Supplementary methods

Tissue sample preparation

The rats were decapitated, and the brains were extracted based on our previous study. Subsequently, bilateral tissue punches of the prefrontal cortex (PFC; 16 gauge) were obtained from about 1 mm thick coronal sections cut in a Reichert-Jung 2800 Frigocut E cryostat at −20ºC. The rostral faces of the coronal sections were about 3.8 mm from the bregma. The tissue punches were homogenized (10–15 s × 3.5 s interval) with an electrical disperser after being lysed with RIPA lysis buffer (Beyotime Biotechnology) for 30 minutes. Afterward, the homogenate was subjected to 10 000 g centrifugation at 4ºC for 20 minutes. All these procedures were performed under low temperature (0–4ºC). The protein concentrations of all samples were determined using the BCA assay kit (Beyotime Biotechnology). The protein concentration was normalized by diluting the samples with RIPA lysis buffer.

Western blot assays

The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide/0.27% N,N’-methylenebisacrylamide resolving gel) for about 30 minutes at 80 V in stacking gel and about 1 hour at 120 V in resolving gel. Proteins were transferred electrophoretically to Immobilon-P transfer membranes (Millipore) at 0.25 A for 3 hours. Membranes were washed with Tris-buffered saline (TBST plus 0.05% Tween-20, pH 7.4) before dipping in blocking buffer (5% skimmed dry milk in TBST) overnight at 4ºC. The membranes were then incubated for 1 hour at room temperature with anti-phospho-GSK3β antibody (1:1000; Cell Signaling Technology), anti-total-GSK3β antibody (1:1000; Cell Signaling Technology), anti-phospho-p70s6k antibody (1:500; Cell Signaling Technology), anti-total-p70s6k antibody (1:500; Cell Signaling Technology), anti-phospho-rps6 antibody (1:500; Cell Signaling Technology), anti-total-rps6 antibody (1:500; Cell Signaling Technology), anti-PSD95 antibody (1:1000; Cell Signaling Technology), and anti-β-actin antibody (1:2000; Cell Signaling Technology) in TBST plus 5% bovine serum albumin. After the membrane was shaken in 4 × 6 minute washes in TBST buffer, the blots were incubated for 45 minutes at room temperature with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or mouse IgG; Santa Cruz Biotechnology and Vector Laboratories, respectively) diluted 1:5000 in blocking buffer. The blots were then shaken in 4 × 6 minute washes in TBST. Afterward, the blots were incubated with a layer of Super Signal enhanced chemiluminescence substrate mixture (Pierce Biotechnology) for 1 minute at room temperature. Finally, the blots were exposed against radiograph film (Eastman Kodak Company). Band intensities were quantified using Quantity One software (version 4.0.3) from Bio-Rad Corporation.

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Intracerebral cannula implantation and intracranial injections

The rats were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal), and guide cannulae (23-gauge, Plastics One) were placed at a 23° angle toward the midline and implanted bilaterally 1 mm above the medial prefrontal cortex (mPFC) with the following stereotaxic coordinates: anterior/posterior (A/P) –3.2 mm; medial/lateral (M/L) ± 2.5 mm; dorsal/ventral (D/V) –3.3 mm. Vehicle, 7-CTKA or LY294002 was intracranially microinjected using 10 μl Hamilton syringes that were connected via polyethylene-50 tubing to 30-gauge injectors (Plastics One). A total volume of 0.5 μl was infused into each side over 1 minute, and the injection syringe was left in place for an additional 1 minute to allow for diffusion. At the end of the experiments, the rats were anesthetized with sodium pentobarbital (100 mg/kg, intraperitoneal) and transcardially perfused. Cannula placements were assessed using Nissl staining with a thickness of 30 μm under light microscopy. Subjects with misplaced cannulae were excluded from the statistical analysis.

Experimental design

Experiment 1: antidepressant-like effect of acute administration of 7-CTKA

Five groups (n = 8–9) of rats received 7-CTKA (0, 0.05, 0.1 and 1 mg/kg, intraperitoneal) or reference antidepressant venlafaxine (40 mg/kg, intraperitoneal). The doses of 7-CTKA were based on our pilot studies. Thirty minutes after drug injections, rats were exposed to the forced swim test (FST), and immobility time was recorded. In addition, a separate subset of rats was injected with 7-CTKA or venlafaxine for measurement of locomotor activity. Another 5 separate groups of rats treated with 7-CTKA (0, 0.05, 0.1 and 1 mg/kg) or venlafaxine (40 mg/kg) were subjected to the novelty-suppressed feeding test (NSFT), in which the latency to feed was determined. In addition, in the learned helplessness paradigm, rats were treated with 7-CTKA (0, 0.05, 0.1 and 1 mg/kg) or venlafaxine (40 mg/kg) 24 hours after shock training and then subjected to inescapable shock to measure the number of failures and latency to escape 24 hours later.

Experiment 2: effects of acute 7-CTKA treatment on depressive-like behaviour in the chronic mild stress (CMS) paradigm

The purpose of this experiment was to investigate whether acute administration of 7-CTKA reverses depressive-like behaviour induced by CMS. Three groups of rats (n = 7 per group) were subjected to CMS for 28 days and received vehicle, 7-CTKA (1 mg/kg, intraperitoneal) or ketamine (10 mg/kg, intraperitoneal) on day 21. Rats in the control group were handled daily without any stress (n = 7). The sucrose preference (SPT) and NSFT were performed on day 22 and day 23, respectively. On day 28, the SPT was conducted again to determine the long-lasting antidepressant-like effects of 7-CTKA. After the completion CMS, rats were decapitated, and their brains were extracted for the subsequent determination of phospho-GSK3β-Ser9 (p-GSK3β) and t-GSK3β in the mPFC and the hippocampus using Western blot. Moreover, the levels of PSD95, p-p70s6k and p-rps6 in the mPFC and the hippocampus were also detected to reflect the synaptic and mTOR function induced by CMS and 7-CTKA treatment.
Experiment 3: effect of acute systemic 7-CTKA treatment on GSK3β activity

To determine whether GSK3β activity is involved in the antidepressant effects of 7-CTKA, rats were acutely injected with 7-CTKA (0 and 1 mg/kg, intraperitoneal) and sacrificed 30 minutes or 1 hour after the drug injections, respectively. The brains were extracted and collected for the following measurement of p-GSK3 and t-GSK3β in the mPFC and the hippocampus using the Western blot assay (n = 6).

Experiment 4: antidepressant-like effect of acute intra-mPFC 7-CTKA administration

To directly determine the antidepressant-like effect of brain-specific administration of 7-CTKA, we used 4 separate groups of rats that received intra-mPFC 7-CTKA (0, 5, 10, 20 µg/side). The rats were subjected to the FST, open field test (OFT) and NSFT, respectively, 30 minutes after drug infusion. In the learned helplessness paradigm, the rats were microinjected with 7-CTKA (0, 5, 10 and 20 µg/side) into the mPFC 24 hours after shock training. The numbers of failures and latency to escape in the active avoidance test were determined 24 hours later. This concentration of 7-CTKA was chosen based on our previous study and other reports that found effective antagonism of the N-methyl-D-aspartate (NMDA) receptor.4,5

Experiment 5: effects of activation of GSK3β on the antidepressant-like effect of 7-CTKA

We then determined whether the antidepressant-like effect of 7-CTKA via inhibition of GSK3β could be blocked by GSK3β activation. LY294002, a specific PI3K inhibitor that activates GSK3β by reducing the phosphorylation of GSK3β (p-Ser9), was used to measure the effect of activated GSK3β on the antidepressant-like effect of 7-CTKA. Four groups of rats (n = 8–11) in a 2 (7-CTKA dose: 0 and 1 mg) × 2 (LY294002: 0 and 10 nM) factorial design were used in the FST. 7-CTKA or its vehicle was administered (intraperitoneal) 30 minutes before the FST. LY294002 or its vehicle was microinjected into the mPFC 30 minutes before 7-CTKA administration. The dose of LY294002 was selected according to prior studies that showed that this dose effectively activated GSK3β.6,7 To exclude the possibility that LY294002 and 7-CTKA induce nonspecific motor effects in the FST, the effects of LY294002 and 7-CTKA on locomotor activity were assessed 5 minutes before the FST. Immobility time in the FST was recorded.

Another 4 groups of rats (n = 8–11) in a 2 (7-CTKA dose: 0 and 1 mg) × 2 (LY294002: 0 and 10 nM) factorial design were used in the NSFT. 7-CTKA or its vehicle was administered (intraperitoneal) 30 minutes before the FST. LY294002 or its vehicle was microinjected into the mPFC 30 minutes before 7-CTKA administration. The latency to take the first bite of food and the weight of food consumed in the home cage 5 minutes after the NSFT were recorded.

Furthermore, a separate 8 groups of rats in a 2 (shock: no shock and inescapable shock) × 2 (7-CTKA: 0 and 1 mg/kg) × 2 (LY294002: 0 and 10 nM) factorial design were used in the learned helplessness test. Four groups of rats were subjected to shock training (i.e., inescapable shock). Another 4 groups of rats were subjected to the chambers but received no footshock. 7-CTKA (1 mg/kg, intraperitoneal) was administered 24 hours after shock training. LY294002 or its vehicle was microinjected into the mPFC 30 minutes before 7-CTKA administration. The rats were then subjected to inescapable shock, and the numbers of failures and latency to escape 24 hours after 7-CTKA treatment were assessed.
In addition, we used 4 groups of rats (n = 8) in a 2 (7-CTKA dose: 0 and 1 mg/kg) × 2 (rapamycin: 0 and 50 μg) factorial design in the FST to determine whether the antidepressant-like effect of 7-CTKA could be reversed by mTOR inhibition. 7-CTKA or its vehicle was administered (intraperitoneal) 30 minutes before the FST. Rapamycin, mTOR inhibitor, or its vehicle was microinjected into the mPFC 30 minutes before 7-CTKA administration. A separate 4 groups of rats in a 2 (7-CTKA: 0 and 1 mg/kg) × 2 (D-serine: 0 and 50 μg) factorial design were used in the FST to investigate the effect of D-serine, an agonist at glycine site of the NMDA receptor, on the antidepressant action of 7-CTKA (n = 8). 7-CTKA or its vehicle was administered (intraperitoneal) 30 minutes before the FST. D-serine or its vehicle was microinjected into the mPFC 30 minutes before 7-CTKA administration. Immobility time in the FST was recorded.

Experiment 6: rewarding effect of 7-CTKA measured by conditioned place preference
In this experiment, we determined whether systemic 7-CTKA (1 mg/kg) treatment has abuse potential at the effective dose of antidepressant actions. Three groups of rats (n = 6–7 per group) were injected with vehicle, 7-CTKA (1 mg/kg, intraperitoneal) or ketamine (10 mg/kg, intraperitoneal), respectively, and trained for conditioned place preference for 8 days and tested for the expression of conditioned place preference on day 9.

References