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*Research Article*

# Differential Expression of mRNAs Coding for Histone Deacetylases (HDACs) in the Nucleus Accumbens of Compulsive Methamphetamine Takers and Abstinent Rats

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**Abstract** Methamphetamine (METH) addiction is a common neuropsychiatric disorder that is associated with loss of control over drug use. The long-term manifestations of METH addiction may be related to epigenetic neuroadaptations in the brain. To test this idea, we used rats of divergent phenotypes triggered by footshocks that helped to distinguish rats that continue to take METH compulsively (shock-resistant, SR) from those that become abstinent (shock-sensitive, SS). Male Sprague-Dawley rats were trained to self-administer METH (0.1 mg/kg/injection, IV) or saline during twenty 9-hour sessions. During training, all rats escalated their intake of METH. Following the training phase, rats were subjected to progressive increases in footshock intensity. This approach led to a split of the rats into the SR and SS phenotypes. Two hours after the last shock sessions the nucleus accumbens (NAc) was dissected and processed to measure mRNA levels. We found significant differences in the expression of HDAC1, HDAC3, HDAC6, and HDAC8 between the SR and SS rats. There was also increased expression of HDAC11 in the SS group in comparison to the SR and control groups. There were also significant differences in the mRNA expression of Sirt1, Sirt2, Sirt3, Sirt5, Sirt6, and Sirt7, with Sirt2 showing the greatest increase in the SS phenotype. Because these HDACs are differentially located in the cytoplasm, mitochondria, and the nucleus, these results suggest that METH self-administration may have impacted signaling pathways in various cellular compartments. Further dissection of these pathways should help us to elucidate molecular events that are involved in the maintenance of abstinence.

**Keywords** self-administration; epigenetics; gene transcription; chromatin modification; sirtuins

## 1. Introduction

Methamphetamine (METH) is a widely abused psychostimulant throughout the world [1]. In humans, large doses of METH can cause strokes, seizures, hyperthermia, and neuropathological changes in the brain [1]. The drug induces the release of dopamine (DA) from DA terminals [2], with secondary stimulation of DA receptors in several brain regions [3]. Noncontingent injections of METH are also accompanied by significant changes in gene expression in the dorsal striatum [4,5]. We have also shown that METH self-administration (SA) is accompanied by substantial changes in gene expression in brain regions that participate in the reward circuitry [6,7,8].

Gene transcription is influenced by epigenetic alterations that consist of post-translational histone modifications and DNA methylation [9,10,11]. Histone tails possess lysine residues that can be acetylated by histone acetyltransferases (HATs) [12] or deacetylated by histone deacetylases (HDACs) [13]. HDACs remove acetyl groups from lysine residues, a process that serves to recruit repressor transcriptional complexes [13]. HDACs are divided into four classes based on sequence similarities [14]. These enzymes include class I (HDAC1, HDAC2, HDAC3, and HDAC8), class II (HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, HDAC10), class III (Sirtuins 1–7), and class IV (HDAC11) HDACs [14]. Classes I, II, and VI HDACs are $Zn^{2+}$-dependent enzymes [15] whereas the sirtuins are $NAD^{+}$-dependent enzymes [16]. Because HDACs participate in gene regulation, we wondered whether their expression might be affected in the nucleus accumbens (NAc) of rats that had undergone METH SA [17].

Our laboratory recently reported that footshocks used as adverse consequences can help to separate rats into shock-resistant (SR) animals that continue to press a lever to take METH and shock-sensitive (SS) rats that suppress their METH intake with increasing shock intensity [17]. We found, in addition, that these rats showed differential changes in DNA hydroxymethylation in the NAc [17]. We used these same rats to test the idea that there might be additional neurobiological alterations in the NAc of these animals. Our results support the view that multiple epigenetic events may mediate the long-term behavioral effects of METH [1].

**Table 1:** List of RT-PCR primers sequences.

| Gene name | Forward | Reverse |
|---|---|---|
| HDAC1 | GCC CTT CCA ATA TGA CTA AC | GAG CAG ATG GAA ATT CGT |
| HDAC2 | TGT TAA GGA AGA AGA CAA ATC CA | ACA GCG AAG GTT TCT TAT C |
| HDAC3 | ATG AAA CAT CTC TGC TGG TA | GGC GGA TCT GGT CTA GAT A |
| HDAC4 | GAA CAA GGA GAA GGG CA | TGT CTT CCC ATA CCA GTA G |
| HDAC5 | TGG ACT GGG ACA TTC AC | CAC GCC ACA TTT ACG TT |
| HDAC6 | GTC TCA TCC TAC CTG CTC | GGC AGA TGT AGA TGG ACT |
| HDAC7 | CTG CTT TCA GGA TAG TGG | CAG CTG CTG TGT CAT GTA |
| HDAC8 | CTC AGG CTG AGT CTG AAA | CTT CAC AAG GGA ATC GCA |
| HDAC9 | TCT GAA CAT CAC TCA CTA CT | GTG CAG CTC ATT CCA AA |
| HDAC10 | GTG CCC TGG AGT CTA TC | CCA AGG CAA CAG CTA TG |
| HDAC11 | TCA CAC TGG CTA TCA AGT T | GTA GAT GTG GCG GTT GTA AA |
| SIRT1 | CCA GAT CCT CAA GCC ATG TT | CCA AAA TTG CTT TCC TTC CA |
| SIRT2 | TTG AAG GAG TGA CAC GCT A | GTA TGG AAG GTG GTA TTT CT |
| SIRT3 | CAA TGT CGC TCA CTA CT | GCA CGT AGC TGA TAC AAA |
| SIRT4 | TCC GGT TAC AGG TTC AT | CTG TCA CTG TGG GTC TA |
| SIRT5 | TGT ACC TCG TGT GGC AAT GT | CAG GAT CCA GGT TTT CTC CA |
| SIRT6 | GCT GAG AGA CAC CAT TC | GTT GAC AAT GAC CAG ACG |
| SIRT7 | CAG CCT CTA TCC CAG ATT | TGT TGC ACC AGC TTA TG |

## 2. Methods

### 2.1. Animals and intravenous surgery

Outbred male Sprague-Dawley rats (Charles River, Raleigh, NC, USA), weighing 350–400 g were used in these experiments. Rats were group-housed in pairs and habituated for 1 week before surgery. Rats had access to food and water ad libitum. Rats were anesthetized with a ketamine/xylazine mixture (50 mg/kg and 5 mg/kg, IP, resp.) and were inserted with catheters into the jugular vein, as described by Krasnova et al. [18] and Cadet et al. [17]. After surgery, rats were individually housed for an additional week and maintained under a reversed 12-hour light/dark cycle. Catheters were flushed with gentamicin (Butler Schein; 5 mg/mL) every 2 days after surgery. All animal procedures were conducted per NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the NIDA Intramural Research Program (IRP).

### 2.2. METH self-administration

This is essentially as reported in [17]. In short, rats were brought to the self-administration room and placed in Med Associates self-administration chambers where they remained throughout the duration of the experiment. Rats were trained to self-administer METH (0.1 mg/kg/injection, IV) on an FR-1 schedule for 20 days using a pattern of three 3-hour sessions/day separated by 30 min breaks. Each chamber was equipped with two levers, with presses on the active lever resulting in reward deliveries paired to a 5-second tone-light cue. Presses on the inactive lever produced no programmed consequences. Rats were trained in 4 cycles of 5 days of METH self-administration with 2 week-end days off. Time off allowed for animals not to suffer excessive weight loss which is a known consequence of METH administration [19].

### 2.3. Footshock phase

Following METH SA training, we added the punishment contingency wherein 50% of the active lever-presses resulted in mild footshocks of 5-second durations through electrified grids. During this phase, rats continued METH self-administration every day (9 hours a day) on the FR-1 reinforcement schedule with tone-light cues for 10 additional sessions. The initial foot-shock session was set at 0.18 mA and was progressively increased to 0.36 mA by increments of 0.06 mA.

### 2.4. RNA isolation

NAc tissues were rapidly dissected, 2 h after the last shock session, put on dry ice and kept frozen at $-80\,^{\circ}\mathrm{C}$. Total RNA was extracted using Qiagen RNeasy Midi kit (Qiagen, Valencia, CA, USA) per the company's protocol. The RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer 2 (Agilent, Palo Alto, CA, USA).

### 2.5. Quantitative PCR

HDAC mRNA expression levels were measured by real-time quantitative polymerase chain reaction (qPCR) as previously reported [17]. Sequences for rat were generated by the LightCycler probe design software v. 2.0 (Roche, Indianapolis, IN, USA) and purchased from Synthesis and Sequencing Facility of Johns Hopkins University (Baltimore, MD, USA). The primer sequences are listed in Table 1. PCR experiments were performed on Lightcycler 480 II, using iQ SYBR Green Supermix (BioRad, Hercules, CA, USA). The relative amounts of messenger RNA were normalized to Clathrin mRNA.

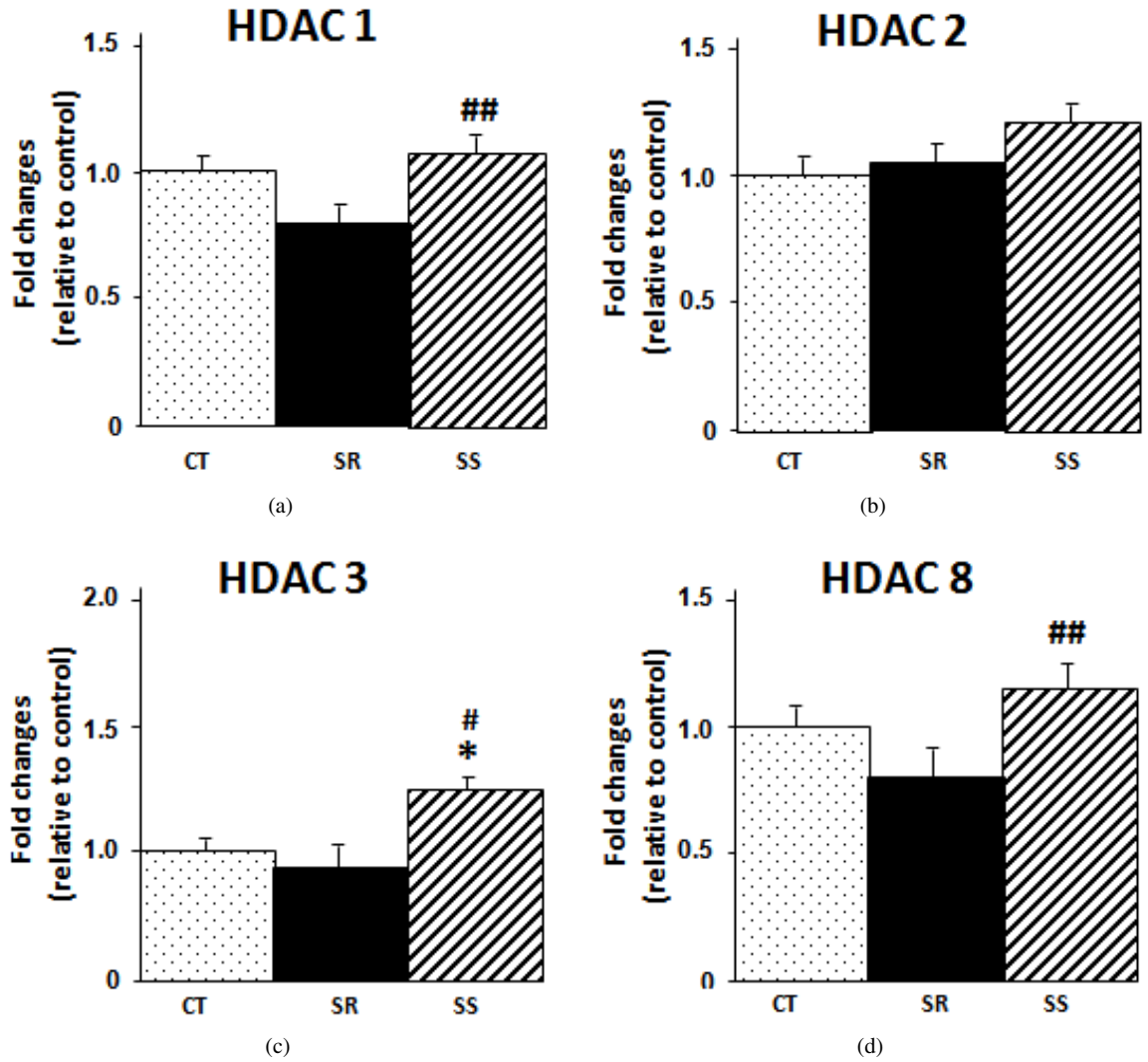



**Figure 1:** Effects of METH SA and footshocks on the expression of class I HDACs. qPCR experiments were conducted as described in the text. Values are means ±SEM in reference to the control animals. (a) HDAC1 expression is higher in the SS group in comparison to the SR phenotype; (b) HDAC2; (c) HDAC3 is higher than both CT and SR groups; (d) HDAC8 expression is higher in the SS than in the SR group. $^{*}P < .05$, in comparison to the control group; $^{\#}P < .05$, $^{\#\#}P < .01$, in comparison to the SR phenotype.

## 3. Results

As reported previously, footshocks caused the METH self-administering into two phenotypes: SR phenotype that continued to press a lever to get METH despite footshocks and SS phenotype that became abstinent in the presence of these adverse consequences [17]. As reported below, mRNA levels of several HDACs were measured in the NAc of the same rats used in these behavioral studies.

### 3.1. Class I HDACs

Figure 1 shows the results of METH and footshock on the expression of class I HDACs. There were significant decreases $[F(2,27) = 4.387,\ P = .0224]$ in the expression of HDAC1 in the SR in comparison to the SS group (Figure 1(a)). HDAC2 mRNA levels showed no significant changes $[F(2,28) = 2.071,\ P = .145]$ (Figure 1(b)). HDAC3 mRNA expression showed significant increases $[F(2,28) = 4.934,\ P = .0146]$ in the SS group in comparison to the control (CT) and SR groups (Figure 1(c)). HDAC8 mRNA levels also showed significant changes $[F(2,27) = 4.249,\ P = .025]$ in the SS group in comparison to the SR phenotype (Figure 1(d)).

### 3.2. Class II HDACs

The results of METH SA on class IIA HDACs are shown in Figure 2. There were no significant differences in the mRNA expression of HDAC4 $[F(2,28) = 2.576,\ P = .094]$ (Figure 2(a)), HDAC5 $[F(2,27) = 1.559,\ P = .229]$

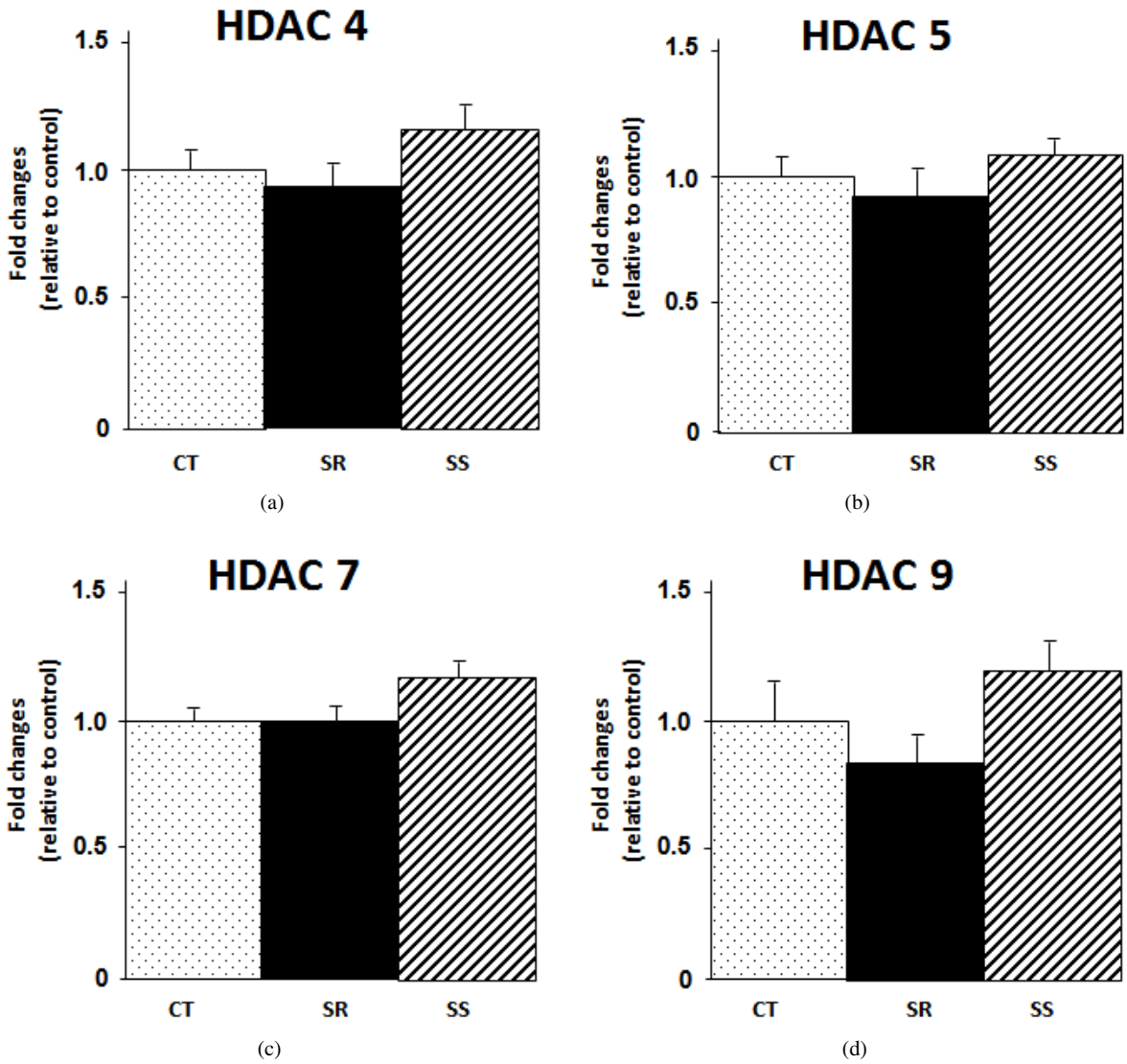



**Figure 2:** Effects of METH SA and footshocks on the expression of class IIA HDACs. (a) HDAC4; (b) HDAC5; (c) HDAC7, and (d) HDAC9. There were no significant changes.

(Figure 2(b)), HDAC7 [$F(2,27) = 3.082$, $P = .0623$] (Figure 2(c)), and HDAC9 [$F(2,23) = 2.425$, $P = .111$] (Figure 2(d)) between the groups.

Figure 3 shows the results of METH SA and footshocks on the expression of class IIB mRNAs in the NAc. HDAC6 showed significant differences [$F(2,27) = 6.629$, $P = .0045$] between the SS groups and the SR and CT groups. HDAC10 did not show any significant changes [$F(2,29) = 1.497$, $P = .2411$] in their expression.

### 3.3. Class III HDACs

Figure 4 shows the results of METH SA and footshocks on the expression of cytoplasmic and nuclear sirtuins. There were significant differences [$F(2,28) = 6.444$, $P = .005$] in the expression of Sirt1 between SR and SS rats (Figure 4(a)). Sirt2 expression was significantly [$F(2,27) = 12.205$, $P = .0002$] higher in the SS phenotype in comparison to both the CT and SR groups. There were significant increases in the expression of Sirt6 [$F(2,27) = 6.020$, $P = .0069$] in the SS group in comparison to the CT and SR group. There were also significant differences [$F(2,27) = 4.066$, $P = .0286$] in the expression of Sirt7 between the SR and SS phenotypes (Figure 4(d)).

The effects of METH SA and footshocks on mitochondrial Sirts are illustrated in Figure 5. There were significant differences [$F(2,29) = 3.959$, $P = .0302$] in the mRNA levels of Sirt3 but no changes [$F(2,27) = 2.513$, $P = .0998$] in Sirt4 expression between the groups. There was also significantly [$F(2,27) = 4.319$, $P = .0236$] higher expression of Sirt5 mRNA levels in the SS rats in comparison to the SR group (Figure 5(c)).

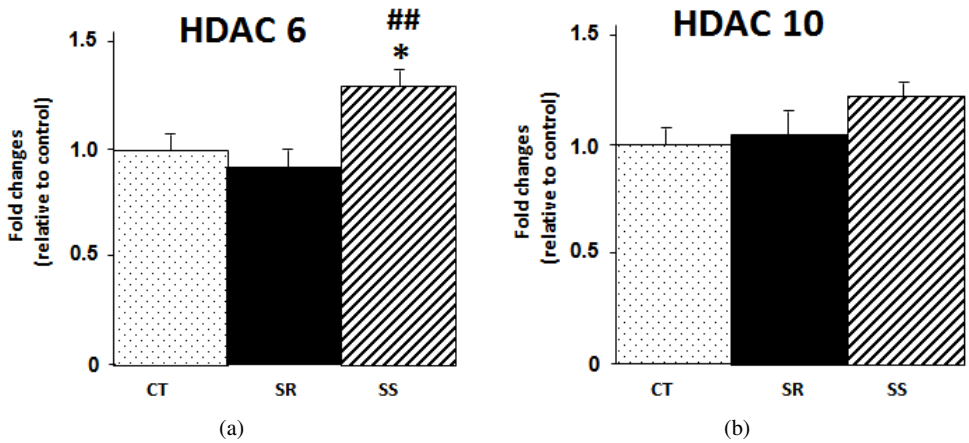



**Figure 3:** Effects of METH SA and footshocks on the expression of class IIB HDACs. (a) HDAC6 expression is higher in the SS group in comparison to the CT and SR phenotypes; (b) HDAC10. $^{*}P < .05$, in comparison to the control group; $^{\#\#}P < .01$, in comparison to the SR phenotype.

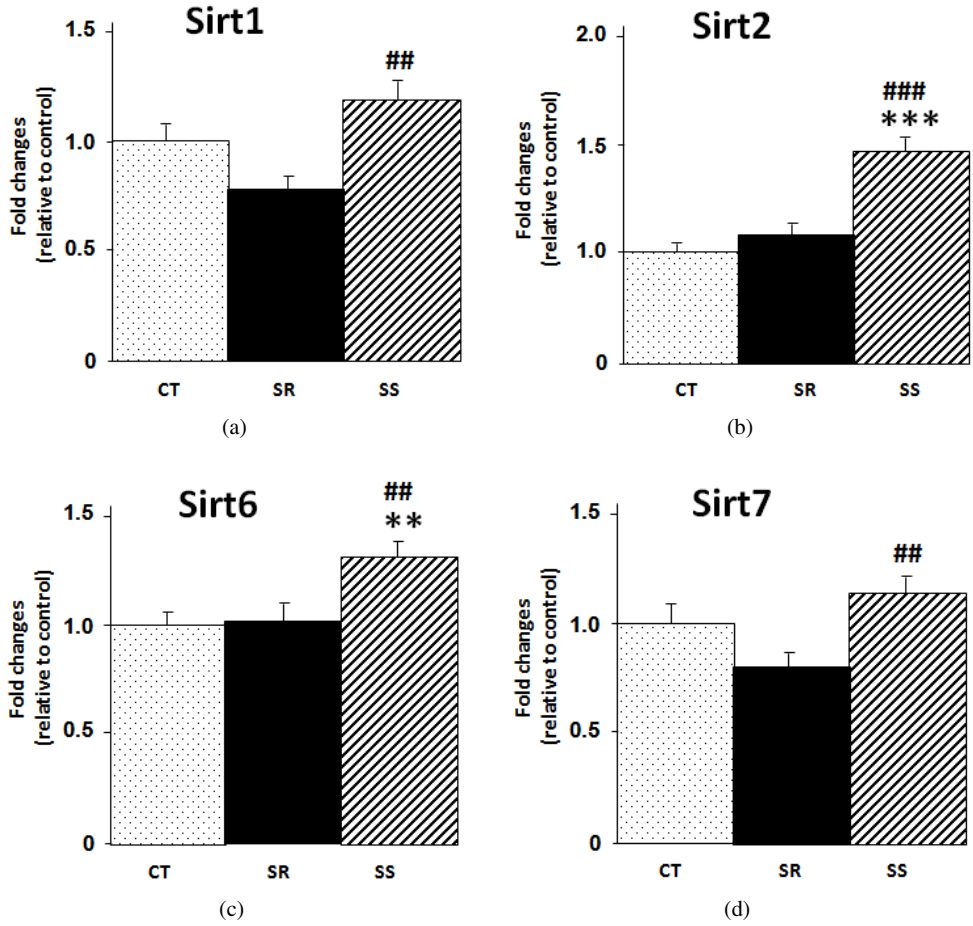
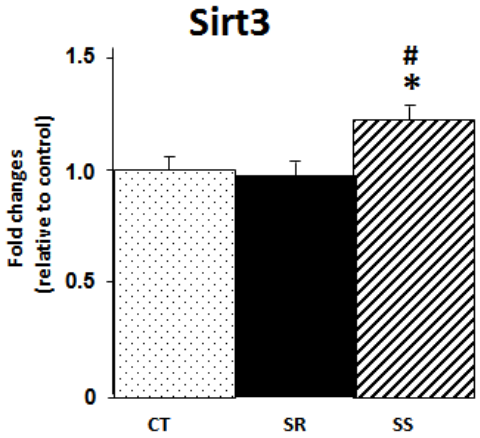
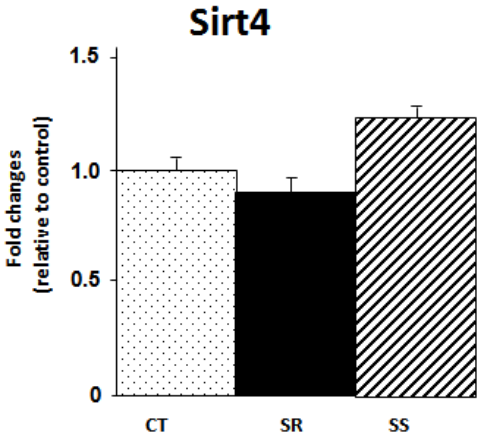


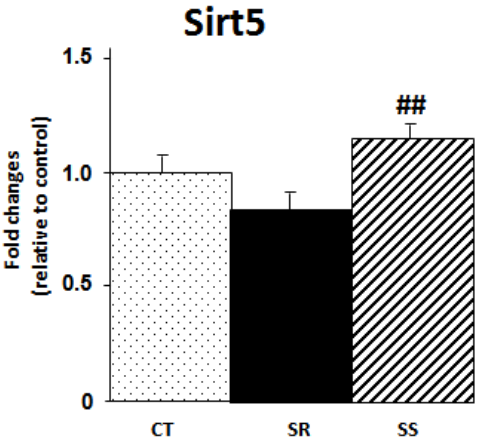

**Figure 4:** Effects of METH SA and footshocks on the expression of cytoplasmic and nuclear class III HDACs. (a) Sirt1 expression is higher in the SS group in comparison to the SR phenotype; (b) Sirt2 and (c) Sirt6 mRNA levels are higher in the SS phenotype in comparison to both CT and SR groups; (d) Sirt7 expression is higher in the SS in comparison to the SR groups. $^{**}P < .01$, $^{***}P < .001$, in comparison to the control group; $^{\#\#}P < .01$, $^{\#\#\#}P < .001$, in comparison to the SR phenotype.




(a)




(b)




(c)

**Figure 5:** Effects of METH SA and footshocks on the expression of mitochondrial class III HDACs. (a) Sirt3 expression is higher in the SS group in comparison to the CT and SR groups; (b) Sirt4 and (c) Sirt5 mRNA levels are higher in the SS phenotype in comparison to the SR group. $^{*}P < .05$, in comparison to the CT group; $^{\#}P < .05$, $^{\#\#}P < .01$, in comparison to the SR phenotype.

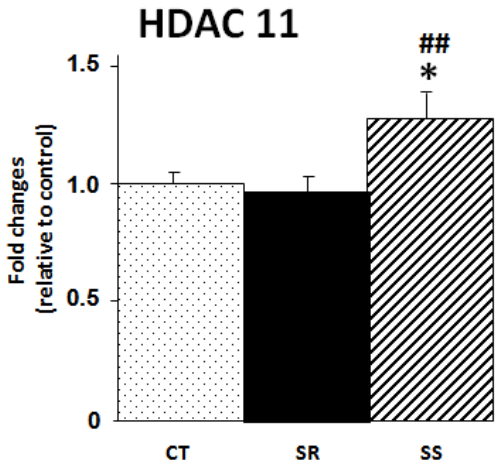



**Figure 6:** HADC11 mRNA expression is increased in abstinent rats. MRNA levels were measured as described in the text. $^{*}P < .05$, in comparison to the CT group; $^{\#\#}P < .01$, in comparison to the SR phenotype.

### 3.4. Class IV HDAC

Figure 6 shows the results of METH SA on the expression of HDAC11. There were significant increases $[F(2,29) = 5.543, P = .009]$ in HDAC11 mRNA expression in the NAc of SS rats in comparison to both SR and control rats (Figure 6).

## 4. Discussion

The main findings of this study are that rats that had undergone self-administration show differential changes in the expression of several HDAC mRNAs based on their responses to footshocks during the duration of METH SA. For example, SS rats showed higher expression of HDAC1, HDAC3, HDAC6, and HDAC8 in comparison to the SR rats. In addition, the mRNA levels of HDAC3 and HDAC6 were higher in the SS rats in comparison to control animals. Interestingly, while SS rats showed higher expression of several Sirts in comparison to the control and SR rats, Sirt2 showed the greatest differences between the groups. Our findings suggest that exposure to METH can exert substantial influence on enzymes that are involved in histone acetylation processes in the rat NAc.

Class I HDACs are known regulators of gene expression [13]. Several laboratories have implicated a role of some of these enzymes in the process of addiction to several drugs of abuse including cocaine, amphetamine, and METH [20,21,22,23,24]. The present study documented higher expression of HDAC1, HDAC3, and HDAC8 in SS in comparison to SR, suggesting that the changes in the expression of these enzymes may be associated with the abstinent state. These results suggest that abstinence from METH may be accompanied by decreased expression of genes that are targets for these epigenetic enzymes in

the NAc and that these proteins might be important to promoting abstinence from METH intake. This idea will need to be tested further by using specific inhibitor of this enzyme and/or by manipulation of NAc HDAC3 mRNA levels via virus-mediated gene expression. It is important to note that the changes in the expression of these enzymes were not due solely to METH exposure since injection of the drug in a binge pattern [25] or in single large doses [26] did not impact their expression in the rodent brain.

It is to be noted that we found increases in HDAC11 expression in the SS group similar to what we observed for the class I HDACs. HDAC11 is the only class IV HDAC [27]. HDAC11 is very highly expressed in the brain [28,29] and is involved in the differentiation of oligodendrocytes [30]. Cocaine SA has also been reported to be associated with increased HDAC11 expression in the cingulate cortex, the dorsal striatum, and the NAC of rats [31]. Thus, the observations of increased HDAC11 in METH and cocaine SA suggest the possibility that this epigenetic enzyme may play an important role in the intake of these stimulants.

Interestingly, we found no significant changes in the expression of class IIA HDACs that included isoforms 4, 5, 7, and 9 [32]. Class IIA HDACs have some features that distinguish them from other HDACs [32]. For example, their participation in transcription regulation depends on their phosphorylation-dependent shuttling from the cytoplasm to nucleus in order to suppress the transcription of their target genes [33,34]. A similar mechanism has been reported for cocaine-induced regulation of HDAC5 [35]. Thus, our findings are consistent with the possibility that METH may regulate these HDACs in a similar fashion.

The increased expression of HDAC6 in comparison to the CT and SR groups is also of interest. HDAC6 belongs to class IIB HDACs that consist of HDAC6 [36] and HDAC10 [14]. HDAC6 is a cytoplasmic HDAC with many biological functions, it forms complexes with a diversity of proteins, has cytoprotective properties, and it can deacetylate tubulin and heat shock protein (HSP)90 [36]. HSP90 is a 90-kDD molecular chaperone that is a soluble protein found in the cytoplasm of the cell [37]. It participates in cellular stress responses and is important for protein folding and degradation, and cellular stability [37]. Interactions of these two proteins might serve to protect the brains of abstinent rats from the protoxic effects of METH [38].

The effects of METH SA on cytoplasmic and nuclear class III Sirt expression were also of interest. Sirtuins are a class of HDACs that require $NAD^+$ as a cofactor to catalyze deacetylation reactions [39,40]. Interestingly, the SS groups showed higher expression of Sirt1, Sirt2, Sirt6, and Sirt7 than the SR group. In addition, Sirt2 and Sirt6 mRNA levels were increased in the SS in comparison to the control group. These results suggest that abstinence from METH may promote decreased expression of genes that are targeted by some of these proteins [41]. Sirt2 deacetylates acetylated histone H4K16 (H4K16Ac) [16], participates in the regulation of the cell cycle [42], is a tubulin deacetylate [43], and is a regulator of metabolism [44]. The participation of Sirt2 in H4K16 deacetylation suggests that there might be decreased acetylation of H4K16 in the SS rats in comparison to both controls and SR rats because there is a Sirt2 increase in the SS phenotype. This argument suggests further that there might be decreased expression of genes that are regulated by H4K16 acetylation since decreased acetylation is, for the most part, associated with decreased gene expression [11]. We have also shown that repeated injections of METH are associated with decreased H4K16 acetylation and decreased expression of glutamate AMPA receptors in the rat dorsal striatum [22]. The higher expression of Sirt6 in the SS group is also interesting because of its involvement in DNA repair, regulation of metabolism, and chromatin modifications [45,46]. Sirt6 deacetylates histone lysine 9 (H3K9) [47] and H3K56 [48] and this process can lead to substantial changes in gene expression. H3K56 acetylation is involved in the DNA damage response [49], suggesting further a role of the activation of these HDACs in a compensatory response to METH-induced damage in that brain region. The observations that the mitochondrial sirtuins Sirt3 and Sirt5 also showed differential expression between the SR and SS groups support the notion that there might be substantial epigenetic differences and associated changes in gene expression between these two phenotypes. More research needs to be done to further identify these potential differences between compulsive METH takers and abstinent rats.

In conclusion, this is the first demonstration that compulsive METH takers show significant differences in the expression of several HDACs that are involved in the regulation of gene expression and mitochondrial functions. These observations suggest that METH SA may be accompanied by altered expression of diverse classes of genes that are targets for various HDACs. Our results suggest further that a detailed examination of specific pathways may help to better understand the molecular substrates responsible for the varied clinical presentation of METH addiction. A better elucidation of the involvement of specific signaling cascades will allow for better data-driven pharmacological approaches to the treatment of stimulant addiction.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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