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Enhancing HIF-1α–P2X2 signaling in dorsal raphe serotonergic neurons promotes psychological resilience

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ABSTRACT

Major depressive disorder (MDD) is a devastating condition. Although progress has been made in the past seven decades, patients with MDD continue to receive an inadequate treatment, primarily due to the late onset of first-line antidepressant drugs and to their acute withdrawal symptoms. Resilience is the ability to rebound from adversity in a healthy manner and many people have psychological resilience. Revealing the mechanisms and identifying methods promoting resilience will hopefully lead to more effective prevention strategies and treatments for depression. In this study, we found that intermittent hypobaric hypoxia training (IHHT), a method for training pilots and mountaineers, enhanced psychological resilience in adult mice. IHHT produced a sustained antidepressant-like effect in mouse models of depression by inducing long-term (up to 3 months after this treatment) overexpression of hypoxia-inducible factor (HIF)-1α in the dorsal raphe nucleus (DRN) of adult mice. Moreover, DRN-infusion of cobalt chloride, which mimics hypoxia increasing HIF-1α expression, triggered a rapid and long-lasting antidepressant-like effect. Down-regulation of HIF-1α in the DRN serotonergic (DRN^{5-HT}) neurons attenuated the effects of IHHT. HIF-1α translationally regulated the expression of P2X2, and conditionally knocking out *P2rx2* (encodes P2X2 receptors) in DRN^{5-HT} neurons, in turn, attenuated the sustained antidepressant-like effect of IHHT, but not its acute effect. In line with these results, a single sub-anesthetic dose of ketamine enhanced HIF-1α–P2X2 signaling, which is essential for its rapid and long-lasting antidepressant-like effect. Notably, we found that P2X2 protein levels were significantly lower in the DRN of patients with MDD than that of control subjects. Together, these findings elucidate the molecular mechanism underlying IHHT promoting psychological resilience and highlight enhancing HIF-1α–P2X2 signaling in DRN^{5-HT} neurons as a potential avenue for screening novel therapeutic treatments for MDD.

1. Introduction

Major depressive disorder (MDD) is a leading cause of disability, with a 4.4 % global prevalence [1]. Considerable progress has been made in the treatment of depression since the first antidepressant was developed in 1950s, and the first-line treatments, selective serotonin and/or noradrenaline reuptake inhibitors, stand out as the most effective therapies. However, less than half of individuals with MDD benefit from these treatments, mostly due to their low response to first-line antidepressants, late-onset of efficacy, and acute withdrawal symptoms [2]. Resilience is the ability to rebound from adversity in a healthy manner [3,4]. Although the prevalence of MDD is high, most people do not show depression in the face of stress and trauma, and even a substantial proportion of patients with MDD show spontaneous recovery [5], suggesting many people have psychological resilience. Therefore, revealing the mechanisms and identifying methods promoting resilience will hopefully lead to more effective prevention strategies and treatments for depression [1,6].

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Intermittent hypobaric hypoxia training (IHHT) is an altitude pre-acclimatization method for training pilots, mountaineers, and athletes with great potential for treating various diseases, including chronic spinal cord injury [7], hypertension [8,9], myocardial infarction [10], coronary heart disease [11], and some neurological disorders [9]. For its ability to alleviate symptoms of psychiatric disorders, IHHT has been proposed as a non-invasive treatment for patients with schizophrenia, post-traumatic stress disorder, and depression [12–14]. In multiple rodent models, at an altitude of 5000 m, IHHT produces antidepressant-like effects [15,16]. These effects have been linked to IHHT through FG-4592, a small-molecule activator of hypoxia-inducible factor-1α (HIF-1α) and a key hypoxia-responsive transcription factor [17]. Moreover, IHHT may have a long-lasting effect because 24 h ambulatory blood pressure decreases after 15 days of IHHT [18]. Whether and how IHHT enhances psychological resilience, however, remains to be determined.

Purinergic 2X (P2X) receptors are adenosine triphosphate (ATP) ion channels broadly distributed in the brain. Among the seven subunits of P2X receptors (P2X1-7); P2X2 and P2X4 are wildly expressed in the brain [19]. Yet, the physiological roles of P2X receptors, especially in the brain, are not fully understood. Nevertheless, a clinical study has found a significant relationship between P2X2 expression and depression [20]. In addition, ATP/P2X2 receptor-induced upregulation of leukemia inhibitory factor expression in astrocytes mediates the efficacy of electroconvulsive therapy in psychiatric disorders [21]. In line with these findings, we have shown that P2X2 receptors in the medial prefrontal cortex (mPFC) mediates the antidepressant-like effects of astrocyte-derived ATP in mice [22]. These reports suggest a link between increased P2X2 receptor activity and antidepressant effects. Considering that P2X2 mediates the ventilatory responses to hypoxia in the carotid body [23], P2X2 could be involved in the antidepressant-like effect of hypoxia. Here, we assessed whether IHHT enhances psychological resilience and whether P2X2 receptors play a key role in mediating this effect.

2. Materials and methods

2.1. Animals

The male C57BL/6J mice used in the present study (2–4 months old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). ePet-Cre mice (JAX stock 012712) and Tph2-iCre^{ERT} mice (JAX stock 016584) were purchased from Jackson Laboratory (Bar Harbor, ME, USA), while *P2rx2*$^{fl/fl}$ mice were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China). All mice were C57BL/6J background. ePet-Cre;*P2rx2*$^{fl/fl}$ mice were generated by crossing ePet-Cre mice with *P2rx2*$^{fl/fl}$ mice, and Tph2-iCre^{ERT}; *P2rx2*$^{fl/fl}$ mice were generated by crossing Tph2-iCre^{ERT} mice with *P2rx2*$^{fl/fl}$ mice. Animals were housed in standard, plastic rodent cages, under controlled conditions, with a constant temperature of 24 ± 1 °C and a 12-h light/12-h dark cycle (lights on at 07:00 h) and *ad libitum* access to food and water. All behavioral tests were conducted in a double-blinded manner. Before the behavioral experiments, the mice were gently handled twice daily for at least 3 consecutive days. All experimental procedures involving animals were conducted following the guidelines of the Chinese Council on Animal Care.

2.2. IHHT protocol

IHHT was performed as previously described, with some modifications [15]. Briefly, 8-week-old mice were placed inside a commercial hypoxia chamber (Eusyn Medical Tech. Co., Guangzhou, China). To simulate a gradual descent from atmospheric pressure (21 % O_2) to 10 % oxygen partial pressure (equivalent to an altitude of ~5000 m), the oxygen levels were gradually reduced over approximately 30 min using a computer program to control a vacuum pump system. The mice were exposed to 10 % oxygen partial pressure for 4 h. After this period, the oxygen levels were gradually restored to normal over 30 min. Subsequently, the mice were returned to their cages. Hypoxia treatment was performed daily for 14 continuous days. In the control group, the animals were kept in the same chamber with regular circulation of room air during the corresponding period (4 h).

2.3. Human brain samples

This study was approved by the research ethics board at Shenzhen University (Shenzhen, China). Postmortem brain samples from depressed suicide subjects, and matched accidental- or natural-death controls were provided by the Suicide Section of the Douglas-Bell Canada Brain Bank (http://www.douglas.qc.ca/page/brain-bank). All psychiatric subjects died by suicide in the context of depressive episode, whereas all control subjects died suddenly and had no psychiatric, neurological, or inflammatory illnesses. Unfixed frozen tissue samples were dissected from Brodmann areas 9 (BA9), BA24, and brain tissue containing the dorsal raphe nucleus (DRN) was stored at – 80 °C until use. The groups were matched by age, tissue pH, and postmortem interval. Detailed subject information is provided in Table S1.

2.4. Chemical reagents

Tamoxifen (TAM) (#T5648, Sigma-Aldrich, St. Louis, Missouri, USA) was dissolved in corn oil (#C8267, Sigma-Aldrich) at a final concentration of 10 mg/ml. To induce Cre recombination, mice were injected with TAM once a day (100 mg/kg) for 5 days. Ketamine (Fujian Gutian Pharma Co., Fujian, China), which contains equal amount of *R*- and *S*-ketamine identified by prof. Xiao-hui Wang (Changchun Institute of Applied Chemistry, Chinese Academy of Sciences), 10 mg/kg, and imipramine (#10899, Sigma-Aldrich), 15 mg/kg, were dissolved in 0.9 % saline, and were administered intraperitoneally (i.p.) for the behavior studies. Cobalt chloride ($CoCl_2$) (#232696, Sigma-Aldrich) was dissolved in artificial cerebrospinal fluid (ACSF) and stereotaxic injected into the DRN. Actinomycin D (#114666, Sigma-Aldrich) and anisomycin (#176880, Sigma-Aldrich) were dissolved in DMSO and added into the medium of cultured HEK293 cells.

2.5. Virus productions

The recombination adeno-associated virus (rAAV)-hSyn-Cre-hGH-pA viral vector (titer: 5.54 × 10^{12} vg/mL) was obtained from BrainVTA (Wuhan, China). The sequence of the short hairpin RNA (shRNA) for *Hif1a* (which encodes HIF-1α) knockdown was 5′-CCAGTTACGATTGTGAAGTTA-3′. The rAAV2/9-CBG-DIO-miR30-*Hif1a*-shRNA-EGFP vector (briefly *Hif1a*-shRNA) (titer: 6.56 × 10^{12} vg/mL) was custom-engineered by Obio Technology (Shanghai, China). For knockdown of *Egln1*, which encodes prolyl hydroxylase domain-containing protein 2 (PHD2), the following shRNA sequence was used: 5′-GCATGAACAAGCACGGCATCT-3′, and the rAAV2/9-hEF1a-DIO-EGFP-*Egln1*-shRNA (titer: 3.70 × 10^{13} vg/mL) was purchased from Shanghai Taitool Bioscience (Shanghai, China). For knockdown of *P2rx2* (which encodes P2X2 receptors), the sequences were 5′- GCAGGGAAATTCAGTCTCATT -3′ and 5′-CCAAAGGTTTGGCCCAACTTT-3′. And the rAAV2/9-CBG-DIO-miR30-*P2rx2*-shRNA-EGFP vector (briefly *P2rx2*-shRNAs) (titer: 5.07 × 10^{12} vg/mL) was custom-engineered by Obio Technology. To construct the *P2RX2* expression vector, the *P2RX2* coding sequence (NM_170682) was amplified with the following primer pair: 5′- CACACAGCGCCCAGTCCTCAGGCCGTTTCCCAG-3′ (forward) and 5′-TTATACGAAGTTATGCTAGCCACCATGGCCGCCGCCCAGCCCAAG-3′ (reverse), and was inserted into to the rAAV2/9-EF1a-DIO-mCherry-WPRE vector by recombination. The rAAV2/9-EF1a-DIO-*P2RX2*-3 × Flag vector (briefly *P2RX2*) (titer: 2 × 10^{12} vg/mL) was custom-engineered by Taitool Bioscience. rAAV-hEF1a (or CBG)-DIO-scrambled sequence-EGFP (SC) (titer: 6.56 × 10^{12} vg/mL) was used as

a control virus.

2.6. Virus injection and cannula implantation

Mice were anesthetized with pentobarbital (intraperitoneally, i.p., 75 mg/kg), and gently secured on a stereotaxic frame (RWD Life Science, Shenzhen, China). The viruses were delivered with a micro-syringe pump (Stoelting, Wood Dale, IL, USA) fitted with a 33-gauge needle at a controlled rate of 0.1 μL/min. After injection, the needle remained in place for 10 min before being slowly removed. To express shRNAs or *P2RX2* in DRN serotonergic neurons, the corresponding viruses (500 nL) were injected into the DRN of ePet-Cre mice at the following coordinates (mm from the bregma): DRN: mediolateral = +0.83, anteroposterior = −4.60, dorsoventral = −3.18 (administered at a 15° angle from caudal to rostral). Cobalt chloride (200 μM, total volume: 1 μL) and artificial cerebrospinal fluid (ACSF) infusion were performed through a unilateral cannula (RWD Life Science) implanted above the DRN. The cannula was secured in place using dental cement and a stainless-steel obturator was inserted into each guide cannula to prevent blockage. Only mice that exhibited correct cannula placement and viral expression were included for further analysis.

2.7. Cell culture and transfection

HEK293 and HEK293T cells (China Infrastructure of Cell Line Resource, Beijing, China) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L D-glucose (Gibco, Life Technologies, Waltham, MA, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, Life Technologies) at 37 °C in 5 % CO_2/95 % air. HEK293T cells were transfected with a HIF-1α expression plasmid or a control plasmid (vector with EGFP) using Lipofectamine 2000 (Gibco, Life Technologies) (ratio of 1 μg: 2 μL). To assess transfection efficiency, HEK293T cells (2×10^5 cells/mL) were seeded in 24-well plates and incubated with the plasmid mixture under 5 % CO_2 at 37 °C for 6 h in medium without FBS. Then, the medium was changed to DMEM containing 10 % FBS and incubation was continued for 24–48 h under the same conditions. Transfection efficiency was visualized under an ECLIPSE FN1 microscope (Nikon Instruments, Japan).

2.8. Cell counting Kit-8 (CCK-8) assay

The HEK293 cell were seeded in 96-well plates; approximately 10^4 cells were seeded per well. After culturing for 24 h at 37 °C in 5 % CO_2, $CoCl_2$ or vehicle was added to the cells, each group had at least 3 repetitions. After 48 h, 10 μL of CCK-8 reagent (#C0037, Beyotime, Shanghai, China) was added to each well and incubated for 1 h under the above conditions. The absorbance at 450 nm was measured by a microplate reader (PerkinElmer, Wellesley, Massachusetts, USA).

2.9. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using RNAiso Reagent (#9109, TaKaRa Bio, Tokyo, Japan) and reverse-transcribed into cDNA using PrimeScript RT Master Mix (#RR047A, TaKaRa Bio). qRT-PCR was performed using TB Green qPCR Master Mix (#RR420A, TaKaRa Bio). The 18S ribosomal RNA gene served as the internal control. The sequences of the primers used were, *Hif1a*: 5′-CGCCTCTGGACTTGTCTCTTT-3′ and 5′-TTCGACGTTCAGAACTCATCC-3′; *18S*: 5′-AGTTCCAGCACATTTTGCGAG-3′ and 5′-TCATCCTCCGTGAGTTCTCCA-3′; and *P2rx2*: 5′-CAGAACTGGCACACAAGGG-3′ and 5′-CAGTCACACAGAAAGGAGCC-3′.

2.10. Western blot analysis

For western blot experiments, two methods were used in the present study, traditional western blot protocol and Jess Simple Western system (Bio-Techne; ProteinSimple, San Jose, CA, USA) procedures. Briefly, protein was extracted from tissue samples using RIPA lysis buffer (#P0013B; Beyotime, Shanghai, China) supplemented with protease inhibitors (#ST506-2; Beyotime). Cytoplasmic and nuclear extracts from brain tissues were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (#78833; Thermo Fisher Scientific, Waltham, MA, USA). The protein concentrations in the resulting lysates were determined using a BCA kit (#23225, Thermo Fisher Scientific). For traditional western blot procedure, the following primary antibodies were used: anti–HIF–1α (1:500, #14179S), anti-NR2A (1:1,000, #4205), anti-NR2B (1:1,000, #4207) (all from Cell Signaling Technology, Danvers, Massachusetts, USA), anti-β-actin (1:1,000, #20536-1-AP, Proteintech, San Diego, California, USA), anti-GluR1 (1:5,000, #ab31232), anti-GluR2 (1:5,000, #ab133477), anti-GADPH (1:5,000, #ab9484) (all from Abcam, USA), anti-P2X2 (1:1000, #APR-003, Alomone Labs, Israel), and anti-Flag (1:1000; #F7425, Sigma-Aldrich). All quantification was performed with AlphaEaseFC software (Alpha Innotech Corporation, USA). For JESS Simple Western system, the following primary antibodies were used: anti-HIF1α (1:50, #14179S; Cell Signaling Technology), anti-PHD2 (1:200, #NB100-2219, Novus Biological, USA), anti-P2X2 (1:100, #APR-003, Alomone Labs), anti-GluR1 (1:5,000, #ab31232, Abcam), anti-GluR2 (1:5,000, #ab133477, Abcam), anti-NR2A (1:200, #4205, Cell Signaling Technology), anti-NR2B (1:200, #4207, Cell Signaling Technology), anti-LaminB1 (1:200, #ab16048, Abcam) and anti-β-actin (1:500, #20536-1-AP; Proteintech). The relative abundance of each protein was determined by quantifying the peak areas detected in the chemiluminescence electropherogram generated by the Compass for Simple Western software (ProteinSimple).

2.11. Forced swim test (FST)

The FST was performed as previously described [4]. The mice were placed in a glass bucket (45 × 19 cm) filled with water (23 ± 1 °C) to a depth of 23 cm under bright light and video recorded for 6-min. To prevent mutual influence, a white baffle was positioned between any two glass buckets. To assess immobility, the time spent motionless during the final 4 min was measured using the Ethovision XT tracking system (Noldus Information Technology, Netherlands). Immobility was characterized as the absence of movement except for that required for the mouse to keep its nose above the water.

2.12. Open field test (OFT)

Mice were placed in the central zone of a white plexiglass open chamber (40 × 40 × 30 cm) (Omnitech Electronics, Inc., Columbus, OH, USA), and were permitted to freely explore for 5 min. The total distance traveled, rearing activity in the arena was analyzed using Fusion software (6.47; Omnitech Electronics, Inc.).

2.13. Chronic mild stress (CMS) paradigm

The CMS paradigm was conducted as previously reported [24]. Initially, a baseline measurement of the sucrose preference test (SPT) was performed three times. Following this, the chronic mild stresses were administered. The CMS protocol involved the sequential application of various mild stressors, including restraint (twice, each lasting for 1 h), a forced swim in ice-cold water (5 min), food deprivation (twice, for 19 and 21 h, respectively), water deprivation (twice, for 7 and 19 h, respectively), an empty water bottle (13 h), cage tilting (twice, for 6 and 12 h, respectively, at a 45° angle), strobe light exposure (twice, for 7 and 17 h, respectively), and a soiled cage (13 h), which were repeated every week. Control mice were kept under the same housing conditions but without exposure to these stressors. For drug treatment, imipramine (15 mg per kg body weight, i.p.) was injection used as the positive control, and CMS-animals injected with saline were served as the controls. For IHHT, IHHT were conducted following the 6-week CMS paradigm, and the CMS-mice which placed in the same chamber, but normoxia was

IHHT-control group. The sucrose preference and coat score were measured weekly.

2.14. Sucrose preference test (SPT)

Before the SPT, the mice were individually housed and acclimated to either water or a 1 % sucrose solution for 4 days. For the SPT, the mice were fasted overnight (both food and water). Subsequently, two bottles were provided for each mouse, one containing 1 % sucrose solution (A) and the other a pure water (B). To prevent any potential bias, the sides of the two bottles were switched between each trial. The amount of each solution consumed within 1 h was determined and the sucrose preference was then calculated using the following formula: SPT index $= 100 \times [{}_{Vol}A/({}_{Vol}A + {}_{Vol}B)]$, where ${}_{Vol}A$ represents the volume of sucrose solution consumed and ${}_{Vol}B$ represents the volume of pure water consumed.

2.15. Social interaction test

Mice were placed in a transparent plexiglas arena (42 × 42 × 42 cm) containing an empty plexiglas cage at one end. The movements of the mice were tracked and recorded for 5 min using the Ethovision XT. This allowed for the assessment of their exploratory behavior and locomotion as a baseline measurement in the absence of a social target (stranger mouse). Subsequently, the mice were removed from the arena, and the entire space was cleaned with 75 % ethanol. A novel social target (stranger mouse) was then placed in the arena inside a small plexiglas cage and the exploratory behavior of the mice was evaluated for 5 min. The time spent in the interaction zone was recorded.

2.16. Fear conditioning test

Fear conditioning test was used to evaluate memory and learning, and was performed as previously described [25]. Mice were placed in an enclosed sound-attenuating chamber (Coulborn Instruct, PA, USA) equipped with speakers (OSD AUDIO, LA, USA). A visual CCD camera system was positioned above the chamber and freezing behavior was recorded and analyzed using FREEZEFRAME software (v.4.07, ACTIMETRICS, Coulbourn Instruments). Two distinct contexts were used. Context A featured aluminum walls with metal grid floors wired to a shock generator (Precision Animal Shocker, Coulbourn Instruments), enabling the delivery of an electric footshock as the unconditioned stimulus (US). Context B involved the use of dark plastic walls and smooth plastic floors. The conditioning and test boxes, as well as the floor, were cleaned with 75 % ethanol before and after each session. On day 1, fear acquisition was carried out under context A. This phase consisted of a 180 s baseline period, followed by four pairings of conditioned stimuli (CS), consisting of a tone (30 s, 2800 Hz, 70 dB, 10 ms rise/fall), and US, consisting of a 1 s scrambled electric footshock (0.70 mA). Each pairing was separated by a 60 s interval, with a subsequent 120 s post-stimulus period. Both stimuli terminated simultaneously. Freezing time in response to the tone during each 30 s CS was measured. The threshold for freezing was determined based on the motion index. Context and cued tests were conducted on days 2 and 3. During the context test, mice were allowed to freely explore the set-up for context A for 180 s and their contextual fear memories were assessed. During the context test, freezing behavior was observed and the average percentage of freezing time was measured throughout the 180 s of context exploration. The cued test was conducted under context B and involved a 180 s baseline period followed by a 180 s long presentation of the CS to evaluate the fear response of the mice to the tone. The percentage of freezing was measured both during the baseline period and CS presentation.

2.17. Quantification and statistical analysis

All data were analyzed using GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA) and expressed as the mean ± SEM. Two-tailed unpaired student's *t*-tests or multiple two-tailed unpaired *t*-tests were used for comparisons between two groups. Three or more groups were compared by one-way ANOVA, followed by Dunnett's multiple comparisons test for post hoc comparisons. Two-way ANOVA, followed by Sidak's multiple comparisons test, was used for repeated measures analysis. Differences with p values < 0.05 were considered significant.

3. Results

3.1. IHHT produces a sustained antidepressant-like effect in adult mice

To assess whether IHHT enhances psychological resilience, we first subjected male C57BL/6J mice to IHHT and the FST, a widely used animal model that is suitable for assessing antidepressant activity [26], was conducted on the 3rd (during IHHT) and 14th (at the end of IHHT) day, respectively (Fig. 1A, a). No effect on immobility was observed in mice under normoxic conditions (henceforth referred to as control animals) or in IHHT-treated mice after 3 days of IHHT. After 14-day of IHHT, the immobility time significantly decreased, but the locomotor activity was not affected (Fig. 1B, and Fig. S1A). These results corroborate the findings of our previous study [15], indicating that IHHT produces an antidepressant-like effect. Mice were then subjected to a 14-day IHHT paradigm, and FST was performed one or three months after IHHT (Fig. 1A and b). IHHT significantly decreased the immobility time on the 42nd and even on the 98th day, as shown by comparing IHHT with control animals (Fig. 1C). These results suggest that IHHT exerts a sustained antidepressant-like effect.

To further characterize IHHT promoting psychological resilience, we generated a mouse model of depression using a well-established chronic mild stress (CMS) paradigm [24]. To this end, mice were randomly divided into five groups, control animals, CMS + saline, CMS + imipramine, CMS + normoxia, and CMS + IHHT groups (Fig. 1D). CMS reduced the index in the SPT and deteriorated the coat condition in saline group after 4 weeks of stress. In contrast, after a 4-week treatment with imipramine, a classic antidepressant, the sucrose preference and coat condition scores of mice subjected to CMS were better than those of the saline group. These results match the findings of previous reports [27,28]. Notwithstanding the above, the effect of imipramine was not long-lasting. The SPT index increased upon imipramine treatment but return to that of the saline group after withdrawing imipramine for one week (Fig. 1E and F). Nevertheless, in line with the FST results, IHHT markedly improved the SPT index and coat condition scores of mice following CMS. Moreover, this effect lasted for at least two weeks in the face of stress because, by the end of CMS paradigm, the increased SPT index and coat score induced by IHHT remained significantly higher than that of CMS-mice under normoxic conditions (Fig. 1G and H). Together, these results clearly show that IHHT enhances psychological resilience in adult mice.

3.2. IHHT induces a long-lasting overexpression of HIF-1α in the DRN

HIF-1α is a master regulator of oxygen homeostasis. Under hypoxic conditions, HIF-1α is stabilized and promotes adaptation to inadequate oxygen supply at both transcriptional and translational levels [29,30]. To investigate the role of HIF-1α in the effect of IHHT, we first examined the mRNA levels of *Hif1a*, which encodes HIF-1α, in brain areas implicated in neuropsychiatric disorders including mPFC, nucleus accumbens (NAc), striatum, amygdala (AMG), hippocampus (Hip), and DRN three months after 14-day IHHT [31]. We found that *Hif1a* mRNA levels were specifically increased in DRN following IHHT, with no significant difference in other brain regions when compared to that of the corresponding brain area of control animals (Fig. 2A). Consistently, using the western blot analysis, we found that HIF-1α protein level significantly increased in the DRN. But not in the other brain regions (Fig. 2B and C). To examine whether HIF-1α enters into the nucleus, we separated the

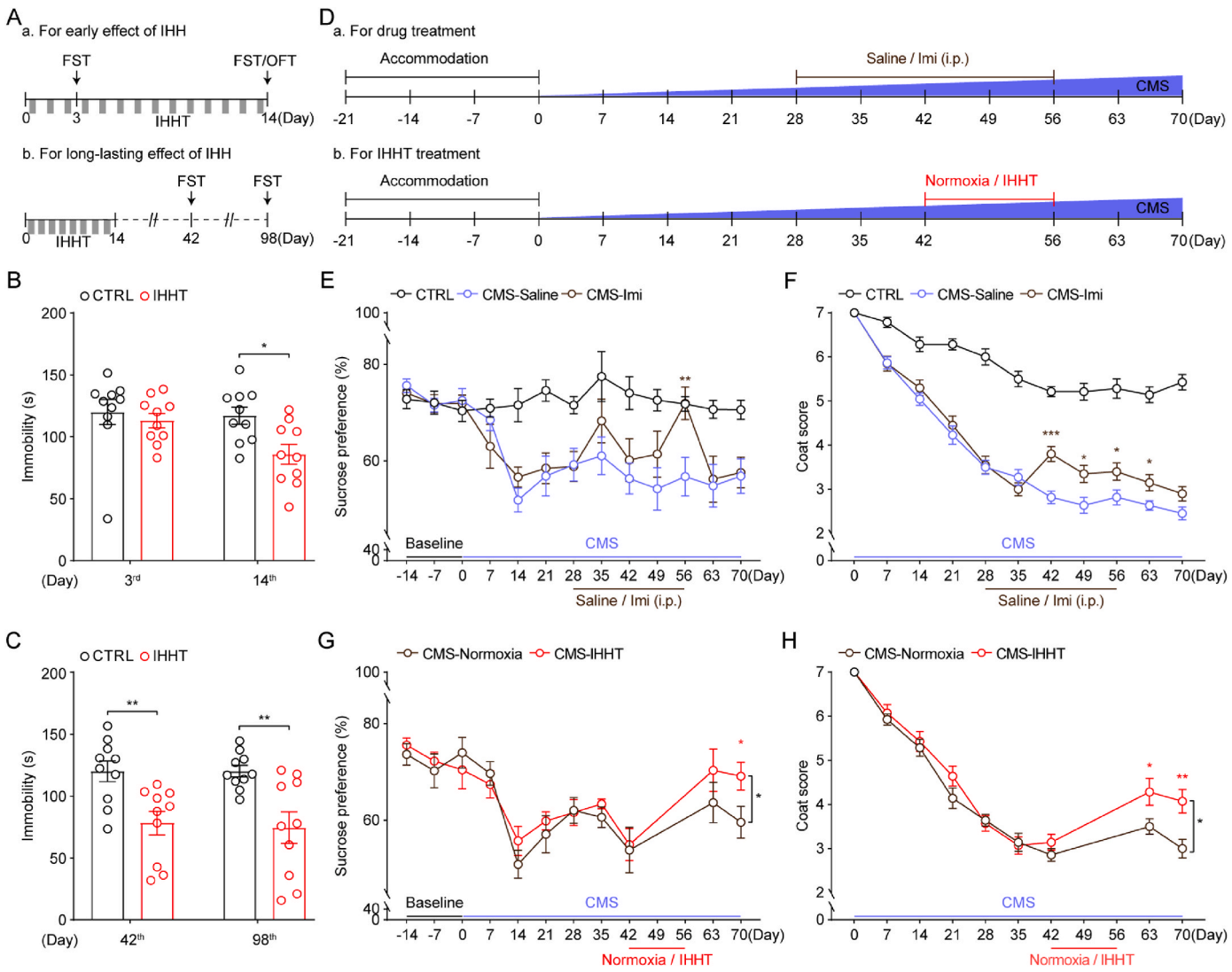

Fig. 1. IHHT produces long-lasting antidepressant-like effects in adult mice. **A** Schematic of IHHT; behavioral analysis was conducted on the 3rd and 14th day (a) and one (42nd day) and three (98th day) months after IHHT (b). B Immobility time of mice on the 3rd and 14th day of IHHT in the forced swim test (FST) ($n = 10$). Two-way ANOVA; $F_{(1,\ 18)} = 6.6250$, $p = 0.0191$; Interaction: $F_{(1,\ 18)} = 2.0940$, $p = 0.1651$. **C** For the long-lasting effect of IHHT, FST was conducted on the 42nd and 98th day ($n = 10$). Two-way ANOVA; $F_{(1,\ 18)} = 23.7700$, $p = 0.0001$; Interaction: $F_{(1,\ 18)} = 0.0399$, $p = 0.8440$. **D** Experimental design. Male C57BL/6J mice were exposed to chronic mild stress (CMS) for 70 days. Sucrose preference and coat scores were assessed per week. Imipramine (Imi, 15 mg/kg) or saline was administered from the 28th to the 56th day **(a)**. IHHT was conducted from the 42nd to the 56th day **(b)**. **E–H** Measurements of sucrose preference (**E**, **G**) and the physical state of the coat (**F**, **H**), showing baseline followed by post-stress measurements conducted each week for the mice treated with saline or Imi (i.p.), or IHHT and normoxic conditions ($n_{CTRL} = 14$, $n_{CMS\text{-}Saline} = 22$, $n_{CMS\text{-}Imi} = 20$, $n_{CMS\text{-}Normoxia} = 14$, $n_{CMS\text{-}IHHT} = 14$). Repeated measures two-way ANOVA; **E**: $F_{(1,40)} = 6.5720$, $p = 0.0170$; **F**: $F_{(1,40)} = 8.2510$, $p = 0.0060$; **G**: $F_{(1.26)} = 4.3800$, $p = 0.0463$; **H**: $F_{(1.26)} = 4.4090$, $p = 0.0460$. All data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

cytoplasmic and nuclear extracts from the DRN and found that both the cytosolic and nuclear protein levels of HIF-1α significantly increased following IHHT compared to that of control animals (Fig. 2D and E). We then examined *Hif1a* expression in DRN at different time points during/after 14-day IHHT and found that *Hif1a* mRNA levels remained upregulated from the 3rd to 98th day following IHHT, peaking at the 7th day of IHHT (Fig. 2F). Intriguingly, we found that 7-day of IHHT significantly decreased the immobility time in the FST (Fig. S1B). Based on these results, that enhanced HIF-1α signaling in the DRN could be involved in IHHT promoting psychological resilience.

3.3. *DRN-infusion of $CoCl_2$ induces a rapid and sustained antidepressant-like effect*

Subsequently, we assessed whether HIF-1α overexpression in DRN produced an antidepressant-like effect. Cobalt chloride is known to stabilize HIF-1α and increase its levels [32]. Accordingly, we infused 200 μM $CoCl_2$ [15] or artificial cerebrospinal fluid (ACSF) into the DRN of adult C57BL/6J mice and conducted FST at 1 and 24 h after infusion. Infusion of $CoCl_2$ significantly shortened immobility at two time points without affecting locomotion (Fig. 3A, and Fig. S2A), indicating that DRN-infusion of $CoCl_2$ induces a long-lasting antidepressant-like effect. We then infused $CoCl_2$ (50–200 μM) into DRN and examined the behavior of the mice 7 days later. A single dose of 200 μM $CoCl_2$ significantly shortened immobility in the FST (Fig. 3B). Moreover, when using the CMS paradigm, including $CoCl_2$ (a single dose of 200 μM) or ACSF infusion into DRN on the 35th day, we found that $CoCl_2$ completely reversed the decrease in SPT index induced by CMS 48 h after a single infusion (Fig. 3C and D). In addition, $CoCl_2$ infusion into DRN had little effect on cognition-related behaviors (Figs. S2B–H).

DRN is the predominant source of serotonergic innervation in the forebrain [33]. Both impaired serotonergic [34,35] and imbalanced

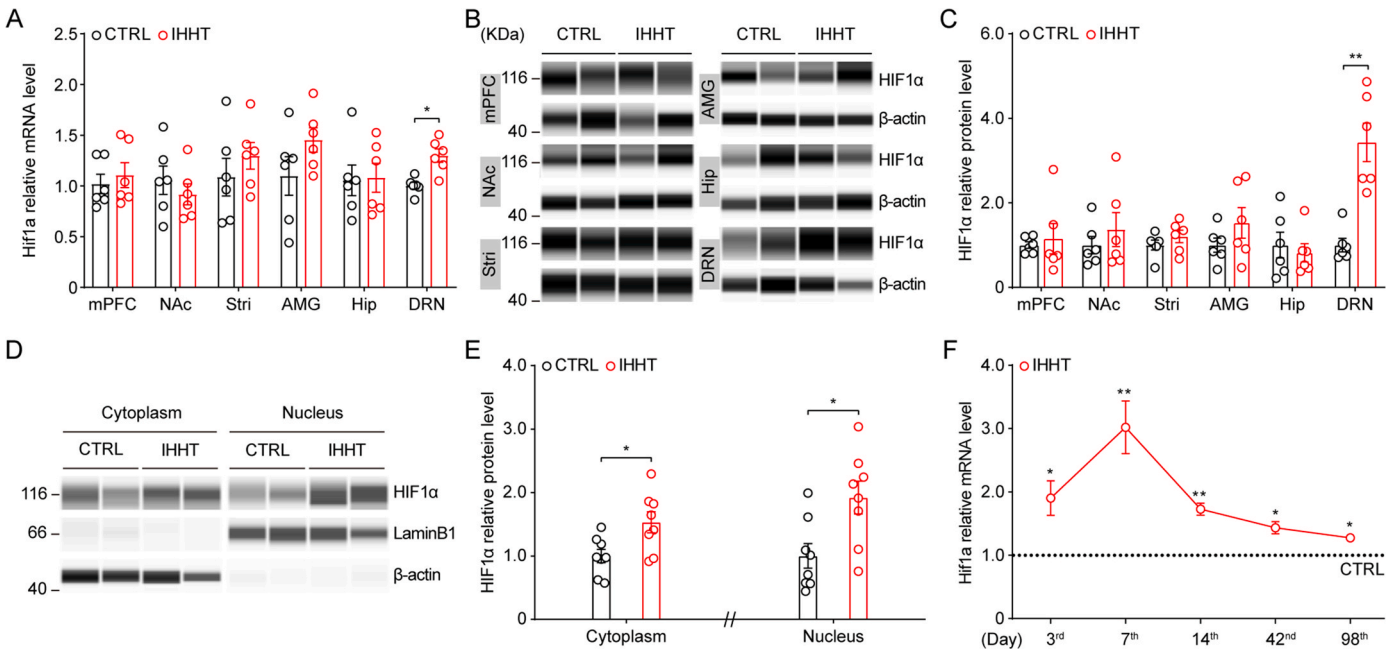

Fig. 2. IHHT induces a long-lasting overexpression of HIF-1α in the DRN. **A** The mRNA levels of *Hif1a* in mouse brain regions implicated in MDD on the 98th day after IHHT ($n = 6$). Multiple two-tailed unpaired *t*-tests following FDR correction; mPFC: $t_{(10)} = 0.5563$, $q = 0.7154$; nucleus accumbens (NAc): $t_{(10)} = 0.8000$, $q = 0.6701$; Stri (Striatum): $t_{(10)} = 0.9221$, $q = 0.6701$; amygdala (AMG): $t_{(10)} = 1.5710$, $q = 0.4462$; hippocampus (Hip): $t_{(10)} = 0.1179$, $q = 0.9176$; DRN: $t_{(10)} = 4.0150$, $q = 0.0149$. **B** Representative bands of western blot for HIF-1α (Jess system). **C** Protein levels of HIF-1α on the 98th day following IHHT ($n = 6$). Multiple two-tailed unpaired *t*-tests following FDR correction; mPFC: $t_{(10)} = 0.4164$, $q = 0.5773$; NAc: $t_{(10)} = 0.8141$, $q = 0.5486$; Stri: $t_{(10)} = 1.0860$, $q = 0.5097$; AMG: $t_{(10)} = 1.3250$, $q = 0.5097$; Hip: $t_{(10)} = 0.4902$, $q = 0.5773$; DRN: $t_{(10)} = 5.0300$, $q = 0.0026$. **D, E** Representative western blot bands and statistics of HIF-1α protein levels in the DRN (cytosolic and nuclear parts) on the 98th following IHHT ($n = 8$). Multiple two-tailed unpaired *t*-tests following FDR correction. Cytoplasm: $t_{(14)} = 2.6940$, $q = 0.0176$; Nucleus: $t_{(14)} = 2.8400$, $q = 0.0176$. **F** *Hif1a* mRNA levels (relative to the control [dark dashed line]) during and after IHHT ($n = 6$). Multiple two-tailed unpaired *t*-tests following FDR correction. Day 3: $t_{(10)} = 2.2250$, $q = 0.0451$; day 7: $t_{(10)} = 3.7120$, $q = 0.0081$; day 14: $t_{(10)} = 5.3750$, $q = 0.0013$; day 42: $t_{(10)} = 2.1630$, $q = 0.0451$; day 98: $t_{(10)} = 2.7760$, $q = 0.0264$. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

glutamatergic neurotransmission and decreased synaptic plasticity have been associated with depression, especially in mPFC where DRN serotonergic neurons are heavily innervated [36,37]. To validate the behavioral effect of $CoCl_2$, we euthanized the mice and measured the expression levels of synaptic plasticity-related proteins in mPFC. In line with previous reports [38,39], we found that the protein levels of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR1 and N-methyl-D-aspartate (NMDA) receptor subunits NR2A and NR2B were lower in the mPFC of ACSF-treated mice treated with CMS than that of mice without CMS, and that $CoCl_2$ infusion reversed this decrease in protein levels induced by CMS (Fig. 3E and F). In addition, DRN-infusion of $CoCl_2$ had little effect on protein levels of GluR1, GluR2, NR2A, and NR2B in the DRN, although the protein levels of GluR1, GluR2, and NR2B were increased in the mPFC of C57BL6/J mice following DRN infusion of $CoCl_2$ (Figs. S2I and J). Together, these results suggest that HIF-1α overexpression in DRN is sufficient to induce a rapid and sustained antidepressant-like effect in adult mice and that increased HIF-1α levels in the DRN contribute to IHHT promoting psychological resilience.

3.4. Down-regulation of HIF-1α in DRN serotonergic neurons attenuates the antidepressant-like effect of IHHT

To characterize the role of HIF-1α in DRN serotonergic (DRN^{5-HT}) neurons contributing the effect of IHHT, we silenced HIF-1α *via* rAAV-mediated expression of shRNA. We tested the efficiency of *Hif1a*-shRNA by injecting hSyn-Cre and *Hif1a*-shRNA or control virus, SC, into the DRN of adult C57BL/6J mice and euthanizing the animals after 28 days. Confocal microscopy images showed that most EGFP-positive cells were strictly localized in DRN. Western blot analysis showed that HIF-1α protein levels were lower in the DRN of mice infused with *Hif1a*-shRNA than that of mice injected with SC (Fig. S3), thereby validating our genetic approach. We then injected *Hif1a*-shRNA or SC into the DRN of ePet-Cre mice and conducted behavioral tests 28 days after virus injection. We found that mice injected with *Hif1a*-shRNA exhibited a depressive phenotype, because knockdown of HIF-1α in DRN^{5-HT} neurons significantly increased the immobility in the FST and decreased the SPT index compare to that of mice injected with SC, and viral manipulations had little effect on locomotion (Fig. S4). Then, these mice were subjected to IHHT and behavioral tests were conducted 24 h and one month after a 14-day IHHT paradigm (Fig. 4A and B). Once again, IHHT significantly shortened immobility on the 1st and 42nd day after training, while *Hif1a*-shRNA injection blocked the IHHT-induced reduction of immobility on both sampling days, without affecting locomotion (Fig. 4C–E). Moreover, IHHT could not rescue the decreased SPT index of mice with knockdown of HIF-1α in DRN^{5-HT} neurons (Fig. 4F). These results suggested that enhancement of HIF-1α signaling in DRN^{5-HT} neurons is crucial for the antidepressant-like effect of IHHT.

3.5. HIF-1α translationally regulates P2X2 expression in vitro

To investigate the downstream mechanism of HIF-1α signaling in the effect of IHHT, we analyzed P2X2 receptors. First, we measured P2X2 expression following IHHT. For this purpose, mice subjected to IHHT were euthanized on the 14th, 21st, or 98th day, and DRN was rapidly removed for biological analysis. IHHT significantly increased *P2rx2* mRNA levels on the 14th day, but not on the 21st and 98th day (Fig. 5A). Conversely, P2X2 protein levels did not change on the 14th day, but increased on the 21st and 98th day (Fig. 5B and C). These results indicate that HIF-1α could post-transcriptionally regulate the expression of P2X2 following IHHT.

To further characterize the role of HIF-1α on P2X2 expression, we

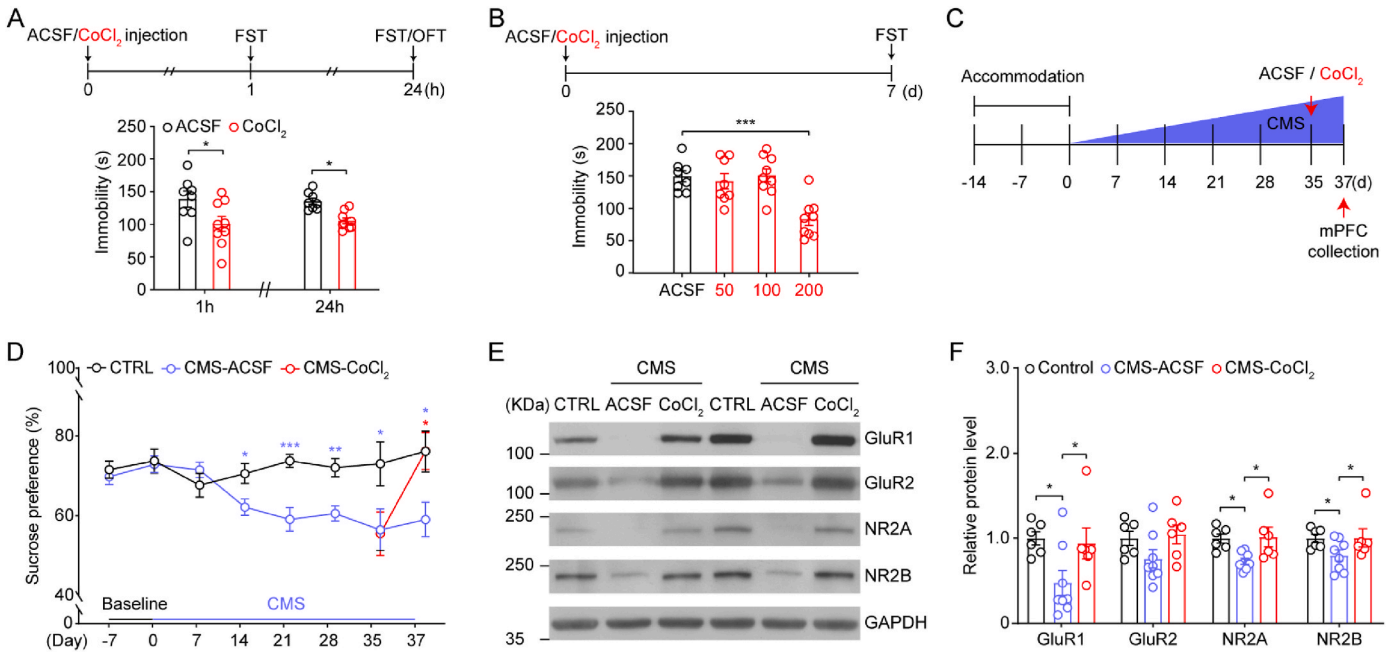

Fig. 3. DRN-infusion of $CoCl_2$ induces a rapid and long-lasting antidepressant-like effect in C57BL/6J mice **A** Effect of $CoCl_2$ infusion into DRN on immobility time in FST 1 and 24 h after treatment ($n_{ACSF} = 8$, $n_{CoCl2} = 9$). Two-way ANOVA with Sidak's multiple comparisons test; $F_{(1, 15)} = 15.4800$, $p = 0.0013$; Interaction: $F_{(1, 15)} = 0.1738$, $p = 0.6826$. **B** The immobility time in the FST 7 days after $CoCl_2$ or ACSF infusion into DRN ($n_{ACSF} = 8$, $n_{50} = 8$, $n_{100} = 9$, $n_{200} = 9$). One-way ANOVA with Dunnett's multiple comparisons test; $F_{(3,30)} = 10.7400$, $p < 0.0001$; ACSF *vs.* 50 μM: $p = 0.9114$; ACSF *vs.* 100 μM: $p = 0.9990$; CTRL *vs.* 200 μM: $p = 0.0002$. **C, D** Sucrose preference was assessed at the start of exposure to chronic mild stress (CMS) and at the end of the 2nd day after $CoCl_2$ infusion ($n_{CTRL} = 11$, $n_{CMS\text{-}ACSF} = 14$, $n_{CMS\text{-}CoCl2} = 13$). Repeated measures two-way ANOVA; $F_{(1, 35)} = 4.6400$, $p = 0.0163$. CMS-ACSF *vs.* CMS-$CoCl_2$: $p = 0.0116$. **E** Representative immunoblots bands for synaptic proteins using traditional western blot protocol. **F** Quantification of synaptic proteins in the mPFC normalized to loading controls. Representative images are from the same gel and lines indicate that they originate from different lanes ($n_{CTRL} = 6$, $n_{CMS\text{-}CTRL} = 8$, $n_{CMS\text{-}CoCl2} = 6$). One-way ANOVA with Dunnett's multiple comparisons test; GluR1: $F_{(2,17)} = 5.4797$, $p = 0.0146$; GluR2: $F_{(2,17)} = 2.3270$, $p = 0.1279$; NR2A: $F_{(2,17)} = 5.5622$, $p = 0.0139$; NR2B: $F_{(2,17)} = 3.8295$, $p = 0.0424$. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

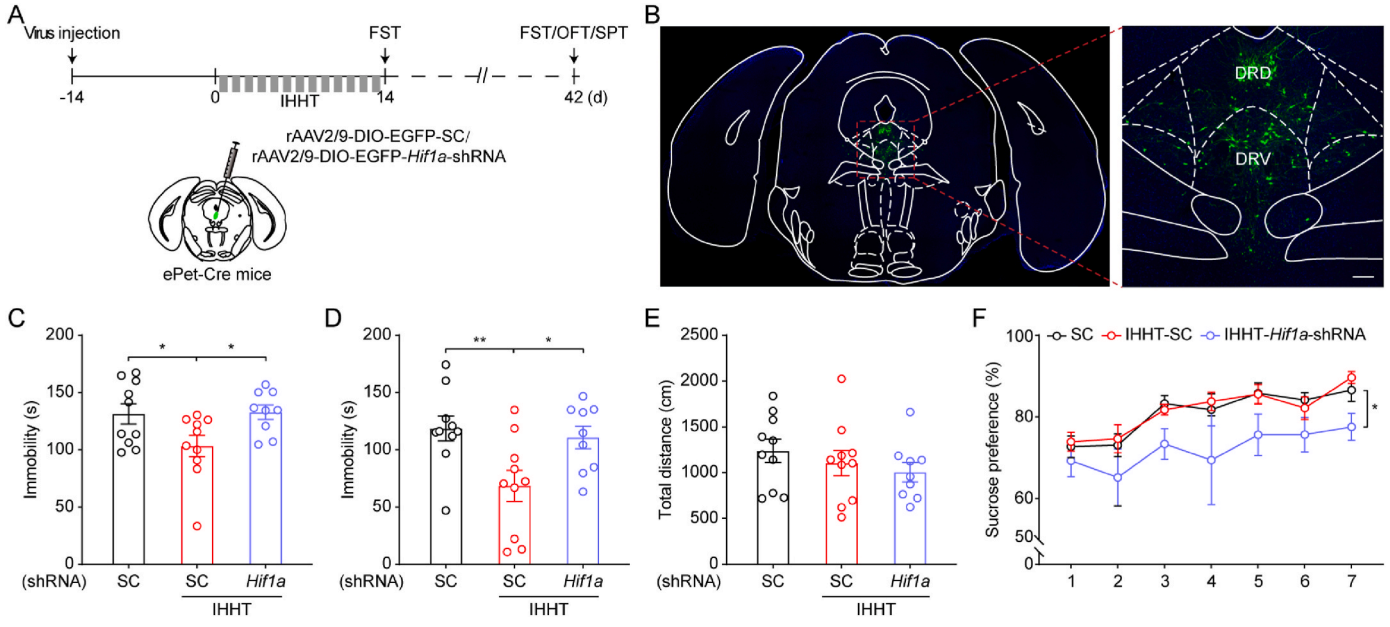

Fig. 4. Knockdown of HIF-1α in DRN^{5-HT} neurons attenuates the antidepressant-like effect of IHHT. **A** Experimental timeline. Fourteen days after viral injection, ePet-Cre mice were subjected to a 14-day IHHT paradigm. Behavioral tests were conducted 24 h (on the 1st day) and 28 days (on the 42nd day) after IHHT. **B** Coronal sections showing EGFP-positive cells were strictly located in the DRN. DRD, dorsal DRN; DRV, ventral DRN. Scale bar = 100 μm. **C, D** Knockdown of *Hif1a* in serotonergic neurons blocked the antidepressant-like effect of IHHT in FST 24 h **(C)** and 28 days **(D)** after IHHT ($n_{SC} = 10$, $n_{IHHT\text{-}SC} = 10$, $n_{IHHT\text{-}Hif1a\text{-}shRNA} = 9$). One-way ANOVA with Dunnett's multiple comparisons test; 24h: $F_{(2,26)} = 4.0330$, $p = 0.0298$; 28 days: $F_{(2,26)} = 5.5170$, $p = 0.0101$. **E** The injection of *Hif1a*-shRNA had little effect on locomotion. One-way ANOVA with Dunn'tt's multiple comparisons test; $F_{(2,26)} = 0.8486$, $p = 0.4395$. **F** Mice injected with *Hif1a*-shRNA showed a lower sucrose preference than control animals. Repeated measures two-way ANOVA; $F_{(2,26)} = 3.9196$, $p = 0.0325$. IHHT-SC *vs.* IHHT-sh*Hif1a*: $p = 0.0319$. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. OFT: open field test; SPT: sucrose preference test.

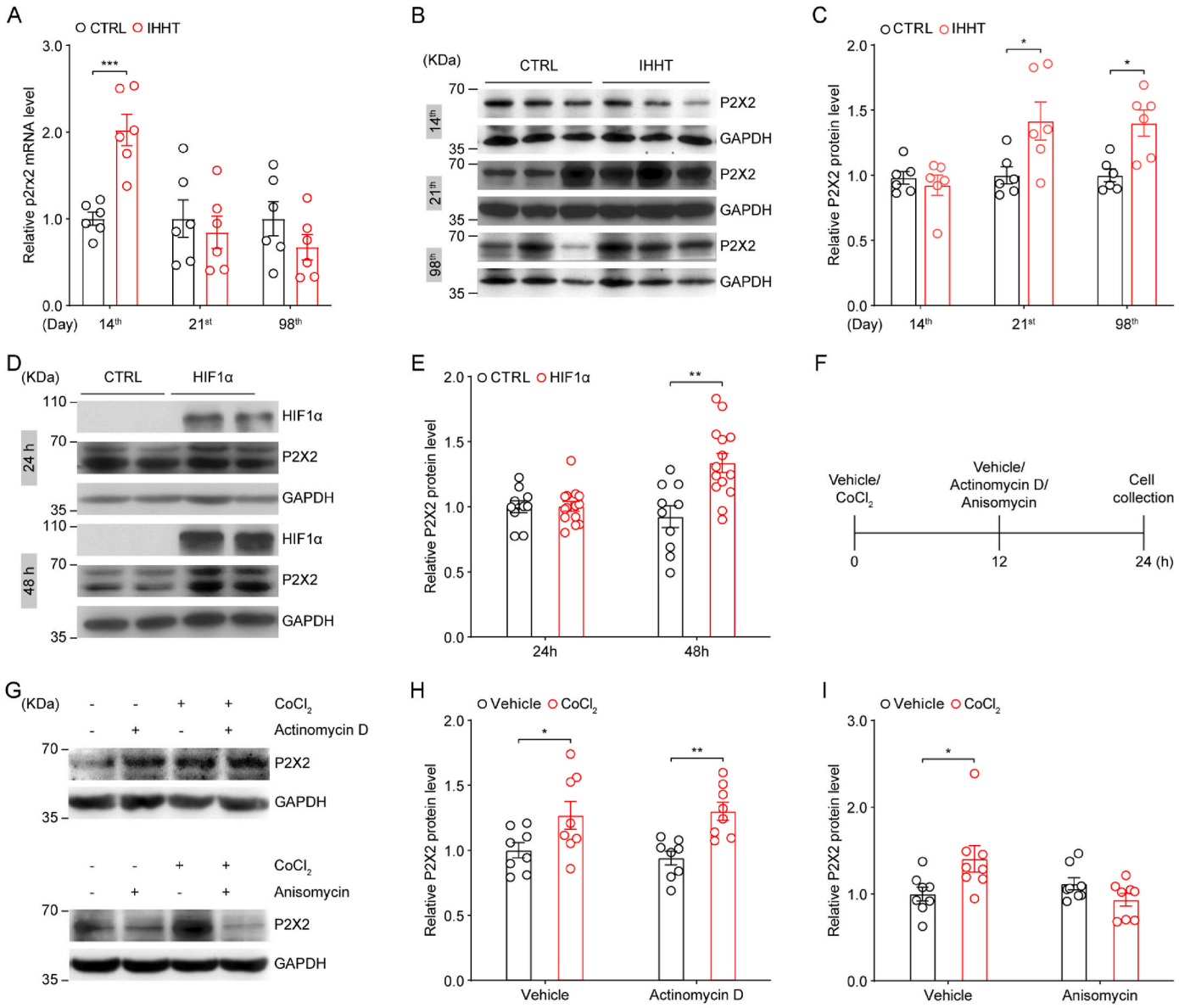

Fig. 5. HIF-1α translationally upregulates P2X2 expression *in vitro*. **A** The mRNA levels of *P2rx2* in DRN following IHHT. Multiple two-tailed unpaired *t*-tests following FDR correction. 14th day: $t_{(10)} = 5.1940$, $q = 0.0008$; 21st day: $t_{(10)} = 0.5547$, $q = 0.3981$; 98th day: $t_{(10)} = 1.3340$, $q = 0.2140$. **B, C** Western blot images and quantification of P2X2 and GAPDH levels in DRN following IHHT ($n = 6$). Multiple two-tailed unpaired *t*-tests following FDR correction. 14th: $t_{(10)} = 0.6117$, $q = 0.5599$; 21st: $t_{(10)} = 2.5940$, $q = 0.0405$; 98th: $t_{(10)} = 3.5900$, $q = 0.0149$. **D, E** HEK293T cells were transfected with a HIF-1α expression plasmid or empty vector (CTRL) and exposed to vehicle or $CoCl_2$ for 24 and 48 h before immunoblotting assays ($n_{CTRL} = 10$, $n_{HIF1\alpha} = 14$). Two-way ANOVA with Sidak's multiple comparisons test; $F_{(1, 44)} = 10.8900$, $p = 0.0019$; Interaction: $F_{(1, 44)} = 10.4900$, $p = 0.0023$. **F** The experimental flow chart. HEK293 cells were incubated with 200 μM $CoCl_2$ for 12 h, following which actinomycin D or anisomycin was added. Cells were then collected 12 h later and subjected to western blot analysis. **G** Representative images of western blot showing the protein levels of P2X2 in the DRN. **H** HIF-1α did not affect the transcription of P2X2. In the control group, both $CoCl_2$ and actinomycin D induced a significant increase in P2X2 protein levels ($n = 8$). Two-way ANOVA with Sidak's multiple comparisons test; $F_{(1, 28)} = 17.6000$, $p = 0.0002$; Interaction: $F_{(1, 28)} = 0.3724$, $p = 0.5466$. **I** HIF-1α affected the translation of P2X2. After anisomycin incubation, there was no significant change in P2X2 protein levels in the $CoCl_2$ group compared with that in the control group ($n = 8$). Two-way ANOVA with Sidak's multiple comparisons test; $F_{(1, 28)} = 4.2060$, $p = 0.0498$; Interaction: $F_{(1, 28)} = 10.6900$, $p = 0.0029$. In this figure, western blots were performed using the traditional western blot protocol. All data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

prepared cell cultures. HEK293 cells were treated with or without $CoCl_2$, and samples were harvested 48 h after treatment. $CoCl_2$ exposure, at concentrations lower than 200 μM, had little effect on cell death. As expected, $CoCl_2$ application significantly increased HIF-1α and P2X2 protein levels. In turn, $CoCl_2$ application increased HIF-1α protein levels in a time-dependent manner, and the initial effect was observed 12 h after treatment. In contrast, P2X2 protein levels remained increased 24 h after $CoCl_2$ application (Fig. S5). We then transfected the HIF-1α vector into HEK293T cells, and as expected, HIF-1α protein levels were significantly higher in cells transfected with HIF-1α vector 24 h after transfection than in cells transfected with control vector. P2X2 protein levels were greatly increased only 48 h after HIF-1α transfection, but no effect was observed at 24 h after HIF-1α vector transfection (Fig. 5D and E), supporting a notion that HIF-1α regulates P2X2 expression at a translational level. To further test out hypothesis, we treated cells with actinomycin D, a RNA polymerase inhibitor, or anisomycin, a protein synthesis inhibitor [40], 12 h after $CoCl_2$ application (Fig. 5F). Actinomycin D application had little effect on the increased expression of P2X2

induced by $CoCl_2$, while anisomycin blocked the $CoCl_2$ effect on P2X2 protein levels (Fig. 5G–I).

3.6. HIF-1α regulates the expression of P2X2 in DRN^{5-HT} neurons of mice

To assess whether HIF-1α regulates P2X2 expression *in vivo*, we performed pharmacological and genetic experiments. Using a pharmacological approach, we found that DRN-infusion of $CoCl_2$ significantly increased P2X2 protein levels 48 h after treatment, while no effect was observed at 24 h after the infusion in comparison to that of vehicle treatment (Fig. 6A and B). PHD2 is the primary enzyme responsible for HIF-1α hydroxylation and subsequent degradation [41]. To further characterize the role of HIF-1α on P2X2 expression, we microinjected *Egln1*-shRNA or SC into the DRN of ePet-Cre mice. Western blot showed that the protein level of PHD2 lowered in the DRN of mice injected with *Egln1*-shRNA, in contrast, HIF-1α level significantly increased. Interestingly, the P2X2 protein level increased by 2-fold following *Egln1*-shRNA injection compared to that of SC-injected mice (Fig. 6C–E). Moreover, we found that *Hif1a*-shRNA significantly decreased P2X2 expression in the DRN of ePet-Cre mice in comparison to that of animals infused with control virus (Fig. 6F).

To characterized HIF-1α-P2X2 signaling in DRN^{5-HT} neurons contributing to the behavioral effect of $CoCl_2$, after validating our genetic approach on P2X2 modulation (Fig. S6), we injected *P2rx2*-shRNAs or SC into the DRN of ePet-Cre mice and conducted behavioral tests 24 h and 48 h after $CoCl_2$ infusion (Fig. 6G and H). Again, we found that, in mice injected with SC, DRN-infusion of $CoCl_2$ decreased the immobility in the FST compared to that of mice infused with ACSF into DRN on both sample days. Intriguingly, although *P2rx2*-shRNA injection had little effect on DRN-infusion of $CoCl_2$ shortening the duration of immobility 24 h after $CoCl_2$ treatment, it indeed attenuated the antidepressant-like effect of $CoCl_2$ infusion when mice were examined 48 h after drug infusion (Fig. 6I–K). These results suggest that HIF-1α-P2X2 signaling in DRN^{5-HT} neurons is involved in IHHT promoting psychological resilience.

3.7. P2X2 receptors in the DRN^{5-HT} neurons are essential for IHHT enhancing psychological resilience

To determine whether P2X2 receptors in DRN^{5-HT} neurons are necessary for IHHT promoting psychological resilience, we generated conditional *P2rx2* knockout mice by crossbreeding Tph2-iCre^{ERT} or ePet-Cre mice with *P2rx2*$^{fl/fl}$ mice, yielding Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ and ePet-Cre;*P2rx2*$^{fl/fl}$ mouse lines. *P2rx2*$^{fl/fl}$ littermates served as control animals. *P2rx2* mRNA levels were significantly lower in the DRN of ePet-Cre;*P2rx2*$^{fl/fl}$ mice than in that of control littermates. In addition, P2X2 protein levels were markedly lower in the DRN of Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ mice following TAM injections than in control animals (Fig. S7). These results indicated that P2X2 signaling in serotonergic neurons was efficiently disrupted in both Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ and ePet-Cre;*P2rx2*$^{fl/fl}$ mice.

Subsequently, we subjected Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ and control littermates to a 14-day IHHT paradigm, and induced Cre-mediated recombination *via* TAM injection starting on the 70th day, conducting behavioral tests 3 months after IHHT (Fig. 7A). Consistently, we found that IHHT produced a long-last antidepressant-like effect in *P2rx2*$^{fl/fl}$ mice, as shown by their shortened immobility in FST in comparison to that of *P2rx2*$^{fl/fl}$ mice under normoxic conditions. In contrast, no difference in immobility time was observed between IHHT-treated-Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ mice and their littermates under normoxic conditions (Fig. 7B). Furthermore, genetic manipulation had little effect on locomotion (Fig. 7C). These results indicate that P2X2 receptors in DRN^{5-HT} neurons are necessary for the sustained antidepressant-like effect of IHHT.

To further characterize the role of P2X2 receptors on DRN^{5-HT} neurons in the effect of IHHT, we assessed whether *P2RX2* expression in DRN^{5-HT} neurons rescues the sustained antidepressant-like effect of IHHT after *P2rx2* deletion. To this end, *P2RX2* or control virus were microinjected into the DRN of adult ePet-Cre;*P2rx2*$^{fl/fl}$ mice (Fig. 7D). As expected, *P2RX2* injection induced a gain-of-function of P2X2 receptors, as shown by Flag expression and by the higher P2X2 protein levels in the DRN of treated mice than that of mice injected with SC when euthanizing the mice 28 days after viral injection (Fig. 7E and F). Once the viral system was validated, ePet-Cre;*P2rx2*$^{fl/fl}$ mice were subjected to a 14-day IHHT paradigm 28 days after viral injection, and behavioral tests were conducted on the 14th and 42nd days following IHHT (Fig. 7D). ePet-Cre;*P2rx2*$^{fl/fl}$ mice showed longer immobility than that of *P2rx2*$^{fl/fl}$ littermates under nomoxic conditions following injection of control virus, displaying a depressive-like phenotype. This phenotype was consistent with that of Tph2-iCreERT;*P2rx2*$^{fl/fl}$ mice following TAM injection. In contrast, IHHT significantly decreased the duration of immobility in all mice tested on the 14th day (Fig. 7G). But when mice were tested one month after IHHT, ePet-Cre;*P2rx2*$^{fl/fl}$ mice injected with control virus did not show decreased immobility following IHHT, while immobility was remained shortened in ePet-Cre;*P2rx2*$^{fl/fl}$ mice injected with *P2RX2*, which showed an antidepressant-like phenotype (Fig. 7H). No significant difference in total distance traveled was detected between groups (Fig. 7I). Together, these results strongly support our hypothesis that HIF-1α–P2X2 signaling in the DRN^{5-HT} neurons is essential for IHHT enhancing psychological resilience.

3.8. HIF-1α–P2X2 signaling in DRN^{5-HT} neurons mediates the antidepressant-like effect of ketamine

Mounting evidence indicates that a single sub-anesthetic dose of ketamine elicits a long-lasting antidepressant effect [42–44]. To verify whether enhancement of HIF-1α-P2X2 signaling in DRN^{5-HT} is related to psychological resilience in human, we examined the effect of ketamine. Consistently with a previous report [40], we found that a single dose of 10 mg/kg ketamine significantly shortened immobility in the FST when mice were tested 1 and 24 h after injection (Fig. 8A and B). HIF-1α protein levels were considerably increased in DRN 1 h after injection and stably increased up to 24 h. Meanwhile, ketamine promoted P2X2 expression in DRN 24 h after injection but had little effect on DRN-P2X2 expression 1 h after injection (Fig. 8C and D). Moreover, the antidepressant-like effect of ketamine in FST was attenuated by *Hif1a*-shRNA injection into the DRN of adult ePet-Cre mice (Fig. 8E and F). These results clearly show that enhanced HIF-1α–P2X2 signaling in DRN^{5-HT} neurons mediates the antidepressant-like effect of ketamine.

3.9. P2X2 signaling is disrupted in the DRN of patients with MDD

To verify the potential role of dysfunctional P2X2 signaling in DRN^{5-HT} neurons in the pathophysiology of MDD, we examined P2X2 protein levels in postmortem brain samples from patients with MDD. To this end, samples containing DRN were compared to BA9 and BA24 samples, two brain areas implicated in MDD [2,26]. We did not detect the expression of P2X2 receptors in BA24 by western blot. And while P2X2 receptors were detected in BA9, their protein levels were much lower than those in DRN, with no difference between patients with MDD and their matched control subjects. Intriguingly, P2X2 protein levels were significantly lower in the DRN of patients with MDD than in that of matched control subjects (Fig. 8G and H), indicating that P2X2 signaling was disrupted in the DRN of patients with MDD.

4. Discussion

The major findings in this study are followings: (1) IHHT produces a sustained antidepressant-like effect in adult mice up to 3 months after this treatment, and this effect is mediated by enhancing HIF-1α–P2X2 signaling in DRN^{5-HT} neurons following IHHT; (2) HIF-1α regulates

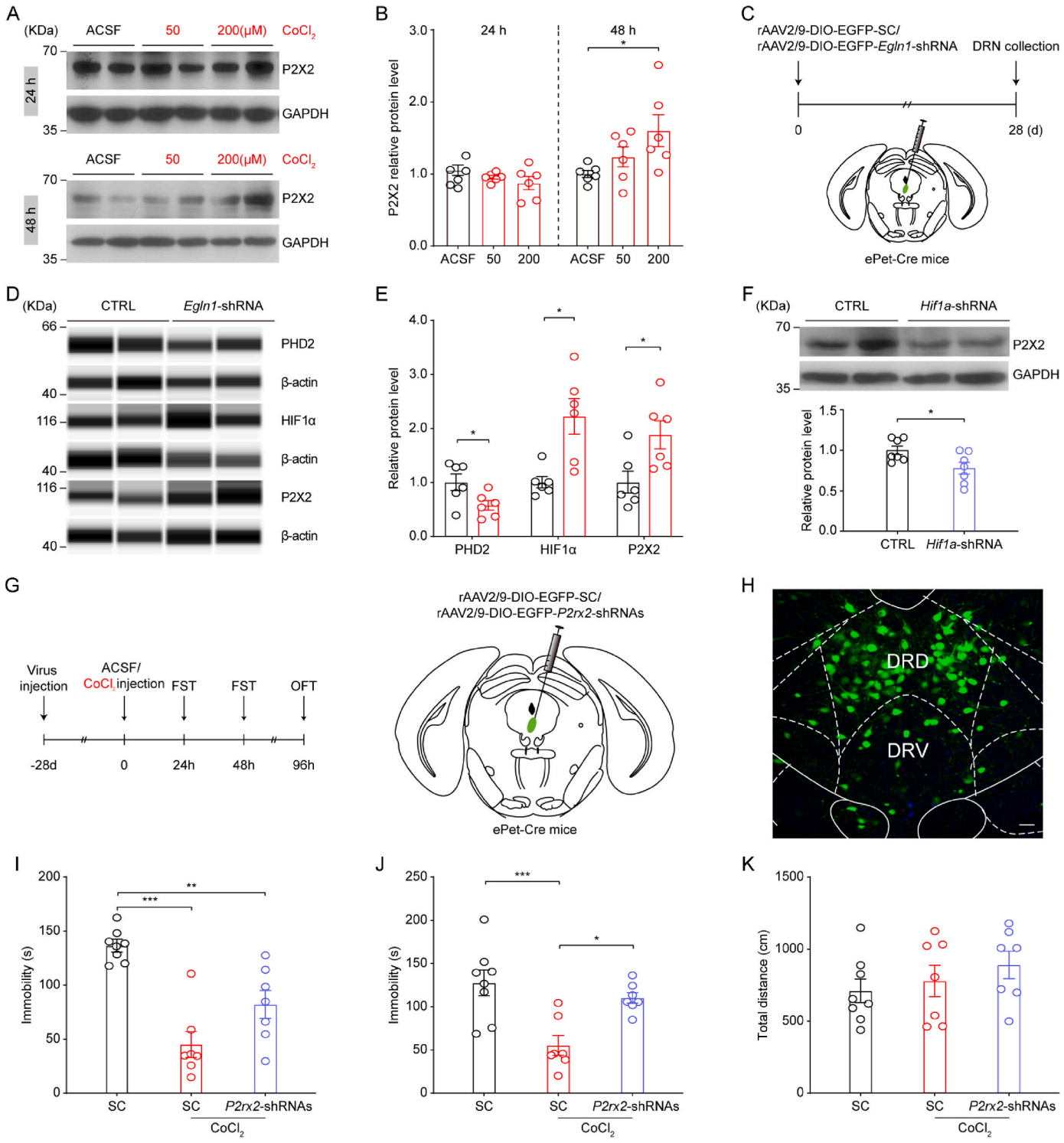

Fig. 6. HIF-1α bidirectionally regulates P2X2 expression in the DRN. **A, B** Western blot analysis (traditional western blot protocol) of P2X2 expression in the DRN of male C57BL/6J mice 24 and 48 h after DRN-infusion of ACSF or $CoCl_2$ (50 or 200 μM). One-way ANOVA with Dunnett's multiple comparisons test ($n = 6$). 24 h: $F_{(2,15)} = 1.6670$, $p = 0.2219$. 48 h: $F_{(2,15)} = 3.8440$, $p = 0.0449$. **C** Experimental timeline for *Egln1*-shRNA or SC injection into the DRN of adult ePet-Cre mice. **D, E** Western blot images (Jess system) (**D**) and quantification (**E**) of protein levels of PHD2, HIF-1α, and P2X2 in the DRN of adult C57BL6/J mice following *Egln1*-shRNA or SC injection ($n = 6$). Multiple two-tailed unpaired *t*-tests following FDR correction. PHD2: $t_{(10)} = 2.4030$, $q = 0.0375$; HIF1α: $t_{(10)} = 3.5180$, $q = 0.0168$; P2X2: $t_{(10)} = 2.6560$, $q = 0.0365$. **F** Western blots (traditional western blot protocol) and quantification of P2X2 in the DRN of mice injected with SC or *Hif1a*-shRNA ($n = 7$). Unpaired student's *t*-test. $t_{(12)} = 2.4940$, $p = 0.0282$. **G** Experimental schedule for virus/drug injection and behavioral tests. **H** Confocal microscopy images showed that most EGFP-positive cells were strictly localized in the DRN. DRD, dorsal DRN; DRV, ventral DRN. **I, J** The immobility time in FST 24 h **(I)** and 48 h **(J)** after $CoCl_2$ or ACSF infusion. **K** Locomotion in the open field test (OFT) ($n_{SC\text{-}ACSF} = 8$, $n_{SC\text{-}CoCl2} = 7$, $n_{P2rx2\text{-}shRNA\text{-}CoCl2} = 7$). One-way ANOVA with Dunnett's multiple comparisons test; **I**: $F_{(2,19)} = 20.6300$, $p < 0.001$; **J**: $F_{(2,19)} = 10.0700$, $p = 0.0010$; **K**: $F_{(2,19)} = 0.9250$, $p = 0.4137$. All data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

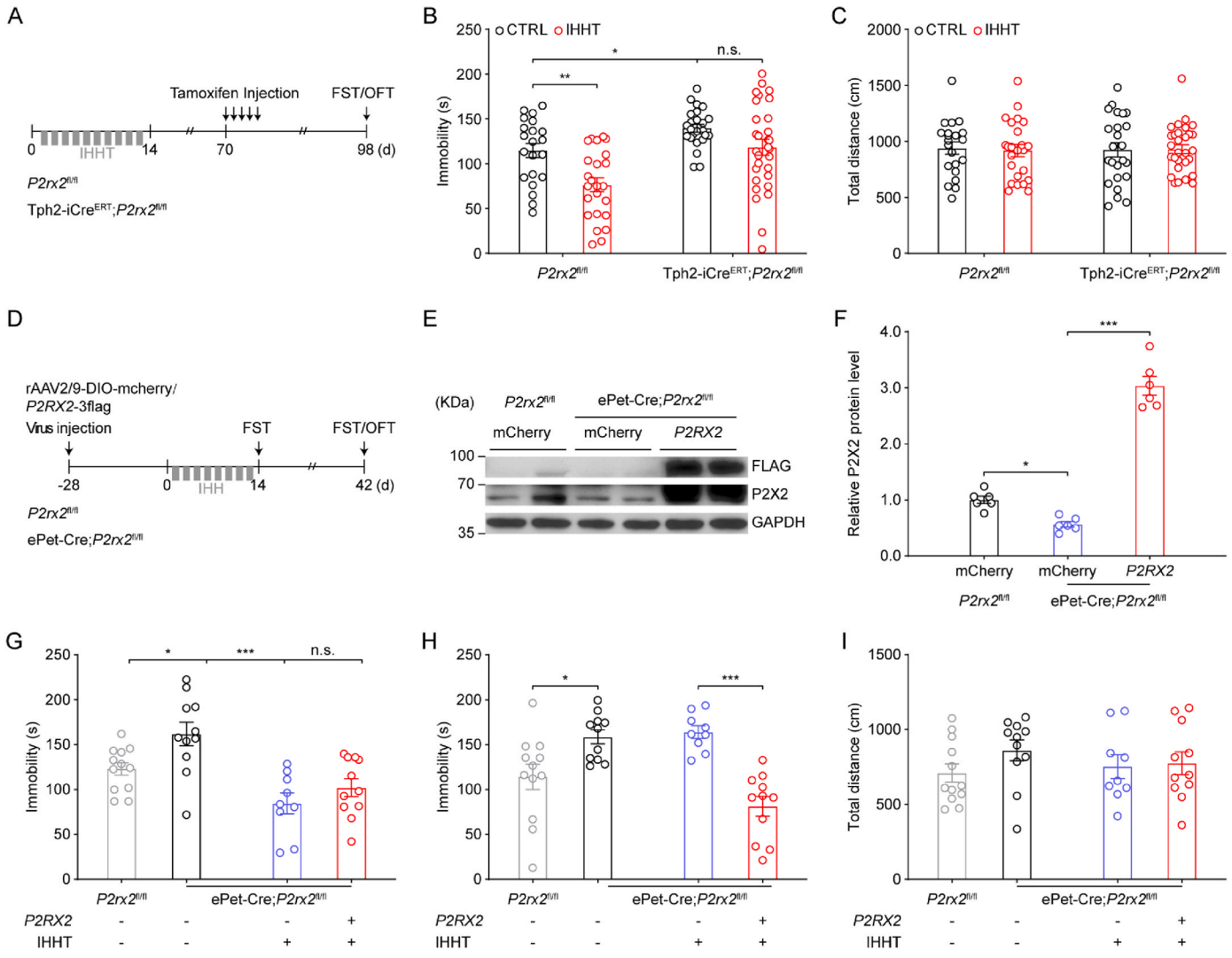

Fig. 7. P2X2 receptors in DRN^{5-HT} neurons mediate the long-lasting antidepressant-like effects of IHHT. **A.** Schematic of tamoxifen-induced *P2rx2* deletion in serotonergic neurons 2 months after IHHT; behavior tests were conducted on the 98th day. **B**, **C** Immobility time **(B)** and total distance **(C)** of IHHT-treated Tph2-iCre^{ERT};*P2rx2*$^{fl/fl}$ mice and their littermate controls ($n_{P2rx2fl/fl\text{-}CTRL} = 21$, $n_{P2rx2fl/fl\text{-}IHHT} = 24$, $n_{Tph2\text{-}iCreERT;P2rx2fl/fl\text{-}CTRL} = 24$, $n_{Tph2\text{-}iCreERT;P2rx2fl/fl\text{-}IHHT} = 30$). Two-way ANOVA with Sidak's multiple comparisons test; **B:** $F_{(1,\ 95)} = 14.9400$, $p = 0.0002$; Interaction: $F_{(1,\ 95)} = 1.1800$, $p = 0.2801$; **C:** $F_{(1,\ 95)} = 0.0279$, $p = 0.8676$; Interaction: $F_{(1,\ 95)} = 0.0614$, $p = 0.8048$. **D.** Schematic representation of *P2rx2* deletion followed by *P2RX2* injection into DRN. Behaviors were assessed 1 h (on day 14th) and 1 month (on the 42nd day) after IHHT. **E**, **F** P2X2 levels in the DRN of ePet-Cre;*P2rx2*$^{fl/fl}$ mice and their littermate controls injected with a viral *P2RX2* expression construct ($n = 6$). One-way ANOVA with Dunnett's multiple comparisons test; $F_{(2,15)} = 145.5000$, $p < 0.0001$. **G**–**I** The effects of *P2RX2* injection into the DRN on the antidepressant-like phenotype observed on the 14th **(G)** and 42nd **(H, I)** day. One-way ANOVA with Dunnett's multiple comparisons test ($n_{P2rx2fl/fl} = 12$, $n_{ePet\text{-}Cre;P2rx2fl/fl} = 11$, $n_{ePet\text{-}Cre;P2rx2fl/fl\text{-}IHHT} = 9$, $n_{ePet\text{-}Cre;P2rx2fl/fl\text{-}P2RX2\text{-}IHHT} = 11$). **G:** $F_{(3,39)} = 9.7640$, $p < 0.0001$. **H:** $F_{(3,39)} = 12.5800$, $p < 0.0001$. **I:** $F_{(3,39)} = 0.8298$, $p = 0.4856$. All data are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; n.s., not significant.

P2X2 expression at a translational level *in vitro* and *in vivo*, suggesting that, to our knowledge, P2X2 is a new downstream factor of HIF-1α; (3) Enhancement of HIF-1α–P2X2 signaling in DRN^{5-HT} neurons is crucial for the long-lasting antidepressant-like effects of ketamine; and (4) P2X2 protein levels are lower in the DRN of patients with MDD than in that of control subjects. Combined with our previous data [15], these findings indicate that IHHT enhanced psychological resilience, may help people to gain ability to healthily rebound in the face of stress and trauma, or even recover from depression, and therefore could be developed as a physical treatment for patients with MDD.

Mounting evidence indicates that HIF-1α and its target genes are involved in the pathophysiology of depression. In a previous study, the mRNA levels of HIF-1α and HIF-1β and those of their target genes *VEGF* (which encodes vascular endothelial growth factor, VEGF) in peripheral white blood cells were higher in patients with depression than in healthy controls [45]. The plasma levels of erythropoietin (which is encoded by *EPO*, another HIF-1α target gene) were also significantly higher in non-suicidal and suicidal patients with MDD than in healthy controls and were higher in suicidal than non-suicidal MDD patients [46]. Moreover, patients with MDD who received erythropoietin reportedly had significantly better memory than patients who received the placebo [47]. In addition, animal studies have shown that VEGF levels are significantly reduced in the hippocampus and frontal cortex of rats with depressive phenotypes [48], while mPFC-infusion of VEGF significantly shortens immobility in mice during FST [49]. These findings implied that the IHHT-induced increase in HIF-1α expression correlated with an antidepressant effect, as supported by the results of this study. In this study, we found that $CoCl_2$, a compound that mimics hypoxia at a biological level and induces HIF-1α expression, shortened immobility in the FST 7 days after its administration into DRN and improved sucrose preference in the CMS paradigm 48 h after treatment. Moreover, shRNA-mediated knockdown of HIF-1α induced a depressive-like

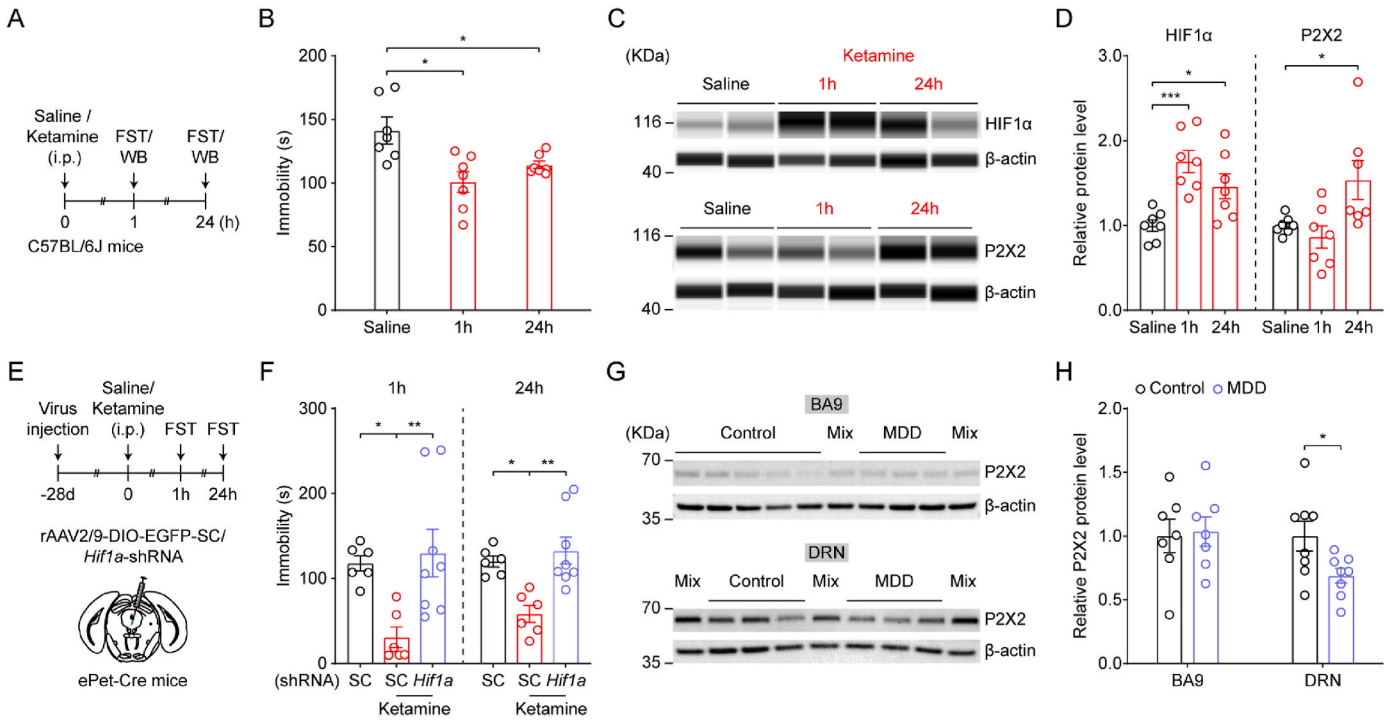

Fig. 8. HIF-1α–P2X2 signaling in DRN^{5-HT} neurons mediate the antidepressant-like effect of ketamine. **A** Schematic representation of ketamine injection (i.p.) and behavioral or biological tests. **B** Antidepressant-like response of ketamine in FST 1 and 24 h after treatment ($n = 7$). One-way ANOVA with Dunnett's multiple comparisons test; $F_{(2,18)} = 8.0750$, $p = 0.0031$. **C, D** Ketamine effect on HIF-1α and P2X2 expression in DRN 1 and 24 h after injection ($n = 7$). Western blot was performed using Jess system. One-way ANOVA with Dunnett's multiple comparisons test; HIF-1α: $F_{(2,18)} = 10.0000$, $p = 0.0012$; P2X2: $F_{(2,18)} = 5.3110$, $p = 0.0154$. **E** Schematic for *Hif1a*-shRNA injected into the DRN followed by ketamine treatment and behavioral tests. **F** Knockdown of *Hif1a* in DRN^{5-HT} neurons blocked the antidepressant-like effect of ketamine in FST 1 and 24 h after ketamine treatment ($n_{SC\text{-}saline} = 6$, $n_{SC\text{-}ketamine} = 6$, $n_{Hif1a\text{-}shRNA\text{-}ketamine} = 8$). One-way ANOVA with Dunnett's multiple comparisons test; 1h: $F_{(2,17)} = 6.3380$, $p = 0.0088$; 24h: $F_{(2,17)} = 9.5710$, $p = 0.0016$. **G, H** Western blot and quantification of P2X2 in the BA9 and DRN of subjects with MDD and matched controls ($n_{BA9} = 7$, $n_{DRN} = 8$). Western blot was performed using the traditional western blot protocol. Unpaired student's *t*-test. BA9: $t_{(12)} = 0.2023$, $p = 0.8431$; DRN: $t_{(14)} = 2.3980$, $p = 0.0310$. All data are expressed as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

behaviors and blocked the antidepressant-like effect of IHHT. Moreover, we found that HIF-1α translationally regulated P2X2 expression in DRN^{5-HT} neurons, and P2X2 was specifically downregulated in the DRN of patients with MDD compared to their matched control subjects. These results suggest that HIF-1α-P2X2 signaling in the DRN^{5-HT} neurons could be involved in the pathophysiology of MDD.

P2X receptors are membrane ion channels permeable to sodium, potassium and calcium that open upon extracellular ATP binding [50]. P2X receptors have a widespread tissue distribution. In the brain, P2X2 receptors play a key role in synaptic transmission, as shown by recent studies: ATP regulates GABAergic synaptic transmission through P2X2 receptors [51], while P2X2 knockdown in mPFC impairs GABAergic neurotransmission [52]. P2X2 receptors also regulate excitatory synaptic transmission in mPFC, in a social stress paradigm [53], and P2X2/P2X4 receptors have slow and long-lasting modulatory effects on the function or surface trafficking of receptors [54]. In addition, activation of post-synaptic P2X receptors by ATP released from glia has been shown to trigger changes in the surface trafficking of AMPA receptors, which leads to long-lasting changes in synaptic efficacy at glutamatergic synapses [55]. Also, previous studies have linked P2X2 receptors to depression [20,22,53,56]. In the present study, however, we found that P2X2 receptors in DRN^{5-HT} neurons are crucial for the sustained antidepressant-like effect of IHHT and ketamine. Both IHHT and ketamine significantly increase P2X2 expression in the DRN, which may result in an ATP-evoked calcium entry into DRN^{5-HT} neurons, thus modulating 5-HT release in their target brain areas, such as mPFC. P2X2 receptors are highly expressed in the DRN of human, but their protein levels remain relative lower in BA9, indicating that P2X2 receptors may be located on the terminal of DRN^{5-HT} neurons projecting to the mPFC. This result is consistent with the findings of a previous report, which detected P2X receptors in presynaptic nerve terminals [57]. The increased P2X2 expression on presynaptic terminals may increase the calcium concentration and rapidly elevate the synaptic concentration of 5-HT, thus reversing the proposed serotonergic neurotransmission deficit in depression and leading to a rapid and sustained antidepressant-like effect. Supporting this hypothesis, we found that $CoCl_2$ infusion into DRN ameliorated the deficit of glutamatergic neurotransmission in mPFC induced by CMS and resulted in rapid and sustained antidepressant phenotypes in FST and CMS without affecting the local neural circuit. Previous study showed that hypoxia induced the expression of HIF-1α [29] and P2 receptor blockade in the ventrolateral medulla augments hypoxia-induced secondary slowing of the respiratory rhythm in rats [58], suggesting a link between HIF-1α and P2X2. In present work, we provided several lines of evidence to show that P2X2 is a downstream factor of HIF-1α following IHHT. First, IHHT induced an overexpression of *Hif1a* in the DRN as early as at 3rd day of IHHT, while the P2X2 protein level increased at 21st and 98th day, indicating that both HIF-1α and P2X2 time-dependently accumulated in the DRN. Second, pharmacologically overexpressing HIF-1α time-dependently increased P2X2 protein levels. Moreover, genetically overexpression of HIF-1α increased the expression of P2X2, while knockdown of HIF-1α lowered P2X2 levels in the DRN. Third, knockdown of HIF-1α blocked the antidepressant-like effect of IHHT, while knockdown of P2X2 only attenuated the long-lasting antidepressant effect of IHHT and $CoCl_2$, not their acute effect. And fourth, our *in vitro* experiment showed that HIF-1α translationally regulated the expression of P2X2. Together, these results suggest that HIF-1α-P2X2 signaling is essential for IHHT promoting psychologic resilience.

The mammalian target of rapamycin (mTOR) signaling can be activated by ketamine [59], and eukaryotic initiation factor 4E-binding

proteins (4E-BPs) are pivotal mTORC1 effectors, controlling mRNA translation. The antidepressant-like effect of ketamine is mediated by 4E-BP2 in excitatory neurons and by 4E-BP1 and 4E-BP2 in inhibitory neurons [60]. Moreover, ketamine can deactivate eukaryotic elongation factor 2 (eEF2), which is a critical catalytic factor for ribosomal translocation during protein synthesis, decreasing eEF2 phosphorylation and reversing the translation suppression of brain-derived neurotrophic factor, which is required for its antidepressant-like effect [40]. These results link protein translation to a rapid antidepressant effect. In the present study, we found that HIF-1α translationally regulates the expression of P2X2, which mediates the effect of IHHT enhancing psychological resilience. Thus, these results suggest that protein translation could be involved in the neurobiological mechanism promoting resilience. Because the *P2RX2* gene is highly conserved during evolution, and human and mouse P2X2 genes show ~77 % identity at DNA and protein levels [61], further studies should be conducted to understand how P2X2, a new downstream player of HIF-1α, was regulated in DRN^{5-HT} neurons, especially in respond to stress.

Hypobaric hypoxia at high altitude entails some risks, such as those associated with pulmonary hypertension, reproductive hazards, and increased erythropoiesis [62]. Studies have shown that uncomfortable "low oxygen doses" can promote tumor metastasis [63], raise blood pressure and cholesterol levels, increase atherosclerosis, impair spatial memory, and increase inflammatory responses. In addition, hypoxia can increase the weight coefficient of the heart, liver, and lung and plasma estrogen levels, decrease estrogen receptor and progesterone receptor mRNA levels, damage ovarian and uterine tissue structure, and cause estrus disturbance [64–70]. However, we and others have demonstrated that IHHT, at an altitude of 5000 m, for 14 days, 4 h per day, did not induce neuronal death in the hippocampus [15,16], and that IHHT, at an altitude of 3000 m, for 28 days, 6-h per day, rescues deficits in spatial and object memory and in synaptic plasticity, in pilocarpine-treated epileptic rats [71]. However, we cannot exclude the acute neurotoxic effect of our IHHT paradigm, because we found that IHHT significantly increased the HIF-1α expression in the DRN of mice 3 days after IHHT, while no significant difference was observed in immobility in the FST between IHHT and control groups on the 3rd day of IHHT. This inconsistency could be due to the reversable neurotoxicity of IHHT [72]. Therefore, optimizing IHHT regimens and evaluating their safety are prerequisites for the clinical application of IHHT.

In summary, IHHT promoted psychological resilience in adult mice, and this effect was mediated by the enhancement of HIF-1α-P2X2 signaling in DRN^{5-HT} neurons. Ketamine, which produced a rapid and long-lasting antidepressant effect, increased HIF-1α-P2X2 signaling in DRN^{5-HT} neurons. Using postmortem brain samples, we found that P2X2 signaling is disrupted in the DRN of patients with MDD. These results support a notion that IHHT could be developed as a physical treatment for MDD and highlight the potential of enhancing HIF-1α–P2X2 signaling in DRN^{5-HT} neurons as an avenue for screening novel therapeutic treatments with a sustained effect on MDD.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper or the supplementary information.

Author contributions

X.–H.Z. designed the study. X.–H.Z., Y.Z., and Y.–D.P. analyzed the data and wrote the manuscript. Y.Z., Y.–D.P., W.–Y. Z., H.-Y.L., and M.-Z.Z. conducted the behavioral experiments. Y.Z. and Y.–D.P. performed the qRT-PCR and western blot. Y.Z. performed cell culture. Y.Z., Y.–D.P., W.–J. OY. and Y.Q. bred the mice and performed the genotyping. G.T. and N. M. characterized and prepared the postmortem brain samples. All authors read and contributed to the final version of the manuscript.

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Declaration of competing interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2023.103005.

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