Withdrawal from cocaine self-administration alters the regulation of protein translation in the nucleus accumbens

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Abstract

Background—Cue-induced cocaine craving "incubates" during abstinence from cocaine self-administration. Expression of incubation ultimately depends upon elevation of homomeric GluA1 AMPA receptors (AMPARs) in the nucleus accumbens (NAc). This adaptation requires ongoing protein translation for its maintenance. Aberrant translation is implicated in CNS diseases, but nothing is known about glutamatergic regulation of translation in the drug-naïve NAc or after incubation.

Methods—NAc tissue was obtained from drug-naïve rats and after 1 or >40 days of abstinence from extended-access cocaine or saline self-administration. Newly translated proteins were labeled using ^{35}S-Met/Cys or puromycin. We compared basal overall translation and its regulation by mGlu1, mGlu5 and NMDA receptors (NMDARs) in naïve, saline and cocaine rats, and compared GluA1 and GluA2 translation by immunoprecipitating puromycin-labeled proteins.

Results—In all groups, overall translation was unaltered by mGlu1 blockade (LY367385) but increased by mGlu5 blockade (MTEP). NMDAR blockade (APV) increased overall translation in naïve and saline, but not cocaine, rats. Cocaine/late withdrawal rats exhibited greater translation of GluA1 (but not GluA2), which was not further affected by NMDAR blockade.

Conclusions—Our results suggest that increased GluA1 translation contributes to the elevated homomeric GluA1 AMPAR levels in NAc that mediate incubation. Additional contributions to incubation-related plasticity may result from loss of the braking influence on translation normally exerted by NMDARs. Apart from elucidating incubation-related adaptations, we found a suppressive effect of mGlu5 on NAc translation regardless of drug exposure, which is opposite to

results obtained in hippocampus and points to heterogeneity of translational regulation between brain regions.

Keywords

AMPA receptor; incubation of cocaine craving; metabolic labeling; nucleus accumbens; protein translation; puromycin

INTRODUCTION

A major challenge in treating addiction is the persistence of vulnerability to relapse. Even after prolonged abstinence, cocaine addicts remain susceptible to relapse, often elicited by cues previously associated with drug-taking (1–3). Aspects of this can be studied in rats using the "incubation of craving" model, in which cue-induced craving progressively intensifies ("incubates") during withdrawal from cocaine self-administration and then remains high for months (4, 5). This models the situation of humans users when heavy cocaine use is interrupted by incarceration or prolonged hospitalization (6). During this interruption, craving may build and, when the addict returns to the "real world", drug-related cues may present an elevated relapse risk. Incubation of craving in human drug users has now been observed during abstinence from cocaine and other drugs of abuse (3, 7–9).

In rats, incubation of cocaine craving involves time-dependent changes in multiple brain regions (5, 10). However, after >1 month of withdrawal, "incubated" craving depends upon strengthening of AMPA receptor (AMPAR) transmission onto medium spiny neurons (MSNs) in the NAc through accumulation of high-conductance GluA2-lacking, Ca^{2+}-permeable AMPA receptors (CP-AMPARs; primarily homomeric GluA1) (5). This is accompanied by profound alterations in group I mGluR-mediated synaptic depression (11, 12). Recently, we identified a novel linkage between these adaptations and protein translation. NAc slices from "incubated" rats were exposed for ~1 h to inhibitors of translation (anisomycin, cycloheximide or rapamycin) prior to patch-clamp recordings. This normalized the state of synaptic transmission, i.e., the contribution of CP-AMPARs to synaptic transmission returned to control levels and alterations in group I mGluR-mediated synaptic depression were reversed (13). This suggests that ongoing protein translation maintains synaptic adaptations in MSNs directly linked to incubation of craving. Consistent with these findings, our recent results demonstrate that expression of incubated cocaine craving during a cue-induced seeking test requires protein translation in the NAc (14).

In many brain regions, including hippocampus, cerebellum and ventral tegmental area (VTA), group I mGluRs are well-established regulators of protein translation, with stimulation of translation by these receptors playing a critical role in long-term depression (LTD) (15). In addition, NMDA receptor (NMDAR) signaling negatively regulates translation in hippocampal and cortical neurons (16–20). Through such studies, aberrant protein translation has been implicated in CNS diseases, including fragile X syndrome and depression, and identified as a potential target for therapeutic intervention (19–25).

Previous studies have demonstrated a role for protein synthesis in the effects of drugs of abuse (e.g. (26–34)) and shown that drugs affect signaling molecules regulating translation

(e.g., mTOR; (35, 36)), but nothing is known about upstream regulation of this signaling by glutamate transmission in the NAc. Based on work in other brain regions summarized above, we focused on determining how NMDAR and group I mGluRs regulate translation in the NAc under control conditions and whether this is altered after incubation of cocaine craving. As protein synthesis-mediated changes in synaptic connections are believed to underlie long-term alterations in neural circuits involved in a variety of behaviors (37), expanding our understanding of the regulation of translation in the NAc should shed light on normal and aberrant learning involving the striatum. Furthermore, if dysregulation of protein translation underlies addiction-related plasticity, normalizing translation may represent a novel therapeutic approach.

METHODS AND MATERIALS

Subjects and drug self-administration

All procedures were approved by the Rosalind Franklin University Institutional Animal Care and Use Committee in accordance with the USPHS Guide for Care and Use of Laboratory Animals. Adult male Sprague-Dawley rats underwent extended-access cocaine or saline self-administration (6 h/d) as described previously (12) and in Supplemental Information.

Metabolic labeling

Procedures were adapted from well-established protocols (22, 24) (see Supplementary Fig. 1 for time-line). Rats were decapitated and the NAc (mainly core) was punched from a 2mm brain slice obtained using a brain matrix. Each hemisphere was chopped into 4 pieces to increase surface area, placed into a net-well, and submerged in a manifold containing artificial cerebrospinal fluid (aCSF) saturated with 95% O_2/5% CO_2. Each hemisphere was counted as an individual sample, with hemispheres from the same rat assigned to different experimental groups, enabling paired t-tests (see Statistical Analysis). Tissue recovered in net-wells for 3 h, enabling optimal and stable protein synthesis (Supplementary Figure 2). Actinomycin D (25 μM; Sigma-Aldrich; St. Louis, MO) was added during the last 30 min of recovery to inhibit new transcription. Then net-wells were transferred to manifolds with aCSF containing actinomycin D and 10 μCi/mL ^{35}S-Met/Cys (EasyTag™ L-[35S]-Methionine, PerkinElmer; Waltham, MA) ± drug, incubated for 1 h, and snap-frozen. To compare ^{35}S-Met/Cys incorporation between experimental groups, labeled tissue was homogenized and processed for autoradiography as described in Supplemental Information.

Measurement of mRNA levels

RNA was extracted from NAc homogenates or synaptoneurosomes (prepared as described previously (38)) and analyzed by quantitative PCR (qPCR) as described in Supplemental Information.

Puromycin labeling

Procedures were adapted from prior studies (39, 40). Pieces of NAc tissue were prepared exactly as described for metabolic labeling experiments and placed in net-wells. Tissue was incubated with 25 μM actinomycin D (Sigma-Aldrich) during the last 30 min of the 3-h recovery period, transferred to manifolds with aCSF containing 1 μM puromycin (Sigma-

Aldrich) and actinomycin D for 1 h, and then snap-frozen. As described in Supplemental Information, overall puromycin incorporation was determined by immunoblotting; puromycin incorporation into GluA1 or GluA2 was determined by Immunoprecipitation of puromycin-labeled proteins.

Statistical analyses

Data were analyzed in Prism (GraphPad Software). Student's t-tests [paired when comparing differently treated hemispheres from a single brain (Figures 1–3), or unpaired when each rat generated a single sample (Figures 4,5)] or ANOVA followed by Tukey's test (Figure 6) were conducted with significance set at p 0.05. Data are presented as mean±SEM.

RESULTS

Measuring protein translation in the NAc

To confirm that ^{35}S-Met/Cys incorporates into newly translated proteins in the NAc, we pre-exposed tissue from naïve animals to the translation inhibitor cycloheximide (60 μM) for 30 min, before moving the tissue into a chamber containing cycloheximide and ^{35}S-Met/Cys for an additional 60 min. We then processed tissue for SDS-PAGE/autoradiography. Figures 1A and 1B show incorporation of ^{35}S-Met/Cys into newly synthesized proteins across multiple molecular weights and demonstrate that cycloheximide significantly reduced ^{35}S-Met/Cys incorporation (Figure 1C, t_3=5.46, p<0.05), validating the assay in NAc tissue.

Regulation of translation in the NAc under control conditions and after incubation

We then tested the hypothesis that the overall rate of protein translation is altered in the NAc of rats after incubation of cocaine craving. Tissue was obtained after extended-access self-administration of saline or cocaine (Figure 1D) and >40 days of abstinence, a period sufficient for maximal incubation of craving and stable CP-AMPAR elevation in NAc core (10, 42–44). Rats subjected to this cocaine regimen and withdrawal period will hereafter be referred to as "incubated" rats. After 3 h of recovery, tissue was incubated with ^{35}S-Met/Cys (60 min) and processed for SDS-PAGE/autoradiography. Measurements of total protein translation, made by scanning the entire lane, were not different between saline controls and "incubated" rats (Figure 1E, t_5=0.30, p=0.77). This result indicates that altered translation of a subset of proteins, rather than gross changes in protein translation, must contribute to maintenance of incubation-related neuroadaptations in the NAc (13). Furthermore, it leaves open the possibility that mechanisms regulating protein translation are altered in the NAc after incubation of craving.

To test the latter possibility, we examined two glutamate receptor subtypes – NMDARs and group I mGluRs – that are well-established as regulators of protein translation in several brain regions (see Introduction). We utilized the strategy of incubating tissue with receptor antagonists to detect tonic regulation of translation by endogenous glutamate. This strategy has been used to study the regulation of protein synthesis in other brain regions (17, 22) and was preferred to incubating with agonists because NMDAR activation can be associated with toxicity, while effects of the group I mGluR agonist DHPG in our hands were quite

variable depending on concentration and time of incubation (data not shown), as has been found previously (Emily Osterweil, personal communication).

First, we examined mGlu5 by co-exposing NAc tissue (from drug-naïve, saline, or "incubated" rats) to vehicle or MTEP, a selective mGlu5 negative allosteric modulator (NAM) (45), and ^{35}S-Met/Cys for 60 min. In contrast to previous studies in hippocampus, where mGlu5 <u>activation</u> increases protein translation (46), <u>inhibition</u> of mGlu5 with MTEP increased ^{35}S-Met/Cys incorporation in both naïve and saline NAc tissue (Figure 2A,B, naïve: $t_7=2.20$, saline: $t_7=2.12$, p<0.05). This is an important result because it shows that protein translation is differentially regulated in these two regions, and thus that regulatory mechanisms operating in the NAc cannot necessarily be inferred from prior studies in hippocampus. Furthermore, the magnitude of the MTEP-induced increase in translation was similar between saline and cocaine animals (Figure 2B,C, cocaine: $t_7=2.69$, p<0.05). These results suggest that mGlu5 activity suppresses protein translation in the NAc and this is not changed after incubation of cocaine craving.

To examine mGlu1, NAc tissue from naïve, saline, or "incubated" rats was labeled with ^{35}S-Met/Cys in the presence or absence of LY367385 (50 μM), a selective mGlu1 competitive antagonist (47). LY367385 did not influence overall translation in any group (Figure 2D,E,F, naïve: $t_7=0.73$, saline: $t_5=0.59$, cocaine: $t_6=0.22$). Thus, group I mGluR-mediated translational regulation in the NAc is primarily driven by mGlu5.

We next asked whether NMDAR transmission regulates protein synthesis in the NAc and if this is altered following incubation of craving. In other brain regions, NMDAR transmission can exert a suppressive influence on translation by stimulating phosphorylation of eukaryotic elongation factor 2 (eEF2; see Discussion). NAc tissue from naïve, saline, or "incubated" rats was labeled with ^{35}S-Met/Cys in the presence or absence of the competitive NMDAR antagonist APV (50 μM). APV increased protein synthesis in naïve and saline rats (Figure 3A,B, naïve: $t_7=2.26$, saline: $t_6=1.90$, p<0.05). This suggests that tonic NMDAR signaling suppresses translation in the NAc, consistent with findings in other regions. Interestingly, this NMDAR-mediated inhibitory tone was lost in the "incubated" rats ($t_6=1.70$, p=0.14; Figure 3C).

GluA1 and GluA2 translation under control conditions and after incubation

As described in the Introduction, homomeric GluA1 CP-AMPARs accumulate in NAc core after ~30 days of withdrawal from our cocaine regimen and thereafter are required for expression of "incubated" craving (5, 12, 42). Their accumulation is associated with increased GluA1 protein levels in NAc homogenates, LP1 fractions and on the cell surface (42, 48), suggesting a role for increased GluA1 translation.

Testing this hypothesis requires measuring the translation rate of individual proteins. Using GluA1 as a test case, we labeled tissue using ^{35}S Met/Cys, performed a highly efficient immunoprecipitation of GluA1 (~95% recovery), subjected the immunoprecipitated material to SDS-PAGE/immunoblotting, and exposed the membrane to a phosphorimager screen for 3–4 weeks (Supplementary Figure 3). Even under these conditions, we could detect only faint isotope incorporation into the GluA1 band that was insufficient for reliable

quantification. We therefore explored an alternative method based on the ability of the antibiotic puromycin, which resembles tyrosyl-transfer RNA, to incorporate into newly translated proteins (39, 40, 49, 50). When puromycin incorporates into the growing peptide chain, it interrupts elongation; however, if used at a low concentration (1 μM), it labels proteins without substantially altering overall protein synthesis (40).

We began by verifying that puromycin and metabolic labeling yielded similar results, using NMDAR regulation of translation as a test case. As observed with metabolic labeling (Figure 3A–C), we found that APV increased translation, measured with puromycin labeling, in NAc tissue from naïve but not cocaine rats (Figures 3D and 3E, naïve: $t_{12}=2.25$, p=0.04; cocaine: $t_7=0.08$, p=0.94). To examine whether this method could be used to study the rate of translation of individual proteins, we prepared puromycin-labeled NAc tissue and immunoprecipitated with puromycin antibody to isolate all newly translated (puromycin-containing) proteins. Then, we used immunoblotting to measure GluA1 and GluA2 protein levels in starting material and the puromycin-immunoprecipitated fraction. By comparison to samples immunoprecipitated with IgG (control condition), we identified puromycin-labeled GluA1 and GluA2 bands (Figure 4A,D). They migrated slightly above their predicted molecular weight, which is not surprising as structural changes due to puromycin incorporation likely affect protein unfolding/SDS binding and thereby alter migration during SDS-PAGE. As expected, the newly translated pool of each protein was small compared to the amount present in starting material (Figure 4A,E). Perhaps for this reason, the alternative strategy – immunoprecipitating with GluA1 antibody and immunoblotting for puromycin – did not yield a quantifiable signal, paralleling results described above for ^{35}S-labeling (see legend to Supplemental Fig. 3).

Puromycin labeling/pull-down followed by GluA immunoblotting was then used to compare GluA1 and GluA2 translation in the NAc of rats that self-administered saline or cocaine. Rats were killed on WD1 (prior to CP-AMPAR elevation) or on >WD40 (maximal incubation and CP-AMPAR accumulation). No group differences were observed on WD1 (Fig. 4B and E, GluA1: $t_{14}=0.04$, p=0.97, GluA2 $t_{14}=0.69$, p=0.50). However, "incubated" rats (>WD40) exhibited a significant increase in GluA1 but not GluA2 translation (Figure 4C and F, GluA1: $t_{14}=1.72$, p=0.05, GluA2: $t_{13}=0.34$, p=0.74). To evaluate a possible role for altered transcription, we used additional "incubated" rats and saline controls. We measured levels of *Gria1* and *Gria2* mRNA extracted from NAc core or shell homogenates, and from synaptoneurosomes prepared from the entire NAc. We found no group differences in mRNA from homogenates (Figure 5A,C, *Gria1*: Core $t_{18}=0.39$, p=0.70, Shell, $t_{18}=0.21$, p=0.83; *Gria2*: Core $t_{18}=0.01$, p=0.99, Shell $t_{18}=0.93$, p=0.36). In synaptoneurosomes, we found no group difference in *Gria1* mRNA levels (Figure 5B, $t_{20}=0.68$, p=0.50) but observed a trend towards decreased *Gria2* mRNA in cocaine rats (Figure 5D, $t_{19}=1.77$, p=0.09). Results for *Gria3* mRNA were similar to those for *Gria2* (Supplementary Table 1). The most important finding is absence of a change in *Gria1* mRNA in synaptoneurosomes because this suggests that the enhanced GluA1 translation observed after incubation of cocaine craving (Figure 4C) is not explained by increased *Gria1* mRNA levels in dendritic compartments. Together, these findings suggest that elevated translation of GluA1 contributes to CP-AMPAR accumulation in the NAc of "incubated" rats.

Finally, given its robust effect on overall translation in the NAc, we asked whether NMDAR blockade, shown to increase dendritic GluA1 translation in hippocampal neurons (17), influenced the translation of GluA1 or GluA2 in NAc, and whether this effect was changed following incubation. Using the puromycin immunoprecipitation approach described above, we compared GluA1 and GluA2 translation in NAc tissue from "incubated" and naïve rats (naïve rats were chosen as the control because the effect of APV was most robust in this group) labeled with puromycin in the presence of either aCSF (vehicle control) or APV. Results in Figure 6 confirm increased GluA1 translation in "incubated" rats (naïve aCSF versus Coc aCSF, Tukey's post hoc, p=0.04). However, in contrast to its ability to enhance overall translation (Figure 3), APV had no effect on GluA1 translation in naïve rats (naïve aCSF versus naïve APV; p=0.39) and did not further augment GluA1 translation in cocaine rats (cocaine aCSF versus cocaine APV; p=0.75). For GluA2 translation, two-way ANOVA revealed a significant main effect of drug history, i.e., cocaine exposure tended to reduce GluA2 translation relative to the drug-naïve condition (F(1,28)=9.04, p<0.01). However, Tukey's post hoc analysis revealed that GluA2 translation did not differ between naïve and cocaine aCSF groups (p=0.15), similar to results in Figure 4F showing no significant difference between saline and cocaine groups. Likewise, there was no difference between cocaine aCSF and cocaine APV groups (p=0.95), although there was a marginally significant decrease in newly translated GluA2 in cocaine APV tissue compared to naïve aCSF tissue (Figure 5B, p=0.05). Together, these results suggest that cocaine exposure and NMDAR blockade differentially influence protein translation in the NAc.

DISCUSSION

This study is the first to characterize regulation of protein translation in the NAc by glutamate transmission. In addition, we tested the hypothesis that this regulation was altered after incubation of cocaine craving. Our results reveal mGlu5 and NMDAR regulation of translation under control conditions, loss of NMDAR regulation of translation after incubation, and increased GluA1 translation after incubation.

We suggest that our results primarily reflect translation in MSNs, as these comprise ~90% of neurons in the NAc (48). However, contributions of other cell types cannot be excluded. Furthermore, we suggest that dendritic translation may contribute to our measures of protein translation based on the fact that glutamate inputs synapse on dendrites/spines of MSNs rather than the soma (48) and on similarities between the present mGlu1/5 results and those we have recently obtained by monitoring translation in processes of cultured MSNs (see next section).

mGlu5 and translational regulation

In hippocampus, it is well established that group I mGluRs stimulate translation (23, 46). In contrast, we found an MTEP-induced increase in protein translation in NAc tissue from both drug-naïve and "incubated" rats (Figure 3), consistent with results obtained in cultured NAc neurons (51). Since MTEP is an mGlu5 NAM, we cannot determine whether it acts by opposing mGlu5 transmission stimulated by tissue glutamate or by opposing constitutive mGlu5 activity (52), although our results in cultured NAc neurons support the latter

possibility (51). Regardless, an important conclusion is that mGlu5 transmission exerts an *inhibitory* influence on protein translation in NAc tissue, opposite from the direction observed in hippocampus. Different "wiring" may reflect the fact that the NAc is comprised mainly of GABAergic MSNs, whereas hippocampal principal neurons are glutamatergic. While this underscores the importance of characterizing mGluR regulation of translation in the NAc, such differences are not entirely unprecedented. For example, mGlu5 stimulation in hippocampus leads to LTD that depends upon dendritic translation in the postsynaptic cell and AMPAR endocytosis (15, 46, 53), while the predominant form of mGlu5-induced LTD in striatum is presynaptically expressed via CB1R stimulation (54, 55). The only study implicating protein translation in striatal mGluR LTD indicated a locus for this translation outside the MSN itself (56), whereas another found no effect of translation inhibition (57).

We previously showed that mGlu5-mediated synaptic depression is disabled in the NAc of "incubated" rats (11). This is accompanied by decreased coupling of long Homer proteins to mGlu5 (12). Homers are critical for regulating group I mGluR association with downstream signaling pathways (58), and changes in association with long Homers can shift mGlu5 signaling (59–61), including signaling regulating protein translation (46, 62). Despite loss of mGlu5 synaptic depression and reduced association with long Homers after incubation (11), the MTEP-induced increase in translation in all groups demonstrates that mGlu5 remains capable of regulating protein translation (Figure 3). This dissociation is consistent with evidence that mGlu5-synaptic depression in NAc MSNs involves the canonical Gq-linked pathway (11, 55) and is independent of protein translation within the MSN (56, 57), whereas mGlu5 regulation of translation in hippocampus is mediated through a distinct beta-arrestin pathway (63, 64). Interestingly, after cocaine exposure and extinction training, mGlu5 remains capable of modulating reinstatement (65–69). It will be important to determine the signaling pathways mediating mGlu5's disparate roles in regulating MSN function (e.g. (70)).

mGlu1 and translational regulation

Both mGlu1 activation and exposure to protein synthesis inhibitors remove CP-AMPARs from NAc synapses of "incubated rats" (11, 13), raising the possibility of overlapping mechanisms. We showed previously that mGlu1 removes CP-AMPARs via a PKC-dependent mechanism (11) and here we show no effect of the mGlu1 antagonist LY367385 on overall translation in any group (Figure 4). Nevertheless, we cannot rule out a role for mGlu1 in regulating translation of key proteins. We note that protein translation is implicated in the mGlu1-mediated removal of CP-AMPARs from the VTA of cocaine-exposed animals (71). In the VTA, as in the NAc of "incubated" rats, this mGlu1 LTD involves a swap in which high-conductance CP-AMPARs are removed and replaced with lower conductance GluA2-containing AMPARs (11, 28, 71–73). In VTA, the swap depends upon mGlu1-stimulated translation of GluA2 (28). Future studies should examine potential mGlu1 regulation of GluA1 or GluA2 translation in the NAc, as we have done here for NMDAR regulation.

NMDAR and translational regulation

NMDAR transmission, including spontaneous transmission (miniature EPSCs), can exert a tonic suppressive effect on translation (16–20). In hippocampus and superior colliculus, this was shown to depend on Ca^{2+} influx through the NMDAR channel and Ca^{2+}-induced Ca^{2+} release, ultimately leading to phosphorylation of eEF2 (74, 75). Phosphorylation of eEF2 inhibits its activity and reduces general translation, a regulated process that contributes to synaptic plasticity (76, 77). In cultured NAc neurons, the NMDAR antagonist APV did not alter translation, probably due to low glutamate tone at NMDARs in this system (51). However, in NAc tissue from drug-naïve animals, our results with APV suggest a suppressive influence of NMDARs on overall protein translation similar to that observed in other regions (Figure 3A,B,D). Interestingly, the ability of APV to increase overall translation is absent in NAc tissue from "incubated" rats (Figure 3C,E). This alteration in NMDAR function may be related to emergence of GluN3-containing NMDARs in NAc MSN during incubation (78). The presence of this atypical subunit confers low Ca^{2+} permeability on NMDARs (79), and, as noted above, Ca^{2+} entry is part of the pathway by which NMDARs reduce translation (74, 75).

What is the functional consequence of loss of NMDAR regulation of translation? In hippocampal neurons, one of the proteins negatively regulated by NMDAR transmission is GluA1, such that NMDAR blockade increases dendritic GluA1 translation and synaptic insertion of homomeric GluA1 CP-AMPARs (17). Therefore, loss of NMDAR negative regulation could in theory account for increased homomeric GluA1 CP-AMPARs in the NAc after incubation of craving. However, in experiments using puromycin labeling to selectively measure GluA1 translation, we found no effect of APV in NAc tissue from drug-naïve or "incubated" rats (Figure 6). These results suggest that the functional significance of the loss of NMDAR regulation is not related to CP-AMPAR upregulation during cocaine withdrawal. Rather, our recent results (14) suggest that its significance may be manifest during the expression of incubated craving, i.e., during a cue-induced seeking test in which rats perform an operant response to deliver cues previously paired with cocaine. Specifically, we conducted immunoblotting studies of signaling molecules regulating translation in the NAc after long withdrawal (>47 days) from saline or cocaine self-administration (i.e., saline and cocaine groups identical to those in the present study) or identically treated rats subjected to a cue-induced seeking test (saline/test and cocaine/test groups). Several robust changes were observed in the cocaine/test group, including dephosphorylation of eEF2 relative to the cocaine group and the saline/test group. This is indicative of increased translation. Indeed, we also found that expression of incubated craving by the cocaine/test group depends on translation in the NAc (14). It is possible that NMDAR plasticity during incubation (78; see previous paragraph) lessens the ability of NMDAR transmission to maintain eEF2 in a phosphorylated/inactivated state, setting the stage for its dephosphorylation/activation during a cue-induced seeking test (14). Other evidence, however, indicates that altered signaling downstream of mTOR may underlie eEF2 dephosphorylation in this group (14). The picture is further complicated by other changes in translation pathways in the cocaine/test group. Most notably, dephosphorylation of eukaryotic initiation factor 2α (eIF2α), which promotes translation, is observed in the cocaine/test group and this dephosphorylation is required for expression of incubation (14). Future studies will probe the relationship between incubation-

related alterations in NMDAR regulation of translation, downstream signaling cascades, and cue-induced cocaine seeking.

Conclusions

These experiments are the first to characterize how glutamatergic transmission regulates protein translation in the NAc under control conditions and after incubation of cocaine craving. Under control conditions, translation in the NAc showed similar regulation by NMDARs but different regulation by group I mGluRs compared to hippocampus, revealing cell-type differences in regulatory mechanisms. While metabolic labeling revealed no difference in overall protein translation between saline and cocaine animals, the ability of translation inhibition to normalize the elevation of homomeric GluA1 CP-AMPARs that mediates expression of incubation (13) indicates that some proteins must be differentially translated in the NAc of "incubated" rats. Indeed, we found elevated translation of GluA1 after incubation, suggesting a simple explanation for increased CP-AMPAR formation. Incubation was also accompanied by loss of NMDAR-mediated suppression of protein translation in the NAc. The functional consequences of this adaptation remain to be determined, but our results suggest that it is not related to increased GluA1 translation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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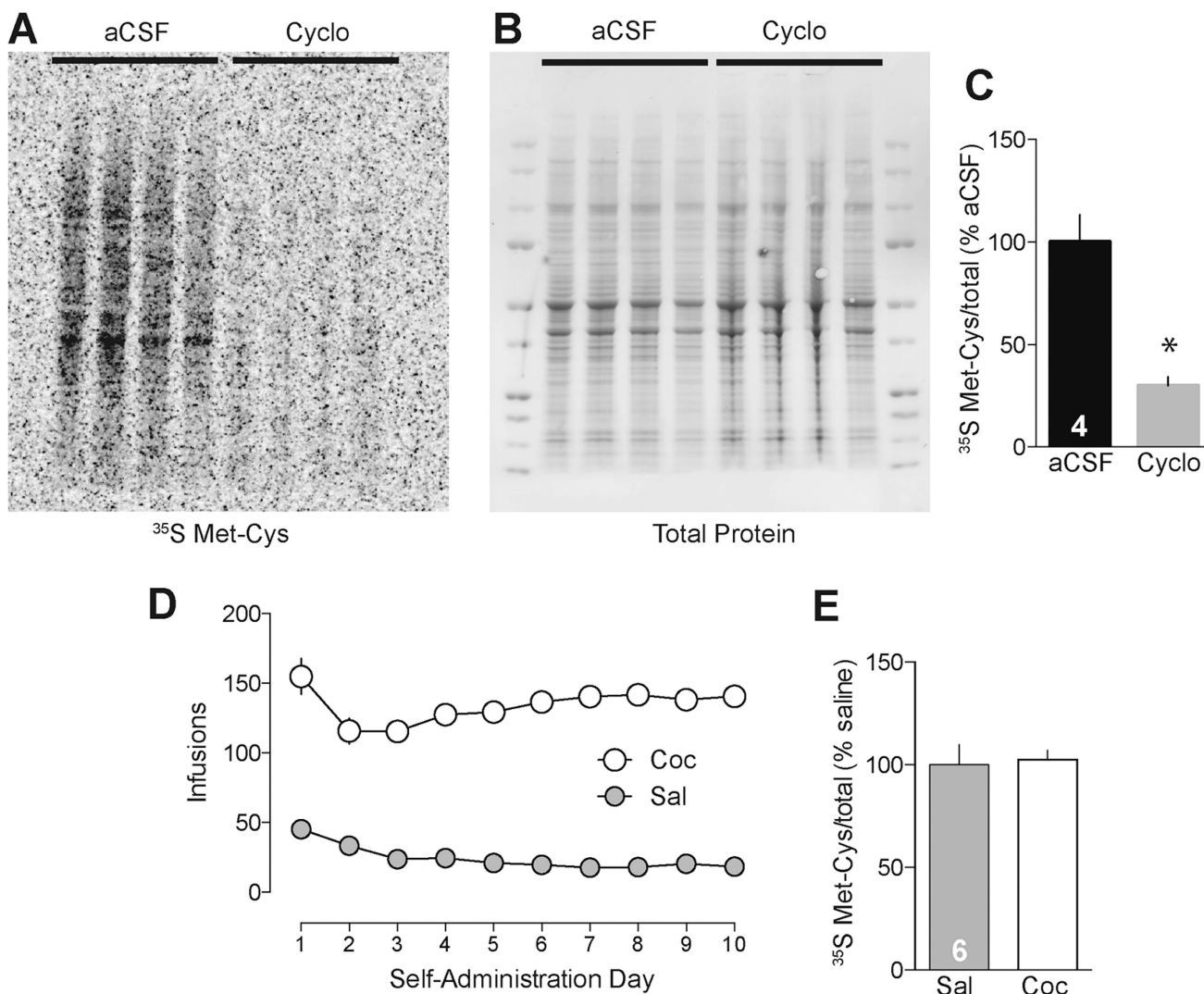
Figure 1.

Monitoring protein translation in the NAc using ^{35}S-Met/Cys incorporation reveals no differences in overall translation between saline controls and rats that have undergone incubation of cocaine craving. **(A)** Representative autoradiograph shows ^{35}S-Met/Cys incorporation into proteins of various molecular weights in freshly dissected NAc tissue. **(B)** Ponceau S stain of the same gel demonstrates equal protein loading in each lane. **(C)** Quantification of ^{35}S-labeled proteins in **A**, normalized to total protein in each lane determined with Ponceau S staining, shows that 30 min of incubation with the protein translation inhibitor cycloheximide (Cyclo) significantly reduces protein translation. **(D)** Mean ± SEM number of cocaine infusions per day over the ten 6-hour daily self-administration training sessions for all animals (163 total rats). **(E)** Quantification of ^{35}S-labeled proteins (normalized to total protein in each lane) shows that overall translation did not differ between cocaine and saline self-administering rats killed after withdrawal day 40. Numbers in black bars (panels **C** and **E**) represent the number of rats in the experiment; for each rat, the NAc from one hemisphere was used for the control group and the NAc from the

other hemisphere was used for the experimental group. *p<0.05 versus aCSF control (paired t-test).
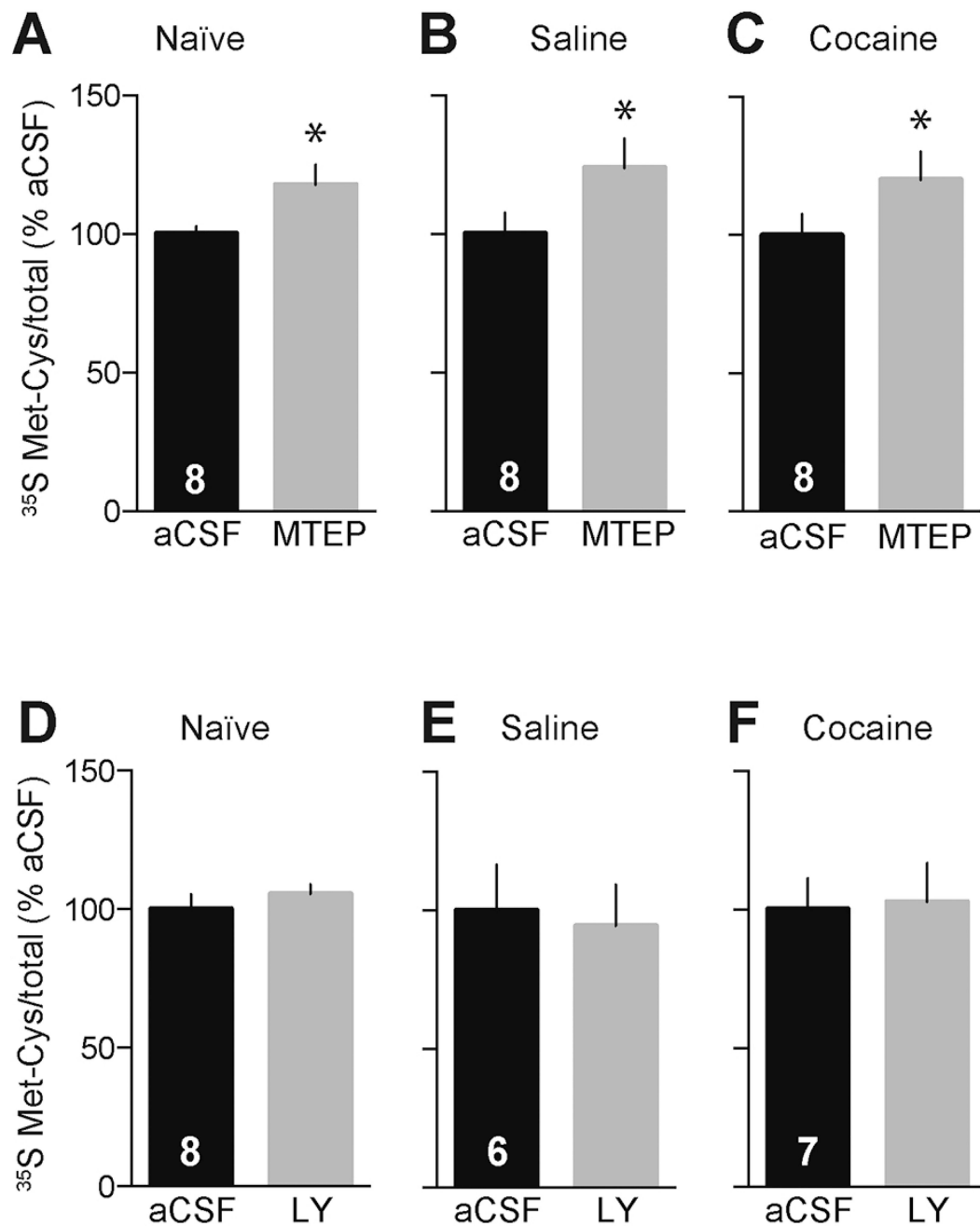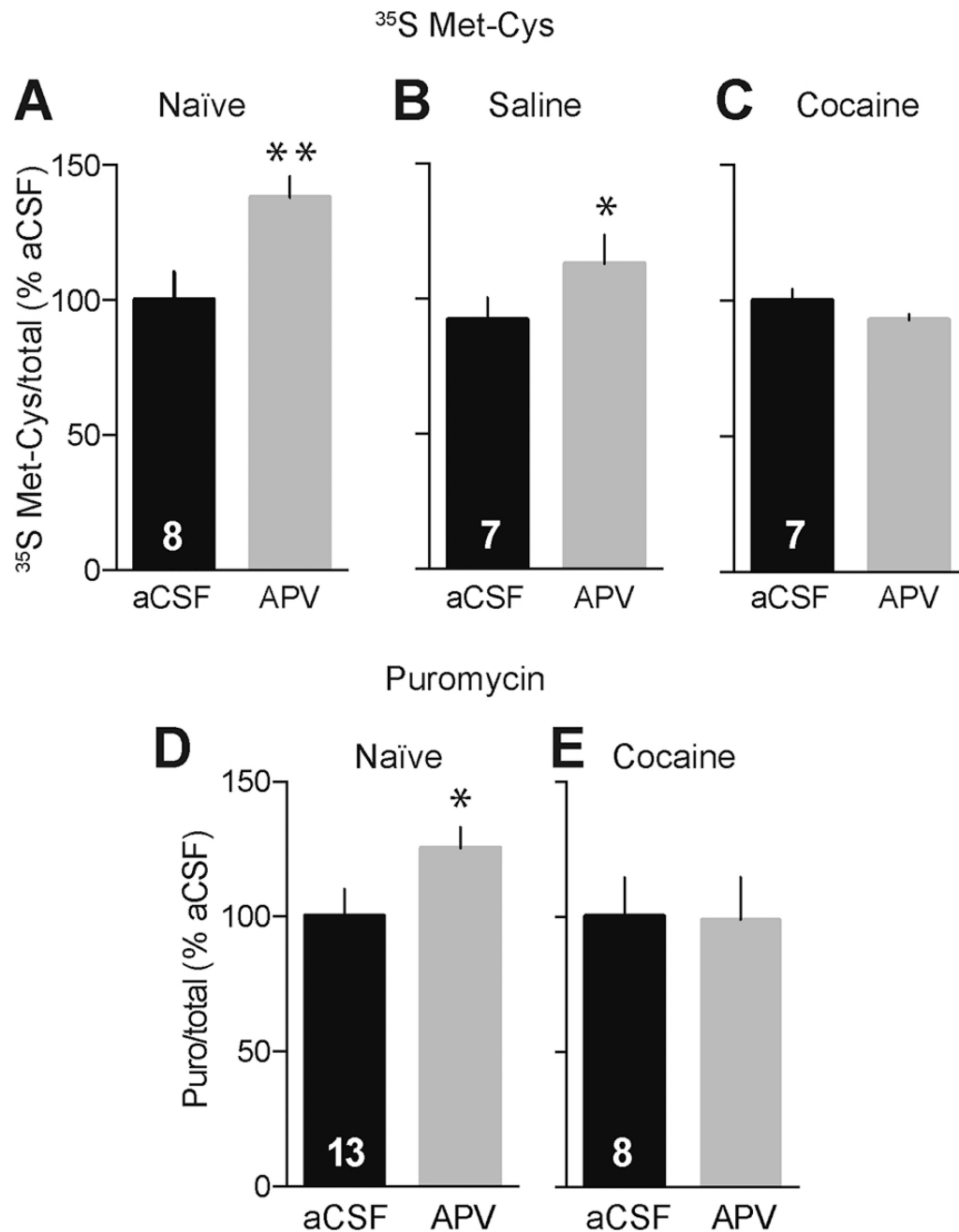
Figure 2.
mGlu5 blockade increases protein translation in the NAc of naïve rats, saline controls, and "incubated" rats while mGlu1 blockade does not influence overall translation in the NAc. Including the mGlu5 antagonist MTEP (25 μM) during the ^{35}S-Met/Cys incorporation period increases protein translation in NAc tissue from naïve rats (**A**), as well as rats that self-administered saline (**B**) or cocaine (**C**) and were killed after withdrawal day 40. Including the mGlu1 antagonist LY367385 (LY; 50 μM) during the ^{35}S incorporation period does not significantly alter protein translation in NAc tissue from the same experimental groups (**D–F**). Numbers in black bars represent the number of rats in the experiment; for

each rat, the NAc from one hemisphere was used for the control group and the NAc from the other hemisphere was used for the experimental group. *$p < 0.05$ versus aCSF control (paired t-test).

Figure 3.
NMDAR blockade increases translation in control rats, but not following prolonged withdrawal from cocaine self-administration. Including the NMDAR antagonist APV (50 μM) during the ^{35}S-Met/Cys or puromycin incorporation period increases protein translation in NAc tissue dissected from naïve rats (**A, D**) and saline self-administering rats killed after withdrawal day 40 (**B**), but not NAc tissue from cocaine self-administering rats killed after withdrawal day 40 (**C, E**). Numbers in black bars represent the number of rats in the experiment. For rats in these metabolic labeling and puromycin experiments, the NAc from one hemisphere was used for the control group and the NAc from the other hemisphere was

used for the experimental group. In both cases, data were normalized to total protein in each lane, determined with Ponceau S staining. **$p<0.01$ and *$p<0.05$ versus aCSF control (paired t-test).
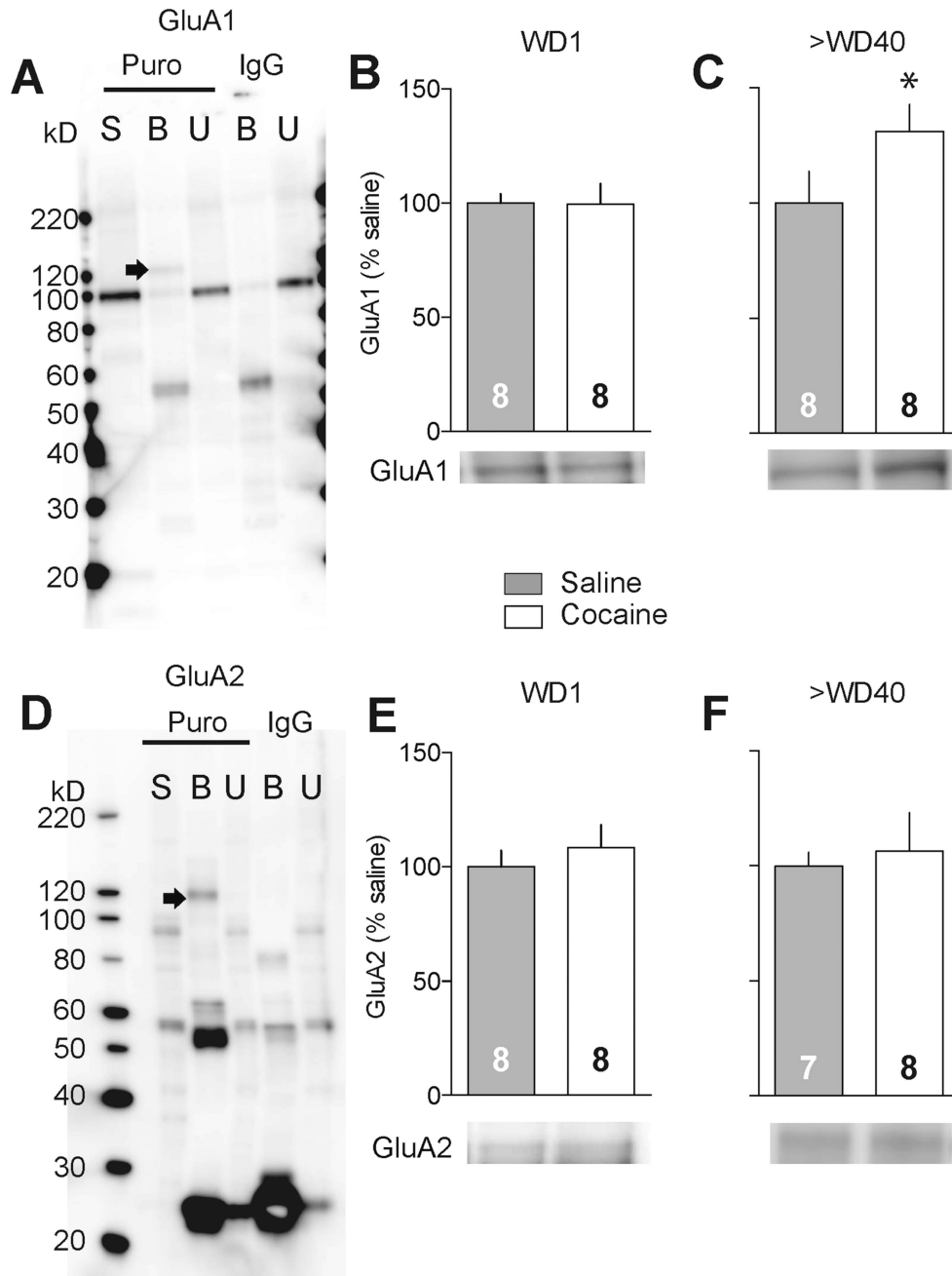
Figure 4.
GluA1 translation in the NAc is unchanged one day after discontinuing cocaine self-administration but is increased after incubation of craving has occurred. NAc tissue was labeled with puromycin and then immunoprecipitated using puromycin antibody or IgG (control condition). Equal amounts (40 μg) of starting material (S), bound material (B; immunoprecipitated material purified by Protein A/G resin), and unbound material (U) were separated by SDS-PAGE and immunoblotted for GluA1 **(A)** or GluA2 **(D)**. Comparison of bound fractions after puromycin versus IgG immunoprecipitation reveals newly translated GluA1 and GluA2 bands that migrate above their predicted molecular weight (arrowheads in

panels **A** and **D**), presumably due to structural changes associated with puromycin incorporation (see Results). Using this approach, we demonstrated that, compared to saline controls, GluA1 translation is unchanged after 1 day of withdrawal from cocaine self-administration **(B)** but increased after prolonged withdrawal (>40 days) **(C)**, whereas GluA2 translation is unchanged at both withdrawal times **(E,F).** For these puromycin experiments, each sample consisted of NAc pooled from both hemispheres of one rat. Numbers in bars represent number of samples/rats in each group. *p<0.05 versus saline controls (unpaired t-test).
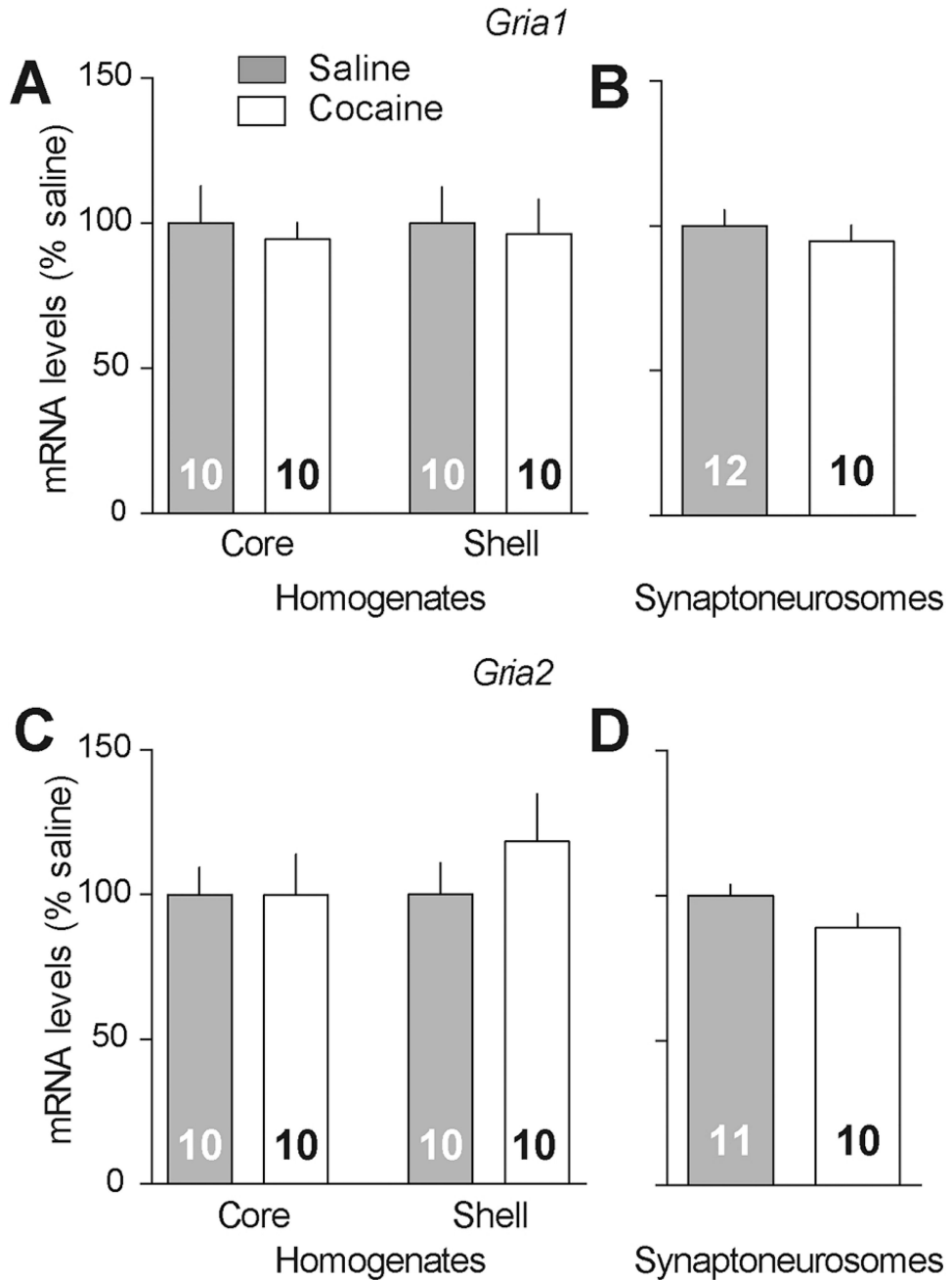
Figure 5.
GluA1 and GluA2 mRNA levels are unchanged in NAc homogenates or synaptoneurosomes after incubation of cocaine craving. **(A and C)** Quantification of *Gria1* and *Gria2* mRNA levels in NAc core and NAc shell homogenates indicates no differences between rats killed after >40 days of withdrawal from saline versus cocaine self-administration. **(B and D)** Likewise, there was no significant difference in *Gria1* or *Gria2* mRNA levels in synaptoneurosomes prepared from the whole NAc of identically treated saline and cocaine rats, although a trend towards a reduction in *Gria2* mRNA (p=0.09) was observed in the cocaine group. For these experiments, each sample consisted of NAc pooled from both

hemispheres of one rat. Numbers in bars represent number of samples/rats in each group. Data analyzed with unpaired t-tests.
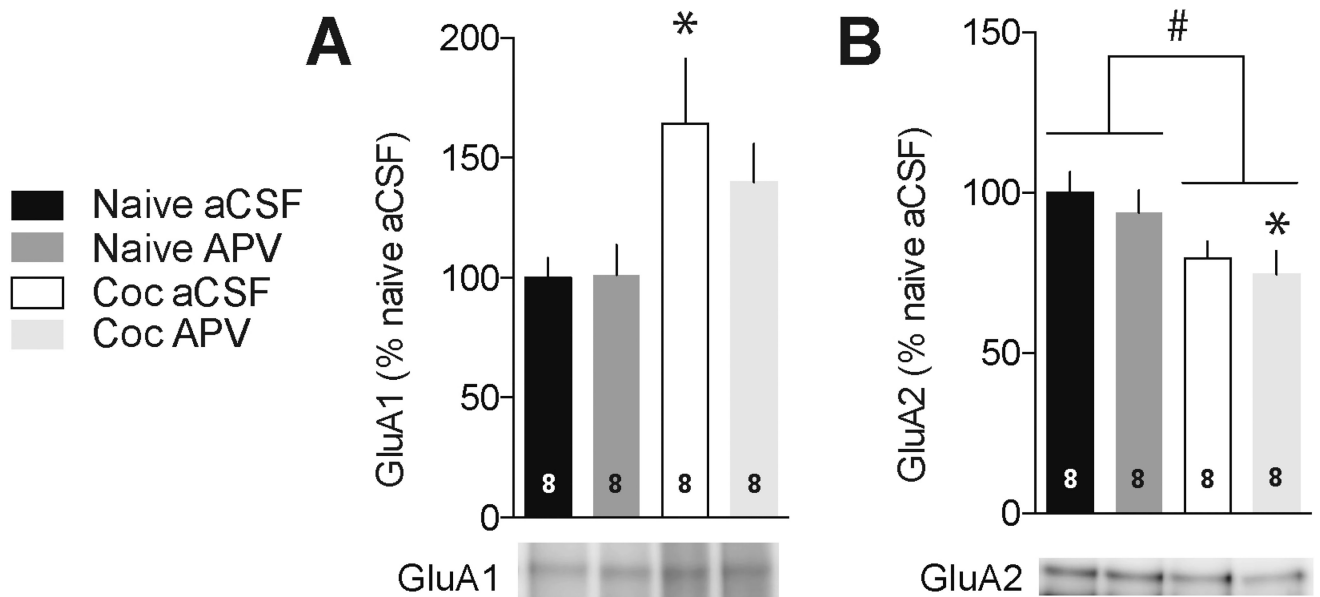
Figure 6.
APV does not influence GluA1 translation in the NAc of naïve or "incubated" rats. NAc tissue from drug-naïve rats or rats killed after >40 days of withdrawal from cocaine self-administration was labeled with puromycin in the presence of aCSF or APV (50 μM). Tissue was immunoprecipitated with puromycin antibody to isolate newly translated proteins, and the immunoprecipitated fraction was immunoblotted for GluA1 (**A**) or GluA2 (**B**). (**A**) Comparison of GluA1 translation in naïve aCSF and cocaine aCSF tissue confirmed results in Figure 4 demonstrating elevated GluA1 translation after cocaine withdrawal, but APV did not influence GluA1 translation in either naive or cocaine groups. (**B**) A two-way ANOVA revealed a main effect of drug pre-exposure on GluA2 translation, as indicated by lines over bars (#$p<0.05$) (see Results for discussion of post hoc comparisons). For these puromycin experiments, each sample consisted of NAc pooled from both hemispheres of one rat. Numbers in bars represent number of samples/rats in each group. *$p<0.05$ compared to naïve aCSF group (Tukey test).