UCSF

UC San Francisco Previously Published Works

Title

A new generation of mTORC1 inhibitor attenuates alcohol intake and reward in mice.

Permalink

<https://escholarship.org/uc/item/46p731rg>

Journal

Addiction biology, 23(2)

ISSN

1355-6215

Authors

Morisot, Nadege
Novotny, Christopher J
Shokat, Kevan M
et al.

Publication Date

2018-03-01

DOI

10.1111/adb.12528

Peer reviewed

A new generation of mTORC1 inhibitor attenuates alcohol intake and reward in mice

Nadege Morisot¹, Christopher J. Novotny², Kevan M. Shokat² & Dorit Ron¹

Department of Neurology, University of California, San Francisco, CA USA¹ and Howard Hughes Medical Institute and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA USA²

ABSTRACT

Alcohol use disorder (AUD) is a chronic condition associated with devastating socioeconomic consequences. Yet, pharmacotherapies to treat behavioral phenotypes such as uncontrolled heavy drinking are limited. Studies in rodents suggest that the mammalian target of rapamycin complex 1 (mTORC1) plays an important role in mechanisms underlying alcohol drinking behaviors as well as alcohol seeking and relapse. These preclinical evidence suggest that mTORC1 may be a therapeutic target for the treatment of AUD. Thus, the aim of the present study was to test the potential use of newly developed mTORC1 inhibitors, RapaLink-1 and MLN0128, in preclinical mouse models of AUD. First, we used the intermittent access to 20 percent alcohol in a two-bottle choice paradigm and tested the efficacy of the drugs to reduce alcohol intake in mice with a history of binge drinking and withdrawal. We found that both inhibitors reduce excessive alcohol intake and preference with RapaLink-1 exhibiting higher efficacy. We further observed that RapaLink-1 attenuates alcohol consumption during the first alcohol-drinking session in naïve mice, and interestingly, the effect was still present 14 days after the initial treatment with the drug. We also found that RapaLink-1 did not alter the consumption of water or saccharin, revealing a specific effect of the inhibitor on alcohol intake. Finally, we report that RapaLink-1 blocks the retrieval but not acquisition of alcohol place preference without affecting locomotion. Together, our findings suggest that RapaLink-1 may be developed as a new medication to treat and prevent the development of AUD.

Keywords alcohol, mTORC1, RapaLink-1.

Correspondence to: Dorit Ron, Department of Neurology, University of California, 675 Nelson Rising Lane, BOX 0663, San Francisco, CA 94143-0663, USA. E-mail: dorit.ron@ucsf.edu

INTRODUCTION

Alcohol use disorder (AUD) is a major public health problem with about 11 percent of worldwide alcohol consumers engaging in weekly heavy episodic drinking (WHO 2014). AUD phenotypes such as excessive and compulsive drinking are thought to result from, and are maintained by, aberrant forms of learning and memory (Hyman, Malenka & Nestler 2006; Torregrossa, Corlett & Taylor 2011). Alcohol promotes learning of environmental stimuli associated with its rewarding properties, while suppressing learning about the adverse consequences of intake (Torregrossa *et al.* 2011). As a result, individuals with an alcohol drinking problem spend an increasing amount of time obtaining and consuming alcohol (APA 2013). The progression of the disease also parallels the transition from goal-directed

alcohol seeking to habitual and compulsive alcohol intake (Everitt & Robbins 2016). Growing literature indicates that AUD and normal learning and memory processes share common neuronal mechanisms (Hyman *et al.* 2006; Torregrossa *et al.* 2011). Thus, targeting molecular participants of such processes may represent a potential strategy for the treatment of the disorder.

An important molecular transducer of learning and memory processes is the mammalian target of rapamycin (mTOR) complex 1 (mTORC1). mTORC1 is a multiprotein complex consisting of the serine and threonine kinase mTOR (Zoncu, Efeyan & Sabatini 2011). mTORC1 plays an essential role in various cellular processes including cell growth, proliferation, autophagy and protein synthesis (Wullschleger, Loewith & Hall 2006; Zoncu *et al.* 2011). In the central nervous system, mTORC1 promotes the dendritic translation of proteins initiating

synaptic plasticity processes and learning and memory (Buffington, Huang & Costa-Mattioli 2014; Santini, Huynh & Klann 2014). Dysregulation of mTORC1 signaling contributes to various disease states including cancer, autoimmune disorders, neurodegenerative diseases, autism and addiction (Wullschleger *et al.* 2006; Lipton & Sahin 2014; Neasta *et al.* 2014; Santini *et al.* 2014).

Accumulating evidence reveal that mTORC1 plays a central role in mechanisms underlying AUD (Neasta *et al.* 2014). Specifically, genetic variants in mTOR signaling-related genes predict heavy consumption in humans (Meyers *et al.* 2015). In rodents, excessive alcohol drinking promotes mTORC1 activation in the nucleus accumbens (NAc) and orbitofrontal cortex (OFC) (Neasta *et al.* 2010; Beckley *et al.* 2016; Laguesse *et al.* 2016), and the recall of alcohol memories triggered by re-exposure to the smell and taste of alcohol activates mTORC1 in the OFC, the prelimbic cortex and central nucleus of the amygdala (CeA) (Barak *et al.* 2013). Activation of mTORC1 by alcohol in the NAc initiates the translation of synaptic proteins, producing long-lasting synaptic and structural modifications that in turn drive alcohol drinking phenotypes (Neasta *et al.* 2010; Beckley *et al.* 2016; Liu *et al.* 2016).

Much of the information regarding the potential role of mTORC1 in AUD stemmed from the use of rapamycin, a selective allosteric mTORC1 inhibitor (Dowling *et al.* 2010). Specifically, systemic administration of rapamycin decreases home cage alcohol intake in mice and rats with a history of heavy alcohol drinking (Neasta *et al.* 2010) and reduces 'binge-like' alcohol intake following the first 4-hour drinking episode in mice (Beckley *et al.* 2016). Systemic administration of rapamycin as well as intra-NAc infusion of the drug attenuates rat operant alcohol self-administration and seeking (Neasta *et al.* 2010). Furthermore, systemic as well as Intra-CeA administration of rapamycin disturb the reconsolidation of alcohol-related memories, resulting in reduced alcohol-seeking and taking behaviors (Barak *et al.* 2013). Finally, systemic administration of rapamycin reduces alcohol-induced locomotor sensitization as well as conditioned place preference (CPP; Neasta *et al.* 2010), without producing place aversion or reward (Neasta *et al.* 2010; Barak *et al.* 2013). Rapamycin does not alter water or sucrose intake, nor does it alter the consumption of quinine and does not affect ambulatory activity or motor coordination (Neasta *et al.* 2010; Barak *et al.* 2013). Together, these data strongly suggest that mTORC1 is a key mediator of molecular adaptations and behavioral phenotypes associated with AUD. Thus, targeting mTORC1 appears to be a very promising therapeutic approach for the treatment of AUD.

Rapamycin is an FDA-approved drug that is used in the clinic for the prevention of organ rejection after

transplantation and has numerous beneficial effects for the treatment of cancer, neurodegenerative diseases and aging (Guertin & Sabatini 2007; Bove, Martinez-Vicente & Vila 2011; Li, Kim & Blenis 2014). However, rapamycin suffers from several limitations. First, the drug is an immunosuppressant (Li *et al.* 2014). In addition, rapamycin has poor solubility and pharmacokinetic profile (Li *et al.* 2014). Furthermore, rapamycin does not block a subset of mTORC1-dependent processes (Shor *et al.* 2009; Li *et al.* 2014), and some tumors in which mTORC1 is hyperactivated are resistant to the drug (Ducker *et al.* 2014; Rodrik-Outmezguine *et al.* 2016). To overcome these issues, second and third generations of mTORC1 inhibitors have been developed. These include MLN0128, which blocks the catalytic domain of mTOR (Hsieh *et al.* 2012), and RapaLink-1, in which rapamycin and an mTOR kinase inhibitor are covalently linked, allowing simultaneous inhibition and a long-lasting full blockade of mTORC1 (Rodrik-Outmezguine *et al.* 2016). Both RapaLink-1 and MLN0128 effectively cross the blood–brain barrier and block mTORC1 activity in *in vivo* models of brain cancer (Fan *et al.* 2017). Thus, the present study was aimed at testing the efficacy of MLN0128 and RapaLink-1 in preclinical mouse models of AUD.

MATERIAL AND METHODS

Animals

Male C57BL/6J and DBA/2J mice (Jackson Laboratory, Bar Harbor, ME, USA) were 8–9 weeks old at the beginning of the experiment. C57BL/6J mice were individually housed under a reversed 12-hour light/dark cycle (lights on at 10:00 PM), and DBA/2J mice were group-housed (four per cage) under normal 12-hour light/dark cycle (lights on at 07:00 AM). Housing rooms were temperature-controlled and humidity-controlled, and food and water were available *ad libitum*. The mice were weighed once a week and again prior to receiving drug treatment. All animal procedures were approved by the University of California San Francisco Institutional Animal Care and Use Committee and were conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care.

Reagents

RapaLink-1 was synthesized as described in Rodrik-Outmezguine *et al.* (2016). MLN0128 was purchased from Tocris Biosciences (Minneapolis, MN, USA). Ethyl alcohol (190 proof) was purchased from VWR (Randor, PA, USA). Dimethyl sulfoxide and saccharin were purchased from Sigma Aldrich (St. Louis, MO, USA). Polyethylene glycol 300 (PEG300) was obtained from Spectrum Chemical (Gardena, CA, USA), and Tween80 was purchased from Fisher Scientific (South San Francisco, CA, USA).

Drug preparation

RapaLink-1 (0.75–1.5 mg/kg) and MLN0128 (1.5 mg/kg) were dissolved in 5 percent dimethyl sulfoxide, 5 percent PEG300 and 5 percent Tween 80 in distilled water, which constitutes the vehicle solution. Drug and vehicle solutions were injected intraperitoneum (i.p.) at a volume of 10 ml/kg. Alcohol solution was prepared from ethyl alcohol solution (190 proof) that was diluted to 20 percent v/v in tap water (voluntary intake) or in 0.9 percent saline (systemic administration). Saccharin (0.03 percent w/v) was prepared in tap water.

Voluntary alcohol intake

Voluntary consumption of alcohol was conducted as described in Warnault *et al.* (2013). C57BL/6J mice had access to one bottle containing 20 percent alcohol and

one bottle of water (two-bottle choice (2 BC)) in their home-cage. Animals were subjected to an intermittent access (IA) 2 BC paradigm for 8 weeks during which the mice had 24-hour access to a bottle of water and a bottle of 20 percent alcohol on Monday, Wednesday and Friday with 24 or 48 hours of alcohol deprivation sessions in between during which the mice had access to water only Fig. 1a. The mice drank significantly more on week 8 as compared with week 1 (week 1: 14.58 ± 0.56 ; week 8: 16.92 ± 0.46 ; $t_{20} = 2.52$, $P < 0.05$, $n = 21$).

Drug administration

Mice were systemically administered with vehicle, RapaLink-1 (0.75 mg/kg) or MLN0128 (1.5 mg/kg) 3 hours prior to the beginning of a 24-hour drinking session, a time point that was previously used to test the effect of rapamycin on alcohol-dependent behaviors

Figure 1 RapaLink-1 and MLN0128 reduce alcohol intake and preference in mice with a history of excessive alcohol drinking. (a) Schematic depiction of alcohol drinking paradigm and drug administration. (b,c) Vehicle, RapaLink-1 (0.75mg/kg), or MLN0128 (1.5mg/kg) were administered systemically (i.p.) 3 hours before the beginning of the 24-hour alcohol-drinking session. Alcohol (g/kg) (bi, ci) and water (ml/kg) (biii, ciii) intake were measured 4 hours (b) and 24 hours (c) after the beginning of the drinking session. Alcohol preference (bii, cii) was calculated as the ratio of consumed alcohol vs. total fluid (alcohol+water). Data are presented as mean±SEM. (c) $n=21$ vehicle, $n=20$ RapaLink-1 and MLN0128. (c) $n=20$ per treatment. * $p < 0.05$ and ** $p < 0.001$ vs. vehicle, Newman Keuls' *post hoc* test.

(Neasta et al. 2010; Barak et al. 2013; Beckley et al. 2016). Specifically, vehicle or drug was administered at 9 AM, drinking sessions started at 12 PM, and alcohol and water intake were measured 4 and 24 hours later. The 4-hour time point was chosen because the mice drink about 30 percent of the total alcohol consumed during the first 4 hours of the 24-hour session (Morisot and Ron, unpublished observation). All subjects received each drug treatment in a counterbalanced manner with one injection per week. An independent cohort of C57BL/6J mice received a single systemic injection of RapaLink-1 (0.75 mg/kg) or vehicle 3 hours prior to the beginning of the first 4-hour alcohol drinking session Fig. 2a.

Figure 2 RapaLink-1 produces a long-lasting attenuation of binge drinking in alcohol naïve mice. (a) Schematic representation of alcohol intake paradigm and drug administration. (b) Alcohol intake (g/kg), (c) preference and (d) water intake (ml/kg) were measured at the end of each of the 4-hour drinking sessions. Data are presented as mean $\pm$ SEM. $n = 7$ vehicle, $n = 6$ RapaLink-1. * $P < 0.05$ versus vehicle, Newman Keuls' post hoc test

Saccharin intake

Mice were treated with RapaLink-1 (0.75 mg/kg) or vehicle as described in the preceding texts but had access to 0.03 percent saccharin instead of alcohol (Fig. 3a). Fluid (saccharin and water) consumption was measured at the end of the drinking session.

Conditioned place preference paradigm

The CPP paradigm (Fig. 4a) was conducted as described in Neasta et al. (2010). The testing apparatus (Columbus Instrument) consists of a rectangular Plexiglas box (length 42 cm, width 21 cm and height 21 cm) divided by a central partition into two equal compartments equipped with horizontal photo beams. The compartments differ by the wall color (black versus white) and the texture of the translucent grip liner that covers the floor (ribbed versus raised-dot pattern). During the conditioning phase, the individual compartments are closed off from each other. During the test sessions, the central partition is elevated 4 cm above the floor of the apparatus, allowing the mice to move freely in both compartments. The time spent in each of the two compartments of the apparatus is quantified by an automated system (Optomax, Columbus Instrument). CPP testing was conducted during the light phase of the 12-hour light/dark cycle in a quiet room dimly illuminated (30 lux). On day 1 (pre-conditioning test), DBA/2J mice were allowed to freely explore the entire CPP apparatus for 15 minutes. The mice were then

Figure 3 RapaLink-1 does not affect saccharin intake and preference. (a) Schematic presentation of saccharin drinking paradigm and drug administration. (b) Saccharine intake (ml/kg/4 hours), (c) preference and (d) water intake (ml/kg/4 hours) were measured at the end of the drinking sessions. Data are represented as mean $\pm$ SEM. $n = 7$ vehicle, $n = 7$ RapaLink-1

Figure 4 RapaLink-1 reduces alcohol-induced conditioned place preference. (a) Mice were daily (6 days) conditioned to receive a systemic injection of alcohol (1.8 g/kg, i.p., 'Alc') or saline solution ('Sal') in the drug-paired or unpaired compartment of the CPP apparatus, respectively. A control group received saline conditioning only. On the day following the last conditioning session, saline-conditioned or alcohol-conditioned mice were treated with vehicle or RapaLink-1 (0.75 mg/kg, i.p.). Three hours later, the post-conditioning test 1 was carried out. On the following day, animals underwent the post-conditioning test 2: Mice that received vehicle on post-conditioning test 1 were treated with RapaLink-1 (1.5 mg/kg) on the post-conditioning day 2 and vice versa. CPP score expressed as percentage of time spent by the mice in the drug-paired compartment on the pre-conditioning test (b), post-conditioning test 1 (c) and 2 (d). (e) Ambulatory activity is expressed as number of infrared beam breaks by vehicle-treated and RapaLink-1 (1.5 mg/kg)-treated mice, during the post-conditioning day 2. (f) An independent experiment was conducted as described in (a), except that mice were treated with vehicle or RapaLink-1 (1.5 mg/kg) prior to the first drug-paired conditioning. CPP score on the pre-conditioning (g) and post-conditioning (h) test was calculated as in (a). Data are represented as mean $\pm$ SEM. (b and c) $n = 8$ Sal/vehicle, $n = 8$ Sal/RapaLink-1, $n = 7$ Alc/vehicle and $n = 9$ Alc/RapaLink-1. (d) $n = 8$ Sal/vehicle, $n = 8$ Sal/RapaLink-1, $n = 9$ Alc/vehicle and $n = 7$ Alc/RapaLink-1. (e) $n = 17$ vehicle and $n = 15$ RapaLink-1. (g and h) $n = 5$ Sal/vehicle, $n = 6$ Sal/RapaLink-1, $n = 7$ Alc/vehicle and $n = 8$ Alc/RapaLink-1. $###P < 0.0005$, main conditioning effect. $*P < 0.05$ versus all other groups, Newman Keuls' *post hoc* test

pseudo-randomly assigned to receive saline or alcohol conditioning. The groups were counterbalanced based on pre-conditioning time values in the two compartments. Conditioning training consisted of six daily sessions (days 2–7) during which the mice were confined to one of the compartments for 5 minutes

immediately following an i.p. injection of saline or 1.8 g/kg of alcohol, a dose that produces a robust CPP response in DBA/2J mice (Neasta *et al.* 2010). On days 2, 4 and 6, mice received a saline injection prior confinement in the 'drug-unpaired' compartment. On days 3, 5 and 7, mice were administered with saline

(saline conditioning group) or alcohol (alcohol conditioning group) and were then confined to the 'alcohol-paired' compartment. On day 8 (post-conditioning test), mice were allowed to freely explore the entire CPP apparatus for 15 minutes. CPP score was calculated as the percentage of time spent in the alcohol or saline-paired compartment during the pre-conditioning or post-conditioning tests.

To test whether RapaLink-1 affects the retrieval of alcohol associative memories, on day 8, mice were pseudo-randomly assigned to receive systemic administration of vehicle or RapaLink-1 (0.75 mg/kg) 3 hours prior to the post-conditioning test 1. On day 9, mice that previously received RapaLink-1 were treated with vehicle, whereas mice that were previously assigned to the vehicle group received an injection of RapaLink-1 (1.5 mg/kg) 3 hours prior to the post-conditioning test 2 (Fig. 4a).

To test whether RapaLink-1 prevents the formation of alcohol associative memories, another cohort of DBA/2J mice were pseudo-randomly assigned to receive systemic administration of vehicle or RapaLink-1 (1.5 mg/kg) 3 hours prior to the first confinement to the 'alcohol-paired' compartment (day 3; Fig. 4f).

Data analysis

Drinking volumes were corrected for spillage by deducting the average loss of fluid from bottles placed in an empty cage. Measurements of twofold above the average consumption data were excluded from the analysis. A one-way or two-way analysis of variance (ANOVA) was used for analysis of the drinking data with drug treatment as a between-subject factor and post-injection days as a within-subject factor when appropriate. A two-way ANOVA was used to examine the CPP score with conditioning and drug treatment as between-subject factors. Newman Keul's post-hoc test was used for individual group comparison. Statistical significance was set at $P < 0.05$.

RESULTS

RapaLink-1 and MLN0128 reduce excessive alcohol drinking

First, we tested whether similar to rapamycin (Neasta *et al.* 2010), the new generation of mTORC1 inhibitors MLN0128 and RapaLink-1 alter alcohol drinking in mice with a history of long-term heavy alcohol consumption. Mice first underwent IA-2 BC for 8 weeks. On test day, vehicle, RapaLink-1 (0.75 mg/kg) or MLN0128 (1.5 mg/kg) was systemically administered 3 hours prior to the beginning of a drinking session and alcohol intake

was measured following a 4-hour binge drinking session and at the end of a 24-hour period (Fig. 1a). Dosage of drugs was based on previous studies (Slotkin *et al.* 2015; Rodrik-Outmezguine *et al.* 2016). Because RapaLink-1 at the dose of 1.5 mg/kg affects body weight in mice (Rodrik-Outmezguine *et al.* 2016), we initially tested a lower dose (0.75 mg/kg). The dose of MLN0128 was chosen based on the dosing range used in Slotkin *et al.* (2015).

Treatment of mice with both MLN0128 and RapaLink-1 produced a significant reduction of alcohol intake at the 4-hour time point (Fig. 1bi, one-way ANOVA: $F_{2,58} = 4.41$, $P < 0.05$) and preference (Fig. 1bii, one-way ANOVA: $F_{2,58} = 3.98$, $P < 0.05$) without affecting water intake (Fig. 1biii, one-way ANOVA: $F_{2,58} = 0.18$, $P = 0.83$). However, the drugs exhibited different profiles when alcohol intake (Fig. 1ci, one-way ANOVA: $F_{2,57} = 8.85$, $P < 0.001$), preference (Fig. 1cii, one-way ANOVA: $F_{2,57} = 6.19$, $P < 0.05$) and water intake (Fig. 1ciii, one-way ANOVA: $F_{2,57} = 4.33$, $P < 0.05$) were measured at the end of a 24-hour session. Specifically, RapaLink-1 reduced the consumption of ($P < 0.001$, Fig. 1ci) and preference for ($P < 0.05$, Fig. 1cii) alcohol, without affecting water intake ($P = 0.15$, Fig. 1ciii), compared with the vehicle-treated group. In contrast, MLN0128 did not alter alcohol intake (Fig. 1ci, $P = 0.1$), and because water intake was increased (Fig. 1ciii, $P < 0.05$), alcohol preference was reduced (Fig. 1cii, $P < 0.05$).

Neither RapaLink-1 nor MLN0128 altered alcohol intake 48 hours after drug administration (one-way ANOVA: $F_{2,53} = 2.18$, $P = 0.12$; data not shown). Similar to what we found on the treatment day, MLN0128 reduced alcohol preference (one-way ANOVA: $F_{2,53} = 4.01$, $P < 0.05$; data not shown) by increasing water intake (one-way ANOVA: $F_{2,53} = 5.48$, $P < 0.05$; data not shown) at the end of the 48-hour time point. Together, these results show that RapaLink-1 reduces excessive alcohol consumption throughout the 24-hour drinking session, whereas MLN0128 only attenuated alcohol intake during the first 4-hours of 2 BC access. Moreover, MLN0128 increased water consumption up to 48 hours following drug administration, which could indicate an adverse reaction due to xerostomia and/or an increase in polydipsia (Cosgray *et al.* 1990; Dundas, Harris & Narasimhan 2007). Thus, the remaining of the study focused on RapaLink-1.

RapaLink-1 reduces the initial binge alcohol drinking session and gates excessive alcohol intake during subsequent drinking episodes

We previously found that the first bout of alcohol intake activates mTORC1 in the NAC of mice and that

rapamycin inhibits alcohol drinking during the subsequent drinking sessions (Beckley *et al.* 2016). Thus, we tested whether RapaLink-1 exhibits a similar profile. To do so, alcohol-naïve mice received a single systemic administration of vehicle or RapaLink-1 (0.75 mg/kg), 3 hours prior to the beginning of the initial binge drinking session (Fig. 2a). We found that systemic administration of RapaLink-1 produces a long-lasting reduction of alcohol intake (two-way ANOVA, drug treatment X RM interaction effect: $F_{7,77} = 2.13$, $P < 0.05$; Fig. 2b) and preference (two-way ANOVA, drug treatment X RM interaction effect: $F_{7,77} = 2.12$, $P = 0.05$; Fig. 2c). Specifically, RapaLink-1-treated mice displayed reduced 'binge-like' alcohol intake ($P < 0.05$, Fig. 2b) and preference ($P < 0.05$, Fig. 2c) without affecting water intake (two-way ANOVA, main drug treatment effect: $F_{1,11} = 0.66$, $P = 0.43$; drug treatment X RM interaction effect: $F_{7,77} = 0.96$, $P = 0.45$; Fig. 2d) for up to 14 days compared with vehicle-treated mice. These results reveal that a single administration of RapaLink-1 prevents the onset of alcohol drinking, which is sustained even 14 days after drug administration.

RapaLink-1 does not alter saccharin intake

Next, to verify that the reduction of alcohol intake by RapaLink-1 is not due to changes in palatability or general reward sensitivity, naïve mice received vehicle or RapaLink-1 (0.75 mg/kg) 3 hours prior to the beginning of a 4-hour access to a sweet saccharin solution, and consumption was determined on the initial drinking session as well as the following day (Fig. 3a). We found that RapaLink-1 did not affect saccharin intake (two-way ANOVA, main drug treatment effect: $F_{1,12} = 2.08$, $P = 0.17$; drug treatment X RM effect: $F_{1,12} = 0.01$, $P = 0.91$; Fig. 3b) or preference (two-way ANOVA, main drug treatment effect: $F_{1,12} = 0.40$, $P = 0.54$; drug treatment X RM effect: $F_{1,12} = 0.93$, $P = 0.35$; Fig. 3c). Water intake was also unaltered (two-way ANOVA, main drug treatment effect: $F_{1,12} = 0.28$, $P = 0.60$; drug treatment X RM effect: $F_{1,12} = 0.13$, $P = 0.72$; Fig. 3d). These results reveal the specificity of RapaLink-1's action on alcohol drinking.

RapaLink-1 prevents the retrieval of alcohol place preference

As mTORC1 plays a role in learning and memory (Buffington *et al.* 2014; Santini *et al.* 2014), we determined whether RapaLink-1 affects the maintenance of alcohol-paired contextual memory by utilizing the CPP paradigm (Fig. 4a). Following the pre-conditioning test,

the mice were assigned to four groups with similar basal CPP score (one-way ANOVA: $F_{3,28} = 0.11$, $P = 0.98$; Fig. 4b). After conditioning, mice received vehicle or RapaLink-1 (0.75 mg/kg), and 3 hours later, mice underwent a post-conditioning test 1 (Fig. 4b). We found that while alcohol conditioning produced a strong place preference response (two-way ANOVA, main conditioning effect: $F_{1,28} = 28.78$, $P < 0.0005$), RapaLink-1 (0.75 mg/kg) did not affect alcohol place preference (two-way ANOVA, conditioning X drug treatment effect: $F_{1,28} = 0.24$, $P = 0.62$, Fig. 4c). Next, mice that received vehicle on post-conditioning test 1 were treated with RapaLink-1 (1.5 mg/kg) on the post-conditioning day 2 and mice that received RapaLink-1 (0.75 mg/kg) on post-conditioning test 1 were treated with vehicle on post-conditioning day 2. We found that RapaLink-1 (1.5 mg/kg) differentially affects the CPP score (two-way ANOVA, conditioning X drug treatment effect: $F_{1,28} = 4.94$, $P < 0.05$, Fig. 4d). Specifically, systemic administration of mice with RapaLink-1 (1.5 mg/kg) produced a robust reduction of alcohol place preference compared with alcohol-conditioned mice treated with vehicle ($P < 0.005$; Fig. 4d). Neither the vehicle ($P = 0.62$) nor RapaLink-1 (1.5 mg/kg; $P = 0.87$) affected place preference in the saline-treated mice, suggesting that the drug by itself is not aversive or rewarding. Finally, RapaLink-1 (1.5 mg/kg) did not affect ambulatory activity as measured by the number of infrared beam breaks on post-conditioning day 2 ($t_{30} = 0.86$, $P = 0.39$; Fig. 4e). Thus, RapaLink-1 (1.5 mg/kg) blocks the retrieval of alcohol-associated memory.

RapaLink-1 does not affect the acquisition of alcohol place preference

Finally, we tested whether a single systemic administration of RapaLink-1 (1.5 mg/kg) would be sufficient to prevent the formation of alcohol-paired contextual memory. To do so, mice with similar pre-conditioning CPP score (one-way ANOVA: $F_{3,22} = 0.13$, $P = 0.94$; Fig. 4g) were assigned to receive vehicle or RapaLink-1 (1.5 mg/kg) 3 hours prior to the first (but not prior to the second or third) 'alcohol-paired' conditioning. We found that RapaLink-1 (1.5 mg/kg) did not affect the expression of alcohol place preference (two-way ANOVA, main conditioning effect: $F_{1,22} = 21.12$, $P < 0.0005$; conditioning X drug treatment effect: $F_{1,22} = 0.02$, $P = 0.88$; Fig. 4h). These findings reveal that a single administration of the inhibitor prior the beginning of conditioning training was insufficient to block learning of alcohol place preference, and thus, mTORC1 activation during the first alcohol-context pairing is not essential to the acquisition of alcohol-paired contextual memory.

DISCUSSION

Our data herein suggest that the new generation of mTORC1 inhibitors and specifically Rapalink-1 could be developed as novel therapeutics to treat AUD. Alcohol addiction is characterized by excessive alcohol drinking, repeated cycles of intoxication, withdrawal, craving and relapse (Koob & Volkow 2010; APA 2013). Phenotypes related to the disorder can be modeled by using the IA-2 BC procedure in which animals intermittently drink large amounts of alcohol for a relatively long period of time (i.e. 2 months; Carnicella, Ron & Barak 2014). Using this model, we show that similar to rapamycin (Neasta *et al.* 2010), Rapalink-1 and MLN0128 reduce alcohol intake in mice with a history of long-term heavy alcohol drinking. Interestingly, the profile of drug effects on drinking of Rapalink-1, MLN0128 and rapamycin is different. While the reduction in alcohol consumption in response to Rapalink-1 and rapamycin (Neasta *et al.* 2010) treatment was observed at both the 4 and 24 hours of 2 BC access, MLN0128 attenuated alcohol drinking only at the beginning of the drinking session (i.e. 4-hour time point). In addition, MLN0128 but not Rapalink-1 and rapamycin increased water intake a finding which may compromise its further development of the compound as treatment for AUD. Furthermore, Rapalink-1 reduces alcohol consumption during the first binge-like episode, whereas rapamycin dampens alcohol intake only on the second binge-drinking session (Beckley *et al.* 2016), and Rapalink-1 but not rapamycin (Beckley *et al.* 2016) produces a long-lasting reduction of binge-like alcohol intake that is still observed 2 weeks after the drug administration. It is possible that the three inhibitors differ in their ability to inhibit mTORC1-dependent protein translation, the mechanism by which mTORC1 contributes to alcohol-drinking behaviors (Neasta *et al.* 2010; Beckley *et al.* 2016; Liu *et al.* 2016). Specifically, protein translation requires mTORC1 phosphorylation of the ribosomal machinery proteins eukaryotic translation initiation factor 4E binding protein (4EBP) and S6 kinase (S6K) (Feldman & Shokat 2010; Hsieh *et al.* 2010; Buffington *et al.* 2014), and Rapalink-1 is more potent than MLN0128 and rapamycin at dampening the phosphorylation of 4EBP and S6K *in cells* (Rodrik-Outmezguine *et al.* 2016). Differences in pharmacokinetics among the three inhibitor could be another reason, and, in line with this possibility, Rapalink-1 has a longer half-life than MLN0128 *in cells* (Rodrik-Outmezguine *et al.* 2016). These potential mechanisms require further exploration.

While a single administration of Rapalink-1 prevents the development of excessive alcohol drinking for a relatively long period (i.e. 2 weeks), its ability to reduce

alcohol consumption in mice with a history of long-term excessive alcohol drinking was apparent 24 hours but not 48 hours following treatment. The difference in duration of Rapalink-1's actions could be explained by the different time course of mTORC1 activation by alcohol. Specifically, we previously found that the level of mTORC1 activation returns to baseline 24 hours following a single binge session of alcohol drinking (Beckley *et al.* 2016), whereas alcohol-induced mTORC1 activation was still present 24 hours following the last drinking session (Neasta *et al.* 2010; Laguesse *et al.* 2016; Liu *et al.* 2016). Furthermore, chronic alcohol intake produces long-lasting neuroadaptations that are harder to overcome as compared with short-term initial molecular alterations induced in response to the first bout of alcohol intake (Beckley *et al.* 2016). Thus, attenuation of the short-term alcohol-induced adaptations by Rapalink-1 may be sufficient to inhibit alcohol drinking for a longer duration of time.

Alcohol-related memories are powerful drivers of alcohol seeking and relapse (Seo & Sinha 2014). We previously showed that mTORC1 contributes to the reconsolidation and retrieval of alcohol-associated memories (Neasta *et al.* 2010; Barak *et al.* 2013; Neasta *et al.* 2014). Herein, we assessed whether Rapalink-1 could prevent the retrieval and learning of alcohol-paired contextual memory. We found that Rapalink-1 inhibited the expression of alcohol place preference, raising the possibility that Rapalink-1 abolishes the retrieval of alcohol-associated memories.

We found that administration of Rapalink-1 at a dose of 1.5 but not 0.75 mg/kg was sufficient to block the expression of alcohol place preference. Interstrain difference may explain why a higher dose of Rapalink-1 is required to block alcohol CPP compared with alcohol drinking. Specifically, we used C57BL/6J mice that display a high level of alcohol intake to assess the efficacy of mTORC1 inhibitor on alcohol consumption and DBA/2J mice that exhibit high alcohol CPP (Cunningham *et al.* 1992; Yoneyama *et al.* 2008) to study the effect of mTORC1 inhibition on alcohol reward processes. We previously found that systemic administration of alcohol (2 g/kg, i.p.) promotes a higher level of mTORC1 activation in the NAc of DBA/2J as compared with C57BL/6J mice (Neasta *et al.* 2010). Therefore, it is plausible that a higher dose of the inhibitor may be required to prevent alcohol-dependent mTORC1 activation in DBA/2J as compared with C57BL/6J mice.

We also showed herein that a single treatment with Rapalink-1 (1.5 mg/kg) administered prior to the first alcohol conditioning session did not affect acquisition of alcohol place preference, revealing intact learning of alcohol-related memory. However, we cannot exclude the possibility that Rapalink-1 treatment prior to each

alcohol conditioning would prevent learning of alcohol-paired contextual memory.

What could be the behavioral mechanism(s) that underlies RapaLink-1-mediated reduction in voluntary alcohol intake? The smell and taste of alcohol are powerful cues that promote the reconsolidation of the memory of alcohol seeking and consumption (Everitt & Robbins 2005; Barak *et al.* 2013). Interestingly, we previously found that mTORC1 plays an essential role in these phenotypes (Barak *et al.* 2013). Thus, it is plausible that RapaLink-1 attenuates the association of alcohol cues, i.e. smell and/or taste with alcohol intake resulting in a reduction in alcohol intake on the initial and subsequent drinking sessions.

In summary, our study puts forward initial evidence supporting the potential use of RapaLink-1 for the treatment of heavy drinkers. Furthermore, RapaLink-1, by reducing the amount of alcohol consumed during initial binge drinking episodes, could be developed as a therapeutic approach to reduce the initial risk of alcohol use and thus the prevalence of AUD. This possibility is of importance because the occurrence of binge pattern of alcohol consumption, especially in young adults, is on the rise (Naimi *et al.* 2003; Margret & Ries 2017). Furthermore, RapaLink-1 may be used as a prevention therapy in vulnerable populations with high propensity of harmful alcohol use. Finally, mTORC1 has been shown to play an important role in neuroadaptations induced by other classes of addictive substances such as stimulants and opiates (Neasta *et al.* 2014); thus, RapaLink-1 may be beneficial for the treatment of several substance use disorders.

Acknowledgements

This research was supported by the National Institute of Alcohol Abuse and Alcoholism, P50AA017072 (K.M.S. and D.R.).

Authors Contribution

NM and DR were responsible for the study concept and design. NM conducted the experiments and analyzed the data. CJN and KMS synthesized RapaLink-1. NM and DR wrote the manuscript. All authors critically reviewed content and approved final version for publication.

References

APA (2013) Diagnostic and Statistical Manual of Mental Disorders, Vol. 619. American Psychiatric Association: Washington, DC.

Barak S, Liu F, Ben Hamida S, Yowell QV, Neasta J, Kharazia V, Janak PH, Ron D (2013) Disruption of alcohol-related memories by mTORC1 inhibition prevents relapse. *Nat Neurosci* 16:1111–1117.

Beckley JT, Laguesse S, Phamluong K, Morisot N, Wegner SA, Ron D (2016) The first alcohol Drink triggers mTORC1-dependent synaptic plasticity in nucleus accumbens dopamine D1 receptor neurons. *J Neurosci* 36:701–713.

Bove J, Martinez-Vicente M, Vila M (2011) Fighting neurodegeneration with rapamycin: mechanistic insights. *Nat Rev Neurosci* 12:437–452.

Buffington SA, Huang W, Costa-Mattioli M (2014) Translational control in synaptic plasticity and cognitive dysfunction. *Annu Rev Neurosci* 37:17–38.

Carnicella S, Ron D, Barak S (2014) Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. *Alcohol* 48:243–252.

Cosgray RE, Hanna V, Davidhizar RE, Smith J (1990) The water-intoxicated patient. *Arch Psychiatr Nurs* 4:308–312.

Cunningham CL, Niehus DR, Malott DH, Prather LK (1992) Genetic differences in the rewarding and activating effects of morphine and ethanol. *Psychopharmacology (Berl)* 107:385–393.

Dowling RJ, Topisirovic I, Fonseca BD, Sonenberg N (2010) Dissecting the role of mTOR: lessons from mTOR inhibitors. *Biochim Biophys Acta* 1804:433–439.

Ducker GS, Atreya CE, Simko JP, Hom YK, Matli MR, Benes CH, Hann B, Nakakura EK, Bergsland EK, Donner DB, Settleman J, Shokat KM, Warren RS (2014) Incomplete inhibition of phosphorylation of 4E-BP1 as a mechanism of primary resistance to ATP-competitive mTOR inhibitors. *Oncogene* 33:1590–1600.

Dundas B, Harris M, Narasimhan M (2007) Psychogenic polydipsia review: etiology, differential, and treatment. *Curr Psychiatry Rep* 9:236–241.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. *Nat Neurosci* 8:1481–1489.

Everitt BJ, Robbins TW (2016) Drug addiction: updating actions to habits to compulsions ten years on. *Annu Rev Psychol* 67:23–50.

Fan Q, Aksoy O, Wong RA, Ilkhanizadeh S, Novotny CJ, Gustafson WC, Truong AY, Cayan G, Simonds EF, Haas-Kogan D, Phillips JJ, Nicolaides T, Okaniwa M, Shokat KM, Weiss WA (2017) A kinase inhibitor targeted to mTORC1 drives regression in glioblastoma. *Cancer Cell* 31:424–435.

Feldman ME, Shokat KM (2010) New inhibitors of the PI3K-Akt-mTOR pathway: insights into mTOR signaling from a new generation of tor kinase domain inhibitors (TORKinibs). In: *Phosphoinositide 3-Kinase in Health and Disease*, pp. 241–262. Springer: New York, NY.

Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. *Cancer Cell* 12:9–22.

Hsieh AC, Costa M, Zollo O, Davis C, Feldman ME, Testa JR, Meyuhos O, Shokat KM, Ruggero D (2010) Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. *Cancer Cell* 17:249–261.

Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, Shi EY, Stumpf CR, Christensen C, Bonham MJ (2012) The translational landscape of mTOR signalling steers cancer initiation and metastasis. *Nature* 485:55–61.

Hyman SE, Malenka RC, Nestler EJ (2006) Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci* 29:565–598.

Koob GF, Volkow ND (2010) Neurocircuitry of addiction. *Neuropsychopharmacology* 35:217–238.

- Laguesse S, Morisot N, Phamluong K, Ron D (2016) Region specific activation of the AKT and mTORC1 pathway in response to excessive alcohol intake in rodents. *Addict Biol*. doi: <https://doi.org/10.1111/adb.12464>.
- Li J, Kim SG, Blenis J (2014) Rapamycin: one drug, many effects. *Cell Metab* 19:373–379.
- Lipton JO, Sahin M (2014) The neurology of mTOR. *Neuron* 84:275–291.
- Liu F, Laguesse S, Legastelois R, Morisot N, Ben Hamida S, Ron D (2016) mTORC1-dependent translation of collapsin response mediator protein-2 drives neuroadaptations underlying excessive alcohol-drinking behaviors. *Mol Psychiatry* 22(1):89–101.
- Margret CP, Ries RK (2017) Assessment and treatment of adolescent substance use disorders: alcohol use disorders. *Child Adolesc Psychiatr Clin N Am* 25:411–430.
- Meyers JL, Salling MC, Almli LM, Ratanatharathorn A, Uddin M, Galea S, Wildman DE, Aiello AE, Bradley B, Ressler K, Koenen KC (2015) Frequency of alcohol consumption in humans; the role of metabotropic glutamate receptors and downstream signaling pathways. *Transl Psychiatry* 5:e586.
- Naimi TS, Brewer RD, Mokdad A, Denny C, Serdula MK, Marks JS (2003) Binge drinking among US adults. *JAMA* 289:70–75.
- Neasta J, Barak S, Hamida SB, Ron D (2014) mTOR complex 1: a key player in neuroadaptations induced by drugs of abuse. *J Neurochem* 130:172–184.
- Neasta J, Ben Hamida S, Yowell Q, Carnicella S, Ron D (2010) Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders. *Proc Natl Acad Sci* 107:20 093–20 098.
- Rodrik-Outmezguine VS, Okaniwa M, Yao Z, Novotny CJ, McWhirter C, Banaji A, Won H, Wong W, Berger M, de Stanchina E, Barratt DG, Cosulich S, Klinowska T, Rosen N, Shokat KM (2016) Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor. *Nature* 534:272–276.
- Santini E, Huynh TN, Klann E (2014) Mechanisms of translation control underlying long-lasting synaptic plasticity and the consolidation of long-term memory. *Prog Mol Biol Transl Sci* 122:131–167.
- Seo D, Sinha R (2014) The neurobiology of alcohol craving and relapse. *Handb Clin Neurol* 125:355–368.
- Shor B, Gibbons JJ, Abraham RT, Yu K (2009) Targeting mTOR globally in cancer: thinking beyond rapamycin. *Cell Cycle* 8:3831–3837.
- Slotkin EK, Patwardhan PP, Vasudeva SD, de Stanchina E, Tap WD, Schwartz GK (2015) MLN0128, an ATP-competitive mTOR kinase inhibitor with potent in vitro and in vivo antitumor activity, as potential therapy for bone and soft-tissue sarcoma. *Mol Cancer Ther* 14:395–406.
- Torregrossa MM, Corlett PR, Taylor JR (2011) Aberrant learning and memory in addiction. *Neurobiol Learn Mem* 96:609–623.
- Warnault V, Darcq E, Levine A, Barak S, Ron D (2013) Chromatin remodeling—a novel strategy to control excessive alcohol drinking. *Transl Psychiatry* 3:e231.
- World Health Organization (2014) Global Status Report on Alcohol and Health. <http://www.who.int/gho/alcohol/en/>.
- Wullschleger S, Loewith R, Hall MN (2006) TOR signaling in growth and metabolism. *Cell* 124:471–484.
- Yoneyama N, Crabbe JC, Ford MM, Murillo A, Finn DA (2008) Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol* 42:149–160.
- Zoncu R, Efeyan A, Sabatini DM (2011) mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 12:21–35.