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Repeated methamphetamine and modafinil induce differential cognitive effects and specific histone acetylation and DNA methylation profiles in the mouse medial prefrontal cortex.

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Abstract

Methamphetamine (METH) and modafinil are psychostimulants with different long-term cognitive profiles: METH is addictive and leads to cognitive decline, whereas modafinil has little abuse liability and is a cognitive enhancer. Increasing evidence implicates epigenetic mechanisms of gene regulation behind the lasting changes that drugs of abuse and other psychotropic compounds induce in the brain, like the control of gene expression by histones 3 and 4 tails acetylation (H3ac and H4ac) and DNA cytosine methylation (5-mC). Mice were treated with a seven-day repeated METH, modafinil or vehicle protocol and evaluated in the novel object recognition (NOR) test or sacrificed 4 days after last injection for molecular assays. We evaluated total H3ac, H4ac and 5-mC levels in the medial prefrontal cortex (mPFC), H3ac and H4ac promotor enrichment (ChIP) and mRNA expression (RT-PCR) of neurotransmitter systems involved in arousal, wakefulness and cognitive control, like dopaminergic (*Drd1* and *Drd2*), α-adrenergic (*Adra1a* and *Adra1b*),

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orexinergic (*Hcrtr1* and *Hcrtr2*), histaminergic (*Hrh1* and *Hrh3*) and glutamatergic (AMPA *Gria1* and NMDA *Grin1*) receptors. Repeated METH and modafinil treatment elicited different cognitive outcomes in the NOR test, where modafinil-treated mice performed as controls and METH-treated mice showed impaired recognition memory. METH-treated mice also showed i) decreased levels of total H3ac and H4ac, and increased levels of 5-mC, ii) decreased H3ac enrichment at promoters of *Drd2*, *Hcrtr1/2*, *Hrh1* and *Grin1*, and increased H4ac enrichment at *Drd1*, *Hrh1* and *Grin1*, iii) increased mRNA of *Drd1a*, *Grin1* and *Gria1*. Modafinil-treated mice shared none of these effects and showed increased H3ac enrichment and mRNA expression at *Adra1b*. Modafinil and METH showed similar effects linked to decreased H3ac in *Hrh3*, increased H4ac in *Hcrtr1*, and decreased mRNA expression of *Hcrtr2*. The specific METH-induced epigenetic and transcriptional changes described here may be related to the long-term cognitive decline effects of the drug and its detrimental effects on mPFC function. The lack of similar epigenetic effects of chronic modafinil administration supports this notion.

Keywords

methamphetamine; modafinil; histone acetylation; DNA methylation; prefrontal cortex; cognition

1. INTRODUCTION

Methamphetamine (METH) is a highly addictive psychostimulant known to negatively impact multiple domains of executive function including decision making, attention, and impulse control (Cadet and Bisagno, 2013). These long-lasting cognitive impairments have been associated with METH-induced neuroplastic changes in the prefrontal cortex (PFC) of humans and in animal models of addiction (Bernheim et al., 2016). In contrast, modafinil is a psychostimulant known to produce cognitive enhancement by its ability to increase PFC function (Rasetti et al., 2010; Gozzi et al., 2012) with little abuse liability (Myrick et al., 2004; Bisagno et al., 2016). Due to its profile as a stimulant and cognitive enhancer, modafinil is being used *off label* as an anti-relapse medication against METH dependence and other addictive disorders (McGaugh et al., 2009; Kalechstein et al., 2010; Mereu et al., 2013). Both METH and modafinil interact with the dopamine transporter (DAT) to increase pre-synaptic dopamine (DA) volume transmission, but with distinct kinetic properties. Specifically, METH elicits profound synaptic increases in DA by reversing the DAT transport (Sulzer et al., 2005), whereas modafinil is a weak DAT blocker that prevents intracellular DA reuptake (Wisor, 2013). Beyond dopaminergic effects, both modafinil and METH interact with norepinephrine (NE) and histamine systems as part of their respective pharmacokinetics (Munzar et al., 2004; Ishizuka et al., 2010; Ferrucci et al., 2013; Wisor, 2013). Additionally, modafinil and METH have been shown to activate orexinergic neurons, which induces wakefulness by stimulation DA, NE and histamine neurotransmission (Estabrooke et al., 2001; Ishizuka et al., 2010; Mahler et al., 2013). Glutamatergic neurotransmission in the corticostriatal pathway is essential for motivated behaviors and it is well known that psychostimulant intake may alter synaptic plasticity (Kauer and Malenka, 2007; Parsegian and See, 2014; González et al., 2016). In the PFC, different subtypes of DA receptors activation modulates the strength of excitatory synapses by increasing or decreasing AMPA and NMDA glutamate receptor subunits trafficking to the membrane of

PFC neurons, and unregulated DA release during repeated exposures to psychostimulants can lead to maladaptive plasticity, cognitive decline and addiction (Kauer and Malenka, 2007; González et al., 2016; Bisagno et al., 2016).

Chronic psychostimulant exposure has been shown to impair cognitive functioning (Moore et al., 2013; Peixoto and Abel, 2013), in part due to long-lasting neuroplastic modifications within mesocorticolimbic circuitry (Robison and Nestler, 2011; Rogge and Wood, 2013). Indeed, increasing evidence suggests that epigenetic mechanisms mediate the prolonged changes induced by drugs of abuse and other psychotropic compounds (Robison and Nestler, 2011). One such mechanism is the acetylation of lysines on histone tails, a highly regulated process of transcriptional activation (Zentner and Henikoff, 2013). Histone acetylation is known to influence gene transcription by cumulative effects and in different ways: the addition of acetyl groups can affect electrostatic protein-DNA and protein-protein interactions, impacting nucleosome positioning and accessibility, and it can also influence transcription factor binding (Li et al., 2007). Also, the lysine “code” can be recognized directly by specific enzymes that act as “readers” of lysine acetylation, translating this signal into various normal or abnormal phenotypes, that not necessarily correlates with gene transcription (Strahl and Allis, 2000; Robison and Nestler, 2011; Zentner and Henikoff, 2013). Similarly, DNA methylation is known to repress gene expression by placing a methyl group onto the 5´ position of cytosine at CpG dinucleotides which inhibits the binding of transcription factors to DNA (Moore et al., 2013).

To date, the epigenetic alterations induced by METH have been documented mainly in the dorsal and ventral striatum (Martin et al., 2012; Jayanthi et al., 2014; Cadet et al., 2015; Torres et al., 2015, 2016). However, the effects of METH on the PFC remain largely unexplored. As for the stimulant modafinil, no information is available regarding its epigenetic effects on the brain or in other tissues. Previously, our laboratory reported that repeated METH treatment in mice leads to visual cognitive impairment evaluated in a Novel Object Recognition (NOR) task (González et al., 2014). Visual memory impairment was linked to impaired ERK phosphorylation in the medial PFC (mPFC) after first presentation of novel objects in NOR training, which was performed four days after last METH injection (González et al., 2014). We also reported that METH detrimental effects in mPFC at this very same wash-out time point (four days after last drug administration) included reduced Ca^{2+} currents and glutamate neurotransmission together with increased hyperpolarization IH current in layer V pyramidal neurons and altered mRNA expression of voltage-gated Ca^{2+} channels, glutamate receptors and IH channels subunits (González et al., 2016). Interestingly, modafinil given before the NOR training restored the METH-induced deficits in memory retention (González et al., 2014). We thus postulated that within the mPFC, modafinil and METH might exert distinct epigenetic and transcriptional profiles associated with cognitive improvement and decline, respectively.

Here, we investigated the effects of repeated METH or modafinil exposure on recognition memory and sensitization. In addition, we studied total protein levels for histone 3 and 4 acetylation (H3ac and H4ac), total H3ac and H4ac enrichment at specific gene promoters, transcriptional changes and 5-methylcytocine (5-mC) levels following a 4-day washout period.

2. MATERIALS AND METHODS

2.1. Animals

C57BL/6 male mice (2–3 months old) from the School of Exact and Natural Sciences of the University de Buenos Aires (UBA) were housed in a light- and temperature-controlled (20–22˚C) vivarium. Mice had *ad libitum* access to standard rodent chow and water except during testing. Principles of animal use and care procedures were followed in accordance with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2003). All experimental protocols were approved by the corresponding IACUC authorities of the UBA (Protocol Number: A5801–01) using OLAW and ARENA directives (NIH, Bethesda, USA).

2.2. Drug treatments and experimental procedures

The drugs used were: (+)-methamphetamine hydrochloride (Sigma, St Louis, MO), and modafinil (racemic mixture of R- and S-enantiomers), generously donated by Laboratorios Beta S.A. (Argentina). METH was diluted in 0.9% saline and administered at 1 mg/kg (s.c.). Modafinil was prepared as a suspension in carboxymethylcellulose with 0.5% saline and administered at 90 mg/kg (i.p.). The repeated effects of each drug were evaluated as previously described (González et al., 2014; 2016). Briefly, mice were injected with METH or modafinil once a day for 7 consecutive days and were euthanized 4 days after the last injection (Figure 1A). Control groups were administered vehicle. This protocol was performed for tissue sampling (see Figure 1A).

2.3. Locomotor sensitization analysis

At day 1 and 7 of treatment, mice were placed in locomotor arenas for 5 min before injection to evaluate basal locomotion, and for 30 min after injection to evaluate acute locomotor responses to the drugs (Figure 1A). Locomotion was recorded and analyzed with Ethovision XT 7.0 tracking software (Noldus, The Netherlands).

2.4. Novel Object Recognition test

The NOR test was performed on a separate cohort of animals, following previously reported methods by our laboratory (Figure 1B) (González et al., 2014). This behavioral test was solely performed to characterize the cognitive effects of modafinil and METH repeated treatments, but no tissue was obtained from these animals. Briefly, exploration occurred in an open-field arena (40 cm^3) made of plexiglass, with the floor covered with clean woodchip bedding. Testing was done in a sound-attenuated room with dimmed illumination. In the absence of objects, all mice were individually habituated to the arena for 5 min during three consecutive days after the last modafinil or METH injection (Figure 1B, Habituation). On day 4, two identical objects were symmetrically fixed onto the floor of the arena and each mouse was allowed to explore the box for 10 min (Figure 1B, Training session). Objects were golf balls and plastic pipes (3 cm diameter, 8 cm high), which were similar in size but different in color, shape and brightness. These sets of objects were chosen based on preliminary experiments and previous study (Gonzalez et al. 2014) that indicated that they were similarly preferred, and also that the left object was similarly preferred than the right

object. Following a 24-hour delay, mice were placed back in the open-field arena for 5 min where one of the familiar objects remained the same and the other was replaced by a novel object (Figure 1B, Retention session). The right or left position of the novel object as well as the objects used as novel or familiar were counterbalanced between the animals in each group and between the control and drug-treated groups. All behavioral sessions were recorded and analyzed using the automated Ethovision XT 7.0 tracking software (Noldus, The Netherlands) with the nose point-tail detection. The percentage of exploratory preference (%EP) was calculated as exploration time of the novel object (TN) divided by the total exploration time of both novel (TN) and familiar (TF) objects [%EP=TN/(TN +TF)*100]. For the training sessions, the following formula was applied: exploration time of the right object (TR) divided by the total exploration time of both right (TR) and left (TL) objects [%EP=TR/(TR+TL)*100]. Because both left and right objects in the training sessions were equally preferred, the %EP for both right and left objects is ~50 %.

2.5. Western Blot

Western blot analyses were conducted as previously described (González et al., 2014). Briefly, mouse mPFC regions were quickly removed and stored at –70°C. Protein samples (20 μg) were separated by 12% SDS-PAGE, and transferred to a PVDF membrane. Blots were incubated with primary antibodies: 1:3000 anti-H3ac (06–599, Millipore) or 1:3000 anti-H4ac (06–866, Millipore). To confirm equal protein loading, blots were re-probed with 1:10000 anti- α-tubulin (Sigma). Immune complexes were detected with secondary antibodies and chemiluminescence reagents (Amersham, NJ, USA). Band density was quantified using ImageJ (NIH) software. Results were calculated as the ratios of protein expression for each drug-treated group in comparison to their respective saline-treated group and are reported as % change relative to controls.

2.6. Chromatin immunoprecipitation assays (ChIP-PCR)

Mouse mPFC tissue was processed for chromatin immunoprecipitation (ChIP) according to published protocols (Jayanthi et al., 2014). Details are listed in Supplemental information. Briefly, minced tissue (25–30 μg) was cross-linked in 1% formaldehyde/PBS for 15 min. Dynabeads (Life Technologies) were blocked with BSA and incubated with anti-H3ac (5 μg, 06–599 Millipore), anti-H4ac (2,5 μg, 06–866 Millipore), or normal rabbit IgG (negative control, 2.5 or 5 μg, 12–370 Millipore) antibodies. Chromatin shearing was carried out in a sonicator (Bioruptor Pico, Diagenode) with 200 μl lysis buffer. After sonication, 20 μl were separated for DNA methylation studies. Immunoprecipitation was carried out overnight at 4 °C, and DNA-protein complexes were then disassociated at 65 °C with proteinase K following treatment with RNaseA (Life Technologies). DNA was isolated using phenol/ chloroform extraction, suspended in 10 mM Tris and quantified in NanoDrop 2000 spectrophotometer (Thermo Scientific). PCR was performed on ChIP-derived DNA using the ABIPrism 7500 sequence detection system (Applied Biosystems). Enrichment of H3ac and H4ac was determined by specific ChIP primers designed to amplify proximal sequences from the transcription start site (TSS) of murine *Drd1*, *Drd2*, *Adra1a*, *Adra1b*, *Hcrtr1*, *Hcrtr2*, *Hrh1*, *Hrh3*, *Gria1,* and *Grin1*, and normalized to *Actb* (sequences are listed on Table S2 in Supplemental Information). H3ac and H4ac enrichment over IgG for *Actb* promoter was determined as a positive enrichment control (see S1 in Supplemental Information).

2.7. DNA extraction and ELISA-based global 5-methylcytosine (5-mC) determination

DNA was extracted from aliquots of sheared input chromatin from ChIP assays. Briefly, 20 µl of sheared chromatin from ChIP assays was brought to 500 µl final volume in lysis buffer and incubated overnight with proteinase K, extracted with phenol/chloroform and precipitated overnight with EtOH 100% at −70 ºC. After suspention in 50 µl Tris-EDTA buffer, DNA was treated with RNAseA prior to purity and concentration quantification in NanoDrop 2000 spectrophotometer (Thermo Scientific). One hundred nanograms of DNA per sample were denatured and added to a commercially available ELISA kit to quantify global 5-mC in DNA samples (D5325, Zymo Research), following manufacturer's protocol. After primary and secondary antibodies incubation, HRP developer was added and the plate was allowed to develop at room temperature for 1 hr. Absorbance was read at 405 nm in a Flex Station 3 microplate reader (Molecular Devices).

2.8. RT-PCR

RT-PCR experiments were conducted as previously described (González et al., 2016). Briefly, mPFC tissue was dissected and stored at −70 °C in RNA later solution (Qiagen). Total RNA was then isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. Five hundred nanograms of RNA were treated with DNAseI (Invitrogen) and reverse-transcribed in a 20 µL reaction using M-MLV reverse transcriptase (Promega) and random hexameres (Biodynamics). QRT-PCR primers were designed for the specific amplification of murine *Drd1a*, *Drd2*, *Adra1a*, *Adra1b*, *Hcrtr1*, *Hcrtr2*, *Hrh1*, *Hrh3*, *Gria1,* and *Grin1* (sequences are listed on Table S2 in Supplemental Information). Each sample was assayed in duplicate using 4 pmol of each primer, 1X SYBR Green Master Mix (Applied Biosystems) and 2–20 ng of cDNA in a total volume of 13 µL. Amplification was carried out in an ABI PRISM 7500 sequence detection system (Applied Biosystems). Expression of mRNA levels for each gene was normalized to the reference gene *Actb*. Results are reported as % changes calculated by the ratios of normalized target genes of each drug-treated group in comparison to the gene expression data of respective control groups.

2.9. Statistical analysis

Data are expressed as the mean ± SEM. Statistics were performed using one-way ANOVAs followed by Bonferroni post-hoc tests. Data were transformed when required. For data that did not comply with parametric test assumptions Kruskal-Wallis ANOVA on ranks was applied. For locomotor analysis, a two-way ANOVA with repeated measures (treatment and day) was applied, followed by Bonferroni post-hoc tests. Statistics were done with the software InfoStat 2010. The null hypothesis was rejected at p values less than 0.05.

3. RESULTS

3.1. Modafinil and METH effects on locomotor sensitization

Figure 2 shows the locomotor effects following Day 1 and Day 7 of treatment in animals that were then sampled for molecular assays. Repeated 7-day injections of either METH or modafinil induced behavioral sensitization, as evidenced by increased locomotor response at Day 7 compared to Day 1 [two-way ANOVA with repeated measures-Bonferroni

$F_{(1,73)}$=95.15, p<0.0001 for Day, $F_{(2,73)}$=21.47, p<0.0001 for treatment by day interaction] (Figure 2A). We also evaluated the basal locomotor activity in the habituation sessions prior to drug injections and the time spent in the center of the locomotor arena (Figure 2B). As expected the experimental groups showed no differences in locomotor activity or time in center on day 1 habituation, prior to any drug administration. At day 7 basal locomotion was increased in METH-treated mice compared to controls [ANOVA-Bonferroni $F_{(2,73)}$=4.409, p=0.016]. However, this effect was absent in modafinil-treated mice.

3.2. Modafinil and METH effects on Novel Object Recognition test

We and others have previously described that mice treated with 7 days-repetitive METH treatment show deficits in recognition memory as evaluated by the Novel Object Recognition (NOR) test (Kamei et al., 2006; Gonzalez et al., 2014). In contrast, modafinil is known to act as a cognitive enhancing agent (Bisagno et al., 2016). However, there is no data available on modafinil repetitive treatment on object recognition memory. As expected, METH-treated mice exhibited visual memory impairment evidenced in equal exploration of novel and familiar objects during the retention session [ANOVA-Bonferroni $F_{(20,2)}$=4.59, p=0.024]. In contrast, modafinil-treated mice behaved like control mice in the NOR task, spending more time exploring novel objects (Figure 3A). We also evaluated total exploration time of both objects and locomotion during training and retention sessions and found no differences among groups (Figure 3B and C). The impaired performance observed in METH-treated mice is not likely to be due to changes in motor or motivational functions induced by METH, since exploratory preference for the objects and total exploration time during the training and retention sessions did not differ between the experimental groups (Kamei et al., 2006; Gonzalez et al., 2014). Figure 3D shows representative captions from Ethovision files, showing exploration paths of mice treated with vehicle, modafinil and METH.

3.3. Modafinil and METH effects on total histone 3 and 4 acetylation expression and global 5-methylcytocine levels in the mPFC.

We evaluated total H3ac and H4ac levels and global 5-mC in DNA within the mPFC following repeated modafinil and METH treatments. Figure 4A shows that both H3ac [ANOVA-Bonferroni $F_{(2,20)}$=8.27, p=0.003] and H4ac [ANOVA-Bonferroni $F_{(2,20)}$=13.48, p=0.0003] showed decreased total protein levels after METH, compared to vehicle and modafinil. Interestingly, METH-treated mice also showed increased 5-mC in genomic DNA compared to Vehicle [ANOVA-Bonferroni $F_{(2,20)}$=4.43, p=0.028] (Figure 4B).

3.4. Modafinil and METH effects after repeated treatments on H3ac and H4ac enrichment at different gene promoters in the mPFC.

We performed chromatin immunoprecipitation (ChIP) assays followed by PCR to evaluate H3ac and H4ac status at different promoters of putative target genes of psychostimulant action in the mPFC. It is important to note that in this study we used well validated pan-acetylated histone 3 and 4 polyclonal antibodies that detect general acetylation increases in all lysine residues of histone 3 and 4. Because genome-wide analyses of histone acetylation patterns in mammalian cells have confirmed the correlation between histone acetylation and

gene activation, we used pan-acetylated antibodies to detect global increases in histone acetylation that are indicative of permissive states of the chromatin (Wang et al., 2008).

We found very different H3 and H4 acetylation patterns after chronic modafinil and METH (Figure 5A and B), where most changes were only present in METH-treated mice. Histone 3-dependent specific METH effects showed decreased H3ac enrichment at promoters of DA receptor *Drd2* [Kruskal-Wallis H=12.95, p=0.001], orexin receptors *Hcrtr1* [Kruskal-Wallis H=8.31, p=0.016] and *Hcrtr2* [Kruskal-Wallis H=8.03, p=0.018], histamine receptor *Hrh1* [Kruskal-Wallis H=6.81, p=0.033] and NMDA receptor subunit *Grin1* [Kruskal-Wallis H=6.23, p=0.044], in comparison to modafinil and Vehicle (Figure 5A). Histone 4-dependent METH effects showed increased H4ac enrichment at promoters of DA receptor *Drd1* [Kruskal-Wallis H=8.13, p=0.017], histamine receptor *Hrh1* [ANOVA-Bonferroni $F_{(2,26)}$=3.94, p=0.033] and NMDA receptor subunit *Grin1* [ANOVA-Bonferroni $F_{(2,26)}$=6.28, p=0.006] compared to Vehicle (Figure 5B). Importantly, we found a specific modafinil effect increasing H3ac at *Adra1b* promoter [Kruskal-Wallis H=6.21, p=0.045] compared to Vehicle and METH. We also found shared modafinil and METH effects for orexin and histamine receptors, which could be indicative of general psychostimulant actions: they both increased H4ac enrichment at orexin receptor *Hcrtr1* [Kruskal-Wallis H=8.21, p=0.016] and decreased H3ac at histamine receptor *Hrh3* promoters [Kruskal-Wallis H=6.02, p=0.049]. Finally, there were no drug-induced changes in H3/H4ac enrichment at α(1B)AR subunit *Adra1a* and AMPA receptor subunit *Gria1*.

3.5. Modafinil and METH effects after repeated treatments on mRNA expression in the mPFC.

We evaluated the mRNA expression profile after repeated modafinil and METH treatment (Figure 6). Repeated injections of METH caused increased expression of *Drd1a* [Kruskal-Wallis H=7.42, p=0.024], AMPA *Gria1* [ANOVA-Bonferroni $F_{(2,15)}$=5.95, p=0.015] and NMDA *Grin1* [Kruskal-Wallis H=6.02, p=0.049]. Repeated modafinil increased *Adra1b* expression compared to vehicle [ANOVA-Bonferroni $F_{(2,15)}$=5.55, p=0.018]. Both METH and modafinil caused decreased *Hcrtr2* mRNA expression compared to vehicle [Kruskal-Wallis H=6.51, p=0.039].

For clarification, we summarized ChIP-PCR and RT-PCR results for acute and chronic modafinil and METH treatments graphically depicting the global tendency of each drug compared to vehicle (Figure 7).

4. DISCUSSION

The main findings of this research are: i) repeated METH and modafinil treatment elicited different cognitive outcomes in the NOR test, where modafinil-treated mice performed as controls and METH-treated mice showed impaired recognition memory; ii) within the mPFC, METH-treated mice showed decreased levels of total H3ac and H4ac and increased levels of total 5-mC; iii) METH-treated mice showed specific decreased H3ac enrichment at several promoters including *Drd2*, *Hcrtr1/2*, *Hrh1* and *Grin1*, and increased H4ac enrichment at *Drd1*, *Hrh1* and *Grin1* receptors; iv) METH-treated mice showed up-regulation of DA and glutamate receptor mRNA levels including *Drd1a*, *Gria1* and *Grin1*; v)

modafinil-treated mice showed specific increase of H3ac enrichment and mRNA expression at *Adra1b*; vi) modafinil and METH showed similar effects linked to histamine and orexin systems including decreased H3ac in *Hrh3*, increased H4ac in *Hcrtr1*, and decreased mRNA expression of *Hcrtr2*.

Addictive behavior is characterized by poor cortical impulse control, and abused psychostimulants like METH induce neuroadaptations in the PFC (Cadet and Bisagno, 2013) that can "lock" it in deficient states for long periods of time (Bisagno et al., 2016). We have previously shown that the repeated METH treatment used here causes detrimental effects on mPFC function by reducing Ca^{2+} currents, glutamate neurotransmission, and increasing hyperpolarization I_H current in layer V pyramidal neurons (González et al., 2016). It needs to be mentioned that these effects on synaptic physiology were observed not only following 4 days of METH wash-out, but were also present when METH was applied on the recording bath of naïve slices, suggesting that they might be related to residual effects of the drug (González et al., 2016). These "locked" deficient cortical states after cessation of psychostimulant use might lead to cortical hypofunction and contribute to cognitive impairment (Chen et al., 2013; Bisagno et al., 2016). Consistent with this idea, repeated METH impaired object recognition memory, which is a task that relies on mPFC integrity (Morici et al., 2015). Recognition memory, measured by the NOR test, requires integration of different features and recruit the mPFC and other subcortical areas (Warburton and Brown, 2010). METH detrimental effects on visual memory was also associated with blunted ERK phosphorylation after novelty at the NOR training (at wash-out day 4) (Kamei et al., 2006; González et al., 2014). It is likely that METH effects on the NOR are long lasting since it was described that this effect persisted for at least 28 days after METH treatment (Kamei et al., 2006). Other cognitive deficits induced by METH were also previously described by other research groups, including passive avoidance (Murnane et al., 2012), spatial working memory (Braren et al., 2014) and memory flexibility (Izquierdo et al., 2010). Those cognitive tasks also rely to some extent on mPFC integrity (Torres-García et al., 2017; Wirt and Hyman, 2017) therefore changes described here on epigenetic markers might also explain deficits induced by METH on other behavioral tasks that depends on PFC cortical networks.

Interestingly, we found increased H4ac enrichment and corresponding mRNA expression after METH treatment in two key receptors involved in mPFC physiology and addictive mechanisms: D1 and GluN1 (Cepeda and Levine, 2006; Gao and Wolf, 2008; Kruse et al., 2009; González et al., 2016). In PFC pyramidal neurons, D1 and NMDA receptors have an important functional interaction, where they interact in a positive feedback loop that potentiates NMDA EPSCs (Cepeda and Levine, 2006). We have previously shown that repeated METH treatment increases *Grin1* expression via a D1-dependent mechanism (González et al., 2016). This positive feedback loop might lead to concomitant over-activation of both the D1 and the NMDA systems with detrimental effects for PFC function and cognition. We found that modafinil had no effects on *Drd1* and *Grin1* as did METH, and showed specific effects linked to increased H3ac enrichment and mRNA expression of *Adra1b*, a receptor that was shown to mediate the behavioral activation caused by this drug (Stone et al., 2002). Early studies in knock-out mice have shown that α1B-AR is involved in modulation of memory consolidation and fear-motivated exploratory activity (Knauber and

Müller, 2000). Due to the different cognitive outcomes of repeated modafinil and METH, we can speculate that the specific effects elicited by each drug on global acetylation and methylation profiles in the mPFC, and the associated changes in NE, DA, orexin, histamine and NMDA receptor genes found here, could be markers of enhanced vs impaired cognitive states elicited by these drugs in the mPFC. Interestingly, Gozen et al. (2013) found that repeated nicotine administration also increased D1 expression in the PFC by increasing H4ac at *Drd1* promoter, further suggesting that this feature could be a common effect of addictive drugs.

Histone acetylation is a highly regulated process controlled by histone acetyl transferases (HATs) and counteracted by deacetylases (HDACs). Increased histone acetylation has consistently been shown to favor learning and memory, and their absence has been causally implicated in cognitive impairment, neurodevelopmental disorders, neurodegeneration and ageing (Gräff and Tsai, 2013; Peixoto and Abel, 2013). We found that chronic METH treatment decreased H3ac and H4ac total protein levels in the mPFC. These reduced levels of histone acetylation after METH could be mediated by decreased HAT and/or increased HDAC activity. In this sense, increased HDAC activity has been found after repeated METH and amphetamine in the PFC (Li et al., 2014; Stertz et al., 2014) and HDAC inhibitors has been shown to interfere with many behavioral and biochemical effects of addictive psychostimulants (Romieu et al., 2008; Moretti et al., 2011; Kennedy et al., 2013; Jayanthi et al., 2014). Also, together with decreased H3ac and H4ac we found increased 5-mC levels after METH. DNA methylation is central in gene silencing mechanisms, and increased 5-mC at certain gene promoters has been shown to participate in both memory and addiction (Bali et al., 2011; Robison and Nestler, 2011). Consistent with our results, Mychasiuk et al. (2013) also found increased global DNA methylation in the mPFC and nucleus accumbens after chronic amphetamine and nicotine treatment. In cocaine studies it has been shown an increase in striatal tissue of the DNA methyl transferase DNMT3A and the methyl-CpG binding protein MeCP2 (Anier et al., 2010), which contributes to gene silencing by recruiting HDACs to methylated DNA (Amir et al., 1999). Thus, it is plausible that histone acetylation and DNA methylation might play a significant role in mechanisms mediating addictive behavior and cognitive impairments in the mPFC. Recent findings showed that global DNA hypermethylation is a key factor in reprogramming the mPFC genome after a history of alcohol dependence. Moreover, the hypermethylation effect was detected in histone modifying epigenetic enzymes and on histones genes, shaping the molecular and behavioral long-term consequences of alcohol abuse (Heilig et al., 2017). Further research is needed to clarify DNA methylation and histone modification contributions to mPFC malfunction and addiction, as well as which changes are due to residual drug treatment effects or to neuroadaptation induced by drug withdrawal.

Consistent with decreased levels of H3ac in the mPFC, we found decreased enrichment of H3ac in many genes analyzed including D2, orexin, histamine and NMDA receptor subunits. It is well established that the cAMP response element binding protein (CREB) binding protein (CBP) is important for memory formation, including object recognition memory, and that CBP works as a transcription factor and as an HAT (Wood et al., 2006). Also, CBP and other HATs such as p300/GCN5/PCAF acetylate all lysines of histone 3 as part of their gene activation mechanisms (Jin et al., 2011). In cortical, midbrain and striatal pathways, CREB

is phosphorylated in response to DA/glutamate stimulation and ERK activation (Dudman et al., 2003; Sarantis et al., 2009), therefore both global and specific decrease in H3ac found after METH could be reflecting deficits linked to these neurotransmitter systems and signaling pathways, as we have previously reported (González et al., 2014; 2016). Beyond direct effects on transcription, it was reported that total H3ac is a marker of permissive chromatin, whereas non-permissive chromatin is exhausted of this signal, and transgene silencing effects of nonpermissive chromatin cannot be completely countered by strong transcription activators, indicating the dominance of the chromatin effects (Yan and Boyd, 2006). Therefore, METH effects decreasing H3ac could be indicative of repressive chromatin states, which are also suggested by increased total 5-mC levels.

Interestingly for *Hcrtr1*, *Hrh1* and *Grin1* we found opposed effects between H3ac and H4ac enrichment profiles after METH treatment. These effects are not surprising, since acetylation of histone 4 rarely overlaps with acetylation of histone 3 or the other histones (Renthal et al., 2009; Martin et al., 2012, Rogge and Wood, 2013). Indeed, many reports have found independent histone-specific effects on transcription factor binding, gene expression and chromatin remodeling (Agricola et al., 2006; Yu et al., 2011; Gansen et al., 2015), suggesting that histone 3 and 4 may be targeted by protein complexes of activators/repressors that contain different HATs and HDACs, and respond to different signaling mechanisms (Rogge and Wood, 2013; Jayanthi et al., 2014).

The epigenetic regulation of gene expression involves both alterations in the steady state expression levels of a set of genes as well as changes in other genes' inducibility - meaning sensitization (priming) and desensitization - without a change in steady state mRNA expression (Wang et al., 2009; Nestler, 2014). Therefore, the epigenetic changes detected here that do not correlate with gene expression might reflect "latent" changes in gene inducibility, as well as "residual" changes at genes that have been regulated via chronic drug treatment. Also, given that several epigenetic mechanisms regulate transcription (Robison and Nestler, 2011) it is possible that other mechanisms are contributing to gene expression, such as DNA methylation and microRNA interactions.

Both modafinil and METH had effects on orexin and histamine receptors. Histaminergic and orexinergic neurons, located in the posterior hypothalamus, have leading distinct and complementary roles in sleep-waking regulation and have been implicated in the behavioral effects of modafinil and METH (Estabrooke et al., 2001; Munzar et al., 2004; Ishizuka et al., 2010; Wisor, 2013). The histamine system serves the maintenance of the waking state, whereas the close neighbor, the orexins system, orchestrates motor and other behavioral aspects of arousal, including cortical activation, feeding behavior and reward processing (Anaclet et al., 2009). While HRH1 mediates histamine actions on waking, HRH3 are autoreceptors damping histamine synthesis, release and firing frequency. Orexin antagonists, especially those that block HCRTR2, clearly promote sleep, and genetic mutations inactivating HCRTRs/orexin function are the pathophysiology behind narcolepsy (Mieda, 2017). Here we have found similar modafinil and METH effects on H4ac at *Hcrtr1* and on H3ac at *Hrh3* suggesting epigenetic "scars" of repeated psychostimulant intake that might relate to wakefulness. Interestingly, we also found specific METH effects on H3ac/H4ac enrichment at *Hrh1*, which in the PFC has been linked to object recognition, novelty and

reward (Dai et al., 2007; Zlomuzica et al., 2008). In addition, we found decreased H3ac in both HCRTR1/2 after METH. Recently, Gentile et al. (2017) showed a positive effect of a dual HCRTR1/2 antagonist on cocaine-evoked impulsivity, suggesting that orexin transmission may be involved in executive functions dependent on PFC physiology. Therefore, the specific epigenetic effects of METH on H3ac enrichment at HCRTRs might be linked to the detrimental effects of METH on PFC functioning.

Concluding remarks

To date there is no available information on the epigenetic targets of modafinil in the CNS. This study identified, for the first time, epigenetic effects on histone 3/4 acetylation and DNA methylation in the mPFC, that could mediate opposed cognitive profiles of repeated modafinil and METH treatment. In addition, information on the effects of METH in cortical brain structures is scarce. Thus, this study also provides evidence of altered chromatin states that may last for a long period of time after stopping METH exposure. Importantly, chronic modafinil was without such effects, suggesting that METH-induced differential effects might be related to cognitive impairment and mPFC malfunction. The results found here expand the knowledge on modafinil effects in CNS related to epigenetic markers. Modafinil is not only being prescribed off label to treat psychostimulant addiction but also increasingly consumed by healthy individuals seeking cognitive enhancement (Bisagno et al., 2016). Also, we provide evidence of behavioral, epigenetic and transcriptional changes associated with a low METH dose. Early stages of human consumption are usually associated with low/moderate doses, before gradual dosing escalation occurs (Madden et al., 2005). The altered epigenetic markers found here after METH may lead to a better chance toward the rational design of new pharmacological treatments aiming at restoring histone acetylation and DNA methylation balance in METH users, as well as to treat other neuropsychiatric disorders that show altered cognitive function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Repeated METH administration impairs object recognition memory whereas repeated modafinil does not show cognitive impairment.
- Only METH decreased total histone 3 and 4 acetylation and increased total DNA methylation in the mPFC.
- Only METH altered histone 3 and 4 acetylation at specific dopamine, orexin, histamine and glutamate receptors promoters and/or mRNA levels in mPFC.
- Only modafinil increased promoter histone 3 acetylation and mRNA levels of alpha(1B)adrenoreceptor in mPFC.
- METH-specific effects may be related to mPFC malfunction and long-term cognitive decline.

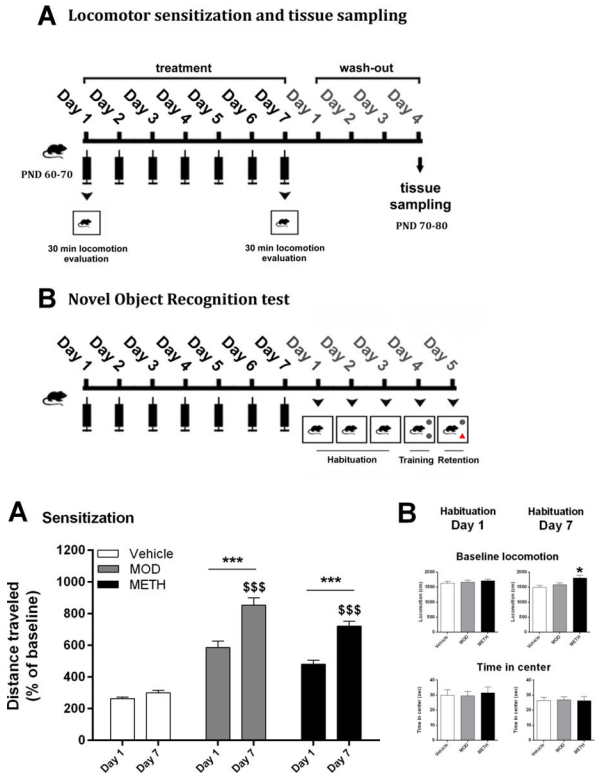

Figure 1: Schematic representation of repeated drug treatments, behavioral analysisand tissue sampling.

A) Locomotor analysis performed before tissue sampling for all molecular assays. This administration protocol was performed for tissue sampling of different animals groups tested for i) western blot, ii) ChIP-PCR/5-mC determination and iii) RT-PCR experiments. PND: postnatal day. B) Novel Object Recognition (NOR) task. Drug- and vehicle-treated mice were habituated to the open field arena 5 min a day for 3 consecutive days. On day 4 of wash-out, mice performed a training session in which they were allowed to freely explore two equal objects for 10 min, and 24 hrs later (day 5) performed a 5-min retention session, where one of the familiar objects was replaced by a novel object.

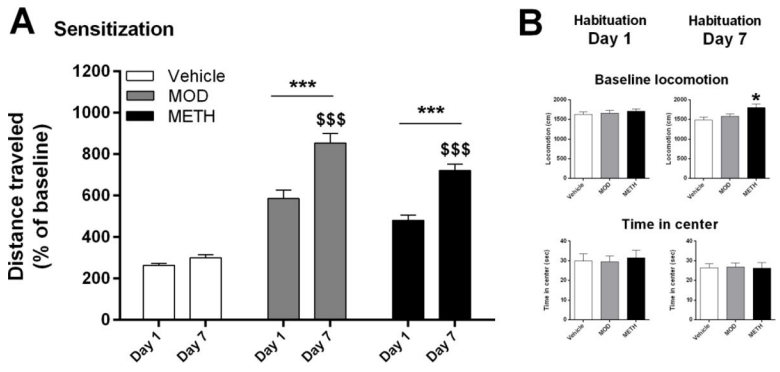

Figure 2: Effect of repeated modafinil (MOD) and methamphetamine (METH) treatment on behavioral sensitization.

A) Locomotor activity evaluated as distance traveled for 30 min after drug injection on Day 1 and Day 7 of treatment. Two-way ANOVA with repeated measures-Bonferroni (N=24–28). *** $p<0.001$ vs Vehicle, $$$ $p<0.001$ vs the corresponding group on Day 1. **B)** Baseline locomotion and time spent in center of the locomotor arena for the habituation sessions (5 min, prior to drug injections) at day 1 and day 7 of treatment. * $p<0.05$ vs Vehicle.

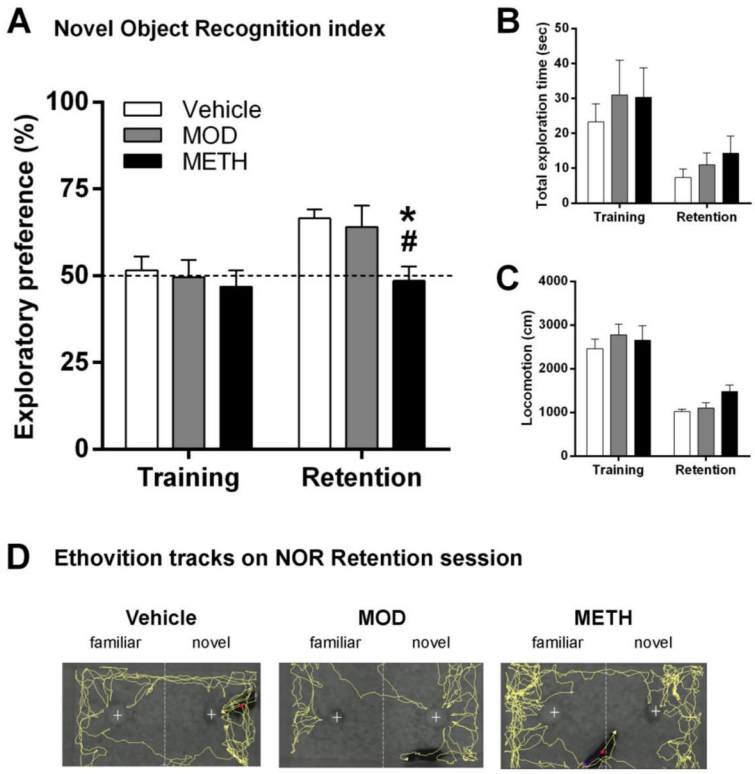

Figure 3: Novel object recognition (NOR) task after repeated treatment with modafinil (MOD) and methamphetamine (METH).

A) Training and retention sessions: ANOVA-Bonferroni (N=7–8), * Different from Vehicle, # different from MOD, $p<0.05$. B) Total exploration time of both objects. C) Locomotion. D) Representative captions from Ethovision files showing the mice nose path on the retention session. + indicates objects position.

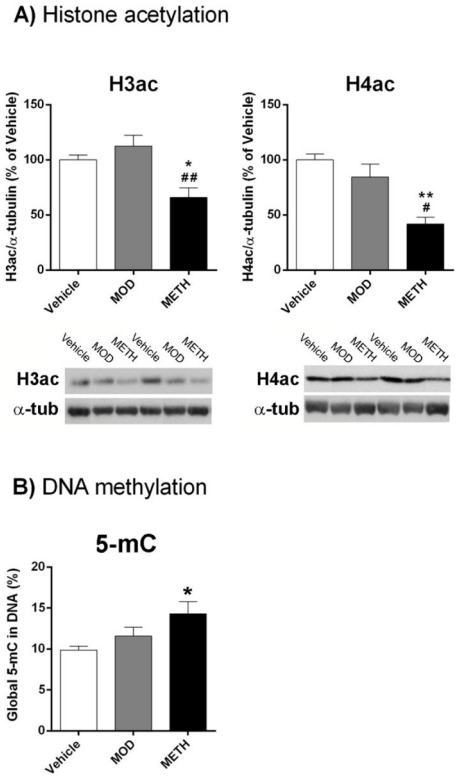

Figure 4: Effect of modafinil (MOD) and methamphetamine (METH) repeated treatment on total acetylated histone 3 and 4 and 5-methylcytocine (5-mC) in the mPFC.

A) Total levels of H3ac and H4ac after 7-day treatment (washout day 4). ANOVA-Bonferroni (N=6–8). B) Global 5-mC levels in genomic DNA were measured using a commercially available ELISA kit. ANOVA-Bonferroni (N=7). * different from Vehicle $p<0.05$, ** different from Vehicle $p<0.01$, # different from MOD $p<0.05$, ## different from MOD $p<0.01$.

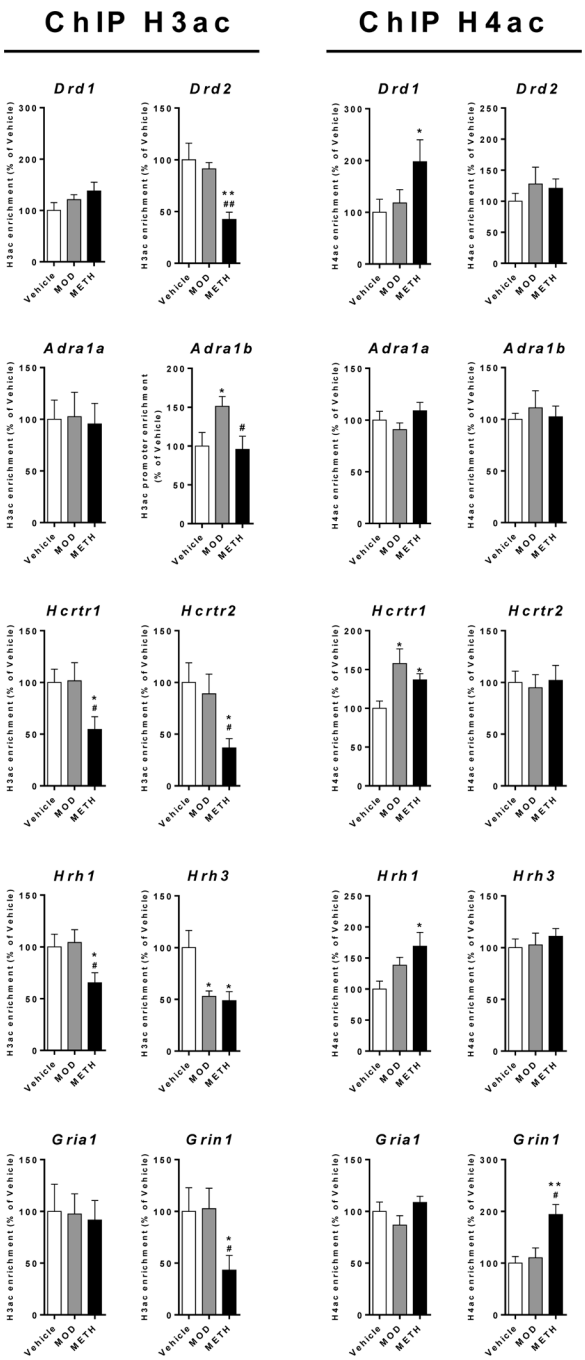

Figure 5: Effect of repeated modafinil (MOD) and methamphetamine (METH) treatment on the enrichment of acetylated histones 3 (A) and 4 (B) at specific promoters.

Genes evaluated: dopamine receptors *Drd1* and *Drd2*, alpha-adrenergic α(1)AR subunits *Adra1a* and *Adra1b*, orexin receptors *Hcrtr1* and *Hcrtr2*, histamine receptors *Hrh1* and *Hrh3,* and glutamate receptor AMPA subunit *Gria1* and NMDA subunit *Grin1.* (N=8–10). * Different from Vehicle $p<0.05$ or ** $p<0.01$, # different from MOD $p<0.05$ or ## $p<0.01$.

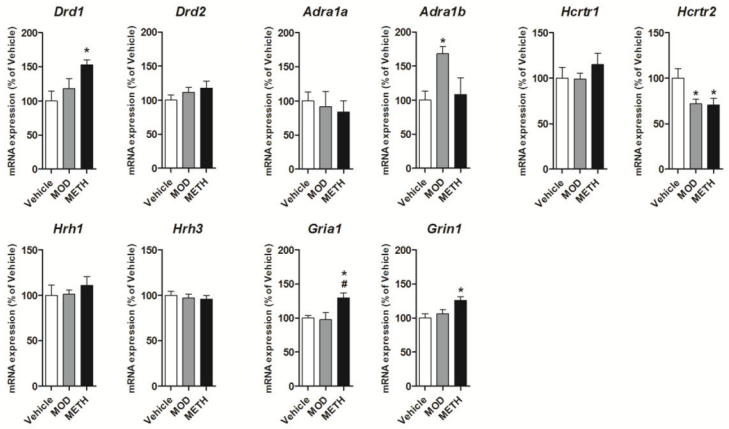

Figure 6: Effect of repeated modafinil (MOD) and methamphetamine (METH) treatment on mRNA expression by RT-PCR. (N=5–6).

* Different from Vehicle $p<0.05$, # different from MOD $p<0.05$.

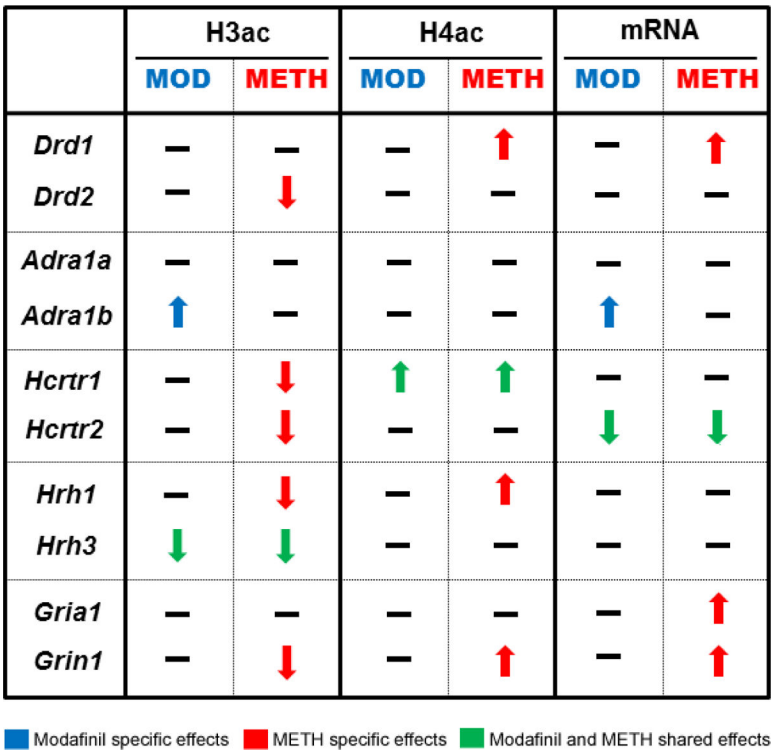

Figure 7: Modafinil (MOD) and methamphetamine (METH) shared and differential histone 3 and 4 acetylation and gene expression profiles in the mPFC.

Results summary showing in blue: MOD specific effects; in red: METH specific effects; in green: MOD and METH shared effects. Upward arrow indicates increase, downward arrow indicates decrease and dash indicates no change compared to vehicle-treated controls.