Encoding of conditioned fear in central amygdala inhibitory circuits

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Supplementary notes

Given that both CE_{l off} and CE_{l on} neurons can form anatomical and functional inhibitory connections in CEm, the question arises why CEm output neurons are disinhibited, rather than inhibited – i.e. why the CE_{l off} pathway predominates over the CE_{l on} pathway during sensory stimulation. A possible explanation could be that unitary synaptic connections made by CE_{l on} and CE_{l off} neurons onto CEm output neurons have distinct properties in terms of their strength or their short-term dynamics. Moreover, based on our data, we cannot exclude that CE_{l on} and CE_{l off} neurons might target distinct subtypes of CEm output neurons. More detailed in vitro electrophysiological and anatomical studies will be required to address these questions.

Our results on CEm cell activity are in accord with most studies on the effects of CEA activation on fear behavior. However, the decrease in firing of brainstem projecting cells upon CS+ presentation reported by Pascoe and Kapp (1985)\(^1\) seems at odds with our results, which can be at least partly traced to methodological differences. Their neuronal groupings were purely functional, and did not distinguish between the subnuclei in which their neurons were recorded. Furthermore, their analysis was on pooled non-normalized data, making it hard to appreciate quantitatively the activity that can be ascribed only to brain-stem projecting cells.

Statistical analysis

**Fig. 1c.** Left: Blue laser light (473 nm) was used for bilateral stimulation of ChR2-expressing CEm neurons (10s periods of light stimulation with inter-stimulation intervals of 30-60s). Time bins for freezing determination were 10 s for light stimulation and 30-60 s for inter-stimulation intervals. Right: The summary data shows significant light-induced freezing in AAV-ChR22A-tdimer infected animals compared to sham-operated controls. There were significant main effects for infection: $F_{(1,50)} = 91.335, P < 0.001$; and light stimulation: $F_{(1,50)} = 39.734, P < 0.001$, two way ANOVA; significant interaction infection x light stimulation: $F_{(1,50)} = 52.523, P < 0.001$. Infected animals spent 15.9 ± 6.2% of time freezing without light, and 63.1 ± 4.0% with light ($n = 5$, $P < 0.05$, post-hoc Bonferroni t-test). Freezing in sham-operated controls: without light, 7.6 ± 1.8%; with light: 4.8 ± 2.4% ($n = 21$, $P > 0.05$ vs. no light, $P < 0.05$ vs. infected animals with light).

**Fig. 1e.** Freezing Values for inactivation of the different subnuclei: CEl: 52.9 ± 5.2%, $n = 8$, $F_{(3,22)} = 6.65, P < 0.01$, one way ANOVA, $P < 0.05$, CEl vs. Ctrl and CEm, post-hoc Bonferroni t-test. CEm: 22.9 ± 3.4%, $n = 8$, $P > 0.05$ vs. Ctrl. CEA: 26.8 ± 4.5%, $n = 5$, $P > 0.05$ vs. Ctrl. Control: 26.2 ± 9.6%, $n = 5$.

**Fig. 1f.** Freezing Values for inactivation of the different subnuclei: Control: 59.4 ± 8.6%, $n = 5$. CEl: 24.4 ± 3.7%, $n = 14$, $F_{(3,31)} = 10.719, P < 0.001$, one way ANOVA, $P < 0.01$, post-hoc Bonferroni t-test. CEA: 27.4 ± 6.4%, $n = 7$, $P < 0.01$. CEm: 54.3 ± 5.5%, $n = 9$, $P > 0.05$.

**Fig. 1g.** Freezing Values for inactivation of the different subnuclei: Control: 65.3 ± 8.0%, $n = 7$. CEm: 39.2 ± 6.0%, $n = 9$, $F_{(3,25)} = 5.345, P < 0.01$, one way ANOVA, $P < 0.05$, post-hoc Bonferroni t-test. CEA: 30.1 ± 10.2%, $n = 7$, $P < 0.01$. CEl: 57.7 ± 7.6%, $n = 8$.
19 trial blocks; generalizing trial blocks: 0.72 ± 0.09, \( n = 23 \), \( P < 0.001 \) vs. discriminating trial blocks; Z-score ratios of phasic responses for discriminating trial blocks: 4.15 ± 1.46, \( n = 19 \) trial blocks; generalizing trial blocks: 1.06 ± 0.35, \( n = 23 \), \( P < 0.05 \) vs. discriminating trial blocks. CElon neurons: freezing ratios for discriminating trial blocks: 3.31 ± 0.63, \( n = 21 \) trial blocks; generalizing trial blocks: 0.80 ± 0.11, \( n = 19 \), \( P < 0.001 \) vs. discriminating trial blocks; Z-score ratios of phasic responses for discriminating trial blocks: 2.97 ± 0.75, \( n = 21 \) trial blocks; generalizing trial blocks: 1.56 ± 0.32, \( n = 19 \), \( P = 0.075 \) vs. discriminating trial blocks.
Supplementary Figure 1

ChR2-mediated optical control of neuronal firing in vivo. a, High-magnification picture illustrating AAV-infected CEm neurons. Scale bar is 50 µm. b, Example raster plot and PSTH illustrating light induced sustained firing of an amygdala neuron recorded in an anaesthetised mouse. Similar light-induced responses were observed in several neurons (n = 5) in AAV-ChR22A-tdimer infected animals.
**Supplementary Figure 2**

**Targeted inactivation of CEA or CEI does not affect US sensitivity and does not lead to permanent damage.**

a, Inactivation of CEA with muscimol does not affect US sensitivity as measured by the US threshold inducing flinching behavior (left, \( n = 5 \), \( P > 0.05 \) vs. control) or vocalization (right, \( n = 5 \), \( P > 0.05 \) vs. control). b, Likewise, inactivation of CEI with muscimol does not affect US sensitivity (left: flinching behavior, \( n = 8 \), \( P > 0.05 \) vs. control; right: vocalizations, \( n = 8 \), \( P > 0.05 \) vs. control).

c, Re-conditioning of mice 24 hrs after inactivation of CEA or CEI. After wash-out of muscimol, mice in which CEA or CEI had been inactivated showed normal acquisition and retrieval of conditioned fear responses. Twenty four hours after muscimol application, mice (\( n = 7 \) in each group) were reconditioned and tested for fear memory retrieval again 24 hrs later (CEA, 65.3 ± 8.0% during CS exposure vs. 17.9 ± 6.6% before CS exposure; CEI, 57.7 ± 7.6% during CS exposure vs. 27.1 ± 4.9% before CS exposure). **\( P < 0.01 \).
Supplementary Figure 3

**Differential role of CEl and CEm in fear expression.** Animals were fear conditioned in the absence of BPY or muscimol-BPY and tested 24 hrs later. BPY or muscimol-BPY was applied before animals were re-tested on the same day. Bar graphs illustrate freezing levels before and during CS exposure in the presence and absence of BPY or muscimol. Consistent with the results obtained in naïve unconditioned animals, we found that inactivation of CEl in conditioned animals resulted in increased baseline (pre-CS) freezing levels. However, when animals with CEl inactivation were exposed to the CS, they exhibited a further increase in freezing levels. This could be explained by several possibilities. First, it is possible that we did not inactivate the entire CEl, and that conditioned freezing was therefore not completely occluded by unconditioned freezing. Alternatively, our findings are also consistent with the notion that conditioned freezing might be elicited through other pathways (e.g. via disinhibition of CEm output neurons by ITCs or by direct excitatory inputs from BLA or thalamus). Data were analyzed by one way ANOVA followed by post-hoc Bonferroni t-test (vs. the no-CS, no-treatment group). CEA-Ctrl, n = 7; CEA, n = 7; CEm, n = 9; CEl, n = 8. Error bars indicate mean ± s.e.m. ns: not significant, **P < 0.01 , ***P < 0.001.
Characterization of fear conditioning-induced plasticity of CS-evoked firing of CEI units. 

**Characterization of fear conditioning-induced plasticity of CS-evoked firing of CEI units.**

a. Coronal section of the amygdala showing the location of the recordings sites of CEI_on (green) and CEI_off (blue) units. Numbers indicate the antero-posterior coordinates caudal to bregma.

b. Summary graph illustrating changes in freezing behavior during fear conditioning. Comparing the first CS with the last CS reveals an increase in freezing behavior during conditioning (1<sup>st</sup> CS: 26.3 ± 4.9% of the time spent freezing; 5<sup>th</sup> CS: 60.0 ± 5.1%).

c, d. Increased freezing levels were associated with significant changes in CS-evoked activity in CEI_on neurons (n = 15 neurons from 14 mice; note that z-scored CS1 responses do not reach significance) and CEI_off neurons (n = 32 neurons from 17 mice). Note that not all units recorded before and after fear conditioning could be reliably identified during conditioning.

e, f. Discrimination between CS<sup>+</sup> and CS<sup>-</sup>. Averaged CS<sup>+</sup>- and CS<sup>-</sup>-evoked responses of all CEI_on units (n = 3) and CEI_off units (n = 3) recorded from a mouse exhibiting behavioral discrimination.

g, h. Analysis of response latencies of CEI_on units. Normalized population peri-stimulus time histograms illustrating CS-evoked responses of CEI_on neurons (n = 50) before (left panel; onset latency of excitation: 10-15 ms) and after fear conditioning (right panel; onset latency: 10-15 ms). 

i, j. Analysis of response latencies of CEI_off units. Normalized population peri-stimulus time histograms illustrating CS-evoked responses of CEI_off neurons (n = 41) before (left panel) and after fear conditioning (right panel; onset latency of inhibition; 35-40 ms). ***P < 0.001.
Supplementary Figure 5

Retrograde tracing of CE1 to CEm projections. a, HSV-GFP injection site in CEm revealed by co-injection of fluorescent latex beads. Antero-posterior coordinates caudal to bregma: -1.34 mm. b, HSV-GFP injection to CEm reveals retrograde labeling of CE1 neurons detected with anti-GFP immunostaining. c, HSV-GFP injection site in CE1 revealed by co-injection of fluorescent latex beads. Antero-posterior coordinates caudal to bregma: -1.22 mm. d, HSV-GFP injection to CE1 reveals local labeling in CE1 as detected with anti-GFP immunostaining. Very few labeled neurons were observed in CEm, indicating that the CE1 to CEm projection is unidirectional. Scale bars: 130 µm.
Supplementary Figure 6

**CEI\textsubscript{on} and CEI\textsubscript{off} units functionally inhibit CEm output neurons.** 

a, Raster plot illustrating CS-evoked responses of an identified CEI\textsubscript{on} unit. 

b, Cross-correlation analysis reveals a short latency inhibitory interaction between the CEI\textsubscript{on} unit taken as a reference and a CEm neuron. Dotted lines indicate 95% confidence intervals. Dashed vertical line indicates time of reference spikes from CEI\textsubscript{on} unit. Because the presynaptic neuron exhibited bursting activity, inhibition of the postsynaptic neuron appears to start before the presynaptic spike. 

c, Raster plot illustrating CS-evoked responses of an identified CEI\textsubscript{off} unit. 

d, Cross-correlation analysis reveals a short latency inhibitory interaction between the CEI\textsubscript{off} unit taken as a reference and a CEm neuron. Dotted lines indicate 95% confidence intervals. Dashed vertical line indicates time of reference spikes from CEI\textsubscript{off} unit. Probabilities for finding inhibitory cross-correlations between identified CEI units and CEm output neurons were: CEI\textsubscript{on} $\rightarrow$ CEm pairs: 1/25; CEI\textsubscript{off} $\rightarrow$ CEm pairs: 2/25. These data were obtained from a single mouse. Because of the low n, additional multi-site unit recordings or other approaches (paired recordings in acute slices) will be necessary in order to obtain a quantitative understanding of the connectivity between defined subtypes of CEI neurons and CEm output neurons.
Supplementary Figure 7

Characterization of fear conditioning-induced plasticity of CS-evoked firing of CEm units. a, Coronal section of the amygdala showing the location of the recordings sites of CEm units. Numbers indicate the antero-posterior coordinates caudal to bregma. b, CS-evoked firing of CEm units changes during fear conditioning. Increased freezing levels were associated with significant changes in CS-evoked activity in CEm neurons (n = 7 neurons from 4 mice). Note that not all units recorded before and after fear conditioning could be reliably identified during conditioning. c, Discrimination between CS⁺ and CS⁻. Averaged CS⁺- and CS⁻-evoked response of a CEm unit recorded from a mouse exhibiting behavioral discrimination. d,e, Analysis of response latencies of CEm units. Normalized population peristimulus time histograms illustrating CS-evoked responses of CEm neurons (n = 15) before (left panel; onset latency of excitation: 10-15 ms) and after fear conditioning (right panel; onset latencies: 10-15 ms and 45-50 ms).
Supplementary Figure 8

Summary of main results. a, Functional connectivity of the CEA microcircuit. The CEl contains two distinct neuronal populations changing responses with fear conditioning: CElon neurons acquire an excitatory response, whereas neurons are inhibited by the CS. CEl off neurons express PKCδ; REF 10). These two populations are reciprocally connected, and in addition project to CEm. Firing of CEm neurons triggers freezing. In addition to these local inhibitory interactions within CEA, both CElon and CEm neurons also receive short-latency excitatory input, presumable from thalamus and/or BLA. Based on our data, we cannot exclude that CElon and CEl off neurons might target distinct subtypes of CEm output neurons. b, Changes in activity of CEl off and CEm neurons with discriminative fear conditioning. During habituation, both populations are spontaneously active but showed no CS response. After conditioning, CEl off neurons acquired an inhibitory CS response, whereas CEm neurons increased their activity in response to the CS. Mice were split into a group with discriminating fear responses and one group displaying stimulus generalization. In discriminating animals, baseline firing rates were very similar to habituation (dashed grey line). In contrast, generalization was associated with changes in baseline firing of opposite polarity for the two neuronal populations: CEl off neurons increased tonic firing, whereas CEm cells displayed decreased tonic activity. The tonic changes were of opposite direction to the phasic CS response in both neuronal populations, and increased the signal-to-noise ratio of both CS+ and CS-, leading to reduced neuronal discrimination between the two stimuli.
Supplementary Figure 9

Correlations of phasic and tonic activity with post-conditioning freezing levels: raw data. a, Phasic z-scored CS-evoked responses of CEm neurons (left), CEI_{off} neurons (middle), and CEI_{on} neurons (right) are highly correlated with behavioral freezing. Correlations were obtained by plotting the averaged z-scored responses for all neurons of a given subtype for each animal over blocks of 2 CSs. Plots include both CS^+ and CS^- responses. P values indicate significance levels of Pearson's correlation coefficients. Dashed lines indicate 95% confidence intervals. b, Absolute levels of tonic activity do not correlate with freezing behavior. Correlations were obtained as described above. c, Z-scored tonic activity does not correlate with freezing behavior. Correlations were obtained as described above.
Supplementary Figure 10

Changes in tonic activity offset phasic CS responses. Direct correlation between fear conditioning-induced changes in tonic activity and in CS-evoked phasic activity (z-scored) in CEm neurons (left, n = 15), CEloff neurons (middle, n = 41), and CEon neurons (right, n = 50). For clarity, data were averaged and binned (bins contain equal number of data points). Linear correlations were performed using the non-binned raw data. P values indicate significance levels of Pearson's correlation coefficients. Dashed lines indicate 95% confidence intervals.
Supplementary Figure 11

Z-scored tonic activity does not correlate with freezing behavior. Z-scored tonic activity at test (24 hrs after fear conditioning) of CEm neurons (left), CEloff neurons (middle), and CElon neurons (right) is poorly correlated with behavioral freezing. Correlations were obtained by plotting the averaged z-scored tonic activity levels for all neurons of a given subtype for each animal over blocks of 2 CSs. For clarity, data were averaged and binned (bins contain equal number of data points). Linear correlations were performed using the non-binned raw data. P values indicate significance levels of Pearson’s correlation coefficients. Dashed lines indicate 95% confidence intervals.
Supplementary Figure 12

Changes in tonic activity during CS⁻ stimulation. Normalized population peri-stimulus (CS⁻) time histograms of CEm neurons (left), CEloff neurons (middle), and CElon neurons (right). Fear conditioning was associated with decreased tonic activity in CEm units ($n=15$) and increased tonic activity in CEloff units ($n=41$).
Supplementary Figure 13

Changes in tonic activity across behavioral sessions. Plots show averaged absolute firing frequencies of CEm, CEloff and CEIon units. Averages were obtained from behavioral trials (blocks of two CS+) in which changes in tonic activity were observed (e.g. trial blocks in which animals exhibited generalization for CEm recordings, n = 8 trial blocks; CEloff, n = 23 trial blocks; or discrimination CEIon, n = 21 trial blocks). Spontaneous activity levels before CS onset ("pre") were measured when animals were first placed in context B.
**Supplementary Figure 14**

**Isolation of unit recordings.** 
**a,** Left: Superimposed waveforms recorded from four different units. Right: Spikes originating from individual units were sorted using 3D-principal component analysis. 

**b,** Quantitative J3 and Davies Bouldin validity index (DB) statistics calculated for CEm, CEoff and CEon neurons. Controls values were obtained using two clusters defined from the centered cloud of points from channels in which no units could be detected. High values for the J3 and low values for the DB are indicative of good single unit isolation.
Supplementary Figure 15

**Time course of freezing behavior during CS stimulation.** a, Freezing probability starts increasing early on during CS⁺ presentation. Freezing probability in relation to CS⁺ presentation in fear conditioned mice. Pooled data from 20 mice with 12 CS⁺ presentations on the day following fear conditioning. Sampling period for freezing was 100 ms. The CS⁺ consisted of a series of 27 pips of 50 ms presented at 0.9 Hz. Vertical red lines indicate the occurrence of the individual pips composing the CS⁺. The whole CS⁺ is displayed. b, Freezing probability to the first few pips is displayed, showing that freezing probability increases around 1.3 s after the onset of the CS. c, Freezing increase following CS⁺ onset can be fitted with an exponential curve rising to a maximum. Baseline freezing was subtracted from total freezing to yield freezing increase linked to CS⁺ presentation. The zero time point was set at 1.3 s following CS⁺ onset, when the freezing starts increasing.
Supplementary Figure 16

Clustering analysis of behavioral discrimination. Discriminating (red) and generalizing (blue) animals were classified according to the maximum likelihood estimates yielded by the EM-GMM algorithm (see Methods). The underlying 2-dimensional Gaussian distributions provided by the model are depicted by the contour lines. Note that a slightly higher threshold (dashed line, slope = 1.4) than the one resulting from the EM analysis was used in order to have a more conservative estimate of the number of discriminating animals.