# ROLE OF EXTRACELLULAR REGULATED KINASE 2 WITHIN LATERAL HABENULA IN MEDIATING ANTIDEPRESSANT RESPONSE AND RESILIENCE DURING ADOLESCENCE

A Dissertation

by

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## ABSTRACT

Approximately 13% of children aged 12-17 are diagnosed with major depressive disorder (MDD). This is particularly troubling since according to the World Health Organization, suicide is the second leading cause of death in individuals aged 15-29, suggesting that there is much left to be understood about the underlying neurocircuitry regulating symptoms of MDD. Previous work has shown that extracellular regulated kinase 2 (ERK2) activity in mesolimbic reward structures such as the ventral tegmental area (VTA), is important in mediating stress- and antidepressant-responding. The VTA receives regulatory input from the lateral habenula (LHb) however little is known about how ERK2 is expressed in the LHb after stress.

To better understand this mechanism, rt-PCR, used to assess changes in mRNA, and western blot, used for protein analysis, was done for ERK2 and showed that both mRNA and protein levels of ERK2 in the LHB were modulated after stress or antidepressant exposure. To assess if ERK2 modulation could buffer stress-induced deficits, adolescent rats were given micro infusions of wtERK2 to increase ERK2 expression in the LHb, and then exposed to the stress and anxiety-eliciting tasks. Increasing ERK2 in the LHb, through a viral-mediated approach, promoted antidepressant-like responses as seen through increased time spent in the open arms of the elevated plus maze and less time immobile in the forced swim test. A separate group of rats was placed through chronic unpredictable stress and then received site-specific infusions of wtERK2 prior to behavioral testing, in an attempt to reverse stress-induced deficits. Similar to infusions in naïve animals, increasing ERK2 in the LHb was sufficient to promote antidepressant-like responses, when compared to GFP-exposed rats. This data suggests that increasing ERK2 in the LHb promotes resilience to stress and can reverse stress-induced deficits. Overall this data highlights the importance of LHb second-messenger signaling in mediating resilience to stress-eliciting stimuli.

To my mom, Bianca Mori, who made me the woman I am proud to be today.

And to my loves, Eric Parise and our Olive.

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# CHAPTER I

## INTRODUCTION AND LITERATURE REVIEW

As one of the leading causes of disability in the world (Kessler, 2012), major depressive disorder (MDD) is an immensely costly and burdensome illness (Kessler, 2012). MDD afflicts up to 20% of the world's population (Manji, Drevets, & Charney, 2001; Nestler, Barrot, DiLeone, Eisch, Gold, & Monteggia, 2002a), and nearly 10% of adolescents suffer from MDD (Lewinsohn, Rohde, Seeley, & Fischer, 1993). Sadly, adolescent rates of depression have increased over the last 10 years, yet very little has been done to address antidepressant availability and efficacy in pediatric populations (Birmaher, Brent, & Benson, 1998; PharmD, PhD, & MSPH, 2009). This is particularly troubling because nearly 50% of adolescents who suffer from MDD do not respond to currently approved treatments (Maalouf, Atwi, & Brent, 2011), and according to the World Health Organization, suicide is the second leading cause of death in afflicted individuals aged 15-29 (World Health Organization, 2015).

Even though MDD has been a public health concern for many years, its etiology and pathophysiology remain poorly understood. There is an abundance of data describing just one particular mechanism or specific pathway that mediate certain aspects of MDD, however, a better understanding of the predisposing factors, potentiating processes, and overall mechanisms of MDD is still lacking. Additionally, most evidence of the mechanisms underlying MDD processes and antidepressant efficacy come from studies in adults, making it extremely difficult to address and treat juvenile mood disorders (Birmaher, 1998; Emslie & Mayes, 2001; Coyle et al., 2003). Taken together, this highlights the need for newer, more efficacious treatment options for this population.

The mechanisms by which antidepressants exert their therapeutic effect varies widely between the different classes of antidepressants (ADs) and is still a major topic of ongoing research. Different classes of ADs may act via modulation of more than one neurotransmitter, such as serotonin, norepinephrine, or dopamine, among others, and influence the release/reuptake of that transmitter, ultimately increasing its bioavailability (Arborelius et al., 1996; Arnone et al., 2018). Most if not all ADs eventually work through binding to particular receptors to initiate subsequent intracellular signaling cascades. At the clinical level, most AD take weeks to months to have a therapeutic effect, and some patients have to try several different ADs to find one that

works at all (Gupta, Gersing, Erkanli, & Burt, 2015). Unfortunately, some commonly prescribed antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), have been shown to have negative side effects that coincide with their therapeutic efficacy (Cheung, Emslie, & Maynes, 2004). Fluoxetine (FLX), the only approved SSRI approved for the treatment of pediatric depression, tends to increase weight gain, promote mood swings, and even increase anxious or impulsive tendencies (Gupta et al., 2015). Despite its negative side-effects, FLX does alleviate some depression-related symptoms in adolescents and when taken responsibly, promotes remission of MDD (MD et al., 2015; Shehab, Brent, & Maalouf, 2016; Warner-Schmidt, Vanover, Chen, Marshall, & Greengard, 2011).

Ketamine (KET) is an *N*-methyl-D-aspartate (NMDA) receptor antagonist that has recently gained much attention due to its ability to elicit rapid antidepressant effects when compared to traditional medications (N. Li et al., 2011). In stark contrast to traditional antidepressants which, as stated, take weeks to months to be effective, patients receiving just one infusion of KET report to have sustained reductions in depressed mood (Murrough et al., 2013), and it has been effective in patients suffering from treatment resistant depression (TRD) (Price, Nock, Charney, & Mathew, 2009) (Price et al., 2014). Importantly, KET has also been shown to be safe for use in adolescent and pediatric populations (Dale, Somogyi, Li, Sullivan, & Shavit, 2012; Nugent et al., 2013; Papolos, Teicher, Faedda, Murphy, & Mattis, 2013). Given KET's rapid and sustained antidepressant action, especially in TRD, it is a very promising candidate tool to uncover the neural mechanisms underlying MDD as well as elucidate more effective therapeutic strategies in adolescent populations.

The mechanisms by which antidepressants exert their therapeutic effects is still a major topic of ongoing research. By identifying where in the brain drugs such as FLX- and KET-induced biological changes occur we can advance our understanding of depression and discover potentially novel therapeutic targets in the process. Most research delineating brain mechanism mediating depression has focused on the hippocampus (HIPP) and prefrontal cortex (PFC), and these regions have been directly implicated in mediating various aspects of depression (Yetnikoff, Lavezzi, Reichard, & Zahm, 2014). More recently, research efforts have focused on the mesolimbic reward pathway (Nestler, Barrot, DiLeone, Eisch, Gold, & Monteggia, 2002a). This reward pathway is known for playing a major role in controlling goal-directed behavior and mood under normal conditions (Naranjo, Tremblay, & Busto, 2001; Nestler & Carlezon, 2006; Wise, 1996). The key

brain substrates that comprise this circuit include the nucleus accumbens (NAc), the HIPP, the amygdala (Amy) and the PFC, which all receive input from the ventral tegmental area (VTA) (Duman & Monteggia, 2006). Post-mortem tissue of individuals with depression or those whom have committed suicide show atrophy in some of these brain regions, which is accompanied by dysregulated signaling of key depression-related molecules, such as brain derived neurotrophic factor (BDNF) (Berton et al., 2006).

The VTA is comprised of mainly dopamine-secreting neurons, however recent studies show evidence for glutamate-, GABA-, and CRH-releasing neurons as well (Yoo et al., 2016). The mesolimbic reward circuit is bi-directionally modulated by the VTA and its target structures to maintain proper functioning in response to stress, and also in the modulation of antidepressant efficacy (Krishnan et al., 2007; Luo, Tahsili-Fahadan, Wise, Lupica, & Aston-Jones, 2011). Current evidence suggests that treatment with KET rapidly changes the structure and enhances the functioning of synapses within the mesocorticolimbic pathway (Murrough et al., 2013). Studies have demonstrated that some of the mechanisms underlying KET's antidepressant effects may depend on rapid activation of the mammalian target of rapamycin (mTOR) pathway, including increases in extracellular signal-regulated kinase (ERK), protein kinase B (PKB/Akt), and brain-derived neurotrophic factor (BDNF) within the HIPP, changes in glutamatergic synaptic strength in the NAc, and leads to an increase in new spine formation within the PFC (Abdallah et al., 2016; Autry et al., 2011; N. Li et al., 2010; Murrough et al., 2013).

Although the VTA has direct control over NAc, PFC, and HIPP, it is important to note that VTA activity is modulated by other structures, one of which is the habenular complex (Mameli, 2013). Specifically, the lateral portion of this complex, the lateral habenula (LHb), has been shown to be important in mediating behavioral responses to both positive and negative stimuli (Stamatakis & Stuber, 2012; Stamatakis et al., 2013). The LHb is a glutamatergic hub which is suspected to inhibit VTA activity by directly increasing inhibitory tone through local VTA GABAergic modulation (Quina et al., 2014). This hypothesized LHb-induced inhibition would ultimately lead to reduced dopamine output by the VTA, thus promoting depressive-like behaviors (Meng et al., 2011). Interestingly, lesions to the LHb have been shown to attenuate anxiety- and depressive-like behaviors in rodents exposed to stress (Gill, Ghee, Harper, & See, 2013; Winter, Vollmayr, Djodari-Irani, Klein, & Sartorius, 2011). Given the evidence supporting the LHb involvement in mediating depressive-like behavior, it is likely that antidepressants may elicit some of their actions,

at least in part, through the LHb. Indeed, a recent study has demonstrated that KET, for example, reverses stress-induced hyperactivity of neurons within the LHb (Y. Yang et al., 2018). However, little is known about how KET influences second messenger signaling within the LHb, and even less is known about the role of ERK2 signaling within the habenula. This is important because there is evidence for a close relationship between ERK2 activity and antidepressant responses (Trentani, Kuipers, Horst, & Boer, 2002; Valjent, Pages, Herve, Girault, & Caboche, 2004; Warren et al., 2014), making ERK2 a likely target candidate for mediating antidepressant responses within the LHb. Therefore, the main goal of my dissertation is to determine the LHb's potential role in the antidepressant effects induced by KET. My decision to investigate the LHb was based on findings indicating: a) its sensitivity to stress, and mediates stress-induced depressive symptomology, b) modulates input to the VTA, and therefore the mesocorticolimbic system, c) mediates stress-induced neuronal hyperactivity, which KET can reverse (Y. Yang et al., 2018), and d) it might contribute to, or even be the region that underlies KET's induced synaptic plasticity in other brain regions.

Given these findings, I hypothesize that antidepressant exposure will influence ERK2 activity within the LHb, and that direct regulation of ERK2 will modulate depression- and anxiety-related behaviors, and that this brain region plays a direct impact on antidepressant efficacy. To test these hypotheses, I will first assess the long-term behavioral effects of chronic stress exposure and compared the stress-induced behavioral profile to that of adolescent rodents exposed to FLX or KET. I will then use rtPCR and Western Blot to compare and contrast the biochemical effect of stress and antidepressant exposure on ERK2-related signaling within the LHb. Given the evidence that ERK2 also acts within the VTA, I will also investigate the consequences of these insults in the VTA. Lastly, I will directly manipulate ERK2 within the LHb, using viral vectors, to functionally determine its role in stress and antidepressant-induced phenotypes during adolescence.

# CHAPTER II

# BEHAVIORAL CONSEQUENCES OF STRESS OR ANTIDEPRESSANT EXPOSURE DURING ADOLESCENCE

## Introduction

Approximately 13% of children aged 12-17 are diagnosed with major depressive disorder (MDD) (Avenevoli, Swendsen, He, Burstein, & Merikangas, 2015). This is particularly troubling because suicide is the second leading cause of death in individuals aged 15-29 according to the World Health Organization. This suggests that there is much left to be understood about the underlying neurocircuitry regulating symptoms of MDD. Early life MDD can be highly debilitating, and its lasting negative consequences, such as increasing risk for conduct and substance abuse disorders, greater likelihood of relapse, and increase susceptibility to post traumatic stress disorder (PTSD), can extend into adulthood (Y. Chen & Baram, 2015; DSc, MRCPsych, & PhD, 2012). Stress and maladaptive coping mechanisms are suggested to be among the major precipitating factors in developing MDD (Juster, McEwen, & Lupien, 2010). Early life stress has been shown to have long lasting negative effects resulting in dysregulated functionality in activity of the hypothalamic pituitary axis (van Bodegom, Homberg, & Henckens, 2017), a major component of the stress response, and a key element of the feedback mechanism necessary for appropriate signaling of the stress hormone, corticotrophin releasing hormone (CRH) [corticotrophin releasing factor (CRF) in rodents; Authement et al., 2018; Inda, Armando, Santos Claro, & Silberstein, 2017] . Early life stress has also been shown to change the volume of key brain regions such as the prefrontal cortex and the hippocampus, responsible for executive function and emotional processing, respectively (Syed & Nemeroff, 2017; King, Humphreys, Camacho, & Gotlib, 2018). This change in volume and the subsequent changes in connectivity between these brain areas could be a potential contributor to the affective abnormalities seen in MDD. Animal models of early life stress have been instrumental in delineating the neurobiology of stress, however they often focus on prenatal maternal stressors or postnatal maternal separation which rely heavily on disruption of social bonds (Alcantara, Parise, & Bolaños-Guzmán, 2017), however human adolescents often undergo social and emotional stress that go beyond this type of insult (Teicher, Samson, Polcari, & McGreenery, 2006). Gaining a better understanding of the pathology of MDD may be facilitated through modeling of stress exposure experienced during adolescence.

Medications approved for use in children are severely limited, with fluoxetine (FLX), a selective serotonin reuptake inhibitor (SSRI), being the only pharmacotherapy approved for use in children and adolescents (Birmaher et al., 1998). A vast majority of what is known about the effectiveness and the side-effects of SSRIs, such as FLX, has been derived from studies in adult populations, while research on the effectiveness and long-term effects of FLX exposure during periods prior to adulthood, is critically lacking (Birmaher et al., 1998; PharmD et al., 2009). In general, it has been reported that the therapeutic efficacy of FLX treatment often coincides with unfavorable side-effects ranging from weight gain to sexual dysfunction (Wernicke, 2005). Aside from unwanted side effects, two major setbacks have been identified for FLX: true clinical efficacy is usually reached after about 3-4 weeks of treatment, and about half of young individuals that are prescribed FLX are non-responsive to treatment and require multiple adjunctive pharmacotherapies (Cipriani et al., 2016; Maalouf et al., 2011; Zhou et al., 2015).

Recently, the non-competitive NMDA receptor antagonist, ketamine (KET), has received attention due to its ability to act as a rapid acting, long lasting treatment, for adult MDD, and has been found to be particularly efficacious in individuals deemed treatment resistant (Diazgranados et al., 2010; Rot, Zarate, Charney, & Mathew, 2012). KET is often administered in a clinic, and while it is rapid acting and its antidepressant effects can be seen to last for multiple days, patients often must return to the clinic for subsequent treatments in order to maintain antidepressant effectiveness. This practice parallels findings in animal studies showing that chronic, as opposed to acute, treatment with KET results in antidepressant effects that lasts up to two months (Parise et al., 2013) suggesting the possibility that chronic administration of KET, similar to FLX, may be more efficacious in patients with MDD. As often is the case, most of the current studies with KET have been done in adult patients, and although its use in adolescents has not been approved, there is reason to believe KET treatment for juvenile depression can be just as effective as FLX (Murrough et al., 2013; Sanacora et al., 2016). Basic research provides the means to assess the potential effectiveness of drugs for therapeutics, thus the following set of experiments were designed to establish the behavioral outcome of chronic unpredictable stress (CUS) in adolescent rats and to further compare the short and long-term behavioral effects of chronic exposure to KET or FLX during the adolescent period.

## Methods

*Materials and Tests*

*Animals.* Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Rats arrived at postnatal day (PD) 28 and were allowed to habituate to the facility for 5-7 days. The age of experimental manipulations in adolescent rats (PD 35) was selected because it roughly approximates adolescence in humans (Spear, 2000). Rats were housed in clear polypropylene boxes containing wood shavings in an animal colony maintained at 23–25°C on a 12 h light/dark cycle in which lights were on between 0700 and 1900 hours. Food and water were provided ad libitum.

*Chronic unpredictable stress*. The chronic unpredictable stress (CUS) paradigm is usually carried out for 2-4 weeks (4 weeks in adolescents) and consists of exposing rats to one stressor/day in a randomized manner, such that the animal does not have time to acclimate to the stress schedule and predict the stressor. This is an important detail as controllability of stressors has been shown to have an impact of the deleterious effects of stress (Christianson et al., 2014). Stressors consisted of alternating periods of food or water deprivation (overnight), continuous cage shaking (1h on an automatic shaker), forced swim stress (15 min, in 18°C water), continuous overnight illumination (12 h), overnight cage-flooding (12 h), exposure to cold temperature (1h at 4°C), and acute restraint stress (40 min) using plastic DecapiCones (restraint bags) (Iniguez et al., 2010; Overstreet, 2011).

*Sucrose Preference.* The sucrose preference test consisted of a two-bottle choice procedure in which rats were given the choice between consuming water and a sucrose solution. This paradigm has been used extensively to assess the effects of stress-induced anhedonia (Nestler, Barrot, DiLeone, Eisch, Gold, & Monteggia, 2002b). Rats were habituated to drink water from two bottles for 5 days. At the start of the experiment, they were exposed to ascending concentrations of sucrose (0.0%, 0.25%, and 0.5%) for two days per sucrose concentration. Water and sucrose consumption was measured at 9 A.M. and 7 P.M. each testing day. The position of the sucrose bottle (left or right) was counterbalanced between groups and changed daily. Preference for sucrose over water [sucrose/(sucrose + water)] was used as a measure for rats' sensitivity to reward.

*Elevated Plus Maze.* The elevated plus maze (EPM) is a behavioral assay commonly used to measure anxiety-like behavior (Montgomery, 1955b). The EPM apparatus is elevated

approximately 3 feet off the ground and consists of two perpendicular intersecting runways (6cm x 25cm); one runway has no walls (open arms) while the other arm has fully encompassing walls on either side of the runway (closed arms; 25 cm tall). Rats are placed into the center of the intersecting runways and can freely explore the arena for 5 min. Rats tend to prefer the safety of the closed arms but will eventually begin to explore the open arm runway. Increased time spent in the closed arms is interpreted as increased anxiety-like behavior.

*Forced Swim Test.* The forced swim test (FST) is a task commonly used to assess antidepressant efficacy; however, the FST has high predictive validity and is used as a behavioral task to assess learned helplessness (Reed, Happe, Petty, & Bylund, 2008a). Mice/rats are individually placed into containers filled with cold water (23 ± 2°C). The containers are filled such that the animal cannot touch the bottom is forced to swim to stay afloat. Eventually, the animal adopts an immobile posture, characterized by motionless floating and the cessation of struggling behaviors. The latency to adopt an immobile posture and the total time spent immobile thereafter are recorded. Rodents with lower latency to immobility or more time spent immobile reflect a depressive-like phenotype.

*Drugs and administration schedule***.** Ketamine (KET) or fluoxetine (FLX) were obtained from Butler Schein Animal Health (Dublin, OH) in an injectable solution (100 mg/ml) or Spectrum Pharmaceuticals (Irvine, CA), respectively. KET (10 mg/kg) was diluted in sterile physiological saline (0.9% sodium chloride) and administered intraperitoneally (IP) at a volume of 1 mL/kg. FLX (10 mg/kg) was dissolved in sterile water and administered IP at a volume of 1mL/kg. Rats were administered treatment twice per day for 15 days from PD 35-49. Behavioral testing to assess reactivity to stressful stimuli was done both at 24hrs [short-term (ST)] and 1 month [long-term (LT)] after drug treatment. Assessments were done in separate groups of rats and for the LT group, testing began at PD80.

*Statistical Analyses.* Behavioral data were analyzed using mixed-design (between and within variables) ANOVA followed by Fisher Least Significant Difference (LSD) post hoc tests. When appropriate, Student's *t* tests were used to determine statistical significance of planned comparisons. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as $p < 0.05$.

**Results**

*Stress-induced changes in depression and anxiety-like behaviors*

*Forced Swim Test (FST).* The FST was used as the last stressor of the CUS paradigm and was scored as an assessment of deficit. As the last stressor of the CUS paradigm, CUS-rats and their control (CON) counterparts were exposed to a final 5-minute FST ($n$ = 20/group; Figure 2.1A-B*)*. An unpaired Student's $t$ -test revealed significant differences as a function of stress-exposure, in both latency to adopt an immobile posture ($t_{38} = 3.798$, $p = 0.0005$) and the total time spent immobile ($t_{38} = 2.661$, $p = 0.0113$). CUS-exposed rats took less time to adopt an immobile posture compared to non-stress controls (i.e., gave up faster) ($p < 0.05$; Figure 2.1A). Similarly, exposure to CUS promoted longer time spent immobile for the duration of the FST ($p<0.05$; Figure 2.1B). Avoidance of the open arms in the CUS-exposed rats exemplifies an exaggerated anxiogenic response to a novel environment.

*Elevated Plus Maze (EPM).* Twenty-four hours after the last day of stress exposure, CUS and their CON counterparts ($n$ = 20/group; Figure 2.1C-D) were exposed to the EPM. An unpaired Student's $t$ -test revealed significant differences in time spent in the open ($t_{38} = 2.56$, $p = 0.014$) and closed arms ($t_{38} = 2.78$, $p = 0.008$) of the EPM, as a function of stress exposure. CS-exposed rats spent significantly less time in the open arms of the EPM when compared to non-stress controls ($p < 0.05$; Figure 2.1C). Similarly, exposure to CS resulted in an increase in the time rats spent in the closed arms of the EPM ($p < 0.05$; Figure 2.1D). Avoidance of the open arms in the CS-exposed rats exemplifies an exaggerated anxiogenic response to a novel stress-inducing environment.

*Stress-induced changes in sucrose preference.* After exposure to CUS, rats were tested for sucrose preference ($n$ = 20/group; Figure 2.1E). A two-way ANOVA revealed a significant effect of stress condition ($F_{1,19} = 13.43$ $p = 0.0003$) and sucrose concentration ($F_{3,19} = 57.63$ $p = 0.0001$) and a significant interaction ($F_{3,19} = 5.226$ $p = 0.0018$) between the two variables. Rats exposed to CUS drank less sucrose (0.125%, 0 .25%, and 0.5%) compared to non-stressed controls ($p < 0.05$; Figure 2.1E). No differences were seen in water consumption between CUS- and control rats.

*Short-term effects of FLX exposure on depression and anxiety-like behaviors*

*Elevated Plus Maze (EPM).* Twenty-four hours after the last day of drug treatment, fluoxetine (FLX)-exposed adolescent rats and their saline (SAL)-exposed counterparts ($n$ = 10/group; Figure 2.2A-B) were exposed to the EPM. An unpaired Student's $t$ -test revealed significant differences, as a function of drug exposure, in the time rats spent in the open ($t_{18} = 2.118$,

$p$ = 0.0483) and closed arms ($t_{18}$= 3.093 $p$= 0.006) of the EPM. FLX-exposed rats spent significantly less time in the open arms of the EPM when compared to SAL pre-treated rats ($p$<0.05; Figure 2.2A). Similarly, exposure to FLX promoted an increase in the time rats spent in the closed arms of the EPM ($p$<0.05; Figure 2.2B). This is not unexpected as previous work from our group and others, has shown that exposure to FLX induces an increase in anxiety-like behavior (Iniguez, Alcantara, et al., 2014a).

*Forced Swim Test (FST).* After being exposed to the EPM, FLX-exposed rats and their SAL counterparts were administered the FST (n= 10/group; Figure 2.2C-D). An unpaired Student's *t* -test revealed significant differences, as a function of drug exposure, in both latency to immobility ($t_{18}$= 4.98, $p$<0.0001) and total immobility in the FST ($t_{18}$= 2.504, $p$= 0.0221). FLX-exposed rats took longer to give up (adopt an immobile posture) compared to SAL pre-treated rats ($p$<0.05; Figure 2.2C). Similarly, exposure to FLX promoted a decrease in the overall time that rats spent immobile in the FST ($p$<0.05; Figure 2.2D).

*Long-term effects of FLX exposure on depression and anxiety-like behaviors*

*Elevated Plus Maze (EPM).* A separate group of adolescent rats were administered FLX from PD 35-49. Four weeks after the last day of drug treatment fluoxetine (FLX)-exposed adolescent rats and their saline (SAL)-exposed counterparts (n=10/group; Figure 2.2E-F) were tested in the EPM. An unpaired Student's *t*-test revealed a significant difference in time spent in the open arms of the EPM ($t_{18}$= 2.453, $p$= 0.0246, Figure 2.2E) however there was no significant differences between FLX or SAL exposed rats in the time spent in the closed arms ($t_{18}$= 3.093 $p$= 0.006, Figure 2.2F) of the EPM.

*Forced Swim Test (FST).* After being exposed to the EPM, FLX-exposed rats and their SAL counterparts were administered the FST (n= 10/group; Figure 2.2G-H). An unpaired Student's *t*-test revealed significant differences in the latency to immobility ($t_{18}$= 4.361, $p$= 0.0004) and total immobility in the FST ($t_{18}$= 3.824, $p$= 0.0012), as a function of drug exposure. FLX-exposed rats took longer to give up compared to SAL pre-treated rats ($p$<0.05; Figure 2.2G). Similarly, exposure to FLX promoted a decrease in the overall time that rats spent immobile in the FST ($p$<0.05; Figure 2.2H).

*Short-term effects of KET exposure on depression and anxiety-like behaviors*

*Elevated Plus Maze (EPM).* Twenty-four hours after the last day of chronic drug treatment, ketamine (KET) exposed adolescent rats and their saline (SAL) exposed counterparts (n=10/group;

Figure 2.3A-B) were exposed to the EPM. An unpaired Student's *t* -test revealed significant differences in time spent in the open ($t_{18}$= 2.205, $p$= 0.0407) and closed arms ($t_{18}$= 2.134, $p$= 0.0327) of the EPM, as a function of drug exposure. KET-exposed rats spent significantly more time in the open arms of the EPM when compared to SAL pre-treated rats ($p$<0.05; Figure 2.3A). Similarly, exposure to KET promoted a decrease in the time rats spent in the closed arms of the EPM ($p$<0.05; Figure 2.3B). Interestingly, KET exposure in rats resulted in a reduction of anxiety compared to those exposed to FLX during adolescence.

*Forced Swim Test (FST).* After being exposed to the EPM, KET-exposed rats and their SAL-exposed counterparts were administered the FST (n=10/group; Figure 2.3C-D). An unpaired Student's *t* -test revealed significant differences in latency to immobility ($t_{18}$= 5.572, $p$<0.0001) and total immobility in the FST ($t_{18}$= 3.792, $p$= 0.0013) as a function of drug exposure. KET-exposed rats took longer to adopt an immobile posture compared to SAL pre-treated rats ($p$<0.05; Figure 2.3C). Similarly, exposure to KET promoted a decrease in the overall time that rats spent immobile in the FST ($p$<0.05; Figure 2.3D).

*Long-term effects of KET exposure on depression and anxiety-like behaviors*

*Elevated Plus Maze (EPM).* A separate group of adolescent rats were administered KET from PD 35-49. Four weeks after the last day of drug treatment, KET-exposed rats and their saline (SAL) -exposed counterparts (n=10/group; Figure 2.2E-F) were tested in the EPM. An unpaired Student's *t* -test revealed significant differences in time spent in the open ($t_{18}$= 2.314, $p$= 0.0327) and closed arms ($t_{18}$= 2.633, $p$= 0.0169) of the EPM, as a function of drug exposure. KET-exposed rats spent significantly more time in the open arms of the EPM when compared to SAL pre-treated rats ($p$<0.05; Figure 2.3E). Similarly, exposure to KET promoted an increase in the time rats spent in the closed arms of the EPM ($p$<0.05; Figure 2.3F).

*Forced Swim Test (FST).* After being exposed to the EPM, KET-exposed rats and their SAL counterparts were administered the FST (n=10/group; Figure 2.3G-H). An unpaired Student's *t* -test revealed significant differences in latency to immobility ($t_{18}$= 3.885, $p$<0.001) and total immobility in the FST ($t_{18}$= 3.203, $p$= 0.0049) as a function of drug exposure. KET-exposed rats took longer to adopt an immobile posture, compared to SAL pre-treated rats ($p$<0.05; Figure 2.3G). Similarly, exposure to KET promoted a decrease in the overall time that rats spent immobile in the FST ($p$<0.05; Figure 2.3H).

**Discussion**

Although treatment for MDD is available, treating adolescents is challenging as evidence-based therapeutic approaches are lacking, and decisions regarding treatment are largely based on data from adults. Even more troubling is the fact that close to 50% of adolescents who suffer from MDD are non-responsive to available treatments, and only fluoxetine (FLX) is currently approved for the treatment of pediatric MDD. Animal models are therefore critical for establishing neurobiological mechanisms and to inform clinical research. Although still far from ideal, animal models of depression have evolved over the years and though not perfect, they are now better at being able to more specifically identify subsets of depression-related phenotypes and also to model more complex mood-related disorders such as bipolar or mania (Alcantara et al., 2017) (Ritov, Boltyansky, & Richter-Levin, 2015; Ma et al., 2017; Logan & McClung, 2016). Of particular interest are animal models that can identify the mechanism of potentially harmful insults that can impede the proper development of stress coping mechanisms, particular during adolescence (Czéh, Fuchs, Wiborg, & Simon, 2016). Therefore, the goals of the studies outlined in this chapter were to establish the behavioral outcome of chronic unpredictable stress (CUS) in adolescent rats, and to delineate in parallel the short and long-term behavioral effects of chronic exposure to KET or FLX during the adolescent period.

Studies using adult rats have shown that CUS induces a robust and persistent depressive-like phenotype (C. Hu et al., 2017). This phenotype is often exemplified by an increase in behavioral despair which is measured through various tasks, such as failure to escape a shock in a learned helplessness model, increased total immobility in the forced swim test (FST), increased avoidance to novel or anxiety-invoking environments or objects, and a decreased in the intake of palatable substances such as sucrose or saccharine (i.e., anhedonia). Here, I demonstrate that similar to adult rats, CUS exposure during adolescence is capable of inducing anhedonia, as seen through a reduction in sucrose preference, and this was accompanied by increases in anxiety-like behavior (i.e., less time spent in the open arms of the EPM), and an increase in behavioral despair (i.e., decreased latency to immobility/ increased total immobility in the FST). Although I found similarities between how adult and adolescent rats respond to stress, it is important note that there is evidence delineating differences in how adult and adolescent rodents react to stressful environments and ultimately manifest depression-like behaviors. For example, adolescent rats show a significantly greater increase in levels of corticosterone (CORT) in response to acute

restraint stress compared to adult rats (Romeo, 2013). Although CORT levels go back to comparable adult concentrations after about 2 hours, it is possible that adolescents are more sensitive to repeated stress insults which may be driven by this heighted initial spike in CORT. This would suggest that repeated exposure to stressors during early life may eventually lead to more devastating long-term behavioral disturbance. In addition, adolescent rats exposed to restraint stress show higher resistance to the extinction of fear memories, as compared to their adult counterparts (Barbayannis et al., 2017). This suggests that in adolescent rats, initial physiological responsiveness to stress may induce neurobiological adaptations that potentiate the lasting impact of negative experiences and therefore affect future responses to stressful stimuli.

Similar to stress models, studies assessing the appropriateness, the efficacy, and the potential consequences of antidepressant medication exposure in adolescents is much needed. Although there are pharmacotherapies available, these are not ideal and the need for safer and more efficacious treatments is still present. Stress-related behavioral assays such as the FST, have shown to be useful in identifying the potential of effective antidepressants for adolescent use. For example, adolescent rats exposed to the selective serotonin reuptake inhibitors, FLX and escitalopram, but not tricyclic antidepressants, show improved scores (i.e., decreased immobility) in the FST (Reed, Happe, Petty, & Bylund, 2008a). This result parallels findings reported in clinical pediatric literature promoting the efficacy of SSRI administration for juvenile depression. However, it should be noted that FLX is still prescribed with caution given reports showing that in some cases it also promotes increases in anxiety, impulsivity and suicidality (Gupta et al., 2015). Interestingly, my data show that adolescent rats exposed to chronic FLX exhibit increased anxiety-like behavior as seen through a reduction in time spent in the open arms of the elevated-plus maze (EPM). My work demonstrates that this anxiogenic response is seen 24 h after the last drug treatment and that it persists into adulthood (postnatal day 80). Interestingly, previous work from my laboratory also shows that re-exposure to a sub-chronic schedule of FLX during adulthood can rescue the anxiogenic responses to the EPM (i.e., increase time spent in the open arms) (iniguez, Warren, & Bolaños-Guzmán, 2010). The biological basis underlying this phenomenon remains to be elucidated, but it suggests that FLX-exposed rodents may develop a physiological and/or behavioral dependence to FLX. This concept is not without precedence as there is emerging evidence suggesting that discontinuation of the use of SSRIs leads to withdrawal symptoms such as increased irritability, sleep disturbances, depression relapse, and many symptoms that have been

equated to that of withdrawal from drugs of abuse (Black, Shea, Dursun, & Kutcher, 2000; Fava, Gatti, Belaise, Guidi, & Offidani, 2015). Although not ideal, it has been suggested that in order to manage the withdrawal symptoms of SSRI discontinuation, patients be temporarily put on FLX then tapered off in order to minimize the side effects of withdrawal (Benazzi, 2008). This practice supports the idea that SSRIs promote behavioral dependence and it is conceivable that some individuals may be on SSRI treatment for longer than necessary just to prevent the emergence of unfavorable symptoms (i.e. withdrawal) (Bennazi, 1998). Despite the negative side effects associated with chronic FLX exposure, my data suggest that FLX is effective at ameliorating behavioral despair, and it can promote long-term reductions in reactivity to stress-eliciting stimuli. More specifically, I find that adolescent rats treated with FLX take significantly longer to give up in the FST compared to their saline-treated counterparts and show less total immobility for the entirety of the test. This effect persists well into adulthood. While this finding is in accordance with other basic and clinical literature of the antidepressant efficacy of FLX (Gupta et al., 2015), the anxiety-inducing side effects of repeated SSRI exposure highlight the need for a better alternative pharmacotherapy for pediatric depression.

To this end, I assessed the effects of short- and long-term KET exposure on mood-related behaviors in adolescent rats, as compared to FLX. Interestingly, chronic KET exposure did not induce the same anxiogenic responses as I observed as a consequence of FLX exposure. Twenty-four hours after the last drug administration, KET-exposed rats tend to spend more time in the open arms of the EPM suggesting that KET treatment does not promote increases in anxiety as observed after FLX treatment. I noted a similar trend (i.e., anxiolytic response) 1 month after the cessation of KET treatment. KET has been proposed for the treatment of generalized anxiety or social anxiety disorder (GAD/SAD, respectively) (J. H. Taylor et al., 2018). Individuals suffering from GAD/SAD show relief of their anxiety symptoms and a general improvement of their social life, however, infusions took place once or twice weekly and over the course of 3 months (Glue et al., 2018). Nevertheless, these patients appeared to be in remission from symptoms. Studies using a chronic KET regimen are not widely available and require further studies in humans. KET-exposed adolescent rats also show improvement in the FST as compared to their saline-exposed counterparts. Specifically, both twenty-four hours and 1 month after drug treatment, KET-exposed rats show an increase in latency to immobility and a decrease in total immobility (i.e., antidepressant-like responses). This is in contrast to work done in mice which shows that an acute

infusion of KET improves swimming behavior in the FST 24 h after drug administration, however when tested again at 1-week post-infusion, immobility scores return to control levels (Autry et al., 2011). Previous work suggests that this KET-induced reduction in depression- and anxiety-like behavior is seen even up to two months after drug exposure (Parise et al., 2013). This supports the idea that chronic, as opposed to acute, KET administration may be more beneficial due to its capability of promoting sustained behavioral improvement in depression-related tasks. Although this behavioral outcome is similar to that seen in FLX-exposed rats, the differences in anxiety-related behavior suggest that KET may be a potential alternative to SSRI treatment in juveniles. Although there is a concern that KET can be abused and used as a dissociative drug, and it's considered a drug of abuse, it should be noted that the doses I used here to promote an antidepressant response do not seem to promote behaviors indicative of increased abusive liability (Parise et al., 2013). Others have shown that it is capable to induced conditioned place preference with KET (Du et al., 2017; Guo et al., 2016) however, these studies are limited.

Overall the results of my experiments show that the CUS model of stress used during adolescence is capable of inducing a depressive-like phenotype when rats are tested in adulthood. Interestingly, adolescent rats exposed to FLX or KET exhibit short- and long-term stress resilient phenotypes, however FLX seems to promote an anxiety phenotype that appears to last over the life of the animals, whereas KET does not promote similar anxiogenic responses. Moving forward I will compare the biochemical profile of these two antidepressants in an attempt to identify a common gene that could be modulating the antidepressant aspect of these drugs.

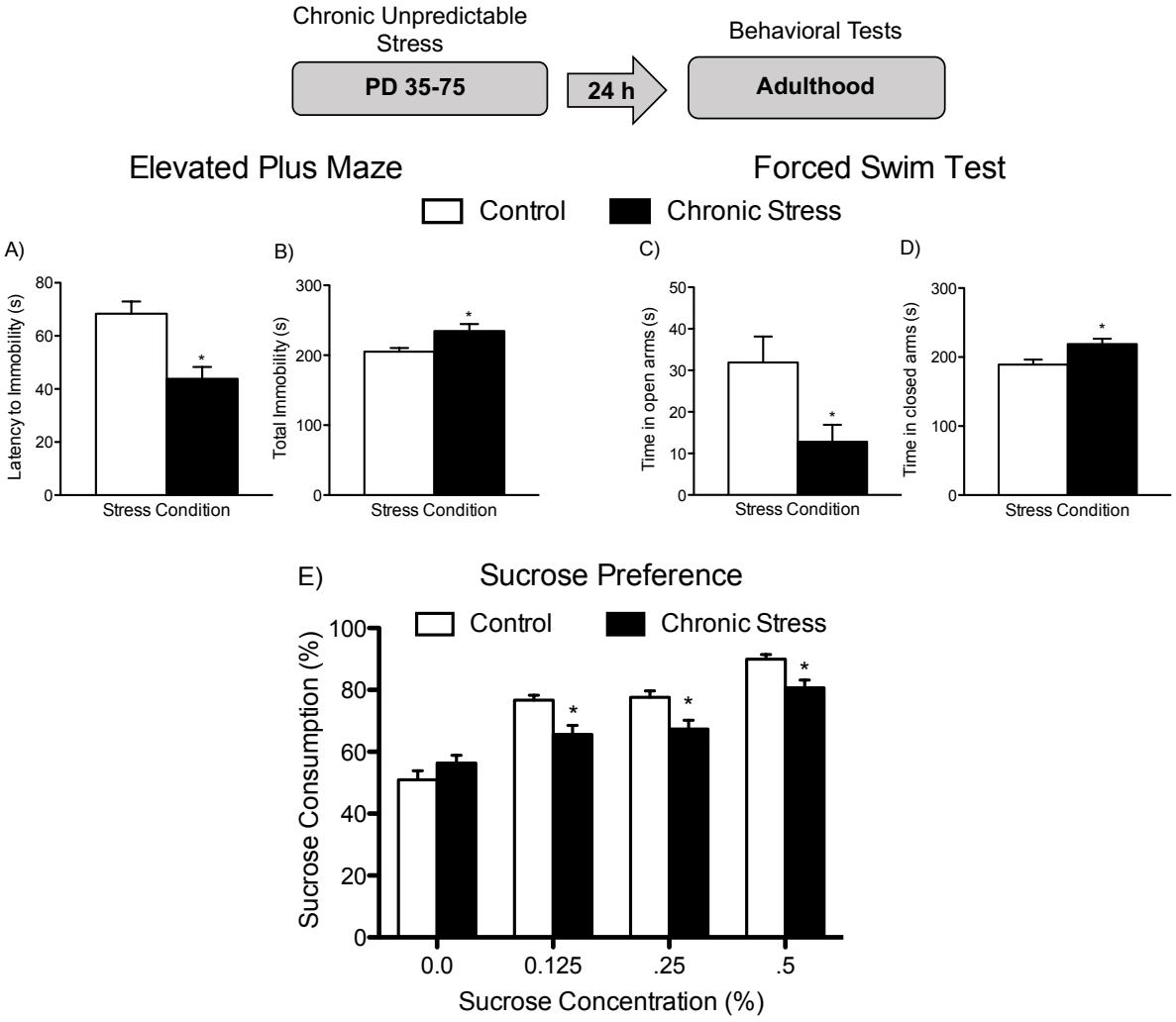



**Figure 2.1** Behavioral effects of Chronic unpredictable stress exposure in adolescence. Adolescent rats exposed to 4 weeks of chronic stress had a decrease in latency to immobility (A) and an increase in total immobility (B) in the FST. Chronic stress-exposed rats also spent less time in the open arms (C) and more time in the closed arms (D) of the EPM. Sucrose preference was also reduced as a function of stress exposure (E). *Significantly different from non-stressed controls ($p<0.05$)

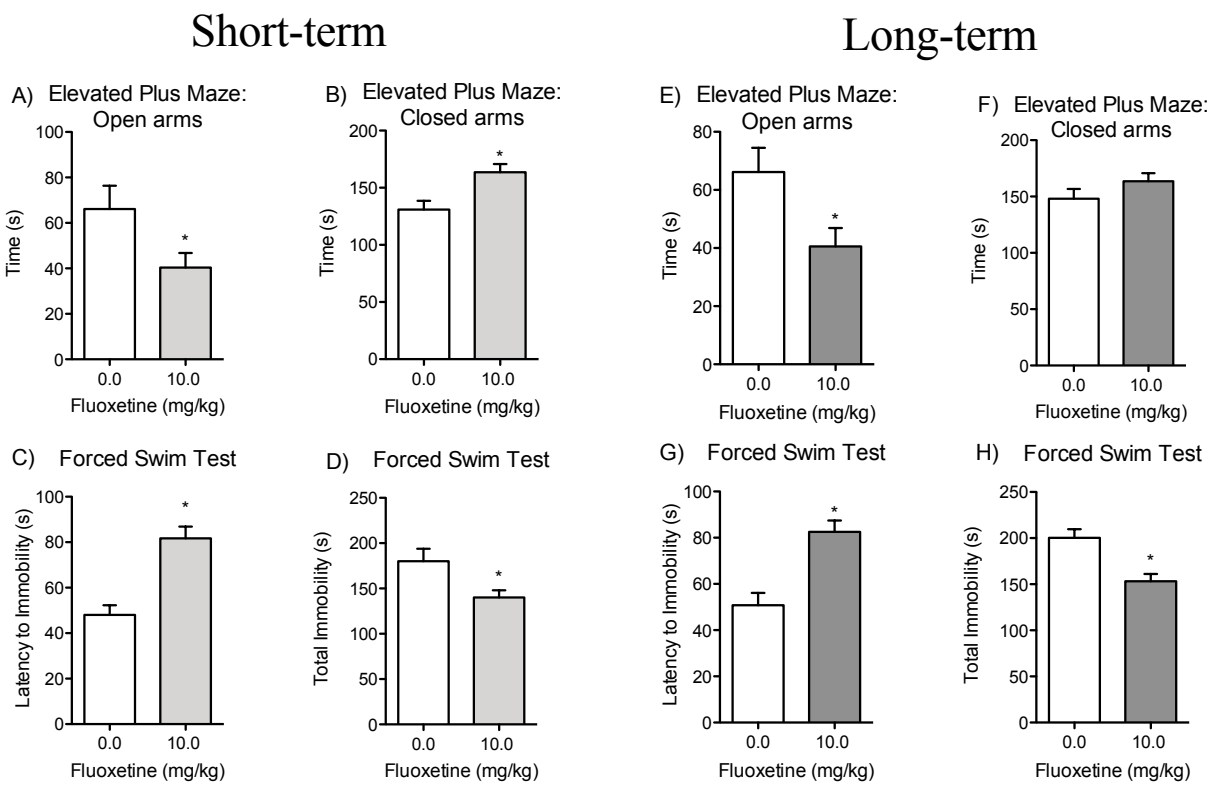



**Figure 2.2** Short- and long-term behavioral effects of adolescent fluoxetine exposure. Adolescent rats were exposed to 15 days of fluoxetine (FLX: 10.0 mg/kg) then test in the EPM and FST either 24hrs (short-term) or 1 month (long-term) after last drug administration. FLX-exposure reduced time spent in the open arms both 24hrs (A) and 1 month (E) after drug treatment. Significant changes in time spent in the closed arm were observed 24hrs (B) but not 1month later (F). FLX exposure promoted and increase in latency to immobility and a reduction in total immobility both 24hrs (C-D; respectively) and 1 month (G-H; respectively) after drug exposure. *Significantly different from saline-exposed controls ($p<0.05$)

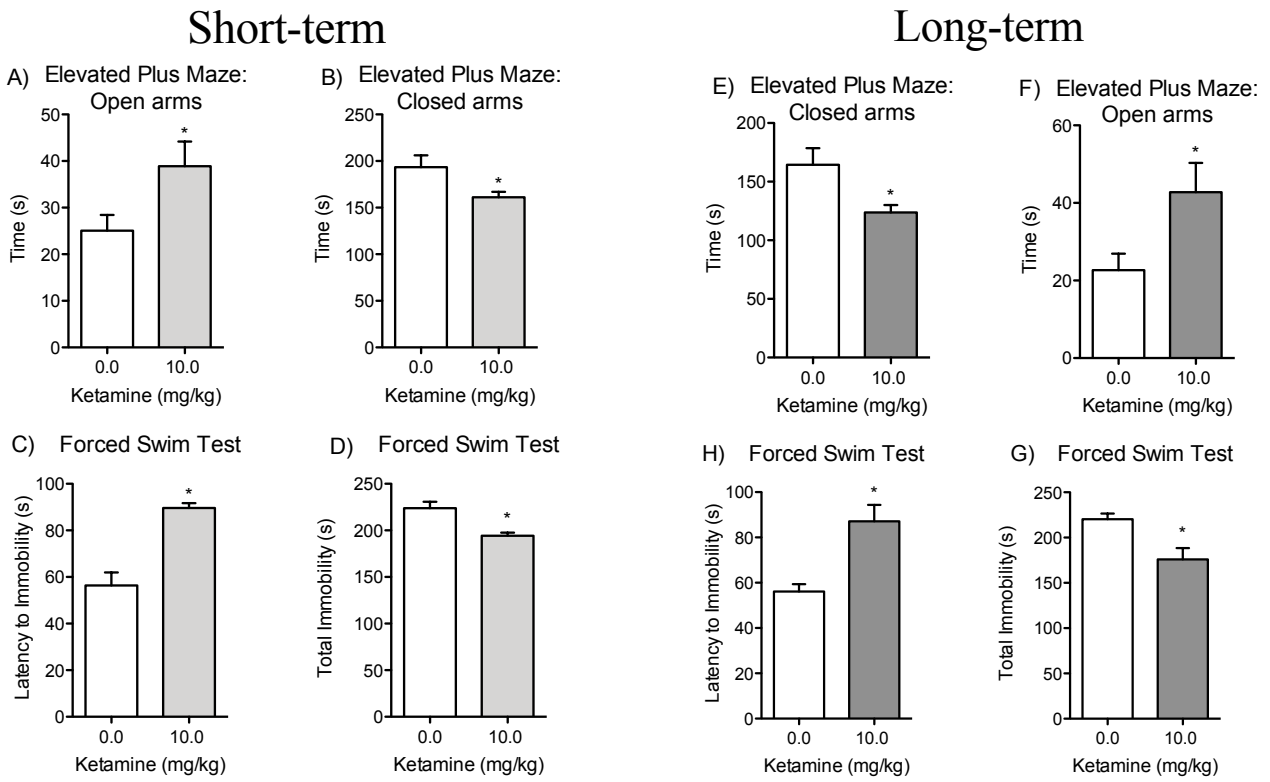



**Figure 2.3** Short- and long-term behavioral effects of adolescent ketamine exposure. Adolescent rats were exposed to 15 days of ketamine (KET: 10.0 mg/kg) and then test in the EPM and FST either 24hrs (short-term) or 1 month (long-term) after last drug administration. KET-exposure reduced time spent in the open arms both 24hrs (A) and 1 month (E) after drug treatment. Similarly, KET exposure reduced time spent in the closed arms of the EPM both 24hrs (B) and 1 month (F) after drig exposure. KET exposure promoted and increase in latency to immobility and a reduction in total immobility both 24hrs (C-D; respectively) and 1 month (G-H; respectively) after drug exposure. *Significantly different from saline-exposed controls ($p<0.05$)

# CHAPTER III

# BIOCHEMICAL PROFILE AFTER STRESS OR ANTIDEPRESSANT EXPOSURE DURING ADOLESCENCE IN REWARD-RELATED BRAIN REGIONS

## Introduction

There has been a significant rise in the diagnosis of mood disorders in children and adolescents (Avenevoli et al., 2015). It has been estimated that about 13% of those between 12-17 years of age are diagnosed with major depressive disorder (MDD) (Van Droogenbroeck, Spruyt, & Keppens, 2018; Merikangas et al., 2010). The World Health Organization reports suicide as the second leading cause of death in individuals aged 15-29 suffering from MDD (World Health Organization, 2015). Although treatments for MDD are available, they are only partially effective and a large portion of adolescents are non-responsive to available treatments, suggesting that there is much left to be understood about the underlying neurocircuitry regulating symptoms of MDD (Cipriani et al., 2016). Stress is a primary factor in precipitating MDD thus necessitating thorough assessments of how stress alters signaling of genes known to be associated with normal and dysregulated mood during early developmental periods (King et al., 2018). One of the hallmark symptoms of depression is a reduction in reward and motivation responding (Der-Avakian & Markou, 2012; Rizvi, Pizzagalli, Sproule, & Kennedy, 2016) and this has led to an increased interest in the study of the limbic dopamine (DA) pathway, as it related to the pathophysiology of depression-related symptoms (Belujon & Grace, 2017; Nestler & Carlezon, 2006). Indeed, there is evidence indicating that there are deficits in DA signaling in patients with MDD (Belujon & Grace, 2017) and it is very likely that these deficits contribute to the changes in motivation and reward valence experienced by individuals suffering with MDD in response to food, sex or social contact (Leigh Gibson, 2006; Melis & Argiolas, 1995; Depue & Collins, 1999; Atzil et al., 2017).

The main dopaminergic hub of the mesolimbic reward pathway is the ventral tegmental area (VTA), and the brain region that has received the most attention in mediating reward-related behaviors is one of the principal connections of the VTA, the nucleus accumbens (NAc) (Carlezon & Thomas, 2008; Galaj & Ranaldi, 2018). Although the VTA and NAc have been shown to play critical roles in reward-responding, these brain areas receive considerable modulation, from other brain regions such as the dorsal raphé nucleus, prefrontal cortex, hippocampus and the lateral habenula (Bourdy & Barrot, 2012; Ikemoto, 2010; Yetnikoff et al., 2014; Amo et al., 2014;

Stamatakis & Stuber, 2012). One growing area of research has been uncovering the contributions of the lateral habenula (LHb) in mediating both depressive-like behavior (Stamatakis & Stuber, 2012; Ootsuka & Mohammed, 2015; Lawson et al., 2016) and, more recently, antidepressant responding (Y. Yang et al., 2018). The LHb is a glutamatergic hub that is thought to be an intermediary signaling region between forebrain and midbrain structures (Yetnikoff et al., 2014). Importantly, the LHb has been shown to play a critical role in mediating activity of the VTA through both direct and indirect control of local VTA GABAergic activity/signaling (Bourdy & Barrot, 2012; Beier et al., 2015). The LHb has a prominent excitatory connection to the rostral medial tegmentum (RMtg), a GABAergic structure, which then acts to inhibit DA neurons in the VTA (Brown et al., 2017). Similarly, LHb projects onto the GABAergic subset of neurons within the VTA (Morales & Margolis, 2017; Brinschwitz et al., 2010). Both of these connections ultimately act to reduce DA output from the VTA (Brown et al., 2017; Sánchez-Catalán et al., 2016), which has been suggested to lead to some of the depression-like behaviors seen after stress exposure (Proulx, Hikosaka, & Malinow, 2014; Stamatakis & Stuber, 2012). The VTA itself also has a unique population of GABA-releasing neurons that feedback to the LHb (Stamatakis et al., 2013). It is suspected that LHb does not have a local regulatory network (Root, Mejias-Aponte, Qi, & Morales, 2014), therefore this feedback from LHb output structures acts to reduce LHb activity to help regulate inhibition and excitation of the LHb-mediated network (Morales & Margolis, 2017). These interactions suggest that there is a complex reciprocal network of communication between these VTA and LHb (Stamatakis et al., 2013).

Intracellular signaling mechanisms within these brain regions also play an important role in MDD and one neurotrophic factor that has gotten much consideration in the pathology of MDD is the neurotrophin, brain derived neurotrophic factor (BDNF) (Shirayama, Chen, Nakagawa, Russell, & Duman, 2002; Eisch et al., 2003; Krishnan & Nestler, 2010). Postmortem tissue of patients with depression has shown deficits in BDNF, and other work has shown that BDNF is an important mediator of antidepressant responses, suggesting that this signaling cascade may be of great interest in both stress-induced deficits and the alleviation of these deficits (Calabrese, Molteni, Racagni, & Riva, 2009; Castrén & Kojima, 2017; Björkholm & Monteggia, 2016). The extracellular signal-regulated protein kinase-1/2 (ERK2), a downstream target of BDNF (Numakawa et al., 2010), has been highly implicated in mediating the deleterious effects of stress (Einat et al., 2003; Gourley, Wu, & Taylor, 2008; Iniguez et al., 2010). Once BDNF binds to its

receptor, tyrosine kinase receptor-B (Trk-B), it initiates activity of ERK2 via a Ras/Raf-dependent mechanism (Tibbles & Woodgett, 1999). Research on how these genes are modulated in the LHb after antidepressant treatment is minimal, however, there is evidence suggesting that deep brain stimulation (DBS) can act in the LHb to promote antidepressant responses (Torres-Sanchez, Perez-Caballero, & Berrocoso, 2017). Even more interesting is a clinical study showing that DBS treatment in the lateral habenula also promotes BDNF activity (Hoyer, Kranaster, Sartorius, Hellweg, & Gass, 2012) potentially mediating, at least in part, remission of depression symptoms. Other studies have implicated the calcium calmodulin kinase (CaMK) family in the LHb as an important regulator of depression-like behavior. Specifically, increasing CaMKII in the LHb increases its synaptic activity and also promotes depression-like behavior (K. Li et al., 2013), while inhibition of CaMKII signaling has shown to be protective against stress-induced behavioral deficits (J. Li et al., 2017a). Interestingly, there is considerable cross talk between CaMKII and BDNF as it relates to antidepressant responding, these findings further suggest that ERK2 may be the common downstream effector which may ultimately mediate antidepressant activity in the LHb (Y. Hu, Liu, Liu, Dong, & Boran, 2014; Brod et al., 2017; Shioda, Sawai, Ishizuka, Shirao, & Fukunaga, 2015).

Attempts at deciphering the molecular underpinnings of the antidepressant effects of FLX or KET have been focused greatly on mechanisms of VTA-mediated activity within the prefrontal cortex, hippocampus, or NAc (Schiena, Ostinelli, Gambini, & D'Agostino, 2015; Duman & Monteggia, 2006; Fumagalli et al., 2005; Autry et al., 2011; Nestler, 2014). Because VTA activity is also regulated by LHb, it is possible that the LHb may be an even more prominent biological substrate for mediating depression and antidepressant-related behaviors. Given the evidence indicating the role ERK2 plays in modulating depression-like behaviors within the mesolimbic reward system (Dwivedi et al., 2001; X. Qi et al., 2009; Iniguez et al., 2010), it is reasonable to postulate ERK2 as a possible facilitator of these stress-related behaviors, however, no work has been done to elucidate how ERK2 is modulated after stress or antidepressant treatment within the LHb. Therefore, the following experiments were conducted in order to reveal how stress, FLX, and KET exposure modulate ERK2-related signaling within the LHb.

## Methods

*Materials and Tests*

*Animals.* Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Rats arrived at postnatal day (PD) 28 and allowed to habituate to the facility for 5-7 days. The age at the of experimental manipulations in adolescent rats (PD 35) was selected because it roughly approximates adolescence in humans (Spear, 2000). Rats were housed in clear polypropylene boxes containing wood shavings in an animal colony maintained at 23–25°C on a 12 h light/dark cycle in which lights were on between 0700 and 1900 hours. Food and water were provided ad libitum.

*Drug Treatment and Experimental Design***.** Ketamine (KET) was obtained from Butler Schein Animal Health (Dublin, OH) in an injectable solution (100 mg/ml). KET was further diluted with saline (0.9% saline) to achieve its desired concentration. Fluoxetine hydrochloride was acquired from Spectrum Pharmaceuticals (Irvine, CA) and was dissolved in sterile water to achieve its desired concentration. Adolescent rats were injected with 10 mg/kg KET or 10 mg/kg FLX, or their respective vehicles, twice per day for 15 days (PD 35-PD 49). These doses are based off of previous studies done in our lab that result in optimal behavioral outcomes (iniguez, Alcantara, et al., 2014a; Parise et al., 2013). Rats were sacrificed 24h after last exposure to treatment. All procedures were in strict accordance with the *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (National Research Council, 2003).

*Chronic Unpredictable Stress (CUS).* Adolescent rats (PD 35 at start) were housed in isolation and exposed to a single unpredictable stressor for 28 consecutive days. The stressors included overnight cage flooding, food and water deprivation, restraint (2 h), overnight light exposure, 45° tilted cages (24 h), cage shaking, lights on overnight, strobe light overnight, cold exposure (4°C for 2 h), and FST. As a control, a group of adolescent rats were double housed and did not receive any stressors during this time. All rats were sacrificed 24hrs after the last stress session.

*Quantitative real-time PCR*. Tissue punches from the ventral tegmental area (VTA) or lateral habenula (LHb) were collected and using the Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions RNA was isolated and stored at −80 °C until use. cDNA was created using qScript cDNA synthesis kit (Quanta) using a C100 Thermal Cycler (Bio-

Rad). Quantitative real-time PCR (rt-PCR) is performed in triplicates using 386 well PCR plates using a CFX384 Real-Time Sytstem:C1000 Touch Thermal Cycler (Bio-Rad), according to the manufacturer's instructions. Threshold cycle [C(t)] values are measured using the supplied software and analyzed with the ΔΔC(t) method (Vialou et al., 2010; Warren et al., 2013). Rt-PCR was performed for the following ERK2-related genes: ERK2 (*Mapk1*), ERK1 (*Mapk3*), MEK1 (*Map2k1*), P90RSK (*Mapkap-k1*), CREB (*Creb1*), GSK3B (*Gsk3b*), AKT (*Akt1*), ELK1 (*Elk1*), and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a normalizing gene. Primer sequences can be found in Table 3.1.

*Western Blotting*: Protein from VTA and LHb tissue punches was also extracted with the Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions and stored at −80 °C until use. Ten micro-grams of protein from each sample are treated with β-mercaptoethanol and subsequently electrophoresed on precast 10 % gradient gels (Bio-Rad). All antibodies were obtained from Cell Signaling (Beverly, Massachusetts). Blots were probed overnight at 4 °C with antibodies against the phosphorylated forms of ERK1/2 and GAPDH. Separate membranes were probed with antibodies against total ERK1/2 GAPDH. All primary antibodies were made to a 1:1,000 dilution (except for GAPDH which was diluted to 1: 20,000). Membranes were washed several times with TBST and were incubated with peroxidase-labeled goat anti-rabbit IgG as the secondary antibody (1: 10,000; Cell Signaling, Beverly, Massachusetts). Bands were visualized with Clarity Western ECL Substrate (Bio-Rad), quantified using ImageJ (NIH), and then normalized to GAPDH.

*Statistical Analyses.* Behavioral data were analyzed using mixed-design (between and within variables) ANOVA followed by Fisher Least Significant Difference (LSD) post hoc tests. When appropriate, Student's *t* tests were used to determine statistical significance of preplanned comparisons. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as $p < 0.05$.

**Results**

*Stress-induced alterations in gene expression within the ventral tegmental area (VTA) and the lateral habenula (LHb)*

*Ventral Tegmental Area.* Twenty-four hours after the last day of CUS, stress-exposed rats and their non-stressed counterparts were sacrificed and tissue was collected for further processing. mRNA was extracted from VTA tissue and then used to run quantitative real-time polymerase

chain reaction assays (rtPCR). Student's *t*-test revealed CUS induced changes in gene expression as a function of stress exposure (*n* = 8/group; Figure 3.1A). Specifically, expression of ERK2($t_{14}$= 4.229, $p$<0.0008), GSK3ß ($t_{14}$= 6.466, $p$<0.001), and CREB ($t_{14}$= 2.553, $p$<0.023) was increased in the VTA of CUS-exposed rats with AKT ($t_{14}$= 3.969, $p$= 0.0014) as the only gene to be decreased, compared to the non-stressed controls (CON). No significant change was observed for ERK1 ($t_{14}$= 1.362, $p$= 1.1946), p90RSK ($t_{14}$= 0.337, $p$<0.001), MEK1 ($t_{14}$= 0.6650, $p$= 0.5169), or ELK1 ($t_{14}$= 1.238, $p$= 0.2362).

*Lateral Habenula.* Twenty-four hours after the last day of CUS, stress-exposed rats and their respective controls were sacrificed and tissue was collected for further processing. mRNA was extracted from VTA tissue and then used to run rtPCR. Student's *t*-test revealed CUS-induced changes in gene expression as a function of stress exposure (n=8/group; Figure 3.1B). Expression of ERK1 ($t_{14}$= 4.015, $p$= 0.0013), ERK2($t_{14}$= 2.286, $p$= 0.0397), MEK1 ($t_{14}$= 2.304, $p$= 0.0371), ELK1 ($t_{14}$= 2.117, $p$= 0.05), and AKT ($t_{14}$= 4.346, $p$= 0.0007) was decreased as a function of stress exposure. Expression of GSK3ß ($t_{14}$= 2.084, $p$<0.059) trended toward an increase however similar to CREB ($t_{14}$= 1.796, $p$= 0.09) and p90RSK ($t_{14}$= 0.8635, $p$= 0.4024), differences did not reach statistical significance.

*Stress-induced changes phospho-ERK2 within the VTA and LHb of adolescent rats*

*Ventral Tegmental Area.* Tissue punches were also used to extract protein from the VTA in order to assess stress-induced changes in ERK1 and ERK2. Student's *t*-test revealed differences in protein expression between CUS-exposed rats and their CON counterparts (n= 8/group; Figure 3.1C). Specifically, increased phosphorylated levels of ERK1 ($t_{14}$= 2.084, $p$= 0.0592) and ERK2 ($t_{14}$= 2.583, $p$= 0.0217) were observed in the VTA as a function of stress exposure. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were detected (data not shown).

*Lateral Habenula.* Tissue punches from the LHb were also used to extract protein and assess stress-induced alterations in ERK1 and ERK2. Student's *t*-test revealed differences in protein expression between CUS-exposed rats and their CON counterparts (n=8/group; Figure 3.1D). Specifically, phosphorylated levels of ERK1 ($t_{14}$= 2.963, $p$=0.0119) and ERK2 ($t_{14}$= 2.196, $p$= 0.0454) were decreased in the LHb as a function of stress exposure. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were observed (data not shown).

*Alterations in gene expression within the VTA and LHb after adolescent administration of chronic FLX*

*Ventral Tegmental Area.* Twenty-four hours after the last day of fluoxetine (FLX) treatment, FLX-exposed rats and their saline (SAL) counterparts were sacrificed and tissue was collected for further processing. mRNA was extracted from VTA tissue and used to run rtPCR. Student's *t*-test revealed a significant difference in gene expression as a function of drug exposure between FLX- and SAL-exposed controls (n= 8/group; Figure 3.2A). FLX administration resulted in decreased expression of ERK2 ($t_{14}$= 2.884, $p$= 0.0120), p90RSK ($t_{14}$= 2.104, $p$= 0.027), MEK1 ($t_{14}$= 4.186, $p$= 0.0009), GSK3b ($t_{14}$= 2.235, $p$= 0.0422), and CREB ($t_{14}$= 3.186, $p$= 0.0066). However, no significant drug-induced changes were seen in expression of ERK1 ($t_{14}$= 0.0843, $p$=0.934), ELK1 ($t_{14}$= 0.01278, $p$= 0.9900), or AKT ($t_{14}$= 1.645, $p$= 0.1194).

*Lateral Habenula.* Twenty-four hours after the last day of FLX treatment, FLX-exposed rats and their SAL counterparts were sacrificed and tissue was collected for further processing (Figure 3.2B). mRNA was extracted from LHb tissue and used to run rtPCR. Student's *t*-test revealed a significant difference in gene expression as a function of drug exposure between FLX- and SAL-exposed controls (n=8/group; Figure 3.2B). Expression of ERK1 ($t_{14}$= 2.180, $p$= 0.0468), ERK2 ($t_{14}$= 2.737, $p$= 0.0161), and GSK3b ($t_{14}$= 2.658, $p$= 0.0187) was increased as a consequence of FLX exposure. Similarly, p90RSK ($t_{14}$= 1.978, $p$= 0.0679) expression trended toward and increase however did not reach statistical significance. Expression of MEK1 ($t_{14}$= 0.1751, $p$= 0.8637) ELK1 ($t_{14}$= 0.9378, $p$= 0.3642) AKT ($t_{14}$= 2.607, $p$= 0.7981) and CREB ($t_{14}$= 1.159, $p$= 0.2673), did not differ significantly as a function of drug exposure.

*Fluoxetine-induced changes phospho-ERK2 within the VTA and the LHb of adolescent rats*

*Ventral Tegmental Area.* Tissue punches were also used to extract protein from the VTA in order to assess FLX-induced alterations in ERK1 and ERK2 protein levels. Student's *t*-test revealed differences in protein expression between FLX-exposed rats and their SAL pre-treated rats (n=8/group; Figure 3.2C). Specifically, phosphorylated levels of ERK2 ($t_{14}$= 2.279, $p$= 0.0388) were decreased in the VTA, whereas levels of phosphorylated ERK1 ($t_{18}$= 0.8924, $p$= 0.3854) showed no change as a function of FLX-exposure. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were observed (data not shown).

*Lateral Habenula.* Tissue punches were also used to extract protein from the VTA in order to assess FLX-induced alterations in ERK1 and ERK2. Student's *t*-test revealed differences in

protein expression between FLX-exposed rats and their SAL pre-treated counterparts (n=8/group; Figure 3.2D). Specifically, phosphorylated levels of ERK1 ($t_{14}$= 2.963, $p$= 0.0119) and ERK2 ($t_{14}$= 2.196, $p$= 0.0454) were increased in the LHb, as a function of drug exposure. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were observed (data not shown).

*Gene expression within the VTA and LHb after adolescent administration of chronic KET*

*Ventral Tegmental Area.* Twenty-four hours after the last day of ketamine (KET) treatment, KET-exposed rats and their SAL-exposed counterparts were sacrificed and tissue was collected for further processing. mRNA was extracted from VTA tissue and used to run rtPCR. Student's *t*-test revealed a significant difference in gene expression as a function of drug exposure between KET- and SAL-exposed controls (n=8/group; Figure 3.3A). Exposure to KET decreased expression of ERK2 ($t_{14}$= 2.341, $p$= 0.0346), MEK1($t_{14}$= 3.028, $p$= 0.0105), and GSK3b ($t_{14}$= 2.486, $p$= 0.0273). A similar trend was seen in expression of p90RSK ($t_{14}$= 1.904, $p$= 0.0776) however group means did not differ enough to reach statistical significance. KET induced and an increase in expression of ELK1 ($t_{14}$= 2.590, $p$= 0.0214) however there were no significant changes in expression seen in ERK1 ($t_{14}$= 0.6047, $p$= 0.5550), AKT ($t_{14}$= 0.7211, $p$= 0.4827), or CREB ($t_{14}$= 0.2714, $p$= 0.7901) mRNA.

*Lateral Habenula.* KET- and SAL-treated rats were sacrificed twenty-four hours after the last day of drug treatment and tissue was collected for further processing. mRNA was extracted from LHb tissue and used to run rtPCR. Student's *t*-test revealed a significant difference in gene expression as a function of drug exposure between KET- and SAL-exposed controls (n=8/group; Figure 3.3B). Exposure to KET increased expression of ERK1 ($t_{14}$= 4.618, $p$= 0.0004), ERK2 ($t_{14}$= 3.376, $p$= 0.0045), p90RSK ($t_{14}$= 2.214, $p$= 0.0439), MEK1 ($t_{14}$= 2.305, $p$= 0.037), and ELK1 ($t_{14}$= 2.290, $p$= 0.038) in the LHb. KET-exposure resulted in decreased expression in AKT ($t_{14}$= 2.341, $p$= 0.0346) however, it induced no significant change in the expression of GSK3b ($t_{14}$= 0.1315, $p$= 0.8973) or CREB ($t_{14}$= 1.1898, $p$= 0.0917).

*Ketamine-induced changes of phospho-ERK2 within the VTA and LHb of adolescent rats*

*Ventral Tegmental Area.* Tissue punches were also used to extract protein from the VTA in order to assess KET-induced alterations in ERK1 and ERK2. Student's *t*-test revealed differences in protein expression between KET-exposed rats and their SAL pre-treated counterparts (n= 8/group; Figure 3.3C). Specifically, phosphorylated levels of ERK1 ($t_{18}$= 2.363,

$p$= 0.0331) and ERK2 ($t_{14}$= 2.798, $p$= 0.0142) were decreased in the VTA, as a function of KET exposure. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were observed (data not shown).

*Lateral Habenula.* Tissue punches were also used to extract protein from the VTA in order to assess KET-induced alterations in ERK1 and ERK2. Student's *t*-test revealed differences in protein expression between the KET-exposed rats and SAL-exposed controls (n=8/group; Figure 3.3D). Specifically, phosphorylated levels of ERK1 ($t_{14}$= 2.202, $p$= 0.0449) and ERK2 ($t_{14}$= 3.069, $p$= 0.0083) were significantly increased in the LHb, after chronic KET administration. Separate membranes were probed for the total forms of ERK1 and ERK2 and no differences in protein levels were observed (data not shown).

**Discussion**

Significant efforts have been devoted to understanding the mechanisms underlying major depressive disorders. Much of this work has focused at deciphering the molecular underpinnings of how drugs such as FLX and KET elicit their antidepressant activity within brain regions such as the VTA and its influence on the prefrontal cortex, hippocampus, or NAc (Schiena et al., 2015; Duman & Monteggia, 2006; Fumagalli et al., 2005; Autry et al., 2011; Nestler, 2014). However, given evidence that the VTA activity is regulated by the LHb, it is conceivable that LHb may be an important biological substrate for mediating depression/anti-depression-like behaviors. Given evidence indicating the role of the extracellular regulated kinase 2 (ERK2) in modulating depression-like behaviors within the VTA, I hypothesized that this kinase is also a facilitator of stress and antidepressant response within the LHb. Thus, the goal of this chapter was to delineate how stress, FLX, and KET exposure modulate ERK2-related signaling within the LHb.

Chronic stress has been shown to cause changes in intracellular signaling within brain regions of the mesolimbic reward system (Ortiz, Fitzgerald, Lane, Terwilliger, & Nestler, 1996; Musazzi, Tornese, Sala, & Popoli, 2017). The ventral tegmental area (VTA) in particular has been the focus of many studies due to its capability of modulating key reward-related structures such at the hippocampus, prefrontal cortex and nucleus accumbens (Duman & Monteggia, 2006; Yetnikoff et al., 2014; Carlezon & Thomas, 2008). Recently, there has been some research demonstrating that the lateral habenula (LHb) may also play an important role in the modulation of depressive-like behaviors (Lawson et al., 2016). This is not surprising as the LHb has prominent connections to the VTA and can therefore modulate its output which as a consequence modifies

the activity of other mesolimbic structures. The importance of the LHb in relation to depression-like behaviors is quickly emerging, and there is still much to be learned about the mechanisms that ultimately promote these stress-induced changes in behavior. The ERK2 signaling pathway has been implicated in mediating both stress-induced behavior and in the mechanism(s) of action of commonly prescribed antidepressants, such as fluoxetine (FLX) (Iniguez, Alcantara, et al., 2014a; Ritt et al., 2016). Specifically, work from our lab and others has shown that ERK2 is mediated in the VTA after chronic stress and that site specific modulation of ERK2 within the VTA or hippocampus (Hipp) can promote susceptibility or resilience to stressful stimuli (Carrier & Kabbaj, 2012; Iniguez et al., 2010). Results from the current set of experiments replicate previous findings demonstrating that ERK2 is increased in the VTA after chronic stress and now expand the current literature to show that ERK2-related signaling is downregulated in the LHb after chronic stress exposure during adolescence. Interestingly, this down regulation of ERK2 signaling in the LHb after stress exposure closely resembles how ERK2 is modulated in the hippocampus and the prefrontal cortex (PFC) (Dwivedi & Zhang, 2016). Similar to Hipp and PFC expression, I find that ERK2 is decreased in the LHb after chronic stress exposure (X. Qi et al., 2009; X. Qi, Lin, Li, Pan, & Wang, 2006). This is in contrast to the increase of ERK2 expression seen within the VTA after stress (Iniguez et al., 2010). It is possible that these dissimilarities are due to the different cell populations that make up the VTA and the LHb. While the VTA is a mainly dopaminergic nuclei, the LHb, Hipp, and PFC are primarily glutamatergic structures (Beier et al., 2015; M. Lin, Hou, Zhao, & Yuan, 2018). Given the differences in cell types found in these brain regions, it is also possible that the differences observed after chronic stress exposure are due to the different varieties of receptors that are found on the cell surface of these neurons. For example, it has been shown that stress can have some of its deleterious effects through activation of glutamatergic receptors (Nasca et al., 2015) however, different subtypes of these receptors are widely distributed and different subtypes are concentrated to a particular region (Jeffrey R Cottrell, 2000; Petralia, Rubio, & Wenthold, 1998). Activity initiated by different receptor subtypes could change how the downstream effectors of these receptors are regulated (J. Q. Wang, Fibuch, & Mao, 2007).

Interestingly, I also find that exposure to either FLX or KET results in the opposite regulation of ERK2 expression, as compared to stress exposure. This supports my hypothesis that both of these medications can at least in part, induce their antidepressant action through a LHb-ERK2 signaling-mediated mechanism. This is not without precedent as ERK2 has been highly

implicated to be involved with underlying the antidepressant mechanism of FLX and KET in other brain regions (X. Qi, Lin, Li, Li, Wang, Wang, & Sun, 2008a; Carrier & Kabbaj, 2012; Caffino et al., 2017). Interestingly, ERK2 has also been shown to be regulated by alternative antidepressant strategies. Specifically, electroacupuncture (EA) administered in cortical-related regions of rats that have undergone CUS not only reverses depression-like behavior but also increases expression of ERK2 while administration of an ERK-inhibitor blocks the antidepressant-like responses to EA (W. Li et al., 2017b).

While it is clear that ERK2 plays a role in the antidepressant mechanism of both these antidepressants it cannot be ignored that the finding from my work in Chapter 2 show that KET and FLX differ on how they influence anxiety-like behavior. While there are many possible explanations for this divergent drug-effect, within the scope of these experiments, GSK3b seems like a likely candidate for mediating the anxiety-like behavior seen after stress or FLX exposure. My data show that mRNA expression of GSK3□ is increased in the LHb after stress or FLX, however it is unchanged by exposure to KET. In the clinical literature it is shown that individuals with MDD who have a particular single nucleotide polymorphism (SNP) of GSK3□ score higher on the Hamilton anxiety rating scale (S. Liu et al., 2012) suggesting a functional role for GSK□-related increases in anxious behavior. In animal models, there is evidence to suggest that maternal separation, a commonly used depression-related paradigm, leads to less time spent in the center of an open field (i.e. increased anxiety-like behavior), and also leads to increased expression of GSK3b without affecting levels of pERK (Sachs et al., 2013). Alternatively, it would seem that KET has a more prominent effect on ELK1 compared to FLX. In addition, it is possible that KET reverses stress-induced reductions of ELK1 in order to promote antidepressant-like responses, including attenuating anxious behaviors.

Overall, my research findings demonstrate that exposure to CUS during adolescence induces differential changes in ERK2 expression and related signaling within the VTA and the LHb. Stress increases ERK2 within the VTA, while it decreases it in the LHb. Similarly, FLX and KET treatment have differential influence on ERK: both drugs decrease ERK2 in the VTA while increasing ERK2 levels within the LHb. The mechanistic circuitry underlying these differential effects is not known. It is possible, as stated, that these findings may be mediated by the prominent type of cell population within each of these brain regions (dopaminergic vs. glutamatergic). Future characterizing the electrophysiological properties of these cell populations and experiments

utilizing optogenetics and other cell-specific types of analysis, such as florescence activated cell sorting (FACS), are needed to assess these possibilities.

Table 3.1. qPCR primer sequences

| Primer Sequence | | |
|---|---|---|
| Gene | Forward | Reverse |
| *Mapk3* | 5’-CAGCTGAGCAATGACCACA-3’ | 5’-CTTAAGGTCGCAGGTGGTG-3’ |
| *Mapk1* | 5’-CACAGCACCTCAGCAATGA-3’ | 5’-GTTCAGCAGGAGGTTGGA-3’ |
| *Mapkap-k1* | 5'-ATGTGTGGCCAAGACTCCC-3' | 5'-TGAACTCTGTCCAGTGGCA-3' |
| *Map2k1* | 5'-TACAGTCACGGCGAGATCA-3' | 5'-AGGCGACATGTAGGACCTT-3' |
| *Akt1* | 5’-GGCGTGGTCATGTACGAGA-3’ | 5’-TGAGCTCGAACAGCTTCTC-3’ |
| *Elk1* | 5'-CCATGGCCCTCAGCTTTTA-3' | 5'-TAGCAGCAGGGTAGGGCT-3' |
| *GSK3B* | 5’-AGCCTATATCCATTCCTTG-3’ | 5’-CCTCGGACCAGCTGCTTT-3’ |
| *Creb1* | 5’-GGCCTGCAGACATTAACCT-3’ | 5’-TCCATCAGTGGTCTGTGCA-3’ |
| *Gapdh* | 5’-AGGTCGGTGTGAACGGATTT-3’ | 5’-TGTAGACCATGTAGTTGAGGT-3’ |

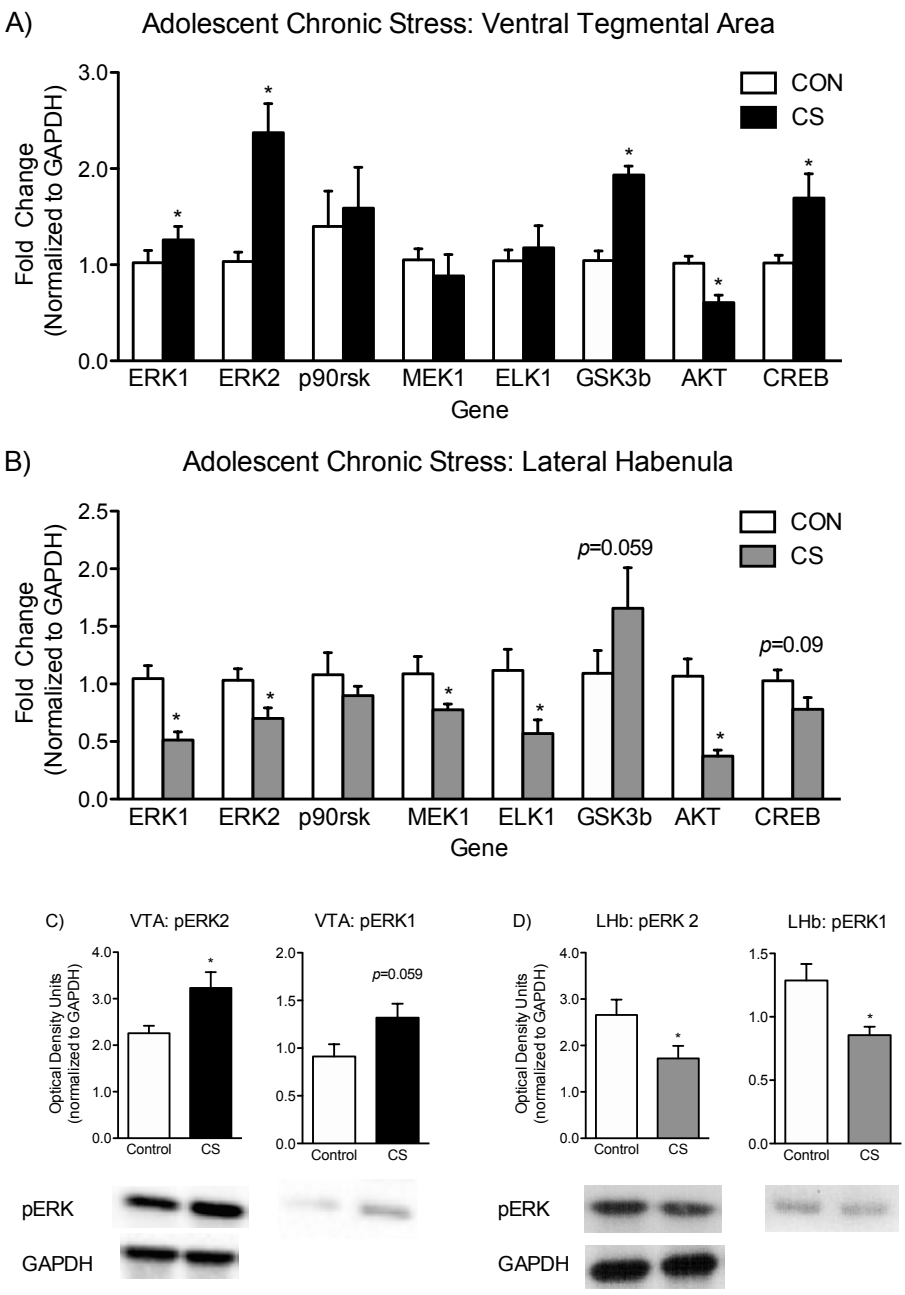



**Figure 3.1** Changes in protein and gene expression in the ventral tegmental area and lateral habenula after adolescent exposure to chronic unpredictable stress. 4 weeks of chronic stress resulted in an increase in mRNA expression of ERK2, GSK3b, and CREB and reduced expression of AKT in the VTA (A). In contrast Chronic stress decreased expression of ERK1/2, p90RSK, MEK1, ELK1, and AKT in the LHb (B). Similarly, stress-induced increase of phosphorylated ERK2 was observed in the VTA (C) while in the LHb (D) there was a decrease in regulation of ERK1/2. *Significantly different from non-stressed controls ($p<0.05$)

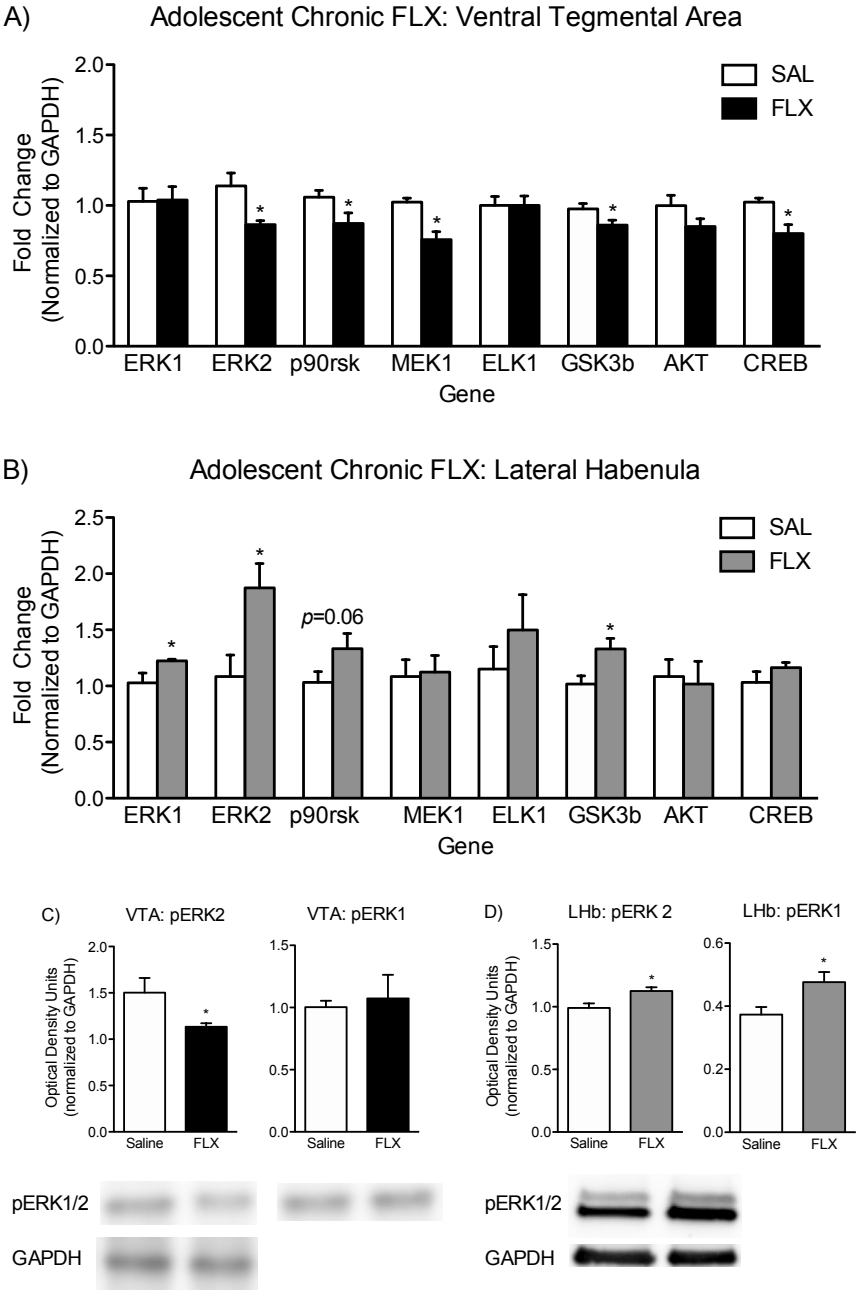



**Figure 3.2** Changes in protein and gene expression in the ventral tegmental area and lateral habenula after adolescent exposure to fluoxetine.15 days of exposure to fluoxetine (FLX; 10mg/kg) resulted in a decrease in mRNA expression of ERK2, P90RSK, MEK1, GSK3b, AKT and CREB in the VTA (A). In contrast in the LHb (B) FLX increased expression of ERK1/2, and GSK3b. Similarly, FLX exposure decreased the phosphorylated levels of ERK2 in the VTA (C) while in the LHb (D) there was an increase in regulation of ERK1/2. *Significantly different from saline-exposed controls ($p<0.05$)

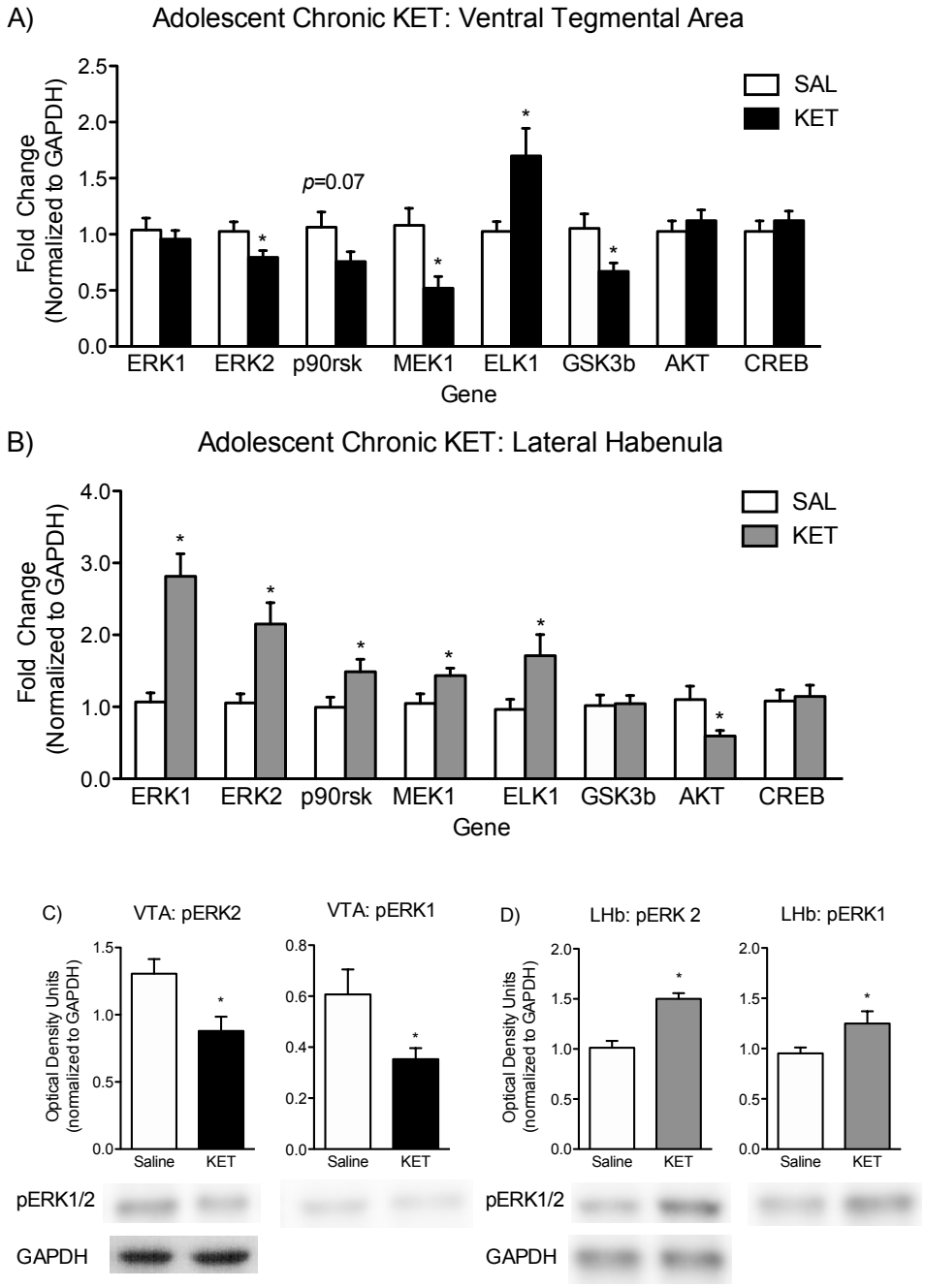



**Figure 3.3** Changes in protein and gene expression in the ventral tegmental area and lateral habenula after adolescent exposure to ketamine. 15 days of exposure to ketamine (KET; 10mg/kg) resulted in a decrease in mRNA expression of ERK2, P90RSK, MEK1, and GSK3b, and increased ELK1 in the VTA (A). In contrast in the LHb (B) KET exposure increased mRNA expression of ERK1/2, p90RSK MEK1, and ELK while decreasing expression of AKT. Similarly, KET exposure decreased the phosphorylated levels of ERK1/2 in the VTA (C), while in the LHb (D) there was an increase in regulation of ERK1/2. *Significantly different from saline-exposed controls ($p<0.05$)

# CHAPTER IV

# ROLE OF EXTRACELLULAR-REGULATED KINASE 2 IN MEDIATING STRESS-RELATED BEHAVIOR WITHIN THE LATERAL HABENULA

## Introduction

It has been demonstrated that the extracellular regulated kinase 2 (ERK2) is a key mediator of stress and antidepressant responses (Carrier & Kabbaj, 2012; Fumagalli et al., 2005; X. Qi, Lin, Li, Li, Wang, Wang, & Sun, 2008a). These experiments have begun to elucidate how ERK2 is modulated in the lateral habenula after exposure to stress or antidepressants. These findings clearly demonstrate that, similar to changes observed within VTA, ERK2 is also modulated within the lateral habenula (LHb) after either chronic stress or antidepressant exposure. As the neuroscience field moves forward, one of the most important goals is the establishment of causation linking experimental manipulations/observations with functional output. Thus, the critical importance of determining whether the alterations observed in mRNA or protein level lead to functionally significant changes in behavior. The use of viral-mediated approaches has facilitated the manipulation of specific genes within highly localized brain regions to determine their significance into mediating specific behavioral effects. This approach has become widely available and it has been previously used in our laboratory to determine the significance of specific gene proteins in modulating behavioral effects in relation to stress and drugs of abuse (iguez et al., 2010; Warren et al., 2011).

To assess the functional significance of gene proteins of interest, our laboratory takes advantage of the gene transfer strategy using the herpes simplex virus (HSV) vector. Previous experiments in our lab and others have validated the use of the HSV viral vector as a valuable tool and we have maintained these conditions in order to assure specificity and reliability of gene expression (Iniguez et al., 2010; Neve, Howe, Hong, & Kalb, 1997). As stated, this vector carries the specific genes (or coding sequences) of interest: wildtype to increase the expression of endogenous genes, or their respective dominant negative form (expression of mutated genes), or the green fluorescence protein (GFP) as control. Expression of the HSV-encoded transgenes is limited to an area of roughly 1 $mm^3$ around the injection site, and no expression is seen in either efferent or afferent regions of the injected area (Neve et al., 1997; Russo et al., 2006). Among the advantages afforded by the HSV vector system is that it is neurotropic (neuron-preferring),

infection expression can be detected within hours (2-3), with maximal expression on day three post-infusion, and expression is short-lived (about five days).

In the previous chapter I demonstrated that levels of ERK2 within the LHb are differentially regulated by stress and antidepressant treatment: chronic stress decreases ERK2 levels whereas chronic exposure to both fluoxetine and ketamine increases ERK2. These biochemical findings set the stage to determine whether direct modulation of ERK2 levels within the LHb is sufficient to induce increased sensitivity to stressful stimuli and induce a depression-like phenotype or if ERK2 modulation is more likely to mimic an antidepressant exposure profile. To this end, I employed two different approaches. First, naïve, non-stressed adolescent rats were microinfused with an HSV-wtERK2 which would increase expression of ERK2 or its dominant negative mutated form (HSV-dnERK2) which would result in blunted expression of ERK2 expression in the LHb and exposed to a battery of aversive stimuli to assess their sensitivity to stress. Another group of rats were exposed to chronic unpredictable stress and then administered ERK2 in the LHb to determine whether it would reverse stress-induced behavioral deficits (i.e., antidepressant-like responses).

**Methods**

*Materials and Tests*

*Animals.* Male Sprague-Dawley rats were obtained from Charles River (Wilmington, MA). Rats arrived at postnatal day (PD) 28 and were allowed to habituate to the facility for 5-7 days. The age at the of experimental manipulations in adolescent rats (PD 35) was selected because it roughly approximates adolescence in humans (Spear, 2000). Rats were housed in clear polypropylene boxes containing wood shavings in an animal colony maintained at 23–25°C on a 12 h light/dark cycle in which lights were on between 0700 and 1900 hours. Food and water were provided ad libitum.

*Experimental Design.* Group1: Adolescent (PD 35) male Sprague Dawley rats were separated into either dnERK2, wtERK2, or GFP (n=5-6 rats/group) groups and administered their respective virus vectors (HSV-dnERK2, HSV-wtERK2, or GFP) into the LHb. On day 3 post-surgery (PD 38), rats were placed in locomotor boxes for 1hr to assess any changes in locomotion due to viral load. Rats were then exposed to the FST and EPM to measure their reactivity to mood- and anxiety-related behavioral challenges. Group 2: Adolescent (PD 35) male Sprague Dawley rats were exposed to 4 weeks of chronic unpredictable stress (CUS) and then assigned to either a

HSV-wtERK2 or HSV-GFP groups (n=5-6 rats/group; CUS-GFP, CUS-wtERK2). A separate group of rats, which had not been exposed to stress but had been handled daily for 4 weeks, was microinfused with HSV-GFP on surgery day and used as a control comparison to control for potential surgery-induced deficits. On day 3 post-surgery rats were exposed to the FST and EPM to measure reactivity to mood- and anxiety-related tasks.

*Viral Vectors and Stereotaxic Surgery.* Viral vectors for ERK2 have been constructed (as previously described) and validated both *in vivo* and *in vitro* (Iniguez et al., 2010; Neve et al., 1997; Robinson et al., 1996). All the behavioral assessments were conducted 3 days post-surgery to ensure testing was performed during maximal viral expression. Rats showing expression in areas outside of the target region (LHb) were excluded from the experiment. Virus microinfusions into the LHb were performed using stereotaxic instruments. Rats were anesthetized with a Ketamine/xylazine mixture (80/10 mg/kg, respectively), after which received bilateral viral microinjections of 1.0 µL per side, at a rate of 0.1 µL/minute. Injections were delivered into established coordinates for the LHb (anteroposterior (AP), −3.4 mm; lateral, +/-1.0 mm; and dorsoventral (DV), −5.0 mm; at a 0° angle) using a 5 µL Hamilton syringe with a 33-gauge needle. After surgery, the local anesthetic bupivacaine was applied directly along the wound edges to minimize any potential postoperative discomfort.

*Chronic unpredictable stress*. As previously described, the CUS paradigm is usually carried out for 2-4 weeks (4 weeks in adolescents) and consists of exposing rats to one stressor/day in a randomized manner, such that the animal does not have time to predict the stressor or acclimate to stress exposure over time. Chronic stress consisted of alternating periods of food or water deprivation (overnight), continuous cage shaking (1h on an automatic shaker), forced swim stress (15 min, in 18°C water), continuous overnight illumination (12 h), overnight cage-flooding (12 h), exposure to cold temperature (1h at 4°C), and acute restraint stress (40 min) using plastic DecapiCones (restraint bags) (Overstreet, 2011).

*Locomotor Activity.* Assessment of virus-induced changes in basal locomotor activity was conducted in automated Truscan Coulbourn boxes (63cm x 63cm). Rats were placed in the boxes and allowed to freely explore the square arena. This test was performed in order to assess for any changes in locomotor activity to virus load and expression. Locomotor activity was measured by beam breaks and reported as distance travelled (cm). Data are reported in 10-minute bins.

*Elevated Plus-Maze (EPM).* As previously described, the EPM apparatus consists of two perpendicular, intersecting runways (12 cm wide x 100 cm long) made from gray plastic. One runway has tall walls (40 cm high), termed "closed arms," while the other has no walls, termed "open arms." The arms are connected together by a central area, and the maze was elevated 1 m from the floor. Testing was conducted between 0900 and 1300 under controlled light conditions (~90 lux) as described previously (iniguez et al., 2009). At the start of the test, rats were positioned in the central area, facing one of the open arms and allowed to explore freely for 5 min. Time in either runway is determined by Noldus Ethovision XT and reported as raw time (seconds) that the rat's center point spent in the open or closed arms.

*Forced Swim Test (FST).* As previously described, once placed in the water filled tubes, rats immediately engage in escape-like behaviors, but eventually adopt a posture of immobility in which they make only the movements necessary to maintain their head above water. After 5 min of forced swimming, rats were removed from the water, dried with towels, and placed in a warmed enclosure for 30 min. All cylinders were emptied and rinsed between rats. Here, the latency to become immobile, total immobility, and swimming, climbing, and immobility counts were measured. Latency to immobility and total immobility were recorded during the 5 min test. Latency to immobility was defined as the time at which the rat first initiated a stationary posture that did not reflect attempts to escape from the water (Lucki, 1997). To qualify as immobility, this posture had to be clearly visible and maintained for ≥ 2.0 seconds.

*Statistical Analyses.* Behavioral data were analyzed using mixed-design (between and within variables) ANOVA followed by Bonferroni post hoc tests. When appropriate, Student's *t* tests were used to determine statistical significance of preplanned comparisons (focused comparisons; Rosenthal and Rosnow, 1985; Rosnow and Rosenthal, 1989). Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as $p< 0.05$.

**Results**

*Group 1*

*Elevated Plus Maze (EPM).* On day 3 post-surgery, rats were tested in the EPM (n=5-6/group; Figure 4.1A-B). A one-way ANOVA revealed significant differences in time spent in the open ($F_{(2,16)}$=4.76; $p$<0.05) and closed arms ($F_{(2,16)}$=5.507; $p$<0.05) of the EPM as a function of virus exposure. Rats microinfused with HSV-wtERK2 spent significantly more time in the open arms of the EPM when compared to rats microinfused with HSV-GFP within the LHb ($p$<0.05;

Figure 4.1 A). Similarly, HSV-wtERK2-infused rats spent significantly less time in the closed arms of the EPM when compared to the HSV-dnERK2-microinfused group ($p<0.05$; Figure 4.1B). Although there was a trend toward increased time in the closed arm of the EPM for the HSV-dnERK2 infused rats, it did not reach statistical significance when compared to the HSV-GFP control rats. Overall, these results indicate that increasing ERK2 levels within the LHb buffers the negative effects induced by anxiety-eliciting circumstances.

*Forced Swim Test (FST).* Twenty -four hours after exposure to the EPM, rats were tested in the FST (n=5-6/group; Figure 4.1 C-D). A one-way ANOVA revealed significant differences in latency to immobility ($F_{(2,16)}=4.791$; $p<0.05$) and total immobility ($F_{(2,16)}=7.523$; $p<0.05$) as a function of virus exposure. Rats microinfused with HSV-wtERK2 showed significantly longer times to adopt an immobility posture (i.e., higher latency to immobility) when compared to rats microinfused with HSV-GFP within the LHb ($p<0.05$; Figure 4.1C). Similarly, HSV-wtERK2-infused rats spent significantly less time immobile for the entirety of the test when compared to both the HSV-dnERK2- and HSV-GFP-infused groups, respectively ($p<0.05$; Figure 4.1 D). No statistical significances were detected in either latency to immobility or total immobility when comparing the HSV-GFP- and HSV-dnERK2-infused groups.

*Locomotor Activity.* As can be seen in Figure 1E, no significant changes in distance traveled were observed between the various viral-infused groups three days after surgery.

*Group 2*

*Elevated Plus Maze (FST).* After 4 weeks of CUS exposure, rats were given intra-LHb infusions of HSV-wtERK2 or HSV-GFP. A separate group on non-stressed rats were microinfused with HSV-GFP and served as a non-stress control. Three days post-surgery, rats were tested in the EPM (n=5-6/group; Figure 4.2A-B). A one-way ANOVA revealed significant differences in time spent in the open ($F_{(2,16)}=3.774$; $p<0.05$) and closed arms ($F_{(2,16)}=3.77$; $p<0.05$) of the EPM as a function of virus exposure. CUS-exposed rats microinfused with HSV-GFP spent significantly less time in the open arms of the EPM when compared to their non-stressed HSV-GFP-infused group ($p<0.05$; Figure 4.2A). Conversely, CUS-exposed rats microinfused with HSV-wtERK2 spent significantly more time in the open arms of the EPM when compared to their respective CUS-exposed rats microinfused with HSV-GFP ($p<0.05$). Similarly, CUS-exposed rats microinfused with HSV-GFP spent significantly more time in the closed arms of the EPM when compared to

their non-stressed HSV-GFP-infused counterparts ($p<0.05$; Figure 4.2B). CUS-exposed rats microinfused with HSV-wtERK2 did not differ from the non-stressed HSV-GFP-infused rats

*Forced Swim Test (FST).* After exposure to the EPM, rats were tested in the FST (n=5-6/group; Figure 4.2C-D). A one-way ANOVA revealed significant differences in latency to immobility ($F_{(2,16)}=11.76$; $p<0.05$) and total immobility ($F_{(2,16)}=7.404$; $p<0.05$) as a function of virus exposure. CUS-exposed rats microinfused with HSV-GFP took significantly less time to adopt an immobility posture (i.e., lower latency to immobility) when compared to its respective non-stressed-HSV-GFP controls ($p<0.05$; Figure 4.2C). CUS-exposed rats microinfused with HSV-wtERK2 showed significantly longer latency to immobility when compared to the CUS-HSV-GFP group ($p<0.05$), but significantly shorter time to immobility when compared to the non-stressed HSV-GFP-infused group ($p<0.05$; Figure 4.2C). Similarly, rats microinfused with HSV-wtERK2 spent significantly less time immobile when compared to the CUS-HSV-GFP- and the non-stressed HSV-GFP-infused groups, respectively ($p<0.05$; Figure 4.2D). No statistically significant differences were detected between non-stressed HSV-GFP- and the CUS-HSV-wtERK2-infused rats.

*Histology and transgene detection.* At the end of the forced swim test, mice were given an overdose of KET and perfused transcardially with 0.9% saline, followed by cold 4% paraformaldehyde. The brains were removed, post-fixed overnight in 4% paraformaldehyde. Coronal sections (40 μm) were taken on a vibratome and stored in 0.1 M sodium phosphate buffer with 0.05% sodium azide. Free-floating coronal sections were processed to examine accuracy of viral injections. Slides were then visualized and photographed using a fluorescence microscope and a digital camera. Data obtained from mice with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses.

**Discussion**

The detrimental consequences of chronic stress exposure are likely experienced throughout the brain, and while it is unlikely that there is one crucial molecule responsible for facilitating stress-responses, the extracellular regulated kinase (ERK2) has been shown to be an important functional regulator of stress and antidepressant response in multiple brain regions such as the hippocampus, the prefrontal cortex, the ventral tegmental area, and the nucleus accumbens. In this chapter, I have now extended those findings to include a truly novel target, the lateral habenula (LHb). My work demonstrated that exposure to chronic unpredictable stress decreases gene

expression of ERK2 within the LHb, whereas antidepressant treatment with either fluoxetine or ketamine results in increased ERK2 (see Chapter 3). In this chapter I have taken advantage of a gene transfer system to assess the potential functional significance of ERK2 within the LHb in regulating behavioral responsiveness to aversive stimuli. Overall, my findings indicate that ERK2 within the LHb plays a significant role in buffering how the against the detrimental effects of stress exposure.

Using HSV viral vectors to manipulate ERK2 expression, I found that increasing ERK2 in the LHb results in attenuated behavioral reactivity to stressful- and anxiety-eliciting situations. Specifically, naïve adolescent rats microinfused with HSV-wtERK2 spent significantly more time in the open arms of the EPM when compared to both the HSV-GFP- or the HSV-dnERK2-infused rats. Given the directionality of the biochemical effects observed after chronic stress and antidepressant treatment, I expected that viral downregulation of ERK2 would have an opposite but equal effect as demonstrated after increasing ERK2. My findings indicate that blocking expression with a dominant negative mutant of ERK2 (HSV-dnERK2) did not increase sensitivity to stress. There is evidence to suggest that certain hub genes, which are the primary modulators of downstream networks may be more important in promoting a specific behavior within a given brain region (Bagot et al., 2016). For example, Neurod2 and Dkkl1 have been shown to promote susceptibility to stress when infused in the ventral hippocampus, but they have no effect when infused in the prefrontal cortex (Bagot et al., 2018). This may be the case within the LHb and ERK2 such that increased ERK2 levels have a functional consequence while downregulation of ERK2 has no effect here. It is also possible that the behavioral assays were not sensitive enough to unmask an effect, or that decreasing ERK mediates another aspect of a depressive-like phenotype that was not tested herein. That being said, some genes are known for working quite specifically in how they elicit behavior effects. For instance, in the nucleus accumbens, delta FosB has been shown to be increased in dopamine receptor type 1 (D1) containing medium spiny neurons (MSN) of resilient mice and increased in D2 MSNs of susceptible mice (Francis & Lobo, 2017). Testing this approach to further assess the effects of ERK2 within the LHb requires a cell-type specific viral delivery, which usually entails the use of transgenic mice. Although the LHb is a mainly a glutamatergic site, it is not entirely homogenous, and cell-type specific delivery of dnERK2 may show differential behavior.

Given that ERK2 in the LHb appears to be more involved in stress resistance instead of susceptibility, I addressed the question of whether direct manipulation of ERK2 would mimic behavioral outputs associated with an antidepressant exposure. To this end, I exposed adolescent rats to 4 weeks of chronic unpredictable stress (CUS), which results in decreased expression of ERK2 (see Chapter 2), and then delivered an intra-LHb microinfusion of HSV-wtERK2, which would reverse the CUS-induced decrease in ERK2 and rescue behavioral deficits. I expected that CUS-HSV-GFP-exposed rats would exhibit similar behaviors to non-surgerized rats that were exposed to chronic stress (i.e., showing a depressive-like phenotype). Supporting my hypothesis, rats receiving microinfusions of HSV-wtERK2 show a reversal of CUS-induced behavioral deficits as measured by the elevated-plus maze (EPM) and forced swim (FST) assays. Namely, HSV-wtERK2-infused rats took significantly more time to give up in the FST and subsequently showed significantly less total immobility for most of the entirety of the test. Similarly, rats microinfused with HSV-wtERK2 spent more time in the open arms of the EPM when compared to CUS-HSV-GFP-microinfused rats, results indicative of decreased anxiety-like behavior. Overall, these data suggest that increasing ERK2 within the LHb is sufficient to reduce stress-induced mood-related behaviors abnormalities.

It is important to note that while it is probable that stress and antidepressants work through similar brain mechanisms and pathways, it is also likely that there are some mechanisms that are more specific to stress while others may be more specific to antidepressant responses. As demonstrated in the previous chapter, both stress and antidepressants have differential effects on ERK2 signaling within the LHb, however these viral-mediated experimental outcomes suggest that decreases ERK2 within the LHb may not be sufficient to promote or underlie functional deficits associated with the pathology of stress-induced behavior. Instead it is possible that ERK2 acts in concert with other downstream targets to ultimately induce depression-like symptomology. For example, in addition to alteration in ERK2, I demonstrate that GSK3ß mRNA is increased within the LHb after exposure to CUS. Within this context, my approach using specific viral manipulation of ERK2 would not have necessarily had an effect on GSK3 signaling, leaving open the possibility that GSK3b, *in addition* to ERK2, would be necessary to mimic behavioral profile seen after exposure to stress. Additionally, increasing ERK2 within the LHb seems to parallel what I would expect to see after antidepressant exposure, suggesting that, functionally, the LHb may have a more significant role in mediating the mechanism(s) of antidepressants action. Among other

important roles in cognitive functioning, the LHb has been shown to promote cognitive flexibility which is often used as a behavioral correlate of antidepressant efficacy (Keefe, 2016). Lesions to the LHb prevent animals from learning how to terminate a foot shock once the parameters for termination are changed (Baker et al., 2016), suggesting that an intact LHb would be necessary to properly learn this task.

Although one of the more well-studied reward-related connections is from the LHb to the VTA, the LHb also has a prominent bidirectional connection to the dorsal raphe(Baker, Oh, Kidder, & Mizumori, 2015). The dorsal raphé is a major source of serotonin, which is the main neurotransmitter targeted by selective serotonin reuptake inhibitors such as Fluoxetine. Although SSRIs ultimately function to increase synaptic levels of serotonin, it is possible that antidepressant-induced action(s) within the LHb help facilitate the efficacy of SSRIs by promoting release of serotonin in the dorsal raphe. Indeed, studies using microdialysis to measure neurotransmitter release have shown that rats exposed to chronic mild stress have decreased levels of serotonin in the dorsal raphe and that this reduction is reversed by inhibition of the LHb (L.-M. Yang, Hu, Xia, Zhang, & Zhao, 2008). Overall these data highlight the need to delve further into the specific functions of LHb efferent connections and, importantly, how ERK2 activity could be affecting the electrophysical properties of LHb neurons which would in turn promote activity, or reduction of activity, to its targets.

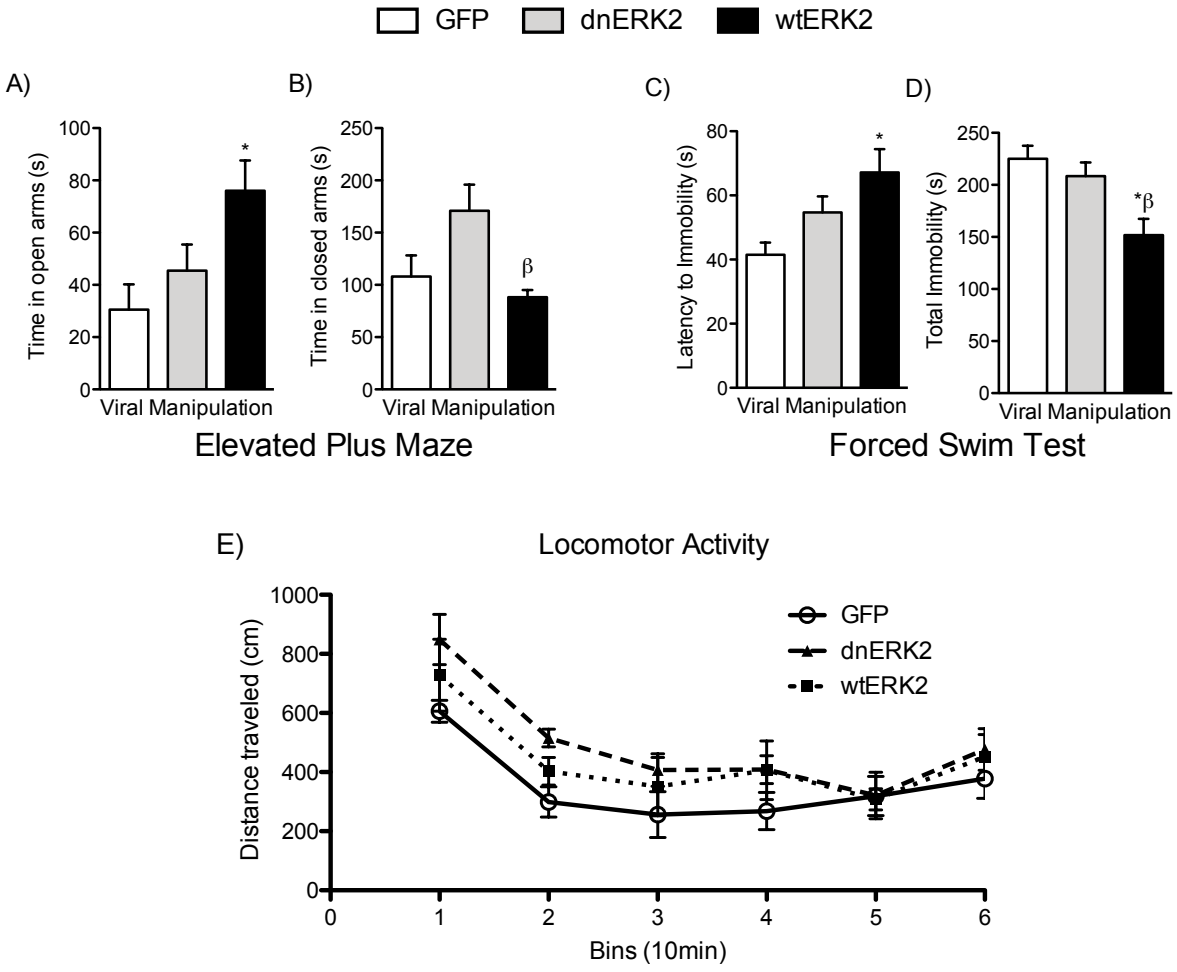



**Figure 4.1** Effect of ERK2 modulation in the lateral habenula on mood related behaviors in naïve rats. Naïve rats were administered microinfusions of either HSV-GSP, HSV-dnERK2, or HSV-wtERK2 in the LHb and then tested in the elevated plus maze (EPM) and the forced swim test (FST). HSV-wtERK2 rats showed an increase in the time spent in the open arms of the EPM (A) and less time in the closed arms (B) and also showed an increase in latency to immobility (C) accompanied by decreased total immobility in the FST (D). No significant changes were seen after infusion of HSV-dnERK2. *Significantly different from HSV-GFP infused rats ($p<0.05$).

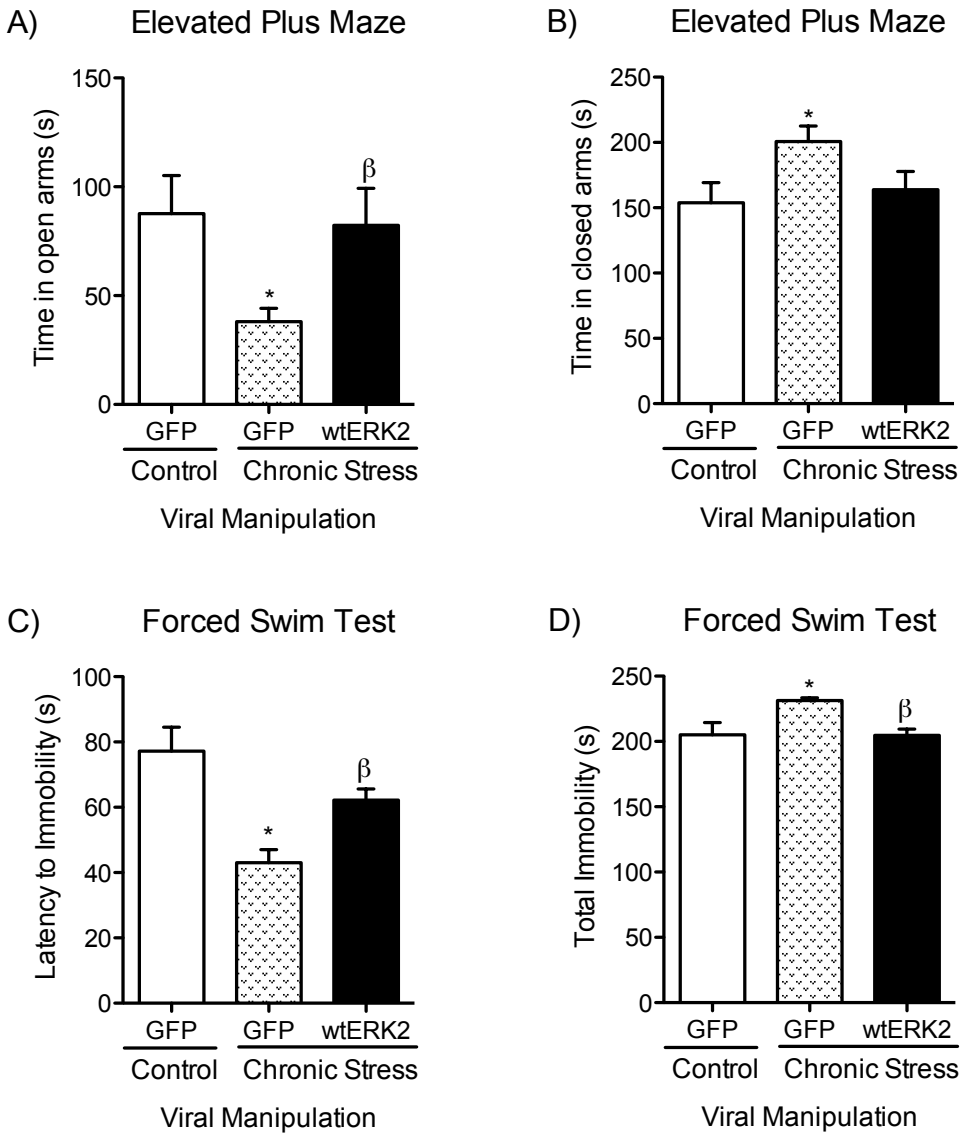



**Figure 4.2** Effect of ERK2 modulation in the lateral habenula on mood related behaviors after exposure to chronic stress. Adolescent rats were exposed to 4 weeks of chronic stress (CS) and then administered microinfusions of HSV-wtERK2 or HSV-GFP in the lateral habenula. After infusions rat were tested in the elevated plus maze (EPM) and the forced swim test (FST). HSV-GFP did not have any effect on stress-induced deficits and were significantly different from non-stressed controls (HSV-GFP CON) however HSV-wtERK2 rats showed an increase in the time spent in the open arms of the EPM (A) and less time in the closed arms (B), while also exhibiting an increase in latency to immobility (C) accompanied by decreased total immobility in the FST (D), suggesting a reversal of stress-induced deficits. $^{\beta}$ Significantly different from HSV-GFP-CS rats ($p<0.05$). *Significantly different from HSV-GFP CON rats ($p<0.05$).

# CHAPTER V

# ESTABLISHING THE ROLE OF HABENULAR EXTRACELLULAR-REGULATED KINASE 2 IN MEDIATING RESILIENCE AFTER SOCIAL DEFEAT STRESS

## Introduction

Exposure to stress is deemed as the primary factor in health dysregulation and in precipitating the development of major depressive disorder (MDD). However, it is important to note that not all individuals whom experience stress will eventually develop MDD (Kalisch, Müller, & Tüscher, 2014). Indeed, the intrinsic response to an acute stressor is meant to be beneficial and actually promote survival. The brain and body are both wired to maintain a certain level of plasticity in response to psychological and environmental insults. This process of "allostatis" follows a particular pattern such that once a reaction to a stressor is initiated, that response is maintained for an appropriate time course to then switched off for a period of recovery (McEwen, 2006). As stress increases, there are certain neurological and biological adaptations that occur to compensate for the increases in allostatic load (Juster et al., 2010). It is possible that the development of MDD could be the result of a maladaptive response to stress, whereas "resilience" can be consider an appropriate maintenance of these mechanisms. Research endeavors have mainly focused in cataloging and delineating potential mechanism(s) underlying susceptibility to stress and its aberrant consequences. More recently, the study of resilience, which is the ability of individuals to withstand severe stress while maintaining physiological and psychological wellbeing, and its potential underlying mechanisms has gained momentum.

Most recently, animal models of stress-induced dysfunction have also tried to identify the possible mechanisms that would promote stress tolerance. In particular the chronic social defeat stress (CSDS) paradigm has been useful in distinguishing between stress susceptible and stress resilient mice. In this paradigm, a robust behavioral syndrome induced in roughly two-thirds of normal C57BL6/J mice by chronic exposure to CSDS. This syndrome includes a mixture of depression- and anxiety-like symptoms, including social avoidance, along with hypothalamic-pituitary-adrenal (HPA) axis abnormalities, disrupted circadian rhythms, overeating, obesity, and related metabolic disturbances (Golden, Covington, Berton, & Russo, 2011; Wells et al., 2017). The mice that display this syndrome are deemed susceptible, and many of the symptoms are long-lived and reversed by chronic, not acute, administration of standard antidepressants (Golden et al.,

2011). This research shows that roughly one third of mice do not express the typical social avoidance expected after defeat stress nor do they show the accompanying deficits exhibited by stress susceptible mice (Golden et al., 2011; Krishnan et al., 2008). This closely mimics the different phenotypes seen in humans, considering that not all individuals that undergo trauma will develop all the core symptoms of clinical depression. While CSDS has been ideal in helping to tease apart the behavioral and biological consequences of social stress, the highly physical nature of CSDS ignores the impact of non-physical stress insults. This is of importance as it has long been known that stress, or trauma, does not need to be physically experienced to induce mood dysregulation (Gleason et al., 2016; Warren et al., 2013). To address this discrepancy, research in our laboratory introduced a modification to the CSDS model to include an "emotional stress" component. In this vicarious social defeat stress model (VSDS), another mouse is forced to watch, or "witness," the antagonistic interaction between a resident mouse and an intruder mouse without being in any direct physical danger (Sial, Warren, Alcantara, Parise, & Bolaños-Guzmán, 2016). Research using the VSDS model in adult mice shows that the biological and behavioral deficits induced by either emotional (ES) or physical (PS) stress result in similar phenotypes. Namely, ES- and PS-exposed mice show increased serum corticosterone after acute or chronic exposure to the stress, increased behavioral despair in the forced swim test, increased anxiogenic responses in the elevated-plus maze, and increased social avoidance. Interestingly, these deficits are seen in both ES- and PS-exposed mice, 1 month after stress exposure, suggesting similar long-term behavioral maladaptation (Warren et al., 2013).

RNA-sequencing data in adult ES- or PS-exposed mice also shows that there are considerable similarities in gene regulation within the ventral tegmental area (VTA) after exposure to either stress condition. As previously stated, the VTA gets considerable input form the lateral habenula (LHb) and it is likely that this is an important hub mediating stress phenotypes. Previous work in our laboratory has shown that ERK2 activity within the VTA can promote susceptibility to CSDS, or reverse some stress-induced deficits in social avoidance (Iniguez et al., 2010). Similarly, as I demonstrated in Chapter 4 using the chronic unpredictable stress model, increasing ERK2 activity within the LHb during adolescence is sufficient to decrease reactivity to stress-eliciting stimuli and reverse stress-induced behavioral deficits. These data suggest that ERK2 signaling within the LHb could also be important in mediating susceptibility and resilience in the VSDS paradigm. Susceptibility and resiliency has not been characterized in the VSDS model, and

these parameters have not been applied to adolescent mice exposed to VSDS. Therefore, I designed the following set of experiments to begin establishing the behavioral profile of susceptibility and resiliency in adolescent mice after exposure to VSDS, and to investigate the role of ERK2 in mediating these behavioral phenotypes.

**Methods**

*Materials and Tests*

*Animals.* Male c57BL/6 and CD1 retired breeder mice were obtained from Charles River Laboratories, and upon arrival were given a 1-week habituation period prior to any experimental manipulation. c57BL/6 mice arrived at postnatal day (PD) 21 and were housed 5/cage until they were separated into their respective experimental conditions. As adolescents (PD 35), mice were separated into control (CON), emotional (ES), or physical (PS) stress group conditions. CD1 mice were single housed before and after behavioral experimentation. All mice were housed in clear polypropylene cages containing wood shavings and had unrestricted access to food and water. The vivarium was maintained at 24°C and mice were on a 12-hour light/dark cycle through the entirety of the experiment.

*Vicarious Social Defeat Stress.* VSDS was done as previously described (Sial et al., 2016; Warren et al., 2013a). Briefly, the VSDS paradigm is a modified version of the chronic social defeat paradigm which allows for assessing the effects of indirectly experienced stress. "Emotionally" stressed (ES) mice are housed with an aggressive mouse (safely separated) and also forced to witness multiple defeat bouts between the aggressor and a smaller subordinate mouse (PS). Briefly, ES-exposed mice were placed into the empty compartment of a divided cage containing a CD1 aggressor, and the PS-exposed mice were placed into the compartment containing the aggressor. During this time, the PS-exposed mouse and the CD1 engage in an aggressive bout, while the ES-exposed mouse witnessed the antagonistic interaction, physically unharmed, from the other side of the compartment. After the defeat, the PS-exposed mouse is left overnight in the compartment adjacent to the recently encountered CD1, while the ES-exposed mouse is moved into a different cage and housed adjacent to a novel CD1. Mice in the CON condition were housed in pairs but separated by a Plexiglas divider such that each mouse is housed in its own compartment. The accelerated VSDS is done similar to the standard VSDS procedure except there are two defeat sessions per day instead of one. These parameters were used given that in the CSDS model, this paradigm induces susceptibility in "normal" mice and is therefore

valuable in assessing stress resilience (Bagot et al., 2018). Additionally, this approach allows for behavioral assessment when working under the restriction of optimal herpes simplex virus (HSV) expression which is limited to 3 days post-infusion.

*Assessing Susceptibility and Resilience.* Time spent with the social target can be expressed as either raw time (seconds) or a ratio of time spent in the interaction zone between the target and no target session (target/no target). In the CSDS model, interaction ratio (IR) scores of 1 or higher have been used to determine stress resilience and lower than 1 indicate stress susceptibility (Krishnan et al., 2008). Given that IR scores can be skewed by the amount of time a mouse spends in the interaction zone within the "no target" session, the use of raw interaction times is often suggested to be utilized as a determining factor as well. For example, if a mouse spends 1s in the interaction zone during the "no target" session and then 2s in the interaction zone during the "target" session it would have an interaction of 1 and if using only IR score as a factor for determining an animals stress phenotype, this animal would be deemed resilient. It could be argued that functionally, this mouse does not exemplify true resilience given that it actually only spent about 1.5% of the total time of the "target" session interacting with the social target. Though this is an extreme example it helps point out the importance of using multiple factors to determine a true behavioral phenotype. To this end, both IR scores and raw time were used to determine stress phenotypes such that animals with an IR score of 1 or higher but also 60s or more spent with the social target, were deemed stress resilient.

*Social Interaction Test.* The social interaction test (SIT) is a two-session test consisting of a "target" and "no target" session. In the "no target" session, a mouse is allowed to explore an open field arena for 2.5 min. The mouse is then removed and a novel CD-1 male mouse is placed into a wire mesh cage, which is situated in an 8cm wide space along one side of the arena. This area is the "interaction zone". For the "target" session, the experimental mouse is placed back into the arena for another 2.5 min and the amount of time spent in the interaction zone is measured. Defeated mice explore the interaction zone significantly less in the presence of the CD-1 mouse (Golden et al., 2011). Data are presented as the ratio of time spent in the interaction zone with and without the target present or as raw time spent in the interaction zone of the "target" session. The SIT was preformed 24h after the last defeat session and then again 1 month later. This was done in order to assess if VSDS resulted long-term behavioral changes as well as to identify if the identified susceptible and resilient phenotypes were maintained.

*Open Field Test.* The open field test is generally used to assess locomotor activity or behavioral reactivity to a novel environment. Mice are placed into the corner of the open field and allowed to explore for 10 minutes. Total distance traveled (cm), time spent in the periphery, latency to enter the center and time spent in the center are recorded. The open field can be used as a measure of anxiety-like behavior. Mice deemed "anxious" will tend to spend less time in the center and will take longer to leave the periphery (D. R. Britton & Britton, 1981).

*Sucrose Preference.* The sucrose preference test consisted of a two-bottle choice procedure in which mice were given the choice between consuming water and a sucrose solution. This paradigm has been used extensively to assess the effects of stress-induced anhedonia (Nestler, Barrot, DiLeone, Eisch, Gold, & Monteggia, 2002b). Mice were habituated to drink water from two bottles for several days. Mice were then exposed to 1% sucrose for two days. Water and sucrose consumption were measured at 9 A.M. and 7 P.M. each testing day. The position of the sucrose bottle (left or right) was counterbalanced between groups and changed daily. Preference for sucrose over water [sucrose/(sucrose + water)] was used as a measure for animals' sensitivity to reward.

*Elevated Plus-Maze.* The elevated plus maze (EPM) is a behavioral assay commonly used to measure anxiety-like behavior and was performed as previously described (Montgomery, 1955a). Briefly, the EPM apparatus is elevated approximately 3 feet off the ground and consists of two perpendicular intersecting runways (6cm x 25cm); one runway has no walls (open arms) while the other arm has fully encompassing walls on either side of the runway (closed arms; 25 cm tall). Mice are placed into the center of the intersecting runways and can freely explore the arena for 5 min. Mice tend to prefer the safety of the closed arms but will eventually begin to explore the open arm runway. Increased time spent in the closed arms is interpreted as increased anxiety-like behavior.

*Forced Swim Test.* The forced swim test (FST) is a task commonly used to assess antidepressant efficacy; however, FST has high predictive validity and is used as a behavioral task to assess learned helplessness (Reed, Happe, Petty, & Bylund, 2008b). Mice were individually placed into 4L Pyrex glass beakers (27 cm x 18 cm) containing 3L of water (23 ± 2°C), for 6 min. Eventually, the mouse adopts an immobile posture, characterized by motionless floating and the cessation of struggling behaviors. The latency to adopt an immobile posture and the total time

spent immobile were recorded. Mice with lower latency to immobility or more time spent immobile reflect a depressive-like phenotype.

*Stereotaxic surgery*

*LHb Cannulation***.** A 26-G guide cannula (Plastics One, Roanoke, VA), was implanted bilaterally 0.5 mm directly above the LHb [anteroposterior (AP) -1.7; mediolateral (ML) 0.4; dorsoventral (DV), -2.0 mm] under 2 to 4% isoflurane in 1liter oxygen/minute inhaled continuously during surgery. Mice were given one week for recovery prior to the initiation to behavioral testing. Mice received a 0.5 µl microinjection at a continuous rate of 0.1 µl/min through their cannulas and then the injector was left in place for 1 min. Twenty-four h after behavioral experiments, 0.5 µg of 4% methylene blue in saline was infused as described above, and animals were then killed 1 h later and their brains extracted and stored in Formalin for histological localization of infusion sites**.** Data obtained from animals with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses.

*Viral-mediated gene transfer.* Mice were anesthetized with 2 - 4% isoflurane in 1 liter oxygen/minute inhaled continuously, placed in a stereotaxic apparatus and their skull was exposed by scalpel incision. For viruses, thirty- three-gauge needles were placed bilaterally at 0° angle into the LHb (AP –1.7; ML +0.4; DV –2.5 in the mm) from bregma and 0.5 µl of virus was infused at a rate of 0.1 µl/ min. Data obtained from animals with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses. I used HSV vectors to directly increase (HSV-ERK2$_{wt}$) Erk2 signaling within the LHb and assess responses to stress- and anxiety-inducing situations. The construction of the vectors (HSV-GFP, HSV-ERK2$_{wt}$) has been thoroughly described, and the HSV-Erk2 virus has been previously validated (Iniguez et al., 2010). Expression of the HSV-encoded transgenes was limited to an area of ~1 mm$^3$ around the injection site (Krishnan et al., 2008).

*Quantitative real-time PCR*: Mice were sacrificed 24hr after lst defeat and 1mm bilateral punches of the LHb were taken with a 16-gauge blunted needle. DNA was created from these samples using qScript cDNA synthesis kit (Quanta) using a C100 Thermal Cycler (Bio-Rad). Quantitative real-time PCR (rt-PCR) was performed in triplicates using 386 well PCR plates using a CFX384 Real-Time Sytstem:C1000 Touch Thermal Cycler (Bio-Rad), according to the manufacturer's instructions. Threshold cycle [C(t)] values are measured using the supplied software and analyzed with the ΔΔC(t) method as previously described (Vialou et al., 2010;

Warren et al., 2013). Rt-PCR will be performed for the following ERK2-related genes: ERK2 (*Mapk1*), ERK1 (*Mapk3*), MEK1 (*Map2k1*), P90RSK (*Mapkap-k1*), GSK3B (*Gsk3b*), AKT (*Akt1*), ELK1 (*Elk1*), Glur2(*Gria2*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as a normalizing gene. Primer sequences can be found in table 5.1.

*Western Blotting*: Protein from LHb tissue punches were isolated using Illustra TriplePrep kit (GE Healthcare) according to the manufacturer's instructions and stored at −80 °C until use. Ten micro-grams of protein from each sample are treated with β-mercaptoethanol and subsequently electrophoresed on precast 10 % gradient gels (Bio-Rad). All antibodies were obtained from Cell Signaling (Beverly, Massachusetts). Blots were probed overnight at 4 °C with antibodies against the phosphorylated forms of ERK1/2, and GAPDH. Separate membranes were probed with antibodies against total ERK1/2, and GAPDH. All primary antibodies were made to a 1:1,000 dilution (except for GAPDH which was diluted to 1: 20,000). Membranes were washed several times with TBST and were incubated with peroxidase-labeled goat anti-rabbit IgG as the secondary antibody (1: 10,000; Cell Signaling, Beverly, Massachusetts). Bands were visualized with Clarity Western ECL Substrate (Bio-Rad), quantified using ImageJ (NIH), and then normalized to GAPDH.

*The effects of pharmacological inhibition of ERK2 within the LHb on the antidepressant efficacy of KET.* A separate group of adolescent mice were cannulated at PD 30 and then exposed to 10 days of CSDS starting on PD35. On the last day of defeat, physically stressed (PS) mice were given KET to reverse stress-induced deficits. Twenty-four hours later, half of the mice were injected with the ERK2 inhibitor, U0126 (2ug/2ul; Iniguez, 2014; Huang and Lin, 2006). A separate groups of non-stressed controls (CON) and a PS group were included as a confirmation of expected behavioral outcomes (i.e.; effectiveness of defeat). Both of these groups of mice were injected with SAL so as to mimic the KET injection of the experimental mice.

*Statistical Analyses.* Behavioral data were analyzed using mixed-design (between and within variables) ANOVA followed by Fisher Least Significant Difference (LSD) post hoc tests. When appropriate, Student's *t* tests were used to determine statistical significance of planned comparisons. Data are expressed as the mean ± SEM. In all cases, statistical significance was defined as $p < 0.05$.

**Results**

*Short-term effects of VSDS on social avoidance and depression- or anxiety-like behavior in susceptible and resilient mice*

*Social interaction.* After 10 days of stress, adolescent mice were tested in the SIT (n=10-18/group; Figure 5.2A-B). A one-way ANOVA revealed a significant difference in interaction time ($F_{(4,58)}$= 15.29; $p$<0.0001) and time in corners ($F_{(4,58)}$= 18.44; $p$<0.0001) as a function of stress condition. Post hoc analyses indicated that ES and PS susceptible mice spent significantly less time interacting with the social target and more time in the corners when compared to the mice in the CON condition ($p$<0.05). In contrast, ESr and PSr mice spent significantly more time in the interaction zone and less time in the corners with the target present when compared to ES and PS mice ($p$<0.05). Importantly, the ESr and PSr mice displayed no significant differences in interaction or corner time when compared to CON mice (p>0.05).

*Open Field Test.* A separate group of adolescent mice exposed to VSDS were tested in the OFT (n= 10-14/group; Figure 5.2C-D). 10 days of VSDS produced a significant difference in time in the center ($F_{(4,44)}$= 4.191; $p$<0.05) and time in the periphery ($F_{(4,44)}$= 3.538; $p$<0.05) as a function of stress condition. ESr and PSr mice spent significantly more time in the center when compared to ES and PS mice (p<0.05). PS but not ES mice spent significantly less time in the center of the OFT when compared to CON mice (p<0.05). As expected, both ES and PS mice spent significantly more time in the periphery of the OFT when compared to CON mice (p<0.05). However, PSr but not ESr mice spent significantly less time in the periphery when compared to their respective susceptible counterpart (p<0.05).

*Long-term effects of VSDS on depression- and anxiety-related behavior in susceptible and resilient mice*

*Social interaction.* Adolescent mice exposed to 10 days of VSDS were re-tested in the SIT 1 month later (n= 10-18/group; Figure 5.3A-B). A one-way ANOVA revealed a significant difference in interaction time ($F_{(4,58)}$= 3.240; $p$<0.05) as a function of stress condition. Post hoc analyses indicated that ES and PS mice spent significantly less time interacting with the social target when compared to the mice in the CON condition ($p$<0.05). In contrast, PSr but not ESr mice spent significantly more time in the interaction zone with the target present when compared to ES and PS mice ($p$<0.05). Importantly, the ESr and PSr mice displayed no significant differences in interaction time when compared to CON mice (p>0.05). Unfortunately, there were

no long-term effects on corner time as a function of stress condition. Interestingly, regardless of stress exposure or stress phenotype, mice show reduced interaction when re-exposed to the SIT. Given that CON mice also show a reduction in interaction, it is unlikely that this is a stress-induced deficit, instead it is possible that this is a reduction in the novelty of the SIT context. There is no significant difference between any group in the magnitude of change of interaction time between SIT 1 and SIT 2 ($p$>0.05; Figure 5.3C)

*Elevated plus maze.* To assess long-term effects of stress on anxiety-like behavior, a separate group of mice were tested in the EPM 1 month after VSDS (n=6-8/group; Figure 5.3D). A One-way ANOVA revealed that time spent in the open arms of the EPM varied as a function of stress exposure ($F_{(4,38)}$= 3.618; $p$<0.05). As expected, ES and PS mice spent significantly less time in the open arms of the EPM as compared to the CON-exposed mice ($p$<0.05). Surprisingly, ESr but not PSr mice spent significantly more time in the open arms of the EPM when compared to their stress-susceptible counterpart ($p$<0.05).

*Sucrose preference.* In order to determine the lasting functional consequences of adolescent exposure to VSDS on anhedonia, I assessed sucrose preference (n= 10-18/group; Figure 5.3E). A two-way ANOVA revealed a significant interaction between sucrose concentration and stress phenotype ($F_{(4,58)}$= 2.647; $p$<0.05) and a significant effect of SUC concentration ($F_{(4,58)}$= 6..295; $p$<0.05). CON, ESr, and PSr mice showed a significant preference for sucrose when compared to water ($p$<0.05) while ES and PS mice failed to show a preference for a sucrose solution ($p$>0.05). Importantly, there were no significant differences in water consumption between the stress conditions.

*Changes in protein and gene expression in the LHb of susceptible and resilient mice after vicarious social defeat stress*

*Rt-PCR*. ERK-related gene expression within the LHb was assessed 24 h after exposure to VSDS using qPCR (n= 6/group; Figure 5.4A-F). One way ANOVA revealed that expression of ERK2 ($F_{(4,29)}$= 4.356, $p$< 0.05; Figure 5.4A), ERK1 ($F_{(4,29)}$= 3.7, $p$< 0.05; Figure 5.4B), P90 RSK ($F_{(4,29)}$= 3.786, $p$ >0.05, Figure 5.4C), MEK1 ($F_{(4,29)}$= 13.63, $p$< 0.01; Figure 5.4D), AKT ($F_{(4,29)}$= 6.325, $p$< 0.001; Figure 5.4E) mRNA varied as a function of stress phenotype. ERK2 and MEK1 mRNA levels were significantly increased in the LHb of ESr and PSr mice when compared to their susceptible counterparts ($p$< 0.05). Interestingly both ERK2 and MEK1 mRNA levels were significantly reduced in PS, but not ES mice when compared to CON ($p$< 0.05). ERK1 mRNA

expression was significantly downregulated in ES and PS mice when compared to CON ($p< 0.05$); and significantly elevated in PSr mice compared to PS mice. AKT gene expression was significantly lower in all stress conditions when compared to CON mice ($p< 0.05$), however PSr mice had significantly higher AKT mRNA expression when compared to PS mice ($p< 0.05$). Interestingly, ELK1 mRNA expression was unaffected by stress phenotype ($p> 0.05$).

*Western Blot.* I further assessed the activity of ERK2-related signaling after adolescent VSDS as inferred from the phosphorylation of ERK1 and ERK2 (Figure 5.4G-H; n= 6/group) Phosphorylation of ERK2 ($F_{(4,29)}= 10.86$, $p< 0.01$; Fig 5.4G) and ERK1 ($F_{(4,29)}= 3.729$, $p< 0.01$ $p< 0.01$; Figure Fig 5.4H) within the LHb was influenced by stress exposure. Both ERK1 and ERK2 phosphorylation levels were significantly increased in PSr mice when compared to CON mice and PS susceptible mice ($p< 0.05$). No changes in total levels of ERK2 and ERK1 protein were detected in any condition ($p> 0.05$, data not shown).

*Effects of viral-mediated upregulation of ERK2 in the LHb in response to accelerated VSDS*

*Social Interaction Test.* The effects of HSV-GFP and HSV-wtERK2 on behavioral responding to accelerated VSDS are shown in Figure 5.5 (n= 6-8/group)**.** Time spent with the social target varied as a function of virus expression ($F_{(1,32}= 0.5025$, $p< 0.05$; Fig 5.5B) and corner time ($F_{1,32}= 0.421$, $p< 0.0216$; Fig 5.5C). Both ES- and PS-GFP mice displayed a significantly reduced interaction ratio and significantly increased corner time when compared to CON-GFP mice ($p< 0.05$). Excitingly, overexpression of ERK2 in the LHb blocked the effects of accelerated VSDS in both ES and PS mice, as ES-wtERK2 and PS-wtERK2 mice had a significantly increased interaction ratio when compared to their GFP counterpart ($p< 0.05$). PS-wtERK2 mice, but not ES-wtERK2 spent significantly less time in the corner when compared to their GFP counterpart ($p< 0.05$). Importantly, there was no significant differences between ES-wtERK2 or PS-wtERK2 and either respective CON condition ($p< 0.05$).

*The effects of pharmacological inhibition of ERK2 within the LHb on the antidepressant efficacy of KET.*

*Social interaction test.* The effect of intra-LHb U0126 on ketamine's antidepressant efficacy in adolescent mice is shown in Figure 6. On the last day of defeat PS mice were given KET to reverse stress-induced deficits. Twenty-four hours later, half of the mice were injected with U0126. Student's *t* test revealed a significant effect of U0126 infusion on social interaction time ($t_{(12)}= 3.341$, $p< 0.05$; Fig 5.6B) and time spent in corners ($t_{(12)}= 4.464$, $p< 0.05$; Fig 5.6C).

Non-stressed controls (CON) and a physically stressed (PS) group were included as a confirmation of expected behavioral outcomes (i.e.; effectiveness of defeat). CON and PS mice were injected with SAL so as to mimic the KET injection of the experimental mice. As expected, PS-mice exposed to SAL avoided the social target compared to their CON-counterparts. Exposure to KET prevented social avoidance in PS-exposed mice and infusion of U0126 blocked the antidepressant effect of KET ($p<0.05$). A separate group of CON mice were injected with U0126 and tested in the SIT and showed no change in interaction time or time spent in corners (data not shown).

*Histology and transgene detection.* At the end of the SIT, mice were given an overdose of KET and perfused transcardially with 0.9% saline, followed by cold 4% paraformaldehyde. The brains were removed, post-fixed overnight in 4% paraformaldehyde. Coronal sections (40 μm) were taken on a vibratome and stored in 0.1 M sodium phosphate buffer with 0.05% sodium azide. Free-floating coronal sections were processed to examine accuracy of viral injections. Slides were then visualized and photographed using a fluorescence microscope and a digital camera. Data obtained from mice with placements outside the intended brain regions (<10% of all experimental animals) were not included in the analyses.

**Discussion**

Early life stress is a major predisposing factor to developing neuropsychiatric disorders (Fagundes, Glaser, & Kiecolt-Glaser, 2013). Most animal models of stress tend to focus on either early postnatal periods or the effects of stress exposure later in adulthood, thus neglecting the adolescent period (Alcantara, Parise, & Bolaños-Guzmán, 2017). This is surprising given that adolescence is a time of behavioral and biological maturation when most neuropsychiatric disorders tend to emerge and is therefore important to acknowledge as a period of sensitivity to developing maladaptive behaviors (Paus, 2008; Slomski, 2012). Most research has also focused on assessing the effects of physical stress, yet it has been known that the effect(s) of emotional stress is just as impactful, if not more so, as physical stress (Teicher, Samson, Polcari, & McGreenery, 2006), suggesting the necessity of investigating models of stress that focus on non-physically invasive/psychological insults. To this end, the chronic social defeat stress (CSDS) paradigm has been adapted to include both a physical (PS) and emotional stress (ES) component. Similar to findings obtained with the CSDS, the vicarious social defeat stress model (VSDS) shows that adult mice exposed to ES exhibit similar behavioral and biological outcomes, such as

increased serum corticosterone, behavioral deficits in the forced swim test, increased anxiety-like responses, and increased social avoidance (Sial et al., 2016; Warren et al., 2013a). More importantly, the CSDS paradigm also lends itself to identifying stress susceptible and stress resilient phenotypes thus providing better parameters for comparing biological mechanisms of stress responding (Krishnan et al., 2007).

I have taken advantage and applied these parameters, in a novel way, to the adolescent period of development. Here I found that mice exposed to the VSDS demonstrate a similar split in susceptibility versus resilience as is observed in adult mice exposed to the CSDS paradigm. In addition, I also found that the phenotype identified (i.e., susceptible or resilient) during adolescence is maintained into adulthood. More specifically, mice deemed susceptible, those showing social avoidance 24 h after exposure to ES or PS, demonstrate similar levels of social avoidant behaviors when re-tested as adults, a deficit that is not present in stress resilient mice at either time point. Similar effects were observed when the mice were tested in anxiety-related behavioral tasks. When tested 24 h after VSDS exposure, the susceptible mice spent significantly less time in the center of the open field (and anxiety-like behavior) apparatus when compared to their resilient counterparts. In addition, when tested one month later in the elevated-plus maze (EPM), the susceptible mice spent significantly less time in the open arms of the EPM as compared to the resilient mice. Long-term deficits in social avoidance and anxiety-like behavior are expected in PS-exposed adolescent mice (Iniguez et al., 2014), however to see resilient mice in the ES condition show maintenance of their stress-resistant phenotype was unexpected. This was based on previous research showing that adult mice exposed to PS demonstrate both short- and long-term deficits, however adult mice exposed to the ES condition do not show robust avoidance when tested 24 h after VSDS exposure. Instead, these mice do exhibit social avoidance one month after stress exposure (Warren et al., 2013). My findings suggest that there is some delayed, or incubation, effects that take place after exposure to ES during adulthood that do not occur in adolescents. Moreover, other work using the CSDS assay in adult mice shows that susceptible and unsusceptible (resilient) mice display a robust anxiety-like phenotype as both spend significantly less time in the open arms of the EPM (Krishnan et al., 2008; Krishnan et al., 2007), findings opposite to what I observed with adolescents. The mechanism(s) underlying these age-dependent behavioral discrepancies are not known. A simple explanation for this age specific differences is that MDD, and perhaps other neuropsychiatric disorders, manifest differently between adults and

adolescents. This appears to be the case, as one of the differences reported in the literature is that adolescents with mood disorders tend to engage in more impulsive behavior compared to adults (Khemakhem et al., 2017; Moustafa, Tindle, Frydecka, & Misiak, 2017). Within this context, more time spent in center of the open field, or more time spent in the open arm of the EPM can have more than one explanation: one interpretation would be as an anxiolytic response, whereas there is an argument to be made for this being representative of an increase in impulsive behavior (Colorado, Shumake, Conejo, Gonzalez-Pardo, & Gonzalez-Lima, 2006; Zaichenko, Vanetsian, & Merzhanova, 2012), partially explaining the differences observed between adult and adolescent anxiety responses. Clearly more behavioral research is needed to decipher these potential explanations.

The long-term maintenance of these phenotypes suggests a biological mechanism that promotes long-term expression of susceptibility or resilience. Given the role ERK2 plays in stress reactivity, I wanted to investigate how ERK2 is expressed in mice deemed susceptible or resilient after ES or PS exposure. After 10 days of stress exposure, mRNA expression of the extracellular regulated kinase 2 (ERK2) was decreased in the lateral habenula (LHb) of both ES- and PS-exposed susceptible mice. This finding is in agreement with reductions in ERK2 within the LHb I observed after adolescent exposure to chronic unpredictable stress (Chapter 4). Interestingly, the mice deemed resilient did not show this reduction in ERK2 mRNA expression within the LHb. This is contrary to my expectation of an increase in ERK2 mRNA levels. While the molecular mechanisms underlying this difference in ERK2 expression is unknown, it is possible that there are compensating mechanisms in the resilient mice that attenuate this decrease in ERK2 levels to promote a more favorable behavioral outcome. Interestingly, however, phosphorylated ERK2 was increased in the LHb of only of PS-exposed resilient mice. It is possible that due to the intense physical nature of the PS condition, resilience to PS necessitates a more prominent biological response than to ES exposure. This is unexpected given the similarities I observed between the ES and PS conditions (in either susceptible or resilient mice), however it is possible that part of the resilient mechanism is the ability to engage in active coping strategies, instead of passive ones, which have been hypothesized to be mediated by distinct mechanisms (Febbraro, Svenningsen, Tran, & Wiborg, 2017; Machida, Lonart, & Sanford, 2018). These findings nevertheless are reminiscent of observations after antidepressant drug exposure. As demonstrated in Chapter 3, increases in phosphorylated ERK2 within the LHb were seen after chronic exposure to both

fluoxetine (FLX) and ketamine (KET) during adolescence, suggesting that functionally, increases in phosphorylated ERK2 protein could be a common mediator of both innate stress resilience and drug-induced antidepressant mechanisms. There are some studies suggesting that gaining controllability of a stressor blunts the potential negative response to subsequent stressors (i.e., active coping) (Amat, Paul, Zarza, Watkins, & Maier, 2006; Kant, Bauman, Anderson, & Mougey, 1992; Breier et al., 1987). There is evidence showing that ERK2 is increased in the PFC in response to controllable but not uncontrollable stressors, suggesting that ERK2 plays a critical role, at least in part, in this stress-adapting mechanism (Christianson et al., 2014).

To further investigate the functional role of ERK2 modulation in the LHb as it relates to resilience to VSDS, I used a viral mediated approach to directly regulate ERK2 levels of expression in the LHb. Given the results of the western blot data suggesting that ERK2 upregulation in the LHb is more correlated with resilient behavior, I decided to utilize an accelerated defeat paradigm in order to assess stress response within the context of ERK2 expression. One of the major advantages of this abbreviated paradigm is the ability to induce social avoidance within only 4 days (e.g., defeats twice per day). This is particularly important when using viral vectors with a short half-life, as overexpression of ERK2 was done with an HSV-wtERK2 vector, which has maximal expression on day 3 after infusion (Barrot et al., 2002; Neve, Howe, Hong, & Kalb, 1997). Adolescent mice microinfused with HSV-wtERK2 or HSV-GFP (control vector) in the LHB and were exposed to twice a day defeat. As expected, the GFP-infused ES- and PS-exposed mice showed avoidance to the novel social target, however, the mice microinfused with HSV-wt-ERK2 showed interaction scores similar to the non-stressed controls (interaction ratios >1.0). This finding suggests that increasing ERK2 in the LHb is capable of promoting resilience to both ES and PS, and further highlights the role of this kinase and the LHb in modulating responsiveness to stress.

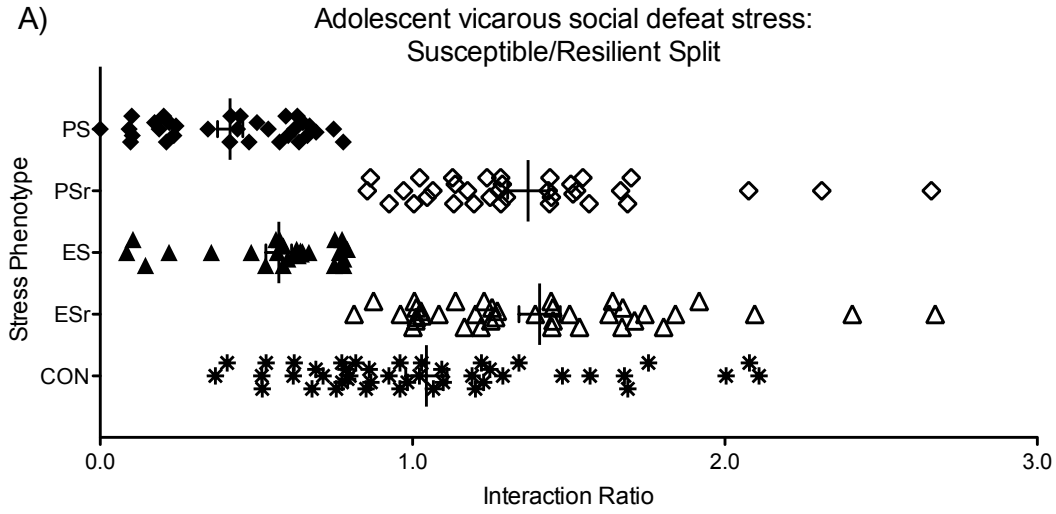
Given the findings of increased ERK2 activity inducing a resilient phenotype, I also assessed whether inhibiting ERK2 activity within the LHb could block the antidepressant effect of KET. To this end, I implanted bilateral cannula in the LHb of adolescent mice and after exposed them to 10 days of CSDS (PS condition only). On the last day of defeat, all PS-exposed mice were given an intraperitoneal injection of 20 mg/kg KET (Autry et al., 2011) (Ramaker & Dulawa, 2017). Twenty-four hours later, social avoidance was measured using the social interaction test (SIT). Thirty minutes prior to the SIT, the KET-exposed mice were given an intra-LHb infusion

of the ERK inhibitor U0126, or its vehicle (as a control). As expected, mice receiving KET and microinfused with vehicle (DMSO) did not display social avoidance indicating that KET was able to reverse the avoidant behavior caused by 10 days of PS exposure, however, inhibition of ERK2 by U0126 blocked the antidepressant effects of KET (i.e., these mice showed social avoidance). This finding further supports my hypothesis that the antidepressant mechanism of KET is in part mediated by ERK2 activity in the LHb.

Overall these data highlight the antidepressant activity of ERK2 in the LHb. Further investigation would be necessary in order to assess whether ERK2 could be used as a biomarker of stress resilience. However, to my knowledge, it is not yet technically possible to measure activity of intracellular signaling molecules within specific brain regions *in vivo*, with the expectation of using it as an indicator of predicting future behavior. It is possible that peripheral levels of ERK2 could be measured and used as an indicator of future behavior, however this would still be an indirect correlation of central activity of ERK2.

Table 5.1. qPCR primer sequences

| | Primer Sequence | |
|---|---|---|
| Gene | Forward | Reverse |
| *Mapk3* | 5'-TCCGCCATGAGAATGTTATAG-3' | 5'-GGTGGTGTTGATAAGCAGAATG-3' |
| *Mapk1* | 5'-GGTTGTTCCCAAATGCTGACT-3' | 5'-CAACTTCAATCCTCTTGTGAGG-3' |
| *Mapkap-k1* | 5'-CCATCACACACCACGTCAAG-3' | 5'-TTGCGTACCAGGAAGACTTT-3' |
| *Map2k1* | 5'-GAGTGCAACTCCCCGTACATC-3' | 5'-TTCTCCCGAAGATAGGTCAG-3' |
| *Akt1* | 5'-ATCCCCTCAACAACTTCTCAT-3' | 5'-CTTCCGTCCACTCTTCTCTTT-3' |
| *Elk1* | 5'-TTGTGTCCTACCCAGAGGTTG-3' | 5'-GCTATGGCCGAGGTTACAG-3' |
| *Gapdh* | 5'-AGGTCGGTGTGAACGGATTT-3' | 5'-TGTAGACCATGTAGTTGAGGT-3' |



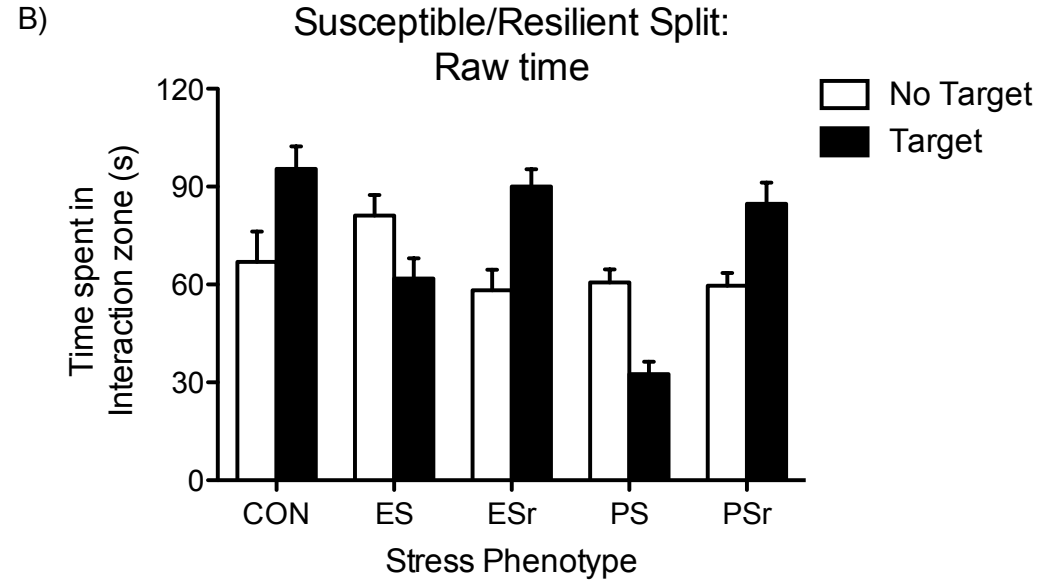




**Figure 5.1** Representation of resilient and susceptible split in adolescent mice exposed to vicarious social defeat stress. Adolescent mice were exposed to 10 days of vicarious social defeat stress and separated according to phenotype, based on social avoidance scores (interaction ration; <1.0= stress susceptible/ >1.0=r stress resilient) (A) and Raw time spent in the interaction zone with and without the target present (B)).

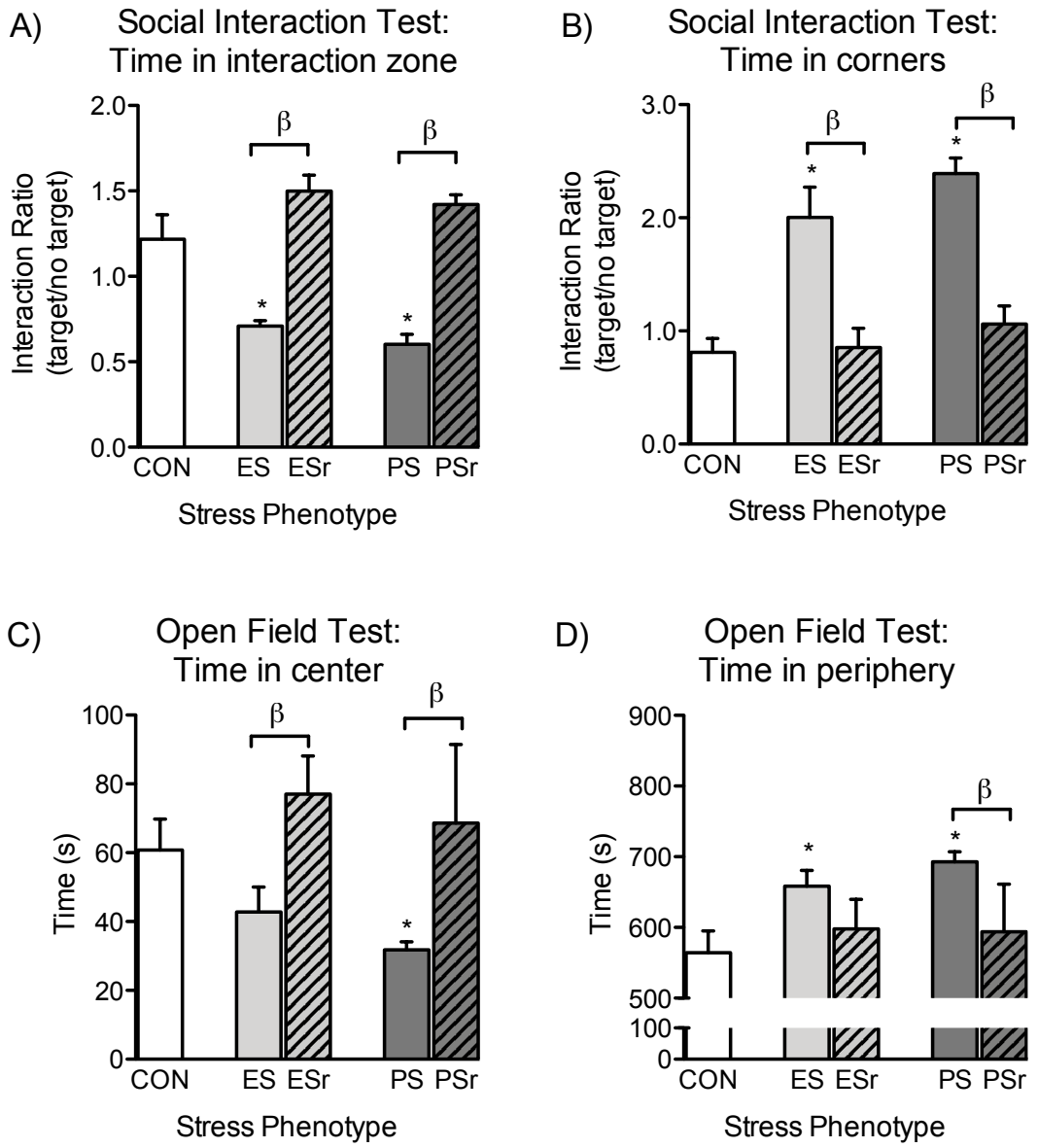



**Figure 5.2** Assessment of social avoidance and anxiety-like behavior in susceptible and resilient adolescent mice 24hrs after vicarious social defeat stress. Exposure to VSDS decreased social interaction (A) and promoted more time spent in the corners (B) in the presence of a novel social target is emotional stress (ES) and physically stressed (PS) mice. ES and PS mice also showed increased anxiety-like behavior in the open field (OFT), spending less time in the center and more time in the periphery of the OFT. These deficits were not observed in ES-and PS-resilient counterparts. *Significantly different from control mice ($p$<0.05). ß significantly different from stress susceptible counterpart ($p$<0.05).

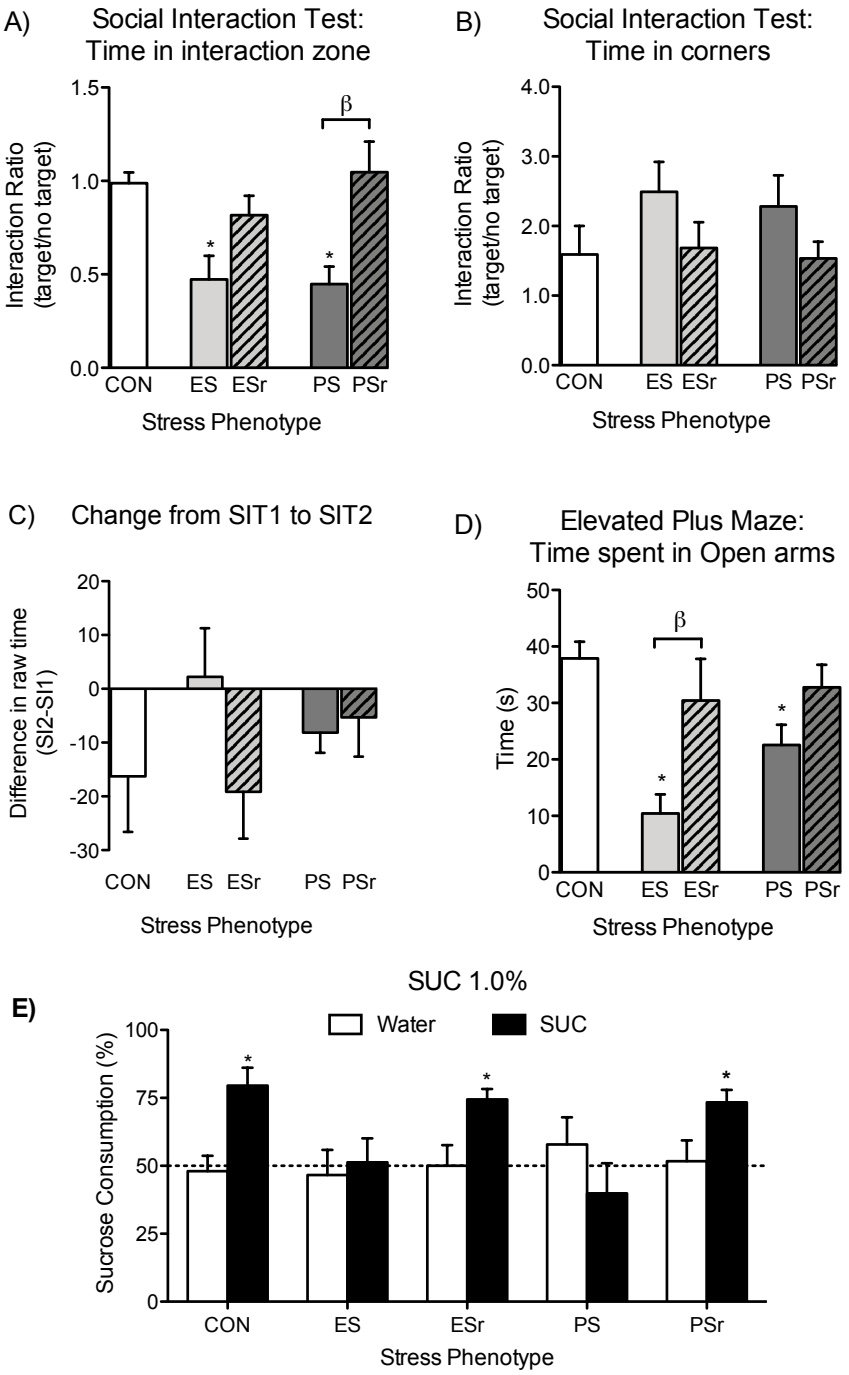



**Figure 5.3** Assessment of social avoidance and anxiety-like behavior in susceptible and resilient mice 1 month after vicarious social defeat stress exposure during adolescence. Exposure to VSDS decreased social interaction (A) and promoted more time spent in the corners (B) in the presence of a novel social target is emotional stress (ES) and physically stressed (PS) mice 1 month after being exposed to VSDS. ES and PS mice also showed increased anxiety-like behavior in the elevated plus maze (EPM), spending less time in the open arms of the EPM. These deficits were not observed in ES-and PS-resilient counterparts. *Significantly different from control mice ($p$<0.05). ß significantly different from stress susceptible counterpart ($p$<0.05).

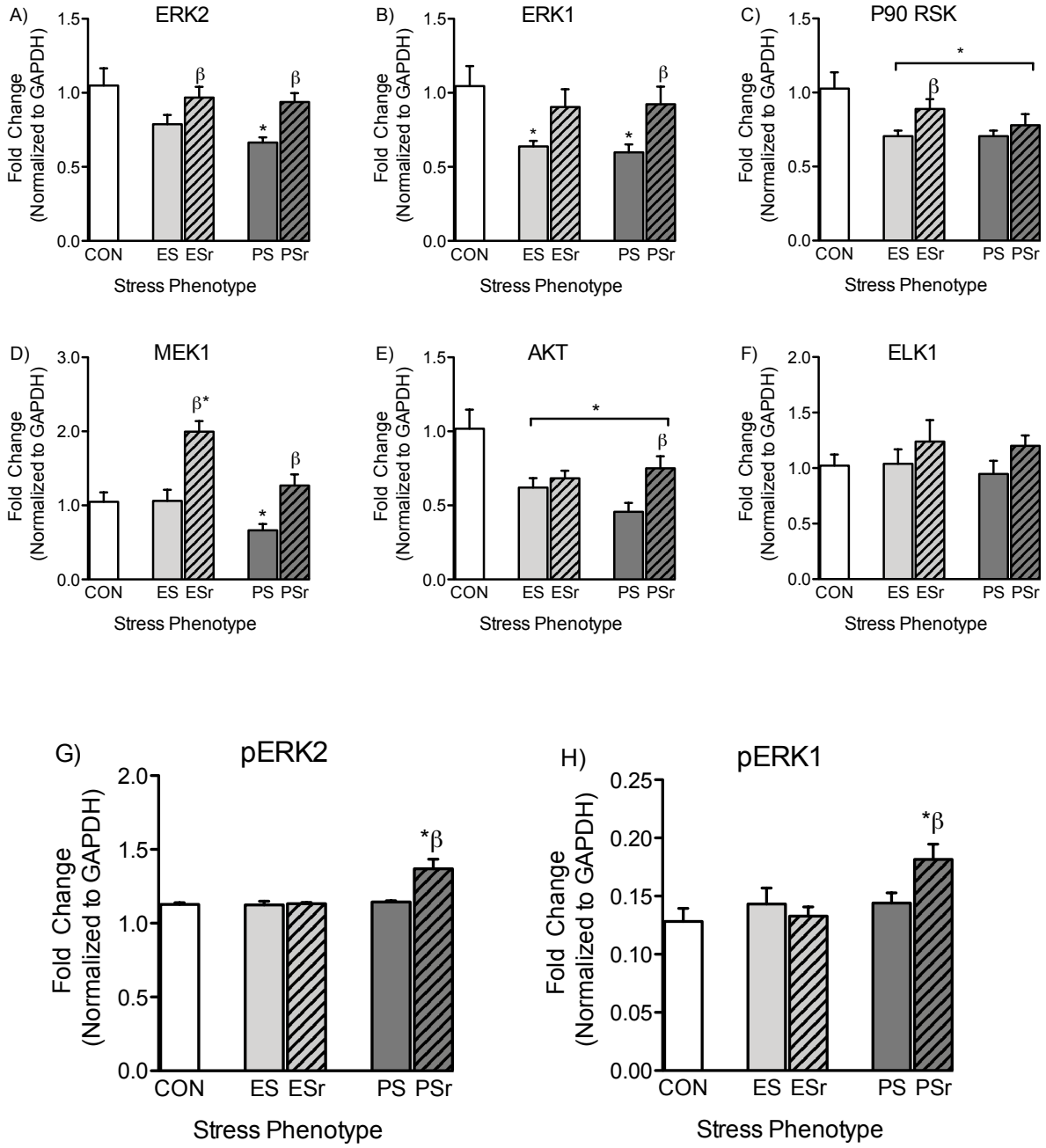



**Figure 5.4** ERK2-related changes in gene and protein expression within the lateral habenula of susceptible and stress adolescent mice. Exposure to VSDS decreased mRNA expression ERK1/2 (AB), P90RSK (C), MEK1 (D) and AKT (E) in PS mice however in ES mice only ERK1, p90RSK and AKT were significantly decreased. P90 RSK and AKT mRNA expression were also decreased in ES and PS resilient mice. No changes in ELK1 were observed in any condition. PS resilient mice showed increased levels of phosphorylated ERk1/2 in the LHb. *Significantly different from control mice ($p$<0.05). ß significantly different from stress susceptible counterpart ($p$<0.05).

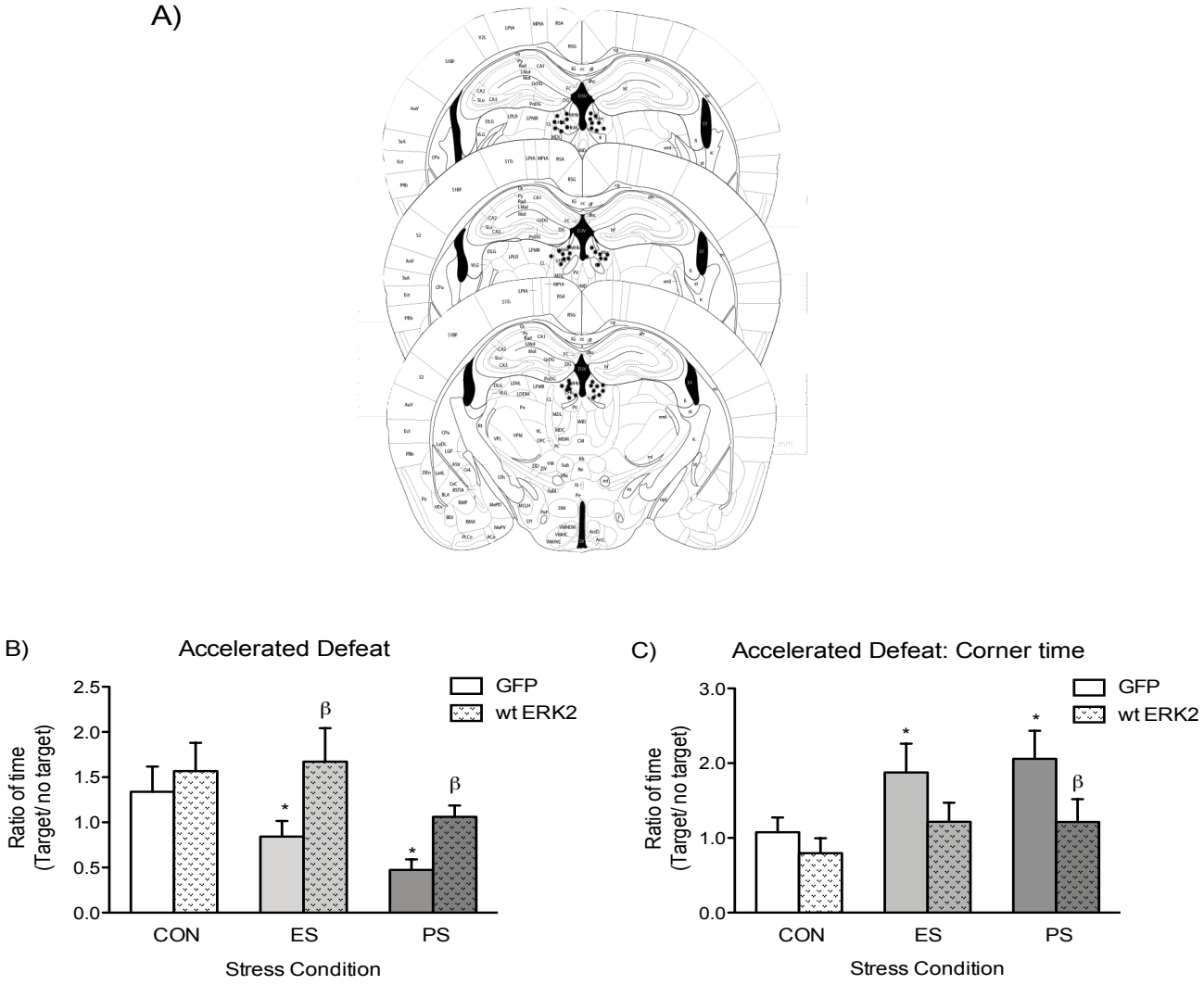



**Figure 5.5** Effects of viral-mediated upregulation of ERK2 within the lateral habenula in accelerated vicarious social defeat stress. Adolescent mice received intra-LHb infusions of either HSV-wtERK2 or HSV-GFP and were then placed through an accelerated VSDS paradigm. Figure (A) shows placement of LHb viral infusion. Administration of wtERK2 attenuated the effects of ES and PS induced social avoidance HSV-wtERK2 mice spent more time in the interaction zone with a target present (B) and less time in the corners (B). *Significantly different from control mice ($p<0.05$). ß significantly different from stress susceptible counterpart ($p<0.05$).

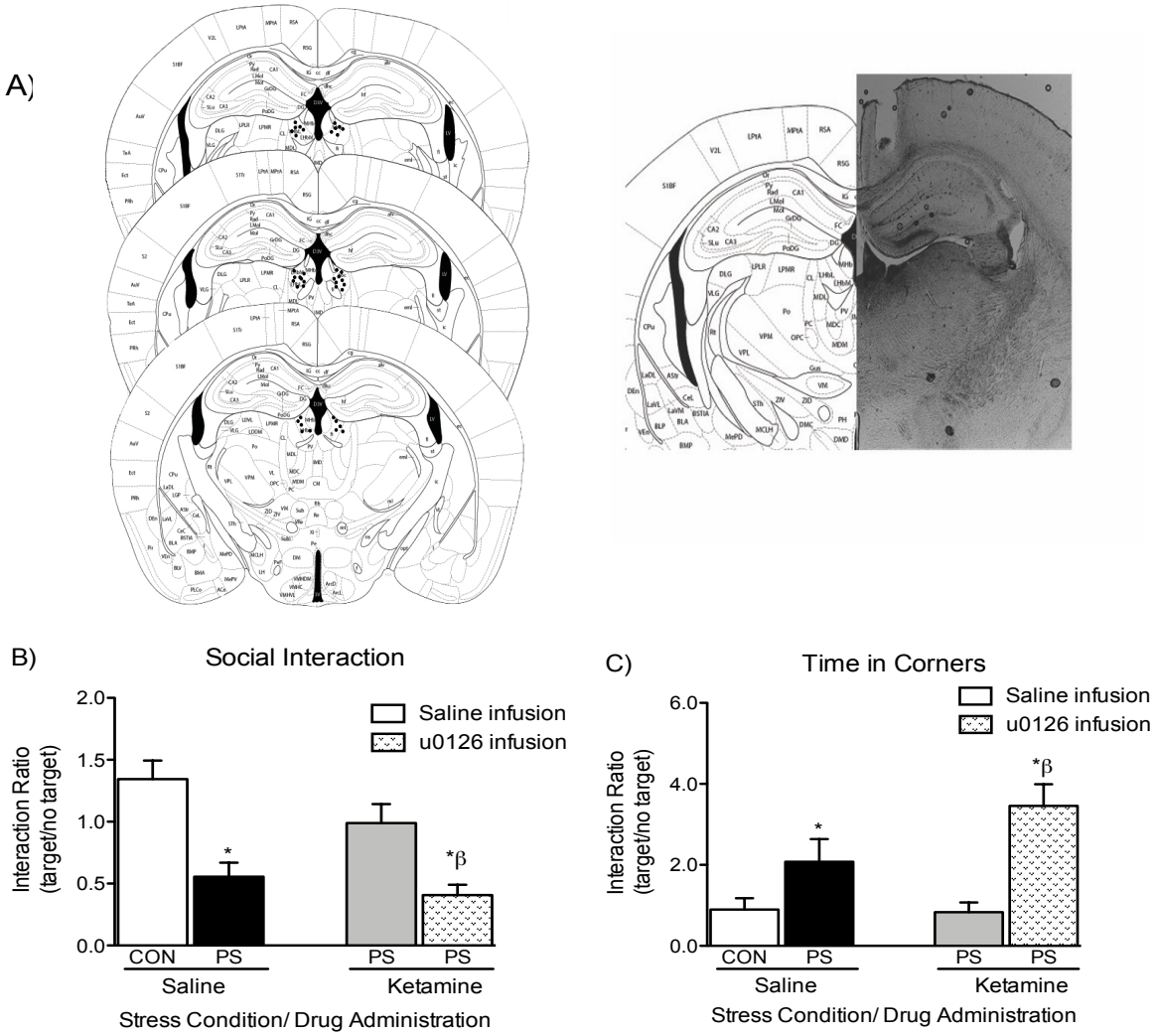



**Figure 5.6** The effects of pharmacological inhibition of ERK2 with U0126 within the lateral habenula on the antidepressant efficacy of ketamine. Adolescent mice were cannulated in the LHb and then placed through 10 days of social defeat stress. On the last day of defeat PS mice were given KET to reverse stress induced deficits. 24hrs later, half of the mice were injected with U0126. Figure A shows placement of LHb infusion cannula. PS mice exposed to saline avoided as expected and spent less time in the interaction zone with a target present (A) and more time in the corners (B). Administration of KET reversed stress-induced avoidance however infusion of U0126 blocked the antidepressant effect of KET. *Significantly different from saline exposed control mice ($p$<0.05). ß significantly different from stress saline infused PS-KET mice ($p$<0.05).

# CHAPTER VI

# CONCLUSIONS AND FUTURE DIRECTIONS

Major depressive disorder (MDD) is projected to be one of the leading causes of disability by 2020, and is currently considered one of the most costly and burdensome illnesses in the world (Kessler, 2012). Until relatively recently, the existence of MDD in children and adolescents was not well recognized. Children who showed signs of what would be considered depression in adults were generally regarded as timid, lazy and disobedient. These symptoms were generally deemed as problems of "adjustment" that represented a "momentary" response to recent stress. It is now well known that children and adolescents can experience MDD. In addition, epidemiologic reports indicate that mood disorders in children and adolescents are quite common, with a proportion of up to 70% of depressed children and adolescents experiencing a recurrence within 5 years of the onset of MDD. Despite this knowledge, most of what is known about MDD and their treatment is based on literature from adult populations. Troubling is also the fact that many afflicted adolescents suffer from treatment-resistant depression, and thus do not benefit from the currently available and approved pharmacological options. To complicate matters, few clinical studies have examined the efficacy, safety, and long-term consequences of exposure to antidepressants during adolescence, with most research focused on adults and findings then applied to adolescent populations.

Aside from our lack of recognition of MDD symptomology in young ages, one of the major problems in basic research addressing pediatric MDD is the lack of valid age-appropriate animal models for the study of depression and antidepressant efficacy (Krishnan & Nestler, 2011). Therefore, as an initial step in this dissertation, I assessed the behavioral effects of a common behavioral paradigm, the chronic unpredictable stress (CUS) assay, during the adolescent developmental window. I also exposed adolescent rats to the novel therapeutic Ketamine (KET) and compared their behavioral outcomes to those seen after exposure to fluoxetine (FLX), a commonly prescribed antidepressant in juvenile populations. I found that exposure to CUS during adolescence promotes a depression-like behavior profile as seen through a development of anhedonia and increased behavioral despair (i.e., reduced sucrose preference and increase immobility in the forces swim test (FST)), and increases in anxiety-like behaviors (i.e., decreased time spent in the open arms of the elevated-plus maze (EPM)). Furthermore, I found that this

stress-induced behavioral profile is ameliorated after administration of KET, which decreases behavioral despair in the FST, and it also increases exploratory time in the open arms of the EPM. Exposure to FLX also improves behavioral reactivity in the FST (an anti-depressant response), however, it induces anxiogenic responses in the EPM. Though troubling, this finding is not surprising as it mirrors clinical evidence showing that one of the side effects of FLX treatment is an increase in anxious behavior (Gupta et al., 2015). The results of my experiment suggest that KET would be a safer, more efficient alternative to FLX, and clinical trials should begin to investigate its use for pediatric MDD (Schiena et al., 2015).

The third chapter of my dissertation sought to investigate a potential molecular mediator for the effects seen after stress or antidepressant exposure. Based on the literature, I chose to investigate the role of extracellular regulated kinase 2 (ERK2) in the lateral habenula (LHb). It has been shown that ERK2 is an important component of the mechanism(s) mediating stress and antidepressant responsiveness in reward-related brain regions such as the ventral tegmental area (VTA), hippocampus, and prefrontal cortex. However, most, if not all of the evidence pertaining ERK2's role in mediating MDD has been derived from work done in adult populations (Iniguez et al., 2010; X. Qi et al., 2009; X. Qi, Lin, Li, Li, Wang, Wang, & Sun, 2008b) leaving open the question as to whether ERK2 would modulate stress-related behaviors during adolescence in a similar manner as in adults. Recent investigations regarding the role played by the LHb point to the importance of second signaling within the LHb in antidepressant responding and suggest that it as promising target for novel therapeutics (Y. Yang et al., 2018), however it is not known how intracellular signaling would work to functionally initiate these antidepressant responses. For this reason, I decided to investigate the role of ERK2 within the LHb as a possible mediator of the antidepressant response. Here, I found that ERK2 was regulated in the LHb after chronic exposure to stress and that administration of both FLX and KET had the opposite effect on ERK2 signaling. Stress increased ERK activity in the VTA while reducing it within the LHb. Conversely, FLX and KET reduced activity of ERK within the VTA, while increasing it within the LHb, to mediate the antidepressant response. Of course, stress responding is a complicated phenomenon and it is unlikely that just one gene is the key regulator of all stress-related behavior (Bagot et al., 2016), but given the inverse relationship between stress- and antidepressant-mediated ERK2 expression, especially within the LHb, it supports the notion that delving deeper into ERK2-related mechanism

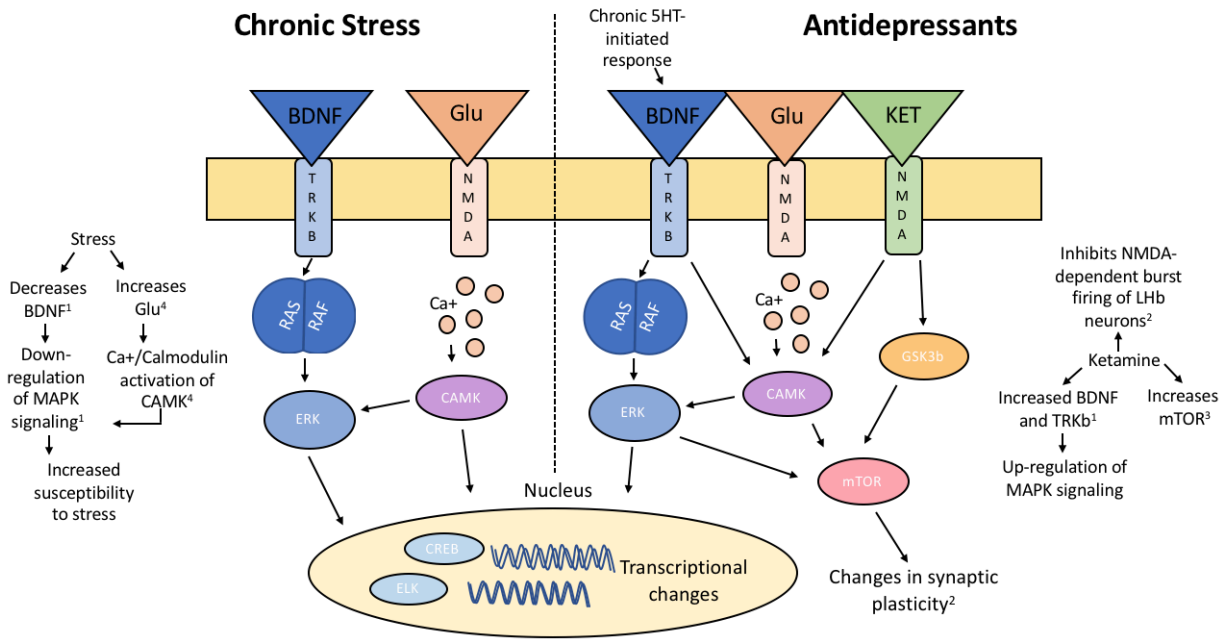
could be a promising area of research for reversing stress-induced deficits in neurocircuitry and finding novel, more specific molecular targets for antidepressant medications.

In the fourth chapter of my dissertation, I investigated the functional role of regulating ERK2 levels within the LHb. Given that ERK2 is part of a larger network of genes, it is possible that ERK2 activity itself would not be sufficient to mimic the behavioral effects that I saw as a consequence of stress or antidepressant exposure. Therefore, a more precise tool is needed to assess the functional role of ERK2. To this end, I employed a viral-mediated approach to be able to regulate ERK2 expression only within the LHb. Interestingly, I found that upregulation of ERK2 promoted antidepressant- anxiolytic-like behaviors (i.e., decreased behavioral despair in the FST and increased exploration in the open arms of the EPM) in stress naïve rats, whereas down-regulation of ERK2 did not promote an opposite (depression-like) response. This finding leads me to believe that even though I observed a downregulation of ERK2 in the LHb after stress exposure (Chapter 3), it is likely that downregulation of ERK2 alone is not sufficient, instead it is only part of a cascade of molecular events that ultimately promote the emergence of depression-related behavior. A potential mechanism for this hypothesis is exemplified in figure 6.1. Although still a hypothesis that needs to be tested, it seems likely the case as ERK2 is also regulated by CaMKII, which has been shown to promote depression-related behaviors when upregulated in the LHb (K. Li et al., 2013). It is thus possible that the changes observed in ERK2 levels after exposure to stress are governed through a CaMKII-dependent mechanism, and that blocking ERK2 by itself may not sufficient to promote the depression-like behaviors.

Given the findings of Chapter 4, I decided to further investigate the role of ERK2 in antidepressant-like behaviors. More specifically, I wanted to see whether ERK2 modulation would have a prominent role in promoting resilience. For these experiments I shifted my experimental design to include an adapted chronic social defeat stress model that includes the emotional stress component. One of the many benefits of this model is the capability of identifying stress susceptible and stress resilient phenotypes via assessment of social avoidance outcomes (Golden et al., 2011; Krishnan et al., 2007) I applied the parameters used to identify stress resilient and stress susceptible phenotypes in adults to adolescent mice, and found that the behavioral reactivity to stress- and anxiety-eliciting tasks was dependent on the identified stress phenotype: as seen in adults, stress resilient mice did not show the same behavioral deficits as seen in their stress susceptible counterparts (Krishnan et al., 2007). These behavioral differences were observed both

24 h, or 1 month after the last defeat exposure. To my knowledge, this is the first study to demonstrate susceptible and resilient phenotypes in adolescent mice exposed to either emotional or physical stress. These findings are exciting as they point to the possibility of eventually developing biomarkers of susceptibility or resiliency at a young age and develop interventions aimed at preventing the deleterious effects of stress. In addition, these findings are supported by my observations in Chapter 3, where I demonstrated that similar to antidepressant exposure, ERK2 levels were increased in the LHb of stress resilient mice and used this knowledge to test the functional role of ERK2 within the LHb in modulating responsiveness to emotional or physical stress. Increasing ERK2 levels within the LHb resulted in an attenuation of stress-induced social avoidance, findings similar to what would be expected after antidepressant treatment. Furthermore, I found that inhibition of ERK2 within the LHb, using the ERK inhibitor U0126, blocked the KET-induced antidepressant effect in the social interaction test further validating the role of ERK2 in the antidepressant response.

These are the first set of experiments to evaluate the role of ERK2 signaling within the LHb and establish a promising path for future development of antidepressant medications. Given that I only did biochemical assessments 24 h after the last stress exposure, it would be important to know whether ERK2 is similarly modulated long-term. The stress-induced changes in protein were much more robust as a consequence of CUS as compared to the VSDS, and it is possible that the duration of the stressor (4 weeks vs 10 days) effected protein expression. Additionally, given that the viral vector approach I used is of a transient nature (expression returns to normal levels after ~4-5 days), it is possible that a more sustained downregulation of ERK2 would in fact result in behavioral outcomes similar to those seen after chronic stress. This approach would require the use of viral vectors capable of inducing extended expression (i.e., adeno associated virus (AAV)) of their gene construct. To my knowledge, the use of these viruses is limited by the viral load they can package, and larger genes (such as ERK2) cannot be packaged in the AAV system making it not a viable approach for my experimental design. Studies have shown that increased neuronal activity in the LHb is what promotes depressive-like behavior (Ootsuka & Mohammed, 2015; L.-M. Yang et al., 2008). For future studies, it would be interesting to take an electrophysiological approach in order to investigate whether upregulation of ERK2 in the LHb decreases neuronal firing to promote antidepressant-like responses.




**6.1 Proposed role for ERK2 in stress and antidepressant mechanisms.** Chronic exposure to stress can have many consequences only one of which include alterations in extracellular-regulated kinase 2 (ERK2) signaling. I propose that while ERK2 modulation in the lateral habenula (LHb) is sufficient to reverse the effects of chronic stress, it is probably an alternate calcium/calmodulin kinase (CAMK)-mediated pathway. CAMK modulates ERK2 activity however it also has other down-stream effectors which can result in transcriptional changes through the transcriptions factors CREB and ELK1, to promote changes in stress-related behavior. Traditional antidepressants such as Fluoxetine act though increasing serotonin (5HT) availability and have been shown to increase ERK2 along with brain derived neurotrophic factor (BDNF). While this is a well-studied pathway, it is not the only modulator of ERK2. My data suggest that the ERK2 inhibition (through U0126) is indeed sufficient to block the antidepressant effects of the non-traditional glutamatergic antidepressant, Ketamine (KET), implying that ERK2 is important in the effectiveness of antidepressant mechanisms of various drug. Abbreviations: tyrosine receptor kinase b (TRKb); glutamate (Glu); *N*-methyl-D-aspartate (NMDA); mammalian target of rapamyacin (mTOR); cyclic AMP response element binding protein (CREB); ETS-domain containing protein (ELK);

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