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Control of the establishment of aversive memory by calcineurin and Zif268

Karsten Baumgärtel[1], David Genoux[1,3], Hans Welzl[2], Ry Y Tweedie-Cullen[1], Kyoko Koshibu[1,3], Magdalena Livingstone-Zatchej[1], Céline Mamie[1] & Isabelle M Mansuy[1]

Emotional memory is a rapidly acquired and persistent form of memory, and its robustness is in part determined by the initial strength of the memory. Here, we provide new evidence that the protein phosphatase calcineurin (CaN), a potent negative regulator of neuronal signaling that is known to constrain learning and memory, critically regulates the establishment of emotional memory through mechanisms involving the immediate early gene *Zif268* (also known as *Egr1*). We found that CaN is inhibited in the amygdala during the establishment of aversive memory, but *Zif268* is activated. Using inducible transgenesis in mice, we further saw that CaN inhibition and *Zif268* overexpression during memory establishment strengthen the memory trace and enhance its resistance to extinction. We found that CaN inhibition correlates with increased *Zif268* expression and that a common pool of proteins is regulated in the amygdala after CaN inhibition and *Zif268* overexpression. Together, these findings reveal a previously unknown mechanism for the control of emotional memory that depends on CaN and Zif268.

Our past experiences and our memories of them are major factors that shape our behavior. Among the multiple memory traces of past experiences, the strongest dominate and are recalled first; thus, they determine behavior. Such dominance is particularly notable for negatively charged memory traces that are formed during and after traumatic events. Thus, the recall of strongly aversive or fear memory often leads to inappropriate behaviors, such as inapt avoidance responses or excessive fear[1]. Although the mechanisms that control the dominance of emotional traces are not known, it is recognized that the degree of dominance of these traces is determined by their initial strength[2,3]. Emotional memory is unique in this respect, as it is extremely strong immediately after acquisition, even though it is usually acquired in a single episode. It may therefore recruit specific mechanisms during acquisition that establish a robust trace of the learned information. Identifying the molecules involved in these mechanisms is necessary to better understand emotional memory and its pathologies.

To fulfill these functions, the molecules that control the establishment and the strength of emotional memory need to be rapidly activated and to efficiently recruit signaling pathways required for long-term memory. Another important prerequisite is that molecular constraints that weaken neuronal transmission and downregulate signaling pathways and nuclear transcriptional events need to be relieved[4,5]. CaN and protein phosphatase 1 (PP1) are such molecular constraints that can strongly suppress learning and memory[6–8]. In particular, CaN has several properties that make it a good candidate for controlling the establishment of strong forms of memory: it is rapidly activated on Ca^{2+} influx[9], it is strategically positioned near ligand-gated[10,11] and voltage-sensitive ion channels[12,13] that are critical for signaling initiation, it controls multiple signaling molecules at the membrane[12,13], in the cytoplasm[14] and in the nucleus[15], and it has a dominant suppressor function over protein kinases[12]. Moreover, CaN is an important regulator of transcription factors[16] and nuclear events involved in the control of transcriptional processes, which are both regulated by phosphorylation-dependent processes[17].

In this study, we show that CaN controls the establishment and the persistence of emotional memory and recruits mechanisms that involve the transcription factor Zif268 (Egr-1, NGFI-A). We found that the proper establishment of aversive memory is associated with the inhibition of endogenous CaN and increased *Zif268* expression in the amygdala. Using inducible transgenesis in mice, we show that decreasing or increasing CaN activity during the establishment of memory is sufficient to strengthen or weaken memory, respectively, and that this determines the course of memory extinction without affecting the mechanisms of extinction *per se*. Furthermore, we demonstrate that CaN inhibition is associated with increased *Zif268* expression in the amygdala and that mimicking *Zif268* overexpression in transgenic mice also strengthens memory. Consistent with a link between CaN and Zif268, we found a common pool of regulated proteins in the amygdala after CaN inhibition and *Zif268* overexpression. Together, these results provide new evidence for the existence of CaN-dependent mechanisms engaging Zif268 that act specifically during memory establishment to determine memory strength.

[1]Brain Research Institute, Medical Faculty of the University of Zürich and Department of Biology, Swiss Federal Institute of Technology, Winterthurerstrasse 190, 8057 Zürich, Switzerland. [2]Division of Neuroanatomy and Behavior, Institute of Anatomy, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland. [3]Present addresses: Department of Physiology, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Road, Auckland, New Zealand (D.G.) and The Queensland Brain Institute, University of Queensland, St. Lucia, Brisbane 4072, Australia (K.K.). Correspondence should be addressed to I.M.M. (mansuy@hifo.uzh.ch).

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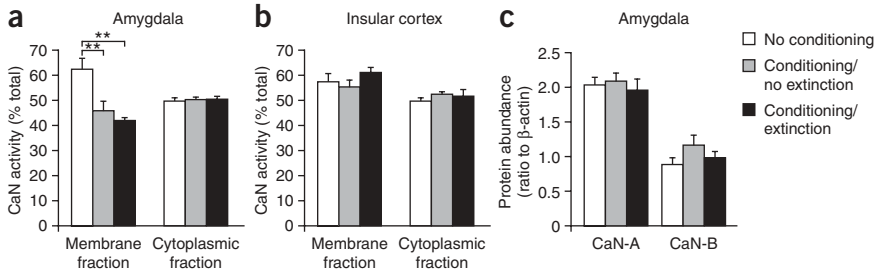

Figure 1 CaN activity is decreased in the amygdala by conditioning in the CTA test. (**a**) Relative CaN activity (percent of total Ser/Thr protein phosphatase activity) in membrane and cytoplasmic fractions of the amygdala 3 d after conditioning followed by extinction (conditioning/extinction, $n = 8$, black bars) or not (conditioning/no extinction, $n = 8$, gray bars). CaN activity was significantly reduced only in the membrane fraction ($F_{2,21} = 10.59$, $P = 0.0007$) in conditioned animals whether they were subjected to extinction training (42 ± 1%, Fischer's PLSD, $P = 0.0003$) or not (46 ± 4%, Fischer's PLSD, $P = 0.0021$; no conditioning, 62 ± 4%, $n = 8$, white bars). (**b**) Data for insular cortex, presented as in **a**. CaN activity was not altered by conditioning or extinction training in membrane (conditioning/extinction, 61 ± 2%; conditioning/no extinction, 55 ± 3%; no conditioning, 57 ± 3%; $F_{2,21} = 1.09$, $P = 0.3533$) or cytoplasmic fractions (conditioning/extinction, 51 ± 3%; conditioning/no extinction, 52 ± 1%; no conditioning, 50 ± 1%; $F_{2,21} = 0.50$, $P = 0.6131$). (**c**) Quantification of CaN-A and CaN-B subunits in the membrane fraction in amygdala. Total CaN-A (conditioning/extinction, 2.0 ± 0.2%; conditioning/no extinction, 2.1 ± 0.1%; no conditioning, 2.0 ± 0.1%; $F_{2,21} = 0.25$, $P = 0.7814$) or CaN-B (conditioning/extinction, 0.9 ± 0.1%; conditioning/no extinction, 1.4 ± 0.3%; no conditioning, 1.2 ± 0.2%; $F_{2,20} = 1.44$, $P = 0.2609$) protein was not changed by conditioning with or without extinction training.

RESULTS

CaN is inhibited and *Zif268* activated in CTA establishment

To investigate the mechanisms that determine the strength and persistence of emotional memory, we used conditioned taste aversion (CTA) in mice. CTA is a form of aversive memory acquired in one trial by association of a novel and appetitive taste (conditioned stimulus, saccharin solution) with visceral malaise (unconditioned stimulus, intraperitoneal injection of LiCl). After acquisition, the association between the conditioned and unconditioned stimuli induces a strong avoidance to saccharin, which persists for several months (**Supplementary Fig. 1** online) or years in mammals[18,19]. The aversive memory trace can, however, be extinguished by the relearning of a new nonaversive trace through repeated exposure to saccharin that is no longer paired with malaise (conditioned stimulus without the unconditioned stimulus)[18,19]. The nonaversive trace is more difficult to acquire because it is weaker by nature, and it is strongly dominated by the aversive trace[3]. It can, nonetheless, be progressively reinforced during extinction training and gain sufficient strength to dominate the aversive trace. The respective degree of dominance of each memory trace during testing determines the animal's behavior toward saccharin and ranges from strong aversion to marked preference[3].

To examine the involvement of CaN in the establishment of aversive memory, we subjected C57BL/6J wild-type mice to conditioning and then extinction training in the CTA task and measured CaN activity and protein levels in the amygdala and insular cortex, two brain areas that are involved in CTA[20,21]. CaN activity was significantly reduced by conditioning selectively in the amygdala (–26%; **Fig. 1a**) but not in the insular cortex (**Fig. 1b**). The decrease in CaN activity was specific to the membrane fraction, where CaN is most abundant through binding to anchoring proteins[22], and was not seen in the cytoplasmic fraction (**Fig. 1a**). CaN activity was not further diminished by extinction training (**Fig. 1a**). The decrease in CaN activity did not result from reduced CaN expression, as CaN catalytic A (CaN-A, α and β isoforms) or regulatory B (CaN-B) subunits were similarly abundant with or without conditioning or extinction training (**Fig. 1c**). The effect of conditioning was specific to CaN and did not involve PP1 (**Supplementary Fig. 2** online), another Ser/Thr protein phosphatase that is partly regulated by CaN (for a review, see refs. 8,23). PP1 activity was not regulated in the amygdala or in the insular cortex (**Supplementary Fig. 2**).

We next examined whether transcriptional changes occur during CTA establishment and looked at the expression of the transcription factors Zif268 and c-Fos in the amygdala and the insular cortex. *Zif268* and *Fos* are immediate early genes (IEGs) that are rapidly activated by neuronal activity and are known to be involved in associative memory[24–26]. *Zif268* expression was selectively increased by conditioning in the amygdala (+50%; **Fig. 2a**), but not in insular cortex (**Fig. 2b**). This increase was specific to *Zif268* and not seen for *Fos* (**Fig. 2a**,**b**). This data therefore indicates that CaN activity is inhibited and *Zif268* mRNA is increased in the amygdala by the establishment of aversive memory.

CaN activity determines memory strength and persistence

To confirm the functional significance of CaN inhibition in aversive memory, we experimentally decreased or increased CaN activity inducibly and selectively in forebrain neurons by expression of an autoinhibitory domain of CaN[27] or an active form of CaN-Aα[28] in transgenic mice using the reverse tetracycline-controlled transactivator (rtTA or rtTA2)[28,29]. CaN inhibition (–35–45%)[27] or overactivation (+50–75%)[28] in adult forebrain neurons did not alter the animals'

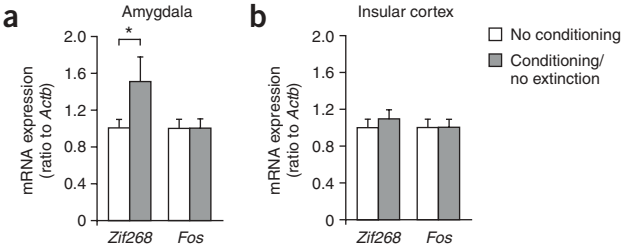

Figure 2 *Zif268* mRNA expression is increased by CTA in the amygdala. (**a**) *Zif268* expression in the amygdala 3 d after conditioning. *Zif268* mRNA expression was significantly increased ($F_{1,14} = 5.66$, $P = 0.0321$) after conditioning (conditioning/no extinction, 1.5 ± 0.3, $n = 6$, gray bars; no conditioning, 1.0 ± 0.1, $n = 10$, white bars), whereas *Fos* mRNA expression was not changed (conditioning/no extinction, 1.0 ± 0.1, $n = 6$; no conditioning, 1.0 ± 0.1, $n = 9$; $F_{1,13} = 0.02$, $P = 0.8984$). (**b**) Data for insular cortex, presented as in **a**. *Zif268* (conditioning/no extinction, 1.1 ± 0.1, $n = 6$; no conditioning, 1.0 ± 0.1, $n = 10$; $F_{1,14} = 1.57$, $P = 0.2313$) and *Fos* (conditioning/no extinction, 1.0 ± 0.1, $n = 6$; no conditioning, 1.0 ± 0.1, $n = 10$; $F_{1,14} = 0.21$, $P = 0.6543$) mRNA expression was not altered after CTA conditioning.

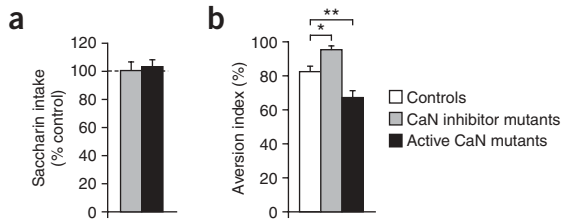

Figure 3 Aversive memory is enhanced by CaN inhibition and weakened by CaN overactivation. (**a**) Natural preference for saccharin was not altered ($F_{1,29} = 0.14$, $P = 0.7140$) by CaN inhibition or overactivation, as shown by a similar ratio of saccharin intake relative to controls in mutants expressing a CaN inhibitor (100 ± 6%, $n = 15$, gray bars; controls, $n = 14$) or an active CaN (103 ± 5%, $n = 16$, black bars; controls, $n = 21$) averaged over several daily presentations of water and saccharin. (**b**) Aversion to saccharin (aversion index defined as volume of water intake over total liquid intake) was significantly ($F_{2,53} = 11.20$, $P < 0.0001$) higher in mutants expressing a CaN inhibitor (93 ± 3%, $n = 6$; Fisher's PLSD, $P = 0.0440$) and lower in mutants expressing an active CaN (68 ± 4%, $n = 18$; Fisher's PLSD, $P = 0.0008$) than in controls (82 ± 2%, pooled, $n = 31$) 2 d after conditioning.

natural preference for saccharin (**Fig. 3a**). When tested for their memory of the conditioned and unconditioned stimuli association, mutants and controls from both transgenic lines strongly avoided saccharin; in the mutants, however, avoidance was increased or decreased depending on whether CaN was inhibited or overactivated, respectively (**Fig. 3b**). Thus, aversion to saccharin was significantly increased by CaN inhibition, whereas it was reduced by CaN overactivation, indicating that the level of CaN activity at the time of aversive memory establishment modulates the strength of memory.

We next examined whether the resistance of aversive memory to extinction is also altered by a change in CaN activity. For this, we repeatedly exposed conditioned mice to saccharin and water in daily trials to induce extinction, and their aversion to saccharin was measured across time. Although extinction training resulted in a gradual decline in aversion to saccharin in controls, it induced no decline in mutants expressing a CaN inhibitor (**Fig. 4a**). In these mutants, the memory of the aversive association was significantly more persistent than in controls and continued to dominate behavior and to induce a strong aversion to saccharin (**Fig. 4a**). This effect of phosphatase inhibition was specific to CaN, as inhibition of PP1 in adult forebrain (–45–65%[7]) during and after conditioning did not alter the establishment nor the extinction of CTA (**Supplementary Fig. 3** online). This lack of effect of PP1 inhibition is consistent with the finding that PP1 activity is not regulated during the establishment of aversive memory.

To examine the persistence of the effect of CaN inhibition on the dominance of the aversive trace, we stopped training for several weeks and then resumed. After this delay, aversion to saccharin was still dominant in mutants expressing a CaN inhibitor, whereas it was partly extinguished in controls (**Fig. 4a**), consistent with the hypothesis that the initial memory trace is most aversive in the mutants. However, following an additional 2 d of extinction training, CTA rapidly extinguished in the mutants and was similar to CTA in controls (**Fig. 4a**), indicating that CaN inhibition did not permanently block extinction training, but rather shifted memory dominance in favor of the aversive trace.

Consistent with these findings, CaN overactivation made memory for the aversive association weaker and less persistent than in controls (**Fig. 4b** and **Supplementary Fig. 4** online). In active CaN mutants, extinction was complete after seven extinction trials and saccharin intake was comparable in conditioned (9 ± 3%) and naive (2 ± 1%, $F_{1,20} = 2.44$, $P = 0.1339$, data not shown) mutants.

Notably, a change in CaN activity not only affected aversive memory, but also modulated fear memory, another form of associative memory[30]. CaN inhibition increased the resistance to extinction of memory for a paired tone footshock, whereas CaN overactivation decreased it (**Supplementary Fig. 5** online). These results suggest that CaN is a general regulator of associative forms of memory.

If CaN has a general function in memory formation, we would expect it to modulate not only the aversive trace, but also the nonaversive trace acquired during extinction training. To test this hypothesis, we examined whether extinction itself is altered by CaN inhibition or overactivation. We took advantage of the inducibility of transgene expression and induced CaN inhibition or overactivation only after conditioning. In the absence of transgene expression during conditioning, aversion to saccharin was similar between mutants and controls (**Fig. 5a,b**), indicating a comparable level of learning. Furthermore, the induction of transgene expression after conditioning did not alter extinction of the aversive trace, as shown by a similar decline in aversion in mutants expressing either a CaN inhibitor or an active CaN and in controls (**Fig. 5a,b**). These results are consistent with the observation that CaN activity is not altered by extinction training in wild-type mice and suggest that the process of extinction *per se* is not regulated by CaN.

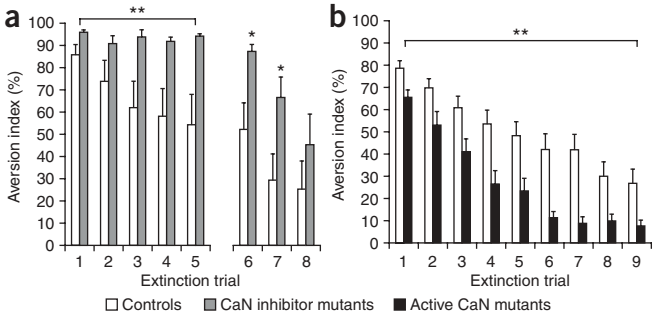

Figure 4 CaN inhibition or overactivation modulate CTA during extinction. (**a**) Extinction of CTA in mutant mice expressing a CaN inhibitor. During the first five extinction trials, aversion to saccharin remained high in CaN inhibitor mutants (2 ± 2% decline in five trials, $n = 6$, gray bars; ANOVA, trial 1 versus trial 5, $F_{1,10} = 1.06$, $P = 0.3272$), whereas aversion gradually declined in controls (42 ± 14% decline in five trials, $n = 9$, white bars; ANOVA, trial 1 versus trial 5, $F_{1,16} = 4.66$, $P = 0.0464$; ANOVA, main effect of genotype on decline, $F_{1,13} = 5.3$, $P = 0.0385$; ** repeated measures ANOVA, trial by genotype interaction, $F_{4,52} = 4.34$, $P = 0.0042$). Aversion was prominent in mutants after five extinction trials (94 ± 1%; controls, 54 ± 14%; $F_{1,13} = 5.32$, $P = 0.0382$) and after 1 month (extinction trial 6, 87 ± 4%; controls, 52 ± 12%; $F_{1,13} = 5.28$, $P = 0.0387$), but was rapidly extinguished following two additional extinction trials (51 ± 16% decline in three trials; repeated measures ANOVA, main effect of trial, $F_{2,10} = 9.68$, $P = 0.0046$) to levels comparable ($F_{1,13} = 0.99$, $P = 0.3377$) to controls (extinction trial 8; mutants, 45 ± 16%; controls, 25 ± 13%). (**b**) Extinction of CTA in mutant mice expressing active CaN. CaN overactivation in mutants resulted in a lower initial aversion index (mutants, 66 ± 3%, $n = 16$, black bars; controls, 79 ± 3%, $n = 21$, white bars; $F_{1,35} = 7.91$, $P = 0.0080$) and a faster return (* main effect of genotype, $F_{1,35} = 13.41$, $P = 0.0008$) to baseline than in controls.

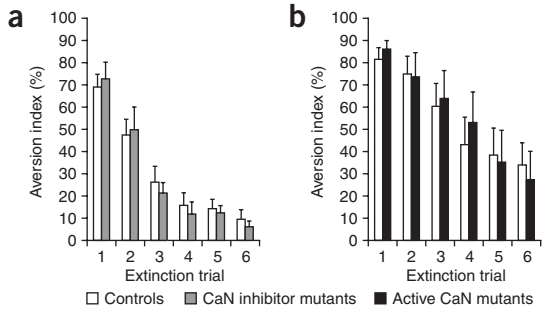

Figure 5 CaN inhibition or overactivation after conditioning does not alter CTA extinction. (**a**,**b**) Transgene induction after conditioning in mutant mice expressing a CaN inhibitor (**a**) did not alter CTA in the first extinction trial (mutants, 73 ± 7%, $n = 8$, gray bars; controls, 69 ± 6%, $n = 16$, white bars; $F_{1,22} = 0.14$, $P = 0.7086$) or extinction (decline in six trials: mutants, 91 ± 3%; controls, 84 ± 7%; $F_{1,22} = 0.52$, $P = 0.4786$; repeated measures ANOVA, trial by genotype interaction, $F_{5,110} = 0.31$, $P = 0.9067$), and in mutant mice expressing an active CaN (**b**) did not alter CTA in the first extinction trial (mutants, 87 ± 4%, $n = 6$, black bars; controls, 82 ± 5%, $n = 11$, white bars; $F_{1,15} = 0.37$, $P = 0.5519$) or extinction (decline in six trials: mutants, 56 ± 13%; controls, 44 ± 10%; $F_{1,15} = 0.58$, $P = 0.4582$; repeated measures ANOVA, trial by genotype interaction, $F_{5,75} = 0.44$, $P = 0.8224$).

CaN activity and *Zif268* expression are functionally linked

Because CaN inhibition and increased *Zif268* expression correlate during the establishment of aversive memory, we examined whether these processes are coupled. For this, we quantified *Zif268* mRNA expression in the amygdala and insular cortex in CaN inhibitor mutants. Expression of the transgene encoding a CaN inhibitor (**Fig. 6a**) significantly increased *Zif268* mRNA in both the amygdala (80%) and insular cortex (50%) ($P = 0.0477$ and $P = 0.0036$, respectively; **Fig. 6b**). The apparent lower increase in *Zif268* mRNA in the insular cortex most likely resulted from the fact that *Zif268* mRNA is already highly expressed in the insular cortex (about five-fold higher than in amygdala; **Supplementary Fig. 6** online). In the amygdala, the effect of CaN inhibition was specific to *Zif268*, as *Fos* expression was not changed (CaN inhibitor mutants, 1.2 ± 0.1; controls, 1.0 ± 0.1; $F_{1,16} = 0.90$; $P = 0.3575$, data not shown). These results suggest a functional link between CaN activity and *Zif268* expression and the possibility that CaN inhibition strengthens aversive memory by regulating Zif268.

To test this hypothesis, we overexpressed *Zif268* in the mouse forebrain using an inducible *Zif268* transgene (**Fig. 7a**). In the resulting mutant mice, *Zif268* mRNA expression was increased in the amygdala, but not in the cerebellum (**Fig. 7b**). When tested for CTA, Zif268 mutants avoided saccharin similarly to controls after conditioning; however, they showed a significantly more persistent CTA during extinction training ($P = 0.0160$; **Fig. 7c**). Zif268 mutants showed a significantly slower aversion decline than controls after five daily extinction trials ($P = 0.0168$; **Fig. 7c**). To further examine the persistence of the effect of *Zif268* overexpression on aversive memory, training was stopped for several weeks and then resumed. After this delay, aversion was still more pronounced in Zif268 mutants than in controls (**Fig. 7c**). However, following an additional 3 d of extinction training, aversive memory was fully extinguished in Zif268 mutants and was similar to that seen in controls (**Fig. 7c**). The persistence of aversion may be explained by a stronger aversive memory that dominates behavior during extinction training or by slower acquisition of the nonaversive trace during extinction training. To distinguish between these possibilities, we examined whether extinction itself is altered by *Zif268* overexpression by inducing transgene expression only after conditioning. In the absence of *Zif268* expression during conditioning, aversion to saccharin was comparable in Zif268 mutants and controls (**Fig. 7d**). Similarly, repeated extinction trials resulted in a comparable decline in aversion in Zif268 mutants and controls, indicating that *Zif268* overexpression does not affect extinction *per se*. These results suggest that, like CaN, Zif268 determines memory strength during CTA establishment but is not involved in extinction. These findings corroborate the suggestion that Zif268 is a downstream effector of CaN-dependent pathways for the regulation of memory strength.

To confirm this suggestion, we examined whether CaN inhibition and *Zif268* overexpression modulate similar targets in the amygdala. We used a comprehensive proteomic approach to identify and quantify proteins in CaN inhibitor and Zif268 mutant mice. As anticipated, the profile of regulated functional groups was comparable in the mutant lines (**Supplementary Fig. 7** online), and among the proteins that were found to be differentially expressed in the amygdala, 14 were coregulated in both lines (**Supplementary Fig. 8** online). These results therefore suggest that CaN and Zif268 share common mechanisms for the regulation of memory strength.

DISCUSSION

Our study demonstrates that the establishment of aversive memory is associated with physiological inhibition of CaN selectively in the amygdala and that the level of CaN activity at the time of memory establishment determines the strength of memory. The enhancing or weakening effect of a decrease or an increase in CaN activity on memory strength highlights a bidirectional role for CaN in the mechanisms of memory establishment and underscores the importance of a tight fine-tuning of CaN activity during learning. This key function may be derived from the strategic positioning of CaN in the postsynaptic density, close to neurotransmitter receptors and ion channels, and upstream of major signaling cascades. This positioning allows for the rapid capture of incoming signals, efficient shaping of signal initiation and activation of downstream signaling cascades[11,31] (**Supplementary Fig. 9** online). In these cascades, CaN may act on various processes. It may apply a molecular constraint on Ca^{2+} influx and neuronal excitability by controlling the activity of L-type voltage-gated Ca^{2+} channels[12] and maintaining the AMPA receptor in a dephosphorylated state to prevent its trafficking[13]. It can also limit cyclic AMP (cAMP) signaling and cAMP-dependent protein kinase

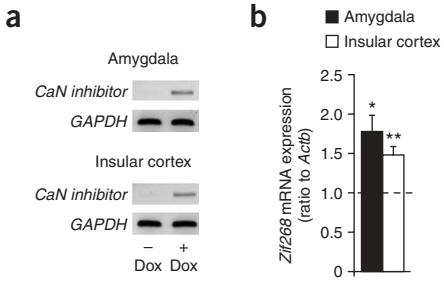

Figure 6 *Zif268* mRNA expression is increased by CaN inhibition in the amygdala. (**a**) *CaN inhibitor* transgene mRNA was detected in amygdala and insular cortex in mutants, but not in controls. *GAPDH* mRNA indicates equal cDNA loading and quality. (**b**) Increased relative expression of *Zif268* mRNA in the amygdala (mutants, 1.8 ± 0.4, $n = 9$, black bar; controls, 1.0 ± 0.2, $n = 10$; $F_{1,16} = 4.60$, $P = 0.0477$) and insular cortex (mutants, 1.5 ± 0.1, white bar; controls, 1.0 ± 0.1; $F_{1,17} = 11.39$, $P = 0.0036$) in CaN inhibitor mutants compared with controls (represented by dashed line). Dox, doxycycline.

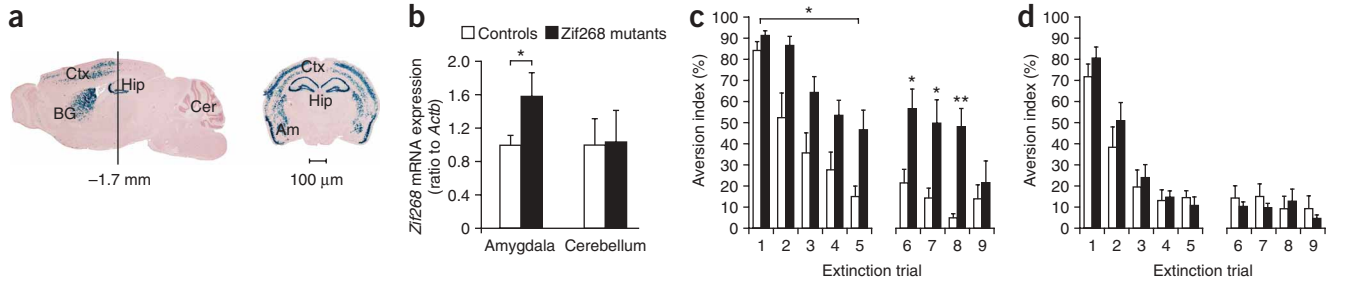

Figure 7 *Zif268* overexpression mimics the effect of CaN inhibition on CTA. (**a**) In Zif268 mutants, the *lacZ* reporter gene was specifically expressed in forebrain, including neocortex (Ctx), hippocampus (Hip), amygdala (Am) and basal ganglia (BG), but not cerebellum (Cer), as shown on a parasagittal section and a representative coronal section at bregma –1.7 mm. (**b**) *Zif268* mRNA expression was significantly increased in Zif268 mutants in the amygdala (1.6 ± 0.3, $n = 11$, black bars; controls, 1.0 ± 0.1, $n = 10$, white bars; $F_{1,19} = 5.41$, $P = 0.0313$), but not in cerebellum (1.0 ± 0.4; controls, 1.0 ± 0.3; $F_{1,17} = 0.01$, $P = 0.9251$). (**c**) CTA is more persistent in Zif268 mutants than in controls (repeated-measures ANOVA, trial by genotype interaction, $F_{8,96} = 2.51$, $P = 0.0160$). Although Zif268 mutants (91 ± 2%, $n = 6$) and controls (84 ± 4%, $n = 8$) avoided saccharin to a similar degree ($F_{1,12} = 1.92$, $P = 0.1913$) when first tested 10 d after conditioning, aversion to saccharin declined much less in Zif268 mutants (decline in five trials, 49 ± 10%; ANOVA, trial 1 versus trial 5, $F_{1,10} = 21.58$, $P = 0.0009$) than in controls (decline in five trials, 83 ± 5%; ANOVA, trial 1 versus trial 5, $F_{1,14} = 112.18$, $P < 0.0001$; ANOVA, decline by genotype interaction; $F_{1,12} = 10.48$, $P = 0.0071$) during the subsequent four extinction trials. Aversion was still strong in Zif268 mutants after 2 weeks (extinction trial 6: mutants, 57 ± 9%; controls, 21 ± 7%; $F_{1,12} = 10.39$, $P = 0.0073$), but was fully extinguished (decline in four trials, 68 ± 10%; repeated measures ANOVA, main effect of trial, $F_{3,15} = 5.98$, $P = 0.0069$) after three additional extinction trials (extinction trial 9: mutants, 22 ± 10%; controls, 14 ± 7%; $F_{1,12} = 0.44$, $P = 0.5206$). (**d**) Zif268 overexpression after conditioning did not modify CTA extinction. In the first extinction trial, the aversion to saccharin was comparable between Zif268 mutants (81 ± 5%, $n = 8$) and controls (72 ± 6%, $n = 10$; $F_{1,16} = 1.18$, $P = 0.2929$). Extinction training had a similar effect in Zif268 mutants and controls (repeated measures ANOVA, trial by genotype interaction, $F_{8,128} = 1.48$, $P = 0.1691$) and resulted in a comparable decline (mutants, 94 ± 2%; controls, 88 ± 6%; $F_{1,16} = 0.69$, $P = 0.4172$).

activity by regulating adenylyl cyclases[32,33]. Furthermore, CaN might weaken signaling to the nucleus by counteracting protein kinases such as Ca^{2+}/calmodulin- and mitogen-activated protein kinases (CaMKs/MAPKs)[34,35], known to regulate transcription factors such as cAMP-response element–binding protein (CREB), serum response factor and Elk1[34,36] (**Supplementary Fig. 9**). Our data demonstrate that CaN regulates transcriptional processes by recruiting mechanisms involving the transcription factor Zif268. We found that CaN inhibition is associated with increased *Zif268* expression in the amygdala and that such an increase occurs naturally during the establishment of CTA. Furthermore, mimicking this increase in *Zif268* expression by transgenesis strengthens CTA in a manner similar to CaN inhibition.

The identification of a common pool of proteins that are similarly regulated by CaN inhibition and *Zif268* overexpression in the amygdala points to a functional link between CaN and Zif268. It is also possible, however, that CaN acts directly on Zif268, as shown for a Zif268-related transcription factor in *Saccharomyces*[37], and that CaN regulates other transcription factors beyond Zif268 (refs. 38,39; **Supplementary Fig. 9**). The mechanisms by which CaN is inhibited during learning have also not been identified but may recruit endogenous inhibitors such as the CaN-binding protein Cabin1/Cain, the calcineurin homolog protein or modulatory calcineurin-interacting proteins, anchoring proteins such as A kinase–anchoring proteins[40], or redox inactivation[41]. The present results point to the importance of a tight fine-tuning of these inhibitory processes for memory, as a complete absence of CaN, such as that induced by genetic knockout, perturbs, rather than improves, memory[42,43]. Finally, the finding that *Zif268* overexpression enhances memory complements previous data showing that *Zif268* knockout impairs learning and memory[25,26]. Taken together, these findings reveal that Zif268-dependent transcriptional processes control memory processes bidirectionally, further highlighting the vital role of Zif268 in cognitive functions.

Our results also reveal that the mechanisms responsible for the establishment and extinction of aversive memory are distinct in that they do not share dependence on CaN or Zif268. Although the establishment of CTA involves both, extinction *per se* does not. This result is important for the understanding of the mechanisms of memory formation because it indicates that extinction, although a form of relearning[18,20], is mechanistically distinct from initial learning. One of the differences reflects the fact that both CaN inhibition and Zif268 activation induced by conditioning are persistent and therefore 'lock' CaN and Zif268 across extinction learning (at least in the amygdala). It should be noted that these mechanisms may be unlocked by recall, as CaN was shown to be activated 1 d after conditioning in the fear-potentiated startle or fear conditioning tasks[44–46]. Notably in the present study, the distinction between the establishment and the extinction of memory was possible because of the unique properties of CTA. It is acquired in a single trial and therefore has distinct acquisition and establishment phases, which is not the case for incremental learning such as fear conditioning, which usually requires several tone-shock pairings[47]. CTA is also extremely robust and consistent across mice but can, nonetheless, be progressively extinguished in a controlled and quantifiable manner. Finally, CTA is ethologically relevant and mimics natural behaviors.

By revealing that CaN critically gates highly salient forms of associative memory, our results extend previous reports showing that CaN acts as a molecular constraint on nonassociative learning and memory such as spatial working, reference memory or object recognition[6,27,28,48]. Our results further highlight a notable dissociation between CaN and PP1, where only CaN is involved in associative memory, whereas both CaN and PP1 in the forebrain control the acquisition and formation of nonassociative memory[7,27]. The present data may provide new perspectives for the development of potential therapeutic treatments against memory disorders, for instance, involving the recall of intrusive memory as in aversive-, fear- or anxiety-associated disorders.

METHODS

Animals. We used adult transgenic mice and control littermates (3–6 months old) and C57BLJOlaHsd wild-type mice (3 months old, Harlan) in our

experiments. Mice were maintained in standard conditions under a reversed light cycle (dark phase, 7 a.m. to 7 p.m.). Doxycycline-supplemented food (6 mg per 100 g of wet food, West-Ward Pharmaceuticals) was administered to transgenic mice and control littermates daily at least 6 d before and throughout experimentation. Habituation, conditioning and test sessions were performed during the second and third quarter of the dark phase. All experiments were carried out in accordance with guidelines and regulations of the cantonal veterinary office, Zürich.

Data analyses. The data were analyzed by one-way or repeated-measures ANOVA, followed by Fischer's protected least-significant-difference (PLSD) *post hoc* test to compare individual groups or group effects over time. When the statistical test is not specifically mentioned, a one-way ANOVA was used and the F and P values refer to the main effect. Data and error bars represent s.e.m. $^{*}P < 0.05$, $^{**}P < 0.005$ in all figures.

Generation of Zif268 mutant mice. A 1,744-bp fragment of *Zif268* coding sequence was excised from the vector *pCMVsport6-Zif268* by *Ava*I/*Hpa*I digest, blunt-ended using T4 polymerase (New England Biolabs) and inserted into the EcoRV site of *pNN265*. From this, a NotI fragment was excised (3,190 bp) and introduced into the NotI site of *pBI-G* (Clontech). *Sal*I/*Ase*I digest resulted in an 8,381-bp fragment that was microinjected into fertilized oocytes with a mixed C57Bl/6J and DBA2 F1 background. Founder mice were backcrossed once to C57Bl/6J mice and their offspring to CaMKIIα-rtTA2 mice[29] to generate Zif268 mutant mice.

CTA test. Singly housed mice were adapted to restricted drinking (two bottles for 20-min once or twice per day for 4 d), then exposed to saccharin (0.5%) for 20-min on conditioning day, followed 40-min later by LiCl injection (intraperitoneal, 0.14 M, 2% of body weight). Control mice were injected with vehicle. CTA was tested by presentation of one bottle of saccharin and one bottle of water (choice test) 2 d after conditioning for the experiments in **Figures 1–4**, and 10 d after conditioning for all other CTA experiments. CTA extinction was induced by successive daily choice tests. Measurements of endogenous CaN activity and *Zif268* mRNA expression were conducted 40-min after the second extinction trial (that is, 3 d after conditioning).

Cued fear conditioning. Mice were habituated to the room and handled for 2 consecutive d before testing. The mice were placed in a box 1 d after habituation for 4 min and then exposed to three 30-s tones (2,800 Hz) paired with an electric foot shock (0.3 mA) during the last second (30-s inter-tone/shock intervals). Cued memory was tested 1, 4, 7, 10, 13 and 16 d after conditioning by placing the animal for 2 min in a novel box, and playing the tone during the second minute. Freezing was determined in 0.75-s bouts and expressed as percent time of tone presentation. Automated boxes and analysis software were used (Freezeframe, Coulbourn Instruments).

CaN activity assay. Amygdala and insular cortex were isolated and processed using the BIOMOL Quantizyme Assay Kit (AK-816) with modifications including centrifugations at 16,000*g* and use of inorganic phosphate–bind resin (Innova Biosciences) for desalting. The first pellet was resuspended in lysis buffer containing 1.2% Nonidet P-40 (vol/vol, Fluka) to obtain a crude membrane fraction. For cytoplasmic and membrane fractions, 0.7 and 1.4 μg of total protein, respectively (as determined by Bradford assay), were used. PSD95 and integral membrane proteins (GluR1, mGluR5), but no nuclear markers (histone 2A, histone deacetylase 1, Zif268, CREB), were detected by western blotting in this fraction.

Western blotting. Proteins from the preparation for the CaN assay (20 μg for cytoplasmic, 30 μg for membrane fraction) were resolved on SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Blocking and antibody dilution were carried out in blocking buffer for near-infrared detection (Rockland). Total CaN protein was revealed using antibody to CaN-A (Abcam, final dilution 1:5,000) or to CaN-B (Upstate, final dilution 1:1,000) followed by IRDye700-conjugated goat antibody to rabbit IgG (Rockland, final dilution 1:5,000) and normalized to β-actin (Upstate, final dilution 1:3,000).

Quantitative RT-PCR. RNA was extracted from amygdala using a Nucleospin RNA II Kit (Macherey-Nagel) followed by DNA digestion (Ambion). RNA (100 ng) was reverse-transcribed using the SuperScript First Strand Synthesis system (Invitrogen). Quantitative PCR was run on a 7500 real-time PCR system (Applied Biosystems) using TaqMan kits (Applied Biosystems) for *Zif268* (Mm00656724_m1), *Fos* (Mm00487425_m1), *Actb* (4352664-0510003) and *GAPDH* (4352662-0509004). Results were normalized to *Actb* or *GAPDH* and expressed as fold change of average of controls.

RT-PCR. cDNA (1 μl), as prepared above, was used for amplification using the primers pNN3050 (5′-CGA TTC TAG AAT TCG CTG TCT GCG AGG GCC-3′) and 211 (5′-GGC ATC CTC TCG TTA ATT CGG-3′). cDNA quality and loading were verified with the housekeeping gene *GAPDH* using the primers GAPDH F1 (5′-CAC TGA GCA TCT CCC TCA CA-3′) and GAPDH R1 (5′-GTG GGT GCA GCG AAC TTT AT-3′).

β-galactosidase staining. Parasagittal and coronal brain sections (14 μm) were processed as described previously[29].

Matrix-assisted laser desorption/ionization tandem mass spectrometry analysis of iTRAQ-labeled samples. Amygdala protein fractions were labeled with iTRAQ, fractionated via two-dimensional liquid chromatography as described previously[49] and analyzed on a 4800 MALDI MS/MS (**Supplementary Methods** online).

Note: Supplementary information is available on the Nature Neuroscience website.

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AUTHOR CONTRIBUTIONS

K.B. was responsible for the project and wrote the manuscript together with I.M.M. He conducted all CTA experiments except those presented in **Figure 3a** and **Supplementary Figure 4**, performed all biochemical and molecular experiments, and generated and characterized the Zif268 mutant mice. H.W. provided the expertise for the behavioral experiments and actively contributed to them. D.G. initiated this project and performed the CTA experiments shown in **Figure 3a** and **Supplementary Figure 4**. R.Y.T.-C. and M.L.-Z. performed the proteomics screen. K.K. was involved in several CTA experiments and in tissue isolation and developed the PP1 assay used to generate **Supplementary Figure 2**. C.M. was involved in one CTA experiment and during several sample preparations. I.M.M. is the principal investigator; she generated the CaN and PP1 transgenic lines, conceived the project, provided continual conceptual input and financial support and wrote the manuscript.

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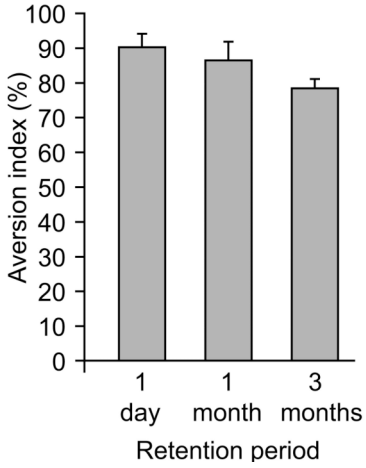
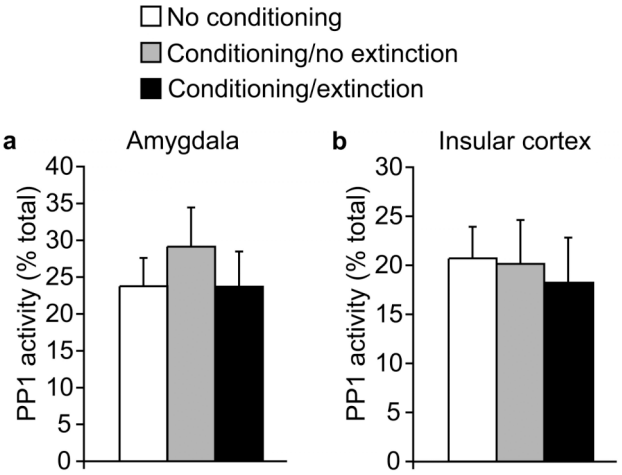
"Control of the Establishment of Aversive Memory by Calcineurin and Zif268"

Karsten Baumgärtel, David Genoux, Hans Welzl, Ry Y. Tweedie-Cullen, Kyoko Koshibu, Magdalena Livingstone-Zatchej, Céline Mamie and Isabelle M. Mansuy

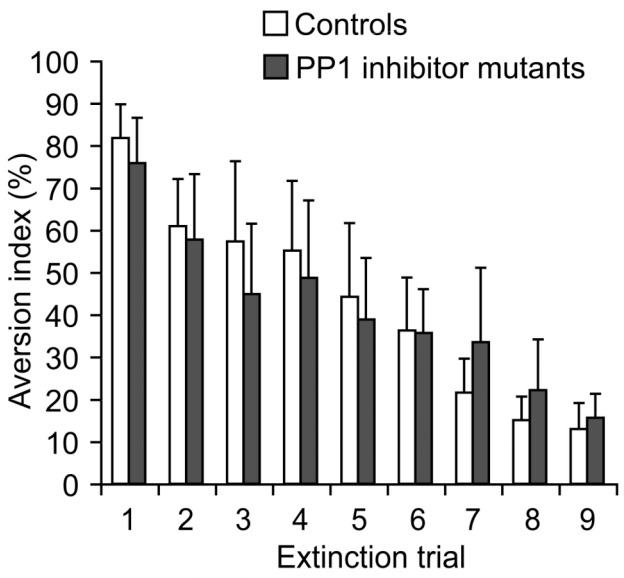
SUPPLEMENTARY FIGURES



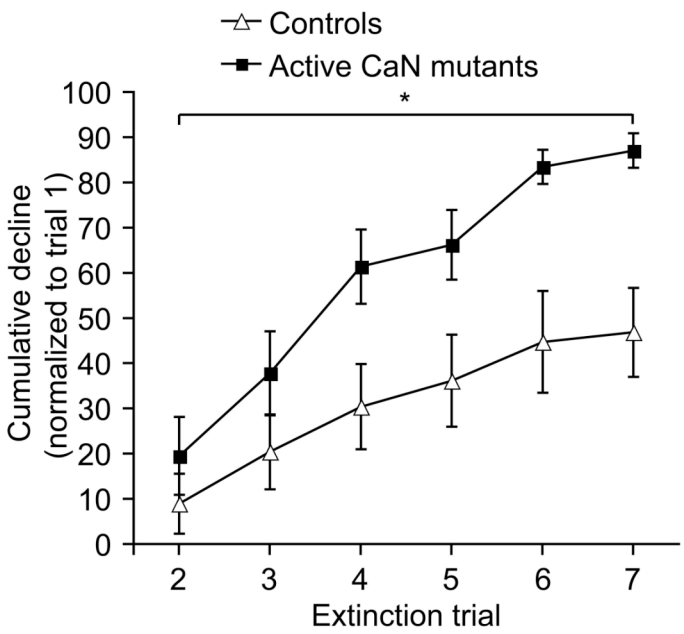
Supplementary Figure 1. CTA memory persists for several months in adult C57Bl/6J mice. One-trial conditioning to saccharin paired with malaise induces a robust CTA as seen by a high aversion index 24 hours after conditioning (90% ± 4%, n = 4). CTA does not significantly decline after 1 month (86% ± 5%, n = 8) or 3 months (78% ± 3%, n = 8; main effect of day: $F_{2,17} = 1.76$; $P = 0.2012$).



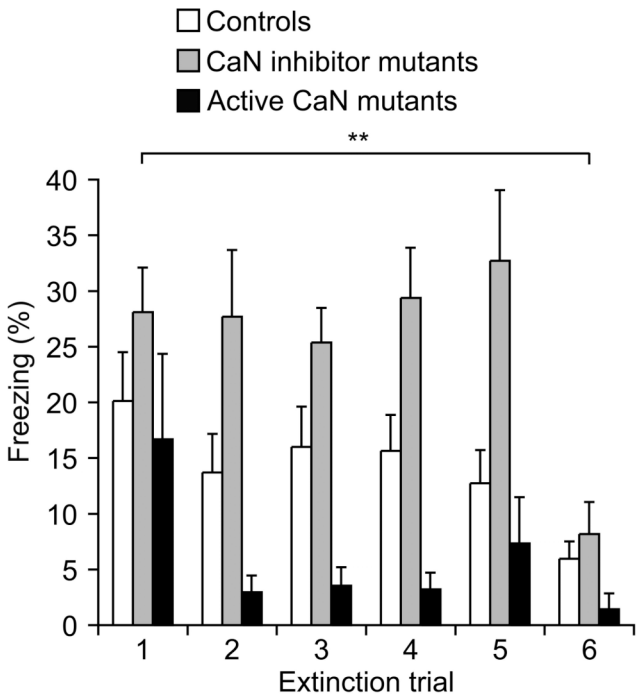
Supplementary Figure 2. PP1 activity is not changed in the amygdala or the insular cortex by conditioning in the CTA test. **(a)** Relative PP1 activity (% total Ser/Thr protein phosphatase activity) in a cytoplasmic fraction of the amygdala three days after conditioning followed by extinction or not. PP1 activity is similar in unconditioned (no conditioning: 24 ± 4%, n = 6, white bars) and conditioned mice whether subjected to extinction training (conditioning/extinction: 24 ± 5%, n = 6, black bars) or not (conditioning/no extinction: 29 ± 5%, n = 6, grey bars). **(b)** Same as a) for insular cortex. PP1 activity is not altered by conditioning or extinction training in a cytoplasmic fraction (no conditioning, 21 ± 3%, n = 4; conditioning/no extinction, 20 ± 5%, n = 5; conditioning/extinction, 18 ± 5%, n = 5).



Supplementary Figure 3. PP1 inhibition does not alter the acquisition or the extinction of CTA. Aversion for saccharin is similar in mutant mice expressing a PP1 inhibitor[1] (76% ± 11%, n = 6, dark grey bars) and control mice (82 ± 8%, n = 5, white bars) 3 hrs after conditioning (extinction trial 1: $F_{1,9}$ = 0.19; P = 06761). Additional extinction trials 15, 24, 39, 48, 63, 72, 87 and 96 hrs following conditioning result in comparable decline in aversion index in PP1 inhibitor mutant (70 ± 15%) and control mice (84 ± 8%; ANOVA, main effect of genotype on decline: $F_{1,9}$ = 0.68; P = 0.4293).

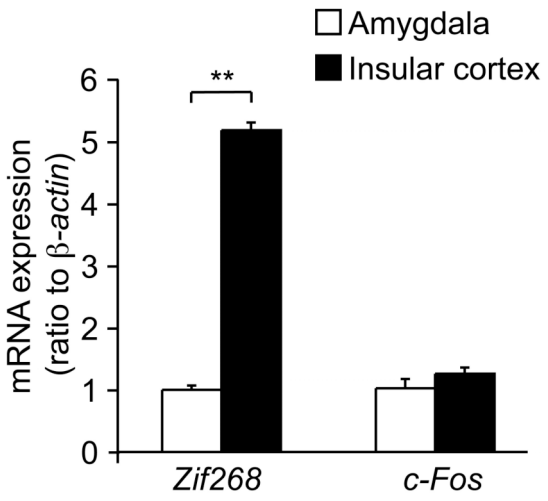


Supplementary Figure 4. CaN overactivation makes CTA memory more susceptible to extinction. Active CaN mutants (n = 16, black squares) show an overall higher cumulative decline (aversion index normalized to aversion index in trial 1 for each individual: 87 ± 4%) than control littermates (47 ± 10%, n = 21, white triangles; ANOVA, main effect of genotype on decline: $F_{1,35}$ = 11.59; P = 0.0017; *repeated measures ANOVA, trial by genotype interaction: $F_{5,175}$ = 2.63; P = 0.0254).

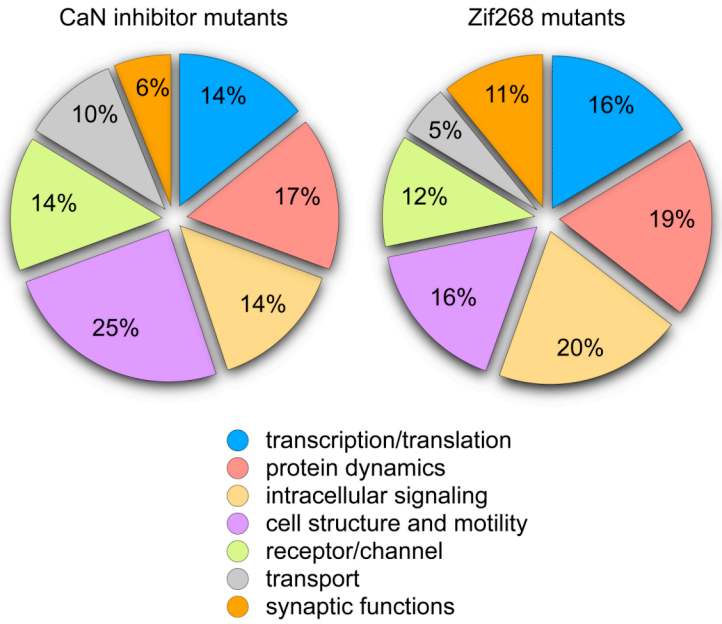


Supplementary Figure 5. CaN inhibition or overactivation modulate fear memory during extinction. The percentage time spent freezing (representing memory for a tone-shock association) is similar ($F_{2,22}$ = 0.96; P = 0.3996) in mutant mice expressing a CaN inhibitor (28 ± 4%, n = 7, grey bars) or an active CaN (17 ± 8%, n = 4, black bars) and control littermates (20 ± 4%, pooled, n = 14, white bars) 1 day after conditioning (extinction trial 1). However, freezing in response to the tone is higher during 3 subsequent extinction trials in CaN inhibitor mutants (extinction trial 2: 28 ± 6%; extinction trial 3: 25 ± 3%; extinction trial 4: 29 ± 5%), but lower in active CaN mutants (extinction trial 2: 3 ± 2%; extinction trial 3: 4 ± 2%; extinction trial 4: 3 ± 2%) than in control littermates (extinction trial 2: 14 ± 3%; $F_{2,22}$ = 5.04; P = 0.0158; extinction trial 3: 4 ± 2%; $F_{2,22}$ = 4.81; P = 0.0185; extinction trial 4: 3 ± 2%; $F_{2,22}$ = 7.33; P = 0.0036; **repeated measures ANOVA, main effect of genotype: $F_{2,22}$ = 8.47; P = 0.0019).

However, after two additional extinction trials, cued fear conditioning is fully extinguished in all groups (extinction trial 6: CaN inhibitor mutants: 8 ± 3%; active CaN mutants: 1 ± 1%; controls: 6 ± 2%; $F_{2,22}$ = 1.56; P = 0.2331).

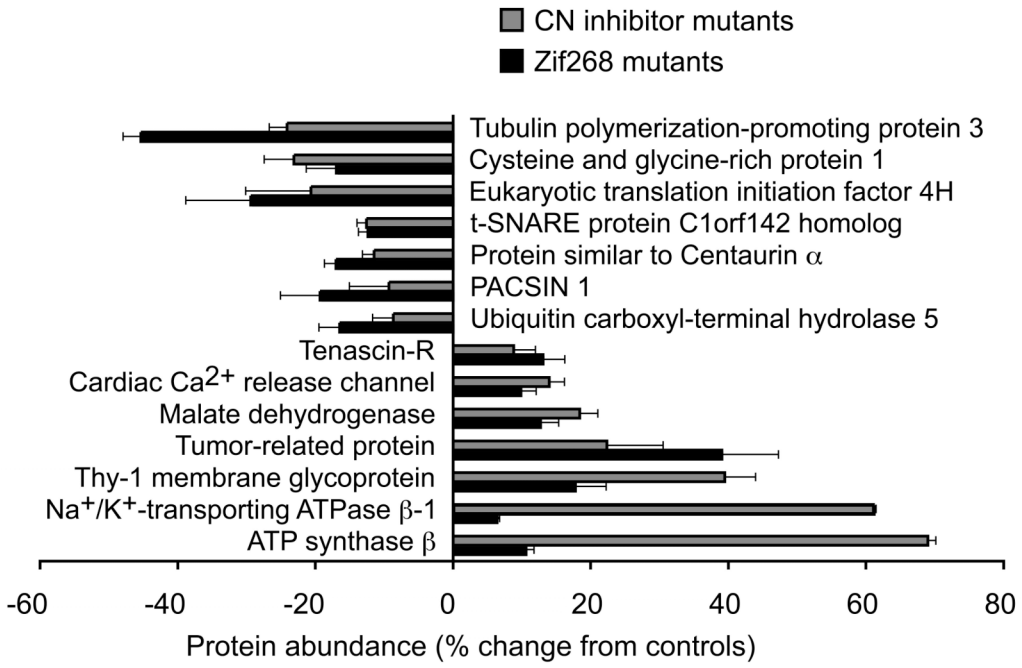


Supplementary Figure 6. Basal expression of *Zif268* is five fold higher in insular cortex than amygdala. Analysis of relative *Zif268* and *c-Fos* mRNA expression in insular cortex and amygdala in basal conditions showing significantly higher ($F_{1,6}$ = 904.22; P < 0.0001) *Zif268* mRNA expression in insular cortex (5.2 ± 0.1; n = 4) than amygdala (1.0 ± 0.1; n = 4), but comparable ($F_{1,6}$ = 1.67; P = 0.2441) *c-Fos* mRNA expression in both structures (insular cortex: 1.3 ± 0.1; amygdala: 1.0 ± 0.2).

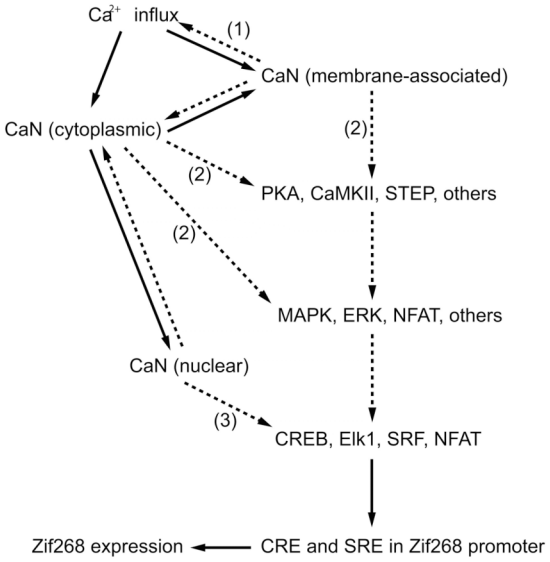


Supplementary Figure 7. Similar functional groups are regulated by CaN inhibition (n = 4, control: n = 4) and *Zif268* overexpression (n = 6, control: n = 6) in the amygdala. All proteins regulated after CaN inhibition or *Zif268* overexpression were classified into 7 functional groups, excluding the group "metabolism", based on GO ontology classification in the Uniprot database. "Transcription/translation" (blue) includes transcription factors, DNA and RNA modifying enzymes such as splicing factors and ribosomal components. "Protein dynamics" (pink) includes proteasomal and ubiquitination machinery, enzymes mediating posttranslational modifications and chaperones. "Intracellular signaling" (ochre) is largely constituted by protein kinases, protein phosphatases and small GTPases. "Cell structure and motility" (purple) include cytoskeletal proteins, actin-like and actin-associated proteins, and extracellular matrix molecules known to control cell motility. "Transport" (grey) applies to molecules

transporting all types of cargo. “Synaptic functions” (orange) includes proteins associated with vesicle release and recycling, and postsynaptic organization.



Supplementary Figure 8. Common target proteins are regulated by CaN inhibition and *Zif268* overexpression. Relative abundance of 14 proteins similarly regulated in the amygdala in CaN inhibitor mutants (n = 4) and Zif268 mutants (n = 6) relative to control littermates (n = 4 for CaN inhibitor mutants; n = 6 for Zif268 mutants; $P < 0.05$).



Supplementary Figure 9. Schematic illustrating potential pathways downstream of CaN. (1) CaN can control ion channels and neurotransmitter receptors and thereby modulate neuronal excitability and Ca^{2+} influx. (2) CaN can directly or indirectly regulate other enzymes involved in postsynaptic organization, secondary messenger generation and signal transduction to the nucleus. (3) CaN may translocate to the nucleus and directly act on TFs. (PKA = cAMP-dependent protein kinase, CaMKII = calcium/calmodulin-dependent protein kinase II, STEP = striatal enriched phosphatase, MAPK = mitogen-activated protein kinase, ERK = extracellular signal-regulated kinase, NFAT = nuclear factor of activated T-cells, CRE = cAMP-response element-binding protein, SRF = serum response factor, SRE = serum response element).

SUPPLEMENTARY METHODS

PP1 activity assay

Amygdala and insular cortex were dissected and homogenized in homogenization buffer (3.75 mM Tris-HCl, pH7.4, 15 mM KCl, 3.75 mM NaCl, 250 µM EDTA, 50 µM EGTA, 30% (w/v) sucrose, 30% (v/v) glycerol, protease inhibitor cocktail (Sigma), 100 µM PMSF) using a Dounce homogenizer. After centrifugation (1,000 g, 10-min), the supernatant (cytoplasmic fraction) and the pellet were resuspended in homogenization buffer without sucrose but 15 mM ß-mercaptoethanol, homogenized using a 26G syringe and desalted using P_i-bind resin (Innova Biosciences). Phosphatase activity was determined by incubating 2 µg sample with 0.15 mM RII substrate either with or without inhibitors, i.e. 5 nM tautomycin (TM), or 5 nM TM + 100 nM okadaic acid (OA) in 50 mM Tris-HCl, pH7.0, 100 µM Na_2EDTA, 5 mM DTT, and 0.01 % Brij 35 at 30°C for 10-min. The reaction was terminated by addition of TCA and centrifugation (13,000 g, 5-min). The amount of free phosphate released in the reaction was measured with BIOMOL Green reagent (BIOMOL International LP) at OD_{620nm} and background subtracted. For total phosphatase activity, TM and OA were excluded from the reaction. PP1 and PP2A activity were calculated from the ratio of phosphatase activity with/without inhibitors as compared to total phosphatase activity.

Sample preparation, iTRAQ labeling and MALDI MS analysis

Mice were sacrificed by cervical dislocation, the amygdala was removed and homogenized in 200 µl 50 mM ammonium bicarbonate pH8, 0.1% SDS, by 10 up and down strokes of a 27G gauge syringe, sonicated for 2-min, and centrifuged at 13,000 g for 10-min to remove insoluble matter. Amygdala protein fractions (100 µg) were desalted by acetone precipitation, solubilized, cysteine-blocked and digested overnight with trypsin (1:13 enzyme:substrate) into peptides. The digested peptides were labeled differentially with iTRAQ reagents according to manufacturer's instructions and as described previously[2]. Labeled samples were combined prior to two-dimensional liquid chromatography and MALDI analysis. The peptide containing solution was separated by strong cationic exchange (SCX) chromatography in the first dimension and reversed-phase (RP) chromatography in the second dimension as described previously[2]. Fractions were automatically deposited onto MALDI target plates that were analyzed on an ABI 4800 Proteomics Analyzer MALDI TOF/TOF system. Database searching of MS/MS spectra was performed using the EBI (European Bioinformatics Institute) mouse protein database. Modifications included MMTS (C, fixed) and iTRAQ (N-termini and K, fixed). Searches using MALDI-MS/MS spectra were performed for mono-isotopic peptides with +1 charge. Error limits were set at 25 ppm for precursor masses and 0.2 Da for fragment ions. GPS (Global Proteome Server) Explorer software (Applied Biosystems) was used for processing spectra and submitting data for database searching using the search parameters described above. Quantification of iTRAQ reporter ion intensity was performed by

integrating the area under reporter ion peaks using Applied Biosystems 4800 Explorer software before combining with database search results. Only peptides with both satisfactory database identification and sufficiently intense iTRAQ reporter ions were selected for subsequent analysis. Peak areas for each iTRAQ signature peak (m/z 114.1, 115.1, 116.1, and 117.1) were obtained and corrected according to the manufacturer's instructions to account for the differences in isotopic overlap and normalized using the GPS Explorer software.

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2. Munton, R.P. et al. Qualitative and quantitative analyses of protein phosphorylation in naive and stimulated mouse synaptosomal preparations. *Mol Cell Proteomics* **6**, 283-93 (2007).