

Supplementary Table 1. Overlap of neuronal marker and PKC- δ expression in CEL.

	Marker/PKC- δ	PKC- δ /Marker
Gad65	87.4 \pm 4.7	50.3 \pm 12.6
CRH	1.2 \pm 1.0	16.9 \pm 15.2
Dyn	1.9 \pm 1.2	4.5 \pm 2.9
Enk	42.8 \pm 7.4	71.3 \pm 6.8
OxtR	65.3 \pm 9.7	81.1 \pm 1.9

Percent cells expressing marker or PKC- δ . Values are means \pm s.e.m ($n = 3-5$).

Supplementary Table 2. Membrane properties of the three types of CEI neurons.

	<i>n</i>	%	R_{input} (M Ω)	$V_{\text{m rest}}$ (mV)	Delay (ms)	AP1 Amp (mV)	Half width (ms)	Threshold (mV)	Rise time (ms)
Late-firing	21	55	391 \pm 45	-61 \pm 2	223 \pm 21	65.4 \pm 2.0	3.6 \pm 1.3	-35.0 \pm 1.2	0.8 \pm 0.1
Regular spiking	14	37	414 \pm 59	-56 \pm 2	60 \pm 8	65.4 \pm 4.1	2.5 \pm 0.3	-41.7 \pm 1.3	0.8 \pm 0.1
Low-threshold bursting	3	8	492 \pm 40	-51 \pm 1	25 \pm 10	67.7 \pm 11.7	1.5 \pm 0.1	-41.0 \pm 1.1	0.7 \pm 0.02
<i>P</i> value			0.77	0.20	<0.0001	0.99	0.49	0.0009	0.94

* Delay is measured from the start of current injection to the start of the first action potential. AP1 Amp, half width, threshold, and rise time are analysed from the first action potential evoked by current injection. *P*-value compares late-firing and regular spiking neurons, 2-tailed t-test. Values are means \pm s.e.m.

Supplementary Table 3. Electrophysiological type of PKC- δ^+ and PKC- δ^- cells in CEI.

	<i>n</i>	Type	<i>n</i>	Incidence
PKC-δ^+		Late-firing	12	86%
	14	Regular spiking	2	14%
		Low threshold	0	0%
PKC-δ^-		Late-firing	5	42%
	12	Regular spiking	6	50%
		Low threshold	1	8%

Retrospective assignment of electrophysiological types to PKC- δ^+ or PKC- δ^- neurons after neurobiotin filling and PKC- δ IFL (see Supplementary Fig. 1m, n).

Supplementary Table 4. Relation of PKC- δ expression and electrophysiological type.

	Late-firing	Other	Total
CEI total	21	17	38
PKC-δ^+	49	8	57

The electrophysiological subtype assignment of the PKC- δ^+ population (Supplementary Table 3) was confirmed by recording from prospectively identified PKC- δ^+ neurons using GluCl α -CFP native fluorescence as a marker. 49 out of 57 PKC- δ^+ cells were late-firing neurons, while 8 out of 57 PKC- δ^+ cells were regular spiking neurons. No low-threshold bursting neurons were represented in the PKC- δ^+ population. Fisher's exact test with significant interaction of PKC- δ expression and firing properties ($P = 0.0016$).

Supplementary Table 5. Tone test data, blocked by infection rate.

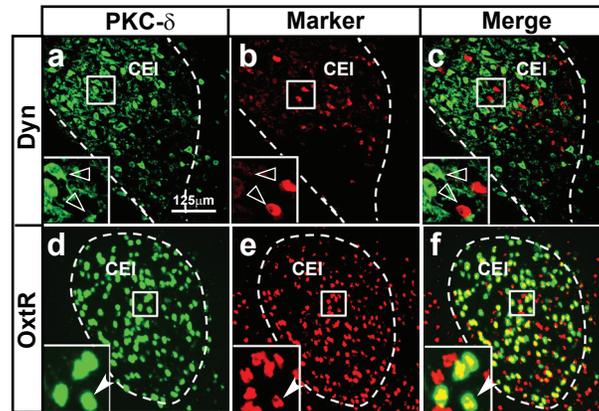
Block	Infection (%)		BL Freezing (%)		CS Freezing (%)		post-CS Freezing (%)	
	Control	Expt	Control	Expt	Control	Expt	Control	Expt
1	0.1±0.1	2.8±0.7	1.3±0.8	8.1±4.0 (n. s.)	70.0±7.4	61.1±11.6 (n. s.)	31.4±10.8	51.1±8.8 (n. s.)
2	2.9±0.6	6.9±0.4	5.0±1.8	4.4±0.8 (n. s.)	83.5±4.3	53.0±6.0 (n. s.)	36.8±7.1	19.2±6.3 (n. s.)
3	6.7±0.5	8.6±0.2	2.5±1.2	1.4±0.8 (n. s.)	21.9±3.9	47.7±10.5 (n. s.)	4.2±1.2	35.6±15.4 (n. s.)
4	10.4±0.7	10.2±0.2	2.3±0.8	5.8±3.1 (n. s.)	42.7±10.9	69.4±12.2 (n. s.)	21.4±6.8	45.3±11.1 (n. s.)
5	13.6±0.2	12.1±0.3	4.1±3.2	2.5±0.8 (n. s.)	44.9±13.9	53.5±12.0 (n. s.)	18.1±6.3	33.6±11.9 (n. s.)
6	17.2±0.5	14.9±0.6	5.2±3.1	2.8±1.0 (n. s.)	58.6±12.1	55.6±13.3 (n. s.)	33.4±12.0	24.4±9.8 (n. s.)
7	27.7±2.1	19.6±0.7	3.7±1.7	8.9±4.0 (n. s.)	67.2±8.0	66.5±11.7 (n. s.)	32.2±6.6	51.9±13.8 (n. s.)
8	35.6±0.9	24.6±1.2	1.7±0.7	1.9±0.8 (n. s.)	44.6±10.8	64.3±12.7 (n. s.)	30.6±8.1	36.5±14.6 (n. s.)
9	40.0±0.8	40.6±3.5	5.8±2.2	2.6±1.5 (n. s.)	49.4±10.4	91.1±4.7 ($P < 0.05$)	19.2±6.0	75.3±8.4 ($P < 0.001$)

Data are mean \pm s.e.m. A Randomized Block ANOVA¹ with 6 experimental and control subjects assigned to each of 9 blocks based on infection rate (total $n = 108$) revealed a significant block \times group interaction for CS ($F_{(8, 90)} = 2.298$, $P < 0.05$; with a significant linear component indicating that the difference between groups tended to increase with infection rate: $F_{(1, 90)} = 22.98$, $P < 0.0001$) and post-CS ($F_{(8, 90)} = 2.459$, $P < 0.05$; linear component: $F_{(1, 90)} = 8.85$, $P < 0.006$), but not BL periods ($F_{(8, 90)} = 1.41$, $P = 0.205$). P is from post-hoc Bonferroni t-test values. n.s., not significant. For graphic representation of the data see Supplementary Fig. 5. “Expt,” experimental group (PKC- δ ::GluCl α -ires-Cre transgenic mice injected with AAV::GluCl β virus and treated with IVM). The control group contains both wild-type animals injected with the GluCl β virus, and transgenic (PKC- δ ::GluCl α) animals injected with the GluCl α virus; these controls were not significantly different from each other (Supplementary Fig. 4) and were pooled for this analysis. All control animals in this comparison were treated with IVM. In some experiments, animals expressing GluCl $\alpha\beta$, but not treated with IVM were included (see also Supplementary Fig. 6 and 7). The number of mice in this additional control group was too small to include in the overall ANOVA shown above. However, among those mice in this group with an infection rate within the range of the animals in block 9 ($37.3 \pm 2.5\%$; $n = 3$), the freezing rates for the different conditions were similar to those in the control group of block 9 (BL = $3.7 \pm 1.0\%$; CS = $57.2 \pm 9.6\%$; post-CS = $18.3 \pm 2.2\%$). This -IVM group was also significantly different from the IVM-treated experimental group, when animals with a bilateral AAV infection rate above the median for the population were compared using non-parametric statistics (Supplementary Fig. 7).

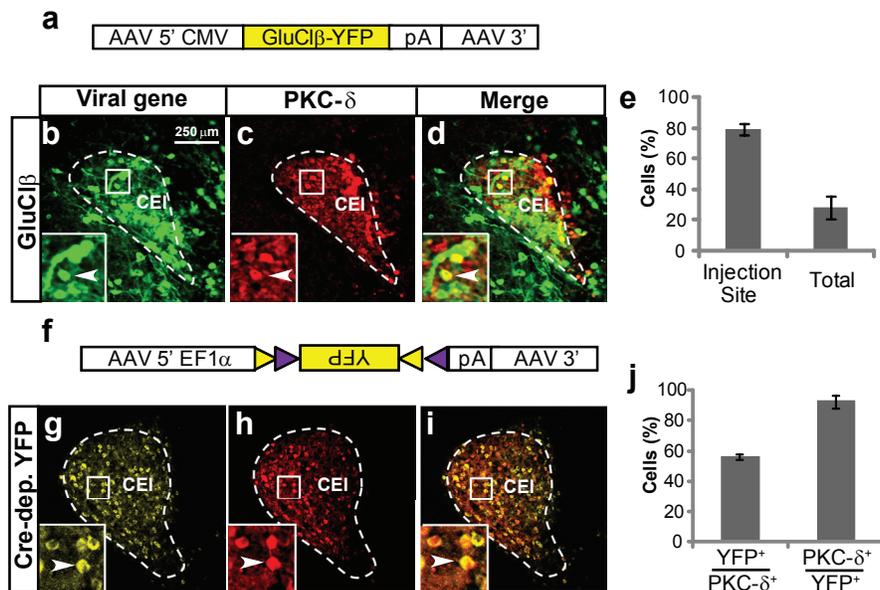
Supplementary Table 6. Injection coordinates.

	Bregma	Lateral	Ventral
CEI	-1.4	2.9	4.8
CEm	-1.0	2.4	4.6
PAG	-4.8	0.5	3.0

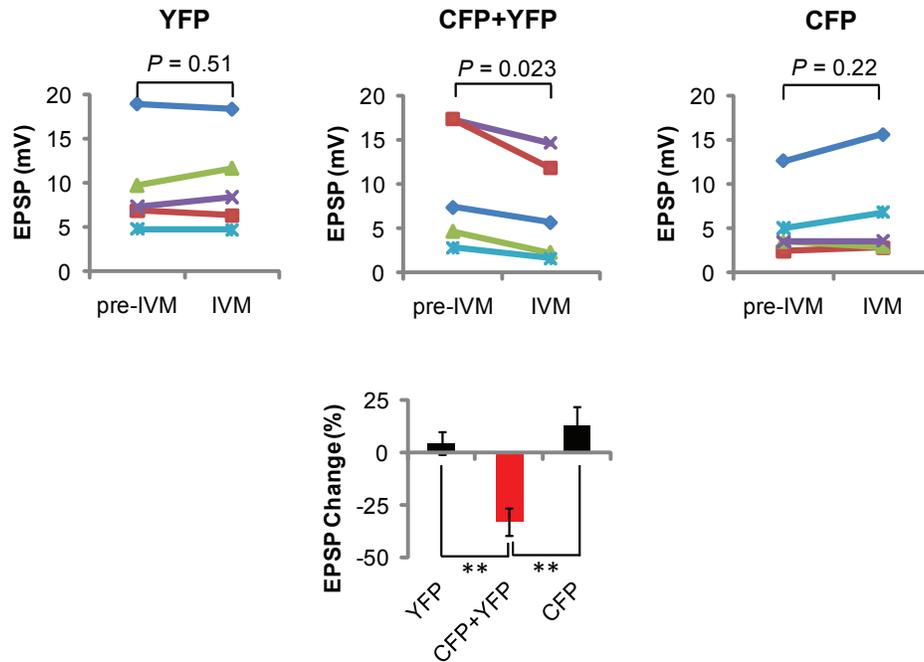
Values in mm.



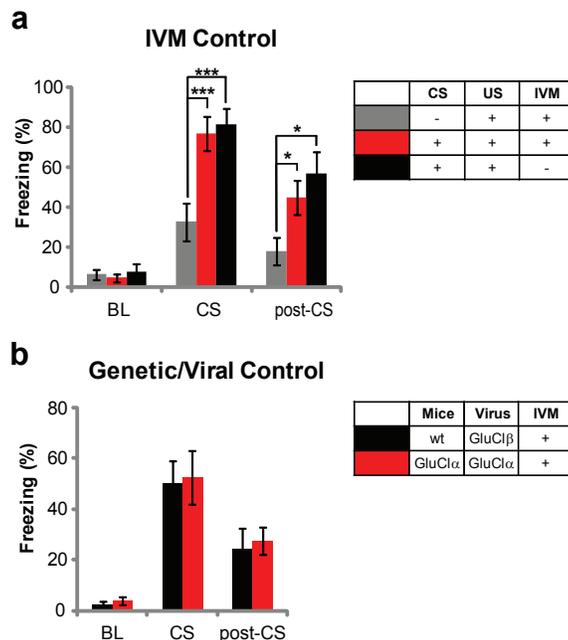
Supplementary Figure 1. Phenotypic characterization of CEI PKC- δ^+ neurons. dIFL (a-c) and dFISH (d-f) reveals that PKC- δ^+ neurons do not express dynorphin (a-c), but express oxytocin receptor (d-f). Filled and open arrowheads, doubly vs. singly labeled cells, respectively.



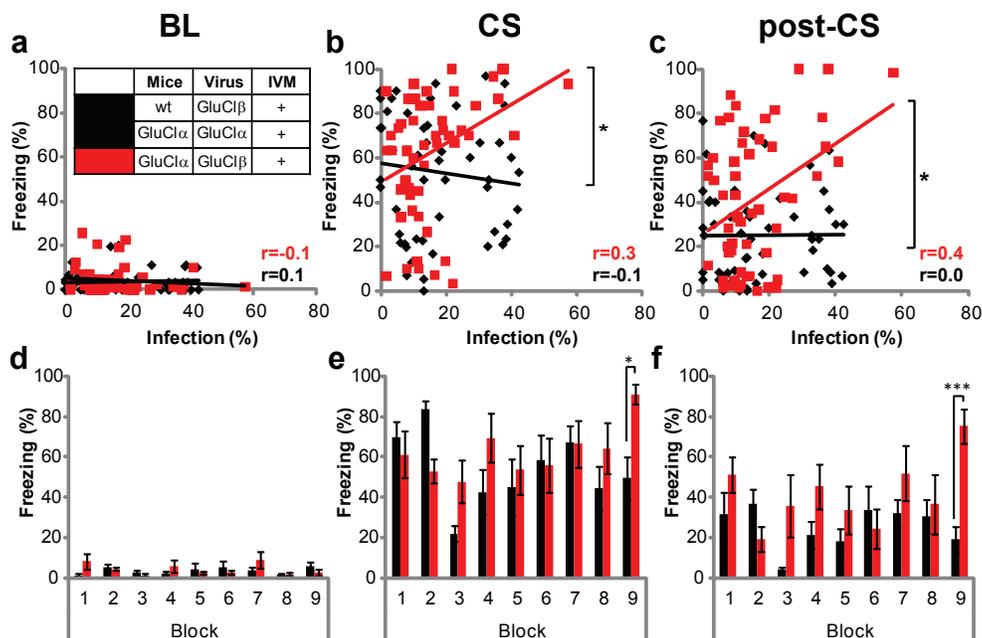
Supplementary Figure 2. Viral targeting and Cre-dependent expression in CEI PKC- δ ⁺ cells. **a-e**, Expression of AAV2::GluCl β -subunit (**a**) after stereotaxic intra-amygdala injections of virus in CEI. **b-d**, GluCl β -YFP and PKC- δ expression were detected by anti-GFP and anti-PKC- δ antibody staining. Wild-type mice were injected to permit immunofluorescent detection of GluCl β -YFP with anti-GFP antibodies, without cross-reaction of the antibody to the transgene-encoded GluCl α -CFP. **e**, Quantification of the fraction of GFP immunoreactive PKC- δ ⁺ cells in (**b-d**), (*left*) within 0.1 mm surrounding the injection site, and (*right*) within CEI as a whole ($n = 4$). **f-j**, Region and cell type specific expression of a Cre-dependent YFP reporter gene in CEI PKC- δ ⁺ neurons by intra-amygdala injection of Cre-dependent AAV (**f**) into PKC- δ ::GluCl α -ires-Cre mice. **g-i**, YFP native fluorescence (**g**) and anti-PKC- δ antibody staining (**h**). **j**, Quantification of data in (**g-i**). *Left*, Efficiency of YFP-delivery ($n = 6$) among CEI PKC- δ ⁺ cells. *Right*, Specificity of Cre-dependent YFP expression for PKC- δ ⁺ cells ($n = 6$). Values are means \pm s.e.m.



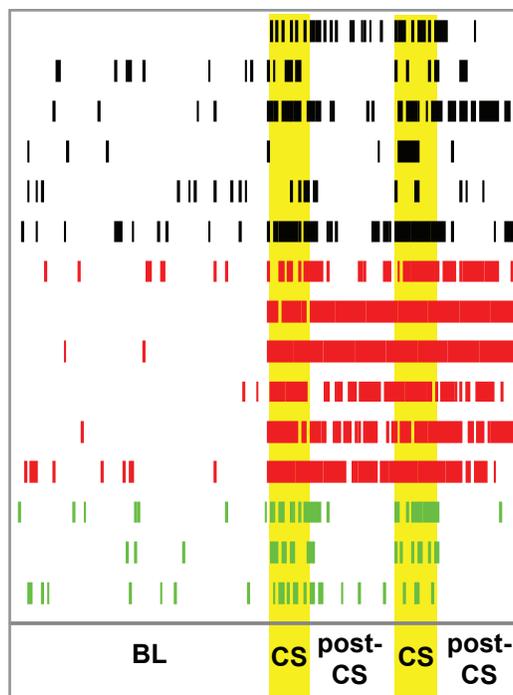
Supplementary Figure 3. EPSPs are significantly decreased in neurons that express both GluCl α -CFP and GluCl β -YFP after IVM treatment. EPSPs of individual cells (*top*) and average (mean \pm s.e.m.; *bottom*) in response to IVM ($n = 5$; One-way ANOVA ($F_{(2, 12)} = 11.36$, $P = 0.0017$) with post-hoc Bonferroni t-test (** $P < 0.01$).



Supplementary Figure 4. IVM, genetic and viral controls of pharmacogenetic silencing *in vivo*. **a**, IVM does not affect fear conditioning. Wild-type mice injected with IVM ($n = 10$) showed significant freezing during the CS exposure and post-CS when compared to US-only controls ($n = 9$), and were indistinguishable from vehicle injected animals ($n = 8$). One-way ANOVA during BL ($F_{(2, 24)} = 0.287, P = 0.753$), CS ($F_{(2, 24)} = 9.091, P = 0.001$) and post-CS ($F_{(2, 24)} = 4.901, P = 0.016$) periods with Fisher's LSD (** $P < 0.01$, * $P < 0.05$). **b**, Freezing in genetic and viral controls is comparable. "Genetic control" is AAV2::GluCl β -injected wild-type animals ($n = 7$; infection rate 37 ± 1.4 PKC- δ^+ cells); "viral control" is PKC- δ ::GluCl α -ires-Cre transgenic mice injected with AAV2::GluCl α rather than with GluCl β virus ($n = 8$; infection rate 36.3 ± 1.3 PKC- δ^+ cells). T-tests during BL ($P = 0.808$), CS ($P = 0.520$) and post-CS ($P = 0.955$) periods. Based on these results, data from these two types of control animals were pooled in subsequent experiments. BL, baseline freezing prior to first CS presentation; CS, conditioned stimulus. Values are means \pm s.e.m.

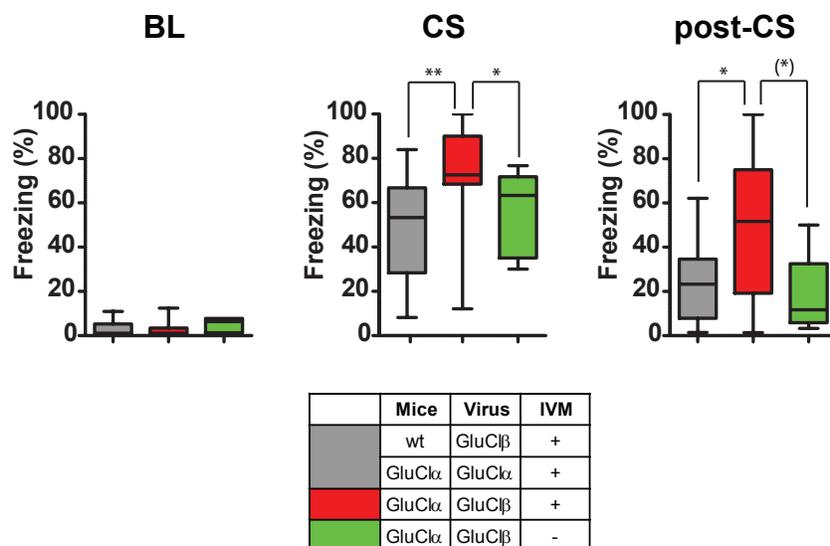


Supplementary Figure 5. Behavioral effect of silencing PKC- δ^+ neurons in CEI is positively correlated with infection. **a-c**, Scatter plots of the experimental data reveal a significant correlation of behavioral phenotype with frequency of infection of PKC- δ^+ neurons in experimental (red line; AAV2::GluCl β -injected PKC- δ ::GluCl α -ires-Cre transgenic mice) but not in genetic and viral control mice (black line; pooled, see Supplementary Fig. 4b). Infection rate was estimated for each animal by immunofluorescence labeling for PKC- δ and GluCl β -YFP fluorescence (see Methods and Supplementary Fig. 10). Statistics for pooled control groups: $r_{BL} = 0.071$, $P_{BL} = 0.608$, $R^2_{BL} = 0.005$, $r_{CS} = -0.11$, $P_{CS} = 0.412$, $R^2_{CS} = 0.013$, $r_{ITI} = 0.009$, $P_{ITI} = 0.949$, $R^2_{ITI} = 0.000$; $n = 54$. Statistics for experimental mice: $r_{BL} = -0.125$, $P_{BL} = 0.639$, $R^2_{BL} = 0.004$, $r_{CS} = 0.360$, $P_{CS} = 0.0075$, $R^2_{ITI} = 0.1294$, $r_{ITI} = 0.375$, $P_{ITI} = 0.005$, $R^2_{ITI} = 0.141$; $n = 54$; with significant differences in slopes (* $P < 0.05$). No significant differences between experimental and control groups were observed during training (not shown). **d-f**, Graphic representation of the blocked ANOVA of the experimental data described in Supplementary Table 5. Values are means \pm s.e.m.

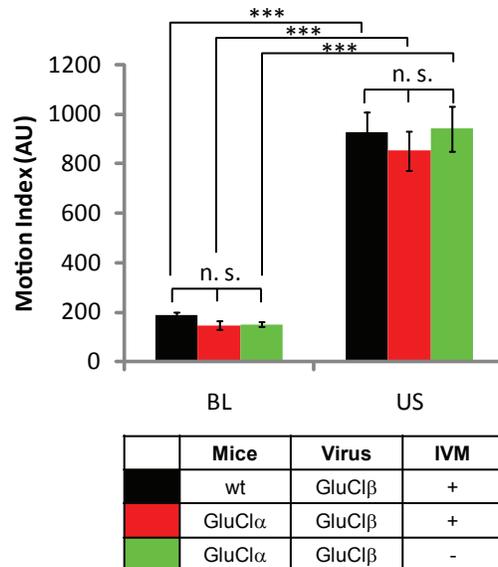


	Mice	Virus	IVM
	wt	GluCl β	+
	GluCl β	GluCl α	+
	GluCl α	GluCl β	+
	GluCl α	GluCl β	-

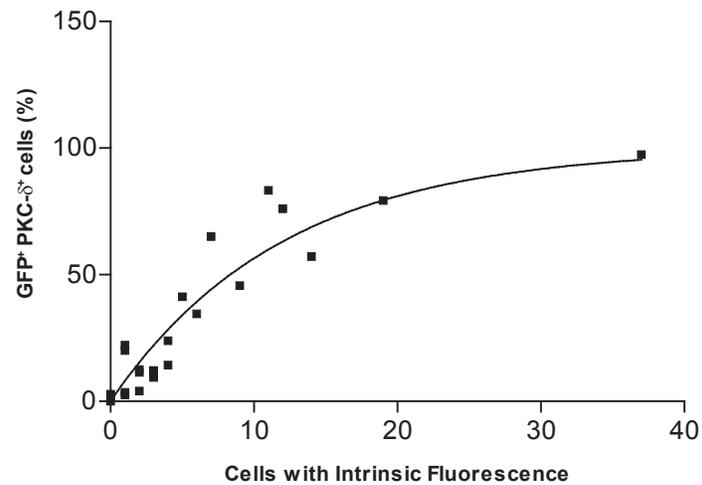
Supplementary Figure 6. Freezing episodes plotted in 2 sec bins during the tone test session of IVM-treated single-subunit (GluCl α - or GluCl β -expressing) control ($n = 6$, black) and IVM-treated experimental (GluCl $\alpha\beta$ -expressing) mice ($n = 6$, red) derived from block 9 in Table I, Supplementary Table 5, Supplementary Fig. 5d-f. The data for GluCl $\alpha\beta$ -expressing control mice not treated with IVM ($n = 3$, green) were taken from animals with equal infection rates to those in block 9 ($35.5 \pm 2.5\%$). The comparison is for illustrative purposes only. See Supplementary Fig. 7 for a statistical analysis of differences between these 3 groups. Note the persistence of freezing into the post-CS period in experimental animals (red rasters).



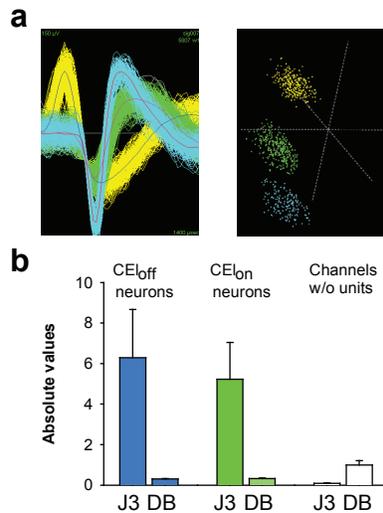
Supplementary Figure 7. Behavioral effect of silencing PKC- δ^+ neurons in animals with bilateral infection. The data represent animals exhibiting AAV infection in CEI in both hemispheres, at a level above the median infection rate of the population, and are pooled from multiple experiments (Supplementary Fig. 5). Box plots show medians and interquartile ranges for the 3 groups described in the table. The single-subunit IVM-treated control mice (wild-type injected with GluCl β virus, or transgenic (“GluCl α ”) mice injected with GluCl α virus) were not significantly different from each other (Supplementary Fig. 4b) and were pooled for this analysis ($n = 21$; gray bar). This control group, the experimental group (GluCl $\alpha\beta$ +IVM, $n = 17$; red bars), and an additional control group expressing GluCl $\alpha\beta$ but not treated with IVM ($n = 6$; green bars), were analyzed using non-parametric statistics, because the data were not normally distributed and the sample sizes were not equal. BL (baseline) period: one-way Kruskal-Wallis ANOVA ($H = 1.94$, $P = 0.38$). CS period: one-way Kruskal-Wallis ANOVA ($H = 11.14$, $P = 0.0038$); * $P < 0.05$, ** $P < 0.01$; one-tailed Mann-Whitney post-hoc test corrected for multiple comparisons. post-CS period: one-way Kruskal-Wallis ANOVA ($H = 6.73$, $P = 0.0346$); * $P < 0.05$, one-tailed Mann-Whitney post-hoc test with Bonferroni correction for multiple comparisons. (*) indicates significant to $P < 0.03$ without correction for multiple comparisons, but not significant ($P < 0.06$) when corrected. No significant difference between the experimental (red) and control (gray) groups was observed, in either the CS or post-CS periods, for those animals whose level of bilateral AAV infection of CEI was below the median of the population. In addition, a permutation test comprising 10,000 simulations was performed using cohorts of animals selected at random from the experimental and control groups, of the same size as those generated using the criterion of bilateral CEI infection rate above the median. This permutation test indicated that the probability of observing differences in the median freezing level equal to or greater than that observed between the cohort selected based on bilateral CEI infection rate, with an equal or lower P -value, was low ($P = 0.005$ and $P = 0.08$ for the CS and post-CS periods, respectively). This analysis supports the validity of using bilateral CEI infection rate above the median as a criterion for inclusion in the comparison groups.



Supplementary Figure 8. Suppression of CEI PKC δ^+ neuronal activity does not change locomotor activity or US sensitivity. Control (black, $n = 9$, average infection rate $53.9 \pm 9.0\%$) and experimental mice 12 h after a single dose of 10 mg/kg IVM (red, $n = 13$, average infection rate $49.9 \pm 6.9\%$), or injected with vehicle (green, $n = 13$, average infection rate $52.0 \pm 6.8\%$), show similar levels of baseline locomotor activity during 20 min in the training context prior presentation of the first CS (*left*) and during exposure to the first US (right; One-way ANOVA ($F_{(2,32)} = 0.46$, $P = 0.64$)), which induced a significant comparable burst in locomotor activity in all groups (Two-way ANOVA ($F_{(1,32)} = 283.85$, $P < 0.0001$) with post-hoc Bonferroni t-test ($*** P < 0.001$). The absence of any substantial differences in US reactivity makes it unlikely that the observed effect on freezing (Table 1, Supplementary Fig. 5 and 7) is merely consequence of increased US sensitivity². Values are mean \pm s.e.m.



Supplementary Figure 10. Relationship between YFP intrinsic fluorescence and GFP-immunoreactivity in CE1 PKC- δ^+ neurons following infection with AAV::*GluCl β -YFP*. The data illustrate the standard curve used to estimate the fraction of PKC- δ^+ cells immunoreactive for GFP per CE1 section based on the number of CFP/YFP $^+$ cells detected by intrinsic fluorescence. The curve was generated using data from *GluCl α* or *GluCl β* virus injections into wt animals and each data point was collected from the same section. See Methods for further details.



Supplementary Figure 11. Isolation of single unit recordings. **a**, *Top left*, Superimposed waveforms recorded from three different units. *Top 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 CEloff 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 Note 1

Several technical factors likely contribute to the variability in behavioral experiments in which PKC- δ^+ neurons are silenced using the combination of PKC- $\delta::$ GluCl α transgene + AAV-driven expression of GluCl β . In addition to the variability inherent in behavioral experiments, the two most important technical variables appear to be the accuracy of the injections, and the quality/titer of the virus preparation. As described in the main text, statistically significant differences between experimental (AAV2::GluCl β \rightarrow PKC- $\delta::$ GluCl α transgenic/IVM) and single subunit-expressing control (AAV2::GluCl α \rightarrow PKC- $\delta::$ GluCl α transgenic/IVM, or AAV2::GluCl β \rightarrow wild-type/IVM) groups were seen only among animals that had a high level and/or efficient bilateral expression of the virus in CEI (Table I, Supplementary Fig. 5 and 7 and Supplementary Table 5). We have also experienced variability according to the particular preparation of AAV used. Initial experiments were performed using a very high-titer ($\sim 10^{13}$ /ml) preparation of AAV2[CMV::GluCl β] made commercially by Virapur, Inc. This virus preparation, which yielded the strongest effects in our experiments, was also successfully used in conjunction with an AAV2[CMV::GluCl α] virus, prepared in parallel, to silence neurons in the striatum in an IVM-dependent manner³, and also yielded strong IVM-dependent electrophysiological silencing of spontaneous spiking in PKC- δ^+ neurons in thalamic slices, when injected into PKC- $\delta::$ GluCl α -ires-Cre transgenic mice (C. Xiao, W.H., W. Lerchner, H. Lester and D.J.A., unpublished data). Subsequent commercial preparations of this virus were less effective. Preparations of AAV GluCl β virus made using serotypes AAV5 or AAV8 did not measurably improve results. In general, it has been more difficult to generate high-titer AAV for the CMV::GluCl β construct, than for many other viral constructs we have produced. Based on our experience, the following technical variables are likely important in achieving significantly increased freezing upon silencing PKC- δ^+ neurons *in vivo*:

1. Strong (above median levels), bilateral expression of AAV::GluCl β in CEI
2. Balanced expression of GluCl α and GluCl β
3. Minimal spread of AAV::GluCl β virus into non-PKC- $\delta::$ GluCl α -expressing regions of the amygdala.

In addition to these technical sources of variation, biological factors may contribute to variability in the results as well. For example, “ceiling effects” may limit the ability to detect significantly increased freezing caused by silencing PKC- δ^+ neurons during the period of CS-exposure, especially in well-trained animals. This is suggested by our observation that the magnitude of the difference between experimental and control groups was greater during the post-CS period, when freezing levels in control animals are returning towards baseline, than during CS-exposure (Supplementary Fig. 6). This may reflect the possibility that activation of PKC- δ^+ neurons plays a particularly important role in extinguishing freezing during the CS-off period, so that silencing these neurons during this interval has a relatively greater effect on freezing. Finally, given the reciprocal inhibition between PKC- δ^+ /CEI_{off} and PKC- δ^- /CEI_{on} units, and the potentially symmetrical inhibition of CEm output neurons by these competing CEI units (but see Supplementary Note 3), it is possible that silencing PKC- δ^+ neurons can, under some conditions, cause opposing influences on CEm activity that cancel each other out. Nevertheless, our *in vivo* recordings indicate that silencing PKC- δ^+ neurons caused only a slight and statistically non-significant increase in CEI_{on} tonic activity, while CEm tonic activity was

significantly increased. However the impact of silencing PKC- δ^+ neurons on CEI_{on} and CEM unit activity during CS exposure remains to be examined.

Supplementary Note 2

There are several differences between the effects of muscimol injections into CEI (Ciocchi et al., submitted), and the effects of IVM/GluCl-mediated silencing of PKC- δ^+ neurons reported here. Injection of muscimol into CEI in untrained animals caused an increase in baseline (unconditional) freezing (Ciocchi et al.), while specific silencing of PKC- δ^+ neurons did not cause any such increase. Furthermore, injection of muscimol into CEI 10 minutes before training strongly attenuated fear conditioning, while silencing of PKC- δ^+ neurons throughout both the training and testing period caused an increase in CS-evoked freezing.

The difference in the effects of the two types of experimental manipulations on unconditional freezing may be explained by differences in their timing, cellular specificity and/or efficiency. Muscimol iontophoresis is performed acutely (10 minutes prior to testing); in contrast PKC- δ :GluCl-expressing animals were tested 24 hrs after IVM administration. Since IVM at the concentration used reaches functional levels in the brain in \sim 12 hrs after administration³, PKC- δ^+ neurons in our experiments have been tonically suppressed in animals examined for baseline freezing; by contrast, muscimol is injected 30 min to 1 hr before testing, and produces an acute inhibition of CEI. Adaptation to tonic inhibition by IVM may explain the lack of an effect on unconditional freezing. In addition, and most importantly, muscimol injection targets all neurons within CEI, while IVM specifically inhibits only the PKC- δ^+ subpopulation. Simultaneous inhibition of multiple CEI populations by muscimol may produce different behavioral effects than silencing of just the PKC- δ^+ population. Finally, the efficiency of muscimol silencing is limited only by diffusion and the amount injected, since all neurons that express endogenous GABA receptors should be equally sensitive to the drug. By contrast, the efficiency of IVM silencing of PKC- δ^+ neurons is limited by the fraction of those cells that express adequate levels of GluCl α + GluCl β , which in turn depends on the efficiency of infection by AAV::GluCl β . Our data indicate that between 30-80% of PKC- δ^+ neurons are typically infected by an AAV injection in CEI, depending on their distance from the injection site (Supplementary Fig. 2e). Incomplete suppression of the PKC- δ^+ population by IVM may explain why silencing this population after training increased the tonic activity of some individual CEM units, as detected electrophysiologically (Fig. 5m-n), but did not produce a measurable increase in baseline freezing. In summary, the difference between the effects of muscimol vs. selective silencing of PKC- δ^+ neurons on baseline freezing may reflect differences in the relative cellular specificity (low vs. high), relative strength/efficiency (high vs. low), or time course (acute vs. chronic) of the two types of experimental manipulations.

Similar factors may explain why muscimol injection into CEI just prior to training inhibited fear conditioning, while silencing of PKC- δ^+ neurons did not (but rather augmented CS-evoked freezing after training). The *in vivo* recordings of Ciocchi et al. suggest that after training, PKC- δ^- neurons are three times more likely to inhibit PKC- δ^+ neurons, than vice-versa (see also Supplementary Note 3). If, during training, CEI_{on} \rightarrow CEI_{off} inhibitory connections become relatively stronger than CEI_{off} \rightarrow CEI_{on} connections, then experimentally silencing PKC- δ^+ /CEI_{off} units would, if anything, only enhance the effect of training to strengthen the influence of CEI_{on}

units. By contrast, since muscimol inhibits both PKC- δ^- and the PKC- δ^+ populations, it would impair the development of dominance by the CEI_{on} population during training. In this way, the difference in the cellular specificity of the two manipulations could yield dramatically different behavioral effects. It is also possible that, due to its relative inefficiency (see above) partial silencing of the PKC- δ^+ population during training does not produce a sufficient overall reduction in inhibitory output from the CEI circuit to impair training, while muscimol silencing has a more highly penetrant effect.

Supplementary Note 3

If PKC- δ^+ neurons inhibit PKC- δ^- neurons, and if both populations inhibit CEm output neurons (see also Ciochi et al.), then why doesn't silencing PKC- δ^+ neurons *decrease* freezing (rather than increase it), via dis-inhibition of PKC- δ^- /CEI_{on} neurons (thereby enhancing feed-forward inhibition by the latter onto CEm)? By the same token, why doesn't activation of PKC- δ^- /CEI_{on} neurons by the CS reduce rather than enhance freezing? There are several possible explanations for this apparent paradox.

First, PKC- δ^+ and PKC- δ^- neurons may inhibit a common population of CEm output neurons, but with different intrinsic strengths. If the PKC- δ^+ /CEI_{off} \rightarrow CEm inhibitory connection is stronger than the PKC- δ^- /CEI_{on} \rightarrow CEm connection, then the net effect of the CS on CEm activity may be dominated by the change in the stronger inhibitory connection, and therefore a net increase in CEm activity would obtain. In the absence of molecular tools to specifically manipulate the PKC- δ^- /CEI_{on} population, we cannot directly compare the relative strength of the PKC- δ^+ \rightarrow CEm and PKC- δ^- \rightarrow CEm inhibitory connections. Nevertheless, even if the two connections are of similar strength, the fact that, after conditioning, the tonic activity of PKC- δ^+ /CEI_{off} neurons is greater than the tonic activity of PKC- δ^- /CEI_{on} neurons (Ciochi et al., Fig. 2) suggests that reducing the strong tonic inhibition by PKC- δ^+ neurons should have a greater influence on net CEm activity, than would enhancing the weak tonic inhibition by PKC- δ^- neurons. Consistent with this idea, Ciochi et al. observe that 85% of CEm units are excited by the CS *in vivo*, and we observe that silencing of PKC- δ^+ neurons leads to a net increase in CEm activity (Fig. 5m-n).

Second, it is not yet certain whether PKC- δ^+ and PKC- δ^- CEI neurons synapse onto the same or different populations of CEm neurons. While we have shown that PKC- δ^+ /CEI_{off} neurons directly inhibit CEm neurons that project to the PAG, where freezing is controlled, there is no such direct evidence for the PKC- δ^- /CEI_{on} population. There is evidence for connectional heterogeneity among CEm output neurons⁴. If PKC- δ^- /CEI_{on} neurons do not directly inhibit CEm output neurons mediating freezing, then enhancing inhibition of their CEm target neurons would not interfere with freezing promoted by dis-inhibition of PAG-projecting CEm neurons controlled by the PKC- δ^+ population.

Third, because CEI_{on} (PKC- δ^-) neurons reciprocally inhibit PKC- δ^+ neurons, enhancing the activity of the former (whether via the CS, or via silencing of PKC- δ^+ neurons), will tend to further depress the activity of the latter population, leading to enhanced dis-inhibition of CEm.

Importantly, the CEI circuit appears inherently “biased” so that the response of the circuit to the CS is dominated by the activation of CEI_{on} neurons, as suggested by two observations:

1) As shown by Ciocchi et al., CEI_{on} → CEI_{off} inhibitory interactions are 3x more frequent than CEI_{off} → CEI_{on} (Ciocchi et al.) connections;

2) PKC- δ^- (CEI_{on}) neurons are rapid-onset neurons, while PKC- δ^+ are delayed-onset firing neurons (this study); therefore activation of PKC- δ^- neurons by the CS will occur prior to any activation of PKC- δ^+ neurons.

Thus, there is both a temporal and a connectional asymmetry in the response of the CEI inhibitory circuit to the CS, with CEI_{on} neurons both responding faster to the CS than CEI_{off} neurons, and inhibiting CEI_{off} neurons more strongly, than vice-versa. Given this asymmetry, and the fact that lateral interactions between CEI_{off} neurons and CEI_{on} neurons may be stronger than their feed-forward inhibition of CEM neurons, increasing the activity of CEI_{on} neurons may have a proportionately greater effect to depress the activity of CEI_{off}/PKC- δ^+ neurons, and thereby dis-inhibit CEM, than to further inhibit CEM directly.

In summary, given that activation of CEI_{on} neurons by the CS is associated with increased freezing, and given that the activity of CEI_{off} (PKC- δ^+) neurons opposes the activity of CEI_{on} neurons, then experimental manipulations that reduce the activity of PKC- δ^+ neurons should enhance the response of CEI_{on} neurons to the CS, and thereby enhance freezing.

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