism: the evolution of empathy. *Annual Review of Psychology*, 59(1): 279–300.
- de Waal FBM, Preston SD. 2017. Mammalian empathy: behavioural manifestations and neural basis. *Nature Reviews Neuroscience*, 18(8): 498–509.
- Du R, Lu GF, Luo WJ, et al. 2024a. Dyadic social interaction paradigm reveals selective role of ovarian estrogen in the caring behavior and socially transferred pain in female mice. *Neuropharmacology*, 261: 110138.
- Du R, Luo WJ, Geng KW, et al. 2020. Empathic contagious pain and consolation in laboratory rodents: species and sex comparisons. *Neuroscience Bulletin*, 36(6): 649–653.
- Du R, Yu Y, Wang XL, et al. 2024b. Social contagion of pain and fear results in opposite social behaviors in rodents: meta-analysis of experimental studies. *Frontiers in Behavioral Neuroscience*, 18: 1478456.
- Euston DR, Gruber AJ, McNaughton BL. 2012. The role of medial prefrontal cortex in memory and decision making. *Neuron*, 76(6): 1057–1070.
- Farias CP, Leite AKO, Schmidt BE, et al. 2024. The 5-HT_{2A}, 5-HT_{5A}, and 5-HT₆ serotonergic receptors in the medial prefrontal cortex behave differently in extinction learning: does social support play a role?. *Behavioural Brain Research*, 463: 114922.
- Ferri BG, de Novais CO, Bonani RS, et al. 2023. Psychoactive substances 25H-NBOMe and 25H-NBOH induce antidepressant-like behavior in male rats. *European Journal of Pharmacology*, 955: 175926.
- Gao J, Chen LL, Li M. 2019. 5-HT_{2A} receptors modulate dopamine D₂-mediated maternal effects. *Pharmacology Biochemistry and Behavior*, 180: 32–43.
- Gao J, Wu RY, Davis C, et al. 2018. Activation of 5-HT_{2A} receptor disrupts rat maternal behavior. *Neuropharmacology*, 128: 96–105.
- Geng KW, Du R, Wei N, et al. 2020. Image-forming visual basis of empathy for pain in mice. *Neuroscience Bulletin*, 36(12): 1563–1569.
- Geng KW, He T, Wang RR, et al. 2016. Ethanol increases mechanical pain sensitivity in rats via activation of GABA_A receptors in medial prefrontal cortex. *Neuroscience Bulletin*, 32(5): 433–444.
- Gong PY, Liu JT, Blue PR, et al. 2015. Serotonin receptor gene (*HTR2A*) T102C polymorphism modulates individuals' perspective taking ability and autistic-like traits. *Frontiers in Human Neuroscience*, 9: 575.
- Gonzalez-Liencre C, Juckel G, Tas C, et al. 2014. Emotional contagion in mice: the role of familiarity. *Behavioural Brain Research*, 263: 16–21.
- Gráf M. 2006. 5-HT_{2c} receptor activation induces grooming behaviour in rats: possible correlations with obsessive-compulsive disorder. *Neuropsychopharmacologia Hungarica*, 8(1): 23–28.
- Gu Y, Zhang NX, Zhu SJ, et al. 2022. Estradiol reduced 5-HT reuptake by downregulating the gene expression of Plasma Membrane Monoamine Transporter (PMAT, *Slc29a4*) through estrogen receptor β and the MAPK/ERK signaling pathway. *European Journal of Pharmacology*, 924: 174939.
- Hadjikhani N, Zürcher NR, Rogier O, et al. 2014. Emotional contagion for pain is intact in autism spectrum disorders. *Translational Psychiatry*, 4(1): e343.
- Haker H, Schimansky J, Jann S, et al. 2012. Self-reported empathic abilities in schizophrenia: a longitudinal perspective. *Psychiatry Research*, 200(2-3): 1028–1031.
- Kalueff AV, Stewart AM, Song C, et al. 2016. Neurobiology of rodent self-grooming and its value for translational neuroscience. *Nature Reviews Neuroscience*, 17(1): 45–59.
- Kaplan AL, Confair DN, Kim K, et al. 2022. Bespoke library docking for 5-HT_{2A} receptor agonists with antidepressant activity. *Nature*, 610(7932): 582–591.
- Kim BS, Lee J, Bang M, et al. 2014. Differential regulation of observational fear and neural oscillations by serotonin and dopamine in the mouse anterior cingulate cortex. *Psychopharmacology*, 231(22): 4371–4381.
- Knutson B, Wolkowitz OM, Cole SW, et al. 1998. Selective alteration of personality and social behavior by serotonergic intervention. *American Journal of Psychiatry*, 155(3): 373–379.
- Kupferberg A, Bicks L, Hasler G. 2016. Social functioning in major depressive disorder. *Neuroscience & Biobehavioral Reviews*, 69: 313–332.
- Kyzar EJ, Stewart AM, Kalueff AV. 2016. Effects of LSD on grooming behavior in serotonin transporter heterozygous (*Sert^{+/−}*) mice. *Behavioural Brain Research*, 296: 47–52.
- Langford DJ, Crager SE, Shehzad Z, et al. 2006. Social modulation of pain as evidence for empathy in mice. *Science*, 312(5782): 1967–1970.
- Li LF, Zhang LZ, He ZX, et al. 2021. Dorsal raphe nucleus to anterior cingulate cortex 5-HTergic neural circuit modulates consolation and sociability. *eLife*, 10: e67638.
- Li Z, Lu YF, Li CL, et al. 2014. Social interaction with a cagemate in pain facilitates subsequent spinal nociception via activation of the medial prefrontal cortex in rats. *Pain*, 155(7): 1253–1261.
- Liu Y, Shan L, Liu T, et al. 2023. Molecular and cellular mechanisms of the first social relationship: a conserved role of 5-HT from mice to monkeys, upstream of oxytocin. *Neuron*, 111(9): 1468–1485. e7.
- Lü YF, Yang Y, Li CL, et al. 2017. The locus coeruleus-norepinephrine system mediates empathy for pain through selective up-regulation of P2X₃ receptor in dorsal root ganglia in rats. *Frontiers in Neural Circuits*, 11: 66.
- Luo WJ, Li CL, Geng KW, et al. 2020. The similar past pain experience evokes both observational contagious pain and consolation in stranger rat observers. *Neuroscience Letters*, 722: 134840.
- Luo WJ, Yang F, Yang F, et al. 2017. Intervertebral foramen injection of ozone relieves mechanical allodynia and enhances analgesic effect of gabapentin in animal model of neuropathic pain. *Pain Physician*, 20(5): E673–E685.
- Martin LJ, Hathaway G, Isbester K, et al. 2015. Reducing social stress elicits emotional contagion of pain in mouse and human strangers. *Current Biology*, 25(3): 326–332.
- Mattson MP, Maudsley S, Martin B. 2004. BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders. *Trends in Neurosciences*, 27(10): 589–594.
- Millan MJ. 2002. Descending control of pain. *Progress in Neurobiology*, 66(6): 355–474.
- Minio-Paluello I, Baron-Cohen S, Avenanti A, et al. 2009. Absence of embodied empathy during pain observation in Asperger syndrome. *Biological Psychiatry*, 65(1): 55–62.
- Mondoloni S, Molina P, Lecca S, et al. 2024. Serotonin release in the habenula during emotional contagion promotes resilience. *Science*, 385(6713): 1081–1086.
- Moul C, Hawes DJ, Dadds MR. 2018. Mapping the developmental pathways of child conduct problems through the neurobiology of empathy. *Neuroscience & Biobehavioral Reviews*, 91: 34–50.
- Odland AU, Kristensen JL, Andreasen JT. 2021. The selective 5-HT_{2A} receptor agonist 25CN-NBOH does not affect reversal learning in mice. *Behavioural Pharmacology*, 32(5): 448–452.
- Okine BN, Rea K, Olango WM, et al. 2014. A role for PPAR α in the medial

- prefrontal cortex in formalin-evoked nociceptive responding in rats. *British Journal of Pharmacology*, **171**(6): 1462–1471.
- Patrick RP, Ames BN. 2014. Vitamin D hormone regulates serotonin synthesis. Part 1: relevance for autism. *The FASEB Journal*, **28**(6): 2398–2413.
- Pitts EG, Minerva AR, Chandler EB, et al. 2017. 3, 4-Methylenedioxymethamphetamine increases affiliative behaviors in squirrel monkeys in a serotonin 2A receptor-dependent manner. *Neuropsychopharmacology*, **42**(10): 1962–1971.
- Price AE, Sholler DJ, Stutz SJ, et al. 2019. Endogenous serotonin 5-HT_{2A} and 5-HT_{2C} receptors associate in the medial prefrontal cortex. *ACS Chemical Neuroscience*, **10**(7): 3241–3248.
- Schreier S, Pijnenborg GHM, Aan Het Rot M. 2013. Empathy in adults with clinical or subclinical depressive symptoms. *Journal of Affective Disorders*, **150**(1): 1–16.
- Shing N, Walker MC, Chang PS. 2022. The role of aberrant neural oscillations in the hippocampal-medial prefrontal cortex circuit in neurodevelopmental and neurological disorders. *Neurobiology of Learning and Memory*, **195**: 107683.
- Smith ML, Asada N, Malenka RC. 2021. Anterior cingulate inputs to nucleus accumbens control the social transfer of pain and analgesia. *Science*, **371**(6525): 153–159.
- Smith ML, Hostetler CM, Heinricher MM, et al. 2016. Social transfer of pain in mice. *Science Advances*, **2**(10): e1600855.
- Sorensen SM, Kehne JH, Fadayel GM, et al. 1993. Characterization of the 5-HT₂ receptor antagonist MDL 100907 as a putative atypical antipsychotic: behavioral, electrophysiological and neurochemical studies. *The Journal of Pharmacology and Experimental Therapeutics*, **266**(2): 684–691.
- Thoma P, Zalewski I, von Reventlow HG, et al. 2011. Cognitive and affective empathy in depression linked to executive control. *Psychiatry Research*, **189**(3): 373–378.
- Unschuld PG, Ising M, Erhardt A, et al. 2007. Polymorphisms in the serotonin receptor gene HTR2A are associated with quantitative traits in panic disorder. *American Journal of Medical Genetics Part B: Neuropsychiatric Genetics*, **144B**(4): 424–429.
- Wallach J, Cao AB, Calkins MM, et al. 2023. Identification of 5-HT_{2A} receptor signaling pathways associated with psychedelic potential. *Nature Communications*, **14**(1): 8221.
- Walsh JJ, Christoffel DJ, Heifets BD, et al. 2018. 5-HT release in nucleus accumbens rescues social deficits in mouse autism model. *Nature*, **560**(7720): 589–594.
- Wan YS, Zhai XJ, Tan HA, et al. 2021. Associations between the 1438A/G, 102T/C, and rs7997012G/A polymorphisms of HTR2A and the safety and efficacy of antidepressants in depression: a meta-analysis. *The Pharmacogenomics Journal*, **21**(2): 200–215.
- Warrier V, Toro R, Chakrabarti B, et al. 2018. Genome-wide analyses of self-reported empathy: correlations with autism, schizophrenia, and anorexia nervosa. *Translational Psychiatry*, **8**(1): 35.
- Weisstaub NV, Zhou MM, Lira A, et al. 2006. Cortical 5-HT_{2A} receptor signaling modulates anxiety-like behaviors in mice. *Science*, **313**(5786): 536–540.
- Wu RY, Davis C, Li M. 2018. Behavioral mechanisms underlying the maternal disruptive effect of serotonin 5-HT_{2A} receptor activation in Sprague-Dawley rats. *Journal of Neural Transmission*, **125**(7): 1065–1075.
- Wu XR, Zhu XN, Pan YB, et al. 2024. Amygdala neuronal dyshomeostasis via 5-HT receptors mediates mood and cognitive defects in Alzheimer's disease. *Aging Cell*, **23**(8): e14187.
- Yang C, Hu YZ, Talishinsky AD, et al. 2022a. Medial prefrontal cortex and anteromedial thalamus interaction regulates goal-directed behavior and dopaminergic neuron activity. *Nature Communications*, **13**(1): 1386.
- Yang GY, Geng HY, Hu C. 2022b. Targeting 5-HT as a potential treatment for social deficits in autism. *Neuroscience Bulletin*, **38**(10): 1263–1266.
- Yu Y, Li CL, Du R, et al. 2019. Rat model of empathy for pain. *Bio-protocol*, **9**(12): e3266.
- Zhang MM, Wu YE, Jiang MP, et al. 2024. Cortical regulation of helping behaviour towards others in pain. *Nature*, **626**(7997): 136–144.
- Zhang X, Zhang X, Hu SJ, et al. 2017. Identification of miRNA-7 by genome-wide analysis as a critical sensitizer for TRAIL-induced apoptosis in glioblastoma cells. *Nucleic Acids Research*, **45**(10): 5930–5944.