Lactic Acid mediates resistance to stress through class I HDAC induction in a chronic social defeat model of depression

By

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To my beloved parents whom I wouldn’t be here if it weren’t for them.
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Lactic Acid mediates resistance to stress through class I HDAC induction in a chronic social defeat model of depression

Rim M. El Ghandour

ABSTRACT

Depression is one of the most common psychiatric disorders and a leading cause of disability worldwide. Environmental factors, mainly stress, play an important role in promoting this disorder by inducing changes in gene expression that are sustained by epigenetic modifications, particularly in the hippocampal brain regions. Transcriptional profiling of the hippocampus revealed a downregulation in the Brain-Derived Neurotrophic Factor (Bdnf) gene expression in animal models of stress, and this downregulation was reversed by antidepressant treatment and physical exercise.

In our study, we use a chronic social defeat paradigm, a validated model of depression in mice, to study the antidepressant effect of the endogenous molecule lactate that is released after physical exercise. We used multiple behavioral tests including open field, T-maze and social interaction tests to show that lactate rescues depression phenotypes such as defeat and anxiety behavior. We also report that lactate activates independent pathways to affect two separate processes: promotion of resilience to stress and protection from depression. Our results reveal an antidepressant-like activity of an endogenous molecule lactate, produced after physical exercise and known to accumulate in the hippocampal regions of the brain and illustrate a novel mechanism that can explain the positive effects of exercise on mood disorders.
Keywords: Depression, hippocampus, Bdnf, chronic social defeat stress, lactate, resilience.
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LIST OF ABBREVIATIONS

*Bdnf* **p1**: brain derived neurotrophic factor promoter 1.

**BDNF**: brain derived neurotrophic factor.

**CSDS**: chronic social defeat stress.

**EPM**: elevated plus maze.

**FNDC5**: fibronectin domain-containing 5.

**HAT**: histone acetyltransferases.

**HDAC**: histone deacetylase.

**LA**: lactic acid.

**PGC1α**: peroxisome proliferator-activated receptor gamma coactivator 1-alpha.

**RNA**: ribonucleic acid.

**RT-PCR**: realtime polymerase chain reaction.

**SAL**: saline.

**SI**: social interaction.
Chapter One

Introduction

1.1. Depression

1.1.1. Depression pathological features

Depression is one the most debilitating disorders (Hollis and Kabbaj, 2014; Ferrari et al. 2013). Almost 15% of the human population is affected by depression. This imposes high costs on both individuals and societies (World Health Organization, 1996). Direct and indirect costs exhaust the countries’ economies due to the loss in productivity of the individuals and the high expense of the available yet ineffective treatments (Blazer 2000; Simon, 2003). Accordingly, it is important to uncover the molecular mechanisms leading to depression in order to identify novel therapeutic targets that can offer cheaper and more effective treatments.

The pathological features of depression are characterized by the persistence of at least one episode of grief or anhedonia in a two-week time span (Phillips, 2017; American Psychiatric Association, 2013). Moreover, one of the well characterized symptoms of depression is the alteration of the social behavior. This has been given great attention when considering animal models of depression (Ottenbreit et al., 2014). Despite the well-known pathological features of depression, there is still much to be investigated about the mechanisms that underlie its clinical manifestations. Indeed, there are several processes that interplay in the occurrence of depression; these include genetic and epigenetic factors (Phillips, 2017; Drevets et al., 2008; Price and Drevets, 2012; Menke and Binder, 2014).
1.1.2. **Brain parts involved**

The brain, the key player in depression, is characterized by altered plasticity demonstrating reduction in density and synaptic connections (Dwivedi, 2009; Frodl et al., 2006; Sheline, 2000). Unlike neurodegenerative disorders like Alzheimer’s disease or Parkinson’s disease, where specific brain regions are affected, there is no consensus about the specific brain site that is affected in depression (Nestler et al., 2002). However, abnormalities in several brain regions including the hippocampus, prefrontal cortex and nucleus accumbens contribute to depression (Nestler et al., 2002; Drevets, 2001; Liotti and Mayberg, 2001; Dolan et al., 1993). These areas function in a series of interacting circuits to formulate the neural circuitry that is involved in depression (Nestler et al., 2002). In addition, these regions exhibit reduction in neuronal number, density and cell body size (Dwivedi, 2009; Rajkowska et al., 2005). The dorsolateral prefrontal cortex is associated with decreased activity and dysfunction, the frontal lobe with anterior lesions, the cortex with reduced cell number, the nucleus accumbens with inhibited dopaminergic activity and the hippocampus with abnormal dendritic morphology (Dwivedi, 2009; Shirayama and Chaki, 2006; Hajszan et al., 2005; Honer, 1999; Dolan et al., 1993).

The hippocampus is a brain region that plays an important role in learning, memory and long-term potentiation (Dwivedi, 2009; Bearden et al., 2006; Horan, 1997). Considering that the hippocampus is responsible for mediating important cognitive functions, the reduction in its volume contributes to explaining the documented cognitive impairments that accompany depression (Sapolsky, 2001; Bremner et al., 2000; Sheline et al., 1996). Moreover, the mechanisms that result in hippocampal volume reduction are major contributors to the progression of major depression disorder (Frodl et al., 2006). In fact,
many studies have focused on the hippocampus since the brain was shown to respond to acute and chronic stress through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Campbell and MacQueen, 2004; Engelmann et al., 2004; Nestler et al., 2002). The activation of the HPA axis involves the production of a cascade of hormones in neurons (corticotropin-releasing hormone, vasopressin and corticotropin) leading to the secretion of the adrenal glucocorticoid hormone cortisol (Smith and Vale, 2006). This pathway is regulated by several feedbacks to control the adrenal secretions during stress and inactivity (Barden, 2004). The HPA system’s normal function is disturbed in depression, in which cortisol secretion is upregulated and shows a deregulated secretory pattern causing the over-activation of this axis. Some antidepressants elevate mood in depressed patients with HPA malfunction through the normalization of its hyperactivity (Holsboer-Trachsler et al., 1991). All these structural and functional alterations explain the pathophysiology of depression from an anatomical point of view, however the exact molecular mechanisms that are activated and mediate depression phenotypes remain less understood.

1.1.3. Epigenetics role in depression

The development of depression is a result of an interplay between genetic predisposition and environmental stimuli (Heim and Binder, 2012; Kendler et al., 2002; Merikangas and Swendsen, 1997; Nestler et al., 2002). For instance, social stress can be a major contributor to the development of depression in susceptible patients (Henriques-Alves et al., 2016). Further evidence of the association between stressful life events and depression has been observed in identical twins whereby the twin subjected to childhood sexual abuse developed major depression as compared to his/her sibling (Heim and
Binder, 2012; Nelson et al., 2002; Kendler et al., 2000). In fact, environmental stressors change gene transcription through the alteration of chromatin structure without altering the DNA code itself. This is referred to as epigenetic regulation of gene expression (Hammels et al., 2015; Landgrave-Gómez et al., 2015; Mill and Petronis, 2007).

Chromatin consists of DNA, the histones and non-histone proteins that are found in the nucleus in a compacted form. Chromatin modulators can regulate gene expression by controlling the access of the transcriptional machinery to DNA (Sandman et al., 1999; Ouzounis and Kyrpides, 1996). This is a dynamic process where epigenetic mechanisms control gene expression through affecting the interactions between the DNA and proteins without altering the genetic code itself (Tsankova et al., 2007). Many studies have associated changes in epigenetic modifications such as changes in histone acetylation/deacetylation, histone methylation and DNA methylation at particular loci, with the development and pathology of several neurodegenerative and psychiatric disorders (Balazs, 2014; Hernandez et al., 2011; Francis et al., 2009; Kontopoulos et al., 2006).

1.1.3.1. Histone acetylation/deacetylation

Histone acetylation is a common form of histone modifications that occurs when specific enzymes called histone acetyl transferases (HATs/KATs) add an acetyl group to the ε-amino group of lysines in the amino-terminal tail of histone proteins (Seto and Yoshida, 2014). It is well-established that this chromatin modification regulates gene transcription (Allfrey et al., 1964). Histone acetylation promotes transcriptional activation by weakening DNA/histone interactions leading to an “open” chromatin structure that allows the recruitment of RNA polymerase II (Struhl, 1998; Sealy and Chalkley 1978;
Histone lysine acetylation is a reversible process (Leipe and Landsman, 1997). Acetylation can be removed by histone deacetylases (HDACs). In contrast to histone acetylation, the deacetylation of histones promotes transcriptional repression (Figure 1) (Struhl, 1998; Sealy and Chalkley 1978; Vidali et al. 1978; Hebbes et al. 1988). Human HDACs include 18 enzymes that belong to four classes: the Class I proteins which include HDAC1, HDAC2, HDAC3, and HDAC8; the Class II proteins which include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10; the Class III Sir2-like proteins which include SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, and SIRT7; and the Class IV proteins which includes HDAC11 (Seto and Yoshida, 2014; Ruijter et al., 2003; Kuo and Allis, 1998). HDACs not only use histone proteins as substrates, but also can deacetylate non-histone proteins (Seto and Yoshida, 2014). This is important to consider when assessing the role of HDACs in different processes. Structural studies on Class I and II HDACs have revealed a conserved region of active residues that suggest a shared mechanism for deacetylation by metal-dependent hydrolysis (Seto and Yoshida, 2014; Kuo and Allis, 1998; Kadosh and Struhl, 1998). On the other hand, Class III HDACs use Nicotinamide adenine dinucleotide (NAD+) to deacetylate acetyl lysine residues forming nicotinamide, the deacetylated product, and another metabolite 2′ -O-acetyl-ADP-ribose (Seto and Yoshida, 2014). HDACs also affect the status of other epigenetic marks since they promote the establishment or removal of other lysine modifications such as methylation, ubiquitination, and SUMOylation (Seto and Yoshida, 2014).
1.1.3.2. HDACs in psychiatric diseases

HDACs are important players in many human diseases including central nervous system (CNS) diseases. Indeed, to expand our understanding of their impact on human health and disease, it is crucial to identify their different roles and describe their mechanisms of action. The discovery of HDAC inhibitors has enhanced our knowledge of HDAC functions and their impact on health. Many HDAC inhibitors are being developed and some are used for the treatment of a number of psychiatric disorders (Kazantsev and Thompson, 2008; Volmar and Wahlestedt, 2015). In fact, HDACs play important roles in brain development, as well as learning and memory (Volmar and Wahlestedt, 2015). For instance, in the adult brain HDACs are involved in the synaptic events that facilitate the formation of long-term memories (Volmar and Wahlestedt, 2015; Morris et al., 2010; Levenson and Sweatt, 2006). In addition, HDAC inhibitors prevent β- amyloid aggregation and rescue phenotypes of a mouse models of Alzheimer’s disease (Morris et al., 2010; Kilgore et al., 2009; Nuutinen et al., 2010). Importantly, many studies have established that HDAC inhibitors are beneficial in models of depression, suggesting that
HDACs play important roles in the pathophysiology of the disease (Volmar and Wahlestedt, 2015; Covington et al., 2009; Schroeder et al., 2006). For example, sodium butyrate, a nonspecific HDAC inhibitor, exerts antidepressant effects in social defeat models of depression (Tsankova et al., 2007; Tsankova et al., 2006; Schroeder et al., 2006). The close association between HDACs and a variety of human diseases has led researchers to look for more epigenetic-based treatments.

1.1.3.3. HDACs have different roles

Among the most relevant HDACs to our study are Class I HDACs and Sirt1. First, Class I HDACs include HDAC1-3 and 8, and are found in the nucleus; hence their role and involvement in epigenetic regulation (Sun et al., 2014; Kazantsev and Thompson, 2008; Abel and Zukin, 2008). HDAC1 regulates neuronal viability by promoting both neuronal survival and death (Bardai et al., 2012; Jeong et al., 2009). Morrison et al. (2006), have reported that the histone deacetylase-related protein (HDRP) promotes neuroprotection by associating with HDAC1. On the other hand, the interaction between HDAC1 with HDAC3 leads to neuronal death. These results suggest that HDAC1 acts as a molecular switch that promotes either neuronal survival or death depending on which protein complex it forms (Bardai et al., 2012). HDAC1 is also considered as a negative regulator for both fear extinction in rodents and mood disorders (Volmar and Wahlestedt, 2015; Schroeder et al., 2013; Bardai et al., 2012; Morrison et al., 2006). HDAC2, another class I HDAC, binds to promoters of synaptic plasticity genes inhibiting their expression and in turn inhibiting learning and memory (Guan et al., 2009). HDAC2 also negatively regulates mood disorders and promotes depression (Sleiman et al., 2016; Volmar and Wahlestedt, 2015; Guan et al., 2009; Broide et al., 2007). Interestingly, studies in mouse
models of depression show a strong link between HDAC2 and stress (Uchida et al., 2011). Chronic social defeat stress (CSDS) upregulates HDAC2 levels causing a reduction in glial-derived neurotrophic factor \textit{Gdnf} expression in the nucleus accumbens (Uchida et al., 2011). Uchida et al., (2011) also showed that by reducing HDAC2 levels in mice depression-like behavior is attenuated. Another study by Wu et al. (2017), showed that HDAC2 is implicated in cognitive decline following chronic stress. The third member of class I HDACs, HDAC3, has the highest expression in the brain (Volmar and Wahlestedt, 2015; Malvaez et al., 2013; McQuown et al., 2011). This HDAC is also a negative regulator of memory, since as previously mentioned, it interacts with HDAC1 to promote neuronal cell death (Volmar and Wahlestedt, 2015; Bardai et al., 2012). Many studies have shown that HDAC inhibitors promote neuroprotection by the inhibition of the pro-apoptotic effects of HDAC1 and HDAC3 (Morris and Monteggia, 2013; Kim et al., 2010; Bardai and D'Mello, 2011). A study by Sleiman et al. (2016) showed that one of the mechanisms by which physical exercise promotes its beneficial effects on learning and memory involves the downregulation of HDAC2 and HDAC3 in the hippocampus. Even though these studies indicate that HDAC3 has negative effects on many neuroprotective processes, HDAC3 normal levels have been shown to be crucial for proper brain development (Norwood et al., 2014). Antidepressant treatments leads to HDAC3 overexpression in both the striatum and cingulate cortex (Volmar and Wahlestedt, 2015; Ookubo et al., 2013). In summary, many studies show that class I HDACs are essential for brain development, but that they also implicated in many neurodegenerative diseases and psychiatric diseases such as depression. As a result, HDAC are important target for novel therapies (Calfa et al., 2012; Convington et al., 2009; Berton and Nestler, 2006).
The class III HDAC, Sirt1 is a NAD+-dependent protein deacetylase. It is highly expressed in the brain and particularly in the hippocampus (Ferland et al., 2013; Chang and Guarente, 2013; Asher et al., 2008). Sirt1 affects many processes in the brain including learning and memory and regulates age-dependent disorders (Ferguson et al., 2015; Ng et al., 2015; Libert et al., 2011; Gao et al., 2010; Michan et al., 2010; Chen et al., 2005). Sirt1 is activated during chronic stress in the hippocampus and its expression is associated with the prevalence of major depressive disorder (Ferland et al., 2013; Ferland and Schrader 2011). A study conducted by Kakefuda et al. (2009) showed that Sirt1 overexpression didn’t display neuroprotection, but rather a deficit in memory. Another study by Libert et al. (2011) showed that Sirt1 deacetylates a transcription factor NHLH2 to activate the gene expression of a molecule, monoamine oxidase A (MAO-A) that normally reduces the serotonin levels in the brain. Several antidepressants inhibit MAO-A to increase the accessibility of serotonin in the brain (Fiedorowicz and Swartz, 2004). This reveals a positive correlation between Sirt1 and stress.

1.2. **Exercise enhances mental health through epigenetic modifications**

1.2.1. **The positive effects of exercise**

Physical exercise plays an important role in enhancing mental health by affecting transcriptional pathways in the brain and particularly in the hippocampus, the center of learning and memory. This role can be considered as part of the environmental impact on changes of gene expression. Both animal and human studies have revealed that physical exercise has a beneficial effect on cognitive function (Kramer et al., 1999; Fordyce & Wehner, 1993). Physical exercise also accelerates functional recovery after brain injury.
and slows mental decline due to aging (Vaynman et al., 2004; Laurin et al., 2001; Grealy et al., 1999). In fact, physical exercise improves the symptoms of patients diagnosed with depression by reducing anxiety and reversing negative moods (Cooney et al., 2014; Blumenthal et al., 2012; Mead et al., 2009; Sharma et al., 2006). However, the endogenous molecular mechanisms through which physical exercise counteracts stress and depression are still not fully elucidated. Many beneficial responses of exercise are due to the induction of brain derived neurotrophic factor (BDNF) (Sleiman et al., 2016; Vaynman et al., 2004; Neeper et al., 1997; Gomez-Pinilla et al., 2001).

One of the mediators of exercise’s positive effects is the transcriptional co-activator PGC-1α (Finck and Kelly, 2006). Wrann et al., (2013) have shown that exercise induces hippocampal PGC-1α, which in turn interacts with ERRα, to induce the expression of myokine Fndc5. This latter molecule is speculated to be cleaved and secreted where it activates unknown signaling pathways that can induce hippocampal Bdnf expression. This pathway links exercise, PGC-1α and FNDC5, with Bdnf gene expression in the brain (Wrann et al., 2013).

Another mechanism that has been reported to explain how hippocampal Bdnf expression is induced by exercise involves epigenetic changes at the Bdnf promoters (Guan et al., 2009; Koppel and Timmusk, 2013). Sleiman et al., (2016) have reported that a ketone body, D-β-hydroxybutyrate, which is produced during exercise as a result of fatty acid oxidation in the liver, is released into blood, reaches the brain by crossing the blood brain barrier and accumulates in the hippocampus where it promotes the induction of Bdnf promoter I (Bdnf pI) expression through a mechanism that involves HDAC2 and HDAC3 inhibition. This work suggests that endogenous metabolites that are produced during
exercise can play the role of energy substrates as well as epigenetic modulators that promote the positive effects of exercise on learning and memory through the induction of BDNF signaling.

1.2.2. Lactate translates exercise’s positive effects

In exercising muscles, the pyruvate produced through glycolysis is converted to lactic acid (LA) through the action of the enzyme lactate dehydrogenase (Figure 2). LA can cross the blood brain barrier via endothelial monocarboxylate transporters (MCTs). LA protects neurons against ischemic stress (Berthet et al., 2009), maintains normal synaptic function in the rat hippocampus (Schurr, 2006; Schurr and Rigor, 1998), aids in memory formation (Pierre and Pellerin, 2005), and improves treatment in patients following traumatic brain injury (Ichai et al, 2009). Our lab has shown that LA produced after exercise accumulates in the hippocampus and enhances learning and memory formation through the induction of hippocampal Bdnf (p1) expression (unpublished data from our lab).

![Figure 2: Reduction of Pyruvate to Lactate. Muscles undergo anaerobic respiration during exercise to result in the reduction of pyruvate to LA oxidizing NADH to regenerate NAD⁺ (Adapted from Akagawa et al., 2016).](image)

1.3. Neurotrophins and depression
Several epidemiological studies have linked alterations in epigenetic modifications resulting from environmental factors to an increased risk of depression (HaoSheng et al., 2013; Hammen, 2005; Kessler, 1997). A wide range of extracellular signals are crucial for the survival and differentiation of the neurons in the central nervous system (CNS) (McAllister, 2001; McAllister et al., 1997; Lewin and Barde, 1996). Among the most relevant are the neurotrophins, which comprise a family of growth factors that share common structural characteristics and which promote neuronal differentiation, the formation of synapses and interconnected circuits, regulation of axonal and dendritic growth and control activity-dependent plasticity (McAllister, 2001; Katz and Shatz, 1996; Snider, 1994). Neurotrophins are dimeric proteins that include: nerve growth factor (NGF) which was the first to be identified, BDNF, neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (McAllister, 2001; Katz and Shatz, 1996; Snider, 1994). The functions of neurotrophins are mediated mostly through the tropomycin receptor kinase (Trk) family of receptors; NGF activates TrkA, BDNF and NT-4 activate TrkB, and NT-3 activates TrkC more strongly (McAllister, 2001; McAllister et al., 1999).

1.3.1. The neurotrophin hypothesis of depression

The neurotrophin hypothesis of depression postulates that the negative outcomes of stress from neuronal atrophy to decreased synaptogenesis are a result of the decrease in neurotrophins, mostly BDNF, and this is due to the importance of neuronal plasticity in the development of depression as well as to responses to antidepressants (Duman and Li, 2011; Bus and Molendijk, 2016). Hence, BDNF has been extensively studied in the context of many mood disorders as it has been considered as an important therapeutic target. BDNF levels are known to be altered in several neurological disorders including
schizophrenia, depression and Alzheimer’s disease (Nieto et al., 2013; Duman 2002; Dwivedi, 2007). Importantly, the hippocampal alterations that occur in depression can be in part a result of the decreased levels of BDNF (Bremner et al., 2000; Videbech, 2004). BDNF mediates neuronal plasticity, formation of memory and dendritic growth (Budni et al., 2015). BDNF signaling involves 2 different receptors: the p75 neurotrophin receptor and tropomysin related kinase B (TrkB) receptor (Boulle et al., 2011; Nagahara et al., 2011). TrkB is essential for BDNF’s effects in the adult brain and binds BDNF with higher affinity as compared to p75 (Nagahara et al., 2011; Yoshii and Constantine-Paton, 2010). When BDNF binds to TrkB, it results in the recruitment of proteins that can activate three different signal transduction pathways. One involves consecutive activation of insulin receptor substrate-1 (IRS-1/2), phosphatidylinositol-3-kinase (PI-3K) and protein kinase B (Akt). The other results in the activation of Shc/Grb2, Ras, Raf, mitogen-activated protein kinase kinases (MEKs) and extracellular signal regulated kinases (ERKs). The third pathway involves phospholipase C (PLC), inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3], diacylglycerol (DAG) and protein kinase C (PKC). These cascades result in the activation of genes that encode proteins involved in neuronal plasticity, survival and stress resistance (Bathina and Das, 2015; Jiang et al., 2008). Moreover, clinically used antidepressants induce the autophosphorylation of TrkB (pTrkB) and this activation is a common step in the mechanism of action of all antidepressants (Lieto et al., 2012; Castren and Rantamaki, 2010).

1.3.2. BDNF altered signaling

Disruptions in BDNF signaling lead to cognitive and neuropsychiatric disorders. The Bdnf gene could be considered a susceptibility gene in the progression of major...
depressive disorder and schizophrenia (Boulle et al., 2011). The Val66met polymorphism is a single nucleotide polymorphism (SNP) isolated in humans and which involves the substitution of Valine at position 66 with Methionine in the Bdnf gene resulting in changes in the structure of the BDNF prodomain. These structural changes alter its interaction with coreceptors leading to defects in signaling and synaptic loss (Chen et al., 2007; Bueller et al., 2006) (Figure 3). This mutation is one of the most important genetic predispositions to depression and anxiety. It also affects the severity of depression symptoms (Ignácio et al., 2014) and correlates with alterations in human carriers where heterozygous individuals for the Met allele have reduced hippocampal volumes (Chen et al., 2006; Bueller et al., 2006; Szeszko et al., 2005) and perform weakly on hippocampal-dependent memory assays (Chen et al., 2006; Hariri et al., 2003; Egan et al., 2003).

**Figure 3:** The Val66Met single nucleotide polymorphism (SNP) is a substitution that changes the structure of the BDNF prodomain and alters its interaction with coreceptors. This change in structure leads to defects in BDNF signaling, synaptic loss as well as lower BDNF levels resulting from defects in its secretion. This SNP predisposes individuals to depression and anxiety. It also significantly affects the reactivity to stress (Modified from Ignacio et al., 2014).
1.3.3. *Bdnf* gene Structure and Transcripts

The *Bdnf* gene is transcribed from several promoters located upstream of multiple 5' noncoding exons to generate heterogeneous *Bdnf* mRNAs. The rodent *Bdnf* gene consists of 9 non-coding exons in the 5' region and 1 coding exon in the 3’ region. Each one of the exons, which is linked to its own promoter, is transcribed and spliced with the coding exon 9 as follows: 1–9, 2a–9, 2b–9, 2c–9, 3–9, 4–9, 5–9, 6–9, 7–9, 8–9, and 9a–9 (Tomotsuka et al., 2014; Pruunsild P et al., 2007). The expression of these splice variants is differentially regulated throughout development in an organ-specific matter (Tomotsuka et al., 2014; Aid et al., 2007). This indicates that different promoters control transcription of these splice variants in response to various environmental conditions (Tomotsuka et al., 2014). The *Bdnf* promoters that are most relevant and expressed in the brain are I, II, III, IV, V and VI. BDNF protein transcribed from promoter-I is neuronal activity dependent and this BDNF protein along with BDNF protein from promoter II regulate the aggressive behavior. Mice lacking expression from these promoters display increased aggressive behavior (Maynard et al., 2015). In fact, these mutant mice displayed severe aggressive behavior that they required to be housed separately from others (Maynard et al., 2015). Epigenetic changes at *Bdnf* pI have been extensively studied in in the depressed brain (Januar et al., 2015; Ikegame et al., 2013). Importantly, chronic depression showed increased methylation at promoter I of *Bdnf* (Januar et al., 2015). DNA methylation status in neuronal cells of promoters I and IV was extensively studied, and they were found to be regulated in many psychiatric disorders like depression and bipolar disorder (Ikegame et al., 2013). BDNF from promoter II is suppressed during consolidation of fear memory and downregulated in depression and Alzheimer’s disease.
BDNF from promoter III and IV play a role in depression in which their expression is repressed in the hippocampi of stressed patients and several classes of antidepressants reverse this repression (Yu and Chen, 2011; Dias et al., 2003). BDNF promoter IV is also neuronal activity dependent and is upregulated in hippocampus following contextual fear conditioning (Mizuno et al., 2012). Similarly, promoter VI is also upregulated after contextual fear conditioning showing involvement in pathophysiology of brain disorders (Mizuno et al., 2012). These studies reveal that several BDNF promoters are involved in psychiatric disorders and each promoter is differentially and separately regulated.

As previously mentioned, the decrease in BDNF is a major cause for stress. It also results in altered learning and memory processes since BDNF is critical for synaptic plasticity and maintenance of long-term memory (Bredy et al., 2007). This reduction in BDNF can be reversed and balanced by exercise which has a de-stressing effect and which enhances learning and memory. Our lab has previously shown that the endogenous metabolite D-B-hydroxybutyrate (DBHB), produced in the liver during exercise is transported to the hippocampus where it accumulates and mediates increases in Bdnf expression through epigenetic regulation of its promoter (Sleiman et al., 2016). In this work, we wanted to test whether another metabolite, LA produced in the muscle during exercise and transported to the hippocampus where it accumulates and mediates increases in Bdnf expression through epigenetic regulation of its promoter promotes resilience to chronic stress. In addition, we wanted to understand whether LA can reverse the decrease in Bdnf expression in the hippocampi of mice models of depression. Finally, we wanted to elucidate the mechanisms by which LA mediates any antidepressive effects.
Chapter Two

Materials and Methods

2.1. Experimental subjects

C57BL/6J male mice (6-8 weeks) were used in the CSDS paradigm. Mice were divided in different groups according to their treatments and every experimental mouse was housed with the aggressor mouse during the paradigm with free access to food and water. Animals were sacrificed on the eleventh day of the CSDS paradigm (day of the behavioral tests) and hippocampus and cortex tissues were collected and frozen on dry ice.

2.2. Treatments

Mice received according to their experimental group intra-peritoneal injections of either 180mg/kg of LA or 30mg/kg of CI-994 or saline (SAL) daily during the 10 days of social defeat (Figure 4). Four hours after the injections, the experimental mouse was exposed to the CD1 aggressor for a seven minute defeat course.

![Social defeat + treatment](image)

**Figure 4:** Experimental design for the CSDS model.

2.3. Chronic social defeat stress

In order to test our hypothesis, an animal model that accurately reflects the symptoms of depression is required. For this, we used the CSDS model of depression in mice (Figure
5). It is a validated model of depression that mimics social stress linking environmental changes to depression in humans (Golden et al., 2011). Social stressors are known to be in part responsible for the vulnerability of depression and its etiology (Fuchs and Flügge, 2002). Hence, the CSDS paradigm has been developed to reflect the CNS changes that occur in the course of depression after social stress (Golden et al., 2011; Fuchs and Flügge, 2002). The repeated exposures of a subordinate C57BL/6J mouse to aggression from a CD-1 dominant mouse cause it to develop a depressive-like phenotype manifested by anxiety, anhedonia and motivational deficit marked by social avoidance (Golden et al., 2011; Rygula et al., 2006; Fuchs and Flügge, 2002; Kudryavtseva et al., 1991). A distinctive aspect of this model is that it results in the activation of the hypothalamic-pituitary-adrenal axis similar to what occurs in depressed humans (Golden et al., 2011; Covington and Miczek, 2005; Fuchs and Flügge, 2002; Koolhaas et al., 1998; Tornatzky and Miczek, 1993). Moreover, this paradigm results in distinguishing between two important features which are susceptibility and resilience to stress (Golden et al., 2011). Some mice deemed “susceptible” will develop pronounced social avoidance behavior and a group of behavioral and physiological deviations indicative of depressive and anxiety symptoms; while others “resilient” will fail to be vulnerable to social stress and present with normal social interaction behavior. This observation also resembles a feature that differentiates humans into individuals that develop depression due to stress vs. those who are resilient and don’t get affected. Resilience is a major aspect that has been focused on when developing novel antidepressants; researchers are now aiming to promote this phenotype when testing new drugs for depression (Southwick and Charney, 2012; Southwick et al., 2005). Moreover, susceptibility is associated with a decrease in BDNF
resulting in neuronal atrophy and progression of depression. On the contrary, resilience is associated with normal BDNF levels and normal neuronal plasticity (Boulle et al., 2011; Neves-Pereira et al., 2002).

The CSDS paradigm consisted of three stages. The first stage was the screening and selection of aggressive mice, agonistic social interaction between selected aggressor mice and screener mice then followed for ten days, after which social avoidance and anxiety-like behavior were tested on the eleventh day (Figure 4). Prior to the screening stage, aggressors were housed singly for seven days with free access to food and water. Screening for the aggressor mice consisted of three days screening sessions in which the screener mouse was placed directly in the home cage of an aggressor mouse for three minutes with the aggressor mouse present. Different screeners were used for three consecutive days after which aggressor mice were selected upon their initiation of at least one aggression episode within the first minute of the screening session. Aggressor mice were screened before every social defeat paradigm. Selected aggressor mice were then housed singly one day before the first defeat session in a cage with a clear perforated Plexiglas divider. For the ten following days, intruder mice were exposed to daily social defeat in the compartment containing the aggressor mouse for seven minutes and then transferred to the other compartment of the cage allowing only vision, audition and olfaction between the intruder and resident mouse. Intruder mice were introduced to a novel resident’s cage every session in order to prevent any habituation between resident and intruder mice (Figure 5). The control animals were also housed in pairs with a resident mouse and alternated every day for ten days, however no physical contact was allowed between the intruder and resident mouse.
2.4. Sociability test

To test the efficiency of the CSDS paradigm and divide experimental mice into susceptible or resilient groups, a sociability test was performed. Mice were habituated for ten minutes in a cage containing three compartments with two compartments having a circular wire enclosure separated by an empty compartment (Figure 6). Each chamber was of 19 cm width x 38.5 cm length x 20 cm height. After the habituation phase, a social stimulus C57BL/6J mouse (ten weeks) was confined to one of the empty chambers and the experimental mouse was reintroduced to the cage in the central chamber. For the following ten minutes, the experimental mouse was allowed to freely explore the cage and its movement was recorded with a camera mounted to the ceiling. The choice of the chamber containing the stimulus mouse was alternated between trials. The time spent in each compartment (central, social and non-social) was measured by the ANY-maze program. The total time spent by the mouse in the social compartment

Figure 5: Representation of the social defeat model of depression (Modified from Henriques-Alves and Queiroz, 2016).
was divided by the total time spent in the non-social compartment and the mouse was considered susceptible if the ratio is <1 and resilient if the ratio is >1.

There are important pathological features that characterize depression and stress, among these are anhedonia and motivation deficit which is manifested mainly by decreased social interaction (Treadway, 2016; Henrique-Alves and Quieroz, 2016; Pizzagalli, 2014; Kessler et al., 1999; Gorman, 1996). Accordingly, some significant behavioral tests have been developed as a measure for depression-related phenotypes, among these is the social interaction test (SI) (Henrique-Alves and Quieroz, 2016; Hager et al., 2014). This test assesses the defeated behavior in which the social behavior of depressed animals will be altered, however unaffected mice will have a normal interaction with the target (Bondar et al., 2009). Another important aspect of this test is that it can be used to separate resilient and susceptible mice through the measurement of the social interaction time. Since it measures the time spent in the compartment with the target vs. in the empty compartment it will result in a ratio that is equal to social interaction time over no interaction time - and the mouse with a ratio <1 is considered susceptible versus resilient if the ratio is >1 (Henrique-Alves and Quieroz, 2016). Consequently, the less stressed the mouse, the more it interacts with the intruder and vice versa.

**Figure 6:** Schematic illustration of the Social Interaction test consisting of habituation stage and sociability test stage.
2.5. Elevated plus maze

Anxiety-like behaviors of experimental mice were tested in the EPM. The apparatus was elevated 70 cm above the floor and consisted of four arms with a plus shape. Each arm was of 50 cm length and 5 cm width and two of the arms were closed with a height of 35 cm and all of the arms were equally illuminated. Each mouse was put in the center of the four arms and was allowed to explore the maze freely for five minutes. The time spent in the closed and open arms along with the number of entries to the open arms was recorded with a camera mounted to the ceiling and measured by the ANY-maze program.

The elevated plus maze (EPM) measures anxiety-like behaviors by using a cross apparatus to measure the time spent in the open arms vs. closed arms. It’s a validated test for anxiety considering both physiological and pharmacological measures (File, 2001). It is used when studying depression because depressed subjects often experience symptoms of anxiety (Varty, 2002). In rodents, their innate motivation will drive them to explore new environments, so the activity recorded in the open arms suggests a conflict between their preference for sheltered areas like the closed arms and their exploration drive (Walf and Frye, 2007). Hence, in the defeat paradigm, it will be useful for determining whether anxiety measures change between defeated mice and resilient/rescued mice.

As previously mentioned, our aim is to decipher the potential antidepressive effects of lactate. We will achieve our aim by investigating whether it can rescue chronic social defeat behaviors and whether it can mediate its therapeutic effects by regulating hippocampal Bdnf gene expression.
2.6. Open field

An open field area (46 cm x 46 cm) was used to assess the tendency of the experimental mice to explore new arena. Each mouse was allowed to freely explore the arena for five minutes. The time spent in the corners, centre and the average distance travelled by each mouse was recorded by a camera mounted to the ceiling and measure by the ANY-maze program.

2.7. Western blot analyses

To determine Sirt1, HDAC1, HDAC2 and HDAC3 protein levels, total cellular proteins were extracted by lysing the hippocampi in RIPA-B (1% Triton X-100, 1% SDS, 50 mm Tris-Cl, pH 7.4, 500 mm NaCl and 1 mm EDTA) in the presence of the protease inhibitors cocktail SIGMA and MG132, followed by benzonase nuclease digestion for 15 min at room temperature. 15 µg proteins for Sirt1, and 10 µg for HDAC1-3 were loaded on a 10 % acrylamide gels and electrophoresed at 90 V for 30 min and 120 V for 60 min. Proteins were transferred from acrylamide gels to PVDF membranes using Trans-Blot SD Semi-Dry Transfer Cell (BioRad) for 30 min. Membranes were blocked using bovine serum albumin diluted in TBS-Tween for 1 hour at room temperature. Antibodies against Sirt1 (ab110304), HDAC1 (Thermo, Fisher, PA1-860), HDAC2 (ab7030, Abcam), HDAC3 (ab16047, Abcam) and b-ACTIN (AC-74; Sigma-Aldrich) were diluted 1:1000, 1:1000, 1:5000, 1:5000 and 1:5000 respectively. Membranes were incubated in primary antibodies overnight at 4 °C then washed three times with TBS-T then secondary antibodies at room temperature for 90 min. They are then washed three times again with TBS-T. A peroxidase conjugated goat anti-rabbit or anti-mouse was used for detection.
Western blots were imaged using the ChemiDoc Imaging System (BioRad) and analyzed with ImageJ software.

2.8. RNA extraction and RT-PCR

Total RNA was extracted from hippocampal samples for all groups using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. Extracted RNA was reverse-transcribed using iScript cDNA synthesis kit (BioRad). Real-time PCRs were performed according to standard PCR protocol using these Primers’ sequences:

$Bdnf$ Var (Rev): GCCTTCATGCAACCGAAGTA

$Bdnf$ Var I (Fwd): CAGGACAGCAAAGCCACAAT

$Gapdh$ (Fwd): CTCTCTGCTCCTCCCTGTTC

$Gapdh$ (Rev): CCGACCTTCACCATTITGTC

$Sirt1$ (Fwd): AAAGGAATTGGTTCATTATTACAGAG

$Sirt1$ (Rev): TTGTGGTTTTTCTTCCACACA

2.9. Statistical analyses

Unpaired t-test, one-way ANOVA were used to measure statistical significance. $p<0.05$ was considered statistically significant.
Chapter Three

Results

3.1. Lactic acid promotes resilience to stress

3.1.1. Lactic acid reverses the social avoidance behavior of defeated mice

The social interaction test was utilized to investigate whether LA affects social avoidance that results from CSDS. During this assay, after mice are exposed to chronic defeat, they are then tested for their social interaction by measuring the time spent in the interaction zone with the social target inside it vs. the non-interaction zone with the empty enclosed container. Accordingly, mice’s exploratory drive will urge them to socially interact with the target as compared to the defeated mice that will be afraid of it. Mice were divided into various groups: Control mice receiving SAL (n=15), Control mice receiving LA (n=16), Defeat mice receiving S (n=26) and Defeat mice receiving LA (n=32). First, locomotor activity was normalized via the open field test in which the average distance travelled was almost the same for all groups. This shows that the exposure to CSDS did not impair locomotion (Figure 7). Defeated mice spent less time in the interaction zone compared to control (One-way anova P<0.05). LA injections reversed the defeated behavior; mice subjected to defeat and receiving LA spend more time in the interaction zone as compared to mice subjected to defeat and receiving SAL (One-way anova *p<0.05) (Figure 8A). Indeed, when we divided mice into susceptible/resilient groups using the social interaction ratio (time spent in interaction zone/time spent in non-interaction zone) where any mouse with a ratio above 1 was considered resilient and below
1 susceptible. We found that 25% of mice subjected to defeat and receiving SAL were resilient to stress and exhibited normal social interaction levels, whereas this percentage increased to 60% in mice subjected to defeat and receiving LA. As a result, LA was able to significantly promote resilience to stress. (Figure 8B).

![Figure 7: Average distance travelled by open field test. All groups of mice (control or subjected to defeat) travelled the same distance in the open field test. This suggests that defeat did not impair the locomotor activity of the mice. Control+SAL n=12, Control+LA n=13, Defeat+SAL n=18 and Defeat+LA n=22.](image)

3.1.2. **Lactic Acid decreases anxiety-related behaviors of defeated mice**

Along with the suppressed social behavior following CSDS, rodents show increased anxiety as measured by the EPM (Golden et al., 2011). The EPM is a behavioral test used to evaluate the exploratory drive of rodents. A decrease in this exploratory drive is deemed to reflect anxiety. Mice naturally prefer to sit next to a wall or a protected area and feel anxious in open arenas (Libert et al., 2011, Shepherd et al., 1994). Consequently, spending more time in closed arms reflects the mice’s preference for protected zones and anxious behavior (Walf and Frye, 2007). Mice were placed at the center of the EPM and
were video-recorded during the test using the Anymaze program. Subsequently, their behavior was analyzed. Mice subjected to defeat and receiving SAL spent significantly less time in the open arms as compared to control mice (one way anova *p<0.05). This suggests that the defeated mice display decreased exploratory behavior and in turn increased anxiety. LA significantly rescued these effects (Figure 8C; one way anova *p<0.05). Consequently, LA also decreased anxiety. Taken together, our results suggest that LA promotes resilience to stress and reduces anxiety in a CSDS model. We were next interested in determining the mechanisms by which LA promotes this resilience to stress.
Figure 8: LA promotes resilience and rescues social interaction deficits and anxiety phenotypes in mice subjected to CSDS.

(A) Intraperitoneal injections of LA reverse the chronic social defeat phenotype as shown by the increase in the time spent in interaction zone of the SI test.

(B) Top panel shows percentage of resilient mice within Defeat+SAL group. Bottom panel shows percentage of resilient mice within Defeat+LA group. The number for Defeat+SAL n=26 and Defeat+LA n=32.

(C) Intraperitoneal injections of LA decreases anxiety as measured by the significant increase in the amount of time spent in the open arms of the EPM. Control+SAL n=15, Control+LA n=16, Defeat+SAL n=26 and Defeat+LA n=32. Significance was measured by 1way anova *p<0.05.

3.1.3. LA counteracts the vulnerability to stress through induction of class I HDACs

Considering that stress leads to changes in gene expression in the hippocampus and considering that epigenetics lies at the intersection between environmental factors and gene expression, we decided to focus on HDACs. In fact, changes in histone acetylation state at promoter of genes is an important mechanism contributing to the development of depression and many neuropsychiatric disorders (Stertz et al., 2014; Fischer et al., 2010; Tsankova et al., 2007; Caspi and Moffit, 2006). So, in order to assess the molecular mechanism by which LA promotes resilience and considering the important and different roles class I HDAC play in psychiatric disorders, we screened for class I HDACs (HDACs 1, 2 and 3) protein levels in the hippocampus. We observed a significant decrease in HDAC
2 and 3 levels in the hippocampi of mice subjected to defeat and receiving SAL as compared to control mice. We also observed restoration of these HDAC protein levels to control levels in mice subjected to defeat and receiving LA (one way anova, *p<0.05, ***p<0.001). Even though we observed a similar trend with HDAC1 protein levels, the results were not statistically significant. Our findings suggest that CSDS deregulates class I HDAC protein levels in the hippocampus and that LA rescues this effect (n=4) (Figure 9). Indeed, this variation particularly in HDAC3 protein levels seems to correlate well with the susceptible versus resilient phenotypes. We find that HDAC3 protein levels in the hippocampi of mice subjected to defeat and classified as susceptible by the social interaction test are significantly lower than those in the hippocampi of mice subjected to defeat and classified as resilient by the social interaction test (Figure 10). A slight similar trend was observed for HDAC2 proteins, but didn’t reach statistical significance (One way anova, **p<0.01).

Since the decrease in class I HDACs levels correlates with the susceptible phenotype, and since LA restored class I HDAC levels, we wanted to understand whether LA promotes resilience to stress by restoring HDAC function. In order to determine whether class I HDACs are the on target effect of LA mediated rescue of CSDS, we decided to test whether LA can still promote resilience and rescue CSDS if class I HDACs are inhibited. In order to achieve our goal, we used selective class I HDAC inhibitor (HDACi) CI-994. CI-994 is a brain permeable benzamide-based HDACi (Gräff et al., 2014). We divided our mice into two groups: one subjected to defeat and one control group. Within these groups mice either received vehicle (combination of 16% SAL, 5% DMSO, 34% cremophor), LA, CI-994 (30mg/kg daily) or LA+CI-994. Mice subjected to
defeat and receiving CI-994 did not show any rescue for the defeated behavior as observed by the significant reduction in interaction time (Figure 13A; n=6, p<0.05 vs. control+vehicle). As expected, LA rescued the social interaction phenotype and promoted resilience. Interestingly, the LA-mediated rescue of social interaction was attenuated when mice received both LA and CI-994 together. Class I HDAC inhibition by CI-994 had a profound effect on mice subjected to defeat and receiving LA, whereby CI-994 limited LA’s ability to reverse avoidance and increase the social interaction time. Instead, mice subjected to defeat and receiving both compounds displayed avoidance behavior, comparable to that of mice subjected to defeat and receiving the vehicle (Figure 11A). Similar results were observed in the EPM suggesting that CI-994 significantly attenuates LA-mediated rescue of anxiety phenotypes (Figure 11A). Indeed, we find that the LA-mediated increase in the time spent in the open arms of the EPM is lost when mice were subjected to defeat and received the combined treatment of LA and CI-994. In conclusion, class I HDAC inhibition prevents LA-mediated resilience to CSDS. These results suggest that LA mediates its pro-resilience effect through upregulation of class I HDAC levels and function.

Next, we assessed hippocampal protein levels of class I HDACs (Figure 11 B, C and D). As expected, HDAC2 and 3 levels were significantly decreased in the hippocampi of mice subjected to defeat and receiving the vehicle and these levels were restored to control levels in mice subjected to defeat and receiving LA or LA+CI-994. These results suggest that CI-994 does not interfere with LA’s ability to restore hippocampal HDAC2 or 3 levels. We did not expect CI-994 to affect HDAC1, 2 or 3 protein levels because it inhibits class I HDAC enzymatic activity without affecting their expression (Wang et al.,
These observations suggest that effects HDAC2 and 3 are downstream of LA and are consistent with a model by which HDAC2 and HDAC3 promote resilience to stress. Indeed, our results support the hypothesis that stress induces a decrease in hippocampal HDAC2 and 3 levels and LA restores these proteins, which promote resilience to stress through their deacetylase activity. (Figure 11 B,C and D).

**Figure 9:** LA induces class I HDACs protein levels. (A), (B) and (C): Representative western blots of HDAC1, HDAC2 and HDAC3 and their corresponding protein quantifications.
Figure 10: The resilient phenotype is characterized by induction of HDAC3, a member of the class I HDACs. (A) A representative western blot showing the HDACs levels from 4 susceptible hippocampal lysates and 5 resilient hippocampal lysates are depicted. (B) Quantification of the HDACs western blot. One way anova, **p<0.01.
3.1.4. Social defeat significantly inhibits *Bdnf* pI expression, whereas lactic acid reverses this effect

Considering LA was identified as an endogenous molecule produced during exercise by the muscle and transported to the brain where it promotes learning and memory through *Bdnf* pI induction, we next wanted to assess whether LA regulates *Bdnf* pI expression in defeated mice. We extracted RNA from the hippocampi of control mice...
and mice subjected to social defeat and receiving either SAL or LA and performed Real-time RTPCR experiments. Our results showed that social defeat resulted in significant downregulation of hippocampal *Bdnf* pI expression, whereas LA reversed this decrease to normal expression levels (Figure 12). Since a decrease in BDNF levels is associated with depression (Smith et al., 1995), our results are consistent with the idea that the LA-mediated restoration of BDNF levels is relevant to its pro-resilience effects. Considering that LA induced the expression of class I HDACs, we ruled out their direct regulation of the BDNF pI expression level and rather focused on other epigenetic modulators. Another class of HDACs that plays a role in regulating *Bdnf*’s expression is the class III HDACs or Sirtuins. These proteins affect many processes in the brain (Libert et al., 2011; Chen et al., 2005) including learning and memory as well as regulation of age-dependent disorders (Libert et al., 2011; Gao et al., 2010; Michan et al., 2010; Ng et al., 2015). Sirt1 is activated during chronic stress in the hippocampus and Sirt1 expression was shown to be associated with the prevalence of major depressive disorder (Ferland et al. 2013; Ferland and Schrader 2011). In addition to its role in depression, Sirt1 was also shown to directly bind to the *Bdnf* gene in the nucleus accumbens and this binding was decreased upon cocaine administration (Ferguson et al., 2015). We found that gene expression analysis showed a significant upregulation in *Sirt1* gene in the defeat group, whereas LA restores Sirt1 expression to control levels (Figure 13A, TTest **p<0.01). This was confirmed with western blots showing matching protein levels (Figure 13B, TTest *p<0.05, ***p<0.001).

Taken together, our results are consistent with the model that CSDS increases hippocampal SIRT1 levels which in turn leads to a decrease in *Bdnf* expression and LA reverses the effects of stress. This hypothesis requires further investigation in order to
show that stress induces hippocampal SIRT1 binding to the Bdnf pl leading to histone deacetylation and inhibition of gene expression, whereas LA treatment decreases hippocampal SIRT1 levels at the Bdnf pl promoter and induces its expression. This hypothesis will ultimately be tested using chromatin immunoprecipitation experiments.

**Figure 12:** CSDS significantly inhibits Bdnf pl expression, whereas LA reverses this effect. Real Time RT-PCR quantification showing the decrease in mRNA expression of Bdnf pl transcript. The n number of control+SAL group and defeat+SAL equals to 8 and 6 to defeat+LA. TTest significance: *p<0.05
Figure 13: CSDS induces Sirt1 mRNA and protein expression in the hippocampus, whereas LA restores control levels. (A) Social defeat induces Sirt1 mRNA levels in the hippocampus, whereas LA restores control levels. The n number is as follows: Control+SAL= 7 and 10 for each of defeat+SAL and defeat+LA groups. TTest significance: **p<0.01. (B): Social defeat induces Sirt1 protein levels in the hippocampus, whereas LA restores control levels. Western blots showing the increase in Sirt1 protein levels in defeat+SAL, in contrast to defeat+LA which shows a repression similar to that of control+SAL levels. (C) Sirt1 western blots quantification.
Chapter Four

Discussion

Studies in post-mortem human brains and various preclinical models have provided data about chromatin-mediated neuroplasticity playing a crucial role in the mechanisms that underlie many psychiatric diseases (Schroeder et al., 2013). In addition, the vulnerability to psychiatric disorders and particularly depression, is in large due to a synergy between genetic factors and environmental stressors (Turner et al., 1995; Zelena et al., 1999; Connor-Smith and Compas, 2002; Southwick et al., 2005; Henriques-Alves and Queiroz, 2015). In fact, not all stress-suffering individuals will develop depression; individuals who are resilient to stress have the ability to tolerate and overcome the damage (Pfau and Russo, 2015; Southwick and Charney, 2012). This feature has been studied in several models of depression in rodents such as CSDS. The repeated exposure to aggression causes the mice to develop depression which is indicated by the decreased social interaction behavior (Henriques-Alves and Queiroz, 2015). However, mice respond to this stress-induced behavior differently and are divided into susceptible or resilient subjects. The therapeutic potential of novel antidepressants is being evaluated through their ability to induce resilience (Bagot et al., 2016). Hence, a very important finding of this study is the identification of a novel endogenous molecule LA, which can mediate resilience by activating epigenetic mechanism involving class I HDACs.

Indeed, our results showed that chronic treatment with LA during the establishment of social defeat promoted resilience to stress by reversing the social avoidance behavior and preventing anxiety. Moreover, our results show that CSDS
induces changes in epigenetic modifiers and LA reverses these effects leading to mood and behavioral changes. We identified class I HDACs (2 and 3) that were regulated by stress and LA in the mouse hippocampus. The induction of HDAC2 and 3 levels and activities plays a role in the establishment of resilience by LA.

First, LA was shown to improve mice behavior by the increasing sociability and decreasing anxiety (Figure 8). Both the social interaction test and EPM are good indicators of the positive effects of LA on defeated mice. Furthermore, we used the social interaction ratio to segregate mice into susceptible or resilient and found that the response to stress changed significantly between vehicle defeated mice and those who received daily LA injections with 25% of mice not receiving LA were resilient as compared to 60% of the mice receiving LA (Figure 8). This resilient behavior was accompanied by the restoration of HDACs 2 and 3 protein control levels that were downregulated by defeat (Figure 9). Additionally, the comparison of the hippocampal levels of these HDACs between susceptible vs. resilient mice from the vehicle defeat group validated our previous findings, in which HDAC protein levels were downregulated in susceptible mice and restored in resilient ones (Figure 10). The restoration of normal levels of class I HDACs vital since they play a crucial role in neuronal health (Morris and Monteggia, 2013). Class I HDACs are highly expressed in the brain and are involved in normal brain development especially in areas associated with learning and memory (Volmar and Wahlestedt, 2015; Graff and Tsai, 2013). HDAC1 acts as a molecular switch crucial for neuronal survival and protection; it protects neurons in cell culture and mouse models of Alzheimer disease and ischemic stroke (Bardai et al., 2012). Normal levels of HDAC2 are essential for normal social behavior (Kumari et al., 2016). HDAC3 is a key player in brain
development. The loss of HDAC3 cannot be compensated for by other HDACs, and it is required for normal brain function (Norwood et al., 2014). As a result, maintaining normal HDAC levels is crucial for signaling in neuronal cells in order to promote neuronal plasticity and mental health. Accordingly, our study has shown that LA doesn’t induce HDACs beyond control levels, but rather restores the average levels which are required for normal brain function and stress resistance.

On the other hand, considering published data (Schroeder et al., 2013; Covington et al., 2011; Covington et al., 2009) indicating that HDAC inhibition is a promising therapy for depression, we believe our results highlight the different roles that class I HDACs play before/during establishment of stress versus after the establishment of depression. So, it becomes critical to decipher the roles of these HDACs during the choice between susceptibility to stress or resilience to stress and their roles after the establishment of susceptibility and depression. Our data suggests that during the choice between resilience and susceptibility, class I HDACs, namely HDAC2 and 3, play important roles in promoting resilience (Figure 12,14); whereas the role becomes negative once the choice has been established. This hypothesis can be tested by using the CSDS paradigm and treating the animals with CI-994 after susceptibility is established to assess the therapeutic potential of HDAC inhibition. It also remains important to address whether LA can be used as a therapy for depression and whether it also mediates its effects by engaging different molecular pathways during the choice between susceptibility and resilience and during the therapeutic phase.
Figure 14: The established model by which LA induces resilience to stress by affecting epigenetic modulators in the hippocampus. LA, as opposed to CSDS (decrease Class I HDAC levels) and CI-994, induces Class I HDACs during stress in order to establish a resilient phenotype characterized by normal levels of BDNF.

Another important finding of this study is that LA induces hippocampal *Bdnf* pI expression when it is injected during the establishment of stress. We show that resilient mice receiving LA that presented with a less anxious and a decreased social avoidance behaviour, had significant increase in *Bdnf* pI expression in their hippocampi (Figure 12). Hippocampal BDNF induction is a common action of many antidepressants since this neurotrophin is a very important mediator of neuroprotection and plasticity (Lieto et al., 2012). Many molecular studies have shown that stress and antidepressants have opposing effects on the expression of neurotrophic factors especially *Bdnf* (Duman and Li, 2013; Castrén and Rantamäki, 2010; Duman and Monteggia, 2006). Indeed, BDNF levels are reduced in depression and restoration of BDNF levels are required in order for patients to respond to antidepressant treatments (Duman and Li, 2013; Castrén and Rantamäki, 2010; Duman and Monteggia, 2006). Moreover, BDNF infusion is enough to induce an
antidepressant response. Our findings verify prior work on the effect of exercise and \textit{Bdnf} on rodent models of depression (Callaghan et al., 2017). Indeed, it is well known that physical exercise enhances mood and protects against depression and many psychiatric disorders (Jackson et al., 2016; Voss et al., 2013); however, our findings are of great relevance because they show that LA, the energy source for the brain during exercise, is one of the molecules that could translate the effects of exercise on the brain leading to resistance to stress. The fact that this induction aids in the protection from stress is also significant, since depressed patients do not tend to exercise as compared to control patients. So, stress resilience is a fundamental feature when studying models of depression and it is becoming of great interest when developing therapies (Southwick et al., 2005).

In order to understand how LA mediates the induction in \textit{Bdnf}, we focused our attention on another class of histone deacetylases, the Sirtuins. Since Sirt1 is a NAD-dependent histone deacetylase, it could link the change in NAD$^+$/NADH ratio after LA production to the epigenetic modulations that occur particularly in the nucleus (Jang et al., 2012; Blander and Guarente, 2004). We observed social defeat upregulates SIRT1 levels and that LA restores normal levels (Figure 13).

In conclusion, we provided evidence of the endogenous molecule, LA, that is increased after physical exercise and can cross the blood brain barrier, is effective in mediating resilience to stress suggesting that it may have an anti-depressant potential.
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