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Compulsive methamphetamine taking and abstinence in the presence of adverse consequences: Epigenetic and transcriptional consequences in the rat brain

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ABSTRACT

Methamphetamine addiction is characterized by compulsive binges of drug intake despite adverse life consequences. A model of methamphetamine self-administration that includes contingent footshocks to constitute adverse consequences has helped to segregate rats that reduce or stop lever pressing for methamphetamine (sensitive) from those that continue to lever press for the drug (resistant) in the presence of negative outcomes.

We have observed differential DNA hydroxymethylation and increased expression of potassium channel mRNAs in the nucleus accumbens of sensitive compared to resistant rats, suggesting a role of these channels in suppressing methamphetamine intake. There were also significant increases in nerve growth factor (NGF) expression and activation of its downstream signaling pathway (NGF-TrkA and p75NTR/MAPK signaling) in only the dorsal striatum of sensitive rats after a month of abstinence. In contrast, oxytocin mRNA expression was increased in only the nucleus accumbens of resistant rats compared to sensitive rats euthanized after that time. These results indicate that footshocks can differentiate two behavioral phenotypes with differential biochemical and epigenetic consequences in the ventral and dorsal striatum.

1. Introduction

Methamphetamine (METH) addiction is a biopsychosocial disorder characterized by loss of control over drug consumption despite adverse consequences (DSM5, 2013). Its abuse is accompanied by negative outcomes on the brain and peripheral organs (Cadet et al., 2014a; Paratz et al., 2016). Specifically, cognitive and psychiatric deficits consequent to structural and functional pathologies have been well documented (Cadet and Bisagno, 2015; Cadet et al., 2014a; Rusyniak, 2013; Scott et al., 2007; Volkow et al., 2001). Although clinical manifestations of addictive states can be observed after abuse of several classes of drugs that include marijuana (Copeland, 2016), opioids (Fattore et al., 2015; Vowles et al., 2015), and psychostimulants (Badiani and Spagnolo, 2013), it is imperative to delineate specific neurobiological processes that are attributable to individual substances of abuse. Without definitive knowledge of the molecular, structural, and other neuroplastic alterations caused by each specific substance, it is going to remain difficult to develop rational therapeutic interventions that are beneficial to groups or subgroups of patients who suffer from the various substance use disorders (SUDs). This conclusion was reached because the accumulated evidence supports the theme that repeated exposure to drugs can lead not only to different neuropsychiatric signs and symptoms (Cadet and Bisagno, 2015) but also to neuropathological abnormalities in distributed networks of potentially dissociable reward and non-reward pathways in the mammalian brain (Cadet et al., 2014a). It is, therefore, not far-fetched to suggest that pathologies outside of the so-called reward network may significantly impact responses to pharmacological therapies that solely target neurotransmitter pathways that lie within reward sub-structures. Thus, targeting just the influences of these drugs on drug intake behaviors during drug self-administration (SA) experiments in rodents might not be sufficient to predict the clinical responses of humans addicted to these drugs.

Behavioral phenomena observed during drug SA experiments are likely to be influenced by distinct but interconnected brain regions that include the nucleus accumbens (NAc), dorsal striatum (DS), the prefrontal cortex (PFC), and the hippocampus, among others (Cadet et al., 2015; Everitt, 2014; Scofield et al., 2016; Volkow et al., 2012; Yager et al., 2015). These regions form nodal connections within circuits that participate in various manifestations of addictive diatheses because they are integral to memory formation (Britt et al., 2012), decision making (Parkes et al., 2015), and habitual behaviors (Everitt and

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Robbins, 2005) that drive various aspects of drug taking (Cadet and Bisagno, 2013; Everitt and Robbins, 2016; Orsini et al., 2015; Vandaele and Janak, 2018) by rodents and humans. Indeed, pharmacological and other manipulations that affect some of these structures have been shown to increase, attenuate, or stop animals from self-administering various drugs of abuse in some models of addiction (Bossert et al., 2007; Fuchs et al., 2004; Yager et al., 2019). As a case in point, dopaminergic neurons from the ventral tegmental area (VTA) (Walsh and Han, 2014) and glutamatergic neurons from the PFC (Berendse et al., 1992) send projections to the NAc and can regulate plastic mechanisms that might underlie some of the substrates of addiction (Cadet, 2016).

In fact, use of the original SA models has helped to identify, in part, regional transcriptional and epigenetic programs that appear to drive the transition to addicted states after initial drug exposure (Cadet, 2016: Cadet et al., 2015; Krasnova et al., 2013; Wright et al., 2015). Nevertheless, models that include only drug SA in rodents may not be enough to recapitulate other complex phenomena that exist in humans addicted to drugs. These phenomena include, among others, loss of control over drug use and compulsive drug taking despite adverse consequences (DSM5, 2013). In fact, using these criteria, only a small percentage of humans who use or abuse drugs meet criteria for SUDs. It is therefore imperative to develop models that include additional criteria like those observed in human patients. These models should help to better characterize the functional neuroanatomy and molecular bases of addiction to specific drugs. Indeed, these approaches should facilitate the discovery of molecular and cellular events that are more closely related to compulsive drug taking behaviors and/or cessation of drug intake, causes of relapses to drug taking behaviors, and/or maintenance of prolonged recovery by some patients. Elucidation of these molecular signatures should have the potential to promote the development of pharmacological interventions to modify some of the varied clinical manifestations of SUDs.

2. Compulsive METH taking in the presence of punishment

2.1. Behavioral aspects of compulsive methamphetamine taking under punishment

In an attempt to address these issues as they relate to METH addiction, we have developed a model that uses contingent footshocks to differentiate rats that continue to self-administer METH compulsively (shock-resistant, SR, addicted) from those that significantly reduce or stop (shock-sensitive, SS, non-addicted) their intake in the presence of punishment (Cadet et al., 2017). These aspects of the model are thought to represent the DSM-V criterion of adverse consequences used to reach a diagnosis of SUDs in humans (DSM5, 2013). The behavioral studies with METH SA are consistent with observations of other groups that have reported cessation of or persistent drug seeking behaviors in the presence of footshocks (Chen et al., 2013; Deroche-Gamonet et al., 2004; Pelloux et al., 2007) or despite environmental signals of potential adversity (Vanderschuren and Everitt, 2004) (see Table 1 for a summary for some studies with cocaine and alcohol wherein investigators have used adverse consequences in an attempt to deter animals from drug taking behaviors).

The training procedure for METH SA has been described previously (Cadet et al., 2017; Krasnova et al., 2017). Briefly, rats are randomly assigned to either METH SA or control groups. Rats are trained to self-administer dl-METH HCl (NIDA) during three 3-h sessions/day (the sessions were separated by 30 min) over 20 days under a fixed-ratio-1 (FR-1) with a 20-s timeout reinforcement schedule. During the shock phase, the rats continued to self-administer METH every day (9-h sessions) under the same FR-1 20-s timeout reinforcement schedule as used during the training phase. Additionally, 50% of the reinforced active lever-presses result in the concurrent delivery of a 0.5-s footshock through the grid floor. Initial foot-shocks are set at 0.18 mA and are increased by 0.06 mA daily up to 0.30 mA. Thereafter, rats can receive footshocks of 0.30 mA for 3–4 days followed by 0.36 mA for another 3–4 days, depending on their individual responses to the shock intensity. We usually stop footshocks when rats meet the criteria of < 20% of their peak METH intake for 3–4 consecutive days. We chose to increase the shock progressively because adverse conditions in humans progress differently over the lifetime of individual patients.

Fig. 1a shows the timeline for a set of experiments during which rats met criteria for stopping footshocks after receiving 0.30 mA for 3 days. All METH-trained rats significantly escalated the amount of drug taken during the first 16 days and then maintained their intake for the remainder of the training session (Fig. 1b). Contingent footshocks usually lead to individual responses to the shocks and thus to the segregation of METH-trained animals into two dichotomous phenotypes: one group continued to compulsively press the lever for METH (resistant) while the other group progressively decreased their intake (sensitive). The separation of the two phenotypes is more pronounced at the higher shock intensity (0.30 mA) as shown in Fig. 1b. We concluded that the resistant rats were addicted to the drug because they took more METH infusions and more total amount of METH than the other rats that were not addicted to the drug (see Fig. 1c). It is important to note that all the rats that underwent METH SA training exhibited substantial escalation of their METH intake, suggesting that there is not a one-to-one correspondence between escalation of drug intake and resistance to footshocks (addiction to drug). Indeed, Fig. 1d illustrates that, during the training phase of METH SA, the average METH intake was similar in all animals that ultimately dichotomized into shock-resistant (SR) and shock-sensitive (SS) rats. However, during the footshock phase, the average METH intake for the SR group (9.9 mg/kg/day) was significantly higher than intake for the SS group (3.7 mg/kg/day) (Fig. 1e). Those observations are noteworthy because escalation of cocaine, heroin, and METH intake in rodent SA models has been suggested to be representative of drug addiction in humans (Ahmed and Koob, 1998; Ahmed et al., 2000; Kitamura et al., 2006). Thus, our results support the notion recently promulgated by several groups of investigators (Belin-Rauscent et al., 2016; Chen et al., 2013; Deroche-Gamonet et al., 2004; Pelloux et al., 2007) that additional criteria, beyond drug SA, are necessary to model human drug addiction in animal studies.

To measure the differential relapse potential of these animals, METH seeking behaviors were measured in some rats after a month of forced abstinence from METH SA and footshocks. Interestingly, resistant rats showed higher number of active lever-pressing than the sensitive rats after a month of withdrawal (Fig. 1c). These behavioral observations further suggest that resistant animals might represent groups of METH-addicted human patients who might be more prone to relapses in clinical situations where they are exposed to environmental cues (Wang et al., 2013).

2.2. Potential role of potassium channels in punishment-induced abstinence

The segregation of the two punishment-induced phenotypes allowed us to first test if there were differences in DNA hydroxymethylation in the NAc of the two groups of rats euthanized at 2 h after cessation of METH SA (Cadet et al., 2017). Cadet et al. (2015) had suggested earlier that epigenetic changes in the brain are important determinants of behaviors that are consequent to repeated exposure to METH. Testing that idea showed that both resistant and sensitive animals exhibited large scale changes in DNA hydroxymethylation in comparison to control animals, with the sensitive rats showing increased DNA hydroxymethylation in genes encoding potassium channels. To further clarify the impact of DNA hydroxymethylation on expression of these genes, we used quantitative PCR and measure mRNA levels for genes that coded for potassium channels in the NAc. Table 2 provides a summary of these results. Sensitive rats show increased expression of KCNA1 (*Kv1.1*) and KCNA2 (*Kv1.2*) expression in comparison to METH-addicted and control groups. In addition, there were significant

Table 1
Summary of data from self-administration protocols using punishment-induced behavioral phenotypes.

References	Animals used in the study	Footshock intensity and duration	Behavior and/or biochemical alterations
Alcohol			
(Campbell et al., 2018)	Male- Control-sired and Alcohol-sired offspring mice	0.2–0.7 mA; 6 days	Reduction in the number of active lever presses after punishment in the alcohol-sired rats
(Jury et al., 2017)	C57BL/6J-background Thy1-EGFP mice	0.4 mA; 6 days	Reduction in the number of active lever presses after punishment in the alcohol-sired rats
(Marchant and Kaganovsky, 2015)	Male alcohol- preferring P rats	0.1–0.7 mA; 7 sessions	Systemic injections of SCH 23390 into NAc shell and core decreased renewal of punished alcohol seeking.
Cocaine			
(Pelloux et al., 2018)	Male Sprague Dawley rats	0–0.7 mA; 8 days	↑ *c-fos immunoreactivity* in PFC, DS, AI, PVN, LHb, SN and BLA and DR in context-induced relapse after shock-imposed abstinence
(Datta et al., 2018)	Male and female Wistar rats	0.2–0.5 mA; 4 × 15 min	Increasing cocaine dose significantly increased compulsive cocaine-seeking behavior in female but not male rats. Estrous cycle has no impact on compulsive behavior
(Chen et al., 2013)	Male Wistar rats	0.4 mA 4 days in 30% of trials	↓prelimbic cortex excitability in compulsive cocaine-seeking (resistant rats)
(Deroche-Gamonet et al., 2004)	Male Sprague Dawley rats	0.8 mA FR5; 34 days	Addiction-like behavior was present only in 17% 0f rats using cocaine and is highly predictive of relapse after prolonged period of withdrawal
Methamphetamine			
(Torres et al., 2018)	Male Sprague Dawley rats	0.18 to 0.36 mA; 10 days	↑ *Egr1; Egr2* in resistant rats ↑ *Bdnf; Gfra2;CrhR1; Crhbp;* in sensitive rats ↓ *TrkA in* DS of sensitive rats
(Krasnova et al., 2017)	Male Sprague Dawley rats	0.18 to 0.36 mA; 10 days	↑ *Oxt* in the NAc of resistant rats ↑ *Cartpt* in dorsal striatum of resistant rats
(Cadet et al., 2016)	Male Sprague Dawley rats	0.18 to 0.36 mA; 10 days	↑ *Pdyn; Penk* in resistant rats

The table summarizes the behavioral or biochemical alterations associated after the segregation of the two phenotypes- addicted and non-addicted in the presence of punishment or adverse consequences. We have selected three drugs of abuse to illustrate this approach: *alcohol*, *cocaine,* and *methamphetamine*. For each reference, the rodent strain, sex and punishment paradigm are shown. The biochemical alterations listed under methamphetamine reflect changes in gene expression in the brain between the shock resistant and shock sensitive rats. Abbreviations: PFC-prefrontal cortex; DS-dorsal striatum; AI- anterior insula; PVN-paraventricular nucleus; LHb-lateral habenula; SN-substantia nigra; BLA-basolateral amygdala; DR-dorsal raphe; NAc-nucleus accumbens.

increases in the expression of KCNAB1 in the non-addicted group in comparison to the addicted rats. KCNB2 expression was also increased in the non-addicted rats in comparison to control and compulsive METH takers.

K+ channels regulate biological processes by controlling K+ flow across membrane pores and neuronal firing (Humphries and Dart, 2015). They can also modify neurotransmitter release (Trussel and Roberts, 2008). Several subfamilies of K+ channels are found throughout the brain where they are located in somas, dendrites, spines, and axon terminals (Misonou, 2018). These locations help them to also impact neuronal signaling (Yuan and Chen, 2006). Our observations that their expression is increased in the NAc of rats that had significantly reduce their drug intake suggest that some of these potassium channels might have suppressed the firing of some neurons that may be intimately involved in promoting compulsive drug taking behaviors. This idea needs to be further tested.

It could be argued, nevertheless, that some of these changes might be related to METH doses because the resistant rats took more METH than the sensitive rats (239.11 ± 21.19 vs 192.13 ± 18.89, respectively; see Fig. 1c). This is a very reasonable point that will need to be investigated in future experiments by using rats that are yoked to METH SA rats in order to receive the same amount of METH as the shock-resistant and -sensitive rats, respectively. This approach will help to identify potential METH dose-dependent consequences on epigenetic and transcriptional markers in rats euthanized early during early abstinence.

2.3. Differential changes in corticotrophin-releasing hormone (CRH) mRNA levels in the NAc of METH SA rats

CRH and its receptors are involved in stress-related neuroplastic changes and disorders including addiction (Regev and Baram, 2014; Sanders and Nemeroff, 2016). Acute METH injections alter *Crh* mRNA levels and these changes are mediated epigenetically (Cadet et al., 2014b; Jayanthi et al., 2018). We thus measured the mRNA expression of *Crh* and of its receptors in the NAc of rats euthanized at 2 h after cessation of drug intake and footshocks. We found increases in *Crh* expression in both resistant and sensitive rats compared to control rats, suggesting that exposure to METH, irrespective of dose, was enough to cause changes in its expression. Both CRH receptors, *CrhR1* and *CrhR2* also showed increased expression in the NAc of both phenotypes. Together, these results indicate that the endogenous CRH system cannot distinguish between METH-addicted states using this experimental approach, a suggestion that is consistent with our previous report that even non-contingent injections of METH can also increase *Crh* expression in the NAc (Cadet et al., 2014b). Together, these findings suggest that exposure to METH by itself can trigger the endogenous CRH-stress system and lead to complications independent of its abuse and addiction potentials.

2.4. Potential role of NAc oxytocin in compulsive methamphetamine taking and withdrawal

Oxytocin is a neuropeptide that regulates social bonding/attachment (Bernaerts et al., 2017; Feldman and Bakermans-Kranenburg, 2017). This neuropeptide has been implicated in addiction to several substances (Baracz and Cornish, 2016; Carson et al., 2010; Cox et al., 2017; Lee and Weerts, 2016). Peripheral oxytocin administration can dose-dependently reduce the number of METH infusions by rats (Carson et al., 2010). Infusions of oxytocin in the NAc can also reduce METH conditioned place preference (CPP) and METH-primed reinstatement (Baracz et al., 2016). Interestingly, METH was reported to increase plasma oxytocin levels in rodents (Baracz et al., 2016). More recently, Krasnova et al. (2017) also found significant increases in oxytocin mRNA in the NAc of resistant rats that had undergone a month of forced abstinence after having been engaged in compulsive lever-pressing for

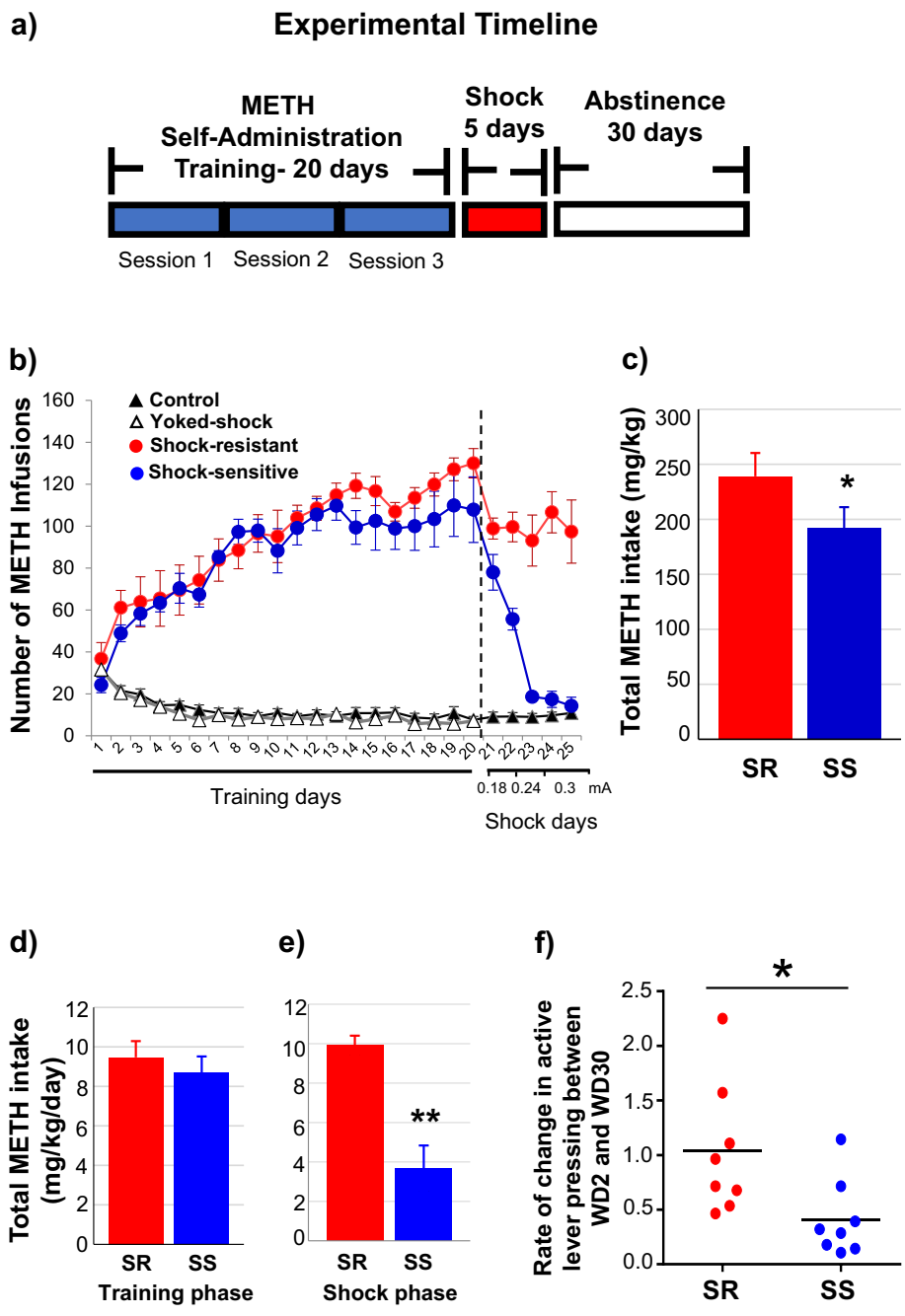

Fig. 1. Prolonged methamphetamine self-administration and contingent footshock produce rats with compulsive drug taking and abstinent phenotypes. (a) Timeline of the experiment. (b) All rats escalate their intake of METH during the training phase of the experiment. Footshocks suppress lever pressing in shock-sensitive (SS) rats but not in shock-resistant (SR) compulsive METH takers. (c) Resistant rats took more METH than sensitive rats during the entirety of the experiment. (d) Importantly, average METH intake per day was similar between SR and SS during the METH SA training phase. (e) During the shock phase, however, SS rats took substantially less METH than SR rats. (f) Resistant rats showed greater incubation of METH seeking than sensitive rats. Key to statistics: * $P < 0.05$, ** $P < 0.01$, in comparison to SR rats.

METH even in the presence of repeated footshocks. When taken together with the other findings detailed above, the observed increases in NAc oxytocin mRNA levels in the resistant rats might have been triggered as an attempt to maintain homeostasis in the NAc by replacing oxytocin that might have been depleted during compulsive METH taking by the addicted rats. This conclusion notwithstanding, it is also possible that changes in oxytocin expression might occur in specific sub-populations of NAc GABAergic neurons that express different dopaminergic receptors (D1 or D2), activation of which may have differential effects on drug-induced behaviors (Carlezon Jr. and Thomas, 2009; Nakanishi et al., 2014). Nevertheless, recent studies have documented the possibility that this dichotomization of NAc D1 and D2 projections may be more complex (Soares-Cunha et al., 2016; Soares-Cunha et al., 2018). More studies are needed to clarify the important issue of the role of oxytocin in mediating responses of specific GABAergic neurons in the NAc during compulsive METH taking.

2.5. Differential expression of neurotrophin in the dorsal striatum of sensitive and resistant phenotypes

Neurotrophins play important roles in regulating diverse neuronal processes including synaptic plasticity, cell survival, differentiation, and neuronal growth (Alder et al., 2003; Bekinschtein et al., 2008). It was thus of interest to test the possibility that there might be differential expression of trophic factors in the DS of sensitive and resistant rats. As a first step towards reaching that goal, we used the unbiased approach of measuring several trophic factors by the Neurotrophins and Receptors RT^2 Profiler™ PCR Array system. There is indeed accumulated evidence for their participation in various aspects of drug-induced effects in the brain (Russo et al., 2009). The choice of using the PCR array system was also supported by previous studies from several laboratories and our own that non-biased approaches might deliver very productive information about the molecular effects of several drugs

Table 2
Effects of METH SA on potassium channel mRNA expression in abstinent (SS) as compared to compulsive (SR) METH taking rats.

Gene	Brain region		
	Nucleus Accumbens	Pre-frontal cortex	Dorsal striatum
Kcna1 (Kv1.1)	↑	–	–
Kcna2 (Kv1.2)	↑	–	–
Kcnb1 (Kv2.1)	↑	–	–
Kcnb2 (Kv2.2)	↑	–	–
Kcnn1 (SK1,KCa2.1)	↑	–	–
Kcnn2 (SK2,KCa2.2)	↑	–	–
Kcnn3 (SK3,KCa2.3)	–	–	–
Kcnma1	↑	–	–

The table summarizes the changes in the expression of potassium channels genes in three different brain regions involved in the brain reward circuit. The upward arrow (↑) indicates up-regulation in gene expression and (–) indicates no significant change in mRNA expression.

including cocaine (Chandra et al., 2013), METH (Torres et al., 2018) and oxycodone (Zhang et al., 2015). We therefore reasoned that these experiments would provide us with a window into ways that these factors might influence drug taking behaviors in the two shock-induced phenotypes described above. We found that these two groups of rats did indeed show differences in gene expression in their DS after a month of withdrawal from METH SA and contingent footshocks (Torres et al., 2017). Specifically, *Bdnf*, *Ngf*, *Vgf*, *Trkb*, and *Ntf3* mRNA levels were increased in the DS of sensitive rats compared to resistant rats at that time. Quantitative PCR analyses validated the PCR array data. Our observations of increased mature BDNF protein levels in SR and SS rats are consistent with previous reports showing increased BDNF expression in rats that self-administered METH chronically (Krasnova et al., 2013; Li et al., 2015). These observations suggest that increased BDNF expression may be a consequence of long-term METH exposure without necessarily reflecting molecular adaptations that could distinguish compulsive from controlled drug taking behaviors.

After observing significant changes in the mRNA levels of several trophic factors, we decided to find out if their protein expression might also be differentially affected in the two phenotypes. We observed significant increases in mature BDNF protein levels in both resistant and sensitive rats. The BDNF receptor, TrkB (Andero et al., 2014), protein levels were also affected. We also found significant increases in mature NGF protein levels in both resistant and sensitive groups in comparison to the control group, with NGF levels being significantly more increased in the sensitive rats than in the resistant rats. Interestingly, phosphorylation of the NGF receptor, TrkA (Marlin and Li, 2015), was only increased in the shock-sensitive rats that had markedly reduced their intake of METH, suggesting that activation of this cascade might be prominently involved in causing cessation of drug intake. The expression of another NGF receptor called p75NTR (Blochl and Blochl, 2007) also showed increases in the sensitive rats compared to resistant and control rats. Indeed, we observed increases in NGF protein levels accompanied by selective increases in phosphorylated TrkA and p75NTR levels in the sensitive phenotype in comparison to the resistant rats. Although NGF was originally described as a neurotrophic factor required for cell proliferation (Cohen et al., 1954), it is now clear that NGF can also participate in a number of neurobiological events (Conner et al., 2009; Manni et al., 2013). For example, within the DS, NGF is produced by GABAergic interneurons (Bizon et al., 1999) and has significant protective and plastic effects on striatal cholinergic neurons under normal and pathological conditions (Fischer et al., 1998; Gratacos et al., 2001). Additionally, NGF can cause hypertrophy of striatal cholinergic neurons, increased levels of choline acetyltransferase (ChAT) mRNA, and reduced spontaneous neuronal activity (Forander et al., 1996). Moreover, the biological effects of NGF occur through its binding to TrkA and p75NTR, with distinct functional and structural interactions between the two receptors (Bucci et al., 2014; Covaceuszach et al., 2015; Matusica et al., 2013). For instance, p75NTR can co-precipitate along with TrkA (Bibel et al., 1999), with TrkA affinity for NGF increasing in the presence of p75NTR (Esposito et al., 2001). p75NTR can also prolong cell-surface TrkA-dependent signaling (Makkerh et al., 2005), thereby enhancing TrkA signaling capacity (Epa et al., 2004; Verdi et al., 1994). Interestingly, knockdown of p75NTR expression significantly reduced excessive drinking (Darcq et al., 2016) whereas our own findings suggest that increased p75NTR might be involved in suppressing METH seeking behaviors since the sensitive rats also show less cue-induced drug seeking (Torres et al., 2018). This statement hints to the possibility that sensitive rats might have a better ability to learn and establish memory for adverse events through the activation of the NGF signaling cascade.

2.6. Activation of the Ras/Raf/MEK/ERK pathway in the rat dorsal striatum and its relationship to reduced drug seeking behavior by shock-sensitive rats

Neurotrophins exert their functions by activation of the Ras/Raf/MEK/ERK intracellular signaling cascade through phosphorylation of several proteins in that cascade (Cargnello and Roux, 2011; Reichardt, 2006). Importantly, it is known that p75NTR can interact with TrkA to potentiate activation of this cascade (Chao et al., 2006; Matusica et al., 2013; Reichardt, 2006), which is known to participate in the modulation of gene expression in response to neuronal activity (Flavell and Greenberg, 2008). We thus thought it likely that the sensitive phenotype might also show increased phosphorylation of proteins of the MAPK cascade. Indeed, sensitive rats showed increased p-Raf abundance in comparison to resistant rats. A partial increase in pERK abundance was also observed in sensitive compared to resistant rats. Interestingly, pMSK1 which can act to phosphorylate histones (Sawicka

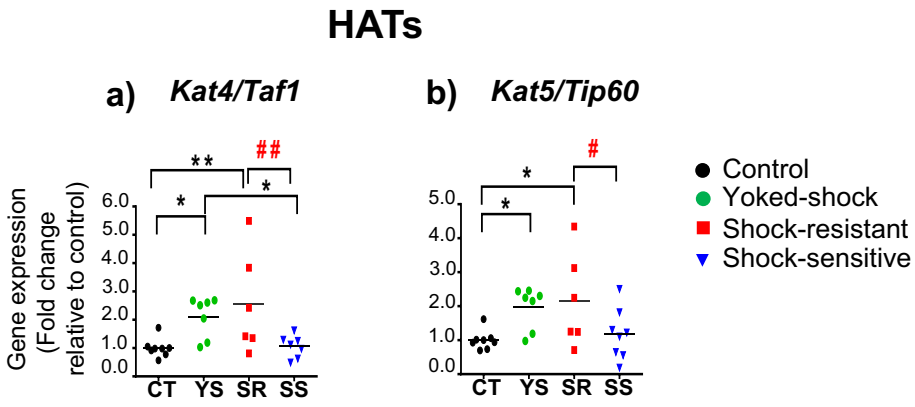

Fig. 2. Incubation of METH seeking in SR rats is associated with increased KAT4 and KAT5 expression in the DS. In the DS, *Kat4* (a) and *Kat5* (b) transcripts showed increases in the SR rats compared to both SS rats and control. There were no significant changes in the expression of KATs in the NAc. Values are means ± SEM fold changes relative to the control group. *, $p < 0.05$, **, $p < 0.01$ vs control; #, $p < 0.05$, ##, $p < 0.01$ vs SR rats.

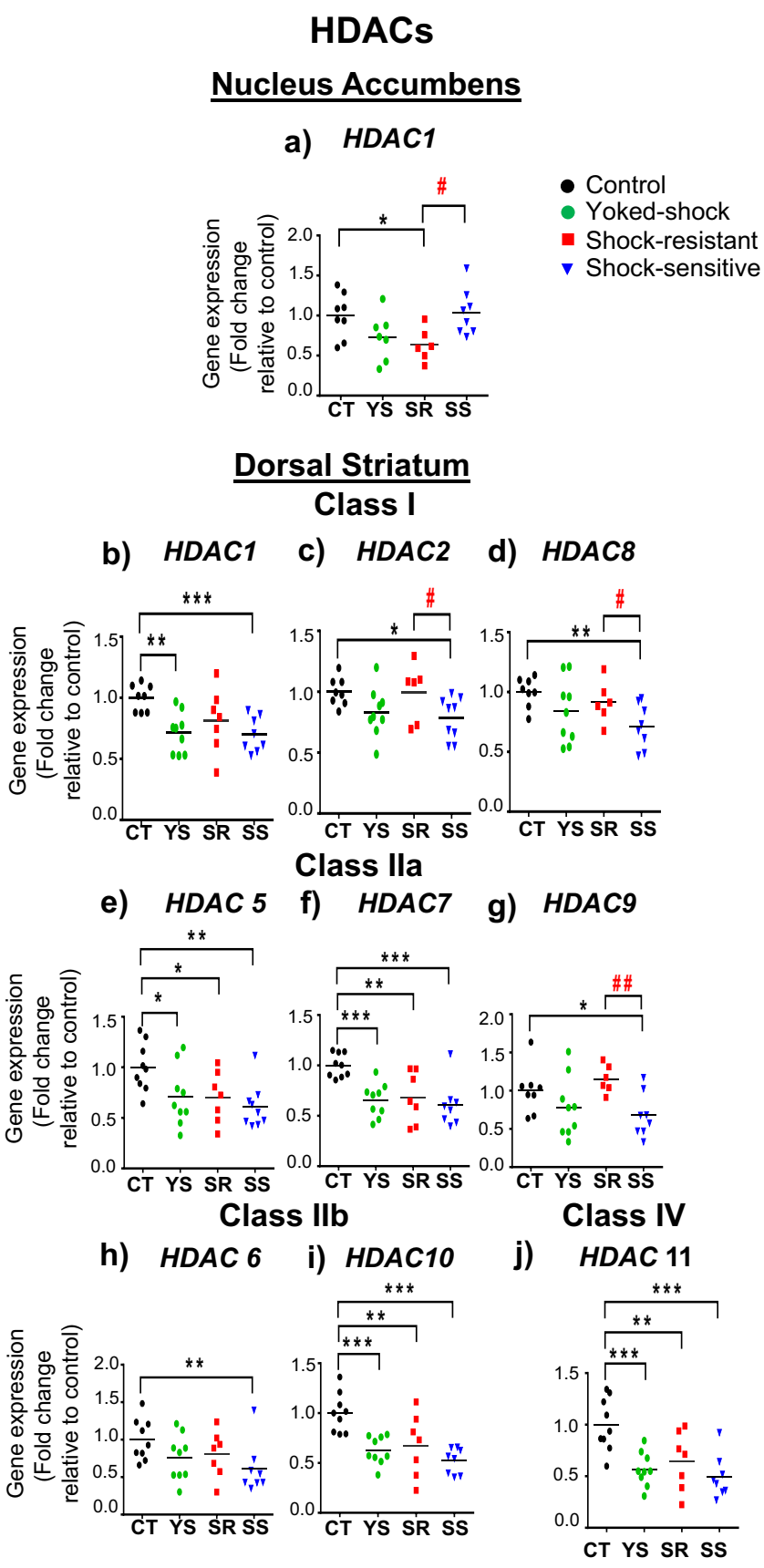

Fig. 3. Effects of withdrawal from compulsive METH taking under punishment on HDAC mRNA expression in both NAc and DS. In the NAc, only *HDAC1* showed significant decreases in the SR rats (a). Other HDAC mRNA levels were not affected in the NAc. In the DS, SS rats showed decreased levels of *Class I HDAC, HDAC1*, *HDAC2* and *HDAC8* (b-d) transcripts compared to control rats. In addition, SS rats showed decreased levels of *HDAC2* (c) and HDAC*8* (d) and *HDAC9* (g) mRNA levels compared to both control and SR rats. Both SR and SS rats showed significant decreases in the mRNA levels of HDAC5 (e), *HDAC7* (f), *HDAC10* (i) and *HDAC11* (j) compared to control rats. Values are means ± SEM fold changes relative to the control group. *, $p < 0.05$, **, $p < 0.01$ vs control; #, $p < 0.05$, ##, $p < 0.01$ vs SR rats.

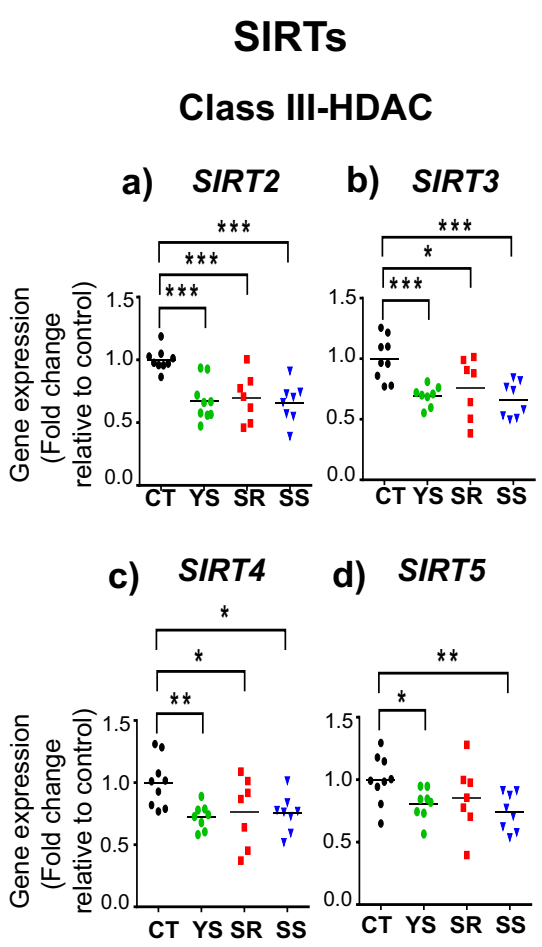

Fig. 4. Sirtuin (SIRT) mRNA levels in the rat DS of SR and SS rats. In the DS, *SIRT2* (a), *SIRT3* (b), and *SIRT4* (c) transcripts were downregulated in both SR and SS rats compared to control. Striatal *SIRT5* mRNA expression exhibited lower levels in SS rats compared to control (d). Values are means ± SEM fold changes relative to the control group. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs control.

and Seiser, 2012) was also increased in the non-addicted rats.

The Ras/MAPK cascade has received much attention due to the prominent role it plays in neuronal plasticity (Bruel-Jungerman et al., 2007; Cargnello and Roux, 2011; Correa et al., 2012), memory formation (Adams and Sweatt, 2002), transcriptional responses (Davis et al., 2000), translation rates, metabolic processes, and synaptic plasticity in neurons (Cho, 2011). In neuronal cells, the binding of mature NGF onto TrkA activates the adaptor GRB2-SOS protein complex which increases the rate of GDP-GTP exchange on Ras, leading to Ras activation (Bucci et al., 2014; Marlin and Li, 2015). Activation of Ras induces a sequential activation of Raf, a MAPK kinase kinase (MAPKKK). Activation of Raf sub-types induces phosphorylation of MEK that then promotes the phosphorylation of ERK1/2 (Ciccarelli and Giustetto, 2014; Plotnikov et al., 2011). Translocation of pERK1/2 into the cell nucleus results in the phosphorylation of MSK1 (Plotnikov et al., 2011). Phosphorylated-MSK1 directly causes the phosphorylation of CREB (Arthur and Cohen, 2000) that binds onto cAMP response elements (CREs). Phosphorylated-CREB then recruits histone acetyltransferases that promote histone acetylation and increase accessibility of the genome for molecular machinery to initiate transcription of several downstream genes. It is also noteworthy that other investigators had reported that another psychostimulant, cocaine, can also increase ERK phosphorylation in DA D1-containing striatal neurons of mice, a process that drove behavioral sensitization in these animals (Valjent et al., 2010). Taken together, these results suggest that ERK phosphorylation may play important roles in various aspects of drug-induced neuroadaptations in the brain (Sun et al., 2016).

In summary, it can be concluded that there was activation of a phosphorylation cascade in the DS of sensitive rats that might have led to suppression of their intake of METH and/or decreased expression of incubated behaviors after several weeks of withdrawal for METH SA. Because trophic factors can mediate plasticity in the brain, it is plausible that some of them, by activating the ERK-dependent pathway, might be partially responsible for the attenuated incubation that we observed in the sensitive rats in comparison to the compulsive METH takers (Torres et al., 2018). As mentioned above, this might occur via enhancement of memory processes that help sensitive rats to better remember adverse events through activation of the ERK signaling pathway.

2.7. Potential role of epigenetic enzymes in compulsive methamphetamine taking

In our continuing efforts to identify potential epigenetic markers of compulsive drug taking behaviors (Cadet et al., 2015), we used the model that describe above to measure potential changes in mRNA levels of several epigenetic enzymes in the NAc and DS after a month of forced abstinence during which time resistant rats showed greater METH seeking than sensitive animals. In animal models of drug addiction, histone acetylation is the most frequently investigated chromatin modification in the brain (Godino et al., 2015; Nestler, 2014). Histone acetylation, in general, correlates with increased gene transcription (Kurdistani and Grunstein, 2003). We thus reasoned that changes in histone acetylation may also play a role in some of the differences in gene expression that we previously detected between resistant and sensitive rats after several weeks of withdrawal from METH SA and footshocks. To test this idea, we decided to measure the mRNA expression of some epigenetic enzymes that mediate histone acetylation (KATs) and deacetylation (HDACs) in these rats.

We found no phenotype-specific changes in the expression of KATs in the NAc. In contrast, both *Kat4* and *Kat5* mRNAs exhibited significant increases in the DS of resistant rats compared to both control and sensitive rats (Fig. 2).

Of the class I HDACs (Fig. 3b–d), *HDAC1* mRNA showed significant decreases in the DS of sensitive rats compared to control rats. In addition, *HDAC2* and *HDAC8* mRNA levels exhibited significant decreases in sensitive rats compared to both control and resistant groups. Class IIa, IIb and IV HDACs, showed significant decreases differences in the sensitive rats as compared to control rats in the DS (Fig. 3e–j). Only *HDAC9* (Fig. 3g) displayed significantly downregulated mRNA expression in the sensitive rats in comparison to control and resistant rats. Other HDACs were not differentially affected in the resistant and sensitive rats.

Only *HDAC1* mRNA expression showed significant changes in the NAc, with resistant rats showing significant *HDAC1* downregulation compared to sensitive rats (Fig. 3a).

Fig. 4 illustrates the results for the Class III HDACs, the sirtuins (SIRTs). In the DS, *SIRT2*, *SIRT3* and *SIRT4* mRNA levels were significantly decreased in both the resistant and sensitive rats compared to control animals. *SIRT5* (Fig. 4d) mRNA expression was decreased only in the sensitive rats in comparison to controls.

Interestingly, most of the changes in the expression of epigenetic enzymes occurred in the DS, suggesting relative increased histone acetylation and potential increased expression of genes that are targets of these enzymes in the sensitive animals. Some of the genes may render these rats more sensitive to shock and/or may be involved in the suppression of behaviors associated with incubation of METH seeking. The observations that few epigenetic enzymes are affected in the NAc suggest that the DS might participate more intimately in the behavioral manifestations of forced abstinence following compulsive METH taking.

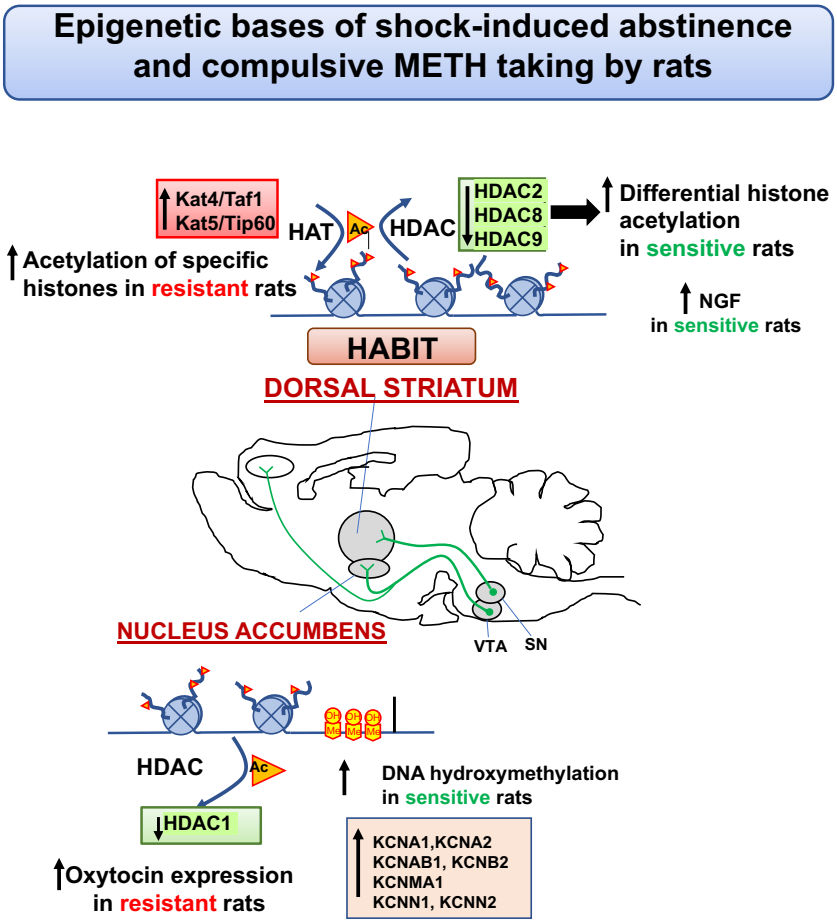

Fig. 5. Schema representing potential involvement of epigenetic enzymes in compulsive METH takers and abstinent rats after prolonged drug withdrawal. Both the NAc and DS are involved in various aspects of drug SA including incubation of METH seeking. In the present study, resistant rats (SR) showed greater incubation of METH seeking in comparison to the sensitive rats. Importantly, sensitive rats (SS) exhibited decreased *HDAC2, HDAC8, and HDAC9* mRNA expression in the DS, a structure thought to be involved in incubation of METH seeking. Sensitive rats also exhibited increased DNA hydroxymethylation and increased mRNA expression of potassium channel genes in the NAc. Resistant rats exhibited increased *Kat4/Taf1* and *Kat5/Tip60* mRNA expression in the DS, These observations suggest that increased expression of KAT4 and KAT5 gene targets might play an important role in the resistant rats and, by extension, in repeated relapses in human METH addicts. These ideas will be tested using vectors to increase or decrease the expression of these epigenetic enzymes in the DS.

2.8. Potential role of striatal epigenetic enzymes in responses to footshock-induced stress

As shown in the figures illustrating the results on epigenetic enzymes, we also found that animals that received only footshocks showed significant changes in the expression of several HDACs (see Figs. 3 and 4). Specifically, there were significant decreases in the striatal mRNA expression of *HDAC1*, *HDAC5*, *HDAC7*, *HDAC10*, and *HDAC11* in the shock-only animals (Fig. 3). These observations indicate that repeated footshocks are accompanied by increased histone acetylation in the rat dorsal striatum. These results are consistent with the demonstration that cells and organisms can adapt to environment stimuli via epigenetic mechanisms that include post-translational modifications of histones (Cadet, 2016; Collins et al., 2019; Sen, 2015; Yap and Greenberg, 2018). Epigenetic changes including DNA methylation and histone modifications have been well documented using the model of fear conditioning that uses various intensities of footshocks to induce the changes in animal behaviors (Jarome et al., 2018; Lubin et al., 2008; Sase et al., 2018). Of specific interest to our findings of decreased expression of several HDACs after repeated footshocks is the report by Sase et al. (2018) who observed increased abundance of H3K9/14 acetylation at the promoter of the CDK5 gene during retrieval of fear memory. Although the authors did not measure the expression of HDACs in their study (Sase et al., 2018), histone H3K9 acetylation is known to be regulated by class I HDACs (Banerjee et al., 2019; Wu et al., 2018) including HDAC1 that is downregulated in the animals that received repeated footshocks in our model (see Fig. 3). This discussion is consistent with the report that inhibition of class I HDACs can enhance fear memory (Zhao et al., 2018).

Another HDAC of interest in our study is *HDAC7* which is significantly decreased in the yoked shock, resistant, and sensitive rats, suggesting that footshocks were driving these changes. These observations are consistent with the report that *HDAC7* expression is decreased in contextual fear conditioning memory formation (Jing et al., 2017). Using a microanalytic approach, other investigators had also reported that *HDAC7* expression was decreased in the hippocampus of fear-conditioned rats (Gupta-Agarwal et al., 2012). When taken together, these observations suggest that the impact of footshocks on HDAC7 were more overwhelming than any potential effects of METH exposure. A similar conclusion can be reached for the effects of footshocks on *HDAC10* and *HDAC11* expression, suggesting a potential role for these two HDACs in fear conditioning.

The effects of footshocks on the expression of four sirtuins are also of interest. As illustrated in Fig. 4, the mRNA expression of *SIRT2*, *SIRT3*, *SIRT4*, and *SIRT5* was significant decreased in all 3 groups that received shocks including the two that had self-administered METH. This suggests that the stress associated with footshocks was more overwhelming than any potential effects of METH. A few studies have implicated the sirtuins in models of neurological and psychiatric disorders including Parkinson's disease, depression, and addiction (Erburu et al., 2017; Esteves et al., 2018; Jayanthi et al., 2014; Liu et al., 2012). Their participation in the models of these disorders may, in part, be secondary to their regulation of cellular metabolism and their roles in mitochondrial function (Kumar and Lombard, 2018; Min et al., 2018). Importantly, however, there is substantial evidence that psychological stress can impact mitochondrial functions and the responses to adverse life events by individuals (Picard and McEwen, 2018a, 2018b). This discussion just suggests potential avenues of research aimed at dissecting further the role of these sirtuins in stress-induced behavioral changes.

3. Concluding remarks

In conclusion, we found that rats that had escalated their intake of METH can be dichotomized into two different phenotypes (shock-resistant and shock-sensitive) in response to the occurrence of contingent footshocks during METH self-administration. We found, in addition, that rats that reduce their METH intake in the presence of punishment show significant increases in DNA hydroxymethylation and increased mRNA expression of genes that code for potassium channels in their NAc. Moreover, sensitive rats that showed attenuated METH seeking behaviors after protracted withdrawal also exhibited increases in striatal NGF levels, TrkA phosphorylation, p75NTR expression, and phosphorylation of the Ras/Raf/MEK/ERK signaling cascade. In contrast, resistant rats showed increases in oxytocin mRNA levels in their NAc after a month of forced abstinence. Moreover, differential expression of epigenetic enzymes including histone acetyltransferases was differentially expressed between the two phenotypes. These molecular adaptations suggest that several processes may be in play to promote the variable responses to contingent footshocks in rats, all of whom had escalated their intake of METH during the SA experiments (see Fig. 5). Therefore, identification of differential molecular and cellular processes in animals that are dichotomized based on their responses to adverse consequences may offer better explanatory windows to neuroplastic events that might occur in humans addicted to METH.

Although we had focused our initial attention on epigenetic and transcriptional consequences that occur in the dorsal and ventral striata as described above, there are other brain regions including the habenula that may regulate responses to punishment/aversion (Hennigan et al., 2015; Wang et al., 2017) and compulsive drug intake (Mathis and Kenny, 2018). The rostromedial tegmental nucleus (RMTg) that contains GABAergic afferent to the midbrain dopaminergic neurons is also of interest because these cells have been reported to encode aversive stimuli and to inhibit motor responses (Jhou et al., 2009). Importantly, cocaine has been shown to modulate aversive conditioning through the activation of dopamine-responsive habenular pathways (Jhou et al., 2013). Therefore, future studies using techniques such as RNAScope (Anderson et al., 2003) combined with immunohistochemistry should help us to characterize potential molecular changes in these structures in our model of METH addiction and shock-induced abstinence. In fact, exposing rats to footshocks has indeed been reported to induce c-fos expression in the RMTg and the medial portion of the lateral habenula (Brown and Shepard, 2013), thus supporting the notion that differential responsivity to shock-induced molecular events in those structures might underlie resistance and sensitivity to footshocks. Finally, the use of similar models to investigate molecular substrates and the neuronal pathways that are impacted during the development and maintenance of addiction to licit and illicit drugs should provide promising leads towards the development of more impactful therapeutic agents in human populations.

Competing interest

All the authors declare no competing financial interests or conflicts of interest.

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