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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.

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

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 C57Bl6/J 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

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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; NFκB, 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.

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(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 μg) using a colorimetric assay kit (Abcam), and expressed as optical density at 405 nm/μg 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 $MgCl_2.6H_2O$, 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 μM 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±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