Epigenetic basis of opiate suppression of \textit{Bdnf} gene expression in the ventral tegmental area

Ja Wook Koo$^1$, Michelle S Mazei-Robinson$^{1,2}$, Quincey LaPlant$^1$, Gabor Egervari$^{1,3,4}$, Kevin M Braunscheidel$^5$, Danielle N Adank$^5$, Deveroux Ferguson$^1$, Jian Feng$^1$, Haosheng Sun$^1$, Kimberly N Scobie$^1$, Diane M Davez-Werno$^1$, Efrain Ribeiro$^1$, Catherine Jensen Peña$^1$, Deena Walker$^1$, Rosemary C Bagot$^1$, Michael E Cahill$^1$, Sarah Ann R Anderson$^{1,3,4}$, Benoit Labonté$^1$, Georgia E Hodes$^1$, Heidi Browne$^1$, Benjamin Chadwick$^{1,3,4}$, Alfred J Robison$^{1,2}$, Vincent F Vialou$^{1,6}$, Caroline Dias$^1$, Zachary Lorsch$^1$, Ezekiell Mouzon$^1$, Mary Kay Lobo$^7$, David M Dietz$^6$, Scott J Russo$^1$, Rachael L Neve$^8$, Yasmin L Hurd$^{1,3,4}$ & Eric J Nestler$^1$

Brain-derived neurotrophic factor (BDNF) has a crucial role in modulating neural and behavioral plasticity to drugs of abuse. We found a persistent downregulation of exon-specific \textit{Bdnf} expression in the ventral tegmental area (VTA) in response to chronic opiate exposure, which was mediated by specific epigenetic modifications at the corresponding \textit{Bdnf} gene promoters. Exposure to chronic morphine increased stalling of RNA polymerase II at these \textit{Bdnf} promoters in VTA and altered permissive and repressive histone modifications and occupancy of their regulatory proteins at the specific promoters. Furthermore, we found that morphine suppressed binding of phospho-CREB (cAMP response element binding protein) to \textit{Bdnf} promoters in VTA, which resulted from enrichment of trimethylated H3K27 at the promoters, and that decreased NURR1 (nuclear receptor related-1) expression also contributed to \textit{Bdnf} repression and associated behavioral plasticity to morphine. Our findings suggest previously unknown epigenetic mechanisms of morphine-induced molecular and behavioral neuroadaptations.

BDNF promotes the neural and behavioral plasticity induced by cocaine or other stimulant drugs of abuse via actions on the mesolimbic dopaminergic system, which is composed of dopamine neurons in the ventral tegmental area (VTA) of the midbrain and their anterior projections to the nucleus accumbens (NAc) and other forebrain regions$^{1-3}$. Previous studies have shown that BDNF-TrkB activity and its downstream signaling cascades are induced in NAc by cocaine exposure$^{4-6}$. In addition, manipulations that enhance BDNF signaling in the VTA-NAc circuit increase rewarding and locomotor responses to cocaine, whereas suppressing BDNF signaling has the opposite effect$^{5,7-10}$.

In marked contrast, we recently found that chronic morphine suppresses \textit{Bdnf} gene expression in mouse VTA and that such a blockade enhances rewarding and locomotor responses to morphine by augmenting dopamine neuron activity$^{11}$. Chronic opiates also induce some unique biochemical and morphological alterations in VTA, such as downregulation of intracellular neurotrophin signaling cascades and reduced soma size of VTA dopamine neurons, which are not seen as downregulation of intracellular neurotrophin signaling cascades and reduced soma size of VTA dopamine neurons, which are not seen as downregulation of intracellular neurotrophin signaling cascades and reduced soma size of VTA dopamine neurons, which are not seen

Despite this evidence for an inverse relationship between BDNF activity in VTA and morphine action, the transcriptional mechanisms underlying \textit{Bdnf} suppression by morphine are largely unknown.

We carried out a comprehensive analysis of epigenetic regulation at the \textit{Bdnf} gene and observed a series of interacting chromatin mechanisms that mediate morphine's downregulation of \textit{Bdnf} transcription in rat VTA. We found that unique binding patterns of RNA polymerase II (Pol II), permissive and repressive histone modifications, their histone-modifying enzymes and related regulatory proteins, and key transcription factors at specific \textit{Bdnf} promoters were associated with morphine-induced \textit{Bdnf} suppression in this brain region and with enhanced behavioral responses to opiates.

RESULTS

Downregulation of \textit{Bdnf} expression in VTA by opiates

We first examined postmortem VTA sections of human brain and observed that heroin addicts, compared with matched controls (Supplementary Table 1), displayed reduced mRNA levels of \textit{Bdnf} exon IX, which represents the protein-coding region of \textit{Bdnf} mRNA that is common to all \textit{Bdnf} transcripts$^{16}$ (Fig. 1a and Supplementary Fig. 1a). \textit{Bdnf} exon IX mRNA levels were also decreased in VTA of rats that chronically self-administered heroin (Fig. 1b and Supplementary Fig. 1b).

To further characterize VTA \textit{Bdnf} gene regulation in opiate action, we used an extensively validated morphine treatment regimen, involving repeated intraperitoneal injections, that is more amenable

---

Received 15 October 2014; accepted 22 December 2014; published online 2 February 2015; doi:10.1038/nn.3932
to higher throughput analyses. Rats received daily morphine injections (5 mg per kg of body weight) for 14 d and were examined 14 d later. Having confirmed the expected sensitizing behavioral effects of chronic morphine in these rats (Supplementary Fig. 1c), we found that Bdnf exon IX expression was suppressed in VTA of chronic morphine-treated rats compared with saline controls (one-way ANOVA, \( F_{1,2} = 5.872, P = 0.0081 \) with Fisher's post hoc test, \( P = 0.0303; \) Fig. 1c). In contrast, acute morphine (5 mg per kg, intraperitoneal) after chronic (14 d) saline treatment had no effect on Bdnf exon IX expression in rat VTA (Fisher's post hoc test, \( P > 0.05 \)). However, subchronic morphine exposure (15 mg per kg, intraperitoneal), during 3 d of training for conditioned place preference (CPP), was sufficient to decrease Bdnf exon IX expression in mouse VTA (Fig. 1d and Supplementary Fig. 1d). These findings indicate that repeated opiate exposure is required for Bdnf mRNA suppression in VTA across species, including human addicts, and supports the relevance of this adaptation to opiate action.

**Stalling of Pol II at Bdnf promoters in VTA by morphine**

Having established opiate suppression of Bdnf exon IX expression in VTA, we investigated the underlying mechanisms involved. Initial quantitative PCR (qPCR) analysis revealed that Bdnf transcripts containing exons III, V, VII or VIII were expressed at very low levels in rat VTA and were not altered after chronic morphine (Supplementary Fig. 1e). Thus, we focused on Bdnf transcripts containing exons I, II, IV or VI in VTA and observed that chronic morphine also reduced Bdnf transcript levels of exons II, IV and VI compared with saline controls (Fig. 2a,b). There was no difference in Bdnf exon I mRNA expression. These data indicate that the chronic morphine–induced suppression of Bdnf exon IX is attributable to reductions in exon II–

To understand the effect of chronic morphine on Bdnf gene transcription at the epigenetic level, we performed quantitative chromatin immunoprecipitation (qChIP) with an antibody that recognizes both non-phosphorylated and phosphorylated forms of Pol II (total Pol II) and with antibodies that specifically recognize Pol II phosphorylated at serine 2 (as in Fig. 2). We focused on Pol II phosphorylated at serine 5 and with antibodies that specifically recognize Pol II phosphorylated at serine 2.

**Figure 1** Opiate-induced downregulation of Bdnf expression in human, rat and mouse VTA. (a) qPCR revealed that mRNA levels of Bdnf exon IX were reduced in VTA of human heroin addicts compared with control subjects (unpaired Student's t test, \( t_{1,2} = 2.623, P = 0.0223, n = 5, 9 \) human samples). (b,c) mRNA levels of Bdnf exon IX were decreased in VTA of heroin self-administering rats (b, t test, \( t_{1,2} = 2.793, P = 0.0106, n = 10, 14 \) rats), and in VTA of rats given 14 daily morphine injections (5 mg per kg, intraperitoneal) and examined after 14 d of withdrawal (c, t test, \( t_{1,2} = 2.923, P = 0.00995, n = 9 \) rats), compared with respective control groups. (d) Morphine CPP (15 mg per kg, intraperitoneal) also decreased mRNA levels of Bdnf exon IX in mouse VTA compared with saline-treated mice (t test, \( t_{1,2} = 2.195, P = 0.0423, n = 12 \) mice). Unpaired t tests, * \( P < 0.05 \) and *** \( P < 0.001 \). Box plots present, in ascending order, minimum sample value, first quartile, median, third quartile and maximum sample value. Primer information is provided in Supplementary Table 2.

**Figure 2** Effect of chronic morphine on expression of Bdnf exons and on binding of Pol II to the Bdnf gene in rat VTA. (a) qPCR revealed that mRNA levels of Bdnf exons II, IV and VI were decreased in rat VTA after chronic (14 d) morphine administration followed by 14 d of withdrawal (as in Fig. 1c) relative to saline controls (two-way ANOVA, drug effect: \( F_{1,2} = 12.898, P < 0.001 \); region effect: \( F_{3,2} = 9.293, P = 0.0435 \); drug \( \times \) region effect: \( F_{3,2} = 0.328, P = 0.805 \), \( n = 9 \) rats). (b) Schematic diagram depicting the relative position of amplicons (thick black lines) generated by primers used to quantify immunoprecipitated chromatin DNA. Exons are represented as boxes and the introns as lines. Numbers of the exons are indicated in roman numerals. The positions of CREB binding sites (gray circles) at Bdnf promoter regions are indicated relative to the transcription start site of exon I (96–84 bp/90–78 bp), exon II (317–307 bp), exon IV (42–33 bp/36–26 bp), and exon VI (84–73 bp). Primer information is provided in Supplementary Table 3. (c) qChIP showed that binding of total Pol II to Bdnf-p2 was increased in response to chronic morphine (drug effect: \( F_{1,2} = 13.279, P < 0.001 \); region effect: \( F_{3,2} = 1.019, P = 0.395 \); drug \( \times \) region effect: \( F_{3,2} = 1.906, P = 0.362 \), \( n = 6 \) rats). (d) Binding of phospho-Ser5-Pol II to Bdnf-p2, Bdnf-p4 and Bdnf-p6 was also increased in VTA of morphine-treated rats (drug effect: \( F_{1,3} = 18.820, P < 0.001 \); region effect: \( F_{3,3} = 6.474, P = 0.002 \); drug \( \times \) region effect: \( F_{3,3} = 2.069, P = 0.069 \), \( n = 5 \) rats). (e) In contrast, binding of phospho-Ser2-Pol II to Bdnf-eII, Bdnf-eIV and Bdnf-eVI was decreased after morphine exposure (drug effect: \( F_{1,2} = 19.921, P < 0.001 \); region effect: \( F_{3,2} = 0.00309, P = 1.000 \); drug \( \times \) region effect: \( F_{3,2} = 0.165, P = 0.919 \), \( n = 5 \) rats). Two-way ANOVA with Fisher’s protected least significant difference (PLSD) post hoc tests, * \( P < 0.05 \), ** \( P < 0.01 \) and *** \( P < 0.001 \). Bar graphs show mean ± s.e.m.
either Ser2 or Ser5 at its C-terminal domain. Ser5 hyper-phosphorylation at gene promoters and Ser2 hypo-phosphorylation in coding regions are thought to be hallmarks of stalled Pol II and are associated with suppressed genes18. Total Pol II binding to Bdnf promoter region 2 (Bdnf-p2), which corresponds to exon II, was higher in VTA of chronic morphine–treated rats relative to saline controls, with no changes seen at the other promoters (Fig. 2c). Phospho-Ser5-Pol II binding to Bdnf-p2, Bdnf-p4 and Bdnf-p6 in morphine-treated rats was increased (Fig. 2d). In contrast, phospho-Ser2-Pol II binding in coding regions was decreased at Bdnf-exon II (eII), Bdnf-eIV and Bdnf-eVI after chronic morphine (Fig. 2e). These findings suggest sustained suppression of Bdnf-p2, Bdnf-p4 and Bdnf-p6 via Pol II stalling in VTA in response to chronic morphine.

**Histone modifications at Bdnf promoters in VTA by morphine**

Next, we conducted qChIP for numerous histone modifications at the four Bdnf promoters: three histone markers of gene activation, acetylated H3 (acH3), acH4 and trimethylation of Lys4 of H3 (H3K4me3); three markers of gene repression, H3K9me2, H3K9me3 and H3K27me3; and one marker of transcription elongation, H3K36me3. Chronic morphine–exposed animals displayed clear patterns of histone regulation at Bdnf promoters in VTA (Supplementary Fig. 2a–g), with the most pronounced regulation occurring, as with Pol II, at Bdnf-p2. The changes observed in morphine-treated rats represent modifications induced by chronic morphine, which persisted despite 2 weeks of abstinence: acH3 was decreased, whereas H3K4me3 and H3K27me3 were increased, at Bdnf-p2 compared with saline controls (Fig. 3a). No changes in acH3, H3K4me3 or H3K27me3 were seen at Bdnf-p1, Bdnf-p4 and Bdnf-p6 after morphine exposure (Supplementary Fig. 2a–g). In addition, we observed no alterations in H3K9me2, H3K9me3 or H3K36me3 at any of the Bdnf promoters that we studied, whereas elevated acH4 levels at Bdnf-p4 were observed in morphine-treated rats.

**Figure 3** Morphine-induced histone modifications at Bdnf promoters in rat VTA. (a) qChIP showed that chronic morphine (14 d of morphine administration followed by 14 d of withdrawal; Fig. 1c) selectively altered H3K27me3 (two-way ANOVA, drug effect: F[1,27] = 0.144, P = 0.708; region effect: F[2,27] = 6.442, P = 0.003; drug × region effect: F[2,27] = 6.178, P = 0.003, n = 4 rats; Supplementary Fig. 2f) at Bdnf-p2, in particular. Chronic morphine changed acH4 (drug effect: F[1,27] = 11.509, P = 0.002; region effect: F[3,27] = 0.482, P = 0.698; drug × region effect: F[3,27] = 0.184, P = 0.906, n = 5, 4 rats; Supplementary Fig. 2b) at Bdnf-p4 in rat VTA, with no changes seen in several other histone modifications analyzed. Additional post hoc analyses with Student’s t tests revealed that chronic morphine also changed acH3 (unpaired t test, t[4] = 2.581, P = 0.0417, n = 4 rats) and H3K4me3 (t test, t[3] = 2.312, P = 0.0495, n = 5 rats) at Bdnf-p2 in VTA. Histone modifications by chronic morphine at other Bdnf promoters (Bdnf-p1, Bdnf-p4 and Bdnf-p6) are available in the Supplementary Figure 2a–g. (b) Binding of mSin3a (two-way ANOVA, drug effect: F[1,36] = 25.829, P < 0.001; region effect: F[3,36] = 0.541, P = 0.653; drug × region effect: F[3,36] = 0.464, P = 0.709, n = 6, 5 rats) and ING2 (drug effect: F[1,28] = 37.786, P < 0.001; region effect: F[2,28] = 2.450, P = 0.064; drug × region effect: F[2,28] = 0.552, P = 0.651, n = 5, 4 rats), core components of a major repressor complex, to Bdnf-p2 were increased by chronic morphine. (c) Consistent with enhancement in H3K4me3 levels, binding of MLL1 (KMT2A) to Bdnf-p2 was increased by chronic morphine (drug effect: F[1,23] = 24.884, P < 0.001; region effect: F[2,23] = 2.285, P = 0.106; drug × region effect: F[2,23] = 2.177, P = 0.118, n = 4 rats). (d) There was no morphine-induced alteration in binding of G9a (EHMT2) to Bdnf-p2 (drug effect: F[1,27] < 0.00384, P = 0.951; region effect: F[2,27] = 0.153, P = 0.927; drug × region effect: F[2,27] = 0.153, P = 0.927, n = 5, 4 rats). (e) Consistent with enhancement in H3K27me3 levels, binding of SUZ12 (drug effect: F[1,24] = 12.662, P = 0.002; region effect: F[3,24] = 1.211, P = 0.327; drug × region effect: F[3,24] = 0.526, P = 0.668, n = 4 rats) and EZH2 (drug effect: F[3,20] = 16.872, P < 0.001; region effect: F[3,20] = 1.209, P = 0.332; drug × region effect: F[3,20] = 1.111, P = 0.368, n = 4, 3 rats), members of PRC2, to Bdnf-p2 was enhanced by chronic morphine. (f) In contrast, binding of PRC1 members, RING1A (drug effect: F[1,23] = 13.708, P < 0.001; region effect: F[3,23] = 0.0154, P = 0.997; drug × region effect: F[3,23] = 2.713, P = 0.067, n = 4 rats) and BMI1 (drug effect: F[1,23] = 10.975, P = 0.002; region effect: F[3,23] = 0.117, P = 0.950; drug × region effect: F[3,23] = 0.0345, P = 0.991, n = 5 rats), to Bdnf-p2 were decreased by chronic morphine, but RING1B binding was unaffected (drug effect: F[1,23] = 1.372, P = 0.253; region effect: F[3,23] = 0.267, P = 0.848; drug × region effect: F[3,23] = 0.298, P = 0.827, n = 4 rats). Morphine-induced epigenetic alterations by key histone-modifying enzymes and related regulatory proteins at other Bdnf promoters (Bdnf-p1, Bdnf-p4 and Bdnf-p6) are available in the Supplementary Figure 2h–p. (g) Representative low-magnification photomicrographs with an inset depicting localized HSV-mediated EZH2 expression (GFP+, green) in dopaminergic neurons (TH+, red) in mouse VTA (scale bar represents 50 µm). The results were replicated in three independent experiments. (h) Intra-VTA HSV-EZH2 suppressed the expression of Bdnf exon IX mRNA in mouse VTA compared with HSV-GFP controls (unpaired t test, t[14] = 2.168, P = 0.0456, n = 9 mice). (i) EZH2 overexpression in mouse VTA markedly enhanced morphine reward (t test, t[22] = 4.134, P = 0.000345, n = 12 mice). Two-way ANOVA with Fisher’s PLSD post hoc tests, * P < 0.05, ** P < 0.01 and *** P < 0.001; t tests, # P < 0.05 and ### P < 0.001. Bar graphs show mean ± s.e.m. Box plots present, in ascending order, minimum sample value, first quartile, median, third quartile and maximum sample value.
As follow-up, we performed qChIP for key histone-modifying enzymes and related regulatory proteins associated with a given histone mark, such as mSIN3a (mammalian SIN3 transcription regulator family member A), ING2 (inhibitor of growth 2), ML1 (mixed lineage, leukemia; KMT2A, lysine-specific methyltransferase 2A), G9a (EHMT2, euchromatic histone-lysine N-methyltransferase 2) and Polycomb group proteins. Given that mSIN3a is a core component of a prominent co-repressor complex implicated in numerous systems, we examined the binding of this protein to Bdnf promoters. We found that mSIN3a binding to Bdnf-p1, Bdnf-p2 and Bdnf-p6 was robustly increased in VTA after chronic morphine (Fig. 3b and Supplementary Fig. 2h). We examined the binding of ING2 to Bdnf promoters, based on our paradoxical observation of increased H3K4me3 levels at Bdnf-p2 in VTA of morphine-treated rats. H3K4me3 typically denotes increased gene transcription, but there is evidence that ING2 represses particular genes by binding to H3K4me3 and recruiting the mSIN3a repressive complex. We found that ING2 binding to Bdnf-p1, Bdnf-p2, Bdnf-p4 and Bdnf-p6 was markedly increased in VTA in response to chronic morphine (Fig. 3b and Supplementary Fig. 2i). Such recruitment of ING2 and mSIN3a to Bdnf promoters explains why an increase in H3K4me3 is associated with gene repression. Consistent with our H3K4me3 data, morphine-treated rats showed elevated binding of an H3K4 methyltransferase, MLL1, to Bdnf-p2 and Bdnf-p6 in VTA (Fig. 3c and Supplementary Fig. 2j).

In contrast, an H3K9 methyltransferase, G9a, exhibited no changes in binding to Bdnf promoters, consistent with the lack of alterations in H3K9me3 levels (Fig. 3d and Supplementary Fig. 2k).

H3K27me3-mediated gene repression is associated with Polycomb group proteins, which reside in two main complexes: PRC1 (Polycomb repressive complex 1) and PRC2. PRC2 contains SUZ12 (zinc finger protein suppressor of zeste 12) and EZH2 (enhancer of zeste homolog 2); the latter catalyzes H3K27me3 (refs. 22, 23). PRC1, which contains BMI1, RING1A and RING1B, contributes to histone H2A monoubiquitination and interactions of Polycomb group proteins with H3K27me3 (refs. 24, 25). Consistent with increased levels of H3K27me3 at Bdnf-p2, the binding of PRC2 complex proteins—namely, SUZ12 and EZH2—to Bdnf-p2 was increased in VTA by chronic morphine (Fig. 3e). High levels of occupancy by EZH2, but not SUZ12, were also found at Bdnf-p4 and Bdnf-p6 (Supplementary Fig. 2l,m). In contrast, binding of PRC1 complex proteins, such as RING1A, to Bdnf-p2 was reduced by morphine, whereas no changes were seen at other Bdnf promoters or in RING1B and BMI1 binding at any promoter (Fig. 3f and Supplementary Fig. 2n-p). This comprehensive analysis of epigenetic regulation of the Bdnf gene in VTA by chronic morphine reveals concerted modifications predominantly at Bdnf-p2 that are consistent with transcriptional repression (Supplementary Fig. 2q).
To directly test whether alterations in H3K27me3 in VTA influence Bdnf expression and morphine-elicited behavioral changes, we generated a herpes simplex virus (HSV) vector to overexpress EZH2 selectively in VTA compared with HSV-GFP controls (Fig. 3g and Supplementary Figs. 3a and 4a). Such EZH2 overexpression, which was selective for VTA and not seen in neighboring brain regions (Supplementary Fig. 4a), reduced Bdnf mRNA levels in VTA (Fig. 3h) and robustly increased morphine CPP (5 mg per kg) in mice (Fig. 3i). In contrast, intra-VTA infusion of a lentiviral vector expressing a short hairpin interfering RNA of Ezh2 (LV-shRNA-EZH2), which repressed Ezh2 mRNA expression (Supplementary Fig. 3b,c), blocked the morphine-induced reduction of Bdnf expression in VTA (Supplementary Fig. 3d).

**Regulation of CREB binding to Bdnf promoters by morphine**

We next examined CREB, which induces Bdnf in other systems.36 Total CREB binding to Bdnf-p6 was increased in VTA in chronic morphine–treated rats, with no changes being observed at the other promoters (Fig. 4a). However, the binding of phospho-CREB (its active form) to Bdnf-p1, Bdnf-p2 and Bdnf-p4 was robustly decreased under these conditions (Fig. 4b). To confirm the direct connection between CREB activity and Bdnf expression in VTA, we infused HSV-CREB into VTA of wild-type mice to overexpress CREB selectively in this region (Fig. 4c). CREB overexpression augmented Creb1 mRNA and protein levels selectively in VTA (Supplementary Figs. 4b and 5a); it also increased Bdnf mRNA expression in VTA compared with HSV-tdTomato (TMT) controls (Fig. 4d). Conversely, knocking down CREB by infusing HSV-Cre into the VTA of mice with loxP-flanked Creb1 mice (Fig. 4e and Supplementary Fig. 5b) decreased Bdnf expression compared with HSV-GFP controls (Fig. 4f).
Regulation of NURR1 binding to Bdnf promoters by morphine

We also examined the transcription factor NURR1 (nuclear receptor related 1), as it regulates several genes including Bdnf in midbrain dopamine neurons\(^{30-33}\). NURR1 binding to Bdnf-p1 and Bdnf-p2 was decreased in VTA of chronic morphine–treated rats (Fig. 5a). Notably, chronic morphine suppressed Nurr1 mRNA expression in VTA, an effect that we also observed in heroin self-administering rats (Fig. 5b, c), which could explain the reduced NURR1 binding to Bdnf promoters. Consistent with Nurr1 suppression, H3K27me3 binding at the Nurr1 promoter in VTA was increased by chronic morphine (Fig. 5d).

Furthermore, we examined the relationship between CREB and NURR1 in response to chronic morphine, as Nurr1 is a known downstream target of CREB in other systems\(^{32,34}\). Chronic morphine decreased phospho-CREB binding to the Nurr1 promoter in VTA (Fig. 5e), whereas CREB overexpression in mouse VTA using HSV-CREB, which induced total and phospho-CREB binding to the Nurr1 promoter (Supplementary Fig. 5c, d), robustly augmented Nurr1 mRNA expression in this region (Fig. 5f). The induced H3K27me3 and reduced phospho-CREB binding at Nurr1 in VTA in response to chronic morphine suggest an antagonistic relationship between H3K27me3 and phospho-CREB, as also observed for the Bdnf promoter under these conditions.

We generated and validated an HSV vector to overexpress NURR1 and examine its functional role selectively in VTA (Supplementary Figs. 4c and 7a). We first infused HSV-NURR1 into rat VTA 10 d after the last injection of our standard 14-d chronic morphine procedure (5 mg per kg, intraperitoneal). Rats were then reexposed to morphine (5 mg per kg, intraperitoneal) 4 d after the HSV-NURR1 infusion (Fig. 5g); such reexposure (M/M) induced higher locomotor responses than a saline challenge (M/S) (Supplementary Fig. 1c). We observed that NURR1 overexpression in VTA blocked this hyper-locomotion by morphine reexposure (Fig. 5h and Supplementary Fig. 7b). We also investigated the influence of NURR1 on morphine reward using the CPP procedure. We first confirmed that NURR1 overexpression reduced Bdnf expression in mice, as observed in rat (Fig. 5i, j), and then found that morphine reward was also blocked by NURR1 overexpression in mouse VTA (Fig. 5k). Notably, NURR1 overexpression in VTA of mice lacking BDNF in this brain region (generated by infusing HSV-Cre into VTA of mice with loxP-flanked Bdnf) did not result in the suppressive effect of NURR1 overexpression on morphine reward (Fig. 5l), which provides a direct link between NURR1 action and regulation of BDNF activity in this brain region.

**Discussion**

Consistent with prior reports of opiate regulation of VTA BDNF expression\(^{11,35}\) (but see ref. 36), we found that Bdnf mRNA levels were robustly reduced in VTA of human heroin addicts, heroin self-administering rats and repeated morphine-treated mice (including those undergoing CPP). We observed that chronic morphine exposure induces a robust and sustained decrease in levels of specific Bdnf exon transcripts (that is, Bdnf exons II, IV and VI) in this brain region in rats. A uniquely comprehensive analysis of epigenetic mechanisms—particularly for a micronucleus such as VTA—revealed that this Bdnf suppression by chronic morphine is associated with a series of interacting and sustained transcriptional and chromatin modifications at the corresponding Bdnf gene promoters. In particular, we found that chronic morphine decreases binding of the active (phospho-Ser2) form of Pol II within Bdnf-eI, Bdnf-eIV and Bdnf-eVI, whereas phospho-Ser5 Pol II binding to Bdnf-p2, Bdnf-p4 and Bdnf-p6 was augmented in VTA. These findings suggest that chronic morphine induces a sustained Pol II stalling at certain Bdnf promoters in concert with the gene's sustained repression.

Such stalling of Pol II is associated with several other key chromatin changes, with the most robust regulation seen at Bdnf-p2 in VTA (Supplementary Fig. 2q). Consistent with repression of Bdnf-p2, we found increased binding of H3K27me3, a major form of repressive histone methylation. Occupancy by the PRC2 complex, including SUZ12 and EZH2, which mediates H3K27 trimethylation\(^{22,23}\), at Bdnf-p2 in VTA was also increased by chronic morphine. Our results provide a causal connection between H3K27me3 and suppression of the Bdnf gene in VTA by showing that EZH2 overexpression in this region decreased Bdnf expression, whereas EZH2 knockdown blocked morphine's suppression of Bdnf. Taken together, these data support a scheme in which PRC2-mediated H3K27me3 reduces Bdnf gene expression (Supplementary Fig. 8). Notably, PRC2 has been shown to interact with other repressor proteins, particularly with mSIN3a and related proteins\(^{37,38}\), which are also enriched at Bdnf promoter in response to chronic morphine. Consistent with our previous study, which linked reduced levels of VTA BDNF with increased morphine reward\(^{11}\), we found that EZH2 overexpression in VTA promotes this morphine-elicited behavior as well.

Repression of Bdnf-p2 was associated with increased levels of H3K4me3, which is typically related to gene activation. This observation illustrates the complexity of chromatin regulatory mechanisms in vivo. Insight into this complexity is provided by our observation that this paradoxical increase in H3K4me3 was associated with induction of ING2 binding to Bdnf-p2 by morphine. ING2, which is also a subunit of the repressive mSIN3a complex, binds with high specificity to H3K4me3 and consequently represses transcription of certain genes\(^{20,21}\). We found that this unusual, but with precedent, interaction between H3K4me3 and the mSIN3a/ING2 also involved the morphine-induced recruitment of the H3K4-specific methyltransferase MLL1 (ref. 39) to Bdnf-p2 (Supplementary Fig. 8). Notably, MLL1, similar to its H3K4me3 mark, has been shown to interact with repressive complexes at particular genes in other systems\(^{40}\).

The morphine-induced Bdnf repression also involves reduced binding of a key transcription factor, phospho-CREB, which has previously been shown to induce Bdnf gene expression in other systems\(^{26}\). However, the reduced phospho-CREB binding to Bdnf promoters in VTA after chronic morphine is surprising, as chronic morphine increases total levels of phospho-CREB and CREB transcriptional activity in VTA\(^{27,28}\). These findings suggest that there are specific features at Bdnf promoters that are responsible for the exclusion of phospho-CREB after chronic morphine. We found that one
mechanism for this exclusion is the induction of H3K27me3— as EZH2 overexpression, which induces H3K27me3—in VTA in the absence of morphine reduces phospho-CREB binding to Bdnf promoters. A recent study has reported that PRC2 occupancy and increased H3K27me3 at certain gene promoters antagonizes CREB binding to silence gene expression in lymphocytes. In contrast, phospho-CREB binding to the promoters of Th and Gria1, two known targets of phospho-CREB in VTA, were increased in response to chronic morphine, as would be expected. It is notable that chronic morphine did not affect H3K27me3 levels at Th and Gria1 promoters and that Th and Gria1 mRNA expression was not altered by EZH2 overexpression in contrast to Bdnf expression. Although a caveat of these studies is potential off-target effects of overexpressed transcription factors, our findings together suggest that reduced phospho-CREB binding to Bdnf promoters is mediated by the selective recruitment of H3K27me3 to key Bdnf promoters during a course of opiate exposure.

Bdnf gene regulation is also known to be controlled by NURR1, which is required for maintenance of adult dopamine neurons in VTA, NURR1 acts downstream of CREB and upstream of BDNF. This connection between CREB and NURR1 is supported by our data that Nurr1 mRNA induction was observed in VTA following CREB overexpression. This finding supports the hypothesis that the morphine-induced reduction in phospho-CREB binding to the Nurr1 promoter that we observed mediates the Nurr1 suppression in VTA by chronic morphine. In parallel, we found that Nurr1 mRNA levels and NURR1 binding to Bdnf promoters were decreased in this region by morphine. Our observation that Bdnf mRNA levels were increased in VTA following NURR1 overexpression is consistent with the scheme that reduced NURR1 binding to Bdnf promoters contributes to Bdnf suppression in response to morphine. This scheme is also supported by our behavioral data: chronic morphine–induced locomotor responses, as well as morphine-elicted reward, were blocked by NURR1 overexpression in VTA. Given that NURR1 overexpression induced Bdnf, these behavioral data are consistent with prior reports that elevated VTA BDNF activity is also associated with suppression of morphine's behavioral effects. Such a connection between NURR1 and BDNF was established by our demonstration that NURR1's ability to suppress morphine-elicted behaviors was lost in mice lacking BDNF selectively in this brain region.

In conclusion, our findings reinforce the complexity of gene and chromatin regulation in adult brain and emphasize the importance of examining numerous chromatin endpoints when investigating epigenetic mechanisms of gene regulation in vivo (Supplementary Fig. 8). Given that morphine's suppression of Bdnf in VTA makes an important contribution to the drug's behavioral effects, our results provide new insight into the detailed molecular mechanisms underlying morphine-induced neural and behavioral plasticity.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
We thank T. Abel (University of Pennsylvania) for helpful discussions. This work was supported by grants from the National Institute on Drug Abuse (E.J.N. and M.S.M.-R.).

AUTHOR CONTRIBUTIONS
J.W.K., M.S.M.-R., S.J.R., Y.L.H. and E.J.N. designed the study. J.W.K., M.S.M.-R., Q.L., G.E., K.M.B., D.N.A., D.F., J.E., H.S., K.N.S., D.M.D.-W., E.R., C.J.P., D.W., R.C.B., M.E.C., S.A.R.A., B.L., G.E.H., H.B., B.C., A.J.R., V.F.V., C.D., Z.L., E.M., M.K.L. and D.M.D.-W. performed the experiments. J.W.K. and R.L.N. generated viral vectors. J.W.K. and Q.L. analyzed data. J.W.K. and E.J.N. wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Lobo, M.K. et al. Cell type-specific loss of BDNF signaling mimics opotgenetic control of cocaine reward. Science 330, 385–390 (2010). 2. Pu, L., Liu, Q.S. & Pos, M.M. BDNF-dependent synaptic sensitization in midbrain dopamine neurons after cocaine withdrawal. Nat. Neurosci. 9, 605–607 (2006). 3. Russo, S.J., Mazei-Robinson, M.S., Ables, J.L. & Nestler, E.J. Neurotrophic factors and neuronal plasticity in addiction. Neuropsychopharmacology 36, suppl. 1, 75–82 (2009). 4. Filip, M. et al. Alterations in BDNF and trkB mRNAs following acute or sensitizing cocaine treatments and withdrawal. Brain Res. 1071, 218–225 (2006). 5. Graham, D.L. et al. Dynamic BDNF activity in nucleus accumbens with cocaine use increases self-administration and relapse. Nat. Neurosci. 10, 1029–1037 (2007). 6. Grimm, J.W. et al. Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. J. Neurosci. 23, 742–747 (2003). 7. Graham, D.L. et al. Tropomyosin-related kinase B in the mesolimbic dopamine system: region-specific effects on cocaine reward. Biol. Psychiatry 65, 696–701 (2009). 8. Hall, F.S., Dongova, J., Goeb, M. & Uhl, G.R. Reduced behavioral effects of cocaine in heterozygous brain-derived neurotrophic factor (BDNF) knockout mice. Neuropsychopharmacology 28, 1485–1490 (2003). 9. Hoger, B.A. et al. Enhancement of locomotor activity and conditioned reward to cocaine by brain-derived neurotrophic factor. J. Neurosci. 19, 4110–4122 (1999). 10. Grimm, J.W. et al. Temporal dependence of brain-derived neurotrophic factor protein levels in the ventral tegmental area produces long-lasting potentiation of cocaine seeking after withdrawal. J. Neurosci. 24, 1604–1611 (2004). 11. Koo, J.W. et al. BDNF is a negative modulator of morphine action. Science 338, 124–128 (2012). 12. Berhow, M.T. et al. Influence of neurotrophic factors on morphine- and cocaine-induced biochemical changes in the mesolimbic dopamine system. Neuroscience 68, 969–979 (1995). 13. Russo, S.J. et al. IRS2-Akt pathway in midbrain dopamine neurons regulates behavioral and cellular responses to opiates. Nat. Neurosci. 10, 93–99 (2007). 14. Sklar-Tavor, L. et al. Chronic morphine induces visible changes in the morphology of mesolimbic dopamine neurons. Proc. Natl. Acad. Sci. USA 93, 11102–11107 (1996). 15. Mazei-Robinson, M.S. et al. Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. Neuron 72, 977–990 (2011). 16. Aid, T., Kazantseva, A., Piirsoo, M., Palm, K. & Timmusk, T. Mouse and rat BDNF gene structure and expression revisited. J. Neurosci. Res. 85, 525–535 (2007). 17. Vanderschuren, L.J. et al. Morphine-induced long-term sensitization to the locomotor effects of morphine and amphetamine depends on the temporal pattern of the pretreatment regimen. Psychopharmacology (Berl.) 131, 115–122 (1997). 18. Komaritsky, P., Cho, E.J. & Buratowski, S. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. 14, 2452–2460 (2000). 19. Laherty, C.D. et al. Histone deacetylases associated with the mSin3 corepressor mediate mid transcriptional repression. Cell 89, 349–356 (1997). 20. Shi, X. et al. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature 442, 96–99 (2006). 21. Ythier, D. et al. Sumoylation of ING2 regulates the transcription mediated by Sin3A. Oncogene 29, 5946–5956 (2010). 22. Czermak, B. et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycrome sites. Cell 111, 185–196 (2002). 23. Müller, J. et al. Histone methyltransferase activity of a Drosophila Polycrome group repressor complex. Cell 111, 197–208 (2002). 24. Simon, J.A. & Kingston, R.E. Mechanisms of polycomb gene silencing: knowns and unknowns. Nat. Rev. Mol. Cell Biol. 10, 697–708 (2009). 25. Cao, R., Tsuchada, Y. & Zhang, Y. Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. Mol. Cell 20, 845–854 (2005). 26. Tao, X., West, A.E., Chen, W.G., Corfas, G. & Greenberg, M.E. A calcium-responsive transcription factor, CaRF, that regulates neuronal activity-dependent expression of BDNF. Neuron 33, 383–395 (2002). 27. Walters, C.L., Kuo, Y.C. & Blendy, J.A. Differential distribution of CREB in the mesolimbic dopamine reward pathway. J. Neurochem. 87, 1237–1244 (2003).
28. Olson, V.G. et al. Regulation of drug reward by cAMP response element-binding protein: evidence for two functionally distinct subregions of the ventral tegmental area. J. Neurosci. 25, 5553–5562 (2005).

29. Xiong, Y. et al. Polycomb antagonizes p300/CREB-binding protein-associated factor to silence FOXP3 in a Kruppel-like factor-dependent manner. J. Biol. Chem. 287, 34372–34385 (2012).

30. Volpicelli, F. et al. Direct regulation of Pitx3 expression by Nurr1 in culture and in developing mouse midbrain. PLoS ONE 7, e30661 (2012).

31. Volpicelli, F. et al. Bdnf gene is a downstream target of Nurr1 transcription factor in rat midbrain neurons in vitro. J. Neurochem. 102, 441–453 (2007).

32. Barneda-Zahonero, B. et al. Nurr1 protein is required for N-methyl-D-aspartic acid (NMDA) receptor-mediated neuronal survival. J. Biol. Chem. 287, 11351–11362 (2012).

33. Kadkhodaei, B. et al. Nurr1 is required for maintenance of maturing and adult midbrain dopamine neurons. J. Neurosci. 29, 15923–15932 (2009).

34. McEvoy, A.N. et al. Activation of nuclear orphan receptor NURR1 transcription by NF-kappa B and cyclic adenosine 5’-monophosphate response element-binding protein in rheumatoid arthritis synovial tissue. J. Immunol. 168, 2979–2987 (2002).

35. Chu, N.N. et al. Peripheral electrical stimulation reversed the cell size reduction and increased BDNF level in the ventral tegmental area in chronic morphine-treated rats. Brain Res. 1182, 90–98 (2007).

36. Vargas-Perez, H. et al. Ventral tegmental area BDNF induces an opiate-dependent-like reward state in naive rats. Science 324, 1732–1734 (2009).

37. Fujii, S., Ita, K., Ito, Y. & Ochiai, A. Enhancer of zeste homologue 2 (EZH2) down-regulates RUNX3 by increasing histone H3 methylation. J. Biol. Chem. 283, 17324–17332 (2008).

38. van der Vlag, J. & Otte, A.P. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat. Genet. 23, 474–478 (1999).

39. Milne, T.A. et al. MLL targets SET domain methyltransferase activity to Hox gene promoters. Mol. Cell 10, 1107–1117 (2002).

40. Xia, Z.B., Anderson, M., Diaz, M.O. & Zeleznik-Le, N.J. MLL repression domain interacts with histone deacetylases, the polycomb group proteins HPC2 and BMI-1, and the co-repressor C terminal–binding protein. Proc. Natl. Acad. Sci. USA 100, 8342–8347 (2003).
Animals. Male 8–10-week-old Sprague Dawley rats (225–250 g, Charles River) were used in all experiments unless otherwise noted, given the larger size of their VTA, which makes detailed epigenetic studies possible. In some experiments, male 7–9-week-old c57BL/6 mice (25–30 g, Jackson) and 9–13-week-old floxed CREB and floxed BDNF mice were used. Floxed CREB mice on a c57BL/6 background and floxed BDNF mice on a B6/sv129 background were generated and maintained, as previously described.41,42 These mice were used for key behavioral experiments to take advantage of genetic mutants. All animals were housed in groups of 2 rats or 2–5 mice per cage at 22–25°C on a 12-h light/dark cycle (lights on 7:00 a.m.) with access to food and water ad libitum. Animals were acclimatized to vivarium conditions for at least 1 week before experimentation and assigned randomly to experimental groups. All behavioral experiments, except self-administration, were performed during the light cycle. All animals used were experimentally naive. All work was in accordance with guidelines of the Society for Neuroscience and the Mount Sinai Institutional Animal Care and Use Committee.

Human postmortem subjects. Postmortem human brain specimens from heroin users and control subjects were collected within ~24 h of death under approved protocols at the Department of Forensic Medicine at Semmelweis University, Hungary, or the National Institute of Forensic Medicine, Karolinska Institutet, Stockholm, Sweden. Brains were immediately frozen using dry ice–cooled isopentane, cryosectioned and thaw-mounted onto poly-L-lysine–treated slides. Cause and manner of death were determined by a forensic pathologist after evaluating the circumstances of death, toxicology data (blood, urine and liver) and autopsy results. Information was also evaluated from police reports, family and friends, and medical records as described in Supplementary Table 1. Inclusion criteria were death associated with heroin intoxication (verified by toxicology), physical signs of heroin use such as needle tracks and history of heroin abuse. Exclusion criteria were postmortem interval (PMI) of >24 h, age <20 years, HIV-positive status and history of alcoholism.

Heroin self-administration. Heroin self-administration in rats was conducted according to published procedures.43

Morphine CPP. An unbiased CPP procedure was used according to published procedures.44

Locomotor activity. Rats were given morphine (morphine sulfate in saline, 5 mg per kg, intraperitoneal) or saline daily for 14 d and then challenged with morphine (5 mg per kg, intraperitoneal) or saline on day 28. On day 0, 14 and 28, morphine or saline was administered after 30-min habituation in the locomotor chamber. Locomotor activity was monitored for 30 min after the administration using the Photobeam Activity System (San Diego Instruments).

HSV vectors. HSV-GFP was generated with the p1005+ HSV amplicon bicistronic plasmid containing a complete CMV-GFP expression cassette as the second cistron. For HSV-NURR1, we designed the forward primer with KpnI restriction sites (5′-GGGTTACCCCATGCTTGTGCTAGGCGGATGATG-3′) and reverse primer with XhoI restriction sites (5′-CCGCTCGAGCGGTTAAGGTGTTTTGGATGTTA GAAAAGTTAAGGTGTCCAGGCAA-3′). The PCR products for Nurr1 were then digested, purified and ligated into the first cistron downstream of the HSV IE4/5 promoter of the HSV ampiclon at KpnI-Xhol. For HSV-EZH2, the coding sequences for Ezh2 from pCMV-HA hEZH2 (Addgene plasmid #24230) were subcloned into the HSV ampiclon at EcoRV-BamHI. Viral titers were determined using qPCR and ~1 × 10^9 particles/site were used. All behavioral experiments were initiated ≥2 d and completed <5 d after viral injection, the time period when HSV expression is maximal. HSV-CREB and HSV-Cre vectors have been described previously.45,46 Viral targeting to VTA was confirmed for all animals; <3% were excluded for anatomically incorrect placements. For validation of viral-mediated gene transfer, rat or mouse VTA bilateral punches (rat, 14 gauge; mouse, 15 gauge) were dissected under fluorescent stereomicroscope (Leica MZ10F) 4 d after intra-VTA infusion of the HSVs and processed for qPCR as described below (Supplementary Figs. 3a, 5a,b and 7a). In addition, rat bilateral 14-gauge punches were collected from substantia nigra and red nucleus (as anatomical controls), and VTA, and processed for western blotting as described below (Supplementary Fig. 4).

Stereotoxic surgery. Mice or rats were anesthetized with ketamine (100 mg per kg) and xylazine (10 mg per kg). 33 gauge needles were used to bilaterally infuse HSVs or LVs into VTA (for mice, AP = −3.2, ML = ±1.0, DV = −4.6; for rats, AP = −5.5, ML = ±2.0, DV = −7.4 from Bregma (mm), 7th angle). An infusion volume of 0.5 μl (mice) or 1.0 μl (rats) was delivered using 5 μl Hamilton syringe over the course of 5 min (at a rate of 0.1 μl min^−1 (mice) or 0.2 μl min^−1 (rats)). The infusion needle remained in place for at least 5 min after the infusions before removal to prevent backflow of the injected material.

RNA isolation and qPCR. Total RNA was isolated from postmortem human brain specimens using Arcturus Pico Pure kit (Life Technologies) and from frozen VTA bilateral punches (rat, 14 gauge; mouse, 15 gauge) using Trizol (Life Technologies) and a micro RNAeasy kit (Qiagen) as described.41 RNA was reverse transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad). Primers were designed to amplify regions of 100–200 bp located in the genes of interest or chosen from previous studies.46,47 All reactions were run in triplicate and analyzed using the ΔΔCt method with glyceraldehyde-3-phosphate dehydrogenase (Gapdh) or 18S ribosomal RNA (Rrn18S) as a normalization control. qPCR primers are listed in Supplementary Table 2.

qChIP. Freshly dissected rat VTA punches were cross-linked with 1% formaldehyde, quenched with 2 M glycine and sonicated to ~500 bp at 4°C. For each ChIP for histone modifications, bilateral 14-gauge VTA punches were used; for each ChIP for transcription factors, bilateral 14-gauge punches were pooled from two rats. Separate groups of animals were used for each qChIP experiment. Sheep IgG antibody to rabbit or mouse magnetic beads (Invitrogen) were prepared by incubating with antibodies of interest. Chromatin samples were immunoprecipitated with the conjugated bead/antibody mixtures, and then reverse cross-linked. DNA was purified and quantified using qPCR in triplicate from independent groups of animals.46,47 Normal mouse or rabbit IgG immunoprecipitations were performed as controls. Primers for most qChIPs, shown in Supplementary Table 3, were designed to amplify 150–200-bp products that include CREB binding sites. All ChIP antibodies were validated using qChIP (Supplementary Fig. 9) with rat primer sets that were designed, based on previous studies validating ChIP antibodies in other systems,46,47 or on validation information provided by the manufacturers (Supplementary Table 4). The primer pairs for NURR1 ChIP validation (for example, Bshf, Ptxc3 and Thb primers) were designed to amplify 150–200-bp products that contain a NBRE (NGFI-B response element)-like sequence, which are putative NURR1 binding sites.47 To further demonstrate the selectivity of antibody to NURR1, we performed western blotting using antibody to NURR1 (rabbit polyclonal, Santa Cruz, sc-30154), antibody to NUR77 (rabbit polyclonal, Santa Cruz, sc-5569) or antibody to NURR1 (rabbit polyclonal, Santa Cruz, sc-991) on samples that were immunoprecipitated with antibody to NURR1 (Supplementary Fig. 7c–e).

Immunohistochemistry. Immunohistochemistry was conducted according to published procedures.47 For GFP/TH and TMT/TH double-labeling, brain sections were incubated in 1:4,000 of antibody to TH (T1299, Sigma), 1:2,000 of rabbit polyclonal antibody to GFP (A1122, Invitrogen) or 1:1,000 of rabbit antibody to dsRed (632496, Clontech), respectively, in block solution overnight at 4°C. The next day, sections were incubated in 1:500 of donkey antibody to rabbit Cy2 (Immuno Research) for antibody to GFP together with 1:500 of donkey antibody to mouse Cy3 for antibody to TH or 1:500 of donkey antibody to rabbit Cy3 for antibody to dsRed, together with 1:500 of donkey antibody to mouse Cy2 for antibody to TH in phosphate-buffered saline for 1 h. For GFP/Cre double-labeling, 1:2,000 of rabbit polyclonal antibody to GFP (A1122, Invitrogen), 1:1,000 mouse antibodies to Cre recombinase (MAB3120, 1:500 of donkey antibody to rabbit Cy2 and 1:500 of donkey antibody to mouse Cy3 were used. All sections were imaged on an LSM 710 confocal microscope (Zeiss) at 10× or 20× magnification. All histological procedures were replicated independently at least two times.

Western blotting. Western blotting analyses were conducted according to published procedures.47 For primary antibody incubation, antibody to Ezh2 was used (1:1,000).
ramp monoclonal, Cell Signaling, 5246S), antibody to total-CREB (1:1,000; rabbit monoclonal, Millipore, 17-600) and antibody to NURR1 were used. Blots were imaged with the Odyssey Infrared Imaging System (Li-Cor) and quantified by densitometry using ImageJ (US National Institutes of Health). The amount of Pruned blasted onto each lane was normalized to levels of tubulin. Western blot analyses were replicated at least twice. Full-length western blots are presented in Supplementary Figure 10.

Experiment 1: Bdnf downregulation by opiates. We examined the effect of opiate exposure on Bdnf mRNA expression in human, rat and mouse VTA. Total RNA was isolated from postmortem brain specimens of human heroin addicts (n = 9) and controls (n = 5) (Supplementary Table 1). VTA punches (bilateral, 14-gauge) were collected from previously frozen brains of rats that were killed 24 h after the final heroin self-administration session (n = 24) (Supplementary Fig. 1b). We also freshly dissected 14-gauge bilateral rat VTA punches 30 min after completing the last locomotor activity test on day 28 (Supplementary Fig. 1c), after 14 d withdrawal from 14 d of prior morphine administration (n = 18). Mouse VTA punches (bilateral, 15 gauge) were freshly dissected 1 h after completing morphine CPP test sessions (n = 24) (Supplementary Fig. 1d).

Experiment 2: stalling of Pol II at Bdnf promoters by morphine. For each ChIP sample, VTA punches were pooled from two rats (four punches) that were killed on day 28, after 14 d withdrawal from 14 d of prior morphine administration (n = 20 or 24). Antibody to total-Pol II (mouse monoclonal, Millipore, 05-623), antibody to phospho-Ser2-Pol II (antibody to Pol II phosphorylated at Ser2 of its C-terminal domain) (mouse monoclonal, Abcam, ab24758) or antibody to phospho-Ser5-Pol II (rabbit polyclonal, Abcam, ab51311) was used for this experiment. Primers for qChIP with antibody to total-Pol II and antibody to phospho-Ser5-Pol II were designed to amplify 150–200-bp products that include CREB binding sites of Bdnf promoters. Primers for qChIP with antibody to phospho-Ser2-Pol II were designed to amplify 150–200-bp products in the coding sequence of the Bdnf gene (Supplementary Table 3).

Experiment 3: histone modifications by morphine. For each ChIP sample for histone modifications, VTA punches (two punches) were collected from one control or chronic morphine–treated rat (14 d of morphine administration followed by 14 d of withdrawal) (n = 8–11 for each ChIP). For each ChIP sample for key histone-modifying enzymes and related regulatory proteins, VTA punches were pooled from two rats (4 punches) (n = 14–22). Antibody to acH3 (rabbit polyclonal, Millipore, 06-599), antibody to acH4 (rabbit polyclonal, Millipore, 06–598), antibody to H3K4me3 (rabbit monoclonal, Abcam, ab1220), antibody to H3K9me3 (rabbit monoclonal, Abcam, ab6002), antibody to H3K27me3 (rabbit monoclonal, Abcam, ab9050), antibody to mSin3a (rabbit polyclonal, Santa Cruz, sc-994X), antibody to G2 (rabbit polyclonal, Santa Cruz, sc-134973), antibody to Kmt2a/Mll (rabbit polyclonal, Millipore, AB240), antibody to Kmt1C/G9a (rabbit polyclonal, Abcam, ab40542), antibody to Suz12 (rabbit monoclonal, Cell Signaling, 3737S), antibody to EzH2, antibody to RInGa (rabbit polyclonal, Abcam, ab32644), antibody to RInGb (rabbit monoclonal, Cell Signaling, 5694S) and antibody to Bmi1 (rabbit monoclonal, Cell Signaling, 6964S) were used.

Experiment 4: role of EZH2 in Bdnf expression and morphine CPP. Stereotactic surgery was performed on adult male c57BL/6 mice (8 weeks) to inject Hsv-Ezh2-GFP or Hsv-GFP into the VTA. The first batch of mice (n = 18) was killed for qPCR for validation (Supplementary Fig. 3a) and Bdnf expression (Fig. 3h) 4 d after stereotactic surgery. Viral infection sites were confirmed using standard histological methods (see Fig. 3g) using VTA sections from a subset of Hsv-Ezh2 infused mice (n = 3). A second batch of rats (n = 14) was used for qPCR for Bdnf expression after chronic morphine treatment (Supplementary Fig. 3d). Briefly, we first injected morphine (5 mg per kg, intraperitoneal) for 14 d and bilateral viral injections with 1Vs were performed 4 d after the last morphine injection. Rats were killed and VTA punches (bilateral, 14 gauge) were collected 10 d after surgery.

Experiment 5: role of CREB in Bdnf expression. For total- and phospho-CREB ChIP experiments, VTA punches were pooled from two chronic morphine–treated rats (14 d of morphine administration followed by 14 d of withdrawal) (n = 16 or 20 for each). Antibody to total CREB and antibody to phospho-CREB (rabbit polyclonal, Millipore, 06–519) were used for immunoprecipitation. In separate groups of animals, we performed stereotactic surgery on male c57BL/6 mice or floxed CREB mice to inject Hsv-CREB or Hsv-Cre and their controls (Hsv-TmT or Hsv-GFP, respectively) into the VTA. The Hsv-CREB/Hsv-TmT infused c57BL/6 mice (n = 17) and Hsv-Cre/Hsv-GFP infused floxed CREB mice (n = 20) were killed for qPCR for validation (Supplementary Fig. 5a,b) and Bdnf expression (Fig. 4d,h) 4 d after stereotactic surgery. Viral injection sites were confirmed using standard histological methods (Fig. 4c,e) using VTA sections from a subset of HSV-CREB or HSV-Cre infused mice (n = 3 each).

Experiment 6: interaction between CREB and H3K27me3. Stereotactic surgery was performed on adult rats to inject Hsv-Ezh2 or Hsv-GFP into the VTA. Two batches of rats were killed for H3K27me3 ChIP (n = 14) and pCREB-Chip (n = 22) at Bdnf promoters (Fig. 4g,h) 4 d after stereotactic surgery. For these ChIP experiments, VTA punches were pooled from two rats. For H3K27me3 ChIP experiment, punches from one rat were used. mRNA levels of Bdnf exon IX were also measured using cDNA derived from VTA of Hsv-Ezh2 or Hsv-GFP infused mice (from Experiment 4) (Supplementary Fig. 3a). ChIP experiments with antibody to phospho-CREB, antibody to total CREB, and antibody to H3K27me3 were also conducted at Bdnf promoter regions with VTA punches from heroin self-administered rats (anti-phospho-CREB, n = 20; antibody to total CREB, n = 14; antibody to H3K27me3, n = 11) (Supplementary Fig. 6d–f).

Another set of ChIP experiments with antibody to phospho-CREB, antibody to total CREB and antibody to H3K27me3 was conducted as a control at Th and Grial promoter regions with control and chronic morphine–treated rats (14 d of morphine administration followed by 14 d of withdrawal) (antibody to phospho-CREB, n = 32; antibody to total CREB, n = 18; antibody to H3K27me3, n = 10) (Supplementary Fig. 6a–c).

Experiment 7: interaction between Nurr1 and CREB. ChIP experiment with antibody to Nurr1 was conducted at Bdnf promoters with chronic morphine–treated rats (14 d of morphine administration followed by 14 d of withdrawal) (n = 18). Another set of ChIP experiments with antibody to H3K27me3 and antibody to phospho-CREB was conducted at Nurr1 promoter regions with chronic morphine–treated rats (antibody to H3K27me3, n = 10; antibody to phospho-CREB, n = 36). In separate experiments, stereotactic surgery was performed on rats to inject HSV-CREB or HSV-TmT into the VTA. Two batches of rats were killed for total (n = 20) and phospho- (n = 20) CREB ChIP at the Nurr1 promoter (Supplementary Fig. 5c,d). For Nurr1 ChIP and total/phospho-CREB ChIP experiments, VTA punches were pooled from two rats. For H3K27me3 ChIP experiment, bilateral punches from one rat were used. Nurr1 mRNA levels in VTA were also measured using cDNA derived from chronic morphine–treated rats from heroin self-administering rats, or from HSV-CREB infused mice generated from prior experiments.

Experiment 8: behavioral and molecular effects of Nurr1. We performed stereotactic surgery on rats to inject HSV-Nurr1 or HSV-TmT into the VTA. The rats (n = 19) were killed for qPCR validation (Supplementary Fig. 7a) and Bdnf expression (Fig. 5j) 4 d after stereotactic surgery. Viral injection sites were confirmed using standard histological methods (Fig. 5i) using VTA sections from a subset of HSV-Nurr1 infused rats (n = 3). Another set of rats was given daily injection of morphine (5 mg per kg, intraperitoneal; n = 27) or saline (n = 11) for 14 d. Each morphine–saline-injected batch was then reexposed to morphine (5 mg per kg, intraperitoneal) or saline on day 28. All of these rats underwent locomotor activity tests on day 0, 14 and 28 and stereotactic surgery with HSV-Nurr1 or HSV-TmT into the VTA 10 d after the last injection of morphine.
chronic morphine or saline (on day 24) (Fig. 5g,h and Supplementary Fig. 7b). In a separate experiment, stereotaxic surgery was performed on adult male c57BL/6 mice (8 weeks) or floxed BDNF mice to inject HSV-NURR1, HSV-TMT, and/or HSV-Cre into the VTA. In case of floxed BDNF mice, we infused 0.25 μl of HSV-NURR1 or HSV-TMT together with 0.25 μl of HSV-Cre. 2 d after the surgery, the HSV-NURR1/HSV-TMT-infused c57BL/6 mice (n = 15) and HSV-NURR1-HSV-Cre/HSV-TMT-HSV-Cre infused floxed BDNF mice (n = 19) were conditioned in a three-chambered CPP box for 3 d. The next day, animals were given a final CPP test. All experimental designs are summarized in Supplementary Table 5.

**Statistical analysis.** Sample sizes were similar to those reported in previous works and based on expected effect sizes and power analyses. Data were collected and processed randomly and analyzed with SigmaPlot 12.5 (Systat) and Prism 5.0 (GraphPad). Data collection and analysis were not performed blind to the conditions of the experiments. Normality (Shapiro-Wilk test) and equal variance (F test) assumptions were confirmed before parametric analysis, unless otherwise indicated. For normally distributed data, Student’s t tests were used to assess differences between two experimental groups. For a two-sample comparison of means with unequal variances, Student’s t tests with Welch’s correction were used. One-way ANOVAs were used for analysis of three or more groups, followed by Fisher’s PLSD post hoc comparison of means with unequal variances, Student’s t tests were used to assess differences between two experimental groups. For a two-sample comparison of means with unequal variances, Student’s t tests with Welch’s correction were used. One-way ANOVAs were used for analysis of three or more groups, followed by Fisher’s PLSD post hoc tests. For all qChIP analyses, two-way ANOVAs with Fisher’s PLSD post hoc tests were used. For locomotor activity and heroin self-administration data, a repeated-measures two-way ANOVA was used followed by Fisher’s PLSD post hoc tests. Main and interaction effects were considered significant at P < 0.05. Mann-Whitney U tests were performed to compare two columns of non-normally distributed data. Outliers were excluded from analysis when identified by Grubbs’ test.

A Supplementary Methods Checklist is available.

41. Rios, M. et al. Conditional deletion of brain-derived neurotrophic factor in the postnatal brain leads to obesity and hyperactivity. *Mol. Endocrinol.* **15**, 1748–1757 (2001).
42. Covington, H.E. III et al. A role for repressive histone methylation in cocaine-induced vulnerability to stress. *Neuron* **71**, 656–670 (2011).
43. Anderson, S.A. et al. Impaired periamygdaloid-cortex dynorphin is characteristic of opiate addiction and depression. *J. Clin. Invest.* **123**, 5334–5341 (2013).
44. Barrot, M. et al. CREB activity in the nucleus accumbens shell controls gating of behavioral responses to emotional stimuli. *Proc. Natl. Acad. Sci. USA* **99**, 11435–11440 (2002).
45. Dietz, D.M. et al. Rac1 is essential in cocaine-induced structural plasticity of nucleus accumbens neurons. *Nat. Neurosci.* **15**, 891–896 (2012).
46. Liu, Q.R. et al. Rodent BDNF genes, novel promoters, novel splice variants, and regulation by cocaine. *Brain Res.* **1067**, 1–12 (2006).
47. Livak, K.J. & Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408 (2001).
48. Schmidt, H.D. et al. Increased brain-derived neurotrophic factor (BDNF) expression in the ventral tegmental area during cocaine abstinence is associated with increased histone acetylation at BDNF exon l-containing promoters. *J. Neurochem.* **120**, 202–209 (2012).
49. Xu, Y.X. & Manley, J.L. Pin1 modulates RNA polymerase II activity during the transcription cycle. *Genes Dev.* **21**, 2950–2962 (2007).
50. Sandoval, J. et al. RNApol-Chip: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res.* **32**, e88 (2004).
51. Egloff, S., Al-Rawaf, H., O’Reilly, D. & Murphy, S. Chromatin structure is implicated in “late” elongation checkpoints on the U2 snRNA and beta-actin genes. *Mol. Cell. Biol.* **29**, 4002–4013 (2009).
52. Khobta, A., Anderhub, S., Kilts, N. & Epe, B. Gene silencing induced by oxidative DNA base damage: association with local decrease of histone H4 acetylation in the promoter region. *Nucleic Acids Res.* **38**, 4285–4295 (2010).
53. Weishaupt, H. & Attewell, J.L. A method to study the epigenetic chromatin states of rare hematopoietic stem and progenitor cells: MiniChIP-Chip. *Biol. Proced. Online* **12**, 1–17 (2010).
54. Nitsche, A., Steinhauser, C., Mucke, K., Paulus, C. & Nevels, M. Histone H3 lysine 4 methylation marks postrepetitive human cytomegalovirus chromatin. *J. Virol.* **86**, 9817–9827 (2012).
55. Griffitt, G.D. et al. Limitations and possibilities of low cell number ChIP-seq. *BMC Genomics* **13**, 645 (2012).
56. Marchesi, I., Fiorentino, F.P., Rizzolio, F., Giordano, A. & Bagella, L. The ablation of HSV-Cre. 2 d after the surgery, the HSV-NURR1/HSV-TMT-infused c57BL/6 mice (n = 15) and HSV-NURR1-HSV-Cre/HSV-TMT-HSV-Cre infused floxed BDNF mice (n = 19) were conditioned in a three-chambered CPP box for 3 d.