A HIPPOCAMPAL CDK5 PATHWAY REGULATES EXTINCTION OF CONTEXTUAL FEAR

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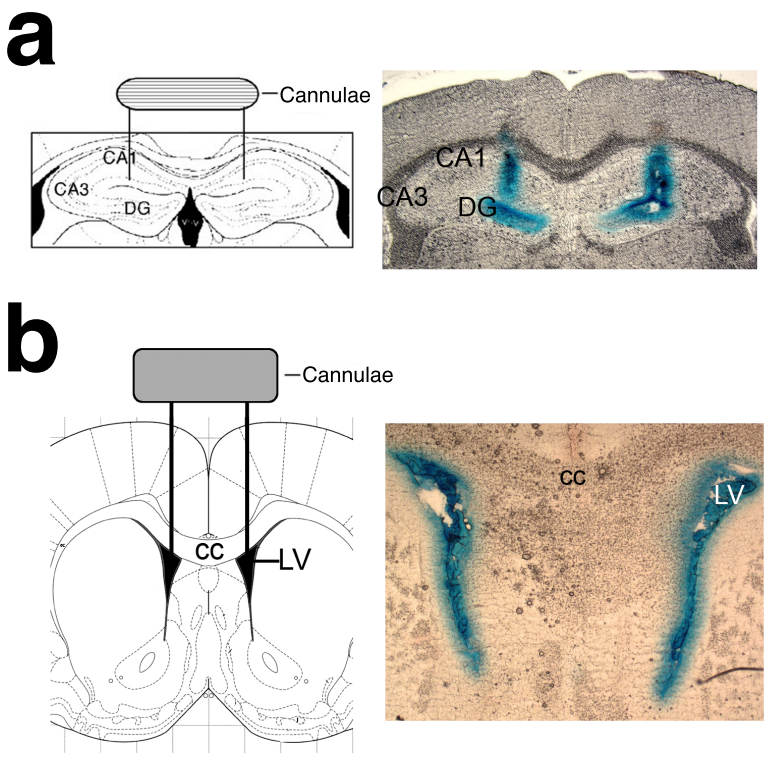
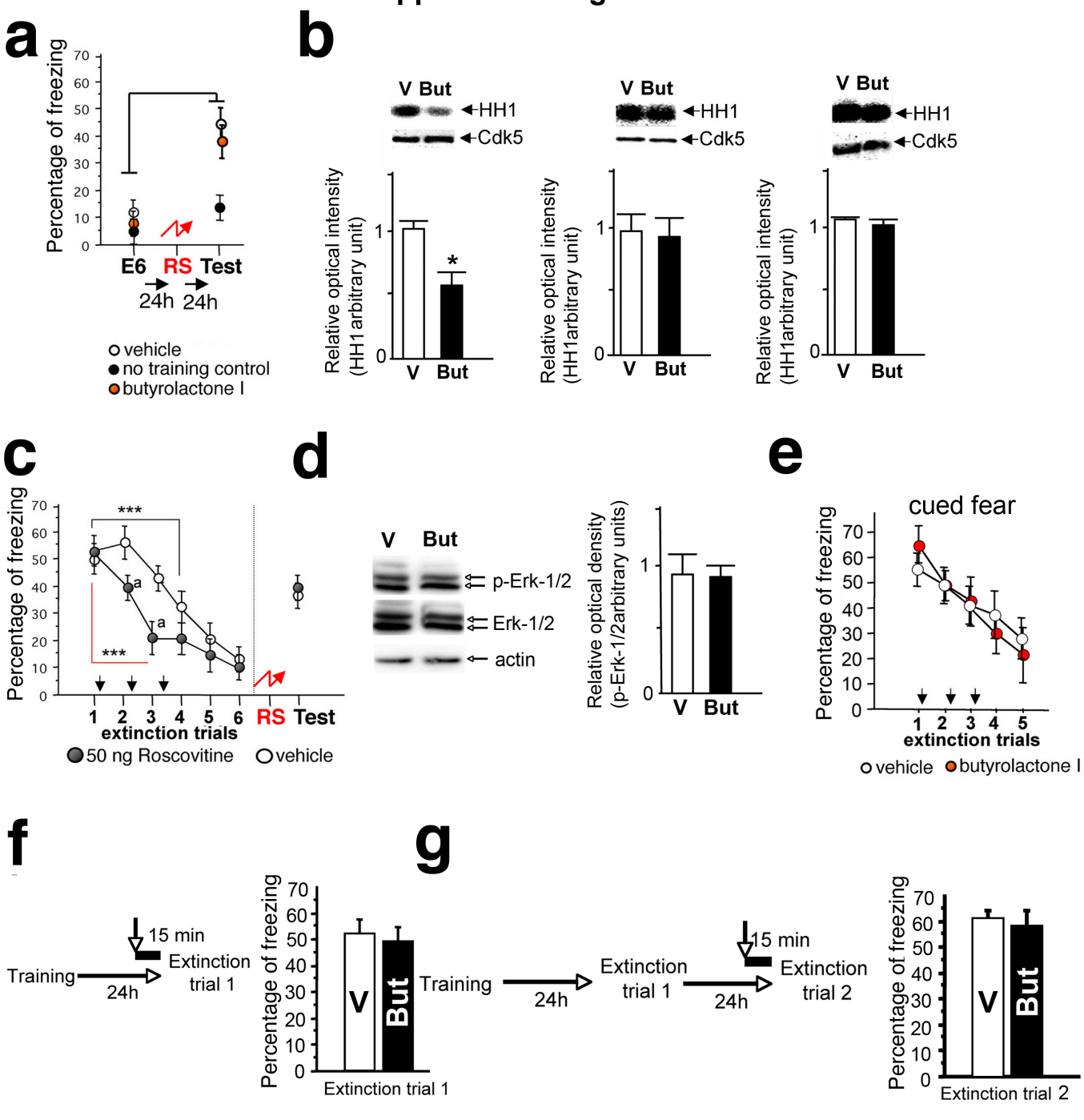
Supplemental Figure 1



Supplemental figure 1. Injection of compounds into the hippocampus and lateral brain ventricles.

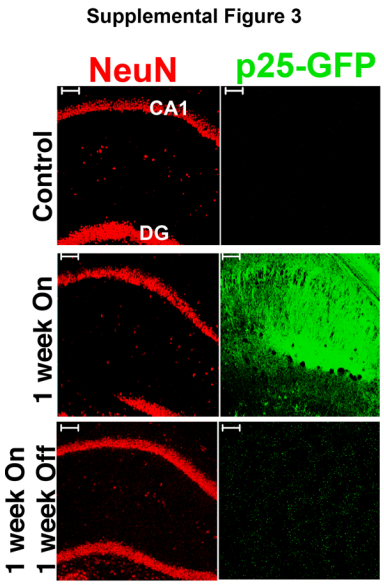
a. Microcanulae (PlasticOne; The gauge of the guide and injection cannulae was 26 and 28) were placed into the hippocampus by stereotaxic surgery using the coordinates AP - 1.5 mm, lateral 1 mm, depth 2 mm relative to the bregma. At the end of each experiment the animals were injected with methylene blue. Then the cannulae were removed and the brain processed to cut 40 μM sections in a cryostat microtome. The right panel displays a representative picture of the injection site. **b.** To insert cannulae into the lateral brain ventricles the coordinates AP +0.5 mm, lateral 1 mm, depth 2 mm relative to the bregma were used. At the end of each experiment the animals were injected with methylene blue to verify the correct location of the canulae. The right panel displays a representative picture of the injection site. LV, lateral brain ventricles; CA1, hippocampal subfield CA1; CA3, hippocampal subfield CA1; DG, dentate gyrus; cc, corpus callosum.

Supplemental Figure 2



Supplemental figure 2. Cdk5 inhibitors affect the consolidation of extinction.

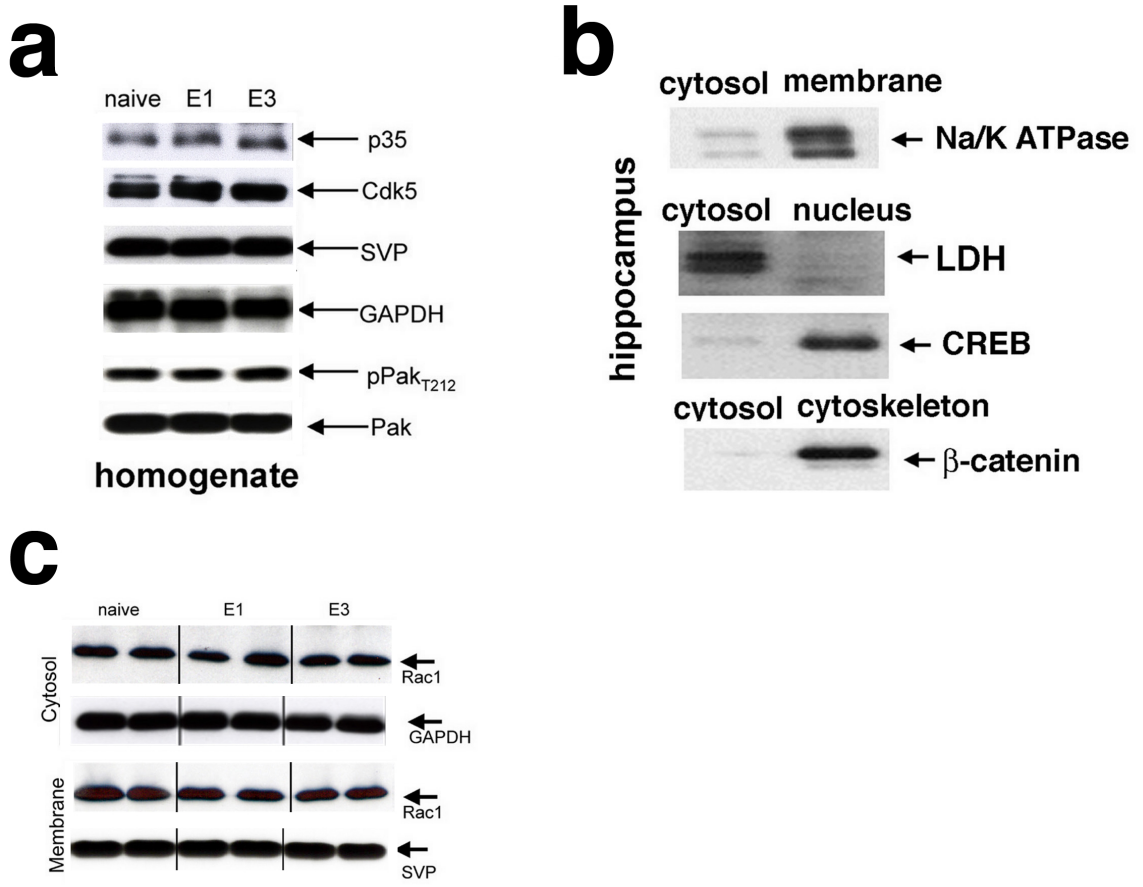
a. The same mice as used in Fig. 1A were subjected to a reminder shock procedure, consisting of an immediate foot-shock in a novel context. When retested in the conditioned context 24 h later, butyrolactone I (But) and vehicle (V) injected mice showed a similar increase in freezing behavior demonstrating that the fear memory was not entirely erased during extinction. **b.** To measure Cdk5 activity, p32 ATP incorporation to the Cdk5 substrate histone H1 was analyzed in hippocampal lysates that were prepared 0.5 h after injection of butyrolactone 1. Note the significant (*P<0.05 Butyrolactone I vs. vehicle) reduction of signal in butyrolactone I (50 ng) injected mice, confirming the inhibition of Cdk5 activity by intrahippocampal injection of butyrolactone I **c.** Mice (10/group) were subjected to our extinction paradigm and injected (i.h) with the Cdk5 inhibitor roscovitine (50ng) immediately after E1-E3. When compared to the vehicle group, roscovitine injected mice showed reduced freezing behavior on E2 and E3 indicating facilitated extinction. A reminder shock procedure reinstated freezing behavior to the same degree in both groups. **d.** The activity of Erk-1/2 kinase was analyzed in the same lysates used in (B) by immunoblotting for phospho-Erk-1/2. No differences in phospho-Erk1/2 levels relative to total Erk-1/2 were observed between the vehicle (set to 1) and butyrolactone I (50 ng) injected group. **e.** Mice were trained in the cued fear-conditioning paradigm and injected into the hippocampus with butyrolactone I (50ng) immediately after E1-E3. This procedure had no effect on the extinction of cued fear, which is amygdala dependent. **f.** Mice (n=9/group) were subjected to our extinction paradigm and injected with butyrolactone I (50ng) or vehicle 15 min before exposure to E1. No difference in freezing behavior during E1 was observed among groups. **G.** Mice (n=9/group) were subjected to our extinction paradigm and injected with butyrolactone I (50ng) or vehicle 15 min before exposure to E2. No difference in freezing behavior during E2 was observed among groups.



Supplemental figure 3. Regulation of p25 expression in bi-transgenic CK-p25 mice.

The expression of p25 in bi-transgenic CK-p25 mice can be switched On and OFF by a doxycyline diet. To this end mice will not express p25 when fed a doxycycline diet. Representative images showing NeuN staining (red) in the hippocampus and the corresponding signal for p25-GFP (green) in a control group, in mice that were induced for 1 week (1 week ON) and in mice that were induced for 1 week and the fed a doxycycline diet for another week (1week ON 1week OFF). Scale bar, 100μm. CA1, hippocampal subfield CA1; DG, dentate gyrus.

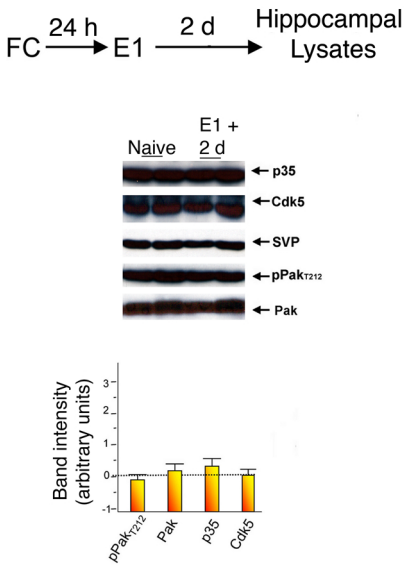
Supplemental Figure 4



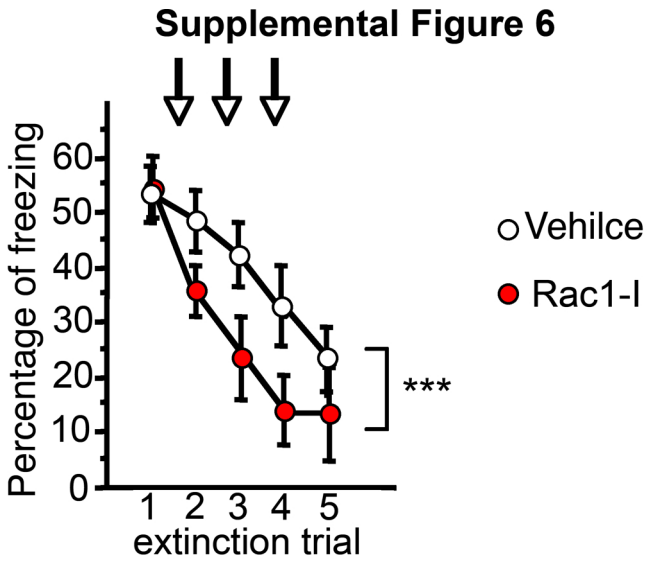
Supplemental figure 4. Total protein levels of p35/Cdk5 and Rac-1 during extinction.

a. RIPA total cell hippocampal lysates were prepared form mice 0.5 after E1, E3 and from naïve mice. Immunoblot analysis demonstrated no differences of p35/Cdk5 or the Cdk5 substrate PAK-1 and PAK-1_{Thr212} levels among groups. **b.** The hippocampus of one mouse was isolated and subcelluar protein fractions were isolated using the calbiochem kit. The quality of different fraction was analyzed by immunblotting for proteins that are expected to be enriched in each fraction. **c.** Membrane and cytosolic fractions were prepared form the hippocampus of mice 0.5h after exposure to E1, E3 or from naïve animals. Immunoblot analysis for total Rac-1 levels revealed no difference among groups.

Supplemental Figure 5



Supplemental figure 5. Membrane-depletion of Cdk5 activity is specific to extinction. To assess whether the reduction of membrane-associated Cdk5 activity was selective for the extinction paradigm mice were trained in the contextual fear conditioning and exposed to E1 24h later. Mice were sacrificed 2 days later without exposure to E2 or E3. Naïve animals served as control. When hippocampal protein lysates were subjected to immunoblot analysis we did not observe a down-regulation of membrane associated Cdk5, p35, PAK-1 or pPAK-1_{Thr212} levels. This data demonstrate that membrane associated down-regulation of Cdk5 activity only occurs when mice are exposed to E1, E2 and E3.



Supplemental figure 6. Intrahippocampal injection of Rac-1 inhibitor facilitates extinction.

Mice were trained in the contextual fear conditioning paradigm and injected into the hippocampus with Rac-1 inhibitor I (NSC23760; 10μg/μl) immediately after E1-E3. Extinction was significantly facilitated when compared to the vehicle group (*P<0.05). Arrows indicate injection.