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N-Methyl-D-aspartate receptor antagonist *d*-methadone produces rapid, mTORC1-dependent antidepressant effects

Manoela V. Fogaça¹, Kenichi Fukumoto¹, Tina Franklin¹, Rong-Jian Liu¹, Catharine H. Duman¹, Ottavio V. Vitolo² and Ronald S. Duman¹

Currently available antidepressants have a delayed onset and limited efficacy, highlighting the need for new, rapid and more efficacious agents. Ketamine, an NMDA receptor antagonist, has emerged as a new rapid-acting antidepressant, effective even in treatment resistant patients. However, ketamine induces undesired psychotomimetic and dissociative side effects that limit its clinical use. The *d*-stereoisomer of methadone (dextromethadone; REL-1017) is a noncompetitive NMDA receptor antagonist with an apparently favorable safety and tolerability profile. The current study examined the rapid and sustained antidepressant actions of *d*-methadone in several behavioral paradigms, as well as on mTORC1 signaling and synaptic changes in the medial prefrontal cortex (mPFC). A single dose of *d*-methadone promoted rapid and sustained antidepressant responses in the novelty-suppressed feeding test (NSFT), a measure of anxiety, and in the female urine sniffing test (FUST), a measure of motivation and reward. *D*-methadone also produced a rapid reversal of the sucrose preference deficit, a measure of anhedonia, in rats exposed to chronic unpredictable stress. *D*-methadone increased phospho-p70S6 kinase, a downstream target of mTORC1 in the mPFC, and intra-mPFC infusion of the selective mTORC1 inhibitor rapamycin blocked the antidepressant actions of *d*-methadone in the FUST and NSFT. *D*-methadone administration also increased levels of the synaptic proteins, PSD95, GluA1, and Synapsin 1 and enhanced synaptic function in the mPFC. Studies in primary cortical cultures show that *d*-methadone also increases BDNF release, as well as phospho-p70S6 kinase. These findings indicate that *d*-methadone induces rapid antidepressant actions through mTORC1-mediated synaptic plasticity in the mPFC similar to ketamine.

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INTRODUCTION

Major depressive disorder (MDD) is a debilitating illness that affects ~17% of the US population and is estimated to be the second leading cause of disability worldwide by 2020 [1, 2]. Approximately a third of MDD patients do not respond adequately to currently available monoamine reuptake inhibitor medications. Moreover, these drugs require several weeks to produce an antidepressant response, a significant limitation for patients experiencing personal suffering and at risk of suicide [3]. These issues highlight the urgent need for new drugs that address the limitations of currently available pharmacological agents (i.e., relatively low efficacy and time lag for therapeutic response).

The discovery that a single dose of ketamine, an N-methyl-D-aspartate (NMDA) receptor antagonist, produces rapid antidepressant effects (within hours) in MDD subjects, including those considered treatment resistant, represents a major advance and a novel therapeutic target [4, 5]. Previous work indicates that ketamine rapidly enhances the function of glutamatergic synapses in the brain, notably the medial prefrontal cortex (mPFC), promoting post-synaptic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA)-mediated calcium influx and release of brain-derived neurotrophic factor (BDNF) by principal pyramidal neurons [6–8]. Extracellular BDNF, in turn, binds to tropomyosin receptor kinase B (TrkB) at the post-synaptic membrane and

activates mammalian target of rapamycin complex 1 (mTORC1) signaling that is involved in protein synthesis and synaptogenesis [9–12]. Ketamine produces a rapid (30 min) and transient (back to baseline by 2 h) activation of mTORC1 signaling, but a sustained increase in synaptic number and function (2 h to 7 days) that is associated with the rapid and sustained antidepressant actions of ketamine [10, 13].

Although ketamine has the potential to revolutionize the treatment of MDD, it also has significant side effects, including dissociative and psychotomimetic actions, as well as abuse potential that limit its clinical use [14]. In this regard, drugs that act as NMDA channel blockers but lack ketamine-like side effects are promising therapeutic candidates to treat MDD. A novel compound that is in current development is the *d*-stereoisomer of methadone (*d*-methadone or dextromethadone). Methadone is a synthetic racemic mixture that is available for clinical use to treat moderate to severe pain and opioid dependence due to its μ -opioid receptor agonism [15]. Both *d*- and *l*-isomers bind non-competitively to the MK-801-labeled site of the NMDAR with low micromolar IC₅₀ values similar to that of ketamine, memantine, and dextromethorphan, known NMDAR antagonists [15]. *D*-methadone has similar, low micromolar affinity at the different GluN2 subunits (2A–2D) of the NMDA receptor, with slightly higher affinity for the GluN2B subunit [16]. However, studies have shown

¹Department of Psychiatry, Yale University School of Medicine, 34 Park Street, New Haven, CT 06520, USA and ²Relmada Therapeutics Inc., 880 Third Ave, 12th floor, New York, NY 10022, USA

Correspondence: Ronald S. Duman (ronald.duman@yale.edu)

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that *d*-methadone has 10–30-fold lower affinity for the μ and δ -opioid receptor subtypes compared with *l*-methadone [17, 18] and does not produce typical opioid-induced effects in humans at doses predicted to exert antidepressant activity [15]. On the contrary, the *l*-isomer accounts for μ -opioid effects of methadone [16–20]. *D*-methadone (REL-1017) is currently in clinical development in a Phase 2a study for the treatment of individuals with depression who have not responded to traditional antidepressants (NCT03051256).

Two Phase 1 studies, a single ascending dose (SAD) study and a multiple ascending dose (MAD) study, showed that *d*-methadone is well tolerated in humans and does not induce dissociative or psychotomimetic adverse events that are observed with ketamine [15]. In addition, a preliminary preclinical study showed that a single dose (10–40 mg/kg) of *d*-methadone induces an antidepressant-like effect in the rat forced swim test (FST), similar to ketamine [21]. The current study extends this work by investigating the effects of *d*-methadone in different rodent models of depression and antidepressant response, mTORC1 signaling, and synaptic number and function in rat mPFC. Our findings highlight the participation of fast cortical mTORC1 signaling and synaptic changes in the antidepressant actions of *d*-methadone. Moreover, the selected behavioral readouts are associated with distinct brain circuits and behavioral domains affected in depression (e.g., anhedonia and reward circuit; anxiety and fear circuit), thus increasing the translational relevance of our findings.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (260–300 g) from Jackson Laboratories (New Haven, USA) were paired-housed at the Ribicoff Facility (Connecticut Mental Health Center, USA) in a temperature-controlled room ($23 \pm 2^\circ\text{C}$) with a 12/12-h light–dark cycle (lights on: 7:00 a.m./lights off: 7:00 p.m.). Animals were single housed 3–5 days prior the drug treatments. Rats received food and water *ad libitum* throughout the study period, except when food or water deprivation was used as stressors in the CUS or when animals were submitted to the novelty-suppressed feeding test (NSFT). Procedures were conducted in compliance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals and were approved by the Yale Animal Care and Ethics Committee.

Drug administration

D-methadone (Mallinckrodt, 20 mg/kg) was administered subcutaneously (s.c.) and ketamine (Ketaset, 10 mg/kg) intraperitoneally (i.p.). Both drugs were diluted in 0.9% sterile saline. Rapamycin (Cell Signaling, 10 nmol, diluted in 10% DMSO) was administered bilaterally intra-mPFC, 30 min before *d*-methadone, with the help of an infusion pump (0.2 $\mu\text{L}/\text{side}$; 0.1 $\mu\text{L}/\text{min}$).

Stereotaxic surgery and cannula placement

Animals were submitted to stereotaxic surgery to implant bilateral cannulae into the mPFC (coordinates: anteroposterior: +1.9 mm; lateral: ± 0.4 mm; depth: -2.7 mm) as previously described [10]. After the behavioral tests, animals were perfused and cannula placement was verified [10].

Chronic unpredictable stress (CUS)

Animals were exposed to a sequence of two random unpredictable stressors per day for 21 days, accordingly to a previous established protocol [22]. At the end of the procedure, stressed animals were divided into three groups and received vehicle, *d*-methadone or ketamine. Control animals (non-stressed vehicle) were handled daily but were not submitted to any stressor.

Behavior studies

Sucrose preference test. At day 18 of CUS the animals were habituated to a palatable solution of 1% sucrose for 48 h, during which the position of the bottle was counterbalanced across days. On the day of the test, rats were water deprived for 6 h and then presented with pre-weighed identical bottles of 1% sucrose and water for 1 h. Sucrose and water consumption were determined by measuring the change in the volume weight of fluid consumed. Sucrose preference was calculated as the ratio of the volume of sucrose versus total volume of sucrose and water consumed during the test.

Female urine sniffing test (FUST). Animals were habituated to the presence of a cotton-tipped applicator in the home cage for 60 min as previously described [23]. Then, each animal was exposed for 5 min to a cotton-tipped applicator infused with water and, 45 later, with fresh urine collected from female rats (9–14-week-old). The test was video recorded and the total time spent sniffing the cotton-tipped applicator, water or urine was measured.

Novelty suppressed feeding test (NSFT). Animals were food deprived for 18 h and placed in a red-light room containing an open field ($76.5 \times 76.5 \times 40$ cm, Plexiglas) with one pellet of food in the center. The latency to feed was measured up to 15 min. Home cage food intake was measured right after the test during 20 min.

Locomotor activity (LMA). The animals were placed in clean home cages with no bedding and activity determined by an infrared automated tracking system and recorded as beam breaks (Med Associates) during 30 min.

Western blot

For brain tissue collection, rats were decapitated and the mPFC and whole hippocampus removed. Tissues were homogenized to protein extraction of the synaptosome fraction as previously described [7, 10]. For primary cortical culture, cells were scrubbed from the wells, collected into RIPA buffer and sonicated. Homogenates were submitted to western blot analysis and the bands quantified as previously described [7, 10] (primary antibodies: rabbit anti-phospho-p70S6K, rabbit anti-p70S6K, rabbit anti-phospho-p44-42 ERK, rabbit anti-p44-42 ERK, rabbit anti-phospho-4EBP1, rabbit anti-phospho-mTOR, rabbit anti-mTOR, rabbit anti-GluA1, rabbit anti-Synapsin1, rabbit anti-PSD95, 1:1000, Cell Signaling. Secondary antibody: anti-rabbit, 1:5000, Vector Laboratories). Total levels of the respective protein or GAPDH (Cell Signaling, 1:5000) were used for loading control and normalization.

Primary cortical culture and BDNF analysis

Primary cortical cultures and BDNF enzyme-linked immunosorbent assay (ELISA) were performed and analyzed as previously described [7]. After 10–12 days, cells were incubated with vehicle (0.9% saline) or *d*-methadone (1–500 nM) for 1 h. For BDNF analysis, medium containing an anti-BDNF antibody (Millipore, #AB1779SP) was collected followed by immunoprecipitation [7]. On the next day, an ELISA assay (BDNF-ELISA E-max; Promega, WI) was performed to detect BDNF levels accordingly to the manufacturer's recommendation.

Electrophysiological recordings

Brain slices were prepared as previously described [10]. Neurobiotin (0.3%) was added to the pipette solution to mark cells for later spines imaging. Pyramidal neurons in mPFC were visualized by videomicroscopy using microscope ($\times 40$ IR lens) with infrared differential interference contrast (IR/DIC). Whole-cell recordings were performed with an Axoclamp-2B amplifier. Postsynaptic

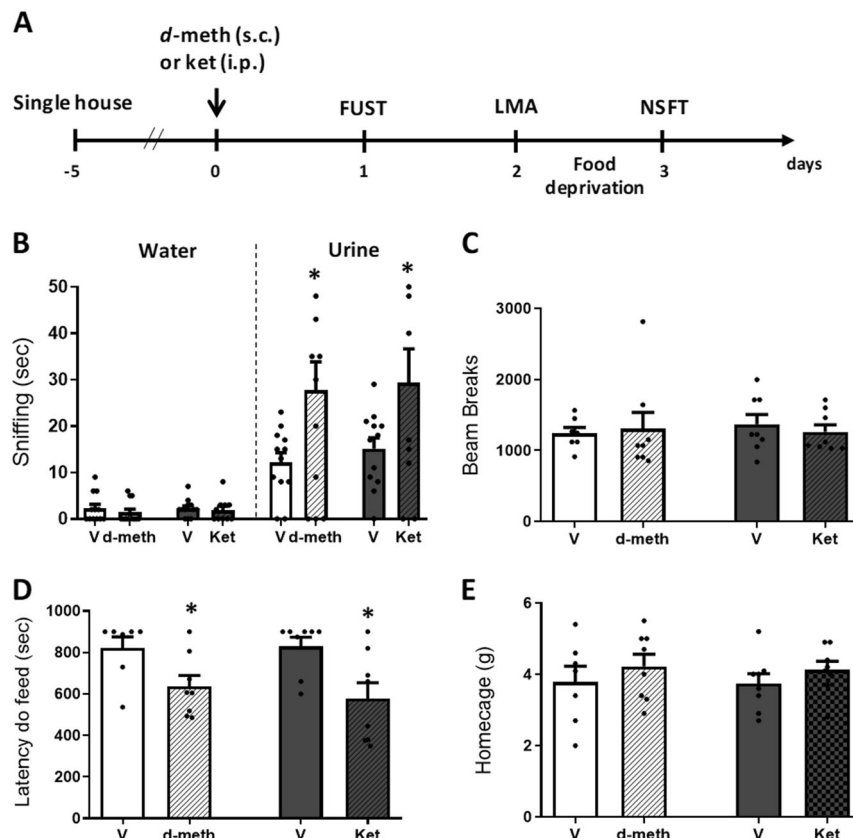


Fig. 1 *D*-methadone induces rapid and sustained antidepressant effects. **a** Experimental time course for *d*-methadone/ketamine injections and the behavioral tests. **b** A single injection of *d*-methadone (20 mg/kg, s.c.) or ketamine (10 mg/kg, i.p.) increased time in the female urine sniffing test (FUST) 24 h after administration (two-way ANOVA followed by Duncan test, effect of drug treatment: $F_{1,88} = 8.89$, $p < 0.05$; effect of water/urine: $F_{1,88} = 64.19$, $p < 0.05$; interaction: $F_{1,88} = 10.50$, $p < 0.05$). **c** There was no effect of either *d*-methadone or ketamine on beam breaks to measure locomotor activity (LMA, one-way ANOVA, $F_{3,27} = 0.13$, $p > 0.05$). **d** Administration of *d*-methadone or ketamine also decreased the latency to feed in the novelty-suppressed feeding test (NSFT, 72 h after dosing; one-way ANOVA followed by Duncan test, $F_{3,26} = 5.29$, $p < 0.05$). **e** There was no effect of either drug on home cage food consumption ($F_{3,26} = 0.56$, $p > 0.05$). Each bar represents the mean \pm standard error of the mean (S.E.M.). * $p < 0.05$ compared with the vehicle groups. $n = 7$ –12/group

currents were studied in the continuous single-electrode voltage-clamp mode (3000 Hz low-pass filter) clamped -65 mV to separate the IPSCs from the EPSCs.

Spine analysis

Neurobiotin-filled mPFC layer V neurons were imaged and spine density was determined as previously described [24]. Due to damage to the cell body during withdrawal of recording pipette and/or incomplete filling, not all recorded cells can be used for morphological analysis. Images were collected on an Olympus confocal laser scanning microscope (FV3000) equipped with a $\times 60$, 1.42 NA objective at a zoom of $4.65 \times$ (XY pixel dimensions 0.095×0.095 mm). Dendrites were sampled within the apical dendritic tuft at sites distal, midway and proximal to distal bifurcation (sampled 60, 90, and 130 μ m from the midline, respectively), and values for spine density were expressed per μ m dendritic length (average dendritic segment, 40–50 μ m). Mean spine density for each sampling location as well as overall mean spine density were calculated for each labeled cell. Computerized analysis of z-stack images was performed in deconvolved confocal image stacks. Spines number and head diameter were quantified by an experimenter blinded to treatment, using NeuronStudio software [25].

Statistical analysis

The results were analyzed by Student's *t*-test, one-, two-, or three-way ANOVA, as appropriate, followed by the Duncan test. For

cumulative distributions it was used the non-parametric Kolmogorov–Smirnov test. Results were expressed as means \pm standard errors, and the significance level was set at $p \leq 0.05$. For all analysis, we used the SPSS Software (version 20.0).

RESULTS

D-methadone induces rapid and sustained antidepressant effects, similar to ketamine

A recent study reports that *d*-methadone (REL-1017) induces dose dependent antidepressant-like effects in the FST at doses of 10, 20, and 40 mg/kg, with effects similar to ketamine [21]. Based on this information and pharmacokinetic simulations that assume equipotency between ketamine 10 mg/kg and the low and intermediate doses of *d*-methadone, as well as safety and tolerability data from the Phase 1 trials, doses were chosen for the ongoing Phase 2 study in depression. For the current study, we chose a dose of 20 mg/kg to conduct additional behavioral tests to verify the antidepressant actions of *d*-methadone, including the FUST, a measure of motivation and reward, and the NSFT, a measure of anxiety that is responsive to chronic administration of typical monoaminergic antidepressants (Fig. 1a). Ketamine was used as a positive control due to its established rapid antidepressant effects in preclinical models. The dose of ketamine used in this study has been widely adopted to study antidepressant effects in rats. In fact, it improves the performance in the FST test without affecting locomotor activity [26] and

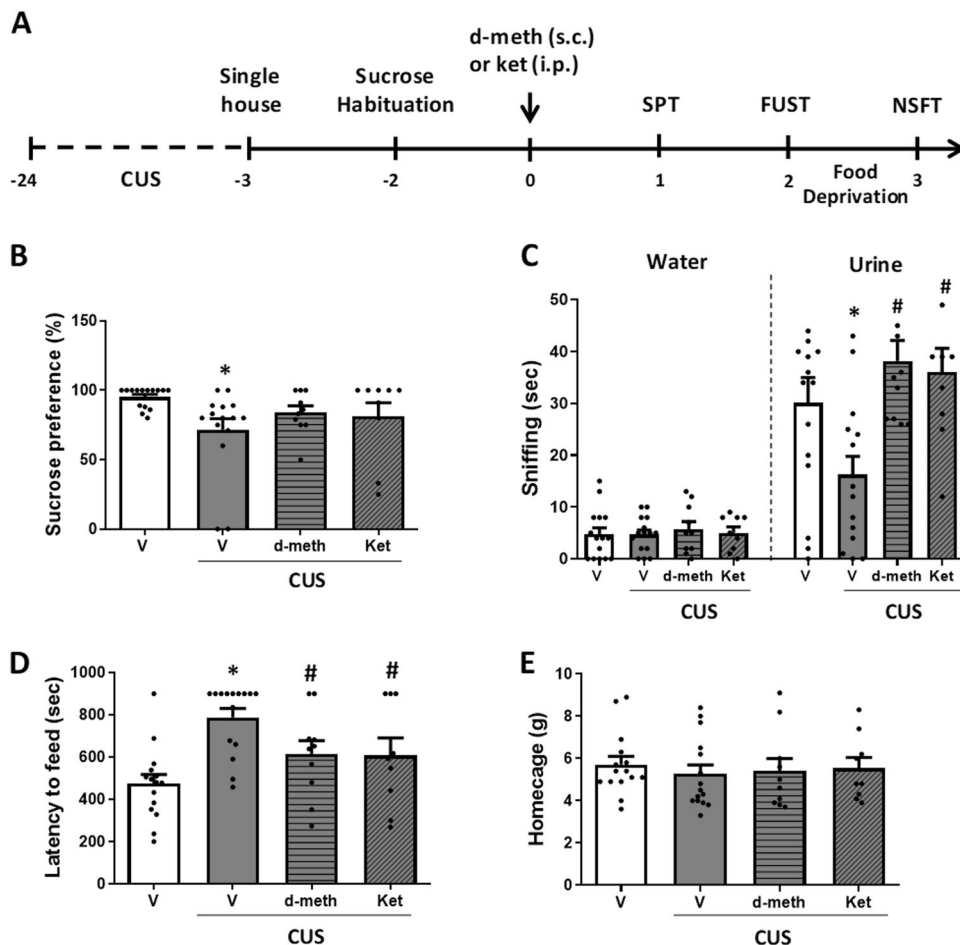


Fig. 2 *D*-methadone rapidly reverses the pro-depressive behaviors induced by chronic unpredictable stress (CUS). **a** Experimental time course for CUS, drug injection and behavioral tests. **b** CUS decreased sucrose preference (one-way ANOVA followed by Duncan test, $F_{3,45} = 2.99$, $p < 0.05$), **c** decreased female urine sniffing time ($F_{3,45} = 5.19$, $p < 0.05$), and **d** increased the latency to feed in the novelty-suppressed feeding test ($F_{3,45} = 6.67$, $p < 0.05$). These effects were reversed by a single injection of *d*-methadone (s.c., 20 mg/kg) or ketamine (i.p., 10 mg/kg). The behavioral tests were conducted 24, 48, and 72 h after dosing as indicated. There was no effect of CUS or drug dosing on (c) time sniffing water ($F_{3,45} = 0.15$, $p > 0.05$) or **e** home cage food consumption ($F_{3,45} = 0.19$, $p > 0.05$). Each bar represents the mean \pm standard error of the mean (S.E.M.). * $p < 0.05$ compared with the non-stressed vehicle group; # $p < 0.05$ compared to the stressed vehicle group. $n = 9$ –15/group

induces mTORC1-dependent synaptic formation [10]. *D*-methadone (20 mg/kg, s.c.) and ketamine (10 mg/kg, i.p.) produced antidepressant effects in the FUST, increasing time sniffing female urine, tested 24 h after drug (Fig. 1b). Both *d*-methadone and ketamine also produced antidepressant/anti-anxiety actions in the NSFT, decreasing latency to feed in an open field, measured 72 h after drug administration (Fig. 1d). There were no effects of either drug on time sniffing water (Fig. 1b), locomotor activity (Fig. 1c) or home cage food consumption (Fig. 1e).

D-methadone reverses the depressive-like effects caused by CUS, similar to ketamine

Next, we tested the ability of *d*-methadone to reverse the depressive-like effects of CUS, a validated model that produces anhedonia, a core symptom of depression that can be measured by preference for a sweetened solution [27]. The CUS-anhedonia model is a rigorous test for rapid-acting agents because a response in this test to typical monoaminergic agents requires 2–3 weeks of dosing [28]. Rats were exposed to a sequence of random stressors for 21 days, then received injections of ketamine or *d*-methadone on day 22, and then were subjected to behavioral testing on day 23 in the SPT (Fig. 2a). CUS exposure decreased sucrose preference in vehicle-treated animals, and this effect was attenuated, but not completely abolished, by a single dose of

either *d*-methadone or ketamine (Fig. 2b). Considering that the vehicle used for *d*-methadone and ketamine is the same (0.9% saline) and there was no statistical difference between the routes of administration (i.e., i.p. \times s.c.), and to avoid excessive/unnecessary number of animals, we combined *d*-methadone and ketamine vehicle-treated animals in the same group (half of vehicle-treated animals received saline i.p. and the other half s.c.). CUS also decreased the female urine sniffing time in the FUST, and this effect was also reversed by previous administration (48 h) of *d*-methadone or ketamine (Fig. 2c). Finally, CUS increased the latency to feed in the NSFT and this effect was reversed by prior treatment (72 h) with *d*-methadone or ketamine, indicating sustained effects up to 3 days after drug dosing (Fig. 2d). There were no effects on water sniffing time (Fig. 2c) or home food cage consumption (Fig. 2e).

D-methadone increases mTORC1-p70S6K signaling in the mPFC and this pathway is required for the antidepressant-like effects of *d*-methadone

Previous studies report that rapid-acting antidepressants, such as ketamine, enhance synaptic function through stimulation of mTORC1 signaling in the mPFC, which includes increased phosphorylation of p70S6K and increased synthesis of synaptic proteins [8, 10, 29]. Here, we collected mPFC 24 h after

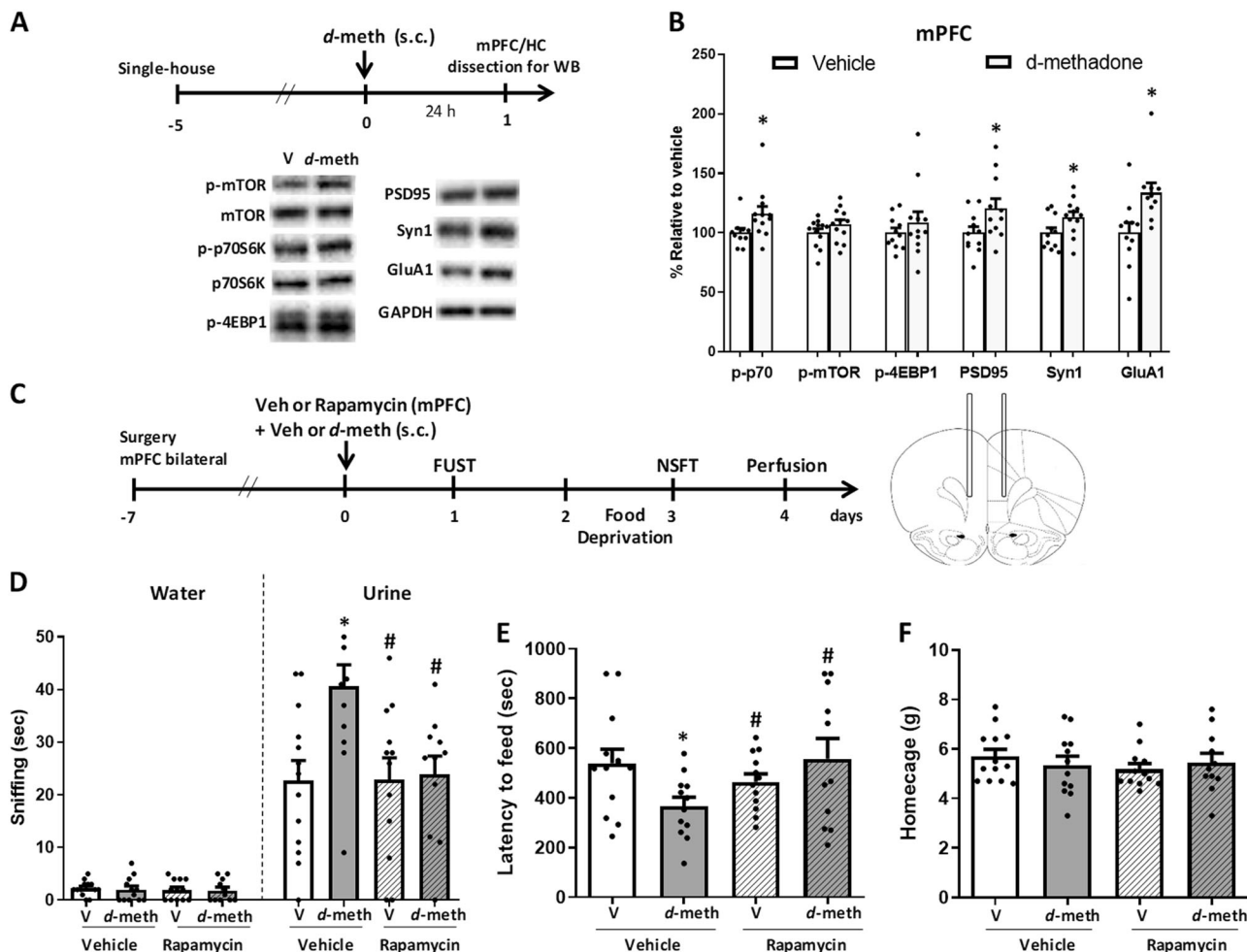


Fig. 3 The rapid antidepressant effects of *d*-methadone are mediated by mTORC1 signaling in the mPFC. **a** Experimental time course for *d*-methadone injection and brain tissues collection. **b** *D*-methadone (*s.c.*, 20 mg/kg) increased the expression of p-p70S6K (Student *t*-test, $t_{20} = 2.08, p < 0.05$), PSD95 ($t_{20} = 2.14, p < 0.05$), Synapsin 1 ($t_{20} = 2.17, p < 0.05$) and GluA1 ($t_{19} = 2.82, p < 0.05$) 24 h after drug administration. No effect was found for p-mTOR ($t_{20} = 1.29, p > 0.05$) and p-4EBP1 ($t_{20} = 0.89, p > 0.05$). **c** Experimental time course for surgery, drug injection and behavioral tests. Intra-mPFC infusions of rapamycin (10 nmol, 0.2 μ L/side) abolished the antidepressant effects of *d*-methadone (*s.c.*, 20 mg/kg) in the **d** FUST (three-way ANOVA followed by Duncan test, effect of treatment 1, vehicle/rapamycin: $F_{1,88} = 4.54, p < 0.05$; effect of treatment 2, vehicle/*d*-methadone: $F_{1,88} = 5.33, p < 0.05$; effect of water/urine: $F_{1,88} = 161.11, p < 0.05$; interaction treatment 1*treatment 2*water/urine: $F_{1,88} = 4.50, p < 0.05$), and **e** NSFT (two-way ANOVA followed by Duncan test, effect of treatment 1, vehicle/rapamycin: $F_{1,44} = 1.14, p > 0.05$; effect of treatment 2, vehicle/*d*-methadone: $F_{1,44} = 0.53, p > 0.05$; interaction treatment 1*treatment 2: $F_{1,44} = 5.98, p < 0.05$). There was no effect on **d** time sniffing water or **f** home cage food consumption (one-way ANOVA, $F_{3,44} = 0.49, p > 0.05$). Each bar represents the mean \pm standard error of the mean (S.E.M.). * $p < 0.05$ compared with the vehicle group; # $p < 0.05$ compared with the *d*-methadone group. $n = 10$ –13/group

administration of *d*-methadone for western blot analysis (Fig. 3a). *D*-methadone administration significantly increased the phosphorylation of p70S6K as well as levels of the synaptic proteins PSD95, Synapsin 1, and GluA1 (Fig. 3b). *D*-methadone did not significantly influence levels of phospho-mTOR or phospho-4EBP1 at the time point analyzed (Fig. 3b). Previous studies have reported a role for hippocampus in the actions of ketamine [30, 31], so this region was also examined. Although there was a trend for increased Synapsin 1 expression in the hippocampus, *d*-methadone did not alter the expression of synaptic proteins in this brain region (Fig. S1).

Since our results showed that *d*-methadone activates mTORC1 signaling in the mPFC (represented by an increase in phospho-p70S6K levels), we conducted additional experiments to determine if the antidepressant effects of *d*-methadone required mTORC1 signaling. For this, animals received microinjections of rapamycin, a selective mTORC1 inhibitor, into the mPFC 30 min prior to administration of *d*-methadone (*s.c.*) (Fig. 3c).

D-methadone increased female urine sniffing time in the FUST 24 h after administration, and this antidepressant response was blocked by infusion of rapamycin (Fig. 3d). Rapamycin infusion also prevented the anxiolytic effect of *d*-methadone in the NSFT measured 72 h after drug administration (Fig. 3e). There were no effects on time sniffing water (Fig. 3d) or home cage food consumption (Fig. 3f).

Influence of *d*-methadone on synapse number and function of mPFC layer V pyramidal neurons
 NMDA receptor blockers, such as ketamine, rapidly enhance the number and function of spine synapses on layer V pyramidal neurons in the mPFC [10, 13, 32, 33]. Here we conducted whole-cell patch clamp studies to evaluate the influence of *d*-methadone on 5-HT-, hypocretin-, and NMDA-induced EPSC frequency and amplitude, as well as on IPSC frequency 24 h after a single injection. *D*-methadone increased the frequency and amplitude of 5-HT- and NMDA-induced EPSCs in comparison to vehicle-treated

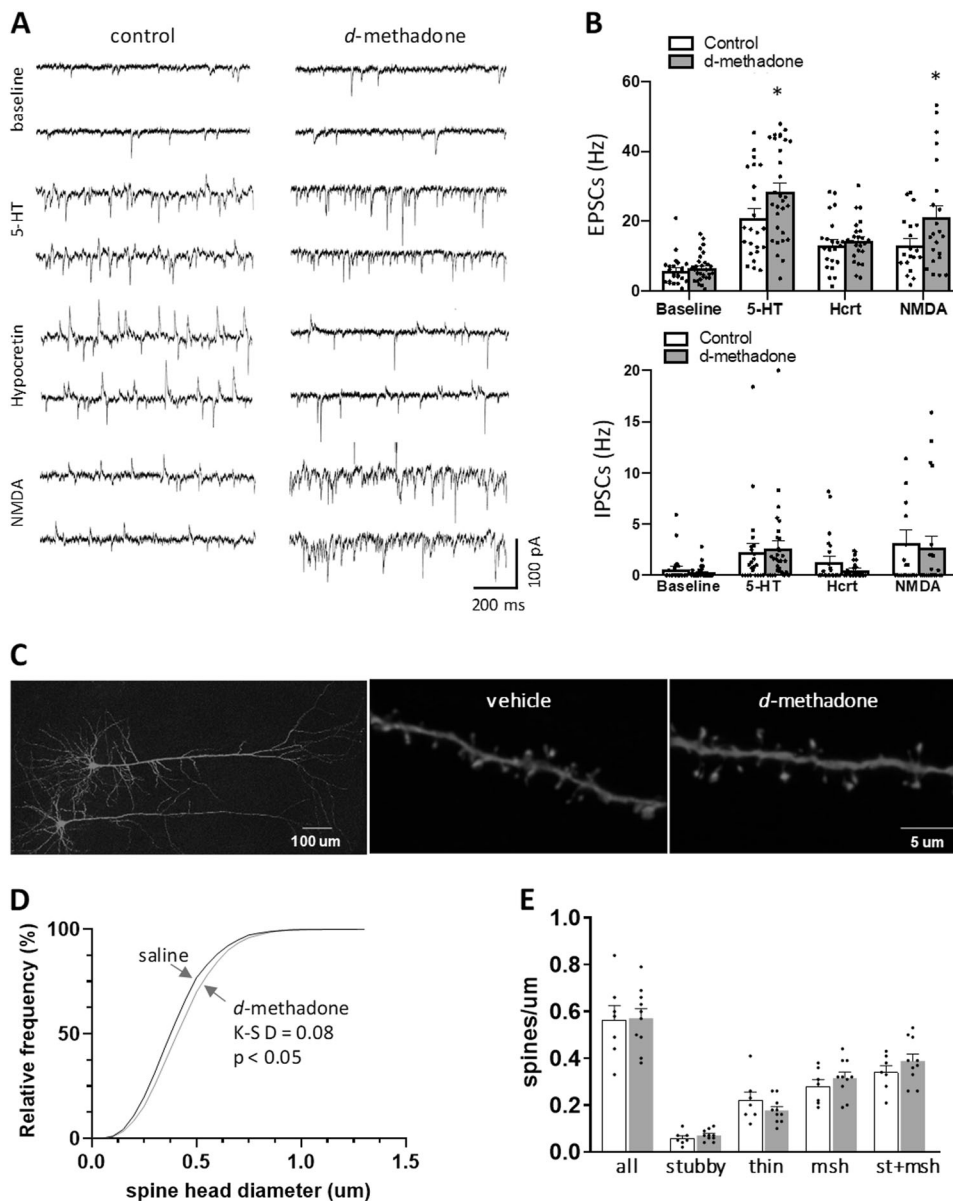


Fig. 4 *D*-methadone enhances 5-HT- and NMDA-induced EPSCs and increases spine head diameter in layer V mPFC pyramidal neurons. Rats were treated with saline or *d*-methadone (s.c., 20 mg/kg) and 24 h later slices of mPFC were prepared for patch clamp recording. Pyramidal neurons were filled with neurobiotin during patch clamp recording. **a** Representative traces of postsynaptic currents (PSCs) in layer V pyramidal cells from saline and *d*-methadone-treated rats. **b** *D*-methadone increased pyramidal cell excitatory postsynaptic current (EPSC) frequency induced by 5-HT (Student's *t*-test, $t_{49} = 2.10$; $p < 0.05$) or NMDA ($t_{33} = 2.03$, $p < 0.05$). There was no effect on hypocretin-induced EPSCs frequency (Student's *t* test, $t_{43} = 2.02$, $p > 0.05$). There was no difference in NMDA-, 5-HT- and hypocretin-induced inhibitory postsynaptic current (IPSC) frequency (Student's *t* test, NMDA: $t_{38} = 2.02$; 5-HT: $t_{31} = 2.04$; Hcrt: $t_{10} = 2.23$, $p > 0.05$; $n = 19$ –23 cells for vehicle group; $n = 22$ –30 cells for *d*-methadone group). **c** Representative confocal image of neurobiotin-filled layer V neurons, and enlarged images of the apical dendrite branch segments from vehicle control and *d*-methadone treated rats (24 h after dosing). **d** *D*-methadone (s.c., 20 mg/kg) increased spine head diameter shown in the cumulative fraction curve (Kolmogorov–Smirnov test, $D = 0.08$, $p < 0.05$; control, $n = 3784$ spines from seven cells; *d*-methadone, $n = 5194$ spines from 10 cells). **e** *D*-methadone did not change dendritic spine density (Student's *t*-test, $p > 0.05$. All: $t_{15} = 0.11$; stubby: $t_{15} = 0.99$; thin: $t_6 = 1.21$; mushroom: $t_{15} = 0.94$; stubby + mushroom: $t_{16} = 1.38$; control, $n = 7$ cells from four rats; *d*-methadone, $n = 10$ cells from six rats). Each bar represents the mean \pm standard error of the mean (S.E.M.). * $p < 0.05$ compared with the vehicle group

rats (Fig. 4a, b, Fig. S2). No effect was found for hypocretin-induced EPSC frequency and there was a reduction in amplitude (Fig. 4a, b, Fig. S2). There was no difference in NMDA-, 5-HT- and hypocretin-induced IPSC frequency (Fig. 4b).

During electrophysiological recording, cells were passively filled with neurobiotin for subsequent evaluation of dendritic spine density and spine head diameter on layer V pyramidal neurons in the mPFC. This technique has the advantage of measuring spine

alterations in the same cells that were used for patch clamp recording of EPSCs. Apical dendrites of filled neurons were scanned in z-stack images taken from a confocal microscope (Fig. 4c). *D*-methadone increased spine head diameter, a measure of more mature spines with greater synaptic efficiency when compared with vehicle-treated controls, measured 24 h after dosing (Fig. 4d), but did not change the density of dendritic spines (Fig. 4e).

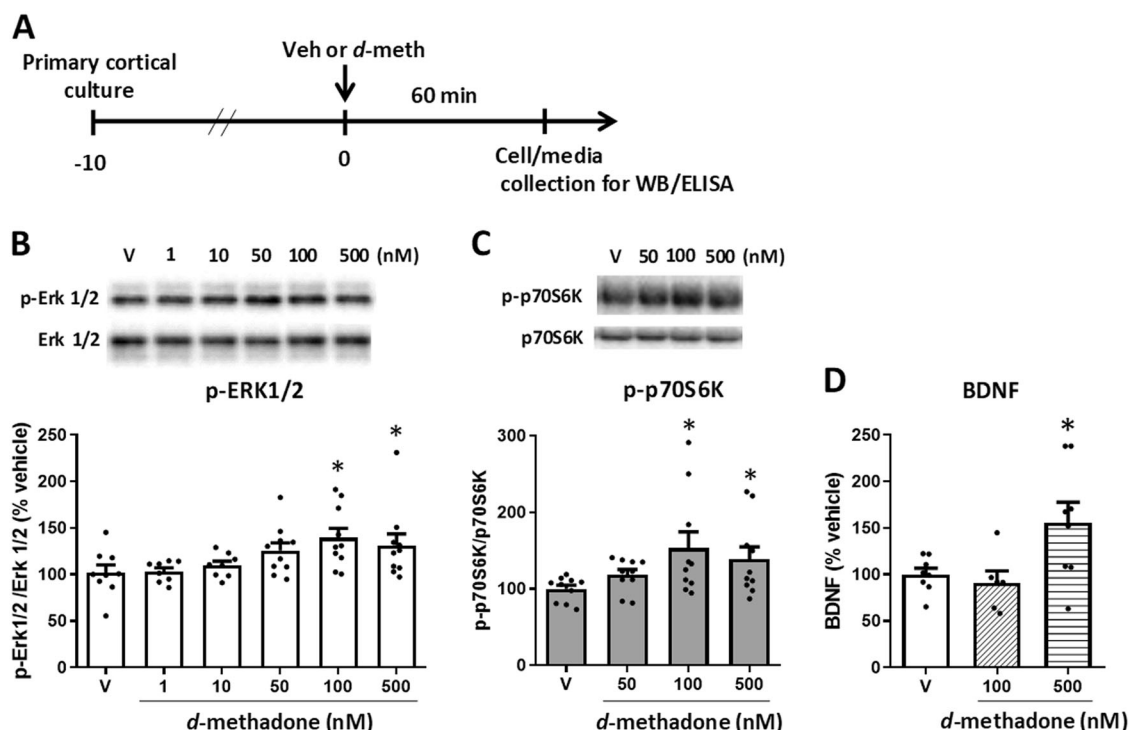


Fig. 5 Incubation with *d*-methadone activates Erk1/2 and mTORC1-p70S6K pathways and increases BDNF release in primary cortical cultures. **a** Experimental time line for *d*-methadone incubation and cell or media collection. *D*-methadone treatment (60 min) induced a concentration-dependent increase in **b** p-Erk1/2 levels (one-way ANOVA followed by Duncan test, $F_{5,49} = 3.10$, $p < 0.05$) and **c** p-p70S6K levels at doses of 100 and 500 nM ($F_{3,36} = 3.15$, $p < 0.05$, $n = 8-10$ wells/group). Representative images of western blots are shown above each bar graph for vehicle and *d*-methadone treatments. **d** *D*-methadone incubation (60 min) increased BDNF release into the media at the higher dose ($F_{2,19} = 5.08$, $p < 0.05$, $n = 6-8$ samples/group). Each bar represents the mean \pm standard error of the mean (S.E.M.). * $p < 0.05$ compared with the control (vehicle) group

D-methadone activates ERK1/2 and mTORC1-p70S6K signaling and induces activity-dependent BDNF release in primary cortical cultures

Previous studies report that ketamine stimulates ERK1/2 and mTORC1 signaling and induces activity-dependent release of BDNF, and that the antidepressant actions of ketamine require BDNF [7-11, 29]. To further characterize the effects of *d*-methadone on these signaling pathways, and because BDNF release is technically difficult to detect in vivo, we performed experiments in vitro using rat primary cortical cultures. For this, *d*-methadone was added into the media and, after 60 min, the cells or media were collected for western blot of phospho-ERK and phospho-p70S6K or BDNF-ELISA (Fig. 5a). Initial studies examined levels of the phosphorylated and activated form of ERK to establish the dose response for *d*-methadone in the primary culture cells. *D*-methadone incubation induced a concentration-dependent increase in levels of phospho-ERK1/2 (Fig. 5b) and increased levels of the downstream mTORC1 target phospho-p70S6K (Fig. 5c) at concentrations of 100 and 500 nM. Moreover, *d*-methadone induced the release of BDNF into the media at the higher concentration of 500 nM (Fig. 5d).

DISCUSSION

Drug development efforts to treat MDD have focused on new fast-acting, ketamine-like antidepressants that are devoid of dissociative and psychotomimetic side effects. In the current study we found that *d*-methadone, a noncompetitive NMDA channel blocker, produces antidepressant behavioral effects and that there is convergence of the signaling and synaptic mechanisms with ketamine [8, 10, 29, 34]. Notably, a single dose of *d*-methadone produced antidepressant effects that required

mTORC1 signaling, increased synaptic protein levels and spine head diameter in the mPFC, and enhanced 5-HT- and NMDA-induced EPSCs. Overall, our results are consistent with the hypothesis that the initial trigger for the antidepressant action of *d*-methadone is antagonism of NMDA receptors, which then rapidly stimulates BDNF-mTORC1 signaling, resulting in rapid and sustained increases in PFC synaptic connectivity and antidepressant behavioral responses.

Based on the NMDAR antagonism similar to that of ketamine, a well-established antidepressant effective in treatment resistant depression, and the behavioral effect previously observed in a FST study [21], *d*-methadone (REL-1017) is now in development for the treatment of depression (Relmada Therapeutics Inc.) and is being tested in a Phase 2a study in individuals with depression who have not responded to traditional antidepressants (NCT03051256). The studies presented here are the first to systematically analyze the synaptic and behavioral activity of *d*-methadone. The results demonstrate that a single dose of *d*-methadone induced rapid and sustained antidepressant responses in two tests in naive rats, the FUST and NSFT (24 and 72 h after administration, respectively). Moreover, a single dose of *d*-methadone reversed the behavioral deficits resulting from exposure to CUS, a widely used animal model of depression. This included reversal of the CUS-induced deficits in sucrose preference (24 h after a single dose), and reversal of the time deficits in the FUST (48 h after dosing), and latency to feed in the NSFT (72 h after dosing); all these responses were similar in magnitude to ketamine. The ability of *d*-methadone to produce sustained antidepressant behavioral actions is presumably due to the sustained induction of synaptic strength, as has been shown for ketamine [10, 13]. These results demonstrate that a single dose of *d*-methadone produces rapid and sustained antidepressant actions in models of anhedonia,

motivation, reward, and anxiety, and that the effects are long-lasting (up to 72 h in the NSFT). However, we acknowledge that it is difficult to study depression and treatment response in rodent models and that the translational activity of *d*-methadone must await the results of the Phase 2, and ultimately Phase 3 clinical trials.

Ketamine, as well as other putative fast-acting antidepressants, including its metabolite (2*R*,6*R*)-hydroxynorketamine (HNK) are reported to increase synaptic number and/or function in the mPFC via stimulation of AMPA-GluA1, activity-dependent BDNF release, and activation of TrkB-mTORC1 signaling, a key pathway required for protein synthesis dependent synaptic plasticity [10, 24, 35–37]. In the current study we found that *d*-methadone increased the phosphorylation of p70S6K, a key downstream protein kinase target of mTORC1 signaling, required for phosphorylation of ribosomal protein S6. We did not observe significant increases in other mTORC1 signaling proteins (i.e., p-mTOR and p-4EBP1) at the 24 h time point; previous studies demonstrate that ketamine produces a rapid (30 min) and transient (back to baseline by 2 h) increase in phospho-mTOR and phospho-4EBP1, but a more sustained increase in phospho-p70S6K [10, 13]. Additional time points between 1 and 24 h are needed to fully characterize the effects of *d*-methadone on these signaling proteins. The results of the Phase 2 study in depressed patients will soon provide information on the time for the antidepressant onset of action, which could indirectly address the issue of time difference between ketamine and *d*-methadone. Nevertheless, studies to directly test the role of mTORC1 signaling showed that infusion of rapamycin, a selective mTORC1 inhibitor, into the mPFC completely blocked the antidepressant actions of *d*-methadone in the FUST and NSFT. Previous studies have reported that rapamycin infusions into the mPFC also blocked the antidepressant effects of ketamine and (2*R*,6*R*)-HNK [10, 35, 36], indicating convergence of mTORC1 signaling for these treatments as well as *d*-methadone.

In addition, we also found that a single dose of *d*-methadone increased levels of the synaptic proteins PSD95, Synapsin 1, and GluA1 in the mPFC, but not in the hippocampus. Consistent with these results, previous studies report that ketamine produces a rapid (2 h) and sustained (72 h) increase in synaptic protein levels in the mPFC [10]. Ketamine is also reported to increase levels of Synapsin 1 in whole rat hippocampus and levels of GluA1 in the CA1 region [38, 39]. While we did not observe significant effects of *d*-methadone in whole hippocampus, we cannot rule out the possibility of effects in specific hippocampal subregions.

Rodent studies report that repeated stress causes dendritic atrophy and decreased spine density of pyramidal neurons in the PFC and hippocampus, which could underlie the reduction in volume of these brain regions in MDD patients [40–43]. Studies in rodents have also found that a single dose of ketamine rapidly increases spine density in layer V pyramidal neurons of mPFC and reverses the CUS-induced spine deficits [10, 12, 24]. In addition to increased levels of synaptic proteins (GluA1, PSD95, and Synapsin 1), we found that a single dose of *d*-methadone increased spine head diameter, indicative of an increase in mature mushroom spines with greater synaptic potential. This is supported by functional studies showing that *d*-methadone also increased 5-HT and NMDA-induced EPSCs in layer V pyramidal neurons. There was no effect of *d*-methadone on the total density of spines, which differs from the effects of ketamine [10], but is similar to the effects of (2*R*,6*R*)-HNK, which also did not increase spine density [36]. Interestingly, a recent study, using two-photon imaging reported that mPFC spinogenesis is required for the sustained, but not rapid antidepressant effects of ketamine [44], and it is possible that *d*-methadone produces a delayed increase in spine density.

Another difference with ketamine is that *d*-methadone increases 5-HT- but not hypocretin-induced EPSCs, while ketamine

increases EPSC responses to both [10, 13, 33]. These results suggest that *d*-methadone exerts more robust effects on corticocortical inputs that mediate 5-HT-induced EPSCs compared with thalamocortical inputs that mediate the hypocretin response. Additional studies will be needed to better elucidate the pharmacological mechanisms responsible for this phenomenon and its relevance to the different safety profiles of *d*-methadone and ketamine.

To further examine the influence of *d*-methadone on mTORC1-p70S6K signaling, as well as BDNF release, we conducted in vitro experiments in rat primary cortical culture. We first performed concentration-response studies of phospho-ERK to identify the effective dose for *d*-methadone, as reported for ketamine [7], and found increased phosphorylation of ERK1/2 as well as p70S6K at concentrations of 100 and 500 nM. The higher concentration of *d*-methadone (500 nM) also stimulated the release of BDNF into the culture media, and is in the range of brain levels of *d*-methadone reported in mice after a dose of 15 mg/kg [45], similar to the dose used in the current study. Ketamine, as well as (2*R*,6*R*)-HNK, are reported to produce similar in vitro effects, although these drugs were more potent than *d*-methadone [7, 35, 36]. Together these studies are consistent with the hypothesis that *d*-methadone, like ketamine increases BDNF release and activates downstream mTORC1-p70S6K signaling. Previous studies have also reported that the antidepressant actions of ketamine and (2*R*,6*R*)-HNK are blocked in BDNF mutant mice or by mPFC infusion of a BDNF neutralizing antibody [8, 30, 35, 36]. It is noteworthy that the above reported findings are consistent with the exposure-dependent increase in plasma BDNF levels observed in a cohort of healthy individuals treated with 25 mg/day of *d*-methadone compared with those treated with placebo in the Phase 1 multiple ascending dose study [46]. In the future, it will be important to determine whether changes of plasma BDNF levels correlate with antidepressant responses. In addition, it will be interesting to determine if the antidepressant behavioral activity of *d*-methadone is blocked by infusing a BDNF neutralizing antibody into the mPFC and/or in BDNF mutant mice.

Taken together, our results demonstrate for the first time that *d*-methadone has similarities to the signaling and synaptic mechanisms underlying the rapid antidepressant actions of ketamine. These findings support the hypothesis that NMDA receptor blockade leads to activity-dependent BDNF release, stimulation of mTORC1 signaling, and increased synaptic connectivity in the mPFC. The current studies provide further support for the potential use of *d*-methadone as a new therapeutic option to treat MDD.

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ADDITIONAL INFORMATION

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