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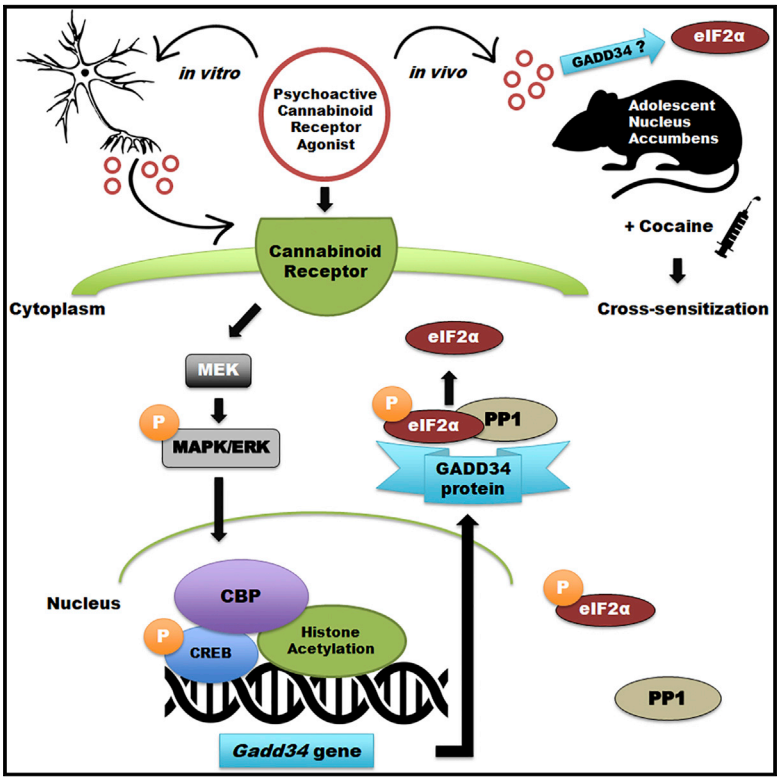
Cannabinoid Modulation of Eukaryotic Initiation Factors (eIF2α and eIF2B1) and Behavioral Cross-Sensitization to Cocaine in Adolescent Rats

Graphical Abstract



Highlights

- Psychoactive cannabinoids affect levels of p-eIF2α and eIF2B1
- The *in vitro* dephosphorylation of eIF2α is mediated by CREB and GADD34
- Brain levels of p-eIF2α are affected in cannabinoid-exposed adolescent rats only
- Cannabinoid-exposed adolescent rats show cross-sensitization to cocaine

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In Brief

Melas et al. show that psychoactive cannabinoids modulate levels of two eukaryotic initiation factors (eIF2α and eIF2B1) known to be involved in protein synthesis, memory formation, and drug sensitivity. Cannabinoid modulation of eIF2α *in vivo* is only observed in adolescent animals, and is associated with cross-sensitization to cocaine.

Data and Software Availability

GSE102946

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Cannabinoid Modulation of Eukaryotic Initiation Factors (eIF2α and eIF2B1) and Behavioral Cross-Sensitization to Cocaine in Adolescent Rats

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SUMMARY

Reduced eukaryotic Initiation Factor 2 (eIF2)α phosphorylation (p-eIF2α) enhances protein synthesis, memory formation, and addiction-like behaviors. However, p-eIF2α has not been examined with regard to psychoactive cannabinoids and cross-sensitization. Here, we find that a cannabinoid receptor agonist (WIN 55,212-2 mesylate [WIN]) reduced p-eIF2α *in vitro* by upregulating GADD34 (PPP1R15A), the recruiter of protein phosphatase 1 (PP1). The induction of GADD34 was linked to ERK/CREB signaling and to CREB-binding protein (CBP)-mediated histone hyperacetylation at the *Gadd34* locus. *In vitro*, WIN also upregulated eIF2B1, an eIF2 activator subunit. We next found that WIN administration *in vivo* reduced p-eIF2α in the nucleus accumbens of adolescent, but not adult, rats. By contrast, WIN increased dorsal striatal levels of eIF2B1 and ΔFosB among both adolescents and adults. In addition, we found cross-sensitization between WIN and cocaine only among adolescents. These findings show that cannabinoids can modulate eukaryotic initiation factors, and they suggest a possible link between p-eIF2α and the gateway drug properties of psychoactive cannabinoids.

INTRODUCTION

The eukaryotic Initiation Factor 2 (eIF2) is central to the regulation of protein synthesis and is required for the initiation of mRNA translation (Wek et al., 2006). eIF2 is a heterotrimer protein consisting of three subunits: alpha, beta, and gamma. The phosphorylated state of the eIF2 alpha subunit (p-eIF2α) constitutes one of the rate-limiting steps in protein translation (Holcik and Sonenberg, 2005). In response to environmental stresses (e.g., amino acid deprivation), different kinases (e.g., PERK and GCN2) phosphorylate eIF2α at serine 51 as part of a cellular stress adaptation process, the integrated stress response (ISR) (Wek et al., 2006). Upon phosphorylation, p-eIF2α binds to and inhibits its own guanine nucleotide exchange factor, eIF2B, whose concentration is much lower than that of eIF2. EIF2B can then no longer return eIF2 to its active guanosine triphosphate (GTP)-bound state, and, as a consequence, general translation comes to a halt, while the translation of a restricted subset of mRNAs (including ATF3, ATF4, and CHOP) is induced (Jiang et al., 2004; Sidrauski et al., 2015). On the one hand, this mechanism allows the cell to conserve resources and reconfigure gene expression to manage stress conditions or, alternatively, to induce apoptosis (Wek et al., 2006). On the other hand, dephosphorylation of eIF2α not only restores general protein synthesis but also has been found to enhance memory formation (Costa-Mattioli et al., 2007).

Recently, the molecular action of a number of drugs of abuse (including cocaine and nicotine) was found to converge on the reduction of p-eIF2α, which accounted for adolescent drug sensitivity (Huang et al., 2016; Placzek et al., 2016a). Moreover, eIF2α was found to regulate the progression from transient to persistent cocaine-induced long-term potentiation (Placzek et al., 2016b). While these findings provide a link between reduced p-eIF2α on the one hand and addiction-related processes on the other, the molecular mechanisms underlying the drug-induced dephosphorylation of eIF2α are still not known. In addition, while cannabinoids have been found to modulate

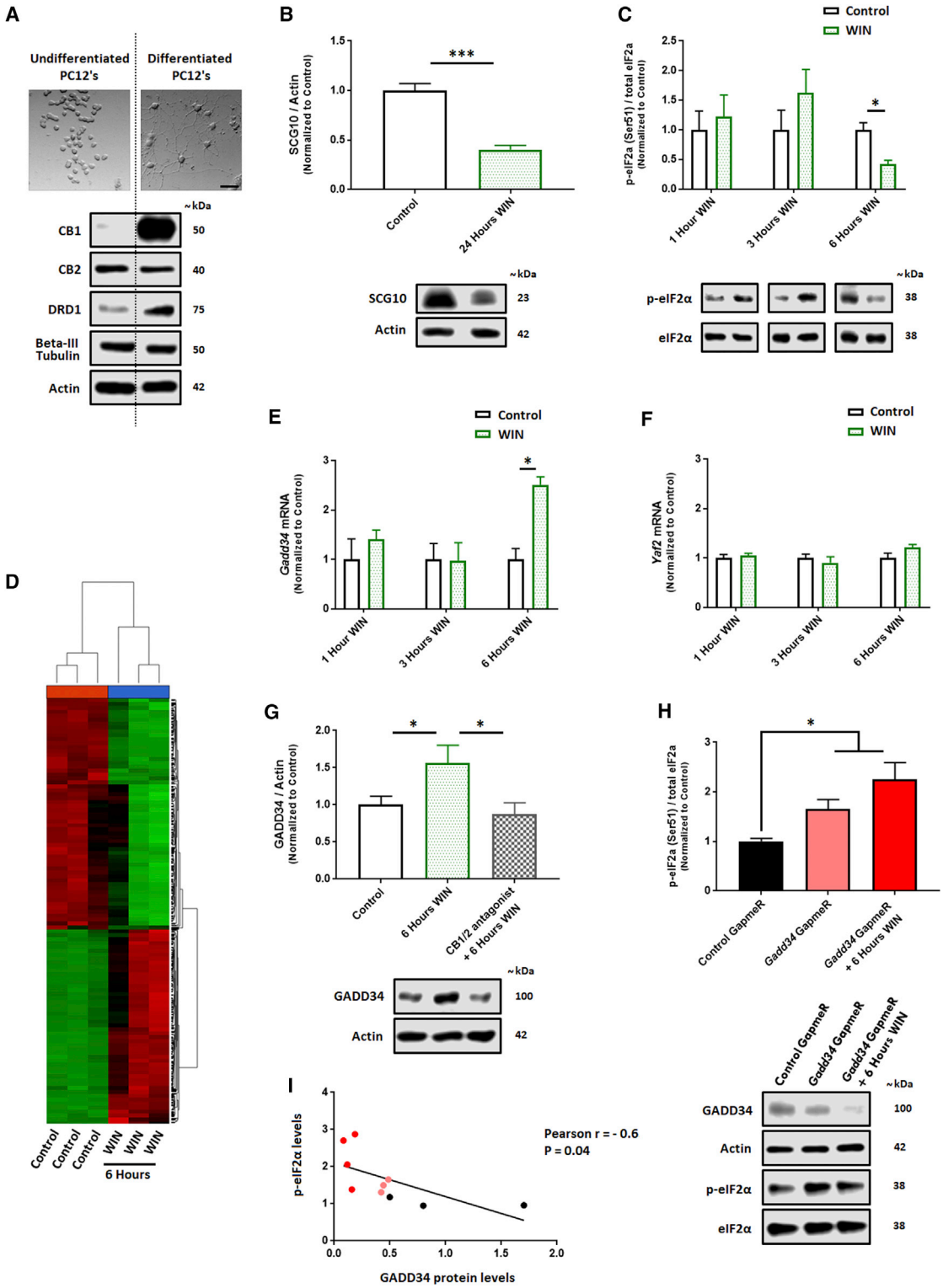

Figure 1. WIN Causes GADD34-Dependent Dephosphorylation of eIF2α *In Vitro*

(A) Top: differential interference contrast (DIC) images showing the morphology of undifferentiated (−NGF) PC12 cells (left image, round cells) and differentiated (+NGF) PC12 cells (right image, cells with neurites). Bottom: western blot images show robust expression of the cannabinoid receptor 1 (CB1) only in differentiated PC12s. Both states express the cannabinoid receptor 2 (CB2), the dopamine receptor D1 (DRD1), beta-III tubulin (a neuronal marker), and actin.

(B) WIN treatment (24 hr, 5 μM) of NGF-differentiated PC12s resulted in a decrease of SCG10/stathmin-2 protein levels (t test, p = 0.0004; n = 4/group).

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memory and protein synthesis via the mammalian target of rapamycin (mTOR) pathway (Puighermanal et al., 2009; Younts et al., 2016), psychoactive cannabinoids have not been studied with regard to p-eIF2α. In the present study, we examined whether p-eIF2α (1) is affected by a synthetic cannabinoid (WIN 55,212-2 mesylate [WIN]) and (2) is associated with the gateway drug properties of cannabinoids, previously observed in adolescent human populations (Kandel, 1975).

RESULTS

PC12: A Neuronal-like Model for Cannabinoid Research

PC12 is a rat cell line that can differentiate into neuronal-like cells when treated with nerve growth factor (NGF). PC12s have traditionally been used as a model for dopaminergic neurosecretion (Westerink and Ewing, 2008) and, to a lesser extent, in cannabinoid studies (Sadri et al., 2010). We therefore started the *in vitro* studies by confirming the suitability of PC12s for cannabinoid research. (1) Since PC12s can attain two states (undifferentiated or NGF differentiated), we examined the presence of cannabinoid receptors in both states by western blot. The antibody against CB1 produced immunoreactivity only in total cell extracts from NGF-differentiated PC12s (Figure 1A). (2) We performed a global proteomic analysis using NGF-differentiated PC12s to uncover molecular changes linked to the activation of cannabinoid receptors (WIN, 24 hr, 5 μM). This revealed a WIN-associated decrease in levels of SCG10/stathmin-2 (Table S1, first worksheet), which was also confirmed by western blot (Figure 1B). SCG10/stathmin-2 was found to be reduced *in vivo*, in the hippocampus of human fetuses exposed to cannabis, as well as *in vitro*, in cortical neurons exposed to WIN or Δ^9-THC (Tortoriello et al., 2014), thus supporting the use of NGF-differentiated PC12s for cannabinoid research.

WIN Causes GADD34-Mediated Dephosphorylation of eIF2α *In Vitro*

To examine whether WIN (5 μM) can affect p-eIF2α, we performed a time course experiment, and we observed a decrease in p-eIF2α after 6 hr of treatment (Figure 1C). At a lower concentration of WIN (500 nM), we found no changes in levels of p-eIF2α (Figure S1A). This suggests that both drug concentration and duration of treatment may be key modulators of WIN's molecular effects on p-eIF2α. When we tested the effect of WIN (5 μM, 6 hr) on p-eIF2α in the presence of CB1/2 antagonists, we observed a lower-than-baseline decrease in *total* levels of eIF2α in the CB1/2 antagonist group (Figure S1B). This suggests that the CB1/2 antagonists may be affecting eIF2α stability and/or the regulation of the *Eif2s1* gene (encoding eIF2α).

To gain insights into how dephosphorylation of eIF2α may be achieved by WIN (5 μM, 6 hr), we performed RNA sequencing and compared controls to WIN-treated cells. WIN treatment resulted in an increase of 397 and a decrease of 471 mRNAs, respectively (Figure 1D). Among the upregulated mRNAs were well-established ISR genes (e.g., CHOP/DDIT3, ATF3, and ATF4; see Table S2 for the top 20 genes and the first worksheet of Table S3 for all significantly expressed genes). *Gadd34* (*Ppp1r15a*) was among the top 20 upregulated genes (Table S2). GADD34 acts as the protein scaffold that independently recruits protein phosphatase 1 (PP1) and eIF2α for dephosphorylation of eIF2α (Choy et al., 2015). GADD34, therefore, provided the best candidate for the observed WIN-induced decrease of p-eIF2α. In a separate time course experiment, we confirmed the upregulation of *Gadd34* mRNA levels after 6 hr of WIN treatment (Figure 1E). As a negative control, we chose *Yaf2* mRNA (Figure 1F), since *Yaf2* did not show any differences in the RNA sequencing (RNA-seq) experiment ($p = 0.9$; Table S3, fifth worksheet). We next confirmed the WIN-induced increase of GADD34 on the protein level, and we found that pretreatment with cannabinoid receptor antagonists was able to block this increase (Figure 1G). Finally, to confirm that the dephosphorylation of eIF2α was linked to the action of GADD34, we performed mRNA knockdown using GapmeRs. We observed that GADD34 knockdown resulted in a significant increase in levels of p-eIF2α (Figure 1H), and, as expected, in the absence of GADD34, WIN was unable to cause a decrease in p-eIF2α levels (Figure 1H). The relationship between GADD34 and p-eIF2α is also shown by a significant negative correlation between the two proteins (Figure 1I).

WIN Induces CBP-Associated Histone Acetylation at *Gadd34*

We next asked, how does WIN cause the increase in levels of GADD34? WIN exposure resulted in an increase of GADD34 not only on the level of the protein but also on the level of the

(C) p-eIF2α levels were reduced after 6 hr of WIN (5 μM) treatment (Holm-Sidak's adjusted t test: 1-hr WIN, $p = 0.6$; 3-hr WIN, $p = 0.5$; 6-hr WIN, $p = 0.03$; $n = 3$–4/group).

(D) Heatmap of differentially expressed transcripts presented by hierarchical clustering (based on RNA-seq data; $n = 3$/group) shows separation between controls (Ctrl) and 6-hr WIN-treated PC12s (red, increase; green, decrease).

(E and F) qRT-PCR showed (E) an upregulation of *Gadd34* mRNA levels after 6 hr of WIN (Holm-Sidak's adjusted t test: 1 hr, $p = 0.6$; 3 hr, $p = 0.9$; 6 hr, $p = 0.01$; $n = 3$/group), whereas (F) no changes were found for *Yaf2* that serves as a negative control (Holm-Sidak's adjusted t test: 1 hr, $p = 0.7$; 3 hr, $p = 0.7$; 6 hr, $p = 0.3$; $n = 3$/group).

(G) GADD34 was also increased on the protein level, and pretreatment with CB1/2 receptor antagonists (AM251/AM630) reversed this increase (one-way ANOVA: $F = 4.337$, $p = 0.04$; Holm-Sidak's multiple comparisons test: WIN versus control group, $p = 0.04$, and WIN versus CB1/2 antagonist-pretreated group, $p = 0.04$; $n = 4$/group).

(H) *Gadd34* mRNA knockdown resulted in an increase in levels of p-eIF2α (one-way ANOVA: $F = 7.694$, $p = 0.01$; Holm-Sidak's post hoc test: Control GapmeR versus *Gadd34* GapmeR, $p = 0.06$; Control GapmeR versus *Gadd34* GapmeR + 6 hr WIN, $p = 0.007$; t test: Control GapmeR versus all *Gadd34* GapmeR samples, $p = 0.01$; $n = 4$/group).

(I) Regression line of normalized GADD34 levels (GADD34/Actin) and p-eIF2α levels (p-eIF2α/total eIF2α) using samples from the *Gadd34* GapmeR experiment, and correlation coefficients (Pearson $r = -0.642$; two-tailed $p = 0.04$; $n = 10$. Samples are color coded to match the groups in (H). Data are represented as mean ± SEM. Scale bar, 60 μm. *$p \leq 0.05$, ***$p < 0.001$.

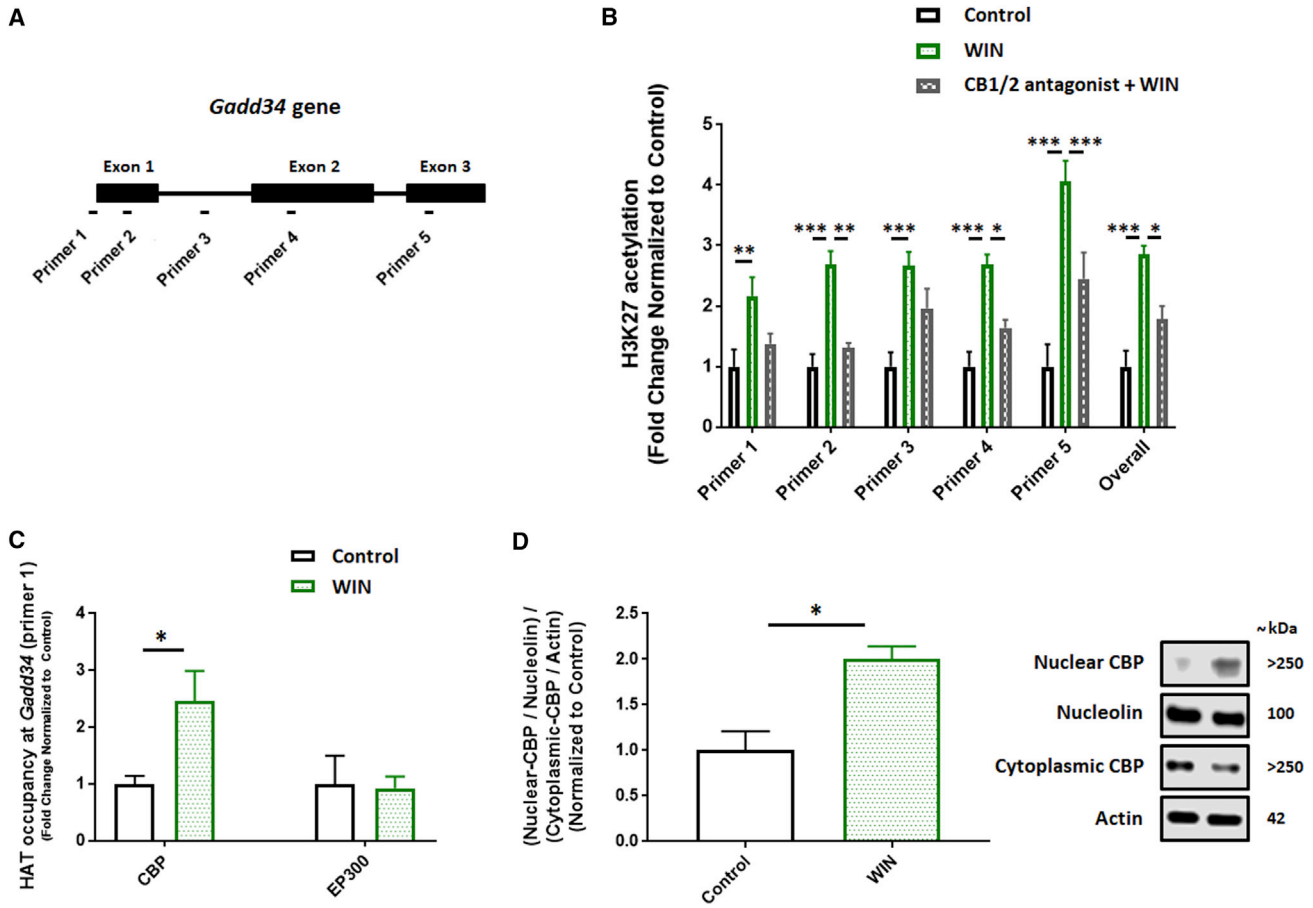


Figure 2. WIN Causes Histone Hyperacetylation and CBP Recruitment at the *Gadd34* Locus *In Vitro*

(A) Schematic representation of the *Gadd34* gene (reverse complement; official gene name: *Ppp1r15a*) and primer placements for the ChIP qRT-PCR experiments.

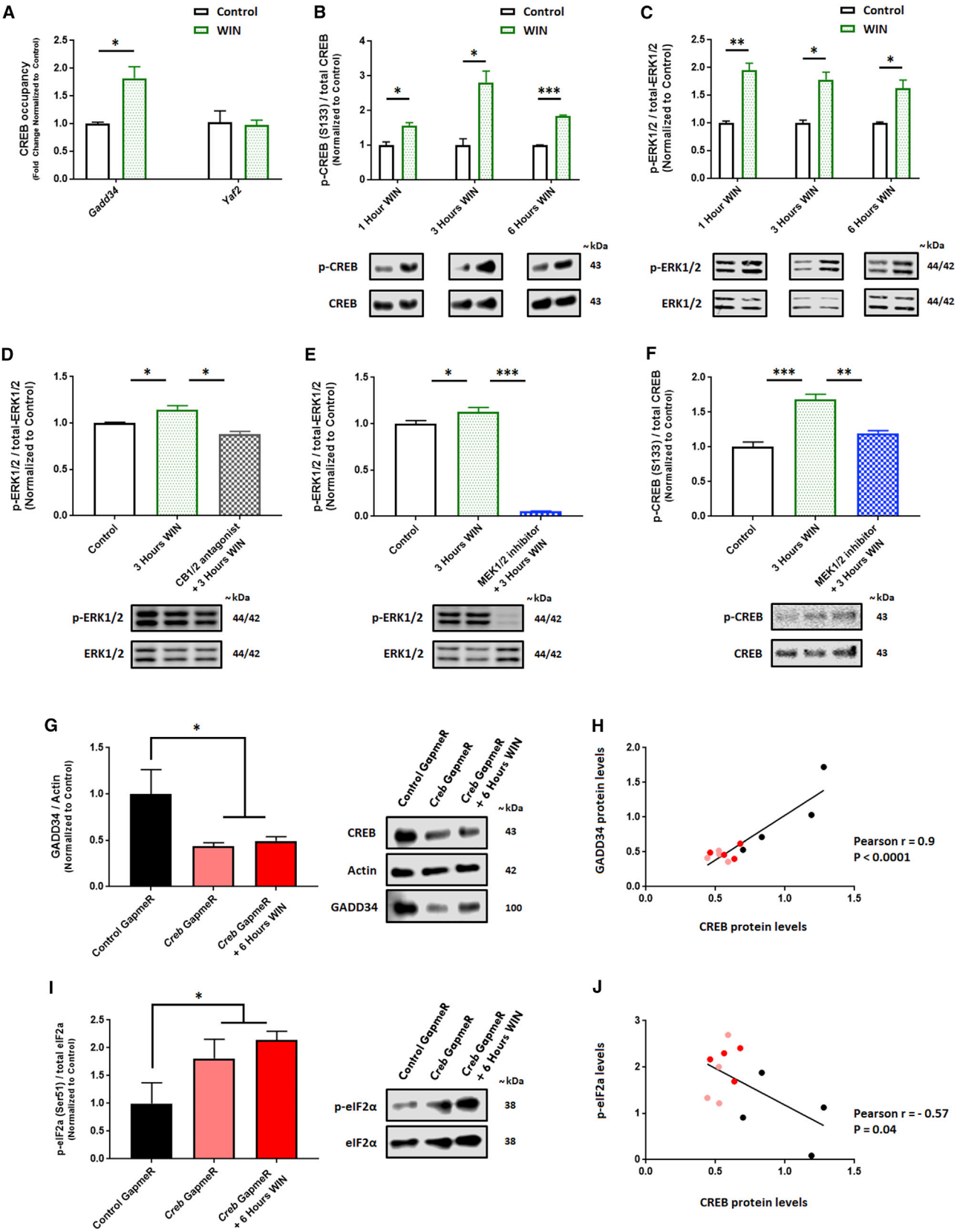
(B) WIN (6-hr treatment, 5 μM) increased levels of H3K27 acetylation throughout the *Gadd34* gene, and CB1/2 antagonists (AM251/AM630) reversed this increase. Asterisks represent significances, following Holm-Sidak's multiple comparisons test, for the comparisons with the WIN group.

(C) ChIP experiments of histone acetyltransferase (HAT) occupancy at the *Gadd34* TSS region (primer 1) showed an increase in binding of CBP, but not EP300, after 6-hr WIN treatment (Holm-Sidak's multiple comparisons test: CBP, $p = 0.01$; n = 5–6/group; EP300, $p = 0.9$; n = 3/group).

(D) The nuclear/cytoplasmic ratio of CBP increased in response to 6-hr WIN treatment (t test $p = 0.01$; n = 3/group). Data are represented as mean ± SEM. TSS, transcription start site. *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$.

mRNA (Figures 1E and 1G). This indicated a transcriptionally dependent regulatory mechanism. We previously found that other drugs of abuse, i.e., alcohol and nicotine, affect gene transcription by increasing levels of histone acetylation (Griffin et al., 2017; Levine et al., 2011). An examination of the epigenetic data from the Encyclopedia of DNA Elements (ENCODE)'s Consortium (ENCODE Project Consortium, 2012) on the human *GADD34* gene (*PPP1R15A*) showed that the gene-activating H3K27 acetyl modification (H3K27ac) is enriched throughout *GADD34*. We therefore performed chromatin immunoprecipitation (ChIP) experiments for H3K27ac, followed by qRT-PCR with primers spanning the entire rat *Gadd34* gene (Figure 2A). We found that WIN increased H3K27ac levels throughout *Gadd34* and, in the presence of CB1/2 antagonists, this increase was reduced (Figure 2B). As a negative control, we again used *Yaf2* and found no significant hyperacetylation at the *Yaf2* locus (Figure S2A). We also measured global histone acetylation levels of lysine residues on H2B, H3, and H4, and we found no significant changes, although there was a trend toward a WIN-induced increase in global acetylation levels of H3K27 (Figure S2B).

We next examined the enzymes that may be responsible for the WIN-mediated increase in H3K27ac at the *Gadd34* locus. There are two verified enzymes with specificity toward H3K27ac (Khare et al., 2012), both of which are histone acetyltransferases (HATs): CREB-binding protein (CBP) and E1A binding protein p300 (EP300). ChIP experiments showed a WIN-induced increase in the occupancy of CBP, but not of EP300, at the *Gadd34* transcription start site (TSS) region (Figure 2C). CBP is located in both the cytoplasm and the nucleus of the cell. In the nucleus it acts as an acetylase with activating effects, whereas in the cytoplasm it acts as a polyubiquitin ligase that destabilizes proteins (Shi et al., 2009). When we examined



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CBP protein levels in the nuclear versus cytoplasmic fractions of the cells, we found that, in response to WIN, CBP increased in the nuclear fraction while it decreased in the cytoplasmic fraction (Figure 2D).

The *In Vitro* WIN-Mediated Increase of GADD34 Is Controlled by CREB

CBP interacts with the transcription factor CREB1 (CREB) and specifically with p-CREB, a phospho-modified form of the protein (phosphorylated at Ser-133). p-CREB is the transcriptionally active form of the protein that binds to cyclic adenosine monophosphate (cAMP)-response elements (CREs). An examination of the nucleotide sequence of *Gadd34* revealed a half CRE site (TGACG) in the promoter region of the gene (132 nt upstream of the TSS). We therefore performed ChIP experiments to examine CREB binding at this locus. After WIN treatment, we found an increase in CREB occupancy at the *Gadd34* TSS, but not at the *Yaf2* locus (Figure 3A). The dual binding of CBP and CREB at *Gadd34* prompted us to measure the levels of p-CREB. We found that WIN resulted in an increase of the protein's phosphorylated state already at 1 hr of WIN treatment, as well as at 3 and 6 hr of treatment (Figure 3B), suggesting a time delay between CREB activation (1 hr) and *Gadd34* induction (6 hr).

CREB is phosphorylated via various signal transduction pathways, including mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPKs/ERKs), protein kinase A (PKA), and Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) (Kida and Serita, 2014). Since there is already evidence that psychoactive cannabinoids, e.g., Δ^9-THC, lead to activation of the ERK pathway (Bouaboula et al., 1995; Tortoriello et al., 2014), we measured levels of activated (phosphorylated) ERK1/2, and we found a WIN-mediated increase in p-ERK1/2 levels again by 1 hr of WIN treatment and up until 6 hr (Figure 3C). We also found that the WIN-induced phosphorylation of ERK1/2 could be blocked by pretreatment with (1) CB1/2 antagonists (Figure 3D) and (2) an inhibitor of the MAPK kinase 1/2 (MEK1/2, the activator of MAPK/ERK; Figure 3E). The WIN-induced phosphorylation of CREB could also be blocked by pretreatment with the MEK1/2 inhibitor (Figure 3F). Finally, to examine the causal relationship between CREB and the activation of the *Gadd34* gene, we knocked down CREB and found a decrease in GADD34 levels (Figure 3G). As expected, in the absence of CREB, WIN was unable to affect GADD34 levels (Figure 3G). The relationship between CREB and GADD34 is also shown as a significant positive correlation between the two proteins (Figure 3H). Using material from the same experiment, we also examined p-eIF2α levels, and we found that CREB knockdown increased levels of p-eIF2α, with or without the presence of WIN (Figure 3I). The relationship between CREB and p-eIF2α is also shown as a negative correlation between the two proteins (Figure 3J).

eIF2B1: An Additional Eukaryotic Initiation Factor that Is Affected by Cannabinoids

An eIF2B subunit (eIF2B1) emerged independently in two of our unbiased methodological approaches: (1) eIF2B1 was significantly upregulated in the proteomic study conducted using PC12s treated with WIN for 24 hr (false discovery rate [FDR] < 0.05; Figure 4A; Table S1, second worksheet), and (2) the mRNA levels of *Eif2b1* were significantly upregulated in the RNA-seq study of PC12s treated with WIN for 6 hr (FDR = 0.00002; Table S3, first worksheet). To confirm the RNA-seq data, we measured *Eif2b1* mRNA levels at different time points of WIN exposure, and we replicated the increase after 6 hr of WIN treatment (Figure 4B). Since eIF2B consists of five subunits that are coded by five different genes (*Eif2b1–b5*), we also measured mRNA levels of the remaining four subunits (*Eif2b2*, *Eif2b3*, *Eif2b4*, and *Eif2b5*), but we found no significant changes in any of them (Figures 4C–4F, respectively), suggesting specificity to *Eif2b1*. We also confirmed that the increase in *Eif2b1* mRNA levels was accompanied by an increase in the protein levels of eIF2B1 (Figure 4G). Since the WIN-induced

Figure 3. GADD34 Is Regulated by CREB1 *In Vitro*

(A) WIN treatment (6 hr, 5 μM) resulted in an increase in CREB1 (CREB) occupancy at the *Gadd34* TSS (Holm-Sidak's adjusted t test, p = 0.03; n = 3/group), but not at the *Yaf2* promoter locus (a negative control region; Holm-Sidak's adjusted t test, p = 0.8; n = 3/group).

(B and C) Levels of (B) p-CREB were elevated following WIN treatment at all three time points tested (Holm-Sidak's adjusted t test: 1-hr WIN, p = 0.01; 3-hr WIN, p = 0.01; 6-hr WIN, p < 0.001; n = 3/group), similar to levels of (C) p-ERK1/2 (Holm-Sidak's adjusted t test: 1-hr WIN, p = 0.006; 3-hr WIN, p = 0.01; 6-hr WIN, p = 0.01; n = 3/group).

(D and E) The WIN-induced phosphorylation of ERK1/2 was blocked by pretreatment with (D) CB1/2 receptor antagonists (AM251/AM630; one-way ANOVA: F = 13.98, p = 0.003; Holm-Sidak's multiple comparisons test: WIN versus control, p = 0.04; WIN versus CB1/2 antagonist + WIN, p = 0.003; control versus CB1/2 antagonist + WIN, p = 0.06; n = 3–4/group) and (E) a MEK1/2 inhibitor (U0126; one-way ANOVA: F = 208.6, p < 0.0001; Holm-Sidak's multiple comparisons test: WIN versus control, p = 0.04; WIN versus CB1/2 antagonist + WIN, p < 0.0001; control versus CB1/2 antagonist + WIN, p < 0.0001; n = 3–4/group), although the MEK inhibition abolished the p-ERK1/2 signal completely, reducing it to well below baseline, suggesting caution in data interpretation.

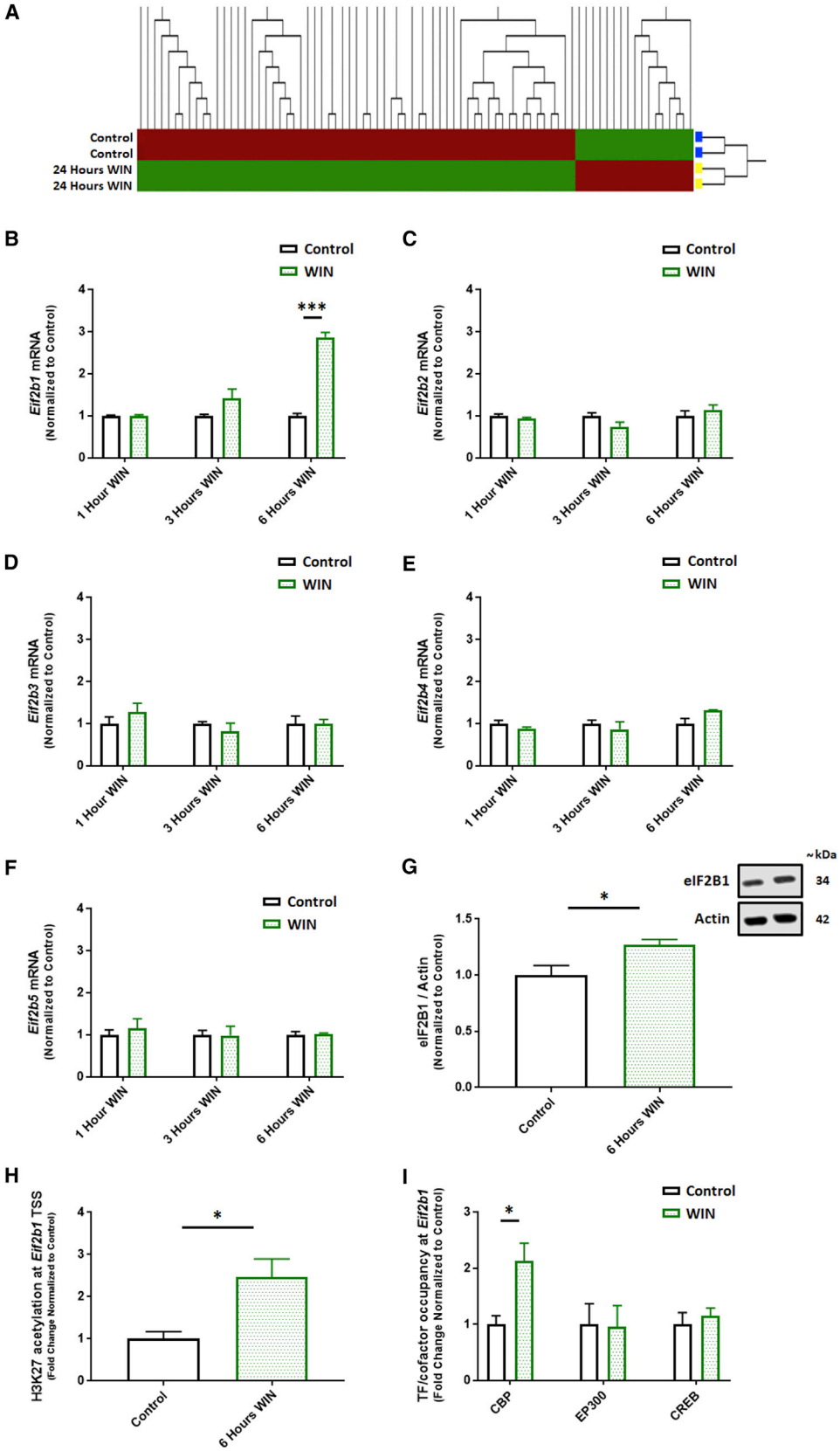
(F) The WIN-induced phosphorylation of CREB was reduced by pretreatment with the MEK1/2 inhibitor (U0126; one-way ANOVA: F = 28.87, p = 0.0002; Holm-Sidak's multiple comparisons test: WIN versus control, p = 0.0002; WIN versus CB1/2 antagonist + WIN, p = 0.002; control versus CB1/2 antagonist + WIN, p = 0.08; n = 3–4/group).

(G) CREB knockdown resulted in reduced levels of GADD34 (one-way ANOVA: F = 3.986, p = 0.05; Holm-Sidak's post hoc test: control GapmeR versus *Creb* GapmeR, p = 0.06; control GapmeR versus *Creb* GapmeR + 6-hr WIN, p = 0.06; t test: control GapmeR versus all *Creb* GapmeR samples, p = 0.01; n = 4/group).

(H) Above also shown as a regression line of normalized CREB (CREB/Actin) and normalized GADD34 (GADD34/Actin) using samples from the *Creb* GapmeR experiment, and corresponding correlation coefficients (Pearson r = 0.9; two-tailed p < 0.0001; n = 12). Samples are color coded to match the groups in (G).

(I) CREB knockdown resulted in an increase in levels of p-eIF2α (one-way ANOVA: F = 3.724, p = 0.06; Holm-Sidak's post hoc test: control GapmeR versus *Creb* GapmeR, p = 0.09; control GapmeR versus *Creb* GapmeR + 6-hr WIN, p = 0.05; t test: control GapmeR versus all *Creb* GapmeR samples, p = 0.02; n = 4/group).

(J) Above also shown as a regression line of normalized CREB (CREB/Actin) and normalized p-eIF2α (p-eIF2α/total eIF2α) using samples from the *Creb* GapmeR experiment, and corresponding correlation coefficients (Pearson r = −0.579; two-tailed p = 0.04; n = 12). Samples are color coded to match the groups in (I). Data are represented as mean ± SEM. *p ≤ 0.05, **p < 0.01, and ***p < 0.001.



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upregulation of eIF2B1 coincided in time with the upregulation of GADD34 (6 hr of WIN treatment), we next asked whether, similar to the regulation of *Gadd34*, the increase of *Eif2b1* mRNA also correlated with levels of H3K27ac. In line with this hypothesis, ChIP experiments targeting the TSS of *Eif2b1* showed WIN-mediated hyperacetylation at this locus (Figure 4H). Similar to the *Gadd34* analyses, we also examined the binding of CBP, EP300, and CREB at *Eif2b1*, and we found again that CBP, but not EP300, was enriched following WIN treatment (Figure 4I). However, we found no significant enrichment for CREB binding (Figure 4I), even though there is a full CRE site (GTGACGTAA) 60 nt upstream of the *Eif2b1*'s TSS. When we used material from our knockdown study to validate this finding, we found no effect of CREB knockdown on protein levels of eIF2B1 (t test, $p = 0.8$; data not shown), also arguing against a CREB-mediated control of *Eif2b1*.

In Vivo WIN Administration Affects p-eIF2α in the Nucleus Accumbens of Adolescent Rats

To address whether cannabinoids can produce similar molecular changes *in vivo*, adolescent (post-natal day [P]42) and adult (P77) male rats were treated sub-chronically with WIN or saline/control (11 days, intraperitoneal [i.p.] injections). Food intake and body weights were monitored throughout the treatment, and, as previously reported with Δ^9-THC administration (Scherma et al., 2016), we found that WIN reduced both parameters similarly in adolescent and adult animals (Figure S3). Rat brain dissections were performed 24 hr after the last drug administration (on abstinence day 1 [AD1]; see schematic, Figure 5A).

We started by examining p-eIF2α levels in five brain regions: the amygdala (AMYG), the dorsal striatum (DSTR), the hippocampus (HPC), the nucleus accumbens (NAcc), and the prefrontal cortex (PFC). In adolescents, we found that WIN led to a significant decrease in p-eIF2α levels in the NAcc only (Figure 5B). No changes in p-eIF2α levels were found in any of the five brain regions of adult animals (Figure 5C). Next, focusing on the NAcc of adolescent rats, we sought to replicate findings derived from the *in vitro* WIN experiments, relating to the regulation of p-eIF2α. In line with the *in vitro* data, we observed (1) a significant upregulation of p-ERK1/2 (Figure 5D), (2) a trend toward upregulation of p-CREB (Figure 5D), and (3) a significant increase in the nuclear/cytoplasmic localization of CBP (Figure 5D). However, levels of GADD34 showed a trend toward decrease (Figure 5D). This finding may reflect the known rapid degradation of GADD34 by the 26S proteasome following dephosphorylation of eIF2α (Brush and Shenolikar, 2008), which may be occurring at the time of brain dissections, i.e., 24 hr after the last WIN administration. Next, we examined levels of eIF2B1, and we found a significant increase in the dorsal striatum of both adolescent (Figure 5E) and adult (Figure 5F) animals. Finally, since chronic exposure to drugs of abuse has been shown to induce the brain accumulation of ΔFosB (Perrotti et al., 2008), a well-established addiction-related molecule (Nestler, 2008), we also examined ΔFosB levels in the same five brain regions. We found significantly increased levels of ΔFosB in the dorsal striatum of both adolescent (Figure 5G) and adult (Figure 5H) animals. A significant increase in ΔFosB was also observed in the prefrontal cortex of adult animals only (Figure 5H).

Behavioral Cross-Sensitization between Cannabinoids and Cocaine in Adolescence

Next, we asked whether the WIN-induced decrease in p-eIF2α, present in the adolescent NAcc, may be associated with the gateway drug properties of cannabinoids previously described in human adolescents (Kandel, 1975). We again pretreated adolescent and adult rats with WIN or saline/control, and (1) 24 hr after the last WIN administration (on AD1) animals were assessed behaviorally; and (2) 48 hr after the last WIN administration (on AD2), neurotransmitter levels were measured in the NAcc (see Figure 6A). Assessment of prepulse inhibition (PPI) of acoustic startle showed no differences in either adolescent (Figures 6B and 6C) or adult (Figures 6D and 6E) animals, suggesting no WIN-induced disturbances of sensorimotor information processing. Assessment of locomotor sensitization, a behavioral paradigm used to model drug-adaptive behaviors, showed significant cross-sensitization between WIN and cocaine in adolescent (Figure 6F), but not adult (Figure 6G), animals. Measurements of dopamine (DA), 3,4- dihydroxyphenylacetic acid (DOPAC), and homovallinic acid (HVA) levels in the NAcc on AD2, 24 hr after the cocaine challenge, revealed no changes in either adolescent (Figures 6H–6J) or adult (Figures 6K–6M) animals. Similarly, there were no changes in levels of glutamate (Figures S4A and S4B). We also observed that, at baseline, WIN-naive adults expressed greater locomotor sensitization than WIN-naive adolescents (Figure S4C). When we compared adolescent and adult samples, irrespective of WIN exposure, we found a trend toward increase in dopamine levels in the adult NAcc that may account for this baseline difference in the effects of cocaine ($p = 0.08$; Figure S4D).

In Vitro Transcriptional Enhancement by a Dopamine Receptor Agonist following WIN Pretreatment

Since PC12s co-express cannabinoid receptors (CBRs) and the dopamine receptor D1 (DRD1) (Figure 1A), and since drugs

Figure 4. *In Vitro* WIN Exposure and Upregulation of eIF2B1

(A) Heatmap of differentially expressed cytoplasmic proteins shows separation between controls (Ctrl, DMSO) and WIN-treated (24 hr, 5 μM) PC12s.

(B) *Eif2b1* mRNA levels increased after 6 hr of WIN treatment (Holm-Sidak's adjusted t test: 1 hr, $p = 0.8$; 3 hr, $p = 0.2$; 6 hr, $p < 0.001$; $n = 3$/group).

(C–F) There were no mRNA changes for (C) *Eif2b2*, (D) *Eif2b3*, (E) *Eif2b4*, and (F) *Eif2b5*.

(G) eIF2B1 was also upregulated on the protein level after 6 hr of WIN treatment (t test, $p = 0.05$; $n = 3$–4/group).

(H) ChIP experiments targeting the transcription start site (TSS) of *Eif2b1* showed WIN-induced hyperacetylation of H3K27 (t test, $p = 0.03$; $n = 3$/group).

(I) ChIP experiments assessing the binding of transcription factors (TFs) and cofactors CBP, EP300, and CREB showed only CBP enrichment at the *Eif2b1* TSS, following WIN treatment (Holm-Sidak's multiple comparisons test: CBP, $p = 0.04$; EP300, $p = 0.9$; CREB, $p = 0.9$; $n = 3$/group). Data are represented as mean ± SEM. *$p \leq 0.05$ and ***$p < 0.001$.

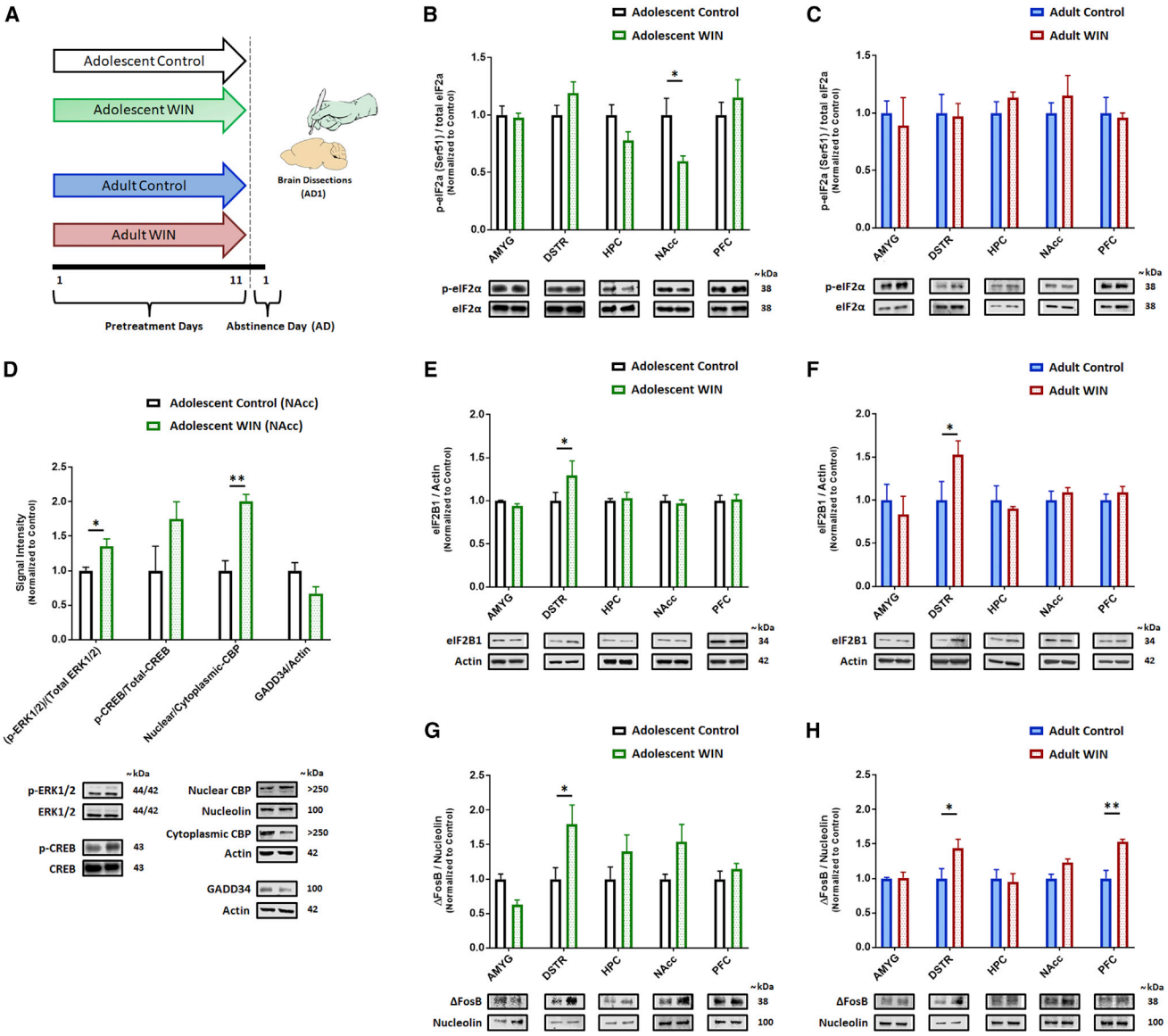

Figure 5. WIN Reduces Levels of p-eIF2α in the NAcc of Adolescent Rats

(A) Schematic representation of the experiment.

(B) In adolescents, WIN pretreatment led to a significant decrease in p-eIF2α levels in the NAcc only (two-way ANOVA: treatment $F(1, 37) = 0.9029$, $p = 0.348$; brain region $F(4, 37) = 2.953$, $p = 0.03$; interaction $F(4, 37) = 2.953$, $p = 0.03$; Holm-Sidak's multiple comparisons test: AMYG, $p = 0.8$; DSTR, $p = 0.5$; HPC, $p = 0.4$; NAcc, $p = 0.04$; PFC, $p = 0.5$; n = 4–5/group).

(C) In adult rats, WIN led to no changes in p-eIF2α levels (two-way ANOVA: treatment $F(1, 38) = 0.04981$, $p = 0.825$; brain region $F(4, 38) = 0.3347$, $p = 0.853$; interaction $F(4, 38) = 0.3347$, $p = 0.853$; Holm-Sidak's multiple comparisons test, $p = 0.9$ for all comparisons; n = 4–5/group).

(D) In the NAcc of adolescent rats, there was also a significant upregulation of p-ERK1/2 (t test, $p = 0.02$; n = 5/group), a trend upregulation of p-CREB (t test, $p = 0.1$; n = 5/group), a significant upregulation in the nuclear/cytoplasmic localization of CBP (t test, $p = 0.001$; n = 4–5/group), and a trend toward decreased levels of GADD34 (t test, $p = 0.07$; n = 4/group).

(E and F) In both (E) adolescents and (F) adults, WIN pretreatment led to a significant increase in eIF2B1 levels in the DSTR (Holm-Sidak's multiple comparisons test, $p = 0.04$ for both adolescents and adults; n = 4–5/group).

(G) In adolescents, WIN pretreatment led to a significant increase of ΔFosB in the DSTR and a trend toward increase in the NAcc (Holm-Sidak's multiple comparisons test: DSTR, $p = 0.017$; NAcc, $p = 0.08$; n = 4–5/group).

(H) In adult rats, WIN pretreatment led to a significant increase in levels of ΔFosB in the DSTR and the PFC (Holm-Sidak's multiple comparisons test: DSTR, $p = 0.017$; PFC, $p = 0.004$; n = 4–5/group). Data are represented as mean ± SEM. *$p \leq 0.05$ and **$p < 0.01$.

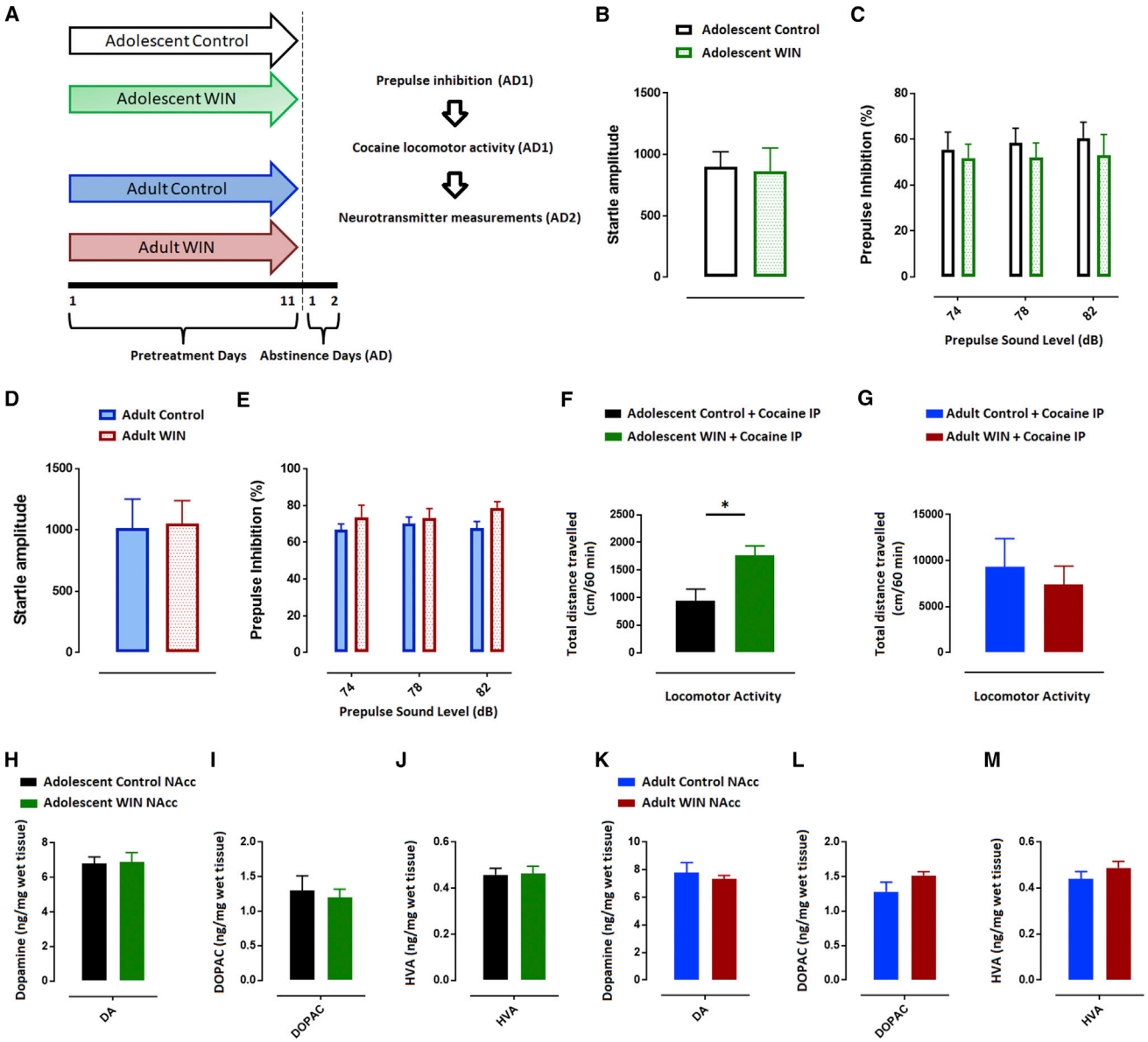

Figure 6. Behavioral Cross-Sensitization between Cannabinoids and Cocaine in Adolescence

(A) Schematic representation of the behavioral experiments.

(B and C) In WIN-pretreated adolescents, no differences in (B) startle amplitude (t test, $p = 0.8$; $n = 5$/group) or in (C) PPI (two-way ANOVA: treatment $F(1, 24) = 1.041$, $p = 0.318$; sound level $F(2, 24) = 0.09625$, $p = 0.909$; interaction $F(2, 24) = 0.03853$, $p = 0.962$; $n = 5$/group) were found.

(D and E) Similarly, in WIN-pretreated adults, no differences in (D) startle amplitude (t test, $p = 0.9$; $n = 5$/group) or in (E) PPI (two-way ANOVA: treatment $F(1, 24) = 3.926$, $p = 0.059$; sound level $F(2, 24) = 0.2147$, $p = 0.808$; interaction $F(2, 24) = 0.4451$, $p = 0.646$; $n = 5$/group) were found.

(F and G) Cocaine locomotor activity assessment showed significant cross-sensitization to cocaine (10 mg/kg, i.p. injection) in (F) WIN-pretreated adolescents (t test, $p = 0.02$; $n = 4$/group), but not in (G) WIN-pretreated adult animals (t test, $p = 0.6$; $n = 5$/group).

(H–J) In adolescents, no WIN-associated changes were found for (H) DA (t test, $p = 0.8$; $n = 5$/group), (I) DOPAC (t test, $p = 0.7$; $n = 5$/group), or (J) HVA (t test, $p = 0.9$; $n = 5$/group).

(K–M) Similarly, in adults, no WIN-associated changes were found for (K) DA (t test, $p = 0.6$; $n = 4$–5/group), (L) DOPAC (t test, $p = 0.1$; $n = 4$–5/group), or (M) HVA (t test, $p = 0.5$; $n = 5$/group). Data are represented as mean ± SEM. *$p \leq 0.05$.

like alcohol and nicotine are known to prime gene expression by cocaine (Griffin et al., 2017; Levine et al., 2011), we asked whether WIN can prime gene expression by a DRD1 agonist *in vitro*. To examine this possibility, differentiated PC12s pre-treated with WIN (6 hr) were also briefly exposed to a DRD1 agonist (SKF 81297, 30 min) and transcriptional changes were assessed. First, we examined the expression of our two main genes of interest (*Gadd34* and *Eif2b1*), and we found

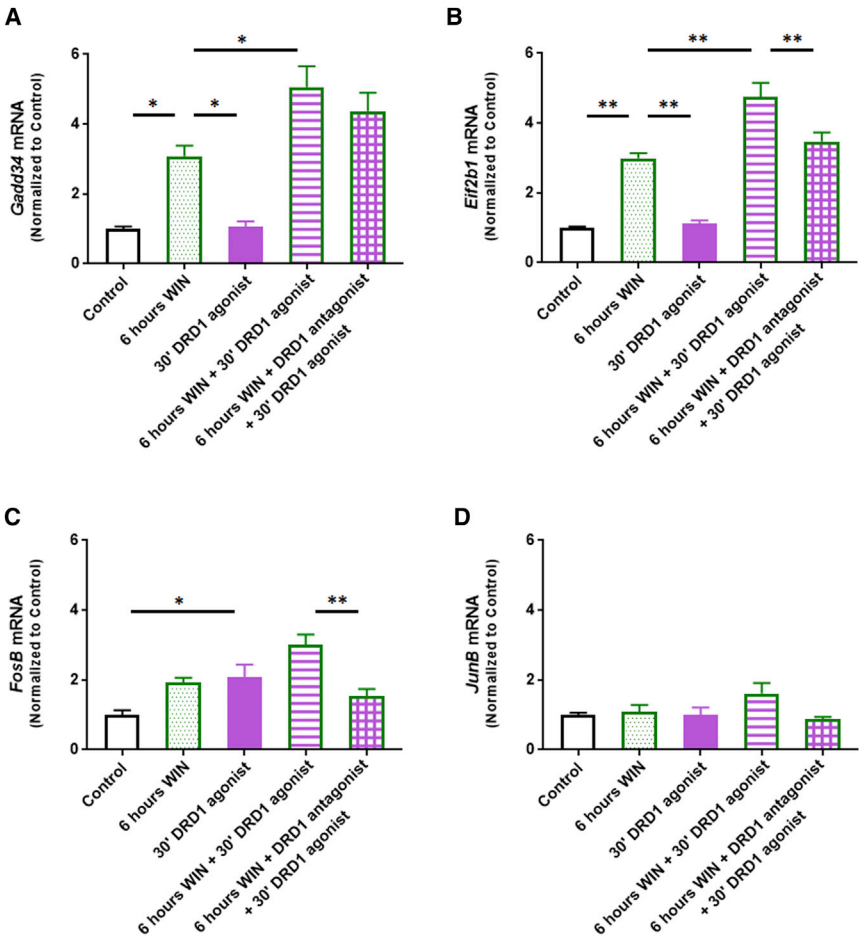

Figure 7. WIN Enhances the Transcriptional Effects of a Dopamine Receptor Agonist

(A and B) Again, (A) *Gadd34* and (B) *Eif2b1* were found to be increased by WIN, and when the WIN group was exposed to a DRD1 agonist, an additional ~2-fold induction of gene expression was observed (*Gadd34*; Holm-Sidak's test: WIN versus control, p = 0.03; WIN versus WIN + DRD1 agonist, p = 0.03; and *Eif2b1*; Holm-Sidak's test: WIN versus control, p = 0.002; WIN versus WIN + DRD1 agonist, p = 0.002; n = 3–5/group). DRD1 antagonist pretreatment produced a significant mRNA reduction in the case of *Eif2b1* (Holm-Sidak's p = 0.005), but not *Gadd34* (Holm-Sidak's p = 0.4).

(C) In the case of *FosB*, the DRD1 and the WIN + DRD1 agonist groups showed a significant induction, which was blocked in the DRD1 antagonist group (Holm-Sidak's test: DRD1 agonist versus control, p = 0.05; WIN + DRD1 agonist versus control, p = 0.0003; DRD1 agonist versus WIN + DRD1 agonist, p = 0.09; WIN + DRD1 agonist versus WIN + DRD1 antagonist + DRD1 agonist, p = 0.004; n = 3–5/group).

(D) For *JunB* there were no changes in any of the tested groups (one-way ANOVA: F = 2.243, p = 0.1; n = 3–5/group). Data are represented as mean ± SEM. Significant differences may not be depicted with asterisks if group comparisons are not of primary interest (e.g., control versus WIN + D1 agonist). *p ≤ 0.05 and **p < 0.01.

that, when the WIN-treated group was also exposed to the DRD1 agonist (i.e., WIN + DRD1), it produced an additional ~2-fold induction of gene expression, while exposure to the DRD1 agonist alone, without WIN exposure, had no such effects (Figures 7A and 7B). To verify that this additional mRNA induction was caused by DRD1 activation, a separate group was pretreated with a dopamine receptor antagonist (SCH 23390 hydrochloride, 30 min). This resulted in a significant reduction only in the case of *Eif2b1* (Figure 7B), but not *Gadd34* (Figure 7A), arguing for putative non-specificity in the case of *Gadd34* overexpression. Second, using the same paradigm, we assessed the mRNA levels of *FosB* and *JunB*, two immediate early genes that are known to be upregulated by acute exposure to cocaine *in vivo* (Zhang et al., 2002). *FosB* is also the gene that gives rise to the *ΔFosB* splice variant. In the case of *FosB*, WIN alone did not produce a significant effect compared to controls, but both the DRD1 agonist alone and the (WIN + DRD1) group showed a significant induction that was blocked with the dopamine receptor antagonist (Figure 7C). Finally, in the case of *JunB*, we found no significant differences in any of the tested groups (Figure 7D), which is in line with data suggesting an additional involvement of dopamine receptor D2 in the case of *JunB* expression (Simpson and Morris, 1994).

DISCUSSION

There is increasing evidence that molecular pathways contributing to normal learning and memory become maladaptive in addiction (Kelley, 2004). However, to date, few molecules that share common roles in both memory formation and addiction have been identified. One shared molecule is ΔFosB, an extensively studied transcription factor that accumulates in the brain after chronic drug use, and which has been found to influence both memory formation (Eagle et al., 2015) and the addiction process (Nestler, 2008). A second shared molecular candidate is p-eIF2α, which has a central role in memory formation (Costa-Mattioli et al., 2005, 2007; Di Prisco et al., 2014) and was recently found to be affected by a number of drugs of abuse (including alcohol, cocaine, methamphetamine, and nicotine) and to account for adolescent drug hypersensitivity (Huang et al., 2016; Placzek et al., 2016a, 2016b).

Psychoactive cannabinoid drugs, e.g., Δ^9-THC that acts as a partial CB1/2 receptor agonist, have previously been studied in relation to ΔFosB (Perrotti et al., 2008), but not in relation to p-eIF2α. In the present study, we found that the synthetic cannabinoid WIN, which acts as a full CB1/2 receptor agonist, was able to reduce p-eIF2α levels *in vitro* by upregulating GADD34, the protein scaffold that recruits PP1 to dephosphorylate eIF2α (Choy et al., 2015). Consistent with previous *in vivo* studies using WIN or Δ^9-THC (Bouaboula et al., 1995; Casu et al., 2005; Tortoriello et al., 2014), we also found that WIN led to the activation of the MAPK/ERK pathway, which, in turn, activated

the transcription factor CREB that bound to the *Gadd34* gene. The CREB-dependent regulation of *Gadd34* is in line with a genome-wide analysis using human hepatocytes and HEK293T cells that also found CREB enrichment at the *GADD34* locus (Zhang et al., 2005). CREB is another molecule known to have a central role in both memory formation and the development of addiction (Kandel, 2012; McPherson and Lawrence, 2007). However, the exact role of CREB in relation to addiction still remains elusive. For instance, there are some data suggesting that CREB can enhance cocaine reinforcement (Larson et al., 2011), while others found a role for CREB in cocaine aversion (Pliakas et al., 2001).

The WIN-induced increase in CREB occupancy at the *Gadd34* gene was found to be accompanied by an increase both in binding of the HAT CBP and in histone acetylation at the same locus. These drug-induced epigenetic effects were also observed in studies on different gateway drugs, with both nicotine (Kandel and Kandel, 2014; Levine et al., 2011) and alcohol (Griffin et al., 2017) affecting histone acetylation levels by inhibiting the action of histone deacetylases (HDACs). Taken together, these studies suggest that drugs can produce the same downstream epigenetic effect, i.e., increased histone acetylation, by two complementary mechanisms: either by decreasing the activity of HDACs (in the case of alcohol and nicotine) or by modulating HATs like CBP (in the case of cannabinoids). Interestingly, when we examined the protein levels of CBP in the nuclear versus cytoplasmic cell fractions, we found that the nuclear/cytoplasmic ratio of CBP increased significantly in response to WIN. Although certain other epigenetic enzymes e.g., class IIA HDACs and the HAT EP300 (Sebti et al., 2014), have been found to translocate between the cytoplasm and the nucleus in response to cellular stimuli, to our knowledge there is no such evidence regarding CBP. Thus, the possibility that CBP also employs a cellular shuttling mechanism warrants further examination.

We also found that *in vitro* WIN treatment resulted in an increase of an eIF2B subunit (eIF2B1), which again correlated with CBP recruitment and histone hyperacetylation. However, the WIN-mediated eIF2B1 upregulation did not appear to be dependent on CREB. The transcription factor (TF)-binding data from the ENCODE Consortium (ENCODE Project Consortium, 2012), which show genomic binding of 119 factors (Gerstein et al., 2012), suggest that two other members of the ATF/CREB family can bind at the promoter of the human *EIF2B1*: ATF2 and ATF3. Interestingly, ATF2 has been found to interact with CBP (Sano et al., 1998). In addition, ATF3 was one of the most upregulated genes in our RNA-seq study of WIN-treated PC12s (Table S2). Since ChIP-validated antibodies against ATF2 and ATF3 with specificity to rats are still lacking, future studies of *Eif2b1*'s transcriptional regulation by these two ATFs are warranted.

From the three molecular markers that were tested *in vivo* (i.e., p-eIF2α, eIF2B1, and ΔFosB), p-eIF2α proved to be a WIN-modulated target that was specific to adolescents and their NAcc. By contrast, both eIF2B1 and ΔFosB were found to be increased by WIN in both adolescents and adults. Our data on ΔFosB are in line with the extensive literature showing ΔFosB brain accumulation following chronic drug administration (Perrotti et al., 2008). In addition, our data on p-eIF2α confirm the two recent publications showing reduced p-eIF2α levels in adolescence after a single exposure to drugs like cocaine and nicotine (Huang et al., 2016; Placzek et al., 2016a), and they suggest that p-eIF2α, similar to, e.g., ΔFosB, may also be a common target for all drugs of abuse, especially when administered during adolescence. When we sought to replicate *in vitro* findings related to the regulation of p-eIF2α, we found again an increase in levels of p-ERK1/2, p-CREB, and nuclear CBP. However, when we examined GADD34 levels, we found a trend toward decrease. A possible explanation for this observation is the known rapid degradation of GADD34 by the 26S proteasome following dephosphorylation of eIF2α (Brush and Shenolikar, 2008). Compared to the *in vitro* system, where we were able to study GADD34 as soon as we observed a decrease in p-eIF2α levels, brain dissections were performed 24 hr after the last WIN administration. Thus, at this late time point, degradation of GADD34 may already have occurred. Future studies are therefore needed to confirm the *in vivo* link between GADD34 and cannabinoid modulation of p-eIF2α. These studies should consider measuring GADD34 closer to the last drug administration and following brain cannulation of proteasome inhibitors that would block the degradation of GADD34. Thus, the possibility that WIN modulates p-eIF2α *in vivo* by mechanisms that are, at least to some extent, different from the GADD34-related mechanisms observed in culture cannot be completely ruled out.

Finally, we also theorized that the cannabinoid-mediated decrease in p-eIF2α, in the adolescent NAcc, could provide a molecular correlate for the gateway drug properties of cannabinoids that have been well described both in human populations and in preclinical models (Biscaia et al., 2008; Cadoni et al., 2001; Dow-Edwards and Izenwasser, 2012; Ellgren et al., 2007; Fergusson et al., 2006; Higuera-Matas et al., 2008; Kandel, 1975, 2003; Manzanedo et al., 2004, 2010; Panlilio et al., 2013; Rodríguez-Arias et al., 2010; Solinas et al., 2004). In line with this hypothesis, cross-sensitization between WIN and cocaine was observed in adolescent animals only. These findings are in line with a cross-sensitization study between Δ^9-THC and cocaine that showed enhanced locomotor effects only in adolescents (Dow-Edwards and Izenwasser, 2012).

The present results enhance our molecular understanding of cannabinoid action, and they suggest a possible link between p-eIF2α and the gateway drug properties of cannabinoids. However, the correlational nature of the *in vivo* findings warrants future investigations using behavioral models that integrate pharmacological and/or genetic perturbations. In addition, since preclinical data suggest that Δ^9-THC exposure during adolescence contributes to cannabis use-like disorder in adulthood (Scherma et al., 2016), the role of p-eIF2α remains to be explored in other-than-gateway drug protocols. Also, while our study focused on the second phase of the p-eIF2α-dependent stress response, i.e., the dephosphorylation of eIF2α, there are also kinases (e.g., PERK and GCN2) that are involved in the initial phase of eIF2α phosphorylation. Besides eIF2α, these kinases can have additional phosphorylation targets and unique neuronal functions, such as the regulation of calcium dynamics (Zhu et al., 2016). Also, since various types of cannabinoids are

known to (1) activate the CB1/2 receptors with different potencies and (2) have non-CB1/2 receptor targets (Pertwee et al., 2010), further *in vivo* studies are needed to examine if drugs of abuse produce their molecular effects on p-eIF2α via specific receptors or if these molecular changes are to a certain extent a by-product of unspecific action when drugs and their downstream-affected neurotransmitters (e.g., dopamine) become available in supraphysiological concentrations in the brain, thus triggering a common endoplasmic reticulum stress response.

EXPERIMENTAL PROCEDURES

PC12 Cell Culturing, Differentiation, and Drug Exposures

All drug treatment experiments, unless otherwise noted, were performed using NGF-differentiated PC12 cells (Sigma-Aldrich, St. Louis, MO, USA). Detailed information on cell culturing, including differentiation and drug exposures, is found in the Supplemental Experimental Procedures.

Global Quantitative Proteomic Analysis

Quantitative (label-free) global proteomic analysis was performed to compare protein levels of NGF-differentiated PC12 controls (DMSO) versus cells treated with WIN (5 μM, 24 hr). Detailed information on this experiment, including data analysis, is found in the Supplemental Experimental Procedures.

Protein Extractions and Western Blotting

Cell lysis and protein extractions were performed using the N-PER Neuronal Protein Extraction Reagent (Thermo Scientific; Thermo Fisher Scientific, Waltham, MA, USA) for total protein extractions, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) for cytoplasmic and nuclear fractionations, and the EpiQuik Total Histone Extraction Kit (EpiGentek, Farmingdale, NY, USA) for histone extractions. Extraction reagents were supplemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (Thermo Scientific). Detailed information on western blotting experiments, including all primary antibodies used, is found in the Supplemental Experimental Procedures. Representative blots are shown in the figures, with the approximate molecular weights of observed band sizes indicated to the right (in kDa).

RNA Extraction and mRNA Real-Time qPCR

Total RNA was isolated using the Direct-zol RNA MiniPrep Plus kit (Zymo Research, Irvine, CA, USA) and was treated with DNase I, Amplification Grade (Invitrogen) to eliminate contaminating DNA. The real-time qPCR procedure and primer sequences are described in the Supplemental Experimental Procedures.

RNA-Seq

RNA-seq was performed to compare mRNA levels of NGF-differentiated PC12 controls (DMSO) versus cells treated with WIN (5 μM, 6 hr), and detailed information, including library preparation and data analysis, is found in the Supplemental Experimental Procedures.

mRNA Knockdown

mRNA knockdown experiments using NGF-differentiated PC12 cells were performed using *in vitro* standard antisense locked nucleic acid (LNA) GapmeRs (Exiqon; QIAGEN, Hilden, Germany) that were delivered by gymnosis (Stein et al., 2010). Specifically, GapmeRs (1 μM) were added to the PC12 (NGF-containing) differentiation media from day 1 and were kept until the end of differentiation at day 7, with one exchange of new differentiation media (including a new aliquot of GapmeRs) at day 4. The sequences of the antisense LNA GapmeRs were as follows: GADD34, GAATCCCAATCACCGT; CREB1, AGCTCCTCAATCAATG; and negative control A, AACACGTCTATACGC.

ChIP and Real-Time qPCR

ChIP experiments were performed with the Chromatin Immunoprecipitation Assay Kit (EMD Millipore) followed by real-time qPCR using primers targeting loci of interest. Detailed information on this experiment, including ChIP antibodies and primer sequences, is found in the Supplemental Experimental Procedures.

Animal Treatments, Behavior, and Neurotransmitter Measurements

Male Sprague-Dawley rats (P35 [adolescents] and P70 [adults]; ENVIGO, Italy) were housed (5 per cage) in a climate-controlled animal room (21°C ± 2°C, 60% humidity) under a reversed 12-hr light/dark cycle (lights on at 07:00 a.m.) and fed standard rat chow and water *ad libitum*. Rats were acclimated for 1 week before starting treatment with WIN or vehicle/control (at P42 for adolescent rats and at P77 for adult rats). All procedures and experiments were carried out in an animal facility according to Italian (D.L. 26/2014) and European Council directives (63/2010) and in compliance with the approved animal policies by the Ethical Committee for Animal Experiments at the University of Cagliari (Sardinia, Italy). Detailed information on drug treatments, brain dissections, behavioral tests, and neurotransmitter measurements is found in the Supplemental Experimental Procedures.

Statistical Analyses

Data are presented as mean values and error bars represent SEM. The number of samples used for statistical analyses is denoted in the legend of the corresponding figure for each experiment. Normality of the data was examined using the Shapiro-Wilk test and parametric or non-parametric tests were used accordingly. Two-group comparisons were performed using two-tailed unpaired Student's t test or Mann-Whitney test for parametric and non-parametric analyses, respectively. Multiple t tests, one-way ANOVAs, and two-way ANOVAs were followed by correction for multiple comparisons using the Holm-Sidak test. Correlation analyses were computed using Pearson correlation coefficients with two-tailed p values. In groups with $n > 3$, likely outliers were identified using the Grubbs test ($\alpha = 0.2$), and, if present, they were excluded from the analyses. Statistical significance was set at $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism 7 (GraphPad, La Jolla, CA, USA).

DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE102946.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.02.065.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.A.M., D.B.K., and E.R.K.; Methodology, P.A.M., J.S.Q., C.U., F.S., M.S., and P.F.; Investigation, P.A.M., J.S.Q., M.D., Y.B.W., F.S., and M.S.; Writing – Original Draft, P.A.M.; Writing – Review & Editing, P.A.M., J.S.Q., C.U., Y.B.W., M.D., F.S., W.F., M.S., P.F., D.B.K., and E.R.K.; Funding Acquisition, P.A.M., W.F., P.F., and E.R.K.; Resources, F.S., W.F., M.S., P.F., D.B.K., and E.R.K.; Supervision, W.F., M.S., P.F., D.B.K., and E.R.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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