Adolescent oxycodone exposure inhibits withdrawal-induced expression of genes associated with the dopamine transmission

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Abstract

Prescription opioid misuse is a major public health concern among children and adolescents in the USA [1]. Opioids are the most commonly abused drugs and are the fastest growing drug problem among adolescents [2]. In humans and animals, adolescence is a particularly sensitive period associated with an increased response to drugs of abuse [3–5]. Our previous studies indicate that oxycodone exposure during adolescence increases morphine reward in adulthood [6]. How early drug exposure mediates long-term changes in the brain and behavior is not known, but epigenetic regulation is a likely mechanism. To address this question, we exposed mice to oxycodone or saline during adolescence and examined epigenetic modifications at genes associated with dopamine activity during adulthood at early and late withdrawal, in the ventral tegmental area (VTA). We then compared these to alterations in the VTA of adult-treated mice following an equivalent duration of exposure and withdrawal to determine if the effects of oxycodone are age-dependent. We observed persistence of adolescent-like gene expression following adolescent oxycodone exposure relative to age-matched saline exposed controls, although dopamine related gene expression was transiently activated at 1-day of withdrawal. Following prolonged withdrawal enrichment of the repressive histone mark, H3K27me3, was maintained, consistent with inhibition of gene regulation following adolescent exposure. In contrast, mice exposed to oxycodone as adults showed loss of the repressive mark and increased gene expression following 28-days of withdrawal following oxycodone exposure. Together our findings provide evidence that adolescent oxycodone exposure has long-term epigenetic consequences in VTA of the developing brain.

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Introduction

The impact of prescription opioid abuse continues to be a serious public health problem. Nonmedical use of prescription opioids remains the number one cause of drug overdose [7]. Current estimates indicate that 10.3 million people in the United States abuse prescription opioids, including 700 thousands afflicted individuals between the ages of 12 and 17 [7]. Use of prescription opioids, such as oxycodone, increase the propensity to initiate heroin use in adulthood [7,8]. In humans and mouse, the association between adolescent opioid use and the propensity to initiate adult heroin use has been well documented [6,8–10]. This underscores the importance of efforts to identify molecular mechanisms that contribute to the transition from prescription opioid use to heroin addiction [6,8].

Adolescence is defined by age-specific behavioral characteristics associated with the transition from childhood to adulthood [3]. Neurobiological correlates to human adolescence, such as synaptic pruning and reductions in dopamine receptor expression, are conserved across the mammalian species. Compared to adult, adolescent dopamine neurons in the ventral tegmental area (VTA) fire at higher rates and release larger pools of dopamine in the NAc, which facilitate the development of drug taking in mice [11,12]. Enhanced dopaminergic neuron activity during adolescence results in increased levels of dopamine in the NAc, relative to adult [13]. A similar trend is observed in the expression of dopamine receptors 1 and 2 (D1 and D2) [14]. These data indicate that molecular adaptions associated with adolescence may render the nervous system more sensitive to environmental stimuli, particularly in the mesolimbic dopamine system [3,15]. However, knowledge regarding the molecular mechanisms that regulate functional changes in the VTA during adolescence remains limited and requires further investigation.

Adolescent structural and molecular remodeling contributes to enhanced drug sensitivity and reward [16]. μ-opioid receptor agonists such as oxycodone and morphine, disinhibit dopamine neurons in the VTA, thereby increasing levels of dopamine in the nucleus accumbens (NAc) [17]., Compared to the transient dopamine release caused by morphine, oxycodone has a greater potential to promote addiction-like behaviors given its long-lasting effect on dopamine release [18]. We previously demonstrated that oxycodone exposure during adolescence enhances rewarding properties of opioids later in life [6]. The effects of oxycodone exposure differ between adolescents and adults, including enhanced striatal dopamine release [19] and oxycodone conditioned place preference (CPP) [10], and attenuated self-administration in adolescent mice [19]. In fact, prolonged exposure to oxycodone during adolescence enhances both behavioral responses to opioids in adulthood and alters gene expression [6,9,20]. These data suggest that the effects of adolescent oxycodone exposure on the mesolimbic reward pathway persist well into adulthood. This long-lasting phenotype led us to hypothesize that adolescent oxycodone exposure induces a persistent adolescent-like state in gene expression and behavior [6].

There is increasing evidence that epigenetic mechanisms mediate the influence of drugs on the CNS and are likely involved in the pathology of drug abuse. Epigenetic changes, such as histone modifications, remain largely plastic during development. However, epigenetic regulation during adolescence is understudied and may underlie the behavioral responses to drugs of abuse. Histone H3 lysine 27 trimethylation (H3K27me3) is a well-studied epigenetic modification that is associated with gene repression [21]. We hypothesized that H3K27me3 regulates drug induced gene expression during adolescence and early adulthood, given its critical role in the maintenance of dopaminergic neurons in the VTA [21]. Given that H3K27me3 is regulated following opioid exposure, its abundance may be associated with important gene expression changes related to drug behavior.

In mouse models, adolescence spans approximately from post-natal day (PND) 28–42, followed by a considerable "gray zone" approaching adulthood (PND 60+) [3,22]. Therefore, we examined gene expression and the enrichment of H3K27me3 at several target genes during adulthood (PND56, PND84, and PND112) relative to the naive adolescent brain at PND 28. In addition, we exposed mice to oxycodone (or saline) during adolescence or early adulthood and examined epigenetic modifications at genes associated with the dopamine transmission during adulthood at 1- and 28-days of withdrawal [6,23]. Adolescent mice were exposed to oxycodone from PND28–56 to ensure coverage of the entire period in which neurobehavioral discontinuities from younger and older mice are evident [3,24]. We utilized two controls to distinguish alterations in normal development from those that occur as a result of withdrawal: (1) all oxycodone groups were compared to the naive adolescent brain (PND28) to define developmentally regulated gene expression changes; (2) oxycodone exposed mice were compared to saline exposed, age-matched controls to define effects of withdrawal and age of exposure. We hypothesized that genes associated with the development of dopamine neurons [25] and opiate reward [6,26], such as, nuclear receptor subfamily 4 group A member 2 (*Nr4a2*), and its downstream target genes, cocaine and amphetamine related transcript (*Cartpt*), tyrosine hydroxylase (*Th*) and the dopamine transporter (*DAT*), would be particularly prone to long-lasting alterations following oxycodone exposure. In sum, we identified an epigenetic mechanism specific to adolescent oxycodone exposure that is associated with the inhibition of withdrawal-induced gene expression.

Materials and Methods

Animals

C57Bl/6NTac mice (Taconic, Hudson, New York) were bred in house at the University of Pennsylvania for a minimum of two generations to eliminate the potential effects of shipping. Male offspring, used in experiments, were grouped housed (3–5/cage) with *ad libitum* access to food and water under a 12h light cycle. All procedures were approved by the University of Pennsylvania Animal Care and Use Committee and in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgery

All surgical procedures were conducted using aseptic techniques under 1–3% isoflurane/oxygen vapor. Osmotic minipumps (model 1004; Alzet, Cupertino, CA) were inserted subcutaneously at either PND 28 or 56 during adolescence or early adulthood, respectively. Minipumps were placed parallel to the thoracic vertebrae with the flow moderator directed away from the wound. Minipumps chronically delivered either oxycodone (oxycodone HCl, Spectrum Chemical, New Brunswick, NJ) dissolved in 0.9 % saline at a dosage of 3 mg/kg/day for 28 days, or saline only for saline control mice. Mice were surgerized at different developmental timepoints to measure the effects of oxycodone exposure on adolescence (PND28 – 56) or adulthood (PND 56 – 84). After 28 days of oxycodone or saline exposure, minipumps were surgically removed from all groups.

Tissue collection

To obtain sufficient numbers of mice in all treatment groups, tissues were collected from 4 different cohorts of mice. Every cohort included mice in each of the following treatment groups; adolescent saline exposure, adolescent oxycodone exposure, adult saline exposure and adult oxycodone exposure. Mice were weaned at PND21 at which time individual littermates (average # of litters per cohort: n = 5–6) were randomly assigned to each of the 9 groups; and every group contained mice from each cohort. Tissue was collected at PND28 in naïve mice or after 1- or 28-days following oxycodone/saline exposure. Mice were euthanized by cervical dislocation and brains were rapidly removed and placed in ice-cold phosphate buffered saline with protease inhibitor cocktail (Roche cOmplete Protease inhibitor). One-millimeter thick sections of the ventral tegmental area were prepared using a cold tissue matrix and regions were bilaterally micro-dissected with a 1.2-mm diameter micro-punch (Harris). Tissue was immediately frozen in liquid nitrogen and stored at −80°C for downstream analysis. During tissue processing, all samples were coded and randomized to reduce experimenter bias and batch effects.

Single Sample RNA extraction and Chromatin Immunoprecipitation (S3EQ)

S3EQ was performed on VTA 1.2 mm bilateral punches dissected as described above (1 mouse/sample), 24 hours after minipump removal or withdrawal. Tissue was homogenized in cell lysis buffer (10mM Tris Hcl pH 8.1, 10mM NaCl, 3mM MgCl2, 0.5% Np-40). Low speed centrifugation (1000xg) separated the cytosol (supernatant) from the nuclei (pellet). RNA was isolated from the supernatant using the RNeasy Mini Kit (Qiagen) according to the manufacturer instructions. RNA concentration and integrity were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and the RNA NanoChip on the Bioanalyzer (Agilent), respectively. All RNA samples were of good quality (RIN above 8) and purity (**1.9 +/− 0.1). cDNA** was synthesized from RNA (100ng) using the iScript cDNA Synthesis Kit (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed on the QuantStudio 7 Flex Real-Time PCR System (ThermoFisher). Ct values of the experimental group to control were analyzed using the Ct method [27]. Primer sequences can be found in Supplementary Table 1.

Nuclei were mildly fixed in 1% formaldehyde for 6 minutes and the reaction was terminated with glycine and pelleted by centrifugation (5500xg). Fixed nuclei were resuspended in

nuclear isolation buffer (50mM Tris pH 8.1, 5mM EDTA, 1% SDS) and chromatin was sheared using a diogenode bioruptor XL at high sonication intensity. For histone modification enrichment, chromatin was sheered for 30 min (30 s on/30 s off) and fragment size was verified at 150–300 bps with an Agilent 2100 bioanalyzer. Sheared chromatin was incubated overnight with H3K27me3 (EMD Millipore 07–449) previously bound to magnetic beads (Dynabeads M-280, Life Technologies): The Dynabeads were washed twice each with 1 ml of Low Salt Wash Buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), High Salt Wash Buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), and TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA). After reverse cross-linking and DNA purification (Qiagen Spin Column), primers were designed to amplify regions ~150–200 within gene promoter regions.

Statistics

The appropriate statistical test was determined based on the number of comparisons being conducted. We utilized two controls to distinguish alterations in normal development from those that occur as a result of withdrawal: (experiment 1 & 2) all oxycodone groups were compared to the naive adolescent brain (PND28) to define developmentally regulated gene expression changes; (experiment 3) oxycodone exposed mice were compared to saline exposed, age-matched controls to define effects of withdrawal and age of exposure. For experiment 1 (Fig 2), multiple two-tailed student ttest were used to compared saline exposed mice at developmental timepoints relative to naïve mice at PND28; we then adjusted the p value using the Two-stage Benjamini, Krieger, & Yekutieli FDR procedure to account for the multiple comparisons being made. For experiment 2 (Fig. 3 and 4), multiple two-tailed student ttest were used to compare naïve mice relative to oxycodone exposed mice at 1- and 28-days of withdrawal; we then adjusted the p value using the Two-stage Benjamini, Krieger, & Yekutieli FDR procedure to account for the multiple comparisons being made. The Two-stage Benjamini, Krieger, & Yekutieli FDR procedure has been shown to have greater power to detect true positives, while still controlling the proportion of type I errors at a specified level (5%) [28]. All seven comparisons to naive control mice (PND28) were used to adjust the p value and account for multiple testing in experiments 1 and 2 (Supplementary Fig. 2). For experiment 3 (Fig. 5 and 6), two-way ANOVAs compared saline exposed mice relative to age-matched oxycodone exposed mice at 1- day or 28-days of withdrawal; when appropriate, a Holm-Sidak's post-hoc test followed to determine significant differences across multiple comparisons. Main and interaction effects were considered significant at $P <$ 0.05. P-values greater than 0.05 and below 0.1 were considered trends. Data are expressed as mean [+ or −] s.e.m. The Grubbs' test was used when appropriate to identify outliers. F-tests of variance were conducted on all data sets to ensure that the data followed a normal distribution.

RESULTS

Developmental expression of genes involved in dopamine transmission

To examine molecular changes associated with the dopamine transmission across developmental time points in the VTA (Figure 1), we focused on the following genes: (1) *Nr4a2* encoding the nuclear receptor NURR1 (Nuclear receptor related-1 protein) known to

be important in dopamine neuron development and neuroprotection in the context of opiate exposure [29], (2) *Cartpt*, encoding the "cocaine and amphetamine regulated transcript' which is a neuropeptide activated acutely by morphine in sensitized and naive animals [30], (3) *Th*, encoding tyrosine hydroxylase, the rate limiting enzyme in catecholamine biosynthesis, which is activated by chronic morphine in the VTA and regulates dopamine release during morphine CPP [26,31] and (4) *Dat*, (*Slc6a3*), which encodes the dopamine transporter that negatively modulates morphine reward [32]. Gene expression was determined from the onset of adolescence (PND 28) to adulthood (PND 112). *Nr4a2* and *Cartpt* were both significantly upregulated at PND 84 (Figure 2B, C), while, *Dat* was downregulated at PND 112 (Figure 2D). There were no significant changes in *Th* expression across the developmental time points examined (Figure 2E).

To interrogate the mechanism by which gene expression is altered across postnatal development, we measured the enrichment of the repressive histone modification, H3K27me3, at promoter regions. In agreement with the upregulation of *Nr4a2* and *Cartpt* (Figure 2B, C), H3K27me3 was significantly depleted at these genes at PND 84 (Figure 2F, G). In addition, the H3K27me3 mark was reduced at the *Cartpt* promoter on PND 56 (Figure 2G). The repression of *Dat* was associated with enrichment of H3K27me3 at its promoter on PND 112 (Figure 2D, H). There were no significant changes in H3K27me3 modified histones at the *Th* promoter (Figure 2I) in line with the absence of a corresponding changes in gene expression (Figure 2E). Taken together, these findings indicate that key genes in dopamine transmission are associated with epigenetic mechanisms during postnatal development.

Adolescent oxycodone exposure alters expression of key genes in dopamine transmission

To determine the effects of adolescent oxycodone exposure on gene expression and epigenetic modifications at key genes in the dopamine system, we exposed adolescent mice (PND28–56) to oxycodone and measured gene expression and H3K27me3 enrichment in the VTA after 1- and 28-days of withdrawal (Figure 3A). All comparisons were made relative to the onset of adolescence (PND 28) to detect deviations from normal development [22]. Adolescent oxycodone exposure dramatically activated *Nr4a2* expression at 1- and 28-days of withdrawal (Figure 3B). There were no changes in *Cartpt* expression at either 1- or 28-days relative to the naïve adolescent brain (PND28; Figure 3C). *Dat* was transiently activated at 1-day but not at 28-days of withdrawal (Figure 3D). There were no significant changes in *Th* expression (Figure 3E).

We next examined the abundance of the H3K27me3 mark at the relevant promoters during withdrawal. We found that although *Nr4a2* expression was activated at both 1- and 28-days of withdrawal (Figure 3B), the repressive H3K27me3 was depleted only at 28-days of withdrawal, suggesting that another mechanism must be responsible for its acute regulation at 1-day of withdrawal (Figure 3F). Consistent with the lack of changes in *Cartpt* mRNA levels following adolescent oxycodone exposure (Figure 3C), we found no changes in H3K27me3 enrichment (Figure 3G). The H3K27me3 mark was increased at the *Dat* promoter at 28-days of withdrawal (Figure 3H), when gene expression returned to baseline (Figure 3D). In contrast, loss of the H3K27me3 modification at the Th promoto*r* (Figure 3I)

corresponded with increased gene expression at 1-day of withdrawal (Figure 3E). These data suggest that adolescent oxycodone exposure disrupts normal developmental gene expression, such that *Nr4a2* is prematurely activated (compare to Figure 2B).

Adult oxycodone exposure alters expression of key genes in dopamine transmission differently than adolescent treatment

To determine if oxycodone-induced changes in gene expression and histone modifications were contingent on exposure during adolescence, we treated adult mice (PND56–84) with oxycodone and measured mRNA and H3K27me3 following withdrawal in the VTA (Figure 4A). All comparisons were made relative to the onset of adolescence (PND 28) to detect deviations from normal development. Adult oxycodone exposure activated *Nr4a2* expression at 1- and 28-days of withdrawal (Figure 4A, B). In contrast to the findings in adolescent exposure, we found *Cartpt* expression was activated specifically at 1- and 28-days of withdrawal (Figure 4C). *Dat* and *Th* expression did not differ at either withdrawal time point relative to the naïve adolescent brain (PND28; Figure 4D, E).

Next, we measured H3K27me3 enrichment at promotor regions following adult oxycodone exposure. H3K27me3 was depleted at *Nr4a2* at 28-days and *Cartpt* at 1- and 28-days of withdrawal (Figure 4F, G), which corresponded with increased gene expression at these time points (Figure 3B, C). We found no significant change in H3K27me3 enrichment at the *Dat* promoter when compared to the naïve brain (PND28, Figure 4H). At Th we found a reduction of H3K27me3 at 28-days of withdrawal (Figure 4I). In conclusion, adult oxycodone exposure prolonged the increased gene expression of *Nr4a2* and *Cartpt*, relative to normal development (Figure 2B, C), which is associated with the abundance of the repressive H3K27me3 mark at gene promoters.

Adolescent oxycodone exposure causes gene expression changes at 1-day of withdrawal.

To determine the age-dependent effects of drug exposure in the VTA, we compared adolescent and adult oxycodone exposed mice relative to age-matched, saline treated controls (Figure 5A). Analysis of gene expression found an age x drug effect following 28-days of oxycodone exposure followed by 1-day of withdrawal (Figure 5B, D, E). Specifically, only adolescent oxycodone exposure activated *Nr4a2*, *Dat* and *Th* expression when compared to saline age-matched controls (PND56). There was no change in the expression of these genes following adult oxycodone exposure relative to saline age-matched controls (PND84). *Cartpt* mRNA levels were unchanged following either exposure paradigm (Figure 5C). Unexpectedly, we did not find any changes in the abundance of H3K27me3 at the promotor regions of *Nr4a2*, *Cartpt*, *Dat*, or *Th* by any of the treatments (Figure 5F–I), suggesting that alternative mechanisms of regulation are responsible for the observed gene expression patterns in adult mice exposed to oxycodone during adolescence.

Adult oxycodone exposure causes gene expression changes after 28-days of withdrawal.

To interrogate long-term effects of adolescent and adult oxycodone exposure we examined the abundance of H3K27me3 and gene expression in the VTA at 28-days of withdrawal following four weeks of opioid treatment (Figure 6A). We found an age x drug effect following oxycodone exposure at 28-days of withdrawal (Figure 6B–E). Specifically, adult

oxycodone exposure activated *Nr4a2*, *Cartpt*, *Dat*, and *Th* expression when compared to saline age-matched controls (Figure 6B–E). Remarkably, at the same time point no changes in steady-state mRNA levels were observed if oxycodone was given to adolescent mice (Figure 6B–E). We then measured corresponding changes in H3K27me3 abundance and found adult exposure reduced and adolescent exposure increased the repressive mark H3K27me3 at selected genes after 28-days of withdrawal (Figure 6F–I). Specifically, adult oxycodone exposure depleted H3K27me3 modified histones at *Nr4a2*, *Cartpt*, *Dat*, and *Th* promoters which corresponded to changes in gene expression (see Figure 6B–I). In contrast, adolescent oxycodone increased the H3K27me3 mark at the *Cartpt* and *Dat* promoters when compared saline age-matched controls (PND84, Figure 6G, H). These data suggest adult oxycodone exposure induces long-term changes in H3K27me3, whereas, adolescent oxycodone exposure abrogates dopamine related gene expression via the maintenance of H3K27me3.

Discussion

Recent studies find that prescription opioid abuse in adolescence causes heroin use in adulthood [8]. To investigate the gene regulatory mechanisms underlying this transition, we exposed either adolescent or adult mice to oxycodone and examined epigenetic modifications at genes associated with dopamine transmission following 1- or 28- days of withdrawal. Using this approach, we identified adolescence-specific gene regulation that may render this developmental phase particularly sensitive to oxycodone exposure. The subtle distinction between naïve mice at PND28 controls and age-matched controls in our study allows one to concluded that oxycodone does not alter normal development of dopamine related gene expression, per se, but rather inhibits abstinence related expression via oxycodone induced epigenetic modifications following adolescent exposure. Specifically, increased gene expression of *Nr4a2* and its downstream target gene, *Cartpt*, was associated with reduced H3K27me3 at PND84. At PND112 the expression of *Nr4a2* and *Cartpt* returned to levels observed at PND28, and *Dat* expression declined below baseline [33]. We examined gene expression during oxycodone exposure relative to PND28 to examine abnormalities in normal development. Both adolescent and adult oxycodone exposure activated *Nr4a2* expression during 1- or 28- days of withdrawal, relative to PND28. The expression pattern of *Cartpt* relative to PND28 was specific to adult exposure.

By comparing oxycodone exposed mice to age-matched, saline exposed mice, we were able to define changes related to both withdrawal and age at initial drug exposure. We discovered epigenetic changes specific to adult oxycodone exposure, such as depleted H3K27me3 and increased expression of *Cartpt*. Adolescent oxycodone exposure led to increased *Th* and *Dat* expression at 1-day of withdrawal relative to age-matched controls, while adult oxycodone exposure resulted in activation of these genes at 28-days of withdrawal relative to age-matched controls. We defined age and length of withdrawal-dependent gene regulation, in which dopamine related gene expression is transient following adolescent oxycodone exposure and delayed following adult oxycodone exposure. We further identified developmental differences in a putative underlying epigenetic mechanism. Adult, but not adolescent, oxycodone exposure led to loss of H3K27me3 at dopamine related genes at 28-days of withdrawal. We hypothesize that the failure of adolescent oxycodone exposure to

induce prolonged dopamine related gene expression may underlie developmental differences in the risk of transition to heroin addiction following oxycodone abuse [6]. Specifically, *Nr4a2* and its downstream target, *Cartpt*, function to repress drug sensitivity via homeostatic control of dopaminergic signaling [26,34,35], and we find that *Cartpt* activation is lost specifically during adolescent oxycodone exposure and withdrawal.

Adaptive changes in cellular and synaptic function after prolonged opiate exposure and withdrawal is well documented [6,36]. For example, consistent with our studies of the VTA, others have observed increased expression of *Nr4a2*, *Cartpt*, *Th* and *Dat* during withdrawal [30,37]. These changes showed a high degree of co-localization with TH+ neurons in the VTA suggesting that *Nr4a2* may be associated with withdrawal induced alterations of dopamine neuron activity [38]. Human studies provide neurological evidence that abnormal expression of *Nr4a2* in heroin abusers may be exacerbated by age [39]. In addition, gene expression changes following adult opioid exposure in mice is correlated with epigenetic changes, including loss of histone H3 lysine 9 acylation (H3K9ac), histone H3 lysine 9 methylation (H3K9me3) and H3K27me3 [36,40,41]. In the VTA, morphine exposure increases histone deacetylase 2 activity in dopamine neurons and reduces levels of H3K9ac genome-wide [40]. These changes are associated with greater disinhibition of dopaminergic neurons which can be restored via increased histone acetylation [40]. Systemic administration of HDAC inhibitors (HDACi) facilitates extinction of morphine CPP, blocks priming-induced reinstatement and attenuates sensitization [42,43]. Opioid-induced withdrawal is also associated with Ezh2 (enhancer of zester homolog 2) expression, which catalyzes H3K27me3 [44]. Virally-mediated overexpression of Ezh2 in the VTA represses genes for dopaminergic neuron survival and enhances morphine CPP [21]. We propose that prolonged exposure to oxycodone during adolescence increases the sensitivity to morphine later in adulthood via the suppression of adaptive gene expression. Conversely, the net effect of adult oxycodone exposure is reward tolerance measured by decreased dopamine output in response to opiates [26,45].

The relative immaturity of the dopamine system during adolescence leads to enhanced behavioral responses to drug exposure [23,46–48]. Innervation of dopamine neurons and striatal dopamine levels gradually increase across adolescence, and then decline throughout adulthood [49,50]. Our current study provides further evidence that adolescence is a specific developmental period with respect to epigenetic modifications at genes associated with dopamine transmission [3,22]. We defined simultaneous effects of age and drug exposure by examining gene expression following adult and adolescent oxycodone exposure across development, relative to both naïve and age-matched controls. Our work is consistent with reports of declining levels of *Nr4a2* [51] and *Dat* [33] during normal aging in the VTA. To our knowledge, we are the first to report age-dependent changes in *Cartpt* expression. Importantly, we found that at 1-day of withdrawal following adolescent exposure, expression of *Nr4a2* and *Dat* were greater than that in adult at PND56, compared to PND28, although we cannot completely exclude effects of intracranial surgery on dopamine related gene expression. During adolescence, mice have larger pools of releasable dopamine and self-administer less oxycodone than adults, which may be associated with an enhanced response to oxycodone [11,19]. This conclusion is further supported by enhanced opioid (oxycodone and morphine) CPP following adolescent self-administration and subcutaneous oxycodone [6,9]. Interestingly, adolescent increase in oxycodone CPP relative to adult, has only been

observed at lower doses, suggesting a leftward shift in the dose response curve following adolescence exposure [9]. We suppose that adolescent oxycodone exposure results in persistence of increased dopamine release and opioid sensitivity that is maintained beyond adolescence [11]. In regard to adult oxycodone exposure, the increased expression of *Cartpt* and *Dat* may reflect a greater capacity for dopamine uptake [6,35,52]. Taken together, we identified a putative epigenetic mechanism by which withdrawal following adolescent oxycodone exposure leads to persistent adolescent dopamine related gene expression into adulthood.

Similar to the enhanced drug-induced behavioral responses observed during adolescence, epigenetic changes in the brain following drug exposure are more salient in adolescents than adults [6]. For example, adolescent ethanol self-administration attenuates dopamine D2 receptor (D2) expression and histone H3 acetylation at 1-day of withdrawal in the NAc [53]. These changes do not occur after adult exposure until 28-days of withdrawal [46,47]. Furthermore, inhibition of histone deacetylation attenuates adolescent specific increases in ethanol self-administration [54]. These data suggest that alcohol-induced gene expression is developmentally regulated. Our study was the first to explore gene expression in the VTA across development and withdrawal from oxycodone. We found that H3K27me3 depletion is only associated with increased gene expression at 28-days of withdrawal or adulthood (PND112). This suggests that H3K27me3 plays a key role in delayed gene activation following drug exposure. We focused specifically on how these changes were associated with long term neuroadaptations in response to drug reward. Increased expression of *Cartpt* following adult oxycodone exposure may be related to its role in homeostatic control of dopamine signaling in adulthood. We found a reduction in H3K27me3, which was associated with increased *Cartpt* expression at PND84, but both H3K27me3 abundance and *Cartpt* expression return to baseline at PND112 (Figure 2C). *Cartpt* was activated in the oxycodone exposed adult brain at 1- and 28-days of withdrawal (PND 84 and 112), when compared to both the naïve brain (PND28; Figure 4C) and age-matched controls (PND112; Figure 6C). However, *Cartpt* was not activated at 1-day or 28-days of withdrawal following adolescent exposure (Figure 5C). Rather, *Cartpt,* expression was suppressed, and H3K27me3 enrichment was maintained when compared to both the naïve brain (PND28; Figure 3C) and age-matched controls (PND56; Figure 6C, G). This finding is significant given that *Cartpt* functions in vivo to reduce levels of dopamine in response to morphine [34,35].

Opioid exposure during adolescence alters normal development of the dopamine reward pathway, and leads to long-term changes in behavior and gene expression [6,10,19]. In the context of development and drug exposure, we discovered specific stages of gene expression which are defined by long-lasting changes in the abundance of the repressive chromatin modification, H3K27me3, at key dopamine related genes. This work lays the groundwork to examine the functional relevance of the H3K27me3 using novel strategies of locus specific epigenetic editing with virally expressed CRISPR/Cas9 fusion enzymes. Future work will interrogate additional epigenetic mechanisms of gene activation that underlie the development of heroin addiction following nonmedical use of prescription opioids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1. Study Design.

Nine tissue sets of VTA were collected at the indicated times and processed within-subject for qChIP and qPCR. To examine adolescent gene expression, we compared dopamine related gene expression at PND 56, 84, and 112 (see Figure 2). To distinguish between epigenetic effects of oxycodone treatment and normal development of gene expression, we normalized gene expression to the naïve adolescent mice following adolescent and adult oxycodone exposure at 1 and 28 days of withdrawal (WD) (see Figure 3 and 4). To determine epigenetic effects of oxycodone exposure at 1 and 28 days of WD, we normalized oxycodone exposed mice to age-matched saline exposed mice and compared adult and adolescent drug exposure (see Figure 5 and 6).

Figure 2. Epigenetic modifications at dopamine related genes across developmental time points in the VTA.

A.) Timeline of developmental time points. **B.)** *Nr4a2* is activated at PND 84, (Students two-tailed ttest; PND 84, p=0.029, adjusted p = 0.012). **C.)** *Cartpt* is activated at PND 84 (Students two-tailed ttest; PND 84, p=0.013, adjusted p = 0.019). **D.)** *Dat* is repressed at PND 112 (Students two-tailed ttest; PND 112, p=0.015, adjusted p = 0.047). **E.)** No significant changes in *Th*. **F.)** H3K27me3 is depleted at the Nr4a2 promotor at PND 84 (Students two-tailed ttest; PND 84, p=0.012, adjusted p = 0.017). **G.)** H3K27me3 is depleted at the *Cartpt* promotor at PND 56 and 84 (Students two-tailed ttest; PND 56, p=0.001, adjusted p = 0.005; PND 84, p=0.002, adjusted p = 0.005). **H.)** H3K27me3 is enriched at the *Dat* promotor at PND 112 (Students two-tailed ttest; PND 112, p=0.006, adjusted p = 0.040). **I.)** H3K27me3 is depleted at the Th promotor at PND 56 (Students two-tailed ttest; p=0.029, adjusted p = 0.012). All data normalized to GAPDH relative to PND28. n = 5 – 12 mice per group. Error bars represent S.E.M. *p<.05 relative to PND28.

Figure 3. Adolescent oxycodone exposure alters dopamine related gene expression in the VTA
A.) Timeline of adolescent oxycodone exposure and subsequent periods of oxycodone withdrawal (WD). **B.)** Adolescent oxycodone activates *Nr4a2* at 1- and 28-days (Students two-tailed ttest; 1-day, p=0.01, adjusted p = 0.007, 28-days,, p=0.001, adjusted p = 0.001). **C.)** No significant changes in *Cartpt.* **D.)** Adolescent oxycodone activates *Dat* at 1-day (Students two-tailed ttest; 28-days, p=0.004, adjusted p = 0.025). **E.)** No significant changes in *Th* expression. **F.)** H3K27me3 is depleted at the Nr4a2 promoter at 28-days (Students two-tailed ttest; 28-days,, p=0.003, adjusted p = 0.007). **G.)** No significant changes in H3K27me3 enrichment at the *Cartpt* promoter (Students two-tailed ttest; p=0.029, adjusted p = 0.012). **H.)** H3K27me3 is enriched at the *Dat* promoter at 28-days (Students two-tailed ttest; 28-days, p=0.015, adjusted p = 0.049). **I.)** H3K27me3 is depleted from the Th promoter at 1-day (Students two-tailed ttest; 1-day, p=0.012, adjusted p = 0.03). All data normalized to GAPDH relative to drug naïve adolescent brain (PND28). n = 5 – 7 mice per group Error bars represent S.E.M. *p<.05 relative to drug naïve adolescent brain (PND28).

Figure 4. Adult oxycodone exposure alters dopamine related gene expression in the VTA
A.) Timeline of adult oxycodone exposure and subsequent periods of oxycodone withdrawal (WD). **B.)** Adult oxycodone activates *Nr4a2* at 1- and 28-days (Students two-tailed ttest; 1-day, p=0.014, adjusted p = 0.007, 28-days,, p=0.001, adjusted p = 0.001). **C.)** Adult oxycodone activates *Cartpt* at 28-days (Students two-tailed ttest; 1-day, p=0.014, adjusted p = 0.019, 28-days,, p=0.003, adjusted p = 0.001). **D.)** No significant changes in *Dat* expression. **E.)** No significant changes in *Th* expression. **F.)** H3K27me3 is depleted at the Nr4a2 promoter at 28-days (Students two-tailed ttest; 28-days, p=0.001, adjusted p = 0.005). **G.)** H3K27me3 is depleted at the *Cartpt* promoter at 1-day and 28-days (Students two-tailed ttest; 1-day p=0.036, adjusted p = 0.038, 28-days, p=0.003, adjusted p = 0.005). **H.)** No significant changes in H3K27me3 enrichment at the *Dat* promoter (Students two-tailed ttest; p=0.029, adjusted p = 0.012). **I.)** H3K27me3 is depleted at the Th promoter at 28-days (Students two-tailed ttest; 28-days, p=0.008, adjusted p = 0.03). All data normalized to GAPDH relative to naïve adolescent brain (PND28). n = 5 – 7 mice per group. Error bars represent S.E.M. *p<.05 relative to naïve adolescent brain (PND28).

Figure 5. Adolescent oxycodone exposure regulates gene expression at 1-day of withdrawal in the VTA

A.) Timeline of oxycodone withdrawal (WD) and tissue collection. **B.)** Adolescent oxycodone exposure activates *Nr4a2* (Two-way ANOVA, n=6 per group, Interaction: $F_{(1,20)} = 22.98$, p=0.0001; Drug: $F_{1,20} = 18.21$, p=0.0004; Age of Exposure $F_{(1,20)} = 18.21$, p=0.0004; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p<0.0001; Adult Saline vs. Oxycodone, p=0.999). **C.)** No significant difference in *Cartpt* expression following adolescent or adult oxycodone exposure (Two-way ANOVA, n=6 per group Interaction: $F_{1,20} = 0.371$, p=0.549; Drug: $F_{1,20} = 0.371$, p=0.549; Age of Exposure $F_{1,20} = 0.011$, p=0.917). **D.)** *Dat* is activated following adolescent oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,19} = 8.010$, p=0.017; Drug: : $F_{1,19} = 4.175$, p=0.052; Age of Exposure : $F_{1,19} = 8.010$, p=0.0176; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.004; Adult Saline vs. Oxycodone, p=0.834). **E.)** *Th* is activated following adolescent oxycodone exposure when compared to saline. (Two-way ANOVA, Interaction: : $F_{1,19} = 7.499$, p=0.0131; Drug: : $F_{1,19} = 7.097$, p=0.015; Age of Exposure : $F_{1,19} = 7.498$, p=0.013; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.002; Adult Saline vs. Oxycodone, p=0.834). **F.)** No significant difference in H3K27me3 enrichment at the Nr4a2 promoter following adolescent or adult oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,20} = 0.396$, p=0.536; Drug: $F_{1,20} = 0.616$, p=0.447; Age of Exposure $F_{1,20} = 0.616$, p=0.447). **G.)** No significant difference in H3K27me3 enrichment at the *Cartpt* promoter following adolescent or adult oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,20} = 0.111$, p=0.741; Drug: $F_{1,20} = 0.111$, p=0.741; Age of Exposure $F_{1,20} = 0.410$, p=0.523). **H.)** No significant difference in H3K27me3 enrichment at the *Dat* promoter following adolescent or adult oxycodone

exposure (Two-way ANOVA, Interaction: $F_{1,17} = 0.067$, p=0.7978; Drug: $F_{1,17} = 0.028$, p=0.884; Age of Exposure $F_{1,17} = 0.410$, p=0.7971). **I.)** No significant difference in H3K27me3 enrichment at the Th promoter following adolescent or adult oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,19} = 1.340$, p=0.7184; Drug: $F_{1,19} = 0.444$, p=0.5130; Age of Exposure: $F_{1,19} = 0.1340$, p=0.7184). All data normalized to GAPDH relative to saline age-matched controls. n = 5–6 mice per group. Error bars represent S.E.M. *p<.05 relative to saline.

Figure 6. Adult oxycodone exposure regulates gene expression at 28-days of withdrawal in the VTA

A.) Timeline of oxycodone withdrawal (WD) and tissue collection. **B.)** *Nr4a2* is activated following adult oxycodone exposure when compared to saline. (Two-way ANOVA, Interaction: $F_{1,17} = 5.468$, p=0.0318; Drug: $F_{1,17} = 12.94$, p=0.002; Age of Exposure $F_{1,17} = 5.470$, p=0.0318; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.7949; Adult Saline vs. Oxycodone, p=0.004). **C.)** *Cartpt* is activated following adult oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,18} = 5.221$, p=0.035; Drug: $F_{1,18} = 7.085$, p=0.016; Age of Exposure $F_{1,18} = 5.221$, p=0.035; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.994; Adult Saline vs. Oxycodone, p=0.009). **D.)** *Dat* is activated following adult oxycodone exposure (Two-way ANOVA, Interaction: $F_{1,18} = 8.290$, p=0.010; Drug: $F_{1,18} = 6.310$, p=0.021; Age of Exposure $F_{1,18} = 8.332$, p=0.009; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.994; Adult Saline vs. Oxycodone, p=0.007). **E.)** Adult oxycodone exposure activates *Th* when compared to saline (Two-way ANOVA, Interaction: $F_{1,17} = 5.938$, p=0.0261; Drug: $F_{1,17} = 5.186$, p=0.036; Age of Exposure $F_{1,17} = 5.258$, p=0.049; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.9918; Adult Saline vs. Oxycodone, p=0.0272). **F.)** Adult oxycodone exposure depletes H3K27me3 at the *Nr4a2* promoter (Two-way ANOVA, Interaction: $F_{1,18} = 5.286$, p=0.0337; Drug: $F_{1,18} = 2.292$, p=0.147; Age of Exposure $F_{1,18} = 5.286$, p=0.0337; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.809; Adult Saline vs. Oxycodone, p=0.039). **G.)** Adolescent oxycodone exposure enriched H3K27me3 and adult oxycodone exposure depleted H3K27me3 (Two-way ANOVA, Interaction: $F_{1,18} = 13.57$, p=0.001; Drug: $F_{1,18} = 0.3661$, p=0.5527; Age of Exposure $F_{1,18}$

= 13.57, p=0.001; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.0143; Adult Saline vs. Oxycodone, p=0.0430). H.) Adolescent oxycodone exposure enriched H3K27me3 when compared to saline (Two-way ANOVA, Interaction: $F_{1,17} = 13.91$, p=0.001; Drug: $F_{1,17} = 1.839$, p=0.193; Age of Exposure $F_{1,17} = 13.91$, p=0.001; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.003; Adult Saline vs. Oxycodone, p=0.12). I.) Adult oxycodone exposure depletes H3K27me3 at the Th promoter (Two-way ANOVA, Interaction: $F_{1,17} = 4.901$, p=0.0261; Drug: $F_{1,17} = 3.074$, p=0.097; Age of Exposure $F_{1,17} = 4.906$, p=0.040; followed by Sidak's multiple comparisons test, Adolescent Saline vs. Oxycodone, p=0.932; Adult Saline vs. Oxycodone, p=0.003). All data normalized to GAPDH relative to saline age-matched controls. n = 5 – 6 mice per group. Error bars represent S.E.M. *p<.05 relative to saline.