Supplemental Material

Materials and Methods

Apparatus

Experiments were conducted in two sets of Med Associates chambers [24 cm (length) x 30 cm (width) x 21 cm (height)] to provide two different contexts. All chambers were enclosed within a sound- and light-attenuating cabinet. A ventilation fan provided constant low-level background noise (~ 58 dB). Presentations of the CS and US were controlled by a computer running Med-PC IV software (Med Associates). Rats were observed using a mounted infrared camera on the rear cabinet wall connected to a DVD recorder and monitor. The chambers were cleaned with tap water after each experimental session.

Rats received conditioning in chambers constructed of a clear Perspex ceiling, rear wall, and hinged front door with stainless steel side walls (referred to as Context A). A stainless steel tray of bedding was underneath a floor made of stainless steel rods (4 mm diameter, spaced 16 mm apart center to center). A clear Perspex divider was placed diagonally in Context A to create a triangle-shaped space. The divider blocked access to the recessed magazine and two inactive nose poke holes located on one of the side walls of the chamber. One side wall contained a speaker located in the top right corner. Infrared lighting was the only source of illumination in Context A. Extinction and test were conducted in a second set of chambers (referred to as Context B), which had different visual features, flooring, size, and lighting to Context A. The diagonal Perspex divider was removed in Context B so that the two contexts differed in size and shape. The ceiling and front wall of the Context B chambers had paper with vertical black and white stripes (2.5 cm width) attached. A clear Perspex insert covered the grid floor and the stainless steel tray under the floor did not contain any bedding. A white light on top of the chamber provided illumination in Context B (~4 lux inside the chambers).

Handling and Context Pre-Exposure.

In those experiments using juvenile and adolescent animals (i.e., Experiments 1 and 5) rats were weaned at P21 or P22 and left unhandled until P24 when some animals received conditioning as

juveniles. The remaining rats received a similar brief handling at P24 during weighing and marking of their tails and later received conditioning as adolescent animals. In these experiments the animals did not receive context pre-exposure. In those experiments using adolescent and adult animals (i.e., Experiments 2-4), rats were handled for 3-4 min for 2 consecutive days before conditioning to habituate to the experimenter. Following handling, on each day, animals were placed in the conditioning chamber for 10 min. In Experiment 5, animals in the experimentally naive group were weaned and then received only brief handling before euthanasia.

Tissue Processing, Immunohistochemistry and Neuronal Counting

In Experiment 5, one hour following the extinction or context-only exposure session (a time-point when pMAPK levels peak after extinction in limbic regions; Fischer et al. 2007; Herry et al 2006) rats were deeply anesthetized with sodium pentobarbital (433 mg/kg, i.p., diluted in 1:2 parts saline). The rats were then transcardially perfused with 0.4 ml of 1:2 parts solution of heparin (5000 IU/ml) and 1% sodium nitrite injected into the left ventricle (adolescent animals only), then 50 ml of 0.9% saline containing 1% sodium nitrite and heparin (5000 i.u./ml), followed by 150-200 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were then removed, postfixed (1 hr), washed in 0.1 M PB saline (pH 7.2), and cytoprotected in 20 % sucrose in PB saline (overnight or up to 48 hr). Brains were blocked using a matrix aligned to a rat brain atlas (Paxinos and Watson 2009) and 40 µm coronal sections were cut using a cryostat (CM1950, Leica Microsystems). Four serially adjacent sets were obtained from each brain and stored in 0.1% sodium azide in 0.1 M PB saline (pH 7.2). One series of sections was selected from each rat and processed for phospho-p44-42MAPK-immunoreactivity (pMAPK-IR). Free-floating sections were washed repeatedly in 0.1 M PB (pH 7.4), followed by two 30 min washes in 50% ethanol, the second of which contained 3% H_2O_2, and were then incubated in 5% normal horse serum (NHS) in PB (pH 7.4), for 30 min. Sections were then incubated in rabbit antiserum against pMAPK (1:2,000; phospho-p44/42 MAPK (Erk1/2) [Thr202/Tyr204] (D13.14.4E) XP® Rabbit mAb, #4370; Cell Signaling Technology, USA) for 48 hr at 4 °C, with gentle agitation. The primary antibody was diluted in 2% NHS in PBT-X, which consisted of 0.1 M PB (pH 7.4) and 0.2% Triton X-100. After washing off unbound primary antibody,

sections were incubated overnight at room temperature in biotinylated donkey anti-rabbit IgG (1:2,000; 711-065-152; Jackson ImmunoResearch Laboratories, USA) diluted in 2% NHS PBT-X, with gentle agitation. After washing, sections were incubated for 2 hr at room temperature in avidin–biotin–peroxidase complex (ABC) (Vectastain Elite ABC kit: 6 µl/ml avidin and 6 µl/ml biotin; PK-6100; Vector Laboratories, USA) in 2% NHS PBT-X. Black immunoreactive (IR) cytoplasm labelled for pMAPK was revealed by a nickel-intensified diaminobenzidine (DAB) reaction, with peroxide being generated by glucose oxidase. In this DAB reaction, sections were washed in PB (pH 7.4), followed by 0.1 M acetate buffer (pH 6.0), and then incubated for 15 min in 0.1 M acetate buffer (pH 6.0) containing 2% nickel sulfate, 0.025% 3,3’ DAB, 0.04% ammonium chloride, and 0.02% D-glucose. The peroxidase reaction was started by adding 0.2 µl/ml glucose oxidase and stopped using acetate buffer (pH 6.0). After the brain sections were washed in PB (pH 7.4), they were mounted onto gelatin-treated slides, air-dried, dehydrated through a graded series of alcohol, cleared with histolene, and coverslipped with Entellan.

Manual blind counts of neurons IR for pMAPK were conducted through the rostro-caudal extent of each brain region using a transmitted light microscope (Olympus BX53) equipped with a digital camera (DP72) at 10 × magnification. Only mid to dark gray/black round or ovoid stains distinct from background staining were counted as pMAPK-positive cells. All sections counted were 160 µm apart. The medial prefrontal cortex (mPFC) was counted from 3 consecutive sections from the left hemisphere (between bregma 3.48 and 3.00 mm). The central amygdala (CeA) and basolateral amygdala (BLA; lateral and basal nuclei) were counted from 6 consecutive sections from the left hemisphere (between bregma -2.28 mm and -3.36 mm). Regions were delineated using clearly visible landmarks and predefined boundaries according to a rat brain atlas (Paxinos and Watson 2009). When landmarks did not clearly delineate regions, boundaries were created with a 10 × 10-grid reticule located in the left eye piece of the microscope. The top of the prelimbic cortex (PL) was defined by aligning the eyepiece grid with dorsal aspect of forceps minor (fmi) corpus callosum and a 1 × 1 mm area between the medial edge of the section and the fmi counted. For the infralimbic cortex (IL), a 1 mm × 0.7 mm (h) area was created with the grid aligned at the point where the fmi and the edge of the section become parallel. The size of the areas examined in this study was kept constant across all

ages. The paraventricular thalamus (PVT) was counted from 2 consecutive sections each for the anterior, middle, or posterior PVT (bregma -1.56 mm to -3.48 mm). The lateral habenula was counted from the 3 most posterior sections. Labelled-IR was expressed as raw counts and summed across sections for each region.

Scoring, Exclusion Criteria and Statistics

Behavior was scored, using a time-sampling procedure every 3 sec, as either freezing or not freezing. Freezing was defined as the absence of all movement except that required for breathing (Fanselow 1980). The total observations scored as freezing were summed and converted to a percentage. A second observer unaware of the experimental condition of each rat scored a 30% sample of the test data. The inter-rater reliability was high (r = .90-.98 across experiments). Six rats were excluded from analysis due to poor fear learning (<12% freezing on the first block of extinction; no more than 2 rats per Experiment and spread across experimental groups) as well as 2 rats from Experiment 4 due to an equipment malfunction. A further two animals were excluded as statistical outliers (freezing at test >2.45 SD away from the mean; one rat each from Experiment 2 and 4).

Pre-CS freezing, conditioning, extinction, and test data, as well as total counts of pMAPK-IR, were analyzed using independent measures *t*-tests or ANOVA with Student-Newman-Keuls (Experiments 1 – 3) or Dunnett's (Experiment 5) *post hoc* tests where appropriate. Type I error rate (α) was controlled at 0.05.

Results

Pre-CS Freezing

Pre-CS freezing before conditioning, extinction, and test was low for all experiments (all means < 11.3%, SEMs < 10.8%). There were no significant group differences in pre-CS freezing in any experiment (see Table S1).

Table S1. Mean (±SEM) percent pre-CS freezing before conditioning, extinction, and test.

Experiment	Group			
Experiment 1	JuvCond-Ext	JuvCond-AdolesExt	AdolesCond-Ext	
Conditioning	0.2 (0.2)	0 (0)	0 (0)	
Extinction	4.8 (1.9)	3.4 (3.4)	4.8 (2.8)	
Test	3.1 (2.6)	1.0 (0.7)	5.6 (2.8)	
Experiment 2	AdolesCond-Ext	AdolesCond-AdultExt	AdultCond-Ext	
Conditioning	0 (0)	0.5 (0.5)	0 (0)	
Extinction	1.0 (0.8)	2.3 (1.2)	2.7 (1.1)	
Test	2.1 (1.5)	1.8 (1.1)	7.3 (3.3)	
Experiment 3	1 day	1 week	2 weeks	4 weeks
Conditioning	0.2 (0.2)	0.3 (0.3)	0 (0)	0 (0)
Extinction	8.5 (5.1)	5.8 (3.5)	8.5 (3.7)	5.0 (2.1)
Test	3.3 (1.9)	1.7 (1.4)	0.3 (0.8)	2.9 (1.3)
Experiment 4	1 day	2 weeks		
Conditioning	0.8 (0.6)	0 (0)		
Extinction	6.9 (4.2)	11.0 (4.1)		
Test	4.4 (2.1)	2.5 (2.1)		
Experiment 5	No Extinction	JuvCond-Ext	JuvCond-AdolesExt	AdolesCond-Ext
Conditioning	0 (0)	0 (0)	0.4 (0.4)	0 (0)
Extinction	9.6 (4.0)	3.8 (1.5)	0.4 (0.4)	11.3 (10.8)

Conditioning and Extinction

In Experiment 1, there was an effect of conditioning trial ($F_{(2,62)} = 67.25$, $p < .001$) and extinction block ($F_{(4,124)} = 105.16$, $p < .001$). There were no effects of group or group-by-trial or group-by-block interactions at either conditioning or extinction ($Fs < 1$), indicating that all groups showed comparable overall levels of fear and rates of fear acquisition and extinction (Fig. S1*A* and *B*).

In Experiment 2, conditioning and extinction proceeded as expected (Fig. S2*A* and *B*), with fear increasing across conditioning trials ($F_{(2,62)} = 65.20$, $p < .001$) and decreasing across extinction blocks ($F_{(4,124)} = 76.28$, $p < .001$). There were no effects of group or group-by-trial or group-by-block

interactions at either time point (*Fs* < 1), indicating that all groups showed comparable overall levels of fear and rates of fear acquisition and extinction.

All groups showed a similar acquisition of fear (trial main effect $F_{(2,78)} = 81.08$, $p < .001$; group main effect $F < 1$; group × trial interaction $F < 1$) in Experiment 3 (Fig. S3*A*). Freezing reduced across extinction training ($F_{(4,156)} = 66.39$, $p < .001$; Fig. S3*B*). No differences in levels of overall freezing (group main effect $F_{(3,39)} = 1.67$, $p > .05$) or the rate of extinction (group × block interaction $F_{(12,156)} = 1.08$, $p > .05$) were detected between the groups.

In Experiment 4, both groups showed an increase in fear across conditioning trials ($F_{(2,44)} = 41.84$, $p < .001$), and were comparable in overall levels of CS-elicited freezing and rate of acquisition (group main effect $F < 1$; group × trial interaction $F_{(2,44)} = 1.12$, $p > .05$; Fig. S4*A*). During extinction, freezing decreased across blocks ($F_{(4,88)} = 58.16$, $p < .001$; Fig. S4*B*). There were no differences in levels of overall freezing (group main effect $F < 1$) or the rate of extinction (group × block interaction $F < 1$) between the groups.

In Experiment 5, there were no group differences or group-by-trial interaction for CS-elicited freezing across conditioning trials in No Extinction rats across the three conditions (largest $F_{(2,9)} = 1.18$, $p > .05$); therefore, data from these rats across conditions were collapsed into a single No Extinction group for subsequent statistical analyses. All groups showed similar acquisition of fear across conditioning (trial main effect $F_{(2,52)} = 40.22$, $p < .001$; group main effect $F_{(3,26)} = 1.64$, $p > .05$; group × trial interaction $F_{(3,26)} = 1.36$, $p > .05$; Fig S5*A*). Freezing significantly reduced across extinction training ($F_{(4,60)} = 25.44$, $p < .001$; Fig. S5*B*). There were no differences in levels of overall freezing (group main effect $F < 1$) or the rate of extinction (group × block interaction $F_{(8,60)} = 1.21$, $p > .05$) between the groups.

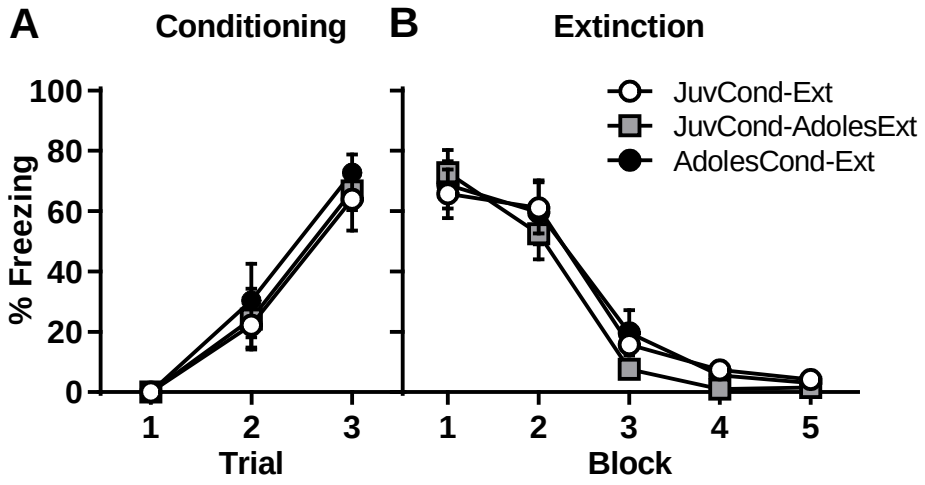

Figure S1. Experiment 1. Mean (± SEM) levels of CS-elicited freezing during conditioning (*A*) and extinction (*B*). Rats were in one of three groups: JuvCond-Ext (*n* = 12), JuvCond-AdolesExt (*n* = 11), or AdolesCond-Ext (*n* = 11).

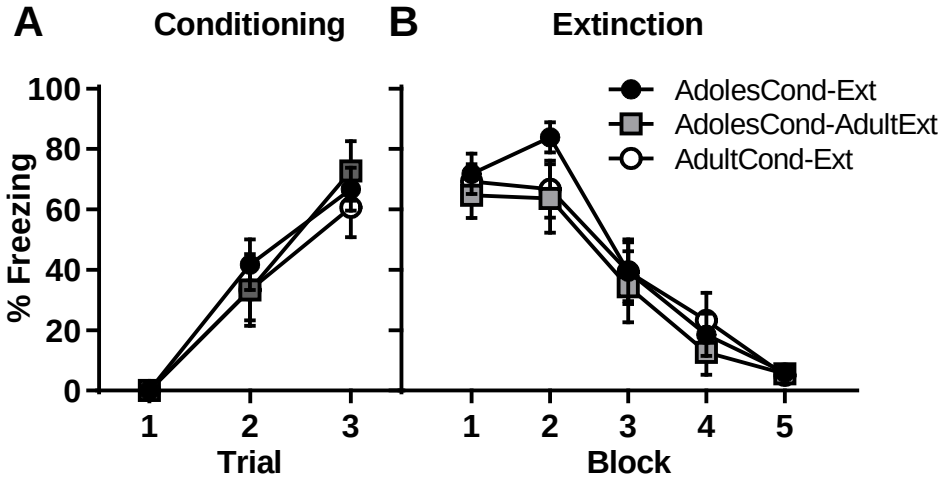

Figure S2. Experiment 2. Mean (± SEM) levels of CS-elicited freezing during conditioning (*A*) and extinction (*B*). Rats were in one of three groups: AdolesCond-Ext (*n* = 12), AdolesCond-AdultExt (*n* = 11), or AdultCond-Ext (*n* = 11).

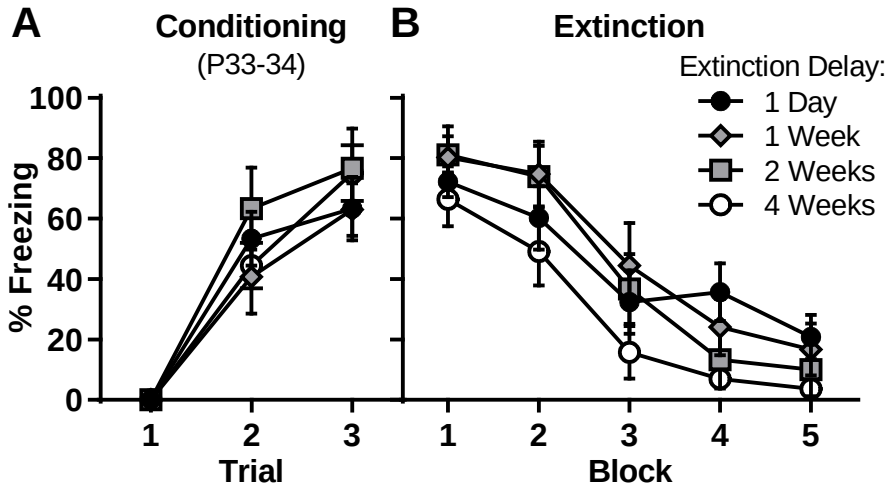

Figure S3. Experiment 3. Mean (± SEM) levels of CS-elicited freezing during conditioning (*A*) and extinction (*B*). Rats were in one of four groups where extinction occurred either 1 day (n = 12), 1 week (n = 9), 2 weeks (n = 10), or 4 weeks (n = 12) after conditioning in adolescence at P33-34.

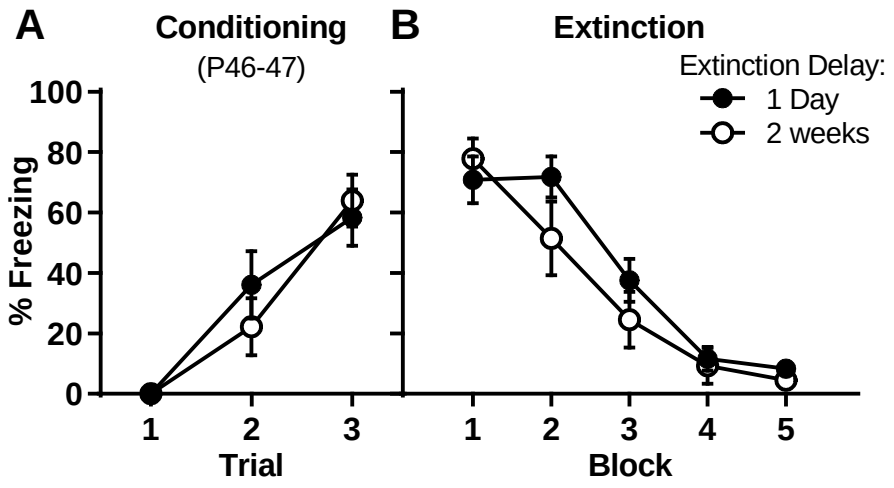

Figure S4. Experiment 4. Mean (± SEM) levels of CS-elicited freezing during conditioning (*A*) and extinction (*B*). Rats were in one of two groups where extinction occurred either 1 day (n = 12) or 2 weeks (n = 12) after conditioning in late adolescence at P46-47.

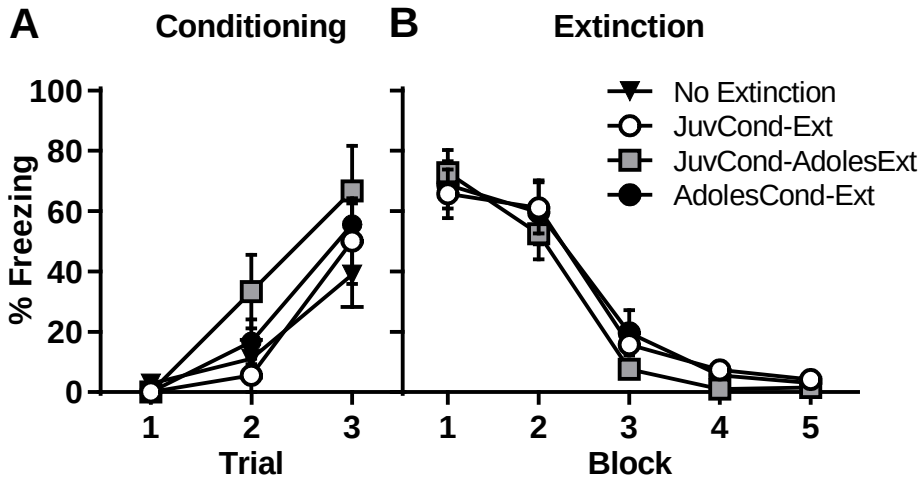

Figure S5. Experiment 5. Mean (± SEM) levels of CS-elicited freezing during conditioning (*A*) and extinction (*B*). Rats were in one of four groups: No Extinction (n = 12), JuvCond-Ext (n = 6), JuvCond-AdolesExt (n = 6), or AdolesCond-Ext (n = 6).

Experiment 5 Analyses of the No Extinction and Naive Groups

One-way ANOVAs showed that there were no significant age differences in pMAPK-IR neuron counts in No Extinction rats across the three conditions for the PL, IL, anterior PVT, middle PVT, posterior PVT, lateral habenula, BLA, CeA, BLA/CeA ratio, rostral BLA, caudal BLA, rostral CeA or caudal CeA (largest $F_{(2,9)} = 2.54$, $p > .05$); therefore, data from the rats in the three conditions were collapsed into a single No Extinction group for each of these regions for subsequent statistical analyses. In a second series of brain sections, there were no significant differences in pMAPK-IR neuron counts in the PL, IL, anterior PVT, middle PVT, posterior PVT, BLA, or CeA between the Naive and No Extinction groups (largest $t_{(12)} = 1.13$, $p > .05$; Fig. S6).

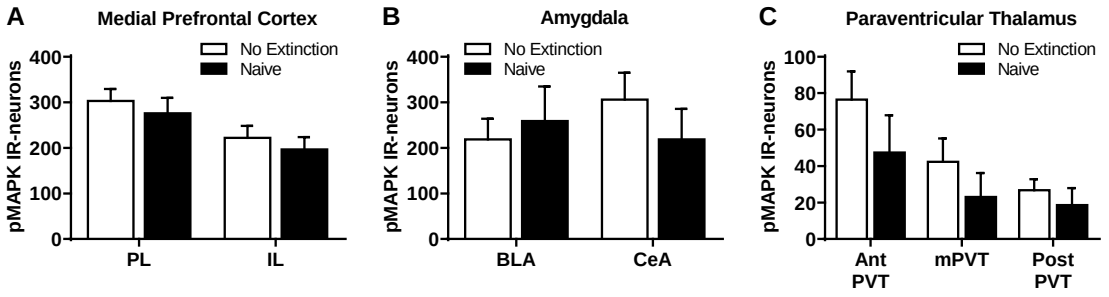

Figure S6. Experiment 5. Mean (± SEM) counts of pMAPK-immunoreactivity (pMAPK-IR) in the (*A*) prelimbic cortex (PL) and infralimbic cortex (IL) subregions of the medial prefrontal cortex (*B*) basolateral amygdala (BLA) and central amygdala (CeA), and (*C*) anterior (AntPVT), middle (mPVT) and posterior (PostPVT) paraventricular thalamus (PVT). Rats were in one of two groups: No Extinction (n = 7), or Naive (n = 7).

Experiment 5 Lateral Habenula Analysis

There were no significant group differences in pMAPK-IR neuron counts in lateral habenula (F < 1; see Fig. S7*B*).

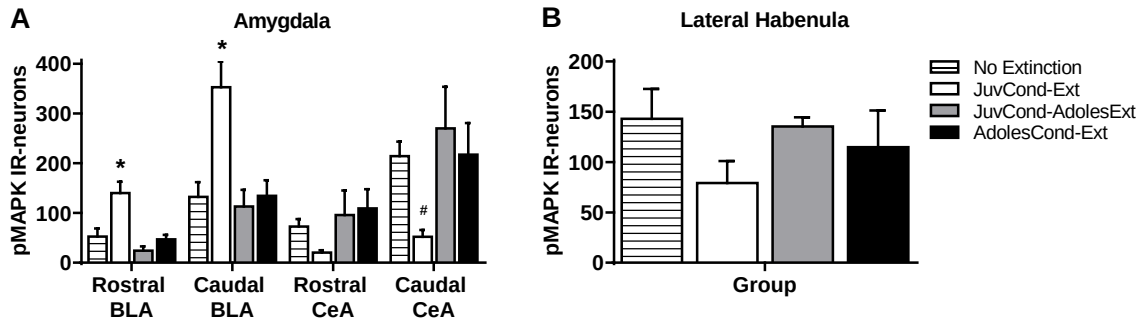

Figure S7. Experiment 5. Mean (± SEM) counts of pMAPK-IR in the (*A*) rostral and caudal extent of the BLA and CeA, and (*B*) lateral habenula. Rats were in one of four groups: No Extinction (n = 12), JuvCond-Ext (n = 6), JuvCond-AdolesExt (n = 6), or AdolesCond-Ext (n = 6). (*) Indicates significant difference compared to No Extinction controls (p < .05). ($^{\#}$) Indicates a trend towards a significant difference compared to No Extinction controls (p = .057).

References

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