Locomotor sensitization to cocaine using a two-injection protocol requires eukaryotic elongation factor-2 kinase (EF2K)-mediated translational control

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Introduction

- The persistence of drug-related memories likely underlies the persistence of addiction. While the transcriptional mechanisms that participate in the formation of these memories are well-characterized, almost nothing is known about the translational control mechanisms involved.
- Locomotor sensitization to cocaine, using either repeated daily injections or a two-injection protocol, is sensitive to disruption by protein synthesis inhibitors (Karler et al. 1993, Shimosato and Saito 1993, Sorg and Ulibarri 1995, Valjent et al. 2010).
- Eukaryotic elongation factor-2 kinase (EF2K) is a Ca^{2+}/calmodulin-dependent protein kinase that phosphorylates and inhibits eukaryotic elongation factor-2 (eEF-2), thereby negatively regulating protein synthesis at the elongation step.
- While phosphorylation of eEF-2 inhibits overall protein synthesis, it appears to activate the translation of a subset of mRNAs. This duality of translational control is manifested as a dual role of EF2K in regulating synaptic plasticity, as hippocampal slices from *EF2K* knockout mice do not express mGluR-LTD, but exhibit enhanced LTP (Park et al., 2008). Similarly, transgenic mice over-expressing EF2K in the hippocampus show impaired consolidation of contextual fear conditioning and LTP (Im et al., 2009), while knock-in mice bearing a kinase-dead mutation of EF2K show impaired conditioned taste aversion learning (Gildish et al., 2012).
- Here, we found that locomotor sensitization to cocaine using a two-injection protocol is impaired in *EF2K* knock-out mice, and that EF2K is engaged during the protein synthesis-dependent phase of this task.

Methods

Animals

All procedures were approved by the Yale Animal Care and Use Committee (YACUC) and followed the NIH Guide for the Care and Use of Animals. Breeding was performed with *EF2K* heterozygous mice (129xC57/Bl6), originally obtained from A. Ryazanov, and tail DNA was genotyped using standard procedures. Adult (aged 2-6 months) homozygous wild-type or knockout littermates were used for all behavior experiments. For biochemical experiments to characterize the timecourse of eEF-2 phosphorylation following acute cocaine, wild-type C57/B6 mice, age 2-3 months, were obtained from Charles River. Mice were maintained on a 12 hr light-dark schedule and received *ad libitum* access to food and water.

EF2K kinase assays

Methods were adapted from Nairn et al. 1985. Briefly, mice were euthanized by rapid decapitation and brains were dissected. Whole brains from wild-type and *EF2K* knockout mice were homogenized in buffer containing 50 mM HEPES, 10 mM MgOAc, 1 mM EGTA, 1.5 mM $CaCl_2$, and phosphatase and protease inhibitor cocktails (Sigma). Samples were sonicated for 20 s and spun at 13,200 rpm. 50 µg of supernatant (determined by BCA) was diluted in the above buffer to a final volume of 1 mL and 5 mM DTT, 10 units/mL calmodulin (Sigma), and 10 µg eEF-2 were added. An aliquot of starting material was removed, then the reaction was initiated by the addition of 50 µM ATP, and the mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 5X SDS-PAGE sample buffer. Samples were then subjected to SDS-PAGE and immuno-blotting with p-eEF-2 (Cell Signaling) and eEF-2 (Angus Nairn) antibodies to assess kinase activity.

Locomotor sensitization to cocaine

For the repeated daily injection procedure, prior to the start of the experiment, mice were habituated to injection stress with one daily i.p. injection of saline (10 mL/kg) for 2 days. Mice were then habituated to the testing chambers (standard mouse cages with bedding, inserted into photobeam frames) and their basal locomotor activity was assessed during a session in which they received an i.p. injection of saline followed by 1 hr of locomotor monitoring, then another i.p. injection of saline and an additional 1 hr of monitoring. The following day, sensitization training began, consisting of 5 daily sessions in which mice were injected with saline, monitored for 1 hr, then injected with cocaine (10 or 20 mg/kg dissolved in saline, 10 mL/kg) and monitored for 1 hr. For the two-injection procedure, mice received three daily habituation sessions in the locomotor monitoring chambers as described. On the following day, mice received an injection of saline and 1 hr of locomotor monitoring, then an injection of cocaine (20 mg/kg) and 1 hr of locomotor monitoring. One week later, this procedure was repeated to assess sensitization. Locomotion was defined as the breaking of 2 adjacent beams, and data are expressed as the ratio between locomotion following cocaine injection and that following saline injection for each mouse on each test day.

Striatal tissue preparation for biochemical experiments

Mice received one daily saline injection in their home cages for 3 days prior to the experiment. Mice were injected with saline and euthanized 10 min later or with cocaine (20 mg/kg) and were euthanized 10, 30, or 60 min later. Euthanasia was performed via focal microwave irradiation (4.5 kW, 1.2 s) to preserve post-translational modifications. Whole striata were then dissected and sonicated in 1X PBS containing 1% SDS and protease and phosphatase cocktails (Sigma). Samples were then boiled at 95°C for 10 min and spun at 14,000 rpm for 10 min to pellet debris. Supernatants were subjected to BCA (Pierce) and diluted with 5X SDS-PAGE sample buffer.

Immuno-blotting and antibodies

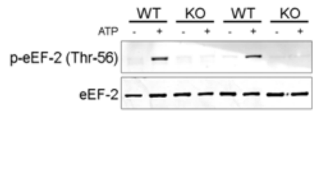
Striatal samples (~30 µg protein) were run on pre-cast 4-12% or 8-16% polyacrylamide Tris-Glycine gradient gels (Invitrogen). Gels were transferred to nitrocellulose membranes (Bio-Rad) and blocked with 5% milk in PBS. Membranes were incubated with primary antibodies at 4°C overnight. All antibodies were diluted in a mixture of equal parts Odyssey blocking buffer (Licor) and PBS with 0.1% Tween-20. The primary antibodies used were: rabbit anti-p-eEF-2 Thr-56 (Cell Signaling, 1:1000), rabbit anti-eEF-2 (Angus Nairn, 1:1000), and mouse anti-GAPDH (Advanced Immuno Chemicals, 1:10000). Membranes were then washed three times with PBS-T, incubated for 1 hr at 4°C with IRDye-800-conjugated goat anti-mouse (Rockland, 1:5000) and IRDye-680-conjugated goat anti-rabbit (Licor, 1:5000), and washed three more times with PBS-T. Blots were imaged with a Licor Odyssey Infrared Scanner, and quantification was performed using Licor Odyssey software. When necessary, membranes were stripped using a solution containing 25 mM glycine and 2% SDS, pH 2.0, at 55°C. Upon confirmation that the signal had been removed, stripped membranes were re-blocked and re-probed with primary and secondary antibodies.

Statistics

Behavior data were analyzed using *t*-test or 2-way repeated measures ANOVA with Bonferroni *post-hoc* tests where appropriate. Biochemical data were analyzed using 1-way ANOVA with Dunnett's *post-hoc* tests. For all experiments, outliers (defined as values exceeding ±2 standard deviations from the mean) were excluded and a *p* value of <0.05 was considered significant. Error bars indicate ±SEM.

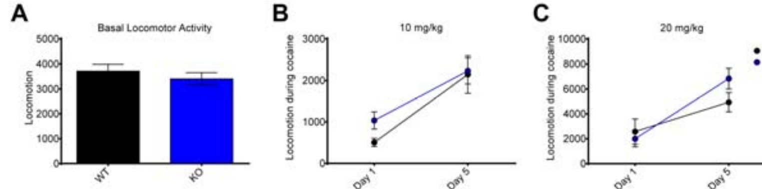
Results

Figure 1: Lack of EF2K activity in *EF2K* knockout brains.



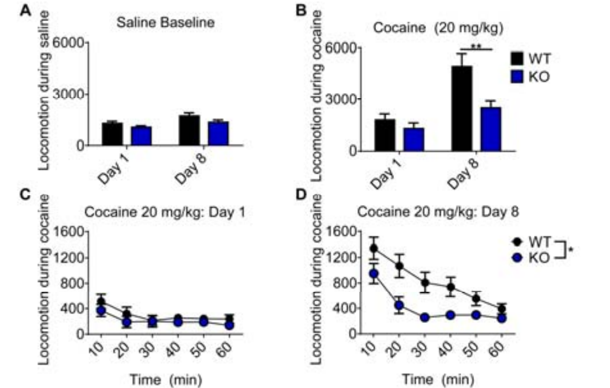
Whole brain homogenates from wild-type and *EF2K* knockout mice (n=6 mice per genotype) were incubated in buffer containing Ca^{2+}, calmodulin, and excess eEF-2, in the absence or presence of 50 µM ATP. Samples were then subjected to SDS-PAGE and immuno-blotting to assess kinase activity. As shown in the representative blot, WT homogenates exhibited robust EF2K activity in the presence of ATP, while KO homogenates did not.

Figure 2: Normal locomotor sensitization induced by repeated daily cocaine injections in *EF2K* knockout mice.



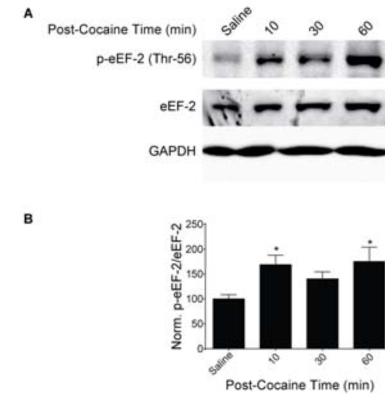
A.) During habituation to the testing chambers, mice (n=9 wild-type and 11 knockout) were injected with saline and locomotor activity was recorded for 1 hr. No significant difference between wild-type and knockout mice in locomotion under these basal conditions was observed (p>0.05). B.) Mice (n=14 wild-type and 15 knockout) received 5 daily injections of cocaine (10 mg/kg) followed by 1 hr of locomotor monitoring. A 2-way repeated measures ANOVA revealed a significant main effect of injection day ($F_{1,30}$=33.72; p<0.0001), but neither a significant main effect of genotype nor a significant injection day x genotype interaction (p>0.05). C.) The repeated daily injection procedure was performed in a separate cohort (n=12 wild-type and 13 knockout mice) with a 20 mg/kg dose of cocaine. A 2-way repeated measures ANOVA revealed a significant main effect of injection day ($F_{1,23}$=23.94; p<0.0001), but neither a significant main effect of genotype nor a significant injection day x genotype interaction (p>0.05).

Figure 3: Impaired locomotor sensitization to cocaine using a two-injection procedure in *EF2K* knockout mice.



Mice (n=18 wild-type and 16 knockout) received a saline injection and 1 hr locomotor monitoring, then a cocaine (20 mg/kg) injection and 1 hr locomotor monitoring during two sessions separated by 7 days. A.) Total locomotion during saline treatment. A 2-way repeated measures ANOVA revealed a significant main effect of day ($F_{1,32}$= 13.65; p<0.001), but not a significant main effect of genotype, nor a significant day x genotype interaction. B.) Total locomotion during cocaine treatment. A 2-way repeated measures ANOVA revealed significant main effects of day ($F_{1,32}$= 35.83; p<0.0001) and genotype ($F_{1,32}$= 5.665; p<0.05), as well as a significant day x genotype interaction ($F_{1,32}$= 7.122; p<0.05) Bonferroni *post-hoc* tests revealed a significant (p<0.01) difference in locomotor activity between genotypes on day 8, but not day 1. C-D.) Locomotion during cocaine separated into 10 min bins. C.) Binned data for Day 1. A 2-way repeated measures ANOVA revealed a significant main effect of time ($F_{1,32}$= 5.315; p<0.001), but not a significant main effect of genotype, nor a significant time x genotype interaction. D.) Binned data for Day 8. A 2-way repeated measures ANOVA revealed significant main effects of time ($F_{1,32}$= 28.02; p<0.0001) and genotype ($F_{1,32}$= 7.376; p<0.05), as well as a significant time x genotype interaction ($F_{1,32}$= 2.482; p<0.05).

Figure 4: Acute cocaine administration increases striatal eEF-2 phosphorylation.



Wild-type mice (n=9-10 per condition) were injected with saline or cocaine (20 mg/kg) and sacrificed 10-60 min later. Whole striata were lysed and samples were subjected to SDS-PAGE and immuno-blot. A.) Representative blot and B.) summary data illustrating the timecourse of eEF-2 phosphorylation following cocaine exposure. A 1-way ANOVA ($F_{3,34}$= 3.551; p<0.05) with Dunnett's *post-hoc* tests revealed significant (p<0.05) increases in p-eEF-2 in the striatum 10 and 60 min following cocaine.

Conclusions

- Phosphorylation of eEF-2 and EF2K activity are undetectable in brains from *EF2K* KO mice.
- *EF2K* KO mice show normal basal locomotor activity and locomotor sensitization to cocaine induced by repeated daily injections.
- *EF2K* KO mice exhibit impaired locomotor sensitization to cocaine induced by a two-injection protocol.
- Acute cocaine injection increases striatal eEF-2 phosphorylation.
- These data suggest that EF2K-mediated translational control is required the plasticity following initial cocaine exposure that produces a sensitized subsequent response.
- In particular, gene-specific *activation* of translation by EF2K may be required. This requirement appears to depend on the parameters of the task, perhaps reflecting a role for EF2K in setting the threshold for plasticity.

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