Supplementary Material for:

Neuronal and astrocytic protein degradation are critical for fear memory formation

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Expanded Methods and Materials

Subjects

All experiments used 8-9 week old male Dawley rats obtained from Envigo (Frederick, MA). Animals were housed two per cage with free access to water and rat chow. The colony was maintained under a 12:12-hr light/dark cycle. All experiments took place during the light portion of the cycle. All procedures were approved by the Virginia Polytechnic Institute and State University Institutional Animal Care and Use Committee and conducted with the ethical guidelines of the National Institutes of Health.

CRISPR guide RNA preparation

The CRISPR guide RNA (gRNA) targeting a 200bp region within the *Uba52* and *Psmd14* promoters were previously designed and described in detail by our group (Devulapalli et al. 2021). The gRNAs and dCas9-KRAB-MECP2 (gift from Alejandro Chavez and George Church; #110821, Addgene) cells were grown in LB Broth with ampicillin and purified with the Endotoxin-free Midi-Prep kit (Takara Bio, Mountain View, CA) according to the manufacturer's instructions. For stereotaxic delivery, plasmids were mixed with In Vivo Jet-PEI (Polyplus Transfection, New York, NY) according to the manufacturer's instructions using a previously described protocol (Devulapalli et al. 2021).

Development of GFAP-dCas9 Plasmid

The lenti SYN-dCas9-KRAB-MeCP2 (Addgene #155365) was altered to generate the GFAP-dCas9-KRAB-MeCP2 plasmid. First, we generated a custom plasmid from IDT containing the truncated GFA_{ABC1D} promoter. Both plasmids were digested with PacI and AgeI for directional cloning and then agarose gel purification was performed. Products were ligated by T4 ligase and

transformed into One Shot TOP10 Chemically Competent E. coli and then positive clones were confirmed through sequencing with unique forward (GFAP-dCas9 F1 Seq - GCGCCAATTCTGCAGACAAA) and reverse primers (GFAP-dCas9 R1 Seq - TACTTCTTGTCGGCTGCTGG). The developed GFAP-dCas9-KRAB-MeCP2 plasmid was submitted to Addgene (#194701).

Surgery

Rats underwent stereotaxic surgeries where CRISPR-dCas9 plasmids were injected into the basolateral amygdala using coordinates relative to Bregma (A/P: -3.0, M/L: +/- 5.0, D/V: -8.8). Animals were anesthetized with 1.5-4% isoflurane and received bilateral injections into the amygdala using a 26-guage Hamilton syringe connected to an automated pump (Harvard Apparatus, Cambridge, MA) at a rate of 0.1µl per minute for a total of 0.5µl per hemisphere. Animals received a subcutaneous injection of carprofen (5mg/kg) and topical lidocaine on the day of surgery.

Apparatus

The 2 identical Habitest chambers used for contextual fear conditioning have been previously described in detail (Orsi et al. 2019). Habitest chamber consisted of a steel test cage with front and back Plexiglas walls and a grid shock floor above a plastic drop pan. The right wall of the chamber consisted of a house light in the top back corner, which remained on during the behavioral procedures, and an infrared light in the top middle, which was not illuminated during this project. The left wall of the chamber consisted of a high-bright light, which was not illuminated during this project. All remaining slots of both walls were filled with blank metal panels. A USB camera was mounted on a steel panel outside the back Plexiglas wall of the chamber, angled at

~45 degrees. The entire chamber was housed in an isolation cubicle with an acoustic liner and a house fan, which remained active during all behavioral procedures. Shock was delivered through the grid floor via a Precision Animal Shocker under the control of FreezeFrame 4 software, which also analyzed animal behavior in real-time. A freezing threshold of 2.0 was used as the scoring parameter for all animals. All video was recorded and stored for later analysis. The chamber walls were wiped with 70% isopropanol before use.

Behavioral Procedures

Rats underwent contextual fear conditioning training and testing as described previously (Orsi et al. 2019) in a Habitest chamber. Animals were handled for 4 days prior to behavioral training; the first two days occurred in the animal housing room and the second two days occurred in an adjacent room where behavioral training were to occur. Following this, animals were placed into the fear conditioning apparatus and after a 1 min baseline, received 4 unsignaled footshock (1.0mA, 1 sec) presentations. After a 1 min post-shock period, the animals were returned to their homecages. Testing consisted of a 5 min exposure to the training context in absence of shock.

Immunofluorescence

Immunofluorescence procedures were performed as described previously with a small-scale modification (Devulapalli et al. 2021). Animals were overdosed on isoflurane and euthanized, after which their brains were removed and placed in 1:10 formalin for 24 hr. One day later, brains were transferred to a sucrose buffer (10% formalin with sucrose) for at least 5 days. Slices (~50-100µM) were taken throughout the basolateral amygdala and stored in 1% PBS in a 12-well dish at 4°C. Slices were fixed with 4% paraformaldehyde for 10 min, followed by antigen retrieval in boiling citric acid buffer. Slices were washed in PBS, blocked for 1 hr (4% normal goat

serum and 3% Triton-X in PBS) and incubated in a primary antibody for NeuN (1:750), 3x FLAG/DDDDK tag (1:500) or GFAP (1:500) overnight at 4ºC. The following day slices were rinsed with PBS and incubated in Alexa Fluor 488 (1:500, #715-025-150, Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 647 (1:200, #715-605-151, Jackson ImmunoResearch, West Grove, PA) secondary antibody for 2 hrs, rinsed with PBS and coverslipped with VectaSheild Mounting Medium with DAPI (#H-1200 Vector Laboratories, Burlingame, CA). Images were taken on a Nikon Eclipse Ci-L microscope (Melville, NY) and adjusted with Image J.

Statistical Analyses

All data are presented as mean with standard error, with scatter plots to identify individual samples (except in line graphs). Data were analyzed with One or Two-way ANOVA and Fisher LSD posthoc tests. Statistical outliers were defined as those samples that were two or more standard deviations from the mean and were determined by the outlier function in Prism.

References

Devulapalli R, Jones N, Farrell K, Musaus M, Kugler H, McFadden T, Orsi SA, Martin K, Nelsen J, Navabpour S et al. 2021. Males and females differ in the regulation and engagement of, but not requirement for, protein degradation in the amygdala during fear memory formation. *Neurobiol Learn Mem* **180**: 107404.

Orsi SA, Devulapalli RK, Nelsen JL, McFadden T, Surineni R, Jarome TJ. 2019. Distinct subcellular changes in proteasome activity and linkage-specific protein polyubiquitination in the amygdala during the consolidation and reconsolidation of a fear memory. *Neurobiol Learn Mem* **157**: 1-11.