Targeting LHb-VTA Brain Reward Circuitry Using Novel Anti-Depressants to Ameliorate the Effects of Early Life Stress

by

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When did I really begin seriously pursuing science as a career? I don't think a lot of kids hold on to this idea that they will be a scientist as an adult. The first person who really lit the spark that I could make it as a scientist was in high school chemistry, Ms. Kimberly Sherman. I was fascinated with chemistry. Ms. Sherman was extremely supportive and gave me many opportunities to enhance my knowledge outside the traditional high school classroom. I will always be thankful for sparking that scientific fire. When I got to undergraduate, I had three amazing professors that really cemented that science was not only going to be a career choice, but a lifestyle choice. My first mentor, Dr. Michelle Hill, went above and beyond the call of duty when I was a student under her. Dr. Hill was the first one who got me interested in psychological studies and research. Many of my first research projects were under her tutorage and she set the foundation for my scientific career not just with studies and research, but also teaching

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Abstract

Targeting LHb-VTA Brain Reward Circuitry Using Novel Anti-Depressants to Ameliorate the Effects of Early Life Stress

Ryan D. Shepard, Doctor of Philosophy, 2019

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According to the World Health Organization, more than 300 million people have been diagnosed with depression making this a serious global healthcare concern. Dysregulation of dopamine (DA) from the ventral tegmental area (VTA) is implicated in neuropsychiatric disorders including depression and addiction. However, research has now identified that the lateral habenula (LHb), a negative regulator of DA signaling, is also involved. Hyperexcitability of LHb neurons have been observed in depression. Although the complete pathophysiology of depression is unknown, early life stress (ELS) predisposes individuals to depression by impacting processes such as histone acetylation. How ELS dysregulates the VTA and LHb in depression is under investigation.

Our previous work showed that maternal deprivation (MD), a single 24hr isolation of male rats from the dam (analogous to child neglect), could disrupt VTA GABAergic plasticity involving alpha-kinase anchoring protein signaling (AKAP150) and histone deacetylase (HDAC) activity. We now show MD decreases histone acetylation at histone-3-lysine-9 (H3K9) and is attenuated by CI-994, a class I HDAC

inhibitor (HDACi). MD impacted mBDNF and AKAP150 abundance, which was ameliorated by HDACi. Lastly, we discovered decreased abundance of synaptosomal protein kinase A (PKA) with no change in calcineurin (CaN) indicating changes in AKAP150 signaling following MD.

Hyperexcitability and increased "burst" firing of LHb neurons is associated with depression. Recently, ketamine has been shown to exert anti-depressant effects. We previously showed that MD-induced LHb hyperexcitability in early adolescent male rats. We sought to see if MD-induced LHb hyperexcitability persisted into late adolescence and whether it would be associated with pro-depressive behavior in the Forced Swim Test (FST). We show that intrinsic LHb hyperexcitability persists into late adolescence with an increased proportion of "bursting" LHb neurons. Following i.p administration of ketamine, MD-induced LHb hyperexcitability and increased "bursting" was normalized. We identified behavioral differences between early and late adolescence. In early adolescence, MD rats exhibited increased climbing behavior while in late adolescence, MD rats exhibited increased immobility; both sets of behavior were attenuated 24hrs later by ketamine. Both sets of results suggest either ketamine or HDACi could be suitable as novel anti-depressants.

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Chapter 1: Introduction

More and more in our society, we are seeing an increased prevalence of depression not only within the US population, but throughout the world. It is estimated that more than 350 million people worldwide are currently afflicted (91). Early life stress has been shown to predispose individuals to develop psychiatric illnesses later in life, such as depression (33; 76; 139). Thus, critical processes occurring during development can be perturbed, which result in later psychopathology.

Historically, the monoaminergic system has been implicated to be involved in the development of these disorders, but now there is contemporary evidence that has highlighted that dysregulation of glutamatergic inputs to these monoaminergic structures are potentially involved (170). Our model of maternal deprivation (MD), a single 24-hour severe early life stressor, is analogous to child neglect and is used as a rodent model to investigate changes in monoaminergic signaling and subsequently observe the changes in behavioral phenotypes in rodent animal models.

The focus of my work and this dissertation is centered on two reward/motivational-related brain regions: the ventral tegmental area (VTA), the origin of the brain reward pathway and the lateral habenula (LHb), a diencephalic negative regulator of monoaminergic signaling. The data presented below addresses four main questions:

1) How does MD affect the neuroepigenome?
2) Do changes within the epigenome have any effect on protein expression?
3) Is there any effect of MD on neurophysiology within both structures and subsequently changes in behavior?

4) Can the use of novel antidepressants amend any MD-induced changes in the epigenome, protein expression, neurophysiology, and behavior?

In the first part of this manuscript, the subject matter will be concerning studies I conducted within the VTA in neonatal Sprague Dawley male rats (post-natal days 14 – 21 [P14-P21]) to assess VTA dopaminergic signaling. Based on work previously published by the Nugent lab detailing the effects of MD on GABAergic metaplasticity within the VTA involving both AKAP150 signaling and HDACs, I investigated specific molecular mechanisms underlying MD-induced changes to the VTA with respect to those previous findings (5). Moreover, I investigated whether a single *in vivo* administration of a histone deacetylase inhibitor (HDACi) was sufficient to recover any MD-induced changes observed up to 24-hours after the administration.

The second part of this manuscript will focus on extending our knowledge based on work that I contributed to demonstrating MD induced LHb hyperexcitability in early adolescence (P21-P28) due to changes in small conductance potassium channel function (SK) (7). My studies examine whether MD affects the neuroepigenome in this early adolescent period when the Nugent lab previously documented LHb hyperexcitability (7). I also raise the question whether epigenetic changes, if any, persist into late adolescence (P42-P50) along with the MD-induced changes in excitability. Lastly, I evaluated whether the use of ketamine, a prototypic N-methyl-D-aspartate receptor (NMDAR) antagonist could ameliorate any changes MD induced in both early and late adolescence. Both studies enhance our knowledge of how early life stress (ELS) affects both dopaminergic and glutamatergic function within the brain reward pathway, but also

whether these systems can be attenuated with the use of novel anti-depressants currently available.

THE RISE OF PSYCHIATRIC DISORDERS AND CURRENT TREATMENTS

The observation of depression in society is not a new phenomenon, but in fact has been observed throughout the ages as far back as Ancient Greece. Originally considered an imbalance in the humors (bodily fluids), the development of better scientific methodology and the introduction of psychological studies further characterized this mental illness, as well as many others (85). Psychological studies and observations from clinicians led to the creation of a psychological diagnosis of Major Depressive Disorder (MDD), a specific form of depression, in the Diagnostic and Statistical Manual for Mental Disorders in which common criteria for individuals suffering from MDD included: sleep disruptions, anhedonia, negative affect, poor concentration, loss of appetite and (in some cases) suicidal ideation (197). Finally, the use of selective serotonin reuptake inhibitors (SSRIs) and monoamine oxidase inhibitors (MAOIs) for pharmacological treatment of MDD suggested a role for involvement of the monoaminergic systems (14).

However, in spite of our increase in the understanding of depression, there has not been a major breakthrough in the development of fast-acting and long-lasting anti-depressants. The current standard of care for depression still primarily revolves around the use of these drugs and psychological therapy (19; 103). One of the biggest challenges facing both clinicians and researchers is the heterogeneity of depression, which creates differences in drug response and efficacy of treatment. My work uses an experimental system to understand one aspect of the heterogeneity of depression via a mechanism

where critical processes in brain homeostasis are disrupted and contribute to the development of psychiatric illness in the context of ELS.

EARLY LIFE STRESS AND THE MATERNAL DEPRIVATION MODEL

Both clinical data and laboratory research has shown that ELS can affect brain development. For example, child maltreatment significantly increases the risk of developing psychiatric issues in later life (79). This evidence points to how stress as an environmental factor can influence development at possible critical and vulnerable periods to influence later on observed psychopathology. For example, one study investigating the role of maternal care found that participants that reported lower levels of maternal care showed increased dopamine signaling, which was observable using raclopride binding assay (156). Thus, studies have explored how ELS and the development of later life psychiatric illnesses might be connected. The role of the infant-mother relationship has also been acknowledged as being significantly important with observed effects of separation on children being conducted mostly by John Bowlby; Bowlby developed theories regarding attachment and how this interaction is crucial for the psychological development of the child (21; 22; 39). To study this effect of ELS, we employ a model of maternal deprivation (MD), the basis of the model is disrupting this infant-mother connection for a 24hr period early in life using the rodent animal model.

The MD model involves separating neonatal rats from the dam at P9. During this time period, neonatal rats receive no nutrients or sensory input for 24hrs and are then reunited with the dam and home cage at P10 (58). This model has been referred to as a "child neglect" model because in addition to the nutrient and sensory deficit, these neonatal rats are also not receiving contact from the dam in the form of nurturing

behavior, such as grooming and licking. Each component that encompasses the MD model has been studied in isolation and all have been sufficient enough to induce changes in animal behavior. The model, as used by the Nugent lab and others, has led to various alterations in behavior (59; 60).

Originally used as a model to study the relationship between ELS and schizophrenia, the MD model was pioneered by Ellenbroek, who initially showed that MD was sufficient to induce observable behavioral changes. One of the initial studies done by Ellenbroek showed that MD slowed the rate of development in Wistar rats, not only in terms of their physical development, but also the development of the dopaminergic system (61). This finding established that there is a link between ELS and dopamine (DA) neurotransmission that could potentially underlie behavioral and neuroadaptations occurring in response to stress. It has also been shown that MD leads to hyperactivity of the hypothalamic pituitary axis (HPA), influencing signaling pathways centering around stress hormones that also lead to changes in coping behavior by rats (124; 158).

Given the amount of studies that have focused on the effects of ELS, and specifically MD, it is now appreciated that early development has critical windows in which developmental processes might be susceptible to maladaptive changes induced by stress and other environmental factors. Based on the understanding that dysfunction of monoaminergic signaling has been historically implicated and empirically observed in depression, the focus of my work centers around the brain reward pathway.

THE BRAIN REWARD PATHWAY

The mesocorticolimbic pathway is the fundamental brain reward pathway in that it modulates decisions within the context of promoting reward seeking behavior. Dysregulation of this pathway is implicated in many different psychiatric illnesses, such as getting "hijacked" by drugs of abuse (159). The primary (but not only) neurotransmitter that is modulated in mental illnesses or after administration of drugs of abuse is DA.

Drugs of abuse, such as heroin and cocaine, can enhance the release of DA throughout the brain reward pathway (207). This enhancement can lead to a change in the rate of neuronal firing of DA neurons within this pathway when a cue is paired with the drug administration. For example, it is thought that tonic firing of DA neurons is the maintenance for steady-state DA release, but in response to a cue and/or drug of abuse, switches to a faster frequency of firing (140; 209). The effects of DA are exerted through two different types of G-protein coupled receptors: dopamine-1 class receptors (D1R) and dopamine-2 class receptors (D2R) (100). These D1R and D2R receptors are located on medium spiny neurons (MSNs) within the striatum and the VTA. This division of D1R and D2R receptor expression on MSNs creates two pathways for circuitry in the context of DA signaling: a direct and indirect (122). In the direct pathway, the activation of D1R leads to an excitatory effect by DA in NAc MSNs whereas in the indirect pathway, the activation of D2R leads to an inhibitory effect (186).

While it is established and understood that both acute and chronic exposure to drugs of abuse change DA signaling, there have also been studies attempting to pinpoint biologic and other genetic markers that could be used to determine whether an individual

is predisposed to developing a psychiatric disorder. For example, typical antipsychotics used to treat schizophrenia work by antagonizing the D2R caused by hypothesized elevated levels of DA. Within the context of drug addiction, studies involving radiotracer labeling of D2R in subjects with drug addiction had significant and persistent decreases in D2R expression in the striatum (206). This role of D2R autoreceptor inhibition and its downregulation is also seen in driving cocaine seeking behavior in mice (86). Another mechanism comes from the use of stimulants, such as methylphenidate, which can block dopamine transporters (DAT) to increase the levels and duration that DA can remain in the synapse (205). For example, genetic knock-out (KO) mice for DAT show upregulation of DA which would be consistent with dysregulation of DA signaling observed in humans (223). Lastly, the mu-opioid receptor (MOR) plays an important role in DA signaling within the VTA and its mechanism sheds light on the current opioid crisis and why opioids are so addictive (67). MOR activation leads to decreased GABA presynaptic release and subsequent disinhibition of DA neurons which yields increased DA release (123). Positron emission tomography (PET) studies show that in comparison to healthy individuals, men with cocaine addiction have increased MOR binding, which in turn suggests increased disinhibition of DA neurons (232). Given the previous mentioned effects of MD on the DA system and reward combined with the evidence above for the role of DA signaling in psychiatric disorders, the scope of my studies in this dissertation will focus on the VTA and its negative controller, the LHb.

THE VENTRAL TEGMENTAL AREA

The VTA is the source of DA neurons within the brain reward circuitry and is important for the context of reward and motivational behaviors (131). Identification of

neurons within the VTA have revealed that it is a heterogeneous structure (128). Tyrosine hydroxylase (TH), the rate-limiting enzyme involved in DA synthesis, is used as a marker to identify the proportion of DA neurons within the VTA, which is approximately 70% (12). Another common marker used for the identification of VTA DA neurons is labeling for the dopamine transporter (DAT) (133). The firing of DA neurons within the VTA is controlled primarily by GABAergic interneurons, which have been identified using glutamic acid decarboxylase (GAD 65/67), an enzyme that converts glutamate to GABA. These GABAergic interneurons constitute approximately 25% (126). Recently, *in situ* hybridization for vesicular glutamate transporter (VGluT2) identified a distinct population of glutamatergic neurons within the VTA composing around 5% of the total neuronal population (216; 217).

The main inputs controlling the rate at which DA neurons fire are the GABAergic inputs (145). These inputs can be from the residing GABAergic interneurons within the VTA itself or from a neighboring local structure, the rostromedial tegmentum (RMTg), sometimes referred to as the Tail of the VTA (98). Previous research from the Nugent lab has shown that both administration of morphine and MD can depress GABAergic transmission, thus affecting the excitability of VTA DA neurons (5; 6).

THE LATERAL HABENULA

While the VTA can be considered the "reward" structure in the neuroanatomy of motivated behaviors and reward seeking, the lateral habenula (LHb) is now referred to as the "anti-reward" structure due to its prominent negative influence on monoaminergic signaling (130). Unlike the heterogeneously composed VTA, this evolutionarily conserved diencephalic structure is comprised primarily of glutamatergic neurons (136).

In addition to its role in mediating aversion and directing other goal-oriented behaviors, the LHb serves as an interface between various forebrain structures and limbic structures. Various inputs from regions such as the medial prefrontal cortex (mPFC) and globus pallidus (GP) coalescence into the stria medullaris, where afferents synapse onto LHb neurons (9). The LHb projects through the fasiculus retroflexus to modulate monoaminergic signaling primarily through the activation of GABAergic structures, such as the GABAergic interneurons of the VTA and the RMTg (16). Although the primary focus of this dissertation is on LHb projecting neurons to the GABAergic neurons in and around the VTA, it is worth noting that there is direct reciprocal innervation of synapses between VTA DA neurons and the glutamatergic LHb neurons (23).

Moreover, research suggests a fundamental role for the LHb in depression and possibly other psychiatric disorders, including drug addiction. One of the first cases came from a patient with prolonged untreatable depression, in whom neuroimaging revealed that there was pronounced activity within the LHb (175). This enhanced activity was subsequently treated with deep brain stimulation and left the patient free of depression (176). Since this intial finding, both clinic and animal models have demonstrated that enhanced activity of the LHb, as well as the firing pattern of LHb neurons, is key to understanding how LHb signaling contributes to the development of depression (24; 99). While the LHb itself is comprised primarily of glutamatergic neurons, there is diversity in their rates of firing. Specifically, research has shown that the "burst" fire neuronal profile seems to be the one most effected and implicated in the context of depression (44; 45; 219).

MONOAMINERGIC VS GLUTAMATERGIC HYPOTHESES OF DEPRESSION

Given the complex role of various brain regions potentially involved in depression, different hypotheses developed based on empirical evidence. One of the first hypotheses to develop was the monoaminergic hypothesis, which suggested that in depression and other psychiatric illnesses, monoamines become dysregulated within the brain, altering neurophysiology in critical brain regions (84). This hypothesis arose from behavioral observations of psychopathology as well as effects of drugs on the monoaminergic system. For example, serotonin (5-hydroxytryptamine, 5-HT) is associated with mood regulation and sleep – both of which are often disrupted in patients with depression (87). In addition, DA is associated with goal-oriented behavior, motivation, and pleasure; alterations in DA signaling may manifest behaviorally as a lack of motivation, anhedonia, etc. It was originally thought that a deficit in these neurotransmitter systems was involved due to the ways in which drugs that target the monoamine circuitry were able to attenuate the behavioral deficits. For example, the use of MAOIs inhibited the breakdown of all monoamines, thus increasing the overall concentration to ameliorate depression in patients (64). Later, the development of SSRIs/SNRIs inhibited re-uptake of monoamines, thus increasing the magnitude of postsynaptic interaction at monoamine receptors due to a longer duration of action (174). Lastly, in the context of other psychiatric illnesses, antagonism of the D_2 receptor using antipsychotic drugs indicated that heightened levels of DA (opposite to what was suggested about 5-HT) was involved in schizophrenia and was used to treat psychosis (115). Thus, the monoaminergic hypothesis has become a favored hypothesis due to the empirical evidence linked by behavioral neuropsychopharmacology.

However, there is still a lack of understanding where monoaminergic dysregulation occurs and what other circuitry is involved. This can be supported by the fact that current anti-depressants are not reliable in terms of their overall efficacy. There are a variety of glutamatergic inputs that come from various structures in the brain, such as the amygdala, prefrontal cortex (PFC), hippocampus, etc. Some of the first pharmacological studies assessing the glutamatergic hypothesis of depression were seen through the use of NMDAR antagonists with anti-depressant mechanisms of action (198; 213). The glutamatergic hypothesis of depression takes into consideration the concept that neurons using the primary excitatory neurotransmitter, glutamate, become dysregulated and this can result in aberrant stimulation of downstream brain structures (170). Observations of increased glutamate levels have been seen post-mortem in patients diagnosed with depression and bipolar disorder (80; 109), Studies have also shown changes in AMPAR composition in post-mortem analysis of depressed patients (55). In spite of this clinical evidence, it is not fully understood how the contribution of dysfunctional glutamatergic circuitry plays a role in psychiatric disorders, such as depression.

In both cases made in the neurobiology of depression, pharmacological evidence indicates that both the monoaminergic and glutamatergic systems are altered in the context of psychiatric illnesses. It is important to note that the changes observed may not be mutually exclusive. The changes observed in both neurotransmitter systems might have a genetic component, as well as affect synaptic neuroadaptations that lead to the development of depression. Taken together, these multiple hypotheses of depression may

explain why clinical depression is heterogeneous in pathophysiology and why there is limited pharmacological efficacy of current anti-depressants.

NEUROEPIGENETICS

Early studies regarding the interaction of genetic structure and the impact of the environment can be summarized as the "nature vs. nurture" debate. However, it became quickly apparent that the development of an individual's phenotype was not one or the other, but rather a combination of traits influenced by the individual's genes and environment. The term, epigenetics, refers to the way in which environmental factors modify the chromatin structure to impact the rate at which genes are expressed and/or repressed (127). Thus, in a way, the environment can have an impact by influencing the rate at which genes are accessed to later influence development.

There are a variety of epigenetic mechanisms that can influence gene expression within the brain, such as histone modifications, DNA methylation, microRNAs, etc (107). For purposes of this dissertation, the focus will be specifically on the impact of histone acetylation. Histone acetylation is one of the more thoroughly understood and documented epigenetic mechanisms within the central nervous system (10; 190). In addition, work done by Dr. Li-Huei Tsai showed that the use of histone deacetylase inhibitors (HDACi) can lead to changes in learning and memory (73; 74). Lastly, work done by Dr. Eric Nestler has highlighted that HDACi can potentially be used as anti-depressants by altering rates of gene expression (42). Histone acetylation is governed by two types of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (105). In brief, HATs enzymatically modify residues on histone tails resulting in a net negative charge that repel deoxyribonucleic acids (DNA) from the histone to

allow increased access to genes (30). In contrast, HDACs enzymatically remove this chemical motif from the histone tail resulting in a net positive charge, which leads to condensation of the DNA and a subsequent decrease in access to genes (179). HDACs can be broken down into four different classes based on their localization. My work centers around class I HDACs (HDAC1, HDAC2, HDAC3, HDAC8), which take up residence inside the nucleus (179).

Stress-induced effects on the epigenome can impact the rate at which critical genes regulate neurophysiology, neuronal excitability, etc. More importantly, it has been shown that inhibition of class I HDACs affect learning and memory during reconsolidation of fear memory (73). Adding to this role of HDACs, previous work from the Nugent lab has demonstrated that the MD model impairs the mechanisms underlying synaptic plasticity of GABAergic neurons within the VTA, but the use of both a pan and class I inhibitor of HDAC (HDACi) is able to alter those MD-induced effects.

ROLE OF SYNAPTIC PLASTICITY

Synaptic plasticity is the cellular basis for learning and memory that is studied in the brain (40). This process is experience-dependent and is crucial for survival in organisms, as it allows neural networks to adapt based on the different intensity and frequency of sensory inputs being received. This change in how strong and/or weak communication is at the synapse can modulate the behavioral output in response to a specific stimulus. This process of "re-wiring" can be broken down into two different processes that influence synaptic strength based on Hebb's rule that "neurons that wire together, fire together": long-term potentiation (LTP) and long-term depression (LTD)

(66; 71; 154). It is also important to note that some forms of plasticity do not conform to Hebb's rule and are thus referred to as anti-Hebbian.

While the first studies regarding memory centered around scientists' search for an engram, scientists realized that processes involving learning and memory dealt with changes in the way that neurons communicated with one another. Traditional protocols used to study synaptic plasticity were conducted in the hippocampus. For example, the use of high frequency stimulation was used to try to induce a sustained increase in synaptic potentiation to yield a LTP (11; 20). In contrast, the use of low frequency stimulation could be used to induce a sustained decrease yielding an LTD (71). Taken together, these first experiments in the field of plasticity highlighted that cellular mechanisms become engaged in which the relative strength of synaptic connections could be altered.

A majority of the first work that was conducted in the field of synaptic plasticity was centered around glutamatergic plasticity. However, researchers also discovered that drugs of abuse, such as opioids, were capable of inducing changes in GABAergic plasticity within the VTA (48; 110; 142). This represented an important scientific finding because the loss of GABAergic tone on DA neurons within the VTA provides evidence for dysregulation of the dopaminergic system in the context of drugs of abuse. Building on this evidence, the Nugent lab showed that MD impairs GABAergic plasticity within the VTA in which GABAergic neurons preferentially underwent LTD with impairment of the LTP mechanism. More importantly, this impairment included an epigenetic mechanism in which HDAC inhibition was involved. Lastly, the mechanism in which this GABAergic plasticity was being impaired involved an orchestration of γ-aminobutyric

acid receptor ($GABA_AR$) trafficking in and out of the synapse involving the alpha kinase anchoring protein (AKAP150) complex (47).

ALPHA KINASE ANCHORING PROTEIN (AKAP150) SIGNALING

There are many different AKAPs expressed, but this dissertation will focus on the AKAP5 family (AKAP150 in rodents, the human ortholog being AKAP79) due to its role of signaling in synaptic plasticity. Foundational work from Drs. John Scott and Mark Dell'Acqua focused on the interaction of protein kinase A (PKA) with cyclic adenosine monophosphate (cAMP), to highlight the importance of AKAP150 as a dynamic signaling complex for mediating processes involved in restructuring synaptic architecture (29). In the context of receptor trafficking, AKAP150 acts as a "molecular switch" docking both PKA and calcineurin (CaN) to regulate either phosphorylation or de-phosphorylation of receptors (41). This finding is important when considering regulation of receptors important for both glutamatergic (AMPAR, NMDAR) and GABAergic ($GABA_AR$) synapses. Therefore, the AKAP150 complex plays a pivotal role in synaptic trafficking and is an important target in the study of how ELS could alter neuronal plasticity and "re-wiring".

Previous work with AKAP150 signaling has focused on the role of this scaffolding protein in glutamatergic signaling. In terms of glutamatergic plasticity, the trafficking of AMPAR is important due to increased Ca2+ influx from NMDAR to promote activity-dependent phosphorylation by kinases, such as PKA (171). Thus, it was found in hippocampal neurons that when the AKAP150-PKA association was disrupted using Ht31 (a small peptide that disrupts PKA-RIIB binding sites to AKAP150 (102)), there was a decrease in the amplitude of miniature excitatory postsynaptic currents

(mEPSC), a measurement of glutamatergic homosynaptic plasticity involving changes occurring on the postsynaptic neuron (165). In addition, biochemical studies and mutations of the AKAP150 complex found that CaN is also involved in the de-phosphorylation of AMPAR that leads to a subsequently formed glutamatergic LTD (5; 13; 120).

However, as a similar theme in the field of synaptic plasticity research, the role of AKAP150 signaling in terms of GABAergic transmission was highly unknown. Previous work from the Nugent lab within the VTA provided a basis for an interaction between AKAP150 signaling and GABAergic transmission (47). It was shown that activation of D2 receptors leads to an inositol triphosphate receptor (IP3) increase in Ca^{2+}, which led to the activation of CaN and subsequent internalization of the $GABA_AR$ to induce a GABAergic LTD in VTA DA neurons. This effect of CaN-dependent GABAergic LTD was also blocked with CaN inhibition. Lastly, using the same methodology to study AKAP150-PKA disruption in earlier studies of glutamatergic plasticity, disruption of the AKAP150-PKA binding site produced a chemically-induced GABAergic LTD. This showed that AKAP150 signaling is involved in both glutamatergic and GABAergic plasticity. We have also shown that MD, an early life stressor, disrupts GABAergic plasticity within the VTA through changes in AKAP150 signaling leading toward a signaling bias in which GABAergic plasticity is impaired (5).

BRAIN DERIVED NEUROTROPHIC (BDNF) SIGNALING

There are many different signaling proteins involved in the development and regulation of the nervous system, but specifically a class of proteins called neurotrophins oversee many of these processes (151). One of these neurotrophins is brain derived

neurotrophic factor (BDNF). BDNF is primarily responsible for regulating aspects related to neuronal maturation, synaptic plasticity, and neuronal excitability. Due to its prominent role in these processes, the role of BDNF has been highly investigated within the context of psychiatric disorders (8; 177).

BDNF is encoded by the *bdnf* gene within the nucleus. Post-transcriptional modifications, such as RNA splicing, are known to occur with this gene to produce different variants (200). Following transcription, the translation of the BDNF transcript results in the formation of the pre-pro-BDNF protein before cleavage to form pro-BDNF (65). While not central to studies conducted in this manuscript, it is important to note that this mature BDNF (mBDNF) precursor signals through the p75 pathway that leads mostly to apoptosis and long-term depression within neurons (119). When required, the pro-BDNF form becomes cleaved to form mBDNF. Once secreted, mBDNF can interact with tyrosine kinase B (TrKB) to initiate different cell programming and signal transduction events within the neuron (118). mBDNF can be secreted not only by neurons, but also by surrounding glial cells like astrocytes (204).

Changes in BDNF expression have been observed in clinical cases. For example, it has been shown that genetics could play a role in the development of depression (117). It has been observed that in patients with depression, the concentration of BDNF in blood serum is lower than non-depressed individuals (95; 178). In addition, a single nucleotide polymorphism (SNP) has been observed in brains from individuals suffering from depression in which the 66-amino acid residue, valine, is converted to a methionine (also referred to as Val66Met) (56; 68; 222). Lastly, postmortem analysis of brains from patients who had been diagnosed with depression have changes in BDNF in discrete

brain regions, indicating that changes observed in BDNF expression are brain region specific (143; 182).

In animal models, the investigation of BDNF signaling has been important to study due to the robust role in the context of its master regulatory properties guiding neural development. However, the role of BDNF within the context of psychiatric illnesses has shown discrepancies in animal studies. Within the context of the VTA, studies have shown that prolonged withdrawal from drugs of abuse, such as opioids, lead to an increased expression of BDNF (201). However, in cases with stress models it has been shown that BDNF can be epigenetically repressed through changes in access to the *bdnf* promoter (96; 108). In both clinical and translational models studying BDNF in the context of mental illnesses, the challenge for researchers is to understand not only the role of BDNF signaling in discrete brain regions, but how perturbation of this important neurotropic factor is involved in the context of brain reward re-wiring in psychiatric illnesses.

DEVELOPMENT OF NOVEL ANTI-DEPRESSANTS

With the incidence of depression on the rise, it is becoming increasingly challenging and costly to treat this disorder. This difficulty is due to a fundamental lack of understanding of how the brain becomes "re-wired" to lead to the hallmark clinical features of depression. This change in circuitry can also impact the heterogeneity of symptoms that are observed in depression. Standard treatment of depression typically includes pharmacotherapy, counseling, and/or a combination of both (146). However, clinical evidence to date has suggested that the current standards of treatment don't work on all patients. One of the biggest complications with depression is when it is

accompanied by suicidal ideation. There are currently no FDA approved anti-depressants that have a robust and rapid mechanism of action or that reduce suicidal ideation/intent in depression. In the context of suicidal thoughts where intervention is critical, this poses a significant problem. Current SSRIs on the market can take as long as 2-6 weeks to work through modulating BDNF signaling and possibly other signaling complexes, making this treatment method ineffective in depression accompanied with suicide (192). The use of SSRIs tend to not be long lasting and this contributes to the problem when a patient becomes treatment-resistant (TRD). TRD patients are classified as patients in which at least two anti-depressant fails to produce efficacious results (63). Ultimately, these failures in pharma may result from the poorly understood and complex changes in key neuronal circuits, as well as discrete brain regions such as the VTA and LHb.

Clinical and animal model evidence suggest that early development is a critical window in which adverse events can disrupt important developmental checkpoints, which in turn could increase the risk of developing a mental illness. One of the key relationships bridging together environmental influence and gene expression is the role of epigenetic modifications. Thus, there could be a critical window during development in which epigenetic mechanisms can be modified to prevent future mental illnesses. One class of experimental pharmaceuticals under investigation is the role of HDAC inhibitors (HDACi).

The general mechanism for HDACis is inhibition of HDACs to promote transcription of genes. Already, HDACi are being used in other therapeutic regiments, such as cancer (116; 211). Different types of HDACi can be used to target various classes of HDACs. One such compound, known as CI-994 (Tacedinaline), has been shown in

animal models to have a wide variety of ameliorating effects caused by injury, stress, drugs, etc. For example in spinal cord injury, the use of CI-994 can lead to better functional recovery (230). In the context of depression, a hallmark paper by Graff et al. demonstrated the role of HDACs in fear memory and consolidation and showed that CI-994 could affect fear memory recall (73). Lastly, work from the Nugent lab showed that within the VTA, the inhibition of HDACs leads to restoration of GABAergic plasticity and AKAP150 signaling within the VTA (5). Taken together, these data create the premise for my research to investigate the effects of CI-994 and ELS within the VTA to understand how HDAC inhibition can be used to pharmacologically target dopaminergic dysfunction in psychiatric illnesses.

While dopaminergic dysfunction and overall monoaminergic dysfunction are implicated in psychiatric illnesses such as depression, it is important to note that monoaminergic neurons make up a smaller proportion of total neurons within the brain compared to both glutamatergic and GABAergic neurons. Studies have indicated that glutamatergic disruption could also be implicated in the context of mental illnesses addiction. For example, during cocaine intake rats have been observed to exhibit diminished glutamatergic neuronal activity within the prefrontal cortex (34). This has also been observed in humans (70). Not surprisingly, NMDAR antagonists can exert anti-depressant properties. Ketamine works by altering glutamatergic transmission as a prototypic NMDAR antagonist.

Ketamine was originally created for use as an anesthetic at higher doses due to the NMDAR receptor antagonism. However, it was noted recently that at lower doses (sub-anesthetic) that the drug exerted a rapid and efficacious anti-depressant mechanism of

action (15). Since this discovery, many clinical studies done on the response of ketamine have yielded reports that ketamine exerts a quick and long-lasting anti-depressant effect (169; 228). The biggest challenges in developing ketamine as a novel anti-depressant treatment are due to its abuse potential and whether it has important non-NMDAR dependent mechanisms of action.

Ethically, ketamine is a drug of abuse that is used recreationally. Ketamine induces a dissociative effect, which may be linked with hallucinations and may be used in combination with or in place of other drugs of abuse (46). Drug delivery of ketamine can occur in many different forms. Due to these reasons, the use of ketamine as a treatment for depression has been under debate due to the fact that it is a drug of abuse. There is also evidence that suggests that chronic NMDAR antagonism can lead to and/or exacerbates schizophrenia (43).

In spite of safety concerns, a wide variety of research is in progress to determine the independent NMDAR mechanisms of action for ketamine. One theory is that ketamine works through influencing levels of BDNF (138) (18). More controversially, research has shown that certain metabolites of ketamine can exert anti-depressant effects on their own (72; 226). For example, hydroxynorketamine (HNK) has been shown to still be systemically available as late as four hours post-administration of ketamine and still detectable up to 24 hours later (227). However, there are significant discrepancies in the time course of ketamine's effects. This inconsistency warrants continued research on the possible role of ketamine and whether it can correct neurophysiological abnormalities associated with ELS by modulating dysregulation of glutamatergic circuitry that work upstream of monoaminergic brain structures.

Chapter 2: Targeting histone deacetylation for recovery of maternal deprivation-induced changes in BDNF and AKAP150 expression in the VTA

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Author Contributions

FN, RS, SG designed the experiments. RS, SG, HK, and WZ performed experiments. FN, RS, SG, HK, AB, WZ, and BM analyzed the data and prepared the figures. FN and RS wrote the manuscript.

ABSTRACT

Severe early life stressors increase the probability of developing psychiatric disorders later in life through modifications in neuronal circuits controlling brain monoaminergic signaling. Our previous work demonstrated that 24h maternal deprivation (MD) in male Sprague Dawley rats modifies dopamine (DA) signaling from the ventral tegmental area (VTA) through changes at GABAergic synapses that were reversible by *in vitro* histone deacetylase (HDAC) inhibition which led to restoration of the scaffold A-kinase anchoring protein (AKAP150) signaling and subsequently recovered GABAergic plasticity (5). Using a combination of *in situ* hybridization, Western blots and immunohistochemistry, we confirmed that MD-induced epigenetic modifications at the level of histone acetylation were associated with an upregulation of HDAC2. MD also increased *Akap5* mRNA levels in the VTA. Western blot analysis of AKAP150 protein expression showed an increase in synaptic levels of AKAP150 protein in the VTA with an accompanying decrease in synaptic levels of protein kinase A (PKA). Moreover, the abundance of mature brain-derived neurotrophic factor (BDNF) protein of VTA tissues from MD rats was significantly lower than in control groups. *In vivo* systemic injection with a selective class I HDAC inhibitor (CI-994) was sufficient to reverse MD-induced histone hypoacetylation in the VTA for 24h after the injection. Furthermore, HDAC inhibition normalized the levels of mBDNF and AKAP150 proteins at 24h. Our data suggest that HDAC-mediated targeting of BDNF and AKAP-dependent local signaling within VTA could provide novel therapeutics for prevention of later-life psychopathology.

KEYWORDS

A-kinase anchoring protein (AKAP), dopamine, histone deacetylase (HDAC), histone deacetylase inhibitor (HDAC inhibitor), brain derived neurotrophic factor (BDNF), ventral tegmental area (VTA), early life stress, maternal deprivation (MD), epigenetics, histone acetylation

ABBREVIATIONS

Histone H3 acetylation at lysine 9, Ac-H3K9; A-kinase anchoring protein, AKAP; Calcineurin, CaN; Dopamine, DA; Histone deacetylase, HDAC; Long-term depression, LTD; mature brain derived neurotrophic factor (mBDNF); Calcium permeable AMPARs, CP-AMPARs; Maternal deprivation, MD; Nucleus accumbens, NAc; NMDA receptor, NMDAR; Non-maternally deprived, non-MD; Prefrontal cortex, PFC; Protein kinase A, PKA; Spike-timing-dependent plasticity, STDP; Type II regulatory subunit of PKA, RII; Ventral tegmental area, VTA.

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INTRODUCTION

Child abuse and neglect are shown to increase the risk of developing stress-related disorders and substance abuse. This increased vulnerability seems to be related to brain monoaminergic dysfunction which includes an altered dopamine (DA) signaling from the ventral tegmental area (VTA) (5; 82; 152; 184; 189). Early life stressors also have a significant impact on epigenome, including histone modifications that may underlie subsequent changes in gene expression, synaptic plasticity and behavior (2; 81; 104; 113; 148; 195). One of the robust long-lasting histone modifications associated with severe early life stress are histone deacetylase (HDAC)-mediated changes in histone acetylation (3; 113; 195; 215). In general, histone deacetylation by HDACs is associated with chromatin condensation and gene repression. On the other hand, blocking histone deacetylation by HDAC inhibitors can increase histone acetylation to possibly promote gene expression through chromatic relaxation. HDAC inhibitors have shown great potential for treatment of age-associated cognitive and memory impairments by improving synaptic plasticity (153). Moreover, HDAC inhibitors have also been shown to have antidepressant properties and ameliorate symptoms of post-traumatic stress disorder, depression, and addiction (42; 104; 148).

We have also shown that acute morphine-induced synaptic plasticities in VTA DA neurons involved HDAC-mediated changes in histone acetylation and were also reversible by *in vitro* application of an HDAC inhibitor through increases in histone acetylation (6; 110). We demonstrated that a 24h early maternal deprivation (MD, an animal model of child abuse), on postnatal day 9 (P9) induces synaptic abnormalities at GABAergic synapses onto VTA DA neurons through disruption of AKAP79/150 (human 79/rodent 150; also known as AKAP5) signaling in juvenile rats that might also be

targeted by HDACs during MD (5). AKAPs were first discovered as the scaffold proteins that principally mediate the crosstalk of cAMP/PKA signaling with other signaling pathways. Although AKAPs identified to bind to the type II regulatory subunit of PKA (RII); it is now known that AKAPs have several binding sites for other signaling molecules including protein kinase C, protein phosphatases including calcineurin (CaN), G-protein coupled receptors, adenylyl cyclases and phosphodiesterases. AKAPs tether these signaling enzymes with their substrates (for example, synaptic AMPA, NMDA and $GABA_A$ receptors and ion channels) within distinct subcellular compartments for specific spatial and temporal interplay of postsynaptic signaling molecules in synaptic plasticity and neuronal function, as well as in synaptic and neuronal dysfunction associated with disease (62; 212; 214). Therefore, therapeutic targeting of AKAP-directed signaling has become an emerging and novel concept in selective normalization of dysfunctional signaling pathways assembled by AKAPs in neurological disorders (212). We found that MD-induced GABAergic metaplasticity (an increased susceptibility of GABAergic synapses to induction of AKAP150-dependent long-term depression, LTD) and dysregulated AKAP signaling could be reversed with local *in vitro* HDAC inhibition in the VTA, suggesting the potential clinical benefits of targeting of AKAP signaling within the VTA by HDAC inhibitors soon after the stress. Given that MD-induced disruption of AKAP signaling was associated with significant increases in the levels of AKAP150 expression in VTA DA neurons (5), this suggested that MD may induce HDAC-mediated transcriptional changes in specific signaling molecules that directly interact with AKAPs or act upstream from AKAP signaling. In fact, activity-dependent alterations of brain-derived neurotrophic factor (BDNF) transcriptional levels and BDNF expression act

upstream to regulate proteasome-dependent synapse remodeling and synaptic protein concentrations including synaptic levels of AKAP150 (93). Moreover, early life adversity results in epigenetic changes in BDNF gene expression and signaling that are critical for synaptic plasticity (3; 49; 148; 166).

Here, we investigated whether reversible HDAC-mediated histone modifications were associated with MD in the VTA and tested the effects of a single *in vivo* injection of a cell permeable potent selective class I HDAC inhibitor (CI-994, also called N-acetyldinaline or tacedinaline) on MD-induced changes at the level of histone acetylation, BDNF protein levels and AKAP gene expression within the VTA. We found that MD indeed increased HDAC2 (a class I HDAC) expression specifically in VTA DA neurons and is associated with a reduction of histone H3 acetylation at lysine 9 (Ac-H3K9). MD also reduced levels of BDNF protein while increased synaptic levels of AKAP150 protein in the VTA, and these changes were reversible by the *in vivo* HDAC inhibition 24h after the injection (see our model in Figure 7). Taken together, our results suggest that a single *in vivo* HDAC inhibition soon after the stress may be sufficient to epigenetically ameliorate MD-induced changes in BDNF signaling that may act upstream to regulate subcellular organization of AKAP150 complexes in VTA synapses.

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 the male pups in litters of Sprague–Dawley rats (Taconic Farms) at P9 were isolated at 10:00 a.m. from the dam and their siblings for 24h (MD group). The isolated rats were placed in a separate quiet room and kept on a heating pad (34° C) and not disturbed until being returned to their home cage 24h later. The remaining non-separated male rat pups received the same amount of handling but were kept with the dam serving as the non-maternally deprived control group (non-MD group). Rats were maintained on a 12-hour light/dark cycle and provided food and water ad libitum. The animals were taken for study during the light period, between 3 and 5 h after light was turned on. Each day, two MD and non-MD rats (age-matched) from the same litter were sacrificed over days P14-21 for electrophysiology recordings, immunohistochemistry and Western Blotting. We blindly performed the analysis with respect to the treatment of the rats to reduce the potential for investigator bias.

Slice preparation for Western blotting

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 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.00 MgSO4, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O_2–5% CO_2. Horizontal slices were cut at 300 μm for Western blot experiments.

HDAC inhibitor treatment

Sprague–Dawley male rats (P14-P21) (non-MD or MD) received either one intraperitoneal (i.p.) injection of CI-994 (10mg/kg) dissolved in 1% Tween80 (vehicle) or

an injection of comparable volumes of 1% Tween80 (vehicle) 24h prior to sacrifice for immunohistochemical or Western blot studies.

Western Blotting

The VTA was dissected bilaterally from horizontal slices (300 μm) of non-MD 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 μg) 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 antibodies recognizing HH3 (1:10,000, Abcam ab1791), antibody against PKA regulatory β2 subunit (1:5000, Abcam, ab75993), antibody against PSD95 (1:500, Cell signaling 362333), antibody against ac-H3K9 (1:1,000 cell signaling 3649), antibody against calcineurin subunit A (1:2,000, Abcam ab3673), antibody against AKAP150 (1:500, Santa Cruz Sc-6445), antibody against mature BDNF(mBDNF, 1:1000 ab108319), antibody against vinculin (1:1,000, Abcam ab129002) and antibody against β-actin (1:10,000, Abcam, ab6276). Secondary antibodies used were HRP-linked specific for rabbit (1:2000, Cell signaling), mouse (1:2000, Cell signaling) and goat (1:5000, Abcam ab97110) IgG. 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. Total abundance of target protein was normalized to appropriate endogenous control. All data were normalized to non-MD group with summary data reported as fold change.

Subcellular Fractionation

The subcellular fractionation method was modified from (78). In brief, VTA tissues were collected from non-MD and MD rats via horizontal sectioning (300 μm). Tissues were homogenized and lysed using TEVP buffer (1 mM Na3VO4, 1mM EDTA, 1mM EGTA, 1M Tris pH 7.5, phosphotase inhibitor, protease inhibitor) containing sucrose (320mM). Samples were then centrifuged at 800g for 10 min at 4°C to yield both P1 and S1 segments. TEVP buffer was then added to S1 segments and centrifuged at 9,200g for 15 min at 4°C to yield S2 and P2 segments. P2 represents the crude synaptosomal membranes. P2 fractions were suspended with TEVP buffer containing sucrose and transferred to polycarbonate microcentrifuge tubes (Beckman Coulter). Samples were centrifugated using Optima Max Ultracentrifuge (Beckman Coulter, Indianapolis, IN, USA). Samples were ultracentrifugated at 25,000g for 20 min at 4°C to yield LS1 and LP1. The LP1 fractions contained the synaptosomal membranes and contents; this fraction was then used to perform western blot for protein expression specifically in the synaptic compartment, as described above.

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 300ml of heparinized 1x phosphate buffered saline (PBS) followed by 250ml of 4% paraformaldehyde (PFA, USB, Cleveland, OH). The brains were dissected and placed in 4% PFA for 24 hr 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 μm) of the midbrain containing the VTA (from -4.92 to -6.72 mm caudal to bregma; Paxinos and Watson, 2007) 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-tyrosine hydroxylase (anti-TH) (1:1000, Calbiochem, San Diego, CA.), 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). For double immunofluorescence of TH and HDAC2, serial coronal sections of the midbrain containing the VTA were fixed in 4% PFA for 5 min, washed in 1x PBS, and then blocked in 10% normal goat serum (NGS) containing 0.3% Triton X-100 in 1x PBS for 1 h. Sections were then incubated in rabbit anti-TH (1:1000; Calbiochem) and mouse anti-HDAC2 (1:1000; Abcam) in carrier solution (0.5% NGS in 0.1% Triton X-100 in 1x PBS) overnight at room temperature. After rinsing in 1x PBS, sections were incubated for 2 h in Alexa Flour 488-labeled goat anti-mouse IgG and Alexa Flour 568-labeled goat 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 or TH/HDAC2 immunoreactive neurons were processed as positive control tissue. Images were captured using a Zeiss Confocal Inverted Microscope System (Carl Zeiss Inc.) 40x/1.4 n.a. oil immersion objective. For HDAC2 density quantification three AP locations (-5.4, -5.7, and -6.0 relative to bregma) were studied. At three AP locations, total of eighteen 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. For each AP location normalized density readings were averaged across the neurons.

***In situ* Hybridization procedure**

An *Akap5* probe corresponding to nucleotides 949–1367 of rat *Akap5* cDNA (accession number NM_133515) was used for all in situ hybridization procedures. ^{35}S-UTP–labeled riboprobes were synthesized using T7 (antisense) RNA polymerase. After incubation at 37 °C for 1 h, probes were treated with DNase I, precipitated and re-suspended. Sections were fixed in 4% formaldehyde followed by two 5 min washes in 1x PBS. They were then placed in 0.25% acetic anhydride/triethanolamine (1.5%) for 10 min and rinsed in 2x SSC twice for 5 minutes each. Sections were dehydrated in ascending ethanol concentrations (70, 80, 95, and 100%) and then air-dried. Antisense-labeled probes (2.04 x 10^6 dpm/100 ul) were hybridized to tissue sections overnight at 55 °C in hybridization buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 300 mM NaCl, 50%

formamide, 10% dextran sulfate,1x Denhardt's). Slide-mounted sections including VTA (from – 5.4 to -6.0 mm caudal to bregma) were rinsed in 4x SSC at room temperature to remove coverslips and then washed four times for 5 min each in 4x SSC containing 1 mM dithiothreitol (DTT). Free probe was removed using 20 mg/mL RNase (Sigma, St. Louis,MO, USA) in buffer (0.5 M NaCl, 0.01 M Tris-HCl, 0.25 mM EDTA) at 37 °C for 30 min. After rinsing twice in 2x SSC/1 mM DTT, then once in 1 x SSC/1 mM DTT and once in 0.5 x SSC/1 mM DTT (all rinses for 5 min), sections were washed twice for 30 min in 0.1x SSC, 1 mM DTT at 65 °C. Sections were cooled in 0.1x SSC, 1 mM DTT at room temperature and then dehydrated in ascending ethanol concentrations (70, 80, 95, and 100%) and then air-dried. Hybridized slides were then exposed to Hyblot CL film (Denville Scientific, Holliston, Ma.) for 5 days. A slide containing 14C microscale standards was also exposed to the film (ARC, St Louis, MO). The autoradiogram was digitized and optical density readings were taken from the VTA at – 5.4, -5.7 and -6.0 mm caudal to bregma. Density readings were also taken from the 14C micro scale standards at 0, 0.63, 0.193, 0.324, 0.453, 0.573, and 0.7 uCi/g in order to generate a standard curve. Data was generated by comparing the sample readings to the standard curve.

Data analysis

Values are presented as means ± SEM. Statistical significance was determined using unpaired or paired two tailed Student's t-test or two-way ANOVA with Bonferroni post hoc analysis. The threshold for significance was set at $*p < 0.05$ for all analyses. All statistical analyses were performed using GraphPad Prism 7.

RESULTS

MD increased HDAC2 expression in VTA DA neurons and induced histone hypoacetylation in the VTA.

Here, we performed HDAC2 double immunofluorescence using antibodies against TH (a marker for DA neurons) and HDAC2 as previously described (6) to examine whether MD induces changes in HDAC2 expression that are limited to VTA DA neurons. We found higher levels of nuclear, but not somatic HDAC2 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, Figure 1A, n=6-7 per group, Somatic HDAC2: $F(1,33)=0.2709$, $p=0.6062$; Nuclear HDAC2: $F(1,33)=8.107$, $p= 0.0075$, two-way ANOVA). To test whether increased HDAC2 expression translates to histone hypoacetylation that is normally associated with transcriptional repression of genes, we performed Western blot assays of VTA homogenates isolated from non-MD and MD rats to quantify acetylated histone H3 (histone H3 acetylation at lysine 9 using an antibody against Ac-H3K9). Consistently, MD-induced increases in HDAC2 expression was associated with significant reduction of Ac-H3K9 (Figure 1B, n=6 per group, $t(10)=2.588$, $p=0.0135$, unpaired Student's t test). We also confirmed that the total level of histone 3 did not change following MD (n=3 per group, non-MD: 1.054 ± 0.1534; MD: 0.9884 ± 0.06021, $t(4)=0.4005$, $p=0.7093$, unpaired Student's t test).

MD increased synaptic levels of AKAP150 protein with significant alterations in synaptic expression of PKA but not CaN.

MD is associated with higher levels of AKAP150 immunoreactivity in VTA DA cells following MD (5), however it is unknown whether the levels of AKAP150 are increased at synapses. To test this, we used biochemical fractionation to examine the synaptic levels of AKAP150, postsynaptic density protein 95 (PSD95, that is shown to colocalize with AKAP150 in complexes with AMPA receptors, AMPARs, at glutamatergic synapses), PKA regulatory subunit IIβ (PKA—RIIβ) and CaN A subunit levels in synaptosomal membrane fractions (LP1) from VTA tissue extracts. We found that MD was associated with higher synaptic levels of AKAP150 and a significant decrease in the synaptic levels of PKA-RIIβ (the total levels of PKA-RIIβ were unchanged). No significant changes were detected in the total or synaptic levels of the CaN A subunit in fractions of VTA tissue extracts from MD rats compared to those from non-MD rats (Figure 2 A-C, AKAP-LP1: n=4 per group, $t(6)=2.837$, $p=0.0148$; total PKA-RIIβ: n=8 per group, $t(14)=1.538$, $p=0.0732$; PKA-RIIβ LP1: n=4 per group, $t(6)=2.094$, $p=0.0406$; total CaN A: n=8 per group, $t(14)=0.03334$, $p=0.4869$, CaN A LP1: n=3-4 per group, $t(5)=0.4414$, $p=0.3387$, unpaired Student's t test). We also did not detect any significant change in levels of PSD95 in synaptosomal membrane fractions following MD (Figure 2A, n=4 per group, $t(6)=0.0866$, $p=0.9338$, unpaired Student's t test). Quantitative *in situ* hybridization also revealed that MD significantly increased *Akap5* mRNA expression in the VTA at three AP levels within the VTA (-5.4, -5.7 and -6 mm caudal to bregma, Paxinos and Watson, 2007, Figure 3, n=6-7 per group, $F(1,33)= 13.38$, $p= 0.0009$, two-way ANOVA).

HDAC inhibition reversed histone hypoacetylation in the VTA.

To determine whether decreases in Ac-H3K9 levels were reversible by a single *in vivo* i.p. injection of CI-994 similar to *in vitro* application of CI-994 (6), we performed Western blots of VTA tissue extracts from non-MD and MD rats with i.p. injection of either vehicle or CI-994. We found that the abundance of Ac-H3K9 was significantly increased in VTA tissues from MD rats treated with CI-994 at 3h and 24h post-injection compared to those from MD rats injected with vehicle. Consistently, histone hypoacetylation was still detectible in Western blots of VTA tissue extracts from vehicle-treated MD rats compared to those from vehicle-treated non-MD rats (Figure 4A-C, 4C represents the quantification of the 24h-post injection Western blot data: n=11-12 per group, $F(1,27)=8.563$, $p=0.0069$, two-way ANOVA).

***In vivo* HDAC inhibition reversed MD-induced AKAP150 upregulation in VTA DA neurons.**

Given that MD triggered reversible changes in histone acetylation for 24h post-injection, we next tested whether MD-induced changes in AKAP150 expression were also normalized at 24h after HDAC inhibitor injection. We performed a double-immunofluorescence staining technique using antibodies against TH and AKAP150 allowing us to visualize DA neurons expressing AKAP150 from non-MD and MD rats that were injected with either vehicle or CI-994 and then sacrificed 24h after the injection. Consistent with our previous result (5), we detected higher levels of AKAP150 immunoreactivity in TH^+ cells of MD+vehicle compared to non-MD+vehicle rats within the VTA. Moreover, we found that AKAP150 expression in TH^+ neurons was returned to normal levels in MD rats injected with CI-994 compared to those from MD rats injected

with vehicle (Figure 5, n=7-8 per group, F(1,27)=9.902 , P= 0.004, two-way ANOVA). Neither MD nor CI-994 altered the levels of expression of TH in the same sections (data not shown).

HDAC inhibition reversed MD-induced decreases in the levels of mBDNF protein in the VTA.

Consistently, we observed that the levels of mBDNF (mature BDNF cleaved from pro-BDNF which preferentially binds and signals through TrkB) in Western blots of VTA tissue extracts from MD rats were lower than controls. Moreover, we found that MD-induced decreases in mBDNF were reversible 24h after a single injection of CI-994 (Figure 6, A: n=5 per group, t(8)=2.55, p=0.0171, unpaired Student's t test, B: represents the quantification of the 24h-post injection Western blot data: n=7-13 per group, F(1,34)=3.028, p=0.0427, two-way ANOVA).

CONCLUSIONS

Early childhood adversities are associated with impaired DA function and signaling which underlie the increased risks of developing drug addiction and other stress-related disorders. The epigenetics of early life stress in relation to DA dysfunction and later-life health outcomes is of great interest considering that DA cell responses to early life stress can be epigenetically modulated during selective and sensitive windows of development. Epigenetic changes significantly impact the expression of genes controlling VTA DA neuronal function and signaling, which subsequently alter motivation and reward-related behaviors (104; 148). Previously, we found that MD triggered an AKAP-dependent metaplasticity at GABAergic synapses onto VTA DA

neurons in response to spike-timing-dependent plasticity (STDP) protocols that rendered GABAergic synapses more susceptible to LTD (5). These GABAergic synaptic modifications were reversed to normal STDP by short-term *in vitro* HDAC inhibition using a pan-HDAC inhibitor (sodium butyrate) or a selective class I HDAC inhibitor (CI-994) (Figure 7). Indeed, adverse experiences in early life, such as MD, are associated with long-lasting changes in the expression of critical synaptic plasticity-associated genes through chromatin remodeling (113; 148; 152; 162; 166; 194; 231). Based on this and our finding of normalization of STDP by acute *in vitro* HDAC inhibition, we hypothesized that MD-induced modifications in histone acetylation and HDAC2 expression in the VTA mediated MD-induced alterations in GABAergic STDP and AKAP signaling (5). Here, we demonstrate that epigenetic mechanisms involved in MD-induced changes in VTA DA function may include HDAC2-mediated decreases in levels of histone acetylation at H3K9 in the VTA which could be restored to normal levels as soon as 3h and up to 24h after a single *in vivo* injection of MD animals with a selective class I HDAC inhibitor, CI-994. We also found that MD increased the expression of the scaffold *Akap5* gene (elevated *Akap5* mRNA levels) which translated to increases in synaptic levels of AKAP150 protein, suggesting MD-induced HDAC-independent transcriptional activation of this gene in the VTA. This increase in synaptic levels of AKA150 by MD was associated with lower synaptic levels of PKA-RIIβ, suggesting an MD-induced reduction in postsynaptic PKA-RII localization and probably reduced availability of AKAP150-anchored PKA at the synapse. This was opposite to our expectation as there is a positive correlation between synaptic AKAP and PKA expression. On the other hand, total and synaptic levels of CaN A, as well as synaptic levels of PSD95 in the VTA, were

unaltered by MD. Consistent with this, recently generated AKAP150-deficient mice that lack PKA anchoring to AKAP150 (by deletion of ten amino acids within the PKA-RII subunit binding near the AKAP C terminus, the AKAP150ΔPKA knockin mice) have reduced PKA-RII levels in hippocampal PSD fractions and AKAP150-anchored PKA signaling. This mutation specifically reduces postsynaptic PKA-RII localization without alterations in the expression levels and localization of other AKAP-associated proteins including CaN A, GluA1 subunit of AMPARs and PSD-95 in the hippocampal synapses (172). In the light of our earlier findings of the functional impairment of AKAP signaling and induction of GABAergic metaplasticity towards a calcineurin-dependent LTD, we suggest a possible dysfunctional association between AKAP150 and PKA affecting proper anchoring of PKA at the synapse which could result in the less availability of AKAP150-anchored pool of PKA at GABAergic synapses. The impaired association of AKAP150 and PKA may also lead to a biased association of AKAP150 with CaN and AKAP targeting of CaN to $GABA_A$Rs to promote CaN-dependent GABAergic LTD (MD-induced GABAergic metaplasticity as previously reported by our team)(5). Indeed, AKAP150 interaction with CaN is found to be necessary for hippocampal LTD (94; 172). Interestingly, PKA anchoring to AKAP150 also plays a critical role in induction of hippocampal LTD (121; 172). In a recent elegant study using knockin mice that were deficient in AKAP-anchoring of either PKA or CaN (AKAP150ΔPKA knockin mice and AKAP150ΔPIX mice), AKAP150-anchored PKA was shown to transiently augment NMDAR Ca^{2+} signaling during NMDAR-dependent hippocampal LTD by recruiting calcium-permeable AMPARs (CP-AMPARs). Once LTD was induced, AKAP150-anchored CaN rapidly removed CP-AMPARs from hippocampal synapses (172).

Whether recruitment and insertion of CP-AMPARs by the PKA-AKAP complex play a transient role in boosting Ca^{2+} signaling during induction of NMDAR-dependent GABAergic STDP, and or in induction of MD-induced GABAergic metaplasticity are open questions that merit further investigation.

Since AKAP150 was upregulated by MD, we hypothesized that the rescue of normal AKAP150 signaling in MD rats by HDAC inhibitors may occur through the reversal of HDAC2-mediated transcriptional changes in the expression of AKAP150-interacting proteins (such as PKA) or signaling molecules upstream to AKAP150 that are necessary for proper functioning, interaction or assembly of AKAP150-anchored complexes of signaling molecules together with their targets at the synapse.

BDNF signaling through its receptor tyrosine receptor kinase B (TrkB) affects neuronal excitability, synaptic transmission and synaptic and structural plasticity. In addition, its expression is regulated by neuronal activity (93; 167). Upregulation of BDNF expression is a common finding after the administration of many drugs of abuse in the mesolimbic system particularly during prolonged drug withdrawal (97; 196; 201; 202). In contrast, a counteractive and opposite role for BDNF is also proposed where suppression of BDNF signaling in the VTA promotes the rewarding effects of opioids (106). Early life stresses can induce long-lasting alterations in BDNF signaling through epigenetic modifications of BDNF expression in the brain. Both increases and decreases of BDNF gene expression in different brain regions through such mechanisms have been shown following early life adversity (49; 166) so we hypothesized that HDAC-mediated transcriptional repression of genes important for synaptic plasticity, such as BDNF, in the VTA might occur in MD rats. We also found that MD decreased the levels of mBDNF

protein in the VTA and this decrease was reversed by *in vivo* HDAC inhibition. Recent evidence also emphasizes the transcriptional regulation of the *Bdnf* gene through histone modifications including Ac-H3K9 that also involves HDAC2 (35; 36; 210). Therefore, we assume that MD increased-HDAC2 expression could lead to transcriptional repression of *Bdnf* gene through increased HDAC2 occupancy at *Bdnf* promoters and histone H3K9 deacetylation in the promoter region of specific *Bdnf* exon/s thus mitigating the synaptic dysfunction induced by MD in the VTA (Figure 7). Activity-dependent changes in synaptic concentrations of AKAP150 is shown through BDNF-TrkB signaling regulation of ubiquitination of AKAP150 (93). Although it is possible that decreases in BDNF expression following MD-induced changes in VTA neuronal activity dysregulates the ubiquitin proteasome system to increase synaptic levels of AKAP150, given the complexity of mBDNF signaling and the myriad of actions of mBDNF it is more likely that mBDNF signaling engages signaling molecules and scaffold proteins interacting with AKAP150 to mediate MD-induced metaplasticity in a complex manner. MD also increased *Akap5* expression in the VTA; we assume that the increased expression of AKAP150 and altered AKAP150-anchored signaling in VTA DA neurons by MD may also be related to HDAC2-mediated epigenetic modifications leading to downregulation of a transcriptional repressor that prevents transcription of *Akap5* gene upon activation. In summary, possible HDAC-mediated epigenetic regulation of BDNF- and AKAP-dependent signaling within VTA highlights the potential power of HDAC inhibitors for prevention of VTA DA dysfunction associated with later-life psychopathology.

FIGURES

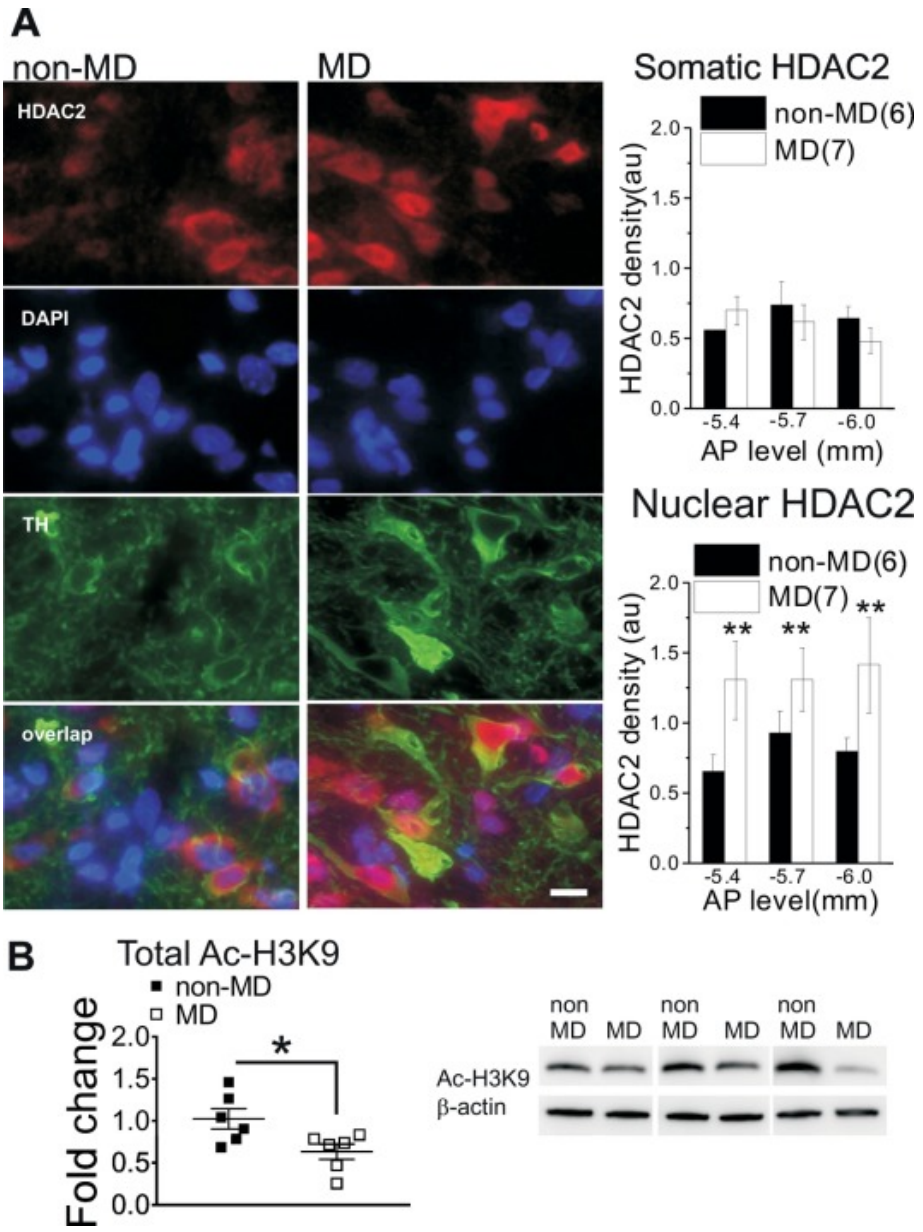

Figure 1

MD increased HDAC2 expression in VTA DA neurons and decreased histone acetylation at H3K9 in the VTA. (A) Examples of brain sections stained with antibodies to TH (green) and HDAC2 (red), and DAPI (blue) with the merged panels, which show the expression of HDAC2 in TH+ neurons in the VTA of non-MD (left column) and MD (right column) rats. Scale bar, 20 μm. Figure also shows the averaged levels of nuclear and somatic HDAC2 expression at three AP levels from non-MD and MD rats. (B) Representative Western blots and quantitative data of total levels of Ac-H3K9 and β-actin (control) in VTA homogenates from non-MD and MD rats. In this and all subsequent figures * denotes statistical significance and average graphs show means ±

SEM from biological replicates or rats for each condition. Also in all western blots, fold-change quantification was pre-normalized to endogenous control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

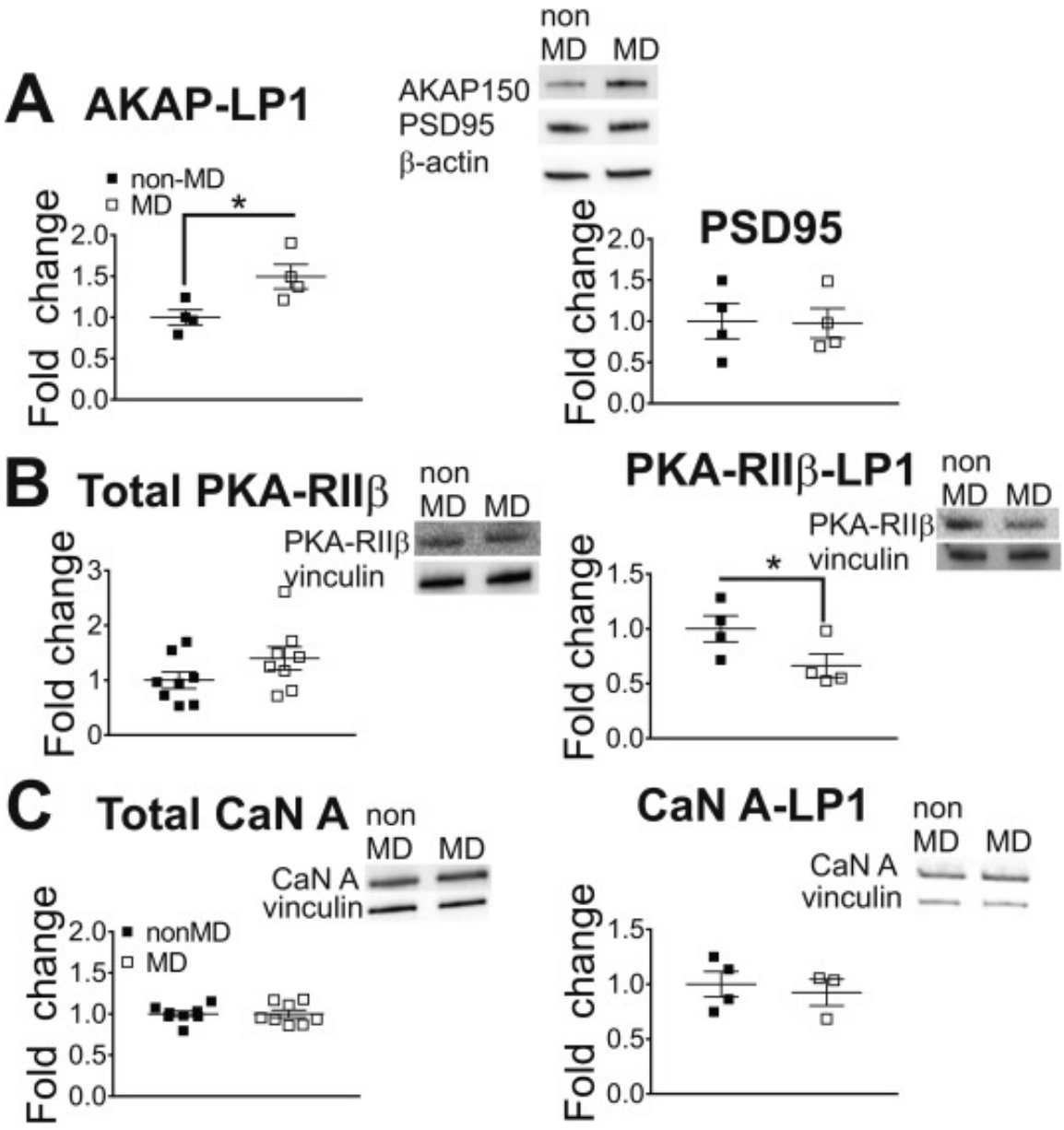

Figure 2

MD increased AKAP150 abundance and decreased PKA abundance in synaptic fractions of the VTA. (A) Representative Western blots and quantitative data of AKAP150, PSD95 (postsynaptic marker) and β actin (control) in synaptosomal membrane fractions (LP1) of VTA homogenates from non-MD and MD rats. (B) Representative Western blots and quantitative data of synaptic (LP1) and total levels of PKA-RIIβ in LP1 fractions or total homogenates of VTA from non-MD and MD rats. (C) Representative Western blots and quantitative data of synaptic (LP1) and total levels of CaN A subunit in LP1 fractions or total homogenates of VTA from non-MD and MD rats.

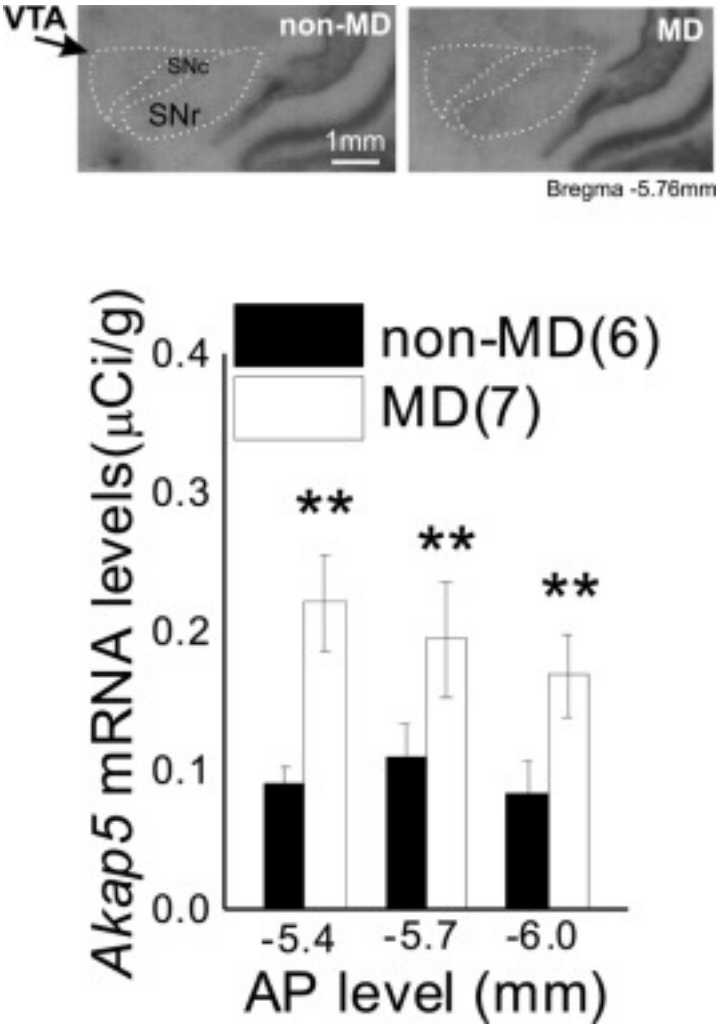

Figure 3

MD was associated with higher levels of Akap5 mRNA in the VTA. Relative expression of Akap5 mRNA with representative images in the VTA from non-MD and MD rats is shown.

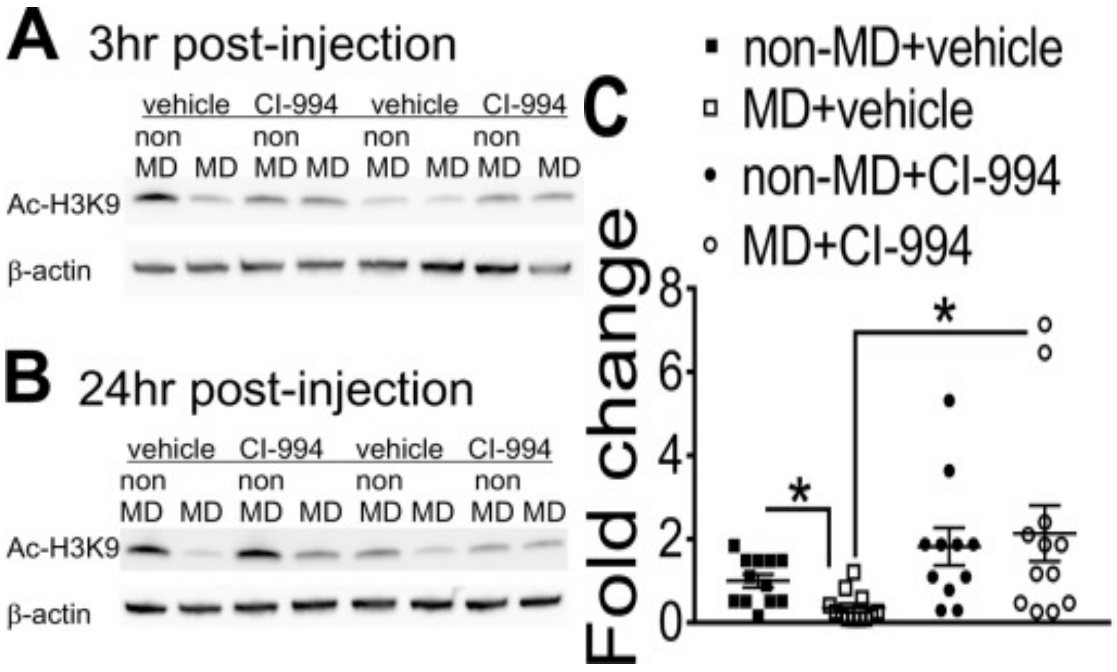

Figure 4

MD-induced histone hypoacetylation at H3K9 was reversible by HDAC inhibition for 24 h after the injection with CI-994. (A) Representative Western blots of Ac-H3K9 and β actin (control) in VTA homogenates 3 h after the injection of non-MD and MD rats with either vehicle or CI-994 (i.p. injection of 10 mg/kg). (B and C) Representative Western blots and quantitative data of Ac-H3K9 and β actin in VTA homogenates from non-MD and MD rats 24 h after the injection with either vehicle or CI-994.

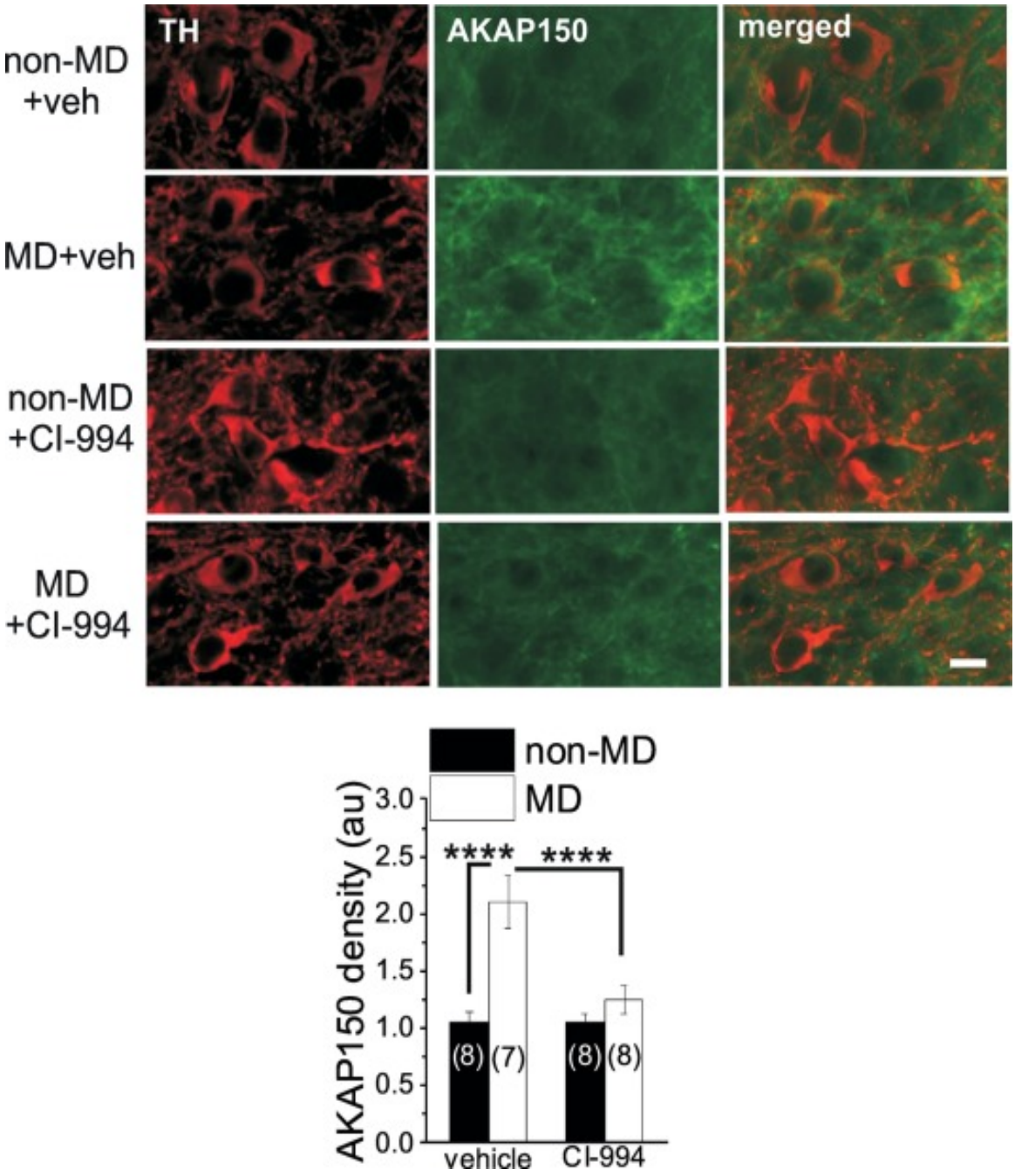

Figure 5

MD-induced upregulation of AKAP 150 was reversible by in vivo HDAC inhibition. Top: Examples of brain sections stained with antibodies to TH (red), and AKAP150 (green) with the merged panels, which show the expression of AKAP150 in TH+ neurons in the VTA of non-MD and MD rats injected with either vehicle or CI-994 (i.p. injection of 10 mg/kg) at 24 h post-injection. Scale bar, 20 μm. Bottom: Graph shows the averaged levels of AKAP150 expression (pooled and averaged data at three AP levels for each

group) from non-MD and MD rats in each group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

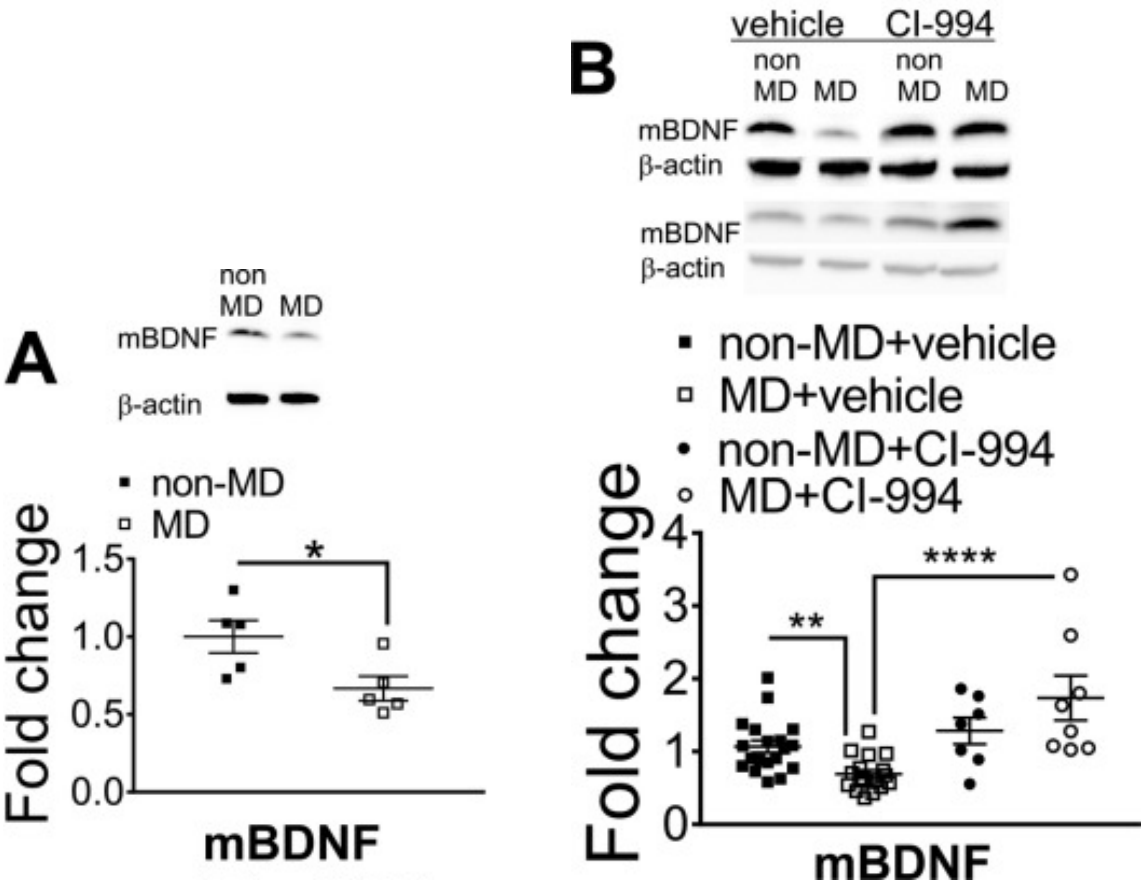

Figure 6

MD-induced decreases in BDNF were reversible by HDAC inhibition for 24 h after the injection with CI-994. (A) Representative Western blots and quantitative data of mBDNF and β actin (control) in VTA homogenates of non-MD and MD rats. (B) Representative Western blots and quantitative data of mBDNF and β actin in VTA homogenates from non-MD and MD rats 24 h after the injection with either vehicle or CI-994 (10 mg/kg, i.p.).

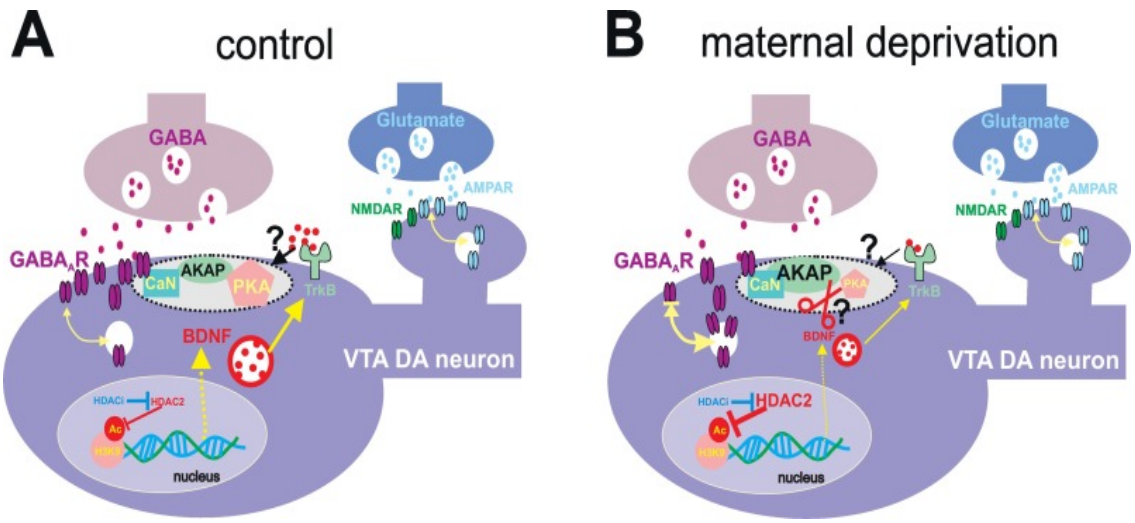

Figure 7

Proposed epigenetic mechanisms supporting MD-induced synaptic modifications in the VTA. MD induces GABAergic metaplasticity in VTA DA neurons through disruption of AKAP150 signaling that renders GABAergic synapses more susceptible to LTD. Our recent findings show that MD increases HDAC2 activity in VTA DA neurons and reduces Ac-H3K9 in the VTA. MD also increases synaptic levels of AKAP150 protein in the VTA with accompanying decreases in synaptic levels of PKA and the levels of mBDNF protein in the VTA. In vivo systemic injection with a selective class I HDAC inhibitor is sufficient to reverse MD-induced histone hypoacetylation and to normalize the levels of mBDNF and AKAP150 proteins in the VTA at 24 h after the injection. Our model suggests that HDAC2-mediated targeting of mBDNF could decrease mBDNF signaling through its receptor tropomyosin receptor kinase B (TrkB). We hypothesize that the reduced local BDNF signaling in turn decreases AKAP150-PKA association and AKAP-dependent PKA anchoring at GABAergic synapses while enhancing AKAP150-CaN association that favors the induction of LTD in VTA DA neurons. These epigenetic and synaptic modifications induced by MD could then affect DA neuronal excitability and DA release in VTA projection areas. → means excitation and ⊣ means inhibition.

Chapter 3: Ketamine Reverses Lateral Habenula Neuronal Dysfunction and Behavioral Immobility in the Forced Swim Test Following Maternal Deprivation in Late Adolescent Rats

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Author Contributions

FN and RS designed the experiments and wrote the manuscript. RS, LL and FN performed electrophysiology experiments. RS and CB performed behavioral experiments. FN, RS, AB, CB and IL analyzed the data and prepared the figures.

ABSTRACT

Mounting evidence suggests that the long-term effects of adverse early life stressors on vulnerability to drug addiction and mood disorders are related to dysfunction of brain monoaminergic signaling in reward circuits. Recently, there has been a growing interest in the lateral habenula (LHb) as LHb dysfunction is linked to the development of mental health disorders through monoaminergic dysregulation within brain reward/motivational circuits and may represent a critical target for novel anti-depressants, such as ketamine. Here, we show that maternal deprivation (MD), a severe early life stressor, increases LHb intrinsic excitability and LHb bursting activity, and is associated with the development of increased immobility in the forced swim test (FST) in late-adolescent male rats. A single *in vivo* injection of ketamine is sufficient to exert prolonged antidepressant effects through reversal of this early life stress-induced LHb neuronal dysfunction and the response in the FST. Our assessment of ketamine's long-lasting beneficial effects on reversal of MD-associated changes in LHb neuronal function and behavior highlights the critical role of the LHb in pathophysiology of depression associated with severe early life stress and in response to novel fast-acting antidepressants.

INTRODUCTION

Exposure to early-life stress is a strong predictor for later life mental disorders, including depression, suicide, post-traumatic stress disorder, schizophrenia and substance use disorder (125; 139). While the exact link between early-life stress and development of mental disorders is unknown, compelling evidence has accumulated implicating

dysregulation of monoaminergic signaling in brain reward/motivational circuits as a culprit (82; 152; 159; 163). Consistently, we found that a single 24 h episode of maternal deprivation (MD, a severe early life stressor as an established rodent model of child abuse and neglect) induces an epigenetic impairment of GABAergic synaptic plasticity within the ventral tegmental area (VTA), a midbrain area involved in reward-related processing, which could significantly contribute to dopamine (DA)-related reward dysregulation following this stress (5). Furthermore, our recent study suggests that MD-induced VTA DA neuronal dysfunction also involves a critical upstream brain area, the lateral habenula (LHb) (7). Because of its rich reciprocal connectivity with forebrain limbic and midbrain structures, the LHb serves as a converging hub for cognitive and emotional signals that are conveyed to midbrain monoaminergic systems and, thus, plays a fundamental role in value-based decision-making and goal-directed behaviors. Not surprisingly, LHb dysfunction contributes to a myriad of cognitive, learning, emotional and social impairments associated with depression, anxiety, psychosis and drug addiction (75; 144; 155).

Recently, ketamine, a non-competitive glutamatergic NMDA receptor (NMDAR) antagonist, has drawn interest due to its rapid and prolonged antidepressant actions after a single administration at low doses in patients suffering from treatment-resistant depression and in animal models of depression (69). Ketamine significantly reduces glucose utilization in the LHb of rats (57). Moreover, the antidepressant effects of ketamine in treatment-resistant major depression have been associated with decreased glucose utilization and metabolism in the habenula (28) suggesting that antidepressant effects of ketamine may be related to reduction of LHb neuronal activity. Consistent with

these findings, a recent study demonstrated that enhanced LHb neuronal bursting codes for behavioral depression and anhedonia in two rodent animal models of depression and that the rapid antidepressant effects of ketamine is mediated through local NMDAR-dependent blockade of this enhanced LHb neuronal bursting (219). Early MD also shifts the balance between synaptic excitation and inhibition towards excitation by increasing LHb intrinsic excitability while impairing responsiveness of LHb neurons to the stress hormone, corticotropin releasing factor (CRF) in juvenile rats (7). Similarly, chronic maternal separation triggers a depressive-like phenotype in adult mice that is associated with LHb hyperexciatbility (193). Here, we extend our observations from juveniles to late adolescent rats where we found that MD-induced LHb hyperexcitability persists into late adolescence and is accompanied by an increase in LHb bursting activity and depressive - like behavior in the FST. Although many studies evaluating antidepressant-like effects of ketamine in animal models use depression models or acute stress paradigms in adults, here we used MD stress to explore the possibility that a single intraperitoneal (i.p.) injection of ketamine is sufficient to exert prolonged antidepressant effects through reversal of LHb neuronal dysfunction and behavioral despair induced by early life stress.

MATERIAL 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. Data analysis was conducted in a blinded manner to ensure reproducibility.

Maternal Deprivation and slice preparation

Maternal deprivation was carried out as previously described (5). Half of the male pups in litters of Sprague–Dawley rats (Taconic Farms) at P9 were isolated at 10:00 a.m. from the dam and their siblings for 24 h (MD group). The isolated rats were placed in a separate quiet room and kept on a heating pad (34° C) and not disturbed until being returned to their home cage 24 h later. The remaining non-separated male rat pups received the same amount of handling but were kept with the dam serving as the non-maternally deprived control group (non-MD group). Rats were maintained on a 12 hour light/dark cycle and provided food and water ad libitum. The animals were taken for study during the light period, between 3 and 5 h after the lights were turned on. Rats were weaned from the mother and housed in pairs in separate cages at P28. Separate cohorts of MD and non-MD (control) rats (age-matched) were used for behavioral experiments over P21-P28 (early-mid adolescent rats) or P42-P50 (late adolescent rats)(132; 187). Separate cohorts of late adolescent rats (P42-P50) were used for electrophysiological recordings in Figure 2C and 2D where one to three neurons were recorded per animal (n represents number of recorded cells/number of rats). On average four rat pups per litter contributed to the study (approximately a total of 42 litters). 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 $NaHCO_3$, 2.5 KCl, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 1.00 MgSO4, 11.1 glucose, 0.4 ascorbic acid, saturated with 95% O_2–5% CO_2. Sagittal slices containing the LHb were cut at 250 μm 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 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 LHb activity, firing patterns and resting membrane potentials (RMP) were assessed using current-clamp recordings of action potentials (APs) for 3 minutes with no injected current immediately after whole cell configuration where neurons remained at their own RMPs. LHb neurons were classified as silent, tonic or bursting based on spontaneous patterns of firing. Neuronal excitability recordings in response to injection of depolarizing currents were conducted as previously described (7). LHb neurons were given increasingly depolarizing current steps at +10pA intervals ranging from +10pA to +100pA, 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 μM), picrotoxin (100μM) and APV (50 μM) to block AMPA, $GABA_A$ and NMDA receptor-mediated synaptic transmission, respectively. Synaptic blockers were present from the start of each recording. The number of APs induced by depolarization at each intensity was quantified and averaged for each experimental group. Measurements of AP threshold, medium afterhyperpolarization (mAHP) and fast afterhyperpolarization

(fAHP) amplitudes, and input resistance (Rin) were conducted as described previously (7). In brief, 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. Rin was determined by injecting a small (50 pA) hyperpolarizing current pulse (5s) and calculated by dividing the steady-state voltage response by the current pulse amplitude. Measurements of AP characteristics were obtained using Clampfit.

Animal Behavior

The modified rat FST was conducted as previously described (52). As a standard method, rats underwent two swim sessions in a glass cylinder (20 cm diameter x 30 cm depth) filled with water (25°C ± 1°C) as it has been shown that juvenile Sprague Dawley rats usually adopt immobility as a passive coping strategy from P20 onwards and need two swim sessions to develop this coping strategy in the FST (129). In the first session (Pre-test), animals were allowed to swim for a total of 15 min. Rats were then dried and returned to the home cage. Rats underwent the second swim session 24 h later, but for only 5 min (Test). With respect to experiments involving ketamine, animals were injected i.p. with either ketamine (20mg/kg) or equivalent amount of vehicle (saline) 1 h after the first session and the second session was conducted 24 h later. We chose this dose because ketamine reliably reduced immobility in the FST at this dose in late adolescent/young adult rats (150). In both the pre-test and test, a time-sampling method was used to quantify three behavioral outputs: climbing, swimming, and immobility. Definitions of behavioral outputs were scored as previously described (26). For electrophysiological

experiments with ketamine, we sacrificed the late-adolescent animals 72 h after a single ketamine administration to investigate the prolonged effects of ketamine on LHb neuronal excitability and firing pattern.

Statistical Analysis

Values are presented as means ± S.E.M. Statistical significance was determined using unpaired Student's t-test or two-way ANOVA with Bonferroni post hoc analysis with a significance level of $p<0.05$. Statistical analysis of electrophysiological recordings was based on the number of recorded cells. In behavioral experiments, pre-planned Bonferroni multiple comparisons were conducted. All statistical analyses were performed using Origin 2016 or GraphPad Prism 7.

RESULTS

Maternal deprivation-induced behavioral immobility was reversible by ketamine

To test whether MD is associated with a depressive-like behavioral phenotype in late adolescence, we subjected non-MD and MD rats (P42-P50) to the FST. MD significantly increased immobility time while decreased time spent swimming or climbing in the FST, suggesting that MD reliably induces a depressive-like phenotype in late adolescence (Figure 8A, lower panel)..

Interestingly, juvenile MD pups (P21-P28) exhibited increased climbing behavior with a significant decrease in immobility in the FST, compared to age-matched non-MD animals (Figure 8B, lower panel). To examine the protracted antidepressant effects of ketamine, we subjected rats (P42-P50) to the FST 24 h after a single *in vivo*

administration of ketamine (20 mg/kg) or saline. Ketamine reduced the MD-induced increase in immobility and increased time spent swimming in late adolescent rats (Figure 8A, lower panel). Strikingly, ketamine was also able to normalize the MD-induced differences in behavior in the FST in juvenile rats (P21-P28). Ketamine increased immobility time while decreasing the exaggerated climbing behavior in MD rats compared to controls (Figure 8B, lower panel). Irrespective of the FST on the test day, both juvenile and late-adolescent non-MD and MD rats quickly adopted the passive coping behavior (immobility) in the first swim session (pre-test) (Figure 8 A- B, upper panels).

MD-induced changes in LHb spontaneous activity and intrinsic excitability were normalized by ketamine

LHb hyperactivity seems to be the common finding in rodent models of depression and in humans with depression (111; 114; 135; 141; 176). Recently we demonstrated that in early-mid adolescent rats (P21-P28), MD dysregulates CRF signaling and leads to a downregulation of SK2 (a Ca^{2+} activated potassium channel) which contributed to LHb hyperexcitability (7). We tested whether MD-induced increases in LHb neuronal excitability persisted into late adolescence (P42-P50). Furthermore, we investigated whether the observed persistent antidepressant behavioral effects of ketamine (Figure 9A, lower panel, 24 h after ketamine administration) were associated with reversal of MD-induced changes in LHb neuronal excitability and firing patterns in late adolescent rats 72 h after ketamine administration. We found that the percentage of neurons which were spontaneously active (tonic or bursting) in current clamp recordings with intact synaptic transmission was larger in late adolescent MD rats

compared to control non-MD rats. In addition, ketamine was able to normalize this effect of MD on LHb neuronal activity (Figure 9A).

In addition to current-clamp recordings of basal neuronal firing, we also tested whether LHb neurons from late adolescent MD rats exhibited enhanced firing in response to depolarization compared to control non-MD rats in the absence or presence of fast synaptic transmission. We did not detect a significant difference in LHb neuronal excitability between non-MD and MD rats in intact synaptic transmission in response to 10-100pA depolarizing current steps (Figure 9C). MD significantly increased LHb intrinsic excitability in response to depolarization when fast synaptic transmission was blocked and ketamine robustly blocked this MD-induced increase in LHb intrinsic excitability 72 h post-injection (Figure 9B and D). Furthermore, the MD-induced increase in intrinsic excitability was associated with a significant increase in input resistance (Rin, without any change in the amplitude of fAHP, mAHP or threshold) that was reversible by ketamine. This suggests possible decreases in potassium conductances underlying membrane resistance in LHb neurons that can also be targeted with ketamine (Figure 9E).

DISCUSSION

We have provided evidence for long-lasting antidepressant effects of a single *in vivo* administration of ketamine in reversal of LHb neuronal dysfunction (72 h post-injection) and depressive-like behavioral abnormalities (24 h post-injection) associated with a severe early life stress (MD). MD rodents and adolescent human subjects reporting low parental care, show heightened impulsivity and exhibit anxiety-and depression-like behaviors associated with altered monoaminergic neurotransmission (83). Consistently,

we also observed that MD induces a pro-depressive behavioral phenotype in the FST in late adolescent rats. Interestingly, we found that juvenile rats showed an increased active coping behavior in the FST (with an increase in climbing behavior) which indicates a developmental shift in the behavior of MD animals in the FST. Ketamine was able to reverse abnormal behavioral phenotypes in mid and late adolescent rats. More importantly, we were able to demonstrate the ability of ketamine in persistently reversing MD-induced changes in LHb neuronal excitability and firing patterns in late-adolescent rats up to 72 h post-injection. MD was accompanied by an increase in LHb intrinsic excitability and enhanced bursting mode of LHb neuronal firing. These aberrant LHb neuronal activity were ameliorated with a single i.p. administration of ketamine 72 h post-injection. Consistent with our findings, a recent study demonstrated that ketamine blocks LHb neuronal bursting that underlies behavioral depression and anhedonia in two rodent animal models of depression through local NMDAR-dependent blockade in the LHb (219). However, the effects of ketamine in this study were evaluated only 4 h after injection. It is yet to be determined how long ketamine-induced suppression of LHb bursting activity persists in these depression models. Furthermore, it is unclear whether NMDAR antagonism underlies ketamine's prolonged effects. In fact, non-NMDAR-mediated glutamatergic potentiation and sustained activation of AMPARs seem to be central to the protracted antidepressant effect of ketamine and its main metabolite, hydroxynorketamine, HNK (25; 137; 225). It is also important to further investigate whether NMDAR- and non-NMDAR-dependent mechanisms of ketamine are involved in MD-induced LHb neuronal dysfunction and behavioral depression. Since LHb bursting involves both NMDARs and low-voltage-sensitive T-type calcium channels (T-VSCCs)

(219), possible developmental delays in NMDAR subunits/function (161; 162) and/or T-VSCCs expression and function following MD could occur and contribute to the increase in LHb bursting activity following MD. However, given that ketamine only blocked MD effects on LHb neuronal excitability and firing patterns without affecting those of non-MD rats suggests that there might be an enhanced contribution of NMDAR after MD. Since MD-induced increase in LHb intrinsic excitability was accompanied by higher input resistance, this also suggests possible decreases in potassium conductances underlying membrane resistance including leaky potassium currents that can also be targeted by ketamine. Given that brain-derived neurotrophic factor (BDNF) signaling is a key mediator of ketamine's antidepressant properties (25; 224) and that BDNF expression is altered following maternal separation or deprivation (161; 183)\, possible BDNF-mediated alterations in potassium channel expression (106) could also be triggered by ketamine to normalize LHb neuronal excitability. In sum, our assessment of ketamine's long-lasting behavioral and neurophysiological effects highlights the critical sensitivity of the LHb in MD-induced depression and subsequent responses to novel fast-acting antidepressants. This model should help to provide a framework for studying the currently limited treatment options available for adolescents suffering from major depressive disorder and higher risk of comorbidity later in life.

ABBREVIATIONS

Lateral habenula (LHb), maternal deprivation (MD), ventral tegmental area (VTA), dopamine (DA), forced swim test (FST), input resistance (Rin), fast afterhyperpolarization (fAHP), medium afterhyperpolarization (mAHP), low-voltage-sensitive T-type calcium channels (T-VSCCs), brain-derived neurotrophic factor (BDNF)

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AUTHOR CONTRIBUTION STATEMENTS

FN and RS designed the experiments. RS, LL, and FN performed electrophysiology experiments. RS and CB performed behavioral experiments. FN, RS, AB, CB, IL analyzed the data and prepared the figures. FN and RS wrote the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENTS

All relevant data is contained within the manuscript: All datasets [GENERATED/ANALYZED] for this study are included in the manuscript and the supplementary files.

FIGURES

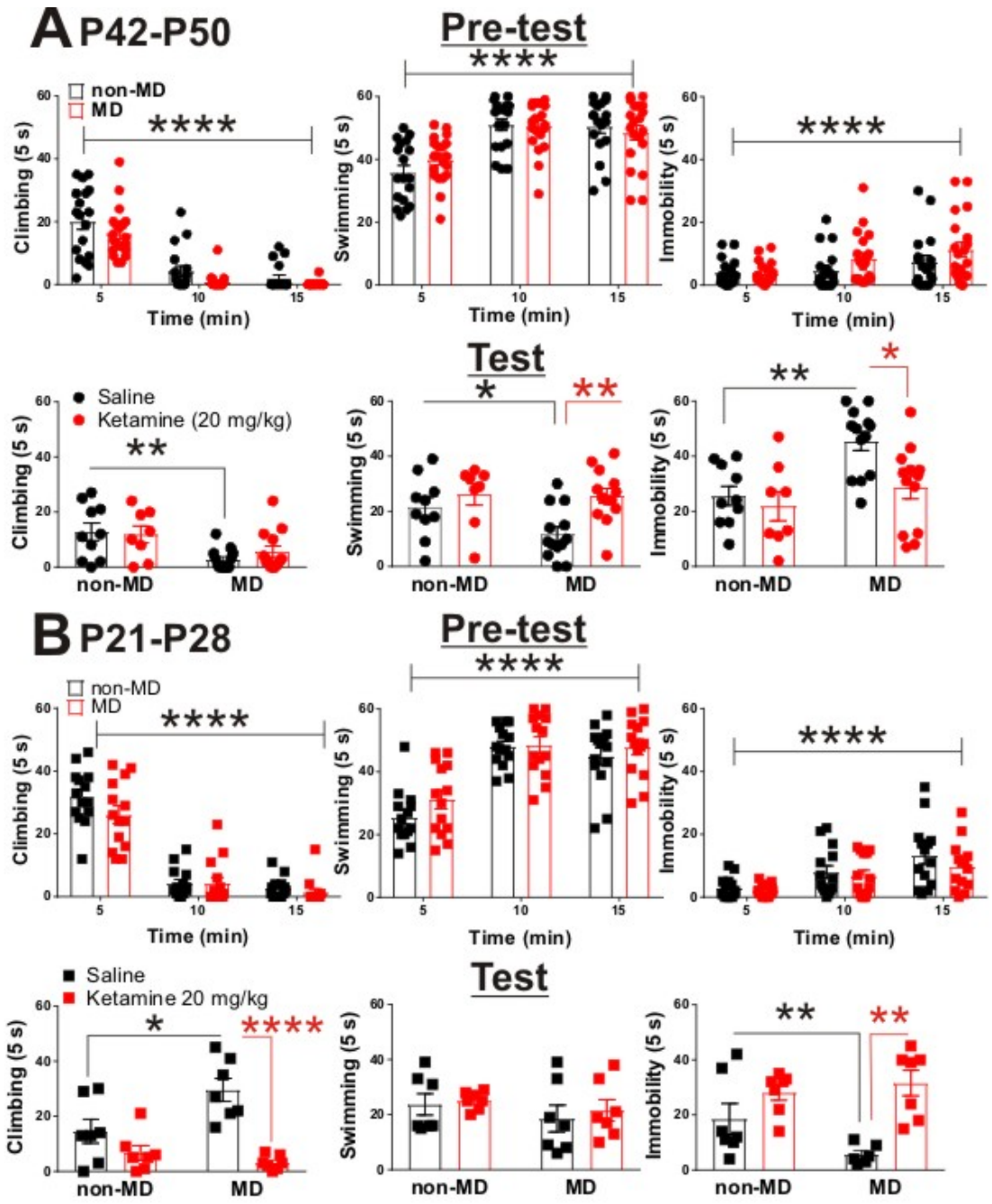
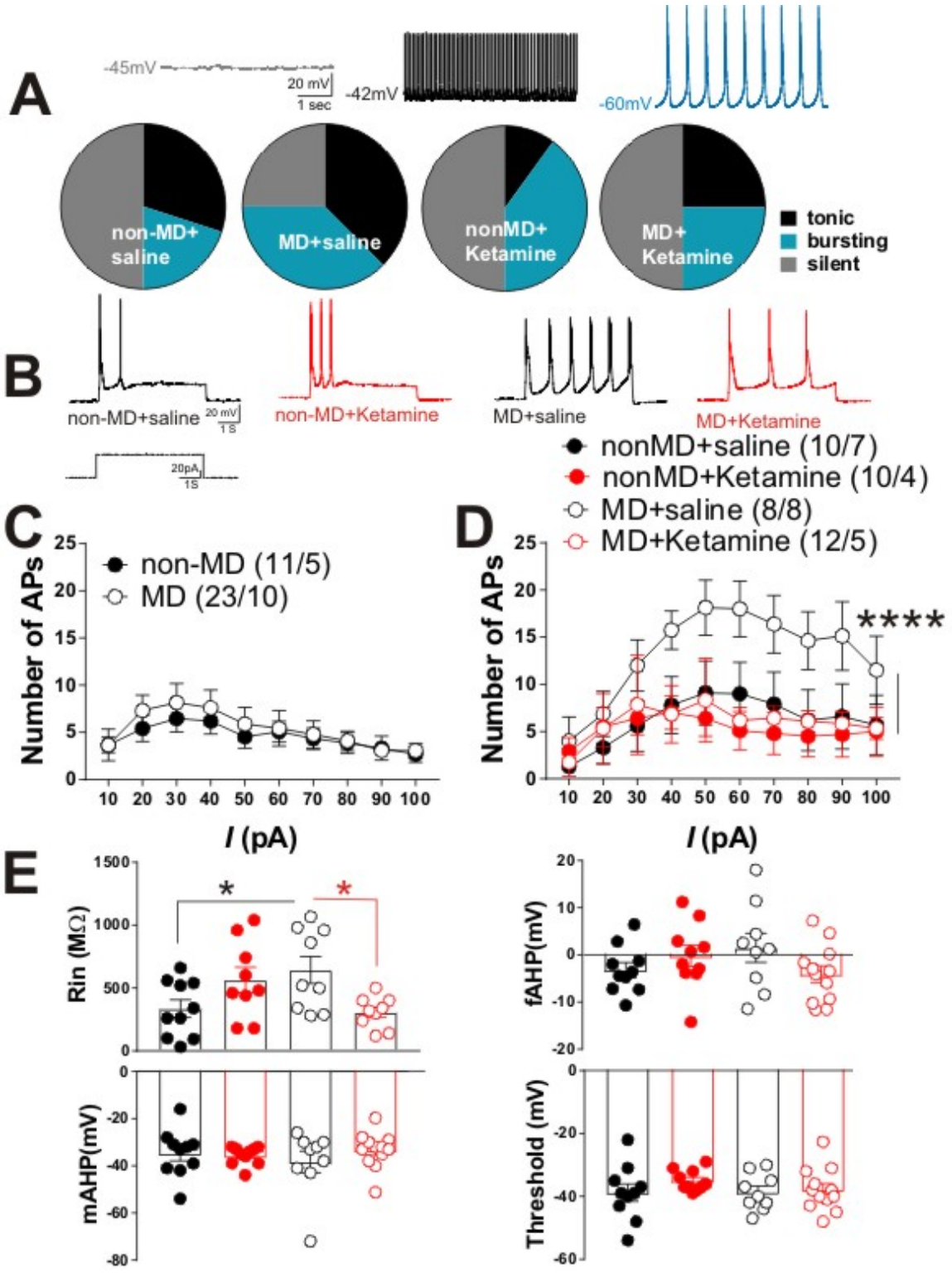


Figure 8

Ketamine normalized maternal deprivation (MD)-induced behavioral changes in forced swim test (FST) in adolescent rats. (A) Upper panel demonstrates the time course of behavioral transition from active (swimming and climbing) to passive (immobility) coping behaviors. Both non-MD and MD late-adolescent rats quickly learned to adopt immobility during the first swim session (Pre-test, non-MD: n = 18, MD: n = 26; climbing time course: $F(2,72) = 107.3$, ****$P < 0.0001$; swimming time course:

F(2,72) = 39.72, ****P < 0.0001; immobility time course: F(2,72) = 11.4, ****P < 0.0001; two-way ANOVA). Lower panel shows that MD induced a significant decrease in climbing behavior and a significant increase in immobility in FST (Test, non-MD + saline: n = 10, non-MD + Ketamine: n = 8, MD + saline: n = 13, MD + Ketamine: n = 13; swimming: *P < 0.05; climbing: **P < 0.01; immobility: **P < 0.01). Administration of ketamine (20 mg/kg) significantly decreased MD-induced immobility 24 h post-injection (swimming: F(1,40) = 8.71, **P < 0.01; immobility: F(1,40) = 6.27, *P < 0.01; two-way ANOVA). (B) Upper panel demonstrates the adoption of immobility in juvenile rats during Pre-test. Similar to late-adolescent rats, non-MD and MD juvenile rats quickly learned to adopt immobility during the first swim session (Pre-test, n = 14 in each group; climbing time course: F(2,52) = 175.4, ****P < 0.0001; swimming time course: F(2,52) = 82.48, ****P < 0.0001; immobility time course: F(2,72) = 23.95, ****P < 0.0001; two-way ANOVA). Lower panel shows that MD induced a significant increase in climbing behavior and a significant decrease in immobility in the FST (Test, n = 7 in each group, climbing: *P < 0.05; immobility: *P < 0.05) in juvenile rats (P21–P28). Administration of ketamine (20 mg/kg) attenuated MD-induced changes in climbing and immobility 24 h post-injection (Test, climbing: F(1,24) = 26.69, ****P < 0.0001; immobility: F(1,24) = 18.76, **P < 0.01; two-way ANOVA). All data is represented as means ± SEM.


A
-45mV
20 mV
1 sec
-42mV
-60mV
non-MD+ saline
MD+saline
nonMD+ Ketamine
MD+ Ketamine
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20 mV
1S
non-MD+Ketamine
MD+saline
MD+Ketamine
20pA
1S
C
Number of APs
non-MD (11/5)
MD (23/10)
I (pA)
D
nonMD+saline (10/7)
nonMD+Ketamine (10/4)
MD+saline (8/8)
MD+Ketamine (12/5)
****
E
Rin (MΩ)
mAHP(mV)
fAHP(mV)
Threshold (mV)

Figure 9

Ketamine normalized MD-induced changes in lateral habenula (LHb) intrinsic excitability and firing patterns in late-adolescent rats. (A) Distribution of LHb neurons based on firing patterns in whole cell current-clamp recordings of spontaneous activity. The insets show example traces from LHb neurons with silent, tonic and bursting patterns recorded from non-MD or MD rats that received either saline or ketamine injections (recorded 72 h post-injection). The number of cells/rats are similar to the ones reported in (D). MD increased spontaneous LHb neuronal activity (specifically bursting) and ketamine reversed these effects of MD. (B) Representative traces from LHb neurons in non-MD and MD rats that received either saline or ketamine (20 mg/kg) injections and sacrificed after 72 h. (C) Figure shows the average of whole cell action potential (AP) recordings in response to depolarizing current injections (I) with intact synaptic transmission in LHb slices from P42 to P50 non-MD and MD rats. MD did not alter LHb neuronal excitability with intact synaptic transmission. (D) Whole cell patch clamp recordings of action potentials (APs) in response to depolarizing current injections (I) with blocked synaptic transmission from LHb neurons in non-MD and MD rats that received either saline or ketamine (20 mg/kg) injections and sacrificed after 72 h. MD increased LHb intrinsic excitability and ketamine reversed this effect ($F(3,360) = 15.84$, ****$P < 0.0001$; two-way ANOVA). (E) Measurements of intrinsic properties of LHb neurons including input resistance (Rin), fast afterhyperpolarization (fAHP), medium afterhyperpolarization (mAHP) and AP threshold. Average amplitude of fAHP, mAHP and Rin were generated from recordings in (D). MD significantly increased Rin and ketamine reversed this effect of MD ($F(1,33) = 3.936$, *$P < 0.05$; two-way ANOVA). Numbers indicated in (C,D) represent the number of cells recorded per rats in each group.

Chapter 4: Discussion

There is an urgent medical need to creating better pharmacological agents for the treatment of depression. Ultimately, the development of novel therapeutics is going to rely on continuing research into understanding the pathophysiology of depression and other related psychiatric disorders. In neuroscience, we are now entering a period where we can investigate genomic markers with greater ease, as well as understand the neuroconnectome to better assess specific circuits involved in behavior and, more specifically, interrogate how these circuits become dysregulated in mental illnesses.

Studies regarding the role of ELS in childhood have shown that there are critical periods in which a child's development can be perturbed with consequences that reappear throughout life, such as in the case of child neglect and/or abuse (33; 76; 79). In my dissertation, I have outlined data and studies in the MD model and characterized how both epigenetic and neurophysiological changes in the VTA and the LHb could ultimately contribute to the long-term stress-induced behavioral abnormalities. My data suggest that there is synergy between both the monoaminergic and glutamatergic hypothesis of depression. Just as most research focuses on one or two genes implicated in an illness, we know that diseases are usually polygenic in which a vast array of genes could be altered resulting in a threshold where pathology occurs. The same concept can be applied to the neurophysiology underlying psychiatric illnesses. Rather than focusing on solely one neurotransmission system or one brain region, it is important to understand how the "re-wiring" process occurs as the result of investigating the relationship between interconnected brain structures and the signaling processes involved.

The focus of this dissertation centers around two interconnected brain structures: the VTA and the LHb. In both the monoaminergic and glutamatergic hypotheses of depression, dysregulation of monoaminergic and glutamatergic transmission, respectively, is implicated (84; 170). The VTA, being the source of DA in the brain reward pathway, is one of the key structures in which extensive studies have been conducted that show disturbances in VTA physiology can occur as a result of stress, drugs of abuse, etc. (110). Thus, common mechanisms can be occurring within the VTA that underpin not just depression, but other mental illnesses. However, it is important to note that the VTA is a heterogeneous structure in which the rate of DA neuronal firing is partly controlled by the GABAergic inhibition (98; 126; 142). Within this context, the activation of GABAergic neurons in the context of DA release is important when discussing dopaminergic dysregulation. The robust role of the LHb as a negative regulator of reward brings into context the role of LHb projections to these GABAergic neurons within and around the VTA; stimulation of GABAergic neurons by these excitatory inputs from the LHb can restrain DA release (92; 220). In conclusion, the question becomes the age-old question of which came first "the chicken or the egg" or specifically – does dysregulation occur first upstream within the LHb or does it begin in VTA DA neurons?

In this discussion, I will further describe our findings on the effects of ELS on histone acetylation, as well as describe the implications of HDACi as a treatment strategy. Secondly, I will describe how ELS impacts neuronal excitability, how this applies to the MD-induced behavioral abnormalities observed in the studies, and whether ketamine can be effective as an anti-depressant.

MD Effects on Histone Acetylation

The rate at which genes are transcribed are important based on when a certain protein is needed. As such, this rate is "plastic" in that during certain periods of development or in response to specific situations, this rate-limiting step to gene expression can be altered. One such mechanism involves the role of histone acetylation in which HATs and HDACs work in a negatively reciprocal matter to either express or repress, respectively, genes through addition or removal of acetyl groups at specific residues (179; 190).

The role of histone acetylation is the most understood and well characterized. Previous work has highlighted that in response to stress or drugs of abuse, there are epigenetic changes that occur (96; 108; 149). The role of chromatin remodeling, and more precisely the removal of acetyl groups by HDACs, has been shown pharmacologically through the use of HDACi to inhibit the re-instatement of fear memory involving HDAC2 (73). Previous work from the Nugent lab has added to this understanding of HDAC involvement in gene regulation within the VTA. Firstly, acute exposure of morphine, a drug of abuse, is sufficient to induce histone hypoacetylation at H3K9 with a subsequent increase in HDAC2 (6). The use of a class I HDACi, CI-994, was able to recover MD-induced changes in acetylation of H3K9. Lastly, we showed previously that MD induces changes in GABAergic metaplasticity in which GABAergic synapses favored LTD and exhibited impaired LTP (5). In both cases of acute morphine exposure and MD, the use of CI-994 was sufficient to amend the electrophysiological abnormalities.

In addition to this study of HDAC inhibition and GABAergic metaplasticity, this previous work by the Nugent lab also identified that an AKAP150-dependent mechanism is required for GABAergic plasticity and is disrupted by MD. One of the important roles for AKAP150 as a scaffolding protein is its regulation of synaptic organization in the context of synaptic plasticity (47; 120; 171). While this study set the precedent for the role of HDACi in synaptic plasticity within the VTA, the molecular mechanisms were still unknown. In addition, this previous study used an acute *in vitro* approach, warranting the question of whether HDACi can work *in vivo*. My study of the effects of MD on the VTA provide a molecular mechanism that could underpin the changes in VTA neurophysiology due to HDACi.

First, we were able to show that the use of CI-994 was able to affect MD-induced changes in GABAergic transmission in acute *in vitro* incubation of slices (5). However, it was unknown if this was due to changes in histone acetylation as observed in our previous morphine study. Through the use of western blot, I was able to determine that, just like acute morphine exposure, MD is sufficient to induce histone hypoacetylation within the VTA at H3K9 (Figure 1B). However, it is known that there are multiple histone tail residues that can be influenced by HDACs. In this regard, I also investigated another histone mark, H3K14. Interestingly, I was unable to detect any changes in this epigenetic mark within the VTA implicating that, at least for H3K9, this is a specific molecular substrate that is modified due to both MD and acute morphine exposure.

With the established MD-induced hypoacetylation within the VTA, I next sought to find which HDAC was engaged in this mechanism. Clues from our previous work were able to shed light on the situation in that 1) acute morphine exposure showed an

increase in HDAC2 expression in TH+ neurons (an established marker for VTA DA neurons) and 2) CI-994, a class I HDACi, was sufficient to amend MD-induced electrophysiological abnormalities (5; 6). Since CI-994 only works on class I HDACs, this provided pharmacological evidence that there were only a few possible HDACs that could be targeted: HDAC1, 2, 3, and 8 (179; 230). Acute morphine exposure was sufficient to increase nuclear expression of HDAC2 in TH+ VTA neurons; therefore, I investigated whether HDAC2 was also upregulated in response to MD. Indeed, MD induces a robust increase in nuclear HDAC2 in TH+ VTA neurons (Figure 1A).

Our previous work showed an effect of HDACi by CI-994 *in vitro*. While this approach provided the findings that there is indeed an epigenetic mechanism that is engaged following MD, the use of *in vitro* incubation lacks a translational approach to novel anti-depressant discovery. Systemic administration of CI-994 has been shown to sufficiently become bioavailable within the brain (73). Therefore, I used an *in vivo* administration (i.p.) to investigate if the same effect of HDAC inhibition could be achieved *in vivo*. Administration of the CI-994 (10mg/kg) was sufficient to recover MD-induced hypoacetylation at H3K9 as quickly as 3hrs and as long-lasting as 24hrs (Figure 4).

Ultimately, this establishes that MD engages epigenetic mechanisms involving an upregulation of HDAC2 that is associated with an observable decrease in H3K9 acetylation. The use of CI-994 was able to ameliorate the MD-induced hypoacetylation *in vivo*. While this association does suggest that HDAC2 is involved, this does not discount other classes of HDACs, such as the class II HDACs that can shuttle between the nucleus and the cytoplasm (179; 191). To determine this, future experiments will involve

immunoprecipitation of ac-H3K9 to determine if there is protein association of HDAC2 that is enriched in MD. Another question that warrants further investigation is the effect of MD on HDACs and histone acetylation in non-TH+ neurons (i.e. either VTA GABAergic or glutamatergic neurons).

ASSOCIATION OF MD-INDUCED HYPOACETYLATION ON VTA PROTEIN EXPRESSION

HDACs generally lead to a transcriptionally repressive effect by removing acetyl groups from histones resulting in chromatin condensation onto the histone (179). HDACi, such as CI-994, had been deemed cognitive enhancers due to the implication that they might promote transcriptional activation of genes important in learning and memory (73). With regards to cellular mechanisms regulating learning and memory, AKAP150 has been shown to be involved due to its role as a scaffolding protein to orchestrate either insertion or removal of receptors from the synapse (47; 165). While the lab originally showed there was no change in AKAP150 expression in whole protein, the question still remained how was MD disrupting AKAP150 signaling?

One initial hypothesis was that the maintenance of $GABA_AR$ in the synapse by PKA-AKAP-CaN association was being disrupted, possibly by biased signaling involving CaN-mediated endocytosis of $GABA_AR$ (5; 47). One consideration was the localization of AKAP150 in that it must be anchored to the synapse for a role as a synaptic re-organizer (101; 157; 171). Therefore, the initial hypothesis was that AKAP150 was being epigenetically regulated and because histone hypoacetylation by HDAC2 leads to transcriptional repression, possibly AKAP150 downregulation leads to overall loss of PKA-anchoring at the synapse and thus, would explain a CaN-mediated preference for impaired GABAergic plasticity.

In order to show an epigenetic mechanism, we employed the use of *in situ* mRNA hybridization to investigate if AKAP150 was being transcriptionally repressed. Interestingly, we observed an opposite effect in that MD induced increased levels of *Akap5* mRNA within the VTA (Figure 3). In congruence with the upregulation of *Akap5*, there was also a significant increase in AKAP150 in the synapse (Figure 2A). In regard to this finding, there was a gap of knowledge in how HDAC inhibition could impact AKAP150 signaling when MD leads to elevated AKAP150 levels. The role of AKAP150 as a "molecular switch" for plasticity could mean that the hypothesis of biased CaN signaling could be correct. One possible piece of evidence that would support this is if there was a lack of PKA association to AKAP150. I observed that in the synapse there was no change in CaN with relation to total CaN expression levels, but there was a decrease in abundance of PKA in the synapse with relation to total PKA (Figure 2B, C). This important finding does support our preliminary work regarding how AKAP150 dysregulation plays a role in GABAergic metaplasticity. In addition, it has now been shown that upregulation of AKAP150 can occur in the basolateral amygdala and this initiates changes in AKAP150 signaling through PKA (77). Future experiments will examine how PKA-AKAP150 association changes in MD through immunoprecipitation of AKAP150 to determine relative levels of both PKA and CaN association to this scaffold.

Given that HDAC inhibition could increase histone acetylation within the VTA, it was important to investigate whether HDACi affected AKAP150 expression. Using TH+ neurons to co-localize changes in AKAP150 expression, MD not only induced increased expression of AKAP150 within these neurons, but interestingly CI-994 decreased

AKAP150 expression in TH+ neurons of MD animals up to 24hrs later (Figure 5). This was an extremely interesting finding because of the canonical role of HDAC inhibitors in increasing gene transcription. This data indicates that CI-994 impacts a protein possibly related to AKAP150 trafficking and thus, this upstream effector is the specific target of HDAC inhibition. One of these possible proteins is BDNF.

BDNF is a master regulator in the CNS with a wide variety of effects. Two forms of BDNF exert cellular signal transduction events: pro-BDNF and mBDNF. mBDNF promotes synaptic plasticity, neuronal maturation, and control over excitability (119). In addition, levels of BDNF have been shown previously to be affected in different stress and drug exposure models (17; 203). Due to the important association of mBDNF with psychiatric illnesses, we investigated whether mBDNF is impaired in MD within the VTA. Indeed, we did find that mBDNF expression is decreased in response to MD and is recoverable following administration of CI-994 24hrs later (Figure 6). Due to the effect of HDACi on mBDNF expression, this suggests that the expression of mBDNF is regulated by the acetylation of H3K9. However, many questions remain such as how BDNF signaling is involved in AKAP150 maintenance at the synapse and whether this phenomenon applies to both TH+ and non-TH+ neurons in the VTA. Future studies will investigate this mechanism as well as employ unbiased ChIP-seq to confirm our hypothesis about H3K9 acetylation being tied to BDNF expression, as well as establishing other candidate genes of interest.

USING HDAC INHIBITORS TO COMBAT DEPRESSION: POSSIBLE MECHANISMS AND INSIGHT

Here, I have provided evidence for an HDAC-mediated effect induced by MD within the VTA that contributes to our understanding of how ELS can possibly

dysregulate dopaminergic signaling. The evidence provided here highlights two possible targets of HDACi: one direct (BDNF) and one indirect (AKAP150) (Figure 7). The use of the HDACi *in vivo* is sufficient to ameliorate MD-induced abnormalities in acetylation and protein expression up to 24hrs post-administration.

An important component of novel anti-depressant discovery and development is translational research through animal models. While there has been severe criticism drawn towards animal models within recent years, they still represent an important tool in understanding molecular and physiological mechanisms of disease that can be investigated in terms of pharmaceutical development. Already HDACi are approved for use within patients, specifically as anti-cancer drugs (116; 211). However, the role of HDAC inhibition in the context of psychiatric treatment is still unknown.

A major consideration is the route of administration and off-target effects. HDAC inhibitors, such as CI-994, have been shown in rodent models to readily penetrate the blood brain barrier and become bioavailable in the brain (73). However, there are a multitude of off-target effects in different systems of the body as well as different circuits within the brain. As a result, more directed therapies of HDAC inhibition would be required in order to minimize impact of drug exposure to periphery systems outside the CNS.

Another important component to consider is the role of discrete brain regions and whether epigenetic changes in response to events such as ELS are ubiquitous throughout the brain, expressed in specific neural pathways, or are only found in specific brain regions. The relationship between the VTA and LHb as “reward” and “anti-reward” structures, respectively is important to understand within the context of depression and

other illnesses. Interestingly, I extended my studies of histone modifications to the LHb to see if there are unified changes in histone acetylation. My data show that within the LHb, there is decreased acetylation of H3K9, just as was observed in the VTA (data not shown). In addition, another epigenetic mark H3K14 also showed a robust decrease (data not shown). This implies that ELS, such as MD, might engage the same epigenetic mechanisms in both brain regions. It is important to point out that this observation of decreased acetylation at both structures was observed at two different time points (VTA: P14-P21, LHb: P21-P28) and therefore it will be important to assess if histone hypoacetylation persists in the VTA or, *vice versa*, can be observed at an earlier time point in the LHb.

In the VTA, I investigated the intersect between HDAC inhibition and AKAP150. I showed that histone hypoacetylation at H3K9 is associated with both increased *Akap5* mRNA and co-localization of AKAP150 at the synapse following MD. HDACi was able to ameliorate these changes. In our previous work on metaplasticity within the VTA, we discovered that GABAergic tone on VTA DA neurons is lost (5). Thus, the assumption could be made that with lack of GABAergic inhibition, there would be increased DA firing. My preliminary unpublished data show that the disruption of PKA-AKAP150 by Ht31 in non-MD animals increases VTA neuronal excitability similar to MD. In addition, the use of CI-994 is able to decrease the excitability of VTA DA neurons in MD animals (data not shown). This seems to suggest that not only is the AKAP150 complex important in regulating synaptic plasticity, but also the excitability of VTA DA neurons. This again also shows that there is a role for HDAC inhibition and the potential cross-talk between HDACi and AKAP150 signaling in mediating not just changes in synaptic plasticity, but

also changes in the intrinsic properties of neurons. This merits further experimentation to determine the role of AKAP150 signaling in MD animals within the context of changes in intrinsic plasticity.

Currently, the role of AKAP150 signaling within the LHb is unknown even though its role in regulating glutamatergic plasticity has been the most studied (171). Coincidentally, my preliminary research into AKAP150 expression in the LHb has yielded the same pattern as my initial studies within the VTA. In response to MD, I found an increased expression of AKAP150 within the LHb. In tandem with future work studying the role of AKAP150 signaling within the VTA, I will determine the role of AKAP150 in LHb hyperexcitability that we previously showed in MD during early adolescence.

In conclusion, both my published data and my preliminary data suggest that the same epigenetic and AKAP150 signaling dysregulation involving HDACs impacts two critical brain structures involved in controlling DA release. These are important findings because they highlight possible unified mechanisms targeted by ELS in two distinct, but connected brain regions. Thus, when considering future behavioral studies, the final output resulting from HDACi could be due to the therapeutic role of HDAC inhibition in both brain structures. Future research will be used to determine if HDACi yields therapeutic efficacy, such as ameliorating MD-induced behavioral phenotypes, as well as teasing out brain region specific mechanisms through either intra-VTA or intra-LHb of specific HDAC inhibitors, such as a HDAC2 specific inhibitor (208). Taken together, these data suggest that with the consideration of off-target effects by HDAC inhibition, unified changes in the VTA and LHb – structures that are implicated to be dysregulated

in depression and other psychiatric illnesses – warrants investigation as a new class of anti-depressant pharmacotherapy.

MD-INDUCED LATERAL HABENULA HYPEREXCITABILITY

Preliminary work from the Nugent Lab showed that in early adolescence, MD induced LHb hyperexcitability that was linked to CRF signaling, impaired endocannabinoid signaling, and downregulation of SK2 (7). I decided to extend our studies to late adolescence (P42-P50) to see if the effect of MD on the LHb persisted. Interestingly, in late adolescent rats there was no significant difference in excitability with synaptic transmission intact; however, when fast synaptic transmission was blocked, there was a robust increase in excitability, suggesting an induction of intrinsic plasticity by MD (Figure 9C, D). Previous literature has shown that the firing pattern is an important component to consider in LHb neurophysiology due to the consistent and increased reporting that in patients with depression and animal models of depression that there is an increase in "burst" firing (44; 219). In congruence with this observed effect, there were a higher proportion of bursting neurons following MD when examining LHb neurophysiology in late adolescence (Figure 9A). However, it is interesting to note that the same mechanisms underlying this increased LHb hyperexcitability seem to be different from those in LHb of early adolescent rats (P21-P28).

Originally, the detection of lower abundance of SK2 in the LHb corroborated the changes in medium afterhyperpolarization (mAHP) in early adolescence. However, in late adolescence, my preliminary data suggest that SK2 expression normalizes in late adolescence (data not shown) with no significant difference in mAHP. In addition, the MD-induced increase in excitability exhibited at late adolescence was accompanied by

changes in input resistance (a measurement usually indicative of changes in channel conductance, most notably potassium channels) in which there was a higher input resistance detected in MD animals (Figure 9E). This could be explained either by channels closing or by channels being removed from neurons. Thus, the examination of this change in input resistance following MD gives me candidate channels of interest to examine, such as two-pore potassium channels whose activation have been implicated to elicit anti-depressant effects.

EFFECTS OF KETAMINE ON MD-INDUCED LHB HYPEREXCITABILITY

The glutamatergic hypothesis of depression suggests that dysregulation of glutamatergic transmission can lead to aberrant excitatory signaling that can disrupt brain regions of interest associated with depression, such as monoaminergic structures. Examination of the LHb, a primarily glutamatergic negative regulator of monoaminergic signaling, is critical to understanding how changes in glutamatergic transmission can impact depression. Ketamine, a prototypic NMDAR antagonist, has promise as a novel anti-depressant; however, the mechanism of action has undergone major scrutiny and investigation.

It has been documented that ketamine decreased "burst" firing of LHb neurons as well as decreased the excitability in animal models of depression (219). The authors of that study implicate both the NMDAR and low-voltage-sensitive T-type calcium channels (T-VSCCs) through pharmacological manipulation. In congruence with these findings, we were able to show that MD increased the proportion of LHb "bursting" neurons and that following systemic administration, ketamine was able to not only reduce this proportion in MD animals, but also decrease the excitability when synaptic transmission

was blocked (Figure 9D). Interestingly, the increased changes in input resistance were also ameliorated by ketamine (Figure 9E). While these two proposed mechanisms may or may not be mutually exclusive of one another, it is important to note striking differences in the studies.

One of the biggest differences in the changes observed by both studies respective models was the time course in which the effects of ketamine were observed. Yang et al. demonstrated the effects of ketamine as an acute exposure. Conversely, my work examines the effects of ketamine at a longer time course (24hrs for behavior, 72hrs for electrophysiology). The cellular mechanisms that ketamine engages to elicit the anti-depressant mechanism of action could be different in terms of time course. The acute study of ketamine provides data regarding NMDAR-dependent and NMDAR-independent mechanisms, specifically focusing on the role of T-VSCC and the contribution of these channels to LHb bursting neurons (219). My study provides a more clinically relevant experimental design; the long-lasting effects of ketamine as an anti-depressant are shown in my study. Behavioral abnormalities were attenuated as late as 24hrs (Figure 8) and changes in LHb hyperexcitability persisted as long as 72hrs (Figure 9). In my studies, I found that changes in input resistance could be involved which contributes to changes seen in neuronal excitability. However, both papers together are able to recapitulate similar findings regarding the role of ketamine as an anti-depressant. Both our studies found that ketamine can decrease the proportion of bursting LHb neurons, as well as excitability. Thus, this provides consistent data that, across multiple animal models, there is translational evidence that ketamine could be working on the same neuronal circuits and utilizing similar cellular mechanisms.

EFFECTS OF KETAMINE ON MD-INDUCED CHANGES IN THE FST DURING LATE ADOLESCENCE

In animal experimental systems, there are many different paradigms in which researchers can mimic aspects of psychiatric disorders. In depression studies, one commonly used experimental paradigm is the use of the FST developed by Porsolt (27). The FST assesses differences in active (swimming, climbing, diving) vs passive behavior (immobility) following exposure to a drug and/or a stress (50; 51). Historically, it had been observed that the use of typical anti-depressants such as SSRIs could shift the behavior of rodents in the FST from passive to more active behavior (185). Therefore, many researchers use the FST as a measurement to interrogate whether a drug elicits anti-depressant effects.

My studies examined the role of ketamine in both early adolescence and late adolescence. In agreement with my electrophysiological studies of MD in late adolescence, I examined whether MD could elicit a pro-depressive behavioral disparity (i.e. increased immobility time) during the FST. Indeed, MD animals do display a more pronounced immobility duration compared to their non-MD counterparts (Figure 8A). However, it is also important to note that during the pre-test there was no significant difference in the time required to achieve immobility between non-MD and MD, indicating that our ELS model doesn't create any observable learning and/or motor deficits in the FST (Figure 8A). Following i.p. administration of either saline or ketamine (20mg/kg), I observed that ketamine induced a shift in the behavior of MD animals from passive behavior (immobility) to active behavior (swimming) (Figure 8A). Interestingly, this effect of decreasing immobility duration was also observed by Yang et al., although both the time course and route of administration were different (219). Interestingly with

the different routes of administration, both our groups were able to observe the same change in behavioral phenotype. However, the limitation of the study done by Yang et al. is that it only represents the effect of ketamine on animal behavior through administration to a discrete brain region, the LHb. My study represents a more translational measure as the administration more closely recapitulates what would be observed in clinic. Lastly, the animal behavior observed in my study is a result of ketamine interacting with multiple brain regions, which is an important consideration when considering the different circuits that could be disrupted downstream of the LHb in depression and other psychiatric illnesses.

EFFECTS OF KETAMINE ON MD-INDUCED CHANGES IN THE FST DURING EARLY ADOLESCENCE

Two studies from the Nugent lab now show that at two periods of adolescence, both early and late, there is increased LHb hyperexcitability, with an associated increase in the population of bursting neurons. In late adolescence, I observed that MD animals preferentially expressed increased immobility that was corrected by ketamine administration 24hrs later (Figure 8B). There is clinical evidence that ketamine can be used as a sedative and anesthetic in children with little to no complication (54). It is also important to note that the safety and efficacy involving administration of ketamine to children is at relatively high doses, whereas ketamine as an anti-depressant is administrated at sub-anesthetic doses. Given the combined behavioral and electrophysiological abnormalities observed, I wanted to investigate if MD animals display increased immobility during early adolescence, a period we had previously documented that is associated with an MD-induced LHb hyperexcitability (7).

Developmentally, in the rodent model some behaviors are not displayed based on the age at which the animal is tested. For example, in the FST male rats on average do not begin to exhibit immobile behavior until approximately P22 (1). In our initial studies, I determined that indeed our early adolescent rats could acquire the immobility phenotype (Figure 8B). In addition, there was no difference in the time it took for the early adolescent rats to learn immobility, indicating that similar to the pre-test results observed in late adolescent rats, MD doesn't affect the rate of learning and acquisition (Figure 8B). Interestingly, early adolescent MD rats had a robust increase in climbing behavior compared to their non-MD counterparts and had significantly lower time spent immobile (Figure 8B). Although opposite to the initial hypothesis, it has been documented that in some instances, stressors and other environmental exposures can elicit a greater active behavior in rodents in the FST model (4; 88). 24hrs after i.p. administration of ketamine, the MD-induced behaviors were attenuated by ketamine in early adolescent rats (Figure 8).

In our model of MD, there seems to be a behavioral phenotype "switch" that occurs in rats between early and late adolescence. The predominant MD-induced behavioral phenotype displayed by early adolescent rats is climbing, whereas in late adolescence it is immobility (Figure 8). Interestingly, in both cases a single i.p. injection of ketamine (20mg/kg) is able to amend this change in behavior associated with MD up to 24hrs later. In addition, both of these periods were accompanied by LHb hyperexcitability (Figure 9)(7). In late adolescence, the ameliorating effect of ketamine was observed on both the behavior and in LHb neurophysiology. Although ketamine can ameliorate the behavioral effects of MD in early adolescence, future studies will

determine if ketamine can also decrease MD-induced LHb hyperexcitability in this age range (P21-P28). The change in behavior observed in the FST could be due to habenular modulation of 5-HT and DA in the raphe nucleus and VTA, respectively. Additional behavioral paradigms will be used in the future to determine how LHb hyperexcitability following ELS could modulate monoaminergic signaling to alter behavior and to determine whether ketamine normalizes habenular input and firing patterns to encode behavioral responses.

USE OF KETAMINE AS A NOVEL ANTI-DEPRESSANT: WHERE THE RESEARCH IS NEEDED

In the search for a new class of anti-depressants, ketamine might represent how we can re-purpose a relatively "old" drug for a new treatment. Studies on safety and efficacy suggest that, with the exception of specific complications, ketamine could be used in both children and adults (54). Evidence has been seen in clinic where intravenous infusion of ketamine can attenuate depressed symptoms and reduce suicidal ideation quickly and long-lasting (15; 53). Lastly, studies on the LHb and use of ketamine highlight glutamatergic hypothesis of depression in which, targeting glutamatergic circuitry might be a way in which depression and other psychiatric disorders can be treated. However, due to ketamine's role in producing hallucinogenic symptoms as a drug of abuse and because the anti-depressant mechanism of action is currently unknown, there is still disagreement about the potential use of ketamine in depression treatment.

When considering the development of pharmaceuticals, metabolism and bioavailability of a candidate drug is important. One of the more controversial hypotheses of how ketamine exerts a rapid and long-lasting anti-depressant effect is through its metabolic breakdown. For example, hydroxy-norketamine (HNK) has been shown to

elicit its own anti-depressant mechanism of action (225; 226). However, this issue becomes somewhat complicated as ketamine has a stereoisomer center meaning that ketamine is delivered as a racemic mixture with both R and S enantiomers. To complicate issues further, it has been shown that R-ketamine has increased efficacy in relieving anti-depressant symptoms, whereas S-Ketamine exhibits little to no anti-depressant properties (229). This has to be taken into consideration when considering the possible mechanism of ketamine; it could be due to metabolic breakdown of ketamine to form its active metabolites. For example, metabolites of R-ketamine seem to still elicit anti-depressant effects (32; 221). Given the fact that my research shows that *in vivo* ketamine has a long-lasting effect on MD-induced behavior and electrophysiology, it cannot be discounted that one of the mechanisms could be due to active metabolites from the breakdown of ketamine. Future experiments will investigate different formulations of ketamine and whether specific enantiomers elicit the same effects as observed with our racemic mixture of ketamine.

The approximate half-life of ketamine is 45 minutes (164). This half-life can vary based on the pharmacogenetics of the individual (i.e. poor metabolizer vs fast metabolizer) and the route of administration. Therefore, it is possible that the acute actions of ketamine on the CNS are due to prototypic action of NMDAR antagonism, but the long-lasting effect is due to the effects of ketamine metabolites. One such mechanism that has been researched is the NMDAR-independent effects of ketamine on AMPAR function (31; 218; 221). Preliminary data from the Nugent lab has shown that when ketamine is bath applied in the presence of APV, another NMDAR antagonist, there is an increase in spontaneous EPSC (sEPSC) (data not shown). This could be through

modulation of AMPAR signaling. Another such mechanism is through BDNF receptor signaling, a typical pathway modulated by anti-depressants. Interestingly, my preliminary data show that MD increases mBDNF expression in the LHb during late adolescence (data not shown). Curiously, one study found that NMDAR antagonism leads to increased BDNF expression in the hippocampus (160). However, the specific role of BDNF in the LHb with respect to controlling neuronal excitability is still unknown. To assess whether AMPAR and BDNF are altered following ketamine administration to elicit anti-depressive behaviors, protein expression for both will be quantified at 24 and 72hrs after i.p. injection to correlate expression with behavioral and electrophysiological data, respectively.

Given that my study used an *in vivo* approach to study the effects of ketamine, it cannot be discounted that the observed behaviors are due solely to the effect of ketamine on the LHb. Ketamine could have also altered LHb-related circuits. When a patient receives an infusion of ketamine, an effect on one brain region of interest might be observed. However, we cannot discount that other brain regions could also be affected in synchrony. LHb hyperexcitability and increased burst firing is a common finding in depression research. I also observed this in my experimental system involving MD. The question is whether the neuroadaptation first occurs within the LHb itself to initiate dysregulation of monoamines or if the dysregulation occurs upstream of the LHb via changes in specific input regions?

One of the main upstream regions also implicated in depression that has direct connections to the LHb is the globus pallidus (GP) [in rats referred to as the entopeduncular nucleus (EP)]. As part of the basal ganglia, the internal segment of the

GP (GPi) is involved with the control of movement, but also modulates reward-related behavior (181; 188). It has also been shown that GPi (EP in rodents) is involved in co-transmission of glutamate/GABA to the LHb and is related to depressive behaviors, as well as those involving drugs of abuse (134; 180). In my work, we show two different behavioral phenotypes involving motor function in which MD induced greater climbing in early adolescence and greater immobility in late adolescence, with ketamine ameliorating LHb hyperexcitability in the latter. In the context of the MD-induced behavior, ketamine was able to alter the MD-induced behavioral changes compared to non-MD counterparts. Given the connectivity and role of GPi and LHb in relation to motivated behavior and aversion, one possible contribution to the changes seen in LHb excitability and the behavioral disparities observed could be due to the effect of ketamine initiating changes in EP signaling. However, it is also important to consider other upstream brain regions, such as lateral hypothalamus (LH) and ventral pallidum (VP), both of which have projections to the LHb and have roles in reward-related behaviors (90). With the development of optogenetics and intra-brain region injections, future work will focus on optogenetic stimulation of input regions to the LHb, such as the EP, to determine how this EP → LHb microcircuit contributes to the observed MD-induced changes in electrophysiology and behavior. This can be combined with our current studies of ketamine both by systemic administration or intra-LHb administration.

Lastly, one of the common mechanisms presumably contributing to changes observed in both the LHb and VTA is changes in acetylation. During early adolescence, decreased acetylation was observed at H3K9 in the VTA (Figure 1), but also H3K14 in the LHb. One of the less understood properties of ketamine is the possibility that it has

HDACi properties. For example, HDAC5, a class II HDAC, has been shown to be downregulated in the nucleus following administration of ketamine in the hippocampus (37; 38). Given the fact that CI-994 can restore GABAergic metaplasticity (5) and VTA DA neuronal excitability within the VTA (data not shown), it is possible that the long-lasting MD-ameliorating effects of ketamine and/or its metabolites are due to epigenetic modulation of the LHb. In addition, it is unknown if any of the ketamine metabolites themselves can induce changes in the epigenome that might account for the long duration of action.

FUTURE DIRECTIONS

In this dissertation, I have highlighted how MD can affect both VTA and LHb neurophysiology. I have also highlighted how the use of novel anti-depressants, HDACi and ketamine, could potentially work to ameliorate the effects of ELS both at a molecular and physiological level in the VTA and LHb, but also related behavioral disparities observed in the FST. In both studies, MD induced changes in excitability, albeit potentially by different mechanisms. My future work will involve continued investigation of these two novel anti-depressants by defining the mechanisms of action in the rodent experimental system to further tease out their potential for use in clinic.

We showed previously that MD disrupted GABAergic plasticity, and this favors the monoaminergic hypothesis of depression in that DA becomes dysregulated. This is also corroborated by the finding that MD induces increased VTA DA neuronal excitability. Also, MD induces increased LHb hyperexcitability and glutamatergic dysregulation has also been implicated in depression. While there is direct innervation of LHb outputs to VTA DA neurons (23; 147), would not increased excitability from LHb

to GABAergic intermediate structures around and/or near the VTA result in greater DA inhibition? One possible mechanism is synaptic scaling.

Synaptic scaling is an activity-dependent process that involves either scaling-up a post-synaptic response in the absence of input or scaling-down when there is too much input (199). Currently, synaptic scaling has only been studied *in vitro* within the hippocampus and there is no information as to whether this phenomenon occurs in other brain regions *in vivo* (89; 112; 168). It has also recently been shown that AKAP150 signaling is involved in calcium-permeable AMPAR insertion within the context of synaptic scaling (173). In my studies of the VTA, I did not examine the effects of MD on non-TH+ neurons, more specifically the GABA interneurons, which make up approximately 25% of the VTA's neuronal population. The LHb has direct projections to these inhibitory neurons. It is possible that as a compensatory mechanism to increased excitatory signaling from LHb glutamatergic neurons that the GABAergic neurons scale-down their response. This in turn contributes to the GABAergic synaptic dysfunction we previously documented. However, one consideration that has to be made is the differences in the different ages in which we documented changes in excitability in both the VTA and the LHb. Therefore, future work will need to interrogate how early changes in excitability start and how long it persists in both the VTA and LHb. It is also possible that HDAC inhibition can alter synaptic scaling just as it does synaptic plasticity through an epigenetic mechanism, as well as the possible role of ketamine in AMPAR trafficking. I will investigate whether synaptic scaling occurs within GABAergic neurons in the VTA, as well as LHb and VTA DA neurons. Lastly, I will investigate whether the novel

anti-depressant mechanism of actions of HDACi and ketamine are related to this phenomenon.

FIGURES

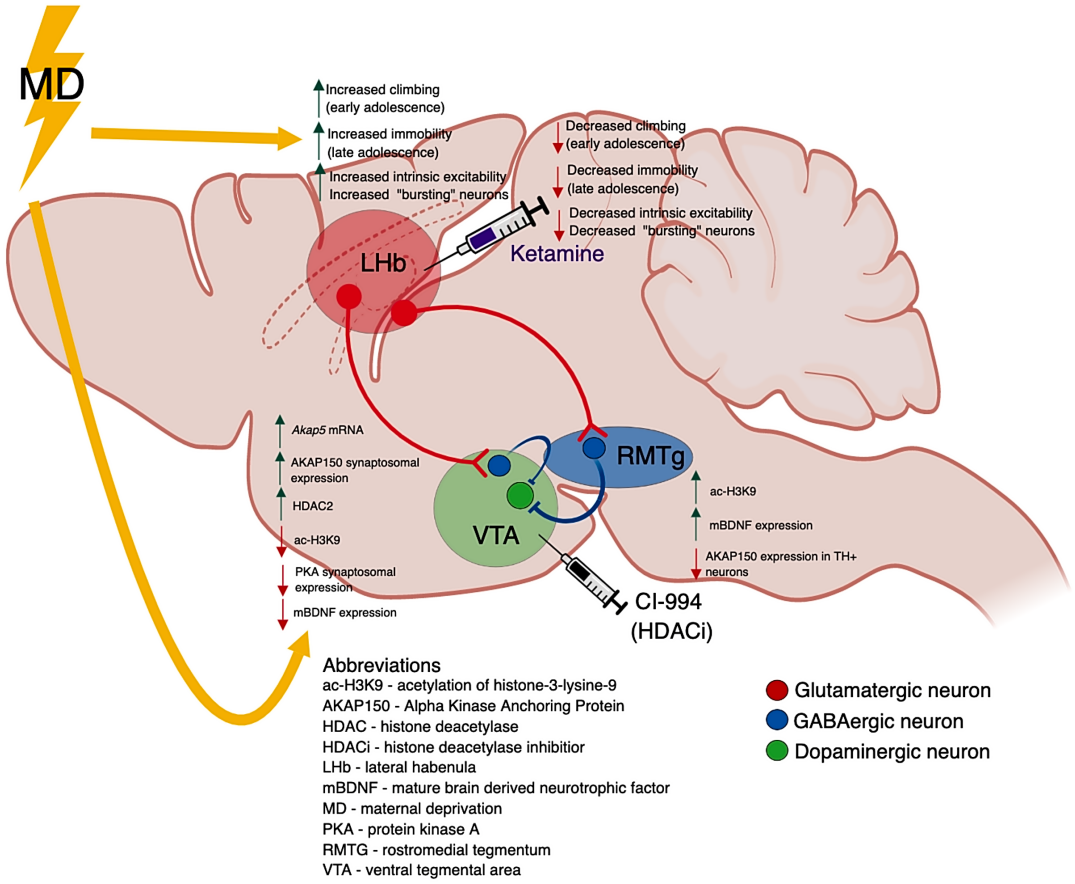

Figure 10

Graphical Abstract Demonstrating the Effects of MD and Novel Anti-Depressant Administration on the LHb and VTA. MD induces decreased ac-H3K9, which is associated with an increase in HDAC2 in the VTA. These epigenetic changes were also associated with increased AKAP150 mRNA and synaptosomal expression, as well as decreased mBDNF. The use of CI-994, a class I HDACi, was sufficient to ameliorate MD-induced changes in ac-H3K9, AKAP150, and mBDNF. MD-induced LHb hyperexcitability persists into late adolescence accompanied by an increased proportion of "bursting" neurons that is attenuated following ketamine administration. Despite persistent LHb hyperexcitability, two different phenotypes were identified at different time points: early adolescence resulted in increased climbing behavior whereas late adolescence resulted in increased immobility. Both MD-induced disparities were ameliorated following ketamine administration. Both studies highlight the possible application of novel anti-depressants, such as HDACi and ketamine, for use in treating patients suffering from depression as a result of early life stress.

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