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Sestrin modulator NV-5138 produces rapid antidepressant effects via direct mTORC1 activation

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Preclinical studies demonstrate that rapid-acting antidepressants, including ketamine, require stimulation of mTORC1 signaling. This pathway is regulated by neuronal activity and endocrine and metabolic signals, notably including the amino acid leucine, which activates mTORC1 signaling via binding to the upstream regulator sestrin. Here, we examined the antidepressant actions of NV-5138, a highly selective small molecule modulator of sestrin that penetrates the blood-brain barrier. The results demonstrate that a single dose of NV-5138 produced rapid and long-lasting antidepressant effects and rapidly reversed anhedonia caused by chronic stress exposure. The antidepressant actions of NV-5138 required brain-derived neurotrophic factor (BDNF) release, as the behavioral responses were blocked by infusion of a BDNF-neutralizing Ab into the medial prefrontal cortex (mPFC) or, in mice, with a knockin of a BDNF polymorphism that blocked activity-dependent BDNF release. NV-5138 administration also rapidly increased synapse number and function in the mPFC and reversed the synaptic deficits caused by chronic stress. Together, the results demonstrate that NV-5138 produces rapid synaptic and antidepressant behavioral responses via activation of the mTORC1 pathway and BDNF signaling, indicating that pharmacological modulation of sestrin may be an attractive approach for the development of rapid-acting antidepressants.

Introduction

Major depressive disorder (MDD) is a chronic, debilitating illness that affects approximately 17% of the population and is one of the leading causes of disability worldwide (1). Currently, MDD is typically treated with drugs that influence monoamine neurotransmission, but large-scale clinical trials (i.e., STAR*D) show that these drugs can take weeks to months to produce a therapeutic response, have limited efficacy, and result in low rates of full remission (2–4). On the other hand, recent studies demonstrate that a single, subanesthetic dose of ketamine produces rapid (within hours) and long-lasting (7 to 10 days) antidepressant effects even in patients who have failed to respond to traditional antidepressants and are considered treatment resistant (5, 6).

Although the exact mechanisms underlying the antidepressant actions of ketamine are not fully known, preclinical studies demonstrate a key role for mTORC1 signaling and increased synapse formation in the medial prefrontal cortex (mPFC) (7, 8). Ketamine rapidly stimulates mTORC1 signaling in the mPFC, and infusion of rapamycin, a selective mTORC1 inhibitor, into the mPFC blocks the synaptic and antidepressant behavioral actions of ketamine (7, 8). A single dose of ketamine also rapidly increases synapse number and function in the mPFC and reverses the synaptic deficits caused by chronic stress exposure (7, 8). A similar role for mTORC1 signaling has been demonstrated in the synaptic and behavioral responses to several other rapid-acting antidepressants tested, including the GluN2B allosteric modulator Ro 25-6981 (7, 8), the mGluR2/3 antagonist LY341495 (9), the metabolite (2R,6R)-hydroxynorketamine (10), the glycine-like partial agonist rapastinel (11), and the muscarinic antagonist scopolamine (12). In addition, viral expression of a constitutively active form of p70S6 kinase, a downstream mTORC1 effector, is sufficient to produce an antidepressant response (13). These studies indicate that mTORC1 signaling is necessary and may be sufficient to produce rapid synaptic and antidepressant behavioral responses.

The mTORC1 signaling pathway regulates cellular protein synthesis and serves as a key sensor of cellular activity, hormonal signals, and nutrient levels (14–16). Amino acids are the primary macromolecular components of proteins, and rates of protein synthesis are regulated by their availability. Leucine is one of the key regulatory amino acids and can promote protein synthesis in large part through regulation of mTORC1 signaling (17–19). Recent studies demonstrate that leucine binds to sestrin isoforms 1 and 2 (hereafter referred to as sestrin), resulting in dissociation from another regulatory multiprotein complex, GATOR2, leading to activation of mTORC1 signaling (20, 21). Further evidence for this pathway is provided by studies demonstrating that mutations of Sestrin2 that block leucine binding also block leucine activation of mTORC1 signaling (20, 21).

This leucine-sensing pathway provides an approach for manipulating mTORC1 signaling and determining whether acti-
vation of this pathway produces rapid antidepressant responses. NV-5138 was designed as a synthetic leucine analogue that readily penetrates the blood-brain barrier and selectively binds sestrin to activate mTORC1 signaling in the brain (22). In addition, NV-5138 is highly selective for sestrin without modulation of other CNS targets, including binding to or functional modulation of NMDA receptors (22). NV-5138 is not a substrate for the key leucine metabolizing pathways and is not incorporated into proteins (22). These unique features of the compound result in rapid induction of mTORC1 activity in the brain via sestrin modulation. This contrasts with leucine, which is rapidly metabolized and incorporated into proteins, resulting in relatively constant levels that are insensitive to dietary augmentation (23). These features, coupled with a short pharmacokinetic half-life of roughly 3 hours in the rat brain and peripheral circulation, result in rapid, but transient, activation of mTORC1 (22). Here, we show that NV-5138 produces rapid synaptic changes in rodent mPFC and antidepressant behavioral responses that are similar to those that result from ketamine, but without NMDA receptor modulation, supporting a key role for mTORC1 signaling and providing a target for antidepressant drug development.

Results
NV-5138 produces ketamine-like antidepressant behavioral responses. The effects of NV-5138 were tested in standard behavioral paradigms that are responsive to antidepressants, including tests of behavioral despair (forced swim test [FST]) and anxiety (novelty suppressed feeding test [NSFT]). In the first study, rats received oral administration of vehicle, NV-5138 (40, 80, or 160 mg/kg, p.o.) or leucine (160 mg/kg, p.o.) and behavioral testing started 24 hours later (Figure 1A). In the FST and NSFT, analyzed 1 or 3 days after dosing, NV-5138 significantly reduced the immobility time and latency to feed, respectively, at 160 mg/kg, but not at 40 or 80 mg/kg (Figure 1, B and D). This dose response is consistent with doses of NV-5138 required to activate mTORC1 signaling in the brain (22). There was no effect of leucine (160 mg/kg) in these paradigms, including the FST and NSFT (Figure 1, B and D). NV-5138 had no effect on locomotor activity or home-cage feeding (HCF), indicating that the effects were specific for behavioral despair in the FST and anxiety in the NSFT and not due to general effects on ambulation or feeding (Figure 1, C and E).

Next, we conducted a female urine sniffing test (FUST), a paradigm used to assess motivation and reward as well as NSFT after NV-5138 (160 mg/kg, p.o.) and compared the response to that seen with the rapid acting antidepressant ketamine (10 mg/kg, i.p.) (Figure 1F). Previous studies demonstrate that this is an effective dose of ketamine for producing antidepressant behavioral responses in naive rodents and in a chronic stress model (7, 8). NV-5138 administration produced a significant increase in time sniffing female urine in the FUST and decreased latency to feed in the NSFT; these responses were comparable to those seen with ketamine (Figure 1, G and I). There were no effects of either NV-5138 or ketamine on locomotor activity, time spent sniffing water, or HCF (Figure 1, G, H, and J).

NV-5138 produces long-lasting antidepressant actions, similar to those of ketamine. Clinical findings demonstrate that ketamine causes long-lasting (7 to 10 days) as well as rapid antidepressant responses in depressed patients (5, 6); similar long-lasting effects have been observed in rodent models (24). To test the duration of the antidepressant action of NV-5138, rats were administered vehicle or NV-5138 (160 mg/kg, p.o.) or vehicle or ketamine (10 mg/kg, i.p.) and behavioral testing started 3 and 7 days later (Figure 2A). Both NV-5138 and ketamine showed significant reduction in immobility times 3 and 7 days after administration in the FST (Figure 2, B and D); there were no effects on latency to feed 10 days after administration in the NSFT (Figure 2, C and E). There were no effects of either NV-5138 or ketamine on HCF (data not shown).

Repeated low-dose NV-5138 (80 mg/kg) also produces antidepressant effects. The antidepressant effects of lower doses of NV-5138 (40 or 80 mg/kg, p.o.) administered daily for a total of 7 days (starting with day 0) were also tested (Figure 2F). Because the antidepressant actions of ketamine begin to reverse after 7 days (24), ketamine (10 mg/kg, i.p.) was administered every other day for 6 days as a positive control. The results demonstrate that 80 mg/kg of NV-5138 showed antidepressant effects by significantly reducing the immobility time and latency to feed in both the FST and NSFT, respectively, without alteration of locomotor activity or HCF (Figure 2, G–J). This was in contrast with the lack of efficacy at this dose when evaluated 24 hours following a single administration (Figure 1, B and D). Ketamine administration also produced significant effects on immobility time and latency to feed in the FST and NSFT, respectively (Figure 2, G and J).

NV-5138 rapidly reverses the behavioral and synaptic deficits caused by chronic stress. The development of anhedonia, a core symptom of depression, with chronic unpredictable stress (CUS) exposure and the requirement for chronic treatment of a typical antidepressant to reverse this effect makes CUS one of the most valid models of depression (25, 26). The CUS model also provides a rigorous test for fast-acting antidepressants, and a single dose of ketamine rapidly reverses CUS-induced anhedonia, determined in a sucrose-preference test (SPT) (8). The results of the current study demonstrate that repeated CUS exposure (21 days) decreases sucrose preference and that a single dose of NV-5138 rapidly reverses this effect (Figure 3, A and B). NV-5138 administration did not influence sucrose preference in nonstressed control rats (Figure 3B). There was no significant difference in the total amount of water or total volume of liquid consumed (Supplemental Figure 1, A and B; supplemental material available online with this article; https://doi.org/10.1172/JCI126859DS1). CUS exposure was continued for subsequent behavioral testing and tissue sampling on day 26. CUS exposure increased latency to feed in the NSFT (day 22), and NV-5138 rapidly reversed this effect (Figure 3C). There were no effects of CUS or NV-5138 on HCF conducted immediately following the NSFT (Figure 3D), indicating that the effects of NV-5138 were not due to general increases in feeding. CUS exposure significantly decreased body weight, a predicted outcome of chronic stress exposure (Figure 3E).

Chronic stress paradigms also profoundly alter brain structure and function in rodents, causing reductions in levels of synapse-associated proteins in the mPFC as well as a reduction in spine synapse number (25–32). Studies were conducted to determine whether NV-5138 reverses these synaptic protein changes. For this study, rats were exposed to an additional 5 days of CUS after
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The initiation of behavioral studies (Figure 3A). CUS exposure decreased levels of GluR1 and PSD95 in synaptosome preparations of mPFC, and a single dose of NV-5138 reversed these synaptic protein deficits (Figure 3, F and G).

NV-5138–induced antidepressant actions require mTORC1 signaling. The effects of NV-5138 on the phosphorylated and activated forms of several mTORC1-signaling proteins in the mPFC were determined 1 hour after dosing (Figure 4, A and B). As expected, a single dose of NV-5138 (160 mg/kg) rapidly increased levels of phospho-mTOR as well as the downstream targets, phospho-p70S6K1, and phospho–4EB-P1, in synaptosomal preparations of PFC (Figure 4, C–E), confirming recent studies (22). A single dose of ketamine (10 mg/kg) produced effects (Figure 4, C–E) similar to those previously reported (7, 8, 11, 24).

To determine whether the behavioral actions of NV-5138, like ketamine and other rapid-acting antidepressants, require mTORC1 signaling, the selective mTORC1 inhibitor rapamycin was infused into the mPFC 30 minutes before NV-5138 (Figure 4F). The cannula placements and infusion sites are shown in Figure 4G. NV-5138 was administered (160 mg/kg), and 24 hours later, behavior was tested (Figure 4F). Note that the preswim was inadvertently conducted 4 hours after NV-5138 administration, which differed from the typical paradigm of 24 hours before dosing. However, NV-5138 still produced a robust antidepressant response by significantly reducing the immobility time and latency to feed in the FST and NSFT, respectively, with no effect on HCF or locomotor activity (Figure 4, H–K). Prior infusion of rapamycin into mPFC completely blocked the behavioral actions (reduction in immobility time and latency to feed) of NV-5138 in both FST and NSFT (Figure 4, H and J). Previous studies demonstrate that infusion of rapamycin alone into the mPFC has no effect on these behaviors (7, 8, 10, 12).

Antidepressant actions of NV-5138 require BDNF. Previous studies demonstrate that the antidepressant actions of ketamine and other rapid acting antidepressant including scopolamine and rapastinel require brain-derived neurotrophic factor (BDNF),
The behavioral actions of NV-5138 were examined in BDNF Val-66Met knockin mice. WT Val/Val littermates, Val/Met heterozygous, and Met/Met knockin mice were examined in antidepressant behavioral models according to the schedule in Figure 5F. There were significant genotype effects between Val/Val and Met/Met littermates in the FST or NSFT (Figure 5, G and I). In WT mice, we observed significant reduction in immobility time and latency to feed upon NV-5138 treatment in both the FST and NSFT, respectively, with no effects on locomotor activity or HCF (Figure 5, H–J). The antidepressant actions of NV-5138 (reduction in immobility time and latency to feed) were completely blocked in the Val/Met and Met/Met mice in both the FST and NSFT (Figure 5, G and I).
NV-5138 increases synaptic number and function in mPFC pyramidal neurons. Previous studies demonstrate that a single dose of ketamine increases spine synapse number as well as function (i.e., increased frequency of 5-HT and hypocretin-induced EPSCs) of layer V pyramidal neurons in the mPFC (7, 8, 10). Using the same approach, whole-cell patch recordings of layer V pyramidal cells, the influence of NV-5138 on 5-HT- and hypocretin-induced EPSCs in layer V neurons was determined at submaximal concentrations (20 μM and 200 nM, respectively). Twenty-four hours following a single dose of NV-5138, the frequency of hypocretin-induced EPSCs was significantly increased and the increase of 5-HT-induced EPSCs approached significance (P = 0.052) (Figure 6, A and B). NV-5138 also significantly increased the amplitudes of both hypocretin and 5-HT-induced EPSCs, as shown in the cumulative fraction graphs (Supplemental Figure 2A).

Layer V pyramidal neurons were passively filled with neurobiotin during recording to allow for subsequent imaging and analysis of dendritic spines. Dendritic segments in the apical tuft of filled neurons were scanned using confocal laser microscopy. In NV-5138–treated rats, spine density was significantly increased in the layer V pyramidal cells due to significantly increased densities of thin and mushroom spines (Figure 6, C and D). Similar effects on spine density have been observed with ketamine (7, 8). There were no significant effects on overall spine-head diameter (see frequency distribution analysis, Supplemental Figure 2B). Levels of synaptic proteins were also examined 24 hours after a single dose of NV-5138 or ketamine. Both agents significantly increased levels of GluR1, synapsin 1, and SV2A; only ketamine significantly increased levels of PSD95, although there was a trend following NV-5138 (Figure 6, E–H). There were no significant differences in levels of GAPDH, which was used for normalization (Supplemental Figure 3). We have recently reported that NV-5138 significantly increases PSD95 in naive animals, confirming the trend observed in the current study (22).

Influence of AMPA receptor blockade on the antidepressant actions of NV-5138. Previous studies demonstrated that the actions of rapid-acting antidepressants require glutamate AMPA receptor activity, as pretreatment with the AMPA receptor antagonist NBQX blocks the antidepressant behavioral responses to ketamine, rapastinel, LY345979, (2R,6R)-HNK, and scopolamine (7, 10, 12, 39, 40). Here, we show that pretreatment (30 minutes) with NBQX (10 mg/kg i.p.) (Figure 7A) did not significantly influence the antidepressant actions of NV-5138 on immobility time in the FST measured 24 hours after dosing (Figure 7B); there was a partial blockade of the NV-5138 response on latency to feed in the NSFT measured 3 days after dosing (Figure 7D). There were no significant effects of NBQX on locomotor activity or HCF (Figure 7C and E).
The results demonstrate that a single dose of the mTORC1 activator NV-5138 produces rapid antidepressant actions in behavioral models of depression and treatment response. In addition, a single dose of NV-5138 increases the number and function of synapses and increases levels of synaptic proteins in the mPFC. The results also demonstrate that these actions of NV-5138 require mTORC1 signaling as well as BDNF. Together, these findings demonstrate a mech-

Figure 4. Antidepressant actions of NV-5138 are dependent on activation of mTORC1 signaling. (A) Rats were administered vehicle or NV-5138 (160 mg/kg) and PFC dissections were collected 1 hour later. (B) Diagram showing postsynaptic signaling. Levels of the phosphorylated and activated forms of (C) mTOR, (D) p70S6K, and (E) 4E-BP1 as determined by Western blot analysis were increased by NV-5138 and ketamine; levels of total proteins or GAPDH were also measured to control for loading differences. Results are shown as mean ± SEM. n = 6/group. *P < 0.05; **P < 0.01, Student’s t test. (F) Rats were implanted with bilateral cannula in the mPFC and allowed to recover for approximately 2 weeks. (G) The mTORC1 inhibitor rapamycin was infused into the mPFC 30 minutes prior to administration of vehicle or NV-5138. Twenty-four hours after NV-5138 administration, behavioral studies were initiated and conducted over the next 3 days (H–K). NV-5138 treatment significantly decreased immobility time and latency to feed, but these effects were blocked by rapamycin in (H) the FST (F2,17 = 20.46, P < 0.001) and (I) the NSFT (F2,17 = 5.36, P < 0.05), respectively. No significant effects were seen in (J) LMA (F2,17 = 0.200, P > 0.05) or (K) HCF (F2,17 = 0.814, P > 0.05). Results are shown as mean ± SEM. n = 6–7. *P < 0.05; **P < 0.01, Tukey’s multiple comparisons test, following significant results of 1-way ANOVA. Rap, rapamycin.

Discussion
The results demonstrate that a single dose of the mTORC1 activator NV-5138 produces rapid antidepressant actions in behavioral models of depression and treatment response. In addition, a single dose of NV-5138 increases the number and function of synapses and increases levels of synaptic proteins in the mPFC. The results also demonstrate that these actions of NV-5138 require mTORC1 signaling as well as BDNF. Together, these findings demonstrate a mech-
anism, direct activation of mTORC1 signaling via the upstream regulator sestrin (22), and are consistent with an mTORC1 requirement for the actions of other rapid antidepressant agents (7, 8, 10, 12).

The results demonstrate that a single oral dose of NV-5138 produces rapid antidepressant actions in 3 standard models of behavioral despair (FST), anxiety (NSFT), and motivation/reward (FUST); these effects were dose dependent and were not associated with general effects on locomotor activity or HCF. In addition, the antidepressant actions of a single dose of NV-5138 persisted for up to 7 days in the FST. The FST is typically used for drug screening, but is responsive to acute administration of typical antidepressants (41); the NSFT is responsive to chronic (~3 weeks) administration of

Figure 5. BDNF is required for antidepressant actions of NV-5138. (A–E) The antidepressant actions of NV-5138 are blocked by infusion of BDNF nAb into the mPFC. (A) BDNF nAb or control IgG (0.5 μg/side) was infused into the mPFC 30 minutes prior to administration of vehicle or NV-5138 (160 mg/kg, p.o.). (B and D) NV-5138 significantly decreased immobility time (B) in the FST (effect of NV-5138: F₁,29 = 14.6, P < 0.001; mPFC infusion: F₁,29 = 13.4, P < 0.001; interaction: F₁,29 = 14.9, P < 0.001) and latency to feed (D) in the NSFT (effect of NV-5138: F₁,29 = 39.4, P < 0.001; mPFC infusion: F₁,29 = 11.2, P = 0.002; interaction: F₁,29 = 18.0, P < 0.001), and these effects were blocked by infusion of the BDNF nAb. (C and E) There were no significant effects on locomotor activity (effect of NV-5138: F₁,30 = 0.01, P = 0.92; mPFC infusion: F₁,30 = 0.07, P = 0.79; interaction: F₁,30 = 0.03, P = 0.86) or HCF (effect of NV-5138: F₁,30 = 0.11, P = 0.74; mPFC infusion: F₁,30 = 2.93, P = 0.1; interaction: F₁,30 = 1.23, P = 0.28). (F–J) The antidepressant actions of NV-5138 are attenuated in BDNF Val66Met knockin mice. (F) Experimental time line for behavioral testing of animals after vehicle or NV-5138 administration. In the BDNF Val/Val mice, NV-5138 treatment significantly decreased immobility time (G) in the FST (effect of NV-5138: F₁,64 = 12.7, P < 0.001; genotype: F₂,64 = 1.88, P = 0.16; interaction: F₂,64 = 3.98, P = 0.02) and latency to feed (I) in the NSFT (effect of NV-5138: F₁,65 = 13, P < 0.001; genotype: F₂,65 = 12.8, P < 0.001; interaction: F₂,65 = 3.4, P = 0.04) that were blocked in Val/Met and Met/Met mice. No significant effects were observed in (H) LMA (effect of NV-5138: F₁,64 = 3.39, P > 0.05; genotype: F₂,64 = 0.59, P = 0.56; interaction: F₂,64 = 0.06, P = 0.94) or (J) HCF (effect of NV-5138: F₁,65 = 0.06, P = 0.80; genotype: F₂,65 = 0.52, P = 0.60; interaction: F₂,65 = 0.58, P = 0.56). Results are shown as mean ± SEM. n = 7–9/group (A–E); n = 5–16/group (F–J). *P < 0.05; **P < 0.01; ***P < 0.001 (A–J), 2-way ANOVA and Tukey’s post hoc test.
typical antidepressants, but is a model of anxiety (42,43). To further test the rapid antidepressant actions of NV-5138, we used a CUS model that results in anhedonia, a core symptom of depression that is only reversed by chronic administration of typical antidepressants (25, 26, 44). The results show that a single dose of NV-5138 reverses the CUS-induced effects on sucrose preference as well as latency to feed in the NSFT. NV-5138, like other antidepressants, does not have an effect on sucrose preference in

Figure 6. Influence of NV-5138 on spine number and function and synaptic proteins in the PFC. (A) Representative traces showing postsynaptic currents recorded from layer V pyramidal neurons in mPFC brain slices from vehicle- or NV5138-treated rats (24 hours after drug treatment). (B) Summary of data showing that frequencies of 5-HT- (20 μM) and hypocretin-induced (200 nM) EPSCs are increased by NV-5138; n = 35 cells/8 rats for control; n = 30 cells/8 rats for NV-5138. *P < 0.05; **P < 0.01, t test. Absolute values for baseline EPSC frequency is 3.58 ± 0.4 (Hz); n = 35 cells/8 rats for control and 2.63 ± 0.48 (Hz); n = 30 cells/8 rats for NV-1538 (C). Representative images of high-magnification Z-stack projections of apical dendritic segments from slices collected 24 hours following vehicle or NV-5138 administration. Scale bar: 5 μm. (D) NV-5138 increased overall spine density (t = 2.72, P = 0.015) due to increases in densities of thin (t = 2.20, P = 0.04) and mushroom spines (t = 2.01, P = 0.05). n = 9 cells/8 rats for control and 9 cells/5 rats for NV-5138. (E–G) Rats were administered vehicle, ketamine (10 mg/kg), or NV-5138 (160 mg/kg), and PFC dissections were collected 24 hours later. Levels of the postsynaptic proteins including (E) GluR1, (F) PSD95, (G) synapsin1, and (H) SV2A were determined by Western blot analysis. GAPDH levels were also determined to control for loading differences. n = 6/group. *P ≤ 0.05; **P < 0.01 Student’s t test.
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**allele is not sufficient to support the actions of NV-5138, indicating that a full complement of mature BDNF is required. Together, the results demonstrate that the behavioral actions of NV-5138 are dependent on BDNF activity.**

Fast-acting antidepressants such as ketamine also produce rapid synaptic changes in the mPFC that are associated with the antidepressant behavioral actions of these agents (45). Moreover, ketamine and other fast-acting agents rapidly reverse the synaptic deficits caused by chronic stress exposure and by depression, demonstrating that agents such as ketamine can rapidly reverse the pathophysiology of depression (8, 46). Here, the results demonstrate that a single dose of NV-5138 also rapidly increases the number and function of spine synapses in layer V pyramidal neurons in the mPFC. This includes increased hypocretin-induced EPSCs and a strong trend for increased 5-HT–induced EPSCs. NV-5138 also increased the number of spines on apical dendrites of layer V pyramidal neurons and increased levels of the synaptic proteins GluA1, synapsin 1, and SV2A. Moreover, in animals exposed to CUS, which caused a reduction in sucrose preference, there was a marked decrease in levels of the synaptic proteins GluA1 and PSD95 and these deficits were reversed by a single dose of NV-5138. Previous studies demonstrate that the ability of ketamine to increase synapse number and function are dependent on mTORC1 signaling (7, 8). Together, these findings demonstrate that NV-5138, like ketamine, can rapidly increase synapse number and function and reverse the effects of chronic stress via stimulation of mTORC1 signaling and synaptic protein synthesis.

Previous studies have demonstrated that mTORC1 signaling is required for the rapid behavioral actions of ketamine as well as other rapid-acting antidepressants. This information inspired the development of an agent that activates mTORC1 signaling via the

naive, nonstressed rats due to a ceiling effect, but does influence latency to feed in the NSFT in both naive and nonstressed rats due to the innate fear of a novel environment even in naive rodents. Together, the results demonstrate that NV-5138 produces rapid antidepressant actions in 4 different behavioral models, similar to the effects of the prototypical rapid-acting agent ketamine (7, 8). The results are consistent with the hypothesis that NV-5138 produces antidepressant actions via stimulation of mTORC1 signaling, although this conclusion is tempered for the CUS study, since we did test the influence of NV-5138 on mTORC1 signaling in CUS-exposed rats.

We also found, as expected, that the antidepressant behavioral actions of NV-5138 were blocked by mPFC infusion of rapamycin, a selective mTORC1 inhibitor, consistent with evidence of increased mTORC1 signaling in the mPFC. We also examined the role of BDNF, since previous studies report that the rapid synaptic and antidepressant behavioral actions of ketamine are dependent on neuronal activity and require BDNF signaling (7, 34, 37, 39, 45). Here, we show that the antidepressant actions of NV-5138 are also dependent on BDNF signaling using 2 different approaches. First, we show that the behavioral actions of NV-5138 in the FST and NSFT are blocked by mPFC infusion of a BDNF Ab that binds and neutralizes BDNF that is released into the interstitial space. Second, we show that the antidepressant actions of NV-5138 are completely blocked in mice with a knockin of the BDNF Met allele, which blocks the processing and activity-dependent release of BDNF (33). This was observed in both Val/Met and Met/Met knockin mice, similar to complete blockade of the antidepressant actions of ketamine and scopolamine in Met/Met mice, and partial or complete block, respectively, in Val/Met mice (37, 38). These findings demonstrate that a single WT Val allele is not sufficient to support the actions of NV-5138, indicating that a full complement of mature BDNF is required. Together, the results demonstrate that the behavioral actions of NV-5138 are dependent on BDNF activity.

Fast-acting antidepressants such as ketamine also produce rapid synaptic changes in the mPFC that are associated with the antidepressant behavioral actions of these agents (45). Moreover, ketamine and other fast-acting agents rapidly reverse the synaptic deficits caused by chronic stress exposure and by depression, demonstrating that agents such as ketamine can rapidly reverse the pathophysiology of depression (8, 46). Here, the results demonstrate that a single dose of NV-5138 also rapidly increases the number and function of spine synapses in layer V pyramidal neurons in the mPFC. This includes increased hypocretin-induced EPSCs and a strong trend for increased 5-HT–induced EPSCs. NV-5138 also increased the number of spines on apical dendrites of layer V pyramidal neurons and increased levels of the synaptic proteins GluA1, synapsin 1, and SV2A. Moreover, in animals exposed to CUS, which caused a reduction in sucrose preference, there was a marked decrease in levels of the synaptic proteins GluA1 and PSD95 and these deficits were reversed by a single dose of NV-5138. Previous studies demonstrate that the ability of ketamine to increase synapse number and function are dependent on mTORC1 signaling (7, 8). Together, these findings demonstrate that NV-5138, like ketamine, can rapidly increase synapse number and function and reverse the effects of chronic stress via stimulation of mTORC1 signaling and synaptic protein synthesis.

Previous studies have demonstrated that mTORC1 signaling is required for the rapid behavioral actions of ketamine as well as other rapid-acting antidepressants. This information inspired the development of an agent that activates mTORC1 signaling via the
target sestrin2. The current study demonstrates that the mTORC1 activator NV-5138 produces rapid synaptic and antidepressant behavioral responses and rapidly reverses the synaptic and behavioral deficits caused by chronic stress. These synaptic and behavioral responses are similar to those found with ketamine, but occur via an initial cellular mechanism that is completely independent of NMDA receptor modulation. While the effects of NV-5138 and ketamine converge on mTORC1 signaling, the actions of ketamine occur via a burst of glutamate and require AMPA receptor activation, based on evidence that pretreatment with an AMPA receptor antagonist (NBQX) blocks the antidepressant actions of ketamine as well as other rapid-acting agents (7, 10, 12, 39, 40). Using the same dose as was used in these previous studies, we found that NBQX pretreatment had no effect on the antidepressant actions of NV-5138 in the FST and only a partial, nonsignificant blockade in NSFT. However, it is possible that a higher dose and/or additional doses of NBQX after NV-5138 could reveal a requirement for AMPA receptor activity.

Further studies will be needed to determine whether NV-5138 produces side effects in rodent models and in primates that overlap with or are different from the dissociative and psychotomimetic effects of ketamine. An obvious issue is whether NV-5138 activation of mTORC1 would influence tumor growth. While it is true that the chronic hyperactivation of mTORC1 induced either genetically or through natural mutations (e.g., TSC1/2 inactivation) has been associated with the development and/or propagation of certain cancers, this is quite different from the observed effects of NV-5138 on mTORC1 activation in the current context.

Importantly, the pharmacokinetics of NV-5138 in rodents is relatively short (~3 hours) and the extent of mTORC1 activation is limited (2- to 3-fold) compared with genetically induced hyperactivation (6- to 8-fold) that has been associated with pathological etiologies of mTORC1 hyperactivation (e.g., development of TSC (47). This is due to the self-limiting activation of mTORC1 by NV-5138 through the amino acid activation pathway due to the upstream regulation imparted by the GATOR2 complex (22). Thus, there is a low probability that treatment with NV-5138 will result in pathologies sometimes associated with mTORC1 pathway hyperactivation.

In conclusion, this is the first evidence, to our knowledge, that direct activation of mTORC1 can have positive effects in various models of antidepressant response and depressive behavior that are associated with changes in synaptic protein expression and morphology, directly confirming the critical role of mTORC1 activation for these processes. Characterization of this agent and mechanism for stimulation of mTORC1 signaling that causes synaptic and antidepressant behavioral response provides an alternative mechanistic approach for the treatment of depression.

Methods

Study design. Studies were primarily designed to determine the antidepressant-like efficacy and mechanism of action of NV-5138 in depression models. For in vivo behavioral studies, Western blot, and electrophysiology experiments, age-matched animals were randomly assigned to drug or vehicle treatment groups. The numbers of animals for each study type were determined on the basis of previous experience with the animal models utilized and based on results of pilot studies. Experiments were performed with at least 2 different batches of animals, and it was confirmed that both experiments show the same trend.

Animals and drug administration. Male Sprague-Dawley rats (Charles River Laboratories) weighing 250–260 g were used for most studies, with the exception of the CUS experiment, which used rats weighing 120–140 g because of the longer duration of the study. In addition, mutant BDNF Val66Met knockin mice (Val/Val WT, heterozygous Val/Met, and homozygous Met/Met mice at 8–12 weeks) generated as previously described were used to test the role of BDNF (33).

Animals were singly housed and maintained in standard conditions with a 12-hour light/12-hour dark cycle and ad libitum access to food and water. Rats received a single oral administration of vehicle (0.5% methylcellulose and 0.1% Tween 80), NV-5138 (40, 80, or 160 mg/kg, p.o., Navitcor Pharmaceuticals Inc.), ketamine (10 mg/kg, i.p.), DMSO (0.5%; 1 ml/kg, i.p.), or NBQX (10 mg/kg, i.p., Tocris Bioscience). For the repeated dose study, NV-5138 (40 or 80 mg/kg, p.o.) was administered daily for a total of 7 days and ketamine was injected (10 mg/kg, i.p.) every other day for 6 days.

Surgical and infusion procedures. Rats were anesthetized with 80 mg ketamine/6 mg/kg xylazine i.p., and bilateral 22-gauge guide cannula were implanted at 0.5 mm above the site of infusion (±3.0 mm AP; ±1.0 mm ML; –4.0 mm DV to the bregma). In previous studies, we found that this anesthetic dose of ketamine did not produce an antidepressant response (7). Following 9 to 14 days of recovery, rats were bilaterally infused with one of a number of different agents, including the mTORC1 inhibitor rapamycin (Cell Signaling; 0.005 mmol/side or 10% DMSO vehicle for control), a function-blocking anti-BDNF Ab (Chemicon; 0.5 μg/side, or normal sheep IgG for control; R&D Systems) at a rate of 0.25 μl/min for 2 minutes. These procedures and doses have been validated in previous studies (7, 8, 34, 35). After the behavioral studies, brains were collected, coronal sections were cut in a cryostat, and cannula tip location was confirmed after Nissl staining.

CUS. CUS was carried out as previously described (8). Animals were exposed to a variable sequence of mild and unpredictable stressors for 21 days. A total of 13 different stressors were used (2 stressors per day). The stressors included rotation on a shaker, placement in a 4°C ambient temperature, lights off for 3 hours (10:00 AM–1:00 PM), lights on overnight, strobe light overnight, forced swim, 45°tiled cages, food and water deprivation, crowded housing, wet bedding, white noise, restraint in plastic bag for 1 hour, and no bedding. The CUS protocol was continued until day 26 for subsequent behavioral testing and tissue sampling.

Behavior studies: FST. The FST was carried out as previously described for rat and mouse (7, 35, 48). Each rat was subjected to a 15-minute preswim in a Plexiglas cylinder (30 cm diameter, 65 cm height) filled with water (25 ± 1°C, 45 cm depth). After 24 hours, rats received drug treatments, and the next day (24 hours later), each rat was again placed in the swim cylinders for a 10-minute period and videotaped. Data were analyzed in a blinded manner by scoring the total immobility time during the entire 10-minute swim period. Each mouse was placed in a 4 l glass beaker (16 cm diameter, 24.5 cm height) containing water (25 ± 1°C, 15 cm depth) for 10 minutes and videotaped. The duration of immobility was scored between 2 and 6 minutes by an experimenter blinded to the treatment groups.

NSFT. The NSFT was performed as previously described (7, 35). Briefly, animals were food deprived overnight and placed in an open
bands were detected using ECL. The blots then were incubated in Ab (1:5000 to 1:10000) for 1 hour. After a final 3 washes with TBST, the stripped blots were kept in blocking solution for 1 hour and incubated with the primary Ab directed against total levels of the respective protein or GAPDH (Cell Signaling, catalog 5174, 1:10000) for loading control. Densitometric analysis of immunoreactivity for each protein was conducted using Image Lab (Bio-Rad). Immunoreactivity was normalized to saline-treated control group values for each protein.

Electrophysiology. Brain slices were prepared as previously described (7, 8, 11, 27). Briefly, 1 day after drug treatments, rats were anesthetized (chloral hydrate, 400 mg/kg, i.p.) and brains removed and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) in which sucrose (252 mM) was substituted for NaCl (sucrose-ACSF). A block of tissue containing prefrontal cortex and coronal slices (400 μm) was cut in sucrose-ACSF with an oscillating-blade tissue slicer. Slices were placed in a submerged recording chamber; bath temperature was then raised to 32°C. Known concentrations of drugs in ACSF were applied through a stopcock arrangement (~4 ml/min) to reach the slice within 7 to 10 seconds. The standard ACSF (pH 7.35), equilibrated with 95% O2/5% CO2, contained 128 mM NaCl, 3 mM KCl, 2 mM CaCl2, 2 mM MgSO4, 24 mM NaHCO3, 1.25 mM NaH2PO4, and 10 mM d-glucose. There was a recovery period of 1 to 2 hours before recording. Pyramidal neurons in layer V were visualized by videomicroscopy using a microscope (×40 IR lens) with infrared differential interference contrast (IR/DIC). Patch pipettes (3-5 MΩ) were pulled from glass tubing with a Flaming-Brown Horizontal Puller. The pipette solution contained the following: 115 mM K gluconate, 5 mM KCl, 2 mM MgCl2, 2 mM Mg-ATP, 2 mM Na-ATP, 10 mM Na-phosphocreatine, 0.4 mM Na-GTP, and 10 mM Hepes, pH 7.33. Neurobiotin (0.3%) was added to the pipette solution to mark cells for later imaging. Whole-cell recordings were made with an Axoclamp-2B amplifier. The output signal was low pass-filtered at 3 KHz and digitized at 15 KHz; data were acquired with pClamp 10.2/Digidata 1550 A software. Series resistance, which was monitored throughout the experiment, was usually between 4 and 8 MΩ. To minimize series-resistance errors, cells were discarded if series resistance rose above 10 MΩ. Postsynaptic currents were studied in the continuous single-electrode voltage-clamp mode (3000 Hz low-pass filter) clamped to 65 mV to separate the IPSCs from the EPSCs. After completion of recording, slices were transferred to 4% paraformaldehyde (0.1 M phosphate buffer) and stored overnight at 4°C. Slices were then processed with streptavidin conjugated to Alexa Fluor 594 (1:1000) for visualization of labeled cells.

Spine analysis. Labeled mPFC layer V neurons were imaged on a confocal laser scanning microscope (Olympus FV1000) equipped with a ×100, 1.42 NA objective at a zoom of ×5 (voxel size 0.049 μm × 0.049 μm × 0.2 μm). Spine density was sampled at tips of tuft branches as they approach the pial membrane at a site approximately 100 μm more proximal and midway between distal and proximal sites (length of dendritic segments was approximately 25 μm). Computerized analysis of z-stack images was performed on deconvolved confocal image stacks (AutoquantX Version 3.0.1, Media Cybernetics), and spines were and quantified using NeuronStudio software by an experimenter blinded to treatment history.

Statistics. Data for Western blot and behavioral studies were analyzed using 1-way or 2-way ANOVA, and differences between individual group means were then assessed by post hoc Tukey’s multiple comparison test. Differences between group means for electrophysiol-
ology and spine analysis were assessed by Student’s t tests. Effects were considered significant at \( P \leq 0.05 \), and results are presented as mean ± SEM. Analysis was conducted using GraphPad Prism 6 software.

**Study approval.** Animal use and procedures were in accordance with NIH guidelines and approved by the Yale University Animal Care and Use Committees.

**Author contributions**

TK designed the study, performed experiments and data analysis, and wrote the manuscript. SP performed experiments and data analysis and assisted in editing/revision of manuscript. RJJ performed electrophysiological experiments and analyzed data. CHD performed spine analysis and analyzed data. RT performed Western blots. GPV, ES, SH, and RSD assisted with study design, data interpretation, and writing the manuscript.

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