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The effects of single-dose injections of modafinil and methamphetamine on epigenetic and functional markers in the mouse medial prefrontal cortex: potential role of dopamine receptors.

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Abstract

METH use causes neuroadaptations that negatively impact the prefrontal cortex (PFC) leading to addiction and associated cognitive decline in animals and humans. In contrast, modafinil enhances cognition by increasing PFC function. Accumulated evidence indicates that psychostimulant drugs, including modafinil and METH, regulate gene expression via epigenetic modifications. In this study, we measured the effects of single-dose injections of modafinil and METH on the protein levels of acetylated histone H3 (H3ac) and H4ac, deacetylases HDAC1 and HDAC2, and of the NMDA subunit GluN1 in the medial PFC (mPFC) of mice euthanized 1 hr after drug administration. To test if dopamine (DA) receptors (DRs) participate in the biochemical effects of the two drugs, we injected the D1Rs antagonist, SCH23390, or the D2Rs antagonist, raclopride,

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30 min before administration of METH and modafinil. We evaluated each drug effect on glutamate synaptic transmission in D1R-expressing layer V pyramidal neurons. We also measured the enrichment of H3ac and H4ac at the promoters of several genes including DA, NE, orexin, histamine, and glutamate receptors, and their mRNA expression, since they are responsive to chronic modafinil and METH treatment. Acute modafinil and METH injections caused similar effects on total histone acetylation, increasing H3ac and decreasing H4ac, and they also increased HDAC1, HDAC2 and GluN1 protein levels in the mouse mPFC. In addition, the effects of the drugs were prevented by pre-treatment with D1Rs and D2Rs antagonists. Specifically, the changes in H4ac, HDAC2, and GluN1 were responsive to SCH23390, whereas those of H3ac and GluN1 were responsive to raclopride. Whole-cell patch clamp in transgenic BAC-Drd1a-tdTomato mice showed that METH, but not modafinil, induced paired-pulse facilitation of EPSCs, suggesting reduced presynaptic probability of glutamate release onto layer V pyramidal neurons. Analysis of histone 3/4 enrichment at specific promoters revealed: i) distinct effects of the drugs on histone 3 acetylation, with modafinil increasing H3ac at *Drd1* and *Adra1b* promoters, but METH increasing H3ac at *Adra1a*; ii) distinct effects on histone 4 acetylation enrichment, with modafinil increasing H4ac at the *Drd2* promoter and decreasing it at *Hrh1,* but METH increasing H4ac at *Drd1*; iii) comparable effects of both psychostimulants, increasing H3ac at *Drd2*, *Hcrtr1,* and *Hrh1* promoters, decreasing H3ac at *Hrh3*, increasing H4ac at *Hcrtr1,* and decreasing H4ac at *Hcrtr2*, *Hrh3,* and *Grin1* promoters. Interestingly, only METH altered mRNA levels of genes with altered histone acetylation status, inducing increased expression of *Drd1a*, *Adra1a*, *Hcrtr1,* and *Hrh1*, and decreasing *Grin1*. Our study suggests that although acute METH and modafinil can both increase DA neurotransmission in the mPFC, there are similar and contrasting epigenetic and transcriptional consequences that may account for their divergent clinical effects.

Keywords

methamphetamine; modafinil; histone acetylation; dopamine receptors; glutamate; prefrontal cortex

1. INTRODUCTION

Methamphetamine (METH) is a popular psychostimulant frequently abused for its euphoric properties, which result from stimulation of the mesolimbic and mesocortical pathways (Bisagno et al., 2016). Modafinil is an alertness promoting agent currently prescribed for the treatment of several sleep disorders and, due to its cognitive enhancing properties, is being investigated as a treatment for stimulant addiction (McGaugh et al., 2009; Kalechstein et al., 2010; Mereu et al., 2013). METH interacts with the dopamine transporter (DAT) by reversing the reuptake of dopamine (DA) and increasing DA release (Sulzer et al., 2005). Similarly, modafinil interacts with DAT, although weakly, and inhibits DA reuptake (Wisor, 2013). Because of their distinct pharmacokinetics, METH is strongly habit-forming whereas modafinil has limited abuse liability (Myrick et al., 2004). Both drugs act on neurotransmitter systems in the prefrontal cortex (PFC) where DA release participates in executive functions including attention, impulse control, and memory processes (Cadet and Bisagno, 2013). Within the PFC, METH induces negative neuroplastic changes associated with cognitive decline and addictive behaviors in humans and animals (Bernheim et al.,

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2016). In contrast, modafinil produces cognitive enhancement by its ability to improve PFC functions including working memory (Rasetti et al., 2010; Gozzi et al., 2012). In both cases, neuroadaptations may be secondary for the ability of each drug to alter gene expression through epigenetic modifications. This suggestion is supported by our previous observation that repeated injections of modafinil and METH over 7 days elicited differential cognitive outcomes assessed by the novel object recognition (NOR), PFC-dependent task, with modafinil-treated mice performing similar to controls, but METH-treated mice showing impaired recognition memory (González et al., 2018). The different cognitive outcomes were accompanied by drug-specific epigenetic modifications including alterations in total histone 3 and 4 acetylation (H3ac and H4ac) in the medial PFC (mPFC), differential H3ac and H4ac promoter enrichment, and altered expression of genes coding for DA receptors (DRs). Repeated injections of modafinil and METH for 7 days also altered H3ac/H4ac promoter acetylation and gene expression in other neurotransmitter systems including norepinephrine (NE), histamine, orexin, and glutamate receptors (González et al., 2018).

Acetylation of histone tails by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs) are common epigenetic modifications thought to participate in cognitive functions (Volmar and Wahlestedt, 2015). HDACs remove acetyl groups from lysine residues in the N-terminal tails of core histones, resulting in chromatin condensation and inhibition of gene expression (Strahl and Allis, 2000). Other regulators of histone modification include bromodomain-containing proteins (termed reader proteins) that recognize specific acetylated histone residues and, in the presence of chemical moieties and chromatin-modifying effectors, regulate transcription (Campos and Reinberg, 2009).

Among members of the HDAC family, HDAC1, and HDAC2 belong to the Class I nuclear proteins that are highly expressed throughout the brain, including the neocortex (Morris et al., 2010; Baltan et al., 2011). HDAC1 is found predominantly in glia cells whereas HDAC2 is highly expressed in neurons (Guan et al., 2009; Baltan et al., 2011; Yao et al., 2013). Recent evidence has suggested that Class I HDACs (particularly HDAC2), exert endogenous restraint on memory formation, with their inhibition improving learning and memory during various cognitive tasks (Alarcón et al., 2004; Fischer et al., 2007; Guan et al., 2009). Related to the thesis of the present paper, a single METH injection altered the levels of Class I HDACs in the nucleus accumbens (NAc) (Torres et al., 2016), impacted the abundance of NAc H3ac and H4ac levels (Martin et al., 2012), and increased the expression of immediate early genes in that structure (Torres et al., 2015). However, the effects of METH and modafinil on HDAC1/2 expression in the PFC have largely remained unexplored.

The effects of DA on PFC function depend on D1-type receptors (D1Rs; D1 and D5) and D2-type receptors (D2Rs; D2, D3, D4), that are located on glutamatergic pyramidal neurons and GABAergic interneurons to modulate fast excitatory and inhibitory synaptic transmission (Santana and Artigas, 2017). In the PFC, D1Rs are primarily located in synaptic spines where dopaminergic and glutamatergic afferents converge to form a "synaptic triad" (Goldman-Rakic et al., 1989; Bordelon-Glausier et al., 2008; Yao et al., 2008). In PFC pyramidal neurons, D1Rs and NMDA receptors have important functional and physical interactions (Seamans et al., 2001; Cepeda and Levine, 2006; Kruse et al., 2009). D1 and GluN1 subunits co-localize and interact physically, with D1 activation

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leading to a positive feedback loop that potentiates NMDA-mediated excitatory transmission (Seamans et al., 2001; Cepeda and Levine, 2006; Kruse et al., 2009). Given the importance of DA and NMDA in mPFC physiology, we quantified potential neuroepigenetic effects of single-dose injections of modafinil or METH, as well as the role of D1Rs and D2Rs, on the protein levels of H3ac, H4ac, HDAC1, HDAC2, and NMDA subunit GluN1 in the mPFC of mice euthanized 1 hr after drug administration. We also quantified H3ac and H4ac enrichment (by ChIP-PCR) at the promoters of DA, NE, orexin, histamine, and glutamate receptors, in addition to measuring associated changes in gene expression.

2. MATERIALS AND METHODS

2.1. Animals

C57BL/6 male mice (10-12 weeks old) from the School of Exact and Natural Sciences of the University de Buenos Aires (UBA) were used in this study. Mice were housed in a light- and temperature-controlled vivarium and had access to food and water ad libitum. Principles of animal care were followed in accordance with the "Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research" (National Research Council, 2003), and approved by the Universidad de Buenos Aires IACUC authorities (Protocol Number: A5801-01) using OLAW and ARENA directives (NIH, Bethesda, USA). For whole-cell patch-clamp recordings we used inbred bacterial artificial chromosome (BAC)-transgenic mice Drd1a-tdTomato (Ade et al., 2011).

2.2. Drug treatments

The drugs used were (+)-methamphetamine hydrochloride (Sigma, St Louis, MO), SCH23390 hydrochloride (TOCRIS bioscience, Ellisville, MO), raclopride (TOCRIS bioscience, Ellisville, MO), and modafinil (racemic mixture of R- and S-enantiomers), generously donated by Laboratorios Beta S.A. (Argentina). METH, SCH23390, and raclopride were diluted with 0.9% sterile saline, and modafinil was administered as a suspension in carboximethylcellulose 0.5% in saline. In the present study, we evaluated the effect of METH (1 mg/kg, s.c.) or modafinil (90 mg/kg i.p.) as a single dose. For vehicle administration, half of the mice received saline s.c. and the other half received 0.5% carboximethylcellulose in saline. All mice were sacrificed 1 hr after METH or modafinil injection. Modafinil and METH administration increased locomotion compared to vehicle (see Figure S1 in Supplemental information). In a separate groups of animals, the D1/5 receptor antagonist, SCH23390 (0.05 mg/kg i.p.) or the D2/3/4 receptor antagonist raclopride (8 mg/kg i.p.), were administered 30 min before the METH or modafinil injection. These doses were chosen based on previous studies by our laboratory using SCH23390 (González et al., 2016), and from others demonstrating that raclopride at this dosage was able to antagonize the effects of METH on an mPFC-dependent NOR task (Kamei et al., 2006).

2.3. Whole-cell patch-clamp recordings

Evoked excitatory postsynaptic currents (EPSCs) were recorded as previously described (González et al., 2016). Mice were anesthetized with tribromoethanol (250 mg/Kg i.p.) followed by transcardiac perfusion with ice-cold low sodium/antioxidant solution, and then

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decapitated. Coronal brain slices, including mPFC (300 μm) were obtained by gluing both hemispheres with the caudal part onto a vibratome stage (Integraslicer 7550 PSDS, Campden Instruments, UK), submerged in a chamber containing chilled low-sodium/high-sucrose solution, and aerated with 95% O_2/5% CO_2 (pH 7.4), as previously described (González et al., 2016). Whole-cell patch clamp recordings were made at room temperature (20-24°C). Patch electrodes were made from borosilicate glass (2-3 MΩ) filled with a voltage-clamp high Cl–, high Cs+/QX314 solution. Signals were recorded using a MultiClamp 700 amplifier commanded by pCLAMP 10.0 software (Molecular Devices, CA, USA). Data were filtered at 5 kHz, digitized and stored for off-line analysis. Capacitance and leak currents were electronically subtracted using a standard pCLAMP P/N substraction protocol. Pyramidal prelimbic cortex deep layer V-VI neurons from BAC-transgenic mice Drd1a-tdTomato showing red fluorescence in D1R expressing cells were used in this study. EPSCs were evoked extracellularly (twice threshold; 40–200 μs; 200–1000 μA) using a bipolar concentric electrode (FHC Inc, ME, USA) attached to a motorized micromanipulator MPC200/ROE200 (Sutter Instrument, CA, USA), and located in the deep layer border of mPFC. Using a high Cl– high Cs+/Qx-314- intracellular solution and an extracellular ACSF solution containing bicuculline (20 μM), sixteen stimuli of a 10 Hz paired pulse protocol were delivered. EPSC paired pulse ratio was calculated as the ratio of EPSC2 /EPSC1 amplitudes.

2.4. Chromatin immunoprecipitation assays (ChIP-PCR)

Mouse mPFC tissue was processed for chromatin immunoprecipitation (ChIP) according to published protocols (Jayanthi et al., 2014; González et al., 2018). Briefly, minced tissue (2 pooled mPFC per sample) was cross-linked in 1% formaldehyde/PBS for 15 min. Dynabeads (Life Technologies, Grand Island, NY) 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 using a temperature controlled cold water bath and rotating sonicator (Bioruptor Pico, Diagenode). Immunoprecipitation was carried out overnight at 4 °C with equal amounts of chromatin lysate (25-30 μg) per sample. DNA-protein complexes were then dissociated at 65 °C with proteinase K for 2 hrs following treatment with RNaseA (Life Technologies). DNA was then isolated using phenol/chloroform extraction and suspended in 10 mM Tris. 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 available in González et al., 2018).

2.5. qRT-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) following 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

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the specific amplification of murine *Drd1a, Drd2, Adra1a, Adra1b, Hcrtr1, Hcrtr2, Hrh1, Hrh3, Gria1, Grin1, Hdac1,* and *Hdac2* (sequences available in González et al., 2018). 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 % change calculated by the ratios of normalized target genes of each drug-treated group in comparison to the gene expression data of respective control groups.

2.6. Western Blot

Western blot analyses were conducted as previously described (Gonzalez et al., 2014). Briefly, mPFC were quickly removed and stored at −70°C for western blot analyses. Protein samples (30-50 μg) were separated by 12% SDS-PAGE, and the separated proteins transferred to a PVDF membrane. Blots were incubated with primary antibodies: 1:3000 anti-H3ac 06-599 Millipore, 1:3000 anti-H4ac 06-866 Millipore, 1:3000anti-HDAC1 05-100-I Millipore, 1:1000 anti-HDAC2 sc-7899 (H54) Santa Cruz, 1:1000 anti- NMDAζ1 sc-1467 (C20) Santa Cruz, and 1:6000 anti-tubulin T6199 Sigma. Immune complexes were detected with secondary antibodies and chemiluminescence reagents (Amersham, NJ, USA). Bands were then visualized using an Amersham Imager 600 equipped with automatic detection. The resulting images were quantified with ImageJ (NIH) software.

2.7. Statistical analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using one-way (treatment) ANOVAs followed by Bonferroni post-hoc test. Data were tested for compliance with parametric tests assumptions by evaluating homogeneity of variances (Levene's test) and normality. Data were transformed when required to comply with parametric test assumptions. For data that did not comply with parametric test assumptions Kruskal-Wallis ANOVA on ranks was applied followed by paired comparisons. Statistics were conducted using the software InfoStat 2010. All data analyses were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Effects of single-dose injections of modafinil or METH on histone 3/4 acetylation, HDAC1/2, and NMDA GluN1 expression in the mPFC: involvement of dopamine receptors.

Mice were given the D1Rs antagonist SCH23390 or D2Rs antagonist raclopride, 30 min prior to each stimulant injection, and total H3ac, H4ac, HDAC1, HDAC2 and GluN1 protein levels were analyzed in the mPFC (Figures 1 and 2). For both experiments, we found similar effects of modafinil and METH alone on protein expression: both drugs increased H3ac (Fig. 1A and 2A), and decreased H4ac levels (Fig. 1B and 2B), and they also increased HDAC1 (Fig. 1C and 2C), and HDAC2 expression (Fig. 1D and 2D), compared to vehicle. In addition, modafinil and METH increased GluN1 expression (Fig. 1E and 2E), compared to vehicle. These observations are consistent with previous reports showing that D1Rs activation in PFC neurons facilitate NMDA EPSCs (Seamans et al., 2001; Gonzalez-Islas and Hablitz, 2003; Gao and Wolf, 2008).

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D1Rs antagonist SCH23390 pre-treatment had no effect on the increased H3ac levels induced by modafinil and METH [Kruskal Wallis H=22.28, p=0.0005] (Fig. 1A), but completely prevented the decreased H4ac levels induced by both psychostimulants [Kruskal Wallis H=20.56, p=0.001] (Fig. 1B). It needs to be noted that SCH23390 on its own increased HDAC1 and 2 expression, and did not prevented the increased HDAC1 expression induced by modafinil and METH [Kruskal Wallis H=13.61, p=0.02] (Fig. 1C), but the increased HDAC2 expression was completely prevented for both psychostimulants [Kruskal Wallis H=16.42, p=0.006] (Fig. 1D). Finally, the increased GluN1 expression induced by modafinil and METH was completely prevented by SCH23390 pre-treatment [Kruskal Wallis H=11.14, p=0.04] (Fig 1E).

D2Rs antagonist, raclopride, pre-treatment counteracted the increased H3ac levels induced by both psychostimulants [Kruskal Wallis H=23.46, p=0.0003] (Fig. 2A). Raclopride pre-treatment effectively prevented METH-induced decreased H4ac levels, but had no effect on modafinil-induced decreased H4ac [Kruskal Wallis H=17.6, p=0.003] (Fig. 2B). The increased HDAC1 expression induced by modafinil and METH was not prevented by raclopride pre-treatment [Kruskal Wallis H=26.77, p=0.0001] (Fig. 2C). On the contrary, raclopride pre-treatment effectively prevented the effects of modafinil on HDAC2, but had no effect on METH-induced increased HDAC2 expression [Kruskal Wallis H=16.98, p=0.004] (Fig. 2D). Finally, the increased GluN1 induced by modafinil and METH was completely prevented by raclopride pre-treatment [Kruskal Wallis H=20.47, p=0.001] (Fig 2E).

Interestingly, the antagonists administered alone also caused significant epigenetic modifications. For example, SCH23390 increased HDAC1 and HDAC2 (Fig. 2C and D), and raclopride increased H3ac expression (Fig. 3A), compared to vehicle. We have previously shown that antagonist treatment on its own can have similar effects as psychostimulants on certain parameters, which can be explained by the U shaped response of D1Rs, but also that SCH23390, under certain conditions, may have D1-agonist like effects (González et al., 2016). In addition, raclopride at elevated doses such as the one used in this study may also bind to D1Rs (Andersen, 1988). Also, it needs to be noted that SCH23390 may interact with serotonin receptors, although the doses required to induce a response *in vivo* are greater than 10-fold higher than those required to induce a D1-mediated response (Bourne, 2001). Furthermore, the acute administration of the SCH23390 at the dose used in this study (0.05 mg/kg) has been found to have a very moderate effect on serotonin receptors in dopaminergic areas (Lappalainen et al., 1991).

3.2. EPSC paired pulse ratio in mPFC D1R-expressing layer V pyramidal neurons after single-dose modafinil or METH

DA also exerts its impact on synaptic transmission by affecting glutamate presynaptic release, and its effects on PFC function are highly dependent on DA concentration in an “inverted U” fashion, where high DA concentrations, as elicited by psychostimulants like METH, can dampen glutamate-mediated excitation (Williams and Castner, 2006; González et al., 2016). We previously showed that repeated administration and bath-applied METH is able to decrease glutamate release probability from pre-synaptic terminals, elevating

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the EPSCs paired pulse ratios (PPRs) in layer V mPFC pyramidal neurons (González et al., 2016). Here, we compared the presynaptic effect of single-dose modafinil or METH in D1R-expressing layer V mPFC pyramidal neurons. Whole-cell patch-clamp recordings were performed in BAC-transgenic mice Drd1a-tdTomato (Figure 3). These transgenic mice carry a BAC transgene with the promoter and regulatory sequences of D1R controlling the expression of the red fluorescent reporter (Ade et al., 2011) (Fig. 3C). Interestingly, we found increased EPSC PPR for METH after 1 hr of treatment compared to vehicle, but not for modafinil [ANOVA-Bonferroni $F_{(2,30)}$=6.74, p=0.004] (Fig. 3A and B). These findings indicate that, although both psychostimulants increase DA volume transmission and GluN1 expression, only METH is able to affect pre-synaptic glutamate release in the mPFC synaptic triad.

3.3. Effects of modafinil and METH after single-dose treatment 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 receptors involved in arousal, cognition, and reward, including: A) DRs D1 (*Drd1*) and D2 (*Drd2*), B) alpha-adrenergic receptor (α(1)AR) subunits α(1A)AR (*Adra1a*) and α(1B)AR (*Adra1b*), C) orexin receptors HCRTR1 (*Hcrtr1*) and HCRTR2 (*Hcrtr2*), D) histamine receptors H1 (*Hrh1*) and (*Hrh3*), and E) glutamate receptor AMPA subunit GluA1 (*Gria1*) and NMDA subunit GluN1 (*Grin1*) (Figures 4 and 5).

We found that modafinil increased H3ac enrichment at the *Drd1* promoter [ANOVA-Bonferroni $F_{(2,29)}$=5.42, p=0.010], and both METH and modafinil similarly increased H3ac enrichment at the *Drd2* promoter [Kruskal-Wallis H=6.91, p=0.032] (Fig. 4A). For the promoters of α(1)AR subunits, we found distinct acute effects of modafinil and METH: METH increased H3ac enrichment at *Adra1a* [ANOVA-Bonferroni $F_{(2,29)}$=6.87, p=0.005], whereas modafinil increased H3ac enrichment at the *Adra1b* promoter [Kruskal-Wallis H=6.09, p=0.048] (Fig. 4B). For orexin receptors, we found similar effects of acute modafinil and METH, with both increasing H3ac enrichment at *Hcrtr1* [Kruskal-Wallis H=8.13, p=0.017], and no effect on *Hcrtr2* (Fig. 4C). For the histamine receptor, we observed similar effects where both modafinil and METH increased H3ac enrichment at *Hrh1* [ANOVA-Bonferroni $F_{(2,27)}$=5.26, p=0.012], and decreased H3ac at *Hrh3* promoters [ANOVA-Bonferroni $F_{(2,28)}$=5.8, p=0.008]. For glutamate receptors, we found no changes in H3ac enrichment for any drug treatment (Fig. 4E).

For histone 4, we found that modafinil and METH treatment increased H4ac at *Drd1* and *Drd2* promoters in a distinctive manner: modafinil increased H4ac enrichment at the *Drd2* promoter [ANOVA-Bonferroni $F_{(2,29)}$=5.22, p=0.015], whereas METH increased H4ac enrichment at the *Drd1* promoter [ANOVA-Bonferroni $F_{(2,29)}$=4.54, p=0.021] (Fig. 5A). For the promoters of α(1)AR subunits, we found no changes in H4ac enrichment for any drug treatment at this time point (Fig. 5B). We found similar effects of acute modafinil and METH where both psychostimulants increased H4ac enrichment at *Hcrtr1* [Kruskal-Wallis H=6.30, p=0.043], and decreased H4ac enrichment at *Hcrtr2* [ANOVA-Bonferroni $F_{(2,27)}$=7.1, p=0.003] orexin receptors (Fig. 5C). For histamine receptors, we observed that

modafinil decreased H4ac enrichment at *Hrh1* [ANOVA-Bonferroni $F_{(2,28)}$=5.74, p=0.009], and both modafinil and METH decreased H4ac at the *Hrh3* promoter [Kruskal-Wallis H=8.02, p=0.018] (Fig. 5D). For glutamate receptors, both modafinil and METH decreased H4ac enrichment at the *Grin1* promoter [Kruskal-Wallis H=10.67, p=0.005], and had no effect on H4ac enrichment at *Gria1* at this time point (Fig 5E).

3.4. Transcriptional alterations induced after single-dose modafinil or METH treatment in the mPFC.

While epigenetic regulation can lead to changes in gene expression, accumulating evidence has shown that altered chromatin states may not directly correlate with transcription (Strahl and Allis, 2000; Wang et al., 2009; Zentner and Henikoff, 2013; Nestler, 2014). Therefore, we measured the mRNA levels of genes previously evaluated for H3ac and H4ac promotor enrichment (Figure 6). We found METH-induced increases in the expression of *Drd1a* [ANOVA-Bonferroni $F_{(2,17)}$=6.72, p=0.008], *Adra1a* [ANOVA-Bonferroni $F_{(2,17)}$=7.12, p=0.007], and decreased *Grin1* [ANOVA-Bonferroni $F_{(2,17)}$=7.52, p=0.006] mRNA levels compared to modafinil- and vehicle-treated groups. Interestingly, METH alone increased *Hcrtr1* and *Hrh1* mRNA levels [Kruskal-Wallis H=6.92, p=0.031], and [ANOVA-Bonferroni $F_{(2,17)}$=8.89, p=0.003], respectively, compared to modafinil. We also analyzed HDAC1 and HDAC2 mRNA expression. In agreement with protein levels, both modafinil and METH increased *Hdac1* [Kruskal-Wallis H=8.18, p=0.016] and *Hdac2* [Kruskal-Wallis H=6.92, p=0.031] mRNA expression compared to vehicle.

Figure 7 shows summarized ChIP-PCR and RT-PCR results for modafinil and METH treatments graphically depicting the global tendency of each drug compared to vehicle. Our results show that, except for *Gria1*, all promoters analyzed were responsive to modafinil and/or METH treatment via mechanisms involving changes in H3ac and/or H4ac enrichment.

4. DISCUSSION

This study provides evidence that DRs mediate epigenetic changes and molecular events associated with acute METH or modafinil treatment. DA D1Rs and D2Rs antagonists inhibited the effects of both modafinil and METH injections. Specifically, alterations in H4ac, HDAC2 and GluN1 were responsive to SCH23390, while both psychostimulants caused raclopride-dependent inhibition of changes in H3ac and GluN1 expression. Analysis of histone 3 and 4 enrichment at specific gene promoters revealed: i) distinct effects on histone 3 acetylation, wherein modafinil increased H3ac at *Drd1* and *Adra1b,* but METH increased it at *Adra1a*; ii) distinct effects on histone 4 acetylation enrichment, where modafinil increased H4ac at *Drd2* and decreased it at *Hrh1*, but METH increased H4ac at *Drd1*; iii) comparative effects of both psychostimulants, increasing H3ac at *Drd2*, *Hcrtr1* and *Hrh1*, decreasing H3ac at *Hrh3*, increasing H4ac at *Hcrtr1* and decreasing H4ac at *Hcrtr2*, *Hrh3* and *Grin1*. Interestingly, only METH administration was associated with altered mRNA levels for genes that also showed altered histone acetylation, exhibiting increased expression of *Drd1a*, *Adra1a*, *Hcrtr1,* and *Hrh1*, but decreased *Grin1 expression.* Both METH and modafinil increased *Hdac1* and *Hdac2* mRNA levels.

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Acute modafinil or METH had similar impact on global histone acetylation, increasing total H3ac and decreasing total H4ac in the mPFC. The contrasting effect on histone 3 and 4 acetylation is in line with accumulating evidence suggesting that each histone responds to different signaling mechanisms, and are targeted by specific protein complexes of activators/ repressors containing different HATs and HDACs (Rogge and Wood, 2013; Jayanthi et al., 2014). Indeed, little overlap has been found between acetylation of histone 4 and histone 3, or the other histones (Renthal et al., 2009; Martin et al., 2012, Rogge and Wood, 2013; González et al., 2018). Also, many reports have described 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; González et al., 2018). Accordingly, we found that acetylation of histone 3 and histone 4 appear to be regulated by different DRs activation, given that in our study H3ac increases were restored by a D2Rs antagonist, and the H4ac decrease by a D1Rs antagonist. Typically, D1Rs and D2Rs exert opposing actions on intracellular signaling molecules: D1Rs couple to $G_{s/olf}$ family, leading to increased cAMP and PKA/PKC activation, while D2Rs couple to $G_{i/o}$, which inhibit cAMP and limits PKA activation (Beaulieu and Gainetdinov, 2011). Therefore, given the different responses found for H3ac and H4ac to D1Rs and D2Rs antagonist pre-treatment, it seems possible that these effects observed in H3ac vs H4ac may reflect different sensitivities of each histone to DRs stimulation. To our knowledge, this is the first study to explore links between DRs and histone 3/4 acetylation in the mPFC, thus future studies are needed to expand this subject.

We also report that single-dose modafinil or METH increased the mRNA and protein expression of HDAC1 and HDAC2. Accumulating evidence has indicated that manipulating HDAC2 vs HDAC1 leads to dissociable memory processes. In their seminal paper, Guan et al. (2009) overexpressed and/or deleted HDAC1and HDAC2 in four novel mouse lines. They identified HDAC2, but not HDAC1, as a regulator of associative and spatial memory where HDAC2-overexpressing mice showed impaired memory performance; HDAC2-knockout mice showed enhanced memory performance. Also, virally mediated overexpression of HDAC2 in mouse frontal cortex induced behavioral alterations that replicated psychotic symptoms and cognitive impairments in patients with schizophrenia (Kurita et al., 2012), which are also common features of methamphetamine abuse (Scott et al., 2007). Moreover, the coREST complex, an important regulator of neuron-specific genes like ion channels, synaptic vesicle proteins and neurotransmitter receptors, preferentially associates with HDAC2 relative to HDAC1 (Guan et al., 2009). HDAC2 binds at CREB and CBP gene promoters, suggesting that it may communicate with a well-established CREB-CBP pathway to regulate activity-dependent gene expression and memory formation (Guan et al., 2009). Interestingly, conditional deletion of HDAC2 was able to prolong the time that CREB-induced early genes remained elevated after METH in the NAc, further supporting HDAC2 as a restrainer of gene expression induced by transcription factors such as CREB (Torres et al., 2015). Here, we found that neither a D1Rs nor D2Rs antagonist prevented HDAC1 increases, whereas HDAC2 increases were counteracted by a D1Rs antagonist. This could be related to the different cell-specific expression reported for these enzymes, where HDAC1 was found predominantly in glia, and HDAC2 highly and ubiquitously expressed in neurons (Guan et al., 2009; Baltan et al., 2011). Therefore, it seems plausible that modafinil and METH may trigger acute acetylation responses though D1Rs activation, increasing HDAC2

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in neurons, whereas the HDAC1 increase may be predominantly in glia were D1-5R expression is scarce (Vincent et al., 1993).

Modafinil and METH-treated mice showed similar responses to a D1Rs antagonist, but some differences in their response to D2Rs antagonist pre-treatment: raclopride failed to counteract modafinil-induced effects decreasing H4ac, and METH-induced effects increasing HDAC2. As was previously mentioned, modafinil and METH increase DA volume transmission with different kinetics, and it was shown that DA concentration is a critical determinant of D1Rs vs D2Rs signaling in the PFC (Trantham-Davidson et al., 2004). The different kinetics of DA release elicited by each psychostimulant could also be related to the different pre-synaptic effects that we found, where METH increased glutamate PPR and modafinil did not show an effect on D1-expressing pyramidal neurons. Therefore, although modafinil and METH show similar effects on some markers, they may exert these changes through differential DRs activation. It needs to be noted that modafinil, at the dose used in this study (90 mg/kg), has been shown to exert many of its action through D2Rs stimulation (Qu et al., 2008). Moreover, D2Rs were found to be important mediators of wakefulness (Qu et al., 2010). We also found that modafinil specifically increased H4ac at the D2 promoter. Also, both psychostimulants increased D1 promoter acetylation, but through different histone modifications: modafinil increased H3ac, whereas METH increased H4ac enrichment and *Drd1a* mRNA levels.

In the PFC, NE innervation from the locus coeruleus increases neuronal activity through the activation of αARs (Santana et al., 2013). As occurs with DA, an increased NE tone is detrimental for PFC function and cognition, an effect that has been linked to activation of the α(1)ARs (Arnsten et al., 1999; Xing et al., 2016). In addition, there is co-localization and signaling crosstalk between D1 and α(1)ARs in dendrites, showing functional interactions of these receptors exerting catecholaminergic control of PFC functions (Mitrano et al., 2014). In the mPFC, α(1)ARs are expressed in pyramidal cells and interneurons, and the expression of α(1A) and α(1B) is segregated to cells located in different layers of the cortex (Santana et al., 2013; Santana and Artigas, 2017). Both modafinil and METH were found to increase NE tone (de Saint Hilaire et al., 2001; White and Ruhut, 2014). In the present study, we found that modafinil increased H3ac on *Adra1b*, while METH increased H4ac on *Adra1a* associated with increased mRNA expression. Importantly, the behavioral activation caused by modafinil was mediated by α(1B) (Stone et al., 2002), and we found the same epigenetic label (increased H3ac at *Adra1b* promoter) together with increased mRNA expression after 7-day repeated modafinil treatment (González et al., 2018). These differences between the effects of METH and modafinil on α(1A) vs α(1B) ARs may reflect different sites of action of these drugs on different cell types located in different cortical layers.

We also found epigenetic and transcriptional effects on orexin and histamine receptors, two neurotransmitter systems that have distinct and complementary roles in sleep-wake regulation and have been implicated in the behavioral effects of modafinil and METH (Munzar et al., 2004; Ishizuka et al., 2010). Both modafinil and METH increased H3ac/ H4ac enrichment at HCRTR1 and HRH1, and decreased it in HCRTR2 and HRH3 promoters. Importantly, many of these effects have also been observed after repeated modafinil and METH treatment (González et al., 2018). The changes found on histamine

and orexin receptors could be related to the arousal and wake-promoting effects of these psychostimulants: HRH1 mediates histamine actions on waking whereas HRH3 are autoreceptors damping histamine synthesis, release, and firing frequency, and HCRTRs/orexin function are the pathophysiology behind narcolepsy (Anaclet et al., 2009; Mieda, 2017). We also found specific effects linked to each psychostimulant: modafinil decreased H4ac on HRH1 promoter, and METH specifically increased the mRNA expression of HRH1 and HCRTR1. In the PFC, HRH1 has been linked to reward processing (Zlomuzica et al., 2008), whereas HCRTR1 was found involved in motivation-dependent goal-directed tasks and the control of glutamatergic input (Aracri et al., 2015). Also, increased HCRTR1 mRNA was found in the striatum of METH self-administrating rats (Krasnova et al., 2016). HCRTR1-mediated transmission appears to play a role in the behavioral aspects of addiction (Boutrel et al., 2013).

5. CONCLUSION

In summary, our study indicates that although METH and modafinil can increase DA neurotransmission in the mPFC, the two drugs were shown to lead to different epigenetic and transcriptional effects. We show that the overall changes induced by modafinil and METH in the mPFC, in the genes studied in this manuscript, were given mostly by histone acetylation epigenetic mechanisms, which could be DRs-dependent, and also be modulated by accompanying DRs-independent mechanisms. These different effects associated to each psychostimulant appear to mirror some of the previous observations regarding their mechanisms of action and consequent biochemical and molecular effects. In addition, our results provide novel information on the acute epigenetic effects of these drugs in the mouse mPFC. Thus, our observations further support the idea that drugs with similar structures/functions to modafinil may be of therapeutic interest against cognitive deficits observed in patients with METH addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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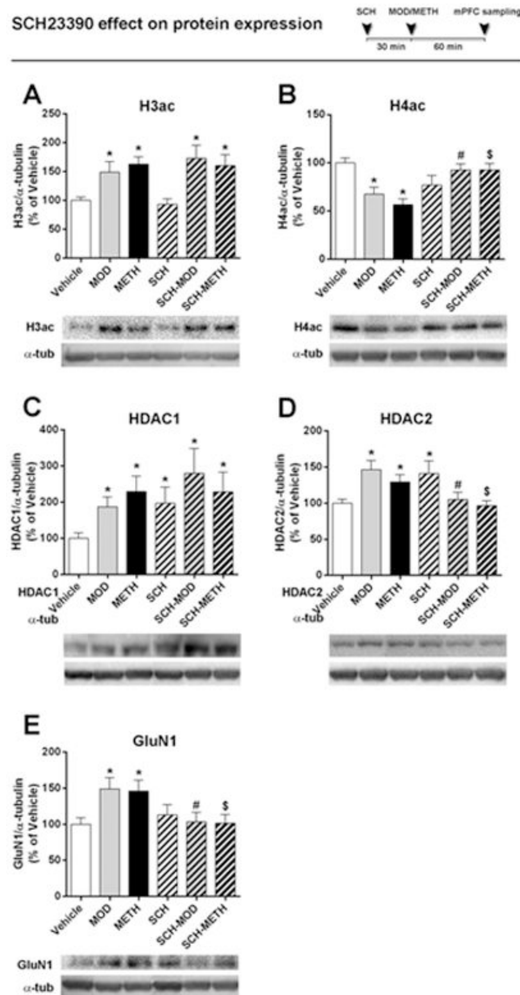

Figure 1. Effects of the D1Rs antagonist SCH23390 (SCH) pre-treatment on single-dose modafinil (MOD) or methamphetamine (METH) action on total acetylated histones 3/4, histone deacetylases HDAC1/2, and NMDA GluN1 protein expression in the mPFC.

Data are expressed as mean ± SEM. Kruskal-Wallis – paired comparisons. * Different from Vehicle, # different from MOD, $ different from METH ($p<0.05$). Numbers above bars indicate N of each experimental group.

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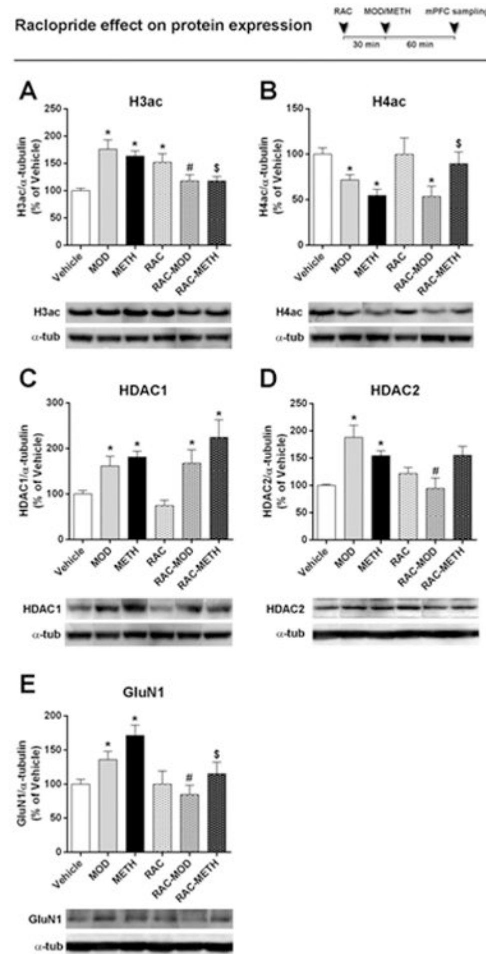

Figure 2: Effect of D2 antagonist raclopride (RAC) pre-treatment on single-dose modafinil (MOD) and methamphetamine (METH) action on total acetylated histones 3/4, histone deacetylases HDAC1/2, and NMDA GluN1 protein expression in the mPFC.

Data are expressed as mean ± SEM. Kruskal-Wallis – paired comparisons. * different from Vehicle, # different from MOD, $ different from METH ($p<0.05$). Numbers above bars indicate N of each experimental group.

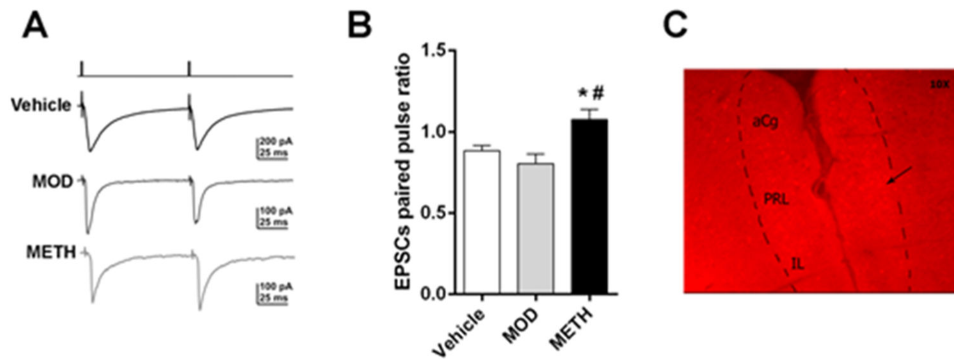

Figure 3: Whole-cell patch clamp in BACDrd1a-tdTomato transgenic mice layer V pyramidal neurons after single-dose modafinil (MOD) or methamphetamine (METH) treatment.

A) Representative excitatory postsynaptic currents (EPSCs) recorded using whole-cell patch clamp of mPFC deep-layer pyramidal neurons, in the presence of bicuculline, DL-AP5, during 10-Hz paired-pulse stimuli for the three different drug treatments. Each trace was obtained averaging 16 stimuli. **B)** Mean EPSC paired pulse ratio (i.e., ratio of EPSC2/ EPSC1 amplitudes). ANOVA-Bonferroni, * different from Vehicle, # different from MOD, numbers above bars indicate N of each experimental group. **C)** The dotted line indicates the localization of the mPFC of BACDrd1a-tdTomato showing neurons positive for DRD1 expression. Electrophysiology experiments were performed in layer V DRD1-positive pyramidal neurons of the prelimbic zone (arrow). aCG: anterior cingulate cortex; PRL: prelimbic cortex; IL: infralimbic cortex.

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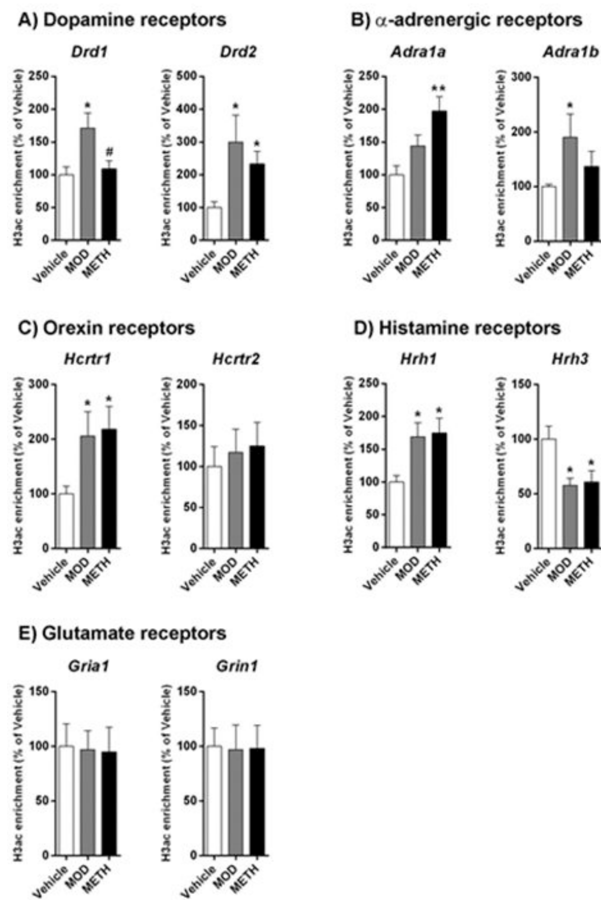

Figure 4: Effect of single-dose modafinil (MOD) or methamphetamine (METH) treatment on the enrichment of acetylated histone 3 (H3ac) at specific promoters in the mPFC.

A) DA receptors *Drd1* and *Drd2*, B) α-adrenergic subunits *Adra1a* and *Adra1b*, C) Orexin receptors *Hcrtr1* and *Hcrtr2*, D) Histamine receptors *Hrh1* and *Hrh3* and E) glutamate receptor AMPA subunit *Gria1* and NMDA subunit *Grin1*. Data are expressed as mean ± SEM. * Different from vehicle $p<0.05$ or ** $p<0.01$, # different from MOD $p<0.05$. Numbers above bars indicate N of each experimental group.

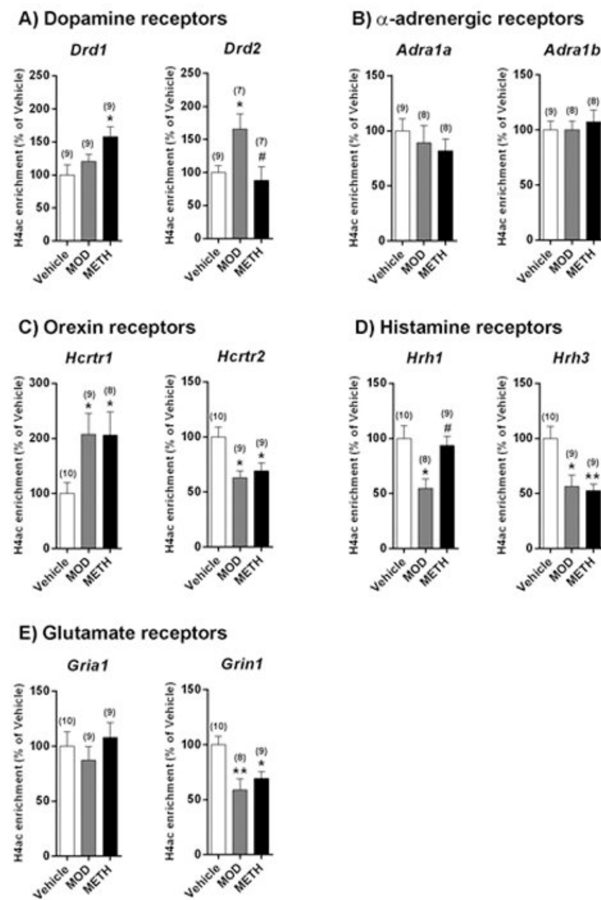

Figure 5: Effects of single-dose modafinil (MOD) or methamphetamine (METH) injections on the enrichment of acetylated histone 4 (H4ac) at specific promoters in the mPFC.

A) DA receptors *Drd1* and *Drd2*, B) α-adrenergic subunits *Adra1a* and *Adra1b*, C) Orexin receptors *Hcrtr1* and *Hcrtr2*, D) Histamine receptors *Hrh1* and *Hrh3* and E) glutamate receptor AMPA subunit *Gria1* and NMDA subunit *Grin1*. Data are expressed as mean ± SEM. * Different from vehicle $p<0.05$ or ** $p<0.01$, # different from MOD $p<0.05$. Numbers above bars indicate N of each experimental group.

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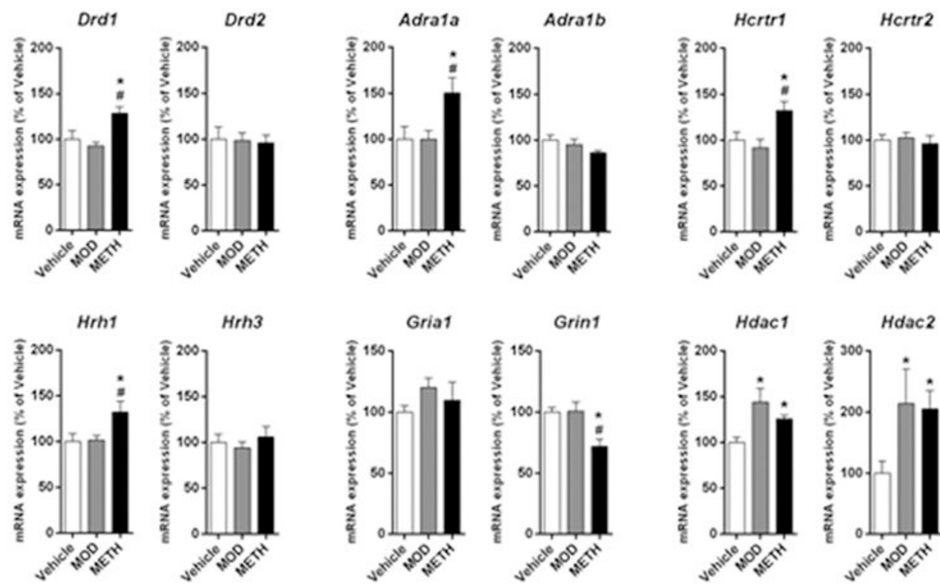

Figure 6: Effect of acute modafinil (MOD) or methamphetamine (METH) treatment on mRNA expression in the mPFC.

Expression of mRNA by RT-PCR. Data are expressed as mean ± SEM. * Different from Vehicle p<0.05, # different from MOD p<0.05. Numbers above bars indicate N of each experimental group.

	H3ac		H4ac		mRNA	
	MOD	METH	MOD	METH	MOD	METH
Drd1	↑	—	—	↑	—	↑
Drd2	⇧	⇧	↑	—	—	—
Adra1a	—	↑	—	—	—	↑
Adra1b	↑	—	—	—	—	—
Hcrtr1	⇧	⇧	⇧	⇧	—	↑
Hcrtr2	—	—	⇩	⇩	—	—
Hrh1	⇧	⇧	↓	—	—	↑
Hrh3	⇩	⇩	⇩	⇩	—	—
Gria1	—	—	—	—	—	—
Grin1	—	—	⇩	⇩	—	↓

Modafinil specific effects METH specific effects Modafinil and METH shared effects

Figure 7:

Modafinil and METH shared and differential histone 3 and 4 acetylation and gene expression profiles in the mPFC. Results summary showing in blue: modafinil specific effects; in red: METH specific effects; in gray: modafinil and METH shared effects. Upward arrow indicates increase, downward arrow indicates decrease, and dash indicates no change compared to vehicle-treated controls.

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