Cannabinoid exposure in rat adolescence reprograms the initial behavioral, molecular, and epigenetic response to cocaine

Maria Scherma[a], Johanna S. Qvist[b,c], Arun Asok[b,c], Shao-shan C. Huang[d], Paolo Masia[a], Matteo Deidda[a], Ya B. Wei[e,f], Rajesh K. Soni[g], Walter Fratta[a], Paola Fadda[a,h], Eric R. Kandel[b,c,i,j,k,1], Denise B. Kandel[k,l,1], and Philippe A. Melas[b,c,m,n,o,1]

[a]Department of Biomedical Sciences, Division of Neuroscience and Clinical Pharmacology, University of Cagliari, 09042 Cagliari, Italy; [b]Department of Neuroscience, Columbia University, New York, NY 10032; [c]Jerome L. Greene Science Center, Mortimer B. Zuckerman Mind Brain Behavior Institute, New York, NY 10027; [d]Center for Genomics and Systems Biology, Department of Biology, New York University, New York, NY 10003; [e]Department of Psychiatry, University of California San Diego, La Jolla, CA 92093; [f]Department of Molecular Medicine and Surgery, Karolinska Institutet, 17176 Stockholm, Sweden; [g]Proteomics Shared Resource, Herbert Irving Comprehensive Cancer Center, Columbia University, New York, NY 10032; [h]Neuroscience Institute, National Research Council of Italy (CNR), 09042 Cagliari, Italy; [i]Kavli Institute for Brain Science, Columbia University, New York, NY 10032; [j]Howard Hughes Medical Institute, Chevy Chase, MD 20815; [k]New York State Psychiatric Institute, New York, NY 10032; [l]Department of Psychiatry, Vagelos College of Physicians and Surgeons and Mailman School of Public Health, Columbia University, New York, NY 10032; [m]Department of Clinical Neuroscience, Karolinska Institutet, 17176 Stockholm, Sweden; [n]Center for Psychiatry Research, Karolinska Institutet, 17176 Stockholm, Sweden; and [o]Center for Molecular Medicine, Karolinska University Hospital, 17176 Stockholm, Sweden

Contributed by Eric R. Kandel, March 11, 2020 (sent for review November 27, 2019; reviewed by Eric J. Nestler and Marina E. Wolf)

The initial response to an addictive substance can facilitate repeated use: That is, individuals experiencing more positive effects are more likely to use that drug again. Increasing evidence suggests that psychoactive cannabinoid use in adolescence enhances the behavioral effects of cocaine. However, despite the behavioral data, there is no neurobiological evidence demonstrating that cannabinoids can also alter the brain's initial molecular and epigenetic response to cocaine. Here, we utilized a multiomics approach (epigenomics, transcriptomics, proteomics, and phosphoproteomics) to characterize how the rat brain responds to its first encounter with cocaine, with or without preexposure to the synthetic cannabinoid WIN 55,212-2 (WIN). We find that in adolescent (but not in adult) rats, preexposure to WIN results in cross-sensitization to cocaine, which correlates with histone hyperacetylation and decreased levels of HDAC6 in the prefrontal cortex (PFC). In the PFC, we also find that WIN preexposure blunts the typical mRNA response to cocaine and instead results in alternative splicing and chromatin accessibility events, involving genes such as *Npas2*. Moreover, preexposure to WIN enhances the effects of cocaine on protein phosphorylation, including ERK/MAPK-targets like gephyrin, and modulates the synaptic AMPAR/GluR composition both in the PFC and the nucleus accumbens (NAcc). PFC–NAcc gene network topological analyses, following cocaine exposure, reveal distinct top nodes in the WIN preexposed group, which include PACAP/ADCYAP1. These preclinical data demonstrate that adolescent cannabinoid exposure reprograms the initial behavioral, molecular, and epigenetic response to cocaine.

cannabis | THC | adolescence | epigenetics | histone acetylation

The brain's endocannabinoid system plays a central role in neurodevelopmental processes, including synaptic plasticity and pruning during adolescence (1). The use of cannabis or synthetic cannabinoids can interfere with endocannabinoid signaling and affect neural pathways that regulate reward and cognition (2). Impaired endocannabinoid signaling has been linked to increased stress responsivity, negative emotional states, and drug craving (3). Moreover, epidemiological studies have found that initiation of cannabis use in adolescence is associated with increased risk for neuropsychiatric disorders, including substance abuse (4), cognitive deficits (5), and psychotic symptoms (6). Both humans and animals are also known to vary in their response to first drug exposures in ways that can predict future drug use (7). Positive first experiences with cocaine, for example, are associated with future cocaine use, shorter latency to second use, and the development of cocaine dependence (7). Although genetic factors may underlie initial drug responses, additional contributors may include environmental and developmental factors. Among adolescents, for example, cannabis use has been associated with an increased risk for later use of cocaine (8–10) and synthetic cannabinoid use has been associated with polydrug use, including psychostimulants (11). Animal studies using both natural cannabinoids—that is, Δ^9-THC, and synthetic analogs, such as WIN 55,212-2 (WIN) or CP-55,940—have found long-lasting neurochemical changes when cannabinoids

Significance

The endocannabinoid system has a modulatory role in brain reward and cognitive processes. It has been hypothesized that repeated interference with endocannabinoid signaling (e.g., through abuse of cannabis or synthetic cannabinoids) can remodel the adolescent brain and make it respond differently to more addictive substances, such as cocaine. In the present study, we demonstrate that a history of synthetic cannabinoid exposure in adolescent animals results in distinct molecular and epigenetic changes following initial exposure to cocaine. These changes were pronounced in the prefrontal cortex and associated with an enhanced response to cocaine's stimulatory effects. The prefrontal cortex is a brain region that still undergoes maturation in adolescence and its dysfunction contributes to the development of addictions.

Author contributions: M.S., W.F., P.F., E.R.K., D.B.K., and P.A.M. designed research; M.S., R.K.S., W.F., P.F., D.B.K., and P.A.M. supervised research; M.S., J.S.Q., P.M., M.D., R.K.S., and P.A.M. performed research; A.A., S.-s.C.H., W.F., and P.F. contributed new reagents/analytic tools; M.S., J.S.Q., A.A., S.-s.C.H., Y.B.W., R.K.S., and P.A.M. analyzed data; M.S. and P.F. edited the paper; and D.B.K. and P.A.M. wrote the paper.

Reviewers: E.J.N., Icahn School of Medicine at Mount Sinai; and M.E.W., Oregon Health & Science University School of Medicine.

The authors declare no competing interest.

Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE134935).

[1]To whom correspondence may be addressed. Email: erk5@cumc.columbia.edu, dbk2@cumc.columbia.edu, or philippe.melas@ki.se.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1920866117/-/DCSupplemental.

are administered in adolescence (12). In addition, animal studies have found that cannabinoids can cross-sensitize to cocaine (13–15), enhance the acquisition of cocaine self-administration (16, 17), and modify cocaine-related withdrawal symptoms (18). Despite these behavioral data, there is still no neurobiological evidence demonstrating that cannabinoids can change the brain's initial response to cocaine. In the present study we utilized a multiomics approach to examine this hypothesis and found that preexposure to cannabinoids in adolescence resulted in a molecular and epigenetic reprogrammed response to the first encounter with cocaine.

Results

WIN Preexposure Results in Cross-Sensitization to Cocaine in Adolescence but Not Adulthood. We recently reported that adolescent rats preexposed to WIN show enhanced cross-sensitization to cocaine both at 1 d (13) and 1 wk (14) after the last WIN administration. Here, by using the same drug-administration protocol, we examined cross-sensitization after a week of WIN abstinence in both adolescent and adult rats. Rat treatments started at postnatal day (PND) 42 for adolescents and at PND 77 for adults (see Fig. 1*A* for a schematic representation of the experiment). The estimated timing of male rat adolescence was according to Schneider (19), and the PND 38 to PND 60 period (puberty) was chosen as a critical developmental period (20). For assessment of cross-sensitization between cannabinoids and cocaine, increasing doses of WIN were given twice a day for 11 consecutive days (2 mg/kg, 3 d; 4 mg/kg, 4 d; 8 mg/kg, 4 d), followed by a week of WIN abstinence and an intraperitoneal challenge with cocaine (10 mg/kg) on abstinence day 8 (for choice of WIN doses, see also *SI Appendix*, *Supplementary Methods*). Cross-sensitization to cocaine was again observed in the WIN preexposed adolescent group (Fig. 1*B*). In contrast, no such effect was observed in adult rats (Fig. 1*C*). Next, we also examined the directionality of this behavioral effect by reversing the drug administration paradigm. For assessment of cross-sensitization between cocaine and cannabinoids, increasing doses of cocaine were given twice a day for 11 consecutive days (10 mg/kg, 3 d; 15 mg/kg, 4 d; 20 mg/kg, 4 d), followed by a week of cocaine abstinence and an intraperitoneal challenge with WIN (0.1 mg/kg) on abstinence day 8 (see also *SI Appendix*, *Supplementary Methods* and Fig. S1*A* for choice of WIN dose). However, we found no evidence for cross-sensitization between cocaine and WIN (*SI Appendix*, Fig. S1*B*). These behavioral data support the existence of unidirectional cross-sensitization between WIN and cocaine in adolescence.

WIN Preexposure Results in Cocaine-Induced Histone Hyperacetylation in the Adolescent Prefrontal Cortex. We next asked whether molecular and epigenetic changes are associated with the behavioral cross-sensitization in adolescence. We first assessed levels of phospho-eIF2α and histone acetylation at H3K27, since we previously found that these specific molecular markers are affected by WIN up to 24 h following the last WIN administration (13). Brain dissections were performed on experimental day 20 (WIN abstinence day 9)—that is, 24 h after the intraperitoneal challenge with cocaine (Fig. 1*A*)—and included the amygdala, dorsal striatum, hippocampus, nucleus accumbens (NAcc), and prefrontal cortex (PFC). We found no significant changes in levels of phospho-eIF2α in any brain regions (*SI Appendix*, Fig. S2). However, there was a 50% increase in H3K27 acetylation in the PFC of the WIN and cocaine groups, and an ∼100% increase in H3K27 acetylation in the WIN–cocaine group, compared to the control group (Fig. 1*D*). When we assessed H3K27 acetylation levels in the adult PFC, we found that only the cocaine group showed histone hyperacetylation and this effect was diminished in the WIN–cocaine group (Fig. 1*E*). Based on these findings, we asked: Is this effect specific to acetylation at H3 or is it observed in additional core histones (e.g., H4 acetylation) and other histone modifications known to be affected by cocaine [e.g., histone methylation and phosphorylation (21, 22)]? To this end, we measured levels of H4 acetylation (K5–K16), H3 trimethylation (K4), and H3 phosphorylation (S10) in the adolescent and adult PFC. Among adolescents, there were significant changes in H4 acetylation (with a pattern similar to H3 acetylation) and no changes in histone phosphorylation or methylation (Fig. 1 *F*, *Left*). Similarly, among adults, there were significant changes in H4 acetylation (with a pattern again similar to adult H3 acetylation) and no changes in histone methylation or phosphorylation (Fig. 1 *F*, *Right*). These data suggest that prior exposure to WIN modulates the effect of cocaine on histone acetylation in the PFC.

WIN Preexposure Results in Cocaine-Induced Enhancement of Chromatin Accessibility in *Npas2*. Since histone acetylation is known to increase chromatin accessibility (23), we next asked whether the acetylation changes observed in the adolescent PFC also translated into changes in open chromatin regions. As an exploratory analysis, we performed a genome-wide characterization of open chromatin regions using an assay for transposase-accessible chromatin using sequencing (ATAC-seq). Descriptive data from the adolescent PFC showed that, on a genome-wide level, the four treatment conditions (control, WIN, cocaine, WIN–cocaine) were indistinguishable from one another, with no discernible changes in the distribution of active regions (i.e., regions of accessible chromatin) across genomic features (*SI Appendix*, Fig. S3*A*) or in the accessibility across transcription start sites (TSS) and gene bodies (*SI Appendix*, Fig. S3*B*). Furthermore, the active regions in the control vs. WIN and the cocaine vs. WIN–cocaine conditions were quantitatively highly correlated (*SI Appendix*, Fig. S3*C*). The Venn diagram of the overlap between active regions in the four conditions showed that the unique active regions in each condition represented less than 10% of the overall active regions (*SI Appendix*, Fig. S3*D*). Nonetheless, data from the top differential active sites (Dataset S1) showed that for certain overlapping active regions, WIN preexposure resulted in cocaine-induced enhancement in chromatin accessibility, for example in promoter or gene body regions (*SI Appendix*, Fig. S3 *E* and *F*). As shown in *SI Appendix*, Fig. S3*F*, one of these regions was an intronic site in *Npas2* (neuronal PAS domain protein 2), a gene coding for a transcription factor that regulates cocaine reward sensitivity (24, 25). Collectively, these data suggest that the observed hyperacetylation in the adolescent PFC of the WIN–cocaine group (Fig. 1*D*) is not associated with aberrant changes in chromatin accessibility on a genome-wide scale. Instead, open chromatin changes appear to be targeted to specific genomic loci.

WIN Preexposure Results in Cocaine-Induced Changes in *Npas2* mRNA Splicing. The ATAC-seq analysis revealed chromatin accessibility changes both in the promoter/TSS and in intragenic regions of the adolescent WIN–cocaine group (Dataset S1). While chromatin accessibility in promoter/TSS regions often correlates with mRNA expression levels, intragenic accessibility can correlate with nucleosome positioning known to affect alternative splicing (26). Thus, we performed a transcriptomic analysis of the adolescent PFC using RNA sequencing (RNA-seq) and analyzed the data with regard to differential gene expression and alternative splicing events. While there were extensive mRNA changes in the (WIN-naïve) cocaine group, preexposure to WIN dampened the cocaine-induced mRNA response in the PFC (Fig. 2*A*). This dampening effect of WIN preexposure on mRNA expression was also confirmed by quantitative real-time PCR experiments focusing on FosB, a known cocaine-responsive gene (*SI Appendix*, Fig. S4). The number of differentially expressed genes, as a result of different treatments, is also shown by pair-wise mRNA comparisons for cocaine vs. control (differentially expressed genes: 1,352) (Fig. 2*B* and Dataset S2), WIN vs. control (differentially

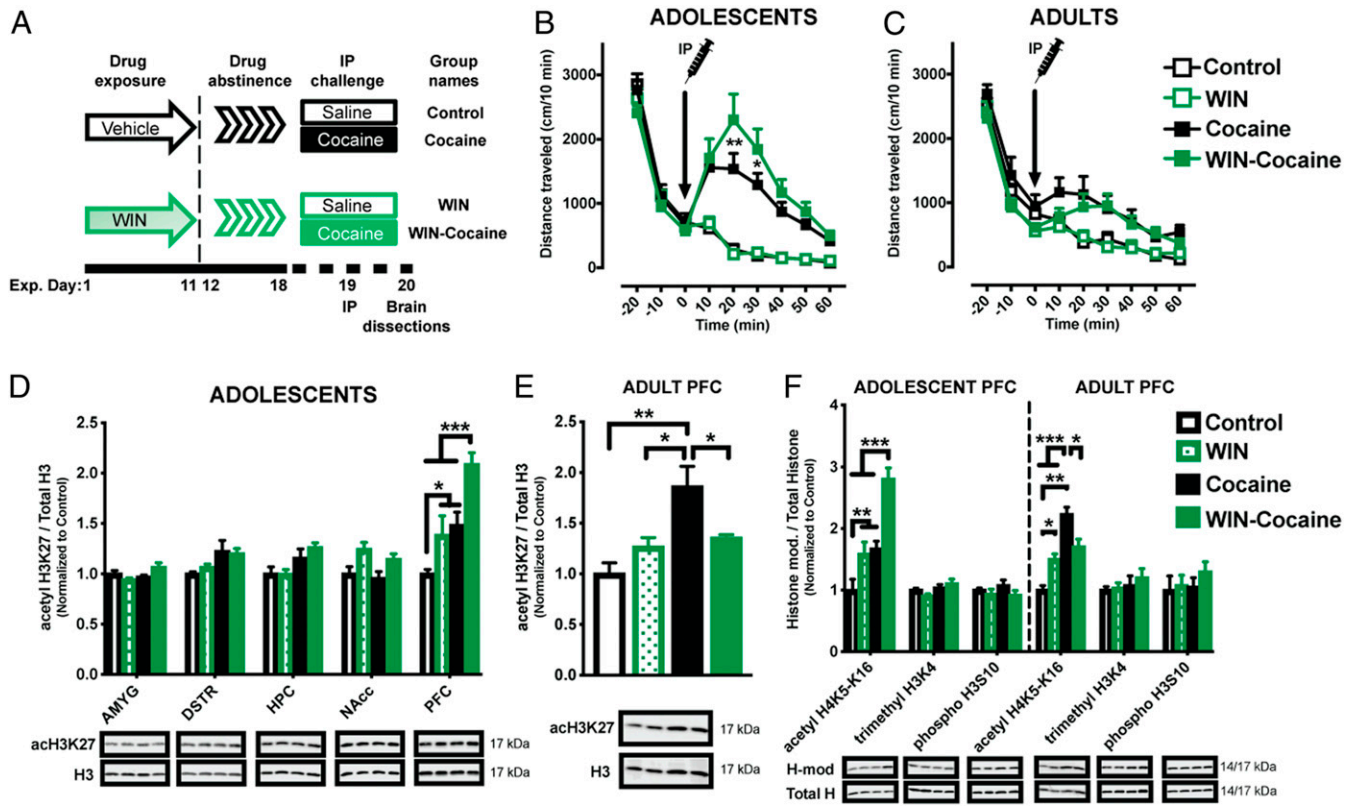

Fig. 1. Cross-sensitization between WIN and cocaine in adolescent rats is associated with histone hyperacetylation in the PFC. (*A*) Schematic representation of the experimental design. Experimental day 1 (exp. d) corresponds to PND 42 and 77 for adolescent and adult rats, respectively. The names used to describe the four treatment groups in all subsequent figures is shown to the right of the schematic diagram. (*B*) Preexposure to WIN in adolescence cross-sensitizes to cocaine [two-way repeated-measures ANOVA: treatment $F(3, 75) = 13.31$, $P < 0.001$; time $F(8, 600) = 123.0$, $P < 0.001$; subject $F(75, 600) = 5.447$, $P < 0.001$; interaction $F(24, 600) = 10.26$, $P < 0.001$; Tukey's multiple comparisons test for groups of interest, cocaine vs. WIN–cocaine: 20 min, $P = 0.002$; 30 min, $P = 0.042$; $n = 18$ to 23 animals per group]. (*C*) Preexposure to WIN in adulthood does not cross-sensitize to cocaine (Tukey's multiple comparisons test for groups of interest, cocaine vs. WIN–cocaine: $P > 0.195$ for all comparisons from 0 to 60 min; $n = 19$ to 20 animals per group). (*D*) Adolescent rats used in *B* were randomly divided up for use in subsequent molecular experiments (see also *Statistical Analyses* in *SI Appendix, Supplementary Methods*). Western blotting experiments show significant increases in global H3K27 acetylation in the adolescent PFC of the WIN and cocaine groups, and additional hyperacetylation in the WIN–cocaine group [two-way ANOVA: treatment $F(3, 75) = 18.68$, $P < 0.001$; brain region $F(4, 75) = 23.35$, $P < 0.001$; interaction $F(12, 75) = 6.231$, $P < 0.001$; Tukey's multiple comparisons test in PFC: control vs. WIN, $P = 0.01$; control vs. cocaine, $P < 0.001$; control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P < 0.001$; $n = 4$ to 6 animals per group with behavioral responses that resembled the overall group outcomes shown in *B*]. (*E*) In the adult PFC, global H3K27 acetylation levels were increased in the cocaine group [ANOVA $F(3, 12) = 8.424$, $P = 0.002$; Tukey's multiple comparisons test: Control vs. cocaine, $P = 0.001$; WIN vs. cocaine, $P = 0.024$; cocaine vs. WIN–cocaine, $P = 0.05$; $n = 4$ animals per group]. (*F*, *Left*) In the adolescent PFC, significant increases are found for H4K5-K16 acetylation but not for H3K4 trimethylation or H3S10 phosphorylation [two-way ANOVA: treatment $F(3, 81) = 19.07$, $P < 0.001$; histone modification $F(5, 81) = 30.45$, $P < 0.001$; interaction $F(15, 81) = 8.327$, $P < 0.001$; Tukey's multiple comparisons test for H4K5-K16ac: control vs. WIN, $P = 0.009$; control vs. cocaine, $P = 0.001$; control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P < 0.001$; $n = 4$ to 6 animals per group]. (*Right*) In the adult PFC, increased H4K5-K16 acetylation is found in the cocaine group, with no changes in H3 phosphorylation and H3 methylation (Tukey's multiple comparisons tests; H4K5-K16ac: control vs. WIN, $P = 0.035$; control vs. cocaine, $P < 0.001$; control vs. WIN–cocaine, $P = 0.001$; WIN vs. cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P = 0.026$; $n = 4$ animals per group). AMYG, amygdala; DSTR, dorsal striatum; H-mod, histone modification; HPC, hippocampus; IP, Intraperitoneal; kDa, kilodaltons; NAcc, nucleus accumbens; PFC, prefrontal cortex; Total H, total histone; WIN, WIN 55,212-2 mesylate. Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$.

expressed genes: 7) (Fig. 2*C* and Dataset S2), and WIN–cocaine vs. cocaine (differentially expressed genes: 21) (Fig. 2*D* and Dataset S2). In contrast, the assessment of five types of alternate splicing events revealed an equivalent number of significant skipped exon (SE) events for the same pair-wise comparisons (Fig. 2 *E*–*G*, first/top rows). For the WIN–cocaine vs. cocaine comparison, one of the significant SE events was again in *Npas2* (Dataset S3). For WIN vs. control, one of the significant SE events was in the histone deacetylase *Hdac4* gene (Dataset S3), which (as for *Npas2*) was also among the top differential genes in the ATAC-seq active region analysis (*SI Appendix*, Fig. S5*A* and Dataset S1). Changes in nuclear levels of HDAC4 could potentially account for the hyperacetylation observed in the WIN group (Fig. 1 *D* and *F*). However, commercially available HDAC4 antibodies do not distinguish among protein isoforms and, indeed, we found no differences in HDAC4 levels using such an antibody (*SI Appendix*, Fig. S5*B*). When we next performed gene ontology (GO) analysis of genes with significant SE events, we found that WIN exposure led to enrichment in GO terms related to neurotransmitter receptor transport to the postsynaptic membrane (Fig. 2*H*). The WIN–cocaine vs. cocaine SE events were also functionally enriched for "broader" terms related to protein transport and localization to the membrane (Fig. 2*H*). This was in stark contrast to the cocaine vs. control SE events that were functionally enriched for the assembly of cell projections, cilium, and organelles (Fig. 2*H*). Collectively, these data demonstrate a reprogramming of the PFC's "normal"

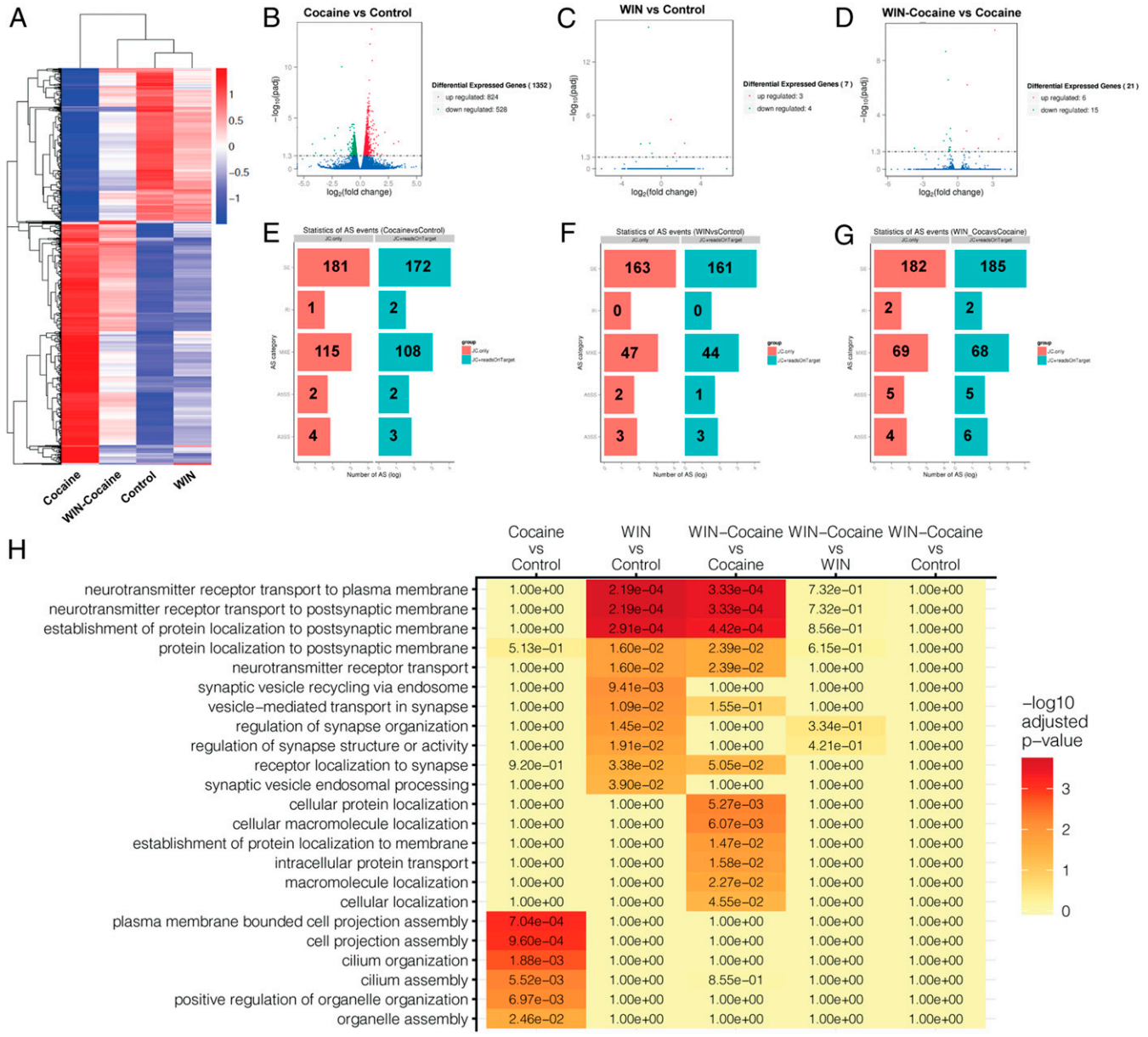

Fig. 2. WIN affects cocaine-induced mRNA changes and SE events. (*A*) RNA-seq–derived heatmap of hierarchical cluster analysis of differentially expressed genes in the PFC of the four adolescent treatment groups (i.e., control, WIN, cocaine, WIN–cocaine) (FDR < 0.05; $n = 3$ animals per group). (*B*) Volcano plot for differentially expressed genes in cocaine vs. control shows 824 up-regulated and 528 down-regulated genes in the cocaine group (see also Dataset S2). (*C*) Volcano plot for differentially expressed genes in WIN vs. control, shows three up-regulated and four down-regulated genes in the WIN group (see also Dataset S2). (*D*) Volcano plot for differentially expressed genes in WIN–cocaine vs. cocaine shows 6 up-regulated and 15 down-regulated genes in the WIN–cocaine group (see also Dataset S2). (*E*) Classification (*y* axis) and total number (*x* axis) of alternative splicing (AS) events for cocaine vs. control (A3SS: Alternative 3′ splice site; A5SS: Alternative 5′ splice site; JC, junction; MXE, mutually exclusive exons; RI, Retained intron; SE, skipped exon). AS-analysis was done by rMATS that uses two quantification methods for evaluating splicing events: That is, with reads spanning splicing junctions only (JC only; graphs in red) and with both reads on target and reads spanning splicing junctions (JC + reads on target; graphs in blue). The numbers inside the graphs denote the total number of significant differences in AS events among groups (at FDR < 0.05) and shows highest number for SE events (for a complete list of SE events, see Dataset S3). (*F*) Classification and total number of alternative splicing events for WIN vs. control. One of the significant SE events was for exon 5 in the *Hdac4* gene (FDR = 1.97E-05) (for a complete list of SE events see Dataset S3). (*G*) Classification and total number of alternative splicing events for WIN–cocaine vs. cocaine. One of the significant SE events was for exon 19 in the *Npas2* gene (FDR = 2.69E-06) (for a complete list of SE events see Dataset S3). (*H*) GO analysis using genes showing significant SE events. Dashed line in *B*–*D* denotes the threshold of significance.

transcriptomic response to cocaine, which results from prior exposure to WIN.

WIN Preexposure Modulates Cocaine-Induced Nucleosome Positioning at SEs. Next, we utilized the ATAC-seq data to assess nucleosome positioning changes and performed a combined analysis with the RNA-seq data. The TSSs of the differentially expressed genes in the WIN-naïve cocaine group were found to be more accessible (i.e., nucleosome free) in all treatment conditions (*SI Appendix*, Fig. S6). By focusing on the most extensive alternative splicing events (i.e., SE) we found that, in cocaine vs. control, cocaine led to nucleosome repositioning that marked SE boundaries (*SI Appendix*, Fig. S7*A*). By comparing WIN and control, we found loss of nucleosome positioning both at boundaries and upstream of SE

events (*SI Appendix*, Fig. S7*B*). In contrast, when comparing WIN–cocaine with other groups, we found that WIN preexposure led to cocaine-induced phased nucleosome positioning upstream of exons, which correlated with exon skipping (*SI Appendix*, Fig. S7*C*). Thus, the transcriptomic reprogramming that occurs in the WIN–cocaine group also coincides with distinct nucleosome positioning events at SEs.

WIN Preexposure Results in Cocaine-Induced Reduction in Nuclear Levels of HDAC6. A question that still remained unanswered was which epigenetic enzyme may underlie the cocaine-induced histone hyperacetylation found in the WIN–cocaine group (Fig. 1 *D* and *F*). Given the numerous known HDACs and histone acetyltransferases, we opted for an unbiased approach to identify a likely candidate. Using adolescent PFC samples from the cocaine and WIN–cocaine groups, we first separated the nuclear fraction from the cytoplasm and then subjected the nuclear extract to quantitative proteomics. We found significantly reduced levels of HDAC6 in the WIN–cocaine group (Fig. 3*A* and Dataset S4). HDAC6 is a class II histone deacetylase (HDAC) that is present both in the cell nucleus and in the cytoplasm. To verify the proteomic HDAC6 findings, we performed Western blotting experiments using both nuclear and cytoplasmic extracts from the adolescent PFC. This analysis confirmed that WIN preexposure in adolescence led to a significant cocaine-induced reduction in levels of nuclear HDAC6 (Fig. 3*B*). In addition, there was a significant negative correlation between nuclear HDAC6 levels and levels of H3 and H4 acetylation (Fig. 3*C*). In contrast, WIN preexposure in adulthood led to a cocaine-induced increase in nuclear HDAC6 levels (Fig. 3*D*), although no significant correlation was found between HDAC6 and histone acetylation in adults (Fig. 3*E*). Nonetheless, the increased levels of nuclear HDAC6 in the adult WIN–cocaine group mirror the dampened effects on histone acetylation found previously in the same group (Fig. 1 *E* and *F*). These data suggest a possible role for HDAC6 in modulating cocaine-induced histone acetylation following WIN preexposure in both adolescence and adulthood.

WIN Preexposure Results in Cocaine-Induced Enhancement of MAPK/ERK Levels. Following proteomic analysis of the nuclear PFC extracts, we also subjected the remaining cytoplasmic fraction for quantitative proteomics. By analyzing both the nucleus and cytoplasm, we aimed at identifying the majority of differentially expressed intracellular proteins, which could then be subjected to GO enrichment analysis, as well as gene function and pathway analyses. *SI Appendix*, Table S1 presents the top five enriched components for each analysis, which was conducted first for up-regulated and then for down-regulated proteins in the adolescent WIN–cocaine group (versus the cocaine group). All five biological processes from the PANTHER GO analysis of up-regulated proteins in the WIN–cocaine group were related to regulation of mRNA processing and splicing, mirroring the abundance of significant splicing events for WIN–cocaine vs. cocaine (Fig. 2*D* vs. Fig. 2G). We next performed two-group comparison using the cytoplasmic data only, which revealed an increase of a mitogen-activated protein kinase (MAPK3, also known as ERK1) in the WIN–cocaine group (Fig. 4*A* and Dataset S5). ERK1 and its homolog ERK2 (collectively referred to as ERK1/2) have an established role in cocaine-induced behavioral sensitization (27). Moreover, recent literature has suggested a reciprocal regulatory interaction between ERK1/2 and HDAC6 (28, 29). Western blotting experiments, using samples from both adolescent and adult animals, confirmed that WIN preexposure led to cocaine-induced increases in cytoplasmic ERK1/2 levels in adolescence but not in adulthood (Fig. 4*B*). Given the reported interaction between ERK1/2 and HDAC6, we also examined whether the decreased HDAC6 levels in the WIN–cocaine group might be linked to the increased levels in ERK. To this end, we first performed correlation analyses and found a close-to-significant negative correlation between nuclear HDAC6 levels and levels of ERK in adolescents ($P = 0.08$) (*SI Appendix*, Fig. S8*A*). In contrast, there was no indication for a correlation between HDAC6 and ERK levels in adults ($P = 0.96$) (*SI Appendix*, Fig. S8*B*). Next, since both HDAC6 and ERK are ubiquitously expressed, we utilized HDAC6 and ERK CRISPR/Cas9 knockout cell lines, as well as HDAC6 and ERK transient overexpression cell lines, to further examine the relationship between HDAC6 and ERK. Western blotting experiments using lysates from HDAC6 knockouts revealed significantly increased levels of ERK1/2 (Fig. 4*C*). Conversely, cell lysates from ERK2 knockouts revealed significantly decreased levels of HDAC6 (Fig. 4*D*). Overexpression of HDAC6 led to increased levels in ERK1 but no changes in ERK2 (Fig. 4*E*), while overexpression of ERK2 led to increased levels of HDAC6 (Fig. 4*F*). These data suggest a dynamic and complex interplay between HDAC6 and ERK, which appears to be affected by cocaine following WIN preexposure in adolescence.

WIN Preexposure Modulates the Effects of Cocaine on Protein Phosphorylation. The cocaine-induced increase in ERK levels in the WIN preexposed adolescent PFC prompted us to perform quantitative phosphoproteomics to examine changes in protein phosphorylation. This analysis revealed a gradient effect in protein phosphorylation changes among the four treatment groups (Fig. 5*A*). For the dephosphorylated residues in the control group (i.e., blue in the lower left quadrant of the heatmap in Fig. 5*A*), the order of change in phosphorylation magnitude was: control < WIN < cocaine < WIN–cocaine. In contrast, the reverse pattern was evident for the hyperphosphorylated residues in the control group (i.e., red in the upper left quadrant of the heatmap in Fig. 5*A*), with the order of change in phosphorylation being: control > WIN > cocaine > WIN–cocaine. In the WIN–cocaine group, gephyrin was among the proteins showing the highest phosphorylation levels (serine residue 337) (Fig. 5*A* and Dataset S6). Gephyrin is a cytoplasmic scaffold protein and a known target of ERK, and at GABAergic synapses ERK has been found to phosphorylate gephyrin at another serine residue (Ser268) (30). While there were no commercially available antibodies to verify the observed increase in gephyrin phosphorylation at Ser337, gephyrin is known to interact with—and to affect the residence time of—α1-subunit–containing GABA(A) receptors at inhibitory synapses (31). Thus, as an indirect measure, we asked whether phosphorylation of gephyrin in the WIN–cocaine group is associated with changes in levels of the α1 GABA(A) receptor subunit (i.e., GABRA1). Western blotting experiments, using cytoplasmic extracts, revealed significantly decreased GABRA1 levels in the PFC of the adolescent WIN–cocaine group and no changes among adults (Fig. 5*B*). Closer examination of the significant phosphoproteomic data also revealed a second event that warrants attention: The dephosphorylation of the HIV Tat-specific factor 1 (HTATSF1) at Ser578 in the adolescent WIN–cocaine group (Fig. 5*A* and Dataset S6). HTATSF1 is a general transcription elongation factor (32) and its dephosphorylated state provides a molecular correlate for the blunted mRNA response to cocaine following WIN preexposure.

WIN Exposure Decreases Synaptic AMPA Receptors in the Adolescent PFC. Next, we asked how WIN may "prime" the PFC to respond differently to cocaine. Since the significant SE mRNA events for WIN vs. control were enriched for neurotransmitter receptor transport to the postsynaptic membrane (Fig. 2*H*), we hypothesized that WIN causes changes in the synaptic composition of the PFC. To examine changes induced by WIN alone, we isolated synaptosomal and cytosolic fractions from the adolescent PFC of

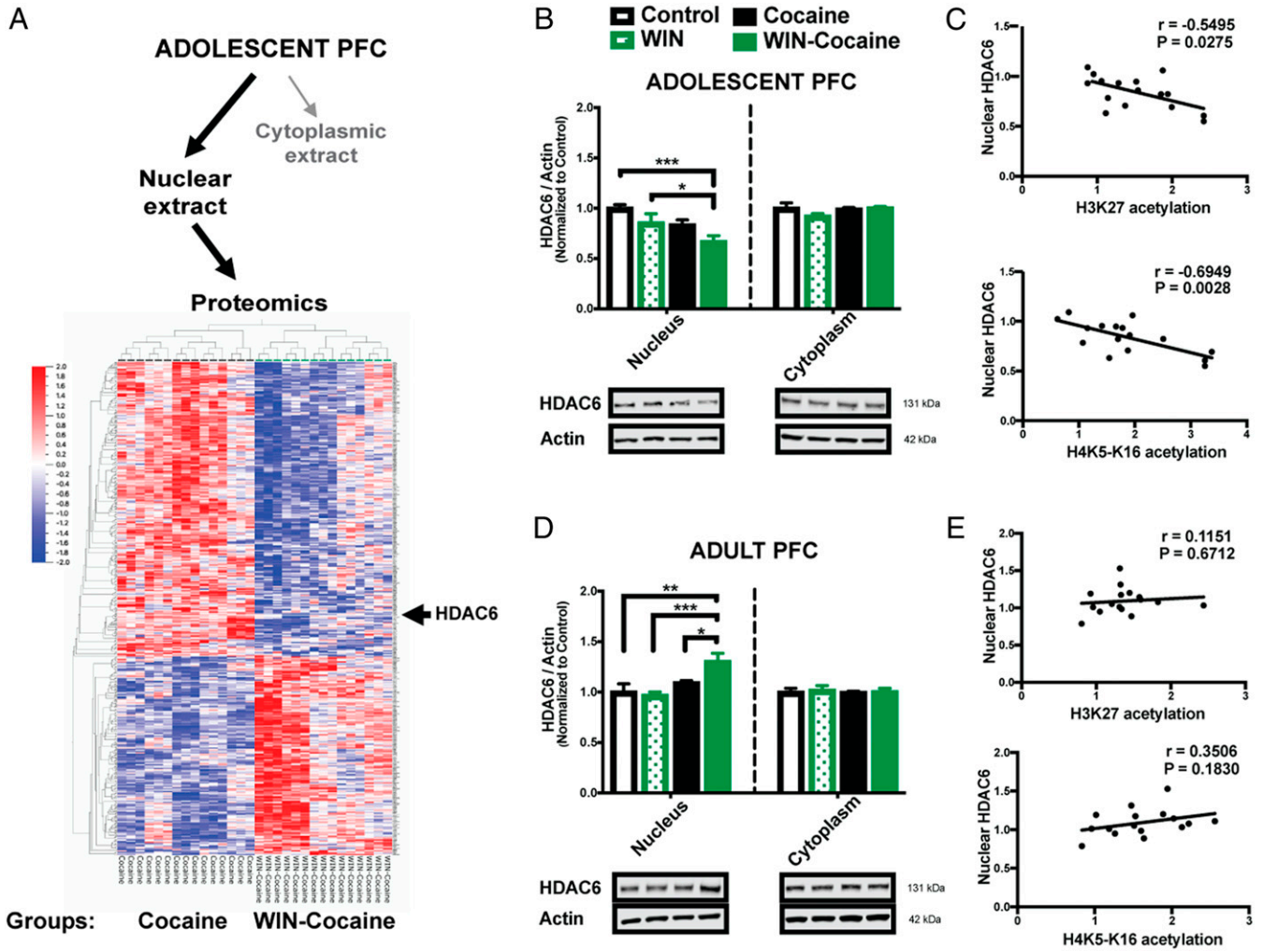

Fig. 3. Nuclear proteomics links WIN preexposure to cocaine-induced changes in HDAC6. (*A*) Nuclear PFC extracts from the adolescent cocaine and WIN–cocaine groups were subjected to labeled TMT-based quantitative proteomic analysis. Hierarchical clustering heatmap is presented for the differentially expressed proteins ($P \leq 7.6e\text{-}4$; $q \leq 0.01$; $n = 5$ animals per group and $n = 3$ technical replicates per animal). The arrow to the right of the heatmap denotes the significant decrease in levels of HDAC6 in the WIN–cocaine group. The whole list of differentially expressed proteins is presented in Dataset S4. (*B*) Western blotting experiments for HDAC6 using nuclear and cytoplasmic PFC fractions from all four adolescent treatment groups confirmed the decrease in nuclear HDAC6 in the WIN–cocaine group [two-way ANOVA: treatment $F(3, 27) = 4.486$, $P = 0.011$; cell fraction $F(1, 27) = 18.36$, $P < 0.001$; interaction $F(3, 27) = 5.086$, $P = 0.006$; Tukey's multiple comparisons test for nuclear fraction: control vs. WIN–cocaine, $P < 0.001$; WIN vs. WIN–cocaine, $P = 0.05$; cocaine vs. WIN–cocaine, $P = 0.09$; $n = 4$ to 6 animals per group]. (*C*, *Upper*) Regression line of normalized nuclear HDAC6 (HDAC6/Actin) and H3 acetylation (H3K27ac/H3) levels, and corresponding correlation coefficients, using data from all four adolescent treatment groups (Pearson's $r = -0.5495$; $P = 0.0275$; $n = 16$ animals for which both histone acetylation and HDAC6 measurements were available). (*Lower*) Regression line of normalized nuclear HDAC6 (HDAC6/Actin) and H4 acetylation (acH4K5-K16/H4) levels, and corresponding correlation coefficients, using data from all four adolescent treatment groups (Pearson's $r = -0.6949$; $P = 0.0028$; $n = 16$ animals). (*D*) Same as in *B* but for the adult PFC revealed a significant increase in HDAC6 levels in the WIN–cocaine group [two-way ANOVA: treatment $F(3, 24) = 4.353$, $P = 0.014$; cell fraction $F(1, 24) = 5.939$, $P = 0.023$; interaction $F(3, 24) = 4.783$, $P = 0.009$; Tukey's multiple comparisons test for nuclear fraction: control vs. WIN–cocaine, $P = 0.001$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P = 0.029$; $n = 4$ animals per group]. (*E*) Same as in *C* but for the adult PFC, showing no significant correlations ($P > 0.05$; $n = 16$ animals). Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. $^*P \leq 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

the control and WIN groups, and subjected them to quantitative proteomics (for details on the synaptosomal fractionation method, see *SI Appendix, Supplementary Methods*). First, we performed GO, gene function, and pathway analyses using the differentially expressed proteins from both extracts. *SI Appendix*, Table S2 presents the top five enriched components for each analysis, which was conducted first for up-regulated and then for down-regulated proteins in the WIN group. For the down-regulated proteins in the WIN group, GO analyses for both biological and molecular processes showed enrichment for glutamate (AMPA) receptor regulation. Similarly, the GO-Slim cellular component analysis showed highest enrichment for the ionotropic glutamate receptor complex, and pathway analysis showed enrichment for the ionotropic glutamate receptor pathway. Next, we performed two-group differential expression analysis of the synaptosomal extracts, which revealed decreased levels of all AMPA receptor subunits (GluR1 to GluR4) in the WIN group (Fig. 6*A*; also note that although GluR1 was not present in the heatmap, its levels were significantly decreased on the unadjusted *P* level; e.g., see Dataset S7). Using Western blotting, we confirmed the WIN-induced decrease in GluR1 and GluR2 in the synaptosomal extracts of the adolescent PFC (Fig. 6*B*). In contrast, there was no WIN-induced decrease in synaptosomal GluR1/2 levels in the PFC of adult animals (Fig. 6*C*). These data suggest that WIN exposure in adolescence leads to reduction of synaptic AMPA receptors in the PFC.

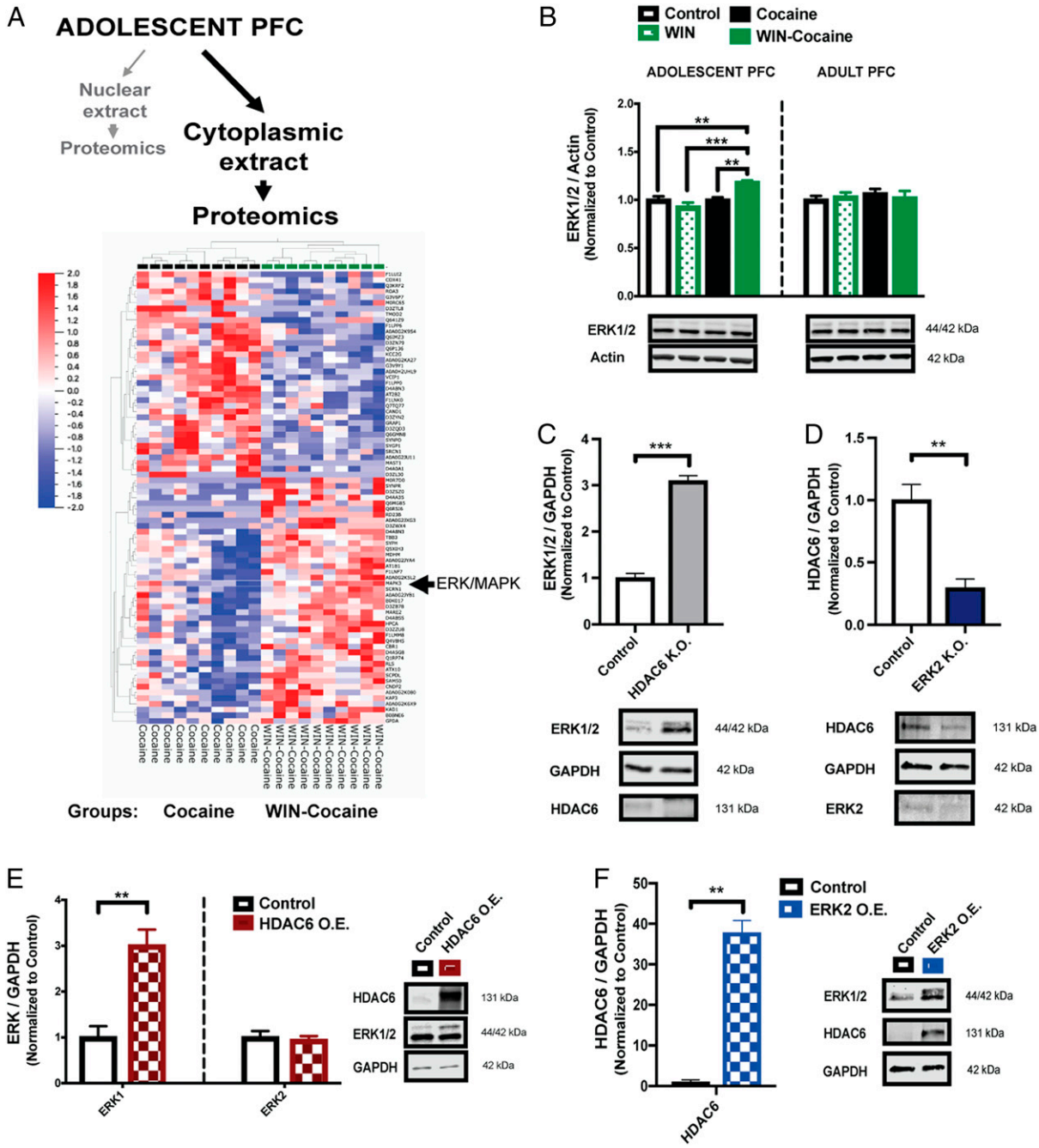

Fig. 4. WIN preexposure leads to cocaine-induced changes in ERK levels. (*A*) Cytoplasmic extracts from the adolescent PFC of the cocaine and WIN–cocaine groups were subjected to unlabeled quantitative proteomics. Hierarchical clustering heatmap is presented for the differentially expressed proteins ($P \leq 0.01$, $q \leq 0.5$; $n = 5$ animals per group and $n = 2$ technical replicates per animal). The arrow denotes changes in levels of ERK1/MAPK3 between groups ($P = 0.009$) (see also Dataset S5 for the complete list of differentially expressed cytoplasmic proteins). (*B*) Western blotting experiments confirmed the increase in ERK1/2 levels in the adolescent cytoplasmic PFC extracts of the WIN–cocaine group (*Left*) [ANOVA: $F(3, 15) = 15.87$, $P < 0.001$; Tukey's multiple comparisons test: Control vs. WIN–cocaine, $P = 0.0019$; WIN vs. WIN–cocaine, $P < 0.001$; cocaine vs. WIN–cocaine, $P = 0.0012$; $n = 4$ to 6 animals per group]. No changes in ERK1/2 levels were found in the adult PFC cytoplasmic extracts (*Right*) [ANOVA: $F(3, 12) = 0.298$, $P = 0.825$; $n = 4$ animals per group]. (*C*) Western blotting experiment using total lysate from a CRISPR-Cas9 HDAC6 knockout cell line (HeLa) showed increased ERK1/2 levels (t test, $t = 14.18$, df = 4; $P < 0.001$; $n = 3$ technical replicates per group). (*D*) Western blotting experiment using total lysate from a CRISPR-Cas9 ERK2 knockout cell line (HeLa) showed decreased HDAC6 levels (t test, $t = 4.875$, df = 4; $P = 0.008$; $n = 3$ technical replicates per group). (*E*) Western blotting experiment using total lysates from a transient HDAC6 overexpression cell line (HEK293T) showed increased levels of ERK1 but no changes in ERK2 levels (ERK1: t test, $t = 4.732$, df = 4; $P = 0.009$; ERK2: t test, $t = 0.355$, df = 4; $P = 0.740$; $n = 3$ biological replicates per group). (*F*) Western blotting experiment using total lysate from a transient ERK2 overexpression cell line (HEK293T) showed increased HDAC6 levels (t test, $t = 11.81$, df = 4; $P < 0.001$; $n = 3$ technical replicates per group). Technical replicates refer to testing of the same (biological) sample multiple times and biological replicates refer to parallel measurements of biologically distinct samples. Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. $^{**}P < 0.01$ and $^{***}P < 0.001$.

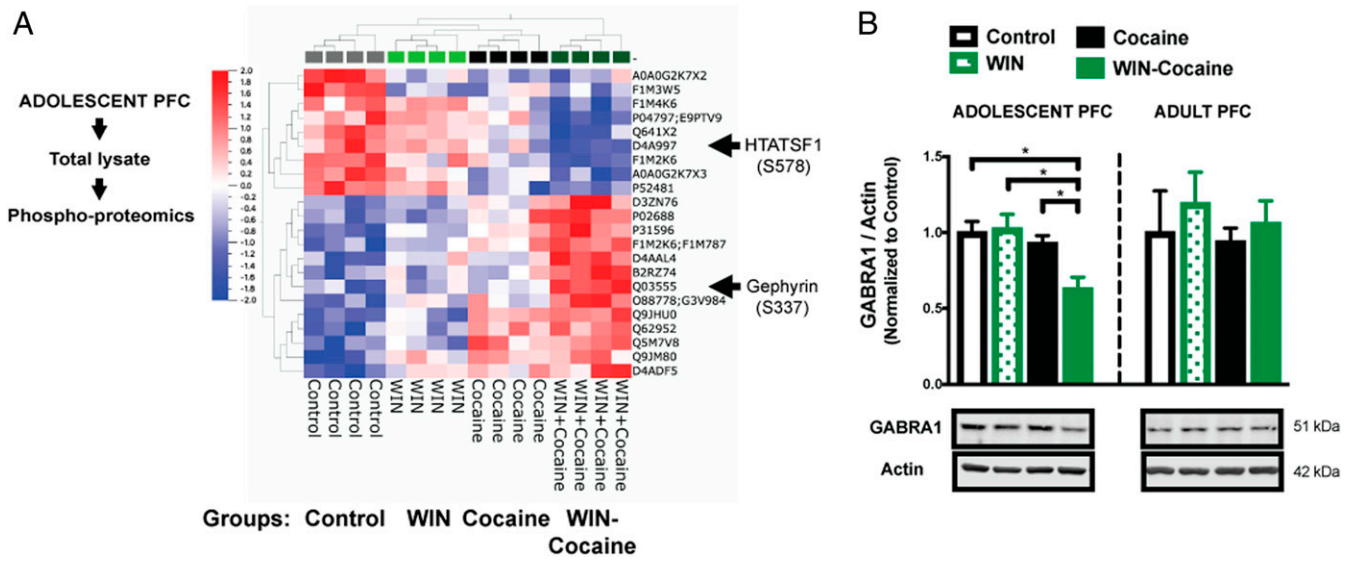

Fig. 5. WIN preexposure enhances cocaine's effect on protein phosphorylation. (*A*) Adolescent PFC samples from all four treatment groups were subjected to labeled TMT-based quantitative phosphoproteomics (separate animal cohorts were used; see also *Statistical Analyses* in *SI Appendix, Supplementary Methods*). Hierarchical clustering heatmap is presented for the differential levels of phosphorylated protein residues (ANOVA, $P \leq 3.9265e\text{-}4$; $q \leq 0.16$; $n = 4$ animals per group). The two arrows to the right of the heatmap denote the dephosphorylation of the HTATSF1 (at serine 578, $P = 5.49e\text{-}5$), and the hyperphosphorylation of gephyrin (at serine 337; $P = 4.91e\text{-}5$) in the WIN–cocaine group (see also Dataset S6 for a complete list of significantly differential phosphorylation events). (*B*) Western blotting experiments showed decreased levels of the GABA receptor subunit α1 (GABRA1) in the cytoplasmic PFC extract of the adolescent WIN–cocaine group (*Left*) [ANOVA: $F(3, 15) = 6.235$, $P = 0.005$; Tukey's multiple comparisons test: Control vs. WIN–cocaine, $P = 0.017$; WIN vs. WIN–cocaine, $P = 0.011$; cocaine vs. WIN–cocaine, $P = 0.044$; $n = 4$ to 6 animals per group]. No changes in GABRA1 levels were found in the adult PFC groups (*Right*) [ANOVA: $F(3, 12) = 0.311$, $P = 0.816$; $n = 4$ animals per group]. Graph data are presented as mean ± SEM. Representative Western blots are shown below the graphs, with the approximate molecular weights of observed band sizes indicated to the right. $^*P < 0.05$.

A Possible Role for mTORC1 in Cocaine-Induced Synaptic AMPAR Up-Regulation. Although WIN led to a decrease in synaptic AMPA receptors in the adolescent PFC, we also observed that the subsequent exposure to cocaine led to GluR1/2 up-regulation in the synaptosomal fraction (Fig. 6*B*). We ruled out a transcriptional involvement in this process, since the RNA-seq experiments showed no significant changes in mRNA expression of the genes coding for GluR subunits, for example, *Gria1* (coding for GluR1), which we also confirmed using qRT-PCR experiments (*SI Appendix*, Fig. S9*A*). However, for all proteins that were up-regulated in the adolescent WIN group, the reactome pathway analysis showed highest enrichment for signaling via the mammalian target of rapamycin complex 1 (mTORC1) (*SI Appendix*, Table S2). The mTOR pathway is known to be modulated by cannabinoid receptor activation (33) and, besides being implicated in locomotor sensitization (34), it has also been found to regulate AMPA receptor surface expression in cortical neurons (35). Thus, given the known control that ERK exerts on mTOR (36), we hypothesized that cocaine may enhance the translation of GluR1 through a primed mTOR pathway that responds to the cocaine-induced increase in levels of ERK (Fig. 4*B*). As an indirect analysis of translational effects, we utilized cytoplasmic PFC extracts from all brain regions under investigation and performed Western blotting experiments to measure intracellular pools of GluR1. First, we found that, in contrast to the decrease in synaptic GluR1 levels in the adolescent PFC, there were no changes for cytoplasmic GluR1 levels between control and WIN groups (*SI Appendix*, Fig. S9*B*). Second, we found a significant increase in cytoplasmic GluR1 levels only in the PFC of the adolescent WIN–cocaine group (*SI Appendix*, Fig. S9*B*), consistent with a putative increase in protein translation.

The Effect of WIN on Synaptic AMPAR Availability Is Brain Region- and Abstinence-Dependent. Changes in synaptic AMPA receptor availability have consistently been demonstrated in animal models of cocaine sensitization and craving (37, 38). These changes have primarily been reported in the NAcc and have been found to depend on the drug abstinence period. Thus, we also examined synaptosomal GluR1/2 levels in additional adolescent and adult brain regions, including the NAcc, the dorsal striatum, the amygdala, and the hippocampus (for details on the synaptosomal fractionation method, see also *SI Appendix, Supplementary Methods*). A marked difference between adolescence and adulthood was found for the NAcc but not for other regions (*SI Appendix*, Fig. S10). Specifically, in adolescence, cocaine decreased synaptic GluR levels in the NAcc and this decrease was blunted by WIN preexposure (*SI Appendix*, Fig. S10*A*). In contrast, in adulthood, cocaine increased synaptic GluR levels in the NAcc and this increase became more significant for GluR1 after WIN preexposure (*SI Appendix*, Fig. S10*E*). Next, we addressed the effect of abstinence by asking whether the WIN-induced decrease in synaptic GluR levels, which were observed on WIN abstinence day 9 in the adolescent PFC (Fig. 6*B*), were also present 24 h after the last WIN administration (i.e., on abstinence day 1). We found no significant changes in synaptosomal GluR1 or GluR2 levels in the adolescent PFC on abstinence day 1 (*SI Appendix*, Fig. S11*A*). However, in the adult PFC there were significantly decreased levels of GluR1 (but not GluR2) on abstinence day 1 (*SI Appendix*, Fig. S11*B*). These data suggest that WIN and cocaine modulate the synaptic AMPA receptor availability in a manner that is dependent on the developmental period, the brain region, and the abstinence period.

The PFC–NAcc Gene–Gene Correlation Network of Cocaine Is Affected by WIN Preexposure. The WIN-associated AMPA receptor changes in the adolescent PFC and NAcc (Fig. 6*B* and *SI Appendix*, Fig. S10*A*), suggested a possible glutamatergic dysregulation that can affect the molecular cross-talk between these two interconnected regions following exposure to cocaine. We hypothesized that we could use PFC–NAcc gene–gene correlation

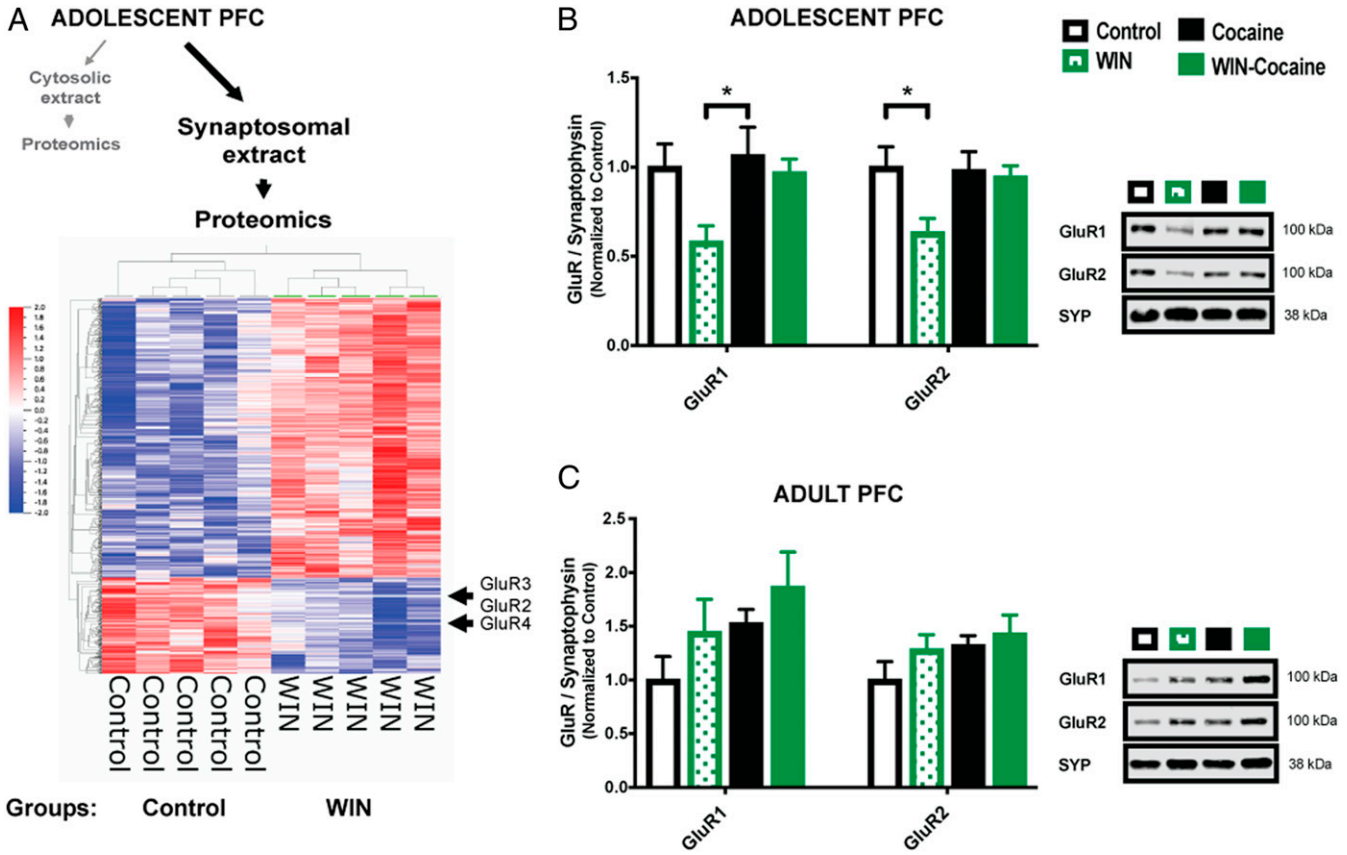

Fig. 6. Synaptosomal proteomics links WIN exposure to a decrease in synaptic AMPA receptors. (*A*) Synaptosomal and cytosolic extracts from adolescent PFC samples of the control and WIN groups were subjected to labeled TMT-based quantitative proteomics (separate animal cohorts were used; see also *Statistical Analyses* in *SI Appendix, Supplementary Methods*). In the hierarchical clustering heatmap for the differentially expressed proteins in the synaptosomal fraction ($P \leq 0.009$, $q \leq 0.07$; $n = 5$ animals per group), arrows to the right denote significantly decreased levels of AMPA receptor subunits (GluRs 2 to 4) in the WIN group (GluR1 levels were also significantly decreased on the nominal P level, $P = 0.026$). For the complete list of differentially expressed proteins in synaptosomes and cytosol, see Dataset S7. (*B*) Western blotting experiments confirmed a decrease in synaptosomal levels of GluR1 and GluR2 in the adolescent WIN group, and suggested that subsequent cocaine exposure led to reinsertion of AMPA receptors into the synaptic fraction [GluR1, ANOVA: $F(3, 20) = 3.437$, $P = 0.036$; Tukey's multiple comparisons test: Control vs. WIN, $P = 0.088$; WIN vs. cocaine, $P = 0.04$, WIN vs. WIN–cocaine, $P = 0.126$; GluR2, ANOVA: $F(3, 20) = 3.56$, $P = 0.032$; Tukey's multiple comparisons test: Control vs. WIN, $P = 0.046$; WIN vs. cocaine, $P = 0.062$, WIN vs. WIN–cocaine, $P = 0.106$; $n = 6$ animals per group]. (*C*) Western blotting experiments assessing GluR1 and GluR2 levels in adult synaptosomal PFC extracts showed no significant differences between groups although a trend for increase was observed in the WIN–cocaine group [GluR1, ANOVA: $F(3, 16) = 1.924$, $P = 0.166$; Tukey's multiple comparisons test: Control vs. WIN–cocaine, $P = 0.12$; GluR2, ANOVA: $F(3, 16) = 1.544$, $P = 0.241$; Tukey's multiple comparisons test: Control vs. WIN–cocaine, $P = 0.2$; $n = 5$ animals per group]. Graph data are presented as mean ± SEM. Representative Western blots are shown to the right of the graphs, with the approximate molecular weights of observed band sizes indicated next to them. *$P < 0.05$.

networks to compare the cocaine and WIN–cocaine groups. To this end, we subjected NAcc samples from the adolescent cocaine and WIN–cocaine groups to RNA-seq. We first performed differential mRNA expression analysis of NAcc data but found no significant changes between the cocaine and WIN–cocaine groups on the false-discovery rate (FDR) level (although a limited number of mRNA changes were found on the nominal P level) (*SI Appendix*, Fig. S12 and Dataset S8). Next, using our already generated RNA-seq data for the PFC, we performed PFC–NAcc gene–gene correlation analyses within each group. We found a pronounced difference between the two treatment groups, with 87 genes vs. 2,086 genes being significantly correlated in the cocaine vs. WIN–cocaine groups, respectively (at FDR < 0.01). To visualize the data, we used a radial network topology analysis that demonstrated the increase in cocaine-induced gene–gene correlation complexity as a result of WIN preexposure (Fig. 7 *A* and *B*). In addition, examination of the four most highly interconnected subnetworks showed that WIN preexposure led to differences in top gene nodes: For example, *Habp2* in the cocaine group (SN1 in Fig. 7*C*) versus *Adcyap1* in the WIN–cocaine group (SN1 in Fig. 7*D*). *Adcyap1* codes for the pituitary adenylate cyclase-activating polypeptide that has previously been implicated in alcohol use (39) and stress disorders (40). GO analysis of the top subnetworks from each group linked the *Adcyap1*-subnetwork of the WIN–cocaine group with biological processes, such as dopamine metabolism, presynaptic assembly, and α1-adrenergic receptor activity (*SI Appendix*, Fig. S13).

Discussion

In the present study, we examined the hypothesis that preexposure to a synthetic cannabinoid (WIN) does not only enhance the behavioral sensitivity to cocaine but may also reprogram key brain regions to respond differently, on the molecular and epigenetic levels, to the first encounter with the drug. To this end, we first confirmed the presence of cross-sensitization between WIN and cocaine only in adolescent (and not in adult) rats. Our previous studies on other "gateway" drugs, such as nicotine and alcohol (41, 42), suggested that drug-priming properties are mediated by epigenetic mechanisms (e.g.,

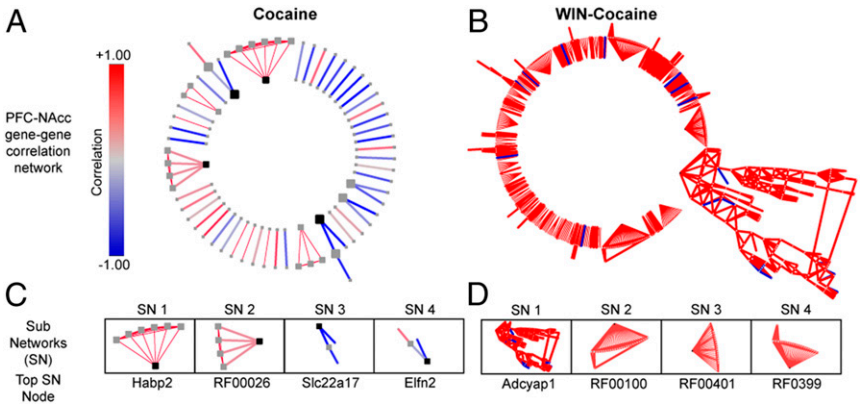

Fig. 7. PFC–NAcc gene correlation network in cocaine vs. WIN—cocaine adolescent groups. (*A*) Radial topology of PFC–NAcc gene–gene correlation network in the adolescent cocaine group ($n = 3$ PFC and $n = 3$ NAcc). Node size represents the rank-stat score of each gene. Edge color represents the positive (red) or negative (blue) correlation between genes. Edge thickness represents the adjusted *P* value (threshold set at FDR < 0.01). Branching represents gene correlations that alternate between regions. (*B*) Radial topology of PFC–NAcc gene–gene correlation network in the adolescent WIN–cocaine group ($n = 3$ PFC and $n = 3$ NAcc). In both figures *A* and *B*, the most highly interconnected gene node in each subnetwork (SN) is colored in black. (*C*) Visual analysis of the top four subnetworks in the cocaine group. The top node in SN1, for example, is *Habp2* (colored in black) with mRNA levels in the NAcc that correlate positively (red lines) with six other genes in the PFC. *Habp2* was up-regulated in the Nacc relative to PFC. Also, note that some of the six (non*Habp2*) genes in the PFC from SN1 correlate positively with each other. GO analysis of genes in SN1 revealed enrichment for biological processes such as amino acid transporter activity (*SI Appendix*, Fig. S13*A*). (*D*) Same analysis as in *C* but for the WIN–cocaine group. The top node in SN1 is *Adcyap1* (up-regulated in the PFC relative to NAcc) and GO analysis of all genes in SN1 revealed enrichment for biological processes, such as dopamine metabolism, presynaptic assembly, and α1-adrenergic receptor activity*SI Appendix*, Fig. S13*B*.

HDACs and histone acetylation) in line with the epigenetic-priming hypothesis in addiction (43). In agreement with this hypothesis, we found that WIN preexposure resulted in cocaine-induced global histone hyperacetylation in the adolescent PFC. This hyperacetylation, however, was not associated with global increases in open chromatin and mRNA levels. Instead, it was associated with enhanced chromatin accessibility and alternative splicing events in a limited number of genes, which included *Npas2*. *Npas2* codes for the neuronal PAS domain protein 2 (also known as member of PAS protein 4, MOP4) and is a circadian transcription factor recently found to regulate cocaine reward sensitivity and glutamatergic transmission (24, 25). Moreover, in our effort to identify epigenetic enzymes that may underlie this histone hyperacetylation, we conducted proteomic analyses of nuclear extracts and found decreased levels in HDAC6. HDAC6 is a class IIb HDAC, which was traditionally thought to localize in the cytoplasm, where it deacetylates microtubules (44). However, recent studies have demonstrated a role for HDAC6 in the nucleus as well, where similar to other HDACs, it takes part in histone deacetylation (45). Other class II HDACs, including HDAC5 (that targets another *Npas* gene, i.e., *Npas4*), have also repeatedly been associated with the rewarding effects of cocaine (46, 47), giving rise to the class II HDAC hypothesis of addiction (48).

Besides the epigenetic-related changes in the nucleus, we also found that WIN preexposure led to a cocaine-induced increase in levels of the MAP kinases ERK1/2 (ERK) in the adolescent PFC. ERK has been linked to cocaine-induced behavioral sensitization (27) and a regulatory interaction between ERK and HDAC6 has also been reported, with ERK phosphorylating HDAC6 and HDAC6 deacetylating ERK (28, 29). Indeed, using knockout and overexpression cell lines, we found a causal link between the two enzymes, which now warrants replication using neuronal systems. The increase in ERK levels also prompted us to conduct a phosphoproteomic analysis, which demonstrated that WIN preexposure intensifies the effects of cocaine on protein phosphorylation. Among the most prominent hyperphosphorylated proteins in the WIN–cocaine group was gephyrin. Gephyrin is a known target of ERK and affects GABRA1-containing GABA(A) receptors (31). When we examined cytoplasmic levels of GABRA1, we found that WIN preexposure led to a cocaine-induced decrease in its levels. GABA(A) receptor subtypes have been associated with addictive behaviors in both humans and preclinical models (49) and, notably, the *Gabra1* gene was recently found to be a transcriptional target of NPAS2 (50). This raises the possibility that the NPAS2-related changes in the adolescent WIN–cocaine group act in concert with ERK and gephyrin to regulate the availability of GABRA1 and the composition of GABA(A) receptors in response to cocaine. Although our findings applied to the adolescent WIN–cocaine group, previous literature has found a functional down-regulation of GABAergic transmission in the adult PFC following adolescent exposure to WIN or Δ^9-THC (51, 52).

When we examined how WIN preexposure affected the synaptic composition of the adolescent PFC, we found a decrease in all glutamate receptor subunits (GluRs) indicative of a reduction in AMPA receptor availability. This synaptic AMPA receptor reduction emerged as a consequence of WIN abstinence (for 9 d), since it was not present 24 h after the last WIN administration. Changes in GluR levels following drug abstinence have consistently been reported in animal models of addiction, especially in the NAcc, although a causal role for GluR subunits is thought to underlie the incubation of drug craving (37) and not the expression of behavioral sensitization (38). Nonetheless, the observed WIN-induced decreases in synaptic AMPA receptors in the adolescent PFC may lead to the formation of silent synapses that can prime the brain to undergo stronger synaptic plasticity upon subsequent experiences (e.g., with cocaine) through an enhanced capacity to recruit AMPA receptors (53). This idea is supported by our findings showing that exposure to cocaine led to increase in GluRs in the synaptic PFC fractions of the WIN preexposed, but not the WIN-naïve, animals. Ras signaling, through NMDA receptor activation, is known to drive synaptic delivery of AMPA receptors in an ERK-dependent manner (54). This may suggest that another downstream action of the cocaine-induced increase in ERK levels is the up-regulation of synaptic AMPA receptors.

When we examined AMPA receptor subunit levels in additional brain regions, we found that cocaine resulted in a decrease in synaptic GluRs in the adolescent NAcc but that WIN preexposure

ameliorated this outcome. In contrast, in the adult NAcc we found that cocaine resulted in an increase in synaptic GluR levels. In line with our adult findings, it has previously been shown that a single cocaine injection increases AMPA receptor surface expression in the adult NAcc (55). The same study also found that AMPA receptor surface expression decreased in the NAcc following injection of a D2-class agonist (55). This may suggest that the observed cocaine-induced decrease in synaptic GluR levels in the NAcc of WIN-naïve adolescent animals occurs through activation of D2 receptors. Indeed, adolescent rats have been found to present with enhanced D2-like receptor control of locomotor behaviors, which transitions to D1-like mechanisms in adulthood (56). D1 receptor stimulation has been found to result in AMPA receptor synaptic insertion in cultured NAcc neurons (57), suggesting that a D1-mediated mechanism underlies the cocaine-induced increase in synaptic GluRs in the adult NAcc. This raises the possibility that WIN exposure in adolescence, which partially blocks the cocaine-induced decrease in synaptic GluR levels in the NAcc, interferes with D2-like mechanisms that may account for the observed cross-sensitization.

Our study demonstrates that preexposure to cannabinoids in adolescence alters the initial behavioral, molecular, and epigenetic response to cocaine. However, a number of limitations need to be acknowledged: 1) The use of experimenter-administered drug regimens instead of self-administration procedures, 2) the use of synthetic cannabinoids instead of Δ^9-THC, 3) the assessment of neurobiological changes in bulk tissue instead of cell type-specific analyses, and 4) the lack of in vivo causality experiments rendering the molecular data correlational in nature. It is also important to emphasize that although initial responses to a drug like cocaine may predict its future use (7), substance use disorders do not develop from a single drug encounter but require repeated exposures that result in enduring epigenetic and synaptic changes (58, 59). Given the current increase in permissive societal and legal attitudes toward cannabis use, our study also highlights the need to further characterize the neurobiological consequences of cannabis exposure in adolescence in order to guide future legislature and public policies.

Methods

Animals and Behavioral Assessments. Male Sprague-Dawley rats were acclimated for 1 wk before starting treatment with WIN, cocaine, or vehicle. Detailed information on drug administration, locomotor experiments, and brain dissections, can be found in *SI Appendix*. Behavioral experiments were performed at the University of Cagliari, Italy, and molecular analyses were performed at Columbia University, New York, unless otherwise stated. All experimental procedures were carried out according to Italian (D.L. 26/2014) and European Council (63/2010) directives, and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments at the University of Cagliari.

Next-Generation Sequencing-Based Analyses, mRNA Validations, and Bioinformatics. Flash-frozen adolescent PFC or NAcc tissue was used to perform ATAC-seq or RNA-seq. Detailed information on next-generation sequencing-based procedures, including validation of mRNA targets, analysis of combined ATAC-seq/RNA-seq data, and network topology analyses, can be found in *SI Appendix*.

Subcellular Fractionations and Protein Extractions. Nuclear, cytoplasmic, and histone extractions were performed as previously described (13). The Syn-PER Synaptic Protein Extraction Reagent was used for synaptosomal versus cytosolic isolation. Detailed information on the fractionation methodologies, including their validation, can be found in *SI Appendix*.

Global Quantitative Proteomics by Mass Spectrometry and Western Blotting. All mass spectrometry-based quantitative proteomic experiments were performed at the Proteomics Shared Resource facility at Columbia University Irving Medical Center (New York City, NY). Detailed information on the experimental procedures, including Western blotting reagents and antibodies, can be found in *SI Appendix*.

Gene Knockout and Overexpression. For gene knockout/overexpression experiments, CRISPR-Cas9 HDAC6 or ERK2/MAPK1 knockout cell lysates and transient HDAC6 or ERK2/MAPK1 overexpression lysates were used. Additional information on the knockout and overexpression cell lines, can be found in *SI Appendix*.

Statistical Analyses. Two-group comparisons were performed using two-tailed unpaired Student's *t* test, and four-group comparisons were performed using ANOVAs, followed by correction for multiple testing. Statistical significance was set at $P \leq 0.05$. Additional information on the statistical analyses can be found in *SI Appendix*.

Data Availability. Sequencing data have been submitted to the Gene Expression Omnibus (GEO) data repository, under the accession number GSE134935, and proteomics data are found as research datasets in the *SI Appendix*.

ACKNOWLEDGMENTS. We thank Dr. Christina Doyle for laboratory administration and management. This work was funded by the Howard Hughes Medical Institute (E.R.K.), the Cohen Foundation (E.R.K.), the Swedish Research Council Diarienummer (Dnr) Grant 350-2012-6535 (to P.A.M.), the Royal Physiographic Society in Lund (P.A.M.), the Sweden-America Foundation (P.A.M.), the National Institute of Mental Health Grant F32MH114306 (to A.A.), and the Department of Biomedical Sciences Project RICDIP_2012_Fratta_01, University of Cagliari (to W.F.).

1. H. C. Meyer, F. S. Lee, D. G. Gee, The role of the endocannabinoid system and genetic variation in adolescent brain development. *Neuropsychopharmacology* **43**, 21–33 (2018).
2. R. Mechoulam, L. A. Parker, The endocannabinoid system and the brain. *Annu. Rev. Psychol.* **64**, 21–47 (2013).
3. L. H. Parsons, Y. L. Hurd, Endocannabinoid signalling in reward and addiction. *Nat. Rev. Neurosci.* **16**, 579–594 (2015).
4. M. Taylor *et al*., Patterns of cannabis use during adolescence and their association with harmful substance use behaviour: Findings from a UK birth cohort. *J. Epidemiol. Community Health* **71**, 764–770 (2017).
5. J. G. Morin *et al*., A population-based analysis of the relationship between substance use and adolescent cognitive development. *Am. J. Psychiatry* **176**, 98–106 (2019).
6. K. S. Bagot, R. Milin, Y. Kaminer, Adolescent initiation of cannabis use and early-onset psychosis. *Subst. Abus.* **36**, 524–533 (2015).
7. H. de Wit, T. J. Phillips, Do initial responses to drugs predict future use or abuse? *Neurosci. Biobehav. Rev.* **36**, 1565–1576 (2012).
8. D. M. Fergusson, J. M. Boden, L. J. Horwood, Cannabis use and other illicit drug use: Testing the cannabis gateway hypothesis. *Addiction* **101**, 556–569 (2006).
9. D. B. Kandel, *Stages and Pathways of Drug Involvement: Examining the Gateway Hypothesis* (Cambridge University Press, Cambridge, England, 2002).
10. D. Kandel, Stages in adolescent involvement in drug use. *Science* **190**, 912–914 (1975).
11. J. J. Palamar, M. J. Barratt, L. Coney, S. S. Martins, Synthetic cannabinoid use among high school seniors. *Pediatrics* **140**, e20171330 (2017).
12. T. Rubino, D. Parolaro, The impact of exposure to cannabinoids in adolescence: Insights from animal models. *Biol. Psychiatry* **79**, 578–585 (2016).
13. P. A. Melas *et al*., Cannabinoid modulation of eukaryotic initiation factors (eIF2α and eIF2B1) and behavioral cross-sensitization to cocaine in adolescent rats. *Cell Rep.* **22**, 2909–2923 (2018).
14. J. Kononoff *et al*., Adolescent cannabinoid exposure induces irritability-like behavior and cocaine cross-sensitization without affecting the escalation of cocaine self-administration in adulthood. *Sci. Rep.* **8**, 13893 (2018).
15. D. Dow-Edwards, S. Izenwasser, Pretreatment with Δ9-tetrahydrocannabinol (THC) increases cocaine-stimulated activity in adolescent but not adult male rats. *Pharmacol. Biochem. Behav.* **100**, 587–591 (2012).
16. A. Higuera-Matas *et al*., Augmented acquisition of cocaine self-administration and altered brain glucose metabolism in adult female but not male rats exposed to a cannabinoid agonist during adolescence. *Neuropsychopharmacology* **33**, 806–813 (2008).
17. A. L. Friedman, C. Meurice, E. M. Jutkiewicz, Effects of adolescent Δ9-tetrahydrocannabinol exposure on the behavioral effects of cocaine in adult Sprague-Dawley rats. *Exp. Clin. Psychopharmacol.* **27**, 326–337 (2019).
18. M. A. Aguilar *et al*., Adolescent exposure to the synthetic cannabinoid WIN 55212-2 modifies cocaine withdrawal symptoms in adult mice. *Int. J. Mol. Sci.* **18**, E1326 (2017).
19. M. Schneider, Adolescence as a vulnerable period to alter rodent behavior. *Cell Tissue Res.* **354**, 99–106 (2013).
20. M. Schneider, Puberty as a highly vulnerable developmental period for the consequences of cannabis exposure. *Addict. Biol.* **13**, 253–263 (2008).
21. K. Brami-Cherrier *et al*., Parsing molecular and behavioral effects of cocaine in mitogen- and stress-activated protein kinase-1-deficient mice. *J. Neurosci.* **25**, 11444–11454 (2005).

22. J. Feng *et al*., Chronic cocaine-regulated epigenomic changes in mouse nucleus accumbens. *Genome Biol.* **15**, R65 (2014).
23. S. M. Görisch, M. Wachsmuth, K. F. Tóth, P. Lichter, K. Rippe, Histone acetylation increases chromatin accessibility. *J. Cell Sci.* **118**, 5825–5834 (2005).
24. A. R. Ozburn *et al*., Direct regulation of diurnal Drd3 expression and cocaine reward by NPAS2. *Biol. Psychiatry* **77**, 425–433 (2015).
25. P. K. Parekh *et al*., Cell-type-specific regulation of nucleus accumbens synaptic plasticity and cocaine reward sensitivity by the circadian protein, NPAS2. *J. Neurosci.* **39**, 4657–4667 (2019).
26. H. Tilgner *et al*., Nucleosome positioning as a determinant of exon recognition. *Nat. Struct. Mol. Biol.* **16**, 996–1001 (2009).
27. L. Lu, E. Koya, H. Zhai, B. T. Hope, Y. Shaham, Role of ERK in cocaine addiction. *Trends Neurosci.* **29**, 695–703 (2006).
28. J. Y. Wu *et al*., Histone deacetylase 6 (HDAC6) deacetylates extracellular signal-regulated kinase 1 (ERK1) and thereby stimulates ERK1 activity. *J. Biol. Chem.* **293**, 1976–1993 (2018).
29. K. A. Williams *et al*., Extracellular signal-regulated kinase (ERK) phosphorylates histone deacetylase 6 (HDAC6) at serine 1035 to stimulate cell migration. *J. Biol. Chem.* **288**, 33156–33170 (2013).
30. S. K. Tyagarajan *et al*., Extracellular signal-regulated kinase and glycogen synthase kinase 3β regulate gephyrin postsynaptic aggregation and GABAergic synaptic function in a calpain-dependent mechanism. *J. Biol. Chem.* **288**, 9634–9647 (2013).
31. J. Mukherjee *et al*., The residence time of GABA(A)Rs at inhibitory synapses is determined by direct binding of the receptor α1 subunit to gephyrin. *J. Neurosci.* **31**, 14677–14687 (2011).
32. X. Y. Li, M. R. Green, The HIV-1 Tat cellular coactivator Tat-SF1 is a general transcription elongation factor. *Genes Dev.* **12**, 2992–2996 (1998).
33. E. Puighermanal *et al*., Cannabinoid modulation of hippocampal long-term memory is mediated by mTOR signaling. *Nat. Neurosci.* **12**, 1152–1158 (2009).
34. J. Bailey, D. Ma, K. K. Szumlinski, Rapamycin attenuates the expression of cocaine-induced place preference and behavioral sensitization. *Addict. Biol.* **17**, 248–258 (2012).
35. Y. Wang, M. F. Barbaro, S. C. Baraban, A role for the mTOR pathway in surface expression of AMPA receptors. *Neurosci. Lett.* **401**, 35–39 (2006).
36. P. Tsokas, T. Ma, R. Iyengar, E. M. Landau, R. D. Blitzer, Mitogen-activated protein kinase upregulates the dendritic translation machinery in long-term potentiation by controlling the mammalian target of rapamycin pathway. *J. Neurosci.* **27**, 5885–5894 (2007).
37. M. E. Wolf, Synaptic mechanisms underlying persistent cocaine craving. *Nat. Rev. Neurosci.* **17**, 351–365 (2016).
38. M. E. Wolf, C. R. Ferrario, AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. *Neurosci. Biobehav. Rev.* **35**, 185–211 (2010).
39. L. Kovanen *et al*., Circadian clock gene polymorphisms in alcohol use disorders and alcohol consumption. *Alcohol Alcohol.* **45**, 303–311 (2010).
40. K. J. Ressler *et al*., Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* **470**, 492–497 (2011).
41. E. A. Griffin, Jr *et al*., Prior alcohol use enhances vulnerability to compulsive cocaine self-administration by promoting degradation of HDAC4 and HDAC5. *Sci. Adv.* **3**, e1701682 (2017).
42. A. Levine *et al*., Molecular mechanism for a gateway drug: Epigenetic changes initiated by nicotine prime gene expression by cocaine. *Sci. Transl. Med.* **3**, 107ra109 (2011).
43. P. Mews, D. M. Walker, E. J. Nestler, Epigenetic priming in drug addiction. *Cold Spring Harb. Symp. Quant. Biol.* **83**, 131–139 (2018).
44. C. Hubbert *et al*., HDAC6 is a microtubule-associated deacetylase. *Nature* **417**, 455–458 (2002).
45. Z. Wang *et al*., Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* **138**, 1019–1031 (2009).
46. M. Taniguchi *et al*., HDAC5 and its target gene, Npas4, function in the nucleus accumbens to regulate cocaine-conditioned behaviors. *Neuron* **96**, 130–144.e6 (2017).
47. W. Renthal *et al*., Histone deacetylase 5 epigenetically controls behavioral adaptations to chronic emotional stimuli. *Neuron* **56**, 517–529 (2007).
48. E. A. Griffin, Jr, P. A. Melas, D. B. Kandel, E. R. Kandel, The class II histone deacetylase hypothesis of addiction. *Biol. Psychiatry* **84**, 165–166 (2018).
49. D. N. Stephens, S. L. King, J. J. Lambert, D. Belelli, T. Duka, $GABA_A$ receptor subtype involvement in addictive behaviour. *Genes Brain Behav.* **16**, 149–184 (2017).
50. A. R. Ozburn *et al*., NPAS2 regulation of anxiety-like behavior and GABAA receptors. *Front. Mol. Neurosci.* **10**, 360 (2017).
51. D. K. Cass *et al*., CB1 cannabinoid receptor stimulation during adolescence impairs the maturation of GABA function in the adult rat prefrontal cortex. *Mol. Psychiatry* **19**, 536–543 (2014).
52. J. Renard *et al*., Adolescent THC exposure causes enduring prefrontal cortical disruption of GABAergic inhibition and dysregulation of sub-cortical dopamine function. *Sci. Rep.* **7**, 11420 (2017).
53. Y. H. Huang *et al*., In vivo cocaine experience generates silent synapses. *Neuron* **63**, 40–47 (2009).
54. J. J. Zhu, Y. Qin, M. Zhao, L. Van Aelst, R. Malinow, Ras and Rap control AMPA receptor trafficking during synaptic plasticity. *Cell* **110**, 443–455 (2002).
55. C. R. Ferrario, X. Li, M. E. Wolf, Effects of acute cocaine or dopamine receptor agonists on AMPA receptor distribution in the rat nucleus accumbens. *Synapse* **65**, 54–63 (2011).
56. J. B. Dwyer, F. M. Leslie, Adolescent maturation of dopamine D1 and D2 receptor function and interactions in rodents. *PLoS One* **11**, e0146966 (2016).
57. X. Sun, M. Milovanovic, Y. Zhao, M. E. Wolf, Acute and chronic dopamine receptor stimulation modulates AMPA receptor trafficking in nucleus accumbens neurons cocultured with prefrontal cortex neurons. *J. Neurosci.* **28**, 4216–4230 (2008).
58. P. J. Hamilton, E. J. Nestler, Epigenetics and addiction. *Curr. Opin. Neurobiol.* **59**, 128–136 (2019).
59. E. J. Nestler, C. Lüscher, The molecular basis of drug addiction: Linking epigenetic to synaptic and circuit mechanisms. *Neuron* **102**, 48–59 (2019).