Supplemental Information

Acute synthesis of CPEB is required for plasticity of visual avoidance behavior in Xenopus

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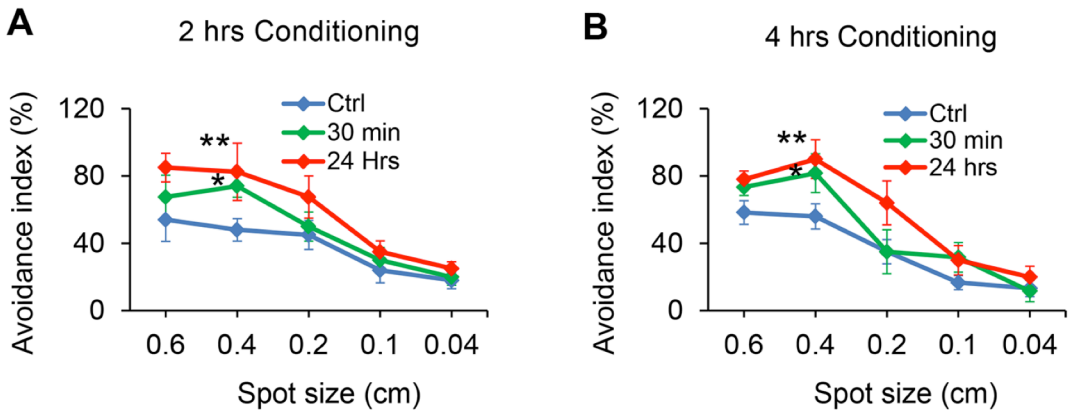

Figure S1. Plots of AI in response to moving spots of a range of sizes, related to Figure 1. The AI in response to 0.4 cm spots is significantly increased when tested 30 minutes (N=6 tadpoles, green line) or 24 hours (N=6 tadpoles, red line) after conditioning for 2 hours (A) or 4 hours (B), compared to control tadpoles without conditioning (blue line). **P<0.01, *P<0.05.

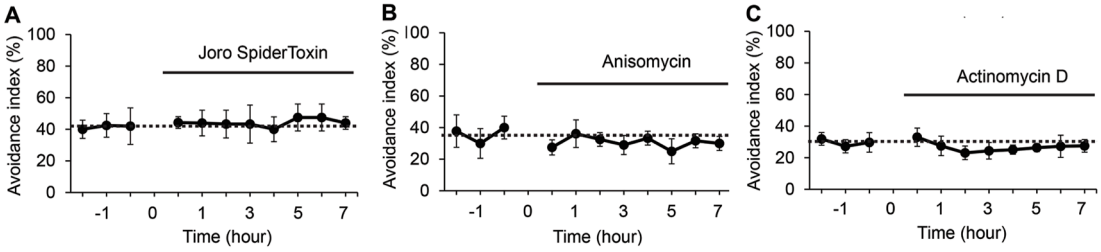

Figure S2. Drug effects on baseline AI, related to Figure 2. Exposure to Joro Spider Toxin (A, JST, 500 nM), Anisomycin (B, 25 μM) or Actinomycin D (C, 25 μM) for 8 hours did not affect the visual avoidance response compared to baseline collected before exposing animals to drugs. N=8 animals/condition.

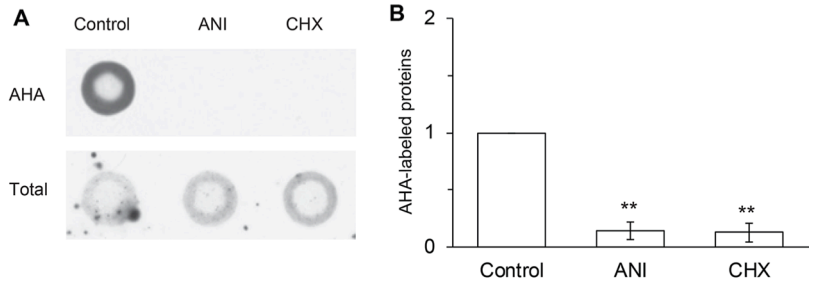

Figure S3. Blockade of protein synthesis by ventricular inhibitor injection, related to Figure 3. Ventricular co-injection of anisomycin (ANI, 25 μM) or Cychoheximide (CHX, 50 μM) with AHA significantly decreased AHA-biotin labeling compared to controls. N=3 independent experiments. **P<0.01.

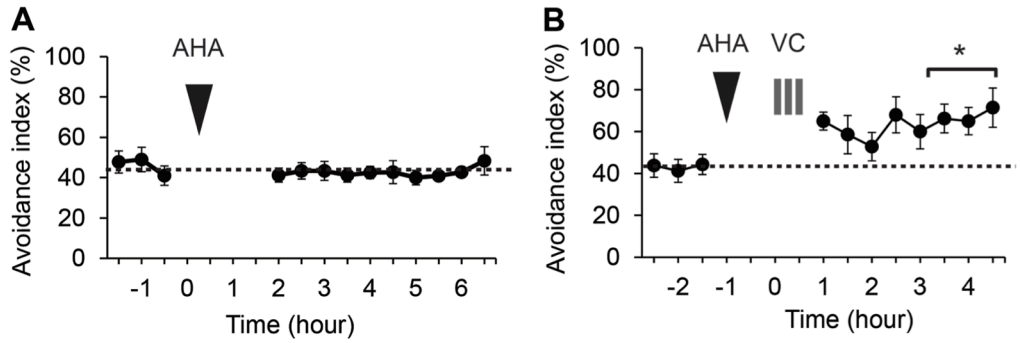

Figure S4. Controls for AHA injection, related to Figures 5 and 6. AHA injection (500 mM, pH 7.4) does not affect tadpole visual avoidance behavior (A) or plasticity of the VC-induced improvement of avoidance behavior (B). **P<0.01.

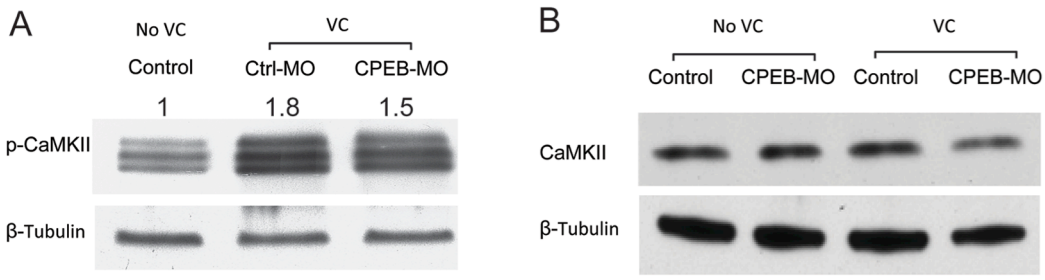

Figure S5. CPEB regulates CaMKII, related to Figure 6. A. Visual conditioning increases phosphorylation of CaMKII compared to control tadpoles without conditioning. CPEB MO decreases the VC-induced CaMKII phosphorylation. B. Electroporation of CPEB MO immediately before VC could decrease AHA-CaMKII by as much as 36% in conditioned animals compared to control conditioned animals. We did not detect decreased AHA-CaMKII in all experiments, consistent with the previous work indicating that activity-dependent CaMKII synthesis is not exclusively regulated by CPEB (Ashraf et al., 2006). The results suggest that CaMKII phosphorylation and newly synthesized CaMKII contribute to visual experience dependent behavioral plasticity.

Table S1. Lists of AHA-labeled proteins identified by direct detection of AHA-modified peptides by tandem mass spectrometry, annotated by cellular location and organelles, as shown in Figure 4A.

Table S2. Lists of AHA-labeled proteins identified by direct detection of AHA-modified peptides by tandem mass spectrometry, annotated by protein function, as shown in Figure 4B.

Supplemental Methods

Visual avoidance assay and visual conditioning

The visual avoidance assay was conducted on individual tadpoles as previously reported (Dong et al., 2009; McKeown et al., 2013; Shen et al., 2011). Single tadpoles were placed in a 8X3 cm tank filled with ~1 cm Steinberg's rearing solution. The bottom of the chamber was mounted with a back-projection screen. Visual stimuli were presented on the screen using a microprojector (3M, MPro110). Videos of tadpoles illuminated by IR LEDs were recorded with a Hamamatsu ORCA-ER digital camera. Visual stimuli were generated and presented by MATLAB 2009b (The MathWorks, Psychophysics Toolbox extensions). Randomly positioned moving spots of 0.04, 0.2, 0.2, 0.4 and 0.6 cm diameter were presented in random order for 60 seconds. Visual avoidance behavior was scored as a change in swim trajectory with the first ten encounters of the tadpoles and moving spots (plotted as avoidance index).

For visual conditioning, animals were exposed to moving bars (1 cm width; 0.3 Hz; Luminance: 25 cd/m2) continuously for 2 or 4 hours or for 30 minutes of 5 minutes of moving bars and 5-minute interval, repeated 3 times. To stimulate all tadpoles positioned in all directions and to minimize the possibility of habituation

when exposed to visual stimuli, the direction of the moving bar was switched between four directions (left to right, right to left, top to bottom, bottom to top).

Electrophysiology

For whole-cell recordings in vivo, tadpoles were anesthetized with 0.02% MS-222 and the tectal lobes were exposed. All recordings were performed at room temperature (20 - 22°C). The brains were perfused with extracellular saline containing (in mM: 115 NaCl, 2 KCl, 3 CaCl2, 1.5 MgCl2, 5 HEPES, 10 glucose, 0.01 glycine and 0.05 Tubocurarine, pH 7.2, osmolality 255 mOsm). Visually evoked currents from tectal neurons in the middle of the tectum were recorded from optic tectal neurons using a K+-based intracellular solution (in mM: 110 K-gluconate, 8 KCl, 5 NaCl, 1.5 MgCl2, 20 HEPES, 0.5 EGTA, 2 ATP, and 0.3 GTP). Recording micropipettes were pulled from borosilicate glass capillaries and had resistances in the range of 7 - 9 MΩ. Liquid junction potential was adjusted during recording. Recordings were accepted for analysis from cells in which the series resistance did not change over 10% and input resistance (0.7 - 2 GΩ) remained relatively constant. Signals were filtered at 2 kHz with a Multiclamp 700A amplifier (Molecular Devices, Palo Alto, CA). Data were sampled at 10 kHz and analyzed using ClampFit 10 (Molecular Devices) or Matlab (Mathworks).

Click chemistry and Analysis of AHA labeling by Western blots

AHA (L-azidohomoalaine, 150-500 mM, pH 7.4, Anaspec) or PBS colored with <0.01 % Fast Green was injected into the tectal ventricle of anesthetized stage 47/48 tadpoles with a Picospritzer. Animals recovered from anesthesia for 30 minutes before they were used for behavioral tests or visual conditioning. For analysis of AHA-biotin tagged proteins by Western blots, tecta were dissected from 60 to 100 anesthetized animals for each group and processed as described for click chemistry labeling (Dieterich et al., 2007; Speers and Cravatt, 2009; Weerapana et al., 2007). For Western blotting, tecta were homogenized in 150 µl PBS containing 1% SDS and 1 µl Benzonase (Sigma). Samples were boiled for 10 min at 96-100 °C and were diluted to 0.1 % SDS by adding 1.35 ml PBS. Samples were adjusted to 0.2 % TritonX-100 and centrifuged for 5 min at 2000 g. The supernatant was transferred to new tubes for Click reaction. For each reaction, 2 µl Triazole ligand (200 mM, Invitrogen), 80 µl Biotin Alkyne (2.5 mM, Invitrogen) and 15 µl Copper Bromide suspension (1% in ddH2O) were added to the supernatant in sequence. After quick and vigorous vibration, samples were incubated overnight at 4°C on a rotator. The next day, samples were centrifuged for 5 min at 2000 g and pellets were discarded. Supernatants were purified on

desalting columns (PD-10 or MidiTrap G-25, GE Healthcare Life Sciences) according to manufacture protocols. To enrich AHA-labeled proteins, the collected solution was mixed with 120 µl Neutravidin Resin and incubated at room temperature for 3-6 hours with rotation. Samples were centrifuged for 5 min at 2000 g and pellets were collected and washed 5 times with 1% NP40 in PBS and 1 time with PBS. To dissociate the proteins from the Neutravidin Resin, pellets were mixed with 80 µl 1X sampling buffer and boiled for 10-15 min. Samples were briefly centrifuged before loading on SDS-polyacrylamide gels for Western Blot analysis.

Western blots

Protein homogenates were made from the same numbers of tecta from control or visually-conditioned animals for each comparison. To ensure comparable inputs were loaded for each paired comparison, protein concentrations were measured and same amount of proteins were loaded for total input and Click reaction. If necessary the volume of homogenate loaded on the gels was adjusted according to the β-tubulin standard. Protein homogenates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Blots were blocked in 2% non-fat milk with 0.05% TBS and TritonX-100 (Sigma) and incubated with primary antibodies diluted in blocking solution. The following antibodies were used in this study. Goat polyclonal anti-biotin antibody (31852, Thermo Scientific), Rabbit polyclonal anti-GABAAR γ2 antibody (ab4073, Abcam, Cambridge, MA), Mouse anti-α CaMKII antibody (NB100-1983, Novus), Rabbit anti-CPEB1 antibody (ab127739, Abcam), Rabbit anti-HDAC3 antibody (ab16047, Abcam), Rabbit anti-GAD65 antibody (G5038, Sigma), Rabbit anti-β-tubulin (sc-9104, Santa Cruz), Mouse anti-PSD95 (ab2723, Abcam), MEK1(NBP1-97542, Novus), Rabbit anti-Syntaxin1A (ab70293, Abcam), Mouse anti-CREB(9104, Cell signaling), Rabbit anti-N-Cadherin(NB200-592SS, Novus), Mouse anti-NF-M(XC106C, Gift from Dr. Ben Szaro). Blots were rinsed and incubated with HRP-linked mouse/rabbit/goat IgG (Bio-rad). The ECL chemiluminescence kit (Amersham Biosciences) was used to visualize labeling. Different exposure periods were used for the same blots to avoid saturation. For quantification of Western blots, labeling intensities were measured from non-saturating exposures with ImageJ or Photoshop.

Dot blots

Samples were prepared as for Western blots. We prepared serial dilutions of samples with 1X TBS and loaded them into the dot blot apparatus (Whatman, Minifold I Dot-Blot System). The samples were transferred to membrane by vacuum and wells were washed 3 times with 1X TBS. Membranes were processed for biotin or C4-actin detection as described for Western blots. The

labeling intensity of the dot blots was analyzed will Gilles Carpentier's *Dot Blot Analyzer for ImageJ* toolset. Experiments and analysis were performed blind to the experimental conditions. We did four serial dilution and two duplicates for each sample. For comparison between different groups, the intensity of the biotin is first normalized to the actin signal obtained after stripping the same membrane and then normalized to the control group in each set of experiments.

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