

NUCLEAR PROTEIN PHOSPHATASE-1: AN EPIGENETIC REGULATOR OF FEAR MEMORY AND AMYGDALA LONG-TERM POTENTIATION

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Abstract—Complex brain diseases and neurological disorders in human generally result from the disturbance of multiple genes and signaling pathways. These disturbances may derive from mutations, deletions, translocations or rearrangements of specific gene(s). However, over the past years, it has become clear that such disturbances may also derive from alterations in the epigenome affecting several genes simultaneously. Our work recently demonstrated that epigenetic mechanisms in the adult brain are in part regulated by protein phosphatase 1 (PP1), a protein Ser/Thr phosphatase that negatively regulates hippocampus-dependent long-term memory (LTM) and synaptic plasticity. PP1 is abundant in brain structures involved in emotional processing like the amygdala, it may therefore be involved in the regulation of fear memory, a form of memory related to post-traumatic stress disorder (PTSD) in human. Here, we demonstrate that PP1 is a molecular suppressor of fear memory and synaptic plasticity in the amygdala that can control chromatin remodeling in neurons. We show that the selective inhibition of the nuclear pool of PP1 in amygdala neurons significantly alters posttranslational modifications (PTMs) of histones and the expression of several memory-associated genes. These alterations correlate with enhanced fear memory, and with an increase in long-term potentiation (LTP) that is transcription-dependent. Our results underscore the importance of nuclear PP1 in the amygdala as an epigenetic regulator of emotional memory, and the relevance of protein phosphatases as potential targets for therapeutic treatment of brain disorders like PTSD. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: PP1, histone posttranslational modifications, fear memory, LTP, epigenetic, amygdala.

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Abbreviations: CREB, cAMP response element-binding protein; CREM, cAMP-responsive element modulator; dox, doxycycline; EGFP, enhanced green fluorescent protein; ERK/MAPK, extracellular signal-regulated kinase/mitogen-activated protein kinase; HATs, histone acetyl transferases; HDACs, histone deacetylases; LA, lateral nucleus of the amygdala; LTM, long-term memory; LTP, long-term potentiation; NF- κ B, nuclear factor kappa B; NIPP1, nuclear inhibitor of PP1; PKs, protein kinases; PPs, protein phosphatases; PP1, protein phosphatase 1; PTMs, posttranslational modifications; PTSD, post-traumatic stress disorder; RT-PCR, reverse transcriptase-polymerase chain reaction.

Fear memory is a form of emotional memory that recruits the amygdala (Rodrigues et al., 2004; Maren, 2005; Sah and Westbrook, 2008), and is often disturbed in individuals suffering from post-traumatic stress disorder (PTSD) (Bremner et al., 2005; Shin et al., 2005; Ressler and Mayberg, 2007). Similar to other forms of memory, the establishment and the maintenance of long-lasting forms of fear memory largely depend on gene transcription. Recent studies have shown that this involves epigenetic mechanisms that regulate posttranslational modifications (PTMs) of histone proteins, in particular acetylation and phosphorylation (Kandel, 2001; Levenson and Sweatt, 2005). Histone acetylation is a PTM that requires histone acetyl transferases (HATs) and deacetylases (HDACs), while histone phosphorylation is regulated by the combined action of protein kinases (PKs) and phosphatases (PPs). In the brain, PKs such as extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and mitogen- and stress-activated protein kinase 1 (MSK1) are known to contribute to the epigenetic regulation of long-term memory (LTM) (Chwang et al., 2006, 2007). These PKs are also known to modulate long-lasting forms of synaptic plasticity such as long-term potentiation (LTP) in the lateral nucleus of the amygdala (LA), a cellular model of fear memory (Huang et al., 2000; Schafe et al., 2008). However to date, the functional role of PPs in the epigenetic regulation of fear memory and amygdala plasticity remains largely unknown.

Among the PPs expressed in the brain, serine/threonine protein phosphatase 1 (PP1) is one of the most important for cognitive functions. It is known to be a potent molecular suppressor of memory formation and hippocampal LTP (Genoux et al., 2002; Munton et al., 2004; Koshibu et al., 2009; Gräff et al., 2010). Here, we newly demonstrate that PP1 is also an essential modulator of fear memory, and a regulator of synaptic plasticity in the amygdala. We show that the nuclear pool of PP1 in neurons of the amygdala is involved in the control of several histone PTMs, and in the expression of specific genes, and that inhibiting this pool by conditional transgenesis enhances fear memory and amygdala LTP.

EXPERIMENTAL PROCEDURES

Animals

Transgenic mice in C57Bl/6J background carrying a fragment of the nuclear inhibitor of PP1 spanning amino acids 143 to 224 (NIPP1*) fused to EGFP (enhanced green fluorescent protein) and linked to a tetO promoter or co-expressed with LacZ via a bidirectional tetO promoter were generated as described previously (Koshibu et al., 2009; Gräff et al., 2010). Adult (3–8 months of age) NIPP1* transgenic males were treated with doxycycline

(dox) (6 mg/g of food for at least 8 days, Westward Pharmaceuticals). For on/off experiments, mice were treated with dox for at least 8 days, then dox was withdrawn for at least 7 days before testing. Mutant off group represents NIPP1* animals not treated with dox. Animals were maintained in accordance with the Federation of Swiss Cantonal Veterinary Office and European Community Council Directive (86/609/EEC) guidelines. Experiments were run in a way to minimize the number of animals and their suffering.

Behavior

Fear conditioning was performed as previously described (Koshibu et al., 2005). Briefly, mice were placed in a box with a shock-grid floor for 2 min then exposed to three pairings of a continuous 30-s tone (80 dB, 2000 Hz) and a 0.5 mA foot-shock delivered during the last 2 s of the tone. Tone-shock pairing was repeated at 1 min interval. The freezing response (time spent with no movement except for breathing) was tested 24 h later in the same context to assess contextual fear memory, or in a new context with a 3-min tone to assess cued fear memory.

Immunohistochemistry and Western blot

For transgene detection, paraformaldehyde-fixed brain sections were incubated in primary antibody against green fluorescent protein (GFP) (1:1000, rabbit, Synaptic Systems) or β -galactosidase (β -gal; 1:1000, Sigma), followed by goat anti-rabbit IgG secondary antibody (1:1000, Jackson ImmunoResearch) and 3,3'-diaminobenzidine (DAB) (Koshibu et al., 2009; Gräff et al., 2010). Images were acquired with an Axiophot microscope (Zeiss) and MCID Elite 7.0 software (MCID). For histone PTMs analyses, nuclear fractions were prepared from the entire amygdala of naive animals, resolved on 10–12% SDS-PAGE, transferred onto a nitrocellulose membrane (Bio-Rad), then incubated in one of the following primary antibody: anti-H3 (1:2000) or -H4 (1:2000) (Upstate); anti-phospho H3T3 (1:1000), -H3T11 (1:1000), -H3S28 (1:1000) (Abcam) or -H3S10 (1:1000), anti acetyl-H3K9 (1:1000), -H3K14 (1:1000) or -H4K5 (1:2000) (Upstate), or anti dimethyl-H3K4 (1:1000) or trimethyl H3K36 (1:2000) (Abcam); H1.0 (1:1000) (Abcam). Secondary antibodies were goat anti-rabbit IRDye 680 nm (1:10000) and goat anti-mouse IRDye 800 nm (1:10000) (Li-Cor Biosciences). The signal was normalized to histone H1.0, then to control littermates.

Quantitative real time RT-PCR (qRT-PCR)

Total RNA was extracted from the entire amygdala from behaviorally naive animals using Macherey Nagel's NucleoSpin Kit II. cDNA was synthesized using the SuperScript First-Strand Synthesis System for reverse transcriptase-polymerase chain reaction (RT-PCR) II (Invitrogen) as described previously (Koshibu et al., 2009). qRT-PCR was performed using Taqman probes (Applied Biosystems) on an Applied Biosystems 7500 Thermal Cycler. The comparative Ct method was used to assess the difference in gene expression between samples (Livak and Schmittgen, 2001). β -actin was used as internal control.

HDAC activity assay

HDAC activity was determined in nuclear extracts (50 μ g) using a colorimetric assay kit (Abcam), and expressed as optical density at 405 nm/ μ g protein. Activity in control samples was used for normalization.

In vitro electrophysiology

Coronal slices for LA recordings were prepared from adult brain in behaviorally naive animals as described previously (Gräff et al.,

2010). Test stimulus intensity was set to evoke 30–50% of the maximum field-excitatory postsynaptic potential (f-EPSP). Recorded signals were amplified with an AXOPATCH 200 B amplifier (Axon Instruments/Molecular Devices) and sampled using pCLAMP. aCSF contained 119 mM NaCl, 1.3 mM $MgCl_2 \cdot 6H_2O$, 1.3 mM NaH_2PO_4 , 2.5 mM KCl, 2.5 mM $CaCl_2$, 26 mM $NaHCO_3$, 11 mM D-glucose. A borosilicate electrode filled with aCSF was placed in the external capsule for stimulation, and the recording electrode was placed in LA. LTP was induced by three trains of high frequency stimulation (HFS; 100 Hz, 1 s, every 20 s) with the stimulation amplitude set at test stimulus intensity. For transcription-dependent experiments, slices were pre-incubated in 25 μ M actinomycin D at least 2 h before recording. Input-output curve and paired pulse facilitation (PPF) were conducted before the LTP experiment. The stimulation intensity for both analyses was set at the test stimulus intensity. The inter-stimulus interval for PPF was 50 ms.

Statistics

ANOVAs and univariate or multivariate general linear model (GLM) were used to determine genotype and treatment effect, and Tukey or LSD post hoc analyses when appropriate. Statistical significance was set at $P \leq 0.05$ (*) and $P \leq 0.01$ (**). All values are expressed as mean \pm SEM.

RESULTS

Inhibition of nuclear PP1 alters histone PTMs and gene expression in the amygdala

We investigated the effect of the inhibition of nuclear PP1 on histone PTMs in the amygdala by examining the level of histone phosphorylation, acetylation, and methylation of several selected residues in the NIPP1* mice. Western blot analyses revealed that phosphorylation of serine 10 (S10) on H3 was specifically increased by inhibition of nuclear PP1. This increase was restricted to S10 as the phosphorylation of other residues including threonine 3 (T3), T11 or S28 was not altered (Fig. 1a, b). Because PP1 can form a complex with HDACs and co-regulate histone PTMs (Canettieri et al., 2003; Brush et al., 2004), we next examined whether histone acetylation is also altered. We found that the acetylation of H3 lysine 14 (H3K14) and H4K5 was increased (Fig. 1a, b). Further, consistent with an increase in acetylation, HDAC activity was significantly reduced in the NIPP1* mice (Fig. 1c), possibly as a result of the dissociation of PP1 and HDACs following PP1 inhibition (Koshibu et al., 2009). H3K9 acetylation was however not changed, most likely due to steric hindrance with the neighboring phosphorylated S10 as previously suggested (Edmondson et al., 2002; Latham and Dent, 2007).

Further, because histone methylation is often co-regulated with histone phosphorylation and acetylation (Latham and Dent, 2007; Li et al., 2007), we also examined whether it is affected by inhibition of nuclear PP1. We looked at di- and trimethylation, which can co-occur on histones and have different effects on gene transcription. Dimethylation of H3K4, a marker of transcriptional initiation, was not altered but trimethylation of H3K36, a marker of transcriptional elongation (Li et al., 2007) was increased by NIPP1* expression (Fig. 1a, b). These results overall

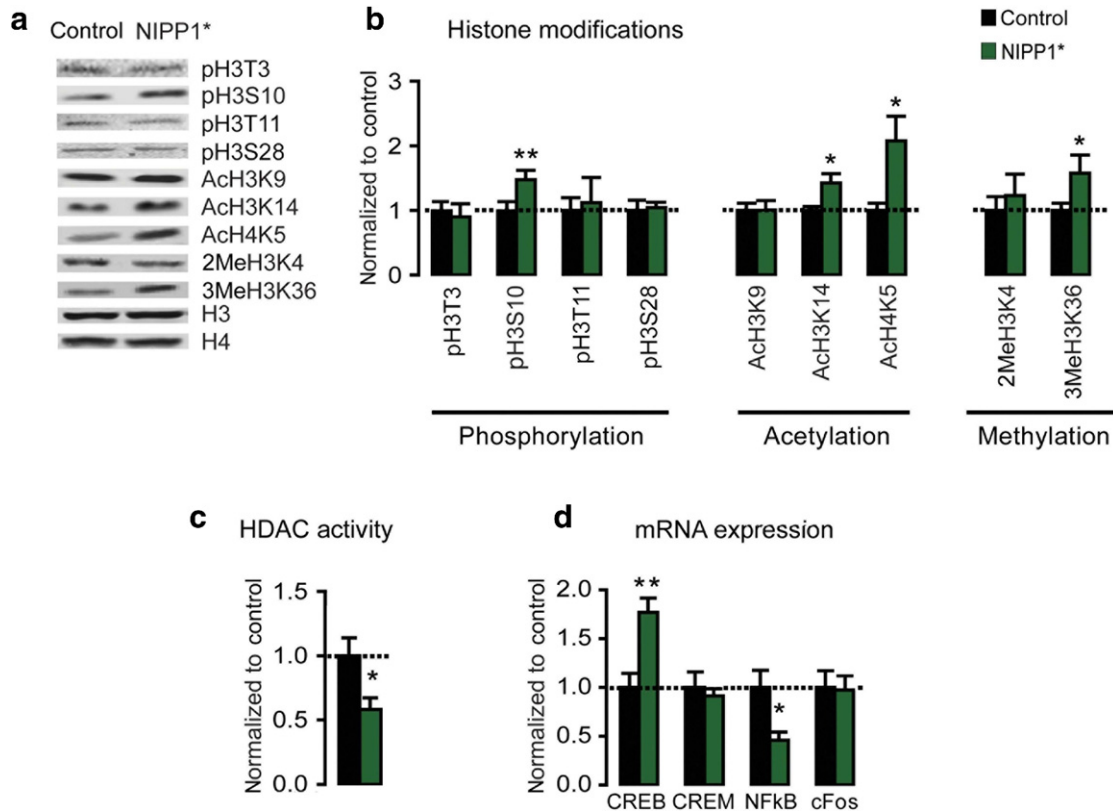


Fig. 1. Histone PTMs, HDAC activity and gene transcription are altered by inhibition of nuclear PP1 in the amygdala. (a) Representative Western blots and (b) corresponding quantitative analyses of phosphorylation of H3T3 (Control, $n=10$; NIPP1*, $n=9$), H3S10 (Control, $n=9$; NIPP1*, $n=9$; $F(1,16)=5.45$, $P<0.05$), H3T11 (Control, $n=10$; NIPP1*, $n=9$), H3S28 (Control, $n=10$; NIPP1*, $n=9$), acetylation of H3K9 (Control, $n=9$; NIPP1*, $n=9$), H3K14 (Control, $n=9$; NIPP1*, $n=8$; $F(1,15)=7.77$, $P<0.05$), and H4K5 (Control, $n=5$; NIPP1*, $n=7$; $F(1,10)=5.53$, $P<0.05$), dimethylation of H3K4 (Control, $n=8-11$; NIPP1*, $n=7-10$), and trimethylation of H3K36 (Control, $n=11$; NIPP1*, $n=7$; $F(1,16)=4.78$, $P<0.05$) in nuclear extracts from the amygdala of NIPP1* expressing mice and control littermates. (c) HDAC activity in the amygdala (NIPP1*, $n=5$, Control, $n=4$; $F(1,7)=6.87$, $P<0.05$). (d) Quantitative RT-PCR showing CREB (Control, $n=7$; NIPP1*, $n=8$; $F(1,13)=13.7$, $P<0.01$), CREM (Control, $n=9$; NIPP1*, $n=8$), NFκB (Control, $n=10$; NIPP1*, $n=9$; $F(1,17)=7.34$, $P<0.05$), and cFos (Control, $n=7$; NIPP1*, $n=6$) mRNA expression in the amygdala. Data are normalized to control littermates. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

indicate that nuclear PP1 regulates specific residues on several histone PTMs in amygdala neurons.

Since histone PTMs are known to contribute to transcriptional regulation, we next examined whether gene expression is altered by inhibition of nuclear PP1 in the amygdala. We focused on four genes known to be important for fear memory: the transcription factors CREB (cAMP response element-binding protein) and NFκB (nuclear factor kappa B), which are involved in fear memory formation and synaptic plasticity (Ploski et al., 2010; Lubin and Sweatt, 2007), the CREB modulator CREM (cAMP-responsive element modulator) (Balschun et al., 2003), and cFos (FBJ osteosarcoma oncogene), an immediate early gene implicated in fear conditioning and extinction in the hippocampus (Radulovic and Tronson, 2010; Tronson et al., 2009). qRT-PCR analyses revealed that while CREB expression was increased, NFκB expression was decreased (Fig. 1d), but CREM and cFos expression was not changed (Fig. 1d). Overall, these results suggest that further to regulating specific histone PTMs, nuclear PP1 also controls the transcription of several genes in the amygdala.

Inhibition of nuclear PP1 enhances long-term contextual and tone fear memory

We next examined the effect of PP1 inhibition on fear memory. The mice were trained on contextual and tone fear conditioning, and fear memory was tested 10 min or 24 h after conditioning. Both contextual and tone fear memory were enhanced by NIPP1* expression 24 h (Fig. 2a, b) but not 10 min (Fig. 2c, d) after conditioning, indicating a selective enhancement of LTM. This enhancement was likely a direct effect of NIPP1* expression since fear memory was normal and comparable to that in control animals in the absence of transgene expression (mutant off). Further, it could be reversed by suppression of NIPP1* expression by dox removal in the mutant mice (Mutant on/off) (Fig. 2a, b). Finally, another transgenic line expressing NIPP1* in forebrain structures but not in LA, and showing enhanced hippocampal-dependent memory (Gräff et al., 2010) had normal fear memory (Fig. 4a, b), pointing to the selective effect of PP1 inhibition in the amygdala on fear memory.

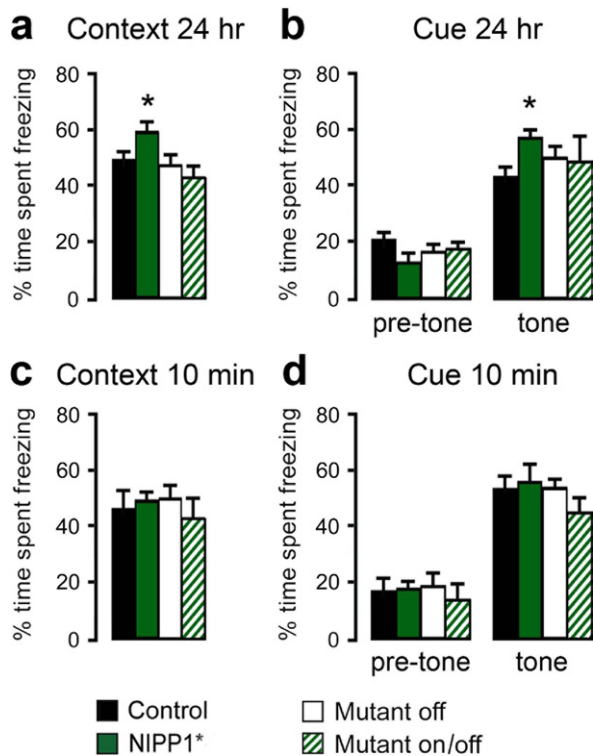


Fig. 2. Fear memory is improved by inhibition of nuclear PP1. (a) Time spent freezing 1 d after contextual fear conditioning in NIPP1* mice ($n=7$), NIPP1* mice off dox ($n=3$), NIPP1* mice on/off dox ($n=6$) and control littermates ($n=8$) ($F(3,20)=2.68$, $P<0.05$, for effect of treatment; Tukey post-hoc: NIPP1* vs. Control, $P<0.05$; NIPP1* vs. Mutant off, $P<0.05$; NIPP1* vs. Mutant on/off, $P<0.05$; all other comparisons, ns). (b) Time spent freezing 1 d after cued fear conditioning in NIPP1* mice ($n=7$), NIPP1* mice off dox ($n=3$), NIPP1* mice on/off dox ($n=6$) and control littermates ($n=8$) before (left panel) and after (right panel) the onset of the tone (ns for the effect of treatment before and after the onset of the tone, $F(3,20)=3.94$, $P<0.05$, for effect of treatment after the onset of the tone; Tukey post-hoc: NIPP1* vs. Control, $P<0.05$; NIPP1* vs. Mutant off, $P<0.05$; NIPP1* vs. Mutant on/off, $P<0.05$; all other comparisons, ns). (c) Time spent freezing 10 min after contextual fear conditioning in NIPP1* mice ($n=5$), NIPP1* mice off dox ($n=3$), NIPP1* mice on/off dox ($n=4$) and control littermates ($n=6$). (d) Time spent freezing 10 min after tone fear conditioning in NIPP1* mice ($n=5$), NIPP1* mice off dox ($n=3$), NIPP1* mice on/off dox ($n=4$) and control littermates ($n=6$) before (left panel) and after (right panel) the onset of the tone, ns for both the effect of treatment before and after the onset of the tone. Cue-dependent memory was specific to the tone since the freezing response before tone onset was normal in the NIPP1* mice. Control data are in black; NIPP1* data are in green; mutant off data are in white; mutant on/off data are in green and white stripes. Statistical significance is indicated by * $P<0.05$. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

LTP in the amygdala is increased by inhibition of nuclear PP1 in a transcription-dependent manner

To determine whether inhibiting nuclear PP1 also impacts synaptic plasticity, we examined LTP in LA *in vitro*. LTP was induced in acute amygdala slices by stimulating the external capsule with three trains of HFS. In these conditions, LTP was significantly increased in the transgenic mice compared to control littermates (Fig. 3a, b). The increased LTP did not result from altered basal synaptic

transmission since input-output and paired pulse responses were normal in the transgenic slices (Fig. 3d, e). Further, it was specific to NIPP1* expression in LA since it was not observed in another transgenic line expressing NIPP1* in other forebrain structures except LA (Fig. 4c, d). Because PP1 has been associated with gene transcription, we also examined whether the LTP enhancement was transcription-dependent. Preincubation of amygdala slices with the transcription inhibitor actinomycin D abolished the LTP increase, and restored LTP to a level comparable to that in control slices (Fig. 3c), indicating that this increase was transcription-dependent.

DISCUSSION

The present results identify nuclear PP1 as a regulator of histone PTMs and a potent suppressor of fear memory and synaptic plasticity in the amygdala. They show that inhibiting the nuclear pool of PP1 in neurons in the amygdala alters the phosphorylation, acetylation and methylation of specific residues on several histone proteins, and enhances long-term fear memory, and amygdala LTP in a transcription-dependent manner. These changes are also shown to be associated with an alteration in the expression of several genes. Together with previous findings (Genoux et al., 2002; Koshibu et al., 2009; Gräff et al., 2010), the present data provides strong evidence that PP1 is a universal negative regulator of memory and synaptic plasticity that acts in several forebrain areas to establish a specific histone code and modulate chromatin remodeling. They show that H3S10 phosphorylation, H3K9 and H3K14 acetylation, and H3K36 are regulated by nuclear PP1 across different brain areas in particular, the hippocampus and the amygdala (Koshibu et al., 2009; Gräff et al., 2010). These histone modifications have previously been reported to be associated with transcriptional activation and can cross-regulate each other (Kouzarides, 2007; Latham and Dent, 2007). In particular, both H3S10 phosphorylation and H3K36 methylation can enhance H3K14 acetylation, while H3S10 phosphorylation can interfere with H3K9 acetylation and methylation. These findings support the notion that these particular histone PTMs may represent a conserved epigenetic signature of LTM, and that nuclear PP1 is a crucial regulator of these key histone modifications.

The expression level of a subset of transcription factors or regulators (i.e. CREB and NF κ B) is altered as a consequence of the inhibition of nuclear PP1. However, these genes are affected differently, and while CREB expression is increased, NF κ B expression is decreased. Notably, other factors such as CREM or cFos are not altered. These results indicate that nuclear PP1 affects several specific genes important for synaptic plasticity and memory formation (Silva et al., 1998; Kida et al., 2002; Yeh et al., 2002, 2004; Bourchouladze et al., 2003), but in an opposite manner. The consequence of the changes in CREB and NF κ B expression is difficult to delineate precisely, but increased CREB expression may provide a mechanistic link for the observed LTM and LTP enhancement since

Fig. 3. LTP in LA is enhanced in a transcription-dependent manner. (a) Coronal section of adult amygdala showing NIPP1* expression (GFP immunostaining, brown signal) and placement of stimulating and recording electrodes. (b, c) LTP in LA in NIPP1* mice and control littermates (b) without actinomycin D (NIPP1*, $n=9$; Control, $n=14$) or (c) with actinomycin D (NIPP1*, $n=6$; Control, $n=9$). $F(4,39)=10.83$, $P<0.001$; Tukey post-hoc for control versus NIPP1*, $P=0.001$; control versus NIPP1* with actinomycin D, ns; actinomycin D effect on control slices, ns; actinomycin D effect on NIPP1* slices, $P<0.001$. LA, lateral nucleus of the amygdala; Ce, central nucleus of the amygdala; BLA, basolateral nucleus of the amygdala. Representative traces before and after LTP are shown above the graphs (scale: 0.5 mV over 5 ms). (d) Input-output curve showing similar basal transmission in LA of NIPP1* mutant and control slices. (e) Paired pulse stimulation in LA showing comparable response in NIPP1* mutant and control slices. Paired pulse responses are normalized to controls. Control data are in black, NIPP1* mutant data are in green. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

CREB is important for long-lasting forms of amygdala-dependent memory (Silva et al., 1998; Josselyn et al., 2001). In contrast, the decrease in NF κ B may rather weaken memory since NF κ B has been reported to positively regulate memory. However, NF κ B activation was also found to correlate with impaired spatial memory in a mouse model of Alzheimer's disease (Arancio et al., 2004), an effect that may be associated with the pro-inflammatory properties of NF κ B known to lead to cell death (Kaltschmidt et al., 2005). This possibility would suggest that downregulation of NF κ B may favor the reduction of inflammatory response and facilitate LTM and synaptic plasticity in the NIPP1* mice. However, until inflammatory and/or cell death responses are assessed in the NIPP1* mice, the significance of decreased NF κ B expression remains uncertain. Alternatively, the decrease in NF κ B expression may be induced to counteract the increased CREB expression, and may explain the relatively small enhancement in fear memory observed in NIPP1* mice.

Finally, the small memory enhancement may result from the fact that PP1 is inhibited only moderately, and selectively in neurons (neuron-specific transgene expression) in the amygdala. Because only a few percent of neurons are activated during fear memory (reviewed in Josselyn, 2010), it is possible that the NIPP1 transgene is expressed in a subpopulation of these neurons, and thus that its beneficial effect is limited.

Overall, these data provide novel potential mechanistic insight into the epigenetic processes involved in complex brain functions, and suggest the possibility that PP1-dependent chromatin regulation may underlie disorders affecting emotional memory. While epigenetic mechanisms have been implicated in numerous neurological and psychiatric disorders including Alzheimer's disease, Rett syndrome, schizophrenia, and depression (Tsankova et al., 2007; Gräff and Mansuy, 2008), their relevance for fear memory disorders such as PTSD has only recently been considered. There is currently no firm evidence for a direct

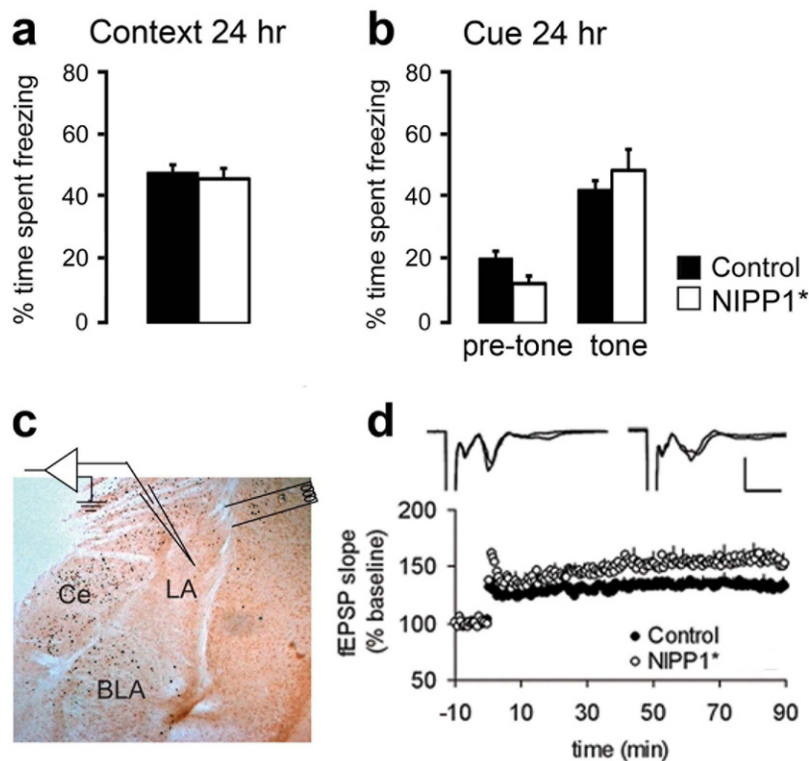


Fig. 4. No change in fear memory or amygdala LTP in another NIPP1* transgenic line. (a, b) In mice expressing NIPP1* in forebrain neurons except in the amygdala, both context and tone fear memory are normal 24 h after training. (c) Immunohistochemistry showing no NIPP1* expression in LA and minimal expression in central (Ce) and basolateral (BLA) nuclei (dark blue). Placement of stimulating and recording electrode is indicated. (d) LTP in LA is normal in the absence of NIPP1* expression. Control data are in black, NIPP1* mutant data are in white. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

involvement of epigenetic processes in PTSD or PTSD risk, but it was recently observed that this risk is associated with childhood adversity and PTSD in mothers, suggesting such involvement (Yehuda and Bierer, 2009). Given the strong relevance of fear memory for PTSD (Bremner et al., 2005; Shin et al., 2005; Ressler and Mayberg, 2007; Koenigs and Grafman, 2009), the present data suggest the possible implication of PP1 in the etiology of PTSD. Interestingly, CREB, whose expression is controlled by PP1-dependent processes, has been considered as a promising target for PTSD treatment (Breithaupt and Weigmann, 2004). Thus, the present findings may provide a novel direction for the development of potential therapeutic approaches for the treatment for brain disorders such as PTSD.

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