THE ROLE OF CHROMATIN REMODELING IN HIPPOCAMPUS IN DEPRESSION AND ANTIDEPRESSANT ACTION

APPROVED BY SUPERVISORY COMMITTEE

Dr. Eric J. Nestler, M.D./Ph.D.

Dr. David Margolis, M.D.

Dr. Gang Yu, Ph.D.

Dr. Jane Johnson, Ph.D.

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Finally, I would like to dedicate this thesis to my close friends struggling with depression. I hope that this thesis will become a small step towards elucidating the enigma of this terrible disease, and will give hope in your lives.

Blagodaria Vi!

# THE ROLE OF CHROMATIN REMODELING IN HIPPOCAMPUS IN DEPRESSION AND ANTIDEPRESSANT ACTION

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NADEJDA MINCHEVA TSANKOVA

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NADEJDA MINCHEVA TSANKOVA, M.D./Ph.D.

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ERIC J. NESTLER, M.D./Ph.D

This thesis presents a novel level by which neuroplastic changes in the brain may be disrupted with depression and reversed by treatment with antidepressants: regulation at the level of chromatin remodeling. The technique of brain chromatin immunoprecipitation was pioneered to directly measure the *in vivo* modifications of histones, a form of chromatin remodeling, at gene promoter regions in the hippocampus after chronic defeat stress, a model of depression, and chronic treatment with the antidepressants imipramine and electroconvulsive seizure (ECS). Chromatin modifications and transcriptional changes were assayed in one gene in particular, the brain-derived neurotrophic factor (BDNF). BDNF is alternatively spliced to generate several mRNA transcripts, driven by unique promoters. I measured the expression levels of each BDNF transcript (I-IV) in rat after ECS, as well as each BDNF transcript (I-V) in mice after chronic stress and

imipramine treatments, and found that these chronic treatments induce lasting changes in the expression of specific BDNF splice variants. These changes correlated with sustained modifications in histones at the exact promoter regions, driving the differential changes in BDNF expression. Chronic defeat stress induced robust enrichment of H3-K27 methylation at BDNF P3 and P4 promoters (modifications expected to repress promoter activity), while chronic imipramine in defeated animals lead to lasting upregulation in the levels of H3 acetylation and H3-K4 methylation at P3 and P4 (modifications expected to stimulate promoter activity). Finally, I discovered a novel role for the histone deacetylase HDAC5 in the therapeutic efficacy of chronic imipramine after defeat stress. I found that chronic imipramine downregulates HDAC5 after stress, that HDAC5 overexpression in the hippocampus blocks the behavioral effects of imipramine in defeated mice, that HDAC5 inhibition exerts a subtle antidepressant-like effect, and that HDAC5 deficiency reduces the pathological response to stress. This unexpected role for HDAC5 provides an important mechanistic link between the adaptive chromatin remodeling changes at genes and the ability of chronic antidepressants to exert therapeutic efficacy after chronic stress. These experiments provide one of the first endeavors to understand the role of chromatin remodeling in modulating long-term adaptive changes in brain associated with complex psychiatric conditions, such as depression.

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## ABBREVIATIONS

Ab: Antibody
AC8: Adenylyl cyclase 8
ACSF: Artificial Cerebrospinal Fluid
ATP: Adenosine Triphosphate
BAC: Bacterial Artificial Chromosome
BDNF: Brain-Derived Neurotrophic Factor
Bl6: Black 6
Bp: base pair
BSP: Bisulfite Specific Primer
$Ca^{2+}$: Calcium
CaMK: Calcium/Calmodulin Mediated Kinase
cAMP: cyclic Adenosine Monophosphate
CBP: CREB Binding Protein
CDK5: Cyclic Dependent Kinase 5
ChIP: Chromatin Immunoprecipitation
CRE: cyclic AMP Response Element
CREB: cyclic AMP Response Element Binding protein
Ct: Threshold cycle
Cy2: Cytochrome 2
DNA: Deoxyribonucleic Acid
ECS: Electroconvulsive Seizure
ECT: Electroconvulsive Therapy
ERK: Extracellular Signal Regulated Kinase
FOS: FBJ Osteosarcoma oncogene
GABA: Gamma-AminoButyric Acid
GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase
GFP: Green Fluorescent Protein
GluR2: Glutamate Receptor subunit 2
H3: Histone 3
H4: Histone 4
HAT: Histone Acetyltransferase
HDAC: Histone Deacetylase
HMTase: Histone Methyltransferase
HPA: Hypothalamic – Pituitary – Adrenal
HSV: Herpes Simplex Virus
ICER: Inducible cAMP Early Repressor
IgG: Immunoglobulin G

Ip: intraperitoneal
K: Lysine
LC: Locus Coeruleus
LTP: Long Term Potentiation
Lys: Lysine
MAPK: Mitogen Activated Protein Kinase
MECP2: Methyl-CpG binding Protein 2
mRNA: Messenger Ribonucleic Acid
NcoR: Nuclear receptor co-Repressor
NIMH: National Institute of Mental Health
NMDA: N-methyl-D-aspartate
NuAC: Nucleus Accumbens
P1-P5: Promoter 1 – 5
PAG: Periaqueductal Gray
PBS: Phosphate-Buffered Saline
PCAF: p300/CBP-associated factor
PCR: Polymerase Chain Reaction
PC12: Pheochromocytoma 12
PKA: Protein Kinase A
PKC: Phosphokinase C
PRMTase: Protein Arginine (R) Methyltransferase
qPCR: quantitative Polymerase Chain Reaction
RT-PCR: Reverse Transcriptase PCR
S: Serine
SB: Sodium Butyrate
SDS: Sodium Dodecyl Sulfate
SEM: Standard Error of the Mean
Ser: Serine
Sin3: Yeast global gene regulator, co-repressor
SMRT: Silencing Mediator of Retinoid and Thyorid Receptors
SWI/SNF: mating SWItching and Sucrose NonFermentation
TAF250: *Drosophila m*. TBP-associated factor 250K
TE: Tris-EDTA
TrkB: Tyrosine Receptor Kinase B
UTR: Untranslated Region
VPA: Valproic Acid
VTA: Ventral Tegmental Area

## INTRODUCTION

> *I have of late, - but wherefore I know not, - lost all my mirth, forgone all custom of exercises; and, indeed, it goes so heavily with my disposition that this goodly frame, the earth, seems to me a sterile promontory: this most excellent canopy, the air,…, this brave o'erhanging firmament, this majestically roof fretted with golden fire, - why, it appears no other thing to me than a foul and pestilent congregation of vapours.*
>
> - Hamlet

### Depression – general overview:

The concept of depression, depicted so eloquently by Shakespeare during the 16th century, has always existed and been recognized by mankind but painted differently over time. The ancient Egyptians living more than four thousand years ago recognized depression as an illness, albeit one of the heart, and prescribed temple sleep therapy for cure. Later, Hippocrates, living in 400 BC, referred to depression as melancholia, meaning literally black bile in Greek, and described it as an internal imbalance of the four body fluids, or humors. He and others at the time insisted that the cause of depression was natural, rather than divine. During the next two millennia, dominated by religious zeal and supernatural believes, mentally ill people were stigmatized as sinners, possessed by the devil, or witches. Fortunately, in the last few centuries, we have returned to the naturalistic view of Hippocrates that depression is not a supernatural illness. While there is much more known about depression today, and there are many different therapies available to alleviate its debilitating symptoms, millions of people are still suffering from the black bile.

Depression is a chronic and a debilitating disease, affecting about 10% of adults in the United States and about a quarter of the population at some point in their lives. Vulnerability to depression has a strong genetic component, 40-50%. Nevertheless, environmental factors such as stress, emotional trauma, several medical illnesses, and viral infections can precipitate symptoms of depression. Some of the most common symptoms include: depressed mood; irritability; feeling of hopelessness, worthlessness, and guilt; decreased ability to concentrate or think; changes in appetite, sleep patterns, or weight; decreased energy; fatigue; anhedonia; and recurrent thoughts of death and suicide. The symptoms and their severity vary widely from patient to patient. Indeed, many believe that depression should not be viewed as a single disease, but rather as a heterogeneous syndrome encompassing many diseases with distinct pathophysiology (1).

**Antidepressants:**

While research in the past few decades has elucidated a great deal about the pathophysiology of depression, the actual mechanisms that precipitate it are still incompletely understood. Nevertheless, there are very effective treatments for depression, including cognitive and behavioral psychotherapy, electroconvulsive therapy (ECT), as well as chemical therapy. The efficacy of chemical antidepressants was discovered serendipitously in the 1950s, when it

was noticed that certain antitubercular drugs as well as certain antihistamines, when given to depressed patients, alleviated many of their symptoms. These drugs later came to be known as monoamine oxidase inhibitors (chemicals that inhibit the catabolism of monoamine neurotransmitters, e.g., iproniazid), and tricyclics (inhibitors of serotonin or norepinephrine reuptake transporters, e.g., imipramine). These, along with newer antidepressant drugs (such as serotonin-selective reuptake inhibitors, e.g., fluoxetine) and ECT, have been very efficacious in alleviating symptoms in as many as 80% of people suffering from depression. However, only about half of all depressed patients show a complete remission with these treatments, which highlights the need for more effective agents. While the acute pharmacological actions of chemical antidepressants are well understood, it is still unclear how they treat depression. This uncertainty arises from the fact that antidepressants increase levels of serotonin and/or norepinephrine acutely, yet the mood-elevating clinical effects of these drugs are seen only after several weeks to months of treatment. Therefore, their efficacy in treating symptoms of depression must involve a mechanism by which the brain undergoes a gradual adaptation to the enhanced neurotransmission (1). This thesis explores possible mechanisms for the lasting neuroadaptations that may occur in the brain after depression and which may underlie the efficacy of chronic antidepressant treatments.

**Stress and depression:**

There are many known medical conditions that cause the depressive syndrome in people. Some of these include: endocrine disturbances, such as hyper- or hypocortisolemia, and hyper- or hypothyroidism; Parkinson's disease; traumatic head injury; diabetes; and stroke (1). An important but not well-understood cause of depression is stress.

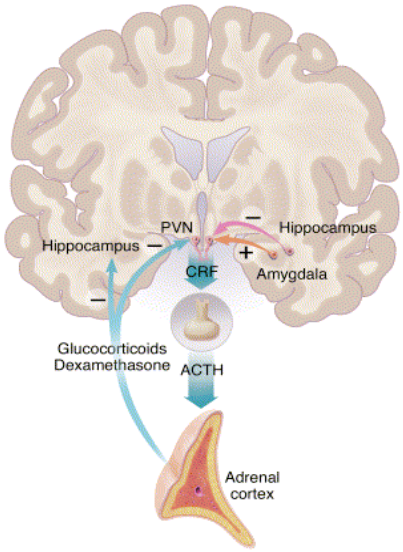



**FIGURE 1**
**(Nestler et. al., 2002)**

Several decades ago, when the stress theory was first formulated, it was believed that stressors precipitating depression are either of endocrine character or noxious stimuli of physical and chemical nature. Further research revealed that psychological stimuli are also potent activators of the endocrine system, and thus can also trigger depression (2). Importantly, major stress events do not necessarily cause depression in every person. Rather, even mild but prolonged stress can precipitate depression in genetically susceptible individuals (1). The goal of much of the current research in the field of depression is to identify the cellular and molecular mechanisms in the brain that predispose certain people to stress-precipitated depression.

The stress response is a normal physiological process by which an animal is alarmed about something unexpected. An acutely stressful stimulus is perceived via numerous pathways, including the following which are the best characterized: 1) activation of the sympathetic nervous system to release norepinephrine from every cell in the body and epinephrine directly from the adrenal medulla into the blood and 2) stimulation of the hypothalamus-pituitary-adrenal (HPA) axis to increase glucocorticoid (cortisol in humans, corticosterone in rodents) release by the adrenal glands. As cortisol begins to accumulate in the brain, it itself can send negative feedback to the hypothalamus to decrease its production (Figure 1). However, repeated stress can lead to deregulation of the HPA axis and to chronic overproduction of glucocorticoids.

Indeed, hypercortisolemia is seen in many patients suffering with depression. In the second pathway by which we respond to stress, especially psychological stress, the limbic system (hippocampus, amygdala, and prefrontal cortex) can become activated and in turn can trigger the hypothalamus and the downstream release of cortisol. One particularly well-studied region of the limbic system that can relay information of stress (such as restraint, fear, novel environment) to the hypothalamus is the hippocampus. The hippocampus normally inhibits hypothalamic activity (Figure 1), but stress blocks this circuitry, leading to high production of cortisol (2).

Chronic stress is the most widely used animal model of depression. Different stress paradigms include chronic unpredictable, restraint, foot shock, and social defeat (psychosocial) stress. In the last stress paradigm, an animal is allowed a brief confrontation with an aggressive opponent daily, after which it is exposed to a continuous stress in the form of a threat, e.g., by having to stay in a compartment beside the aggressor in between fights, exposed to the visual and olfactory cues of its defeater. Recent evidence in rodents and the tree shrew (*Tupaia belangeri*) suggests that chronic psychosocial stress may represent a more naturalistic and more physiological paradigm to study the causal mechanisms of stress-related disorders. Social defeat stress mimics many of the physiological symptoms of stress as well as some of the psychological attributes of depression in humans: elevated glucocorticoid activity, tachycardia, and hyperthermia which take hours to recover even after a brief confrontation with an aggressive opponent; gonadal atrophy and adrenal hypertrophy; compromised immune system; exposure to a combat attack by a dominant aggressor leading to eventual subordination; fear; increased risk of injuries; disruption of normal circadian rhythm cycle; reduced feeding and water intake; diminished general motor activity; and decreased self-grooming (3, 4). Chronic social defeat stress also causes several important cellular changes in the limbic system, and particularly in the hippocampus.

**The hippocampus and neuroplasticity at a cellular level:**

Brain imaging studies, autopsies, and investigations of animal models have suggested the involvement of several brain regions in the pathophysiology of depression (5, 6). The most extensively studied of these is the hippocampus. The hippocampus of patients suffering from depression shows decreased blood flow, decreased glucose metabolism, and atrophy (7-9). The hippocampus is part of the limbic system, and it is involved in emotional processing, in learning, and in memory formation. The hippocampus receives its major input from the entorhinal cortex (via the perforant pathway), which in turn receives input from areas of the association cortex. This information enters the hippocampus into a region called the dentate gyrus, which consists of tightly packed granule cells. Granule cells synapse onto CA3 hippocampal pyramidal cells, which in turn synapse onto CA2, and CA1 pyramidal cells. A unique feature of the hippocampus is the presence of cells in its subgranular zone of the dentate gyrus, which continue to regenerate in adult brain, a process known as adult neurogenesis. It is estimated that 6% of the granule cell layer of the dentate gyrus, or approximately 250,000 new neurons, are formed each month in the rodent hippocampus (10). In addition, high-frequency electrical stimulation in the hippocampus of rodents induces long-term

potentiation, a phenomenon implicated in memory formation and neuroplasticity (11).

Chronic stress causes a decrease in neurogenesis and synaptic plasticity in the hippocampus of rodents (7). Decreased neurogenesis has been reported in different animal models of stress, including inescapable stress following foot shock, chronic mild stress, as well as chronic defeat stress. In the absence of stress, antidepressants induce neurogenesis, increasing cell proliferation as well as cell survival in a chronic-dependent treatment, consistent with the time course of their therapeutic action. Most importantly, in many of the stress paradigms, the reduction in neurogenesis in stressed animals is reversed by antidepressant treatments (12). In fact, neurogenesis may be necessary for the action of antidepressants in behavioral models of depression. This has been recently suggested in a study where blocking cell proliferation, basal as well as antidepressant-induced, lead to corresponding blockage of the behavioral response to antidepressant treatment in two behavioral paradigms of depression (13). In addition to reducing neurogenesis, chronic exposure to stress results in atrophy of CA3 pyramidal neurons, an effect that is also blocked by antidepressant treatment (14). The presence of neurogenesis, neuroplasticity, the inhibitory action of the hippocampus onto the hypothalamus, and its deregulation and atrophy with stress, are some of the important attributes of the hippocampus for studying its role in depression (Figure 2). This capacity of the hippocampus

for long-term neuroadaptations can be manifested at a cellular, as well as at a molecular level. However, the mechanisms of the molecular basis of neuroplasticity in the hippocampus are still poorly understood.

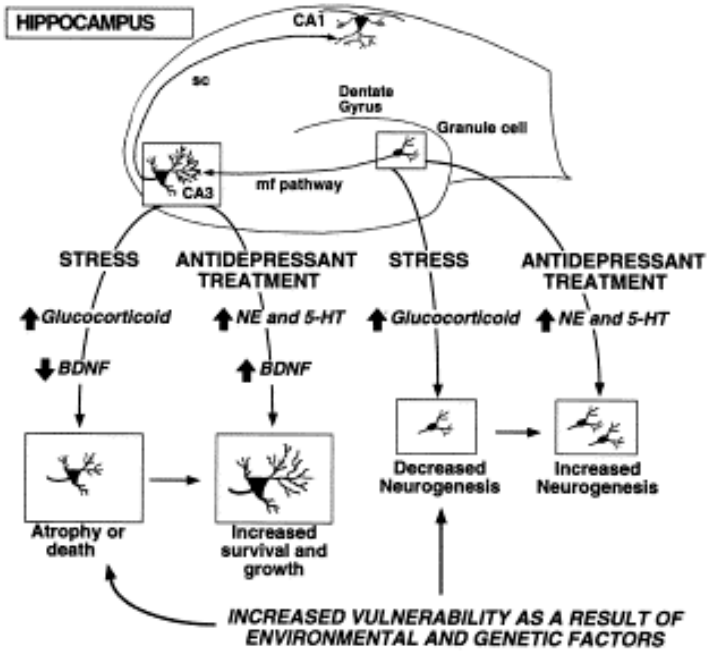



**FIGURE 2**
**(Duman et. al., 1999)**

**Neuroplasticity at a molecular level and the role of BDNF:**

Regulation at the level of gene expression is one possible mechanism that can explain the neuroplastic changes in the hippocampus seen with depression, and reversed by treatment with antidepressants. To this effect, several genes have

been implicated in the molecular pathophysiology of depression and antidepressant efficacy. One of these is the brain-derived neurotrophic factor (BDNF). BDNF has long been studied for its role in neuronal growth and differentiation, but is now known to also be important in regulating plasticity and survival of adult neurons and glia (15). In the context of depression, several findings suggest that neurotrophin deficiency may contribute to hippocampal pathology and thus to the development of depression, and that antidepressant treatment might reverse this pathology and ameliorate the symptoms of depression (1, 16). Human studies have reported decreased serum BDNF levels in patients diagnosed with severe depression, as well as increased BDNF immunoreactivity in post-mortem hippocampal tissue from patients treated with antidepressants at the time of death (17). In animal models, high doses of exogenous corticosterone or chronic stress downregulate BDNF mRNA and protein levels in the dentate gyrus and CA1 hippocampal regions (18). Importantly, chronic, but not acute, treatment with several different antidepressants, including ECS, upregulates BDNF expression, or reverses the stress-induced downregulation of BDNF (15, 18, 19). In addition, BDNF infusions in the hippocampus produce antidepressant-like effects in two animal models of depression, forced-swim test and learned helplessness (20), whereas deficiency of endogenous BDNF blocks the antidepressant-like effects in the forced swim test (21). The pathogenic effects of stress and their reversal by

antidepressants in the hippocampus, and the regulation of BDNF in these paradigms, have contributed to the formulation of the neurotrophic hypothesis of depression. According to this hypothesis, upregulation of BDNF by antidepressants may help repair some of the stress-induced damage to hippocampal neurons as well as protect neurons from further harm. Furthermore, the antidepressant effects on BDNF may work to increase synaptic plasticity in the hippocampus, maintaining its normal function. Nonetheless, while the experiments outlined above provide a strong correlation for the involvement of BDNF in stress-induced pathology of the hippocampus and repair via antidepressants, the mechanisms by which these BDNF changes are brought about are still unclear. There is some evidence that the cAMP response element binding protein, CREB, may at least partly mediate the induction of BDNF by antidepressants (22-24) (Figure 3).

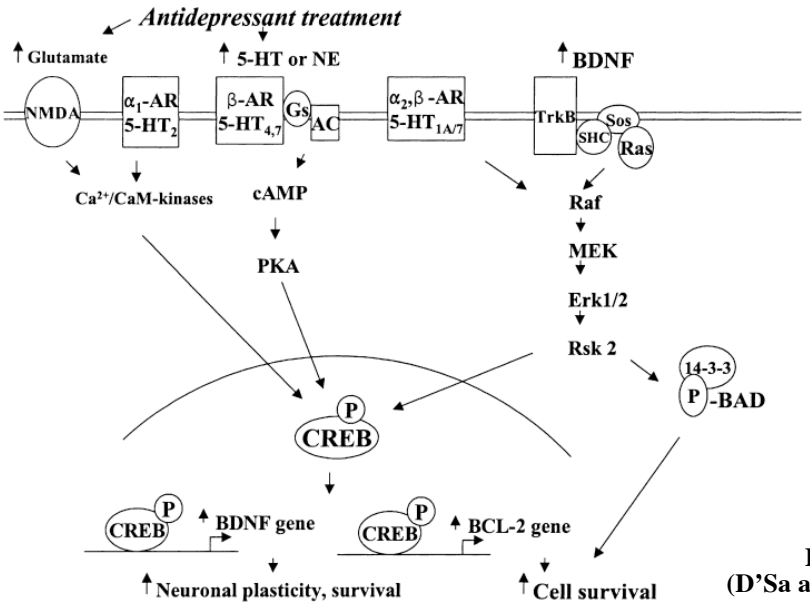
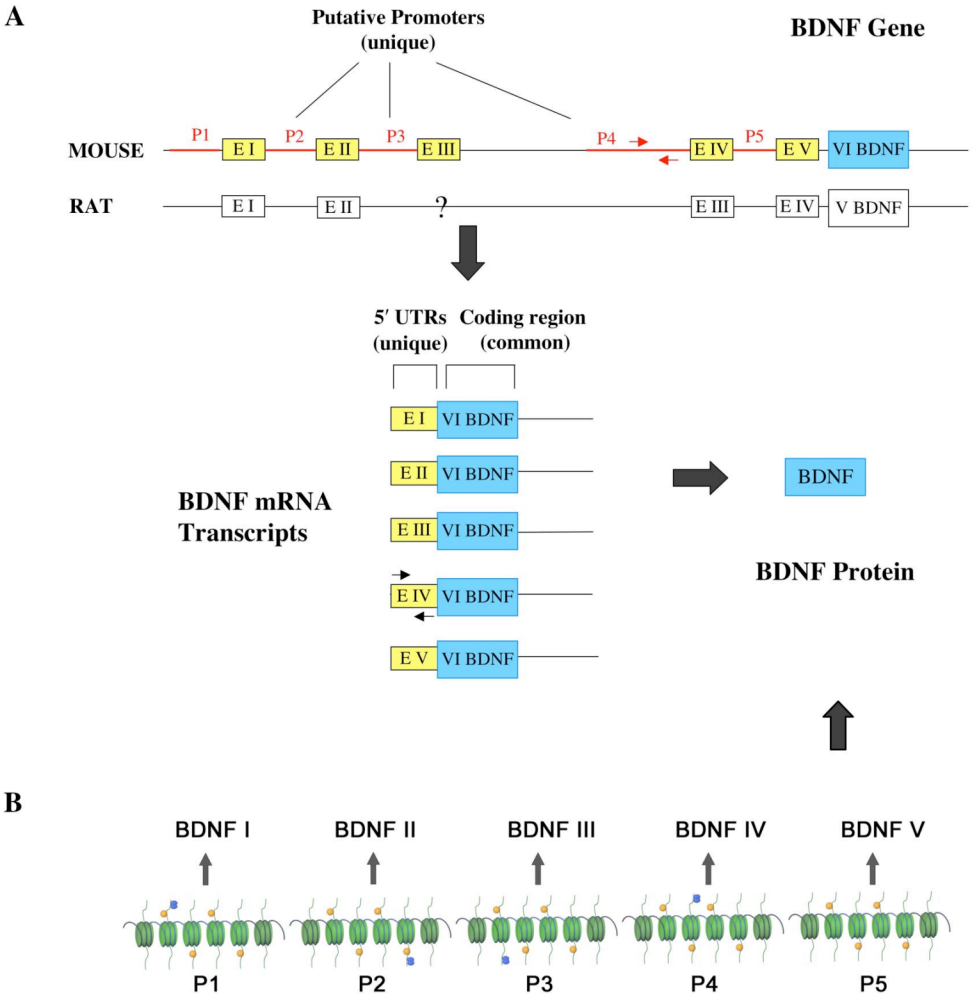



**FIGURE 3**
**(D'Sa and Duman, 2002)**

Still, the regulatory mechanisms leading to long-lasting changes in BDNF expression are not well understood.

One reason for the lack of better-defined transcriptional mechanisms of regulation for BDNF is its complex gene structure. BDNF contains at least four short 5′ non-coding exons (exons I-IV in rats and I-V in mice), each of which can be alternatively spliced next to the common coding exon (exon V in rats and VI in mice) (Figure 4-A). Each non-splicing exon contains a unique promoter region, where distinctive transcription factors can be recruited to modulate the expression of one splice variant over another (Figure 4-B). The use of alternative spliced mRNA transcripts with distinct promoter regions allows for temporal- and spatial-specificity of BDNF expression (15, 25). The stress and antidepressant effects on the differential regulation of the different BDNF splice variant transcripts have not been studied. Detailed understanding of such regulation and the mechanisms by which it is accomplished requires an analysis of the expression of each BDNF transcript variant as well as the factors at the level of chromatin remodeling that bring about possible transcriptional changes. My thesis provides, for the first time, such detailed description of the differential regulation for BDNF, and it underscores the importance of chromatin remodeling in modulating stable changes at the promoters driving specific BDNF transcripts after chronic stress and chronic antidepressant treatments.


A
Putative Promoters
(unique)
BDNF Gene
MOUSE
P1
E I
P2
E II
P3
E III
P4
E IV
P5
E V
VI BDNF
RAT
E I
E II
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E III
E IV
V BDNF
5′ UTRs
(unique)
Coding region
(common)
E I
VI BDNF
E II
VI BDNF
E III
VI BDNF
E IV
VI BDNF
E V
VI BDNF
BDNF mRNA
Transcripts
BDNF
BDNF Protein
B
BDNF I
BDNF II
BDNF III
BDNF IV
BDNF V
P1
P2
P3
P4
P5

**FIGURE 4**

**Chromatin Remodeling:**

Chromatin remodeling is a dynamic process, which modulates gene expression. The fundamental subunit of chromatin is the nucleosome, which consists of approximately 165 base pairs (bp) of DNA wrapped around a core histone octamer (two of each histones H2A, H2B, H3, and H4). The nucleosomal structure of chromatin allows for tight packaging of genomic DNA into the nucleus by several organized events of folding. In addition, the highly controlled process of chromatin remodeling permits single nucleosomes to become more or less open, allowing or inhibiting access of the transcriptional machinery to specific promoter regions. In this manner, chromatin remodeling modulates gene expression with high temporal- and spatial- resolution. The process of chromatin remodeling is quite complex. In the past few years, momentous experiments in yeast have yielded vast information about the intricate interactions of molecules that, alone or in combination, can influence the architecture of chromatin to allow changes in gene expression. Three general ways have emerged that can alter the chromatin structure and thus affect gene expression: ATP-dependent nucleosomal remodeling, replacement of basic core histones by other histone variants, and post-translational modifications at histone tails. These methods are not self-exclusive and independent of each other. In fact, an increasing number of studies suggest that there is interplay between histone modifications and ATP-dependent

remodeling that may be crucial for the appropriate timing of expression of specific genes (26). ATP-dependent restructuring of chromatin is accomplished by the continuous, energy-dependent shuffling of the positions of nucleosomes by molecules, which are part of the SWI/SNF remodeling complex. This shuffling allows accessibility of different nucleosomes to the transcriptional machinery at any particular time (26). Substitutions of core histone variants, such as H2AZ and H3.3 for H2A and H3, have been shown to have functional consequences as well. Finally, the third method of chromatin remodeling, post-translational modifications at histone tails, has been shown in numerous experiments to modulate gene expression, and it will be the major focus of my thesis (27, 28).

There are numerous post-translations modifications at different animo acid residues on the N-terminal tails of histones. These include histone acetylation, methylation, ubiquitination, phosphorylation, ADP-ribosylation, and sumoylation. Some common post-translational modifications include acetylation at lysine (K) residues 5, 8, 12, and 16 on histone H4, acetylation at lysine residues 9 and 14 on histone H3, phosphorylation at serine (S) residue 10 on histone H3, and methylation at lysine residues 4, 9, 27, and 36 on H3 (Figure 5).

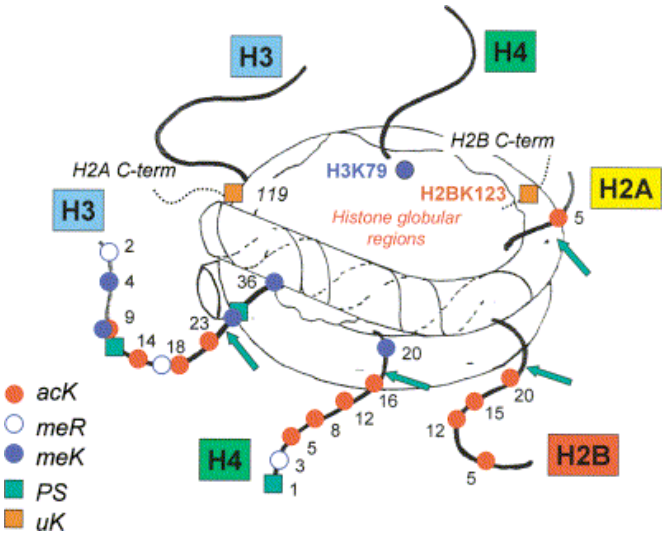



**FIGURE 5**
**(Turner 2002)**

In general, histone acetylation loosens the structural interactions between DNA and histones, allowing greater accessibility of the transcriptional machinery to the promoters of genes. Hyperacetylation promotes an increase in gene activity, whereas hypoacetylation marks a decrease in activity. In contrast, histone methylation can correlate with either gene activation (H3-K4, H3-K36, and H3-K79 methylation) or gene repression (H3-K9, H3-K27, and H4-K20 methylation), depending on the lysine residue being methylated (29). Distinct modifications of histones may also act in combination ("cross-talk") to turn genes on and off. For example, phosphorylation of H3-S10 appears to induce acetylation at K9 and/or K14 during mitogenic and hormonal stimulation in mammalian cells, marking transcriptional activation. Conversely, K9 methylation antagonizes S10 phosphorylation, leading to mitotic chromosome dysfunction (27). The

realization that histone modifications are associated with distinct patterns of gene expression, DNA-repair, or replication has lead to the development of the so called “histone code hypothesis”. According to this theory, different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state, which encodes gene activation versus gene silencing, and cell proliferation versus cell differentiation (27, 28).

There are several different enzymes that catalyze the addition or removal of acetyl, methyl, phosphate, and other groups to histone tails. Histone acetyltransferases (HATs) induce acetylation. Several transcriptional activators, such as Gcn5, p300/CBP, PCAF, TAF250, and the p160 family of nuclear receptor coactivators, contain intrinsic HAT activity. Histone deacetylases (HDACs) catalyze deacetylation, and they associate with several transcriptional repressors, such as Sin3 and NcoR/SMRT. Methylation at lysine residues is mediated by histone methyltransferases (HMTases), whereas arginine residues are methylated by protein Arginine methyltransferases (PRMTases) (30). Several months ago, an enzyme with histone demethylase activity was identified as well (31). In general, histone lysine methylation is regarded as a more robust and epigenetically stable modification than histone acetylation (32). In addition, patterns of histone methylation are intricately linked to patterns of DNA methylation. An important player in this interaction is the methyl-CpG-binding protein 2, MECP2 (33).

Despite the great progress in elucidating the mechanisms of chromatin remodeling in yeast and cancer cells, its role in the regulation of gene expression in post-mitotic adult cells like neurons, and its implication in complex physiological and behavioral phenomena in the brain, are much less clear. Nevertheless, several studies in the last few years have begun to explore the role of chromatin remodeling in the brain. Histone acetylation was shown to be important in modulating the molecular components of the circadian clock (34). Pilocarpine-induced status epilepticus seizure triggers changes in histone H4 acetylation that correlate with seizure-induced changes in the expression of BDNF and GluR2 in the hippocampus (35). Contextual fear conditioning, a hippocampus-dependent learning model, induces a significant increase in H3, but not H4, acetylation (36). CREB binding protein, CBP, which contains intrinsic histone acetyltransferase activity, was shown to be important for normal learning and memory function. Imbalance of HAT/HDAC activity in mice deficient in CBP results in memory deficits (37, 38). Histone deacetylases inhibitors enhance memory formation in two different behavioral models of long-term memory (36, 39). Synaptic plasticity, believed to underlie the formation of long-term memories, appears to have an epigenetic component as well. Elegant studies in the mollusk *Aplysia* have shown that levels of H4 acetylation around the C/EBP promoter are altered after long-term facilitation and long-term depression (40). Induction of mammalian synaptic plasticity leads to ERK signaling cascade-

dependent increase in H3 acetylation and phosphorylation (36, 41). Perturbations in HAT activity are also implicated in the pathology of polyglutamine diseases, such as Huntington's disease (42, 43). Finally, epigenetics may play a role, at least partly, in the pathology of several disorders of human cognition, such as Rubinstein-Taybi syndrome, Rett syndrome, Fragile X mental retardation, and even schizophrenia (44). The above experiments illustrate the importance of chromatin remodeling in the processes of neuronal activity, neurodegeneration, in learning and memory, in circadian rhythm, and even in human cognition. Clearly, chromatin remodeling plays an important role in mediating dynamic as well as stable changes in expression of genes, implicated in the above conditions.

Such stable changes in chromatin remodeling may also underlie some of the mechanisms of neuronal adaptations in the hippocampus that are important for its physiological function, which are maintained by antidepressants, and which may be disturbed in chronic stress and depression. No studies so far have explored the role of epigenetics in the pathophysiology of depression. My thesis provides the first look at the role of chromatin in disrupting and repairing neuroplasticity in the hippocampus as a result of chronic defeat stress and chronic treatment with chemical antidepressants and ECS. Chapter 1 describes a pioneering study to measure histone modifications at several gene promoter regions in rat hippocampus after acute, as well as chronic ECS. It also provides proof that chromatin immunoprecipitation (ChIP) assays can be reliably used to

measure levels of H3 and H4 acetylation in brain tissue. Chapter 2 describes an extensive study of the transcriptional regulation of all alternatively spliced variants of BDNF in mice after chronic defeat stress alone, or followed by treatment with the tricyclic antidepressant imipramine. In addition, it includes a detailed study of the chromatin architecture at five BDNF promoter regions and describes evidence for stable changes in modifications after chronic defeat stress, and after treatment with imipramine. Chapter 2 also explores the role of chromatin remodeling enzymes, in particular the histone deacetylase HDAC5, in modulating the efficacy of imipramine in the context of social defeat stress. The second chapter concludes with a detailed discussion, including future studies that will further elucidate the mechanistic role of chromatin remodeling in depression. Finally, the thesis offers concluding remarks about my overall findings and their significance as well as limitations. Enjoy!

## FIGURE LEGEND

**Figure 1. Regulation of HPA axis**

**Figure 2. Morphological changes in hippocampus after stress and antidepressant treatment**

**Figure 3. Intracellular signal cascade events in antidepressant action**

**Figure 4. Structure of the BDNF gene in mouse and rat.** The BDNF gene contains five non-coding exons I-V, upstream of the coding exon VI in mouse. Exons I-V can be each alternatively spliced next to exon VI, to form the 5′ UTR region of different mRNA splice variants, BDNF I-V. The BDNF gene also contains unique promoter regions upstream of each exon I-V, BDNF P1-5, which can promote the expression of their corresponding transcript variants. Representation of the homology in the BDNF gene between rat and mouse is also illustrated. Exon I, II, IV, V, and VI in mouse are homologous to exons I, II, III, IV, and V in rat, correspondingly. Exon III in mouse does not have a reported and annotated homologous sequence in rat, however exon III in mouse is homologous to a human BDNF exon. The homology analysis is based on data available in NCBI. For mRNA analysis of total BDNF, primers amplified exon VI. For mRNA analysis of BDNF I-V, primers were designed to amplify each exon I-V. For ChIP analysis, primers were designed around the putative promoters, P1-P5, which are located upstream of exons I-V. The small arrows at P4 and E IV exemplify our primer design strategy.

**Figure 5. Post-translational modifications at the tails of histones**

# CHAPTER I

# HISTONE MODIFICATIONS AT GENE PROMOTER REGIONS IN RAT HIPPOCAMPUS AFTER ACUTE AND CHRONIC ELECTROCONVULSIVE SEIZURES

**ABSTRACT**

The mechanism of action of electroconvulsive seizures (ECS), one of the most effective treatments of major depression, may involve the regulation of gene expression. Chromatin remodeling at gene promoter regions is increasingly recognized as a key control point of gene expression and may, therefore, partly mediate acute and chronic effects of ECS on gene activity. Here we assayed how post-translational modifications of histones, a major form of chromatin remodeling, are altered at several gene promoters in rat hippocampus at 30 min, 2 hr, and 24 hr after acute or repeated ECS. Chromatin immunoprecipitation assays were performed to measure levels of histone H3 and H4 acetylation and phosphoacetylation at the promoters of the c-Fos, BDNF, and CREB genes, whose expression is altered by ECS. It was found that, with few exceptions, levels of H4 acetylation correlated with mRNA levels for c-Fos, BDNF, and CREB throughout the acute and chronic time course study, while acetylation and phosphoacetylation of H3 were detected more selectively. These findings suggest that the chronic down-regulation of c-Fos transcription, observed in this study, may be achieved at the level of H4 acetylation, whereas chronic upregulation of BDNF transcription may be sustained via control of H3 acetylation, selectively at the BDNF P3 and P4 promoters. These data provide the first *in vivo* demonstration of the involvement of chromatin remodeling in ECS-induced regulation of gene expression in the brain, and will help in understanding the mechanisms underlying the efficacy of ECS in the treatment of depression.

## INTRODUCTION

Chromatin remodeling is a dynamic process, which modulates gene expression in dividing as well as non-dividing cells, such as neurons (35, 40, 41). Histone modification represents one prominent form of chromatin remodeling. According to the "histone code theory," different modifications of histones at a particular promoter region, alone or in combination, define a specific epigenetic state, which encodes gene activation versus gene silencing (27). Acetylation of histones loosens the structural interactions between DNA and histones, allowing the transcriptional machinery access to the promoters of particular genes. Hyperacetylation at promoters indicates an increase in gene activity, whereas hypoacetylation marks a decrease in activity (45). Acetylation at multiple lysine residues on the N-terminal tail of histone H4, as well as acetylation at Lys9 and 14 on histone H3, are common modifications enriched at transcriptionally active genes (28). Furthermore, Ser10 phosphorylation of H3 is synergistically coupled to its acetylation at Lys14 (46).

Here, I examine how acute or chronic ECS alters these histone modifications in the hippocampus, a brain region implicated in the pathophysiology of depression in humans and in models of depression in animals (1, 7, 16). Acute seizures increase neuronal activity and stimulate the expression of numerous genes, including the immediate-early gene c-Fos and brain-derived

neurotrophic factor BDNF. Levels of c-Fos mRNA peak within minutes, while levels of BDNF mRNA peak a few hours after the onset of ECS, but both increases are transient (19, 47). Repeated administration of ECS is one of the most effective treatments of depression, but the molecular mechanisms underlying its clinical effects are incompletely understood (1, 7, 16, 48, 49). Several molecules, however, have been implicated to play a role in chronic ECS action, including BDNF and the transcription factor CREB (cAMP-response element binding protein). Levels of BDNF and CREB are upregulated in hippocampus by chronic ECS (19, 23), effects directly linked to antidepressant activity in animal models (20, 22). Furthermore, chronic ECS causes a long-term downregulation of c-Fos mRNA levels (50).

One way ECS could induce long-term changes in the expression of particular genes is through alterations in histone modifications at the promoter regions of these genes. This study provides the first endeavor to characterize such chromatin modifications in brain after acute and chronic ECS. Using chromatin immunoprecipitation assays, I measured levels of H4 acetylation, H3 acetylation, and H3 phosphoacetylation at the c-Fos, BDNF, and CREB promoters in the hippocampus of rats at 30 min, 2 hr, and 24 hr after an acute or repeated ECS. Our data indicate complex, time-dependent changes in histone modifications at these promoters, which provide new insight into the mechanisms governing ECS-induced regulation of gene expression in the brain.

## MATERIALS AND METHODS

### ECS Treatment:

Adult male Sprague Dawley rats, 250-275 g, received a single ECS via ear-clip electrodes (acute group) or daily ECS for 7 days (chronic group) between 11 am-12 pm each day (ECS frequency, 100 pulses/sec; pulse width, 0.5 ms; shock duration, 0.5 sec; current, 50 mA). Control animals received sham treatments: they were handled identically to ECS-treated animals but without electrical stimulation. Animals were sacrificed by decapitation 30 min, 2 hr, or 24 hr after the last seizure.

### Chromatin immunoprecipitation assays:

Brain tissue was processed into chromatin by use of published protocols (51, 52) with some modifications. Whole hippocampus, including subiculum, was removed from decapitated rats by gross dissection, minced to ~1 mm-sized pieces, and immediately cross-linked in 1% formaldehyde for 15 min at room temperature. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M. The tissue was washed 4-6 times in cold PBS

containing proteinase inhibitors (1 mM PMSF, 1 μg/ml apoprotin, and 1 μg/ml pepstatin A) and then frozen on dry ice.

The chromatin was solubilized and extracted by detergent lysis followed by sonication. First, minced, fixed hippocampal tissue was homogenized twice, for 10 sec, in a cell lysis buffer [10 mM Tris, 10mM NaCl, 0.2% Nonidet P-40]. The homogenate was centrifuged at 5500xg for 5 min. The supernatant, containing extracellular debris, was decanted, and the pellet was homogenized two more times, for 10 sec, using nuclear lysis buffer (Upstate Biotechnology, Lake Placid, NY, ChIP kit #17-295). Next, the extracted chromatin was sheared to 400-600 bp using the Sonic Dismembrator 550 (Fisher, Hampton, NH). Each sample was sonicated 5 times on ice, 20 sec each, at 25% of maximum power.

Chromatin immunoprecipitation assays were performed to measure the levels of histone acetylation or phosphoacetylation at various promoter regions. A protocol outlined in Upstate Biotechnology Inc. ChIP Kit was used, with some modifications. After the chromatin lysate was extracted and properly fragmented to 400-600 bp, the optical density of each sample was determined. Equal amounts of chromatin lysate, 60 μg, were diluted with ChIP dilution buffer (Upstate, #17-295) to a final volume of 1.5 ml. 100 μl of the pre-immunoprecipitated lysate were saved as "input" for later normalization.

The chromatin solution was pre-cleared with salmon sperm DNA/protein A-agarose 50% gel slurry (Pierce, Rockford, IL, #22811) for 45 min. It was then

immunoprecipitated overnight at 4°C with 5 μg of antibody directed against H3 acetylated on Lys9 and 14 (#06-599), 7 μg of antibody directed against H3 phosphorylated at Ser10 and acetylated at Lys14 (#07-081), and 5 μg of antibody directed against H4 acetylated at Lys5, 8, 12, and 16 (#06-866) (Upstate). The specificity of these antibodies in chromatin immunoprecipitation assays has been established (35, 46). As a control, samples were immunoprecipitated with 5 μg non-immune rabbit IgG (Upstate, #12-370). Following immunoprecipitation, the DNA-histone complex was collected with 40 μl salmon sperm DNA/protein A-agarose beads for 2 hr. The beads were sequentially washed once with low salt, high salt, LiCl, and twice with TE buffers (Upstate, # 17-295). The DNA-histone complex was then eluted from the beads with 500 μl $NaHCO_3$/SDS elution buffer. DNA and histones were dissociated at 65°C for 4 hr under high-salt conditions. Proteins were digested using Proteinase K treatment for 1 hr at 45°C. The DNA, associated with acetylated and phosphoacetylated histones, was extracted with phenol/chlorophorm/isoamyl alcohol, precipitated with 100% ethanol, and finally resuspended in 80 μl of PCR-grade water. Most ChIP experiments were performed twice, on two independent tissue samples, for confirmation.

**Quantification of DNA by real-time PCR:**

Levels of specific histone modifications at each gene promoter of interest were determined by measuring the amount of acetylated or phosphoacetylated histone-associated DNA by real-time qPCR (Applied Biosystems (ABI) Prism 7700, Foster City, CA). Specific primers were designed to amplify proximal promoter regions, less than 200 bp long. For c-Fos, the primers 5′ TTCTCTGTTCCGCTCATGACGT 3′ and 5′ CTTCTCAGTTGCTAGCTGCAATCG 3′ amplified a region 140 bp upstream of the start codon, which contains a TATA box and a CRE consensus sequence. The following primers were used to selectively amplify portions of the BDNF P1, P2, P3, or P4 promoter: for BDNF P1, 5′ TGATCATCACTCACGACCACG 3′ and 5′ CAGCCTCTCTGAGCCAGTTACG 3′; for BDNF P2, 5′ TGAGGATAGGGGTGGAGTTG 3′ and 5′ GCAGCAGGAGGAAAAGGTTA 3′; for BDNF P3, 5′ GCGCGGAATTCTGATTCTGGTAAT 3′ and 5′ GAGAGGGCTCCACGCTGCCTTGACG 3′; for BDNF P4, 5′ TGCAGGGGAATTAGGGATAC 3′ and 5′ TCTTCGGTTGAGCTTCGATT 3′. For CREB, the primers 5′ CATTTACTAACCCAGCCACCACA 3′ and 5′ GGAGGAGCGTTACAAGCCCTAC 3′ amplified a region of the CREB promoter at –100 bp. Finally, β-tubulin (5′ TAGAACCTTCCTGCGGTCGT 3′ and 5′ TTTTCTTCTGGGCTGGTCTC 3′), synaptophysin (5′ TCATCTGGTAGAACTGAGCGGTC 3′ and 5′

GAGGCTGTGGGTTTTAGAGGAA 3′), and ε-globin (5′ TGACCAATAGTCTCGGAGTCCTG 3′ and 5′ AGGCTGAAGGCCTGTCCTTT 3′) were used as controls. Input and immunoprecipitated DNA amplification reactions were run in triplicate in the presence of SYBR-Green (ABI). Ct values from each sample were obtained using the Sequence Detector 1.1 software. Relative quantification of template was performed as described earlier by Chakrabarti et al. (53) and by the ABI manual with some modifications. Briefly, a ΔCt value, representing the difference between Control Ct and Experimental Ct (acute or chronic) was calculated, using the formula: $\mathbf{\Delta Ct} = (\mathbf{N}_{\text{acute,chronic}} - \mathbf{Nave}_{\text{control}})*\mathbf{Ctave}_{\text{control}}$, where **N** is the normalized Ct value of H4 [Ct(H4)/Ct(Input)] or of H3 [Ct(H3)/Ct(Input)], **Nave** is the mean N value for the control, and **Ctave** is the mean Ct value for the control. Fold differences (Acute or Chronic ChIP relative to Control ChIP) were then determined by raising 2 to the ΔCt power. Mean and standard error of mean values were determined for each fold difference, and these values were used in two-tailed paired *t* tests (which were adjusted for multiple comparisons) to determine statistical significance ($P<0.05$). Each PCR reaction, run in triplicate for each brain sample, was repeated at least two independent times.

**Measuring mRNA levels by RT-PCR:**

Whole hippocampus of rats receiving acute or chronic ECS treatments was collected for RNA quantification. RNA was extracted using Trizol reagent from RNA STAT-60™ and precipitated with isopropanol. The purified RNA was DNase-treated (Ambion, Austin, TX). mRNA was reverse-transcribed to cDNA using Invitrogen's first strand synthesis kit (Carlsbad, CA). The amount of cDNA was also quantified using real-time PCR. The following primers were used to amplify specific cDNA regions of the transcripts of interest: c-Fos (5′ GGAATTAACCTGGTGCTGGA 3′ and 5′ TGAACATGGACGCTGAAGAG 3′), BDNF (total) (5′ CCATAAGGACGCGGACTTGTAC 3′ and 5′ AGACATGTTTGCGGCATCCAGG 3′), CREB (5′ AGTGACTGAGGAGCTTGTACCA 3′ and 5′ TGTGGCTGGGCTTGAAC 3′), and GAPDH (5′ AACGACCCCTTCATTGAC 3′ and 5′ TCCACGACATACTCAGCAC 3′). Primers for BDNF exons I-IV, driven by promoters P1 through P4, respectively, were used as previously described (54). GAPDH quantification was used as an internal control for normalization. Fold differences of mRNA levels over control values were calculated using the ΔCt method as described previously (ABI manual). PCR reactions were repeated at least two independent times.

# RESULTS

## 1. Initial standardization of the chromatin immunoprecipitation assay for brain tissue

In order to examine the chromatin architecture at the promoters of genes regulated by ECS, I took advantage of a recently developed technique: the chromatin immunoprecipitation (ChIP) assay. This technique had already been used to study chromatin remodeling in cell lines and in yeast. However, when I started my studies, only one paper had reported the use of ChIP for brain tissue. Therefore, my first project in the lab was to standardize chromatin immunoprecipitation for measuring histone modifications in brain.

There are several essential steps in performing the ChIP assay: 1) extraction of the brain region of interest; 2) formaldehyde cross-linkage of DNA to histones (and other factors bound to the DNA at the time of brain extraction); 3) extraction of the nuclear, fixed chromatin material (referred later as the lysate); 4) sonication of the chromatin into pieces less than 1kb; 5) determination of the concentration of lysate; 6) immunoprecipitation with an antibody specific for a histone N-terminal tail modification or a transcription factor; 7) collection of the immune complex using protein A (or G) beads; 8) washes of the beads to remove non-specific antibody binding, and elution of the antibody-chromatin complex.

Next, one can analyze either the amount of protein in the immunoprecipitated complex by 9) Western blot or the amount of DNA, associated with the protein, by 10) PCR. In order to quantify the amount of antibody-associated DNA, the protein must be reverse-cross linked at high temperature and high salt concentration; then it must be degraded by proteinase treatment; finally, the DNA must be extracted and purified. Below, I provide detailed information about the optimization of each of the above-mentioned steps in chromatin immunoprecipitation for brain tissue.

1. Extraction of brain tissue

The important factors in extracting brain tissue for chromatin immunoprecipitation are similar to the general considerations in processing tissue for protein analysis. The extraction should proceed as fast as possible, and the brain should be placed immediately in ice-cold artificial cerebrospinal fluid, ACSF, or phosho-buffered saline, PBS. The cold temperature is important, as this will prevent the degradation of proteins by proteinases. Addition of proteinase inhibitors is also important to ensure that proteinases are inactivated. I have obtained better yields by saturating ACSF or PBS with 100% oxygen for anywhere between 15minutes to 45 minutes just prior to the experiment. The presence of O2 in cold ACSF decelerates the death of brain tissue. Some people

have suggested that adding sodium butyrate, a histone deacetylase inhibitor, to cell lines before fixation also improves the yield. However, I found that the addition of sodium butyrate, either before or after cross-linking, did not improve, and may have even decreased my chromatin yield, and it did not appear to affect levels of H4 acetylation at the c-Fos promoter. Thus, I decided not to add this inhibitor in my further studies. Nevertheless, I only analyzed two animals, so it cannot be concluded that addition of sodium butyrate may not be beneficial in some experiments.

2. Formaldehyde cross-linking

Most protocols in cell culture use 1% formaldehyde for cross-linking, and the incubation varies from 5-30 minutes at either room temperature or 37°C. Longer incubations at 25°C are analogous to shorter incubations at 37°C. Since it was inconvenient to perform cross-linking at 37°C, I standardized cross-linking times at room temperature only. I cross-linked tissue for 5, 10, 15, 20, 25, and 30 minutes, and assayed levels of H4 acetylation at the c-Fos promoter. I found that 5 minutes, 25 minutes, and 30 minutes yielded lower levels of c-Fos H4 acetylation than 10 and 20 minutes (notebook#1, page 128). My future cross-linking reactions were at either 12 or 15 minutes for histone modification analysis, or 10 minutes for transcription factor analysis.

3. Extraction of nuclear lysate

The extraction of the chromatin lysate from the nucleus is an important step. The standard ChIP protocol from Upstate Biotechnology, used by many for cell culture experiments, contains a single step for extraction of nuclear material using 1% SDS buffer. However, I have found that using cell lysis buffer, containing 0.2% Nonidet P-40, prior to the nuclear lysis buffer increases my eventual chromatin yield by more than two-fold (notebook#1, page 123). Thus I first homogenized my brain tissue in cell lysis buffer, collected the lysate pellet at very low speed (500-2000xg), and then resuspended and homogenized the pellet again, this time in nuclear lysis buffer. I added proteinase inhibitors to both lysis buffers, and kept the tissue on ice in between homogenizations.

4. Sonication

After the nuclear material has been extracted, chromatin must be vigorously sonicated in order to shear the DNA into as small fragments as possible. Since the building unit of chromatin, the nucleosome, encompasses approximately 200bp of DNA, sonication does not yield fragments smaller than 200bp. I have spent considerable amount of time standardizing the process of

sonication, and found that I obtain optimal size fragments, between 400-600bp, when I sonicate 5 times, 20 seconds each time, in a volume of 500μL into a 1.5mL eppendorff tube, using the Sonic Dismembrator 550 (Fisher, Hampton, NH) at maximal setting, #4. Sonication for 10 seconds, 4 times, yielded fragments between 400-1300bp (notebook#1, page 17; notebook#2, page 25). Also, sonication in a tube bigger than 1.5mL produced a lot of froth, which impeded proper sonication (notebook#1, page 57). Sonication in bigger volume can be performed, but appropriate mixing of the lysate during and after sonication is required in order to achieve homogenous level of shearing.

5. Quantification of the sheared chromatin lysate

It is important that similar amount of chromatin is used in all immunoprecipitation reactions. I use the Nanodrop® spectrophotometer to measure $A_{260}$ absorbance of DNA in order to quantify the amount of chromatin extracted. Typically, I obtain an $A_{260/280}$ ratio of ~1. My usual yield for whole hippocampus is around 250-300ng/μL (notebook#2, page 109). For chromatin immunoprecipitation reactions of hippocampus, I have used successfully as little as 40μg of chromatin and as much as 100μg (notebook#1, page 90). Typically, I used 60μg. The lysate is diluted in a buffer to a final concentration of 1.5mL. From each diluted sample, I extract 100μL, the so-called "input," which is non-

immunoprecipitated lysate. Because quantification by measuring optical density is not extremely precise, this input was used as an additional normalizer of our starting lysate material for immunoprecipitation. The "input" chromatin is processed identically to immunoprecipitated chromatin.

For proteins that are less abundantly bound to DNA, such as transcription factors, higher starting amount may be necessary than for modified histones. In most of my studies, I have used whole hippocampus as my starting tissue material. Using smaller brain fragments can be challenging, due to their low yield of chromatin. Nevertheless, I have been successful in using as little as 3 punches (15gauge) for ChIP. Such amount yielded about 20μg of chromatin, sufficient to immunoprecipitate with one antibody (of high specificity) and assay binding levels at 1-3 promoter regions (notebook#2, pages 35-37).

6. Antibody immunoprecipitation

The specificity of antibody binding is, I believe, the most crucial factor for the validity of this assay. For this reason, it is important to use an antibody that has already been shown to have good specificity, or to check the specificity first. One way to control for antibody specificity is to immunoprecipitate an equal amount of lysate with a non-specific IgG antibody or without an antibody (protein beads only). It is then advisable to test for the presence of the antibody target in

the negative IgG or no-antibody controls by Western blot, or by PCR. I have performed a western blot for acetylated H4 (Figure 10-A), and I routinely perform an IgG and no-antibody negative controls in all of my immunoprecipitation reactions followed by PCR analysis (notebook#1, page 98, Figure 6-B, Figure 10-B). An immunoprecipitation reaction with an antibody that does not yield significantly more DNA than an IgG control, at least 4-fold difference in my opinion, at an expected binding site within a promoter points to the lack of specificity in the antibody used. It is also important to determine the optimal dilution of each antibody used for ChIP. My preliminary optimization experiments indicated that 1:100 and 1:200 dilution factors of the anti-histone H4 antibody yielded higher level of H4 acetylation at the c-Fos promoter than 1:500 and 1:1000 (notebook#1, page 89). In a separate experiment, using 10μL (1:150) of the same antibody resulted in higher levels of H4 acetylation at c-Fos than using 15μL (1:100) (notebook#1, page 93). In future experiments, I used 7.5μL of antibodies against acetylated histones and obtained high levels of acetylation compared to IgG controls (Figure 6-B).

7. Collection of the antibody-chromatin complex

I have used Protein A beads supplied by Upstate (ChIP Kit, #17-295, or Salmon Sperm DNA/Protein G Agarose, #16-201) and by Pierce (ImmunoPure®

Immobilized Protein A Plus, #22812), and have not found a difference in the specificity for antibody pull down by any one of these three beads. The agarose beads are pre-incubated with salmon sperm DNA, which appears to reduce (although it does not always eliminate) non-specific DNA binding. I have noticed that using a lesser amount of beads can reduce the background due to non-specific binding. In this regard, I noticed that using 40μL of Protein A beads + 3μL of salmon sperm DNA yielded better specificity than using 60 + 4μL beads/sperm mixture. I have used 43μL mixture in most of my studies.

8. Washes

Washing the immunoprecipitated complex is important for reducing non-specific antibody binding. I have followed the washing protocol outlined in the Upstate ChIP Kit protocol (Upstate Cell Signaling Solutions, #17-295) with minor modifications. These included the addition of a ChIP dilution buffer wash prior to the "Low Salt" wash and two washes with TE buffer, rather than one. All washes were performed for 7 minutes, on a rotator, at room temperature, and without the addition of proteinase inhibitors, unless the chromatin complex was used for subsequent protein analysis, in which case I used proteinase inhibitors and performed the washes at 4°C.

9. Western blot analysis

The Western blot analysis was performed using standard protocol.

10. Quantification of immune complex-associated DNA by PCR

Many ChIP experiments using cell culture have used regular PCR to quantify the amount of chromatin-associated DNA. The disadvantage to this method is that the reaction must be stopped during the linear phase of amplification and before it reaches saturation. In addition, analysis of the amount of DNA involves densitometry, which is not a very sensitive method to detect small but significant differences in levels of DNA. Given that many changes in the brain are more subtle than seen with cells stimulated in culture, I chose to analyze my data using real-time PCR. Real-time PCR allowed detection of significant changes in levels of DNA of less than 2-fold. The disadvantage to using real-time PCR is that primers must have exceptional efficiency for binding to their amplicon. Primers with moderate binding efficiency often bind to each other or to themselves as well, forming the so-called "primer-dimers." Primer-dimers are unacceptable in real-time PCR analysis, since they incorporate the same fluorescence as the amplifying product, and thus distort the actual output data. Designing and optimizing primers has been very challenging at times. All

primers that have been designed have been run on a gel to ensure the absence of primer-dimers. In addition, the primer efficiency has been determined for most by running standard curves. In general, primers with efficiency less than 90% and greater than 110% have not been used in subsequent data analysis. The "Primer3" website (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) has been very helpful in designing primers with acceptable binding and amplification efficiencies. Additional modulation of primer concentration, magnesium concentration, and annealing temperature has been used to optimize primer conditions (notebook#1, page 115). Finally, deciding the exact promoter region to be amplified has, in some cases, been challenging as well due to the lack of well-defined promoter regions for some genes. In such cases, I have used promoter- or transcription factor- prediction programs, TATA sequences, and literature on previous promoter analyses, to define the most likely promoter region for a gene of interest.

### 2. Quantification of histone modifications by chromatin immunoprecipitation assays

To better understand the molecular actions of ECS, I studied the histone architecture at the promoter regions of the c-Fos, BD NF, and CREB genes in the hippocampus after acute or repeated ECS. Since chromatin remodeling is a

dynamic process, I studied histone modifications at these promoters at several different time points (30 min, 2 hr, and 24 hr after acute ECS, and 2 and 24 hrs after chronic ECS) to clearly differentiate between acute and chronic ECS effects. At these time points, the hippocampus was extracted, minced, and fixed in 1% formaldehyde to cross-link proteins bound to DNA *in vivo*. The cross-linked chromatin was then sheared to fragments of approximately 500 bp in length via vigorous sonication (Figure 6-A). I then performed chromatin immunoprecipitation assays with antibodies against polyacetylated H4 (acH4), acetylated H3 (acH3), or phosphoacetylated H3 (p/acH3), and quantified the amount of DNA associated with the modified histones using real-time PCR.

Several controls were performed to confirm the specificity and validity of our assays. To control for the specificity of antibody binding, I immunoprecipitated chromatin samples with non-immune IgG, which precipitated negligible levels of the various genes studied (Figure 6-B). To ensure that our technique allows us to measure acetylation of histones only at promoter regions in the genome where acetylation is present *in vivo* (i.e., active genes), I confirmed that acetylation is absent in hippocampal tissue at transcriptionally silenced genes, such as the ε-globin gene, which is inactivated within neurons early in development (Figure 6-B). Finally, I measured levels of histone acetylation in hippocampus at the promoters of the β-tubulin and synaptophysin genes, which are expressed in adult hippocampus but whose expression levels are reportedly

unchanged in this region after ECS (55, 56). Indeed, levels of histone modifications at these loci did not differ between control and ECS-treated animals (Figure 6-B and Figure 7). These findings indicate that the observed changes in histones at the c-Fos, BDNF, and CREB genes, which are presented below, are not global but are rather limited to genes whose expression varies as a result of ECS treatment.

### 3. Histone modifications at the c-Fos promoter

I first assayed histone modifications at the c-Fos promoter after acute and chronic ECS and found that H4 acetylation at the c-Fos promoter in hippocampus increases more than 3-fold over control values as soon as 30 min after an acute ECS (Figure 7 and Figure 8-A; Table 1). This finding was not surprising, given that c-Fos is an immediate-early gene that is activated within minutes of neuronal stimulation. In fact, seizures have been shown to induce the rapid and transient increase in c-Fos mRNA levels in hippocampus, peaking 45 min after seizure onset (47). At 2 hr after an acute ECS, H4 acetylation at the c-Fos promoter was still about 3-fold higher compared to control animals. In addition, I was able to detect a 3.7-fold increase in phosphoacetylated H3 at this time point. By 24 hr, the level of H4 acetylation had returned to control, and the level of H3 phosphoacetylation was decreased below the level of detection. Levels of

acetylated H3 (i.e., H3 acetylation without concomitant phosphorylation) were assayed as well. I did not find any statistically significant changes in H3 acetylation at the c-Fos promoter 30 min, 2 hr, or 24 hr after acute ECS, although this measure was highly variable for c-Fos.

Chronic ECS generally elicited similar histone modifications at the c-Fos promoter in hippocampus. Levels of H4 acetylation and H3 phosphoacetylation were both increased 2 hr after chronic ECS, and measures of H3 acetylation were highly variable. However, one prominent difference between the acute and chronic setting was observed: levels of H4 acetylation were significantly decreased 24 hr after chronic ECS, an effect not observed in the acute situation (Figure 7 and Figure 8-A; Table 1). Given the earlier report that chronic ECS desensitizes the c-Fos gene in hippocampus (50), the reduction I observed in H4 acetylation after chronic ECS conditions implicates this step of chromatin remodeling as a feature of the desensitization process.

To test the degree to which H4 acetylation correlates with the transcriptional activity of the c-Fos gene, I measured c-Fos mRNA levels in hippocampus after acute or chronic ECS. Levels of c-Fos mRNA were quantified using real-time PCR, and normalized to GAPDH mRNA levels which were unaffected by ECS treatments (not shown). As reported previously (47), c-Fos mRNA expression was dramatically (30-fold) induced 30 min after an acute ECS, remained elevated (15-fold) at 2 hr, and returned to control values by 24 hr

(Figure 8-A; Table 1). In contrast, 24 hr after chronic ECS, mRNA levels were significantly decreased to ~60% of control levels. These results replicate downregulation of c-Fos transcription after chronic ECS (50). In addition, the results provide further evidence that, for the c-Fos gene, levels of H4 acetylation correlate with levels of transcription, while no such general correlation was apparent between c-Fos transcription and levels of H3 phosphoacetylation or acetylation. It is important to emphasize, of course, that these are correlations only, and that causal relationships between histone modification and gene expression in the brain *in vivo* will require further investigation.

### 4. Histone modifications at the BDNF promoters

I next examined how histone modifications are altered in hippocampus by acute and chronic ECS at the BDNF gene, which has been directly implicated in the antidepressant actions of this treatment (see Introduction). I first examined how histone modifications are altered at the BDNF P2 promoter, one of four BDNF promoters (P1 through P4) that can differentially regulate BDNF expression in brain (57). Each promoter drives the expression of a distinct mRNA, although each transcript encodes an identical BDNF protein. Compared to the other BDNF promoters, P2 shows the most prominent increase in H4 acetylation in hippocampus after an acute episode of pilocarpine-induced seizure

(35), suggesting that acute ECS might similarly increase histone acetylation preferentially at this BDNF promoter. Thirty min after an acute ECS, I did not detect any significant change in H4 acetylation, H3 acetylation, or H3 phosphoacetylation at the P2 promoter (Figure 9; Table 1). Levels of total BDNF mRNA were increased at this time point, but only by 1.8-fold. Two hr after acute ECS, however, I detected an almost 3-fold increase in H4 acetylation, but no change in H3 acetylation or phosphoacetylation. The increase in H4 acetylation at the P2 promoter 2 hr after acute ECS correlates with the nearly 5-fold increase in total BDNF mRNA levels at this time point. This induction of BDNF mRNA expression in hippocampus is consistent with previous findings (19). Twenty-four hr after acute ECS, when total BDNF mRNA levels had returned to control values, levels of H4 acetylation at the P2 promoter had partially recovered toward control levels as well.

Examination of the effects of chronic ECS revealed several dramatic shifts in the regulation of histone modifications at the BDNF gene (Figure 9; Table 1). Again focusing on the BDNF P2 promoter, levels of H3 acetylation and phosphoacetylation were increased 2 hr after chronic ECS, while they were not affected by acute ECS at this time point. By 24 hr after chronic ECS, however, these modifications no longer showed a significant change over control levels. Furthermore, while levels of H4 acetylation at the P2 promoter were induced 2 hr after chronic ECS (as seen also for the acute situation), by 24 hr levels of H4

acetylation were dramatically (~4-fold) decreased. This decrease did not correlate with the sustained ~2-fold increase in total BDNF mRNA levels observed 24 hr after chronic ECS administration. This dissociation prompted us to, first, measure the mRNA levels driven by each distinct BDNF promoter and, second, to study how ECS affects the histone architecture at the other BDNF promoters: P1, P3, and P4, particularly under chronic treatment conditions.

Quantitative RT-PCR analysis of BDNF mRNA driven by the P1 through P4 promoters revealed that 30 min and 2 hr after acute ECS the mRNA levels of all four splice variants were significantly upregulated (data not shown). These data confirm previous studies of the promoter-specific regulation of BDNF mRNA following acute ECS or kainic acid treatments (57, 58). Chronic ECS, however, showed more restricted BDNF upregulation. While BDNF mRNA driven by the P1, P2, or P3 promoter was not significantly increased 24 hr after chronic ECS, BDNF mRNA driven by P4 showed a sustained 2-fold increase, which was highly significant (209 ± 29% of control ± SEM, $n=4$, $p<0.05$). This suggests that the increase in total BDNF mRNA levels seen 24 hr after chronic ECS treatment is due to the selective activation of the BDNF P4 promoter.

Next, I examined changes in histone modifications at the BDNF P1, P3, and P4 promoters after acute or chronic ECS and compared these changes with our mRNA data. In contrast to the observed increase in H4 acetylation at the BDNF P2 promoter after acute ECS, there were no changes in H4 acetylation, but

trends for induction of H3 acetylation, at the P3 and P4 promoters (Figure 9; Table 1). Moreover, these latter promoter regions showed significant chromatin regulation after chronic ECS. In particular, I observed a several-fold increase in H3 acetylation at the BDNF P3 and P4 promoters 24 hr after chronic ECS, which correlated with the increase in BDNF mRNA levels driven by the P4 promoter at this time point. H4 acetylation and H3 phosphoacetylation did not change significantly at the P3 or P4 promoters 24 hr after chronic ECS.

### 5. Histone modifications at the CREB promoter

CREB is a transcription factor that regulates the expression of many genes and has been implicated directly in the antidepressant effects of ECS treatment in hippocampus (as mentioned above). Furthermore, CREB is known to regulate the transcription of the c-Fos and BDNF genes by binding to CRE sites within their promoters in its active (dimerized, phosphorylated) form. It was, therefore, of particular interest to study histone modifications at the CREB gene at varying time points after acute or chronic ECS.

I found a significant, ~3-fold decrease in H4 acetylation at the CREB gene promoter in hippocampus 30 min after an acute ECS (Figure 8-B; Table 1). There was no significant change in H3 acetylation or phosphoacetylation at this time point. Histone modifications at the CREB promoter at 2 hr after acute ECS were

more divergent. First, there was a large, 5-fold increase in H4 acetylation relative to control animals. This increase amounted to an 8-fold change in H4 acetylation from 30 min to 2 hr after acute ECS. In contrast, levels of H3 acetylation showed a significant 2.7-fold decrease at 2 hr after acute ECS. At 24 hr after acute ECS, no parameter of histone modification was significantly affected at the CREB promoter. I also observed no significant change in CREB mRNA levels at 30 min, 2 hr, or 24 hr after acute ECS, although there was a trend toward a small (50%) increase in CREB expression at 2 hr (Figure 8-B; Table 1).

A similarly complicated pattern of histone modification at the CREB promoter in hippocampus was observed after chronic ECS. Chronic ECS no longer increased H4 acetylation levels at the CREB promoter at the 2 hr time point as seen for acute ECS. In fact, a ~2.5-fold decrease in H4 acetylation levels were apparent by 24 hr after chronic ECS. In addition, levels of H3 acetylation were reduced at 2 hr, but increased at 24 hr, after chronic ECS. Finally, as seen for most measures, levels of phosphoacetylated H3 were low and variable and showed no significant regulation by chronic ECS. I also found that chronic ECS causes a small, but significant, decrease in CREB mRNA levels in hippocampus at 24 hr. This was a surprising finding, given the previous report that chronic ECS increases CREB mRNA expression in this brain region (23). The explanation for this divergent finding is unknown. One possibility is that Nibuya et al. (23) had demonstrated increased CREB expression in specific subfields of

hippocampus by in situ hybridization, whereas the regulation of CREB mRNA levels and histone modifications reported here would reflect global changes in these measures throughout the hippocampus.

## DISCUSSION

ECS remains one of the most effective treatments for major depression. Like chemical antidepressants, ECS exerts its clinical effects only after repeated administration, suggesting that long-term adaptations at the level of gene expression might be involved. The goal of the present study was to determine whether some of the genomic effects of ECS could be mediated at the level of chromatin remodeling, which has been shown in recent years to be a major determinant of gene regulation. I studied the effect of acute and chronic ECS on several common post-translational modifications of histones H3 and H4 at three ECS-regulated gene promoters: the immediate-early gene c-Fos, the neurotrophic factor BDNF, and the transcription factor CREB, in the hippocampus. I found that, with few exceptions, levels of H4 acetylation correlate best with the expression of c-Fos, BDNF, and CREB mRNA levels at several time points after acute and chronic ECS. This important observation supports the hypothesis that histone hyperacetylation is associated with increased levels of gene activity in brain, and proposes that measuring levels of H4 acetylation at a promoter might serve as a novel marker for the dynamic, *in vivo* state of a gene's activity. There are two exceptions to this observed correlation, however, that should be highlighted.

First, levels of H4 acetylation were not a reliable measure for CREB expression during acute ECS treatment: I did not observe a significant increase in CREB mRNA levels after acute ECS, even though H4 acetylation increased several fold at the CREB promoter under these conditions. One possible explanation for this discrepancy is that other modifications at the CREB promoter counterbalance the effect of H4 acetylation. Chromatin remodeling is a dynamic process, in which several histones can be modified within close temporal and spatial proximity. As suggested by the histone code hypothesis, a combination of several histone modifications may ultimately determine the outcome of gene expression. In support of this possibility, I observed a significant decrease in H3 acetylation at the CREB promoter after an acute ECS.

The second exception to the observed link between H4 acetylation and transcript levels was noticed at the BDNF P2 promoter 24 hr after chronic ECS, when total BDNF mRNA levels were increased, as previously reported (19), while H4 acetylation was significantly decreased. A likely explanation for this discrepancy is that BDNF transcription is being regulated differently chronically than it is acutely. In support of this, I found that while acute seizure induces all four BDNF promoters as shown earlier (57, 58), chronic ECS selectively induces the P4 promoter only. Moreover, I showed that among the histone modifications examined, only H3 acetylation showed a significant increase after chronic ECS and this increase was specific for the P4 and P3 promoters. Recently, H4

acetylation at the BDNF P2 promoter was shown to increase significantly after an acute episode of pilocarpine-induced seizure, whereas no change was evident at the P3 promoter and a slight decrease was seen at the P4 promoter (35). In our study, we have confirmed that acute seizure, in this case ECS, increases H4 acetylation selectively at the BDNF P2 promoter. Interestingly, such chromatin remodeling appears to shift toward the P3 and P4 promoters under chronic ECS conditions. It is noteworthy that while only BDNF P4 mRNA levels were significantly increased 24 hr after chronic ECS, H3 acetylation was induced at both the P4 and P3 promoters. This increase at P3, in the absence of increased BDNF P3 mRNA levels, could be explained by the fact that the P3 and P4 promoters are only 0.8 kb apart in the primary BDNF transcript (25), thus permitting some of the histone enrichments at P4 to also be detected at the P3 promoter.

H3 acetylation at Lys9 and 14, similar to H4 acetylation, is found in transcriptionally active promoters (28). Our findings suggest that while acetylation of H4 modulates BDNF expression through the P2 promoter in acute seizure conditions, acetylation of H3 at the P4 and P3 promoters may be the more important determinant of BDNF expression after chronic ECS. The decrease in H4 acetylation at the BDNF P2 promoter after chronic ECS could, in fact, indicate that this promoter is being suppressed chronically to allow the other promoters to override control of BDNF expression. Previous studies have

proposed a role for CREB in mediating the sustained induction of BDNF expression in hippocampus after chronic ECS (16, 24, 48). It will be important in future studies to determine whether the induction of H3 acetylation observed here is related to this CREB-dependent mechanism of BDNF gene regulation.

In contrast to the regulation of H4 acetylation, which was seen at all three genes studied, regulation of H3 phosphoacetylation was much more restricted. Levels of phosphoacetylated H3 showed a large increase at the c-Fos promoter specifically 2 hr after an acute ECS. A recent study reports that the seizure-inducing drugs pilocarpine and kainic acid induce rapid, transient phosphorylation of H3 at Ser10 in hippocampal neurons (41). This modification was coupled to H3 acetylation at nearby Lys14, and was correlated with the activation of the mitogen-activated protein (MAP) kinase pathway and the induction of c-Fos transcription. These and other findings have led to the suggestion that phosphoacetylation of H3 is the most direct link between signal transduction and histone modification (41, 46, 59, 60). Here, we similarly show that acute ECS treatment increases H3 phosphoacetylation; however, this change was delayed compared to the induction of H4 acetylation and c-Fos mRNA levels. Moreover, the only other significant change in H3 phosphoacetylation observed in this study was at the BDNF P2 promoter 2 hr after chronic ECS, an effect not observed after acute ECS when BDNF mRNA levels are greatly induced. Therefore, it appears that H3 phosphoacetylation *in vivo* occurs transiently at the promoters of some

highly reactive genes, i.e. c-Fos, but not all such genes, i.e. BDNF. Moreover, some unique changes in H3 phosphoacetylation may occur after chronic perturbations, such as observed here for BDNF at the P2 promoter. I did not observe a significant change in H3 phosphoacetylation 24 hr after acute or chronic ECS at either the c-Fos or BDNF promoters. This suggests that even if H3 phosphoacetylation occurs acutely or chronically, it is not a lasting effect.

Chromatin remodeling is normally described as a dynamic process induced by transient histone modifications. However, I observed several chromatin modifications that were changed in chronic ECS conditions and persisted 24 hr after the last seizure. Thus, it is likely that adaptations in chromatin structure exert not only short-term, transient effects, but also longer-term effects on gene activity. Specifically, the downregulation of H4 acetylation at the c-Fos and CREB promoters, and the upregulation of H3 acetylation at the BDNF P3 and P4 promoters, provide proximal mechanisms by which chronic ECS might alter the expression of these three genes in hippocampus. Such changes may well play an important role in modulating neuroplasticity in the adult brain. Indeed, the sustained activation of BDNF expression after chronic ECS, which we hypothesize may be mediated in part via H3 acetylation at the P3 and P4 promoters, could contribute to the antidepressant effects of ECS (see Shirayama et al. (20). Similarly, the sustained inactivation of the c-Fos gene after

chronic ECS (50) may be mediated in part via reduced H4 acetylation at the gene's promoter.

The mechanisms by which these sustained changes in histone modifications occur are not known. Chronic ECS-induced changes in histone acetylation could include recruitment of specific histone deacetylases (HDACs; enzymes that decrease histone acetylation), histone acetyltransferases (HATs; enzymes that increase histone acetylation), or proteins that regulate these enzymes. It is generally believed that HDACs and HATs are controlled mainly at the level of their recruitment to target promoters, but some evidence suggests that at least CBP (CREB-binding protein), a type of HAT, may be regulated directly through $Ca^{2+}$ signaling (61). Our findings demonstrate the importance of better understanding the role of these enzymes and associated proteins, and the signaling pathways that regulate them, not only in activity-dependent transcription but also in models relevant for chronic adaptation in the brain.

The results of the present study must be seen as a necessary first step in elucidating the types of changes in histone modifications that occur in the nervous system after acute and chronic ECS. Our findings reveal that acute and chronic ECS cause histone-, time- and promoter-specific changes in hippocampus, and that some of these modifications are highly correlated with alterations in gene expression observed under these conditions. Further characterization of these changes in histone modifications will contribute to our understanding of the

mechanisms of action of ECS as a leading treatment for severe depression and, more generally, shed light on the molecular mechanisms governing gene regulation in the brain *in vivo*.

**TABLE 1**

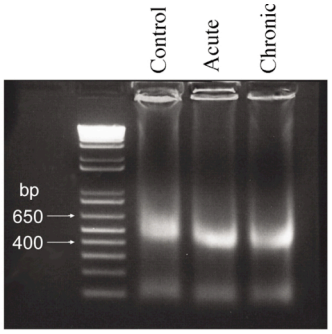
| Promoter/ Time Course | c-Fos | | BDNF P2 | | BDNF P3 | | CREB | |
|---|---|---|---|---|---|---|---|---|
| | Acute | Chronic | Acute | Chronic | Acute | Chronic | Acute | Chronic |
| **H4 Acetylation** | | | | | | | | |
| 30 min: | 330 ±41 | | -220 ±130 | | 156 ±42 | | -283 ±30 | |
| 2 hr: | 295 ±53 | 298 ±71 | 278 ±60 | 268 ± 50 | 114 ±23 | 131 ±16 | 490 ±62 | -125 ±19 |
| 24 hr: | -132 ±32 | -205 ±49 | 185 ±30 | -359 ±79 | -139 ±51 | -138 ±14 | -134 ±32 | -246 ±37 |
| **H3 Acetylation** | | | | | | | | |
| 30 min: | ND | | ND | | *220 ±98* | | -351 ±142 | |
| 2 hr: | 100 ±16 | 295 ±222 | -188 ±86 | 195 ±43 | -133 ±15 | -123 ±11 | -265 ±60 | *-216 ±69* |
| 24 hr: | 183 ±67 | 98 ±29 | ND | | *339 ±116* | 411 ±54 | 153 ±69 | *236 ±47* |
| **H3 Phospho-acetylation** | | | | | | | | |
| 30 min: | ND | | ND | | 344 ±276 | | -239±117 | |
| 2 hr: | 372 ±110 | 424±82 | 130 ±47 | 290 ±39 | 154 ±53 | -148 ±24 | 113 ±31 | 103 ±18 |
| 24 hr: | ND | | ND | | ND | | ND | |
| **mRNA Levels** | | | **( Total BDNF )** | | | | | |
| 30 min: | 3237 ±728 | | 185 ±24 | | | | 107 ±16 | |
| 2 hr: | 1514 ±418 | | 482 ±44 | | | | 149 ±19 | |
| 24 hr: | 131 ±28 | -176 ±10 | -123 ±11 | 214 ±24 | | | -119 ±2 | -178 ±31 |

Red: Increase in acetylation/phosphoacetylation, $p<0.05$
Blue: Decrease in acetylation/phosphoacetylation, $p<0.05$
Grey: Large variability among samples, no trend for regulation
*Italicized*: Trend towards increase or decrease(-), $p<0.1$
ND: Not detectable

**Table 1. Regulation of histone modifications at the c-Fos, BDNF, and CREB promoters in hippocampus by acute and chronic ECS.** The Table provides a comprehensive report of our data, expressed as percent increase (red) or decrease (blue) over control, ± SEM (n=4-6 animals in each treatment group). Values shown in red or blue represent statistically significant changes ($p<0.05$ by paired t-test). Italicized percentages indicate trends: changes that did not reach a level of significance (usually due to large variability in the fold increase or the fold decrease among the samples) but where all samples showed a change in the same direction and were significant by $\chi 2$ test. Values shown in gray represent conditions for which there was large variability and hence no apparent regulation by ECS. Ct values were too high in some samples indicating that the anti-histone antibody precipitated negligible levels of that gene, and was therefore not detectable (ND).

**FIGURES 6 - 10**

A




B

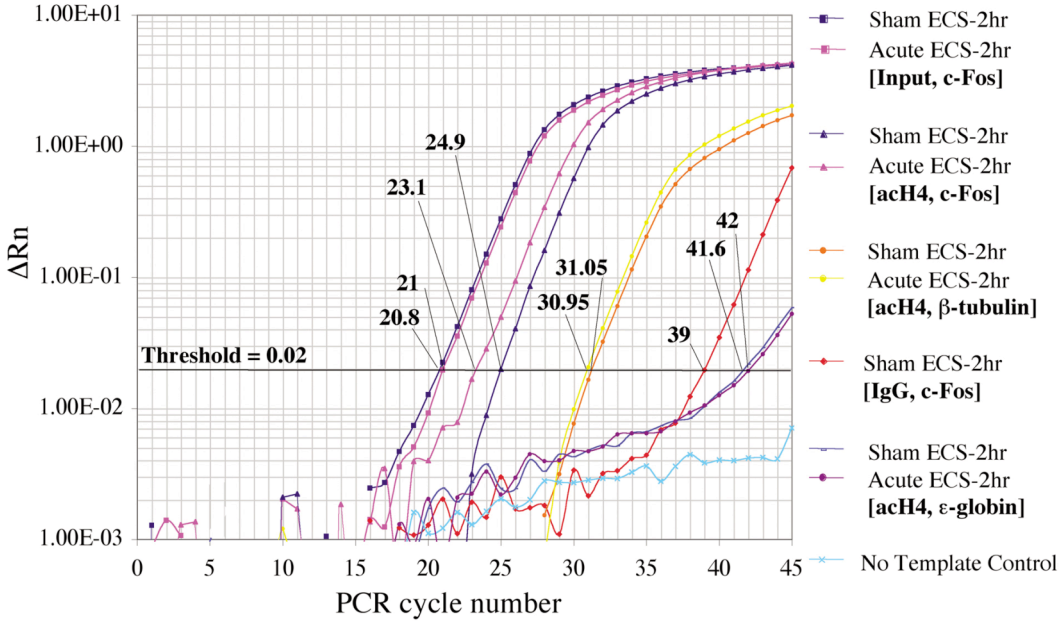


**Figure 6. Representative steps in the chromatin immunoprecipitation assay used to measure histone modifications in hippocampus of ECS-treated and control rats.** **A**. Chromatin was sonicated to fragments of 400-600 bp in size and run on a 2% agarose gel. Ethidium bromide staining of a resulting, representative gel is shown. **B**. Levels of acetylated H4 at various gene promoters in hippocampus were quantified using real-time PCR, by comparing relative Ct values for the various genes of interest at a threshold of 0.02. (Parallel experiments, not shown, were carried out for acetylated and phosphoacetylated H3.) Ct values of immunoprecipitated samples (Control and Acute ECS-2 hrs with antibody specific for acetylated H4 and PCR amplified at the c-Fos gene promoter) were normalized to Ct values obtained from "Input", or non-immunoprecipitated genomic DNA, where there is no difference between Acute ECS and Control samples, as expected. Comparison of these Ct values revealed a 3.5-fold increase in levels of H4 acetylation at the c-Fos gene in Acute ECS vs. Control (Sham ECS) samples. In aliquots of the same samples, Ct values from the β-tubulin gene differed by a negligible 0.1, reflecting no regulation by Acute ECS. Immunoprecipitation with non-immune IgG yielded much higher Ct values, typically 16 cycles higher, indicating a ~65,000-fold difference, i.e., negligible precipitation of the c-Fos gene in the absence of specific antibody. Finally, analysis of ε-globin, a gene that is silenced in adult animals and contained within transcriptionally inactive heterochromatin, reveals virtually non-detectable levels of H4 acetylation.

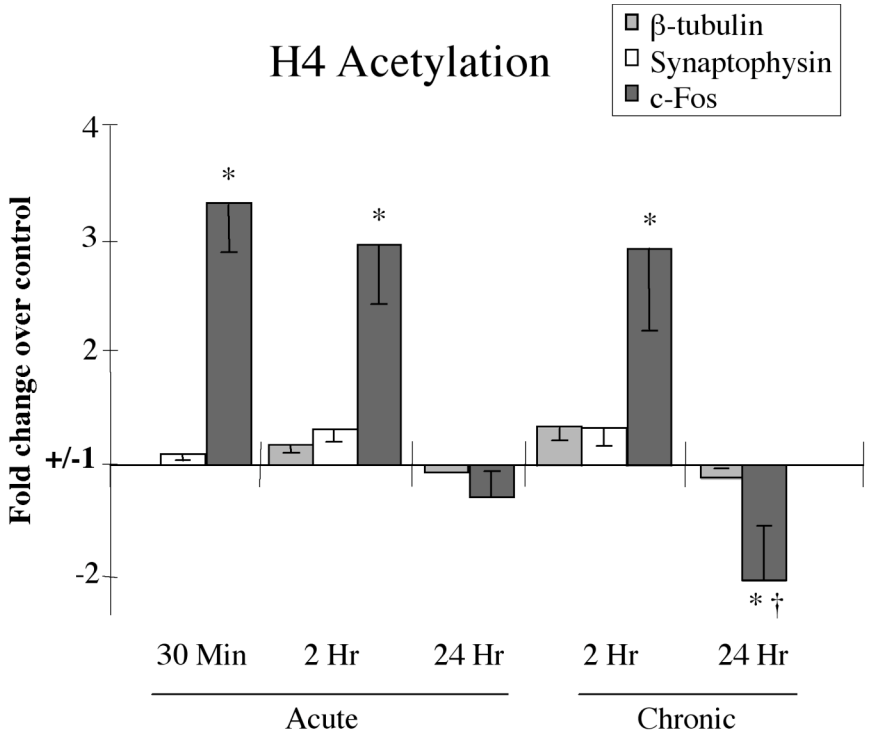
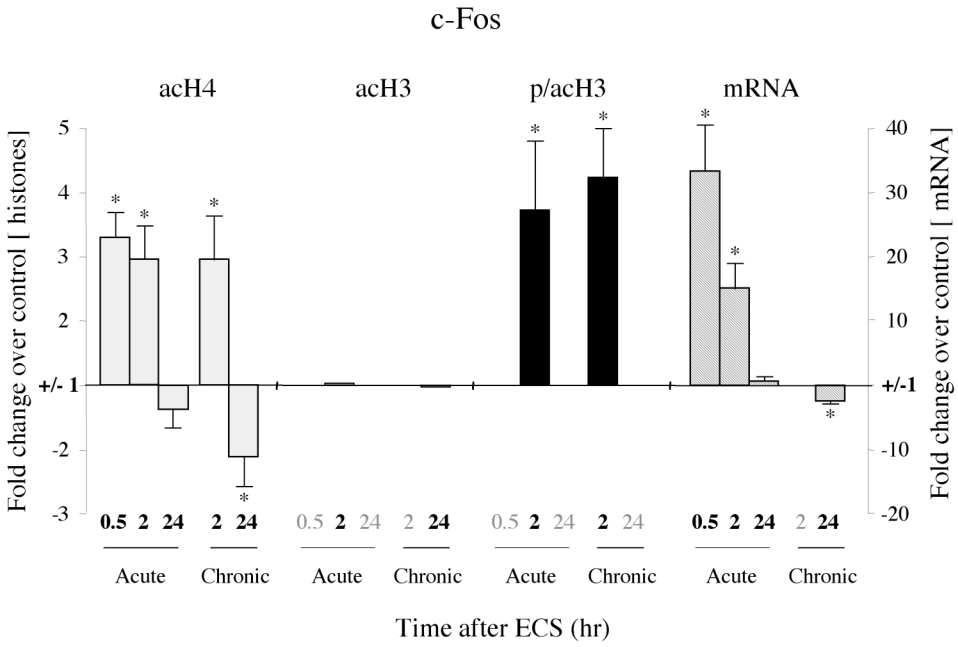



**Figure 7. Regulation of H4 acetylation at gene promoters after acute or chronic ECS.** Levels of H4 acetylation at the synaptophysin and β-tubulin promoters, two genes whose expression is not regulated by ECS, are compared to the levels of H4 acetylation at the c-Fos promoter at 30 min, 2 hr, and 24 hr after acute or chronic ECS. Note the robust, time-dependent regulation of H4 acetylation at the c-Fos promoter, but lack of regulation at the synaptophysin and β-tubulin promoters, under these conditions. Data are expressed as mean ± SEM (n=4-6 in each treatment group). Control (Sham-ECS) values are 1 ± the following SEM: 0.02 (c-Fos, 30 min), 0.05 (c-Fos, 2 hr), 0.04 (c-Fos, 24 hr), 0.04 (β-tubulin, 2 hr), 0.06 ((β-tubulin, 24 hr), 0.05 (synaptophysin, 30 min), and 0.03 (synaptophysin, 2 hr). *$P < 0.05$ different from control, $^{\dagger}P < 0.05$ different from Acute ECS-24 hr, by t-test.

**A.**


c-Fos
acH4
acH3
p/acH3
mRNA
Fold change over control [ histones]
Fold change over control [ mRNA]
5
4
3
2
+/- 1
-2
-3
40
30
20
10
+/-1
-10
-20
0.5 2 24
2 24
0.5 2 24
2 24
0.5 2 24
2 24
0.5 2 24
2 24
Acute
Chronic
Acute
Chronic
Acute
Chronic
Acute
Chronic
Time after ECS (hr)

**B.**

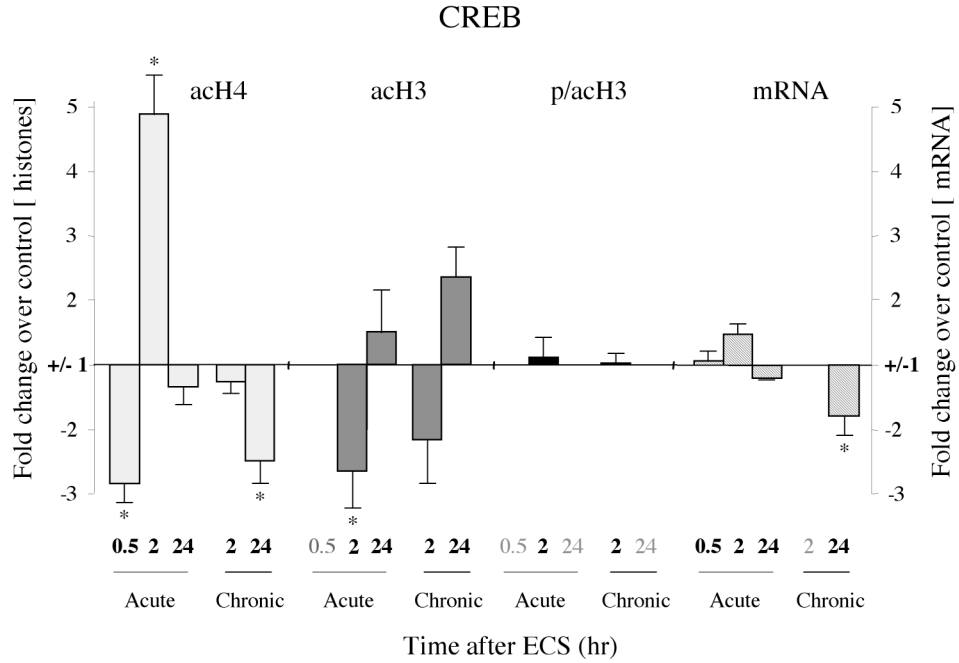

CREB
acH4
acH3
p/acH3
mRNA
Fold change over control [ histones]
Fold change over control [ mRNA]
5
4
3
2
+/- 1
-2
-3
0.5 2 24
2 24
0.5 2 24
2 24
0.5 2 24
2 24
0.5 2 24
2 24
Acute
Chronic
Acute
Chronic
Acute
Chronic
Acute
Chronic
Time after ECS (hr)

**Figure 8. Patterns of histone modifications after acute or chronic ECS at the c-Fos and CREB promoters.** Levels of mRNA for these genes were measured as well. Changes in histone modifications and mRNA are represented as mean ± SEM of fold change over control (control = ±1 ± SEM) (n=4-6; *$p < 0.05$ from control). Values with large variability (shown in gray in Table 1) are not depicted in the Figure; these data points (not shown in the figure) correspond to time points written in gray. Table 1 presents all of the data in quantitative form. **A**. Changes at the c-Fos promoter: At 30 min after an acute ECS, there was a 3.3-fold increase in H4 acetylation (acH4) and a 32.4-fold increase in levels of c-Fos mRNA. At 2 hr after acute ECS, there was a 3-fold increase in H4 acetylation, a 3.7-fold increase in H3 phosphoacetylation (p/acH3), and a 15.1-fold increase in mRNA levels. At 24 hr after acute ECS, there were no significant changes in histone modifications or c-Fos mRNA levels. After chronic ECS, the same histone modifications were observed at 2 hr as seen in the acute situation. However, at 24 hr, chronic ECS caused a 2-fold decrease in H4 acetylation which correlated with a 1.8-fold decrease in levels of c-Fos mRNA. **B**. Changes at the CREB promoter: At 30 min after an acute ECS, there was a 2.8-fold decrease in H4 acetylation, large variability in levels of H3 acetylation (acH3) and phosphoacetylation, and no change in CREB mRNA levels. At 2 hr after acute ECS, there was a 4.9-fold increase in H4 acetylation acutely, but a 2.7-fold decrease in H3 acetylation. CREB mRNA levels showed a trend toward a small (1.5-fold) increase. At 24 hr after acute ECS, no significant changes in histone modifications or CREB mRNA levels were observed. After chronic ECS, the major differences from the acute situation were seen at 24 hr, when a 2.5-fold decrease in H4 acetylation, but a 2.4-fold increase in H3 acetylation were observed along with a 1.8-fold decrease in CREB mRNA levels.

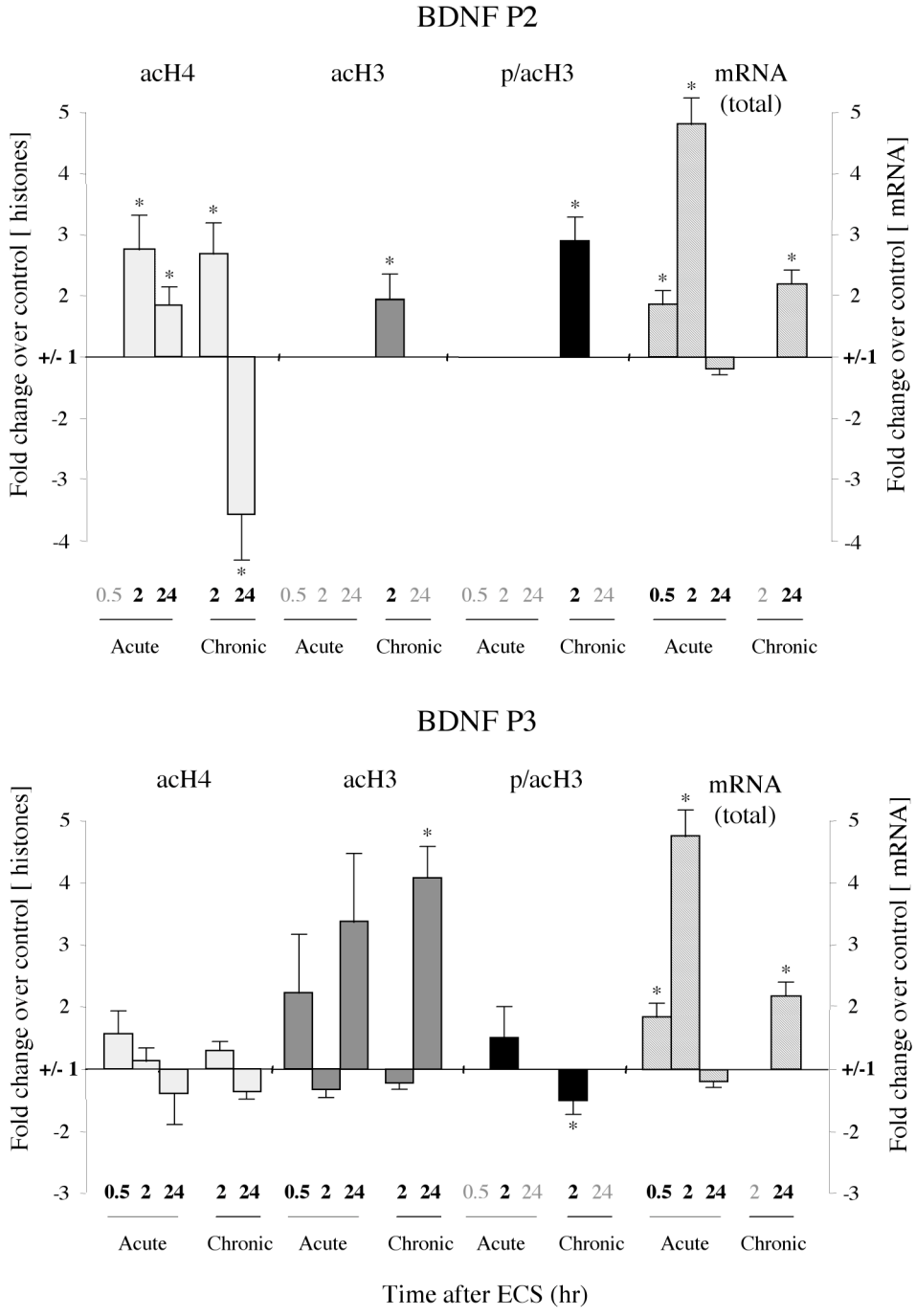
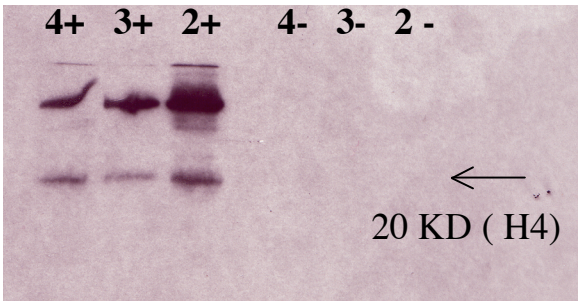

BDNF P2
acH4
acH3
p/acH3
mRNA (total)
Fold change over control [ histones]
Fold change over control [ mRNA]
5
4
3
2
+/- 1
-2
-3
-4
+/-1
0.5 2 24
2 24
Acute
Chronic
BDNF P3
Time after ECS (hr)

**Figure 9. Patterns of histone modifications after acute or chronic ECS at the BDNF P2 and P3 promoters.** At 30 min after an acute ECS, no significant changes in histone modifications were observed at the P2 or P3 promoter, while total BDNF mRNA levels were increased by 1.8-fold. At 2 hrs after acute ECS, H4 acetylation increased 2.8-fold at the P2 promoter, with no changes seen in H3 at either the P2 or P3 promoters, while total BDNF mRNA levels increased by 4.8-fold. At 24 hr after acute ECS, when BDNF mRNA levels had returned to control values, levels of H4 acetylation were increased 1.9-fold at the P2 promoter, whereas H3 acetylation tended to increase at the P3 promoter. After chronic ECS, the major differences from the acute situation were seen at 24 hr, when a 3.6-fold decrease in H4 acetylation at the P2 promoter, but a 4.1-fold increase in H3 acetylation at the P3 promoter, were observed along with a 2.1-fold increase in total BDNF mRNA levels. In general, the P4 promoter showed similar alterations in histone modifications as seen for the P3 promoter: 2 hr Acute ECS, H4 acetylation -336 ± 79%(*), H3 acetylation 134 ± 31%; 24 hr Acute ECS, H4 acetylation -202 ± 96%, H3 acetylation 132 ± 5%; 24 hr Chronic ECS, H4 acetylation -119 ± 19%, H3 acetylation 184 ± 11%(*) (n=6-8; *$p<0.05$).

**A.**




**B.**

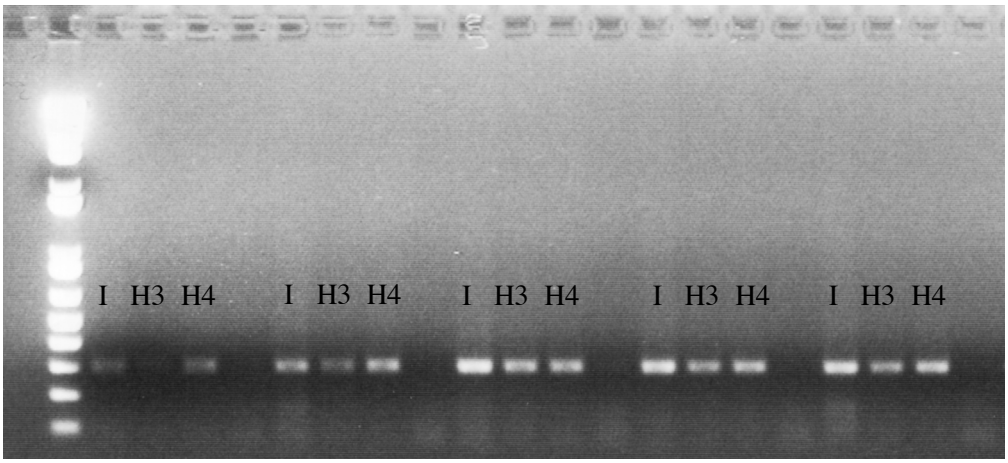



**Figure 10. Standardization of the ChIP assay technique for brain. A.** Immunoblot for acetylated H4 is shown, after chromatin immunoprecipitation assay with anti-acetylated H4 in the presence of proteinase inhibitors (2+), the absence of proteinase inhibitors (3+, 4+), and immunoprecipitation with protein A beads but without anti-H4 antibody (no antibody control) (2-, 3-, 4-). Proteinase inhibitor use resulted in a much higher yield of immunoprecipitate (2+), and no-antibody control did not display any background binding, suggesting that the anti-H4 antibody is highly specific. **B.** PCR bands are shown, demonstrating the levels of enrichment of H3 acetylation (H3) and H4 acetylation (H4) at the c-Fos promoter (300bp mark). Amplification of non-immunoprecipitated input (I) was used as a normalizer. Immunoprecipitation with a non-antibody control (protein A beads, PA) did not amplify the c-Fos gene, suggesting again that the two antibodies have excellent binding specificity.

# CHAPTER II

# STABLE CHANGES IN TRANSCRIPTION AND HISTONE MODIFICATIONS AT THE BDNF GENE IN HIPPOCAMPUS IN DEPRESSION AND ANTIDEPRESSANT ACTION: POSSIBLE ROLE FOR HDAC5

**ABSTRACT**

Depression is a chronic and debilitating disease, yet the molecular mechanisms of its pathophysiology are incompletely understood. Chronic social defeat stress, an attractive animal model of depression, was administered for 10 days, followed by chronic treatment with a tricyclic antidepressant (4 weeks imipramine, 20mg/kg/day) in mice to study neuroplastic adaptations at the levels of chromatin remodeling and gene expression. A social interaction/avoidance test was used to monitor depression-like behavior. Chronic treatment with imipramine reversed the avoidance behavior precipitated after the defeat stress. To study the stable molecular changes induced by this paradigm, I measured levels of mRNA and histone modifications at five BDNF exon variants (I-V) and their unique promoter regions (P1-5) in the hippocampus. RT-PCR analysis revealed a significant decrease in the expression of BDNF exons III and IV, which persisted for at least four weeks after the cessation of stress. Importantly, this decrease was reversed by treatment with imipramine. Other BDNF exons did not show such changes. Next, I examined if the long-lasting changes in BDNF expression are accompanied by stable modifications in chromatin. ChIP assays were performed to measure changes in H3 and H4 acetylation, as well as H3-K4, H3-K9, and H3-K27 methylation at BDNF promoters P1-5. We found that H3-K27 di-methylation was very robustly enriched at BDNF P3 and less so at BDNF P4 after chronic defeat stress, paralleling the downregulation of BNDF III and IV after this treatment. In addition, H3 acetylation and H3-K4 methylation but not H4 acetylation were increased at BDNF P3 and P4 in chronically stressed mice treated with imipramine, correlating with the reversal of BDNF III and IV downregulation by imipramine. To test the hypothesis that the sustained change in H3 acetylation may be mediated through specific histone deacetylases (HDACs), I measured mRNA levels of several HDACs and found that only HDAC5 was decreased by chronic imipramine in stressed mice. I directly examined a role for HDAC5 in this model in several ways. Viral-mediated HDAC5 overexpression in hippocampus blocked imipramine's ability to reverse avoidance behavior, whereas chronic administration of the HDAC inhibitors sodium butyrate and valproic acid (3 weeks, 200mg/kg ip, twice/day) displayed subtle antidepressant-like effects in defeated mice. Finally, HDAC5 knockout mice showed diminished responsiveness to stress. We propose that downregulation of HDAC5 is critical for the clinical efficacy of imipramine. These experiments provide one of the first endeavors to understand the role of chromatin remodeling in modulating long-term adaptive changes in brain associated with complex psychiatric conditions, such as depression.

## INTRODUCTION

Chronic stress affects our physiology, and it can precipitate symptoms of depression. Still, it is not well understood why certain people are more susceptible to the pathological effects of stress than others. Chronic social defeat stress in animals mimics many of the physiological symptoms of stress, including tachycardia, elevated glucocorticoid activity, and adrenal hypertrophy. In addition, it induces some of the psychological symptoms of depression, such as anhedonia (measured by decreased preference to sucrose), diminished self-grooming and locomotor activity, increased anxiety in the elevated plus maze test, and disruptions in normal circadian rhythm (3). Several brain regions have been implicated in the pathophysiology of stress and depression (5, 6). The most extensively studied of these is the hippocampus. Chronic stress, including social defeat stress, decreases neurogenesis in the hippocampus of rodents. Most importantly, these effects are reversed by ECS and antidepressant treatments (7, 12). The molecular basis by which defeat and other chronic stress paradigms induce long-lasting behavioral changes, some of which are reversible only by chronic antidepressants, is poorly understood.

Regulation at the level of gene expression may mediate these long-term adaptive changes in the hippocampus. One gene that has been highly implicated in the molecular neuroplasticity of the hippocampus and the pathophysiology of

depression is the brain-derived neurotrophic factor, BDNF (1). BDNF infusions in the hippocampus produce antidepressant-like effects in two animal models of depression, forced-swim test and learned helplessness (20), whereas deficiency of endogenous BDNF leads to impaired antidepressant response in the forced-swim test (21). Levels of BDNF expression are decreased after chronic stress (18, 62). Moreover, chronic, but not acute, treatment with several different antidepressants increases BDNF expression, and reverses the stress-induced downregulation (19, 63). Still, the regulatory mechanisms leading to long-lasting changes in BDNF expression due to stress and antidepressant treatments are unknown. One reason for the lack of better-defined transcriptional mechanism for BDNF regulation is its complex gene structure. BDNF contains at least four short 5′ non-coding exons (exons I-IV in rats and I-V in mice), each of which can be alternatively spliced next to the common coding exon (exon V in rats and VI in mice) to form several mRNA transcripts (15, 25). Each non-coding exon contains a unique promoter region with a distinct chromatin architecture, which could modulate the expression of one splice variant over another.

Post-translational modification of histones, a form of chromatin remodeling, can alter the chromatin architecture at promoter regions by controlling its permissiveness for transcription. In general, histone acetylation (H3 and H4 acetylation) loosens the DNA-histone interactions, allowing the transcriptional machinery to bind, and increase transcription (26). Histone

methylation, on the other hand, can correlate with either transcriptional activation (H3-lysine (K) 4, H3-K36) or repression (H3-K9, H3-K27, H4-K20), depending on the animo acid residue being methylated. Histone methylation may also play a role in mediating DNA methylation (33). The process of chromatin remodeling is increasingly recognized as a crucial mechanism in mediating important phenomena in the nervous system, including neuronal differentiation, neurodegeneration, circadian rhythm, seizure, long-term potentiation, memory formation, and synaptic plasticity (44).

Here, I employed the social defeat stress paradigm followed by chronic antidepressant treatment to induce a physiologically suitable model of depression, and to study, for the first time, how each alternatively spliced BDNF variant is regulated at the levels of gene expression and chromatin remodeling. I find that chronic defeat stress is sensitive to chronic antidepressant treatment with imipramine, that this paradigm regulates specific expression of BDNF III and IV, and that this regulation is modulated by long-term adaptive changes of the chromatin architecture at the promoters of BDNF, P3 and P4. In addition, I find that control of HDAC5, possibly via imipramine, is critical for the clinical efficacy of imipramine as an antidepressant.

## METHODS

### Animal Housing and Injections:

Adult male Bl6/C57 or C129x Bl6/C57 HDAC5 +/+ and HDAC5 -/- mice (9-12 weeks old) were used in all experiments. Animals were single-housed and maintained on a 12 hr light-dark cycle with access to food and water ad labium. All animal procedures were carried out in accordance with the IACUC. Animals were injected intraperitoneally with either imipramine (20mg/kg once daily), saline (once or twice daily), sodium butyrate (200mg/kg twice daily), or valproic acid (200mg/kg twice daily). Animals were sacrificed 24 hrs after their last injections.

### Chronic Defeat Stress:

Resident aggressor mice used were retired breeders of the CD1 strain. These mice were screened for level of aggressiveness by measuring latency to attack, where only mice that attacked in less than 3 minutes on 3 consecutive days were kept, which amounted to about 15% of all aggressors. Each test cage was divided into two compartments, one for the aggressor and the other for the defeated mouse. The separator allowed visual, auditory, and olfactory, but not

tactile contact between the aggressor and the test mouse. Mice were exposed to a different aggressor each day for 10 minutes, for a total of 10 days. During the brief exposure, all mice displayed signs of stress and subordination, including vocalization, flight response, and a submissive posture. After the fight, mice were returned to the adjacent compartment of their daily defeater, where they were exposed to chronic stress in the form of threat for the next 24 hrs. Control mice were housed in double-compartment cages as well. Twenty-four hrs after the last defeat, mice were socially isolated for 4 weeks, during which they received imipramine (20mg/kg/day ip) or equivalent volume of saline (ip). At the end of the 4 weeks, the long-term behavioral consequences of chronic stress were tested using a measure of interaction/avoidance towards one of the aggressors used during the defeat experiment. Mice were placed in a box with a cage, and their movement was tracked for 2.5 minutes in the absence of the aggressor, followed by 2.5 minutes in the presence of the aggressor, encaged. Using the Ethovision 3.0 software, the following measurements were performed in the presence and absence of the aggressive target: duration in an interaction zone delineated around the cage, duration at the two far corners from the cage, latency of first approach into the interaction zone, and total movement.

**mRNA Analysis:**

Whole hippocampus was extracted for RNA quantification from the following groups of mice, 24 hrs after their last injection and 2 days after the behavioral test: chronic defeat stress plus 4 weeks saline, chronic defeat stress plus 4 weeks imipramine (chronic treatment), chronic defeat stress plus 4 weeks saline but imipramine on the last day (acute treatment), and non-stressed controls plus saline, acute imipramine, or chronic imipramine. RNA was processed as described previously, except that the ABI 7500 machine and software were used instead (see Methods section in Chapter I). Primers were designed complementary to each non-coding exon, I-V, in order to assay for the level of expression of each individual transcript. Please see Table 2 for all primers used.

**Chromatin Immunoprecipitation:**

The chromatin immunoprecipitation technique used was identical to the one described in Chapter I with the exception of the primers used, some of the antibodies, and a slight difference in the method of analysis. The primers were designed around the putative promoter regions of BDNF P1-5, upstream of each exon I-V (Please see Table 2). The antibodies used were against: acetylated H3 (K9, 14); acetylated H4 (K5, 8,12,16); di-methylated H3 (K4) and H3 (K9); and mono- and di-methylated H3 (K27). The real time PCR ChIP data was analyzed

identically to the mRNA data, using the ΔΔCt method, except the data was normalized to "input", rather than to GAPDH.

**DNA Methylation by Sodium Bisulfite Treatment:**

The DNA methylation analysis was performed as outlined in the Dennis et al., Weaver et al., Clark et al., and Chemicon DNA methylation Kit protocols, with few modifications (Dennis et al., personal communication) (64, 65). To extract DNA, the hippocampus was incubated at 55°C overnight in lysis buffer with Proteinase K (10μg/mL Proteinase K, 10% SDS, 1M Tris, Ph 7.5, 0.5M EDTA, 5M NaCl, 1.5 DTT, 100mM Spermadine), and then at 37°C for 2 hr with RNase A (10μg/mL). DNA was extracted using phenol/chlorophorm/isoamyl alcohol (25:24:1), ethanol precipitated, and resuspended in 50μL 1X TE. The DNA was then treated with sodium bisulfite to convert all non-methylated cytosines into thymidines. For sodium bisulfite treatment and desalting, the Chemicon kit and protocol were used without any changes. Modified DNA was then amplified using primers specific for bisulfite-treated DNA, which did not include any CpG sites where possible methylation could be present. The primers used to amplify the BDNF P3 promoter and BDNF ex3 are listed in Table 2 and illustrated in Figure 24. The following PCR amplification conditions were used: 95°C/2 min × 1 cycle; 95°C/1 min, 50°C or 54°C/2 min, 72°C/3 min, × 5 cycles;

95°C/ 45 sec, 50°C or 54°C/1.5 min, 72°C/1.5 min, × 25 cycles; 72°C/6 min, × 1 cycle. The amplified DNA products were cloned directly using TOPO-TA® cloning kit and transformed into TOP10 competent cells. Five different colonies from each DNA amplification reaction were then analyzed for possible methylated CpG sites using direct sequencing from the TOPO plasmids, containing the insert.

**HSV Viral Preparation and Overexpression:**

HDAC5 and HDAC4 cDNAs, gift from Dr. Eric Olson at UTSW, were each subcloned into a previously generated and published bicistronic HSV-GFP virus (66). The virus was packaged by Dr. Rachel Neve at Harvard. HSV-HDAC4, HSV-HDAC5, or control HSV-GFP viruses were injected into the dentate gyrus region of the hippocampus by stereotaxic surgery. The following coordinates were used: -0.2 posterior, +0.16 lateral, and -0.1 ventral followed by -0.2 ventral (relative to dura). Mice were anesthetized with isofluorane during the entire surgery. The virus was delivered using a pump (Harvard Apparatus, *PicoPlus*), at a rate of 0.1μL/min for a total volume of 2.4μL per mouse (1.2μL for each hippocampus (0.6μL at -0.1 ventral and an additional 0.6μL at -0.2 ventral)). All mice were fully awake and functional within ten minutes after the anesthesia was discontinued. Mouse behavior was tested at day 3 of viral

infection. Infection levels were confirmed by measuring exogenous mRNA levels of HDAC5 in the dentate gyrus of animals receiving HSV-HDAC5 vs. HSV-GFP.

## RESULTS

### 1. Standardization of the social defeat stress and the social interaction/avoidance test paradigms

Social defeat was used to induce chronic stress. In this paradigm, the Bl6/C57 test mice were allowed a brief, 10 minute, encounter with a highly aggressive mouse. During this encounter, the aggressor attacked the test mouse, trying to inflict bites or otherwise show superiority. The test mice displayed several signs of defeat as soon as 30 seconds after the first encounter, and continued to show these signs during each subsequent combat episode for the next 10 days. These included submissive posture, vocalization, and attempts to escape the combat situation by jumping or climbing up the cage. There were no signs of sensitization or desensitization in the behavioral responses of the test or aggressor mice on each subsequent day through day 10. After the brief combat, the mice were separated from the aggressors with a plastic divider with holes, thus allowing visual, olfactory, and auditory, but not tactile, input from the aggressor. This created a chronic "threat" for the mouse. During this time, mice often displayed an avoidant behavior by running away from the aggressor as he approached them through the holes of the plastic separator. The selection of an aggressor was crucial. All aggressors needed to display similar levels of

combative behavior, so that each mouse was stressed equally. In some initial experiments performed, aggressors varied in strain and age, which lead to a large variation in the level of defeat, as measured later by the social interaction/avoidance test paradigm. In order to standardize the level of aggression, mice from the CD1 strain, retired breeders, were screened for level of aggressiveness, by measuring their latency of initial attack of an unfamiliar Bl6/C57 mouse. Any aggressor that did not begin to fight within a period of 3 minutes, on 3 consecutive days, was screened out, and was not used in the defeat experiments. To further insure that all mice are exposed to similar levels of combat, mice were introduced to a different aggressor each day. Twenty-four hours after the last defeat stress, mice were single-caged. Their behavior was tested one day after the end of defeat stress, and again 28 days later, in the interaction/avoidance test paradigm. The experimental mouse was placed in a box for 2.5 minutes first in the absence and then in the presence of an encaged aggressor, which it had met during one of the 10 days of defeat. A video camera tracked its movement and, via the Ethovision 3.0 software, it captured its position in the XY coordinate at each second. Using this software, I could measure several different parameters, including time spent, latency, frequency, and total distance spent in any user-defined region of the box. The experiments performed measured time spent and latency to approach an interaction zone, defined around

the cage (with or without the aggressor target), as well as total distance traveled in both 2.5-minute sessions.

**2. Chronic social defeat stress induces behavioral avoidance, which is reversed by chronic, but not acute, treatment with imipramine**

Chronic social defeat stress has been shown to mimic some of the pathological dimensions of depression; however, the response of defeated animals to chronic antidepressant treatment had not been studied previously. Wild type Bl6/C57 male mice were subjected to chronic defeat stress for 10 consecutive days. Thereafter, they were socially isolated into single cages, where they received daily intraperitoneal injections of the tricyclic antidepressant imipramine (4 wks, 20mg/kg/day) or saline. Control, non-defeated, animals were housed similarly and received either imipramine or saline as well. At the end of the four weeks of injections, all animals were tested for their social interaction/avoidance behavior to an aggressor by measuring the time spent interacting/avoiding the encaged aggressor (Figure 11-A). I found that control animals spend equal time in the interaction zone regardless of whether there was an aggressor present or not (~100% interaction). On the other hand, chronically defeated animals spent only about 40% of the time in the interaction zone when an aggressor was introduced into the cage, compared to when the cage was empty. Administration of chronic

imipramine reversed this avoidance behavior, increasing the interaction time close to that of a non-defeated animal (from 40% to 100% interaction) (Figure 11-B, 11-C). Moreover, a similar behavioral response was seen with chronic, but not acute, fluoxetine, another type of chemical antidepressant (not shown). Imipramine injections in control animals did not have a significant effect on interaction time. These results indicated that the social interaction/avoidance test is sensitive to chronic defeat stress, and to the effect of a chronic (but not acute) antidepressant. Furthermore, these results validated chronic social defeat stress as a relevant animal model of depression. Finally, this test provided a useful paradigm to study molecular adaptations induced by a physiological stressor for which chronic imipramine provided effective treatment.

### 3. Chronic defeat stress causes downregulation of specific BDNF splice variants, BDNF III and IV, which is reversed by treatment with imipramine

Next, I assayed whether chronic defeat stress and imipramine administrations induce long-lasting changes in gene expression, specifically at the BDNF gene. The gene structure of BDNF is unusual in that it contains five different non-coding exons in mouse, I-V, each of which is alternatively spliced upstream of the protein-coding exon VI to form distinct mRNA splice variants, BDNF I-V, with

unique 5′ untranslated regions (UTRs) (Figure 4). Since the promoter regions upstream of each 5′ UTR are also unique, I hypothesized that chronic stress and antidepressant treatments may regulate BDNF expression through events that promote the expression of distinct transcript variants. Prior results in rat have shown, for example, that ECS causes promoter-specific effects in brain (58, 67). I designed primers specific for exons I, II, III, IV, V, or VI and quantified the mRNA levels of each BDNF transcript (I-V) as well as levels of total BDNF (VI) using RT-PCR in whole hippocampus after chronic defeat alone or followed by chronic treatment with imipramine. Non-stressed animals receiving saline or chronic imipramine were included as controls. Our detailed analysis revealed indeed changes in only two out of the five BDNF transcript variants, BDNF III and BDNF IV. First, chronic defeat stress induced a significant almost 3-fold downregulation in the overall levels of BDNF (Figure 12-A). When I examined the mRNA levels of each BDNF transcript, I found that this downregulation was due to changes at BDNF III and IV, but not at any of the other splice variants (I, II, V), amounting to the overall 3-fold downregulation seen for total BDNF (Figure 12-B). In addition, chronic imipramine increased the overall BDNF level of expression 2.5-fold in non-stressed animals (Figure 12-A). Again, this increase was mediated via BDNF III and IV transcription (Figure 12-B). Most importantly, chronic imipramine reversed the lasting BDNF downregulation after chronic stress, mediated by BDNF III and IV, to a baseline level (Figure 12-A).

Our results indicated that chronic defeat stress, even a month after its cessation, induces lasting changes in BDNF expression, which are mediated specifically at BDNF III and BDNF IV, and which are reversed by chronic treatment with imipramine. This sustained regulation of BDNF III and IV after stress and imipramine was intriguing and it prompted us to further investigate the mechanism by which the corresponding promoters of BDNF III and IV, P3 and P4, might be differentially regulated to bring about the selective changes in gene expression.

**4. Histone methylation, a repressive marker for transcription, is highly and lastingly enriched after defeat stress at the promoters driving BDNF downregulation**

In order to study the chromatin architecture at the promoters of BDNF, which might be modulating the selective transcriptional changes of its splice variants, I assayed the levels of several histone post-translational modifications at the different BDNF promoter regions in the hippocampus of mice, which underwent identical treatments to the ones used for the mRNA analysis outlined above. I performed a series of chromatin immunoprecipitations (ChIP) assays with antibodies to acetylated H3 (K9, K14), acetylated H4 (K5, 8, 12, 16), di-methylated H3 (K4) and H3 (K9), and mono- and di-methylated H3 (K27) to

measure changes in these histone modifications at BDNF P1-5. I found several long-lasting changes in histone modifications that correlated precisely with the changes observed for BDNF III and IV mRNA regulation. Firstly, I detected an extremely robust increase in H3-K27 di-methylation, a repressive histone modification marker, at the BDNF P3 promoter after defeat stress of more than 50-fold when quantified by qPCR (Figure 13-B). In this analysis, the levels of H3-K27 antibody binding in non-defeated control animals were similar to or even lower than the levels of non-specific IgG binding (i.e. Ct values were at or above that for IgG controls). In contrast, defeated animals showed level of H3-K27 binding that was ~6 Ct values lower, on average, from controls (Figure 13-A). Levels of H3-K27 di-methylation were significantly increased at BDNF P4 as well, albeit to a smaller level (~3-fold). Other histone modifications examined that also correlate with transcriptional repression, H3-K9 di-methylation and H3-K27 mono-methylation, were not increased after chronic defeat stress at these promoters. In fact, H3-K27 mono-methylation was slightly but significantly decreased at BDNF P3 and P4 after chronic defeat (~4-fold) (Figure 13-B). Therefore, the significant downregulation of BDNF III and IV correlated with a quite robust increase in levels of H3-K27 di-methylation, but not with either H3-K27 mono-methylation or H3-K9 di-methylation.

**5. Histone modifications that mark transcriptional activation are stably enriched at the promoters driving BDNF de-repression after chronic imipramine treatment of defeat**

Next, I examined how histone modifications, known to correlate with transcriptional activation, are altered after chronic stress and treatment with imipramine. I found that acetylation of H3 was significantly increased by 2-fold only at BDNF promoters P3 and P4, and only in animals that had received chronic defeat stress followed by treatment with chronic imipramine (Figure 14-A, 14-B). Acetylation at H3 was not increased in any other group of animals, and not at any of the other BDNF promoters (Figure 14-C). Di-methylation of H3-K4, another histone modification that correlates with transcriptional activation, showed similar patterns of enrichment to acetylated H3 at BDNF P3 (Figure 15-A). Acetylation of H4, which similarly to H3 acetylation and H3-K4 methylation correlates with transcriptional activation, did not show corresponding long-lasting changes as observed for H3 (Figure 16-A). Instead, I observed a decrease in H4 acetylation at BDNF P3 2 hrs after acute defeat stress reactivation, when H3 acetylation was unaffected (Figure 16-B). In conclusion, we found long-lived changes in histone modifications (H3-K9,14 acetylation and H3-K4 methylation), selective for the promoters that drive BDNF III and IV expression after chronic defeat stress followed by chronic imipramine treatment. No changes were detected at other

BDNF promoters (ex: P1, P2, P5), consistent with the observation that BDNF I, II, and V mRNA expression, driven by these promoters, was also unaffected. In addition, chronic stress followed by imipramine treatment lead to a stable increase in levels of H3 acetylation and methylation, but no stable changes were observed for H4 acetylation. Given these results, I wanted to explore further the causal mechanisms of this selective and long-term increase in H3 acetylation at BDNF P3 and P4 that is evident only in chronically defeated animals treated with imipramine. I hypothesized that levels of H3 acetylation may be modulated by specific histone deacetylases (HDACs).

### 6. HDAC5 mRNA level is downregulated in chronically stress animals treated with imipramine

In order to investigate the possible role of HDAC enzymes in controlling levels of acetylation after chronic defeat stress and imipramine treatments, I first assayed whether any HDACs are transcriptionally regulated in the above chronic paradigms. I measured transcript levels of HDAC 1,2,4,5,7 and 9 (both class I and class II HDACs) in the identical conditions used for assaying BDNF transcription and histone modifications. Significant changes of expression were observed in only two of these: HDAC5 and HDAC9 (Figure 17-A, 17-B, 17-C). HDAC9 was significantly decreased in non-stressed animals receiving

imipramine (Figure 17-B), whereas HDAC5 mRNA levels were significantly decreased in chronically stressed animals receiving imipramine, correlating with the hyperacetylation of H3 in this group of animals (Figure 17-A). The fact that HDAC5 is downregulated in the same group of animals that showed increased H3 acetylation, but not in any of the other groups, suggested that HDAC5 may play a role in regulating H3 acetylation, and that this regulation may be mediated by imipramine after defeat stress. Recently, HDAC5 had been implicated to play a role in long-term plasticity (40, 68). Thus, its possible role in chronic antidepressant action in the hippocampus was worth exploring further.

### 7. HDAC5 overexpression into the hippocampus blocks the effectiveness of imipramine as an antidepressant

Chronic imipramine induced HDAC5 downregulation in defeated animals but it had no effect on HDAC5 levels in non-stressed controls. This selectivity of antidepressant action lead us to speculate that an imipramine-induced downregulation of HDAC5 might be important for the therapeutic efficacy of this drug. If so, increasing levels of HDAC5 beyond the control of imipramine might render the antidepressant less effective in reversing the behavioral effects of defeat stress. In order to test this hypothesis, exogenous HDAC5 was overexpressed into the dentate gyrus region of the hippocampus and the behavior

of defeated mice with or without imipramine on board was tested after the overexpression (Figure 18-B). We chose to target the dentate gyrus region because it has shown the greatest magnitude of BDNF mRNA regulation in other studies (19). Also, the dentate gyrus mediates neurogenesis and neuroplasticity, phenomena that play crucial roles in maintaining long-term neuroadaptive changes, which could be imperative for the chronic actions of antidepressants.

Human HDAC5 cDNA was subcloned into a bicistronic HSV-GFP virus, and then packaged for infection (Figure 18-A). First, I confirmed that the virus is able to overexpress HDAC5 *in vitro,* and *in vivo*. *In vitro*, the virus was transfected into PC12 cells, which resulted in robust overexpression of HDAC5 protein (not shown). *In vivo*, qRT-PCR revealed that viral overexpression in the dentate gyrus induces a many-fold increase in HDAC5 mRNA levels four days after the infection (Figure 18-C). This confirmed that the HDAC5-GFP virus successfully overexpresses HDAC5 in the brain. We then proceeded to virally overexpressing HDAC5 into the dentate gyrus of the following groups of animals: defeated animals receiving 4 weeks saline, defeated animals treated with 4 weeks of imipramine, and non-defeated animals receiving 4 weeks of saline. Infection with virus alone (HSV-GFP) was performed as control in the identical animals groups. The social interaction/avoidance behavior of all animals was tested on the 3$^{rd}$ day after infection, when the expression levels of this HSV virus had been shown to be maximal (66). The avoidance behavior of the mice was scored by two

methods. The first method measured the amount of time spent in the interaction zone around the encaged aggressor mouse (Figure 19-A). The second method attributed a score for the level of interaction/avoidance by looking blindly at the tracks of the mice (Figure 19-B). Analysis by both methods revealed similar results: control HSV-GFP infection did not significantly alter the behavior in any of the animal groups, while HSV-HDAC5 affected the behavior of only defeat animals receiving imipramine. Neither control virus nor HDAC5 overexpression affected significantly the behavior of non-stressed mice; they displayed a high degree of social interaction, as expected (~120% interaction). Similarly, mice that were stressed but did not receive imipramine showed typical avoidant behavior, after both infection with a control or HDAC5-overexpressing virus (~20% interaction). Finally, mice that were stressed but received chronic imipramine displayed more social interaction and less avoidance when infected with HSV-GFP (62% interaction), again as expected (Figure 19-A). Therefore, simply infecting mice with a virus did not significantly affect the behavior that we had observed previously: defeated mice still showed avoidance, non-defeated mice still interacted, and imipramine was still able to ameliorate this avoidance behavior. In addition, HDAC5 overexpression had no significant effect on the avoidance behavior of defeated mice or on the social interaction of control mice. However, HDAC5 overexpression had a profound effect on defeated mice treated with imipramine, where it blocked the ability of imipramine to reverse avoidance

and increase social interaction (Figure 19-B). These animals displayed very robust avoidance behavior, similar to that of defeated mice not treated with imipramine (18% interaction). Interestingly, infection of defeated animals receiving imipramine with HSV-HDAC4, another class II HDAC that is structurally quite similar to HDAC5, did not display similar reductions in the efficacy of imipramine (68% interaction) (Figure 19-A). These results corroborated our hypothesis that overexpression of HDAC5 into the hippocampus reduces the therapeutic efficacy of imipramine. Furthermore, they suggested that this effect might be specific to HDAC5, since overexpression of HDAC4 did not show similar results.

**8. HDAC inhibitors induce subtle antidepressant-like effects in defeated mice**

Overexpression of HDAC5 in the dentate gyrus blocked the ability of imipramine to reverse avoidance behavior in chronically defeated mice. I speculated that inhibition of HDAC5 might yield the opposite results, to potentiate the efficacy of the antidepressant. In order to address this question, two important experiments were performed. First, defeated mice were treated chronically (3 weeks) with two histone deacetylase inhibitors, sodium butyrate (SB) and valproic acid (VPA), and their interaction/avoidance behavior was

assayed identically to the chronic imipramine studies. Both of these agents are broad HDAC inhibitors (69, 70) and were used due to the lack of more selective inhibitors. The results suggested that both sodium butyrate and valproic acid show trends towards partial anti-depressant like effects in defeated mice. Chronically defeated mice injected with saline (3 weeks) displayed a characteristic decrease in social interaction as seen in previous experiments (12 seconds or 20% of interaction time compared to non-defeated animals in the presence of an encaged aggressor). Chronic treatment with SB and VPA induced a less pronounced decrease in social interaction of defeated animals (28 seconds or 27 seconds, or 50% and 42% compared to their respective controls) (Figure 20). However, this trend did not reach a level of statistical significance ($p<0.05$) when compared to defeated animals treated with saline. Some of the limitations in this experiment, which could have accounted for the lack of significance, are discussed later.

**9. Deficiency of endogenous HDAC5 induces weaker response to stress**

The second method by which I addressed the question of whether HDAC5 inhibition shows opposite behavioral effects to HDAC5 overexpression is by using mice deficient in HDAC5. Constitutive HDAC5 -/- mice were obtained from Dr. Eric Olson's laboratory, and bred in our own facility. Wild type mice of

the same background were bred simultaneously and used as controls. The HDAC5 -/- mice did not show any obvious phenotypes during development, except for slightly smaller size and weight than some but not all of the wild types. Both HDAC5 +/+ and -/- mice were subjected to chronic defeat stress, using the same procedure and aggressors as for the overexpression studies. Throughout the defeat experiment, both wild type and knock out mice displayed greater levels of stress/fear during the brief daily combat: they vocalized more than previous Bl6/C57 mice, were more actively trying to escape during the first few days of defeat, and, during the later days of defeat, assumed submissive postures more frequently. The social interaction/avoidance test in these mice revealed strong avoidant behavior in both HDAC5 +/+ and -/- defeated mice. HDAC5 wild type and knock out mice spent, respectively, 6% and 15% (1-day after defeat) and 1.5% and 11% (28 days after defeat) time in the interaction zone when an aggressor was present in the cage, compared to when the cage was empty (Figure 21-A, 21-B). Interestingly, the social avoidance of these mice was exacerbated with time, a phenomenon we had observed for other mice as well. Previously tested Bl6/C57 mice of similar age reduced their time spent in the interaction zone to approximately 20 to 40% in the presence of an aggressor, indicating that both HDAC5 +/+ and -/- mice display a greater avoidant behavior than the previously tested strain. This behavior was not due to a general strain-specific level of social interaction or a difference in the test conditions, since non-stressed mice, both +/+

and -/-, interacted similarly to previously tested control mice of different strains (92% and 125% at 1-day and 100% and 130% at 28-days of being single-caged for +/+ and -/- mice, respectively) (Figure 21-A, 21-B). While both +/+ and -/- mice showed profound avoidance behavior as measured by % time spent in the interaction zone, other measurements indicated that the HDAC5 knock out mice might be less stressed/fearful than wild types. In particular, I detected significant differences between +/+ and -/- defeated mice when examining their total distance traveled inside the test box, in the absence and then in the presence of an aggressor. HDAC5 +/+ mice displayed characteristic and significant decrease in locomotor activity when an aggressor was introduced (30% of no aggressor), and compared to non-stressed +/+ mice (40%). On the contrary, mice deficient in HDAC5 displayed much smaller reduction in locomotion when an aggressor was present (73% of no aggressor) and this reduction was similar to that seen in HDAC5-/- non-stressed controls (Figure 22). Therefore, HDAC5 -/- mice displayed a diminished responsiveness to stress compared to HDAC5 +/+ as assessed by their total locomotion in the presence of an aggressor.

**10. HDAC5 overexpression downregulates a marker of neurogenesis**

The results from the HDAC5 overexpression and HDAC5 inhibition and deficiency studies provide strong support for an important, repressive role of

HDAC5 in mediating the therapeutic effects of imipramine after induction of chronic stress. I wanted to further explore the possible mechanisms by which HDAC5 excess blocks the therapeutic efficacy of imipramine in the hippocampus. Since antidepressants have been shown to reverse the inhibition of stress-induced neurogenesis in the dentate gyrus, I asked whether HDAC5 overexpression might alter the levels of cell proliferation, an attribute of neurogenesis. I measured the mRNA levels of one cell-proliferation marker, Ki-67, which had been previously shown to correlate with the amount of cell proliferation seen in hippocampal neurogenesis. I found that HDAC5 overexpression in animals receiving defeat followed by chronic imipramine was decreased by almost 2-fold (Figure 23). Thus, it is possible that HDAC5, in excess, blocks the effects of imipramine by modulating levels of neurogenesis. However, a much more thorough analysis of cell survival and the morphological effects on neurogenesis, in many different conditions, is necessary to substantiate this finding.

### 11. DNA methylation is not present at the BDNF P3 promoter after chronic defeat stress

Chronic stress induced a very robust increase in the levels of H3-K27 di-methylation. Several recent reports have suggested that histone methylation facilitates methylation of DNA at specific promoter regions. Therefore, I wanted

to see if strong enrichment of H3-K27 di-methylation at BDNF P3 also correlates with an increase in DNA methylation at CpG sites within the BDNF P3 promoter. To explore this, I measured the levels of methylation at all CpG sites within BDNF P3 using the sodium bisulfite treatment method. Treatment with sodium bisulfite modified DNA by converting all non-methylated cytosines into thymidines. Methylation-specific primers, encompassing the entire BDNF P3 promoter as well as BDNF exon 3, were designed and used to amplify the modified DNA (Figure 24). Each PCR reaction was analyzed by TOPO-TA cloning followed by direct sequencing of several different colonies carrying the PCR-vector plasmids. The analysis revealed that there were no CpG sites within BDNF P3 as well as exon III that were consistently methylated after chronic defeat stress (Figure 24). Therefore, histone H3-K27 di-methylation did not induce DNA methylation at the BDNF promoter P3 or exon III after chronic defeat stress.

## DISCUSSION

The mechanisms by which chronic stress disrupts the normal physiological balance in the brain, leading to maladaptive responses manifested in symptoms of depression, are not well understood. While chronic treatment with a variety of different antidepressants has proved to be highly therapeutic in many, the manner by which these drugs are able to restore the disrupted balance in the brain is also unclear. The acute mechanism of action of chemical antidepressants is to increase levels of serotonin and norepinephrine in the brain, thus enhancing synaptic neurotransmission. However, these effects cannot explain the therapeutic efficacy of these drugs, for it takes several weeks to months of repeated treatment with antidepressants before patients start showing clinical improvement. This phenomenon has prompted many to speculate the involvement of a mechanism by which the brain undergoes a gradual adaptation to the enhanced neurotransmission (1). In this study I have explored the possibility that regulation at the level of chromatin remodeling and its control on gene expression are a possible mechanism that can explain some of the long-term adaptive changes in the brain seen with depression, and reversed by antidepressants. In particular, I employed the paradigm of chronic social defeat stress and found that chronic, but not acute, treatment with the antidepressant imipramine reverses social avoidance behavior caused by defeat. I then studied if

chronic defeat stress is changing the expression levels of the BDNF gene and all of its transcriptional variants in the hippocampus, how the chromatin architecture at the promoter regions of BDNF are altered in association with these transcriptional changes, and finally how chronic imipramine treatment is able to reverse such adaptations. I found that chronic defeat stress leads to the selective downregulation of the BDNF III and IV splice variants, and that chronic imipramine treatment is able to reverse this downregulation. Furthermore, I detected long-lasting changes in chromatin modifications at the BDNF promoter regions, which drive the specific downregulation of BDNF III and IV after stress (increased H3-K27 di-methylation) and upregulation after chronic imipramine (increased H3 acetylation and H3-K4 di-methylation). Finally, I discovered that the histone deacetylase enzyme HDAC5 plays an important repressive role in the stress-relieving effects of imipramine.

Our first important discovery, the fact that chronic defeat stress causes a social avoidance behavior in mice that is reversed by chronic, but not acute treatment with the antidepressant imipramine, implicates, for the first time, the use of this behavioral paradigm as a valid animal model of depression. The most widely currently used animal model of depression is the forced-swim test, in which mice are placed in a bucket of water from where they cannot escape by swimming. The test measures the latency of the mice to give up their escape efforts. The main limitation of this test is that it is sensitive to both acute and

chronic treatment with antidepressants, and, as a result, it does not parallel the clinical efficacy of antidepressants, which is achieved only after repeated administration (1). Moreover, the forced swim test utilizes normal mice to study antidepressant efficacy of drugs. Other often-used behavioral "models" of depression, such as learned helplessness, have similar limitations (71). The chronic defeat animal model surpasses these limitations. Firstly, chronic defeat stress mimics closely several attributes of depression. For instance, this stress causes reduced preference in mice for sucrose, modeling the depressive symptom of anhedonia in humans. Defeat stress has been shown to also disrupt normal circadian rhythm cycle, to diminish general motor activity, to increase anxiety, and to reduce self-grooming behavior (3). Here we reproduced another pathological aspect of depression, in particular the decrease in social interaction and the increase in avoidance behaviors in defeated mice. Many people suffering from depression display such social isolation and withdrawal, which exacerbate even further their agony. The social avoidance in defeated mice was still present even four weeks after the cessation of the stress, mimicking the chronic nature of depression in humans. Secondly, we showed that the long-lasting behavioral changes in defeated mice can be reversed only by chronic treatment with the antidepressant imipramine, rendering our paradigm sensitive to chronic but not to acute antidepressant treatment. Importantly, these chronic effects were not selective only to imipramine. In fact, similar behavioral effects have been

observed with another antidepressant, fluoxetine. Finally, we found that chronic imipramine increases social interaction only in animals that have been previously stressed, but it does not affect the behavior of non-stressed mice. One limitation of the chronic defeat stress paradigm that we encountered in this study was the difficulty to standardize the response to stress in different strains of mice. I observed that Bl6/C57 mice vocalized less during the defeat stress, and subsequently displayed somewhat less social avoidance and greater receptiveness to imipramine than 129XC57/Bl6 cross-bread mice (used in the HDAC5 +/+ vs. -/- experiment) of similar age and body weight. There are many parameters that could have contributed to such differences, including variations in genetics as well as nurturing. While this was a limitation for the analysis of stress-related downstream molecular neuroadaptations, it does parallel the variability in human susceptibility and response to stress.

In the second part of the study, I investigated the molecular adaptations at the BDNF gene, both at the level of gene expression and chromatin remodeling, that are affected by chronic defeat stress and reversed by chronic treatment with imipramine in the hippocampus. I made three important observations for the regulation of specific BDNF splice variants in these chronic paradigms: 1) BDNF was significantly downregulation after chronic defeat, which was mediated by changes in the expression levels of only two splice variants, BDNF III and IV; 2) chronic imipramine in defeated animals reversed the downregulation of BDNF III

and IV; and 3) chronic imipramine in non-stressed animals significantly upregulated BDNF, again at only BDNF III and IV. The defeat-induced downregulation of BDNF III and IV was present four weeks after the cessation of stress, and it was reversed by chronic treatment with imipramine. Therefore, these transcriptional changes at specific BDNF splice variants were very long lasting in nature. I hypothesized that such distinctive and stable regulation might be driven by the unique putative promoter regions of BDNF, P1-P5, each of which is located upstream of exons I-V.

To examine the mechanism of chronic and long-lasting BDNF downregulation, I measured the levels of several histone modifications thought to be important in transcriptional repression: H3-K9 di-methylation, H3-K27 mono-methylation, and H3-K27 di-methylation. Histone lysine methylation is thought to be a very robust modification, and therefore important in epigenetic gene control. The role of histone methylation in mammalian transcriptional regulation has not been explored extensively, and its role in the brain, *in vivo*, after chronic stress has never been examined. Here, we find the first evidence for a transcriptionally repressive role of histone methylation after defeat stress. In particular, I found a very robust increase in the levels of H3-K27 di-methylation at the BDNF P3 promoter in defeated mice, four weeks after the end of their stress. H3-K27 was increased at BDNF P4 as well, but to a lesser extent. This finding was quite significant, and it implicated, for the first time, a repressive role

for histone methylation, at least at the BDNF gene, after chronic stress. Importantly, this marker was so highly enriched four weeks after the animals were stressed, suggesting that chronic defeat stress induces very long-lasting changes not only in the expression of genes, but also in the chromatin architecture of their promoters. Interestingly, the repressive nature of H3 histone methylation was specific for a particular H3 residue (K27), as well as for a specific methylation state (di-methylation). The other H3 residue that was examined, H3-K9, showed no changes in its di-methylated form after defeat. Furthermore, I detected a small but significant decrease in H3-K27 methylation in its mono-methylated form at both BDNF P3 and P4. It was not completely surprising that di-methylation of H3-K27 rather than mono-methylation correlated with transcriptional repression of BDNF. It has been shown that the di-methylated state of H3-K27 is present in euchromatin, whereas the mono-methylated state of this lysine residue is enriched in pericentric heterochromatin (72). In addition, di- and tri-methylation of H3-K27, but not mono-methylation, have been shown to facilitate binding of Polycomb (PC), a component of the polycomb repressive complex 1, which functions to maintain long-term gene silencing (29, 73). Overall, these studies suggest that the di-methylated state of H3-K27 correlates best with euchromatic gene silencing. Our results corroborate these findings, and provide further evidence that di-methylation at H3-K27 is important for stress-induced gene repression *in vivo*.

This thesis does not explore further the mechanism by which methylation at H3-K27 is regulated so dramatically after chronic stress. Nevertheless, examining the role of histone methyltransferases (HMTases) and Polycomb repressor complex binding after chronic stress will be extremely important in order to elucidate the mechanisms of this observed robust increase. Equally important will be to study if chronic antidepressants affect the levels of enrichment for this repressive marker. As discussed further below, we have provided evidence that the antidepressant imipramine induces hyperacetylation in chronically stressed animals, and it downregulates levels of HDAC5. However, acetylation markers are less thermodynamically stable than methylation markers, and thus more easily removed (32). Therefore, it is likely that antidepressants will be unable to reverse the stress-induced hypermethylation at H3-K27. The robust and stable nature of histone methylation and the likely inability of antidepressants to reverse it provide a possible explanation for the recurrence of depression in patients that discontinue treatment with antidepressants. Until very recently, it was believed that histone methylation is irreversible. There is now some evidence for the existence of a histone demethylase (31), although its exact role remains to be established. If, on the other hand, antidepressants are able to downregulate the robust increase in H3-K27 di-methylation, this could implicate a role for a histone demethylase as well in antidepressant efficacy.

While the effect of chronic antidepressant treatment on levels of repressive histone methylation remains unexplored, we did explore its role in controlling levels of activating histone modifications. I measured the levels of the most commonly described transcriptionally activating histone modifications (H3 and H4 acetylation, and H3-K4 di-methylation) at all BDNF promoter regions after chronic stress alone or following treatment with imipramine. I detected long-lived changes in H3 acetylation and H3-K4 di-methylation, selectively at the promoters that drive BDNF III and IV expression after chronic defeat stress followed by imipramine treatment. H3 acetylation was increased at both P3 and P4, while H3-K4 methylation was enriched at P3 only. These changes were not detected at the other BDNF promoters (ex: P1, P2, and P5), consistent with the observation that BDNF I, II, and V mRNA expression, driven by these promoters, was also unaffected in that animal group.

Most studies of chromatin remodeling have reported changes in histone modifications after acute, dynamic phenomena (26). Here, we report changes that are present in a very chronic paradigm, suggesting that chromatin remodeling at gene promoters might be important in the maintenance of dynamic as well as more robust and long-term stimulations. The role of chromatin in maintaining stable and lasting regulation of gene expression, especially for histone acetylation, has not been well defined, and this study provides one of the first evidence of the importance of chromatin in this regard. We believe that such stability is

maintained at some but not all histone modifications. In this analysis, I found that while H3 acetylation is chronically upregulated at BDNF P3, acetylation of H4, another modification that marks transcriptional activation, remained unchanged at the same promoter. On the other hand, I observed changes in H4 acetylation after acute stress reactivation at BDNF P3, when H3 acetylation was unaffected. This interesting phenomenon of apparent switch from H4 to H3 acetylation in acute to chronic stimulations at BDNF was observed previously by our lab in two other separate investigations. We had detected such switch after acute vs. chronic ECS administration (see Chapter I) (67), as well as after acute vs. chronic cocaine treatments (Kumar et al., in review). In all cases, the chronic stimulations correlated with changes in H3 acetylation, while the acute stimulations induced changes in H4 acetylation at BDNF promoter regions. In addition, a separate group has recently reported that contextual fear conditioning, a hippocampal-dependent learning model, correlates with an overall increase in H3, but not H4, acetylation (36). The accumulating evidence for this switch necessitates further studies to define the mechanism by which this is achieved. One possibility is that specific histone modifying enzymes, HATs or HDACs, are recruited to execute the switch. The specificity of individual HATs or HDACs for a particular histone modification is still not well defined. A recent *in vitro* targeting study revealed that the two different co-repressor complexes, Sin3/HDAC and N-CoR/SMRT, which contain HDAC1,2 and HDAC3 respectively, target specific histones.

Sin3/HDAC deacetylates both H3 and H4, while N-CoR/SMRT is only able to deacetylate H3 (74). It is therefore possible that the selective H3 acetylation seen after chronic stimuli in the brain is a result of the recruitment of specific co-repressor complex with distinct HDAC activity. Further studies are needed to elucidate the mechanisms of this intriguing switch phenomenon. Another interesting observation in our chromatin modification study was the fact that both H3 acetylation and H3-K4 methylation were upregulated at BDNF P3. The significance of this finding was not explored further, and remains undefined. However, it is not entirely unexpected. In the last few years, accumulating research in the basic mechanisms of chromatin remodeling has suggested that distinct modifications of histones may act alone or in combination ("cross-talk") to turn genes on and off (27). One possible explanation for the presence of both H3 acetylation and H3-K4 methylation at BDNF P3 is that these two modifications somehow cross-talk to establish the long-lasting message for transcriptional activation. Alternatively, it is possible that the upregulation of both modifications represents redundancy in the histone code. One study has in fact suggested that acetylated and methylated lysine residues at histone H3 have redundant functions in controlling gene expression in yeast (75).

In order to study the possible mechanisms leading to the sustained hyperacetylation of H3 at the BDNF promoters, I examined if the regulation of any histone deacetylases, enzymes that decreases acetylation of histones, is

altered by imipramine. I measured the mRNA levels of both class I and class II HDACs (HDACs 1, 2, 4, 5, 7, and 9), and found HDAC5 to be slightly but significantly downregulated in chronically stressed animals treated with imipramine but not in any other group of animals. This downregulation correlated with the increase in H3 acetylation seen after imipramine. Recent studies had implicated a role for HDAC5 in another neuroadaptive process, long-term depression (40). Thus, I decided to pursue further the possible role of this enzyme in mediating the action of chronic imipramine. Since imipramine was shown to reverse the robust social avoidance behavior in defeated mice, I hypothesized that the molecular effects of imipramine in defeated mice, in particular the downregulation of HDAC5, might be directly related to its antidepressant efficacy to mitigate stress-related behaviors. Further, I speculated that if imipramine normally downregulates HDAC5, then increasing the levels of this enzyme beyond the control of imipramine might render the antidepressant less effective in reversing the behavioral effects of defeat stress, while decreasing its levels might allow greater permissiveness for imipramine efficacy.

Indeed, when HDAC5 was overexpressed into the dentate gyrus of the hippocampus on the forth week of imipramine injections, we found that the imipramine efficacy in defeated animals was profoundly blocked three days after the introduction of exogenous HDAC5. Importantly, HDAC5 did not alter the social interaction behavior in non-stressed mice and the social avoidance behavior

in defeated mice not treated with imipramine, compared to mice injected with control virus. In addition, surgery itself did not significantly alter the social behavior in non-stressed mice; however, I did observe a non-significant trend towards a greater level of avoidance for defeated mice that underwent surgery, compared to non-injected mice tested previously. This trend was most likely due to variations in the aggressors used in the non-injected vs. injected mice, since the two experiments were performed several months apart. In addition, in later experiments, I utilized more stringent conditions, in which defeated mice that do not show avoidance behavior (interaction time > 100%) at 1 day after defeat were not tested 28 days later. Finally, surgery with a control virus decreased slightly, but not significantly, the level of imipramine efficacy compared to non-injected animals. Again, we believe that these differences are due to variations in the aggressors and the stringency of testing. Overall, surgery did not alter significantly the interaction/avoidant behavior in mice, and the repressive effect of overexpressing HDAC5 was specific for defeated mice treated with imipramine.

Given that HDAC5 overexpression blocks severely the efficacy of imipramine, I wanted to test if inhibition of HDAC5 induces the opposite effects. I treated control and defeated mice with two chemicals known to cause inhibition of HDAC activity, sodium butyrate (SB) and valproic acid (VPA). Valproic acid is active in the central nervous system and is clinically used as anticonvulsant and

antimanic mood stabilizer drug. Recently, it has also been characterized as an HDAC inhibitor (76). Both SB and VPA have been shown to cross the blood brain barrier after oral or intraperitoneal administration, and to increase histone acetylation in the brain (77, 78). However, one limitation to the currently available HDAC inhibitors, including SB and VPA, is that they are highly non-specific: they inhibit all forms of HDACs and exert other effects as well (69, 70). These inhibitors were administered intraperitoneally, twice daily, for a total of 3 weeks. Sodium butyrate and valproic acid treatments did not affect the social interaction behavior of non-defeated controls, compared to controls injected with saline. In chronically defeated animals, however, both sodium butyrate and valproic acid were found to reduce the severity of avoidance behavior, suggesting a partial antidepressant-like efficacy. While defeated mice injected with saline displayed characteristic avoidance behavior (reduced interaction to 20% in the presence of an aggressor compared to controls), sodium butyrate and valproic acid treatments showed less pronounced avoidance (50% and 42% reduction in interaction time compared to controls treated with the respective HDAC inhibitors). However, while both drugs showed a strong trend towards reducing social avoidance after defeat, the effect did not reach a level of significance ($p<0.05$). There were several limitations to this experiment, which could account for the subtle effects of the HDAC inhibitors compared to the more robust HDAC5-overexpression behavioral effects. First, these chemicals were injected

into the peritoneum rather than directly into the hippocampus. While the drugs can freely cross the blood-brain barrier, their bioavailability is certainly limited, and their effects on the hippocampal molecular structure cannot mimic the robustness of a direct overexpression-like test paradigm. Effects in hippocampus could also be opposed by effects in other brain regions. In addition, the actions of these drugs are not specific to inhibition of HDACs; in fact, they have several other effects on the brain pharmacology. For example, valproic acid modulates levels of GABA in the brain, which is responsible for its anticonvulsant-like effects (76, 79). Finally, HDAC inhibition by these drugs is not specific to HDAC5. Rather, these drugs have been shown to cause non-specific hyperacetylation in brain (36, 77). In the HSV overexpression study, we showed that HDAC4, another class II HDAC that is also inhibited by SB and VPA, does not block the behavioral consequences of imipramine. Here, we see that general HDAC inhibition is not fully capable of mimicking imipramine's antidepressant effects on defeat stress. These observations further support the possibility that the effect of imipramine on histone deacetylation is specific for HDAC5.

To examine the consequences of specific HDAC5 inhibition, traditional HDAC5 knock out mice were used to test their susceptibility to stress compared to wild type controls of the same strain. We found that 1 day after the end of chronic defeat stress, HDAC5 -/- mice show similar avoidance behavior to HDAC5 +/+, as measured by the % time spent in the interaction zone in the

absence vs. the presence of an aggressor, but that HDAC5 -/- do not show a characteristic stress-induced decrease in locomotor activity in the presence of an aggressive target. In fact, the total distance traveled by HDAC -/- defeated mice was similar to that of non-defeated mice of the same genotype when an aggressor was present, while HDAC5 +/+ defeated mice moved much less compared to their non-defeated controls. Chronic defeat stress has been shown to decrease total locomotor activity (3), indicating that the behavior of the HDAC5 -/- mice was unusual. Therefore, while HDAC5-deficient mice displayed robust social avoidance as a result of chronic defeat stress, there were more subtle differences in their responsiveness to stress compared to HDAC5 wt mice. It would be interesting to know whether the ability of imipramine to reverse social defeat is affected by the loss of Hdac5. However, we were unable to determine this, because the usual effect of chronic imipramine was not observed in the +/+ mice. This was not entirely surprising: during the defeat administration, I noticed that both HDAC5 +/+ and -/- mice displayed greater signs of defeat than the previous Bl6/C57 strain. When tested in the social interaction/avoidance test, defeated mice not treated with imipramine, both +/+ and -/-, spent even less time in the interaction zone in the presence of an aggressor compared to previously defeated Bl6/C57 mice. Thus, the HDAC5 129XC57Bl6 mixed strain of mice were much more susceptible to defeat. This experiment should now be repeated where mice receive less-severe stress, so that their response to defeat, and the response of the

+/+ mice to imipramine, can mimic that of Bl6/C57 mice. An important limitation of looking at mice developmentally deficient in HDAC5 is that other histone deacetylases could compensate for its absence. This may be the reason why the effects we observe are rather subtle. Still, our results implicate that even if other HDACs do compensate, they do not have complete redundancy for the effects of HDAC5, since we are able to observe some behavioral differences in +/+ vs. -/- mice.

Future experiments that will better address the question of HDAC5 necessity include developing a conditional knock out mouse for HDAC5 or blocking HDAC5 via RNA interference (RNAi). Both experiments will allow inhibition of HDAC5 in adulthood and directly into the hippocampus. We speculate that these more precise HDAC5-inhibitory experiments will yield more robust behavioral differences and provide better evidence for the role of HDAC5 in depression. If we find clear evidence that deficits in HDAC5 are changing some dimensions of the stress response and/or imipramine efficacy, it will be important to see whether such changes are reversed or rescued by addition of HDAC5. These experiments will define more clearly if HDAC5 is necessary and sufficient to mediate the effects of antidepressant efficacy after chronic stress, and will allow for better molecular manipulation in order to further investigate the downstream targets of HDAC5. Finally, it will be important to establish if other HDAC enzymes have roles similar to that of HDAC5. We provide evidence that

a very structurally and functionally similar HDAC, HDAC4, does not appear to block the efficacy of imipramine after chronic stress. Still, it is important to examine if other class I and class II HDACs are regulated by imipramine by means other than changes in transcription, and whether they affect its efficacy when overexpressed. For example, it will be very interesting to study whether HDAC9 overexpression, which was noticed to be downregulated only in control animals treated with imipramine, affects the social interactive behavior in these mice (discussed further below). In addition, it has been shown that HDAC5 associates with HDAC3, a class I histone deacetylase, *in vivo* (80). This association may be necessary for HDAC5's activity (Keystone meeting on chromatin remodeling, personal communication). Therefore, it would be also important to examine the role of HDAC3 in our animal model. As we gain more knowledge of the general mechanism of regulation of HDAC enzymes to the promoters of genes, it will be necessary to reevaluate our data and search for other possible regulators of chromatin remodeling that are important in establishing long-term molecular changes in the brain after chronic stress and as a result of chronic antidepressant administration.

There are several possible mechanisms that could account for the interaction between imipramine and HDAC5. I have provided evidence that chronic imipramine downregulates the mRNA level of HDAC5. To study the mechanism of such regulation, the chromatin structure of the HDAC5 promoter

and the possible repressive role of transcription factors implicated in chronic antidepressant actions must be analyzed further. The acute pharmacological action of imipramine is to increase the extrasynaptic levels of serotonin and norepinephrine by inhibiting their plasma membrane transporters. These molecules, in turn, bind to their membrane-bound receptors, stimulating a cascade of intracellular events, including the activation of $Ca^{2+}$/CaM-kinases and protein kinase A (PKA) (see Figure 3). Antidepressant regulation of these signaling cascades, by eventually signaling to the cell nucleus, could alter the expression of HDAC5 like they alter the regulation of numerous other genes (48). The chromatin architecture and transcription factor regulation at the HDAC5 promoter have not been studied so far, except in one paper, characterizing the human HDAC5 gene. This report suggests the likely presence of a cAMP response element (CRE) binding site within the promoter region of human HDAC5, just upstream of its 5′ UTR (81). I analyzed the mouse promoter using the TFSEARCH program and also found a likely (95%) presence of a CRE site at the same position. This raises the possibility for a repressive role of the transcription factor CREB, or one of its isoforms, after chronic treatment with imipramine. Since CREB has been shown to be upregulated after chronic imipramine treatment (82), its binding to a CRE site within the HDAC5 promoter would not downregulate HDAC5 expression. Rather, it is more likely that the repression at the HDAC5 promoter is mediated via inhibition of CREB binding or competition

for binding at the putative CRE site. An example of a molecule with such passive repressive action is the inducible cAMP early repressor, ICER. The ICER protein can bind to the same CRE site that CREB normally binds to, preventing the binding of and the transcriptional activation by CREB (83). Further research is needed to understand the mechanisms by which chronic blockage of monoamine reuptake leads to reduced HDAC5 expression, and whether CREB or one of its isoforms plays a repressive transcriptional role in this process. HDAC5 activity might be regulated not only at its level of mRNA expression, but also by its translocation from the cytoplasm to the nucleus. In fact, that appears to be the main mode for regulation of class II HDACs (HDACs 4,5,7, and 9) in the heart (84), as well as in cultured hippocampal neurons (HDAC4 and HDAC5) (85). In the heart, activation of the calcium/calmodulin-dependent protein kinase (CAMK) as well as the phosphokinase C (PKC) can phosphorylate HDAC5 at a N-terminal kinase-binding domain, leading to its nuclear export and thus inactivation (86, 87). In cultured hippocampal neurons, stimulation of calcium flux through synaptic NMDA receptors also induces HDAC5 translocation to the cytoplasm, a process found to be partially blocked by a CaM kinase inhibitor (85). Imipramine induces the activation of CaM kinase as well, which could phosphorylate, among other downstream molecules, HDAC5. Thus, chronic imipramine might repress HDAC5 activation via regulation of its subcellular localization in addition to downregulating its level of mRNA expression. Unfortunately, I was not able to

study the regulation of HDAC5 translocation by chronic imipramine due to the lack of specific antibodies for HDAC5 in general and especially for its kinase-binding domain. This is an important and necessary experiment that should be performed as soon as such antibodies become available.

The final result from the BDNF I-V expressional analysis was that chronic imipramine in non-stressed mice increases BDNF transcription, and that this upregulation is also mediated via specific changes in splice variants III and IV. To our knowledge, this is the first report of imipramine-induced upregulation of specific BDNF transcripts in mice. Our results are consistent with several recent studies, which show that different antidepressants and ECS increase specific BDNF splice variants in normal, non-stressed rats (58, 67). Chronic ECS was found to increase BDNF IV transcript in whole hippocampus extract in rat (which corresponds to BDNF V in mouse) 24 hrs after the last seizure (67). A separate study found all four BDNF transcripts to be upregulated in rat dentate gyrus 2 hrs after the last seizure, with the largest increase seen at BDNF I and II. Moreover, chronic tranylcypromine, a monoamine oxidase inhibitor antidepressant, increased BDNF II in rat hippocampus (58). It is important to point out that while I observed a 2.5-fold upregulation of total BDNF, the combined upregulation of BDNF III and IV amounted to a 1.5-fold increase. This one fold discrepancy in the results is most likely due to differences in primer efficiencies of amplification

but might also indicate the presence of a sixth splice variant that is increased by chronic imipramine as well, one which has not been annotated yet.

The exact mechanisms, by which chronic antidepressants result in transcript-specific upregulation of BDNF in normal, non-stressed mice, are not well understood. We have previously shown that the increase in BDNF IV in rat after chronic ECS correlates with an increase in H3 acetylation at the BDNF P4 promoter (67). In the present study, I did not observe significant changes in the levels of H3 and H4 acetylation, nor in the levels of H3 methylation, at any of the BDNF promoters in non-stressed animals treated with saline or imipramine. However, I observed a significant downregulation of HDAC9 mRNA, of similar magnitude as the HDAC5 downregulation, but only in non-stressed animals treated with imipramine. This finding is quite intriguing since it suggests a fundamentally different mechanism by which imipramine increases BDNF expression in normal vs. defeated animals, and should be explored further. The downregulation of HDAC9 expression in control animals treated with imipramine corresponded to the increase in BDNF III and IV mRNA levels in this animal group, but paralleling changes at the chromatin architecture of the P3 and P4 promoter regions were not detected. While this chromatin modification study was quite detailed, it did not include measurement of every single histone modification at these promoters. It is possible that the upregulation of BNDF III and IV, and the corresponding downregulation of HDAC9 in control animals treated with

imipramine, is mediated via chromatin changes in some of these unexplored histone modifications. It would be interesting to measure acetylation of other H3 residues, for example K18 and K23, as well as to measure acetylation of only H3-K9 and H3-K14 (in these studies, I measured only the combined levels of these two modifiers). A more detailed understanding of the chromatin structure driving BDNF expression by imipramine in the absence vs. the presence of defeat will allow us to better understand the nature of the therapeutic efficacy of imipramine, as well as its differential regulation of histone deacetylases. Studying the behavior of mice after overexpression of HDAC9, as suggested above, would contribute to understanding better the mechanisms of imipramine action in normal vs. pathological states. Unfortunately, at present we do not have an explanation for the mechanism of BDNF upregulation in non-stressed animals.

Based on our chromatin immunoprecipitation data, mRNA data, and HDAC5 overexpression and inhibition behavioral data, we propose a model for a possible mechanism by which chronic stress induces repression and by which chronic imipramine, via regulation of HDAC5, can alleviate this repression (Figure 25). This thesis has explored the transcriptional regulation of one gene highly implicated in the pathophysiology of stress and antidepressant actions, BDNF. However, there are other genes, whose expression levels might be similarly modulated after stress and antidepressant treatment. Thus, we regard the data for BDNF as a prototypical example of a stress-related gene whose

expression levels are being regulated at the level of chromatin remodeling. In the absence of stress, the chromatin state of stress-related genes is at a level that neither promotes nor represses transcription ("basal level"). Our ChIP data revealed that neither acetylation nor methylation of histones was enriched at BDNF in control animals. In these animals, HDAC5 might function to repress unnecessary activation of genes and maintain a chromatin balance. Chronic defeat stress induces the specific di-methylation of histone H3 at residue K27, which remains long after the end of stress. This creates a more "closed" chromatin state, mediating the repression of stress-related genes. Acetylation and HDAC5 regulation are not affected after chronic stress alone, corroborating the idea that repression is mediated mainly via histone methylation. Chronic imipramine induces hyperacetylation after chronic defeat, and it downregulates HDAC5 expression. Inhibition of HDAC5 leads to a more permissive (or "open") chromatin state at the promoters of the stress-related genes, whose upregulation is important for antidepressant efficacy. Since histone methylation is a very thermodynamically stable modification and thus not easily removed, an opening of the chromatin via histone hyperacetylation provides a more plausible mechanism of de-repression than histone demethylation. We believe that these hyperacetylating effects of imipramine are highly exaggerated in defeated animals, due to a more highly repressive state of gene expression as a result of histone methylation. Thus, imipramine may always exert an effect on HDAC5

expression/translocation, which translates into modulation of expression of stress-related genes, but these effects are only visible in an off-balanced system, such as after chronic stress, when imipramine must exert greater effects in order to restore homeostasis. This could explain why we see HDAC5 downregulation and H3 hyperacetylation only in defeated animals treated with imipramine but not in non-defeated controls. When levels of HDAC5 are oversaturated, for example after overexpressing exogenous HDAC5 in the hippocampus, the normal inhibitory action of imipramine is insufficient, HDAC5 is present in the nucleus to a greater level, thus maintaining the repressive state of genes. Contrary, if HDAC5 is absent, the antidepressant effects of imipramine might be observed even in defeated animals not treated with the antidepressant, provided that there is no compensation from other histone deacetylases.

In this proposed mechanism, the acute antidepressant actions of imipramine, i.e. the increased levels of serotonin and norepinephrine, translate into more gradual but stable downstream molecular adaptations at the levels of chromatin and gene expression, which can affect behavior. Such mechanism can account for the chronic therapeutic efficacy of imipramine. Since every cell in our body contains the same DNA sequence, it is possible that the pharmacological actions of many different antidepressants can be integrated at the level of chromatin remodeling to exert a common effect on gene transcription. Microarray experiments would be important to elucidate which genes are being

regulated after chronic imipramine in the hippocampus, as well as after other antidepressants. It would be interesting to uncover the genes being differentially regulated, and to also identify which of them are possible targets of HDAC5. One way to identify specific targets of HDAC5 would be to perform a ChIP on Chip experiment for HDAC5. In its essence, this technique involves chromatin immunoprecipitation (ChIP) with an appropriate HDAC5 antibody, followed by hybridization of the HDAC5-bound immunoprecipitated DNA on a genome-wide microarray (Chip) in order to identify specific HDAC5-binding DNA regions. Genome-wide ChIP on Chip experiments for modified histones in human cells and in yeast are already a reality. In addition, microarrays containing the entire mouse genomes have also been recently developed. Therefore, it is just a matter of time before such an experiment can be performed to reveal *in vivo* targets of HDAC5 important not only for normal but also for antidepressant-treated mice.

In this study, I present suggestive but indirect data that BDNF might be a gene target of HDAC5. I have shown that specific BDNF transcripts are downregulated by chronic stress, that chronic imipramine reverses this, that acetylation at H3 is increased at the specific promoters, driving this re-repression, and that this corresponds to a downregulation of HDAC5 mRNA. I have not, however, shown that HDAC5 binds directly to the hyperacetylated BDNF promoters. Again, this is due mainly to the lack of specific HDAC5 antibody. In the future, it would be important to examine whether one or more of the BDNF

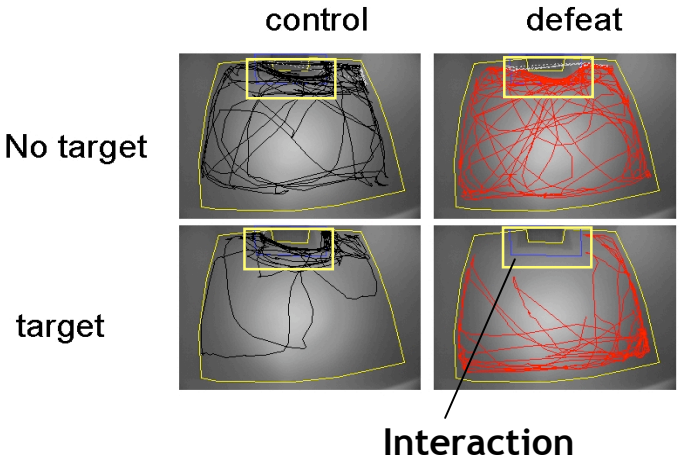
promoters serve as direct binding targets for HDAC5. Other genes that are regulated by chronic antidepressant treatments might also be downstream targets of HDAC5, for example genes that are involved in the process of neurogenesis. In support of this, I observed that HDAC5 overexpression in the dentate gyrus of defeat animals treated with imipramine lead to a significant downregulation of the nuclear antigen Ki-67, a marker of cell proliferation, in the hippocampus. This finding is in line with previous studies showing that HDAC5 overexpression in cell culture induces growth suppression and apoptosis (88). However, further and more detailed studies are necessary to establish a direct role for HDAC5 in neurogenesis.

**ACKNOWLEDGMENTS**

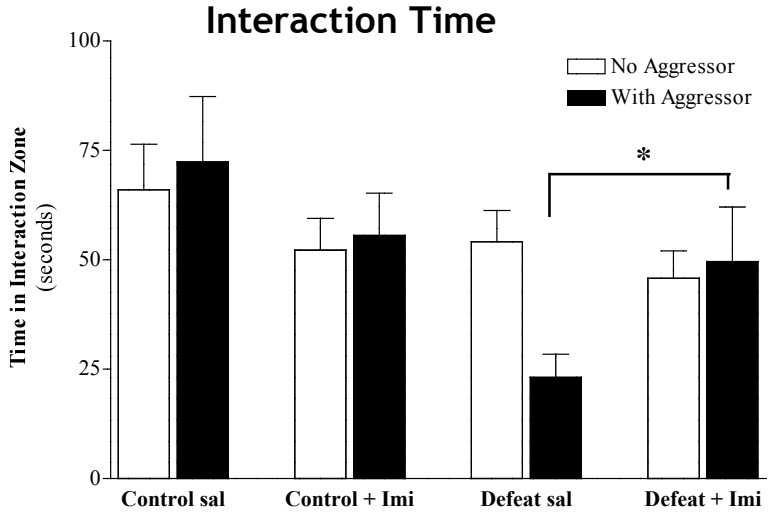
I would like to thank the following people who contributed to this study: members of the Dr. Eric Olson's laboratory for providing HDACs cDNA and HDAC5 -/- mice; William Renthal and Dr. Arvind Kumar for helping with the HSV-HDAC subcloning and performing the in vitro transfection assay; Cathy Steffen for breeding the HDAC5 mice; David Theobald and Teresa Sasaki for helping with some of the mouse injections; Rachael Neve at Harvard for packaging the HSV virus; Dr. Kathleen Dennis at Vanderbilt for supplying the sodium bisulfite protocol for DNA methylation, and for her advice; Georgi Petrov for graphic design expertise. In addition, we would like to thank the NIMH for financial support.

**FIGURES 11 - 25:**

**A.**




**B.**




**C.**

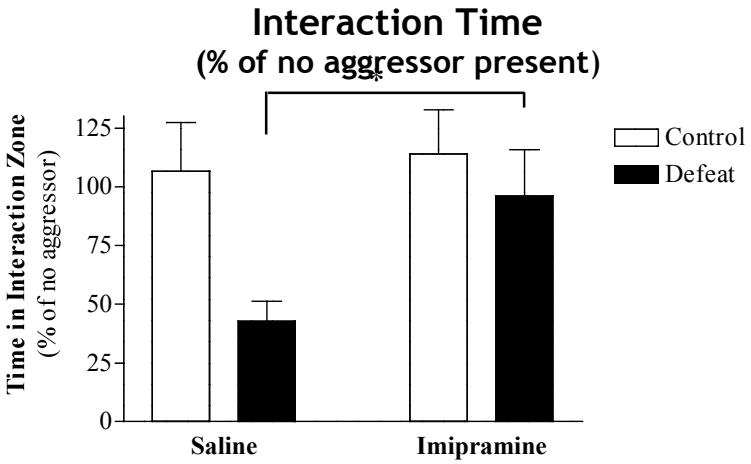
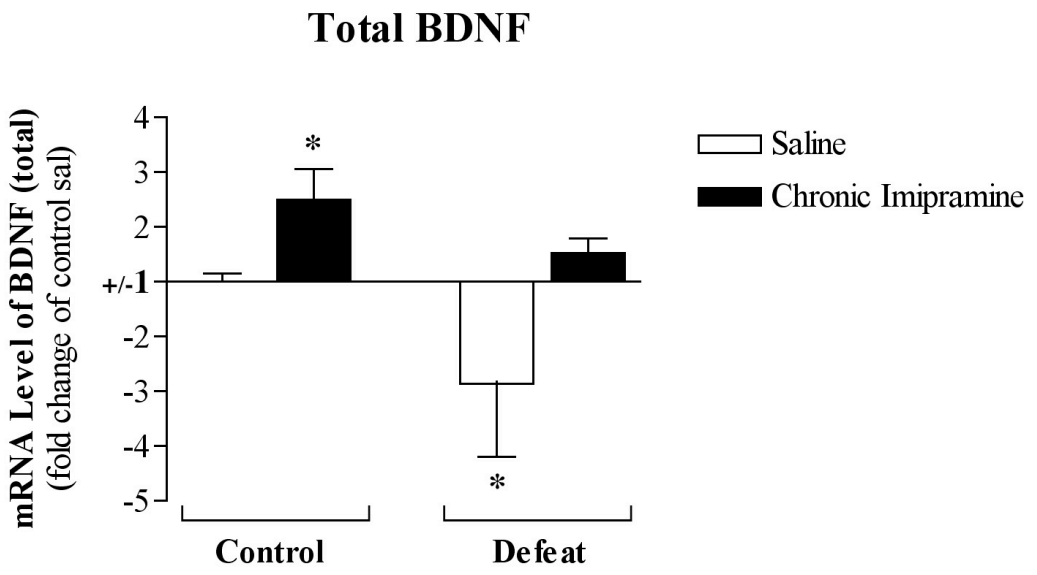


**Figure 11. Social interaction/avoidance after chronic defeat and imipramine treatments.** (**A**) Schematic representation of the interaction/avoidance test paradigm. The interaction zone is defined around a cage which is either empty, or it contains an aggressor (target). The black and red lines represent the tracks of non-stressed (control) and stressed mice in the box in the absence or the presence of an aggressor target. Control mice continue to spend time in the interaction zone in the presence of an aggressor, whereas defeated mice avoid the interaction zone once an aggressor has been introduced. Time spent in the interaction zone in seconds (**B**) and % of no aggressor (**C**) was measured for control and defeated mice treated with chronic imipramine. In the absence of an aggressor, all four groups of animals spent comparably equal times in the interaction zone: (66 ± 10 sec for control-saline, 52± 7 sec for control imipramine, 54± 7 sec for defeat-saline, and 46± 6 sec for defeat imipramine). When an aggressor was introduced into the cage, chronically defeated mice not treated with imipramine displayed a reduction in their interaction time by more than half (23 ±5 sec, or 42 ± 8%). Importantly, the control saline and control imipramine animals did not show a reduction in the time spent in the interaction zone (72 ± 15 sec, 107 ± 20%, and 52 ± 7 sec, 114 ±19%). Chronic imipramine (4 weeks) treatment in defeat mice was able to reverse the stress-induced decrease in interaction (to 50±12 sec, or 96 ± 19%). N=12. Two-way ANOVA and Bonferroni post-test were used to determine statistical significance ($p < 0.05$).

**A.**


Total BDNF
mRNA Level of BDNF (total)
(fold change of control sal)
4
3
2
+/-1
-2
-3
-4
-5
*
*
Saline
Chronic Imipramine
Control
Defeat

**B.**

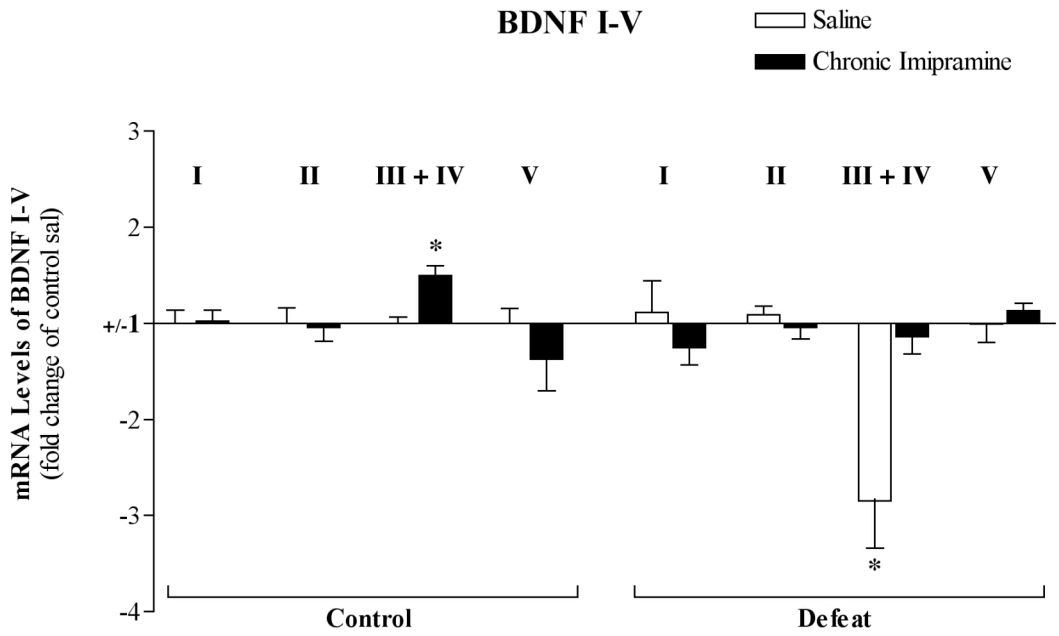
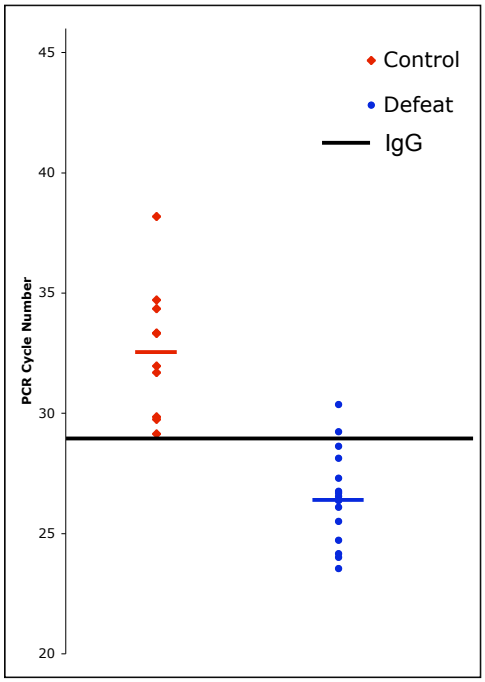

BDNF I-V
Saline
Chronic Imipramine
mRNA Levels of BDNF I-V
(fold change of control sal)
3
2
+/-1
-2
-3
-4
I
II
III + IV
V
I
II
III + IV
V
*
*
Control
Defeat

**Figure 12. Differential regulation of BDNF III and IV after chronic defeat stress and imipramine treatments.** The mRNA levels of total BDNF and BDNF transcripts I-V were measured in the hippocampus of control or defeated mice receiving either saline or chronic imipramine, and were quantified by qRT-PCR. **A**. Total BDNF was significantly decreased (2.9 ± 1.3 fold) at 4 weeks after defeat stress, compared to control saline animals. Chronic imipramine treatment in control animals induced a significant increase in BDNF (2.5 ± 0.5 fold). Chronic imipramine treatment in defeated animals reversed the stress-induced downregulation to slightly above the levels of control saline (1.5 ± 0.3 fold). **B**. Measurement of the five BDNF splice variant transcripts, BDNF I-V, revealed significant changes in only BDNF III and BDNF IV, amounting to a combined 2.85 ± 0.5 fold decrease compared to control saline. In non-stressed control animals, again only BDNF III and IV in combination, but not any other splice variants, alone or in combinations, showed small but significant increase after chronic imipramine treatment (1.5 ± 0.1 fold). N=5-6. T-tests were used to determine statistical significance ($p<0.05$).

**A.**

**Di-Methylation of H3-K27 at BDNF P3**




**B.**

**Histone Methylation at BNDF P3 and P4 after Chronic Defeat**

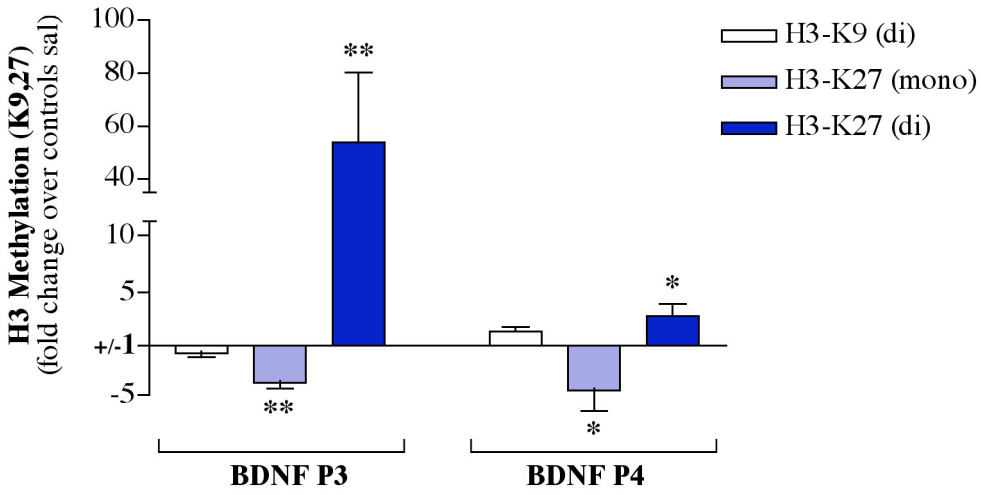
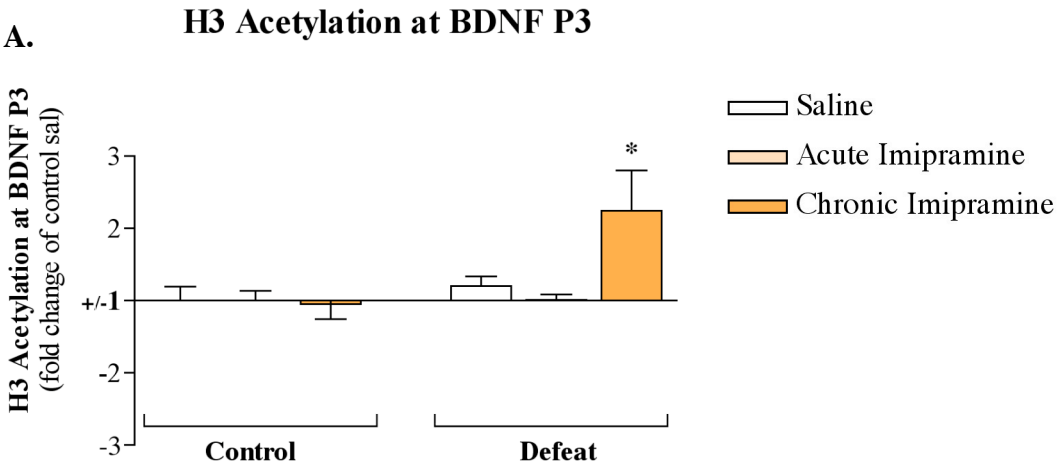
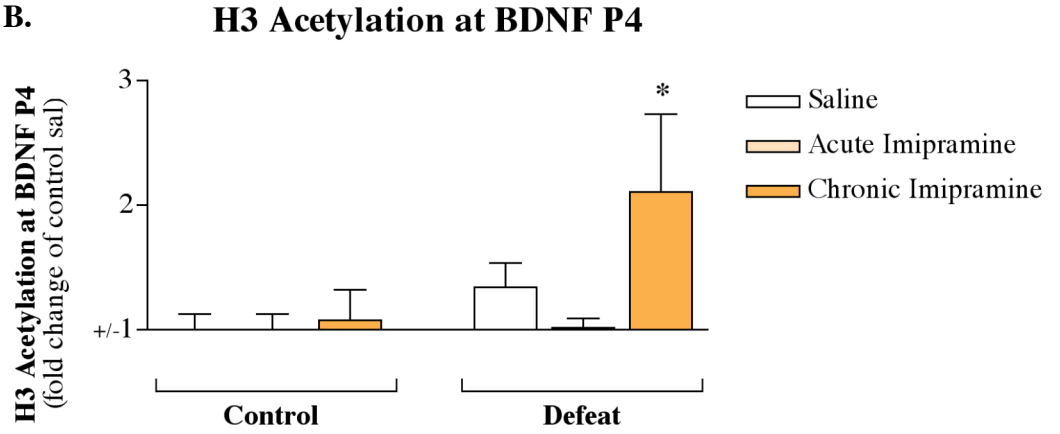


**Figure 13. Histone methylation is robustly and lastingly enriched at BDNF P3 after chronic defeat stress.** Chromatin immunoprecipitation (ChIP) assays were performed to measure the levels of histone modifications for several methylation states: H3-K9 di-methylation, H3-K27 mono-methylation, and H3-K27 di-methylation. Antibodies used were specific for each modification state. Levels of enrichment were quantified at the BDNF P3 and P4 promoters by qPCR. Levels of histone H3-K27 di-methylation were significantly increased at BDNF promoters P3 and P4 in chronically defeated animals, four weeks after the cessation of stress. Two different graphical representations depict this increase. **A.** Histone H3-K27 di-methylation levels at BDNF P3 after defeat yielded an average PCR cycle numbers of linear amplification (Ct values) significantly below that of non-specific antibody control (black line), whereas control animals showed Ct values at or above the threshold level for antibody specificity. **B.** Levels of H3-K27 di-methylation after chronic defeat were increased by more than fifty fold at BNDF P3 (54 ± 26 fold) and less so at P4 (2.9 ± 1 fold) compared to controls. H3-K9 di-methylation was not changed at BDNF P3 or P4. H3-K27 mono-methylation was significantly decreased after chronic defeat stress, but to a more subtle level than H3-K27 was increased at P3 (3.9 ± 0.5 fold for at P3 and 4.6 ± 1.8 fold at P4).

A.


H3 Acetylation at BDNF P3
H3 Acetylation at BDNF P3
(fold change of control sal)
3
2
+/-1
-2
-3
*
Control
Defeat
Saline
Acute Imipramine
Chronic Imipramine

B.


H3 Acetylation at BDNF P4
H3 Acetylation at BDNF P4
(fold change of control sal)
3
2
+/-1
*
Control
Defeat
Saline
Acute Imipramine
Chronic Imipramine

C.

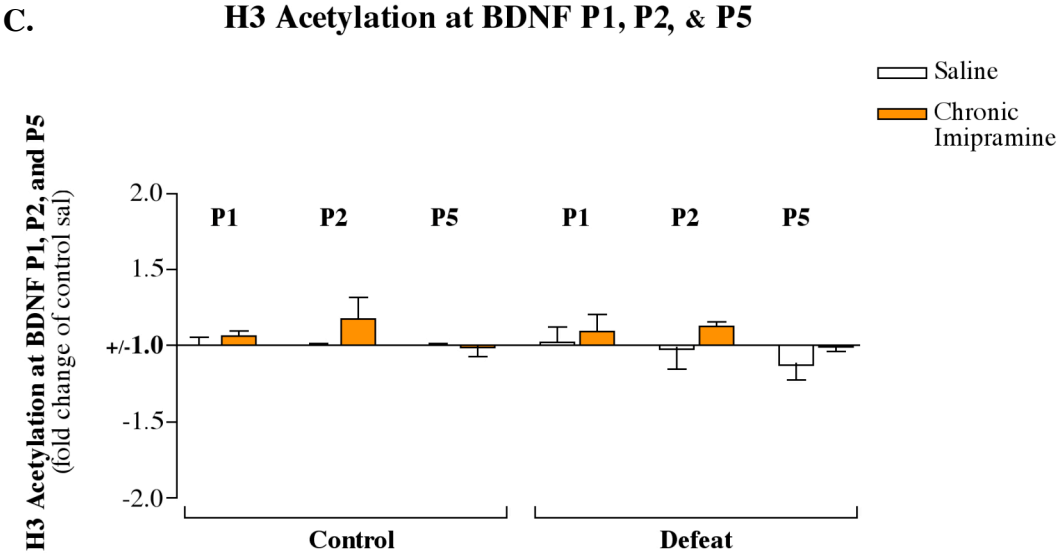

H3 Acetylation at BDNF P1, P2, & P5
H3 Acetylation at BDNF P1, P2, and P5
(fold change of control sal)
2.0
1.5
+/-1.0
-1.5
-2.0
P1
P2
P5
P1
P2
P5
Control
Defeat
Saline
Chronic
Imipramine

**Figure 14. Sustained changes in H3 acetylation at BDNF P3 and P4 after chronic treatment with imipramine.** Levels of H3 acetylation (K9, 14) were measured by ChIP after the following treatments: control + saline, control + acute imipramine, control+ chronic imipramine, defeat + saline, defeat + acute imipramine, and defeat + chronic imipramine. H3 acetylation-associated DNA was quantified by qPCR. Defeated animals treated with chronic imipramine displayed significant increase in the level of H3 acetylation at the promoter regions BDNF P3 (**A**) and BDNF P4 (**B**). H3 acetylation was enriched by 2.2 ± 0.5 fold at BDNF P3 and by 2.1 ± 0.6 fold at BDNF P4. None of the other animal groups examined showed enrichment that was significantly different from control saline. **C**. Other BDNF promoters (BDNF P1, P2, and P5) did not show significant differences in H3 acetylation neither in defeat + chronic imipramine animals (as seen for BDNF P3 and P4) nor in any of the other animal groups examined. N=6. T-tests were used to determine statistical significance ($p<0.05$).

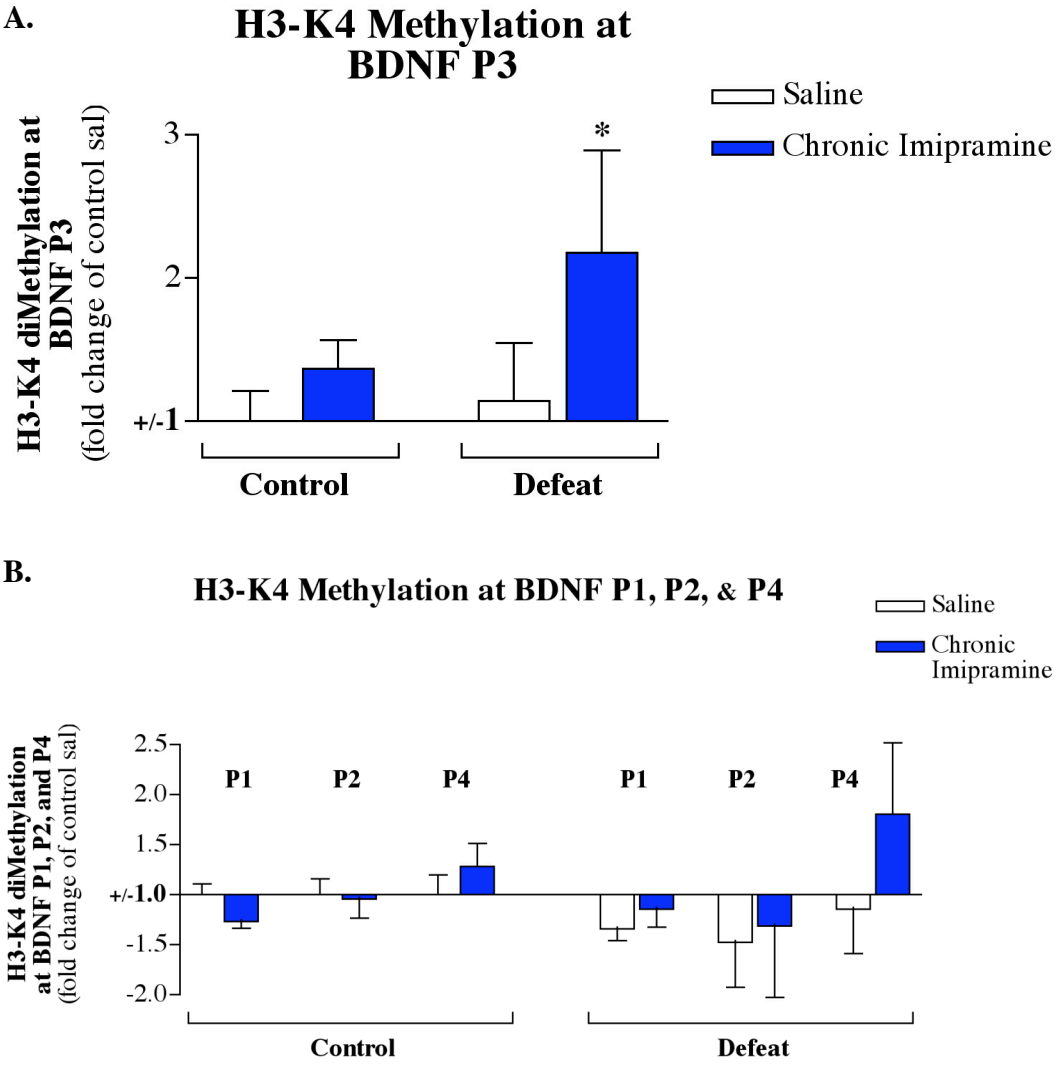



**Figure 15. Sustained changes in H3-K4 di-methylation at BDNF P3 after chronic treatment with imipramine.** Levels of H3-K4 di-methylation were measured by ChIP after the following treatments: control + saline, control+ chronic imipramine, defeat + saline, and defeat + chronic imipramine. H3-K4 di-methylation-associated DNA was quantified by qPCR. **A.** Defeated animals treated with chronic imipramine displayed significant increase in the level of H3-K4 di-methylation at BDNF P3 (2.2 ± 0.7 fold). Other animal groups did not show changes in H3-K4 di-methylation at P3. **B**. Significant changes were not detected at other BDNF promoter regions (P1, P2, and P4). N=6. T-tests were used to determine statistical significance ($p<0.05$).

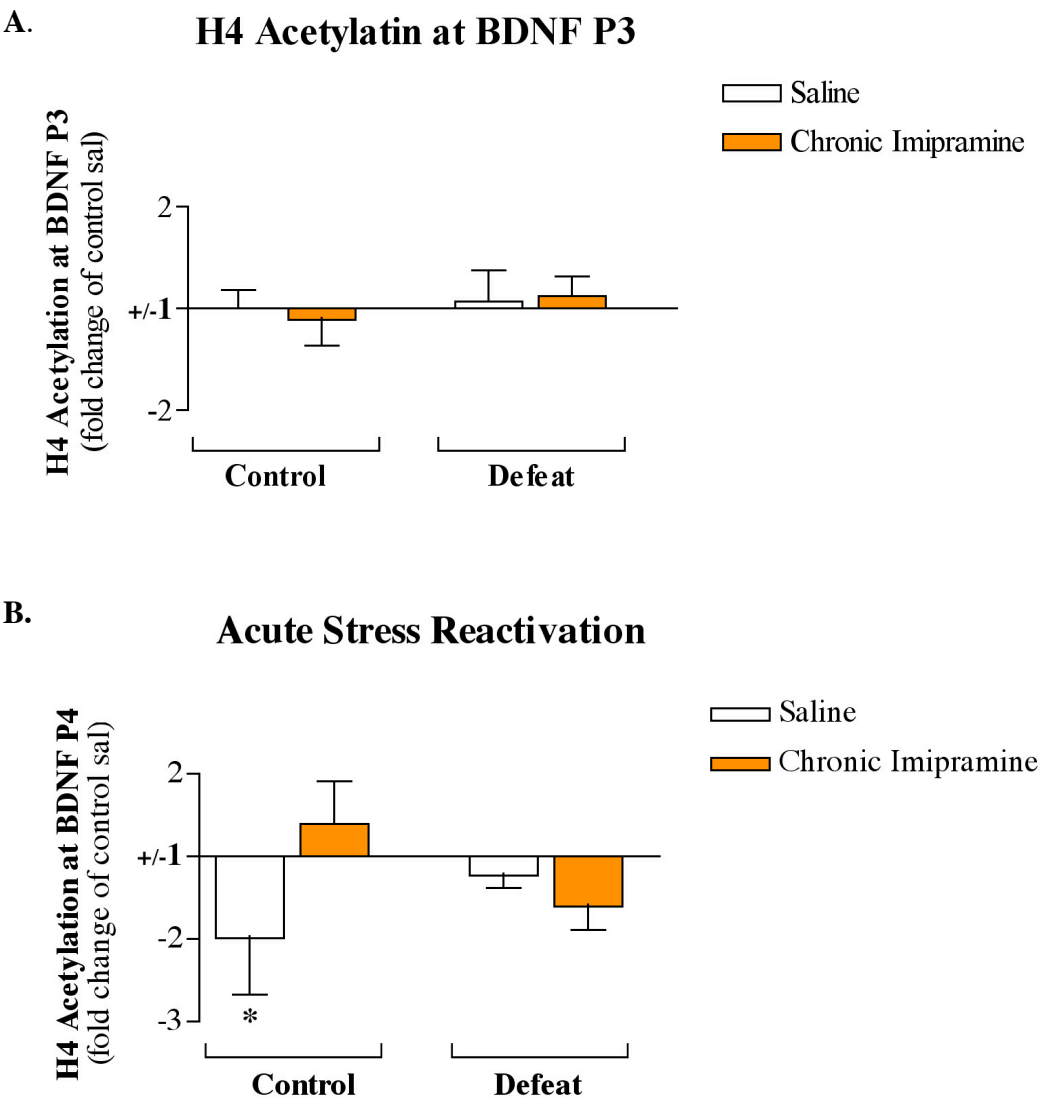
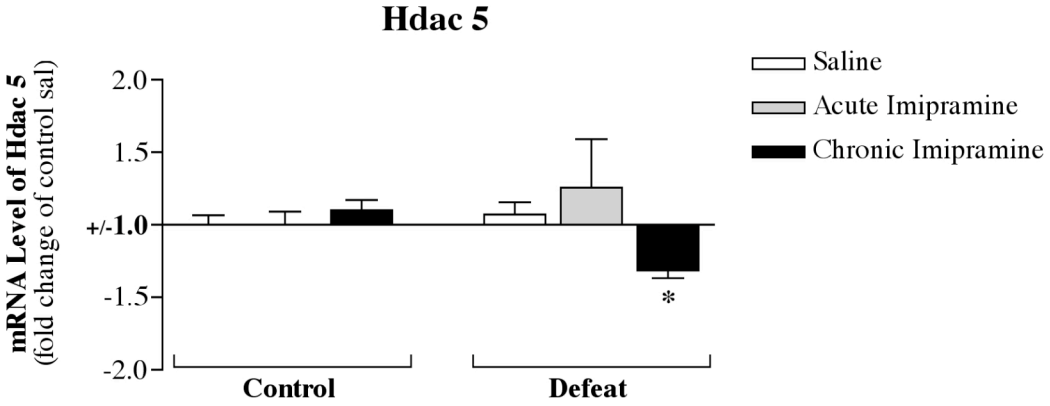
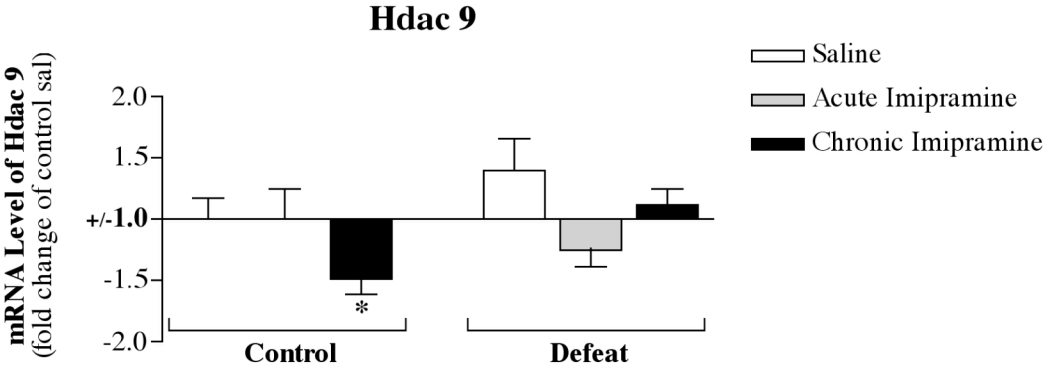



**Figure 16. Changes in H4 acetylation after chronic defeat and acute defeat stress reactivation. A**. Chronic defeat and imipramine treatments did not induce changes in H4 (K5, 8, 12, 16) acetylation at BDNF P3. **B.** Acute reactivation of defeat stress, however, induced a significant downregulation of H4 acetylation at BDNF P4 only in control animals treated with saline, compared to non-reactivated controls (2 ± 0.6 fold). N=6. T-tests were used to determine statistical significance ($p<0.05$)

**A.**


Hdac 5
mRNA Level of Hdac 5
(fold change of control sal)
2.0
1.5
+/-1.0
-1.5
-2.0
*
Control
Defeat
Saline
Acute Imipramine
Chronic Imipramine

**B.**


Hdac 9
mRNA Level of Hdac 9
(fold change of control sal)
2.0
1.5
+/-1.0
-1.5
-2.0
*
Control
Defeat
Saline
Acute Imipramine
Chronic Imipramine

**C.**

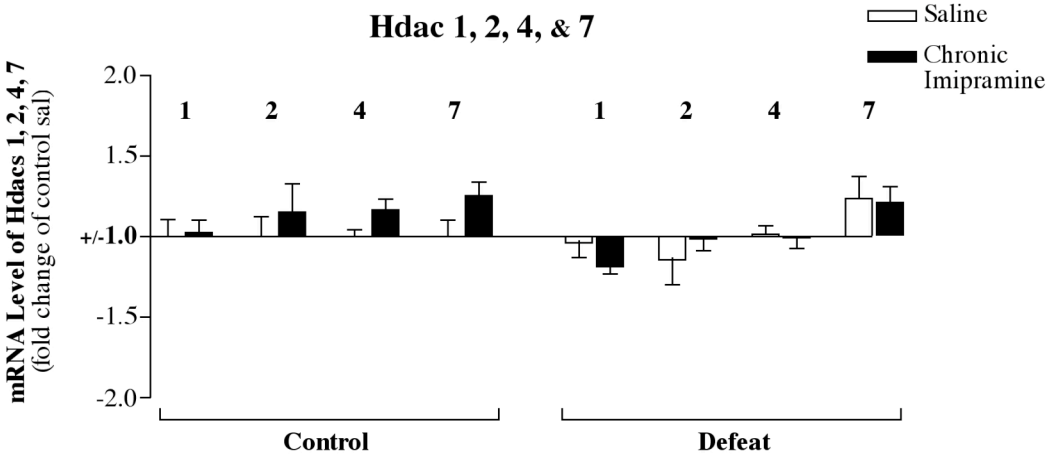
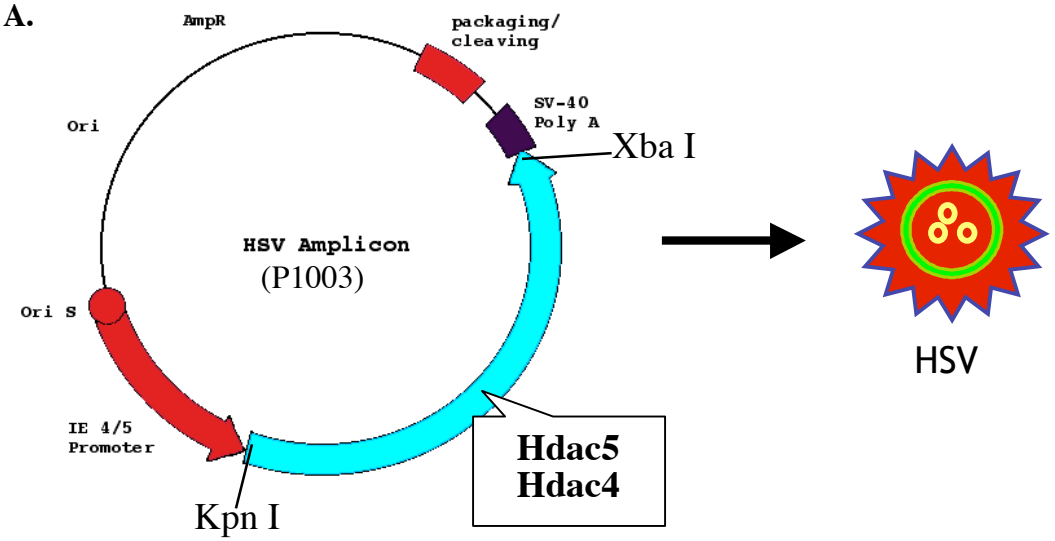
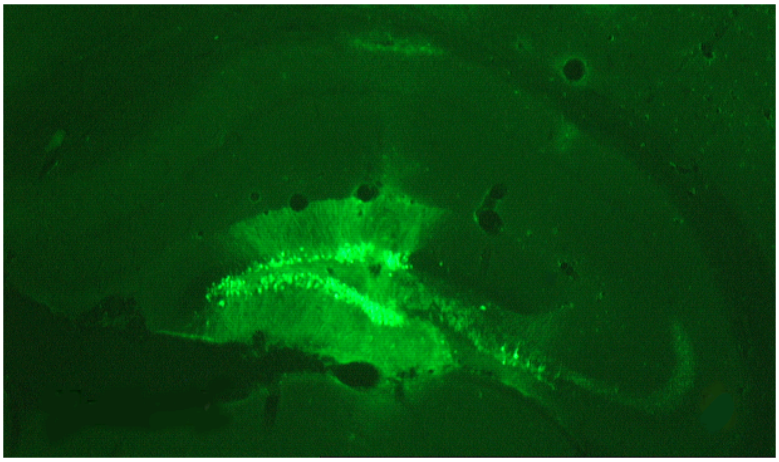

Hdac 1, 2, 4, & 7
mRNA Level of Hdacs 1, 2, 4, 7
(fold change of control sal)
2.0
1.5
+/-1.0
-1.5
-2.0
1
2
4
7
1
2
4
7
Control
Defeat
Saline
Chronic Imipramine

**Figure 17. HDAC5 mRNA expression is downregulated after chronic imipramine treatment in defeated animals.** The expression levels of HDACs 1, 2, 4, 5, 7, and 9 were measured by RT-PCR in non-stressed and defeated animals receiving either saline, acute imipramine (HDAC5, 9), or chronic imipramine. Only HDAC5 and HDAC9 showed significant changes in expression levels. **A**. HDAC5 mRNA was slightly but significantly downregulated in chronically stressed animals receiving chronic imipramine (1.3 ± 0.05 fold). This experiment was repeated twice, yielding similar results. HDAC5 mRNA was not changed in any of the other animal groups. **B**. HDAC9 was downregulated only in non-stressed animals treated with chronic imipramine (1.5 ± 0.12 fold). **C**. No significant changes were observed for HDACs 1, 2, 4, and 7 by any of the described treatments. N=6. T-tests were used to determine statistical significance ($p<0.05$).

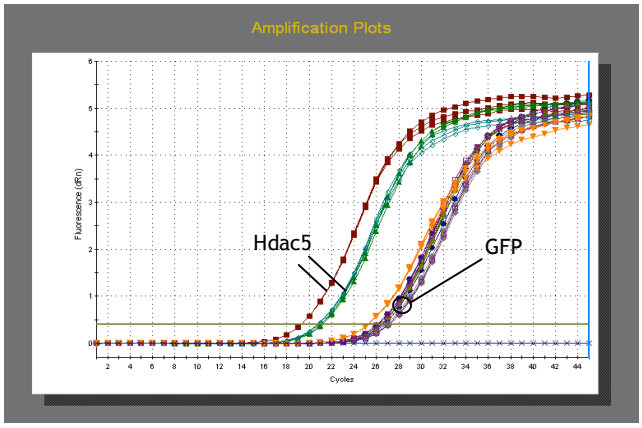
**A.**


AmpR
packaging/
cleaving
SV-40
Poly A
Xba I
Ori
HSV Amplicon
(P1003)
Ori S
IE 4/5
Promoter
Kpn I
Hdac5
Hdac4
HSV

**B.**



**C.**


Amplification Plots
Hdac5
GFP
Cycles

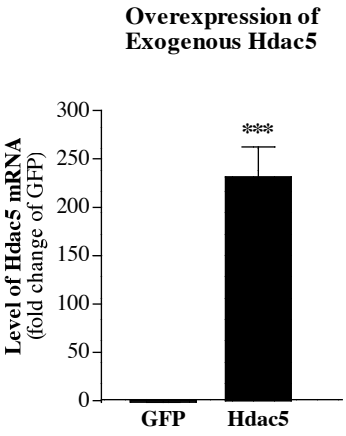
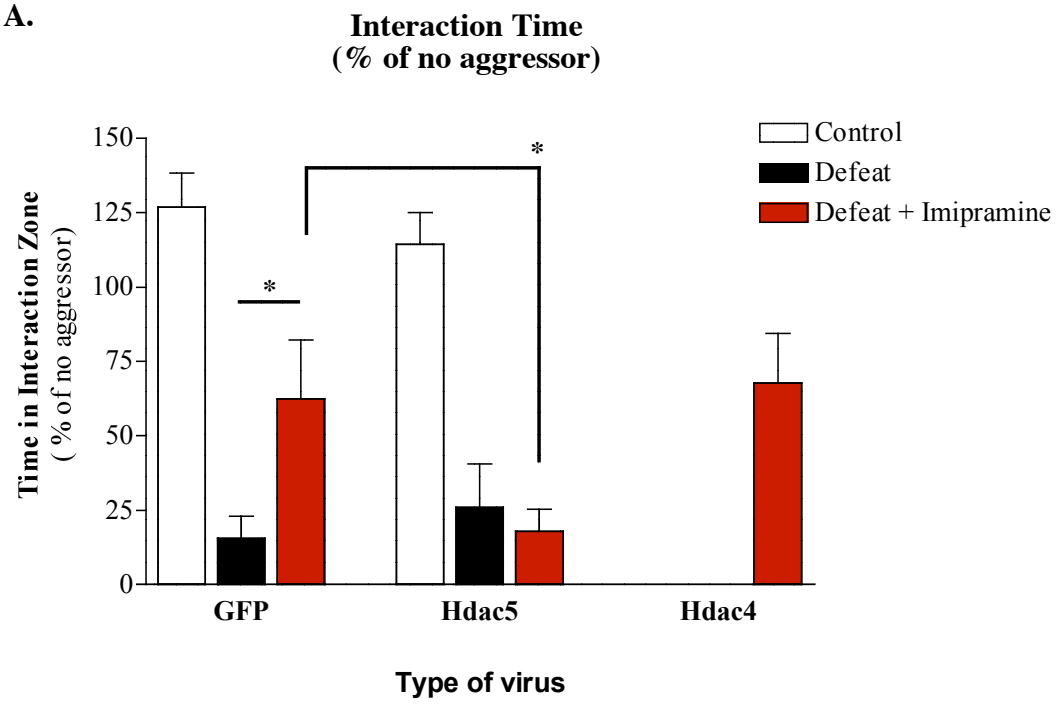

Overexpression of
Exogenous Hdac5
Level of Hdac5 mRNA
(fold change of GFP)
300
250
200
150
100
50
0
***
GFP
Hdac5

**Figure 18. Viral-mediated overexpression targets the dentate gyrus of the hippocampus and induces robust increase in HDAC5 expression.** **A**. HDAC5 cDNA was subcloned into HSV-GFP expression virus, and packaged for infection. **B**. The HSV virus was introduced into the hippocampus via stereotaxic surgery, and it infected specifically the dentate gyrus region, as assayed by immunohistochemical staining for GFP. **C**. HSV-HDAC5 infection induced very robust overexpression of exogenous HDAC5 mRNA (240 ±42 fold compared to HSV-GFP) in the dentate gyrus, four days after infection. Primers used were specific for exogenous HDAC5.

**A.**


Interaction Time
(% of no aggressor)
Time in Interaction Zone
( % of no aggressor)
150
125
100
75
50
25
0
*
*
Control
Defeat
Defeat + Imipramine
GFP
Hdac5
Hdac4
Type of virus

**B.**

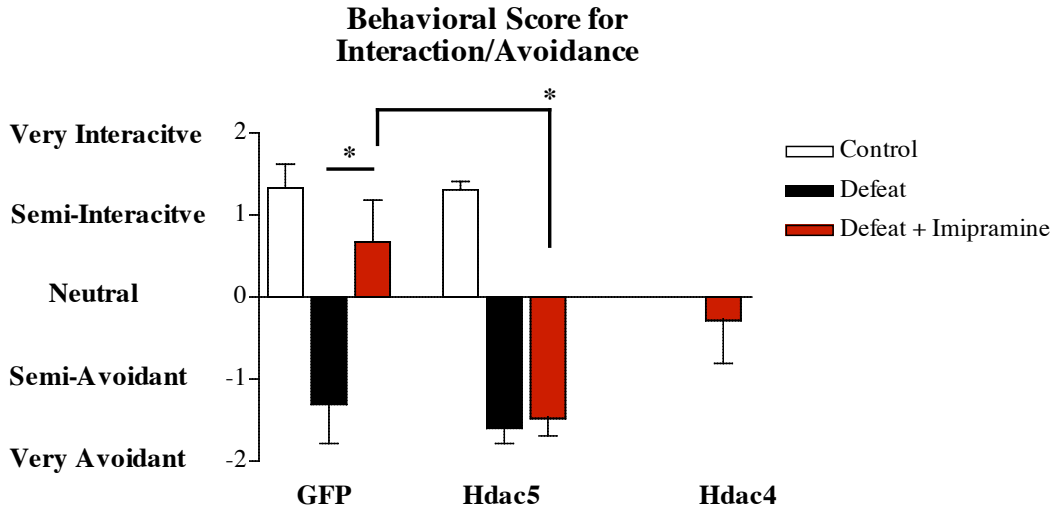

Behavioral Score for
Interaction/Avoidance
Very Interacitve
Semi-Interacitve
Neutral
Semi-Avoidant
Very Avoidant
2
1
0
-1
-2
*
*
Control
Defeat
Defeat + Imipramine
GFP
Hdac5
Hdac4

**Figure 19. HDAC5 overexpression blocks the ability of chronic imipramine to reverse deficits of social avoidance, while HDAC4 does not.** HSV-HDAC5 was injected into the dentate gyrus of mice after 10 days of chronic defeat or no defeat (control), followed by 4 weeks of saline or imipramine treatment. HSV-HDAC4 was only injected into the dentate gyrus of defeated animals treated with chronic imipramine. The defeated behavior of mice was tested in the social interaction/avoidance paradigm 3 days after infection. Their behavior was assessed by two different methods: measurement of time spent in the interaction zone as a % if no aggressor present (**A**), and blindly assigning a qualitative score (0 to +/- 2) for the level of social interaction/avoidance (**B**). Infection with HDAC5 did not significantly alter the interaction time of control animals (126 ± 11 % vs. 114 ± 10%; 1.3 ± 0.3 vs. 1.3 ± 0.1 scores; GFP vs. HDAC5) or defeated animals (16 ± 7 % vs. 26 ± 14%; –1.3 ± 0.4 vs. –1.6 ± 0.2 scores; GFP vs. HDAC5) treated with saline. HSV-HDAC5 infection induced significant changes in the behavior of defeated animals treated with imipramine compared to HSV-GFP controls, where it completely blocked imipramine's effect on reversing social avoidance after defeat (62 ± 20% vs. 18 ± 7 %; 0.7 ± 0.5 vs. –1.5 ±0.2 scores; GFP vs. HDAC5). HSV-HDAC4 infection did not show similar reduction, as seen for HDAC5 (62 ± 20% vs. 68 ± 17%; 0.7 ± 0.5 vs. –0.3 ± 0.5 scores; GFP vs. HDAC4). N=10 (defeat + imipramine, GFP and HDAC5); N=7 (defeat + imipramine, HDAC4); N=9 (control, GFP and HDAC5); N=8(defeat, GFP); N=5(defeat, HDAC5). Two-way ANOVA and Bonferroni post-test were used to determine statistical significance ($p < 0.05$).

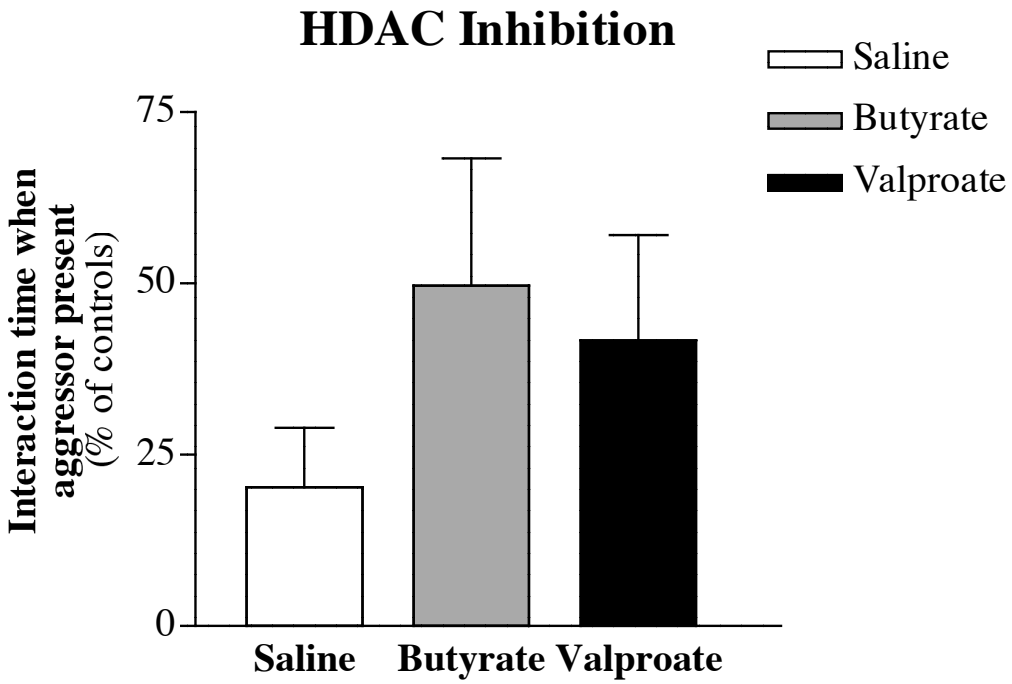
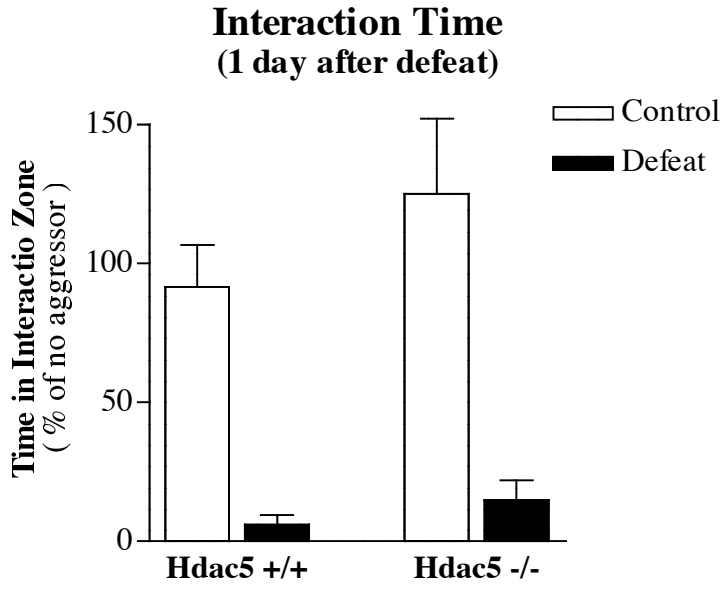



**Figure 20. HDAC inhibition with sodium butyrate and valproic acid induces subtle antidepressant-like effect in defeated animals.** Control and chronically defeated animals were treated with either sodium butyrate, valproic acid, or saline (twice daily, each time with 200mg/kg ip) for 3 weeks. At the end of the treatment, the behavior of these mice was measured in the social interaction/avoidance test paradigm. Defeated animals receiving saline showed a characteristic decrease in the time spent in the interaction zone in the presence of an aggressor (12 ±5 sec or 20% compared to their control). Defeated animals treated with either butyrate or valproate displayed a less pronounced decrease in interaction time compared to their controls, in the presence of an aggressor target (28 ± 10 and 27 ±10 sec or 50% and 42% for SB and VPA, respectively). N=8

**A.**


Interaction Time
(1 day after defeat)
Control
Defeat
Time in Interactio Zone
( % of no aggressor )
150
100
50
0
Hdac5 +/+
Hdac5 -/-

**B.**

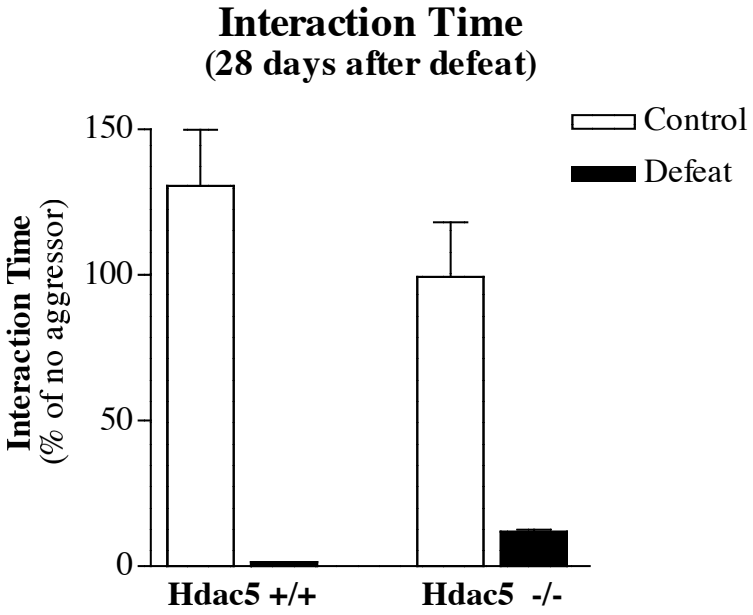

Interaction Time
(28 days after defeat)
Control
Defeat
Interaction Time
(% of no aggressor)
150
100
50
0
Hdac5 +/+
Hdac5 -/-

**Figure 21. Social interaction of HDAC5 wild type and knock out mice after defeat.** Chronic defeat decreased profoundly the interaction time of both HDAC5 +/+ and HDAC5 -/- mice when an aggressor was introduced into the test cage. When tested at 1 day after the end of defeat (**A**), HDAC5 +/+ and -/- defeated mice decreased their interaction to 6 ± 3% and 15 ± 7%, respectively. When tested at 28 days after the end of defeat (**B**), the interaction of the same mice decreased even further, to 1.5% and 12 ± 1%. Non-defeated mice of both strains did not show significant reduction in their interaction under an aggressive target during both tests (92 ± 15% and 125 ± 27% at 1-day and 100 ± 18% and 130 ± 19% at 28-days of being single-caged for +/+ and -/- mice, respectively). N=5-11.

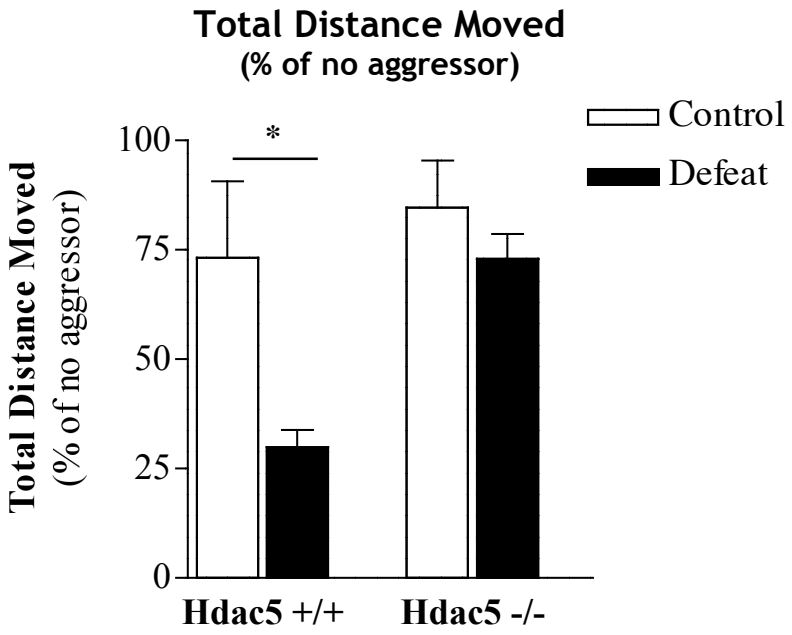



**Figure 22. HDAC5-deficient mice show a weaker response to stress as measured by their locomotor activity in the presence of an aggressor.** The total distance traveled in the box, in the absence and presence of an aggressor, was measured for both HDAC5 +/+ and -/- mice one day after the end of the defeat stress. HDAC5 +/+ mice displayed a robust reduction in their overall locomotor activity (distance traveled in the box) when an aggressor mouse was introduced (30% of distance (cm) traveled compared to no aggressor, and 40% less compared to HDAC5+/+ non-defeated mice). Contrary, HDAC5 -/- did not show a strong stress-induced reduction in locomotor activity. Their distance traveled in the presence of an aggressor was lowered to only 73% of that in the absence of an aggressor, which was similar to the reduction in distance traveled by non-defeated HDAC5 -/- mice (85 ± 11%). N=18

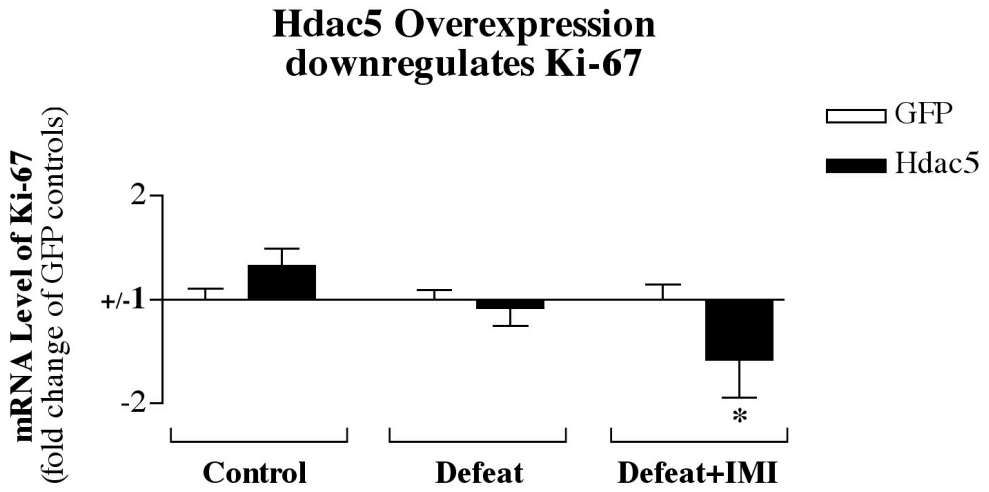
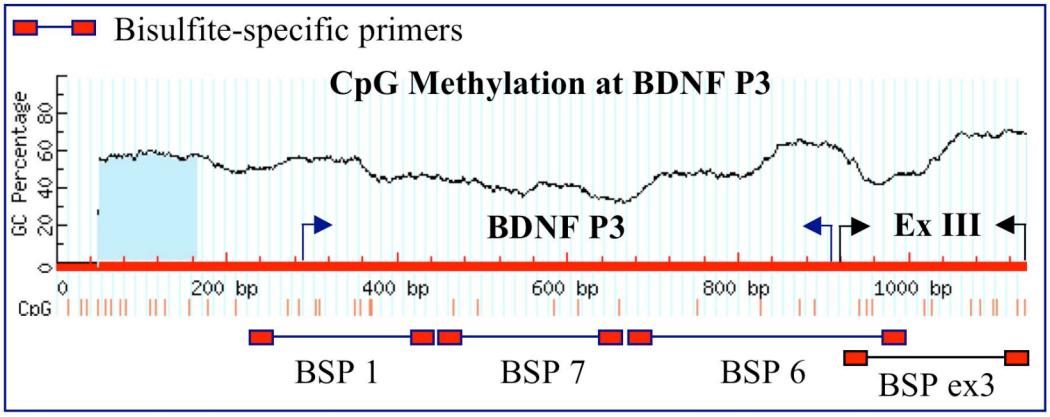


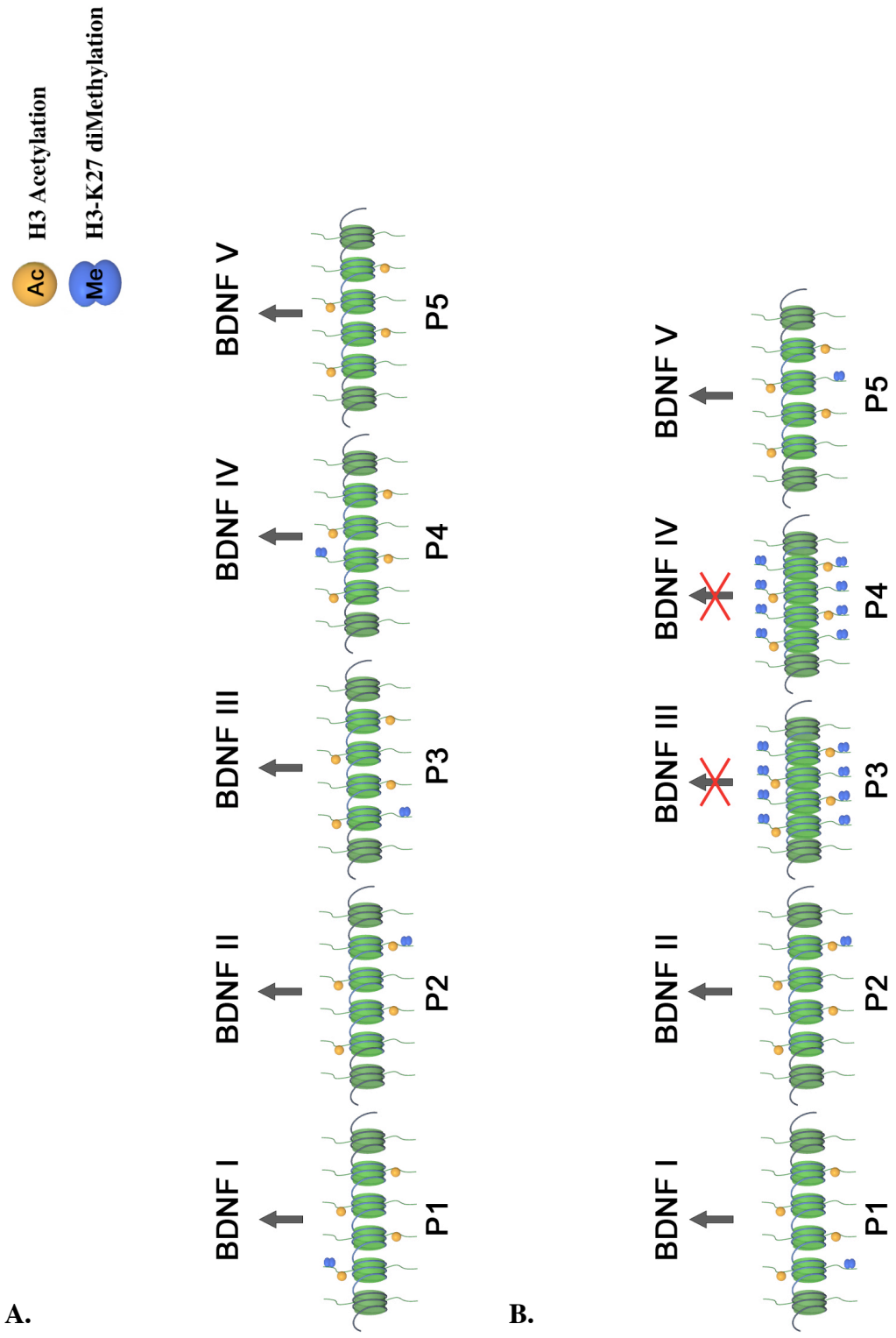

**Figure 23. HSV-HDAC5 overexpression into the dentate gyrus of the hippocampus decreases the expression of Ki-67, a marker of cell proliferation and neurogenesis.** The mRNA expression of Ki-67 was measured by RT-PCR in the dentate gyrus on day 4 after infection with the HSV-HDAC5 overexpressing virus or the control HSV-GFP virus. Levels of Ki-67 for control, defeated, and defeated mice treated with chronic imipramine were compared after either surgery with HSV-GFP or HSV-HDAC5. HSV-HDAC5 overexpression caused a slight but non-significant increase in Ki-67 expression in control animals (1.3 ± 0.16 fold, $p>0.05$), did not affect Ki-67 expression in defeat animals (-1.1 ± 0.2 fold), but it induced a significant downregulation of Ki-67 in defeated animals treated with imipramine (-1.6 ± 0.3 fold). N=6. T-tests were used to determine statistical significance ($p<0.05$).




**Number of methylated CpG out of total sites and colonies examined**

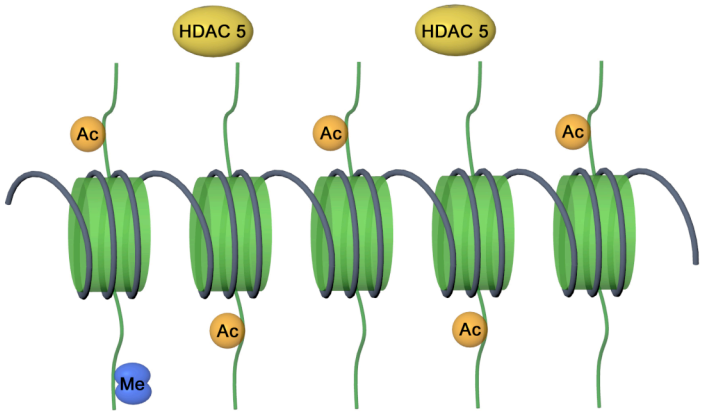
| Primer/ Treatment | BSP 1 | BSP 7 | BSP 6 | BSP ex3 |
|---|---|---|---|---|
| Control | **8 / 48** | **0 / 60** | **1 / 84** | **N / A** |
| Defeat | **7 / 48** | **0 / 60** | **2 / 84** | **2 / 150** |

**Figure 24. Methylation at CpG sites is not increased after defeat at BDNF P3.** Levels of methylation at CpG sites at the BDNF P3 promoter and BDNF exon III were assayed using the sodium bisulfite method in non-defeated controls and chronically defeated mice, 4 weeks after then end of stress. Several different primers were used (BSP1, BSP7, BSP6, and BSP ex3) to amplify small regions of the BDNF P3 promoter and exon III. The PCR products were cloned into TOPO-TA vector, and sequenced. Methylation was compared in 3 control and 3 defeat animals; 4-5 different colonies carrying the amplification insert were sent for sequencing from each animal. The table depicts the number of CpGs found to be methylated using the sodium bisulfite treatment out of total number of CpGs examined (total # of animals/group **x** total # of insert/animal **x** total # of CpGs/insert). N=12-15.


H3 Acetylation
H3-K27 diMethylation
Ac
Me
A.
BDNF I
BDNF II
BDNF III
BDNF IV
BDNF V
P1
P2
P3
P4
P5
B.
BDNF I
BDNF II
BDNF III
BDNF IV
BDNF V
P1
P2
P3
P4
P5

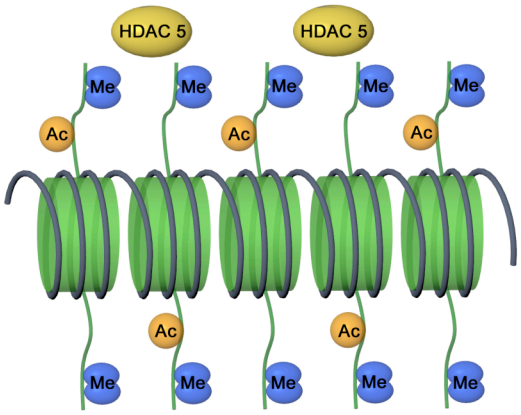
C.

Normal




D.

Defeat




E.

Antidepressant

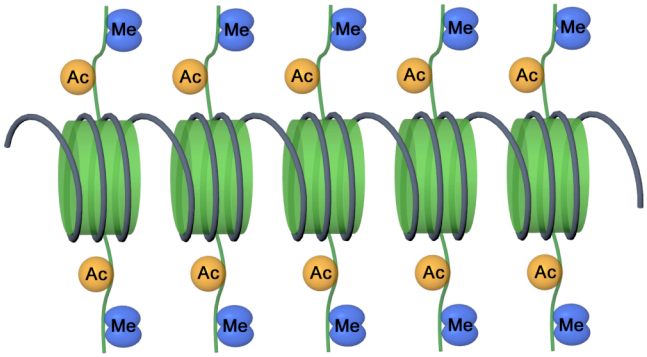


**Figure 25. Model of "open/closed" chromatin state modulation in chronic defeat and after treatment with chronic imipramine at the BDNF gene.** Based on our chromatin immunoprecipitation data, mRNA data, and HDAC5 overexpression and inhibition behavioral data, we propose a model for a possible mechanism by which chronic stress induces repression at BDNF P3 and P4 and by which chronic imipramine, via regulation of HDAC5, can alleviate this repression. In the absence of stress, the chromatin state of BDNF is at a level that neither promotes nor represses transcription ("basal level"), characterized by a basal level of H3 acetylation and little to none enrichment of H3-K27 dimethylation (**A**). In these animals, HDAC5 might function to repress unnecessary activation of BNDF and maintain a chromatin balance (**C**). Chronic defeat stress induces the specific di-methylation of histone H3 at residue K27, which remains long after the cessation of the stress. This induces a more "closed" chromatin state, and the repression of BDNF III and IV (**B**). Acetylation and HDAC5 regulation are not affected after chronic stress alone, corroborating the idea that the main repressive marker after chronic stress is histone methylation (**D**). Chronic imipramine induces hyperacetylation after chronic defeat, and downregulates HDAC5 expression. This imipramine-induced inhibition of HDAC5 leads to a more permissive chromatin state at BDNF P3 and P4, an upregulation of BDNF mRNA level, and an overall antidepressant efficacy (**E**).

**TABLE 2:**

| Experiment | Name | Sequence |
|---|---|---|
| mRNA | BDNF I | CCTGCATCTGTTGGGGAGAC<br>GCCTTGTCCGTGGACGTTTA |
| ChIP | BDNF II | CTAGCCACCGGGGTGGTGTAA<br>AGGATGGTCATCACTCTTCTC |
| | BDNF III | CTTCCTTGAGCCCAGTTCC<br>CCGTGGACGTTTACTTCTTTC |
| | BDNF IV | CAGAGCAGCTGCCTTGATGTT<br>GCCTTGTCCGTGGACGTTTA |
| | BDNF V | TTGGGGCAGACGAGAAAGCGC<br>AGGATGGTCATCACTCTTCTC |
| | BDNF total | ACTTGGCCTACCCAGGTGTG<br>CCAAAGGCACTTGACTGCTG |
| | HDAC1 | TGCGTGGAAAGAAAACAACC<br>ACCCAGACCCCTCCTAAATG |
| | HDAC2 | GGGACAGGCTTGGTTGTTTC<br>GAGCATCAGCAATGGCAAGT |
| | HDAC4 | CAATCCCACAGTCTCCGTGT<br>CAGCACCCCACTAAGGTTCA |
| | HDAC5 | TGTCACCGCCAGATGTTTTG<br>TGAGCAGAGCCGAGACACAG |
| | HDAC7 | GGTGGACCCCCTTTCAGAAG<br>TGGGTAGCCAGGAGTCTGGA |
| | HDAC9 | GCGAGACACAGATGCTCAGAC<br>TGGGTTTTCCTTCCATTGCT |
| | Human HDAC5 | GTAGCCATCACCGCAAAACT<br>GTCCTCCACCAACCTCTTCA |
| | Ki-67 | GCCAAGTCAGTGGGAAAAGA<br>TCTGGACCTCAAACACCTAACA |
| | BDNF P1 | TGATCATCACTCACGACCACG<br>CAGCCTCTCTGAGCCAGTTACG |
| Methylation | BDNF P2 | CCGTCTTGTATTCCATCCTTTG<br>CCCAACTCCACCACTATCCTC |
| | BDNF P3 | GTGAGAACCTGGGGCAAATC<br>ACGGAAAAGAGGGAGGGAAA |
| | BDNF P4 | CTTCTGTGTGCGTGAATTTGCT<br>AGTCCACGAGAGGGCTCCA |
| | BDNF P5 | ACTCACACTCGCTTCCTCCT<br>GCACTGGCTTCTCTCCATTT |
| | CDK5 | GCTGAAGCTGTCAGGAGGTC<br>GTGCCCCGCTCTTGTTATTA |
| | CREB | GTCGAGCTCGGCTGTTTC<br>ACTCACCAACACTCCGCTTC |
| | Substance P | GCGGAAGTTATTTGGCTGTC<br>ACAATCTGACGCCCTCCTC |
| | BSP P3 1 | TTTGGGAAATGTAAGTGTTTATTATTAG<br>CCAAATTCTCACCTAAATCAATTTAA |
| | BSP P3 6 | AAGAGTATGGAGGGAATGTG<br>ACCCAAACATAAAAAACTATCC |
| | BSP P3 7 | ATTTAGGTGAGAATTTGGGGTAAAT<br>AATACAAAATCAAATCTTAAAAAATC |
| | BSP Ex3 | GTTTGGAGGGTTTTTGTTTTTTAA<br>TAAAAATATTCTTCTCCACCTCCAC |

**Table 2. Comprehensive list of primer sequences used for mRNA and ChIP analysis.**

# THESIS CONCLUSION

As Hippocrates scientifically observed two and a half millennia ago, depression disrupts the internal imbalance in our body and mind. We now know that this disease causes perturbations in the endocrinological, neuropharmacological, electrical, morphological, and the molecular structure of the brain, which amass to lasting changes in the physiology and psyche of susceptible individuals. This thesis discussed yet another level at which the balance in the brain might be disrupted in depression, the level of chromatin regulation of gene expression. The thesis explored the plastic capacity of the brain, and in particular the hippocampus, to prevent such imbalance at this level of regulation or to restore the system to homeostasis with the help of antidepressant drugs. Chromatin modifications provide an epigenetic control on cellular memory; they allow cells to remember what was, what is, and what might be without affecting the structure of the DNA. In this manner, epigenetics can maintain plasticity. Here, we propose that the cellular memories encoded by epigenetic modifications of histones can transform into behavioral memories as well.

We have shown that chronic ECS, an effective antidepressant treatment in many people with severe depression, leads to lasting changes in the expression of the BDNF, c-Fos, and CREB genes, and that these changes are mediated via

sustained alterations in the levels of histone acetylation at their promoter regions. Next, we discovered that chronic defeat stress, which we validated as an animal model of depression, induces the long-lasting downregulation of two splice variant transcripts of the BDNF gene, BDNF III and IV. Furthermore, we provided intriguing evidence that the promoter regions driving this downregulation are highly enriched in one particular histone methylation marker (di-methylated H3-K27), which is present at least four weeks after the cessation of stress. This thesis explored even further the role of antidepressants not only in normal animals, but also in mice, which had been chronically stressed with consequential changes in their behavior (social interaction) and their molecular homeostasis (downregulation of BDNF). When treating such mice with the tricyclic antidepressant imipramine, we saw a reversal of the sustained downregulation of BDNF III and IV with corresponding sustained changes in the chromatin structure of the BDNF promoters P3 and P4 (increased H3 acetylation and H3-K4 methylation). Finally, the thesis provides substantial evidence for the role of a histone-modifying enzyme, HDAC5, in the maintenance of the long-term antidepressant efficacy of imipramine.

This thesis offers evidence for the disruptive role of histone methylation after chronic stress, as well as for the role of antidepressants, both imipramine and ECS, in maintaining neuroplastic adaptations at the level of histone acetylation in normal and stressed animals. However, it does not explore the role of

antidepressants in regulating levels of histone methylation, a marker that might be more closely related to long-lasting pathological states of stress. Other questions remain to be answered as well. For example, is there a chromatin pattern that governs which BDNF splice variants are expressed after ECS vs. different types of antidepressants, and how much do individual animal differences affect the differential expression of BDNF I-V? In addition, what causes certain histone modifications to be prevalent in acute stimulations vs. more chronic ones? We have proposed that acute to chronic stimulations might be mediated via a H4 to H3 acetylation switch at BDNF, but the mechanisms governing this switch are unclear. We have provided evidence that dysregulation of HDAC5 levels leads to perturbations in the therapeutic efficacy of imipramine. Still, we don't know if this effect is specific for HDAC5, as well as if the efficacy of other antidepressants is affected similarly. Finally, we do not yet understand if chromatin remodeling is a process by which many different drugs can act, or if it is specific for imipramine and ECS.

For every answer provided in this thesis, we have raised several new questions. This illustrates the intricate and complicated nature of how chromatin is regulated, how it affects plasticity in the brain to maintain an internal balance, and how depression can disrupt this process, and how antidepressants can restore it. Nevertheless, with each new question that we ask, we come one step closer to understanding the mystery of our biology.

# APPENDICES

During my Ph.D. research, I was involved in several other projects. While these are not directly related to the main focus of this thesis, I am including a description of this work for completeness.

APPENDIX I: *In Vivo* Regulation of Adenylyl Cyclase VIII After Chronic Morphine Administration and Morphine Withdrawal

APPENDIX II: Chromatin Remodeling at the Promoter of Genes Implicated in Contextual Fear Conditioning

APPENDIX III: *In vivo* Binding of FosB and ΔFosB at the Substance P Promoter After Repeated Foot Shock

# APPENDIX I

## *In Vivo* Regulation of Adenylyl Cyclase VIII After Chronic Morphine Administration and Morphine Withdrawal

**Background:**

Drug addiction is a chronic disease that debilitates the body and the mind. Acutely, drugs interfere with the normal functions of the brain to induce a feeling of pleasure by activating the mesolimbic dopaminergic reward system. Chronic drug exposure causes behavioral abnormalities in rodent models, such as tolerance, dependence, and sensitization. These behavioral changes are due to long-term effects of brain metabolism and activity. Chronic exposure to opiates is a well-studied example of addiction that leads to stable cellular and molecular changes in the brains of rodent models (89). One pathway that is very important in mediating these changes is the cyclic-AMP (cAMP) pathway. Chronic opiate administration leads to a direct upregulation of the cAMP pathway in the locus coeruleus (LC) (90-92), and may lead indirectly to upregulation in the periaqueductal gray (PAG), nucleus accumbens (NuAC), and the ventral tegmental area (VTA) (89, 93, 94). This response is believed to be compensatory;

it counteracts the acute inhibitory effects of opiates on adenylyl cyclase, a molecule that is part of the cAMP pathway (91). In the nucleus accumbens and the VTA, two areas that are part of the mesolimbic reward pathway, upregulation of cAMP manifests as dysphoria during early withdrawal periods (89, 93, 94). In the locus coeruleus, the major noradrenergic nucleus in the brain implicated in controlling vigilance and the autonomic nervous system (95), upregulation of the pathway results in physical dependence and withdrawal (89). In the locus coeruleus, chronic morphine administration leads to increased expression of cyclic-AMP response-element-binding protein (CREB) (96-98), a transcription factor that binds to cAMP response element (CRE) consensus sites [TGACGTCA] and mediates the transcriptional regulation of many genes (99). Two such genes are adenylyl cyclase 8 (AC8) and tyrosine hydroxylase (TH). AC8 and TH contain CRE-sites within their promoters, and are also upregulated in the LC during chronic morphine administration (100-102). Several experiments including cell-culture analysis of AC8 promoter activity upon cAMP stimulation, expression of constitutively active or dominant-negative forms of CREB, deletions of specific promoter sites, as well as gel-shift assays, implicate a direct role of CREB for the regulation of AC8, at least *in vitro*. While there is some evidence that CREB regulates AC8 promoter activity *in vivo* as well (103), the precise mechanism by which CREB activation leads to increased levels of AC8 transcription during chronic morphine and morphine withdrawal remains

unclear (89). This study was initiated in order to examine how CREB regulates AC8 expression *in vivo* after chronic morphine administration and morphine withdrawal.

**Experimental Design, Results, and Discussion:**

The model for this study were two transgenic mouse lines, which carried a 35 kb fragment of the AC8 promoter linked to a GFP reporter. Some lines carried the wild-type promoter fragment (wtAC8_GFP) while others carried a promoter in which the CRE consensus site had been mutated (mAC8_GFP). Mutations in the CREB-binding site, as above-mentioned *in vitro* data indicates (103), was hypothesized to prevent the binding of CREB to the AC8 promoter and thus to prevent any CREB-mediated regulation of AC8 expression. The conditions under which AC8 expression was analyzed included chronic morphine and morphine withdrawal. Previous studies had indicated that chronic opiate administration and opiate withdrawal lead to upregulation of CREB and AC8 in the LC (89, 96, 100). CREB is a transcriptional regulator for AC8, at least *in vitro* (103). Therefore, we suspected that the levels of AC8-induced GFP immunofluorescence would increase after chronic morphine administration in the LC, as well as in other brain areas where there is a change in the activity of the cAMP pathway, such as the NuAC, VTA, and the PAG. If CREB regulates the transcription of AC8 *in vivo*,

as hypothesized, the AC8-induced GFP immunofluorescence after chronic morphine would be reduced in the line of mice that carry a promoter with a mutated CRE site. Finally, since opiate withdrawal leads to even further (and unopposed) upregulation of the cAMP pathway, we hypothesized similar changes of AC8 expression for morphine withdrawal as for chronic morphine, but of much higher amplitude.

The above-mentioned constructs had already been inserted into a BAC, and the BAC DNA had been microinjected into oocyte pronuclei of B6BAF1 intercrosses, and transferred into pseudopregnant Swiss Webster females. Mice positive for the insert were weaned. These adult mice received chronic morphine for 5 days via subcutaneous placement of a morphine pellet every other day. Withdrawal was precipitated by intraperitoneal injection of 100mg/kg of the morphine antagonist naltrexone. Five hours later, mice were perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, post-fixed for 24 hrs, and then cryoprotected in 20% glycerol at 4°C for up to 1 week. Brains were then cut in 35 nm slices using a microtone. Slices were used for immunohistochemical staining with an anti-rabbit primary antibody to GFP, followed by donkey-anti-rabbit secondary antibody conjugated with cytochrome 2 (Cy2). The Cy2-GFP staining was detected by fluorescent microscopy. I tried several different antibody dilutions: 1:100, 1:200, 1:500, 1:1000, and 1:2000 of the primary anti-rabbit GFP antibody. I found that the

dilution between 1:200 and 1:500 yielded the best staining with minimal amount of background.

Using the above-mentioned experimental design, I began to analyze the wtAC8_GFP line for GFP staining throughout the brain. Unfortunately, I did not detect any AC8 expression in the brain areas of interest for addiction studies: LC, NuAC, VTA, and PAG in controls, chronic morphine, and withdrawal animals. However, AC8 expression was detected consistently in several other areas, including the habenula, olfactory bulb and tubercle, lateral olfactory tract, superior colliculus, dorsal lateral geniculate nucleus, some cortical areas, medial habenular nucleus of the thalamus, the hippocampus, and the deep mesencephalic nucleus (Table 3, Figure 26). Previous experiments confirmed the expression of AC8 in some of these areas (olfactory cortex, habenula, and hippocampus), but not in others (superior colliculus, dorsal lateral geniculate nucleus, and deep mesencephalic nucleus). In addition, previous experiments had reported expression of AC8 in the cerebellum, amygdala, hypothalamus, arcuate nucleus, and the locus coeruleus, which I did not see (96, 104) (Table 3).

It was possible that the AC8_GFP vector had inserted into a region of the genome that is normally transcriptionally silenced, thus preventing the expected expression of GFP under the AC8 promoter in the region of the LC. Therefore, we decided to generate more lines of transgenic mice using the same BAC vector. These injections were carried out, and we obtained several new founders. I

analyzed the new founders similarly to the first ones, and I saw expression again mainly in the habenula, superior colliculus, and hippocampus. Unfortunately, once again, I did not find any expression of AC8 driving GFP in any of regions of interest for our lab: LC, NuAC, VTA, and PAG. Since I did not find a line, which expresses AC8 in brain regions implicated in the pathophysiology of addiction, I did not analyze further the differences of AC8 expression after chronic morphine and morphine withdrawal, as well as the effects of AC8 expression when CREB binding is disabled in the mutant AC8_GFP lines. AC8 expression in the LC had been reported previously; however, our transgenic model did not recapitulate this expression, for reasons that are not completely understood.

# APPENDIX 2

## Chromatin Remodeling at the Promoters of Genes Implicated in Contextual Fear Conditioning

**Background:**

Contextual fear conditioning is a behavioral paradigm that tests associative learning of the context in which an animal receives an aversive stimulus, such as electric foot shock. Lesion studies have shown that the hippocampus is necessary for acquisition of declarative memories, and this region, as well as the amygdala, have both shown to be critical for acquisition of contextual memories associated with fear (105, 106). Long-term potentiation (LTP), a widely studied model of memory in the hippocampus, is also implicated in fear conditioning (107). LTP depends on the *de novo* gene transcription and protein synthesis. LTP induces the activation of several kinases, and results in the activation of transcription from CRE-containing gene promoters (108). Thus, it is believed that the transcription factor CREB is key in regulating protein synthesis necessary for LTP formation. Mice deficient in CREBα and CREBδ isoforms show impairments in hippocampal contextual fear conditioning learning (109). In

addition, inducible transgenic mice have shown that CREB is important for the stability of new and reactivated fear memories (110). Another gene, which has been implicated to play a role in contextual fear conditioning and has been shown to be transcriptionally regulated by CREB, is BDNF (54, 111). BDNF expression is increased by synaptic stimulation (112). Mice deficient in BDNF show abnormal LTP, and mice deficient in the BDNF receptor, TrkB, show deficits in hippocampus-dependent learning tasks (113, 114). BDNF expression is rapidly induced in the CA1 region of the hippocampus but not in the amygdala during contextual learning (115). While both CREB and BNDF appear to be important in mediating the molecular mechanisms of fear-related memory formation, it has not been established if the upregulation of BDNF is CREB-dependent, and which, if any of the BDNF promoters, is selectively targeted by CREB. In addition, the role of chromatin remodeling in fear conditioning had not been studied at the time this experiment was carried out, except for a recent study, which reported an increase in histone acetyltransferase activity in the insular cortex during novel taste learning (116).

We were interested in exploring the role of chromatin remodeling, in particular histone acetylation, at the promoters of CREB and BDNF, as well as other genes that show altered gene activity as a result of fear-dependent learning. We hypothesized that histone acetylation would be enriched at some of the BDNF promoters after contextual learning. This process may be mediated via CBP at the

BDNF P4 promoter in mouse, which has been shown to be transcriptionally regulated by CREB after calcium stimulation. Since there are no reports of changes in CREB transcriptional activity during and immediately after fear conditioning, we were curious to see what, if any, changes in acetylation would occur at the CREB promoter. Finally, BDNF transcription was shown to peak at 30 minutes after foot-shock at the CA1 hippocampal region. By 3 hours after foot shock, however, protein synthesis necessary for fear-associated memory formation is completed, and we suspected that learning-dependent gene expression would return to baseline at that point (115). Therefore, we expected that levels of acetylation and CBP binding at the BDNF promoters would similarly peak at 30 minutes but return to baseline by 3 hrs.

**Experimental Design, Results, and Discussion:**

To induce contextual learning associated with fear conditioning, mice received a one-time foot shock in a novel environment. Control mice were placed in the same environment but did not receive a shock. This behavioral test had been previously validated in our lab. Mice were sacrificed 30 minutes and 3 hours after the shock or control treatments (six mice per group). Whole hippocampus was extracted and processed as previously described (see Methods section from Chapter I). Chromatin immunoprecipitation assays were performed

with antibodies against acetylated H3, acetylated H4, and CBP. The amount of antibody-associated DNA was assessed at the promoters of CREB, BDNF P1, BDNF P3, BDNF P4, and CDK5 using quantitative RT-PCR. Our results revealed that 30 min after shock, there was a decrease in levels of H3 acetylation at BDNF P1, CREB, and CDK5 promoters of approximately 2-fold. There were no significant changes in H3 acetylation at BDNF P3 and P4 at this time point. By 3 hrs, no significant changes in H3 acetylation were detected at any of the studied promoters (Figure 27). Levels of H4 acetylation and CBP were very low in both control and shock-treated animals. The low levels of CBP could be attributed to low specificity of binding for this antibody. I obtained similar Ct values when we immunoprecipitated with CBP as when we immunoprecipitated with a non-specific IgG antibody. Therefore, I was unable to obtain data for the binding of CBP at any of the assayed promoters. The low levels obtained after immunoprecipitation with acetylated H4 were puzzling. This antibody is well standardized and has worked well in our hands before. In addition, the enrichment of acetylated H4 was much lower than normally seen in the control groups as well as in the shock groups of animals, indicating that the reason for its low yield is not a result of fear conditioning shock. The most likely explanation is that the particular antibody batch was defective. The decrease in levels of H3 acetylation at BDNF P1, CREB, and CDK5 indicates that these are not likely to be promoter regions that are being transcriptionally activated during contextual

fear-dependent learning. In fact, they seem to be downregulated. We had hypothesized that at least one of the other BDNF promoters, most likely BDNF P4, is upregulated during this contextual-learning process, and that this promoter should show increased levels of H3 acetylation. However, we did not detect significant changes in the levels of H3 acetylation at the BDNF P4 promoter either at 30 min or 3 hr after the shock. In conclusion, the results of this experiment did not reveal a learning-associated increase of H3 acetylation, as hypothesized, at any of the promoters analyzed, including the promoter of CREB, CDK5, and three of the mouse BDNF promoters P1, P3, and P4. In fact, the CREB, CDK5, and BDNF P1 promoters showed a significant decrease in H3 acetylation 30 minutes after shock, indicating that these genes might be downregulated as a result of fear-associated learning. In addition, we obtained inconclusive results about the levels of CBP binding and H4 acetylation at these promoters. I did not pursue further this experiment, due to the lack of time. Nevertheless, there are several suggestions for improvements of the experiment. First, mRNA levels for the genes whose promoters are being assayed should be measured first, to ensure that there are changes in gene expression in whole hippocampus, and in the particular paradigm employed. If BDNF shows a significant change as a result of fear-conditioned learning, the expression levels of each transcriptional splice variant should be measured, as well as the levels of H3 and H4 acetylation at each promoter driving the corresponding splice variant.

Finally, the behavioral paradigm used here, shock vs. no shock, does not distinguish whether the changes observed are a result of fear-associated contextual memory, a new spatial memory upon exposure to a novel environment (not associated with shock), or simply a stress response to the shock. Therefore, another animal treatment should be included in the experiment, one that controls for the formation of non-contextual memory formation. In the latent inhibition training paradigm, animals are pre-exposed to a novel environment prior to receiving the unconditioned stimulus (electric shock). Latent inhibition has been shown to induce a spatial memory of the novel context, but not an associative contextual fear memory. Therefore such control should be able to distinguish between fear-associative learning and spatial learning by itself.

# APPENDIX 3

## *In vivo* Binding of FosB and ΔFosB at the Substance P Promoter After Repeated Foot Shock

A recent and exciting new method to measure transcription factor binding *in vivo* is by chromatin immunoprecipitation. This technique has been successfully used in yeast and cell lines, but its use in the brain has been very limited. I have performed several experiments to assay the binding for several transcription factors to the specific promoters of genes implicated in long-term plastic changes in the brain. For example, I have completed a study where I have measured *in vivo* binding of the transcription factors FosB and ΔFosB, a truncated form that lacks the C-terminal domain of FosB, at the Substance P gene promoter in the raphe nucleus in mice after administration of repeated foot shock. This experiment was very challenging due to the small size of the brain region that we were interested in, and it required that I pool the raphe nucleus of six mice per sample. The experiment involved a sequential immunoprecipitation with an antibody that recognizes only full-length FosB (C-terminus Ab), followed by an antibody against both FosB and ΔFosB (N-terminus Ab). Theoretically, after immunoprecipitation with full-length FosB, all of the FosB- but none of the

ΔFosB-associated DNA should be precipitated. This first immunoprecipitate was eluted, reverse cross-linked, and used to analyze levels of FosB binding at the Substance P promoter by qPCR. I then used the remaining non-immunoprecipitated supernatant for a second round of immunoprecipitation with an antibody, which recognizes the N-terminal domain of both FosB and ΔFosB. Since all of the FosB was pulled during the first round of immunoprecipitation, the second immunoprecipitation allowed collection of only ΔFosB-associated DNA. Using this method of sequential immunoprecipitation, I was able to analyze the binding of both FosB and ΔFosB at the Substance P promoter in the raphe of mice 2 hr and 24 hr after the administration of repeated foot shock. Previously, our lab has shown that the FosB protein increases acutely after ECS and cocaine administrations, but that repeated administrations of these treatments leads to the preferential accumulation of the ΔFosB protein several hours later, likely due to the higher stability of this truncated form of FosB (117). Thus, we expected that in our chronic conditions of repeated foot shock, there will be more ΔFosB than FosB available for binding at the substance P promoter at 24 hrs but not at 2 hrs, and that if this promoter is in fact an *in vivo* binding target for these transcription factors, we will observe greater ΔFosB binding at that time point. This experiment has been repeated twice, yielding similar results both times. The results indicate that indeed, binding of ΔFosB is robustly increased at the Substance P promoter 24 hr after repeated foot shock, whereas FosB binding is

decreased at this time point. Contrary, at the 2 hr time point, binding at both FosB and ΔFosB was not significantly altered (Figure 28). These findings corroborated the hypothesis that repeated stimulation leads to the gradual accumulation, and in this case binding, of ΔFosB to DNA. In addition, these results provided evidence that the Substance P promoter is an *in vivo* target for ΔFosB. In addition, this *in vivo* DNA binding experiment is consistent with previous gel-shift data that shows binding of ΔFosB at the Substance P promoter. Finally, it provides one of the first experimental evidence for using ChIP to assay levels of transcription factor binding in the brain, *in vivo*.

**TABLE 3**

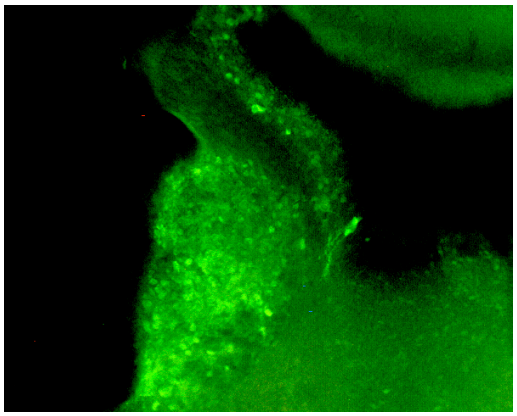
| Brain region of AC8 expression | Intensity of AC8 expression ( 1 to 10 [strongest] ) | Seen in previous studies |
|---|---|---|
| Habenula: Medial Habenular Nucleus | 9: strong and specific staining | √ |
| Olfactory bulb: glomerular or external plexiform layer | 8, also autofluorescence | √ |
| Olfactory tubercle | 8, also autofluorescence | √ |
| Superior colliculus | 8 | |
| Dorsal lateral geniculate nucleus of the thalamus | 7 | |
| Deep mesencephalic nucleus | 6 | |
| Ventral spinocerebellar tract | 5 | |
| Cingulate cortex | 5 | |
| Pretectal nucleus | 4 | |
| Hippocampus | 3-4 | √ |
| Somatosensory cortex | 3, with high background | √ |
| Periaqueductal gray area | 0-1: Very low expression, hard to distinguish from background | |
| Rhinal cortex | ? | |
| Piriform cortex | ? | √ |
| Nucleus accumbens | 0: No expression detected | |
| Ventral tegmental area | 0 | |
| Locus coeruleus | 0 | √ (withdrawal) |
| Hypothalamus (Supraoptic and Paraventricular nuclei) | 0 | √ |
| Amygdala | 0 | √ (chronic morphine) |
| Arcuate nucleus(with chronic morphine) | 0 | √ |
| Pontine nucleus | 0 | √ |
| Cerebellum | 0 | √ |

**Table 3. Brain-wide mapping of AC8 promoter-mediated expression.** Transgenic mice, carrying a 35 kb fragment of the AC8 promoter linked to a GFP reporter were used for this mapping experiment. The intensity of AC8-mediated expression was measured by GFP immunofluorescence in consequent 35-micron sections of the entire brain. Strong AC8 expression was detected in the following brain regions: habenula, olfactory bulb, olfactory tubercle, superior colliculus, and dorsal lateral geniculate thalamic nucleus. Moderate AC8 expression was detected in the deep mesencephalic nucleus, ventral spinocerebellar tract, cingulated cortex, pretectal nucleus, the hippocampus, and the somatosensory

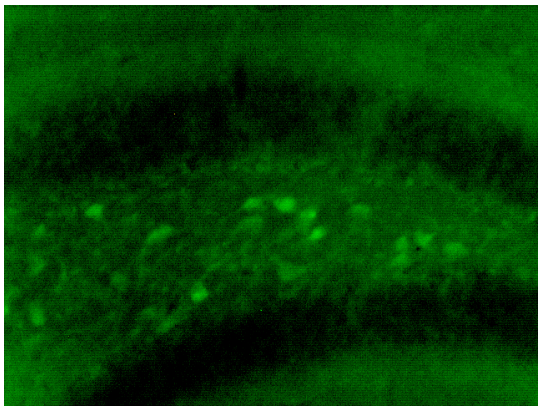
cortex. Expression above the level of non-specific GFP binding was not detected in any of the following brain regions: periaqueductal gray, nucleus accumbens, ventral tegmental area, locus coeruleus, hypothalamus (supraoptic and paraventricular nuclei), amygdala, arcuate nucleus, pontine nucleus, and the cerebellum.

**FIGURES 26 - 28**

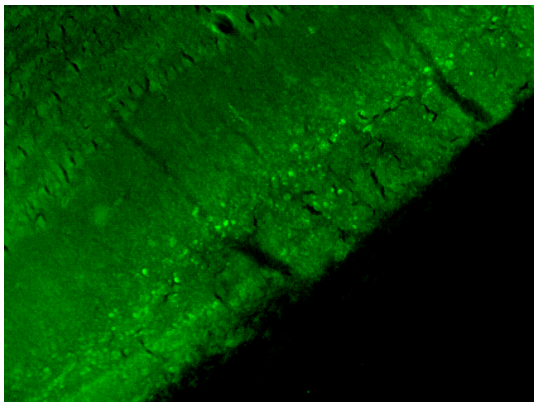
Habenula

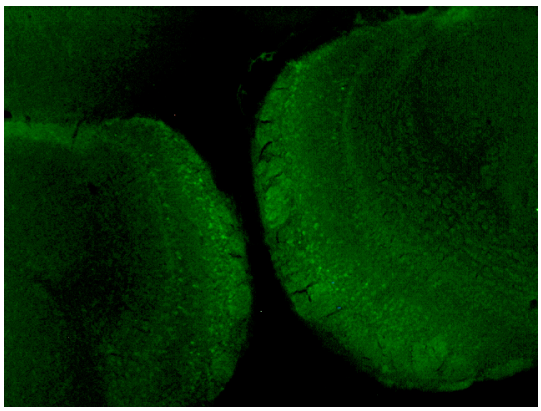


Hippocampus

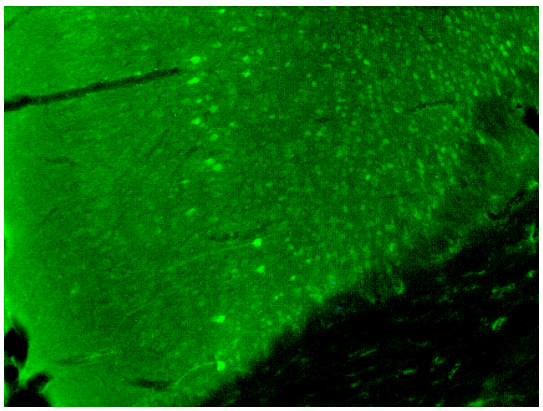


Olfactory bulb

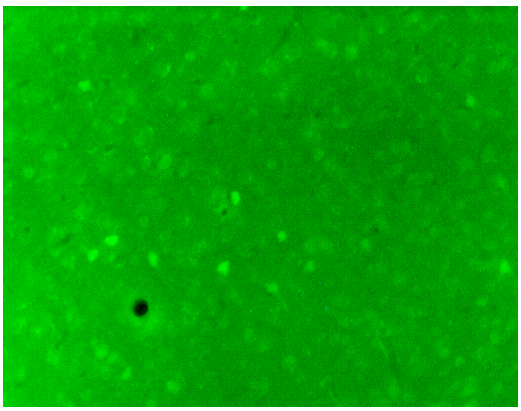




Cingulate cortex



Cortex



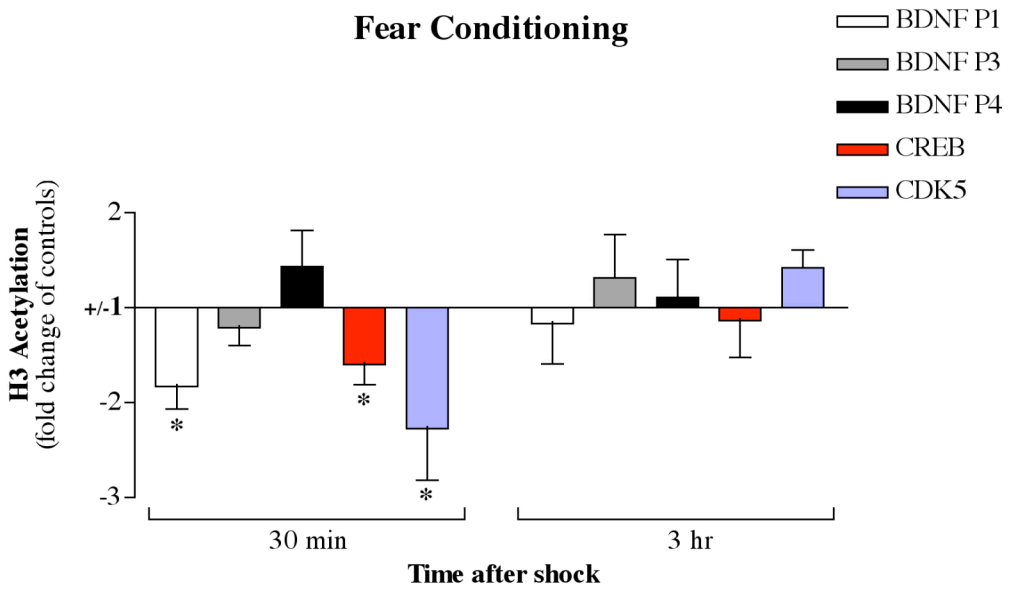



**Figure 27. Fear conditioning induces changes in the levels of H3 acetylation at the promoters of CREB, CDK5, and BDNF P1.** Contextual fear conditioning, associated with an electric shock in a novel environment, induced a significant decrease in the levels of H3 acetylation at the promoters of CREB (1.6 ± 0.2 fold), CDK5 (2.3 ± 0.5 fold), and BDNF P1 (1.8 ± 0.2 fold) 30 min following a single shock when compared to non-shocked control animals handled in a similar environment. H3 acetylation at BDNF P3 and P4 promoters was not changed at this time point. At 3 hrs following the same shock paradigm, levels of H3 acetylation at CREB, CDK5, and BDNF P1 had returned to basal levels, and there were still no significant changes at BDNF P3 and P4. N=5-6. T-tests were used to determine statistical significance ($p<0.05$).

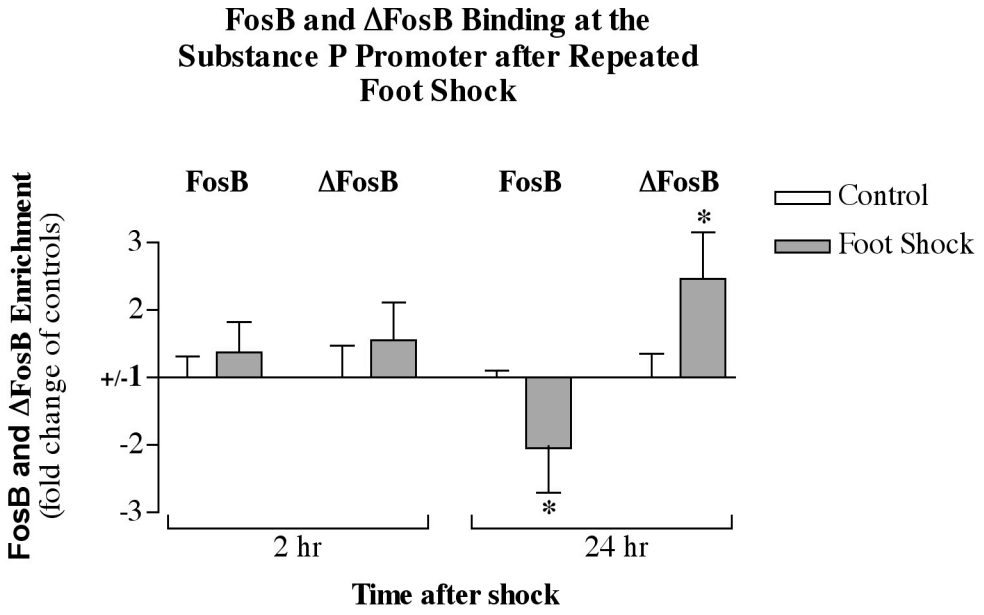



**Figure 28. Repeated foot shock induces ΔFosB binding *in vivo* at the Substance P promoter.** Levels of FosB and ΔFosB binding at the Substance P promoter in the raphe region of the brain were assayed by two sequential rounds of chromatin immunoprecipitation (ChIP) at 2 hrs and 24 hrs following repeated foot shock. Immunoprecipitation with a C-terminus antibody specific to FosB yielded a 2-fold decrease in the level of FosB binding at Substance P at 24 hrs after repeated foot shock (2 ± 0.6 fold). Subsequent immunoprecipitation with a N-terminus antibody, common to both FosB and ΔFosB, resulted in a significant increase in the levels of ΔFosB binding, again 24 hrs after the shock (2.5 ± 0.7 fold). Significant changes in the binding of FosB or ΔFosB were not detected at the Substance P promoter 2 hrs after repeated foot shock. N=6. T-tests were used to determine statistical significance ($p<0.05$).

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# VITAE

Nadejda (Nadia) Mincheva Tsankova was born in Sofia, Bulgaria, on October 10th, 1977, the daughter of Rossitsa Antonova Tsankova and Mincho Alexandrov Tsankov. She moved permanently to the United States of America at the age of 14, where she attended high school. After completing her high school education at Georgetown High School, Georgetown, Texas in 1996, she entered the University of Texas at Austin, Texas. She received the degree of Bachelor of Science with highest honors in zoology from the University of Texas at Austin in May, 2000. In June, 2000, she entered the Medical Scientist Training Program at the University of Texas Southwestern Medical Center at Dallas. She completed her first two years of medical training, after which she joint the laboratory of Dr. Eric Nestler, where she is currently pursued a doctorate in philosophy degree in neuroscience. After successfully defending her dissertation in June of 2005, she returned to complete her medical training and will graduate with a combined M.D/Ph.D degree. She plans to continue her medical training in Neurology or Neuropathology and further her research career in basic Neurobiology.

Permanent Address: 3269 Carlisle Street apt # 171
Dallas, TX 75204