

The consequences of early life stress on midbrain reward circuitry

by

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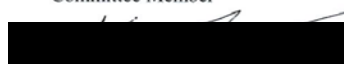
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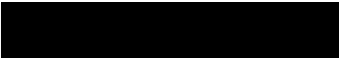
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DEDICATION

This work is dedicated to my parents whose support and encouragement took me this far,
my sister Emily, and my wife Carly.

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ABSTRACT

The consequences of early life stress on midbrain reward signaling

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Adverse early life experiences such as child neglect and abuse increase the risk of developing addiction and stress-related disorders through alterations in relevant brain systems including the mesolimbic dopamine (DA) pathway and extrahypothalamic corticotropin releasing factor (CRF) system. We first investigated whether a severe early life stress (i.e., maternal deprivation, MD) promotes DA dysregulation through an epigenetic impairment of synaptic plasticity within ventral tegmental area (VTA) DA neurons. MD selectively induces long-term depression (LTD) and shifts spike timing-dependent plasticity (STDP) toward LTD at GABAergic synapses onto VTA DA neurons through epigenetic modifications of postsynaptic scaffolding A-kinase anchoring protein

79/150 (AKAP79/150) signaling. Histone deacetylase (HDAC) inhibition rescues GABAergic metaplasticity and normalizes AKAP signaling in MD animals.

We next demonstrate for the first time that extrahypothalamic CRF neurotransmission exists in the lateral habenula (LHb), an epithalamic brain region involved in stress evasion. CRF-CRF receptor 1 (CRFR1) signaling increases the intrinsic excitability of LHb neurons through modulation of small- and large-conductance SK- and BK-type K⁺ channels. In addition, CRF reduces GABAergic synaptic transmission onto LHb neuron promoting LHb hyperactivity. MD increases LHb neuronal excitability and blunts the excitatory actions of CRF on LHb neurons due to down-regulation of SK2 channel expression. Increasing the function of SK channels by positive allosteric modulation prevents CRF excitatory action on LHb intrinsic excitability and reverses the effects of MD on LHb intrinsic excitability.

Additionally, we investigated the function of histone acetylation and HDAC2 in the VTA in recovery of morphine-induced synaptic modifications following a single *in vivo* exposure to morphine. Morphine increased HDAC2 activity in VTA DA neurons and reduced histone H3 acetylation at lysine 9 (Ac-H3K9) in the VTA 24 hours after injection. Morphine-induced synaptic changes at glutamatergic synapses involved endocannabinoid (eCB) signaling, reducing GABAergic synaptic strength onto VTA DA neurons. Both plasticities were recovered by *in vitro* incubation of midbrain slices with a class I specific HDAC inhibitor (HDACi), CI-994, through an increase in acetylation of histone H3K9. Our results suggest that acute morphine-induced changes in VTA DA activity and synaptic transmission engage HDAC2 activity in the VTA to maintain synaptic modifications through histone hypoacetylation.

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CHAPTER 1: INTRODUCTION

Adverse early life experiences increase the risk of cognitive impairment and later life mental health disorders such as depression, schizophrenia, and substance abuse [1-4]. Maternal deprivation (MD) is one particularly potent early life stressor with long-term developmental consequences [5-7]. Increased susceptibility to such mental illnesses suggests that MD can lead to dysfunction of brain monoaminergic systems, including dopamine (DA) brain reward systems. However, the exact mechanisms of how early life stress predisposes children to mental health disorder in adolescence and adulthood are unknown. This dissertation details the investigation of two key brain areas involved in DA signaling and how they are affected by early life stress: the ventral tegmental area (VTA) and lateral habenula (LHb). Given that corticotropin releasing factor (CRF) signaling is involved in stress responses, I also investigated contribution of CRF signaling to LHb neuronal excitability. In addition, this dissertation also includes an investigation of a morphine-induced epigenetic and synaptic mechanism altering VTA DA signaling.

Maternal deprivation as a model for early life stress

Exposure to stressors early in life can have consequences on adult behavior [7]. This is likely due to the introduction of these stressors during critical windows of brain development, when different brain regions and neurotransmitter systems are highly susceptible to insults. In humans, correlational studies have associated these early-life adverse experiences with an increased susceptibility to mental illnesses, including depression, addiction, anxiety and psychosis [8, 9]. To further study the causal

relationship between early-life stress and later-life mental illness, researchers have developed animal models that aim to replicate certain aspects of early life stress. MD is a widely-used animal model of severe early life stress which mimics the parental neglect that many children experience. Currently, there are a variety of rodent models of early-life parental separation that differ in duration, frequency and ages at which the separation occurs [7, 10]. However, a 24 h period of maternal deprivation at postnatal day 9 (P9) is a highly significant model of maternal separation in rodents due to the variety of impairments it induces that mimic aspects seen in human psychopathology [11]. MD is a combination of stressors including a lack of maternal care (licking/grooming, nursing) and lack of nutrients. Together, these stressors produce a variety of behavioral impairments.

Behavioral impairments induced by MD include abnormalities associated with schizophrenia, such as disturbances in prepulse inhibition of startle in adult rodents as well as an inability to ignore irrelevant stimuli as evidenced by deficits in latent inhibition [12]. Additionally, MD has been shown to produce depressive-like phenotypes as assessed by the forced swim test [13, 14]. In our own unpublished work, we observe a reduced latency to immobility and increased immobility times in adult MD rats compared to nonMD controls. Furthermore, these observations are supported by the aforementioned studies investigating the emergence of depressive symptoms following MD conducted in both adolescent and adult rodents. MD has also been associated with sex-dependent behavioral consequences. For example, adult MD males showed enhanced risk-taking/reduced anxiety behavior by spending more time exploring the open

arms of the elevated plus maze while adult females showed increased aggressive behaviors [14, 15].

Considering the behavioral abnormalities associated with MD, it is not surprising that several studies have demonstrated that MD impairs neurotransmitter systems known to underlie these behaviors including dopaminergic and serotonergic systems [7, 16]. Due to impaired dopaminergic neurotransmission following early-life stress, and its association with mental illnesses such as depression and addiction, this study focuses on the consequences of MD on brain areas involved in the mesocorticolimbic dopamine system.

Mesocorticolimbic dopamine system

The brain's reward circuitry, the mesocorticolimbic dopamine system plays an important role in mental illnesses such as addiction and depression [17, 18]. The name of this dopamine system derives from the major brain regions that belong to it: the midbrain VTA (meso), the prefrontal cortex (PFC) (cortico), and the nucleus accumbens (NAc) (limbic). This system is highly involved in motivation, goal-directed behavior, and natural reward and reinforcement processing. However, this area is known for mediating the effects of drugs of abuse.

All addictive drugs of abuse increase DA levels in the mesocorticolimbic system. Drugs of abuse accomplish this by mimicking the phasic firing (>15Hz) of DA neurons that normally occurs during presentation of natural rewards and their predictive cues [18]. DA is released into the NAc where it binds to D1 and D2 receptors (D1R and D2R) expressed on GABAergic medium spiny neurons (MSNs). MSNs expressing excitatory D1R comprise the direct pathway which is associated with reward, while MSNs

expressing inhibitory D2R comprise the indirect pathway which is commonly associated with punishment [18]. Although both D1R and D2R are GPCRs, activation of D1R typically results in neuronal excitation while activation of D2R results in inhibition. Thus, the increase in DA released onto these neurons leads to stimulation of the direct pathway and inhibition of the indirect pathway. Drug induced DA release mediates the acute reinforcing effects of drugs of abuse and triggers long-lasting neuroadaptations that are critical for the development of addiction. However, these neuroadaptations and reinforcing effects are not sufficient for the development of addiction.

Genetic predispositions and environmental factors can increase the susceptibility for developing addiction. For example, genes modulating monoamines such as catechol-*O*-methyltransferase (*COMT*) and the serotonin transporter (*SLC6A4*) have been implicated in susceptibility to addiction [19]. Environmental factors such as stress can also increase addiction susceptibility. MD is an environmental factor that has been suggested to impair the development of the mesocorticolimbic dopamine system [7]. This impairment may explain why certain individuals are more susceptible to developing addiction; MD has been shown to increase dopamine turnover and induce a regionally dependent modulation of D2 receptor expression in this dopamine system [7]. Furthermore, adults reporting low early life maternal care showed increased dopamine release in the NAc in response to an aversive stressful task compared to individuals reporting high early life maternal care [20]. Due to these well-established impairments of DA signaling following MD, I chose to focus on two brain regions directly involved in the release of DA in the mesocorticolimbic system: the VTA and the LHB.

The Ventral Tegmental Area

The mesocorticolimbic dopaminergic pathway originates from the VTA, an area consisting mostly (60- 70%) of tyrosine hydroxylase (TH)-expressing DA neurons. This pathway is highly involved in mood, motivation, and reward learning [21-23]. The VTA is a locus for drug reward. Enhanced DA transmission from the VTA is an early neuroadaptation in response to a single exposure to drugs of abuse and triggers subsequent synaptic changes in projection areas of the VTA, such as the NAc and PFC [24, 25]. Although most VTA neurons are dopaminergic, synthesizing and releasing dopamine, there also exists a sizable population of GABA and glutamate neurons. GABA neurons expressing glutamate decarboxylase 65/67 (GAD 65/67) are distributed throughout the VTA and comprise approximately 23% of VTA neurons [26]. Vesicular glutamate transporter 2 (VGLUT2)-expressing glutamate neurons are mainly distributed in the rostral and medial portions of the VTA [27]. GABA and glutamate VTA neurons synapse locally onto VTA DA neurons, but also send projections to other brain regions including the nucleus accumbens, lateral habenula, and medial prefrontal cortex [28]. Furthermore, some VTA neurons co-release neurotransmitters. Dopamine and GABA releasing VTA neurons have been found to project to NAc, while dopamine and glutamate releasing VTA neurons project to both NAc and mPFC [18]. In addition, GABA and glutamate releasing neurons have been shown to project to LHb neurons [29].

The activity of VTA neurons dictates the release of DA at downstream targets and is determined by the intrinsic neuronal properties of the neuron itself as well as the balance of excitatory (glutamatergic) to inhibitory (GABAergic) inputs. Glutamatergic inputs to the VTA arise mainly from the PFC, but also from subcortical areas such as the lateral hypothalamus, laterodorsal tegmental nucleus (LDTg), pedunculopontine

(PPT), and bed nucleus of the stria terminalis (BNST) [30]. These glutamatergic inputs release glutamate to stimulate postsynaptic AMPARs, NMDARs, and mGluRs of VTA DA neurons. GABAergic inputs to the VTA mainly arise from the NAc and globus pallidus which are involved in generating burst in VTA DA neurons [31]. Additionally, a sizable amount of GABAergic input is received from the neighboring tail of the VTA, the rostromedial tegmental nucleus (RMTg), which is believed to play a pivotal role in the influence of opiates on VTA DA neuronal firing [32]. And as mentioned previously, GABAergic interneurons within the VTA also synapse onto VTA DA neurons. These GABAergic inputs release GABA to stimulate both ionic GABA_ARs and metabotropic G-protein coupled GABA_BRs. Stress and also drugs of abuse are known to influence the strength of both glutamatergic and GABAergic synapses onto VTA DA neurons through the induction of synaptic plasticity [33].

Synaptic plasticity onto VTA DA neurons

Synaptic plasticity is the cellular basis for learning and memory, and is observed at synapses across the brain. The long term strengthening of synaptic strength is referred to as long-term potentiation (LTP), while the long term weakening of synaptic strength is referred to as long-term depression (LTD). The relative strength of excitatory and inhibitory synaptic inputs onto VTA dopamine neurons can influence the activity of these neurons. For example, a LTP of glutamatergic transmission and/or LTD of GABAergic transmission will shift the activity towards excitation while the opposite would shift activity towards inhibition. In fact, acute exposure to drugs of abuse has been shown to induce synaptic plasticity at both glutamatergic and GABAergic synapses onto VTA DA. Such drug-evoked synaptic plasticity at glutamatergic and GABAergic synapses onto VTA DA neurons is crucial to the development of addiction. Much like drugs of abuse,

stress has been shown to similarly dysregulate plasticity in the brain reward system including the VTA, thus promoting the development of stress-related disorders such as depression, anxiety, and addiction [34].

LTP and LTD can be induced by traditional as well as spike-timing dependent plasticity protocols. Our lab found that A-kinase anchoring protein 150 (AKAP150) signaling was necessary for a form of postsynaptic GABAergic LTD and furthermore that disruption of AKAP binding to protein kinase A (PKA) could induce a chemical LTD that occluded the induction of GABAergic LTD. We then established induction protocols for spike-timing-dependent plasticity (STDP) at these same synapses. STDP protocols utilize repetitive and coincident pre- and postsynaptic firing within a time window of 5 to 25ms to elicit long term changes in synaptic strength. In the typical Hebbian form of STDP, presynaptic spiking preceding post synaptic spiking (pre-post) induces a LTP (STD-LTP) of the synapse, while postsynaptic firing preceding presynaptic firing (post-pre) induces LTD (STD-LTD). At GABAergic synapses onto VTA dopamine neurons, STDP is heterosynaptic, requiring NMDAR activation at glutamatergic synapses onto VTA DA neurons [35]. This STDP is also bidirectional, with a pre-post time window of 15ms inducing STD-LTP and a post-pre time window of 5ms inducing STD-LTD.

In addition, the laboratory has previously investigated the consequences of morphine administration on synaptic plasticity in the VTA. Exposure to morphine, like other drugs of abuse, induces a glutamatergic LTP onto VTA DA neurons as evidenced by an increase in AMPAR/NMDAR ratio after a single *in vivo* injection of morphine. The AMPAR/NMDAR ratio is an assessment of glutamatergic potentiation that reveals

whether AMPAR function is increased by comparing it to NMDAR function which is assumed to not change. This LTP is NMDAR-dependent and is a result of increased postsynaptic insertion of inwardly-rectifying Ca^{2+} permeable GluA2-lacking AMPARs following acute exposure to morphine [33]. Our previous work demonstrated that a single injection of morphine 24 hours before brain slice recording blocked the induction of GABAergic LTD onto VTA dopamine neurons. How these synaptic plasticities are maintained after acute morphine exposure is not entirely known; however, epigenetic mechanisms are likely candidates for maintenance of drug-induced synaptic plasticity.

Epigenetic mechanisms in addiction

Although transcription factors such as cAMP response element binding protein (CREB) and deltaFosB that bind to the promoter region of genes are the classical mechanism of gene expression regulation, regulation of gene expression via modification of DNA and chromatin structure is equally important [36]. Such modifications are mechanisms of epigenetic modulation. The major epigenetic modifications are DNA methylation, histone tail methylation, and acetylation. Of these, histone tail acetylation is of interest due to a large amount of experimental evidence that this epigenetic modification underlies functional abnormalities seen in addiction. Acetylation of histone tails is mediated by acetylating and deacetylating enzymes, namely histone acetyltransferases (HATs) and histone deacetylases (HDACs). The balance of activity between HATs and HDACs determines the acetylation profile of the chromatin.

Histone tail acetylation by HATs can encode environmental stimuli changes in mRNA expression levels by chromatin remodeling; thus, promoting gene transcription. Such chromatin modification has been shown to alter gene expression in neurons and

neuroplasticity underlying memory formation [37, 38]. Acute and chronic administration of psychostimulants and morphine elicits histone modifications such as histone acetylation at a specific subset of learning-associated genes in the brain reward circuitry that could facilitate and consolidate lasting neural and behavioral plasticity induced by drug abuse. Moreover, increasing histone acetylation through pre- or co-administration of HDACis in conjunction with abused drugs promotes or opposes drug-induced synaptic and behavioral abnormalities suggesting that this epigenetic mark might be required to potentiate or prevent an addicted state [39, 40]. In this dissertation I have analyzed an epigenetic modification associated with acute morphine administration that maintains morphine induced synaptic plasticity in VTA DA neurons.

Lateral habenula

The habenula is an epithalamic brain area that is well conserved in vertebrates and believed to be part of a common vertebrate mechanism to regulate monoaminergic systems. It is divided into a medial and lateral portion, named medial habenula (mHb) and LHb, respectively. The mammalian LHb has recently received appreciation for its role in processing aversive and reward-related information as well as its involvement in emotion and cognitive flexibility. The LHb is well equipped to perform these functions in mammals, as it acts as a bridge between limbic system inputs and monoaminergic systems. In phylogenetically older vertebrates, the LHb receives inputs containing sensory information as opposed to emotional [41].

The LHb receives both glutamatergic and GABAergic innervation from the basal ganglia and some limbic forebrain regions. Glutamatergic afferents to the LHb arise primarily from the entopeduncular nucleus (EPN), lateral hypothalamus area, anterior

cingulate, and medial PFC [42-45]. GABAergic afferents arise from the EPN, diagonal band of Broca, VTA, NAc, substantia innominata, and ventral pallidum [44-47]. The LHb can be divided into 15 subnuclei based on morphological features which consist mostly of glutamatergic neurons-- as evidenced by a widespread expression of vesicular glutamate transporter VGluT2 [43, 48, 49]. However, small scattered amounts of GABAergic neurons have been reported in the lateral-most subnuclei of the LHb.

Axons from the LHb project to both the VTA and raphe nuclei (RN) through the fasciculus retroflexus (FR), a descending fiber tract [50, 51], making the LHb one of the few brain areas able to directly influence the release of both dopamine and serotonin. Aversive stimuli and reward omission activate the LHb, which in turn leads to the inhibition of VTA DA neurons. Overall, the LHb exerts a strong inhibitory influence on the VTA through activation of an intermediary GABAergic structure: the RMTg as well as through stimulation of VTA GABAergic interneurons [52, 53]. When stimulated, LHb neurons inhibit 90% of putative VTA DA neurons from firing [53, 54]. When the LHb is lesioned, DA turnover is increased in terminal fields. Also, depressive-like behavior is reduced in rodent models of depression after LHb lesioning [55, 56].

Extrahypothalamic corticotropin releasing factor signaling

CRF, a 41-amino acid neuropeptide, mediates behavioral responses to stress and is implicated in a multitude of stress and mood related disorders. Although first recognized for its participation in stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, CRF also acts centrally within the brain (extrahypothalamic CRF system) where it directly contributes to a behavioral state of anxiety. CRF expressing neurons are found throughout the brain with especially high levels in the hypothalamus,

hippocampus, bed nucleus of stria terminalis (BNST), extended amygdala, and raphe nuclei [57]. Many of these brain areas are implicated in illnesses such as major depressive disorder (MDD), post-traumatic stress disorder (PTSD), and addiction. It is believed that dysfunctional CRF signaling in these areas may underlie such disorders [58-60]. Indeed, CRF levels are elevated in CSF collected from humans with either MDD or PTSD [61, 62].

The receptors for CRF, CRFR1 and CRFR2, are both G-protein coupled receptors (GPCRs). Activation of these receptors has a diverse range of neuronal effects depending on the brain region in which they are expressed [57]. CRF modulates neuronal cell excitability, synaptic transmission, and plasticity through its action on post- and/or pre-synaptic CRF receptors. This diversity can result from regional dependent interaction with G protein systems and signaling downstream of receptor activation. Indeed, while activation of CRFR1 most commonly engages the cAMP-PKA pathway, it has been demonstrated that in regions such as the VTA, CRF activates PLC-PKC signaling and enhances the hyperpolarization activated cationic current (ih) to increase dopamine neuron firing ; and furthermore, in these same neurons, CRF also regulates the intracellular calcium concentration which in turn potentiates the activity of calcium-sensitive small conductance potassium (sK) channels to influence neuronal excitability [57, 63, 64]. In some brain regions, the response of neurons to CRF is dependent on previous exposures to stress, with stress increasing GABAergic transmission in CRF neurons in the CeA and BNST, and switching the action of CRF from appetitive to aversive in the NAc after severe stress; once again highlighting the importance of both region and experience in determining the action of CRF [65, 70].

In general, activation of CRFR1 is anxiogenic. Both overexpression and drug-induced activation of CRFR1 have been shown to increase anxiety-like behavior in rodents [66, 67]. Furthermore, rodents with blockade of CRFR1 or a deficiency of CRFR1 display decreased anxiety-like behavior [68]. The role of CRFR2 signaling is not as well defined as CRFR1. The prevailing view of CRFR2 signaling is that it promotes stress recovery by counteracting CRFR1 signaling to achieve homeostasis. However, recent studies have shown that CRFR2 activation in the lateral septum and the BNST is anxiogenic, complicating matters further [69]. In addition, both CRFR1 and CRFR2 have several splice variants with differential expression in tissues throughout the body. These receptors also bind other CRF-related neuropeptides: in mammals, urocortins, and in non-mammals, sauvagine and urotensin-1. While urocortin 1 binds with similar affinity to CRFR1 and CRFR2, urocortin 2 and 3 both bind with higher affinity to CRFR2 [57].

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CHAPTER 2: Histone deacetylase inhibition rescues maternal deprivation-induced GABAergic metaplasticity through restoration of AKAP signaling

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SUMMARY

Adverse early life experiences such as child neglect and abuse increase the risk of developing addiction and stress-related disorders through alterations in motivational systems including the mesolimbic dopamine (DA) pathway. Here we investigated whether a severe early life stress (i.e., maternal deprivation, MD) promotes DA dysregulation through an epigenetic impairment of synaptic plasticity within ventral tegmental area (VTA) DA neurons. Using a single 24h episode of MD and whole-cell patch clamp recording in rat midbrain slices, we show that MD selectively induces long-term depression (LTD) and shifts spike timing-dependent plasticity (STDP) toward LTD at GABAergic synapses onto VTA DA neurons through epigenetic modifications of postsynaptic scaffolding A-kinase anchoring protein 79/150 (AKAP79/150) signaling. Histone deacetylase (HDAC) inhibition rescues GABAergic metaplasticity and normalizes AKAP signaling in MD animals. MD-induced reversible HDAC-mediated GABAergic dysfunction within the VTA may be a mechanistic link for increased propensity to mental health disorders following MD.

Running title: GABAergic metaplasticity in the VTA by MD

INTRODUCTION

Child neglect and abuse are pervasive and costly public health concerns with serious negative long-term effects on child health and development including an increased risk of mental health disorders and substance abuse in later life [1]. Understanding the consequences of child abuse and neglect on neural processes that shape memories and guide behaviors is critical for developing psychological and pharmacological interventions to improve outcomes of these children. Synaptic plasticity (changes in synaptic strength) is an experience-dependent learning mechanism of the brain used to process and store information vital for survival. However, prolonged exposure to stress and addictive drugs dysregulates synaptic plasticity within mesocorticolimbic brain systems (i.e., the brain reward pathway), thereby shaping pathological learning of anxiety-, depressive-, and addictive-like behaviors [2]. A single 24h episode of early MD in rodents has been widely used to model severe early life stress such as child abuse and neglect. This model was shown to promote behavioral impairments with disturbances in stress responsiveness that resemble anxiety-, depressive-, psychotic-, and addictive-like symptoms [3-8]. During MD, the hypothalamic-pituitary-adrenal (HPA) stress response is persistently upregulated and the ability of MD animals to cope with stressful situations is significantly diminished with symptoms evident from adolescence and into adulthood [5]. Moreover, MD rodents, and adolescent human subjects reporting low parental care, show heightened impulsivity and exhibit anxiety-like behaviors associated with increased levels of monoamine neurotransmitters including DA in the mesocorticolimbic dopaminergic pathway [1, 9-12].

The mesocorticolimbic dopaminergic pathway originating from the VTA controls mood and motivation. DA release from the VTA is a key detector of reward response and is

involved in reward-related learning. Dysregulation of DA signaling from the VTA has been linked to neuropsychiatric disorders. However, whether early MD affects the reward learning processes and synaptic function in the VTA is unknown. Strengthening (long term potentiation, LTP) and weakening (LTD) of excitatory and inhibitory synapses onto VTA DA neurons critically influences DA cell firing and release [13]. Investigations are currently aiming to identify patterns of learning-related DA neuronal activity and signaling mechanisms that drive activity-dependent plasticity of VTA DA neurons. Such knowledge will lead to a better understanding of the function DA neurons serve in reward-related behaviors and how the brain reward circuitry is remodeled by stress and addictive drugs. There is an increasing interest in the functional roles of synaptic plasticity at inhibitory GABAergic synapses in brain circuits; particularly the brain reward pathway where GABAergic dysfunction significantly contributes to the pathophysiology of depression, addiction, anxiety, and schizophrenia [13, 14].

Epigenetic mechanisms regulate gene expression without altering the DNA sequence. Many of these are activated by early environmental experiences and shape neuronal plasticity, and hence behavior. Epigenetic modifications in the VTA such as DNA methylation are critical for the formation of reward learning [15]. Chromatin modifications by enzymes such as HDACs also alter gene expression in neurons and neuroplasticity underlying memory formation [16, 17]. GABA_A receptors (GABA_AR) are epigenetically regulated in response to early life stress and addictive drugs [18, 19]. GABA_AR signaling in DA neurons plays a key role in reward-motivated learning [20, 21]. However, it is unknown which epigenetic mechanisms influence GABAergic plasticity and mediate maladaptive forms of such plasticity in the VTA. Because of the

importance of GABA_AR signaling in reward learning, and the critical implications of epigenetic regulation of memory, stress-related disorders, schizophrenia, and addiction [17, 22, 23], we hypothesized that MD would trigger HDAC-mediated epigenetic changes in the gene encoding the scaffold protein AKAP150 (human 79/rodent 150, also referred to as AKAP5) in the VTA. AKAP150 is an important integrator of signaling molecules to glutamatergic and GABAergic synapses that control the synaptic trafficking underlying synaptic plasticity [24-28]. Epigenetic changes leading to altered AKAP signaling would in turn selectively reduce GABAergic inhibition in the VTA and thus contribute to the development of later psychopathology following MD. Indeed, AKAP proteins are epigenetically regulated [29, 30] and global knockout of AKAP150 is associated with synaptic and behavioral abnormalities [31]. Therefore, we tested whether a single 24h episode of MD, on postnatal day 9 (P9), would affect synaptic function and plasticity of VTA DA neurons through reversible epigenetic modifications of genes involved in AKAP signaling and learning mechanisms in the VTA. Our results suggest that MD induces an aberrant GABAergic metaplasticity through reversible epigenetic mechanisms that alter the AKAP signaling associated with memory formation in the VTA.

Results

Repeated or prolonged exposure to stress increases DA levels in projection areas of the VTA leading to heightened impulsivity and anxiety-like behaviors from adolescence to adulthood [32]. MD-induced changes in synaptic transmission onto VTA DA neurons are likely to occur but have yet to be described. Stress effectively modulates synaptic plasticity at both glutamatergic and GABAergic synapses onto DA neurons [33, 34], thus we first tested whether MD induces an excitatory LTP in VTA DA neurons.

MD did not alter glutamatergic synaptic transmission and plasticity of VTA DA neurons.

Although acute stress potentiates glutamatergic synapses onto VTA DA neurons [33], we found that AMPAR/NMDAR ratios (commonly used as a reliable measurement for glutamatergic plasticity) measured in VTA DA neurons from 14-21 days old non-MD and MD rats showed no significant difference (Fig. 1a-c, non-MD, $n=8$: 0.86 ± 0.23 ; MD, $n=9$: 0.81 ± 0.20 ; unpaired Student's *t*-test: $p=0.87501$). To further test whether MD induced changes in the function and/or surface expression of AMPARs (postsynaptic plasticity) or glutamate release from glutamatergic terminals (presynaptic plasticity) onto VTA DA neurons, we recorded AMPAR-mediated miniature EPSCs (mEPSCs) in the presence of the sodium channel blocker, tetrodotoxin (TTX, $1\mu\text{M}$) in slices prepared from non-MD and MD rat pups. We found that the average and cumulative probability of AMPAR-mediated mEPSC amplitudes and frequencies did not differ between MD and non-MD rats suggesting that MD did not change AMPAR function in VTA DA neurons (Fig. 1f-j, non-MD, $n=8$: 13.47 ± 0.7 pA, 3.848 ± 0.36 Hz; MD, $n=9$: 12.75 ± 0.59 pA, 3.249 ± 0.36 Hz; unpaired Student's *t*-tests: $p=0.19573$ for amplitude, $p=0.12262$ for frequency; Kolmogorov-Smirnov (KS) tests for cumulative distribution curves: $P=0.927$ for amplitude, $p=0.989$ for frequency). MD alters AMPAR and NMDAR subunit composition in the hippocampus [3, 35, 36] and given that such changes in AMPAR subunit composition could occur without alteration in overall synaptic strength measured by mEPSC amplitude or AMPAR/NMDAR ratios, we recorded evoked AMPAR-mediated EPSCs at different holding potentials while intracellularly applying spermine. This allowed us to detect the relative contribution of inwardly rectifying Ca^{2+} -

permeable/GluA2-lacking AMPARs to synaptic transmission. AMPAR rectification was unaltered after MD and EPSC IV plots were linear in both groups. This is consistent with the presence of GluA2 in most synaptic AMPARs in MD rats similar to non-MD rats (Fig. 1d, e, AMPAR rectification indices: non-MD, n=5: 1.444 ± 0.23 ; MD, n=5: 1.4677 ± 0.33 ; unpaired Student's *t*-test: $p=0.9548$; two way ANOVA for IV curves, $F_{(1,5)}=0.01102$, $p=0.92047$).

MD induced pre- and postsynaptic LTD at GABAergic synapses onto VTA DA neurons.

Acute stress was previously shown to block a presynaptic form of LTP at GABAergic synapses onto VTA DA neurons [34, 37]. We tested the effects of MD on GABA_AR-mediated miniature IPSCs (mIPSCs) to identify whether MD per se induced any form of GABAergic plasticity. We found that the frequency and amplitude of mIPSCs were significantly reduced in MD compared to non-MD rats. (Fig. 2a-b, non-MD, n=6: 30.25 ± 0.244 pA, 4.92 ± 0.38 Hz; MD, n=8: 22.33 ± 1.06 pA, 3.42 ± 0.45 Hz; unpaired Student's *t*-tests: $p=0.0067$ for amplitude, $p=0.0316$ for frequency; KS tests for cumulative distribution curves: $P < 0.0001$ for amplitude, $p=0.036$ for frequency). These findings suggest that MD suppressed both pre- and post-synaptic function of GABAergic synapses onto VTA DA neurons.

MD shifted STDP at GABAergic synapses onto DA neurons toward LTD.

Previous experiences such as *in vivo* exposure to addictive drugs, stress, sensory or visual deprivation can change the ability of synapses to undergo subsequent plasticity in response to LTP and/ LTD induction protocols. This phenomenon is referred as metaplasticity [38]. Although traditional induction protocols of LTP and LTD have been successfully used to

induce synaptic plasticity, near coincidental pre- and post-synaptic firing can also trigger a form of plasticity where the precise timing and patterns of spikes are critical (i.e., STDP). This is an attractive induction protocol since it mimics naturally occurring neuronal activity required for plasticity [39]. Recently, we showed that GABAergic synapses onto VTA DA neurons were capable of exhibiting a bidirectional postsynaptic Hebbian STDP (i.e., STD-LTP_{GABA} and STD-LTD_{GABA} in response to repeated pre-post spike pairings at positive interval of +15ms and post-pre spike pairings at negative interval of -5ms, respectively). Interestingly, this STDP was triggered by correlated activities of the presynaptic glutamatergic input and postsynaptic DA cells where NMDARs served as coincident detectors for this heterosynaptic plasticity [40]. Given that early MD is a potent stressor, we hypothesized that MD induces metaplastic changes in GABAergic synapses that subsequently alter the response of DA neurons to STDP protocols. Therefore, we used STDP protocols to induce GABAergic STDP in midbrain slices prepared from non-MD and MD rats. To define the temporal window for STDP induction, we tested three additional STDP protocols with different time intervals (-15ms, +5ms and +25ms). Data for time courses of STDP at these three additional intervals are shown in Fig. S1 and the averaged magnitude of synaptic change for all STDP experiments with a given Δt are depicted in Fig. 3e. Pairings at a negative interval of -15ms and a positive interval of +25ms did not induce any form of plasticity in slices from non-MD or MD rats demonstrating a short temporal window for GABAergic STDP in the VTA. Interestingly, a robust STD-LTD_{GABA} was induced in response to pre-post positive pairing at +5ms intervals in slices from MD rats while non-MD rats did not exhibit STDP at this time interval. Moreover, slices prepared from MD animals did not exhibit STD-LTP_{GABA} at +15ms (in fact, the

polarity of STDP in response to pre-post pairings at this interval shifted toward LTD) whereas non-MD rats showed robust STD-LTP_{GABA}. STD-LTD_{GABA} (induced in response to negative pairings at -5 ms) was also absent in slices from MD rats, whereas plasticity was intact in slices from non-MD rats. Altogether these data suggest that MD-induced metaplastic changes impaired the ability of GABAergic synapses to exhibit normal bidirectional STDP, narrowed the STDP window and shifted STDP toward LTD. In Fig.3, the time courses of STDP are only shown at the negative timing of -5ms and positive timing of +15ms. In the remainder of the paper only these two intervals are used for STDP induction and are designated as “pre-post” for positive timing of +15ms and “post-pre” for negative timing of -5ms (Fig.3a,d, for STD-LTD_{GABA} , non-MD, n=6: 64±2.4% of pre-STDP values, $F_{11.53,57.67}=11.09$, $p<0.0001$, MD, n=7: 96±2.7% of pre-STDP values, $F_{9.43,37.73}=0.478$, $p=0.886$, unpaired Student's *t*-test for STD-LTD_{GABA} between MD and non-MD rats, $p<0.001$; Fig. 3b,c for STD-LTP_{GABA}: non-MD, n=8: 151±8.4% of pre-STDP values, $F_{5.26,36.87}=3.369$, $p=0.012$, MD, n=8: 85±21% of pre-STDP values, $F_{3.32,23.26}=3.368$, $p=0.032$, unpaired Student's *t*-test for STD-LTP_{GABA} between MD and non-MD rats, $p<0.001$; Fig. 3e shows the temporal windows of STDP in non-MD and MD rats).

GABAergic STDP in the VTA required protein kinase A (PKA)-AKAP-calcineurin (CaN) signaling complex.

Although we identified a shift in GABAergic STDP toward LTD following MD, the cellular and molecular mechanisms underlying this metaplasticity remained unknown. To identify potential mechanisms of metaplasticity, we first characterized STDP. Previously we discovered that the AKAP150 complex selectively controlled GABA_AR trafficking-mediated GABAergic plasticity in the VTA. Postsynaptic disruption of PKA-AKAP

association or PKA inhibition induced a CaN-dependent long-lasting reduction in synaptic GABA_A responses that mimicked LTD_{GABA} (induced in response to a traditional LTD pairing protocol) without affecting AMPAR-mediated glutamatergic transmission in VTA DA neurons [24, 41]. Here, we determined whether the PKA-AKAP-CaN complex also underlies GABAergic STDP. Consistent with our previous results, intra-pipette AKAP inhibitor Ht31 (1 μ M) resulted in a remarkably rapid rundown of synaptic transmission whereas control peptide Ht31p (1 μ M) did not (Fig. 4a, Ht31p cells, n=19: 93 $\pm$ 4% of the first 5 min baseline values, $F_{10.387, 46.161} = 1.035$, $p = 0.44$; Ht31 cells, n=14: 58 $\pm$ 1% of the first 5 min baseline values, $F_{15.902, 47.707} = 6.614$, $p < 0.00001$). Once Ht31-induced depression plateaued, STDP protocols were used to trigger STD-LTP_{GABA} or STD-LTD_{GABA}. Intra-pipette Ht31 prevented the induction of STD-LTP_{GABA} while Ht31p-filled cells expressed STD-LTP_{GABA} (Fig. 4b,c, Ht31p cells, n=7: 140 $\pm$ 2% of pre-STDP values, $F_{10.127, 9.922} = 6.124$, $p < 0.0001$; Ht31 cells, n=7: 99.3 $\pm$ 2.8% of pre-STDP values, $F_{11.78, 47.151} = 0.93$, $p = 0.453$). Moreover, intra-pipette Ht31-induced rundown occluded STD-LTD_{GABA} whereas Ht31p did not affect the plasticity (Fig. 5a, Ht31p cells, n=12: 71 $\pm$ 2% of pre-STDP values, $F_{9.192, 45.962} = 6.316$, $p < 0.0001$; Ht31 cells, n=7: 105 $\pm$ 3% of pre-STDP values, $F_{14.945, 59.779} = 0.878$, $p = 0.591$) suggesting that the disruption of association between PKA and AKAP is sufficient to induce STD-LTD_{GABA}. Consistent with our previous results, postsynaptic inhibition of PKA by intracellular inclusion of PKI₍₆₋₂₂₎ (10 μ M), a membrane impermeant PKA inhibitor, significantly reduced IPSC amplitude (Figure 4d,e; PKI cells, n=6: 63 $\pm$ 2% of the first 5 min baseline values, $F_{11.37, 45.48} = 21.145$, $p < 0.0001$). PKI₍₆₋₂₂₎-induced depression of IPSCs not only inhibited STD-LTP_{GABA} but also shifted the polarity of plasticity toward LTD (Figure

4e,f; control STD-LTP for PKI₍₆₋₂₂₎, n=7: 121±1.4% of pre-STDP values, $F_{8.58,34.34} = 3.356$, $p = 0.005$; PKI₍₆₋₂₂₎ cells, n=6: 76.6±1.1% of pre-STDP values, $F_{16.95,50.86} = 3.147$, $p = 0.01$). This suggests that PKA association with AKAP150 promotes LTP and may control the polarity of STDP. We also confirmed that similar to LTD_{GABA} induced by our LTD pairing protocol [24], STD-LTD_{GABA} was postsynaptic, AKAP- and CaN-dependent, and involved clathrin-mediated endocytosis. We attempted to induce STD-LTD_{GABA} induction in the presence of intra-pipette CaN inhibitors (1 μ M cyclosporin A or FK506). Both CaN inhibitors blocked STD-LTD_{GABA} (Fig. 5c, control STD-LTD_{GABA} for cyclosporin A, n=5: 60±0.5% of pre-STDP values, $F_{8.94,26.84} = 7.026$, $p < 0.0001$; cyclosporine A cells, n=6: 98±1.3% of pre-STDP values, $F_{7.5,15} = 0.870$, $p = 0.557$; Fig. 5d, control STD-LTD_{GABA} for FK506, n=6: 61±2.8% of pre-STDP values, $F_{7.92,31.69} = 6.277$, $p < 0.0001$; FK506 cells, n=4: 92±2.5% of pre-STDP values, $F_{14.84,44.54} = 0.663$, $p = 0.830$). Postsynaptic inhibition of clathrin was achieved by loading cells with 50 μ M Pitstop2TM and prevented STD-LTD_{GABA} whereas cells loaded with 50 μ M Pitstop-control peptide still exhibited STD-LTD_{GABA} (Fig. 5b, control peptide cells, n=6: 68±1% of pre-STDP values, $F_{7.38,29.15} = 6.592$, $p < 0.0001$; Pitstop cells, n=6: 109.5±1.1% of pre-STDP values, $F_{7.98,23.95} = 1.210$, $p = 0.352$).

MD disrupted the AKAP150 signaling complex at GABAergic synapses in the VTA.

Since AKAP150 signaling was implicated in GABA_AR-mediated plasticity in the VTA (including STDP here, Figs. 4&5) and MD affected GABAergic transmission and plasticity without affecting AMPAR-mediated glutamatergic transmission (Figs.1-3), we assumed that MD-induced metaplasticity (Fig. 3) involved AKAP signaling. Similarly to the experiments shown in Fig. 4a, we attempted to induce chemical LTD_{GABA} using intra-

pipette Ht31 in slices from MD and non-MD animals. Intra-pipette Ht31 did not induce a significant rundown of IPSCs in cells recorded from MD animals whereas cells recorded from non-MD animals showed a robust and rapid rundown of IPSCs. Control Ht31p-filled cells recorded from MD or non-MD rats did not show any rundown of IPSCs (Fig. 6a,b, non-MD: Ht31p cells, $n=7$, $96\pm3\%$ of the first 5 min baseline values, $F_{18.37,36.75} = 1.474$, $p = 0.155$; Ht31 cells, $n=7$, $67\pm2.8\%$ of the first 5 min baseline values, $F_{4.36, 13.079} = 13.938$, $p < 0.0001$; Fig. 6a,c, MD rats: Ht31p cells, $n=7$, $101\pm4\%$ of the first 5 min baseline values, $F_{3.67, 14.678} = 0.377$, $p = 0.807$; Ht31 cells, $n=9$, $91\pm5.8\%$ of the first 5 min baseline values, $F_{5.468, 32.808} = 1.442$, $p = 0.232$, unpaired Student's *t*-test for Ht31-induced LTD between MD and non-MD rats, $p < 0.001$). Furthermore, we confirmed that the LTD-induced by pre-post protocol in MD animals was indeed STD-LTD_{GABA} and CaN-dependent. Intra-pipette cyclosporin A (1 μ M) prevented the induction of LTD in response to pre-post protocols (+15ms) in MD animals but did not rescue STD-LTP_{GABA} (Fig. S2) suggesting that MD-induced metaplastic disruption of AKAP150 signaling blocked the induction of STD-LTP_{GABA} and facilitated the induction of STD-LTD_{GABA}.

HDAC inhibition reversed MD-induced GABAergic metaplasticity through restoration of normal AKAP signaling.

HDACs contribute to the formation of condensed and transcriptionally silenced chromatin, which decreases gene transcription. Blocking histone deacetylation using HDAC inhibitors (HDACi) has been shown to improve learning, memory and synaptic plasticity in rodents indicating that site- and cell-specific HDAC inhibition may provide a selective and promising target for enhancing memory [16, 17]. Interestingly, adverse experiences in early life such as MD and low maternal care are also associated with long-

lasting changes in the expression of critical synaptic plasticity- and GABAergic signaling-associated genes through chromatin remodeling [36, 42, 43]. Therefore, we sought to determine whether MD-induced GABAergic metaplasticity and impairment of AKAP signaling involve HDACs. We first tested whether local HDAC inhibition in the VTA (via *in vitro* incubation of midbrain slices in HDACi or vehicle) could rescue the GABAergic synaptic abnormalities that we detected in mIPSC recordings from slices of MD animals (MD-induced GABAergic LTD). We used a selective class I HDACi (CI-994, 20 μ M) which has previously been shown to attenuate remote fear memories [44]. Consistent with our previous findings (Fig. 2), significant reductions in the average amplitude and frequency of mIPSCs were detected in DA cells from vehicle (DMSO)-incubated non-MD and MD slices (Fig. 7, non-MD+DMSO, n=6: 38.89 $\pm$ 5.58 pA, 5.39 $\pm$ 1.13 Hz; MD+DMSO, n=6: 23.54 $\pm$ 2.55 pA, 3.55 $\pm$ 0.36 Hz; unpaired Student's *t*-tests: p=0.0023 for amplitude, p=0.018 for frequency). A 2-4hr *in vitro* incubation of midbrain slices prepared from MD animals in CI-994 reversed these GABAergic abnormalities (Fig. 7b, MD+DMSO, n=6: 23.54 $\pm$ 2.55 pA, 3.55 $\pm$ 0.36 Hz; MD+CI-994, n=9: 31.52 $\pm$ 1.83 pA, 5.88 $\pm$ 0.93 Hz; unpaired Student's *t*-tests: p=0.021 for amplitude, p=0.042 for frequency; KS tests for cumulative distribution curves: P <0.0001 for amplitude, p<0.0001 for frequency). CI-994 significantly reduced the amplitude and frequency of mIPSCs in HDACi-incubated midbrain slices from non-MD rats although no significant difference between the average frequency of mIPSCs was detected after HDACi treatment (Fig. 7a, non-MD+DMSO, n=6: 38.89 $\pm$ 5.58 pA, 5.39 $\pm$ 1.13 Hz; non-MD+CI-994, n=8: 28.63 $\pm$ 1.38 pA, 4.74 $\pm$ 0.47 Hz; unpaired Student's *t*-tests: p=0.045 for

amplitude, $p=0.568$ for frequency; KS tests for cumulative distribution curves: $P < 0.0001$ for amplitude, $p= 0.043$ for frequency).

Next we investigated the effects of *in vitro* HDAC inhibition on MD-induced metaplasticity of STDP using CI-994 and a widely-used HDACi, sodium butyrate (NaB, 300 μ M) [45]. We found that 2-4 hr *in vitro* incubation of midbrain slices prepared from MD animals in either NaB or CI-994, also recovered STDP to normal levels in these slices (Fig. 8b, STD-LTD_{GABA}: MD, $n=7$: $96 \pm 2.7\%$ of pre-STDP values, $F_{9.43, 37.73}=0.478$, $p=0.886$, MD+NaB, $n=7$: $73 \pm 2\%$ of pre-STDP values, $F_{9.705, 48.523}=8.523$, $p<0.0001$; Fig. 8d, MD+DMSO, $n=4$: $108 \pm 2.7\%$ of pre-STDP values, $F_{34, 104}=0.013$, $p=0.765$, MD+CI-994, $n=6$: $63 \pm 2.4\%$ of pre-STDP values, $F_{3.86, 19.32}=7.255$, $p<0.001$; Fig. 8f, STD-LTP_{GABA}: MD rats, $n=8$: $85 \pm 21\%$ of pre-STDP values, $F_{3.32, 23.26}=3.368$, $p=0.032$, MD+NaB, $n=8$: $150 \pm 6.5\%$ of pre-STDP values, $F_{12.19, 24.39}=4.01$, $p<0.001$; Fig. 8h, MD+DMSO, $n=6$: $65 \pm 0.5\%$ of pre-STDP values, $F_{29.8, 119.25}=7.044$, $p<0.0001$, MD+CI-994, $n=4$: $142 \pm 2\%$ of pre-STDP values, $F_{21.72, 43.45}=3.144$, $p<0.001$). HDAC inhibitors had very different effects on VTA GABAergic synapses in slices from non-MD versus MD animals. Although these drugs inhibited GABAergic transmission and disrupted STDP in non-MD animals, synaptic transmission and plasticity in MD animals were normalized (Fig. 8a, STD-LTD_{GABA}: non-MD, $n=6$: $64 \pm 2.4\%$ of pre-STDP values, $F_{11.53, 57.67}=11.09$, $p<0.0001$, non-MD+NaB, $n=6$: $75 \pm 3\%$ of pre-STDP values, $F_{15.23, 45.69}=2.436$, $p<0.01$; unpaired Student's *t*-tests: $p<0.001$ for LTD levels between non-MD and non-MD+NaB; Fig. 8c, non-MD+DMSO, $n=5$: $60 \pm 3.1\%$ of pre-STDP values, $F_{11.35, 22.7}=6.087$, $p<0.0001$, non-MD+CI-994, $n=4$: $99 \pm 0.6\%$ of pre-STDP values, $F_{30.9, 92.75}=1.266$, $p=0.243$; Fig. 8e, STD-LTP_{GABA}: non-MD, $n=8$: $151 \pm 8.4\%$ of pre-STDP values, $F_{5.26, 36.87}=3.369$, $p=0.012$,

non-MD+NaB, n=9: $117 \pm 11\%$ of pre-STDP values, $F_{4.74,9.485}=0.778$, $p=0.583$; Fig. 8g, non-MD+DMSO, n=6: $139 \pm 5.8\%$ of pre-STDP values, $F_{21.59,86.39}=5.022$, $p<0.0001$ non-MD+CI-994, n=5: $119 \pm 2.4\%$ of pre-STDP values, $F_{8.49, 25.48}=1.23$, $p=0.332$).

To confirm whether the HDACi-mediated reversal of GABAergic metaplasticity induced by MD is due to normalization of AKAP signaling by HDAC inhibition, we tested the effects of HDACis (NaB and CI-994) on Ht31-induced depression in slices from non-MD and MD rats. HDACi incubation also restored the Ht31-induced rundown of IPSCs in midbrain slices from MD animals (Fig. 6c, MD+Ht31, n=9: $91 \pm 5.8\%$ of the first 5 min baseline values, $F_{5.468, 32.808} = 1.442$, $p = 0.232$; MD+Ht31+NaB, n=11: $60 \pm 2.8\%$ of the first 5 min baseline values, $F_{4.596,13.787}= 13.77$, $p < 0.0001$; Fig 6e, MD+Ht31+DMSO, n=5: $99.5 \pm 2.5\%$ of the first 5 min baseline values, $F_{4.54,13.63} = 0.58$, $p = 0.701$; MD+Ht31+CI-994, n=6: $65 \pm 1.3\%$ of the first 5 min baseline values, $F_{17.97,84.86}= 15.133$, $p < 0.0001$). Ht31-induced run-down remained unchanged after NaB or CI-994 treatment in slices from non-MD rats (Fig. 6b, non-MD+Ht31, n=7: $67 \pm 2.8\%$ of the first 5 min baseline values, $F_{4.36, 13.079} = 13.938$, $p < 0.0001$; non-MD+Ht31+NaB, n=6: $68 \pm 1.3\%$ of the first 5 min baseline values, $F_{6.76,33.82}= 11.517$, $p < 0.0001$; Fig. 6d, non-MD+Ht31+DMSO, n=6: $64.9 \pm 1.4\%$ of the first 5 min baseline values, $F_{3.85,15.42} = 12.973$, $p < 0.0001$; non-MD+Ht31+CI-994, n=4: $56 \pm 1.4\%$ of the first 5 min baseline values, $F_{11.2,22.3}= 4.999$, $p < 0.0001$).

Given that local HDAC inhibition (through two structurally different HDACis) was sufficient to reverse metaplastic abnormalities of GABAergic transmission, we hypothesized that sustained HDAC-mediated downregulation of AKAP150 gene expression may occur in VTA DA neurons following MD. A decrease in the availability

of AKAP proteins at GABAergic synapses in the VTA could promote less confinement of GABA_ARs at the synapse thereby favoring LTD (MD-induced metaplasticity). To test this hypothesis, we performed a double-immunofluorescence staining technique using antibodies against TH (marker for DA neurons) and AKAP150 allowing us to visualize DA neurons expressing AKAP150. Surprisingly, we detected higher levels of AKAP immunoreactivity in TH-positive cells of MD compared to non-MD rats at three AP levels within the VTA (-5.4, -5.7 and -6 mm caudal to bregma, Paxinos and Watson, 2007) (Fig. 6f, g, non-MD, n=5; MD, n=6; two-way ANOVA, $P=0.0016$). To further determine AKAP expression in the VTA, we performed Western blots of VTA homogenates isolated from non-MD and MD rats. There was no significant difference in AKAP expression in the VTA between non-MD and MD rats (Fig. 6h, i, non-MD, n=5; MD, n=5, unpaired Student's *t* test, $p=0.9056$). Thus, the enhanced AKAP immunofluorescence may be related to translocation of AKAP away from the synapse, uncoupling AKAP from GABA_ARs. Together, our data provide the first experimental evidence linking the scaffolding protein, AKAP150, to induction of metaplasticity by severe early life stress at inhibitory synapses. The restoration of normal levels of GABAergic transmission, and STDP by HDACis in slices from MD animals is achieved locally in the VTA through epigenetic rescue of AKAP signaling to VTA DA neurons.

Discussion

Our study demonstrated that severe early life stress may promote the dysregulation of DA signaling and reward circuitry through an epigenetic-mediated GABAergic metaplasticity in the VTA. This epigenetic modification involved the postsynaptic scaffolding protein AKAP150 and was potentially reversible if targeted during the early stages following trauma.

A number of studies have found a strong link between the dysregulation of DA signaling by early post-natal life stress such as child maltreatment and a later propensity to develop stress-related disorders and addiction. This link raises the possibility that alterations in VTA DA neuronal activity and synaptic function may occur following childhood trauma [1]. Moreover, childhood adversities trigger dysfunction in epigenetic control of neuronal activity and synaptic plasticity, thus having a significant impact on the brain and behavior [36]. Histone modifications are induced by MD through HDAC activity [46-48] suggesting the potential therapeutic benefits of early life epigenetic intervention. These observations led us to hypothesize that early life adversity engages epigenetic mechanisms regulating synaptic activity and learning mechanisms in VTA DA neurons and that these synaptic maladaptations may be reversed by HDACis if targeted soon after the insult. HDACis have proved effective in attenuating neurodegenerative-associated cognitive declines, and fear-related memories by affecting epigenetic mechanisms involved in neuronal plasticity [44, 49]. Here, we provide the first evidence that GABAergic synaptic abnormalities occur in the VTA in response to an episode of prolonged severe early life stress (i.e., MD). In addition, our data suggest that these abnormalities are at least partly mediated through epigenetic modifications of genes involved in the AKAP signaling complex. Previously, we reported that AKAP150 signaling plays a selective role in regulating a D2 dopamine receptor (D2R)-mediated CaN-dependent GABAergic LTD. We found that disruption of PKA-AKAP association mimics and occludes this LTD without altering glutamatergic transmission in VTA DA neurons [24, 41]. Remarkably, AMPAR-mediated glutamatergic transmission and subunit composition of synaptic AMPARs were also unaffected after early MD while GABAergic transmission and plasticity in DA neurons were significantly

impacted by MD. This is consistent with the findings that maternal care significantly influences the epigenetic regulation of GABAergic signaling in the brain [43]. Some of our observations here implicate MD-induced GABAergic metaplasticity in VTA DA neurons through signaling modifications that involve AKAP150. Inhibition of PKA-AKAP interaction inhibited STD-LTP_{GABA} and occluded further induction of STD-LTD_{GABA} suggesting that AKAP-anchored PKA and CaN also underlie postsynaptic STDP. Although MD per se induced a postsynaptic LTD (suggested by the reduced amplitude of mIPSCs indicative of postsynaptic modifications) and occluded STD-LTD_{GABA} in response to post-pre protocol, MD changed the learning rules of bidirectional Hebbian STDP at GABAergic synapses in the VTA to enable LTD. We found that GABAergic synapses of MD rats exhibited LTD in response to pre-post pairing protocol at +5ms while non-MD rats did not show any form of GABAergic STDP. Moreover, MD not only prevented the induction of STD-LTP_{GABA} in response to pre-post pairing at +15ms but also shifted the polarity of this plasticity towards LTD. MD-induced LTD in response to pre-post protocols at +15ms was CaN-dependent. More importantly, CaN blockade did not rescue STD-LTP_{GABA}. The fact that LTP was not present even after the blockade of CaN-dependent LTD confirms that the induction of LTP was inhibited by MD and not simply masked by MD-induced LTD. Thus MD-induced metaplasticity facilitated CaN-dependent STD-LTD_{GABA} while inhibiting STD-LTP_{GABA}.

Postsynaptic AKAP inhibition was insufficient to trigger chemical LTD in MD slices suggesting that disruption of AKAP signaling by MD may be the mechanistic link for MD-induced metaplasticity and synaptic deficits. Local HDAC inhibition in the VTA was sufficient to reverse metaplastic abnormalities of GABAergic transmission by MD.

However, MD did not change the levels of AKAP150 protein in VTA extracts suggesting that MD disrupted AKAP signaling without transcriptional repression of the AKAP150 gene. Interestingly, we detected higher levels of AKAP immunoreactivity in VTA DA neurons in MD pups. Thus, MD likely dysregulated normal AKAP postsynaptic targeting at GABAergic synapses onto VTA DA neurons which led to the translocation of AKAP or AKAP-interacting proteins away from the synapse. AKAP complexes are not stationary and can be redistributed dynamically in response to stimuli such as NMDAR activation or rises in intracellular Ca^{2+} [50, 51]. Consistent with this hypothesis, NMDAR-dependent glutamatergic LTD in the hippocampus involves a CaN-dependent translocation of AKAP150 and its anchored pool of PKA away from synapses. This in turn uncouples AKAP-PKA complexes from CaN-dephosphorylated AMPARs and prevents their subsequent re-phosphorylation by PKA [51]. AKAP150 is also required for PKA phosphorylation of GABA_ARs in the hippocampus [52] although it is unknown how AKAP150 signaling complexes are formed at GABAergic synapses and coordinate the opposing functions of kinases and phosphatases under physiological and pathological conditions. The interaction between AKAP150 and the GABA_AR subunits also occurs in the Golgi apparatus [53] which raises the possibility that MD may have triggered a redistribution of AKAP that influenced the subcellular localization and anchoring of PKA and CaN at GABAergic synapses. Therefore, the rescue of normal AKAP signaling and GABAergic plasticity in slices from MD rats by HDACis may occur through the reversal of HDAC-mediated transcriptional changes in the expression of AKAP-interacting proteins or signaling molecules upstream to AKAP that are necessary for proper functioning, interaction or localization of AKAP at the synapse.

Similar to what we observed with MD animals, postsynaptic inhibition of PKA alone is sufficient to shift the polarity of STDP toward LTD in response to pre-post firing suggesting that changes in PKA activity downstream to neuromodulators such as DA may be a critical determinant for the direction of STDP. Given that D2R stimulation through PKA inhibition promotes postsynaptic LTD_{GABA} in VTA DA neurons [24], it will not be surprising that part of MD-induced facilitation of LTD and disruption of AKAP signaling involve reversible epigenetic changes in DA-D2R- PKA mediated signaling that facilitates CaN activity. Although the levels of NMDAR activation and the ensuing increase in intracellular Ca^{2+} are considered the main determinants of the polarity of synaptic changes, neuromodulators including DA can control this polarity and change the rules of STDP induction [54]. Therefore, it will be worthwhile to investigate whether MD increases dendrodendritic release of DA in the VTA and promotes D2R-mediated modulation of GABAergic metaplasticity involving AKAP. In a scenario where dendrodendritic DA release is elevated by MD, this can also lead to presynaptic inhibition of GABA release from GABAergic terminals onto DA neurons (suggested by the decrease in the frequency of IPSCs in MD slices which is also recovered by HDAC inhibition in our experiments) through activation of presumably presynaptic D2Rs and engaging endocannabinoid signaling and presynaptic cAMP-PKA pathway enabling a presynaptic LTD [55, 56].

MD-induced GABAergic synaptic modifications could shift the balance of glutamatergic/ GABAergic synaptic inputs onto DA neurons toward a decreased GABAergic inhibition, increasing DA neuronal excitability and DA release. In our STDP experiments, postsynaptic firing is controlled by direct somatic current injections through patch

pipettes while holding the cells at -65mV to avoid random postsynaptic firing. It is plausible to assume that the increase in excitability of DA neurons *in vivo* by MD could lead to irregular and random pre-post and post-pre firing pairs. The polarity of plasticity would then favor LTD as the direction of synaptic plasticity induced in response to more complex STDP protocol is toward LTD [40].

In summary we demonstrated that MD may alter motivated- and reward-related learning later in life partly through epigenetic modifications that disrupt AKAP signaling and trigger GABAergic metaplasticity in the VTA. MD-induced metaplastic and epigenetic changes in the learning rules governing spike timing GABAergic plasticity of VTA DA neurons may reinforce association of irrelevant synaptic inputs which would have not been correlated under physiological conditions, thereby falsely exaggerating the incentive salience of stimuli such as drug-associated cues. Further mechanistic and epigenetic studies of early life stress-induced neuroplasticity within the VTA are warranted to identify novel signaling cascades in regulation of DA signaling and reward learning.

EXPERIMENTAL PROCEDURES

Male juvenile Sprague-Dawley rats were used for all studies. All procedures were performed according to approved protocols by the Uniformed Services University Animal Care and Use Committee in accordance with the guidelines set forth by the National Institutes of Health. Detailed experimental procedures can be found in supplemental methods.

Maternal deprivation procedure

For 24h MD, 5 rat pups (half of the pups from normal litters) at P9 were isolated at 10:00 AM from the dam, and siblings and placed in a separate cage with a heating pad (34°C) located in another room to avoid disturbance by vocalization in the original animal room

(MD group) as described previously [10]. The pups were not disturbed and were returned to the home cage after 24h. The remaining non-separated rat pups (5 pups) received the same amounts of handling and served as the non-maternally deprived control group (non-MD group). Each day two rats (age-matched MD and non-MD rats of the same litter) were sacrificed over P14-P21 for electrophysiology, immunohistochemistry and Western blotting. Only one cell per animal (non-MD or MD) was recorded to demonstrate that the MD effects are consistently observed across multiple animals, therefore all reported n values represent the number of animals recorded.

Slice preparation for electrophysiology

Horizontal midbrain slices (250 μ M) were prepared from 14- to 21- day old rats. Slices were cut and incubated at least 1h at 34°C before recording in artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.0 MgSO₄, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O₂/5% CO₂. Slices were then transferred to recording chamber and submerged in warm (28°C) ascorbic acid-free ACSF.

Electrophysiology

GABA_A receptor mediated IPSCs were evoked, isolated and recorded in ACSF containing 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μ M), and strychnine (1 μ M). For mIPSC recordings, tetrodotoxin (TTX, 1 μ M) was also added to the perfusion bath. The patch pipettes (3–6 M Ω) were filled with (in mM): 125 KCl, 2.8 NaCl, 2 MgCl₂, 2 ATP-Na⁺, 0.3 GTP-Na⁺, 0.6 EGTA, and 10 HEPES (pH adjusted 7.28 with KOH, osmolarity adjusted to 275-280 mOsm). Cells were voltage-clamped at -70 mV. Paired GABA_A IPSCs were stimulated at 0.1 Hz (duration 100 μ s, 50 ms inter-stimulation interval). To induce STDP, cells were taken to current clamp and received trains of a presynaptic stimulation paired with back-propagating action potentials

at 5 Hz. STDP protocols consisted of 30 trains of five bursts repeated at 0.1 Hz. For STD-LTP_{GABA}, each burst was composed of three bAPs at 50 Hz and preceded by a single presynaptic stimulation (positive timing, +15ms). For STD-LTD_{GABA}, each burst was composed of three bAPs at 50 Hz followed by a single presynaptic stimulation (negative timing, -5ms).

Combined excitatory postsynaptic currents (EPSCs) were evoked and recorded in the presence of ACSF containing picrotoxin (100 μ M) at +40mV. Patch pipettes were filled with (in mM): 117 Cs-gluconate, 2.8 NaCl, 5 MgCl₂, 2 ATP-Na⁺, 0.3 GTP-Na⁺, 0.6 EGTA and 20 HEPES (pH adjusted to 7.28 with CsOH, osmolarity adjusted to 275–280 mOsm). APV (50 μ M) was added to obtain AMPAR-only EPSCs at +40mV. For AMPA receptor-mediated mEPSC recordings, APV (50 μ M) and TTX (1 μ M) were also added to ACSF that contained picrotoxin and cells were voltage-clamped at -70mV. For AMPAR rectification, AMPAR EPSCs were pharmacologically isolated and evoked as described previously [25] in the presence of ACSF containing 50 μ M APV and 100 μ M picrotoxin and inclusion of 10 μ M spermine in the patch pipette using the Cs-based internal. AMPAR EPSCs were then recorded at different holding potentials (>10/neuron) and normalized IV curves were generated by dividing the AMPAR EPSC peak amplitudes by the mean of AMPAR EPSC peak amplitude at -65mV. AMPAR rectification was determined by dividing the peak amplitudes of AMPAR EPSCs recorded at -65 mV by those recorded at +40 mV for each neuron.

Western Blots

The VTA was dissected from midbrain slices of non-MD and MD rat pups and lysed in 1% RIPA buffer containing protease inhibitors. Protein extracts were prepared, run on

SDS-PAGE gels, transferred and probed as described in supplemental methods. The primary antibody was anti-AKAP-150.

Immunohistochemistry and image analysis

Non-MD and MD rat pups were perfused with 4% paraformaldehyde and coronal sections (20 μ m) of the midbrain from MD and non-MD rat pups, containing the VTA (from -4.92 to -6.72 mm caudal to bregma) were dissected. Sections were incubated overnight with TH and AKAP150 antisera, followed by incubation with appropriate secondary antisera as described in supplemental methods.

Data analysis

Values are presented as means $\pm$ s.e.m. Statistical significance was determined using repeated measures ANOVA with significance level of $p < 0.05$ for STDP, and chemical LTD (PKI₍₆₋₂₂₎ or Ht31) experiments. Unpaired Student's *t* tests were performed subsequent to significant repeated measure ANOVAs for those experiments to make comparisons between MD and non-MD rats. Levels of STDP are reported as averaged IPSC amplitudes for 5min just before STDP induction compared with averaged IPSC amplitudes during the 5min period from 35 to 40 min after the protocol. The magnitude of STDP in Fig. 3 was expressed as percentage of synaptic change. Levels of PKI₍₆₋₂₂₎-/Ht31-induced depression are reported as averaged IPSC amplitudes for 5 min just before the emergence of drug-induced synaptic depression compared with averaged IPSC amplitudes during the last 5 min period of the peak response. The statistical differences for AMPAR/NMDAR ratio and AMPAR EPSC rectification index were evaluated using unpaired Student's *t* test. Two-way ANOVA with Bonferroni post hoc analysis was used to detect the statistical difference between non-MD and MD rats for AMPAR EPSC IV plots. Unpaired Student *t* test was used to analyze the

statistical differences in the means of amplitude and frequency of mEPSCs and mIPSCs in the represented bar graphs. The KS test was performed for the statistical analyses of cumulative probability plots of mEPSCs and mIPSCs. MD effects on AKAP intensity were compared by a two-way ANOVA and Bonferroni post-hoc analysis. AKAP expression was normalized to β -actin expression in Western blot analysis and an unpaired Student's *t* test was used to evaluate the statistical difference between the two groups.

Author contributions

F.S.N designed the research; M.E.A., J.N.K, S.G, M.R. and F.S.N performed the experiments; M.E.A., J.N.K, S.G., M.R., A.J.S, B.M.C and F.S.N analyzed the data and prepared the figures; M.E.A., J.N.K, S.G, A.J.S, B.M.C and F.S.N wrote the paper. The authors declare no conflict of interest.

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FIGURES

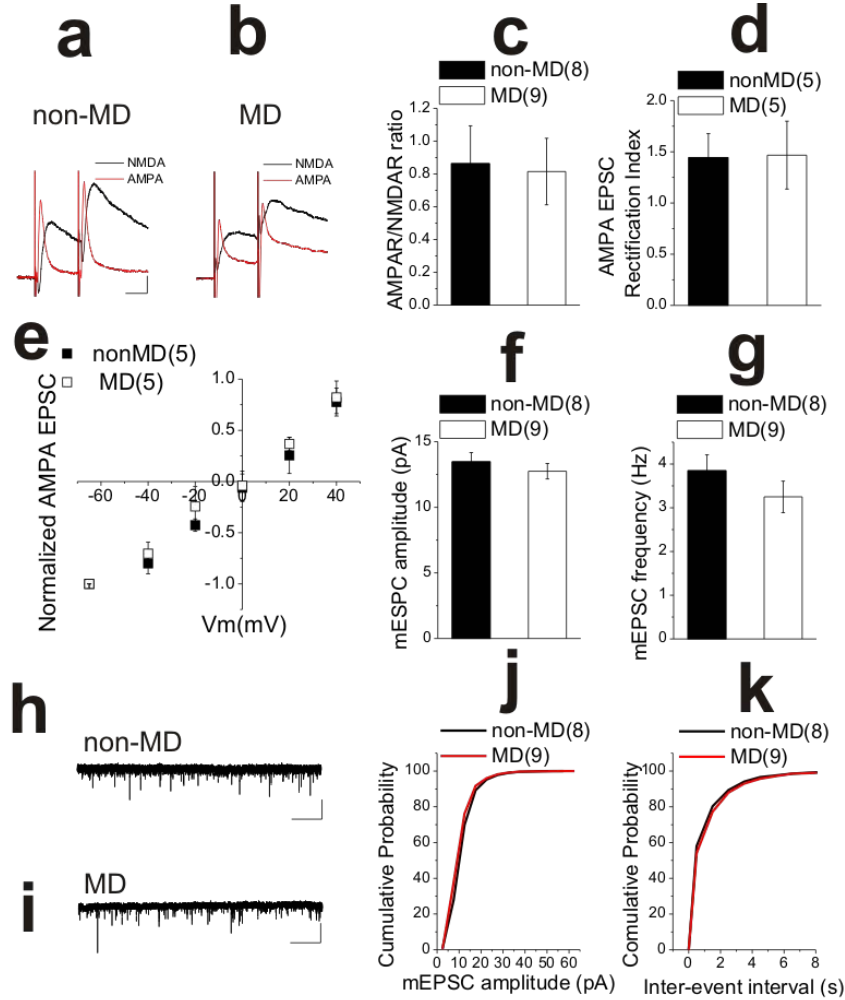


Figure 1: MD did not alter basal excitatory synaptic function or AMPAR subunit composition in VTA DA neurons. a, b: Sample traces of AMPA (red) and NMDA (black) EPSCs recorded at +40mV from DA neurons of non-MD and MD rat pups (calibration bars: 50 pA/25ms). **c:** Summary of AMPAR/NMDAR ratios obtained from non-MD and MD animals. **d:** MD did not change AMPAR EPSC rectification. **e:** Normalized I-V plots of AMPAR EPSCs in VTA DA neurons of non-MD and MD rat pups. In both **d** and **e**, AMPAR EPSC recordings were performed with intra-pipette

inclusion of spermine. **f, g:** Average bar graphs of mEPSC amplitude and frequency from non-MD and MD rats. **h, i:** Sample AMPAR-mediated mEPSC traces from non-MD and MD rats (calibration bars: 20 pA/5s). **k, j:** Cumulative probability plots of amplitude and frequency (inter-event interval) for all mEPSCs in non-MD and MD rats. In this and all subsequent figures, numbers within the figure legends indicate the number of animals examined.

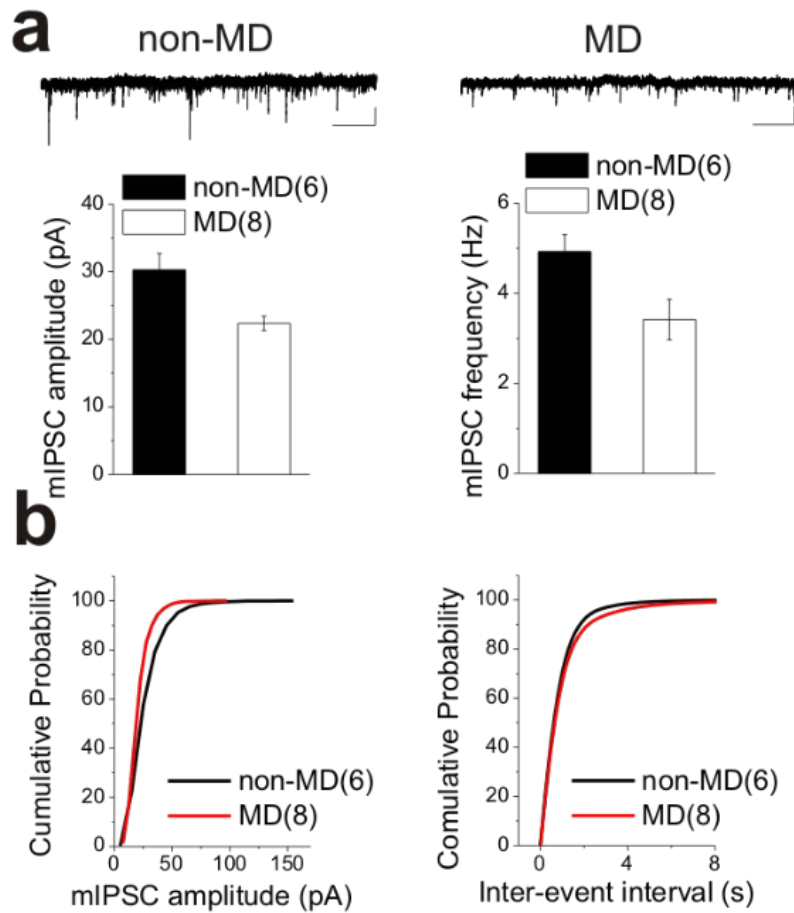


Figure 2: MD induced pre- and post-synaptic LTD at GABAergic synapses onto VTA DA neurons. a: Sample GABA_AR-mediated mIPSC traces from non-MD and MD rats (top), and average bar graphs of mIPSC amplitude and frequency from non-MD and MD rats (bottom) (calibration bars: 20 pA/5s). **c:** Cumulative probability plots of amplitude and frequency (inter-event interval) for all mIPSCs in non-MD and MD rats.

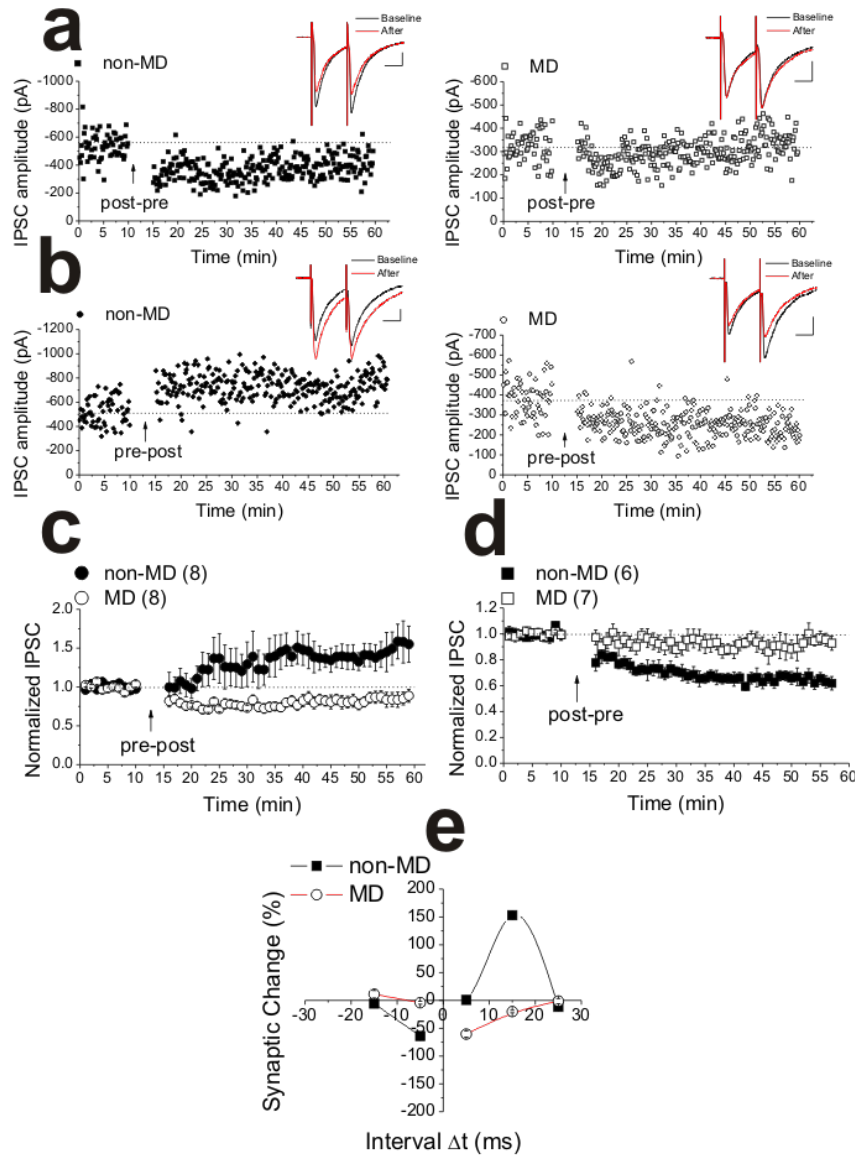


Figure 3: MD triggered metaplasticity of GABAergic STDP in VTA DA neurons.

a, b: Single experiments showing induction of STDP in response to pre-post (+15ms) and post-pre (-5ms) STDP protocols in slices from non-MD and MD rats. At the arrow, STDP was induced using STDP protocols. Insets: averaged IPSCs before (black) and 25 min after STDP protocol (red). In this and all figures, ten consecutive traces from each condition were averaged for illustration as inset. Calibration: 100 pA, 25 ms. **c,d:** Average experiments from DA neurons of non-MD (filled symbols) and MD (open symbols) rats in

response to STDP protocols. **e**: Summary of STDP induction with various time interval (Δt) STDP protocols in non-MD and MD rats. Each data point represents the averaged STDP levels for all experiments with a given Δt .

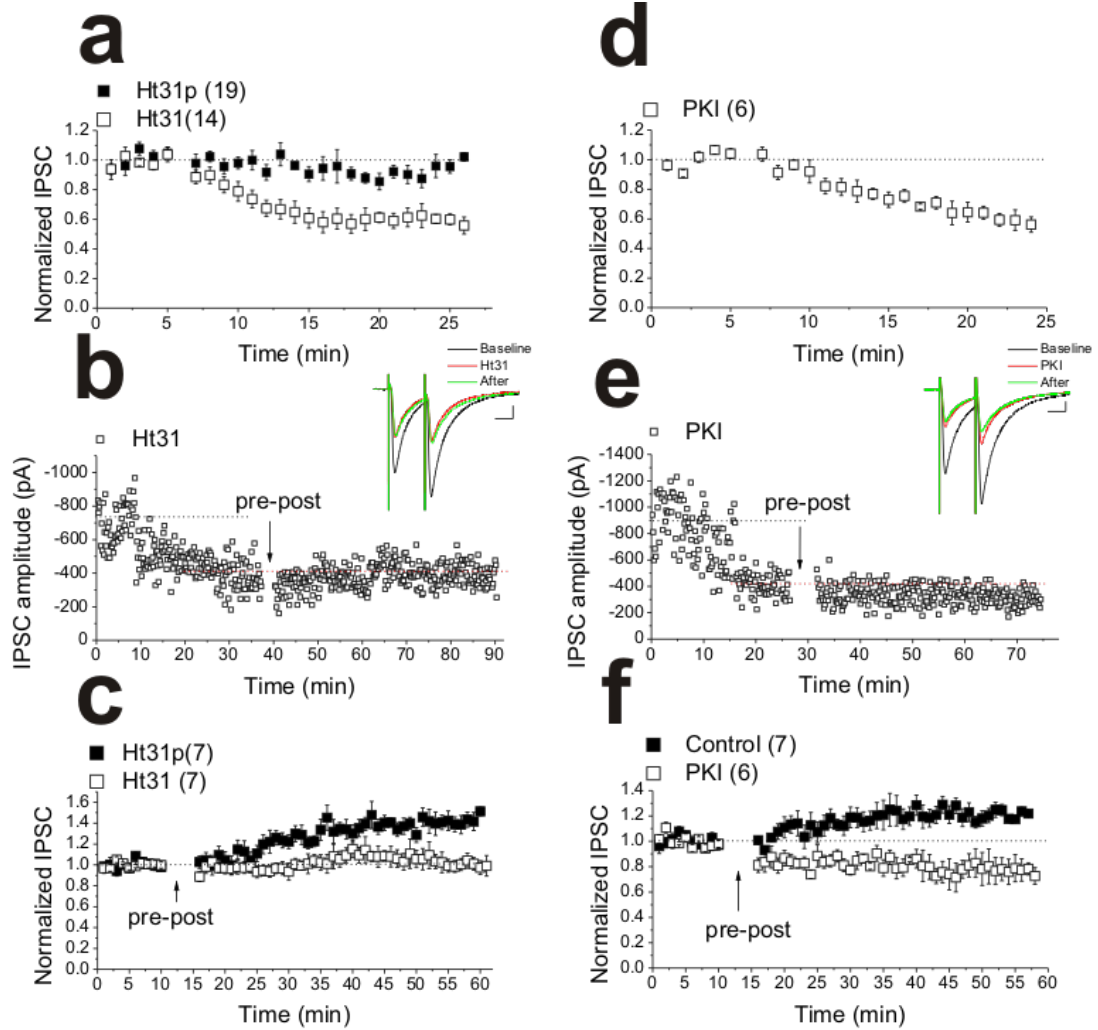


Figure 4: The induction of STD-LTP_{GABA} required the association of PKA with AKAP150. Only non-MD rats were used for this set of experiments. a: Averaged experiments with intra-pipette 1 μ M Ht31 (open symbols) or intra-pipette of 1 μ M Ht31p (filled symbols). **b:** Single experiment illustrating the effect of intra-pipette Ht31 on basal synaptic transmission and the induction of STD-LTP_{GABA} in the Ht31 loaded cell (only the last 5 min of the baseline responses before the emergence of the Ht31-induced depression

is shown in this sample experiment). Inset: averaged IPSCs before (black) or the peak response of Ht31 (red) or 25 min after pre-post protocol (green). Calibration: 100 pA, 25 ms. **c:** Averaged STD-LTP_{GABA} experiments with intra-pipette Ht31 (open symbols) or Ht31p (filled symbols). **d:** Averaged experiments with intra-pipette 10 μ M PKI₍₆₋₂₂₎ (open symbols). **e:** Single experiment illustrating the effect of intra-pipette PKI₍₆₋₂₂₎ on basal synaptic transmission and the induction of STD-LTP_{GABA} in the PKI₍₆₋₂₂₎-loaded cell (only 5 min of the baseline before the emergence of the PKI₍₆₋₂₂₎-induced depression is shown in this sample experiment). Inset: averaged IPSCs before (black) or the peak response of PKI₍₆₋₂₂₎ (red) or 25 min after pre-post protocol (green). Calibration: 100 pA, 25 ms. **f:** Averaged STD-LTP_{GABA} experiments with (open symbols) or without (filled symbols) intra-pipette PKI₍₆₋₂₂₎.

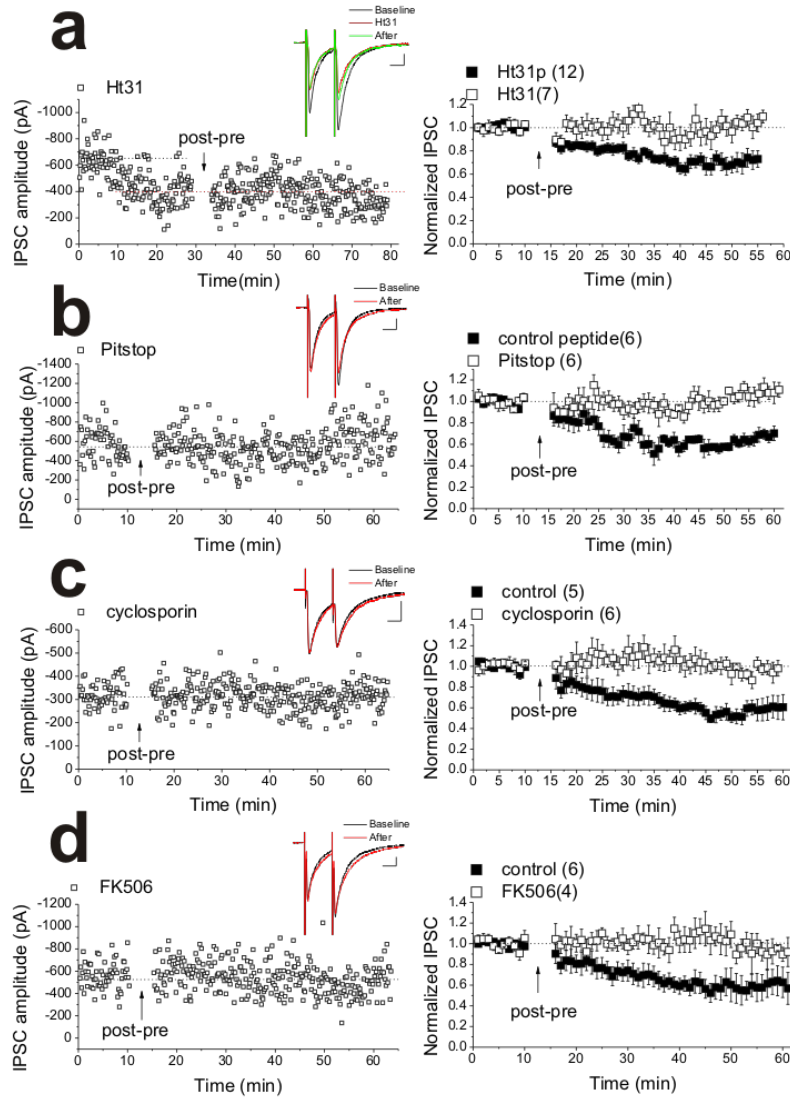


Figure 5: STD-LTD_{GABA} was AKAP- and CaN-dependent and required clathrin-mediated internalization of GABA_A receptors for its expression. Only non-MD rats were used for this set of experiments. **a:** Single experiment (left) illustrating the effect of intra-pipette Ht31 on basal synaptic transmission and the induction of STD-LTD_{GABA} in the Ht31 loaded cell (only the last 5 min of the baseline responses before the emergence of the Ht31-induced depression is shown in this sample experiment). Inset: averaged IPSCs

before (black) or the peak response of Ht31 (red) or 25 min after post-pre protocol (green). Calibration: 100 pA, 25 ms. Averaged STD-LTD_{GABA} experiments (right) with intra-pipette Ht31 (open symbols) or Ht31p (filled symbols). **b**: Single experiment (left) illustrating the block of STD-LTD_{GABA} by intra-pipette Pitstop2TM. Inset: averaged IPSCs before (black) or 25 min after post-pre protocol (red). Calibration: 100 pA, 25 ms. Averaged STD-LTD_{GABA} experiments (right) with Pitstop2TM control peptide (filled symbols) or Pitstop2TM (open symbols). **c, d**: Single experiments (left) illustrating the block of STD-LTD_{GABA} by intra-pipette CaN inhibitors, cyclosporine A and FK506. Insets: averaged IPSCs before (black) or 25 min after post-pre protocol (red). Calibration: 100 pA, 25 ms. Averaged experiments (right) with (open symbols) and without (filled symbols) 1 μ M CaN inhibitor in the pipette solution.

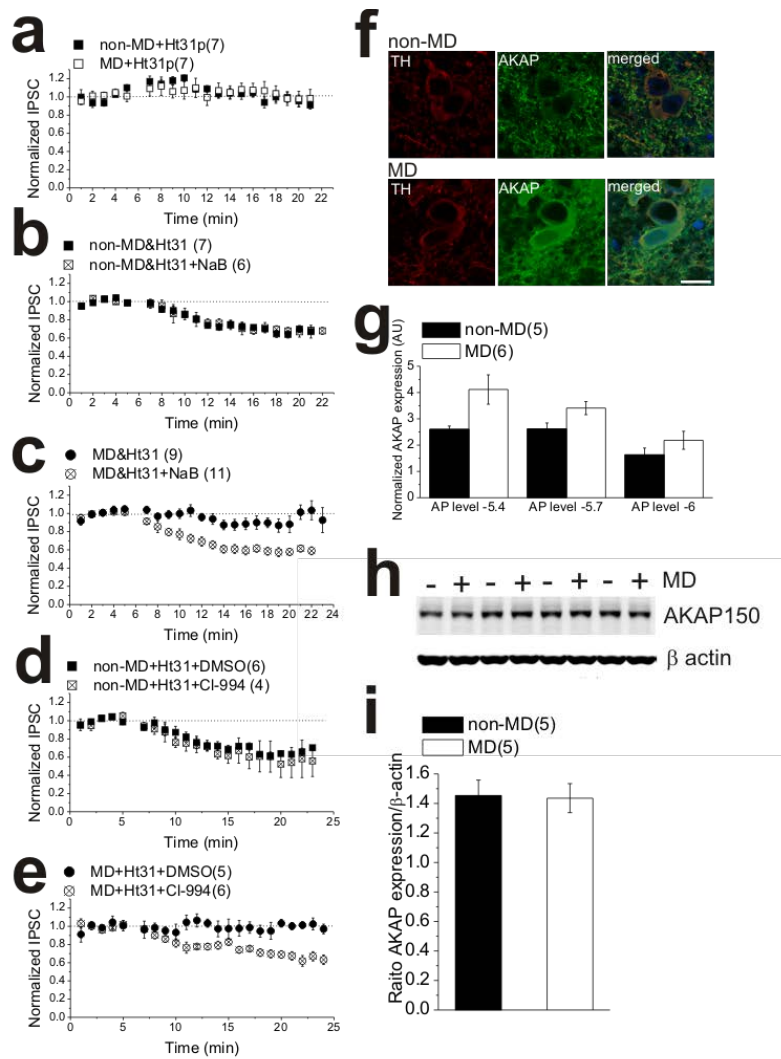


Figure 6: MD induced reversible HDAC-mediated disruption in AKAP150 signaling in VTA DA neurons. **a:** Averaged experiments with intra-pipette 1 μ M Ht31p on GABA_A IPSCs recorded from DA neurons in slices from non-MD rats (filled symbols) or MD rats (open symbols). **b, d:** Averaged experiments with intra-pipette 1 μ M Ht31 (filled symbols) or intra-pipette Ht31+*in vitro* HDACi incubation (either NaB or CI-994, crossed symbols) on GABA_A IPSCs recorded from DA neurons in slices from non-MD rats. **c, e:** Averaged experiments with intra-pipette 1 μ M Ht31 (filled symbols) or intra-pipette Ht31+*in vitro*

HDACi incubation (NaB or CI-994, crossed symbols) on GABA_A IPSCs recorded from DA neurons in slices from MD rats. **f**: Examples of brain sections stained with antibodies to TH (red), AKAP150 (green), and DAPI (59) with the merged panels, which show the expression of AKAP150 in TH neurons in the VTA of non-MD (top) and MD (bottom) rats. Scale bar, 20µm. **g**: Averaged levels of AKAP150 expression at three AP levels from non-MD and MD rats. **h, i**: Representative Western blots (top) and quantitative data (bottom) of AKAP 150 and β actin (control) in VTA homogenates from non-MD and MD rats are shown.

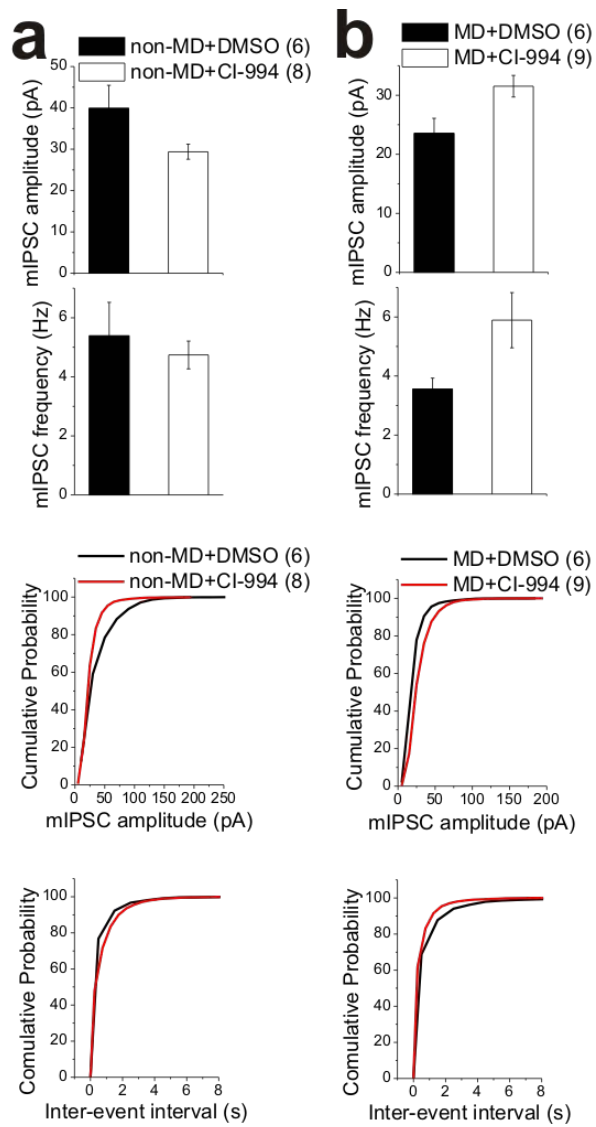


Figure 7: MD-induced GABAergic LTD was reversed by HDAC inhibition. a: Average bar graphs and Cumulative probability plots of mIPSC amplitude and frequency from non-MD rats with DMSO (vehicle) and CI-994. **b:** Average bar graphs and Cumulative probability plots of mIPSC amplitude and frequency from MD rats with DMSO (vehicle) and CI-994.

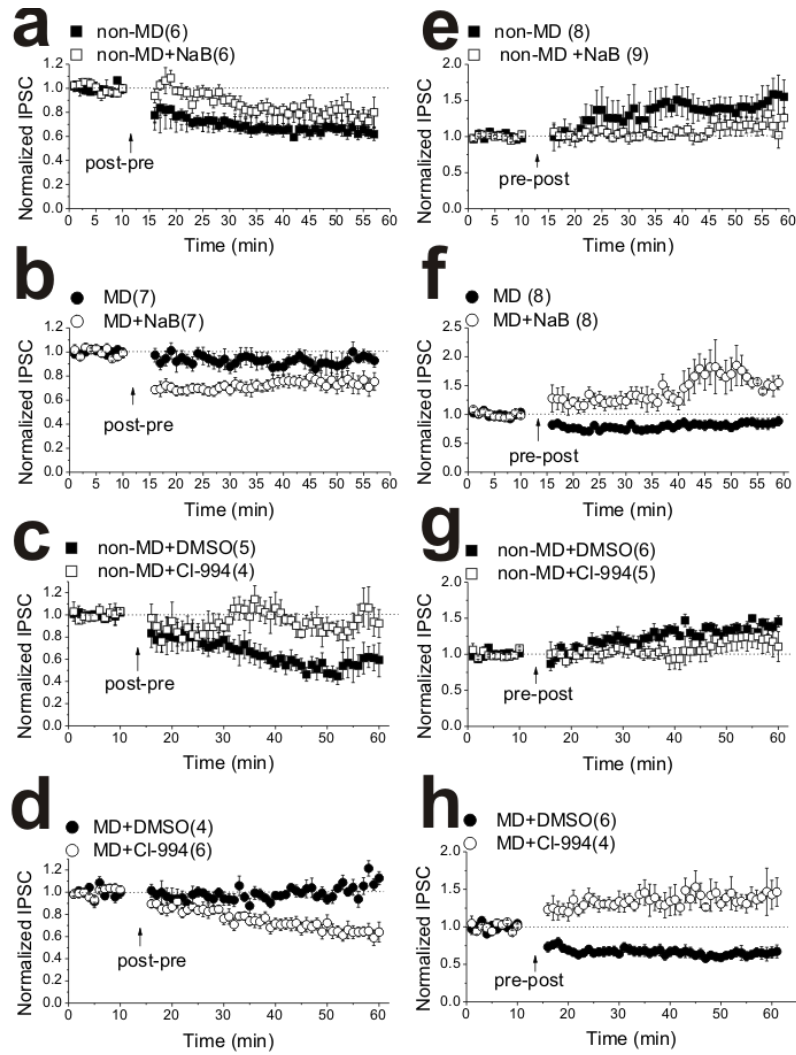


Figure 8: MD-induced GABAergic metaplasticity was reversed by HDAC inhibition.

a, c: Averaged STD-LTD_{GABA} experiments with (open symbols) or without (filled symbols) HDACi (either NaB or Cl-994) treatment in slices from non-MD rats. **b, d :** Averaged STD-LTD_{GABA} experiments with (open symbols) or without (filled symbols) HDACi (NaB or Cl-994) treatment in slices from MD rats. **e,g:** Averaged STD-LTP_{GABA} experiments with (open symbols) or without (filled symbols) HDACi (either NaB or Cl-994) treatment in slices from non-MD rats. **f, h:** Averaged STD-LTP_{GABA} experiments with

(open symbols) or without (filled symbols) HDACi (either NaB or Cl-994) treatment in slices from MD rats.

Supplemental Experimental Procedures:
Slice preparation for electrophysiology

Horizontal midbrain slices (250 μM) were prepared from 14- to 21- day old rats. Slices were cut and incubated at least 1h at 34°C before recording in artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.0 MgSO₄, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O₂/5% CO₂. Slices were then transferred to recording chamber and submerged in warm (28°C) ascorbic acid-free ACSF.

Electrophysiology

Whole cell recordings from VTA DA neurons were performed using a patch amplifier (Multiclamp 700B) under infrared-differential interference contrast microscopy filtered at 3 kHz and digitized at 10 kHz. Data acquisition and analysis were carried out using DigiData 1440A, pClamp 10 (Molecular Devices, Union City, CA), Mini Analysis 6.0.3 (Synaptosoft Inc., Decatur, GA.), Origin 8 (OriginLab Corporation, Northampton, MA) and IBM SPSS Statistics 22. GABA_A receptor mediated IPSCs were evoked, isolated and recorded in ACSF containing 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), and strychnine (1 μM). For mIPSC recordings, tetrodotoxin (TTX, 1 μM) was also added to the perfusion bath. The patch pipettes (3–6 M Ω) were filled with (in mM): 125 KCl, 2.8 NaCl, 2 MgCl₂, 2 ATP-Na⁺, 0.3 GTP-Na⁺, 0.6 EGTA, and 10 HEPES (pH adjusted 7.28 with KOH, osmolarity adjusted to 275-280 mOsm). Cells were voltage-clamped at -70 mV. Paired GABA_A IPSCs were stimulated using a bipolar stainless steel stimulating electrode placed 200-500 μm rostral to the recording site in the VTA at 0.1 Hz (duration 100 μs , 50 ms inter-stimulation interval). GABAergic STDP was induced as described previously (Kodangattil et al., 2013). Briefly, after obtaining a stable baseline, DA cells were taken to current clamp and received trains of a subthreshold presynaptic stimulation paired with back-propagating action

potentials (bAPs/postsynaptic spiking) at 5 Hz. The bAPs were evoked by somatic injection of direct depolarizing currents of 1.5 nA for 5ms through patch pipettes while holding the cells at $\sim -65\text{mV}$. STDP protocols consisted of 30 trains of five bursts repeated at 0.1 Hz. To induce STD-LTP_{GABA}, each burst was composed of three bAPs at 50 Hz and preceded by a single presynaptic stimulation (positive timing, +15 ms). To induce STD-LTD_{GABA}, each burst was composed of three bAPs at 50 Hz followed by a single presynaptic stimulation (negative timing, -5ms). To define STDP window, additional positive (+5ms and +25ms) and negative (-15ms) time intervals were also tested with similar pairing STDP protocols as described above.

In experiments evaluating glutamatergic synaptic transmission and plasticity, combined excitatory postsynaptic currents (EPSCs) were evoked and recorded in the presence of ACSF containing picrotoxin (100 μM) at +40mV as previously described (Ungless et al., 2001). Patch pipettes were filled with (in mM): 117 Cs-gluconate, 2.8 NaCl, 5 MgCl₂, 2 ATP-Na⁺, 0.3 GTP-Na⁺, 0.6 EGTA and 20 HEPES (pH adjusted to 7.28 with CsOH, osmolality adjusted to 275–280 mOsm). After a 10min stable baseline recording of combined EPSC, APV (50 μM) was added to obtain AMPAR-only EPSCs at +40mV. For AMPA receptor-mediated mEPSC recordings, APV (50 μM) and TTX (1 μM) were also added to ACSF that contained picrotoxin and cells were voltage-clamped at -70mV. For AMPAR rectification, AMPAR EPSCs were pharmacologically isolated and evoked as described previously (Sanderson et al., 2012) in the presence of ACSF containing 50 μM APV and 100 μM picrotoxin and inclusion of 10 μM spermine in the patch pipette using the Cs-based internal. AMPAR EPSCs were then recorded at different holding potentials ($>10/\text{neuron}$) and normalized IV curves were generated by dividing the AMPAR EPSC peak amplitudes by the mean of AMPAR EPSC peak amplitude

at -65mV. AMPAR rectification was determined by dividing the peak amplitudes of AMPAR EPSCs recorded at -65 mV by those recorded at +40 mV for each neuron. Putative DA neurons in dorsal and caudal VTA regions were identified by measurement of a hyperpolarization-activated current (I_h , ≥ 50 pA) in response to stepping cells from -50 mV to -100 mV. The cell input resistance and series resistance were monitored through the experiment and if these values changed by more than 10%, data were not included.

Drug treatment

Drugs were present in ACSF perfused slices in recording chamber or in the patch pipette at indicated concentrations for at least 15-20 min before STDP protocols. Interleaved control experiments were performed with experiments in which drugs were bath applied. Slices were preincubated in NaB or CI-994 for 2-4h before being transferred into the recording chamber. CI-994 was dissolved in DMSO and diluted to the final concentration in ACSF. Most drugs were purchased from Sigma, Tocris, or Calbiochem. Ht31 and Ht31p (control peptide) were obtained from Promega. Pitstop2TM and its control peptide were purchased from Abcam.

Western Blots

The VTA was dissected bilaterally from horizontal midbrain slices (400 μ M) prepared from non-MD and MD rat pups and snap frozen. Tissue was thawed in 1% RIPA lysis buffer containing protease inhibitors, sonicated on ice for 20 seconds, incubated for 30 mins at 4°C then centrifuged at 1600g, for 20 minutes. Protein concentration in the supernatant was determined by the BCA protein assay (Pierce, Rockford, IL, USA). 36 μ g protein was combined with loading buffer, boiled for 5 min, and loaded onto an SDS-polyacrylamide gel. Separated proteins were transferred onto nitrocellulose membranes, and probed with goat anti-AKAP 150 antiserum (1:1000, SCB, sc-6445), or mouse anti-

β -actin (1:4000, Sigma, #A1978-200), followed by incubation with the appropriate HRP-linked secondary antibody (Cell Signaling, MA). Blots were developed with super-signal enhanced chemiluminescence reagent (Thermo Scientific) and detected using a Fuji LAS-3000 image acquisition system (Fuji, Stamford, CT, USA) equipped with a cooled CCD camera.

Immunohistochemistry and image analysis

Non-MD and MD rats were anesthetized with an intraperitoneal injection containing ketamine (85 mg/kg) and xylazine (10 mg/kg) and perfused through the aorta with heparinized 1x phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, USB, Cleveland, OH). The brains were dissected and placed in 4% PFA for 24 hrs and then cryoprotected by submersion in 20% sucrose for 3 days, frozen on dry ice and stored at -70 C until sectioned. Sections of the VTA were cut using a cryostat (Leica CM1900) and mounted on slides. Serial coronal sections (20 μ m) of the midbrain containing the VTA (from -4.92 to -6.72 mm caudal to bregma; Paxinos and Watson, 2001) were fixed in 4 % PFA for 5 minutes and washed in 1x PBS then blocked in 10% normal horse serum (NHS) containing 0.3 % Triton X-100 in 1x PBS for 1 hr. Sections were incubated in rabbit anti-TH (1:1000, Calbiochem, San Diego, CA.) and goat anti-AKAP150 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA.) in carrier solution (0.5 % NHS in 0.1 % Triton X-100 in 1x PBS) overnight at room temperature. After rinsing in 1x PBS, sections were incubated for 2 hours in Alexa Fluor® 488 labeled chicken anti-goat IgG and Alexa Fluor® 568 labeled donkey anti-rabbit IgG (both diluted 1:200). Finally sections were rinsed in 1x PBS, dried, and cover slipped with Prolong mounting medium containing DAPI to permit visualization of nuclei. Background staining was assessed by

omission of primary antibody in the immunolabeling procedure (negative control). VTA tissue sections of rats with previously established presence of TH/AKAP150 immunoreactive neurons were processed as positive control tissue. Images in Fig. 6f were captured using a Zeiss Confocal Inverted Microscope System (Carl Zeiss Inc.) 40x/1.4 n.a. oil immersion objective.

Data analysis

Values are presented as means $\pm$ s.e.m. Statistical significance was determined using repeated measures ANOVA with significance level of $p < 0.05$ for STDP, and chemical LTD (PKI₍₆₋₂₂₎ or Ht31) experiments. Unpaired Student's *t* tests were performed subsequent to significant repeated measure ANOVAs for those experiments to make comparisons between MD and non-MD rats. Levels of STDP are reported as averaged IPSC amplitudes for 5min just before STDP induction compared with averaged IPSC amplitudes during the 5min period from 35 to 40 min after the protocol. The magnitude of STDP in Fig. 3 was expressed as percentage of synaptic change. Levels of PKI₍₆₋₂₂₎-/Ht31-induced depression are reported as averaged IPSC amplitudes for 5 min just before the emergence of drug-induced synaptic depression compared with averaged IPSC amplitudes during the last 5 min period of the peak response. NMDAR-only EPSCs were computed by digital subtraction of EPSCs at +40mV before and after application of APV using Clampex 10 (10 EPSCs for each).

AMPA/NMDAR ratio was then calculated by dividing the peak of the AMPAR-only EPSC by the peak of the NMDAR-only EPSC. The statistical differences for AMPAR/NMDAR ratio and AMPAR EPSC rectification index were evaluated using unpaired Student's *t* test. We performed two-way ANOVA with Bonferroni post hoc analysis to detect the statistical differences between non-MD and MD rats for AMPAR EPSC IV plots. For mIPSC and

mEPSC analyses, detection parameters were set at 5 pA amplitude and <3 ms rise time and events were also screened and verified by visual examination before the inclusion in the final data analysis. Unpaired Student *t* test was used to analyze the statistical differences in the means of amplitude and frequency of mEPSCs and mIPSCs in the represented bar graphs. The KS test was performed for the statistical analyses of cumulative probability plots of mEPSCs and mIPSCs. Effects of MD on AKAP levels in TH containing VTA neurons were evaluated at three AP levels and five neurons were quantified for each AP level. Images for densitometric quantification were obtained using a Leica (Nussloch, Germany) DM-RXA fluorescence microscope with a 40x objective (0.75 n.a.). After merging the AKAP and TH images, a 12 μm x 12 μm square was placed in the soma of the TH/AKAP positive neuron and the AKAP fluorescence intensity was determined. Fluorescence intensity within this box was measured on an arbitrary unit scale. Background and normalization corrections of the density reading were based on fluorescence intensity measurements from a location on the slide containing no tissue. MD effects on AKAP intensity were compared by a two-way ANOVA and Bonferroni post-hoc comparisons. AKAP expression was normalized to β -actin expression in Western blot analysis and an unpaired Student's *t* test was used to evaluate the statistical difference between the two groups.

Supplement References

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Supplemental Figures

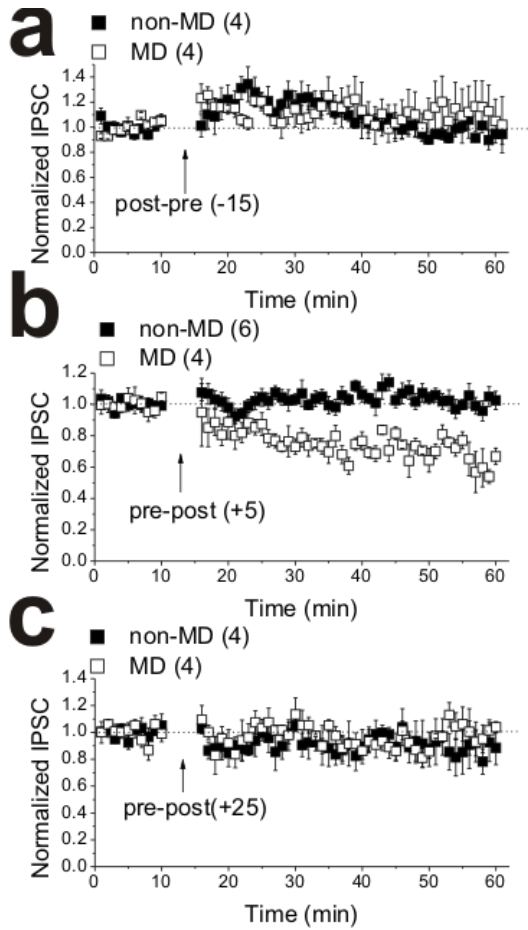


Figure S1

Fig. S1. Induction of GABAergic STDP in VTA DA neurons at different time

intervals (Δt). **a:** Average experiments from DA neurons of non-MD (filled symbols)

and MD (open symbols) rats in response to post-pre pairing at -15ms intervals (non-MD,

$n=4$: $94.36 \pm 4.4\%$ of pre-STDP values, $F_{12.24,36.74}=1.235$, $p=0.139$, MD, $n=4$: $101 \pm 6.7\%$ of

pre-STDP values, $F_{7.34,22.04}=0.629$, $p=0.733$). **b:** Average experiments from DA neurons

of non-MD (filled symbols) and MD (open symbols) rats in response to pre-post pairing

at +5ms intervals (non-MD, $n=6$: $101 \pm 4.1\%$ of pre-STDP values, $F_{22.25,89.03}=0.958$,

$p=0.523$, MD, $n=4$: $60.4 \pm 5.4\%$ of pre-STDP values, $F_{8.06, 24.197}=0.3.476$, $p=0.008$). **c:**

Average experiments from DA neurons of non-MD (filled symbols) and MD (open

symbols) rats in response to pre-post pairing at +25ms intervals (non-MD, $n=4$:

$88.45 \pm 5.9\%$ of pre-STDP values, $F_{8.4, 25.2}=0.817$, $p=0.600$, MD, $n=4$: $98.7 \pm 4.5\%$ of pre-

STDP values, $F_{9.6, 28.83}=0.991$, $p=0.472$).

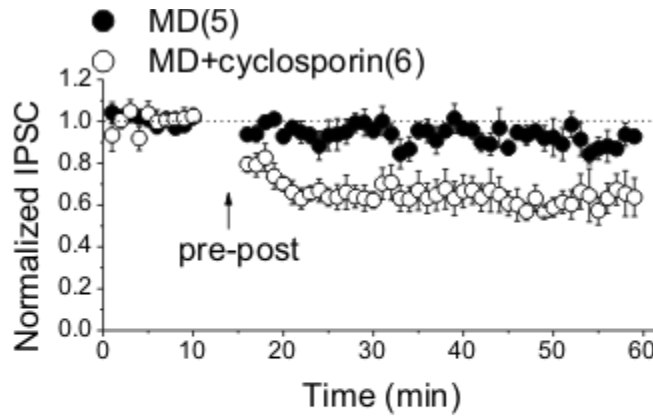


Figure S2

Fig. S2. Calcineurin inhibitor, cyclosporine A, blocked the induction of LTD in response to pre-post STDP protocols at +15ms in MD rats. Averaged experiments with (open symbols) and without (filled symbols) 1 μ M cyclosporin A in the pipette solution in slices from MD animals (MD, n=5: $63.4 \pm 1.7\%$ of pre-STDP values, $F_{8.94,26.84}=7.026$, $p<0.0001$; MD+cyclosporin A, n=6: $89.7 \pm 1.4\%$ of pre-STDP values, $F_{7.5,15}=0.870$, $p=0.557$).

CHAPTER 3: A novel role for corticotrophin releasing factor signaling in the lateral habenula and its modulation by early life stress

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This paper has been submitted to Science Signaling; it is now in revision. Many of the changes in the revised version have been incorporated here.

Abstract

Centrally released corticotropin releasing factor (extrahypothalamic CRF) acting within various brain regions is involved in behavioral and emotional responses to stress. Here, we demonstrate for the first time that extrahypothalamic CRF neurotransmission exists in the lateral habenula (LHb), an epithalamic brain region involved in stress evasion. Dysfunction of the CRF system and LHb is critical for development of stress-related disorders and addiction, however no study has investigated whether CRF signaling plays a role in activity of the LHb. In fact, the LHb shows immunoreactivity for both CRF and its receptor, CRFR1, and receives inputs from the structures that comprise the extrahypothalamic CRF system, but its involvement in this central CRF system is unexplored. Using whole cell patch clamp recording from rat LHb neurons, we found that CRF-CRF receptor 1(CRFR1) signaling increases the intrinsic excitability of LHb neurons through modulation of small- and large-conductance SK- and BK-type K⁺ channels. In addition, CRF reduces GABAergic synaptic transmission onto LHb neuron promoting LHb hyperactivity. Maternal deprivation (MD), a severe early life stress known to predict later-life psychiatric disorders, increases LHb neuronal excitability and blunts the excitatory actions of CRF on LHb neurons due to down-regulation of SK2 channel expression. Increasing the function of SK channels by positive allosteric modulation prevents CRF excitatory action on LHb intrinsic excitability and also reverses the effects of MD on LHb intrinsic excitability suggesting that SK channels may serve as novel pharmacological targets in reversal of central CRF dysregulation in stress-responsive brain regions such as the LHb.

One-sentence summary

CRF-CRFR1 signaling in the LHb increases intrinsic neuronal excitability through a G-protein dependent activation of Ca^{2+} signaling and modulation of large and small conductance BK and SK K^+ channels and is dysregulated by severe early life stress.

Introduction

Corticotropin releasing factor (CRF) mediates behavioral responses to stress and is implicated in a multitude of stress and mood related disorders. Although widely known for its participation in stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis, CRF also acts centrally within the brain where it directly contributes to a behavioral state of anxiety. This extrahypothalamic CRF system includes brain regions whose activities are implicated in mental illnesses [1-4]. Many of these brain regions, including the ventral tegmental area (VTA), extended amygdala, raphe nuclei, the bed nucleus of the stria terminalis (BNST) and medial prefrontal cortex (mPFC) have fallen under intense investigation as to their role in the extrahypothalamic CRF system [5-8]. To date, these studies have demonstrated that the action of CRF is not only region-specific, owing to circuit, cell-type, and pathway specificity, but that CRF's action is also dependent on the organism's previous exposures to stress [9-11].

To extend knowledge of the extrahypothalamic CRF system to other brain regions, we sought to investigate the role of CRF in the lateral habenula (LHb). The LHb is a highly stress-responsive brain region due to its involvement in motor and motivational control of behavior by conveying negative reward information to midbrain monoaminergic systems [12]. Dysfunction of the LHb is implicated in stress-related disorders [13-16]. This area is activated by reward omission and aversive stimuli but inactivated by unexpected reward. The LHb influences behavior by its inhibitory control of midbrain dopamine (DA) neurons. Indeed, optogenetic activation of LHb neurons projecting to rostromedial tegmental nucleus (RMTg) GABAergic neurons is sufficient to elicit behavioral avoidance [17]. Other studies have demonstrated the role of fast synaptic transmission mediated by GABA and glutamate and neuromodulatory effects of serotonin,

norepinephrine and DA in the LHb [18]; however, no study to date has investigated CRF neurotransmission in the LHb. The receptors for CRF, CRFR1 and CRFR2, are both G-protein coupled receptors (GPCRs). Activation of these receptors has a diverse range of neuronal effects depending on the brain region in which they are expressed. In general, activation of CRFR1 is associated with stress-like behaviors while CRFR2 activation seems to reduce stress-related responses [4]. Although, the LHb shows immunoreactivity for both CRF and CRFR1 [19, 20], and receives inputs from the structures that comprise the extrahypothalamic CRF system, its involvement in this system is unexplored.

This study investigates the role of central CRF signaling on synaptic transmission and neuronal excitability in the rat LHb. We also examined the experience-specific role of CRF signaling in the LHb using an established model of severe early life stress, maternal deprivation (MD), that is associated with persistent hyperactivity of the HPA axis and central CRF signaling and DA dysfunction [21-23]. In fact, MD impairs GABAergic control of VTA DA neurons through epigenetic impairment of GABAergic plasticity which in part contributes to MD-induced dysregulation of DA neurotransmission [24]. CRF can modulate DA signaling and alter mesolimbic reward function [25, 26].

Therefore, an altered CRF signaling within the LHb as an upstream input to the VTA could play a crucial part in DA dysfunction associated with MD stress model. Our work here demonstrates that CRF-CRFR1 signaling in the LHb increases intrinsic neuronal excitability through a G-protein dependent activation of Ca^{2+} signaling and modulation of afterhyperpolarizations (AHPs) mediated by Ca^{2+} -activated K^{+} channels; BK and SK channels. Additionally, we found that SK channel down-regulation mediates the increases in LHb intrinsic neuronal excitability by MD, which in turn blunts the excitatory action

of CRF within the LHb after MD. Our study provides the first evidence for CRF signaling within the LHb which may mediate part of stress-relevant effects of CRF through changes in synaptic transmission and intrinsic excitability of LHb neurons. The recruitment of the CRF stress systems within the LHb may play a key role in the development of stress-, addiction- and depressive-related behaviors.

Results

Extrahypothalamic CRF mediates behavioral responses to stress. The region-specific actions of CRF have been defined in brain regions that are known to be involved in stress-related behaviors, but until now, no study has investigated the role of CRF signaling in one such brain region: the LHb. Therefore, we first tested the action of CRF on the excitability of LHb neurons.

CRF increases the excitability of LHb neurons

Neuronal excitability is influenced by both synaptic transmission and neuronal intrinsic properties. Action potentials (APs) generated by depolarizing steps were first measured in LHb neurons with intact fast synaptic transmission. We found that CRF significantly increased the firing of neurons, suggesting an increase in LHb neuronal excitability in response to CRF that may involve fast synaptic transmission as well as changes in intrinsic excitability (Figure 9 A,B). To isolate the action of CRF on the intrinsic excitability of the cell, we repeated the same experiment in the absence of fast synaptic transmission (GABA_AR, AMPAR and NMDAR blockers were present in the perfusate) and found that CRF still significantly increased neuronal excitability (Figure 9C). The excitatory effect of CRF on neuronal excitability of LHb neurons was maximally achieved by 20-30min of bath application and remained stable over time. With fast

synaptic transmission blocked, we then analyzed CRF's effects on different characteristics of APs and intrinsic neuronal excitability including fast AHPs (fAHPs), medium AHPs (mAHPs), threshold potential, half width and amplitude of AP (Table 1 and Figure 9D). Of these, only fAHP, mAHP and AP threshold measurements were significantly different after CRF. AHPs are mainly mediated by multiple Ca^{2+} -dependent K^+ channels that tend to be inhibitory; i.e., reduce neuronal excitability [27]. Although fAHPs were increased after CRF application, CRF was associated with decreased mAHPs. CRF also lowered AP threshold (Figure 9D).

CRF does not affect glutamatergic synaptic transmission, but does decrease GABAergic synaptic transmission

Although CRF increased neuronal excitability in the absence of fast synaptic transmission, it was likely that CRF also altered synaptic transmission to promote LHB hyperexcitability. To test this, we examined the effects of CRF on the frequency and amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) and GABA_AR-mediated miniature inhibitory postsynaptic currents (mIPSCs) recorded from LHB neurons. CRF did not have a significant effect on either the frequency or amplitude of mEPSCs, suggesting that CRF does not affect the presynaptic release of glutamate, nor the function of postsynaptic AMPARs (Figure 10A). However, we found that mIPSCs were significantly reduced in the frequency and amplitude (only a significant shift in cumulative probability plot for amplitude), suggesting that CRF reduced GABAergic inhibition in LHB neurons through significant decreases in the release of GABA from presynaptic terminals and postsynaptic GABA_AR number/function (Figure 10B). Altogether, these findings suggest that CRF shifts the

balance of synaptic transmission onto LHb neurons towards excitation by depressing fast GABAergic synaptic transmission.

CRF activates CRFR1 and acts through postsynaptic Ca^{2+} signaling

Since the LHb is involved in the processing of aversive stimuli and has also been shown to be hyperactive in depressed and stress-related behavioral states, we hypothesized that CRF's ability to increase LHb neuronal excitability is achieved through binding to postsynaptic CRFR1. In all the following experiments characterizing the mechanisms underlying CRF-induced increases in LHb intrinsic excitability, AP recordings were performed in the presence of synaptic blockers similar to Figure 9C. Indeed, in the presence of antalarmin, we found that CRF's action on intrinsic excitability was completely blocked (Figure 11A). To further confirm CRF's action through postsynaptic CRFR1, a G-coupled protein receptor, we blocked postsynaptic G-protein signaling in the neuron by intracellular administration of GDP β S, a non-hydrolyzable GDP analog, through the patch pipette. Intra-pipette GDP β S prevented CRF-induced increase in LHb intrinsic excitability (Figure 11B). Both intracellular release of Ca^{2+} from internal stores and extracellular Ca^{2+} influx have previously been shown to be involved in CRFR1 signaling in other brain areas [28, 29]. To further investigate whether increase in postsynaptic Ca^{2+} signaling is involved downstream of CRFR1 activation, BAPTA (a fast Ca^{2+} chelator) was used intracellularly through the patch pipette. Intra-pipette application of BAPTA blocked CRF's ability to increase intrinsic excitability of LHb neurons (Figure 11C). We next sought to determine how exactly Ca^{2+} signaling through CRFR1 activation could lead to increased intrinsic excitability of LHb neurons.

CRF affects SK and BK channel function in LHb neurons

Changes in intrinsic conductances through K^+ channels such as SK and BK channels influence the firing of neurons [30]. In our earlier experiments evaluating the effects of CRF on intrinsic excitability of LHb neurons, we observed that CRF altered AHPs and significantly lowered AP threshold in LHb neurons. The fAHP amplitude was significantly enhanced by CRF suggesting an increase in BK channel function by CRF. However, the mAHPs measured in response to depolarization was decreased after bath application of CRF, suggesting decreased function of SK channels by CRF which could support CRF-induced increases in LHb intrinsic excitability. Given that an increase in BK channel function can inactivate other K^+ channels and subsequently support high frequency neuronal firing [31], we focused on the role of SK channels in CRF action on LHb intrinsic excitability. First, we tested whether a SK channel blocker, apamin, could mimic and occlude CRF-induced increases in LHb intrinsic excitability. We found that apamin significantly increased LHb intrinsic excitability and furthermore occluded CRF's action on LHb neuronal excitability (Figure 12A). The effect of apamin on intrinsic excitability was also accompanied by an increase in the amplitude of fAHP, and a decrease in the amplitude of mAHP (Figure 12A). To further confirm that increasing SK channel function could prevent CRF-induced increase in intrinsic excitability, we used a positive modulator of SK channels (CyPPA). CyPPA did not affect intrinsic neuronal excitability by itself, but blocked the excitatory effect of CRF on LHb intrinsic excitability (Figure 12B). CyPPA did not significantly affect fAHP or mAHP measurements (Table 1).

Early-life stress increases the spontaneous firing and excitability of LHb neurons through decreasing the function of SK channels

Given that early life stresses including MD induce long-lasting alterations in CRF-CRFR1 signaling in the brain [23]; it was feasible to assume that an adverse early life experience such as MD would affect the function of LHb neurons and alter their responsiveness to CRF. In fact, MD modifies DA signaling from the VTA through epigenetic modifications of critical signaling pathways involved in trafficking of GABA_A receptors onto VTA DA neurons [24]. Given the potent inhibitory control of VTA DA neurons by the LHb, we chose to use the same model of severe early life stress in this work to determine whether the response of LHb neurons to extrahypothalamic CRF is affected by exposure to early life stress. First, we hypothesized that MD would increase LHb neuronal excitability. To test this hypothesis, we recorded LHb neuronal spontaneous activity using cell-attached recordings in voltage-clamp mode to determine the percentage of neurons which were spontaneously active and of those, the average spike frequency. In this set of experiments, synaptic transmission was intact. We observed that the portion of spontaneously active LHb neurons was significantly larger in MD rats compared to control non-MD rats. Moreover, LHb neurons displaying spontaneous activity had remarkably higher spike frequencies in MD rats compared to controls (Figure 13B).

In addition to cell-attached recordings of basal neuronal firing, we also tested whether LHb neurons from MD rats exhibit enhanced firing in response to depolarization compared to control non-MD rats in the absence or presence of fast synaptic transmission. Regardless of whether fast synaptic transmission was blocked, MD increased LHb neuronal excitability (Figure 13A and C). Furthermore, the MD-induced increase in intrinsic excitability was associated with a significant reduction in mAHP

amplitude without any change in the amplitude of fAHP, suggesting that this increase may be due to a decreased function and/or expression of SK channels (Figure 13D). Except mAHP, all the other measured passive and active properties of LHb neurons including Vm were unaffected following MD (Table 1). In addition, western blot analysis of LHb tissues from non-MD and MD rats demonstrated that SK2 protein levels were significantly decreased in MD rats compared to controls. In contrast, we did not observe any significant change in protein levels of SK1 and SK3 (Figure 13E).

MD potentiates both glutamatergic and GABAergic synaptic transmission

MD-induced changes in glutamatergic synapses support increases in LHb neuronal excitability as we found that LHb neurons from MD rats exhibited significantly higher AMPA/NMDA ratios compared to control non-MD rats (Figure 14A). In support of this postsynaptic glutamatergic potentiation, there was a significant increase in the cumulative probability of AMPAR-mediated mEPSC amplitudes after MD, although the average amplitudes were not significantly different between MD and non-MD rats. Moreover, we detected a marked increase in the average and cumulative probability of frequencies of mEPSCs suggesting an increase in presynaptic glutamate release by MD (Figure 14D). We also recorded evoked AMPAR-mediated EPSCs at different holding potentials to measure AMPAR rectification and determine whether MD induced any changes in synaptic AMPAR subunit composition. AMPA EPSC rectification and EPSC IV plots were unchanged after MD (Figure 14B and C).

We also found that although the average amplitudes of GABA_A receptor-mediated mIPSCs were not significantly different between MD and non-MD rats, the cumulative probability of mIPSC amplitudes was significantly increased after MD. Moreover, the

average and cumulative probability of frequency of mIPSCs were significantly increased in MD compared to non-MD rats. This suggests that MD potentiated GABAergic synaptic transmission pre- and postsynaptically (Figure 14E).

The effects of CRF and apamin on LHb intrinsic excitability are blunted following MD

Depending of the timing and severity of the stress, CRF release can have long lasting regional effects and change subsequent responses to CRF exposure. Here we tested whether LHb neurons' responsiveness to CRF was affected following MD, with the hypothesis that CRF is excessively released during the episode of MD, leading to a dysregulation of central CRF signaling within the LHb. Although CRF significantly increased neuronal excitability of non-MD neurons in both the presence and absence of synaptic blockade (Figure1), in MD rats, CRF's action on neuronal excitability was blunted both in the presence and absence of fast synaptic transmission. In fact, there was no significant increase in LHb neuronal excitability in MD rats after adding CRF (Figure 15A and B). Since we previously established that CRF signaling involved the activity of postsynaptic SK channels and that following MD, SK2 channel levels were diminished, we sought to determine whether an impaired function in SK channels in MD rats underlies the blunted effect of CRF on LHb intrinsic excitability (synaptic transmission was blocked here). The first piece of evidence to support this hypothesis was that bath application of apamin had a diminished effect on the intrinsic excitability of MD LHb neurons (Figure 15C). We also tested the effects of another positive SK channel modulator, 1-EBIO, which similar to CYPPA blocked the excitatory action of CRF on intrinsic excitability of LHb neurons (data not shown). Bath application of 1-EBIO

significantly decreased intrinsic neuronal excitability of LHb neurons at the lower current steps in slices from MD rats despite of the lower SK2 channel protein expression in MD rats (Figure 15D).

Discussion

This study is the first demonstration of the role of an extrahypothalamic CRF-CRFR1 system in the LHb. We demonstrate that the stress neuropeptide CRF increases the excitability of LHb neurons through changes in intrinsic neuronal excitability as well as GABAergic transmission onto LHb neurons. CRF increases LHb intrinsic neuronal excitability through a G-protein-dependent CRFR1 activation of Ca^{2+} signaling and subsequent modulation of BK and SK channels. The ability of CRF to increase neuronal excitability in LHb neurons was consistent across all recorded neurons and rats; however, the response of LHb neurons to CRF was modulated by previous exposure to severe early life stress. Using an established model of child abuse and neglect associated with the hyperactivity of the CRF neuronal system (i.e., a single episode of MD at P9), we found that MD per se induces adaptations in neuronal membrane properties as well as at glutamatergic and GABAergic synapses onto LHb neurons. An impairment in the function of SK channels in MD rats underlies the alteration in LHb intrinsic excitability and blunts the excitatory effects of CRF on LHb neurons. Increasing the function of SK channels by positive allosteric modulation not only prevents CRF's excitatory action on LHb intrinsic excitability but also reverses the effects of MD on LHb intrinsic excitability suggesting that SK channels may serve as novel pharmacological targets in reversal of dysregulated stress-responsivity within the LHb during severe early life stress.

CRF is a key neuromediator of the stress response and dysregulation of CRF neuronal systems has also been strongly linked to depressive-like behaviors and drug-seeking behaviors [2]. However it is unknown whether CRF in the LHb plays a role in stress; while CRF- and CRFR1-like immunoreactivity have been detected in moderate levels in the LHb [20], their function is unknown. Here, we found that CRF had a potent excitatory effect on LHb neuronal excitability; significantly increasing the excitability of these neurons in the presence or absence of fast synaptic transmission. The increase in neuronal excitability induced by CRF was associated with a significant reduction in the amplitude of the mAHP. Consistently, we found that blocking SK channels with apamin mimicked and occluded CRF's effect while positive enhancement of SK channel function with 1-EBIO and CYPPA blocked CRF's effect. CRF increases neuronal excitability in the basolateral amygdala, paraventricular nucleus of the hypothalamus (PVN) and VTA DA neurons [32-34]. Consistent with our finding, decreases in the amplitude of AHPs and K^+ channel function including SK type is associated with increase in neuronal excitability [35, 36]. On the other hand, CRF receptor signaling in VTA DA neurons potentiates the function of SK channels [28]. Interestingly, we also found that CRF increased the amplitude of fAHPs (mostly mediated by large conductance, BK, Ca^{2+} -activated K^+ channel). fAHPs may facilitate high-frequency firing of LHb neurons similar to that shown in CA1 hippocampal pyramidal neurons by limiting activation of other K^+ channels and Na^+ channel inactivation [31, 37-40]. In fact, in addition to an increase in the amplitude of fAHP, CRF significantly lowered AP threshold suggesting that a hyperpolarized inter-spike membrane potential during high AP firing rates might be

maintained by fAHPs to prevent voltage-gated Na^+ channel inactivation and promote suppression of SK channel function by CRF.

Although CRFR1 and CRFR2 expression often overlaps, CRFR2 does not seem to be expressed in the LHb [41]. Consistent with this anatomical evidence, antalarmin completely blocked the excitatory effect of CRF on intrinsic excitability. To determine whether CRFR1 activation engaged postsynaptic G-protein and Ca^{2+} signaling to increase LHb intrinsic excitability, we disrupted postsynaptic G-protein signaling using intra-pipette $\text{GDP}\beta\text{S}$ or blocked postsynaptic Ca^{2+} signaling using intra-pipette BAPTA. CRF was unable to increase intrinsic excitability in both sets of experiments. All together our findings suggest that postsynaptic G-protein-mediated CRFR1 signaling requires intracellular Ca^{2+} signaling to increase LHb neuronal excitability. How the intracellular Ca^{2+} signaling leads to a decrease in SK channel function has yet to be determined. Gating (opening and closing) of SK channels is dependent on intracellular Ca^{2+} and although SK channels open in response to increases in postsynaptic Ca^{2+} , voltage-dependent blockade of these channels by intracellular divalent cations including Ca^{2+} has been shown indicating a complex and dynamic Ca^{2+} -dependent gating of SK channels [42]. It is also likely that signaling downstream to postsynaptic CRF-CRFR1- Ca^{2+} first involves BK channels which subsequently inactivate SK channels or trigger additional steps to regulate the trafficking as well as the permeation characteristic of SK channels in LHb neurons.

Other studies have shown that the action of CRF is dependent on the organism's previous exposure to stress [9-11]. In our previous study, we established that MD triggers a dysregulation of DA and GABAergic signaling within the VTA which could play a

critical role in the development of substance abuse, and stress-related psychiatric disorders later in life [24]. CRF modulates DA signaling and mesolimbic reward function [25, 26]; thus, an alteration of CRF signaling within the LHb and subsequent changes in LHb function could significantly affect DA release from the VTA. Here we found that MD per se increased both basal and depolarization-induced AP firing of LHb neurons, with or without synaptic transmission. In addition, the increase in LHb intrinsic excitability following MD was accompanied by a decrease in mAHP. Indeed, protein levels of SK2 were lower in MD rats, while SK1 and SK3 levels were unaffected. Interestingly, positive modulation of SK channels did not reduce firing rate of LHb neurons in control non-MD rats suggesting that the basal activity of these channels may be relatively high. On the other hand, 1-EBIO normalized intrinsic excitability in LHb neurons of MD rats at the lower depolarizing current steps, despite of lower expression of SK2 channels in MD rats. A loss of apamin-sensitive SK channels due to SK channel heteromerization in MD rats may also occur and confer apamin insensitivity [30]. Given that the effects of CRF on LHb intrinsic excitability are mediated through SK channels, the blunting of CRF's excitatory effects in the LHb of MD rats is partly due to MD-induced reduction in expression and gating of SK channels.

Although it has been shown that CRF pre- or post-synaptically potentiates GABAergic transmission in some brain areas such as dorsal raphe (DR), central amygdala (CeA), and BNST [43-45], we found that CRF depressed GABAergic synaptic transmission onto LHb neurons by decreasing GABA release from presynaptic terminals. AMPAR-mediated glutamatergic synaptic transmission, however, was unaffected by CRF. This indicates that CRF increases LHb neuronal excitability not only through changes in

membrane properties (intrinsic excitability) but also by depressing GABAergic synaptic transmission.

Both AMPA/NMDA ratios and the amplitude of mEPSCs were larger in LHb neurons from MD rats suggesting an induction of a postsynaptic glutamatergic potentiation by MD. This glutamatergic potentiation was not associated with a change in AMPAR subunit composition. Reduction in SK2 protein levels by MD likely facilitates potentiation of glutamatergic synapses in LHb neurons. In fact, long-term potentiation (LTP) at Schaffer collaterals to CA1 synapses involving recruitment of GluA1 containing AMPAR to the plasma membrane was accompanied by a PKA-dependent endocytosis of SK2 channels [46]. Similarly, potentiation of glutamatergic synapses and reduction of K⁺ conductance underlie LHb hyperexcitability after repeated injections of cocaine [47]. MD also increased presynaptic glutamate release in LHb neurons similar to what was found in LHb neurons projecting to the VTA in learned helplessness animal models of depression [16]. Although glutamatergic synaptic modifications induced by MD support LHb hyperexcitability, MD was associated with significant increases in GABAergic synaptic transmission both pre- and post-synaptically. Alteration in GABAergic synaptic strength could have been triggered in response to long-term changes in neuronal excitability induced by MD to stabilize neuronal activity (i.e., homeostatic synaptic scaling) [48]. However, it is also possible that changes in LHb intrinsic excitability is a neuroadaptation triggered by MD-induced changes in GABAergic synaptic inputs to the LHb. Main GABAergic structures projecting to the LHb include the entopeduncular nucleus (EPN)/the internal globus pallidus (GPi, in primates) and the VTA which are shown to co-release GABA and glutamate onto LHb neurons [15, 49]. Therefore, an increase in

glutamate and GABA release from such inputs onto LHb neurons following MD may elicit a homeostatic increase in LHb neuronal intrinsic excitability observed here.

Whether MD alters the way CRF affects the strength of synaptic inputs onto LHb neurons is also an important question that merits further investigation given the increasing interest in the role of the extrahypothalamic CRF system in health and disease. Recent advances in optogenetic interrogation of neuronal circuitries and the availability of promoter driven Cre mouse and rat lines will help us to elucidate the specific contributions of synaptic inputs to LHb dysfunction after MD and further delineate mechanisms underlying MD-induced dysregulation of intra-LHb CRF signaling.

Materials and Methods

All experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Uniformed Services University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Maternal Deprivation Procedure

Half of Sprague–Dawley male rat pups at P9 were isolated at 10:00 a.m. from the dam and siblings for 24hr (MD group). The isolated rats were placed in a separate empty room and kept on a heating pad (34° C) and not disturbed until being returned to their home cage. The remaining non-separated male rat pups received the same amount of handling and served as the non-maternally deprived control group (non-MD group). Each day, two MD and non-MD rats (age-matched) of the same litter were sacrificed over P21-28 for electrophysiology recordings and Western Blotting. Except Figure 13 where two or three neurons were recorded per animal, (n represents number of recorded cells/number of

rats), one cell per animal was recorded to demonstrate that the MD effects were consistently observed across multiple animals; therefore, all reported *n* values in those graphs represent the number of animals.

Slice preparation for Electrophysiology

Rats were anesthetized with isoflurane and immediately decapitated. The brains were quickly dissected and placed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.00 MgSO₄, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O₂–5% CO₂. Sagittal slices containing the LHb were cut at 250 μ m and incubated in ACSF at 34°C for at least 1 h. Slices were then transferred to a recording chamber and perfused with ascorbic acid-free ACSF at 28°C.

Electrophysiology

Whole cell and cell-attached recordings were performed on LHb slices using a patch amplifier (Multiclamp 700B) under infrared-differential interference contrast microscopy. Data acquisition and analysis were carried out using DigiData 1440A, pClamp 10 (molecular devices, Union City, CA), Clampfit and Mini Analysis 6.0.3 (Synaptosoft Inc.). Signals were filtered at 3 kHz and digitized at 10 kHz. The recording ACSF was the same as the cutting solution except that it was ascorbic acid-free. Spontaneous activity was monitored using cell-attached voltage-clamp recordings of action potentials (APs) at $I = 0$ pA for 5 minutes. Number of APs was counted over 5 min and spike frequency was calculated. Neuronal excitability recordings in response to depolarization were performed in whole-cell current-clamp mode. LHb neurons were given increasingly

depolarizing current steps across two ranges of current intensities: one at +10pA intervals ranging from +10pA to +100pA, and the other at +50pA intervals ranging from +50pA to +500pA, allowing us to measure AP generation in response to membrane depolarization (5 sec duration). Current injections were separated by a 20s interstimulus interval and neurons were kept at -65 mV with manual direct current injection between pulses. Synaptic transmission blockade was achieved by adding 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μ M), picrotoxin (100 μ M) and APV (50 μ M) to block AMPA, GABA_A and NMDA receptor-mediated synaptic transmission, respectively. The number of APs induced by depolarization at each intensity was counted and averaged for each experimental group.

Since AHP amplitude increases with increasing number of APs, we measured AP threshold, mAHP and fAHP amplitudes at the current step that was sufficient to generate the first AP/s [50]. AP threshold was measured at the beginning of the upward rise of the AP. mAHP was measured as the difference between AP threshold and the peak negative membrane potential at the end of the current step. fAHPs were calculated as the difference between AP threshold and the peak negative potential following the AP (Figure 9D). Resting membrane potential (V_m) was assessed at the beginning of the recording by quickly switching to $I=0$. Reported AP half width, AP peak amplitude were obtained from measurements of AP characteristics in Clampfit.

Whole-cell recordings of GABA_AR-mediated mIPSCs were isolated in ACSF perfused with DNQX; 10 μ M, strychnine (1 μ M) and tetrodotoxin (TTX, 1 μ M). The patch pipettes (3–6 M Ω) were filled with 125 mM KCl, 2.8 mM NaCl, 2 mM MgCl₂, 2 mM ATP-Na⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA, and 10 mM HEPES (pH adjusted to 7.28

with KOH, osmolarity adjusted to 275–280 mOsm). AMPAR-mediated mEPSCs were isolated in ACSF perfused with picrotoxin (100 μ M), D-APV (50 μ M) and TTX (1 μ M). Patch pipettes for mEPSC recordings were filled with 117 mM Cs-gluconate, 2.8 mM NaCl, 5 mM MgCl₂, 2 mM ATP-Na⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA, and 20 mM HEPES (pH adjusted to 7.28 with CsOH, osmolarity adjusted to 275–280 mOsm). For both mIPSCs and mEPSCs, LHb neurons were voltage-clamped at –70 mV and recorded over 10 sweeps, each lasting 50 seconds.

EPSCs were evoked with a stimulating electrode placed in the stria medullaris. Evoked EPSCs were recorded in ACSF perfusion containing picrotoxin (100 μ M) while the cell was voltage-clamped at +40 mV. Internal solution for patch pipettes was similar to that used for mEPSC recordings (Cs-gluconate-based) but also included intracellular spermine (10 μ M). AMPAR-mediated currents were isolated with D-APV (50 μ M), a selective NMDA receptor antagonist. Isolated AMPAR-mediated currents were then subtracted from the combined EPSC to provide the NMDA receptor-mediated current and thus, the AMPA/NMDA ratio. AMPAR EPSCs were also recorded at holding potentials ranging from –65 to +40 mV in the presence of picrotoxin (100 μ M), APV (50 μ M) and intracellular spermine (10 μ M) included in Cs-gluconate-based internal. Normalized current-voltage (I-V) curves were then generated by dividing the AMPAR EPSC peak amplitudes by the mean of AMPAR EPSC peak amplitude recorded at –65 mV. AMPAR EPSC rectification was determined by dividing peak AMPAR EPSCs amplitudes recorded at –65 mV by those recorded at +40 mV. The cell input resistance and series resistance were monitored through all the experiments and if these values changed by more than 10%, data were not included.

Antalarmin, CYPPA and 1-EBIO were prepared as stock solution in DMSO and were diluted (1:10000) to final concentration in ACSF of 1 μ M, 10 μ M, and 300mM, respectively. Stock solutions for apamin and CRF were prepared in distilled water and diluted (1:1000) to final concentration in ACSF of 100nM and 250nM, respectively. The intra-pipette concentrations of BAPTA and GDP β s were 300 μ M and 30mM, respectively. In some of our control interleaved experiments, AP recordings were continued for an hour without addition of drugs and no significant changes were observed over time. For all drug experiments, a baseline depolarization-induced AP recording/mIPSC/mEPSC was recorded in each neuron, and then the appropriate drug was added to the slice via the perfusate and AP generation in response to depolarizing current steps was again tested 25-30 min after. For apamin and CYPPA experiments, a second baseline with apamin or CYPPA was obtained before the addition of CRF and AP generation was re-evaluated 25-30min after CRF application.

Western Blotting

The LHb was dissected bilaterally from sagittal slices (300 μ M) of nonMD or MD rats in ACSF and then snap frozen in liquid nitrogen and stored at -80°C. Tissues were thawed, washed in ice-cold PBS and lysed in RIPA buffer containing protease inhibitors (Sigma). Samples were then sonicated, incubated on ice for 30 minutes and centrifuged at 10,000g for 20 min at 4°C. Protein concentration in the supernatant was determined by Pierce BCA Protein Assay Kit (Life Technologies). Equal amounts of protein (20 μ g) were combined with loading buffer, boiled for 5 min, and loaded onto 4–20% precast polyacrylamide gel (Bio-Rad Laboratories). Separated proteins were transferred onto nitrocellulose membranes, blocked with casein-based blocking reagent (I-Block, Life

Technologies) for 60 minutes at room temperature and then incubated overnight at 4°C with rabbit anti-SKs (Anti-KCa2.1 for SK1, Anti-KCa2.2 for SK2, and Anti-KCa2.3 (C-term) for SK3, 1:300, Alomone Labs), or mouse anti- β -actin (1:10,000, Abcam, ab6276). After incubation, the membranes were washed with PBS-T and exposed to the appropriate horseradish peroxidase-linked secondary antibody (Cell Signaling). Blots were developed with Clarity Western ECL Substrate (Bio-Rad Laboratories) and detected using a BioRad ChemiDoc Touch image acquisition system (BioRad Laboratories, Hercules, CA, USA). Data was analyzed using ImageJ software.

Data analysis

Values are presented as means $\pm$ s.e.m. Statistical significance was determined using unpaired or paired two tailed Student's t-test, two way ANOVA or repeated-measures ANOVA (RMANOVA) with Bonferroni post hoc analysis with significance level of $p < 0.05$. The peak values of the evoked paired EPSCs were measured relative to the same baseline. A stable baseline value was considered in each sweep of paired pulses starting at 20-50ms right before the emergence of the EPSC current using p-Clamp 10 software. Mini Analysis software was used to detect and measure mIPSCs and mEPSCs using preset detection parameters of mIPSCs and mEPSCs with an amplitude cutoff of 5 pA. The Kolmogorov–Smirnov test (KS test) was performed for the statistical analyses of cumulative probability plots of mEPSCs and mIPSCs. All statistical analyses were performed using IBM SPSS Statistics 24 or Origin 2016.

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Competing interests: The authors declare no conflict of interest.

Figures

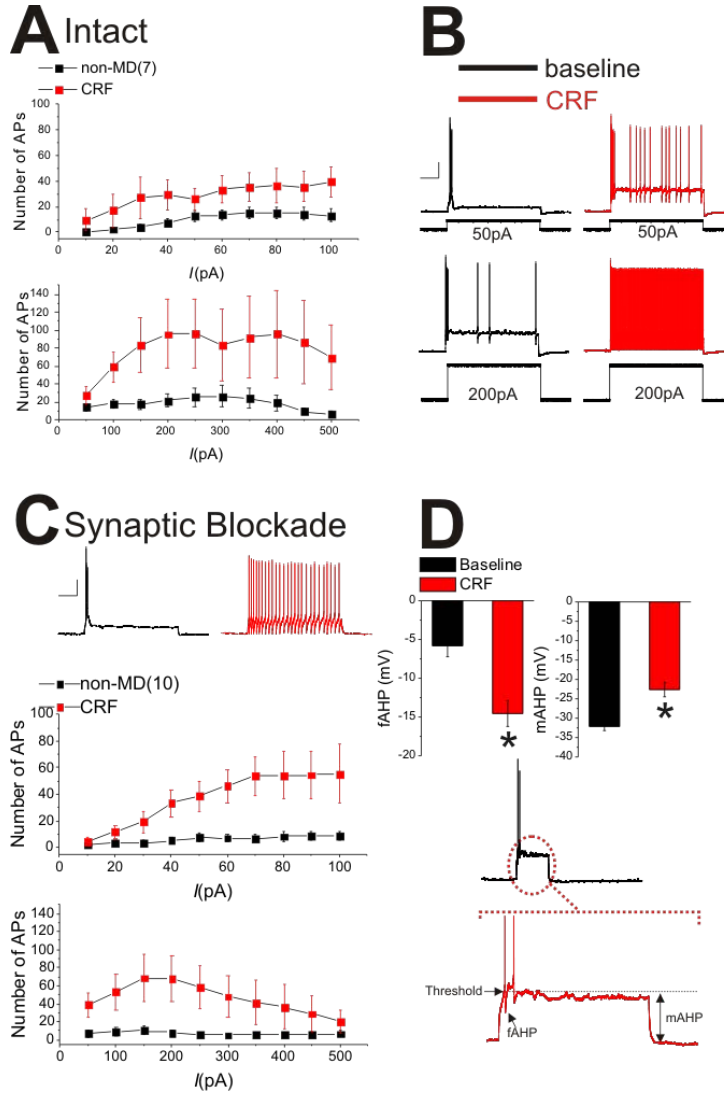


Figure 9. CRF increases the excitability of LHB neurons. **A:** Depolarizing current injection elicited more APs in LHB neurons after CRF (250nM) application with intact synaptic transmission. In this and all the other relevant figures throughout the paper, LHB neuronal excitability in response to depolarization was evaluated across two ranges of current intensities: 10-100pA and 50-500pA ($n=7$, 10-100pA: $F_{(1,6)}=12.3$, $p=0.013$, 50-500pA: $F_{(1,6)}= 5.34$, $p=0.05$; two-way RMANOVA) **B:** Sample AP recordings of LHB

neurons in response to depolarizing current steps before (baseline, black) and after CRF (red) application (calibration bar: 20mV/1s). **C:** LHB neurons fire more APs in response to depolarizing current steps after application of CRF with fast synaptic transmission blocked (n=10, 10-100pA: $F_{(1,9)}=13.901$, $p=0.0013$, 50-500pA: $F_{(1,9)}=7.963$, $p=0.023$; two-way RMANOVA). Top traces show sample AP recordings in response to a 50pA depolarizing current step before and after CRF application (calibration bar: 20mV/1s). **D:** Average bar graphs of the amplitude of fAHP and mAHP with synaptic transmission blockade. CRF lowers AP threshold, increases the amplitude of fAHP and decreases the amplitude of mAHP (n=10, AP threshold: baseline= -37.37 ± 1.26 mV, CRF= -45.09 ± 0.9 mV, $p=0.003$; fAHPs: baseline= -5.79 ± 1.42 mV, CRF= -14.53 ± 1.66 mV, $p=0.004$, mAHPs: baseline= -32.08 ± 1.08 mV, CRF= -22.59 ± 1.87 mV, $p=0.033$; paired Student's t-tests). Bottom traces demonstrate the method used to measure AP threshold, mAHP, and fAHP in a sample AP recording in response to the lowest depolarizing current step that generated the first AP/s. In this and all the other graphs * represents a statistical significant difference.

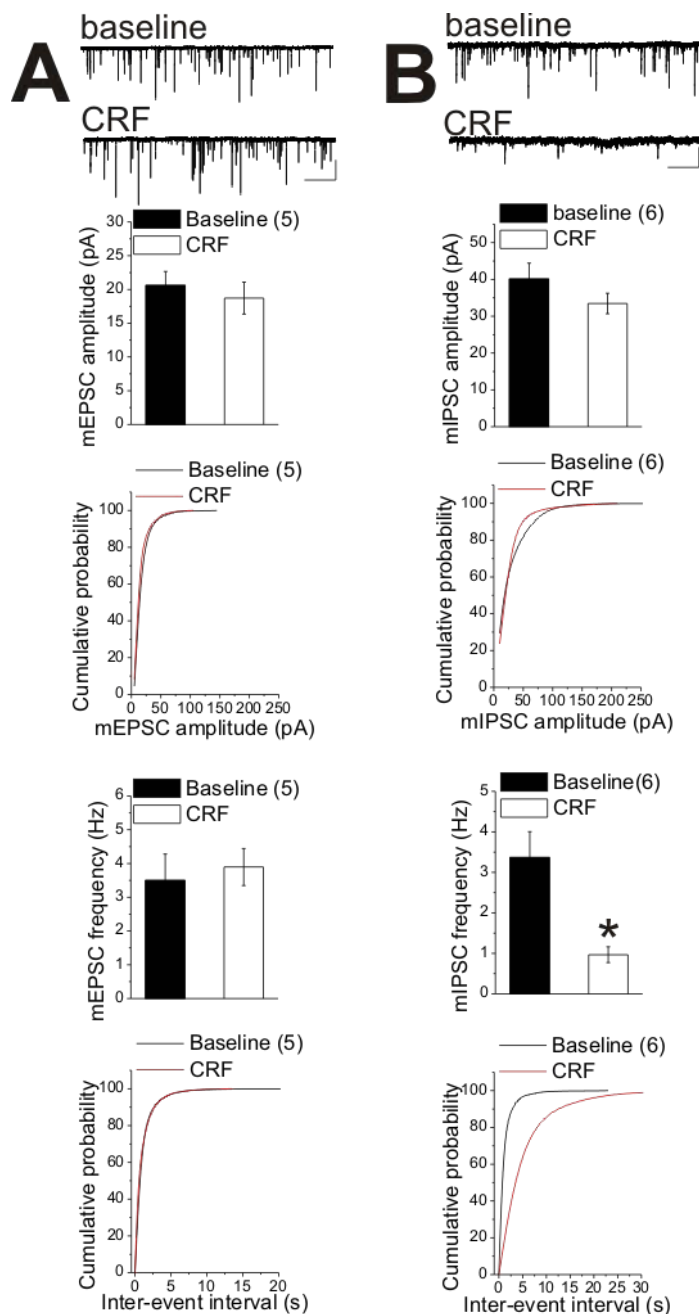


Figure 10: CRF depresses GABAergic transmission without affecting glutamatergic synaptic transmission. A: Sample AMPAR-mediated mEPSC traces before (baseline) and after CRF (250nM) application (calibration bars: 30 pA/5s). Average bar graphs of mEPSC amplitude and frequency, and cumulative probability plots of amplitude and frequency (inter-event interval) for all mEPSCs before and after CRF application. CRF did not alter

the amplitude or frequency of mEPSCs in LHb neurons (n=5, baseline: 20.61 ± 4.58 pA, 3.5 ± 1.73 Hz; CRF: 18.72 ± 2.36 pA, 3.89 ± 1.22 Hz; paired Student's *t*-tests: $p = 0.071$ for amplitude, $p = 0.54$ for frequency; KS tests for cumulative distribution curves: $p = 0.5$ for amplitude, $p = 0.5$ for frequency). **B:** Sample GABA_AR-mediated mIPSC traces before and after CRF (250nM) application (calibration bars: 50 pA/5s). Average bar graphs of mIPSC amplitude and frequency, and cumulative probability plots of amplitude and frequency (inter-event interval) for all mIPSCs before and after CRF application. CRF significantly reduced the amplitude and frequency of mIPSCs in LHb neurons (n=6, baseline: 40.13 ± 4.26 pA, 3.37 ± 0.63 Hz; CRF: 33.458 ± 2.77 pA, 0.96 ± 0.19 Hz; paired Student's *t*-tests: $p < 0.05$ for amplitude, $p = 0.022$ for frequency; KS tests for cumulative distribution curves: $p = 0.094$ for amplitude, $p < 0.001$ for frequency).

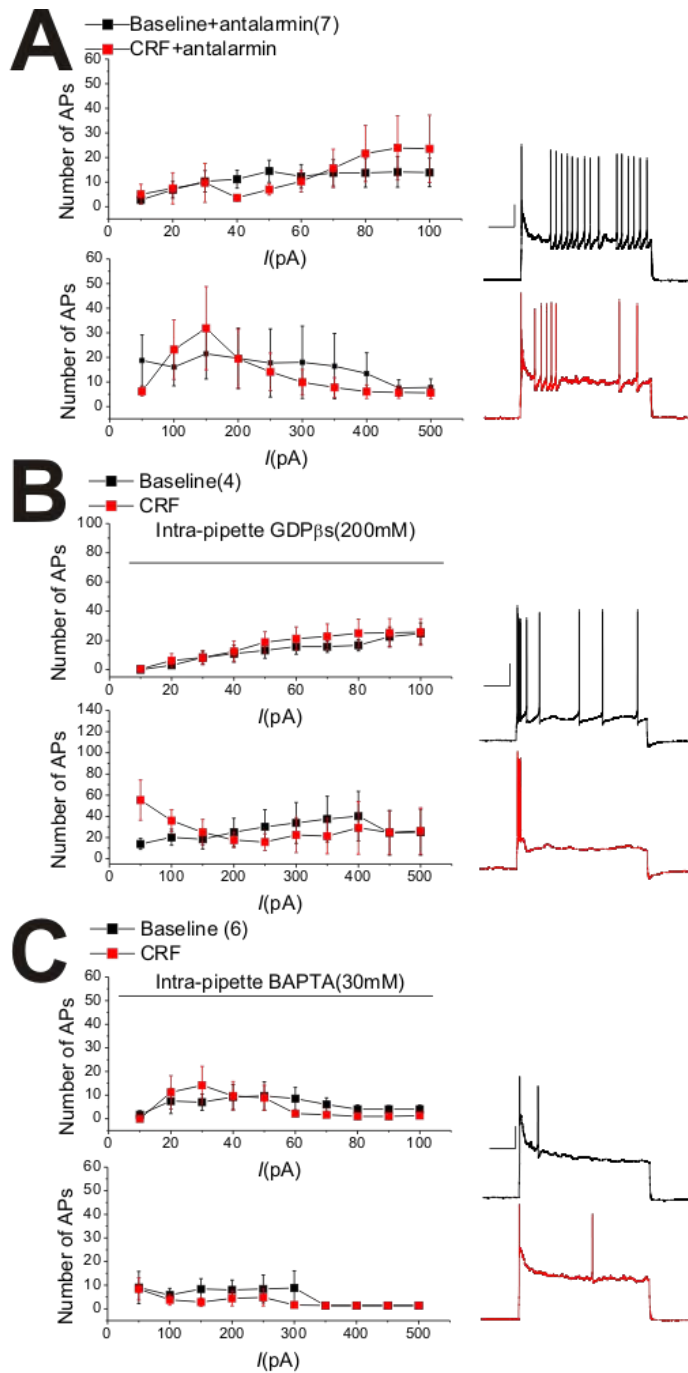


Figure 11: CRF increases LHb intrinsic excitability through G-protein-dependent CRFR1 signaling and requires intracellular Ca^{2+} . All recordings in this graph were performed with fast synaptic transmission blocked. Each row shows AP recordings in

response to depolarizing step currents with representative AP traces (in response to a 100pA current step) before (baseline) and after CRF application. There was no significant difference in the number of APs recorded from LHb neurons after application of CRF (250nM) when CRFR1 antagonist antalarmin (1 μ M) was present in the perfusate throughout the experiment (**A**, n=7, 10-100pA: $F_{(1,6)}=0.222$, $p=0.654$, 50-500pA: $F_{(1,6)}=0.961$, $p=0.364$; two-way RMANOVA), or when 300 μ M GDP β S (**B**, n=4, 10-100pA: $F_{(1,3)}=0.86$, $p=0.422$, 50-500pA: $F_{(1,4)}=0.104$, $p=0.768$; two-way RMANOVA) or 30mM BAPTA (**C**, n=6, 10-100pA: $F_{(1,5)} = 0.327$, $p= 0.592$, 50-500pA: $F_{(1,5)} = 4.87$, $p= 0.092$; two-way RMANOVA) was included in patch pipette.

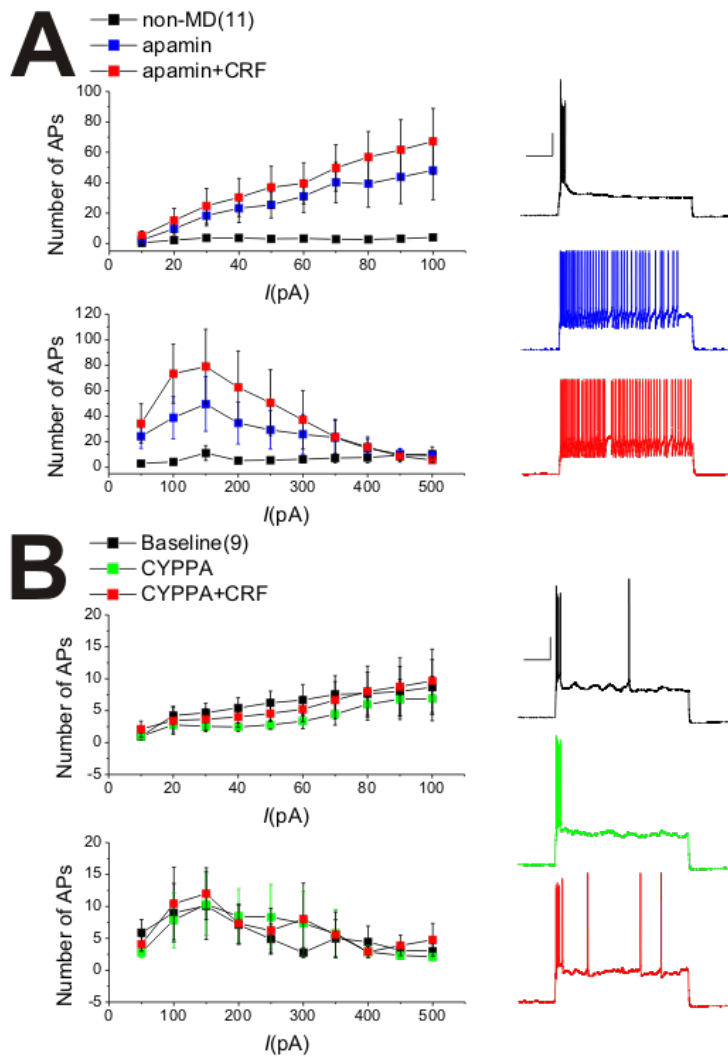


Figure 12: Pharmacological modulation of SK channels alters CRF-induced increases in LHb intrinsic excitability. All recordings in this graph were performed with fast synaptic transmission blocked. **A.** AP recordings in response to depolarizing current steps with representative AP traces (in response to a 100pA current step) before (baseline, black), after apamin (100nM, blue) and subsequent CRF (250nM, red) bath applications. Apamin mimics and occludes CRF-induced increases in intrinsic excitability (n=11, 10-100pA:

$F_{(2,20)}=9.127$, $p=0.015$, 50-500pA: $F_{(2,20)}=5.673$, $p=0.037$; two-way RMANOVA; basal vs. apamin, $p=0.014$; apamin vs. CRF, $p=0.612$). Effects of apamin on the amplitudes of fAHP and mAHP (n=11, fAHPs: baseline= -1.14 ± 2.5 mV, apamin= -8.47 ± 2.13 mV, $p=0.018$, mAHPs: baseline= -36.63 ± 1.8 mV, apamin= -27.81 ± 1.73 mV, $p < 0.0001$; paired Student's t-tests). **B.** AP recordings in response to depolarizing current steps with representative AP traces (in response to a 100pA current step) before (baseline, black), after CYPPA (10 μ M, green) and subsequent CRF (250nM, red) bath applications. CyPPA does not affect LHb intrinsic excitability but blocks the effect of CRF on LHb neuronal excitability (n=9, 10-100pA: $F_{(2,16)}=2.058$, $p=0.198$, 50-500pA: $F_{(2,16)}=0.49$, $p=0.632$; two-way RMANOVA).

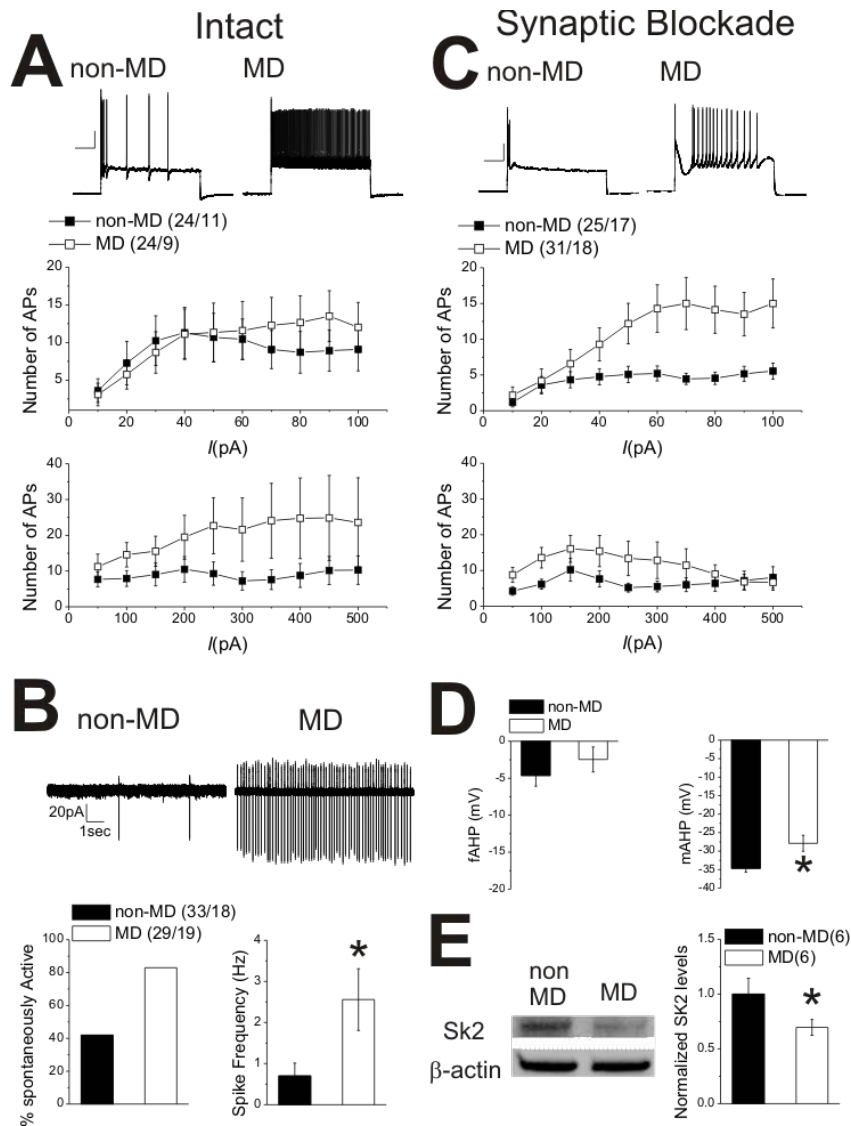


Figure 13. MD increases the excitability of LHb neurons. **A.** Sample AP recordings of LHb neurons with intact synaptic transmission from non-MD and MD rats in response to depolarizing current steps (calibration bar: 20mV/1s). Depolarizing current injection elicited more APs in LHb neurons from MD rats compared with non-MD rats with intact synaptic transmission (non-MD: n= 24 cells/ 11 rats; MD: n=24 cells/ 9rats, 10-100pA: $F_{(1,460)}=1.69$, $p=0.803$, 50-500pA: $F_{(1,460)}=11.329$, $p=0.001$). **B.** Sample spontaneous AP recordings of LHb neurons from non-MD and MD rats using cell-attached voltage clamp;

Cell-attached recordings over 5 minutes revealed more spontaneously active neurons with higher spike frequency in the LHb from MD versus non-MD rats (non-MD: n= 33 cells/ 18 rats; MD: n=29 cells/ 19rats, spontaneously active LHb neurons detected in non-MD versus MD rats: 42% vs 83%, spike frequencies: non-MD: 0.7 ± 0.31 ; MD: 2.56 ± 0.75 , unpaired Student's t-tests: $p=0.018$) **C.** Sample AP recordings of LHb neurons with blocked fast synaptic transmission from non-MD and MD rats in response to depolarizing current steps. Depolarizing current injection elicited more APs in LHb neurons from MD rats compared with non-MD rats with synaptic transmission blocked (non-MD: n= 25 cells/ 17 rats; MD: n=31 cells/ 18 rats, 10-100pA: $F_{(1,600)}=35.84$, $p<0.0001$, 50-500pA: $F_{(1,600)}=10.53$, $p=0.013$; two-way ANOVA). **D:** Average bar graphs of the amplitude of fAHP and mAHP derived from AP recordings in **C.** MD leads to a significant decrease in the amplitude of mAHP with no change in the amplitude of fAHP (non-MD: n= 25 cells/ 17 rats, fAHPs = -4.63 ± 1.42 mV, ; MD: n=31 cells/ 18 rats, fAHP= -2.46 ± 1.68 mV, $p=0.348$, nonMD: mAHPs= -34.66 ± 1.0 mV, MD: mAHP= -27.87 ± 2.17 mV, $p= 0.011$; unpaired Student's t-tests). **E:** Representative Western blots and quantitative data of SK2 and β -actin (control) in LHb homogenates from non-MD and MD rats are shown. MD had significantly lower total SK2 protein levels in LHb tissue homogenates (non-MD: n= 6, MD: n=6, $p= 0.032$; unpaired Student's t-test). In contrast, we did not observe any significant change in protein levels of SK1 and SK3 (non-MD: n= 6, MD: n=6, SK1: $p= 0.509$; non-MD: n= 8, MD: n=8, SK3: $p= 0.674$; unpaired Student's t-tests).

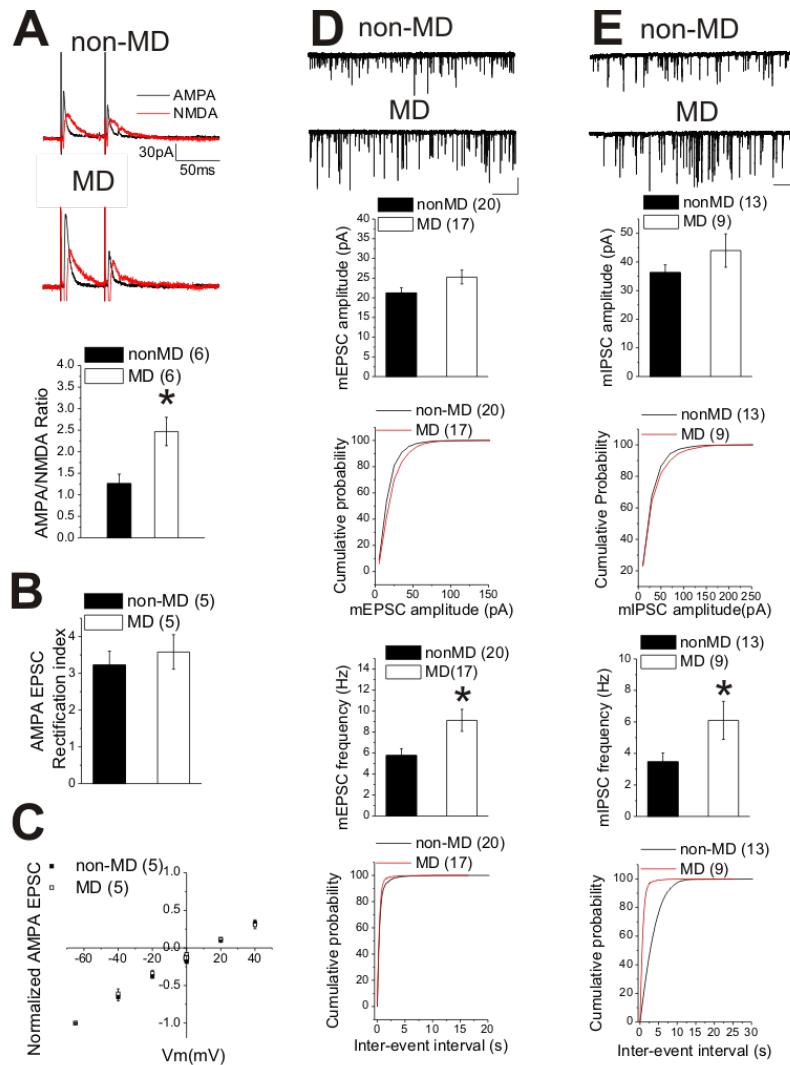


Figure 14. MD potentiates both glutamatergic and GABAergic synaptic transmission in LHB neurons. **A.** Sample traces of AMPA (black) and NMDA (red) EPSCs recorded at +40mV from LHB neurons of non-MD and MD rats. Summary of AMPA/NMDA ratios obtained from non-MD and MD rats. AMPA/NMDA ratios were significantly increased in MD rats (non-MD: $n=6$, 1.31 ± 0.42 ; MD: $n=6$, 2.52 ± 0.34 ; unpaired Student's t -test: $p=0.046$). **B.** Average bar graphs of AMPAR EPSC rectification indices. MD did not change AMPAR EPSC rectification (non-MD: $n=5$, 3.22 ± 0.37 ; MD: $n=5$, 3.58 ± 0.46 ;

unpaired Student's *t*-test: $p = 0.569$) **C.** Normalized I-V plots of AMPAR EPSCs in LHB neurons of non-MD and MD rats (non-MD: $n=5$, MD: $n=5$, two way ANOVA for IV curves, $F_{(1,53)} = 2.28837$, $p = 0.136$). **D.** Sample AMPAR-mediated mEPSC traces from non-MD and MD rats (calibration bars: 30 pA/5s). Average bar graphs of mEPSC amplitude and cumulative probability plots of amplitude for all mEPSCs in non-MD and MD rats. mEPSC amplitude is increased by MD as evidenced by cumulative probability plots. Average mEPSC amplitude however, is unaffected. Cumulative probability plots of frequency (inter-event interval) for all mEPSCs in non-MD and MD rats. MD significantly increases mEPSC frequency as evidenced by both average frequency and cumulative probability plot (non-MD: $n=20$, 21.24 ± 1.33 pA, 5.76 ± 0.64 Hz; MD: $n=17$, 25.26 ± 1.75 pA, 9.11 ± 1.04 Hz; unpaired Student's *t*-tests: $p = 0.072$ for amplitude, $p = 0.008$ for frequency; KS tests for cumulative distribution curves: $p < 0.05$ for amplitude, $p < 0.05$ for frequency). **E:** Sample GABA_A-mediated mIPSC traces from non-MD and MD rats (calibration bars: 50 pA/5s). Average bar graphs of mIPSC amplitude and cumulative probability plots of amplitude for all mIPSCs in non-MD and MD rats. Average amplitudes of GABA_A receptor-mediated mIPSCs are not significantly different between MD and non-MD rats, but the cumulative probability of mIPSC amplitudes is significantly increased after MD. Average bar graphs of mIPSC frequency and cumulative probability plots of frequency (inter-event interval) for all mIPSCs in non-MD and MD rats. Both average frequencies and cumulative probability inter-event intervals of mIPSCs are significantly increased in MD rats (non-MD: $n=13$, 36.30 ± 2.69 pA, 3.46 ± 0.55 Hz; MD: $n=9$, 43.98 ± 5.82 pA, 6.1 ± 1.21 Hz; unpaired Student's *t*-tests: $p = 0.2$ for amplitude, $p = 0.04$ for frequency; KS tests for cumulative distribution curves: $P < 0.05$ for amplitude, $p < 0.0001$ for frequency).

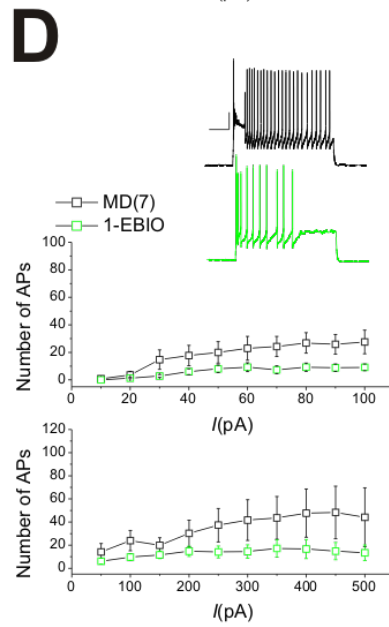
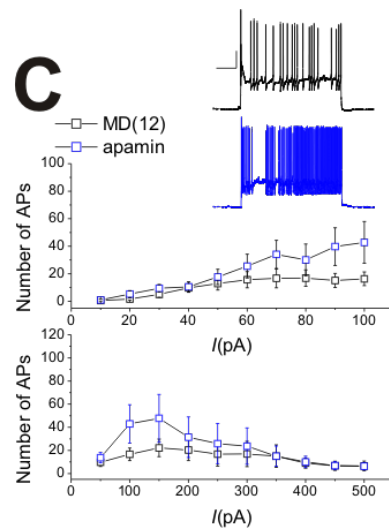
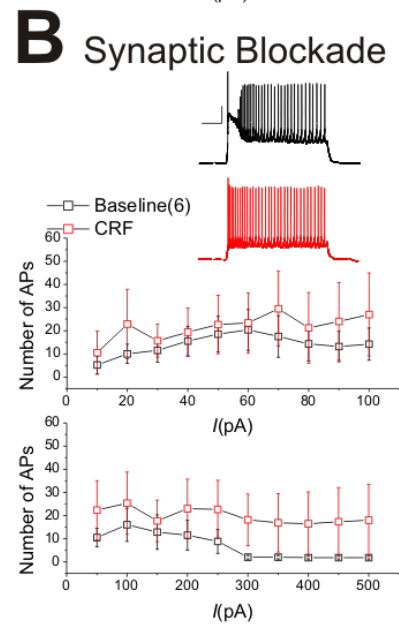
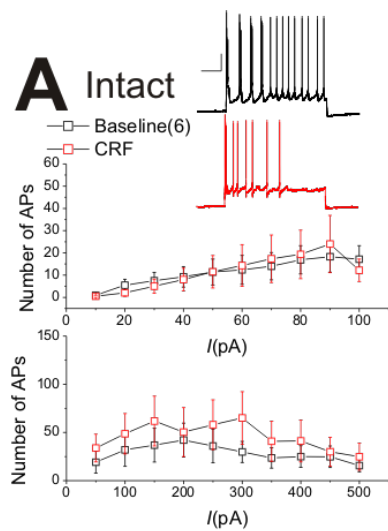


Figure 15. The effects of CRF and apamin on LHb intrinsic excitability are blunted following MD. AP recordings in response to depolarizing current steps with representative AP traces (in response to a 100pA current step) before (baseline, black) and after CRF (red) application in intact fast synaptic transmission (**A**, n=6, 10-100pA: $F_{(1,5)}=0.38$, $p=0.565$, 50-500pA: $F_{(1,5)}=2.353$, $p=0.209$) and blockade of fast synaptic transmission (**B**, n= 6, 10-100pA: $F_{(1,5)}=2.335$, $p=0.187$, 50-500pA: $F_{(1,5)}=4.789$, $p=0.08$; two-way RMANOVA). There is no significant difference in the number of APs in response to depolarization recorded from LHb neurons of MD rats after application of CRF (250nM) with or without fast synaptic transmission. **C.** AP recordings in response to depolarizing current steps with representative AP traces (in response to a 100pA current step) before (baseline, black) and after apamin (100nM, blue) application with fast synaptic transmission blocked. There was no significant difference in the number of APs in response to depolarization recorded from LHb neurons of MD rats after apamin application (n=12, 10-100pA: $F_{(1,11)}=1.816$, $p=0.205$, 50-500pA: $F_{(1,11)}= 3.112$, $p=0.108$; two-way RMANOVA). **D.** AP recordings in response to depolarizing current steps with representative AP traces (in response to a 100pA current step) before (baseline) and after 1-EBIO (300mM, green) application with fast synaptic transmission blocked. A positive SK channel modulator, 1-EBIO, significantly decreases LHb intrinsic excitability at current intensities ranging from 10-100pA (n=7, 10-100pA: $F_{(1,6)}=5.677$, $p=0.05$, 50-500pA: $F_{(1,6)}=5.460$, $p=0.058$; two-way RMANOVA).

	non-MD				MD			
	Baseline	CRF	apamin	CYPPA	Baseline	CRF	apamin	1-EBIO
AP threshold(mV)	-37±0.8	-45±0.9	-43±1.4	-37±1.5	-40±1.5	-43±1.6	-42±1.8	-42±1.8
AP peak(mV)	132±2	119±3.7	138±2.6	110±5.3	131±2.8	111±6.1	133±4.4	134±8
AP half width(ms)	2.85±0.6	1.97±0.9	1.98±0.1	2.38±0.1	2.08±0.1	2.4±0.2	1.71±0.1	1.4±0.14
fAHP(mV)	-4.6±1.4	-14±1.6	-6.02±3	-9.8±4.5	-2.4±1.6	-8.6±0.9	-6.1±3.1	-12±2.2
mAHP(mV)	-34 ±1	-22 ±2	-27 ±1	-33.8 ±1	-27.8 ±2	-23 ±2	-28 ±1.8	-30±2

Table 1. Summary of changes in AP characteristics of LHb neurons from non-MD and MD rats before and after drug application. Measurements of the baseline parameters are all derived from AP recordings in response to depolarization depicted in Figure 13C while synaptic transmission was blocked. Except mAHP, all the other measured passive and active properties of LHb neurons including Vm were unaffected following MD (nonMD: Vm=-37±1.2, MD: Vm=-39±1.2, p=0.22 unpaired Student's t-test).

CHAPTER 4: Morphine-induced synaptic plasticity in the VTA is reversed by HDAC inhibition

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Running title: Reversal of morphine-induced plasticity by HDACi

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Abstract

Dopamine (DA) dysfunction originating from the ventral tegmental area (VTA) occurs as a result of synaptic abnormalities following consumption of drugs of abuse and underlies behavioral plasticity associated with drug abuse. Drugs of abuse can cause changes in gene expression through epigenetic mechanisms in the brain that underlie some of the lasting neuroplasticity and behavior associated with addiction. Here we investigated the function of histone acetylation and histone deacetylase (HDAC2) in the VTA in recovery of morphine-induced synaptic modifications following a single *in vivo* exposure to morphine. Using a combination of immunohistochemistry, Western blot and whole-cell patch clamp recording in rat midbrain slices, we show that morphine increased HDAC2 activity in VTA DA neurons and reduced histone H3 acetylation at lysine 9 (Ac-H3K9) in the VTA 24 hours following the injection. Morphine-induced synaptic changes at glutamatergic synapses involved endocannabinoid (eCB) signaling to reduce GABAergic synaptic strength onto VTA DA neurons. Both plasticities were recovered by *in vitro* incubation of midbrain slices with a class I specific HDAC inhibitor (HDACi), CI-994, through an increase in acetylation of histone H3K9. Interestingly, HDACi incubation also increased levels of Ac-H3K9, and triggered GABAergic and glutamatergic plasticities in DA neurons of saline-treated rats. Our results suggest that acute morphine-induced changes in VTA DA activity and synaptic transmission engage HDAC2 activity locally in the VTA to maintain synaptic modifications through histone hypoacetylation.

The New & Noteworthy section

Learning mechanisms in brain reward pathways are hijacked after experience with drugs of abuse, shaping drug-related memories that underlie compulsive drug-taking behavior. Ventral tegmental area (VTA) dopamine neuronal dysfunction is an initial step for the development of addiction. Drugs of abuse may silence genes in the VTA while compounds called histone deacetylase (HDAC) inhibitors may oppose this. We demonstrate that acute morphine-induced synaptic plasticities in the VTA are reversible by an HDAC inhibitor.

Keywords: VTA, synaptic plasticity, electrophysiology, synaptic transmission, HDAC

Introduction

Although opioids are among the most effective pain relievers, their potential for abuse is undeniable. Certain brain areas are known loci for drug-induced changes in activity, gene expression and synaptic transmission [1]. These include the nucleus accumbens (NAc), prefrontal cortex (PFC) and VTA, all of which are key parts of the brain's reward circuitry. The VTA is of particular interest as a locus for drug reward since enhanced DA transmission from the VTA is an early neuroadaptation in response to a single exposure to drugs of abuse and triggers subsequent synaptic changes in projection areas of the VTA, such as the NAc and PFC [2, 3]. Drug-evoked synaptic plasticity at glutamatergic and GABAergic synapses onto VTA DA neurons significantly contributes to drug-induced changes in DA cell excitability and DA signaling. Long-term potentiation, LTP, of glutamatergic synapses onto VTA DA neurons can be triggered 24 hours after a single

in vivo exposure to opioids and other drugs promoting DA cell excitability and behavioral sensitization [4-6]. Moreover, a single exposure to a drug of abuse such as morphine has been shown to block LTP and may occlude long-term depression (LTD) at GABAergic synapses onto VTA DA neurons, further enhancing DA signaling from the VTA [7, 8].

Neuroplastic changes in response to drugs of abuse also include drug-induced transcriptional changes. Drugs of abuse such as morphine are most likely enacting these transcriptional changes via epigenetic mechanisms. In the VTA, such modifications are necessary for reward-learning [9]. Epigenetic mechanisms such as histone lysine-tail acetylation are able to encode environmental stimuli into a change in mRNA expression levels by chromatin remodeling, promoting gene transcription. Such chromatin modification has been shown to alter gene expression in neurons and neuroplasticity underlying memory formation [10, 11]. Acute and chronic administration of psychostimulants and morphine elicits histone modifications such as histone acetylation at a specific subset of learning-associated genes in the brain reward circuitry including the VTA that could facilitate and consolidate lasting neural and behavioral plasticity induced by drug abuse. Moreover, increasing histone acetylation through pre- or co-administration of HDACis in conjunction with abused drugs promotes or opposes drug-induced synaptic and behavioral abnormalities suggesting that this epigenetic mark might be required to potentiate or prevent an addicted state [12-22]. This opens the possibility of targeting epigenetic mechanisms during initial learning or memory retrieval of drug-associated cues as a novel pharmacological therapy for extinction of drug-seeking behaviors. It is worthwhile to mention that in all studies investigating the effects of HDAC inhibition on addictive behaviors, HDACis have been used as pretreatment drugs

or in conjunction with drugs of abuse, therefore it is unknown whether HDAC inhibition per se is sufficient to reverse a drug-induced synaptic plasticity after a drug exposure. Stress and drugs of abuse tap into a common synaptic mechanism to alter DA signaling [5, 7, 23]. Recently, we demonstrated that a severe early life stress (i.e., 24 hour maternal deprivation) induces synaptic abnormalities within the VTA that are reversible by HDAC inhibition suggesting that early-life stress-induced neuroplasticity in the VTA involves HDACs to trigger long-lasting GABAergic and DA dysfunction [24]. Here, we tested whether HDAC inhibition locally in the VTA is sufficient to reverse epigenetic modifications and synaptic plasticities associated with a single *in vivo* injection of morphine 24h after exposure.

Materials and Methods

All experiments were carried out in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Uniformed Services University Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

Morphine treatment

Sprague–Dawley male rats (P14-P21) received either one intraperitoneal injection of morphine sulfate (10mg/kg) dissolved in 0.9% saline or an injection of comparable volumes of saline 24h prior to sacrifice for electrophysiological recordings or immunohistochemical studies. Only one cell per animal (saline or morphine) was recorded, therefore all reported n values represent the number of animals recorded.

Immunofluorescence and image analysis

Morphine- and saline-treated rats were anesthetized with an intraperitoneal injection containing ketamine (85 mg/kg) and xylazine (10 mg/kg) and perfused through the aorta with heparinized 1x phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA, USB, Cleveland, OH). The brains were dissected and placed in 4% PFA for 24 hrs and then cryoprotected by submersion in 20% sucrose for 3 days, frozen on dry ice and stored at -70 C until sectioned. Sections of the VTA were cut using a cryostat (Leica CM1900) and mounted on slides. Serial coronal sections (20 μ m) of the midbrain containing the VTA (from -4.92 to -6.72 mm caudal to bregma; Paxinos and Watson, 2001) were fixed in 4 % PFA for 5 minutes and washed in 1x PBS then blocked in 10% normal horse serum (NHS) containing 0.3 % Triton X-100 in 1x PBS for 1 hr. Sections were incubated in rabbit anti-TH (1:1000, Calbiochem, San Diego, CA.) and mouse anti-HDAC2 (1:1000, abcam, Cambridge, MA) in carrier solution (5 % NHS in 0.1 % Triton X-100 in 1x PBS) overnight at room temperature. After rinsing in 1x PBS, sections were incubated for 2 hours in Alexa Fluor® 488 labeled goat anti-rabbit IgG and Alexa Fluor® 568 labeled goat anti-mouse IgG (both diluted 1:200). Finally sections were rinsed in 1x PBS, dried, and cover slipped with Prolong mounting medium containing DAPI to permit visualization of nuclei. Background staining was assessed by omission of primary antibody in the immunolabeling procedure (negative control). VTA tissue sections of rats with previously established presence of TH/HDAC2 immunoreactive neurons were processed as positive control tissue. Images in Fig. 16 were captured using a Zeiss Confocal Inverted Microscope System (Carl Zeiss Inc.) 40x/1.4 n.a. oil immersion objective. At three AP locations (-5.4, -5.7, and -6.0 relative to bregma) six TH positive neuron were identified within the VTA. From each TH positive neuron two HDAC2

density readings (3 μm x 3 μm) were taken from the somatic region (clearly labeled with TH) and nuclear region (clearly labeled with DAPI). Three background density readings were taken from an area clearly not labeled with HDAC2. All density readings were normalized to background.

Slice preparation for Electrophysiology and Western Blot

Saline or morphine-treated rats were anesthetized with isofluorane, and immediately decapitated. The brains were quickly dissected and placed into ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 21.4 NaHCO₃, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.00 MgSO₄, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O₂–5% CO₂. Horizontal midbrain slices containing the VTA were cut at 250 μm (for electrophysiology) and incubated in ACSF at 34°C for at least 1 h. Slices were then transferred to a recording chamber and perfused with ascorbic acid-free ACSF at 28°C. Midbrain slices were cut at 400 μm for Western blot experiments.

For HDACi treatment, slices were incubated in the presence of CI-994 dissolved in DMSO and diluted to the final concentration in ACSF. The final concentration of CI-994 (20 μM) was selected for brain slice incubation because it is approximately the concentration measured in the brain following a systemic, intraperitoneal administration of 30 mg/kg CI-994 [25]. Controls were incubated in the same dilution of DMSO (1:1000), which did not affect synaptic transmission. These slices were allowed to incubate for 2-4 hours before being transferred to the recording chamber or dissecting the VTA for Western blots. In some of our control recordings, slices were only incubated in DMSO-free ACSF and the data for both DMSO and DMSO-free groups were pooled

together as controls in the graphs. For chemically induced GABAergic LTD experiments, drugs were bath applied after 10 min of stable baseline recordings of evoked IPSCs and remained in the bath throughout the experiment. The CB1 receptor agonist, R-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo- [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN 55,212-2) was dissolved in DMSO (1:1000 dilution) and diluted to 2 μ M in ACSF. Stock solutions for the group I mGluR agonist, dihydroxyphenylglycine (DHPG, 50 μ M), was made by dissolving in deionized water. All the drugs were purchased from Sigma, Tocris, or Calbiochem.

Western Blot

The VTA was dissected bilaterally from horizontal midbrain slices (400 μ M) of morphine- or saline-treated rats. For HDACi treatment, slices were incubated in ACSF in the presence of CI-994 dissolved in DMSO or the same dilution of vehicle (DMSO) for 2-4 hours before being snap frozen in liquid nitrogen and stored at -80°C. Tissues were thawed, washed in ice-cold PBS and lysed in RIPA buffer containing protease inhibitors (Sigma). Samples were then sonicated, incubated on ice for 30 minutes and centrifuged at 10,000g for 20 min at 4°C. Protein concentration in the supernatant was determined by Pierce BCA Protein Assay Kit (Life Technologies). Equal amounts of protein (20 μ g) were combined with loading buffer, boiled for 5 min, and loaded onto 4–20% precast polyacrylamide gel (Bio-Rad Laboratories). Separated proteins were transferred onto nitrocellulose membranes, blocked with casein-based blocking reagent (I-Block, Life Technologies) for 60 minutes at room temperature and then incubated overnight at 4°C with rabbit anti-Acetyl-Histone H3K9 (Ac-H3K9, 1:1000, Cell Signaling, #9649), or mouse anti- β -actin (1:10,000, Abcam, ab6276). After incubation, the membranes were

washed with PBS-T and exposed to the appropriate horseradish peroxidase-linked secondary antibody (Cell Signaling). Blots were developed with Clarity Western ECL Substrate (Bio-Rad Laboratories) and detected using a Fuji LAS-3000 image acquisition system (Fuji, Stamford, CT, USA).

Electrophysiology

Whole cell recordings were performed on midbrain slices using a patch amplifier (Multiclamp 700B) under infrared-differential interference contrast microscopy. Data acquisition and analysis were carried out using DigiData 1440A, pClamp 10 (molecular devices, Union City, CA) and Mini Analysis 6.0.3 (Synaptosoft Inc.). Signals were filtered at 3 kHz and digitized at 10 kHz. The recording ACSF was the same as the cutting solution except that it was ascorbic acid-free. The appearance of an I_h current (≥ 50 pA) in response to stepping cells from -50 mV to -100 mV was used to identify VTA DA neurons as previously described [26]. Paired AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) were stimulated using a bipolar stainless steel stimulating electrode placed 200-500 μ m rostral to the recording site in the VTA at 0.1 Hz (100 μ s). Combined EPSCs were evoked and recorded in ACSF perfusion containing picrotoxin (100 μ M) while the cell was voltage clamped at +40 mV. Patch pipettes were filled with 117 mM Cs-gluconate, 2.8 mM NaCl, 5 mM MgCl₂, 2 mM ATP-Na⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA, 20 mM HEPES and intracellular spermine (10 μ M) (pH adjusted to 7.28 with CsOH, osmolarity adjusted to 275–280 mOsm). D-APV (50 μ M) was added to the perfusion to block NMDAR-mediated currents and isolate AMPAR-mediated currents. Subtraction of AMPAR-mediated currents from combined excitatory currents allowed us

to measure AMPA/NMDA ratios. Pharmacologically isolated AMPAR-mediated evoked EPSCs were also recorded to measure AMPAR rectification. These recordings were performed in the presence of picrotoxin (100 μ M), D-APV (50 μ M) and intracellular spermine (10 μ M) included in Cs-gluconate-based internal solution. AMPAR EPSCs were recorded at several holding potentials ranging from -65 to +40 mV. AMPAR rectification was determined by dividing peak AMPAR EPSCs amplitudes recorded at -65 mV by those recorded at +40 mV. Paired GABA_A receptor mediated inhibitory post synaptic currents (IPSCs) were similarly evoked (at 0.1 Hz, duration 100 μ s, 50 ms inter-stimulation interval), isolated and recorded in ACSF containing 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μ M), and strychnine (1 μ M). The internal solution was similar to mIPSC recordings as stated below. Cells were voltage clamped at -70 mV for these recordings.

Whole-cell recordings of GABA_A receptor-mediated miniature IPSCs (mIPSCs) were performed in ACSF perfused with DNQX (10 μ M), strychnine (1 μ M) and tetrodotoxin (TTX, 1 μ M). The patch pipettes (3–6 M Ω) were filled with 125 mM KCl, 2.8 mM NaCl, 2 mM MgCl₂, 2 mM ATP-Na⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA, and 10 mM HEPES (pH adjusted to 7.28 with KOH, osmolarity adjusted to 275–280 mOsm).

Similarly, AMPAR-mediated miniature EPSCs (mEPSCs) were isolated in ACSF perfused with picrotoxin (100 μ M), D-APV (50 μ M) and TTX (1 μ M). Patch pipettes for mEPSC recordings were filled with 117 mM Cs-gluconate, 2.8 mM NaCl, 5 mM MgCl₂, 2 mM ATP-Na⁺, 0.3 mM GTP-Na⁺, 0.6 mM EGTA, and 20 mM HEPES. VTA neurons were voltage-clamped at -70 mV and recorded over 10 sweeps, each lasting 50 seconds. The cell input resistance and series resistance were monitored through all the experiments and if these values changed by more than 10%, data were not included.

Data analysis

Values are presented as means $\pm$ s.e.m. Statistical significance was determined using unpaired Student's t-test and one way or two way ANOVA with Bonferroni post hoc analysis with significance level of $p < 0.05$. The peak values of the evoked paired EPSCs or IPSCs were measured relative to the same baseline. A stable baseline value was considered in each sweep of paired pulses starting at 20-50ms right before the emergence of the EPSC or IPSC current using p-Clamp 10 software. Paired pulse ratio (PPR, 50 ms inter-stimulus interval) was measured over five minute epochs of 30 EPSCs or IPSCs each. The average value for the amplitude of all 30 EPSC2/IPSC2 was divided by the average value for the amplitude of the corresponding 30 EPSC1/IPSC1 and reported as the mean PPR for that epoch. Unpaired Student's t-test was used to determine statistical significance for baseline PPRs of EPSCs or IPSCs between saline and morphine animals. Levels of drug-induced depression in response to WIN55212-2 and DHPG are reported as averaged IPSC amplitudes for 5min just before bath application compared with averaged IPSC amplitudes during the 5min period from 35 to 40 min after the application. The magnitude of chemical LTD in Fig. 19C was expressed as percentage of synaptic change. Statistical significance was determined using repeated measures ANOVA with significance level of $p < 0.05$ for chemical LTD (WIN55212-2 and DHPG) experiments. Mini Analysis software was used to detect and measure mIPSCs and mEPSCs using preset detection parameters of mIPSCs and mEPSCs with an amplitude cutoff of 5 pA. The Kolmogorov–

Smirnov test (KS test) was performed for the statistical analyses of cumulative probability plots of mEPSCs and mIPSCs.

Results

Acute morphine exposure induces epigenetic modifications at the level of histone acetylation through HDAC2

Exposure to drugs of abuse has been shown to modify histone acetylation in brain reward regions [27]. Given that HDAC2, a member of the class 1 HDACs, is the most highly expressed HDAC in neurons and it plays a critical role in synaptic plasticity and memory [28], we hypothesized that acute morphine exposure decreases histone acetylation through an increase in HDAC2 expression in VTA DA neurons. Using a double-immunofluorescence staining technique using antibodies against TH (marker for DA neurons) and HDAC2, we found higher levels of both somatic and nuclear HDAC2 immunoreactivity in TH-positive cells of morphine-treated compared to saline-treated rats at three AP levels within the VTA (-5.4, -5.7 and -6 mm caudal to bregma, Paxinos and Watson, 2007) (Fig. 16, saline, n=5; morphine, n=6; two-way ANOVA, $P<0.0001$). To determine whether increased HDAC2 expression leads to changes in epigenetic modifications, we performed Western blot assays of VTA homogenates isolated from saline- and morphine-treated rats to quantify acetylated histone H3 (using an antibody against Ac-H3K9). There was a significant decrease in levels of H3K9 acetylation in morphine-treated rats (Fig. 17A, saline, n=3; morphine, n=3, unpaired Student's *t* test, $p<0.0001$). Given that our previous work demonstrated the reversal of maternal-deprivation-induced synaptic abnormalities of VTA by *in vitro* CI-994 treatment, we sought to determine whether the changes in Ac-H3K9 were similarly reversible. Our

results here confirm that a 2-4hr incubation of brain slices with the HDACi does indeed increase histone acetylation. We found that levels of AC-H3K9 were significantly higher in brain slices from morphine-treated rats incubated in CI-994 compared to vehicle (DMSO) (Fig. 17C, morphine +DMSO, n=5; morphine+CI-994, n=4, unpaired Student's *t* test, $p<0.0001$). Interestingly, this treatment also significantly increased Ac-H3K9 levels in saline-treated animals (Fig. 17B, saline+DMSO, n=6; saline+CI-994, n=6, unpaired Student's *t* test, $p<0.001$). Given that acute morphine increased HDAC2 activity and triggered reversible changes in histone acetylation, we next tested whether morphine-induced synaptic modifications in response to a single morphine exposure were also reversible by the HDAC inhibitor.

Alteration of glutamatergic transmission by morphine was reversed following HDAC inhibition.

Potentiation of glutamatergic transmission onto VTA DA neurons in rodents is a well characterized adaptation in both acute and long-term exposure to drugs of abuse, including cocaine and morphine [5]. Here we tested whether a single injection of morphine was sufficient to trigger glutamatergic LTP in VTA DA neurons of rats. To accomplish this, we measured AMPA/NMDA ratios, a commonly used indicator of glutamatergic potentiation [5] and AMPAR-mediated mEPSCs. AMPA/NMDA ratios from morphine-treated animals were significantly increased compared to those from saline-treated controls (Fig. 18C-D). Our cumulative probability analyses showed that both amplitude and frequency of mEPSCs were significantly increased in morphine-treated rats relative to saline-treated control rats although we detected no significant

difference between the average amplitude and frequency of mEPSCs in morphine-treated rats versus saline controls (Fig. 18A and B, saline, n=7: 12.43 ± 1.25 pA, 1.18 ± 0.22 Hz; morphine, n=7: 12.91 ± 1.09 pA, 3.03 ± 0.87 Hz; unpaired Student's *t*-tests: $p = 0.77$ for amplitude, $p = 0.06$ for frequency; KS tests for cumulative distribution curves: $P < 0.05$ for amplitude, $p < 0.01$ for frequency). We also calculated the baseline PPRs from paired evoked EPSCs in saline and morphine treated rats (PPR for saline: n=6, 1.33 ± 0.17 ; PPR for morphine: n=6, 1.2 ± 0.08 ; unpaired *t*-test, $p = 0.49$). There was no significant difference in PPRs between two groups suggesting that the change in mEPSC frequency is consistent with a postsynaptic effect (unsilencing of postsynaptic receptors).

We next tested whether HDAC inhibition was sufficient to reverse this morphine-induced plasticity. To accomplish this, we incubated midbrain slices prepared from both morphine-treated and saline-treated rats sacrificed 24h after *in vivo* injection in either vehicle (DMSO) or CI-994 for 2-4 hours and measured AMPA/NMDA ratios. When incubated in CI-994, AMPA/NMDA ratios in slices from morphine-treated rats were significantly reduced compared to morphine-treated controls incubated in carrier solution. In fact, CI-994 treatment fully reversed the elevated AMPA/NMDA ratios after morphine treatment back to the normal levels as seen in control rats. Furthermore, AMPA/NMDA ratios recorded in slices from saline-treated rats incubated in CI-994 showed no significant difference from saline-treated controls incubated in carrier solution (Fig. 18 C-D, saline+DMSO, n=6: 0.52 ± 0.108 ; morphine+DMSO, n=6: 2.02 ± 0.34 ; saline+CI-994, n=7: 0.63 ± 0.14 ; morphine+CI-994, n=7: 0.79 ± 0.09 ; two way ANOVA, treatment: morphine vs. saline: $F_{(1,22)} = 19.12$, $p < 0.0001$; drug: DMSO vs. CI-994: $F_{(1,22)} = 8.7$, $p = 0.007$; interaction: $F_{(1,22)} = 12.56$, $p = 0.0018$; Bonferroni post-hoc analyses after one-way

ANOVA showed significant differences between saline-DMSO vs. morphine-DMSO treated slices: $p < 0.0001$ and morphine-DMSO compared to morphine-CI-994 treated slices: $p < 0.0001$; one way ANOVA, $F_{(3,22)} = 12.72$, $p < 0.0001$).

To further investigate the effects of HDAC inhibition after acute *in vivo* morphine exposure on AMPAR-mediated synaptic function, we recorded evoked AMPAR-mediated EPSCs at different holding potentials (while intracellularly applying spermine) from DA neurons in midbrain slices incubated in either vehicle or CI-994 prepared 24h after saline or morphine treatment. This allowed us to measure the relative contribution of Ca^{2+} permeable/GluA2-lacking AMPARs to synaptic transmission. AMPA EPSC rectification index was significantly higher in morphine-treated animals compared to saline controls (Fig.3E). An increase in rectification suggests that morphine induced a loss of GluR2-containing AMPARs that were replaced by GluR2-lacking AMPARs. In spite of the demonstrated recovery of AMPA/NMDA ratios by HDAC inhibition in slices from morphine-treated rats, the morphine-induced increase in the rectification index could not be reversed to normal levels by the HDAC inhibitor suggesting that the mechanisms underlying the increases in AMPA/NMDA ratio and rectification index may differ. However, there was also a significant increase in rectification indices measured from DA neurons in CI-994-incubated slices prepared from saline-treated rats compared to those from saline-treated controls incubated in carrier solution. The level of increase in rectification after CI-994 in control rat slices was comparable with those seen in slices from morphine-treated rats, potentially masking the anticipated reversal effects of HDAC inhibition on rectification indices in morphine-treated rats (Fig.3E, saline+DMSO, $n=6$: 1.53 ± 0.34 ; morphine+DMSO, $n=6$: 2.45 ± 0.12 ; saline+CI-994, $n=6$: 2.54 ± 0.16 ;

morphine+CI-994, $n=7$: 2.57 ± 0.07 ; two way ANOVA, treatment: morphine vs. saline: $F_{(1,21)} = 5.79$, $p = 0.025$; drug: DMSO vs. CI-994: $F_{(1,21)} = 8.25$, $p = 0.009$; interaction: $F_{(1,21)} = 5.17$, $p = 0.033$; Bonferroni post-hoc analyses after one-way ANOVA showed significant differences between saline-DMSO vs. morphine-DMSO treated slices: $p = 0.022$ and saline-DMSO compared to saline-CI-994 treated slices: $p = 0.01$ whereas morphine-DMSO and morphine-CI-994 treated slices did not show a significant difference: $p = 1.01$; one way ANOVA, $F_{(3,21)} = 6.29$, $p = 0.003$).

Morphine-induced pre- and postsynaptic changes of GABAergic synaptic transmission was reversed by HDAC inhibition.

So far, our data suggest the possible involvement of HDAC2 and H3K9 acetylation in maintenance of morphine-induced glutamatergic plasticity that could be reversed by HDAC inhibition. We further examined the effects of CI-994 treatment on morphine-induced GABAergic plasticity. Midbrain slices containing the VTA from both saline- and morphine-treated animals sacrificed 24h after saline/morphine exposure were incubated in either CI-994 or the vehicle (DMSO) for 2-4 hours as described previously [24]. The carrier solution, DMSO, did not have any significant effect on either mIPSC amplitude or frequency, therefore the data from DMSO-free control experiments were pooled together with DMSO controls. Both amplitude and frequency of mIPSCs were significantly reduced in morphine-treated rats relative to saline-treated control rats (Fig. 19A, B and D, saline, $n=12$: 38.13 ± 2.67 pA, 6.46 ± 0.68 Hz; morphine, $n=13$: 30.07 ± 2.94 pA, 2.76 ± 0.40 Hz; unpaired Student's t -tests: $p = 0.02$ for amplitude, $p = 0.0012$ for frequency; KS tests for cumulative distribution curves: $P < 0.0001$ for amplitude, $p < 0.0001$ for frequency).

Consistent with a presynaptic GABAergic LTD, baseline PPRs from paired evoked IPSCs calculated in morphine treated rats were significantly higher than those from saline-treated rats (PPR for saline: $n=10$, 1.02 ± 0.05 ; PPR for morphine: $n=11$, 1.27 ± 0.09 ; unpaired t-test, $p = 0.023$).

To investigate the possibility that morphine-induced plasticity at glutamatergic synapses could trigger subsequent presynaptic depression of GABAergic transmission in VTA DA neurons, we examined the role of eCB signaling in this inhibitory plasticity. We found that the GABAergic depression induced in response to the CB1 agonist, WIN 55212-2 ($2\mu\text{M}$), is absent in slices from morphine-treated rats while WIN 55212-2 successfully induced depression of evoked IPSCs of DA neurons in slices from saline-treated animals. Given that mGluRs have been shown to trigger LTD at GABAergic synapses through production of eCBs [29], we also tested the effects of mGluR agonist, DHPG, on GABAergic transmission in VTA DA neurons. Interestingly, DHPG ($50\mu\text{M}$) was still able to induce LTD at GABAergic synapses in both saline- and morphine-treated animals suggesting that morphine affects CB1 receptor-mediated GABAergic plasticity independently from mGluR-mediated LTD. In fact, it has been shown that mGluR-mediated LTD induced in response to DHPG is postsynaptic and independent of eCB signaling, while eCBs only mediate the presynaptic short-term depression induced by DHPG [30] (Fig. 19C, saline+WIN55212-2, $n=4$, $71.5 \pm 1.4\%$ of the baseline values $F_{8,18,16.35} = 4.55$, $p = 0.004$, morphine+WIN55212-2, $n=5$, $99.5 \pm 1.4\%$ of the baseline values, $F_{10,08,30.25} = 0.67$, $p = 0.74$; saline+DHPG, $n=6$, $58.2 \pm 1.08\%$ of the baseline values $F_{6,64,19.94} = 3.93$, $p = 0.015$, morphine+DHPG, $n=6$, $74 \pm 2.2\%$ of the values, $F_{3,8,19.05} = 3.11$, $p = 0.04$).

Midbrain slices from morphine-treated rats incubated in CI-994 displayed larger amplitudes and higher frequencies of mIPSCs when compared to morphine-treated rats whose slices were incubated either in DMSO or DMSO-free solutions. This suggested that CI-994 was able to recover normal GABAergic synaptic transmission onto VTA DA neurons following acute morphine exposure (Fig. 19A,B, and F, morphine, n=13: 30.07 ± 2.94 pA, 2.76 ± 0.4 Hz; morphine+CI-994, n=11: 42.46 ± 3.41 pA, 4.40 ± 0.49 Hz; unpaired Student's *t*-tests: $p = 0.011$ for amplitude, $p = 0.017$ for frequency, KS tests for cumulative distribution curves: $P < 0.0001$ for amplitude, $p < 0.0001$ for frequency). Interestingly, saline-control rats treated with CI-994 also showed significant decreases in both mIPSC amplitude and frequency although no significant difference between the average amplitude of mIPSCs was detected after HDACi treatment (Fig. A, B and E, saline, n=12: 38.13 ± 2.67 pA, 6.46 ± 0.68 Hz; saline+CI-994, n=5: 31.91 ± 1.88 , 2.46 ± 0.46 Hz; unpaired Student's *t*-tests: $p = 0.092$ for amplitude, $p < 0.0001$ for frequency, KS tests for cumulative distribution curves: $P = 0.012$ for amplitude, $p < 0.0001$ for frequency).

Discussion

The acute effects of morphine on VTA synaptic plasticity are well documented [5-7, 31]. Here we provide the first demonstration of the involvement of HDAC2-mediated changes in histone acetylation profile upon a single *in vivo* morphine exposure in this phenomenon. We show that morphine increased HDAC2 activity in VTA DA neurons and reduced histone H3K9 acetylation in the VTA 24 hours following injection. We further demonstrated that 2-4hr *in vitro* incubation of midbrain slices with a class I specific HDACi significantly increased acetylation of histone H3K9 in morphine-treated

rats (Figs. 1-2). Consistent with these reversible epigenetic modifications, HDAC inhibition reversed morphine-induced glutamatergic potentiation while also reversing depression of GABAergic synaptic transmission onto VTA DA neurons. HDACi incubation also increased levels of Ac-H3K9, and triggered plasticity at GABAergic and glutamatergic synapses onto VTA DA neurons of saline-treated rats. Furthermore, we also provide the first evidence that morphine-induced depression of GABAergic transmission is subsequent to morphine-induced potentiation of glutamatergic transmission and mediated by eCB signaling. Our results suggest that acute morphine-induced changes in VTA DA activity and synaptic transmission engage HDAC2 activity locally in the VTA to maintain synaptic modifications through histone hypoacetylation. This work highlights the fact that reversible epigenetic dysregulation through HDACs may serve as a possible mechanism by which initial VTA synaptic plasticities in response to opioids could be maintained.

Consistent with findings in mice and rats, demonstrating NMDAR-dependent LTP of glutamatergic transmission onto VTA DA neurons and increased postsynaptic insertion of inwardly-rectifying Ca^{2+} permeable GluA2-lacking AMPARs following acute exposure to morphine [5, 6, 32], we found that a single exposure to morphine increased AMPA/NMDA ratios, the amplitude of mEPSCs and rectification indices in DA neurons (Fig. 18). Previous studies have also shown that a single *in vivo* injection of morphine is able to trigger long-lasting changes in synaptic strength of GABAergic synapses [7, 8]. Here we demonstrated that morphine per se induced a reduction in GABAergic synaptic strength by suppressing GABA release and decreasing the number or function of postsynaptic GABA_ARs (Fig. 19). Consistently, a recent study established that a single

dose of morphine was able to elicit a decrease in presynaptic GABA release facilitated by orexin signaling [32]. However, their results did not support an induction of a postsynaptic change in GABA_AR number or function by morphine, which is likely due to the use of higher amplitude cutoff for detecting mEPSCs. Interestingly, we provide new evidence that the presynaptic component of morphine-induced suppression of GABA release is in fact eCB-mediated (Fig. 19). This suggests that eCB signaling arising from morphine-induced glutamatergic plasticity retrogradely depresses GABAergic signaling onto VTA DA neurons. Consistently cocaine-induced LTD of GABAergic synapses onto VTA DA neurons also required eCB signaling [33].

Increasing evidence suggests that HDACs play an important role in memory and cognition [34]. Histone acetylation is tightly regulated by the opposing actions of histone acetyltransferases (HATs), which promote histone acetylation, and HDACs, which promote histone deacetylation. Typically, histone acetylation promotes expression of genes that positively regulate synaptic plasticity while HDAC activity (deacetylation) negatively regulates plasticity. Furthermore, other studies have shown that inhibition of HDAC activity through the use of HDAC inhibitors such as sodium butyrate, SAHA and CI-994, is able to facilitate LTP induction and increase LTP magnitude in the hippocampus and amygdala [28, 35-37]. We recently demonstrated that *in vitro* HDAC inhibition with CI-994 was able to reverse VTA GABAergic synaptic abnormalities in a rodent model of severe early life stress; i.e., a 24h maternal deprivation procedure [24]. Here, we confirmed that this short-term *in vitro* HDACi treatment of the VTA is indeed sufficient to increase histone H3K9 acetylation. Morphine increased HDAC activity selectively in VTA DA neurons which subsequently resulted in decreased levels of Ac-

H3K9 (Figs 1, 2). CI-994 treatment reversed this histone modification as well as glutamatergic and GABAergic plasticity induced by a single injection of morphine (Figs. 2- 4). In closer relation to our work, early repeated maternal deprivation increases opiate consumption in adult rats through epigenetic modifications that enhance HDAC expression and activity in the NAc, and treatment with the HDAC inhibitor sodium valproate normalized opioid consumption [38]. HDAC inhibitors have also been shown to facilitate extinction of CCP and reduce development of morphine behavioral sensitization in rodents exposed to morphine [12, 21]. Furthermore, specific knockdown of class 1 HDACs suppresses cocaine-induced behavioral plasticity through a chromatin-mediated decrease in GABAergic inhibition in the NAc [14]. In contrast, HDAC inhibition and HDAC3 deletions enhance morphine-induced locomotor sensitization and drug-induced CPP [19, 39]. It must be noted that the timing and regimen of HDACi administration differs in these studies which may account for the conflicting results. While in some studies animals were pretreated with HDAC inhibitors, other studies used HDAC inhibitors in conjunction with addictive drugs and during addiction behavioral assays. Our work here supports the hypothesis that post-morphine HDAC inhibition in the VTA per se is sufficient to reverse acute morphine-induced epigenetic dysregulation and synaptic adaptations.

The exact mechanisms linking morphine-induced synaptic changes and HDAC activity remains unknown. However, it is likely that morphine-induced changes in VTA DA neuron activity and synaptic transmission precede upregulation of HDAC2. The increased levels of HDAC2 and hypoacetylation of histones may then be maintaining the morphine-induced changes in synaptic transmission. Therefore, when HDAC activity is

inhibited, this maintenance is no longer present, allowing synaptic transmission to return to levels comparable to saline-treated animals. Transcriptional repression of mechanisms governing AMPAR trafficking could maintain morphine-induced postsynaptic glutamatergic LTP. The opposing actions of kinases such as protein kinase A (PKA) and phosphatases such as calcineurin (CaN) regulate synaptic insertion and maintenance of AMPARs and GABA_ARs during synaptic plasticity through phosphorylation and dephosphorylation of synaptic receptors [40, 41]. In fact, CaN seems to be a negative regulator of memory whereas PKA activity enhances learning and memory [42, 43]. CaN overexpression also prevents behavioral sensitization to amphetamine and morphine suggesting that decreased CaN expression and activity at glutamatergic synapses may allow PKA activity to favor glutamatergic LTP underlying sensitization [44]. Therefore, we assume that HDAC2-mediated transcriptional repression of PKA regulatory subunits (which promotes the formation of a persistently active PKA) or of CaN at glutamatergic synapses could favor phosphorylation of AMPARs and maintain morphine-triggered LTP [44, 45]. Our previous work in the VTA demonstrated that HDAC inhibition was able to reverse early life stress-induced GABAergic synaptic abnormalities through restoration of AKAP150 signaling. AKAP150 is a scaffolding protein that functions as an integrator of signaling molecules to glutamatergic and GABAergic synapses where it can modulate synaptic trafficking and plasticity [46-49]. However, AKAP150 in the VTA has been shown to only modulate synaptic trafficking at GABAergic synapses [26]. Therefore, it is reasonable that treatment with HDACi, CI-994, is recovering a morphine-induced disruption in AKAP-mediated trafficking of GABA_ARs, thereby reversing morphine-induced reduction in the number of postsynaptic GABA_ARs.

We also found that HDACi incubation of midbrain slices from saline-treated animals increased histone H3K9 acetylation and reduced the strength of GABAergic inhibition in DA neurons pre- and post-synaptically (Figs. 2 and 4). This is consistent with our previous findings that HDACi incubation of midbrain slices from non-maternally deprived animals induced a postsynaptic LTD at GABAergic synapses, and modulated GABAergic spike timing-dependent plasticity [24]. CI-994 treatment may promote the insertion of GluA2-lacking AMPARs into the postsynaptic membrane allowing an increased influx of Ca^{2+} to the postsynaptic neuron facilitating plasticity at the synapse. HDACi-induced histone modifications and plasticity at GABAergic synapses and glutamatergic plasticity in the VTA could both increase levels of DA which is shown to be necessary for hippocampal LTP and memory formation [50]. These findings reinforce the idea that HDAC inhibitors can act as cognitive enhancers [28, 34, 36].

Given that the pattern and nature of epigenetic modifications induced by drugs of abuse vary from one brain region to another, it is critical to identify cell- and brain region-specific epigenetic mechanisms underlying drug-triggered DA dysfunction thereby advancing our knowledge of epigenetics of addiction.

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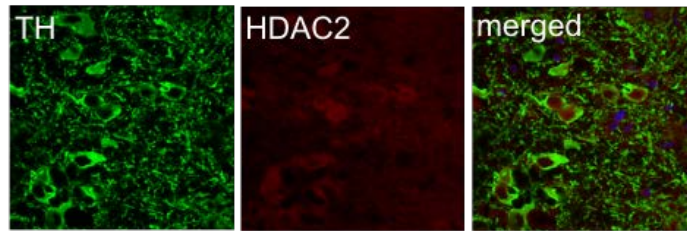
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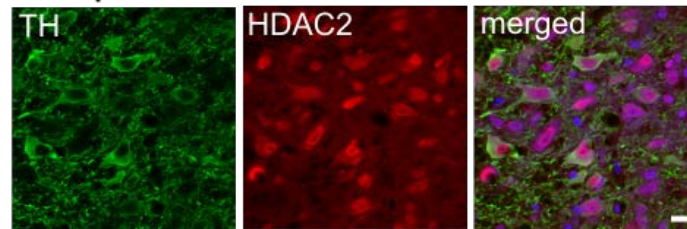
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Figures

A saline



morphine



B

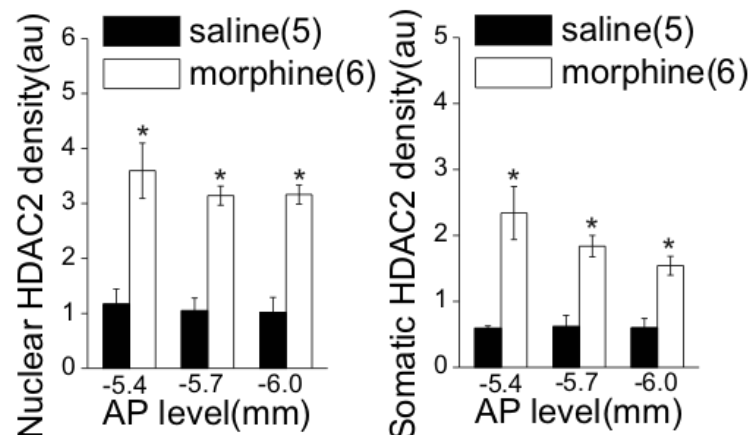


Figure 16. Morphine increases HDAC2 expression in VTA DA neurons

(A) Examples of brain sections stained with antibodies to TH (green), HDAC2 (red), and DAPI (59) with the merged panels, which show the expression of HDAC2 in TH neurons in the VTA of saline- (upper row) and morphine-treated (lower row) rats. Scale bar, 20 μ m.

(B) Averaged levels of nuclear and somatic HDAC2 expression at three AP levels from saline- and morphine-treated rats. In this and all subsequent figures, numbers within the

figure legends indicate the number of animals examined and * denotes statistical significance.

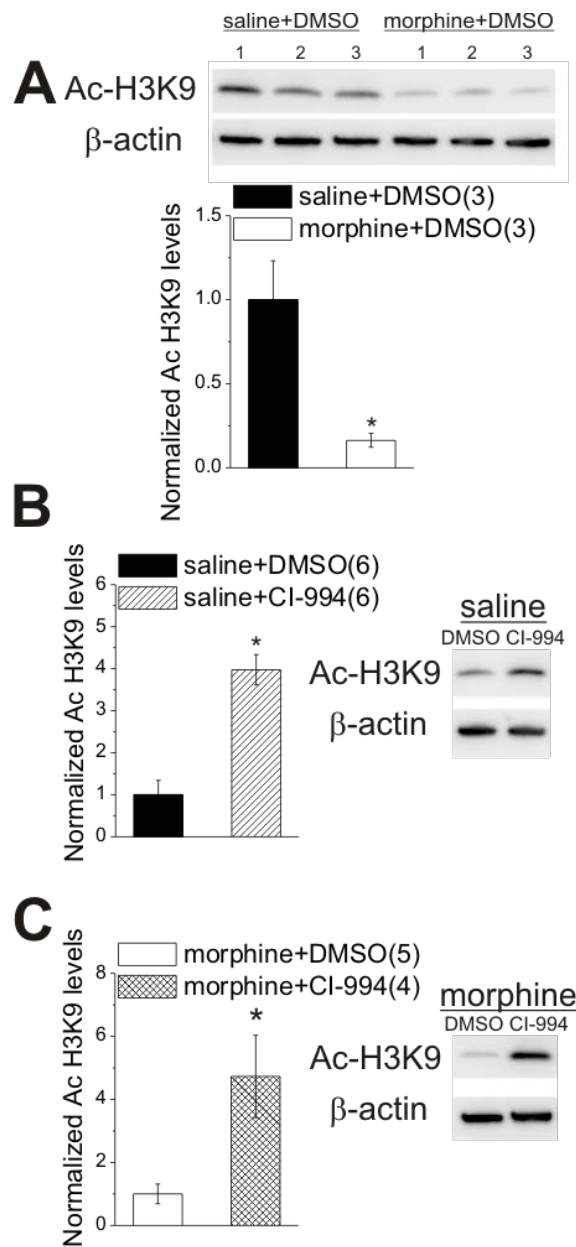


Figure 17. Histone acetylation at H3K9 is decreased following a single exposure to morphine and is recovered by *in vitro* HDAC inhibition with CI-994

(A) Representative Western blots (top) and quantitative data (bottom) of Ac-H3K9 and β -actin (control) in VTA homogenates from saline- and morphine-treated rats. (B and C) Representative Western blots and quantitative data of Ac-H3K9 and β -actin in VTA

homogenates from saline- and morphine-treated rats incubated either in DMSO or CI-
994.

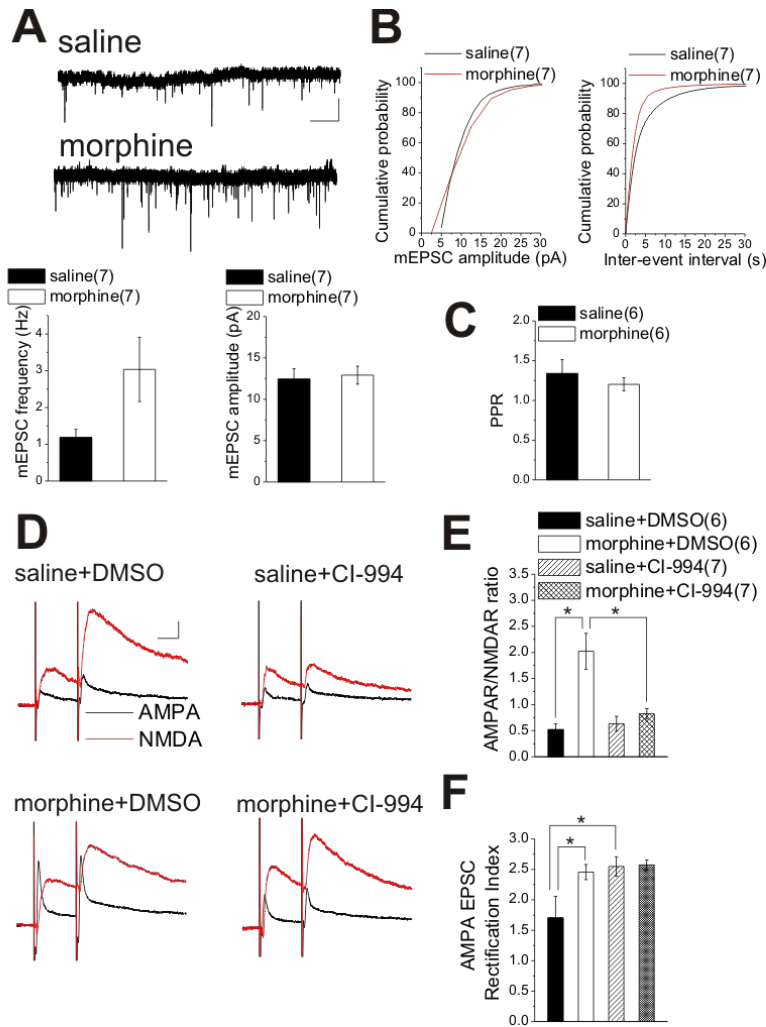


Figure 18. Morphine-induced glutamatergic plasticity is reversed with HDAC inhibition

(A) Sample AMPAR-mediated mEPSC traces and average bar graphs of mEPSC amplitude and frequency from saline-treated and morphine-treated rats (calibration bars: 10 pA/5s). (B) Cumulative probability plots of amplitude and frequency (inter-event interval) for all mEPSCs in saline-treated and morphine-treated rats. Morphine induced an increase in amplitude and frequency. (C) Sample traces of AMPA (black) and NMDA (red) EPSCs recorded at +40mV from VTA DA neurons of saline-treated and morphine-

treated rats (calibration bars: 50 pA/25ms). (D) Summary of AMPAR/NMDAR ratios obtained from saline-treated and morphine-treated rats with DMSO (vehicle) or CI-994 (20 μ M). The morphine-induced increase in AMPAR/NMDAR ratio was reversed by incubation with CI-994. (E) Summary of AMPAR EPSC rectification indices obtained from saline-treated and morphine-treated rats with DMSO (vehicle) and CI-994. CI-994 did not reverse the morphine-induced increase in rectification index but increased this index in slices from saline-treated rats. All recordings (C-E) were performed with intrapipette inclusion of spermine.

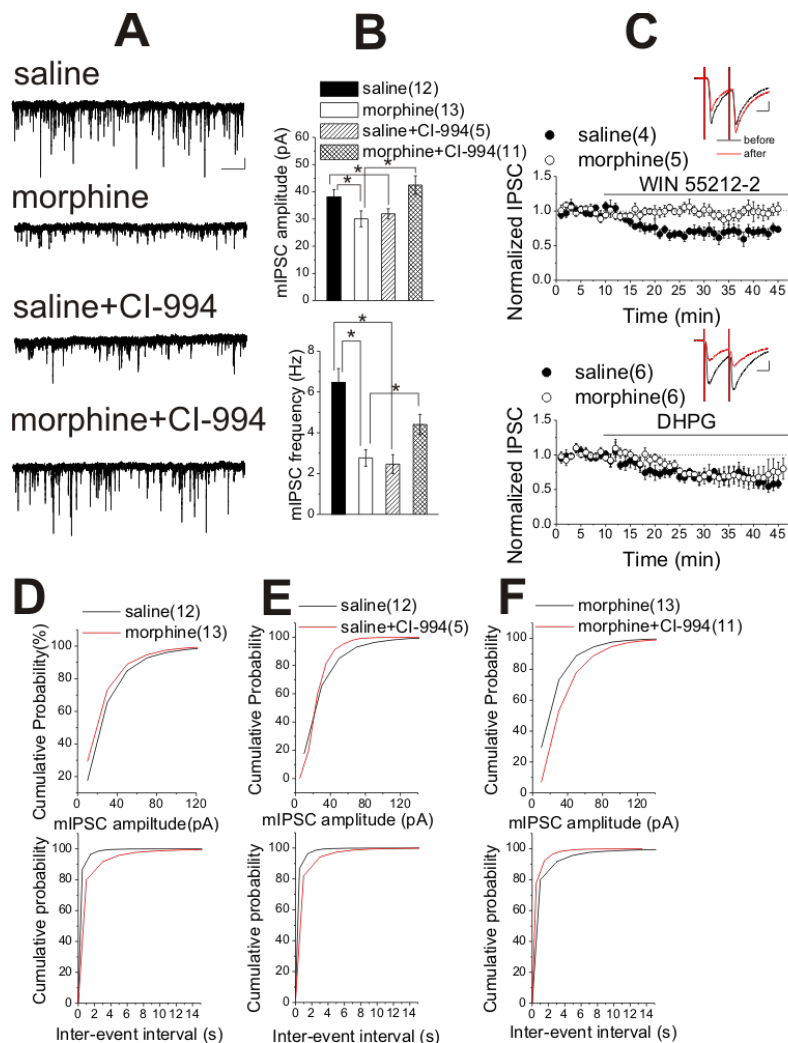


Figure 19. Morphine-induced changes of GABAergic synaptic transmission onto VTA DA neurons is reversed by HDAC inhibition.

(A) Sample GABA_AR-mediated mIPSC traces from saline-treated and morphine-treated rats (calibration bars: 30 pA/5s). (B) Average bar graphs of mIPSC amplitude and frequency from saline-treated and morphine-treated rats with DMSO and CI-994. (C) Insets: representative traces from saline with bath application of WIN 55212-2 (upper row) or DHPG (lower row): averaged IPSCs before (black) or the peak response 35 min after WIN 55212-2 or DHPG (red). Calibration: 100 pA, 25 ms. Averaged experiments

with bath applied WIN 55212-2 (2 μ M) or DHPG (50 μ M) in saline (filled symbols) or morphine (open symbols). WIN 55212-2 induced a significant rundown of IPSCs in saline but not morphine-treated rats. DHPG induced a significant LTD in both saline and morphine-treated rats. (D-F) Cumulative probability plots of amplitude and frequency (inter-event interval) for all mIPSCs in saline-treated and morphine-treated rats. CI-994 (20 μ M) reversed morphine-induced pre- and postsynaptic LTD.

CHAPTER 5: DISCUSSION

The broad goal of this dissertation was to identify physiological changes induced by MD at the cellular level that could increase the likelihood of later life development of mental disorders. Due to the prevailing evidence that MD and other early life stressors affect mesocorticolimbic DA signaling, we investigated two interconnected brain regions either directly or indirectly involved in the release of DA: the VTA and LHb, respectively. In doing so, we not only characterized GABAergic STDP in the VTA and CRF signaling in the LHb, but also identified key molecular targets underlying the physiological consequences of MD on these newly characterized systems. In fact, the third aim of my proposal and fourth chapter of this dissertation focused on one of these molecular targets, HDAC2, and examined its role in the acute effects of morphine administration in the VTA. Ultimately, this work lays the foundation for future studies to examine these molecular targets; investigate the interconnectivity of the LHb and VTA, in order to give a more complete picture of how MD exerts its effects in these regions; and determine how the neuroadaptations within VTA-LHb circuitry leads to long-lasting changes in DA signaling following early life stress. In the following paragraphs, I discuss the individual findings of this dissertation, examine how they interact, and the future directions I envision will be taken with this foundation. Finally, I include a summary model of the major findings of this dissertation as well as some speculation of how these changes are precipitated (Fig. 20).

Consequences of MD on GABAergic transmission and STDP in the VTA

We initially chose to investigate the effects of MD in the VTA due to the well-documented impairment in DA signaling observed following early life stress [1-4].

Furthermore, VTA DA neurons are highly involved in processing reward and aversion-related stimuli and are therefore key in the rewarding effects of drugs of abuse [5]. How exactly early life stress predisposes an individual to addiction and other related disorders is unknown, however, it stands to reason that early life stress (i.e. MD) may induce changes at the cellular or molecular level in VTA DA neurons that result in an increased release of dopamine in response to rewarding stimuli, increasing the reinforcing effects of drugs of abuse. (Fig. 20).

We observed a decrease in GABAergic transmission onto VTA DA neurons following MD. This decrease in inhibitory tone could be solely responsible for the increase in DA release in response to rewarding stimuli following MD; however, since the brain, even in adulthood, has a remarkable ability to engage Hebbian plasticity to change the strength of such synapses, we sought to determine how MD affects this plasticity. As we had previously characterized GABAergic STDP in the VTA, and since the strengthening or weakening of GABAergic transmission onto VTA DA neurons strongly influences the excitability of VTA DA neurons, we chose to examine this form of plasticity. Important to note is that while the presence of an ih current is our criteria for identifying VTA DA neurons, previous studies have demonstrated that non-DA neurons also express ih currents [18, 19]. However, the presence of non-DA ih-expressing cells in the lateral VTA is much lower and is where our recordings are performed [20]. Our previous work established STD induction protocols that could either induce STD-LTP or STD-LTD at GABAergic synapses onto VTA DA neurons, depending on the timing and order of pre- and postsynaptic spiking [6]. We found that MD affected STDP in such a way that promoted the LTD of GABAergic synapses onto

VTA DA neurons while preventing LTP at these synapses. By establishing the ordering and time window between pre- and postsynaptic spiking that could elicit either LTP or LTD in control animals, we were able to compare these windows for STDP to those of MD animals, and found that MD shifted the window of plasticity to enable LTD. This is an example of a metaplasticity. Metaplasticity describes the ability of a priming activity to modulate subsequent synaptic plasticity. In this case, the priming activity was MD.

Involvement of HDAC2 and AKAP150 in MD induced metaplasticity

Both stress and drugs of abuse have been shown to engage epigenetic mechanisms including transcription factors such as Δ FosB, DNA methylation marks, and histone tail methylation and acetylation marks [7]. Due to the involvement of HDAC2 in learning and memory [8], we chose to investigate its involvement in MD induced metaplasticity with the hypothesis that MD triggered HDAC-mediated epigenetic changes in the gene encoding the scaffold protein AKAP150. Our lab previously found that AKAP150 was involved in the LTD of GABAergic synapses onto VTA DA neurons [9].

Here we found that inhibition of AKAP with Ht31 in the intracellular patch pipette not only mimicked GABAergic STD LTD in VTA DA neurons but also occluded its induction. In MD animals, AKAP inhibition did not induce LTD of GABAergic transmission. This was likely due to an impaired AKAP signaling following MD which may have been caused by epigenetic changes involving HDAC2. We were able to recover STDP in MD animals following inhibition of HDAC activity with two separate HDACi's: sodium butyrate, a pan HDAC inhibitor and CI-994, a class-I specific HDAC inhibitor. Furthermore, HDACi recovered the ability of Ht31 to induce a chemical LTD at GABAergic synapses in MD animals. This led us to hypothesize that the epigenetic

changes induced by MD and mediated by HDAC2 resulted in a repression of the AKAP150 gene. However, when we performed immunohistochemistry, AKAP150 levels were instead increased in VTA DA neurons following MD, suggesting that the impairment of AKAP150 signaling may be due to a mislocalization of this scaffolding protein, rendering it unable to properly coordinate the phosphorylation and dephosphorylation of GABA_ARs, by protein kinase A (PKA) and calcineurin, respectively. This mislocalization may be the consequence of HDAC2-mediated suppression of genes coding for proteins integral to the trafficking of AKAP150 to GABAergic synapses. Additionally, AKAP150 may not receive appropriate post-translational modifications to localize to the synapse. In fact, palmitoylation of AKAP150 is required for AMPAR trafficking during LTP induction in rat hippocampal neurons [10]. Whether this also holds true for GABAergic synapses has yet to be determined.

LHb-VTA microcircuitry

Although my initial study on the effects of MD focused on VTA DA neurons, I also wanted to determine whether the LHb was also affected by this early life stress. The LHb exerts a potent inhibitory control over the firing of VTA neurons. Because early life stress is so strongly correlated with impaired DA signaling, and the VTA has reciprocal connections with the LHb, I decided to investigate how MD affects this structure.

The LHb consists mostly of glutamatergic neurons which send projections to VTA GABAergic interneurons and also the RMTg, an intermediary GABAergic nucleus (Fig. 20). The RMTg in turn inhibits VTA DA neurons when the excitatory drive from LHb neurons is increased. The LHb is activated by aversive stimuli and reward

omission, resulting in inhibition of VTA DA neurons. Conversely, LHb neurons are inhibited in response to rewarding stimuli which decreases the inhibitory drive onto VTA DA neurons, resulting in increased DA signaling. Furthermore, TH-expressing neurons send projections back to the LHb. Although these TH-expressing neurons produce DA, they are not able to package it into vesicles due to the lack of vesicular DA transporter 2, and therefore they are not able to release dopamine into the LHb. Instead, it was found that these VTA neurons release GABA into the LHb, establishing the potential for a feedback loop between the VTA and LHb [11]. Activation of these VTA-GABA neurons projecting to the LHb promotes reward-related behaviors.

While the interaction between LHb and VTA, and whether the dysfunction of one is necessary for or contributes to the dysfunction of the other is an important question to address, my work in this dissertation regarding the LHb focuses on first establishing what changes occur in the LHb following a severe early life stress. In addition, I investigated the involvement of CRF signaling in MD-induced cellular and molecular changes in the LHb. Early-life stress, has previously been associated with hyperactivity of the brain's extrahypothalamic CRF system and a subsequent down-regulation of CRF receptors later in life in both humans and animal models [12-14]. Therefore, CRF may precipitate the changes induced by MD in the LHb, and perhaps contribute to long-lasting reward dysregulation into adulthood. However, the only work concerning CRF signaling in the LHb were immunoblot studies which simply reported the presence of CRFR1, but not CRFR2 in the LHb. The questions pursued were: Does CRF signaling occurs in the LHb? Does MD induce any intrinsic or synaptic plasticities in LHb neurons? And finally, if CRF signaling is present in the LHb, is it affected by MD?

A novel role for CRF signaling in the LHb

Until now, no study had investigated the role of CRF signaling in the LHb, despite the presence of CRFR1 in the LHb. We showed that CRF increased the excitability of LHb neurons intrinsically, through a G-protein and Ca^{2+} -dependent modulation of BK and SK-channel functioning, as well as through a decrease in GABAergic synaptic transmission. In addition, we found that CRF's effect seems to be mediated by activation of CRFR1, as antalarmin, a CRFR1 antagonist, completely blocked this effect. Although CRF's ability to increase neuronal excitability is consistent with its action in other brain areas including the VTA and basolateral amygdala, the way it does so is unique to the LHb.

By decreasing the function of SK channels, CRF consistently reduced the mAHP in LHb, resulting in an increased intrinsic excitability (Fig. 20). A decrease in amplitude of the mAHP is commonly associated with an increase in neuronal excitability. Apamin, a SK channel blocker, replicated the effects of CRF on excitability and occluded CRF signaling. Furthermore, pharmacological positive modulation of SK channel function with CyPPA and 1-EBIO was sufficient to block CRF's effect on increasing intrinsic excitability. CRF also increased the fAHP in LHb neurons, which may allow high-frequency firing by suppressing the inactivation of Na^+ channels and decreasing the activation of other K^+ channels.

Aside from enacting changes on the intrinsic excitability of LHb neurons, CRF also increased excitability through depressing GABAergic synaptic transmission as evidenced by a decrease in the amplitude and frequency of mIPSCs, a measure of

GABAergic transmission reflecting vesicular release (frequency) and GABA_A function. Glutamatergic transmission, however, was unaffected by CRF.

MD blunts the excitatory effects of CRF and affects synaptic transmission onto LHb neurons

Previous studies have demonstrated that MD is associated with an increased activity of extrahypothalamic central CRF signaling as well as HPA axis, as evidenced by an increase of CRF levels in CSF and blood, respectively. This led us to investigate not only the effects of MD on LHb neuronal excitability, but also whether MD altered subsequent CRF signaling. Compared to their non-MD counterparts, MD animals showed increased excitability of LHb neurons both with and without synaptic transmission. Additionally, glutamatergic transmission was potentiated as evidenced by an increase in AMPAR/NMDAR ratio and an increase in amplitude of miniature EPSCs, a measure of glutamatergic transmission reflecting vesicular release (frequency) and AMPAR function. Compared to non-MD animals; however, the rectification index of evoked EPSCs was unchanged in MD animals, suggesting that this potentiation was not due to a change in AMPAR subunit composition. GABAergic transmission, on the other hand, was significantly increased in MD animals, both pre- and postsynaptically. It is possible that either the increase in LHb intrinsic excitability or the increase in GABAergic transmission is a homeostatic plasticity.

As stated previously, MD also increased the intrinsic excitability of LHb neurons through a decrease in protein levels of SK2. The potentiation of glutamatergic transmission by MD (increase in amplitude and AMPA/NMDA ratio) may be facilitated by the decrease in SK2 levels as seen in the hippocampus where glutamatergic LTP involves a PKA-mediated endocytosis of SK2 channels. Not only were LHb neurons in

MD animals insensitive to apamin, but positive modulation with 1-EBIO normalized intrinsic excitability in LHb neurons of MD rats at lower depolarizing current steps. Although there were functional SK2 channels in MD animals for 1-EBIO to act upon, the sensitivity of these remaining SK2 channels to apamin may be diminished due to decreased expression of SK channels. Additionally, heteromerization has been shown to confer apamin insensitivity.

The effect of CRF on neuronal excitability was blunted in MD animals, which may be a result of the MD-induced reduction in expression and gating of SK channels and/or expression of CRFR1 (Fig. 20). Since MD and other forms of early life stress are associated with an increased activity of centrally acting extrahypothalamic CRF, the reduction of SK channel functioning may be a compensatory mechanism undertaken by LHb neurons in response to overstimulation by CRF during the episode of stress. Other studies have associated MD with a later-life reduction in CRFR1 levels in response to hyperactive CRF signaling during the stress episode (Fig. 20). Although it is yet to be determined whether CRFR1 downregulation occurs with MD, the reduction in SK channel functioning may serve a similar function in the LHb as downregulation of CRFR1 does in other brain areas. The consequences of blunted CRF signaling and increased LHb excitability, and whether they persist into adulthood are important to more completely understand how early life stress can lead to later life mental disorders.

Involvement of HDAC2 on morphine-induced synaptic plasticity in the VTA

Activation of the brain's reward circuitry, originating in the VTA, is a common mechanism in the acute administration of drugs of abuse, including cocaine and opiates. Even acute administration of such drugs triggers plasticity at both glutamatergic and

GABAergic synapses onto VTA DA neurons as seen in the well-established increase of the AMPA/NMDA ratio following acute administration of cocaine or various opiates [15]. Chronic exposure to these acute drug-induced plasticities is critical for the development of addiction. Neuroplastic changes in response to drugs of abuse also include drug-induced transcriptional changes such as DNA methylation and histone lysine-tail acetylation/methylation. Previous work in my dissertation identified one such epigenetic modification, histone acetylation, to be involved in the consequences of early life stress. Since stress and addiction share common mechanisms, I chose to investigate whether HDAC2 was involved in the acute effects of morphine on synaptic plasticity in the VTA.

We first replicated results reported in other studies (that examined) on the effects of morphine on both glutamatergic and GABAergic synaptic transmission. Consistent with findings in mice and rats, demonstrating NMDAR-dependent LTP of glutamatergic transmission onto VTA DA neurons and increased postsynaptic insertion of inwardly-rectifying Ca^{2+} permeable GluA2-lacking AMPARs following acute exposure to morphine [15-17], we found that a single exposure to morphine increased AMPA/NMDA ratios, the amplitude of mEPSCs and rectification indices in DA neurons. We also confirmed that a single *in vivo* injection of morphine triggered a long-term depression of GABAergic transmission, both pre- and postsynaptically. Our study was the first to report the induction of a postsynaptic change in GABA_A R function by morphine. Furthermore, we also provided the first evidence that the presynaptic component of morphine-induced GABAergic depression arises subsequently to morphine-induced

glutamatergic plasticity which retrogradely suppresses GABA release via eCB signaling similar to that seen in acute cocaine administration.

This is the first study to demonstrate that a single *in vivo* injection of morphine can reduced acetylation of H3K9 through an increase in HDAC2 activity. This hypoacetylation was reversed by incubation of the brain slice with the same HDAC inhibitor used in my previous study, CI-994. CI-994 was also able to reverse the morphine-induced changes in synaptic transmission at both glutamatergic and GABAergic synapses onto VTA DA neurons. Although we do not know exactly how morphine-induced changes in synaptic transmission and HDAC activity are linked, it is likely that the increased levels of HDAC2 and hypoacetylation of histones may be subsequent to morphine-induced changes in synaptic transmission and DA neuronal excitability, thus enabling a long-lasting maintenance of these synaptic changes (Fig. 20).

Future directions

Although the work of this dissertation has established foundation for understanding the effects of early life stress on the brain's reward pathway, several additional experiments are essential to form a more complete picture (Fig. 20). Firstly, it is important to characterize the behavioral outputs of MD. Previous studies have shown that MD reduces prepulse inhibition and impairs spatial learning ability in adulthood in Wistar rats. Our lab is now investigating the effects of MD using the behavioral paradigms of forced swim test (FST) and open field test. At adolescent ages, MD rats exhibit increased climbing behavior, an active coping strategy, compared to nonMD rats. However, at adult ages, MD animals exhibit a more pro-depressive behavior in the FST as evidenced by an increase in immobility.

With regard to the first chapter, several routes of investigation could be taken to more fully characterize the MD-induced changes; namely, MD-induced upregulation of HDAC2 and mislocalization of AKAP150. Our proposed model is that MD induces an increase in HDAC2 which affects expression of genes critical for AKAP150 signaling and synaptic plasticity. Therefore, it is important to determine whether histone lysine tails are hypoacetylated following MD. Additionally, we should perform a CHIP assay to determine which genes are affected by the MD-induced histone hypoacetylation. This may reveal downregulation of genes associated with synaptic plasticity such as *c-fos* and the plasticity-associated activity-regulated cytoskeletal-related (*Arc*) gene. In addition, brain-derived neurotrophic factor (BDNF), which is important for the maintenance of plasticity, may also be downregulated. Gephyrin, a scaffolding protein at inhibitory synapses responsible for anchoring GABA_ARs and glycine receptors to the synapses, may also be downregulated due to histone lysine-tail hypoacetylation. The CHIP assay may also reveal downregulation of genes crucial for the localization of AKAP150 to GABAergic synapses, as proposed by our model. Genes mediating post-translational modifications of AKAP150 are strong candidates for HDAC2-affected genes, as it has been shown that AKAP150 requires post-translational modifications such as palmitoylation to localize properly and participate in LTP at glutamatergic synapses [10]. It is possible that acetylation may be a post-translational modification necessary for AKAP150 to localize to GABAergic synapses in VTA DA neurons and for induction of LTP at these synapses, however, no study to date has identified acetylation as a post-translational modification for AKAPs. To determine if AKAP150 is acetylated in VTA DA, we would need to employ a technique such as mass-spectrometry, which may be

beyond the scope of our lab and require collaboration with a lab with this expertise.

Additionally, HDACi may be affecting other proteins necessary for receptor trafficking to synapse and/or localization of AKAP150 to GABA synapses as HDAC6 for example has been shown to deacetylate tubulin [21-23].

Additionally, we should establish whether elevated CRF signaling during the episode of MD results in the increased excitability and blunted CRF signaling seen at later ages (P21-P28), as speculated in the summary model (Fig. 20). Previous studies have reported hyperactivation of the CRF system during MD, however, several experiments could be performed by our lab to more completely answer this question. First, an intra-LHb injection of CRF at P9 could be delivered to rat pups and determine whether this could replicate any of the changes we see associated with MD. Second, an intra-LHb injection of antalarmin, a CRFR1 antagonist, in combination with the MD procedure could be given. If antalarmin prevents MD-induced changes in the LHb, this would suggest that CRF signaling during the MD episode is essential for the formation of long lasting synaptic and neuronal changes. It would also be interesting to see whether the injection of CRF into the LHb would replicate any of the MD-induced changes in the VTA, and shed light on whether dysfunction of the LHb by MD is implicated in VTA dysfunction.

Going forward with the CRF study in the LHb, it is critical to further characterize CRFR1 associated Ca^{2+} signaling, and how this signaling leads to a decreased function of SK channels. Although we know that Ca^{2+} signaling is essential for the CRFR1 mediated increase in LHb neuronal excitability, we have not yet determined the source of this Ca^{2+} . While it is possible that CRFR1 activation triggers an influx of external Ca^{2+} through

membrane-associated Ca^{2+} channels, it is also likely that CRFR1 activation recruits Ca^{2+} signaling from internal stores (endoplasmic reticulum). Therefore, intracellular application of heparin, an IP3 blocker, would be a potential method of narrowing down the source of Ca^{2+} . In addition, it will also be worthwhile to determine the synaptic effects of CRF in MD animals through recordings of mIPSCs and mEPSCs.

Furthermore, although we found that CRF reduced amplitude and frequency of mIPSCs in nonMD rats, we did not determine whether this was through CRF acting on presynaptic GABAergic neurons, or whether CRF stimulated the postsynaptic neuron to produce a retrograde signaling molecule which could then affect the presynaptic neuron.

Summary model

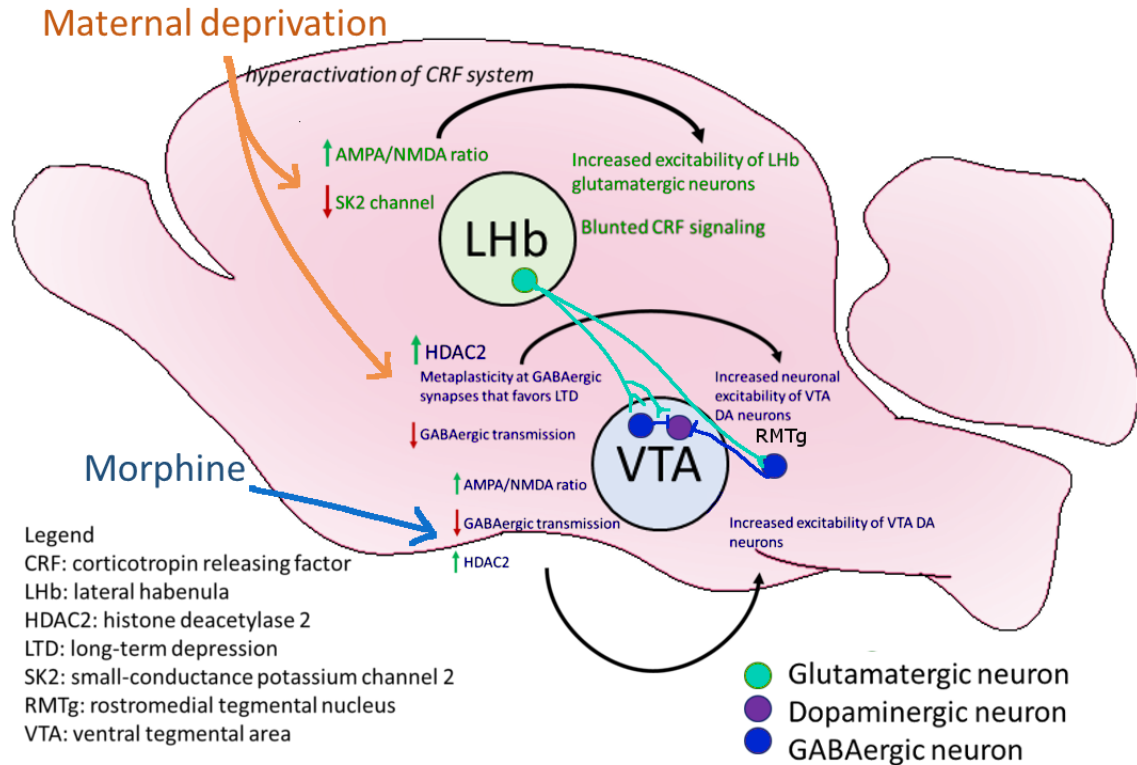


Figure 20: Summary model of major dissertation findings. MD increases AMPA/NMDA ratio and decreases SK2 protein levels in the LHb, leading to increased excitability of LHb neurons and a desensitization (blunting) of CRF signaling. These changes may be precipitated by a hyperactivation of the CRF system during the episode of MD, but this remains speculative. MD increases HDAC2 levels in VTA DA neurons, leading to a decrease in postsynaptic GABAergic transmission and inducing a metaplasticity at GABAergic synapses that favors LTD. Morphine similarly increases HDAC2 levels, which may maintain acute morphine induced plasticity at glutamatergic and GABAergic synapses onto VTA DA neurons. Both the effects of morphine and MD lead to an increased excitability of VTA DA neurons which can affect DA release in downstream regions of the midbrain reward circuitry.

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