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Research Article

Cocaine Regulates the Salt-Inducible Kinase (SIK1) by Inducing Protein Phosphatase-2A Expression in Rat Brain

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Abstract We have recently documented that cocaine-induced *MEF2C* expression in rat brain is mediated by the activation of the salt-inducible kinase SIK1, which is itself regulated by phosphorylation mechanisms. We report here that acute or chronic treatment of rats with cocaine increased the expression of the catalytic subunit of protein phosphatase PP2A in the prefrontal cortex and striatum. Cocaine treatment also reduced the number of cortical neurons expressing SIK1 phosphorylated on Ser-577, but not on Thr-182. To further support the hypothesis that phospho-SIK1-S577 is a substrate of PP2A, we used Neuro-2A cell cultures overexpressing either the wild-type PP2A, in which the amount of phospho-SIK1-S577 was found to be decreased, or a mutant PP2A devoid of enzymatic activity, in which the level of phospho-SIK1-S577 was increased when compared to control cells. The data indicate that, by inducing PP2AC, cocaine regulates the nuclear location and activity of SIK1 and HDAC5, which ultimately govern the activity of CREB and MEF2C transcription factors. The results highlight PP2A as a novel target for regulating long-term effects of cocaine.

Keywords cocaine; salt-inducible kinase 1; protein phosphatase type 2A; histone deacetylase 5; rat brain

1. Introduction

The Ser/Thr protein kinase SIK (salt-inducible kinase) was first identified as an enzyme induced in adrenal glands of rats fed with a high-salt diet [28]. Three SIK isoforms (SIK1–3) have been identified today as belonging to the family of AMP-activated protein kinases [14,24]. SIK1 is encoded by an immediate early gene which is induced by depolarization in PC12 cells [9]. The *SIK1* gene is expressed in rat brain, predominantly in cortex and hippocampus, in which it is also induced by depolarization. While SIK1 has been shown to be involved in the development of cortical neurons by controlling TORC1 phosphorylation [9,17], only few data have unveiled a role for SIK1 in the mature brain. We have recently shown that acute cocaine injection to rats induces the expression of MEF2C transcription factor in the striatum through a mechanism that involves the subsequent activation of SIK1 and the phosphorylation of HDAC5, a member of the class IIa of HDACs [8]. Cocaine activates SIK1 by phosphorylation, which is accompanied by the nuclear import of the kinase. In the nuclear compartment, SIK1 then phosphorylates HDAC5 causing the shuttling of its phospho-form from the nucleus to the cytoplasm of cells, a process which finally results in the activation of MEF2C [4,18]. Activation of the MEF2C transcription factor is a likely candidate mechanism for participating in the long-term effects of cocaine, including the induction of an addictive behavior, since it is well recognized that these effects are brought about by long-lasting changes in gene expression.

Not much is known on how the pathway activated by SIK1 phosphorylation is ultimately reversed. Given that protein phosphatase-type 2A (PP2A) controls many cellular functions and that it represents a major constitutively expressed protein phosphatase in the brain [15], we checked in the present study whether PP2A would be able to regulate the levels of phospho-SIK1 and/or phospho-HDAC5. PP2A holoenzyme is a multiprotein complex composed by the catalytic subunit PP2AC and a structural subunit A, to which a regulatory subunit B associates, conferring substrate specificity to the enzyme [27]. Its enzymatic activity is regulated by the reversible methylation of the PP2AC subunit and various inhibitors have been characterized, including the simian virus 40 small tumor antigen (SV40 small t) [5,22]. We first examined the effect of cocaine on PP2AC expression in the prefrontal cortex (PFCx) and striatum, two dopaminergic structures playing a crucial role in the brain reward system [6,21]. We then checked whether cocaine treatment was able to regulate the amount of phospho-SIK1, using antibodies directed against the phospho-forms of the proteins. Our results suggest that SIK1 and PP2A may act as possible opposite effectors that

subtly control the synthesis of MEF2C transcription factor in response to cocaine in dopaminergic brain structures.

2. Materials and methods

2.1. Animals and tissue preparation

Male Wistar rats (175–200 g; Janvier, France) were housed with a fixed 12 h light-dark cycle and free access to food and water. All procedures involving animal care were conducted in compliance with national laws and policies (Council directive 87848, 1987, Service Vétérinaire de la Santé et de la Protection Animale; permission 67–165 to JZ) and with international guidelines (NIH publication 5586-23, 1985). Rats were intraperitoneally (IP) injected either acutely or repeatedly for 10 days (one injection per day) with 20 mg/kg cocaine hydrochloride (Cooper, Melun, France) or an equivalent volume of saline (NaCl 0.9%). Fifteen hours after injection, or after the last injection of a series of ten, animals were sacrificed by an overdose of pentobarbital (100 mg/kg, IP) and perfused transcardially with 50 mL saline followed by 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.2; 250 mL). The brains were kept overnight at 4 °C in 15% sucrose, frozen in isopentane at −40 °C, and then stored at −80 °C. Coronal tissue sections (30 μm) were obtained using a vibratome (Leica VT 1000S) and stored in Watson cryoprotective medium (30% sucrose, 1% polyvinylpyrrolidone 40, 30% ethyleneglycol, and 0.9% NaCl in 0.1 M phosphate buffer, pH 7.2) before use. For immunoblotting experiments, striatum and medial PFCx (mPFCx) were dissected from rats treated similarly with cocaine, but not perfused with paraformaldehyde.

2.2. Antibodies

The following primary antibodies were used for both immunohistochemistry and immunoblotting experiments: rabbit polyclonal antibodies against HDAC5 (Abcam, UK) and against PP2AC (Millipore Biotech., Billerica, MA, USA); goat polyclonal antibodies against HDAC5, PP2AC, and SIK1 (Santa Cruz Biotech., CA, USA). Rabbit polyclonal antibodies against SIK1 and against phospho-SIK1 (phospho-SIK1-S577 and phospho-SIK1-T182) were from the lab of Dr. H. Takemori. Secondary antibodies used were donkey-antirabbit and donkey-antigoat peroxidase-conjugated IgG (Jackson Laboratories, West Grove, PA, USA).

2.3. Immunohistochemistry

Free-floating sections were washed in 600 μL of 50 mM Tris-buffered saline, pH 7.4 (TBS) and in the same volume of blocking buffer consisting of TBS supplemented with 5% bovine serum albumin and 0.5% (v/v) Triton X-100 (Euromedex, Souffelweyersheim, France). Sections were incubated overnight in primary antibody against PP2AC (diluted 1:200), against phospho-SIK1-S577 (diluted 1:500) or against phospho-SIK1-T182 (diluted 1:500). After 5 washes with TBS, they were incubated with the secondary peroxidase-conjugated antibody (diluted 1:25,000) for 2 h and the immunochemistry was carried out essentially as previously described [3]. Following immunohistochemical labeling, sections were incubated for 15 min in 2.5 μM Hoechst 33258 (Sigma-Aldrich) solution to label nuclei and covers lipped with Mowiol. Photomicrographs were taken with an Axiocam camera (Zeiss, Germany). Percentage of immunoreactive cells in the mPFCx or striatum was calculated by counting an average of 800 cells from each section. Each counting was performed twice by an investigator blind to the identity of the samples.

2.4. Gel electrophoresis and immunoblotting

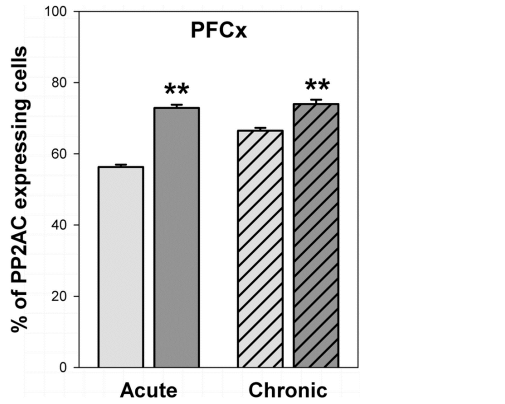
SDS-PAGE of brain tissue extract and immunoblotting were performed as described previously [7]. Precision Plus Protein Kaleidoscope prestained protein standards were obtained from Bio-Rad (Hercules, CA, USA). Homogenate proteins (30 μg) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The resulting blots were treated with blocking buffer supplemented with 0.05% (v/v) Tween-20 (Pierce, Rockford, IL, USA) for 1 h at room temperature under agitation, followed by incubation overnight at 4 °C with following antibodies: anti-PP2AC diluted 1:200, anti-SIK1 diluted 1:500, anti-pSIK1-S577 diluted 1:200 or anti-pSIK1-T182 diluted 1:350 in blocking buffer. Blots were washed 5 times in 0.05% (v/v) Tween-20 in TBS and then incubated for 1 h at room temperature in secondary donkey antigoat peroxidase-conjugated antibody or secondary donkey antirabbit peroxidase-conjugated antibody diluted 1:25,000. Detection was carried out using the chemiluminescence method (Pierce, Rockford, IL, USA) with 0.5 min to 5 min exposure to Kodak Biomax MR autoradiographic film (Eastman Kodak, Rochester, NY, USA). Equal loading was assessed using Ponceau red staining.

2.5. PP2A-SIK1 or PP2A-HDAC5 interaction studies

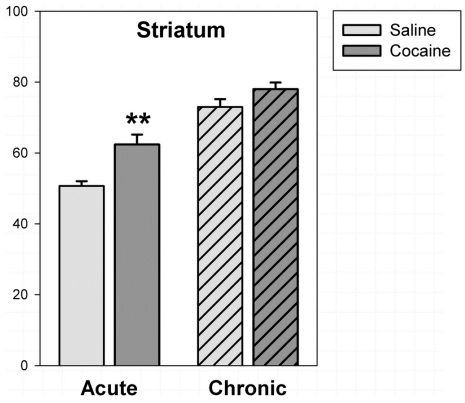
To study the interaction between SIK1 and PP2AC proteins, 100 μg of rat mPFCx (or striatum) homogenate were incubated in 500 μL RIPA buffer in the presence of 0.5, 1 or 2 μg anti-SIK1 antibody at 4 °C overnight. After the addition of 25 μL of Protein A-Sepharose beads and incubation at 4 °C for 3 h, the immunoprecipitated material was washed three times with RIPA buffer and analyzed by SDS-PAGE followed by western blotting using anti-PP2AC antibody (diluted 1:100). The interaction between HDAC5 and PP2AC proteins was analyzed similarly, except that 1.5 μg to 6 μg anti-HDAC5 antibody was used.

2.6. Cell culture and gene transfection

Control and transfected Neuro-2A cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen,



(a)



(b)

Figure 1: Effect of cocaine on the expression of PP2AC in the mPFCx and striatum of cocaine-treated rats. (a) The number of PP2AC immunoreactive cells was quantified in the mPFCx 15 h after a single IP injection of saline or 20 mg/kg cocaine (acute) or 15 h after the last injection of a series of 10 daily injections (chronic). Coronal sections were incubated with an anti-PP2AC antibody, followed by secondary peroxidase-conjugated donkey antirabbit IgG. (b) The same quantification was achieved in the dorsal striatum from the same animals. Each bar represents the percentage of immunoreactive cells calculated from seven counts on three sections from three separate rats and expressed as means $\pm$SD; $^{**}P < .01$ (Student's t-test).

Carlsbad, CA, USA) containing 2.5 mM Hepes buffer, pH 7.4, 10% fetal bovine serum (HyClone, Logan, UT, USA) and 10 mg/mL gentamycin (Invitrogen). Cell transfection was performed using Metafectene ProTM reagent following the manufacturer's instructions (Biontex laboratories, Munich, Germany). Neuro-2A cells stably overexpressing either the hemagglutinin (HA)-tagged wild-type PP2AC, the HA-tagged methylation-site L309ΔC mutant or the SV40 small t have been thoroughly characterized previously [20, 23]. Total homogenates were prepared from the stable cells as described previously [23].

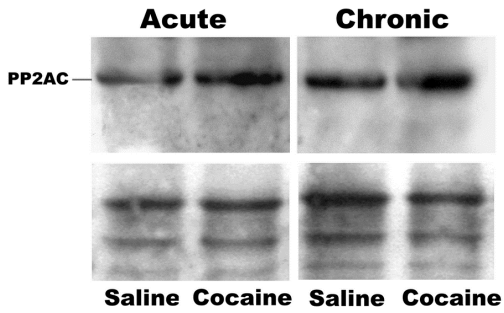


Figure 2: Effect of cocaine on PP2AC expression analyzed by western blot experiments. Equivalent amounts of proteins (20 μg) from rat mPFCx homogenates were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. Rats were given either a single IP injection of saline or 20 mg/kg cocaine (acute) or a series of 10 daily injections (chronic). Immunostaining was carried out with peroxidase-conjugated donkey antirabbit IgG. PP2AC was revealed as a band of Mr 36,000 as indicated (upper panel). Equal loading was assessed by Ponceau red staining of the blot (lower panel).

2.7. Statistical analysis

Results are expressed as means $\pm$SD. For immunohistochemistry experiments, 4 to 6 animals were used for each group. For immunoblotting experiments, 4 animals were used per group. Data were analyzed by standard two-tailed Student's t-test. Significance was set at $P = .05$.

3. Results

3.1. PP2AC expression is increased by cocaine in the cortex and striatum of rat brain

Using immunohistochemistry performed on coronal sections from rat PFCx, we found that the expression of the catalytic subunit of PP2A was increased in response to cocaine treatment. Figure 1(a) shows that the expression was increased by 28% 15 h after a single cocaine injection ($P < .01$), while a lower, but still significant increase of 13% was found 15 h after the last injection of a series of ten daily injections ($P < .01$). An increase in PP2A expression was also found in the dorsal striatum, although to a lesser extent and the effect was only statistically significant in response to a single cocaine injection (23% increase; $P < .01$; Figure 1(b)). The smaller effect observed under the chronic condition may be attributed, at least partially, to the increased PP2A expression found in the repeated saline groups.

The increase in cortical PP2A content in response to cocaine treatment was confirmed by western blot analysis (Figure 2). We found that the anti-PP2AC antibody detected a protein of Mr 36,000, as expected considering the molecular weight of PP2AC. The intensity of the band was enhanced in the mPFCx from rats treated acutely or

repeatedly for 10 days with 20 mg/kg cocaine. Quantitative analysis revealed that PP2A expression increased by 53% and 21%, respectively. Animals were killed 15 h after the injection, or after the last injection of a series of ten, but interestingly, the PP2AC induction was already observed 3 h after the cocaine treatment (data not shown).

3.2. PP2AC interacts with SIK1 and HDAC5 in the mPFCx of rat brain

In order for PP2A to dephosphorylate SIK1, the two proteins would have to physically interact. To demonstrate such a direct interaction between the two enzymes, SIK1 was immunoprecipitated from an extract of mPFCx from cocaine-treated rats, using increasing amounts of anti-SIK1 antibody. We then searched by SDS-PAGE for the presence of the catalytic subunit of the protein phosphatase in the precipitated material, using a western blot analysis with an antibody directed against PP2AC. Figure 3(a) shows that the immunoprecipitates contained increasing amounts of PP2AC, characterized by a band of Mr 36,000, strongly suggesting that the two proteins were actually physically interacting. Very similar results were obtained when an extract from striatum was used instead of mPFCx (data not shown).

Since we previously demonstrated that HDAC5 is a nuclear substrate of activated SIK1, we also examined the possibility that PP2A associates with HDAC5. For that, similarly to what we did for SIK1, HDAC5 was immunoprecipitated from a cortical extract using increasing amounts of anti-HDAC5 antibody. Figure 3(b) shows that the immunoprecipitates contained actually increasing amounts of PP2AC, strongly suggesting that the two proteins physically interact and that phospho-HDAC5 is probably another substrate of PP2A.

3.3. Cocaine downregulates the number of phospho-SIK1-S577 expressing neurons

If PP2A does dephosphorylate activated SIK1, one would expect that cocaine, by inducing PP2A, would decrease the amount of cellular phospho-SIK1. To test this hypothesis, we used phospho-specific antibodies to SIK1 phosphorylated on either Ser577 (phospho-SIK1-S577) or on Thr182 (phospho-SIK1-T182) to perform immunohistochemistry on coronal brain sections. Figure 4(a) shows that the expression of phospho-SIK1-S577 diminished by about 37% in cortical sections of rats that were injected with a single dose of cocaine. A less pronounced effect but still significant (25% decrease; $P < .01$) was noticed 15 h after the last injection of a series of ten daily cocaine injections. On the opposite, no significant reduction in the number of cells expressing phospho-SIK1-T182 was observed in adjacent sections from the mPFCx of the same animals (Figure 4(b)), suggesting that PP2A dephosphorylates SIK1 at the S577, but not at the T182 residue in cortical neurons.

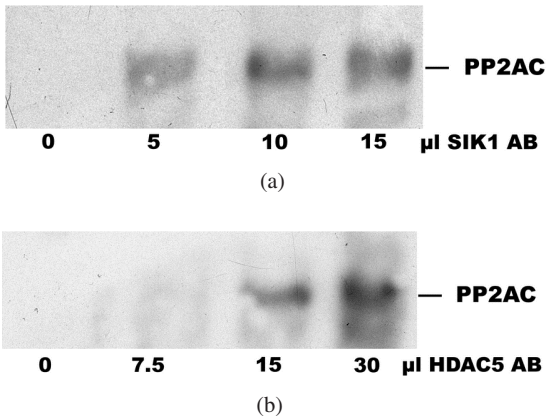


Figure 3: PP2AC associates with SIK1 (a) and HDAC5 (b). To show the association of PP2A with SIK1 or HDAC5, respectively, increasing amounts of SIK1 antibody or HDAC5 antibody were used to immunoprecipitate the enzyme from mPFCx homogenates from rats injected IP with 20 mg/kg cocaine. Equivalent amounts of proteins from each immunoprecipitate were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Blots were probed with anti-PP2AC antibody, which revealed the presence of the catalytic subunit of PP2A in the immunoprecipitate, as a band of Mr 36,000. Secondary antibody was peroxidase-conjugated donkey antirabbit IgG.

3.4. Overexpression of PP2AC in Neuro-2A cells and effect on phospho-SIK1-S577 expression

In order to strengthen our claim that phospho-SIK1 is actually a substrate of PP2A, we shifted to Neuro-2A cell cultures overexpressing either PP2A or a PP2A methylation mutant displaying altered substrate specificity. Cells were also treated with the SV40 small t antigen, which strongly and specifically inhibits PP2A enzymatic activity [22]. Figure 5 depicts the expression of phospho-SIK1-S577, phospho-SIK1-T182, and total SIK1 analyzed by western blot using Neuro-2A cell extracts. Ponceau red staining of the blot is also shown. A much higher level of phospho-SIK1-S577 was noticed in cells overexpressing the PP2AC mutant, as compared to the level reached in cells overexpressing the wild-type PP2AC. In cells treated with the SV40 small t antigen, a significant increase in the expression of phospho-SIK1-S577 was also observed, when compared to control cells. A slight increase in the expression of SIK1 phosphorylated on T182 could be observed in cells expressing the mutant enzyme, compared to cells expressing the wild-type PP2AC. The increase was not noticed in small t antigen-treated cells, when compared to the control cells. Anti-SIK1 antibody, together with Ponceau red staining, revealed the equal loading of proteins in the various lanes. The data are therefore in favor of PP2A dephosphorylating the S577 residue to a greater extent than the T182 residue.

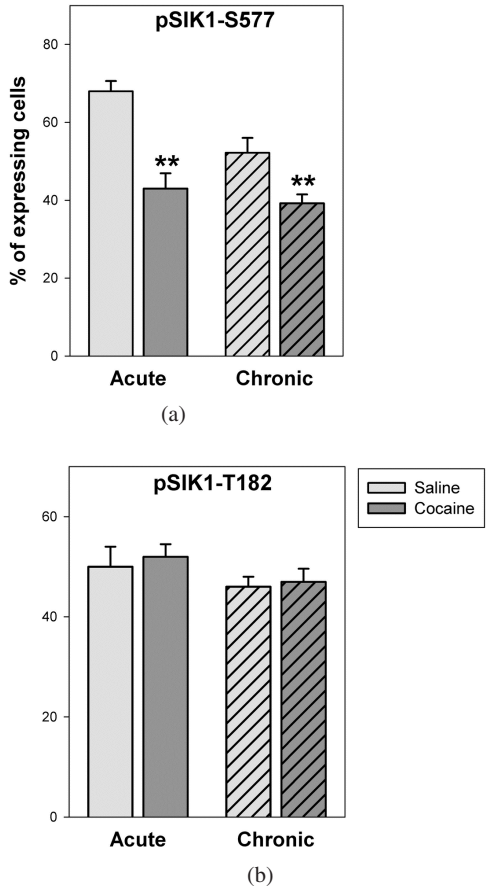


Figure 4: Effect of cocaine on the expression of phospho-SIK1-S577 and phospho-SIK1-T182 in the mPFCx of cocaine-treated rats. The number of phospho-SIK1-S577 (a) or of phospho-SIK1-T182 (b) immunoreactive cells was quantified in the mPFCx 15 h after a single IP injection of saline or 20 mg/kg cocaine (acute) or 15 h after the last injection of a series of 10 daily injections (chronic). Coronal sections were incubated with antibodies against the phosphoforms of the kinase, then by secondary peroxidase-conjugated donkey antirabbit IgG. Each bar represents the percentage of immunoreactive cells calculated from seven counts on three sections from three separate rats and expressed as means $\pm$SD; $^{**}P < .01$ (Student's t-test).

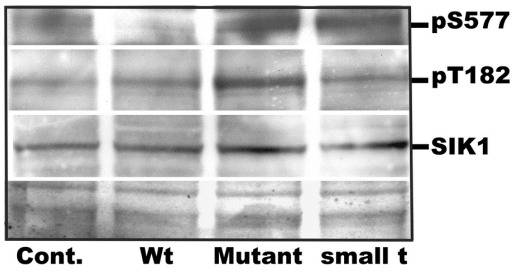


Figure 5: Expression of phospho-SIK1-S577, phospho-SIK1-T182, and SIK1 in Neuro-2A cells expressing wild-type PP2AC or mutant PP2AC deprived of activity. Control cultured Neuro-2A cells, cells stably expressing the wild-type PP2AC (wt) or the mutant PP2AC (mutant) and cells stably expressing the SV40 small t antigen (small t) were homogenized and analyzed by western blot experiments using anti-phospho-SIK1-S577, anti-phospho-SIK1-T182 or anti-SIK1 antibodies, with the latter antibody recognizing the total amount of SIK1. Secondary antibody was peroxidase-conjugated donkey antirabbit IgG. The lower panel presents the Ponceau red staining of the blot.

4. Discussion

We have recently documented that cocaine-induced *MEF2C* expression was mediated by the activation of the salt-inducible kinase SIK1 [8]. This kinase is known to be activated by mechanisms that mainly implicate phosphorylation of the enzyme. The observation prompted us to investigate the effect of cocaine on the expression of the protein phosphatase PP2A, since this phosphatase has already been shown to bind to SIK kinases in non-neural cells [26]. In the present study, we report that acute or chronic treatment of rats with cocaine increased the expression of the catalytic subunit of the protein phosphatase (PP2AC) in the mPFCx and striatum, two dopaminergic projection structures involved in processing the reinforcing properties of drugs of abuse. Interestingly, the data are in agreement with those reporting that cocaine treatment enhanced *PP2AC* expression at the messenger level in the ventral tegmental area [1], which contains cell bodies of neurons projecting to the PFCx and nucleus accumbens.

Several phosphorylation regulatory sites have been identified in SIK1 protein. The cAMP-dependent protein kinase (PKA) phosphorylates SIK1 on Ser-577 residue, inside the nuclear import/export domain which spans between positions 567 and 612 of the amino acid sequence [10]. As demonstrated by site-directed mutagenesis, this phosphorylation by PKA is responsible for the transfer of SIK1 to the cytoplasm by nuclear export [12, 24]. Ca^{++}/calmodulin-dependent protein kinase phosphorylates SIK1 on Thr-322, while LKB1, a tumor suppressor kinase involved in many pathophysiological processes, phosphorylates it on Thr-182 residue [13], with the latter phosphorylation being responsible for enzymatic activation and for the shuttling of the kinase towards the nuclear compartment [8]. Active SIK1 plays an important role in repressing CREB activity via the phosphorylation of TORC (transducer of regulated CREB activity), a CREB-specific coactivator [12, 24]. We found that acute or chronic cocaine treatment reduced the number of cortical neurons expressing the phospho-form of SIK1 on Ser-577 residue (phospho-SIK1-S577). In contrast, no significant reduction in the number of neurons expressing the phospho-form of

SIK1 phosphorylated on Thr-182 (phospho-SIK1-T182) was observed upon cocaine treatment. The data suggest that PP2A actually dephosphorylated S577 but not T182 residue of SIK1. The hypothesis is supported by the observation that PP2AC physically interacted with SIK1 in rat mPFCx. Interestingly, no such interaction could be demonstrated between SIK1 and the catalytic subunit of PP1, another protein phosphatase largely expressed in the brain (data not shown). By dephosphorylating phospho-SIK1-S577, increased PP2A expression by cocaine is expected to provoke the accumulation of active SIK1 in the nuclei and to halt the SIK1-induced CREB inhibition, which would then be followed by the transcription of a whole array of genes. The data are also in agreement with our former report showing that cocaine increased the nuclear level of phospho-SIK1-T182 [8].

PP2AC was also found to physically interact with HDAC5, a histone deacetylase belonging to the class IIa of HDACs, thus controlling the activity of the transcription factor MEF2C. Class IIa of HDACs comprises HDAC4, -5, -7, and -9. It is noteworthy that some of its members, like HDAC4 and -5, are able to form homo- and hetero-oligomers via a conserved coiled-coil domain near their amino terminals [2]. MEF2C activation is achieved as a consequence of the phosphorylation of HDAC5 by SIK1 in the nuclear compartment, causing the HDAC5 shuttling from the nucleus to the cytoplasm. In the opposite, nuclear import of HDAC5 requires a signaling mechanism that involves the transient PP2A-dependent dephosphorylation of Ser-279 residue located within the HDAC5 nuclear localization sequence [25]. Dephosphorylation of HDAC5 increases its nuclear accumulation, by accelerating its nuclear import rate and reducing its nuclear export rate. The cocaine-induced PP2A activation we report here may therefore lessen MEF2 activation in cortical neurons through the targeted PP2A-induced dephosphorylation of HDAC5. This regulation may participate to the long-term effects of cocaine. Interestingly, dephosphorylation of phospho-HDAC5-S279 in the nucleus accumbens was found to suppress the development, but not the expression, of cocaine reward [25]. Additional arguments for HDAC5 being indeed a substrate of PP2A derive from the observation that class IIa HDACs were found in an in vivo macromolecular complex with active PP2A in endothelial cells [11]. PP2A was also reported as dephosphorylating HDAC7 in HEK293 cells [19] as well as HDAC4 in chondrocytes [16].

To better support our hypothesis that phospho-SIK1-S577 is actually a substrate of PP2A, we used Neuro-2A cell cultures overexpressing either the wild-type PP2A, thus displaying about 40% increased enzymatic activity, or a mutant PP2A protein devoid of enzymatic activity. When compared to control cells, the amount of phospho-SIK1-S577 was lower in cells overexpressing the wild-type PP2A, and higher in cells expressing the mutant form. Interestingly, the selective PP2A inhibitor SV40 small t antigen also yielded increased SIK1 phospho-form. Concerning the phospho-SIK1-T182 level, it was found to be somehow elevated only in cells expressing the mutant form of PP2A. The data therefore argue in favor of PP2A dephosphorylating phospho-SIK1-S577 and, to a much lesser extent, phospho-SIK1-T182. It should be considered here that cocaine was not found to reduce the level of phospho-SIK1-T182 in rat mPFCx.

5. Conclusion

In conclusion, it appears that cocaine treatment, by inducing PP2AC in mPFCx and striatum, had only a minor effect on SIK1 activity, since we noticed only a small or no effect on the dephosphorylation of phospho-SIK1-T182. In contrast, the effect was rather directed towards the dephosphorylation of phospho-SIK1-S577, indicating a strong regulation of the nuclear availability of the kinase. In addition, the dephosphorylation of HDAC5 that is expected from PP2A induction may cause a lower MEF2 activation in response to cocaine in the same brain structures. Clearly, our results highlight PP2A as a novel target for regulating the cocaine effects. Pharmacological agents targeting the protein phosphatase, for instance, would provide unique tools for interacting with the SIK1-HDAC5 pathway and therefore regulating the chromatin-modifying activity that this pathway controls. By ultimately governing the induction of CREB and MEF2C transcription factors, this approach represents a new avenue to regulate the long-term effects of cocaine, including drug dependence.

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