mTORC1 Inhibition in the Nucleus Accumbens 'Protects' Against the Expression of Drug Seeking and 'Relapse' and Is Associated with Reductions in GluA1 AMPAR and CAMKIIα Levels

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The mechanistic target of rapamycin complex 1 (mTORC1) is necessary for synaptic plasticity, as it is critically involved in the translation of synaptic transmission-related proteins, such as Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIα) and AMPA receptor subunits (GluAs). Although recent studies have implicated mTORC1 signaling in drug-motivated behavior, the ineffectiveness of rapamycin, an mTORC1 inhibitor, in suppressing cocaine self-administration has raised questions regarding the specific role of mTORC1 in drug-related behaviors. Here, we examined mTORC1's role in three drug-related behaviors: cocaine taking, withdrawal, and reinstatement of cocaine seeking, by measuring indices of mTORC1 activity and assessing the effect of intra-cerebroventricular rapamycin on these behaviors in rats. We found that withdrawal from cocaine self-administration increased indices of mTORC1 activity in the nucleus accumbens (NAC). Intra-cerebroventricular rapamycin attenuated progressive ratio (PR) break points and reduced phospho-p70 ribosomal S6 kinase, GluA1 AMPAR, and CAMKIIα levels in the NAC shell (NACsh) and core (NACc). In a subsequent study, we treated rats with intra-NACsh infusions of rapamycin (2.5 μg/side/day for 5 days) during cocaine self-administration and then tracked the expression of addiction-relevant behaviors through to withdrawal and extinction. Rapamycin reduced drug seeking in signaled non-drug-available periods, PR responding, and cue-induced reinstatement, with these effects linked to reduced mTORC1 activity, total CAMKIIα, and GluA1 AMPAR levels in the NACsh. Together, these data highlight a role for mTORC1 in the neural processes that control the expression and maintenance of drug reward, including protracted relapse vulnerability. These effects appear to involve a role for mTORC1 in the regulation of GluA1 AMPARs and CAMKIIα in the NACsh.
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INTRODUCTION

Dysregulated signaling through a number of neuronal surface receptors on nucleus accumbens (NAC) medium spiny neurons (MSNs), including dopamine, ionotropic, and metabotropic glutamate receptors, as well as neurotrophin receptors, has been linked with expression of addiction-relevant behaviors (Brami-Cherrier *et al*, 2002; Dayas *et al*, 2012; Russo *et al*, 2009). These receptor systems can signal through the MAPK-ERK and PI3K/AKT pathway to influence gene transcription and protein translation, with subsequent effects on neural activity and behaviors (McGinty *et al*, 2008; Russo *et al*, 2009). Interestingly, recent studies indicate that these receptor systems can recruit the mechanistic target of rapamycin (mTOR; Hoeffer and Klann 2010), a serine-threonine kinase that, together with Raptor and other interacting proteins, forms the mechanistic target of rapamycin complex 1 (mTORC1; Hoeffer and Klann 2010). mTORC1 is expressed in both neuronal cell bodies and neuronal dendrites and controls key steps in protein translation initiation via the phosphorylation and activation of translation initiation factors such as the p70 ribosomal S6 kinase (p70s6k) and eukaryotic

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translation initiation factor-4E-binding protein (Hoeffer and Klann 2010). It is now believed that mTORC1 plays a role in the synaptic plasticity processes of LTP (Cammalleri et al, 2003) and LTD (Mameli et al, 2007). The role of mTORC1 in these mechanisms is linked to the function of this complex in regulating the translation of specific synaptic proteins, such as Ca^{2+}/Calmodulin-dependent kinase II alpha (CAMKIIα) and AMPAR receptor subunits (GluA1s; Schratt et al, 2004; Slipczuk et al, 2009).

The formation of mTORC1 can be blocked by rapamycin, an allosteric mTOR inhibitor that acts by displacing Raptor, inhibiting its capacity to phosphorylate mTOR substrates (Hoeffer and Klann, 2010). Studies using rapamycin have implicated mTORC1 in late phase (protein synthesis dependent) LTP and LTD (Cammalleri et al, 2003; Stoica et al, 2011), as well as associative learning processes, including inhibitory avoidance memory and fear conditioning (Blundell et al, 2008; Parsons et al, 2006). More recently, experiments using rapamycin have implicated mTORC1 signaling in addiction-relevant behaviors and in alcohol-related memory consolidation (Barak et al, 2013). Both acute systemic and intra-NAC injections of rapamycin attenuated alcohol-drinking behaviors and conditioned place preference (CPP) for alcohol (Neasta et al, 2010). Acute systemic rapamycin treatment also suppressed the expression of cocaine CPP, behavioral sensitization to cocaine (Bailey et al, 2012), and the sensitization of meth-amphetamine CPP (Narita et al, 2005). These findings indicate that mTORC1 may have a role in controlling the processes responsible for the strengthening of drug/cue associations. NAC mTORC1 inhibition also attenuated cue-induced reinstatement of cocaine seeking but, interestingly, had no effect on cocaine self-administration under a fixed-ratio 1 (FR1) schedule of reinforcement (Wang et al, 2010b).

In the present study, we investigated the role of mTORC1 in drug-related behaviors by first assessing the effects of cocaine withdrawal on indices of mTORC1 activity. Next, we determined the role of mTORC1 in drug seeking by examining the effects of central (intra-cerebroventricular; i.c.v.) mTORC1 inhibition on lever pressing for cocaine using a progressive ratio (PR) schedule of reinforcement, a procedure thought more likely to reveal the neural processes underlying drug-motivated behavior. In these experiments, we assessed changes in mTORC1-signaling pathway proteins in the NAC shell (NACsh) and core (NACc). Cocaine-motivated behaviors have been shown to require proteins that enhance glutamate signaling in NAC MSNs (Anderson et al, 2008; Conrad et al, 2008; Wolf, 2010), so we assessed GluA1 AMPAR subunit and CAMKIIα levels in these brain regions after rapamycin treatment. Importantly, given the potential for mTORC1 inhibition to prevent neuroadaptations that promote synaptic plasticity and memory (Barak et al, 2013; Lin et al, 2014), we also tested whether rapamycin delivered to the NACsh during the cocaine-taking phase might have protracted effects on the expression of addiction-relevant behaviors including relapse-like behavior. We then assessed changes in NAC mTORC1 signaling pathway and GluA1 and CAMKIIα proteins, as they have recently been found to also play a role in cocaine relapse (Anderson et al, 2008).

MATERIALS AND METHODS

General Methods

For details on intravenous and intracranial surgery, drug preparation and behavioral testing equipment, see Supplementary Material (S1).

Self-Administration Training

Rats were trained to respond on the right lever for a cocaine infusion (0.25 mg/0.1 ml i.v. delivered over 4 s; (James et al, 2011a, 2010)) on an FR1 schedule (3 h/day, 5 days/week). Infusions were followed by the illumination of a white cue light above the active lever signaling a 20-s timeout period. Rats were limited to earning a maximum of 20 infusions during these sessions to prevent overdosing. Once stable responding was established ($\pm 10\%$ rewarded responses over three sessions), the 3-h daily sessions were divided into alternating 'drug-available' and 'non-drug-available' periods, as described elsewhere (Brown et al, 2011; Deroche-Gamonet et al, 2004; Kasanetz et al, 2010). Briefly, the 40-min drug-available periods were signaled by a constant 70 dB white noise, whereas the 20-min non-drug-available periods were signaled by constant illumination of the white house-light. Cocaine infusions were only possible during drug-available periods, whereas lever presses during non-drug-available periods were recorded but had no scheduled consequences. An FR1 schedule was employed during the initial drug-available sessions and was then increased to FR5 with unrestricted access to drug. During these sessions, responses on the left 'inactive' lever were recorded but did not result in a drug infusion. Following the FR5 discrimination training sessions, rats underwent intracranial surgery (see Supplementary Material S1). After 7 days recovery from surgery, rats underwent an additional 5 days of cocaine self-administration on the FR5 drug-available/non-drug-available program.

For saline control groups, rats were prepared with an intravenous catheter and received 'yoked' saline infusions over a similar period of time to rats trained to self-administer cocaine. The number and temporal pattern of saline infusions was calculated based on the same parameters averaged across cocaine self-administering rats.

PR Testing

PR testing was conducted under drug-available conditions using the schedule 5, 10, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, 268, 345, 445, 573, 737, 947, 1218, 1566, 2012, 2585 presses required for one infusion. PR testing was performed over 5 h, however, PR sessions were programmed to shut down if a lever press was not recorded within 45 min of a previous lever press.

Spontaneous Locomotor Activity Assay

Locomotor activity was assessed in cocaine-naive rats using previously published procedures (James et al, 2011b). Rapamycin or vehicle was administered 3 h before testing.

Extinction and Cue-Induced Reinstatement Testing

Lever pressing was extinguished in 2 h extinction sessions until responding reached ⩽6 active lever presses over three consecutive sessions. Discriminative stimuli were withheld, along with drug infusions. One day after the last extinction session, rats were tested for reinstatement of drug seeking for 1 h by re-exposing rats to the cues paired with drug availability during self-administration training. Lever presses during reinstatement tests did not result in cocaine infusions but did result in illumination of the white cue light above the active lever (20 s).

Experiment One

Effect of cocaine self-administration on mTORC1 activity in the NAC. A group of rats ($n = 6$) were trained to self-administer cocaine for a period of approximately 5 weeks. A separate group of rats received yoked saline over the same period ($n = 6$). Both groups of rats were killed 24 h after the final self-administration (or yoked) session, and brains were processed for markers of mTORC1 activity in the NAC.

Experiment Two

Effect of i.c.v. rapamycin on PR responding. Rats acquired stable self-administration behavior on an FR1 schedule before being tested on a PR schedule. On days 1–3 of PR testing, baseline PR scores were obtained. On days 4–6, rats received an infusion of rapamycin (12.5 µg/µl, i.c.v., 1 µl/min for 2 min; $n = 10$) or vehicle ($n = 9$) 3 h before PR testing through an injector (28 gauge; Plastics One, VA, USA), which protruded 1 mm below the guide cannulae. The timing and dose of rapamycin was based on previous studies (Cota *et al*, 2008; Neasta *et al*, 2010). The injector remained in place for 60 seconds to ensure diffusion of the injectate. Rats were killed 24 h after the final PR session and brains were processed for markers of mTORC1 activity.

The effect of i.c.v. rapamycin on locomotor activity was assessed in a separate cohort of cocaine-naive rats (Supplementary Material S1). Rats from this group were killed 24 h after the final locomotor test and brains were assessed for markers of mTORC1 activity.

Experiment Three

Effect of intra-NACsh-directed rapamycin infusions on addiction-relevant behaviors. Rats were trained to discriminate between periods of signaled drug availability and non-drug availability, and then underwent cranial surgeries, as described in Supplementary Material S1. To facilitate intra-NACsh infusions, rats were gently restrained, stylets were removed and an injector (20.2 mm; 28 gauge; Plastics One) was inserted into each of the two guide cannulae. Rapamycin ($n = 17$) or vehicle ($n = 17$) was infused at a volume of 2.5 µg/0.5 µl/side over 1 min. For both cannuale, the injector remained in place for an additional 60 s to ensure diffusion of the injectate, after which stylets were replaced. Infusions took place once per day for 5 consecutive days. As rapamycin may interfere with memory consolidation processes when administered before learning (Parsons *et al*, 2006), approximately half of the rats ($n = 8$)

received rapamycin or vehicle 3 h before testing, whereas the remaining half ($n = 9$) received treatment immediately following testing (immediately after the 3 h cocaine self-administration session). Importantly, no differences were observed between these two treatment regimes across all parameters measured (non-drug available responding ($F_{1,14} = 0.03$, $P = 0.98$); PR responding: ($F_{1,14} = 7.12$, $P = 0.32$); cue-induced reinstatement: ($F_{1,9} = 0.60$, $P = 0.97$), data not shown), and as such, data were collapsed across both rapamycin treatment groups.

To assess the effects of intra-NACsh rapamycin treatment on addiction-like behavior, rats were assessed on three well-established behavioral indices of addiction vulnerability (Brown *et al*, 2011; Deroche-Gamonet *et al*, 2004; Kasanetz *et al*, 2010; for timeline, see Figure 4). First, across the 5 days of rapamycin treatment, rats were assessed for active lever responding during signaled periods of non-drug-availability. Second, on the three days following the last treatment session, rats were tested for motivation to obtain drug on a PR schedule identical to *Experiment 2*. Finally, in order to assess the long-term effects of rapamycin, we assessed reinstatement behavior after extinction training by re-exposing rats to cues paired with drug availability during self-administration training. Rats were killed 24 h after reinstatement testing and brains were processed for protein analysis.

We also sought to assess the effect of intra-NACsh treatment on the activity of the mTORC1 pathway in rats with no history of cocaine self-administration but that had similar exposure to the operant environment. To do this, a separate group of rats ($n = 6$) received yoked saline infusions. Rats were killed 3 weeks after the final saline yoking session, which equated to the average time between the final self-administration session and kill in the cocaine-exposed group. Brains from the saline-yoked group were processed for markers of mTORC1 activity in a similar manner to cocaine-exposed rats.

Details of animal kill, western-blot analysis, and statistical analysis are outlined in Supplementary Material S1.

RESULTS

Experiment One

Withdrawal from cocaine self-administration increased indices of mTORC1 activity. Rats trained to self-administer cocaine consumed on average a total of 542.59 (± 24.86) mg/kg of cocaine over the training period. We compared the levels of total and phosphorylated mTOR protein in the NACc and NACsh of all treatment groups 24 h after the last saline or self-administered cocaine infusion. Cocaine self-administration increased both total mTOR ($t(10) = -2.33$, $P = 0.042$) and phosphorylated mTOR levels ($t(10) = -3.43$, $P = 0.006$) in the NACsh. In contrast, there was no effect of cocaine on total or phosphorylated mTOR levels in the NACc (P's > 0.05). Total p70s6k levels in the NACc were significantly elevated in cocaine self-administering rats compared with yoked saline controls ($t(10) = -4.69$, $P < 0.001$). No differences were observed in phospho-p70s6k, GluA1, and CAMKIIα levels in either subregion (Figure 1).
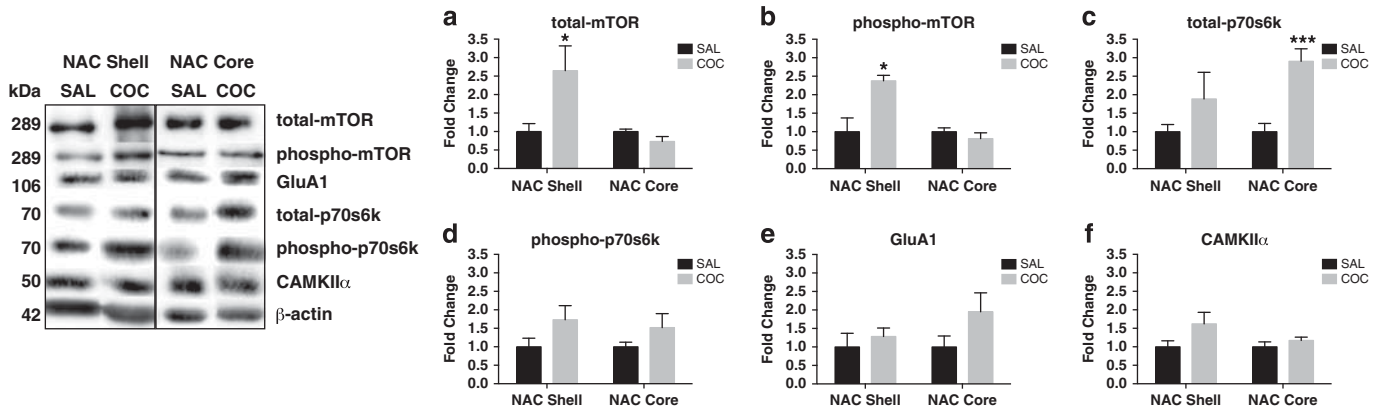
Figure 1 Withdrawal from cocaine self-administration increased indices of mTORC1 activity. Compared with saline controls, rats with a history of cocaine self-administration demonstrated increased total-mTOR (a) and phospho-mTOR (b) in the NACsh but not NACc. Cocaine-exposed rats also demonstrated an increase in total-p70s6k in the NACc (c). No significant changes were observed in phospho-p70s6k (d), GluA1 (e) or CAMKIIα in the NAC (f). Data represent means ± SEM. $*P < 0.05$; $***P < 0.001$. $n = 6$/group.

Experiment Two

Effect of mTORC1 inhibition on motivation to obtain cocaine under PR reinforcement conditions. A comparison of treatment groups revealed no differences in FR5 responding ($P > 0.05$; Figure 2a) or the number of cocaine infusions before rapamycin treatment ($P > 0.05$; data not shown). The effect of i.c.v. rapamycin on motivation to obtain cocaine was then assessed using PR tests over 3 consecutive days. Rapamycin-treated rats exhibited significantly lower break points than vehicle-treated controls on days 2 ($F_{1,16} = 5.03$, $P = 0.043$) and 3 ($F_{1,16} = 5.14$, $P = 0.041$) of PR testing (Figure 2b). Of note, i.c.v. rapamycin treatment was associated with a modest decrease in body weight over the treatment period ($F_{1,16} = 4.65$, $P = 0.047$; Figure 2c).

Effect of mTORC1 inhibition on CAMKIIα and GluA1 levels. In i.c.v.-treated rats, rapamycin significantly reduced total mTOR levels in the NACsh ($t(10) = 2.27$, $P = 0.047$) but not the NACc ($P = 0.13$; Figure 3a). Interestingly, no differences were observed between treatment groups in terms of total p70s6k levels in either NAC subregion (P's > 0.05; Figure 3b), but significantly reduced phosphorylated p70s6k levels were observed in both the NACsh ($t(10) = 11.13$, $P < 0.001$) and NACc ($t(10) = 4.209$, $P = 0.002$) of rapamycin-treated rats (Figure 3c). We also assessed the effects of rapamycin treatment on the expression of synaptic proteins GluA1 and CAMKIIα. Rapamycin treatment reduced GluA1 levels in both the NACsh ($t(10) = 9.05$, $P < 0.001$) and NACc ($t(10) = 3.66$, $P = 0.004$; Figure 3d) and reduced total CAMKIIα levels in the NACsh ($t(10) = 4.76$, $P < 0.001$) and NACc ($t(10) = 2.58$, $P = 0.03$; Figure 3e).

For data outlining the effect of mTORC1 inhibition on CAMKIIα and GluA1 levels and locomotor activity in cocaine naive rats, see Supplementary Material S2.

Experiment Three

Effect of intra-NACsh mTORC1 inhibition on drug seeking in the signaled non-drug-available period and under PR conditions. Details of guide cannulae placement are described in Supplementary Material S3.

As with *Experiment 2*, rats were randomly allocated to treatment groups following self-administration training. Treatment groups did not differ in terms of overall cocaine self-administration ($P = 0.83$, data not shown) or number of days taken to reach the treatment phase ($P = 0.57$, data not shown). Importantly, rats from both groups were able to discriminate between drug-available and non-drug-available cues, as responding was significantly higher in drug-available sessions ($F_{1,26} = 491.09$, $P < 0.001$). Over the treatment period, rapamycin-treated rats did not differ to controls in terms of lever responding during drug-available periods ($P = 0.58$; Figure 4a) nor amount of cocaine consumed ($P = 0.84$, data not shown). However, rapamycin-treated rats exhibited significantly fewer active lever responses in the non-drug-available periods as compared with controls across the treatment period ($F_{1,26} = 6.89$, $P = 0.014$; Figure 4a). Throughout the treatment period, no differences were observed between treatment groups in terms of responding on the inactive lever ($P > 0.05$; data not shown). In addition, rapamycin treatment had no effect on body weight over the treatment period ($P > 0.05$, data not shown).

One day following rapamycin treatment, rats were assessed for motivation to obtain cocaine on a PR schedule. Rapamycin-treated rats exhibited a significantly lower average break point than vehicle-treated controls across the 3 days of PR testing ($t(27) = 2.553$, $P = 0.017$; Figure 4b). A similar analysis revealed no effect of treatment on inactive lever responding ($P > 0.05$, data not shown).

Effect of intra-NACsh mTORC1 inhibition on cue-induced reinstatement of drug seeking. Two rapamycin-treated and two vehicle-treated rats did not complete the entire experimental protocol, precluding collection of extinction and reinstatement data for these rats. All other rats took an average of 20 days to reach the extinction criterion, and this did not differ between treatment groups ($F_{1,25} = 1.841$, $P = 0.188$; Figure 4c). All rats exhibited a significant reinstatement of responding on the active lever following presentation of the drug-available cue during reinstatement testing, as compared with extinction responding. Reinstatement on the active lever was significantly attenuated in
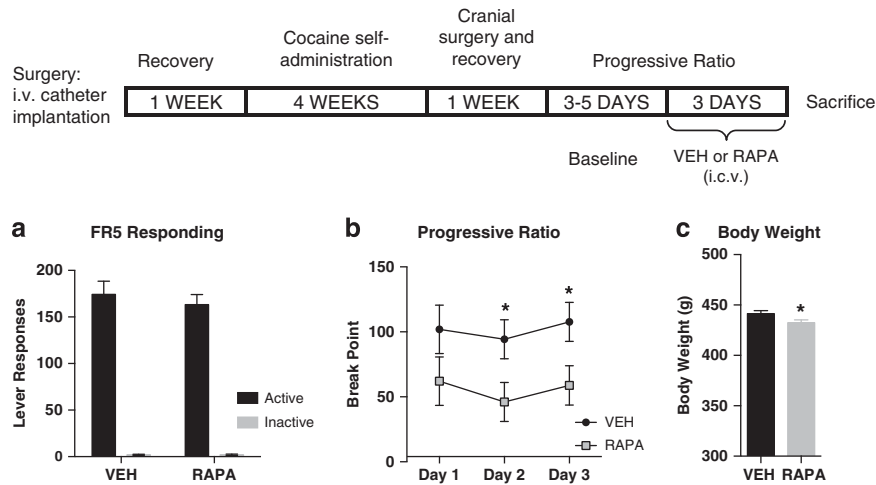
Figure 2 Effect of mTORC1 inhibition on motivation to obtain cocaine under progressive ratio reinforcement conditions. Vehicle- or rapamycin-injected rats did not differ in terms of active or inactive lever responding in cocaine self-administration sessions before treatment (a). Rats that received i.c.v. rapamycin exhibited significantly lower break points on days 2 and 3 of progressive ratio testing as compared with controls (b). i.c.v. administration of rapamycin was associated with modest decreases in body weight on the final day of treatment (c). Data represent means ± SEM. $*P < 0.05$; $***P < 0.001$. $n = 9–10$/group.
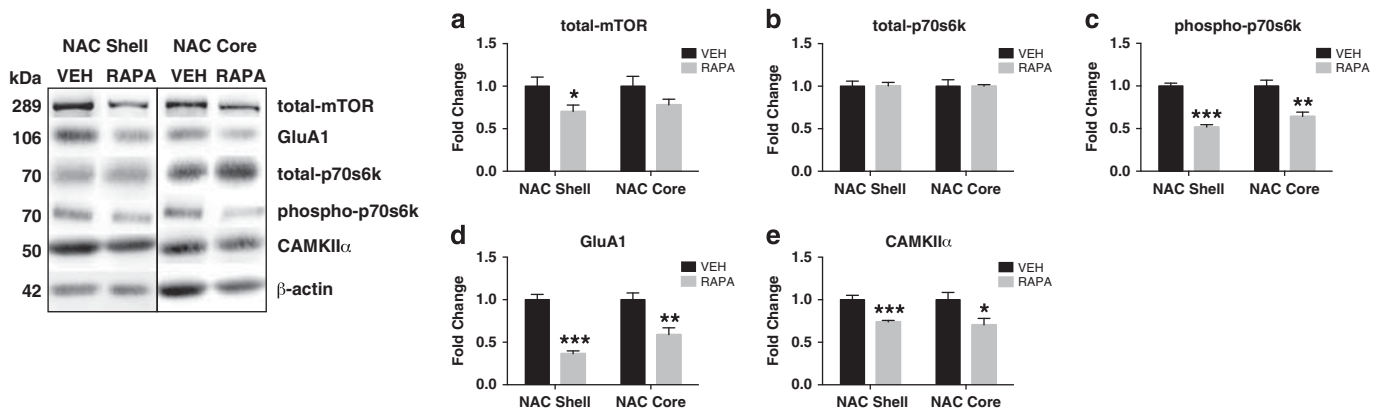

Figure 3 Effect of i.c.v. mTORC1 inhibition on CAMKIIα and GluA1 levels. i.c.v. rapamycin administration significantly attenuated total mTOR levels in the NACsh but not NACc (a). Rapamycin had no effect on total p70s6k levels (b), but significantly reduced phospho-p70s6k levels in both the NACsh and NACc (c). Similarly, rapamycin treatment was associated with reduced GluA1 (d) and CAMKIIα (e) expression in NACsh and NACc. Data represent means ± SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 6$/group.

rapamycin-treated rats, as compared with vehicle-treated controls ($F_{1,25} = 5.929$, $P = 0.023$; Figure 4d). No differences were observed in terms of responding on the inactive lever during reinstatement testing ($P = 0.544$; data not shown).

Effect of intra-NACsh mTOR inhibition on mTORC1 signaling and GluA1 and CAMKIIα levels after reinstatement. Intra-NACsh rapamycin-treated rats displayed no changes in total mTOR or p70s6k levels in either NAC subregion (P's > 0.05; Figure 5a and b). Importantly, however, in rapamycin-treated rats, significantly reduced levels of phosphorylated p70s6k within the NACsh were observed ($t(10) = 3.34$, $P = 0.008$; Figure 5c). Rapamycin treatment was also associated with significantly attenuated total GluA1 ($t(10) = 2.91$, $P = 0.016$) and total CAMKIIα ($t(10) = 2.66$, $P = 0.024$) levels in the NACsh, but had no effect on these parameters in the NACc (P's > 0.05; Figure 5d and e).

For data outlining the effect of mTORC1 inhibition on CAMKIIα and GluA1 levels and locomotor activity in cocaine naive rats, see Supplementary Material S4.

DISCUSSION

In the present study, we found evidence of increased mTORC1 activity in the NAC after withdrawal from cocaine self-administration. Further, we show that mTORC1 inhibition using rapamycin reduced the motivation to lever press for cocaine under PR conditions, with these effects being linked to a reduction in GluA1 AMPARs and CAMKIIα in the NAC. Importantly, in a separate experiment where we tracked the effect of mTORC1 inhibition on the expression of addiction-relevant behaviors over time, we found that intra-NACsh rapamycin did not alter cocaine self-administration under FR5 conditions. In contrast, in the week
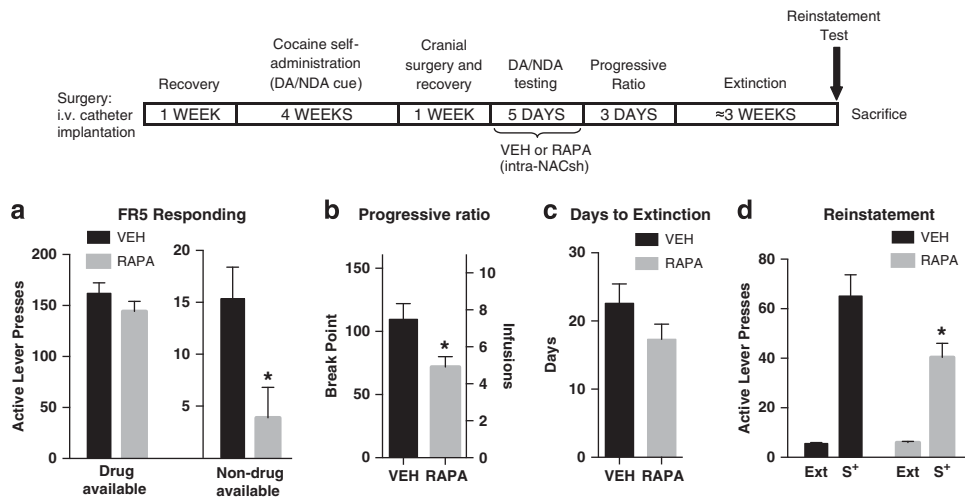
Figure 4 Effect of intra-NAC mTORC1 inhibition on drug-seeking and taking behaviors and progressive ratio responding. In both treatment groups, responding during periods of signaled drug availability was significantly higher than during periods of signaled non-drug availability. Treatment groups did not differ in their responding during periods of drug availability. However, intra-NACsh rapamycin treatment significantly reduced responding during periods of signaled non-drug-availability (a). In the week following treatment, rats that received intra-NACsh rapamycin exhibited significantly reduced break points (b). Treatment groups did not differ in terms of days taken to reach the extinction criterion (c). Rapamycin-treated rats exhibited significantly lower levels of cue-induced reinstatement of extinguished cocaine seeking, despite treatment occurring approximately 4 weeks prior (d). Data represent means ± SEM. *$P < 0.05$. $n = 14-15$/group. DA, Drug-available; NDA, Non-drug-available; S$^+$, Presentation of 'drug-available' discriminative stimulus.
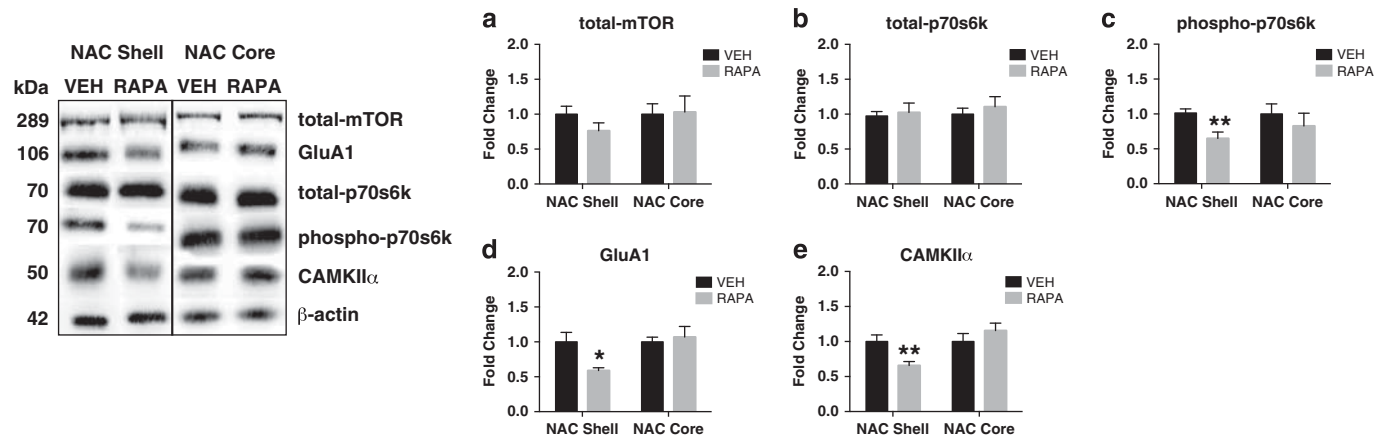

Figure 5 Effect of intra-NACsh mTORC1 inhibition on cue-induced reinstatement and CAMKIIα and GluA1 levels. Intra-NACsh vehicle- or rapamycin-treated rats did not differ in terms of total mTOR levels in the NAC (a). Similarly, total p70s6k levels did not differ between groups (b), but rapamycin treatment resulted in reduced phospho-p70s6k in the NACsh (c). Interestingly, rapamycin treatment resulted in reduced total GluA1 (d) and CAMKIIα (e) levels in the NACsh, but had no effect on these parameters in the NACc. Data represent means ± SEM. *$P < 0.05$; **$P < 0.01$. $n = 6$/group.

following treatment, intra-NACsh rapamycin reduced drug seeking during periods of signaled non-drug-availability and attenuated motivation to lever press for cocaine on a PR schedule. Most notably, a 40% reduction in cue-induced reinstatement of drug seeking was observed in rapamycin-treated rats, despite the fact that treatment ceased up to 4 weeks before testing. The protracted rapamycin-induced reductions in drug-seeking behavior were accompanied by biochemical evidence of reduced mTORC1 activity and reduced total GluA1 and CAMKIIα levels. These data suggest that mTORC1 reduces the translation of synaptic plasticity proteins in the NAC, an effect that 'protects' against the expression of drug-seeking behavior.

Effect of Cocaine Withdrawal on Indices of mTORC1 Activity

Based on changes in the levels of phospho-mTOR, our study indicates that after 24 h of cocaine withdrawal, mTORC1 activity is increased in the NACsh. These findings are highly consistent with the report of Neasta et al (2010), who showed that markers of mTORC1 activity were increased after withdrawal from alcohol. It is possible that changes in mTORC1 activity manifest on a different time scale in the NACsh versus NACc. Further work will be required to understand the significance of these findings given the different roles ascribed to NAC core in shell in drug-motivated behavior (Ito et al, 2004).

Intra-cerebroventricular mTORC1 Inhibition Reduces PR Responding and GluA1 and CAMKIIα Levels

In accordance with findings of Lu and colleagues (Wang *et al*, 2010b), we found that cocaine self-administration (FR5) was unaffected by intra-NAC rapamycin injections (*Experiment 3*). However, i.c.v. rapamycin reduced lever pressing for cocaine when PR conditions were required to obtain cocaine infusions. To date, data supporting the role for mTORC1 in psychostimulant reinforcement has been limited to CPP and sensitization studies (Bailey *et al*, 2012; Narita *et al*, 2005). However, mTORC1 inhibition has been reported to reduce low-effort alcohol drinking in mice and FR1 alcohol self-administration in rats (Neasta *et al*, 2010). With respect to biochemical changes induced by i.c.v. rapamycin, we found significant reductions in indices of mTORC1 activity and GluA1 AMPARs and CAMKIIα within the NAC. The magnitude of these effects was similar to previous studies (Barak *et al*, 2013; Neasta *et al*, 2010). These findings are interesting given that dopamine D1-like agonists increase AMPAR insertion into NAC MSNs through a process involving CAMKIIα (Anderson *et al*, 2008; Sun *et al*, 2008) and that both dopamine and CAMKIIα have been shown to be important for the expression of psychostimulant sensitization and drug-seeking behavior (Licata and Pierce, 2003; Loweth *et al*, 2008; Pierce *et al*, 1998). In fact, lentiviral-mediated knockdown of CAMKIIα in the NACsh reduced motivation to self-administer cocaine on a PR schedule (Wang *et al*, 2010a). Collectively, these data support a hypothesis in which mTORC1 acts as a key effector in the molecular pathway controlling the dynamic regulation of synaptic proteins within the NAC that are required for the expression and maintenance of drug reward. However, we cannot rule out effects of mTORC1 inhibition after i.c.v. rapamycin in other parts of the reward circuit, including the VTA (Mameli *et al*, 2007).

Inhibition of mTORC1 in the NACsh Reduces Drug-Seeking Behavior Including Reinstatement

We found that mTORC1 inhibition in the NACsh significantly reduced lever-pressing behavior during periods of signaled non-drug-availability—a putative measure of compulsive drug seeking. Further, in the week following rapamycin, treated rats demonstrated reduced PR break points. These findings are in line with our i.c.v. rapamycin data and support a role for mTORC1 in the neural processes that are invoked to elevate motivational performance. Importantly, after extinction training, rapamycin-treated rats demonstrated close to a 40% reduction in reinstatement of cocaine seeking. Notably, the time to reach the extinction criterion did not differ between vehicle and treatment groups, indicating that there was no impairment in extinction learning produced by prior rapamycin injections.

The exact mechanism responsible for the reduction in reinstatement behavior exhibited after rapamycin treatment is unclear. However, our data showing reduced synaptic protein levels are interesting on several levels. Cocaine seeking has been shown to require an increase in translation, trafficking, and signaling through GluA1 AMPARs in the NAC, and CAMKIIα has a well-established role in this

process (Anderson *et al*, 2008; Conrad *et al*, 2008; Ferrario *et al*, 2011; Lisman *et al*, 2012; Opazo *et al*, 2010). Given the functional link between these glutamate signaling molecules and reinstatement, the protective effects of intra-NACsh rapamycin on drug seeking might be mediated by a disruption in withdrawal-induced increases in synaptic GluA1s that increase the propensity for drug seeking (Anderson *et al*, 2008; Conrad *et al*, 2008; Mameli *et al*, 2007; Wolf, 2010). An important additional consideration is the possibility that rapamycin treatment disrupted associative memory processes during the drug-taking phase. For example, an important recent study identified a role for mTORC1 in alcohol-cue memory reconsolidation (Barak *et al*, 2013). Further, intra-amygdala or hippocampal rapamycin suppresses the consolidation and reconsolidation of fear memories (Blundell *et al*, 2008; Parsons *et al*, 2006) and mTORC1 is required for cocaine-induced plasticity in VTA dopamine neurons (Mameli *et al*, 2007).

Effect of mTORC1 Inhibition in Cocaine Naïve Rats

To assess the effects of rapamycin on mTORC1 signaling in cocaine naive rats, we performed biochemistry on NAC tissue in rats that received i.c.v. or intra-NAC rapamycin at equivalent time points to cocaine-trained rats. Although subtle differences in mTORC1 signaling were observed between i.c.v.- and intra-NAC rapamycin-treated cocaine naive controls, in general these experiments revealed that rapamycin treatment produced a generalized perturbation in mTORC1 activity and consequently GluA1 and CAMKIIα levels.

A particularly interesting observation in the present study was the effect of rapamycin on mTORC1 activity seen more than 1 month after treatment. These effects were more pronounced in cocaine-treated rats. The apparent long-term effects of rapamycin on mTORC1 activity might involve rapamycin-induced reductions in mTOR complex 2 and consequently AKT activity or other compensatory changes in this signaling pathway produced by sub-chronic rapamycin (Huang *et al*, 2013). Indeed, acute but not chronic rapamycin is known to reduce mTOR complex 2-induced phosphorylation of AKT at Ser473 (Huang *et al*, 2013). However, because cocaine appears to upregulate mTORC1 activity, a more likely explanation for the reductions in mTORC1 activity and addition-relevant behaviors after rapamycin treatment is a restoration of *normal/baseline* function in this signaling pathway.

Notably, rapamycin treatment did not affect locomotor activity or produce other non-specific effects on baseline motivational performance (ie, FR5 cocaine responding), findings that support recent demonstrations that rapamycin does not affect responding for a natural rewards (Neasta *et al*, 2010; Wang *et al*, 2010b). Thus, reductions in baseline mTORC1 in drug naive rats may be insufficient to alter general motivation status.

CONCLUSIONS

The present study extends our understanding of the role of mTORC1 in the neural processes that control the expression and maintenance of drug reward, including protracted

vulnerability to reinstatement. These findings are significant given the recent demonstrations that mTORC1 effectively reduces alcohol-motivated behaviors in rats (Barak *et al*, 2013; Neasta *et al*, 2010) and that acute systemic rapamycin suppressed cue-induced drug craving in abstinent heroin addicts (Shi *et al*, 2009). Although it is important to note the need to directly test the hypothesis that the rapamycin-induced reductions in mTORC1 activity and glutamate signaling molecules suppressed cocaine-seeking behavior, the present study indicates that mTORC1 inhibition can have long-lasting protective effects against drug-seeking behavior.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)