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# Positive allosteric modulation of mGlu1 reverses cocaine-induced behavioral and synaptic plasticity through the integrated stress response and oligophrenin-1

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## Abstract

**Background:** Cue-induced cocaine craving progressively intensifies (‘incubates’) during abstinence from cocaine self-administration. Expression of ‘incubated’ cocaine craving depends on elevated calcium-permeable AMPARs (CP-AMPARs) on medium spiny neurons in the nucleus accumbens (NAc) core. After incubation has occurred, stimulation of NAc mGlu1 receptors or systemic administration of mGlu1 positive allosteric modulators (PAM) removes CP-AMPARs from NAc synapses via dynamin-dependent internalization (mGlu1-LTD) and thereby reduces ‘incubated’ cocaine craving. As mGlu1 PAMs are potential therapeutics for cocaine craving, it is important to further define the mechanism triggering this mGlu1-LTD.

**Methods:** Male and female rats self-administered saline or cocaine (10 days) using a Long Access regimen (6 hours/day). Following ≥40 days of abstinence, we assessed the ability of an mGlu1 PAM to inhibit expression of ‘incubated’ craving and remove CP-AMPARs from NAc synapses under control conditions, after blocking the Integrated Stress Response (ISR), or after knocking down oligophrenin-1, a mediator of the ISR that can promote AMPAR endocytosis. AMPAR transmission in NAc MSNs was assessed with *ex vivo* slice recordings.

**Results:** mGlu1 stimulation reduced cue-induced craving and removed synaptic CP-AMPARs. When the ISR was blocked prior to mGlu1 stimulation, there was no reduction in cue-induced craving nor were CP-AMPARs removed from the synapse. Further, selective knockdown of oligophrenin-1 blocked the mGlu1-LTD.

**Conclusions:** Our results indicate that mGlu1-LTD in the NAc and consequently the reduction of cue-induced seeking occur through activation of the ISR, which induces translation of

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oligophrenin-1. We also demonstrate CP-AMPAR accumulation and mGlu1 reversal in female rats, as previously shown in males.

**Keywords**

AMPA receptor; incubation of cocaine craving; mGluR-LTD; nucleus accumbens; OPHN1; Integrated Stress Response

## INTRODUCTION

Individuals suffering from substance use disorder (SUD) have difficulty remaining abstinent, as evidenced by the high incidence of relapse. One key cause of relapse is exposure to drug-associated cues that provoke craving. In fact, cue-induced craving can progressively intensify across a drug-free abstinence period. This is termed ‘incubation of craving’ and occurs following cocaine use in rodents (1) and humans (2). Incubation likely contributes to continued vulnerability to relapse long after the cessation of drug use (3).

Incubation of cocaine craving involves synaptic plasticity in the Nucleus Accumbens (NAc), a critical region regulating reward and motivation (4). The NAc is comprised primarily of medium spiny neurons (MSN) that receive inputs from cortical and limbic regions and send projections to downstream regions of the Basal Ganglia to control action selection and motivated behavior. Following cocaine self-administration and a subsequent drug-free abstinence period, we demonstrated an increase in high-conductance $Ca^{2+}$-permeable AMPA receptors (CP-AMPARs; homomeric GluA1) at excitatory synapses on MSNs in the core subregion of the NAc; very low CP-AMPAR levels are found in MSNs of drug-naïve rats (5). Additional studies showed that CP-AMPARs in both core and shell subregions of the NAc are required for expression of incubated cocaine seeking (5–9). Taken together this suggests that CP-AMPAR elevation strengthens NAc glutamatergic synapses, which produces an augmented synaptic response to glutamate release elicited by presentation of cocaine-associated cues and leads to intensified cocaine seeking (4).

The group I metabotropic glutamate receptors (mGluR), mGlu1 and mGlu5, are primarily located post-synaptically and couple to the Gq-like class of G-proteins that play important roles in plasticity, including LTD in many brain regions (10). Interestingly, there is a special relationship between mGlu1 and CP-AMPARs: in synapses containing high CP-AMPAR levels, across multiple brain regions, mGlu1 stimulation elicits a form of LTD expressed via CP-AMPAR internalization (11). Critically, enhancing mGlu1 transmission onto NAc MSNs with a positive allosteric modulator (PAM) elicits this mGlu1-LTD and thereby reduces incubated cocaine seeking (6). This effect, as well as mGlu1’s ability to reverse cocaine-induced synaptic plasticity in the ventral tegmental area (VTA) (12–18), suggests mGlu1 PAMs as potential anti-craving therapeutics, making it important to understand their mechanism of action.

The Integrated Stress Response (ISR) is a conserved signaling network that restores protein homeostasis by regulating protein synthesis (19). In response to a variety of environmental stressors, phosphorylation of the α subunit of the eukaryotic initiation factor 2 (eIF2α) decreases the rate of general translation, but at the same time increases translation of a

subset of mRNAs with an upstream open reading frame (uORF) in the 5' untranslated region (20, 21). Proteins regulated by the ISR have diverse functions, including modulation of synaptic plasticity and memory storage in hippocampus and other brain areas associated with reward (19, 22). One such protein is OPHN1, a Rho-GTPase activating protein implicated in X-linked mental retardation (23). OPHN1 also interacts with endophilin A2/3, a component of the clathrin-dependent endocytic machinery, and through this interaction mediates mGluR-LTD in hippocampus (24, 25). OPHN1 is also critical for reversing cocaine-induced synaptic plasticity in VTA (15, 16).

Here we examined the role of the ISR and OPHN1 in mGlu1-mediated removal of synaptic CP-AMPARs in NAc core and the resultant reduction of incubated cocaine seeking. We found that mGlu1 stimulation reduced seeking and removed synaptic CP-AMPARs, in agreement with our previous work (6). However, after acute treatment with a small molecule inhibitor of the p-eIF2α translational program, ISRIB (26), or shRNA-mediated knockdown of OPHN1 protein (16, 24, 27), mGlu1 stimulation no longer prevented the expression of incubation nor removed synaptic CP-AMPARs. These results indicate that the ISR is required for the ability of mGlu1 PAMs to reverse incubation and underlying synaptic plasticity.

## METHODS AND MATERIALS

### Subjects and drug self-administration

Procedures were approved by the Oregon Health & Science University IACUC in accordance with the USPHS Guide for Care and Use of Laboratory Animals. Adult male and female Long-Evans rats underwent Long Access cocaine or saline self-administration (6 h/d × 10d; infusions paired with a light cue). After 40–60 days of forced abstinence, rats were tested for cue-induced seeking or slices were prepared for electrophysiological recordings after manipulations of the ISR (see Supplemental Information).

### Virus infusion

*Ophn1* shRNA virus or scrambled shRNA virus was infused bilaterally into NAc core during the week after discontinuing drug self-administration (see Supplemental Information).

### Whole-cell patch-clamp recordings

Procedures adapted from our previous studies were used to determine the contribution of CP-AMPARs to synaptic transmission based on the rectification index (RI) and sensitivity to the CP-AMPAR antagonist NASPM (see Supplemental Information).

### Statistical analyses

Linear mixed models (LMM) analyses were used for all repeated measure behavioral data and one-way and two-way ANOVAs were used to analyze the rectification index and NASPM sensitivity data obtained from the ISRIB+SYN slice electrophysiology experiments. Corrections for multiple comparisons were done for all planned post-hoc tests. Statistical significance was set at $p \leq 0.05$ (see Supplemental Information).

# RESULTS

## Cocaine self-administration

Fig. 1A shows a timeline for behavioral experiments and Fig. 1B shows self-administration data from all rats depicted in Fig. 1 (n=73). The number of infusions increased progressively across sessions, indicating 'escalation of intake' [effect of session: $F(9,122)=3.308$, $p=0.001$; Fig. 1B,C]. Males and females did not differ in number of infusions [effect of sex: $F(1,431)=0.124$, $p>0.1$; Fig. 1C] nor in the degree of escalation [session X sex interaction: $F(9,122)=0.410$, $p>0.1$]. After 10 self-administration sessions, rats received a cue-induced seeking test on withdrawal day (WD) 1 to establish baseline craving. They were then divided into comparable groups, destined for studies described below, based on the number of infusions (Fig. 1D) and nose-pokes during the WD1 seeking test.

## The mGlu1 PAM SYN119 prevents expression of 'incubation of craving'

Following the WD1 seeking test, rats underwent forced abstinence and then received a seeking test on WD40. Prior to the test, rats received intraperitoneal (IP) injection of a potent and specific pharmacological inhibitor of the ISR, ISRIB (1.25 mg/kg or 3.0 mg/kg), or vehicle, followed 70 min later by injection of the mGlu1 PAM SYN119 (10 mg/kg) or vehicle (Fig. 1A). Here we describe results from vehicle+vehicle and vehicle+SYN119 groups; effects of ISRIB are described in the subsequent section.

Rats in the vehicle+vehicle group poked more in the previously active port on WD40 than WD1 ['incubation of craving'; effect of test session: $F(1,122)=5.991$, $p=0.016$; Fig. 1E,G]. Rats administered SYN119 prior to the test (vehicle+SYN119) showed no increase in responding on WD40, i.e., SYN119 blocked the expression of incubation [effect of test session: $F(1,122)=1.039$, $p>0.1$; Fig. 1F,G], consistent with our prior results (6). There was no main effect of the abstinence period on inactive responding ($p>0.1$; Fig. 1H). Analysis of locomotor activity during the tests, measured using photobeams within the operant boxes, did not reveal differences between WD1 and WD40 or between groups (main effect of test session: $p>0.05$; main effect of treatment group: $p>0.1$; Supp. Fig. 1A,B).

Across all treatment groups, male and female rats did not differ in responding at the previously active port [effect of sex: $F(1,122)=0.188$, $p>0.1$]. Also, there were no sex differences on WD1 ($p>0.1$) or WD40 ($p>0.1$) (Supp. Fig. 2A), nor in the extent to which SYN119 reduced seeking on WD40 ($p=0.1$; Supp. Fig. 2B).

## Inhibiting the ISR via ISRIB blocks the effect of SYN119

To determine if SYN119's ability to prevent expression of incubation depended on the ISR, some rats were treated with ISRIB, which inhibits the translational effects mediated by the ISR, prior to SYN119 injection and a WD40 seeking test (Fig. 1A). We tested two doses of ISRIB (see Supplemental Methods). Rats administered the lower dose of ISRIB (1.25 mg/kg, IP) prior to SYN119 did not express incubation of craving [effect of test session: $F(1,122)=0.211$, $p>0.1$; Fig. 1H,I]. Thus, SYN119 was still capable of blocking expression of incubation. However, rats administered the higher ISRIB dose (3.0 mg/kg) prior to SYN119 exhibited greater seeking on WD40 than on WD1 [effect of test session:

F(1,122)=3.778, p=0.05; Fig. 1H,I]. Thus, the higher dose of ISRIB blocked the ability of SYN119 to prevent expression of incubated cocaine craving.

Rats treated with ISRIB alone (ISRIB+vehicle) showed greater seeking on WD40 versus WD1, similar to the vehicle+vehicle group (effect of test session: p=0.011 for ISRIB (3.0 mg/kg) group). These data are shown in Fig. 1H,I for the ISRIB (3.0 mg/kg)+vehicle group and Supp. Fig. 3A,B for the ISRIB (1.25 mg/kg)+vehicle group.

Because there is considerable variability in responding at the previously active nose port on WD1, we normalized the WD40 active port responses for each rat to the average WD1 response for its experimental group (Fig. 1I). As found for the raw data, there was an effect of test session in the vehicle+vehicle group [F(1,121)=5.767, p=0.018], the ISRIB+vehicle group [F(1,121)=4.981, p=0.027], and the ISRIB (3.0 mg/kg)+SYN group [(F(1,121)=4.477, p=0.036], indicating incubation of craving, but no significant effect of test session in the other groups.

## Incubation is accompanied by elevation of synaptic CP-AMPARs in males and females

Additional male and female rats self-administered cocaine or saline followed by a 40–60-day abstinence period (Fig. 2A,B). Rats that self-administered cocaine were assigned to one of four experimental groups [Coc/no other treatment (hereafter referred to as cocaine or Coc), Coc/vehicle+vehicle, Coc/SYN, Coc/ISRIB+SYN] based on equivalent self-administration data (Supp. Fig. 4A). First, we will describe the cocaine and saline groups. On WD40–60, whole-cell patch-clamp recordings were conducted from NAc core MSNs. AMPAR-mediated electrically evoked EPSCs (eEPSC) were assessed at different holding potentials. We have previously demonstrated robust CP-AMPAR elevation across this period in male cocaine rats (28). Whereas MSNs from saline rats showed linear current-voltage relationships, MSNs from cocaine rats exhibited inwardly rectifying AMPAR currents indicative of an increased contribution of CP-AMPARs to synaptic transmission (Fig. 2D;Fig. 2E, left bars), quantified as a significant increase in the RI [effect of group: q(1,67)=10.8, p<0.001; Fig. 2C–E]. Importantly, there was no main effect of sex (p>0.1) in the analysis of RI data. These results extend our prior findings after incubation of cocaine craving in male rats to females (5, 6, 29, 30).

## The mGlu1 PAM SYN119 removes synaptic CP-AMPARs and pre-treatment with ISRIB blocks this effect

We showed previously that SYN119 (10 mg/kg, IP), administered to cocaine-incubated rats 20 min before a cue-induced seeking test, normalized elevated CP-AMPAR levels and reduced cue-induced craving for ~24 h (6), and that CP-AMPAR normalization reflected dynamin-dependent internalization (30). Given the ability of ISRIB to block the SYN119-induced reduction of seeking (Fig. 1H,I), we tested ISRIB's effect on the SYN119-induced removal of CP-AMPARs.

Along with the cocaine and saline groups described in the previous section, we also recorded from groups that, on the day of recording, received 10 mg/kg SYN119 alone (Coc/SYN group), 3 mg/kg ISRIB prior to SYN119 (Coc/ISRIB+SYN group), or double vehicle injections (Coc/vehicle+vehicle group). The Coc/vehicle+vehicle group exhibited an

elevated RI relative to saline rats [q(1,60)=7.505, p<0.001] and did not differ from Coc rats receiving no treatment on the day of recording [q(1,57)=2.248, p>0.1; Fig. 2C–E). This is consistent with a prior study where we recorded MSNs from cocaine-incubated rats injected with the SYN119 vehicle and demonstrated no effect of the vehicle on the RI (6). As expected, SYN119 decreased the RI compared to cocaine rats that received no SYN119 injection [Coc/SYN vs. Coc: q(1,67)=9.669, p<0.001; Fig. 2C–E]. Furthermore, the RI of Coc/SYN rats did not differ from the RI of saline controls [Coc/SYN vs. Sal: q(1,70)=1.164, p>0.1; Fig. 2C–E]. In cocaine-incubated rats treated with ISRIB+SYN119 on the day of recording, MSNs showed an elevated RI relative to rats receiving SYN119 alone [Coc/ISRIB+SYN vs. Coc/SYN: q(1,70)=8.483, p<0.001; Fig. 2C–E] and the RI in the Coc/ISRIB+SYN rats did not differ from cocaine rats that received no SYN119 (Coc/ISRIB+SYN vs. Coc: q(1,67)=1.439, p>0.1; Fig. 2C–E). Together these results indicate that SYN119 removed synaptic CP-AMPARs in cocaine-incubated rats, and ISRIB pretreatment prevented this effect of SYN119. Post-hoc comparisons revealed no effect of sex within any of the groups tested (Sal, Coc, Coc/Veh+Veh, Coc/SYN, Coc/ISRIB+SYN; p>0.1) (Fig. 2E).

To confirm that the elevated RI resulted from increased synaptic CP-AMPARs, we recorded AMPAR-mediated eEPSCs before and after application of the selective CP-AMPAR antagonist NASPM (100 μM), as in previous studies (5, 6, 30, 31). As expected, NASPM significantly reduced eEPSC amplitude in MSNs from cocaine rats but not saline rats [Coc vs. Sal: q(1,18)=9.042, p<0.001; Fig. 2F–H], whereas NASPM sensitivity was reduced to saline control levels in rats treated with SYN119 on the day of recording [Coc/SYN vs. Sal: q(1,21)=0.069, p>0.1; Fig. 2F–H] but not rats treated with ISRIB+SYN119 [Coc/ISRIB+SYN vs. Sal: q(1,22)=7.414, p<0.001; Fig. 2F–H]. These findings recapitulate the RI results described above.

Next, we examined if CP-AMPAR dynamics were accompanied by altered synaptic strength by measuring AMPAR/NMDAR ratios. We observed a higher AMPA/NMDA ratio in cocaine rats than saline rats [Coc vs. Sal: q(1,60)=4.598, p<0.01; Fig. 2I]. SYN119 injection decreased the AMPA/NMDA ratio to saline control levels [Coc/SYN vs. Sal: q(1,66)=1.633, p>0.1; Fig. 2I], while pretreatment with ISRIB blocked SYN119's effect [Coc/ISRIB+SYN vs. Sal: q(1,62)=5.230, p<0.01; Fig. 2I]. Together with the RI and NASPM data, these findings demonstrate that SYN119 removes CP-AMPARs and normalizes the incubation-induced enhancement of synaptic strength, and that ISRIB prevents these effects of SYN119. In addition, we observed a correlation between AMPA/NMDA ratio and RI in cells for which both were measured, suggesting that CP-AMPAR insertion accounts for the elevated AMPA/NMDA ratio after cocaine (Supp. Fig. 4B).

### OPHN1 knockdown prevents removal of synaptic CP-AMPARs by SYN119

In hippocampus and VTA, activation of the ISR via mGluR-LTD results in an increase in *Ophn1* translation and OPHN1 mediates AMPAR internalization (15, 16, 24, 25). To test if OPHN1 was similarly involved in removing NAc CP-AMPARs, we used a lentivirus expressing shRNA targeting OPHN1 (24, 27). To confirm virus efficacy, the *Ophn1* shRNA virus or a scrambled shRNA control virus was injected into the NAc of drug-naïve rats.

The *Ophn1* shRNA virus reduced OPHN1 protein, relative to the scrambled shRNA [t-test(18)=2.55, p=0.01; Fig. 3B and Supp. Fig. 5B].

In a separate cohort of rats, either *Ophn1* shRNA virus or scrambled shRNA virus was injected bilaterally into the NAc during the first week after discontinuing cocaine self-administration. On WD40–60, rats were injected with SYN119 (10 mg/kg, IP) prior to recordings (Fig. 3A). NAc core MSNs from rats that received the *Ophn1* shRNA virus had an elevated RI compared to rats receiving the scrambled shRNA [t-test(37)=9.148, p<0.001; Fig. 3C–E]. To confirm these findings, we used NASPM. MSNs from rats that received *Ophn1* shRNA showed a robust reduction in eEPSC amplitude following NASPM application compared to rats that received the scrambled shRNA [t-test(11)=4.914, p<0.001; Fig. 3F–H]. We then examined the effect of OPHN1 knockdown on the AMPA/NMDA ratio. We detected a higher AMPA/NMDA ratio in MSNs from SYN119-injected cocaine rats that previously received the *Ophn1* shRNA compared to rats that received the scrambled shRNA [t-test(43)=7.408, p<0.05; Fig. 3I]. Taken together with the RI and NASPM data, this demonstrates that SYN119 treatment removed synaptic CP-AMPARs and attenuated synaptic strength in rats that received the scrambled shRNA, but was incapable of doing so after OPHN1 knockdown.

## DISCUSSION

Protein translation is important for synaptic plasticity in addiction models (32), including incubation of craving (33–35). Consistent with prior findings in male rats (5, 6), here we show that incubation of cocaine craving in both sexes is accompanied by accumulation of synaptic CP-AMPARs in NAc core MSNs, and these receptors can be removed from the synapse by systemic treatment with the mGlu1 PAM, SYN119. Further, pharmacologically blocking protein translation that is induced by activation of the ISR with the drug ISRIB prior to treatment with SYN119 blocked both the behavioral and synaptic effects of SYN119. Thus, both the mGlu1-induced reduction in incubated seeking and removal of CP-AMPARs depend on activation of the ISR. We then showed that OPHN1, a protein that is translationally controlled by the ISR, is critical for this mGlu1-LTD in the NAc. After knocking down OPHN1, mGlu1 stimulation no longer removed synaptic CP-AMPARs. Taken together, our results reveal the mechanism through which mGlu1 stimulation removes synaptic CP-AMPARs on MSNs in the NAc and thereby reduces incubated cocaine seeking. Along with prior work, this suggests a conserved mechanism that exists across several brain regions for mediating mGlu1-LTD and that is important in reversing a subset of the long-term effects of cocaine use, including effects relevant to craving and relapse.

### The Integrated Stress Response

In response to different stresses, ISR kinases phosphorylate eIF2 at its α subunit (see Introduction) to restore protein homeostasis. Interestingly, different drugs of abuse or exposure to drug-associated cues/contexts inhibit the ISR (reduce p-eIF2α) and this is causally linked to maladaptive responses. For example, in VTA, reduced p-eIF2α is associated with greater vulnerability to cocaine-induced LTP and conditioned place preference (16). In the NAc of cocaine-incubated rats, exposure to a cocaine-paired context

and cues (during a seeking test) is associated with reduced p-eIF2α and intra-NAc infusion of Sal003, a selective inhibitor of eIF2α dephosphorylation, prevents the expression of incubation (34). Taken together, these findings provide stong evidence that inhibition of the ISR promotes vulnerability to motivating effects of drugs of abuse in animal models of SUD. Potential translational relevance of these findings is indicated by functional magnetic resonance imaging studies demonstrating that allelic variability of the gene encoding eIF2α modulates responses to reward in human smokers (36). Finally, group I mGluR activation, which induces the ISR, leads to AMPAR internalization in both the hippocampus and VTA dopamine neurons, and inhibition of the ISR prevents both mGluR-LTD and the removal of AMPARs (16, 25).

The present studies, the first to link ISR activation resulting from mGluR-LTD to the reduction of drug craving, demonstrate that mGlu1 stimulation in the NAc core removes CP-AMPARs and reduces incubated cocaine seeking by activating the ISR (Fig. 4). After pharmacologically blocking the ISR with ISRIB, mGlu1 stimulation no longer exerted these effects. Thus, when the ISR is activated by mGlu1 PAMs, there is a reduction in incubated seeking.

In the NAc, little is known about the role the ISR plays in addiction-like behaviors beyond data shown here. In naïve rats, LTD in NAc core MSNs elicited by the non-selective group I mGluR agonist dihydroxyphenylglycine (DHPG) is driven by mGlu5 and expressed presynaptically; after incubation, this form of LTD is abolished and instead DHPG elicits mGlu1-dependent LTD expressed via CP-AMPAR removal (29). It is possible that the ability of mGlu1 and the ISR to regulate CP-AMPAR levels emerges during incubation of craving. This regulation would occur via the LTD demonstrated here and perhaps also by decreasing *Gria1* translation (37). It is also possible that the mGlu1/ISR-LTD pathway is operative in naïve rats and working tonically to maintain very low levels of synaptic CP-AMPARs in MSNs, and that reduced function of this pathway occurs in incubation. Supporting this, mGlu1 surface expression declines in NAc core during incubation, preceding and enabling CP-AMPAR accumulation, although pharmacological activation of remaining mGlu1 elicits internalization of these CP-AMPARs (6). Interestingly, CP-AMPARs are not detectable for ~24 h (after a single SYN119 injection) or ~72 h (repeated SYN119 injections), but then re-emerge and return to ‘incubated’ levels. This suggests that mGlu1 is not the master switch controlling CP-AMPAR synaptic expression, but rather a dimmer capable of acutely removing them, and that the master switch remains ON (yes to CP-AMPARs) even after mGlu1 stimulation. This is important when considering mGlu1 PAMs as a potential clinical treatment for SUD, as it suggests that they could be used prophylactically to provide a short-term reduction of cue-induced craving whereas chronic treatment would be required to provide longer-term protection.

## OPHN1

In hippocampus, mGluR-LTD requires rapid protein synthesis (in contrast to NMDAR-LTD) (38, 39). The proteins upregulated by mGluR stimulation (known collectively as “LTD proteins”) are believed to be synthesized in dendrites containing active synapses and increase the rate of AMPAR endocytosis (10, 40). More specifically, mGluR-LTD induces a

protein synthesis program controlled by the ISR (25). One ISR-regulated protein is OPHN1, previously shown to play a critical role in hippocampal mGluR-LTD (see below) and in cocaine-induced plasticity in VTA (15, 16).

The mechanism through which OPHN1 contributes to LTD has been best studied in hippocampus. OPHN1 can influence synaptic strength through its activity as a rhoGAP, its interaction with Homer1b/c, and its interaction with endophilin A1 and A2/3 (24, 27, 41, 42). OPHN1's interaction with endophilin A2/3, which promotes dynamin-dependent endocytosis, has been established as the mechanism underlying AMPAR internalization during hippocampal mGluR-LTD (24). Given that mGlu1 PAM-induced removal of CP-AMPARs in the NAc of cocaine-incubated rats occurs via dynamin-dependent endocytosis (30), OPHN1 interaction with endophilin A2/3 is likely responsible for this LTD as well. It should be noted that our work suggests that OPHN1-dependent LTD in the NAc is specific to mGlu1-mediated internalization of CP-AMPARs [present findings; (6, 29)], consistent with similar studies in VTA (15, 16), whereas the original hippocampal studies did not distinguish mGlu1 from mGlu5 and did not specify the type of AMPAR internalized (24, 25). Endophilin A1 is primarily located presynaptically and its interaction with OPHN1 is involved in synaptic vesicle endocytosis (27), so this interaction is unlikely to mediate the NAc mGlu1-LTD described here. The activity of OPHN1 as a rhoGAP and its interaction with Homer1b/c are critical for baseline synaptic strength and development, but are not involved in mGluR-LTD in the hippocampus (24, 41, 42). We note that our data do not address whether OPHN1 downregulation affects basal synaptic transmission in NAc MSNs.

Future studies will determine if translation of *Ophn1* can be detected in response to mGlu1 activation in the NAc of cocaine-incubated rats and where this occurs in the neuron. Given the rapidity of the response (CP-AMPAR internalization within 60 min after SYN119 injection; Fig. 2) it is likely that the effect of mGlu1 depends on protein translation in dendrites. It would also be of interest to determine if OPHN1 upregulation is sufficient to elicit CP-AMPAR internalization after incubation of cocaine craving.

## Sex differences

In our prior studies demonstrating CP-AMPAR upregulation in the NAc core during incubation of cocaine craving and reversal of CP-AMPAR plasticity and incubation by mGlu1 PAMs we used male rats (5, 6). Here we show for the first time that female rats exhibit the same synaptic and behavioral plasticity. Although no sex differences were observed in our study, we acknowledge that it was not powered to detect small differences nor was it designed to uncover latent effects beyond the mGlu1-LTD that was our focus, e.g., sex differences in mechanistic underpinnings of incubation or CP-AMPAR accumulation. Further, we did not monitor estrous cycle in this study. Female rats in estrus show greater cocaine-seeking than males and non-estrus females, which do not differ (43–45).

## Conclusions

In cosidering potential pharmacological treatments for SUD, it is important to understand the mechanisms through which they work. It is widely held that neuroplasticity mechanisms underlying memory processes are "hijacked" by drugs of abuse, contributing to SUD. The

ISR not only regulates the two major forms of plasticity in the mammalian brain, namely LTD and LTP, but is an universal regulator of long-term memory formation (22). Therefore it is not surprising that the ISR is implicated in addiction-related plasticity by prior work in the VTA (15, 16) and by the present study. Specifically, we show that mGlu1 PAMs elicit internalization of CP-AMPARs in the NAc core leading to reduced cocaine craving through the ISR and specifically OPHN1 (Fig. 4). These results extend previous work suggesting mGlu1 PAMs as a promising target for treatment of SUD (6, 11). While our studies have focused on reversal of cocaine incubation in the NAc (6, 29, 30, 37), mGlu1 stimulation also reverses cocaine-induced synaptic potentiation in VTA (12–18) and cocaine-induced synaptic and behavioral plasticity involving prefrontal cortex (46, 47). In addition, mGlu1 PAMs reduce incubation of methamphetamine craving and associated CP-AMPAR plasticity (31). Interestingly, they also exhibit antipsychotic-like effects (48, 49). Together, these results indicate mGlu1 PAMs as a potential treatment that is highly compatible with the view that drugs of abuse produce long-lasting changes across a number of brain regions leading to addiction, involving alterations in both mesolimbic motivation and top-down cortical control (50, 51).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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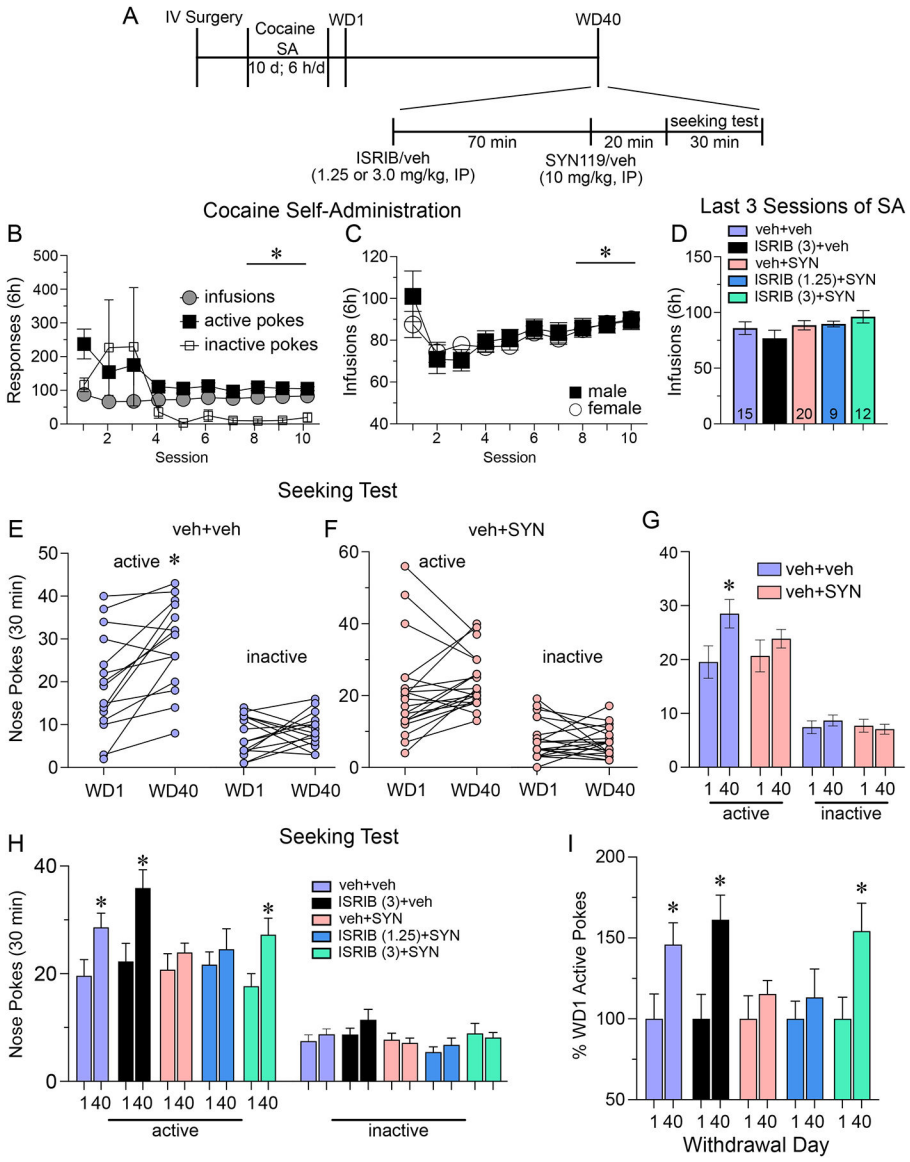


**Figure 1. Treatment with SYN119 reduces 'incubated' seeking after protracted abstinence from cocaine self-administration, and pre-treatment with ISRIB prior to SYN119 blocks this effect.**

A, Experimental timeline. All rats underwent 10 days of intravenous (IV) cocaine self-administration (SA) and a subsequent 40-day, cocaine-free abstinence period. On Withdrawal Day 1 (WD1) and WD40 all rats underwent a seeking test during which nose-pokes in the previously active hole resulted in the presentation of the cue previously paired with cocaine, but no cocaine was delivered. Prior to the WD40 seeking test, rats were treated with ISRIB (1.25 mg/kg or 3.0 mg/kg, IP) and SYN119 (SYN; 10 mg/kg, IP) or the respective vehicle (veh).

B, Cocaine self-administration training over 10 sessions. Pokes in the active hole resulted in an IV infusion of cocaine (0.5 mg/kg) paired with a light cue. *Main effect of session on infusions [$F(9,122)=3.308$, $p=0.001$] with post-hoc comparison showing more infusions on sessions 8–10 than sessions 1–3, $p=0.028$.

C, The number of infusions self-administered by male (n=37) and female (n=36) rats used in behavioral studies shown here. Male and female rats did not differ in the number of infusions self-administered and both sexes showed an equivalent increase in the number of infusions self-administered across sessions [no significant sex by session interaction: $F(9,122)=0.410$, $p>0.1$]. When analyzed separately, post-hoc comparisons showed that both males ($p=0.045$) and females ($p=0.021$) self-administered more infusions on sessions 8–10 than sessions 1–3.

D, The average number of infusions taken over the last 3 self-administration sessions in rats destined for the indicated experimental groups (n values for each group shown within bars). We also generated an additional control group that received the lower dose of ISRIB [ISRIB (1.25)+veh group; n=6 rats]. This group did not differ from groups shown here in infusions over the last 3 self-administration sessions (see Supp. Fig. 3 for these data and seeking test data).

E and F, Nose-pokes in the previously active and inactive ports during WD1 and WD40 seeking tests from cocaine rats treated with only vehicle on WD40 (veh+veh) (E) and rats treated with only SYN119 on WD40 (veh+SYN) (F). Each point and line represent an individual rat. Rats in the veh+veh group made more responses on the previously active port on WD40 than WD1 (‘incubation of craving’). This effect was not present in the veh+SYN group.

G, Summary data from individual data points shown in panels E and F.

H, Nose-pokes in previously active and inactive ports during WD1 and WD40 seeking tests in rats from the indicated WD40 treatment groups. Rats treated with only vehicle (veh+veh) and only ISRIB [ISRIB (3)+veh] poked more on WD40 than on WD1 (‘incubation of craving’). Incubation of craving was not evident when SYN119 was injected alone (veh+SYN) or when SYN119 was administered with the lower dose of ISRIB [ISRIB (1.25)+SYN], but the ability of SYN119 to inhibit the expression of incubation was blocked by the higher ISRIB dose [ISRIB (3)+SYN].

I, Pokes in the previously active nose port on WD40 normalized to the number of pokes made on WD1. The same groups expressed incubation of craving (or failed to express incubation) when the data are analyzed in this way.

Panels E-I: *$p<0.05$, paired t-tests with Bonferroni correction.

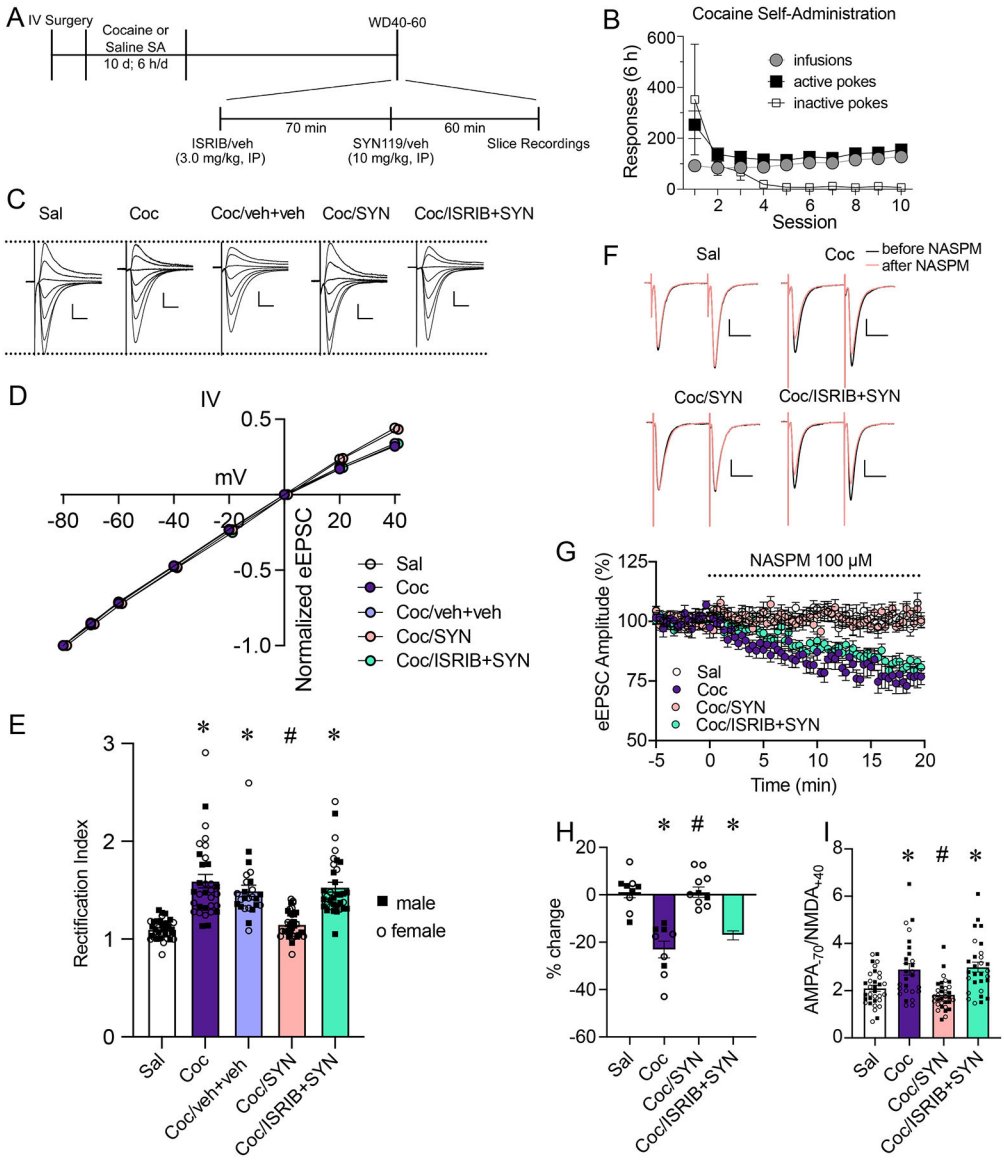


**Figure 2. Pretreatment with ISRIB blocks SYN119-mediated removal of synaptic CP-AMPARs in NAc core medium spiny neurons from cocaine-incubated rats.**

A, Experimental timeline. All rats underwent 10 days of intravenous (IV) saline (Sal) or cocaine (Coc) self-administration (SA) training and a subsequent cocaine-free abstinence period. On Withdrawal Day 40–60 (WD40–60) rats were treated with either no drug, SYN119 (SYN) alone, ISRIB+SYN, or both respective vehicles (veh+veh) prior to being euthanized for slice recordings.

B, Cocaine self-administration training over 10 sessions. Pokes in the active hole resulted in an IV infusion of cocaine (0.5 mg/kg) paired with a light cue.

C, Representative traces of AMPAR-mediated eEPSCs recorded from medium spiny neurons (MSN) at membrane potentials from −70 mV to +40 mV, normalized to peak amplitude at −70 mV. Scale bars, 200 pA, 10 ms.

D and E, I-V relationships of normalized eEPSCs (D) and mean rectification index (RI) (E) for each group. MSNs from Coc and Coc/ISRIB+SYN rats showed a higher RI compared

to Sal and Coc/SYN rats, indicating that SYN119 reverses the cocaine-induced elevation of the RI and that ISRIB prevents SYN's effect. Sal 35 cells (22M, 13F)/6 rats (3M, 3F); Coc 32 cells (13M, 19F)/7 rats (3M, 4F); Coc/veh+veh 25 cells (12M, 13F)/4 rats (2 M, 2F); Coc/SYN 35 cells (17M, 18F)/6 rats (3M, 3F); Coc/ISRIB+SYN 35 cells (23M, 12F)/6 rats (4M, 2F). For E: Black squares, males; open circles, females. *p<0.01 relative to Sal, #p<0.01 relative to Coc; one-way ANOVA (Tukey).

F, Representative eEPSC traces before (black; averaged 15 sweeps, −5–0 minutes) and after (red; averaged 15 sweeps 15–20 min) bath application of NASPM (100 μM), a specific antagonist of CP-AMPARs. Scale bars, 200 pA, 20 ms.

G, Sensitivity to NASPM is evident in MSNs from Coc but not Sal rats, indicating CP-AMPAR elevation after cocaine incubation. SYN eliminates NASPM sensitivity and this effect is blocked by ISRIB. Sal 9 cells (5M, 4F)/6 rats (3M, 3F); Coc 9 cells (4M, 5F)/7 rats (3M, 4F); Coc/SYN 12 cells (4M, 8F)/6 rats (3M, 3F); Coc/ISRIB+SYN 13 cells (7M, 6F)/6 rats (4M, 2F).

H, Summary of NASPM effects on eEPSCs in each group measured after 15–20 min of NASPM application. Black squares, males; open circles, females. *p<0.01 relative to Sal, #p<0.01 relative to Coc; one-way ANOVA (Tukey).

I, AMPA/NMDA ratios for each group. Coc and Coc/ISRIB+SYN rats showed higher AMPA/NMDA ratios than Sal and Coc/SYN rats. *p<0.01 relative to Sal, #p<0.01 relative to Coc; one-way ANOVA (Tukey). Sal, 33 cells (14M, 19F)/6 rats (3M, 3F); Coc 27 cells (13M, 14F)/ 7 rats (3M, 4F); Coc/SYN 33 cells (17M, 16F)/6 rats (3M, 3F); Coc/ISRIB+SYN 29 cells (18M, 11F)/ 6 rats (4M, 2F). Black squares, males; open circles, females.

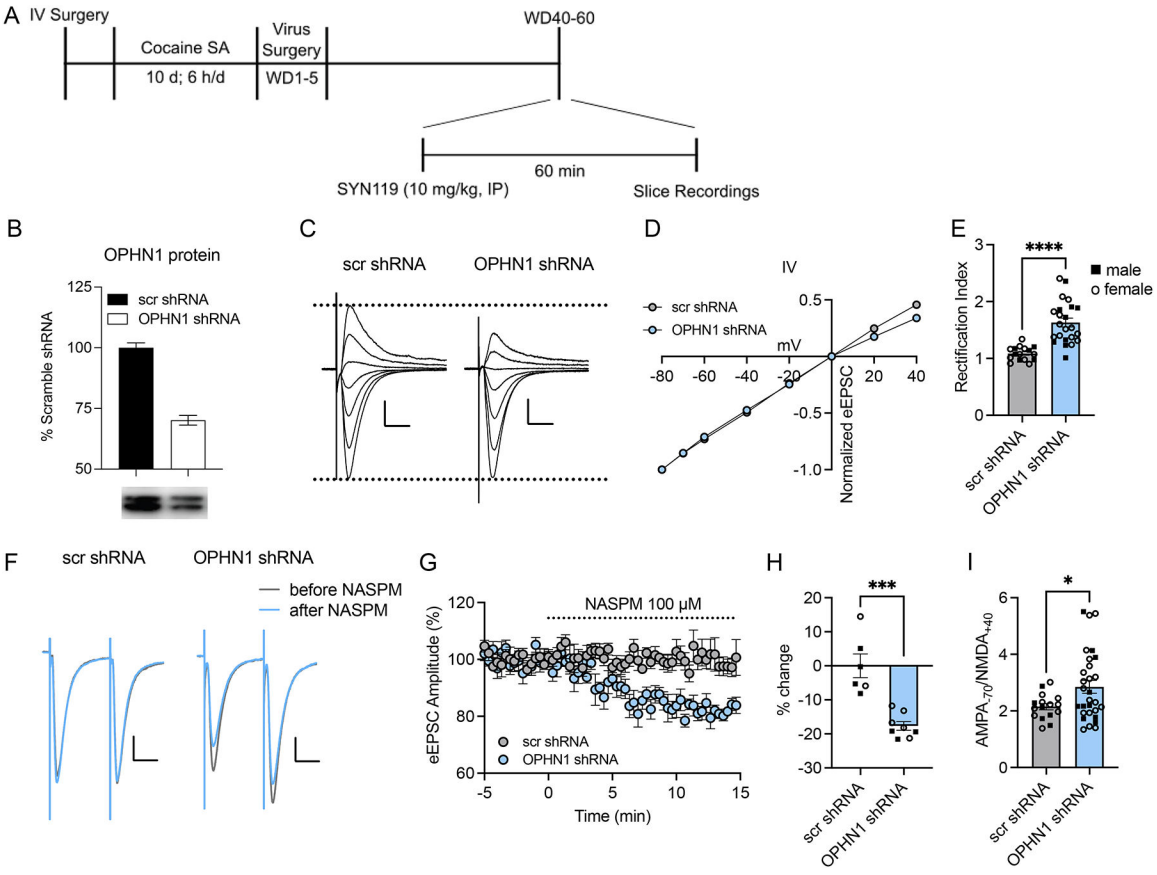


**Figure 3. Knockdown of OPHN1 prevents the SYN119-mediated removal of synaptic CP-AMPARs in NAc core medium spiny neurons from cocaine-incubated rats.**

A, Experimental timeline. Rats underwent 10 days of intravenous (IV) cocaine self-administration (SA) training and a subsequent cocaine-free abstinence period. Rats were infected with scrambled (scr) or *Ophn1* shRNA viruses in the first week after SA. On Withdrawal Day 40–60 (WD40–60), rats received an injection of SYN119 (SYN) (10 mg/kg IP) prior to being euthanized for slice recordings.

B, Summary data and representative immunoblots showing expression of OPHN1 protein in the NAc core of drug-naïve rats 4–5 weeks after infection with scrambled (scr) shRNA virus or *Ophn1* shRNA virus. All OPHN1 bands were normalized to a GAPDH loading control and then this normalized value was compared between lanes.

C-E, Rats infected with scr or *Ophn1* shRNA viruses were injected with SYN 60 min prior to slice recordings to assess the rectification index (RI). SA data for these rats (not shown) were very similar to data presented in Figs. 1 and 2. Shown are representative traces (C) and IV curves (D) of AMPAR-mediated eEPSCs from medium spiny neurons (MSN) recorded at holding potentials from −70 to +40 mV, and the mean RI (E). The scr shRNA group showed a linear IV relationship, indicating that SYN removed CP-AMPARs from synapses. Inward rectification (elevated RI) was observed in the *Ophn1* shRNA group, indicating a block of SYN's effect. Both viruses had a GFP tag that enabled identification of transfected cells. scr 16 cells (6M, 10F)/3 rats (1M, 2F); OPHN1 23 cells (8M, 15F)/6 rats (2M, 4F). For C: Scale bars, 200 pA, 10 ms. For E: Black squares, males; open circles, females.

F-H, Rats were treated exactly as described in C-E except that sensitivity to the CP-AMPAR antagonist NASPM was assessed 60 min after an injection of SYN. Shown in F are example traces of AMPAR eEPSCs measured in SYN-injected rats before (gray; averaged 15 sweeps, −5–0 minutes) and after (blue; averaged 15 sweeps 10–15 minutes) bath application of NASPM (100 μM) (Scale bars, 200 pA, 20 ms). Shown in G is the time course of normalized eEPSCs before and during NASPM application (mean ± SEM). Shown in H is a summary of NASPM effects on eEPSC amplitudes after 10–15 min of NASPM application. NASPM failed to decrease eEPSC amplitude in the scr shRNA group, indicating that SYN had removed CP-AMPARs from synapses, whereas a significant effect of NASPM was found in MSNs expressing *Ophn1* shRNA, indicating a block of SYN's effect by OPHN1 knockdown. scr 6 cells (4M, 2F)/3 rats (1M, 2F); OPHN1 8 cells (3M, 5F)/4 rats (1M, 3F). For H: Black squares, males; open circles, females.

I, AMPA/NMDA ratios for each group. After SYN injection, MSNs expressing *Ophn1* shRNA showed a higher AMPA/NMDA ratio (i.e., SYN was not able to remove CP-AMPARs) than MSNs expressing scrambled shRNA (SYN was able to remove CP-AMPARs). scr 16 cells (6M, 10F)/3 rats (1M, 2F); OPHN1 29 cells (10M, 19F)/4 rats (2M, 4F). Black squares, males; open circles, females.

**p<0.01, ***p=0.005, ****p<0.0001, two-sample t-test

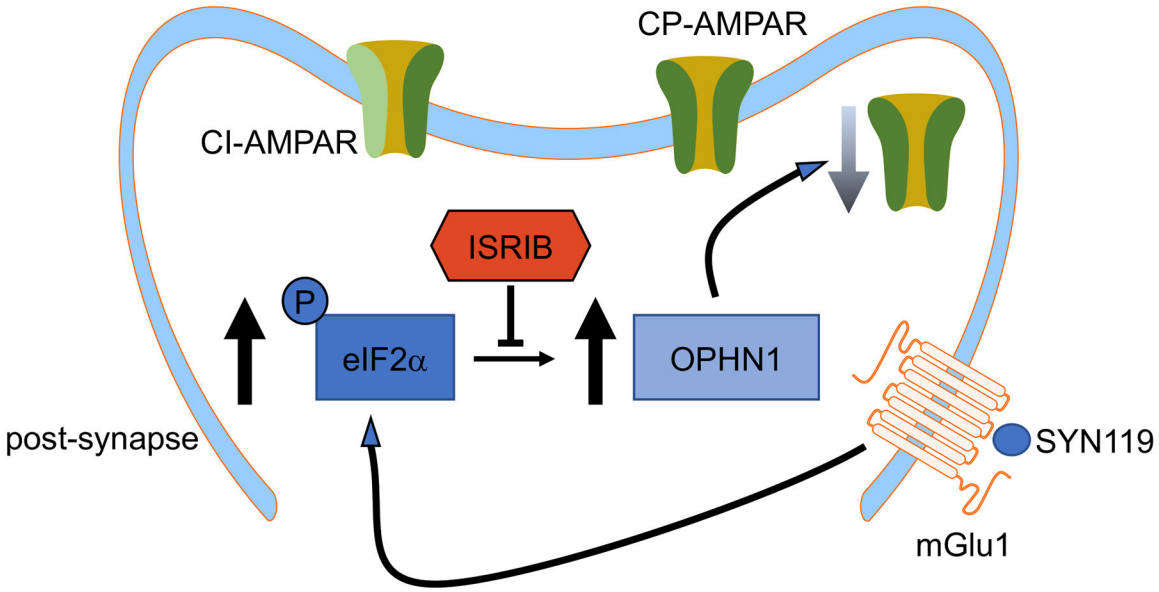


**Figure 4. Proposed mechanism through which mGlu1 positive allosteric modulation mediates LTD in MSNs of the NAc after incubation of cocaine craving.**

SYN119, an mGlu1 positive allosteric modulator, binds to mGlu1 leading to increased phosphorylation of eIF2α, a critical mediator of the Integrated Stress Response (ISR). Activation of the ISR inhibits general translation but increases translation of a subset of mRNA containing a 5' upstream open reading frame (uORF) (see Introduction). One such protein, oligophrenin-1 (OPHN1), has been shown in hippocampus to interact with the endocytic machinery and promote AMPAR internalization (24). In the NAc of 'cocaine-incubated' rats, we show that ISR activation and OPHN1 are required for mGlu1-LTD expressed via CP-AMPAR internalization. Inhibition of the ISR with ISRIB, when given prior to SYN119 injection, inhibits the translational events mediated by the ISR and thus inhibits removal of CP-AMPARs and blocks the reduction in cocaine seeking. The present findings complement prior work in which we have demonstrated that mGlu1-mediated removal of synaptic CP-AMPARs depends on canonical group I mGluR signaling (postsynaptic PKC-dependent mechanism), and dynamin-dependent internalization of CP-AMPARs coupled to insertion of lower conductance $Ca^{2+}$-impermeable AMPARs (29, 30). Note: Although the studies presented here did not directly measure p-eIF2α phosphorylation or *Ophn1* translation, support for these components of the depicted signaling cascade has been provide by other studies (15, 16, 24, 25).

## KEY RESOURCES TABLE

| Resource Type | Specific Reagent or Resource | Source or Reference | Identifiers | Additional Information |
|---|---|---|---|---|
| Add additional rows as needed for each resource type | Include species and sex when applicable. | Include name of manufacturer, company, repository, individual, or research lab. Include PMID or DOI for references; use “this paper” if new. | Include catalog numbers, stock numbers, database IDs or accession numbers, and/or RRIDs. RRIDs are highly encouraged; search for RRIDs at https://scicrunch.org/resources. | Include any additional information or notes if necessary. |
| Antibody | rabbit anti-OPHN1 | Cell Signalling Technology | CST Cat# 11939 | |
| Antibody | mouse anti-GAPDH | MilliporeSigma | CB1001 | |
| Bacterial or Viral Strain | *Ophn1* shRNA cloned into: pTRIPΔU3-EF1α-EGFP lentiviral vector | Supplied courtesy of the Costa-Mattioli Lab; doi: 10.1101/gad.1783809 | N/A | |
| Chemical Compound or Drug | Integrated Stress Response Inhibitor (ISRIB) | Sigma-Aldrich; doi: 10.7554/eLife.00498 | SML0843 | |
| Chemical Compound or Drug | SYN119 [9H-Xanthene-9-carboxylic acid (4-trifluoromethyl-oxazol-2-yl)-amide] | Custom synthesized by EAG Laboratories | N/A | |
| Chemical Compound or Drug | Cocaine HCl | Supplied by NIDA | N/A | |