# Preclinical evidence to support repurposing Everolimus for craving reduction during protracted drug withdrawal

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# Abstract

Cue-elicited drug-craving is a cardinal feature of addiction that intensifies (incubates) during protracted withdrawal. In a rat model, these addiction-related behavioral pathologies are mediated, respectively, by time-dependent increases in PI3K/Akt1 signaling and reduced Group 1 metabotropic glutamate receptor (mGlu) expression, within the ventromedial prefrontal cortex (vmPFC). Herein, we examined the capacity of single oral dosing with Everolimus, an FDA-approved inhibitor of the PI3K/Akt effector mTOR, to reduce incubated cocaine-craving and reverse incubation-associated changes in vmPFC kinase activity and mGlu expression. Rats were trained to lever-press for intravenous infusions of cocaine or delivery of sucrose pellets and then subjected to tests for cue-reinforced responding during early (3 days) or late (30-46 days) withdrawal. Rats were gavage-infused with Everolimus (0-1.0 mg/kg), either prior to testing to examine for effects upon reinforcer-seeking behavior, or immediately following testing to probe effects upon the consolidation of extinction learning. Single oral dosing with Everolimus dose-dependently blocked cocaine-seeking during late withdrawal and the effect lasted at least 24 h. No Everolimus effects were observed for cue-elicited sucrose-seeking or cocaine-seeking in early withdrawal. Additionally, Everolimus treatment, following initial cue-testing, reduced subsequent cue hyper-responsivity exhibited observed during late withdrawal, arguing a facilitation of extinction memory consolidation. Everolimus' "anti-incubation" effect was associated with a reversal of withdrawal-induced changes in indices of PI3K/Akt1/mTOR activity, as well as Homer protein and mGlu1/5 expression, within the prelimbic (PL) subregion of the prefrontal cortex. Our results indicate mTOR inhibition as a viable strategy for interrupting heightened cocaine-craving and facilitating addiction recovery during protracted withdrawal.


# Introduction

Drug-craving is a cardinal feature of addiction that can be elicited by re-exposure to drug-associated cues. Insidiously, the intensity of cue-elicited drug-craving both incubates (i.e., intensifies) during protracted withdrawal (1,2) and becomes resistant to extinction (3-5). These phenomena are theorized to contribute substantially to the chronic, relapsing, nature of addiction by driving perseverative cue hyper-reactivity in drug-abstinent individuals (6,7). The functional neuroanatomy underpinning the incubation of cue-elicited cocaine-craving involves neuroadaptations within glutamatergic corticostriatal projections, particularly those from the prelimbic cortex (**PL**) subregion of the ventromedial prefrontal cortex (**vmPFC**) to the core subregion of the nucleus accumbens (**NAc**) (3-10). In humans with Cocaine Use Disorder, the vmPFC exhibits a time-dependent hyperactivity in response to drug-associated stimuli (e.g.,11,12), and interrogation of the vmPFC and NAc in rodent models of incubated cocaine-craving highlights anomalies in Group1 metabotropic (**mGlu**) and calcium-permeable AMPA-type glutamate receptor expression which may reflect, for instance, deregulated mammalian target of rapamycin (**mTOR**) function (13,14), as a major driver of this phenomenon.

Indeed, mTOR-related signaling has received considerable experimental attention in humans with Cocaine User Disorder (15) and in animal models of addiction (c.f.,16), including incubated cocaine-craving (14) due to its ability to critically regulate intracellular signaling networks. mTOR activity is regulated upstream by phosphoinositide 3-kinase (**PI3K**) and Akt1 (a.k.a. protein kinase B) (17,18). Additionally, activated PI3K recruits Akt1 to the plasma membrane, where it can be phosphorylated at Ser473 by mTOR (18-20), among other kinases (e.g., 21). Recently, we identified increased PI3K/Akt1 signaling within vmPFC as a biochemical correlate of incubated cocaine-craving in rats that is necessary for the expression of this behavioral phenomenon (4). Of potential relevance to anti-craving medications development, there exists a number of FDA-approved, orally bioavailable, medications that inhibit the PI3K/Akt1/mTOR signaling pathway (c.f., 22,23). This fact led us to examine the effects of acute, oral, pretreatment with the FDA-approved and commercially available allosteric mTOR inhibitor Everolimus (formerly RAD 001; a.k.a., Zortress, Certican, Afinitor, Votubia, Evertor) upon the incubation of cue-elicited cocaine-craving in a rat model of addiction and its relation to the activational state of PI3K/Akt1/mTOR signaling within vmPFC subregions. Cocaine-incubated rats also exhibit persistently high drug-seeking across days (3-5), which may reflect extinction failure and relates to a deficit in vmPFC Group1 mGlu function (3). Thus, the present study also examined the impact of Everolimus upon the consolidation of extinction learning. Lastly, relation between the behavioral effects of inhibitor pretreatment and changes in the expression of Group1 mGlu receptors and their associated Homer scaffolding proteins within vmPFC subregions were also determined.

Our results show that acute, oral, pretreatment with Everolimus blocks incubated cocaine-craving in rats over days and reverses incubation-related protein adaptations within vmPFC subregions. Such findings demonstrate the therapeutic potential of systemic treatment with current FDA-approved PI3K/Akt1/mTOR inhibitors for reversing vmPFC neuroadaptations driving perseverative cocaine-craving.

# Materials And Methods

**Subjects.** Subjects were adult, male, Sprague–Dawley rats (275–325 g; Charles River Laboratories, Hollister, CA). Details of housing and animal care are summarized in the Supplement.

**Surgical Procedures, cocaine and sucrose self-administration.** The surgical procedures to implant jugular catheters were identical to those described previously (3-5,9,24-26). The procedures employed to train rats to self-administer either intravenous cocaine or 45 mg banana-flavored sucrose pellets are detailed in the Supplement.

**Gavage Infusion Procedures and Cue Testing.** Details of the gavage infusion procedures are provided in the Supplement. Experiment 1 determined the impact oral dosing with Everolimus on cocaine-seeking during early withdrawal versus late (i.e. incubated) withdrawal (**Fig.1**). For this, rats were gavage-infused with vehicle (**VEH**; 1% DMSO in water; vol: 1 ml/kg) or Everolimus (1.0 mg/kg in VEH) on withdrawal day 3 (**WD3**) or withdrawal day 30 (**WD30**). This Everolimus dose is either at, or below, those demonstrated to exert therapeutic effects in other rodent models of neurological disease (22,23,27-36). Testing for cocaine-seeking occurred 30 min post-gavage, was conducted in the same operant chamber as that employed during the self-administration phase of the study and was 2-h long, as in prior immunoblotting work (1). During these cue tests, depression of the formerly reinforced, "active", lever resulted in the presentation of the 20-sec tone/light stimulus, while depression of the inactive lever had no programmed consequences.

Experiment 2 determined the dose-response function for Everolimus' inhibition of incubated cocaine-seeking and examined for carry-over effects across repeated testing. At WD30-46 from cocaine self-administration, rats were pretreated with VEH or a range of Everolimus doses (0.01, 0.1 and 1.0 mg/kg) at 30 min prior to testing (Cue Test 1). A group of VEH-treated rats were also tested at WD3 to provide a baseline of cue-elicited responding for ascertainment of incubation, as in prior work (5,6). Next, rats underwent a 2nd cue-test session (Cue Test 2) 24 h after Test 1 with no experimental manipulation between tests. Lastly, we examined the possibility that 1.0 mg/kg Everolimus might facilitate the consolidation of extinction learning by administering Everolimus (1.0 mg/kg) to the WD30-46 VEH-treated rats immediately *after* Cue Test 2 and these rats were tested again the next day (Cue Test 3) and their behavior compared to that exhibited on Cue Test 2, using a within-subjects design. A schematic of the procedural time-line for Experiment 2 is provided in **Fig.2**.

Experiment 3 examined off-target effects by assessing potential impact of Everolimus (1.0 mg/kg upon cue-reinforced sucrose-seeking during late withdrawal (9). As Everolimus did not affect cocaine-seeking behavior on WD3, its influence on sucrose-seeking at the earlier timepoint was not examined. Additionally, rats were re-tested at 24 h post-treatment to assess potential carry-over or delayed effects.

**Immunoblotting.** To relate the "anti-incubated craving" effects of Everolimus to the activational state of PI3K/Akt1/mTOR signaling and changes in mGlu/Homer expression within vmPFC subregions, we collected tissue from the rats upon the completion of Experiment 1. Immediately following the cue-elicited cocaine-seeking test, rats were euthanized by rapid decapitation and the PL and IL were dissected out over ice (see **Suppl. Fig.1**). The procedures for detecting and quantifying the expression of mGlu1/5 and Homer1/2, as well as the phosphorylated and non-phosphorylated forms of Akt1, P70S6 kinase (**P70S6K**)

and its major downstream effector riboprotein S6 (**rpS6**) (18,37) were similar to those employed previously by our group (3,4,25, 38-40) and are detailed in the Supplement.

**Statistical Analyses:** The majority of the data were analyzed separately at each withdrawal time-point group using Tukey-Kramer multiple comparison tests between VEH and Everolimus-pretreated groups. Between-group (WD3 vs. WD30) comparisons of behavior and protein expression were also conducted in the VEH-treated animals to confirm the presence/absence of an incubated response. A within-group (Test 2 vs. Test 3) comparison of behavior was conducted in the VEH from the sucrose study. A significant ANOVA is not a prerequisite for planned pairwise comparison procedures, which provides conservative protection against Type I error while maximizing statistical power (41-43). In studies such as this that contain multiple *a priori* comparisons of interest, this statistical approach is particularly powerful, and often considered preferable to the traditional ANOVA (44-48). Pearson's correlational analyses were also conducted to determine the relationship between drug-seeking behavior during Experiment 1 and protein expression within vmPFC subregions. Given the unbalanced design of Experiment 2, the data were analyzing using mixed design ANOVA, with Cue Test as a within-subjects factor, followed by LSD *post-hoc* tests along the Drug factor. As the initial analyses indicated no Cue Test effect (see Results), the data were collapsed across test day for *post-hoc* comparisons using LSD tests. Alpha was set at 0.05 for all analyses.

# Results

**Everolimus pre-treatment decreases cocaine-seeking only following incubation.** Following 10 days of cocaine self-administration, rats were pretreated with VEH or 1.0 mg/kg Everolimus (**E1.0**) prior to a cue-elicited cocaine-seeking test on either WD3 or WD30. Prior to Everolimus treatment, no group differences were apparent for the average active- or inactive-lever presses or for the number of cocaine reinforcers earned (**Suppl.Table 1**). VEH-pretreated rats emitted more cue-reinforced lever-presses on WD30 versus WD3 (**Fig.1a**; $p<0.0001$), indicative of incubated craving. As depicted in **Fig.1a**, Everolimus blocked cue-elicited responding in rats tested on WD30 ($p<0.0001$), with no effect detected on WD3 ($p=0.29$). In contrast, inactive lever-pressing did not vary as a function of withdrawal in VEH-pretreated rats (**Fig.1b**; $p=0.88$) and Everolimus did not affect inactive lever-pressing on either WD3 or 30 (for WD3, $p=0.76$; for WD30, $p=0.63$). From this single-dose study, we concluded that Everolimus abolishes incubated cocaine-seeking, without altering cue-elicited cocaine-seeking during short-term withdrawal.

**Everolimus pretreatment dose-dependently blocks incubated cocaine-seeking.** Consequently, we determined the dose-response function for Everolimus suppression of incubated cocaine-seeking in late withdrawal. All groups exhibited comparable lever-responding and cocaine intake history prior to testing (**Suppl.Table 1**).

Acute Everolimus pretreatment dose-dependently reduced incubated cue-elicited responding (**Fig.2a**; Drug effect: $F_{4,38}$=11.14, p<0.0001) and this effect persisted, unchanged, for at least 24 h (**Fig.2b**; Test effect and interaction: $F_{4,38}$<1.0, p's>0.09). Given the persistence of responding, the data were collapsed across cue tests for LSD post-hoc analyses. WD30-VEH rats exhibited significantly greater responding than WD3-VEH controls (p<0.0001), indicating the presence of an incubated response in protracted withdrawal. Relative to WD30-VEH controls, Everolimus significantly lowered incubated lever-responding at all doses (for 0.01 mg/kg, p=0.02; for 0.1 mg/kg, p<0.0001; for 1.0 mg/kg, p<0.0001). Further, the responding exhibited by the 30WD rats pretreated with the two higher Everolimus doses was not different from the 3WD-VEH controls (for 0.1 mg/kg, p=0.54; for 1.0 mg/kg, p=0.32), indicating a block of incubated cocaine-craving at these doses. The Everolimus effect was selective for active lever-responding as no group differences were detected for inactive lever-pressing on either cue test (**Suppl.Fig.1a,b**; Drug X Test ANOVA: $F_{4,38}$<0.25, all p's>0.40).

**Everolimus post-treatment facilitates the consolidation of extinction learning.** Next, we probed whether treating rats with 1.0 mg/kg Everolimus immediately *following* Cue Test 2 might facilitate the consolidation of extinction learning (see **Fig.2**, top). Pairwise comparisons of the active lever-pressing behavior between Test 2 (post-treatment) and Test 3 (no further treatment) confirmed no change in responding for VEH rats tested in late withdrawal (p=0.34), indicating its persistence. In contrast, Everolimus post-treatment significantly reduced responding exhibited on Cue Test 3 (**Fig.2c**; p=0.04), without affecting inactive lever-pressing (**Suppl.Fig.1c**; p's>0.12). These latter data argue that the "anti-incubation" effect of Everolimus may involve a facilitation of extinction memory consolidation.

**Everolimus pre-treatment does not alter sucrose-seeking.** Finally, we determined the impact of Everolimus on sucrose-seeking at late withdrawal. No group differences in operant responding or reinforcement were noted prior to treatment with 1.0 mg/kg Everolimus (**Suppl. Table 1**). We detected no evidence of incubated sucrose-seeking in *ad libitum*-fed and –watered rats or effects of 1.0 mg/kg Everolimus upon cue-reinforced responding (**Fig.2e,f**; Drug X Test ANOVA: $F_{1,20}$ or $F_{2,20}$<0.70, all p's>0.50) or inactive lever-pressing (**Suppl.Fig.1d,e**; Test effect: $F_{1,20}$=6.00, p=0.02; Drug effect and interaction: $F_{1,20}$ and $F_{2,20}$<4.0, p's>0.07). Thus, acute pretreatment with 1.0 mg/kg Everolimus does not impact cue-elicited sucrose-seeking.

**Everolimus pretreatment blocks or reverses incubation-related changes in mTOR/Akt1 signaling within vmPFC subregions.** Using the tissue obtained from Experiment 1 rats, we also determined Everolimus' effects upon indices of mTOR/Akt1 signaling within the PL and IL and its temporal selectivity. No time-

dependent changes were detected for the total protein expression of Akt1, P70S6K or rpS6 within either subregion of VEH-pretreated rats (**Suppl.Table2**) nor were any effects of Everolimus detected upon total protein expression at either withdrawal time-point (**Suppl.Table2**; **Suppl.Fig.2**).

A time-dependent increase in p(Ser473)-Akt1 levels was observed within both the PL (**Fig.3a**; p=0.04) and the IL (**Fig.3b**; p=0.03) of VEH-pretreated rats. Everolimus did not alter p(Ser473)-Akt1 expression within either subregion on WD3 (for PL, p=0.7; for IL, p=0.82), lowered phospho-protein levels within both subregions on WD30 (for PL, p=0.0001; for IL, p=0.002) (**Fig.3a,b**). Similarly, p(Thr389)-P70S6K expression also incubated within the PL and IL of VEH controls (**Fig.3c,d**; for PL, p=0.003; for IL; p=0.03). However, Everolimus pretreatment lowered the PL levels of p(Thr389)-P70SK6 at both withdrawal time-points (**Fig.3c**; for WD3, p=0.02; for WD30, p=0.001), without altering phospho-protein expression within the IL (**Fig.3d**; p's>0.11). The time-dependent increase in expression within vmPFC subregion exhibited by VEH controls was statistically unreliable (**Fig.3e,f**; for PL, p=0.08; for IL, p=0.1). Everolimus pretreatment lowered p(Ser235/236)-rpS6 within the PL at both withdrawal time-points (**Fig.3e**; for WD3, p=0.4; for WD30, p=0.003), but only lowered phospho-protein expression within the IL in rats tested on WD3 (**Fig.3f**; for WD3, p=0.02; for WD30: p=0.98). These immunoblotting data support the brain-penetrance of Everolimus and confirm that acute oral dosing with 1.0 mg/kg Everolimus inhibits incubation-related increases in Akt1 and P70S6K activation within both the IL and PL subregions of the vmPFC.

**Everolimus pretreatment reverses incubation-related changes in mGlu1/5 expression within the PL.** VEH-pretreated rats exhibited a time-dependent reduction in the PL levels of the monomer (**Fig.4b**; p=0.02), but not the dimer (**Fig.4a**; p=0.12).  forms of mGlu1, with reductions detected also for both the dimer and monomer forms of mGlu5 ((**Fig.4c,d**; for dimer, p=0.048; for monomer, p=0.01. In contrast, mGlu1 monomer expression within the IL increased as a function of withdrawal (**Fig.4f**; p=0.006), while no other time-dependent receptor changes were detected in the IL of VEH-pretreated rats (for mGlu1 dimer, p=0.13, **Fig.4e**; for mGlu5 dimer, p=0.56, **Fig.4g**; for mGlu5 monomer, p=0.15, **Fig.4h**). Although Everolimus pretreatment did not affect PL levels of mGlu1/5 on WD3 (p's>0.30; see **Suppl.Table2**), pretreatment elevated both the monomer and dimer forms of mGlu1 and mGlu5 on WD30 (**Fig.4a-d**; p's≤0.0001; see **Suppl.Table2**). With the exception of an increase in mGlu1 dimer expression (**Fig.4e**, p=0.001), no Everolimus effects were detect for receptor expression within IL (**Fig.4f-h**; p's>0.45; see **Suppl.Table2**). Taken together, these immunoblotting data indicate that incubated cocaine-craving is associated with a PL-selective reduction in mGlu1/5 expression and that acute, oral, dosing with 1.0 mg/kg Everolimus is sufficient to reverse this neuroadaptation and to augment mGlu1 expression within the IL.

**Everolimus pretreatment reverses incubation-related changes in Homer expression within the PL.** Time-dependent increases in both Homer1b/c (**Fig.5a**; p=0.001) and Homer2a/b (**Fig.5b**; p=0.005) were

observed within the PL of VEH-pretreated rats, with no changes detected within the IL (**Fig.5c,d**; p's>0.40, see **Suppl.Table2**). On WD3, Everolimus pretreatment did not alter Homer1/2 expression within either the PL (p's>0.55) or the IL (p's>0.06; see **Suppl.Table2**). In contrast, pretreatment on WD30 significantly lowered Homer2a/b expression within the PL (**Fig.5b**; p=0.002), with less statistically reliable reductions detected also for Homer1b/c within the PL (Fig.5a; p=0.09) and for Homer2a/b within the IL (**Fig.5d**; p=0.06). No Everolimus effect was detected for IL expression of Homer1/c on WD30 (**Fig.5c**; p=0.64). These immunoblotting results for Homer proteins indicate that incubated cocaine-seeking is associated with elevated Homer1b/c and Homer2a/b expression selectively within the PL subregion of the vmPFC, the latter of which is reversed by acute, oral, dosing with Everolimus.

**Protein correlates of cocaine-seeking within vmPFC subregions.** When the entire vmPFC is examined, cue-elicited cocaine-seeking is positively correlated with indices of PI3K/Akt1 signaling (4) and Homer2 expression (25), but inversely correlated with the expression of the monomer forms of mGlu1 and mGlu5 (3). To determine the subregional selectivity of these correlates of cocaine-seeking, comparable correlational analyses were performed on the PL and IL tissue from the rats in Experiment 1. As detailed in the Supplement, we replicated a predictive relationship between cocaine-seeking and indices of mTOR/Akt1 activation within both the PL and IL (**Suppl.Fig.4**). Homer2 expression in the PL also predicted drugs-seeking (**Suppl.Fig.6**), while an inverse relationship between cocaine-seeking and mGlu1/5 monomer *and* dimer expression was detected in the PL only (**Suppl.Fig.5**). Thus, just as incubated cocaine-seeking generalizes across long- versus short-access self-administration procedures, so too do their biochemical correlates.

# Discussion

Rat models of incubated cocaine-craving indicate an important role for PI3K/mTOR signaling within the PL-NAc projection in heightened drug-cue reactivity during protracted drug withdrawal (4,14). While intracranial drug delivery approaches facilitate understanding of the neural loci & molecular mechanisms involved in mediating a particular drug effect, such approaches are currently not experimentally, let alone clinically, feasible in humans. Further, such approaches cannot inform as to issues associated with drug bioavailability and provide much less insight into potential off-target and side-effects than do systemic routes of drug administration. Here, we examined the effects of systemic treatment with the FDA-approved allosteric mTOR inhibitor Everolimus upon cue-elicited cocaine-craving during early and protracted withdrawal. Consistent with a significant body of clinical and preclinical literature (22,23,27-36), our findings support the oral bioavailability, brain penetrance and relative safety of Everolimus by demonstrating that acute, oral, dosing reduces cue-elicited craving only in rats exhibiting incubated cocaine-seeking as well as indices mTOR pathway activation within the vmPFC. When administered at doses equal to, or below, those employed in rodent models of other disease (22,23,27-36), Everolimus dose-dependently reduced incubated cocaine-craving for at least a 24-h period. Everolimus did not affect responding on the inactive lever in cocaine-experienced rats and the maximum dose did not impact cue-

elicited sucrose-craving or cocaine-craving during early withdrawal, despite the 1.0 mg/kg Everolimus being sufficient to reduce mTOR-related signaling within the vmPFC of cocaine rats tested in early withdrawal. Thus, we conclude intact mTOR/P70S6K/rpS6 function is not required for cue-elicited drug-craving behavior *per se*, but is critical for its intensification during protracted withdrawal. While it remains to be determined (1) whether Everolimus might reduce cue-or drug-elicited responding in other models of cocaine-taking or -craving and (2) how long the “anti-incubation” effect persists, the present data argue that acute, oral dosing with low-dose Everolimus can produce a relatively long-lasting reduction in cocaine cue reactivity during protracted withdrawal, with no obvious off-target motivational or motor effects in either cocaine-naïve or –experienced subjects.

### Everolimus blocks incubated cocaine-craving and concomitant Akt1 activation.

In both the appetitive and aversive domains, a dorsal-ventral distinction is reported with respect to how vmPFC regulates the expression of conditioned behavior (49-57). Extending prior immunoblotting results for the entire vmPFC (4), we observed increased PI3K/Akt1/mTOR activation within both subregions of rats expressing incubated cocaine-seeking (here on out referred to as cocaine-incubated rats). Notably, the present incubation-specific molecular alterations based on a relatively limited cocaine intake are strikingly similar to those produced with prolonged daily access (4). Thus, the perturbation of Akt1 activation during protracted withdrawal appears to generalize across intake histories in rats and strengthens their relevance for the human condition, in which the duration and patterning of drug-taking history is highly variable. Further, the “anti-incubation” effects of Everolimus were associated with a blockade of this increased PI3K/Akt1/mTOR signaling activation. Of note, Everolimus did not affect p(Ser473)-Akt1 expression in rats tested in early cocaine withdrawal. In line with this observation, site-directed inhibition of PI3K/Akt1 signaling within the PL is sufficient to block incubated cocaine-craving and to do so for at least a 24-h period (4). Although the functional relevance of PI3K/Akt1 hyper-activity within the IL for incubated responding has not been vetted, inhibiting glutamate release within this subregion reduces incubated cocaine-craving to a similar extent as that produced by comparable manipulation of the PL (26). Taken together, these findings argue time-dependent increases in PI3K/Akt1 activity within both vmPFC subregions as drivers of incubated drug-craving and pose systemic treatment with an allosteric mTOR inhibitor as a means by which to mitigate this excessive signaling and normalize drug cue reactivity.

### Everolimus reverses incubation-related changes in Homer2 expression.

When the entire vmPFC is considered, incubated cocaine-seeking is associated with near-doubling of the expression of the glutamate receptor scaffolding protein isoform Homer2a/b (25). Here, this effect occurs selectively within the PL and again, it is notable that this molecular correlate of incubation generalizes between limited and excessive cocaine intake histories. Aligning with our earlier study (25), Homer1b/c

expression was less sensitive to cocaine withdrawal as we detected a relatively small (<50%) increase in protein expression within the PL of cocaine-incubated rats. Homer proteins interact with the long isoform of the GTP-ase PI3K Enhancer (PIKE) to activate PI3K-dependent signaling (58) leading to the phosphorylation of Akt1 on Ser473 (17-19). Thus, it is tempting to speculate that increased Homer(2)-PIKE scaffolding may contribute to the heightened Akt1 phosphorylation detected in cocaine-incubated rats. Indeed, Homer scaffolding to mGlu5 is required for receptor-mediated activation of PI3K/Akt1/mTOR signaling in brain (59). Although Homer1/2 levels did not vary within the IL as a function of cocaine withdrawal, Everolimus reduced Homer2 expression within both subregions of cocaine-incubated animals, which might contribute to inhibitor effects upon Akt1 phosphorylation in both subregions. However, arguing against a Homer-PIKE-PI3K/Akt1 mechanism as the sole driver of incubated craving, virus-mediated knockdown of Homer2 within the PL does not impact the magnitude of cocaine-incubated responding nor does it affect cue-induced reinstatement of cocaine-seeking following extinction (25). It remains to be determined if interrupting Homer2-PIKE interactions within both vmPFC subregions or selectively within the IL would exert a more pronounced effect upon cue-elicited craving.

### Everolimus facilitates the consolidation of extinction learning and increases mGlu1/5 expression.

As highlighted in several reviews (56,57,60,61), there is little debate in the clinical literature that drug-cue hyper-reactivity is a major cordon to addiction recovery, with recommendations for therapeutics promoting extinction learning as a means to inhibit pathogenic memories in Substance Use Disorder and reduce relapse vulnerability. The drug-cue hyper-reactivity that constitutes incubated craving persists across days of drug-cue re-exposure in rats with a history of extended-access cocaine self-administration (3-5). Herein, we replicate both incubated craving and its persistence despite repeated drug cue re-exposure in rats with a less extensive cocaine-taking history. As with the molecular findings above, this pattern of behavior generalizes across limited verses excessive intake histories. Consistent with the putative cognitive-enhancing potential of mTOR inhibitors (19,26,31-33), Everolimus administration, either prior to, or immediately following, a drug-cue re-exposure session, reduces cue-reactivity the next day. Such findings align with the interpretation that acute oral Everolimus treatment may reduce drug-cue hyper-reactivity, at least in part, by facilitating the consolidation of extinction learning during initial drug-cue re-exposure. To further this hypothesis, it will be important to determine the critical period for extinction learning consolidation in drug-incubated animals and compare the relative efficacy of Everolimus post-treatment inside and outside this window for reducing cue-elicited responding.

Positron Emission Tomography studies reveal a reduction in mGlu5 radioligand binding throughout the mesocorticolimbic system in Cocaine Use Disorder (62,63). Akin to our animal model of incubated cocaine-craving (3), this mGlu5 deficit worsens in humans over the course of drug abstinence (64). The consolidation of extinction learning requires intact mGlu1/5 function within vmPFC (56,61) and we have amassed both correlative and causal evidence supporting reduced mGlu1/5 expression within vmPFC as

key to maintaining the persistently high levels of cue reactivity exhibited by cocaine-incubated rats (3). Here, both the monomer and active dimer forms of mGlu1/5 are reduced within the PL of cocaine-incubated rats. Further, acute Everolimus pretreatment augmented mGlu1/5 expression in the PL and mGlu1 expression within the IL of cocaine-incubated rats *above* that expressed by rats tested in early withdrawal and both Everolimus pre- and post-treatment reduced subsequent cue-reactivity in rats tested on WD30.

Given the putative role for mTOR signaling in activating protein translation (c.f.,65,66), it is not clear at present if and how mTOR inhibition directly relates to increased vmPFC mGlu1/5 expression. However, mTOR is reported to inhibit the activity of the translational repressor fragile X mental retardation protein (FMRP) (67,68), which normally stalls the translation of mGlu5 (as well as other important postsynaptic scaffolding proteins to include PIKE) (66). This raises the intriguing possibility that Everolimus may rescue what appears to be a difficulty in extinction learning/memory by relieving translational repression on mGlu1/5. Alternatively, mGlu1/5 receptors undergo rapid phosphorylation-dependent desensitization upon their activation (68). While we did not examine the phosphorylation status of mGlu1/5, the complexity of the mGlu5 interactome, which includes mTOR (69,70), raises the alternate possibility that Everolimus attenuate subsequent cue-reactivity by blunting kinase-dependent receptor down-regulation. The precise mechanism(s) through which acute Everolimus blocks incubated cocaine-craving require further investigation. Equally, it will be important to determine whether or not the present findings generalize to female subjects (whom exhibit similar incubation but also estrous cycle modulation of drug-seeking behavior independent of cocaine intake histories (71-72)), to animals of different ages, to other models of Cocaine Use Disorder, particularly those that appear to better model the addictive state, as well as to other drugs of abuse. Nevertheless, the present data indicate the potential for repurposing current FDA-approved PI3K/Akt1/mTOR inhibitors for craving reduction during protracted recovery in Cocaine Use Disorder and provide a foundation for preclinical investigation of how PI3K/Akt1/mTOR signaling gates behavioral reactivity to drug-associated cues.

# Declarations

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## AUTHOR CONTRIBUTIONS

The authors contributed to this report in the following ways: conceptualization, ASC, CBS, TEK and KKS; methodology, ASC, CLJC,CBS, TEK, and KKS; formal analysis, ASC, MCK, LHS,TEK, and KKS; investigation, ASC, MCK, LHS, AMF, KNH, BDB, KNE, and CLJC.; data curation, ASC, MCK, LHS, and KKS; writing—original draft preparations, ASC, MCK, LHS, AMF, KNH, BDB, KNE and KKS; writing—review and editing, ASC, LHS, CLJC, TEK, KKS; visualization, MCK, LHS, and KKS; supervision, CBS, CLJC, TEK, and KKS; project administration, ASC, CLJC and KKS.; funding acquisition, ACS, MCK, ANF, CLJC, BDB, TEK and KKS.

# References


1. Grimm JW, Hope BT, Wise RA, Shaham Y. Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature. 2001; 412: 141-142.

2. Li X, Caprioli D, Marchant NJ. Recent updates on incubation of drug craving: a mini-review. Addict Biol. 2015; 20: 872-876.

3. Ben-Shahar O, Sacramento AD, Miller BW, Webb SM, Wroten MG,Silva HE, et al. Deficits in ventromedial prefrontal cortex Group1 metabotropic glutamate receptor function mediate resistance to extinction during protracted withdrawal from an extensive history of cocaine self-administration. J Neurosci. 2013; 33: 495-506.

4. Szumlinski KK, Ary AW, Shin CB, Wroten MG, Courson J, Miller BW et al. PI3K activation within ventromedial prefrontal cortex regulates the expression of drug-seeking in two rodent species Addict Biol. 2018; 24:1216-122619.

5. Miller BW, Wroten MG, Sacramento AD, Silva HE, Shin CB, Vieira PA, et al. Incubation of cocaine-craving requires PKCε priming within vmPFC. Addiction Biol. 2017; 22: 629-639

6. Szumlinski KK, Shin CB. Kinase interest you in treating incubated drug craving? A hypothetical model for treatment intervention during protracted withdrawal. Genes Brain Behavior. 2018; 17:e12440.

7. Dong Y, Taylor JR, Wolf ME, Shaham Y. Circuit and Synaptic Plasticity Mechanisms of Drug Relapse. J Neurosci. 2017; 37: 10867-10876.

8. Koya E, Uejima JL, Wihbey KA, Bossert JM, Hope BT, Shaham Y. Role of ventral medial prefrontal cortex in incubation of cocaine craving. Neuropharmacology. 2009; 56 Suppl 1:177-85.

9. Shin CB, Serchia M, Shahin J, Ruppert-Mejor M, Szumlinski KK. Incubation of cocaine-craving relates to glutamate over-flow within ventromedial prefrontal cortex. Neuropharmacology. 2016; 102:103-110.

10. Ma Y, Lee BR, Wang X, Guo C, Liu L, Cui R, et al. Bidirectional modulation of incubation of cocaine craving by silent synapse based remodeling of prefrontal cortex to accumbens projections. Neuron. 2014; 83: 1453-1467.

11. Gawin FH, Kleber HD. Abstinence symptomatology and psychiatric diagnosis in cocaine abusers. Clinical observations. Arch Gen Psychiatry. 1986; **43:** 107–113.

12. Parvaz MA, Moeller SJ, Goldstein RZ. Incubation of Cue-Induced Craving in Adults Addicted to Cocaine Measured by Electroencephalography. JAMA Psychiatry. 2016; 73: 1127-1134.

13. Scheyer AF, Wolf ME, Tseng KY. A protein synthesis-dependent mechanism sustains calcium-permeable AMPA receptor transmission in nucleus accumbens synapses during withdrawal from cocaine self-administration. J Neurosci. 2014; 34:3095–3100.

14. Werner CT, Stefanik MT, Milovanovic M, Caccamise A, Wolf ME. Protein Translation in the Nucleus Accumbens Is Dysregulated during Cocaine Withdrawal and Required for Expression of Incubation of Cocaine Craving. J Neurosci. 2018; 38: 2683-2697.

15. Álvaro-Bartolomé M, García-Sevilla JA. Dysregulation of cannabinoid CB1 receptor and associated signaling networks in brains of cocaine addicts and cocaine-treated rodents. Neuroscience. 2013; 247:294-308.

16. Neasta J, Barak S, Hamida SB, Ron D. mTOR complex 1: a key player in neuroadaptations induced by drugs of abuse. J Neurochem. 2014; 130: 172-184.

17. Ruggero D, Sonenberg N. The akt of translational control. Oncogene. 2005;24:7426–7434.

18. Hay N, Sonenberg N. Upstream and downstream of mTOR. **Genes Dev. 2004;**18: 1926–1945.

19. King D, Yeomanson D, Bryant HE. PI3King the lock: targeting the PI3K/Akt/mTOR pathway as a novel therapeutic strategy in neuroblastoma. J Pediatr Hematol Oncol. 2015; 37: 245-251.

20. SarbassovDD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science. 2005; 307:1098-1101.

21. ChuaBT, Gallego-Ortega D, Ramirez de Molina A, Ullrich A, Lacal JC, Downward J. Regulation of Akt(ser473) phosphorylation by choline kinase in breast carcinoma cells. Mol Cancer. 2009; 8:131.

22. Moes DJ, Guchelaar HJ, de Fijter JW. Sirolimus and everolimus in kidney transplantation. Drug Discov Today. 2015; 20:1243-1249.

23. Asati V, Mahapatra DK, Bharti SK. PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. Eur J Med Chem. 2016; 109:314-341

24. Szumlinski KK, Cohen M, Wroten MG, Miller BW, Sacramento AD, Kippin TE, et al. Incubation of cocaine-seeking relates to elevated GluN2b within dmPFC. J Drug Abuse. 2016; 2(2). pii: 22.

25. Gould AT, Sacramento AD, Wroten MG, Miller BW, Klugmann M, Ben-Shahar O, et al. Extended access to intravenous cocaine imbalances ventromedial prefrontal cortex Homer1 versus Homer2 expression: Implications for relapse. Addiction Biol. 2015; 20: 148-157

26. Shin CB, Templeton TJ, Chiu AS, Kim J, Vieria PA, Gable ES, et al. Endogenous glutamate within the prelimbic and infralimbic cortices oppositely regulate the incubation of cocaine-seeking in rats. Neuropharmacology. 2018; 128: 293-300.

27. Lin F, de Gooijer MC, Hanekamp D, Chandrasekaran G, Buil LC, Thota N, et al. PI3K-mTOR Pathway Inhibition Exhibits Efficacy Against High-grade Glioma in Clinically Relevant Mouse Models. Clin Cancer Res. 2017; 23: 1286-1298.

28. Mueed Z, Tandon P, Maurya SK, Deval R, Kamal MA, Poddar NK. Tau and mTOR: The Hotspots for Multifarious Diseases in Alzheimer's Development. Front Neurosci. 2019; 12:1017.

29. Zhu Z, Yang C, Iyaswamy A, Krishnamoorthi S, Sreenivasmurthy SG, Liu J, et al. Balancing mTOR Signaling and Autophagy in the Treatment of Parkinson's Disease. Int J Mol Sci. 2019; 20(3). pii: E728.

30. Siracusa R, Paterniti I, Cordaro M, Crupi R, Bruschetta G, Campolo M, et al, Neuroprotective Effects of Temsirolimus in Animal Models of Parkinson's Disease. Mol Neurobiol. 2018; 55: 2403-2419.

31. Magaway C, Kim E, Jacinto E. Targeting mTOR and Metabolism in Cancer: Lessons and Innovations. Cells 2019; 8: pii: E1584.

32. O'Reilly T, McSheehy PM, Kawai R, Kretz O, McMahon L, Brueggen J, et al. Comparative pharmacokinetics of RAD001 (everolimus) in normal and tumor-bearing rodents. Cancer Chemother Pharmacol. 2010; 65: 625-639.

33. Ehninger D. From genes to cognition in tuberous sclerosis: implications for mTOR inhibitor-based treatment approaches. **Neuropharmacology. 2013;** 68: 97–105.

34. Inoki K, Corradetti MN, Guan KL. Dysregulation of the TSC-mTOR pathway in human disease. **Nat Genet. 2005;** 37: 19–24.

35. Bockert J, Marin P. mTOR in Brain Physiology and Pathologies. Physiol Rev. 2015; 95:1157-87.

36. Perluigi M, Di Domenico F, Butterfield DA. mTOR signaling in aging and neurodegeneration: At the crossroad between metabolism dysfunction and impairment of autophagy. Neurobiol Dis. 2015; 84:39-49.

37. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem J. 2012; 441:1-21.

38. *Ary AW, Lominac KD, Wroten MG, Williams AR, Campbell RR, Ben-Shahar O, et al.* Imbalances in prefrontal cortex CC-Homer1 versus –Homer2 expression promote cocaine-seeking behavior. J Neurosci. 2013; 33:8101-8113

39. Cozzoli DK, Courson J, Caruana AL, Miller BW, Thompson AB, Wroten M, et al. Accumbens shell metabotropic glutamate receptor 5-associated signaling regulates binge alcohol drinking: Evidence from Drinking-in-the-Dark studies. Alcohol Clin Exp Res. 2012; 36: 1623-1633.

40. Bailey J, Ma D, Szumlinski KK. Rapamycin attenuates the expression of cocaine-induced place-preference and behavioral sensitization. Addiction Biol. 2012; 17: 248-258.

41. Cardinal RN, Aitken MRF. ANOVA for the Behavioural Sciences Researcher. 2006. L. Erlbaum, Mahwah, N.J.

42. Hayter AJ. A proof of the conjecture that the tukey-kramer multiple comparisons procedure is conservative. Ann. Stat. 1984; 12, 61-75.

43. Kramer CY. Extension of multiple range tests to group means with unequal numbers of replications. Biometrics. 1956; 12: 307-310.

44. Games PA. Multiple comparisons of means. Am. Educ. Res. J. 1971; 8: 531-565.

45. Hancock GR, Klockars AJ. The quest for alpha: developments in multiple comparison procedures in the quarter century since Games (1971). Rev. Educ. Res. 1996; 66: 269-306.
46. Rosnow RL, Rosenthal R.Statistical procedures and the justification of knowledge in psychological science. Am. Psychol. 1989; 44: 1276-1284.

47. Ruxton GD, Beauchamp G. Time for some a priori thinking about post hoc testing. Behav. Ecol. 2008; 19: 690-693.

48. Wilkinson L, Inference TFS. Statistical methods in psychology journals - guidelines and explanations. Am. Psychol. 1999; 54: 594-604.

49. Pelloux, Y, Murray JE, Everitt BJ (2013) Differential roles of the prefrontal cortical subregions and basolateral amygdala in compulsive cocaine seeking and relapse after voluntary abstinence in rats. Eur. J. Neurosci. 38: 3018-3026.

50. Stefanik MT, Kupchik YM, Kalivas PW (2015) Optogenetic inhibition of cortical afferents in the nucleus accumbens simultaneously prevents cue-induced transient synaptic potentiation and cocaine-seeking behavior. Brain Struct. Funct. 221: 1681-1689.

51. Stefanik MT, Moussawi K, Kupchik YM, Smith KC, Miller RL, Huff ML, Deisseroth K, Kalivas PW, Lalumiere RT (2013) Optogenetic inhibition of cocaine seeking in rats. Addict. Biol. 18: 50-53.

52. LaLumiere RT, Smith KC, Kalivas PW (2012) Neural circuit competition in cocaine-seeking: roles of the infralimbic cortex and nucleus accumbens shell. Eur. J. Neurosci. 35: 614-622.

53. Peters J, LaLumiere RT, Kalivas PW (2008) Infralimbic prefrontal cortex is responsible for inhibiting cocaine seeking in extinguished rats. J. Neurosci. 28: 6046-6053.

54. Müller Ewald VA, De Corte BJ, Gupta SC, Lillis KV, Narayanan NS, Wemmie JA, LaLumiere RT (2019) Attenuation of cocaine seeking in rats via enhancement of infralimbic cortical activity using stable step-function opsins. Psychopharmacology 236: 479-490

55. Moorman DE, James MH, McGlinchey EM, Aston-Jones G. (2015) Differential roles of medial prefrontal subregions in the regulation of drug seeking. Brain Res. 1628(Pt A):130-46.

56. Peters J, Kalivas PW, and Quirk GJ (2009) Extinction circuits for fear and addiction overlap in prefrontal cortex. Learning and Memory 16: 279-288.

57. Quirk GJ, Mueller D (2008) Neural mechanisms of extinction learning and retrieval. Neuropsychopharmacology 33: 56-72.

58. Rong R, Ahn JY, Huang H, Nagata E, Kalman D, Kapp JA, et al. (2003) PI3 kinase enhancer-Homer complex couples mGluRI to PI3 kinase, preventing neuronal apoptosis. Nat Neurosci. 6:1153-1161.

59. Ronesi JA, Huber KM. (2008) Homer interactions are necessary for metabotropic glutamate receptor-induced long-term depression and translational activation. J Neurosci. 28: 543-547.

60. Taylor JR, Olausson P, Quinn JJ, Torregrossa MM (2009) Targeting extinction and reconsolidation mechanisms to combat the impact of drug cues on addiction. Neuropharmacology 56(Suppl): 186–195.

61. Cleva RM, Gass JT, Widholm JJ, Olive MF (2010) Glutamatergic targets for enhancing extinction learning in drug addiction. Curr Neuropharmacol 8: 394-408

62. Milella MS, Marengo L, Larcher K, Fotros A, Dagher A, Rosa-Neto P, Benkelfat C, Leyton M (2014) Limbic system mGluR5 availability in cocaine dependent subjects: a high-resolution PET [(11)C]ABP688 study. Neuroimage 98: 195-202.

63. Martinez D, Slifstein M, Nabulsi N, Grassetti A, Urban NB, Perez A, et al. (2014) Imaging glutamate homeostasis in cocaine addiction with the metabotropic glutamate receptor 5 positron emission tomography radiotracer [(11)C]ABP688 and magnetic resonance spectroscopy. Biol Psychiatry 75: 165-171

64. Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N. (2009) Translational control of long-lasting synaptic plasticity and memory. Neuron 61:10-26.

65. Liu-Yesucevitz L, Bassell GJ, Gitler AD, Hart AC, Klann E, Richter JD, et al. (2011) Local RNA translation at the synapse and in disease. J Neurosci. 31: 16086-16093.

66. Ashley CT Jr, Wilkinson KD, Reines D, Warren ST.(1993) FMR1 protein: conserved RNP family domains and selective RNA binding. Science 262:563-566.

67. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, et al., (2011) FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. Cell 146: 247-61.

68. Jong YJ, Sergin I, Purgert CA, O'Malley KL (2014) Location-dependent signaling of the group 1 metabotropic glutamate receptor mGlu5. Mol Pharmacol. 86: 774-785.

69. Kalinowska M, Francesconi A (2016) Group I Metabotropic Glutamate Receptor Interacting Proteins: Fine-Tuning Receptor Functions in Health and Disease. Curr Neuropharmacol. 14: 494-503.

70. Mahato PK, Ramsakha N, Ojha P, Gulia R, Sharma R, Bhattacharyya S (2018) Group I Metabotropic Glutamate Receptors (mGluRs): Ins and Outs. Adv Exp Med Biol. 1112: 163-175.

71. Nicolas C, Russell TI, Pierce AF, Maldera S, Holley A, You ZB, McCarthy MM, Shaham Y, Ikemoto S. Incubation of Cocaine Craving After Intermittent-Access Self-administration: Sex Differences and Estrous Cycle. Biol Psychiatry. 2019; 85:915-924.
72. Kerstetter KA, Aguilar VR, Parrish AB, Kippin TE. Protracted time-dependent increases in cocaine-seeking behavior during cocaine withdrawal in female relative to male rats. Psychopharmacology. 2008; 198:63-75.

# Figures

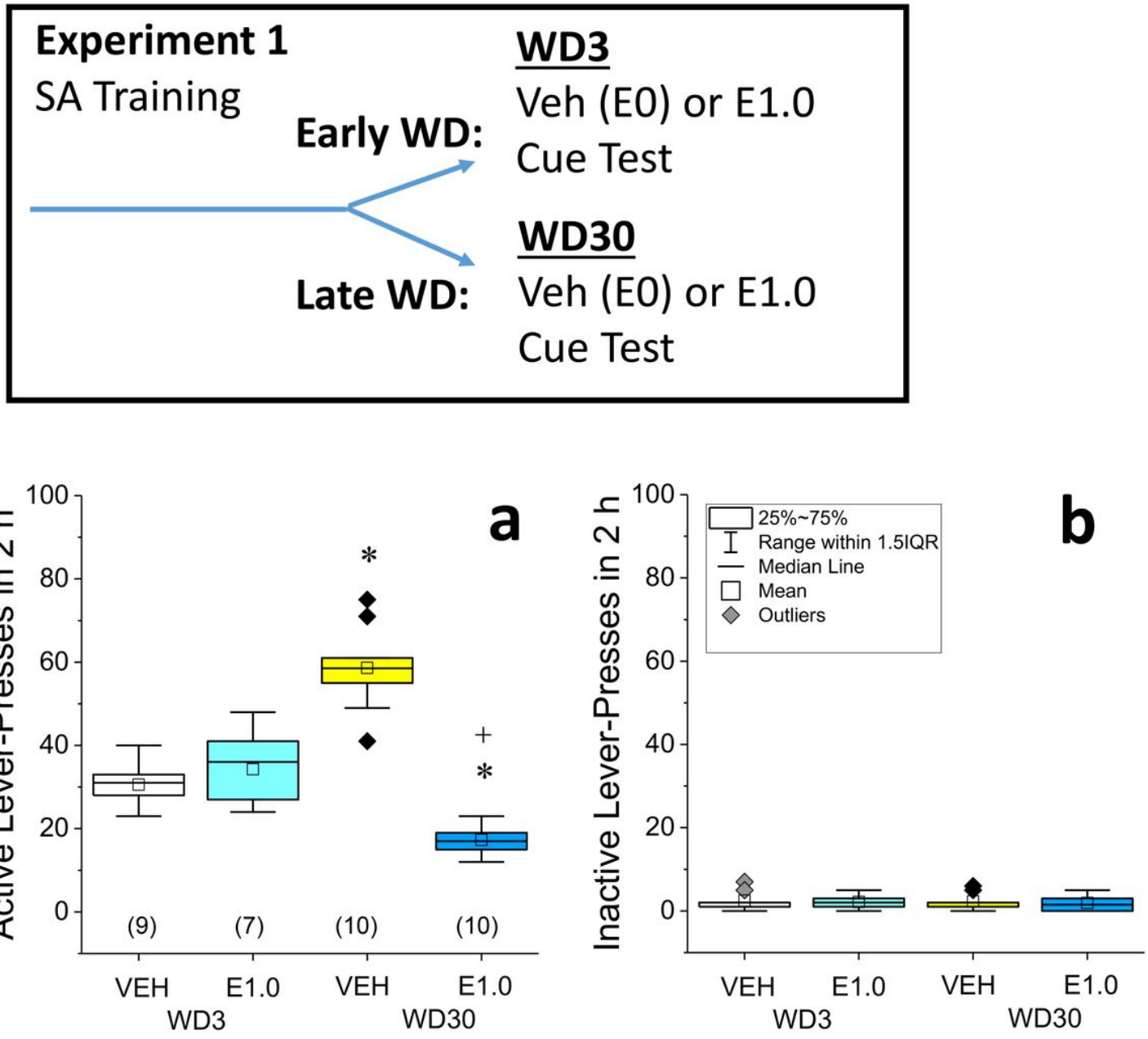
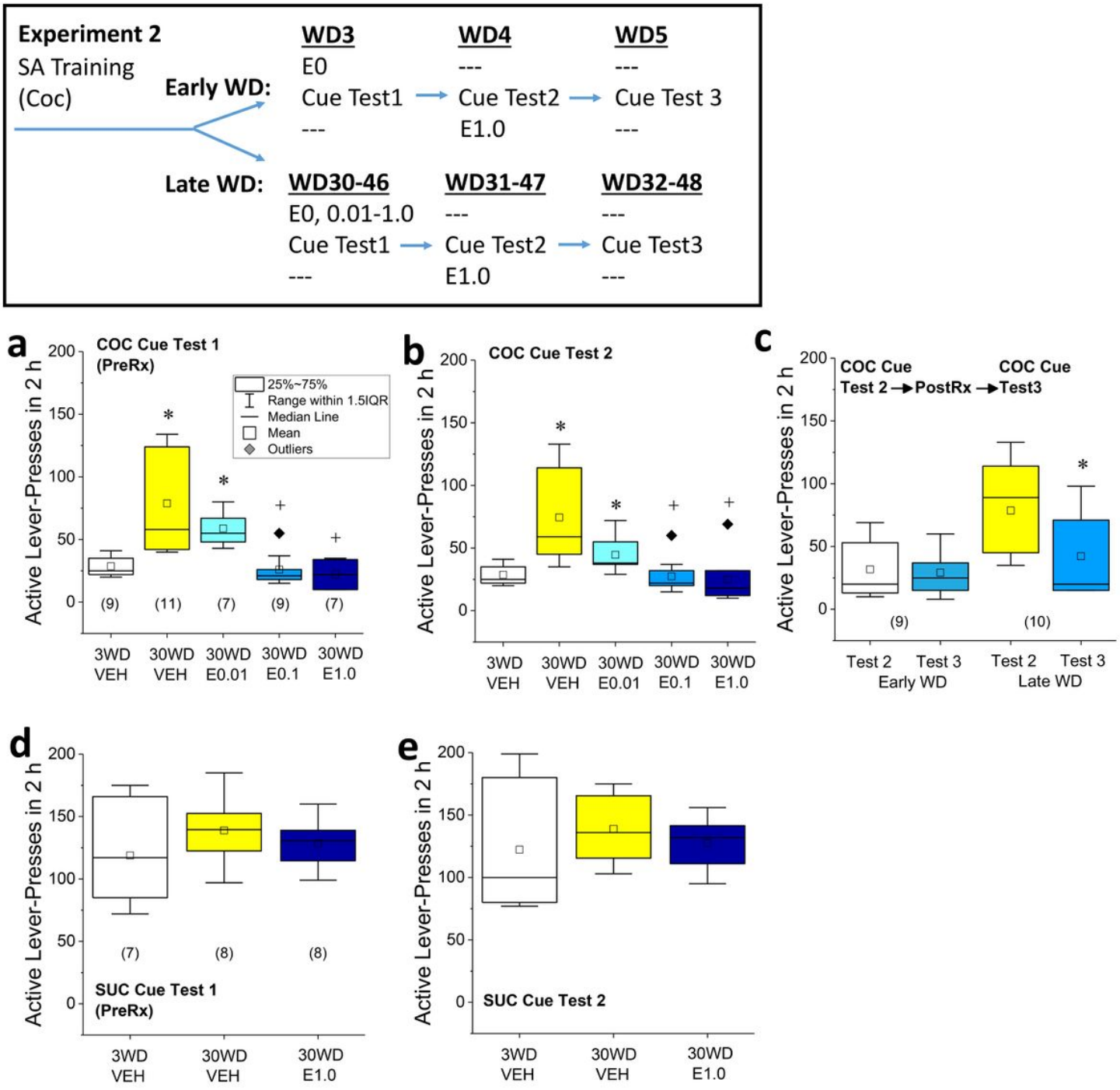



**Figure 1**

Acute Everolimus pretreatment selectively blocks incubated cocaine-seeking. Inset: Summary of the procedural time-line of Experiment 1. a. Vehicle (VEH)-pretreated rats exhibited increased active lever-pressing on withdrawal day (WD) 30 versus WD3 and this incubated responding was reduced by pretreatment with 1.0 mg/kg Everolimus (E1.0). b. No Everolimus effects were observed for inactive presses during cue-testing. As summarized in Panel b, the data are presented as box plots in which the mean is represented by square, the median by -, outliers are indicated by •'s, the box represents the interquartile range (IQR) and the error bars represent 1.5 X IQR. The sample sizes are indicated in

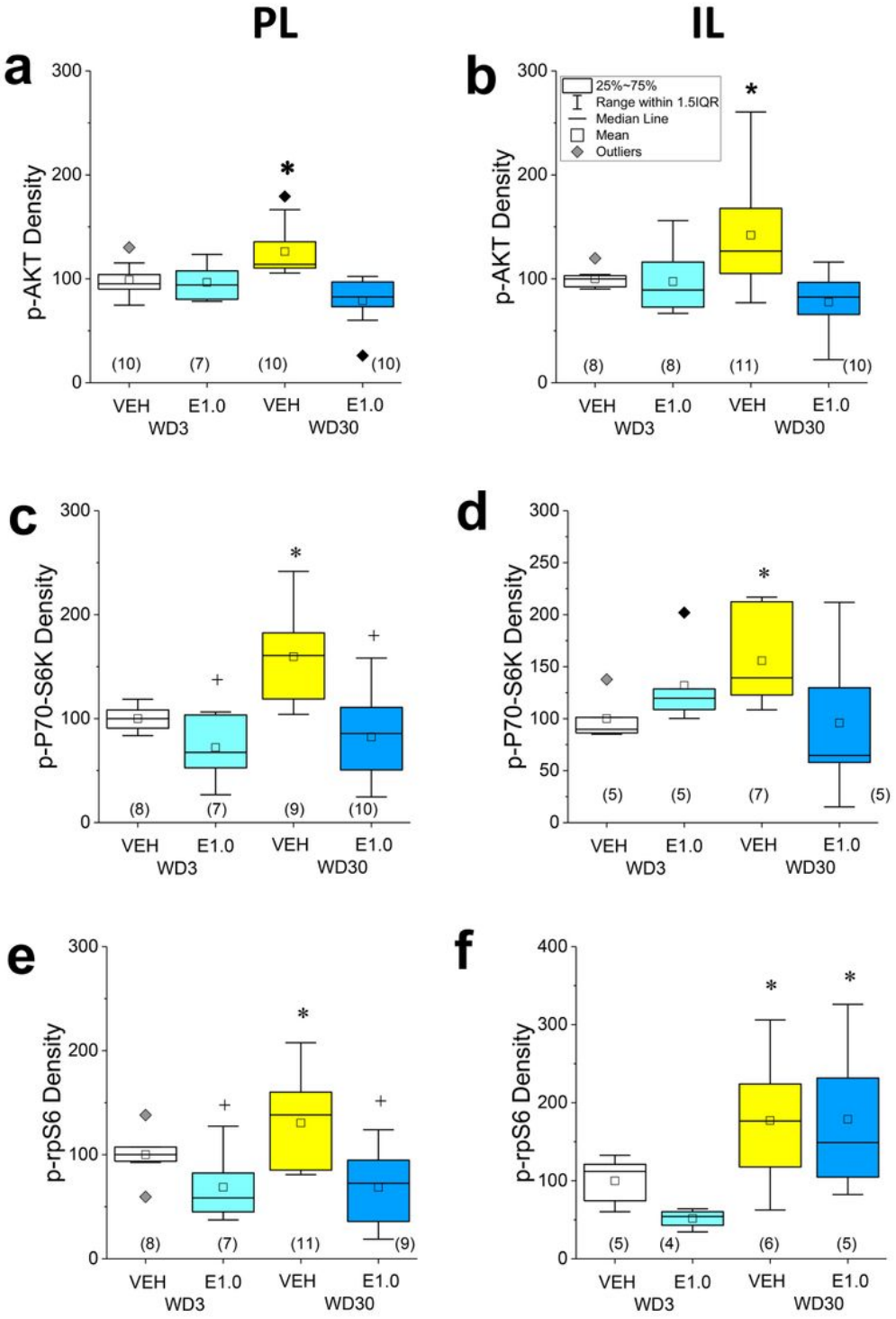
parentheses in Panel a. *p<0.05 vs. WD3-VEH (incubation); +p<0.05 vs. VEH (Everolimus effect) as determined by a priori contrasts.




## Figure 2

Acute Everolimus pretreatment blocks the incubation of cocaine-craving. Inset: Summary of the procedural time-line of Experiment 2. a. Acute Everolimus (E) pretreatment (PreRx) dose-dependently reduced incubated cue-elicited responding on the active lever, relative to vehicle (VEH)-pretreated rats and (b) this effect persisted the next day on Cue Test 2. c. Pairwise comparisons of the active lever-pressing behavior between Test 2 (post-treatment) and Test 3 (no further treatment) confirmed no change in

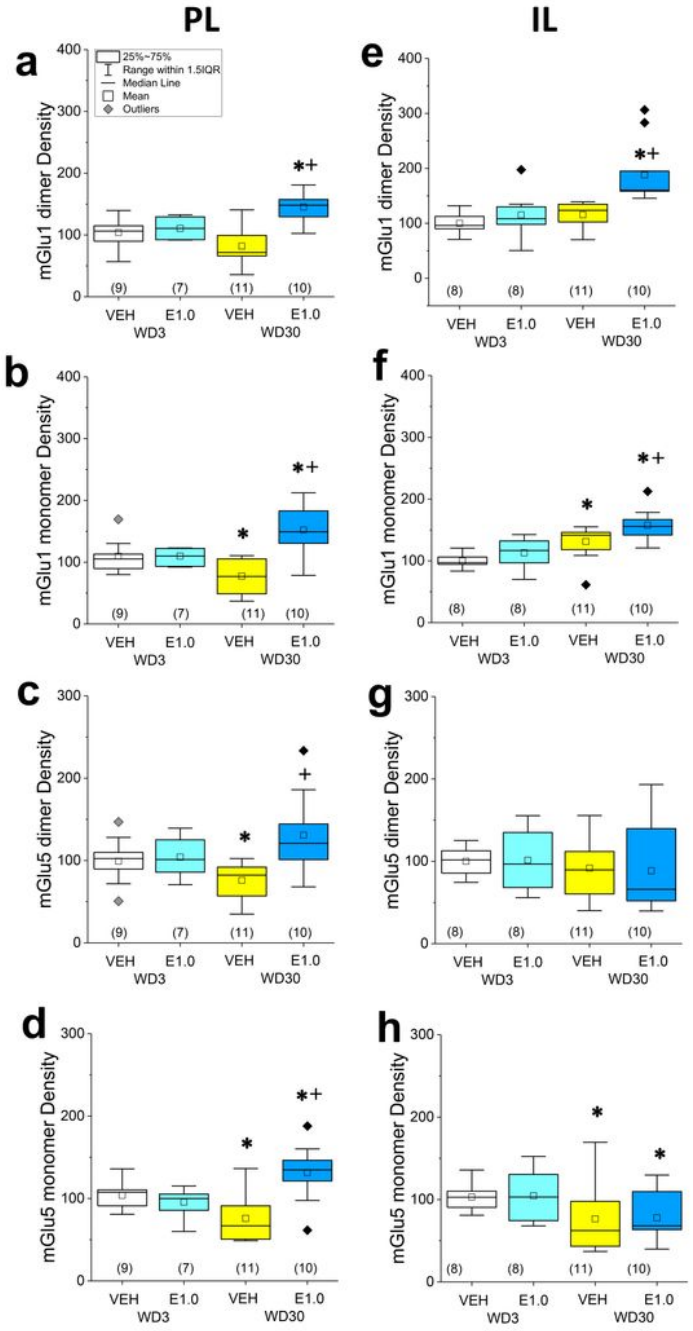
responding for VEH rats tested in late withdrawal, while Everolimus post-treatment significantly reduced responding exhibited on Cue Test 3 in incubated animals. d. In rats trained to self-administer sucrose, cue-elicited sucrose-seeking was unaffected by withdrawal or 1.0 mg/kg Everolimus on Cue Test 1 or (e) Cue Test 2 (no further treatment). As summarized in Panel a, the data are presented as box plots in which the mean is represented by square, the median by -, outliers are indicated by •'s, the box represents the interquartile range (IQR) and the error bars represent 1.5 X IQR. The sample sizes are indicated in parentheses in their respective panels. *$p<0.05$ vs. WD3-VEH (incubation); +$p<0.05$ vs. VEH (Everolimus effect) as determined by LSD post-hoc tests.




## Figure 3

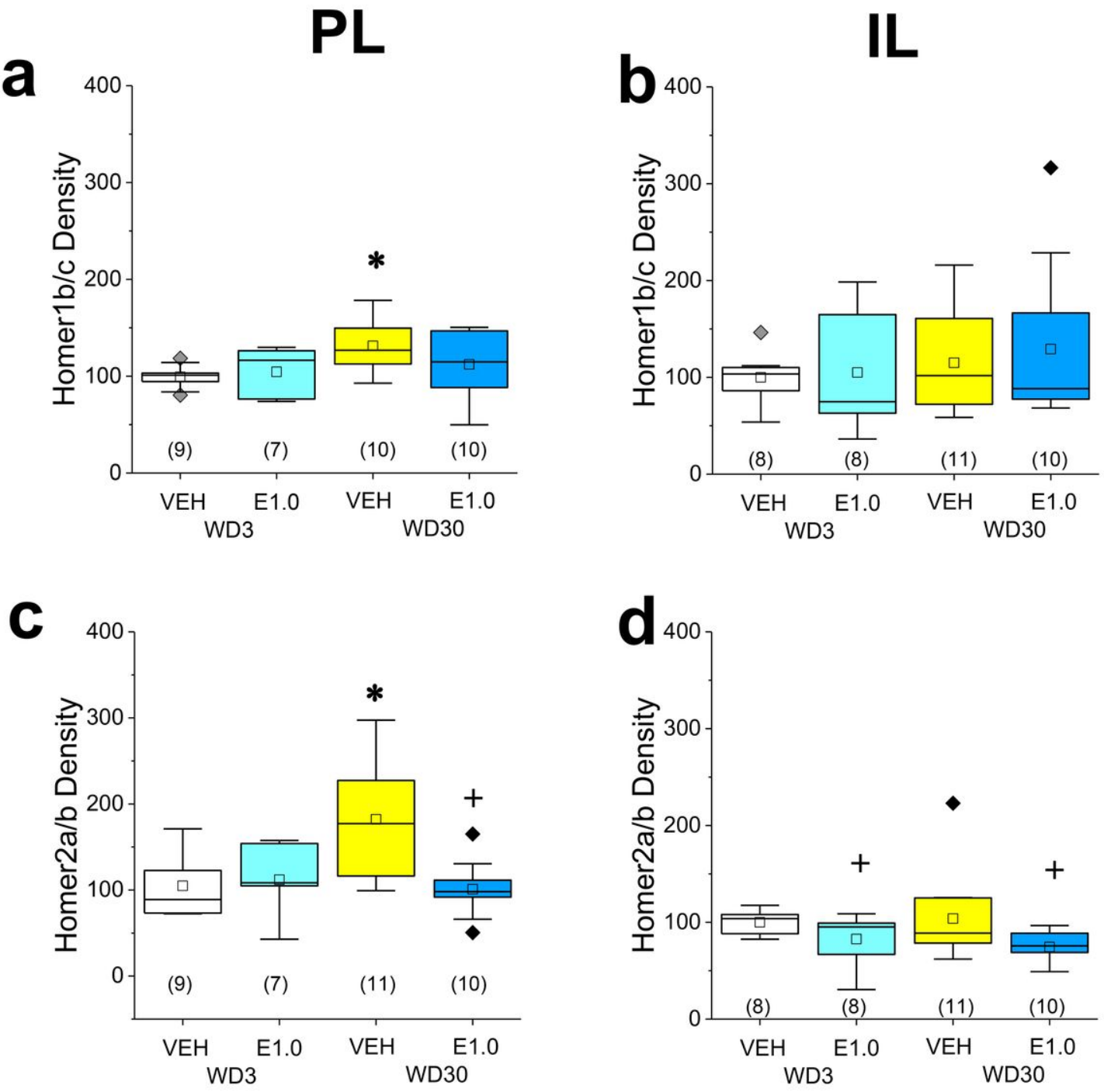
Acute Everolimus pretreatment reverses incubation-related activation of Ak1/mTOR signaling in vmPFC subregions. The immunoblotting results for p(Ser473)-Akt1, p(Thr389)-P70S6K, and p(Ser234/235)-rpS6 expression within the PL are presented in the left panels. For direct comparison, the results for the IL are presented in the right panels. 1.0 mg/kg Everolimus (E1.0) selectively reduced p(Ser473)-Akt1 within the (a) PL and (b) IL of cocaine-incubated rats tested on WD30. P(Thr389)-P70S6K levels incubated in both

the PL (c) and IL (d), but Everolimus reduced this incubation only in the PL. The levels of p(Ser235/236)-rpS6 were not significantly elevated in either the PL (e) or IL (f) of cocaine-incubated rats, although Everolimus reduced protein expression in rats tested on WD30. As indicated in panel b, the data are presented as box plots in which the mean is represented by square, the median by -, outliers are indicated by •'s, the box represents the interquartile range (IQR) and the error bars represent 1.5 X IQR (see panel d'). *p<0.05 vs. WD3-VEH (incubation); +p<0.05 vs. VEH (Everolimus effect) as determined by a priori contrasts.



## Figure 4

Everolimus pretreatment reverses incubation-related changes in mGlu1/5 expression within the PL. The immunoblotting results for the expression of the monomer and dimer forms of mGlu1 and mGlu5 within the PL are presented in the top panels. For direct comparison, the results for the IL are presented in the bottom panels. a-d, In the PL, vehicle (VEH)-pretreated rats exhibited a time-dependent reduction in the expression of both the dimer and monomer forms of each receptor. Everolimus increased mGlu1/5 expression in rats tested on WD30. e. Everolimus pretreatment elevated IL mGlu1 dimer expression only on WD30. f. mGlu1 monomer levels increased in VEH-pretreated rats during withdrawal, but Everolimus did not affect monomer expression. g-h, No group differences were detected regarding IL expression of the dimer or monomer forms of mGlu5. As indicated in panel a, the data are presented as box plots in which the mean is represented by square, the median by -, outliers are indicated by •'s, the box represents the interquartile range (IQR) and the error bars represent 1.5 X IQR (see panel d'). *$p<0.05$ vs. WD3-VEH (incubation); +$p<0.05$ vs. VEH (Everolimus effect) as determined by a priori contrasts.




## Figure 5

Everolimus pretreatment reverses incubation-related changes in Homer expression within the PL. The immunoblotting results for the expression of Homer1b/c and Homer2a/b within the PL are presented in the top panels. For direct comparison, the results for the IL are presented in the bottom panels. PL expression of both Homer1b/c (a) and Homer2a/b (b) increased as a function of withdrawal in vehicle (VEH)-pretreated rats, and Everolimus lowered the levels of Homer2a/b on WD30. c. No group differences were detected for Homer1b/c within the IL. d. Although IL Homer2a/b levels did not vary as a function of

cocaine withdrawal, Everolimus pretreatment lowered IL Homer2a/b expression at both withdrawal time-points. As indicated in panel a, the data are presented as box plots in which the mean is represented by square, the median by -, outliers are indicated by •'s, the box represents the interquartile range (IQR) and the error bars represent 1.5 X IQR. *p<0.05 vs. WD3-VEH (incubation); +p<0.05 vs. VEH (Everolimus effect) as determined by a priori contrasts.

## Supplementary Files

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