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Brief Report

# Transgenic inhibition of neuronal calcineurin activity in the forebrain facilitates fear conditioning, but inhibits the extinction of contextual fear memories

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## Abstract

It is unclear whether protein phosphatases, which counteract the actions of protein kinases, play a beneficial role in the formation and extinction of previously acquired fear memories. In this study, we investigated the role of the calcium/calmodulin dependent phosphatase 2B, also known as calcineurin (CaN) in the formation of contextual fear memory and extinction of previously acquired contextual fear. We used a temporally regulated transgenic approach, that allowed us to selectively inhibit neuronal CaN activity in the forebrain either during conditioning or only during extinction training leaving the conditioning undisturbed. Reducing CaN activity through the expression of a CaN inhibitor facilitated contextual fear conditioning, while it impaired the extinction of previously formed contextual fear memory. These findings give the first genetic evidence that neuronal CaN plays an opposite role in the formation of contextual fear memories and the extinction of previously formed contextual fear memories.
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Understanding the pathways underlying the elimination of fear memories has clinical relevance for the treatment of anxiety disorders like post traumatic stress disorder. Since the early 1960s Pavlovian fear conditioning is used as a generally accepted model to study the cellular events underlying these processes (for review, Maren & Quirk, 2004). When a context (conditioned stimulus, CS) is paired with an aversive stimulus such as a footshock (unconditioned stimulus, US), a fear memory for this specific context is formed. As a result, re-exposure to the same CS in the absence of the US results in a strong fear response. Ongoing exposure to the CS gradually results in a decay of the fear response, a process referred to as fear extinction.

The mechanisms underlying the formation of long-term fear memories have been studied extensively (for review, Maren & Quirk, 2004). Fear conditioning is suggested to be mediated by changes in synaptic strength in the hippocampus and at sensory inputs to the lateral nucleus of the amygdala (for review, Martin, Grimwood, & Morris, 2000; Sigurdsson, Doyere, Cain, & LeDoux, 2007). A potential mechanism controlling synaptic strength is the balance between phosphorylation and dephosphorylation of specific substrates by, respectively, protein kinases and protein phosphatases (for review, Tokuda & Hatase, 1998). One of the protein kinases known to be crucially involved in the formation of long-term fear memories is the cAMP-dependent protein kinase (PKA) (Abel et al., 1997). The protein phosphatase calcineurin (CaN, also known as protein phosphatase 2B) counteracts actions of PKA and is suggested to impair the formation of long-term memories (Mansuy, Mayford, Jacob, Kandel, & Bach, 1998a; Mansuy et al., 1998b; Malleret et al., 2001).

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In contrast to the role of CaN in memory formation, the involvement of CaN in the extinction of memories remains unclear. A first indication that changes in CaN activity in the basolateral amygdala attenuates the extinction of fear memories came from pharmacological studies (Lin et al., 2003). Using a genetic knock out approach, Zeng et al. (2001) showed that ablation of forebrain CaN in distinct areas of the forebrain (including CA1 pyramidal cells, most of the granular cells and neurons in layers 2 and 3 of the cortex) did not affect the formation and extinction of memories for contextual and tone cued fear (Zeng et al., 2001). In this study CaN was absent during both conditioning and extinction training. Therefore, it cannot be excluded that there was an interaction effect between the loss of CaN during conditioning and extinction training affecting the freezing levels. Furthermore, additional studies revealed various behavioral abnormalities in these mice (Miyakawa et al., 2003).

To get more insight into the role of forebrain CaN in the extinction of contextual fear memories, we used a conditional transgenic system, in which we could selectively reduce CaN activity in the forebrain during extinction training by expressing the autoinhibitory domain in the C terminus of the CaN catalytic subunit (Malleret et al., 2001). Using this system, we could avoid interactions between loss of CaN during conditioning and extinction, since CaN activity levels were unaffected during the conditioning of contextual fear.

Double transgenic male mice carrying the tetO promoter-Al transgene and the CaMKIIα promoter-rtTA2 transgene were maintained on a C57/Bl6j background. Genotype verification was determined by PCR as described previously (Malleret et al., 2001; Michalon, Koshibu, Baumgartel, Spirig, & Mansuy, 2005). Mice bred from initial breeding couples (generously provided by Dr. I.M. Mansuy (Brain Research Institute, University of Zürich and Swiss Federal Institute of Technology, Zürich, Switzerland)), were housed under standard laboratory conditions (12 h light/12 h dark cycle with light on at 07:00 h; room temperature 21 ± 1 °C).

Doxycycline administration (Dox, West-ward Pharmaceutical, Eatontown, NJ, 6 mg/g food) was used to induce the expression of the CaN inhibitor. RT-PCRs were performed as described previously using oligonucleotides specific for tetO-Al (Malleret et al., 2001). A pilot experiment revealed that after seven days of Dox-treatment, mRNA levels of the CaN inhibitor (260 bp) were detectable in the hippocampus, while the band was absent in control mice that did not receive Dox (Fig. 1a). These results are in line with previous studies by Malleret et al. (2001) and show that one week of Dox-treatment is sufficient to induce the expression of the CaN inhibitor in the forebrain.

To test whether the expression of the CaN inhibitor in the forebrain would affect the formation of contextual fear, two groups of 16–24 weeks old double transgenic male mice underwent a single training trial in a contextual fear paradigm. One group received Dox in their food (Dox-Ret group, $n = 8$) whereas a second group received
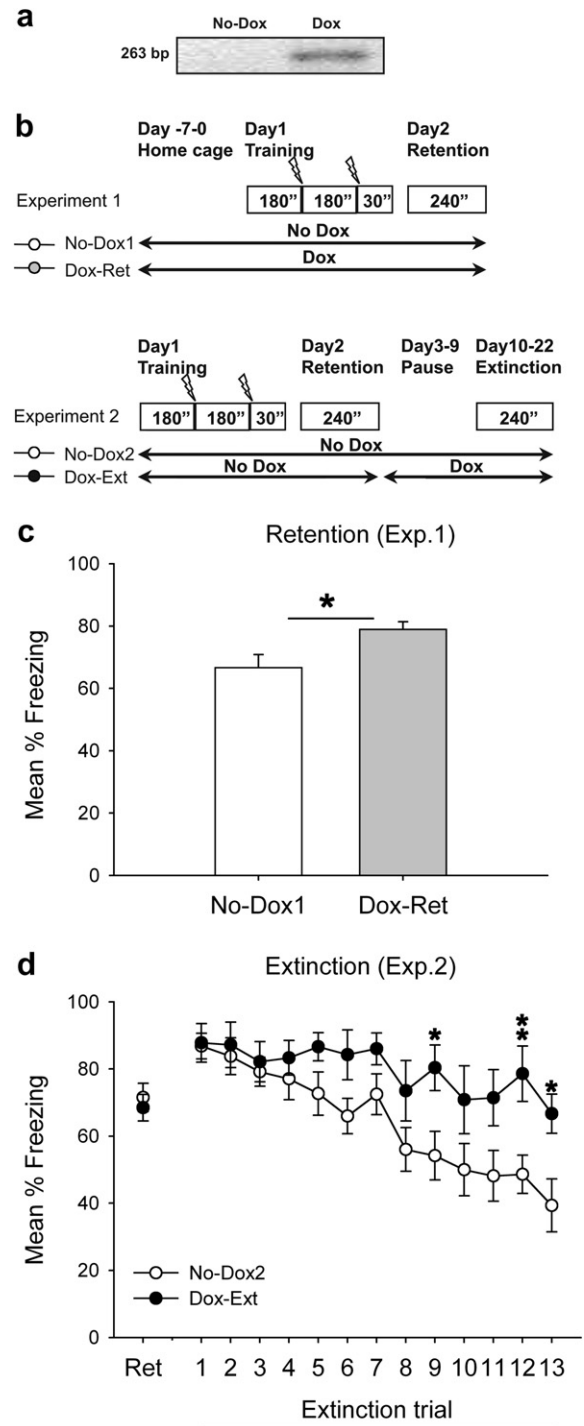


Fig. 1. Inhibition of forebrain CaN activity reduces the rate of extinction of previously acquired contextual fear memory. (a) Double transgenic mice fed with Dox express the CaN inhibitor in the hippocampus. (b) Experimental protocol for the fear conditioning and extinction test. After the conditioning and retention test, mice from the Dox-Ext group ($n = 7$) received Dox (day 3–22) to induce the expression of the CaN inhibitor. Mice of the control group (No-Dox group, $n = 9$) did not receive Dox. (c) Freezing behavior measured during a 4 min session, 24 h after the contextual fear conditioning. (d) Freezing behavior measured during daily 4 min sessions for 13 consecutive days. Error bars indicate ± SEM.

food that was prepared in the same way but without the addition of Dox (No-Dox1 group, $n = 9$). Dox-treatment

started seven days before the start of the experiment. Contextual fear conditioning was performed in a plexiglas cage ($44 \times 22 \times 44$ cm) with constant illumination. The four sides of the cage and the floor were cleaned with 70% ethanol before each trial. The conditioning protocol is shown in Fig. 1b. Mice were exposed to the conditioning context for 180 s followed by an electric shock (0.7 mA, 2 s, constant current) delivered through a stainless steel grid floor. This training cycle was repeated once more and after a 30 s rest period, animals were returned to their home cages. Twenty-four hours after conditioning, contextual fear memory was tested in the fear conditioning box for 4 min without electric shock presentation. Freezing, defined as the lack of movement except for respiration and heart beat, was assessed by a time-sampling procedure every 10 s throughout the memory tests. In addition, mean activity was measured with the Ethovision system (Noldus, The Netherlands). One factor ANOVAs were used to analyze the velocity and freezing behavior during respective conditioning and retention tests. $p < .05$ was considered as significant. Data are expressed as means ± SEM. The procedures described in the present study were approved by the Dutch Animal Experiment Committee of the University of Groningen in compliance with Dutch law and internal regulations in accordance with Dutch biotechnology rules (GGO 03-167).

There was no difference between both groups in activity during training ($F_{(1,16)} < 1$, data not shown). However, enhanced freezing levels were found in the Dox-Ret group compared to the No-Dox1 group in the retention test 24 h after training ($78.90 \pm 2.47\%$ and $66.67 \pm 4.19\%$, respectively, $F_{(1,16)} = 5.912$, $p < .05$, Fig. 1c). These data indicate that reducing forebrain CaN activity facilitates the formation of an associative memory between context and the received foot-shock. In line with these findings, Ikegami & Inokuchi (2002) showed that bilateral ventricular administration of antisense DNA against calcineurin facilitates the formation of a contextual fear memory. Although the activity levels between the two groups did not differ during training, we cannot completely rule out the possibility that enhanced freezing was a result of secondary causes such as altered anxiety or pain sensation.

Next, we assessed whether the expression of the CaN inhibitor would affect the extinction of contextual fear. The conditioning protocol is shown in Fig. 1b. Two groups of 16–24 weeks old double transgenic male mice underwent a single training trial and retention test as described above (both groups without Dox administration). Again, there was no difference between both groups in activity during training ($F_{(1,15)} < 1$, data not shown). The next day, both groups showed similar freezing behavior during the retention test ($71.5 \pm 4.2$ and $68.5 \pm 4.0$ %, $F_{(1,15)} < 1$, Fig. 1d). After the retention test, both groups were left in their home cage for one week. During this week, one group received Dox-treatment to initiate the expression of the CaN inhibitor (Dox-Ext group, $n = 7$). The other group was left untreated (No-Dox group2, $n = 9$). After this week, mice

were daily re-exposed to the conditioning chamber for 4 min and freezing behavior was scored. A repeated measures ANOVA with treatment as between factor and trial as within factor were used to analyze freezing behavior during extinction followed by *post hoc t-tests with Bonferroni correction* when appropriate. In both groups, freezing behavior during the first extinction trial (day 10) was further enhanced compared to the levels found during the retention test (No-Dox: $86.8 \pm 3.8$, Dox-Ext: $87.8 \pm 5.7\%$, $F_{(1,145)} = 42.836$ $p < .001$, Fig. 1d). This finding indicates that the 4 min exposure to the CS during the retention test given 24 h after conditioning possibly initiated further consolidation or reconsolidation rather than extinction of the previously formed CS–US association. In agreement with these findings, Pedreira and Maldonado (2003) showed that depending on the duration of the exposure to the conditioned stimulus either reconsolidation or extinction takes place. Dox-treatment did not affect the freezing levels during the first extinction trial suggesting that reduced CaN activity does not affect further consolidation or reconsolidation.

With ongoing daily exposure to the conditioning chamber, freezing levels gradually decreased in both groups ($F_{(12,168)} = 19.611$ $p < .001$). The No-Dox group showed faster extinction of the freezing response compared to the Dox-Ext group as indicated by a significant interaction between treatment and trial ($F_{(12,168)} = 3.900$ $p < .001$, Fig. 1d). *Post hoc* analysis revealed that mice of the No-Dox group showed significantly lower freezing levels during extinction trial 9, 13 ($p < .05$) and 12 ($p < .01$). Untreated animals decreased their freezing levels 50% after 13 days of re-exposure to the CS without the US, while Dox-treated animals reduced their freezing levels only 20%. These findings suggest that forebrain CaN plays a crucial role in the extinction of contextual fear memories.

In this study, we examined whether decreased neuronal CaN activity in the forebrain affected memory formation of contextual fear as well as the extinction of a previously formed contextual fear memory. Using a genetic approach, we showed that reduced neuronal CaN activity in the forebrain facilitated the formation of a contextual fear memory. CaN counteracts several of the PKA actions through dephosphorylation of the same substrates (Mansuy, 2003). Since both proteins target the same substrates with opposing results, facilitation of one of these two proteins should have a similar effect on learning and memory processes as inhibition of the other of these two proteins. In line with this assumption, Abel et al. (1997) showed that reducing PKA activity in the forebrain reduced the formation of contextual fear memory.

A recent study by Isiegas, Park, Kandel, Abel, & Lattal (2006) revealed that reduced PKA activity in the forebrain facilitated fear extinction. They argued that decreased PKA activity might contribute to the removal of an inhibitory constraint imposed by PKA on CaN signalling during extinction. Based on this hypothesis, one would suggest that decreased CaN activity during extinction training would result in diminished extinction of fear. Indeed, we

showed that a reduction of CaN signalling in forebrain neurons deteriorates fear extinction. Our data give the first genetic evidence for a role of CaN in extinction learning and suggest that CaN plays an active and crucial role in extinction learning rather than acting as a mere passive molecular constraint.

The benefits of using a temporarily regulated system to study different phases of the learning process become particularly clear when our data are compared with the data from Zeng et al. (2001). They could not detect any changes in conditioning or extinction using mice in which CaN activity is continuously disrupted in distinct forebrain regions from five weeks of age onwards. We used a conditional transgenic system in which we could selectively reduce CaN activity in the forebrain during the different phases of the learning process. This allowed us to distinguish between conditioning and extinction and thus to circumvent any interaction processes between these two learning phases.

The question remains at which site (or sites), CaN inhibitor expression facilitates the formation of a memory for contextual fear and reduces the rate of contextual fear extinction. It is widely accepted that, depending on the learning task, different brain areas are involved in extinction learning (Myers & Davis, 2002). Due to the use of the CaMKII promoter, the inhibitor was selectively expressed in various forebrain regions including the hippocampus, prefrontal cortex and amygdala (Michalon, Koshibu, Baumgartel, Spirig, & Mansuy, 2005). In case of fear extinction, CaN in the amygdala might play a crucial role, since pharmacological intervention of CaN activity in this brain region inhibits the extinction of tone cued fear conditioning in rats (Lin et al., 2003). To our knowledge, there have no experiments been conducted yet to test whether inhibition of CaN activity in the hippocampus or prefrontal cortex inhibits the extinction of contextual fear memory. Future experiments using a transgenic or pharmacological approach should clarify whether CaN in the hippocampus, amygdala and/or prefrontal cortex plays a crucial role in the extinction of this type of fear. Understanding the signalling pathways underlying the extinction of previously learned responses could ultimately lead to new therapeutic strategies to treat anxiety related disorders.

## Acknowledgments

## References

Abel, T., Nguyen, P. V., Barad, M., Deuel, T. A., Kandel, E. R., & Bourtchouladze, R. (1997). Genetic demonstration of a role for PKA in the late phase of LTP & in hippocampus-based long-term memory. *Cell, 88*, 615–626.

Ikegami, S., & Inokuchi, K. (2002). Antisense DNA against calcineurin facilitates memory in contextual fear conditioning by lowering the threshold for hippocampal long-term potentiation induction. *Neuroscience, 98*, 637–646.

Isiegas, C., Park, A., Kandel, E. R., Abel, T., & Lattal, K. M. (2006). Transgenic inhibition of neuronal protein kinase A activity facilitates fear extinction. *Journal of Neuroscience, 26*, 12700–12707.

Lin, C. H., Yeh, S. H., Leu, T. H., Chang, W. C., Wang, S. T., & Gean, P. W. (2003). Identification of calcineurin as a key signal in the extinction of fear memory. *Journal of Neuroscience, 23*, 1574–1579.

Malleret, G., Haditsch, U., Genoux, D., Jones, M. W., Bliss, T. V., Vanhoose, A. M., et al. (2001). Inducible & reversible enhancement of learning, memory, & long-term potentiation by genetic inhibition of calcineurin. *Cell, 104*, 675–686.

Mansuy, I. M. (2003). Calcineurin in memory and bidirectional plasticity. *Biochemical and Biophysical Research Communications, 311*, 1195–1208.

Mansuy, I. M., Mayford, M., Jacob, B., Kandel, E. R., & Bach, M. E. (1998a). Restricted & regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell, 92*, 39–49.

Mansuy, I. M., Winder, D. G., Moallem, T. M., Osman, M., Mayford, M., Hawkins, R. D., et al. (1998b). Inducible & reversible gene expression with the rtTA system for the study of memory. *Neuron, 21*, 257–265.

Maren, S., & Quirk, G. J. (2004). Neuronal signalling of fear memory. *Nature Reviews Neuroscience, 5*, 844–852.

Martin, S. J., Grimwood, P. D., & Morris, R. G. (2000). Synaptic plasticity & memory: An evaluation of the hypothesis. *Annual Reviews Neuroscience, 23*, 649–711.

Michalon, A., Koshibu, K., Baumgartel, K., Spirig, D. H., & Mansuy, I. M. (2005). Inducible & neuron-specific gene expression in the adult mouse brain with the rtTA2S-M2 system. *Genesis, 43*, 205–212.

Miyakawa, T., Leiter, L. M., Gerber, D. J., Gainetdinov, R. R., Sotnikova, T. D., Zeng, H., et al. (2003). Conditional calcineurin knockout mice exhibit multiple abnormal behaviors related to schizophrenia. *Proceedings of the National Academy of Sciences USA, 100*, 8987–8992.

Myers, K. M., & Davis, M. (2002). Behavioral and neural analysis of extinction. *Neuron, 36*, 567–584.

Pedreira, M. E., & Maldonado, H. (2003). Protein synthesis subserves reconsolidation or extinction depending on reminder duration. *Neuron, 38*, 863–869.

Sigurdsson, T., Doyere, V., Cain, C. K., & LeDoux, J. E. (2007). Long-term potentiation in the amygdala: A cellular mechanism of fear learning and memory. *Neuropharmacology, 52*, 215–227.

Tokuda, M., & Hatase, O. (1998). Regulation of neuronal plasticity in the central nervous system by phosphorylation & dephosphorylation. *Molecular Neurobiology, 17*, 137–156.

Zeng, H., Chattarji, S., Barbarosie, M., Rondi-Reig, L., Philpot, B. D., Miyakawa, T., et al. (2001). Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity & working/episodic-like memory. *Cell, 107*, 617–629.